fermentation

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Comparative Fatty Acid Compositional Profiles of *Rhodotorula toruloides* Haploid and Diploid Strains under Various Storage Conditions

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Abstract: Microbial-based fatty acids (FAs), biofuels and oleochemicals are potential alternatives to fossil fuels and other non-renewable resources. *Rhodotorula toruloides* (formerly *Rhodosporidium toruloides*) is a basidiomycetous oleaginous yeast, and cells of the wild-type diploids can accumulate lipids to over 70 wt% on a dry cell weight basis in nutrient-limited conditions. Meanwhile, several haploid strains have been applied as hosts for producing high-value fatty acid derivatives through genetic modification and metabolic engineering. However, the differences in fatty acid compositional profiles and their stability between diploid and haploid strains remain unknown in this oleaginous yeast. Here, we grew a haploid strain *R. toruloides* NP11 and its parental diploid strain *R. toruloides* CGMCC 2.1389 (4#) under identical conditions and compared the profiles in terms of cell growth, lipid production, fatty acid compositions of lipids as well as storage stability of fatty acid methyl esters (FAMEs). It was found that lipids from *R. toruloides* composed of fatty acids in terms of chain length ranged from short-chain FAs (C6–C9) to very long-chain FAs (VLCFAs, C20–C24) and some odd-chain FAs (C15 and C17), while long-chain fatty acids (C14–C18) were the most abundant ones. In addition, NP11 produced a little more (1 wt%) VLCFAs than that of the diploid strain 4#. Moreover, no major changes were found for FAMEs being held under varied storage conditions, suggesting that FAMEs samples were stable and robust for fatty acid compositional analysis of microbial lipids. This work revealed the fatty acid profiles of lipids from *R. toruloides* haploid and diploid strains, and their stability under various storage conditions. The information is valuable for reliable assessment of fatty acid compositions of lipids from oleaginous yeasts and related microbial cell factories.

Keywords: *Rhodotorula toruloides*; haploid; diploid; fatty acid; storage conditions

Citation: Zhang, Y.; Kamal, R.; Li, Q.; Yu, X.; Wang, Q.; Zhao, Z.K. Comparative Fatty Acid Compositional Profiles of *Rhodotorula toruloides* Haploid and Diploid Strains under Various Storage Conditions. *Fermentation* **2022**, *8*, 467. https://doi.org/10.3390/fermentation8090467

Academic Editor: Timothy Tse

Received: 8 August 2022
Accepted: 13 September 2022
Published: 18 September 2022

1. Introduction

Increasing concerns over energy security, unsustainability, and climate change related to the use of fossil fuels and oleochemicals are leading to a comprehensive exploration of bio-based diesel and related chemicals which are renewable, biodegradable and clean substitutes for petroleum-based counterparts [1,2]. However, the current biodiesel and oleochemicals production from different edible and non-edible plant feedstocks puts huge stress on the food market, arable land, water consumption, and eventually on the economy [2,3]. The recent past has therefore extensively focused on microbial lipids as an alternative feedstock for biodiesel production [4,5]. Microorganisms with over 20 wt% lipids accumulating capabilities of their total cell weight are known as oleaginous microorganisms. The fuels produced from microbial lipids have higher energy density and better miscibility than other fuels [6] and are exempted from the key limitations related to plants and vegetable oil, such as the fact that they are seasonal, have a long production cycle,

require huge arable land and water resources, and more importantly that they compete with edible oils affecting the community [7]. Therefore, extensive research had been conducted on oleaginous microorganisms to address the potential challenges in high titer lipid production through the integration of synthetic biology, metabolic engineering, and systems biology [8,9].

Compared with other oleaginous microorganisms, oleaginous yeasts present several leads such as higher growth frequency, adaptation to diverse substrates, and higher lipid production potentials [5,7]. The robust stress tolerance, work on a broad range of substrates and capacity to accumulate lipids to over 75 wt% per dry cell weight give *Rhodotorula toruloides* (formerly *Rhodosporidium toruloides*) an ascendant place among other oleaginous yeasts [10,11]. Previous studies have already explored the mechanism of lipid and fatty acid accumulation by *R. toruloides* by using multi-omics approaches including genomics, proteomics, metabolomics and systems analysis [8,12–14]. Most studies usually analyzed these known long-chain fatty acids, such as C14, C16, and C18 [15,16], or very-long-chain monounsaturated fatty acids produced by engineered *R. toruloides* [17]. Nevertheless, fatty acid compositional profiles of lipids produced by *R. toruloides* are still missing.

The diploid strain *R. toruloides* CGMCC 2.1389 (4#) is a wild type that had been domesticated in corn stover hydrolysates to give a strain *R. toruloides* Y4, which were used to isolate the haploid strains, NP11 (MAT A1) and NP5-2 (MAT A2) [12]. In this work, we compared fatty acid compositions of lipids from *R. toruloides* 4# and NP11 by incorporating from short chain fatty acids (SCFAs, C6–C8) to very long chain fatty acids (VLCFAs, C20–C24). Moreover, we confirmed the presence of odd chain fatty acids (OCFAs) such as C15, C17, and VLCFAs C22, C24 in lipids produced by *R. toruloides* naturally, as well as the storage stability of the fatty acid methyl esters (FAMEs) samples at different temperatures for up to 2 months. Identification of endogenous synthesis of OCFAs and VLCFAs paves the way for further engineering *R. toruloides* for high value-added fatty acid derivatives and chemicals. Moreover, this work also provides standard methods for the preservation of FAMEs being widely used for fatty acid compositional analysis of lipids produced by oleaginous microbes.

2. Materials and Methods

2.1. Strains, Media and Culture Conditions

Rhodotorula toruloides CGMCC 2.1389 (4#), which is identical to CBS 6016, was originally acquired from the China General Microbiological Culture Collection Center (CGMCC). The haploid NP11 (MAT A1, GDMCC 2.224) was stored in the Guangdong Microbial Culture Collection Center (GDMCC), and was isolated from basidiospores that were germinated from teliospores of Y4 which were derived from *R. toruloides* CGMCC 2.1389 [12].

The yeast strains were activated at 28 °C on YPD plates containing: glucose 20 g/L, peptone 20 g/L, yeast extract 10 g/L and agar 15 g/L. Precultures were prepared in a YPD broth (glucose 20 g/L, peptone 20 g/L, yeast extract 10 g/L as seed culture) at 30 °C and 200 rpm for 24 h. The nitrogen-limited lipid production medium with a C/N ratio (mol/mol) of 413 contained [10,15]: 70 g/L glucose·H_2O, 0.1 g/L $(NH_4)_2SO_4$, 0.75 g/L yeast extract, 1.5 g/L $MgSO_4{\cdot}7H_2O$, 0.4 g/L KH_2PO_4 and supplemented with 10 mL/L trace element solution containing g/L: $CaCl_2{\cdot}2H_2O$ 4.0, $FeSO_4{\cdot}7H_2O$ 0.55, citric acid·H_2O 0.52, $ZnSO_4{\cdot}7H_2O$ 0.10, $MnSO_4{\cdot}H_2O$ 0.076 and 100 µL 18M H_2SO_4. Moreover, the media also included 50 mM MES to maintain a pH of 6.0. All culture media were sterilized at 121 °C for 20 min before use. Shake-flask batch cultures were conducted for lipid productions. 250 mL Erlenmeyer flasks containing 45 mL nitrogen-limited medium were inoculated with 10 vol% (5 mL) 24-h-old preculture seed (around 1.0×10^7 cells/mL), incubated in an orbital shaker at 30 °C 200 r/min, and sampled every 24 h for residual glucose and OD_{600} analysis, as well as cells microscopic examination with a fluorescence microscope (Evos FL AMF4300, Thermo Fisher Scientific, Bothell. WA. USA). Each time the culture lasted until glucose exhaustion, or until it reached a glucose concentration below 10 g/L.

2.2. Cell Mass and Total Lipids

Upon completion of the culture, cells in 30 mL of culture broth were collected by centrifuging at 8000× *g* at 4 °C for 5 min using pre-weighed 50 mL PP centrifugal tubes; the cells were then washed twice with 50 vol% ethanol to prevent bulky cell loss in the supernatant due to high lipid content. The collected cells were dried in an oven at 105 °C for 24 h to constant weight; then the dry cell weight was measured gravimetrically.

Total lipids were extracted by the classical acid heat method according to a previous report [15]. The dried cells were digested with 4 M HCl (3 mL per 0.5 g dry cell) in a shaking water bath at 78 °C, 200 r/min for 1 h. Then total intracellular lipid was extracted three times with chloroform-methanol (1:1, *v*/*v*). The chloroform extracts were washed with 0.1% NaCl (*w*/*v*) solution and passed over an anhydrous Na_2SO_4 pad; the chloroform was then eliminated by reduced pressure rotary evaporation and the pre-weighed round bottom flasks containing the lipid concentrates were dried at 105 °C to constant weight and the total lipid was measured gravimetrically.

The lipid titer was expressed in g/L (culture broth volume), and the lipid contents in weight percentage (wt%) were measured as total lipid produced per total dry cell weight. The lipid yield was calculated as gram lipid produced per gram of consumed glucose (g/g consumed glucose); the lipid productivity was calculated as lipid titer per day (g/L/d) [18].

2.3. Fatty Acid Composition Analysis

The aforesaid extracted lipids were transformed to FAMEs according to a previously established method [19], and the FAMEs samples were held at different temperatures ranging from −20 °C to room temperature for up to 2 months.

To analyze the fatty acid composition differences in lipids from different hosts as well as potential changes over time, the FAMEs samples were detected by a Gas Chromatography/Triple Quadrupole Mass Spectrometer (GC-MS, Agilent 7890B/7000D system, Agilent Technologies, Santa Clara, CA, USA) as reported [20,21] with modifications, using an HP-5MS column (30 m × 0.25 mm × 0.25 μm), FID detector. Gaseous nitrogen was used as a carrier gas (1.1 mL/min). 1 μL sample was injected and the split ratio was 1:100 (250 °C), and the oven temperature was set at 80 °C at the beginning for 1 min; increased to 230 °C with a ramp rate of 8 °C/min; then increased to 245 °C with a ramp rate of 3 °C/min. Lastly, it was increased to 280 °C with a ramp rate of 10 °C/min, held for 5 min, post-run to 320 °C, and held for 2 min. The temperatures of the MS transfer line and ion source were set at 250 °C and 230 °C, respectively. These setting conditions can detect all the short-, medium-, long-, and very-long-chain fatty acids of *R. toruloides*. Fatty acid methyl esters were qualitatively identified by standard samples (methyl lauric acid, C12:0; methyl myristic acid, C14:0; methyl palmitic acid, C16:0; methyl palmitoleate, C16:1; methyl heptadecanoate, C17:0; methyl stearic acid, C18:0; methyl oleic acid, C18:1; methyl linoleic acid, C18:2; methyl tetracosanoate, C24:0;) and mass spectrometric ion peaks, and their relative mass percentage (wt%) content was determined by the area normalization method.

2.4. Statistical Analyses

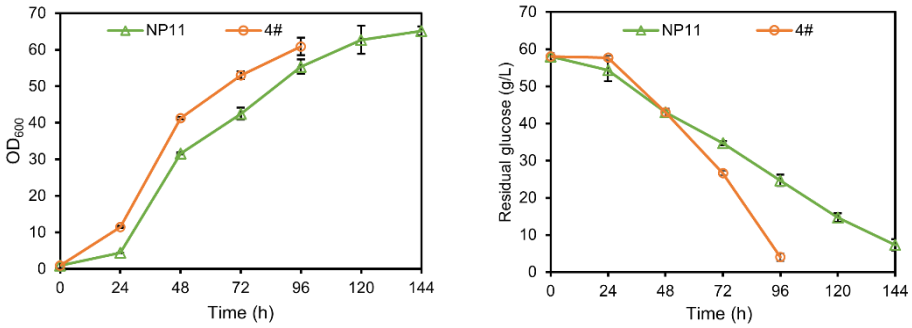
Statistical analysis was performed using the Student's *t*-test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; the data is normally distributed.) by Microsoft Excel software. The Benjamini–Hochberg (BH) method was used for the correction of the *p*-value after multiple comparisons and obtaining FDR (False Discovery Rate); FDR < 0.05 was accepted as significant. All data are presented as a mean ± SD of biological triplicates.

3. Results

3.1. Difference in Growth Patterns of Haploid and Diploid Strains

Previous reports suggested the presence of discrepancies in cell growth and sugar consumption by the diploid and haploid strains [16,22]. To verify these observations, culture broths were analyzed for cell density (OD_{600}) and residual glucose every 24 h. When the initial OD_{600} was 1.0, the diploid strain *R. toruloides* 4# grew faster than the

haploid NP11 (Figure 1a). Relative differences were observed in the OD_{600} from 24 h between both strains as Figure 1a showed and confirmed by the Student's *t*-test (Table S1). The μ_{max} (specific growth rate) of the strain *R. toruloides* 4# (0.14 ± 0.02 h^{-1}) and NP11 (0.11 ± 0.01 h^{-1}) also confirmed it (Figure S1). When *R. toruloides* 4# was in the stationary lipid production phase, its medium residual glucose was significantly decreased compared with that of NP11. As a result, 4# reached the end of the culture faster compared to NP11 in a nitrogen-limiting medium with the same sugar content; the glucose consumption rate in Figure 1b of 4# (10.8 g/L/d) was significantly higher than that of NP11 (7.1 g/L/d).



(**a**) (**b**)

Figure 1. Results of cell density (OD_{600}) and glucose consumption time course in shaking flask culture of haploid strain *R. toruloides* NP11 and diploid strain *R. toruloides* 4#. (**a**) The growth curve of strains in nitrogen-limited culture; growth was measured as the turbidity of culture broth at 600 nm every 24 h. (**b**) Residual glucose concentration curves of strain NP11 and 4#.

Therefore, we concluded that the diploid strain has an inherent growth advantage and higher sugar utilization efficiency than the haploid strain in the culture process. In addition, its cell density increased rapidly after 24 h until the end of the culture (Figure 1a). Though the haploid strain grew a little slower, its cell density could reach the same level as the diploid strain at the end of the culture.

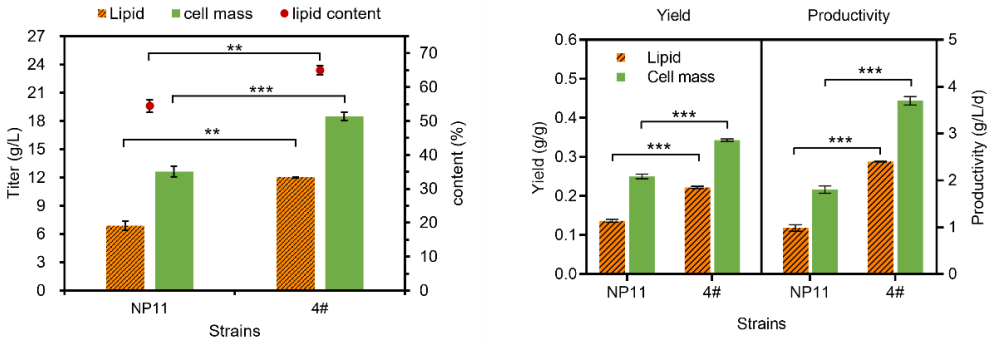
3.2. Lipid Accumulation Potential of Haploid and Diploid Strains

The haploid strain NP11 can accumulate over 50 wt% lipid contents of its dry cell mass [16,23], while the diploid strain could produce more than 70 wt% of lipid contents of its dry cell mass in shake flask cultures using glucose as a carbon substrate [10,15,22].

NP11 showed good lipid accumulation potential with a lipid titer of 6.9 g/L, and lipid contents of 54.4 wt% with a comparatively lower cell mass of 12.6 g/L (Figure 2a, Table S2). The strain 4# undoubtedly produced a higher cell mass of 18.5 g/L, a lipid titer of 12.0 g/L, and a lipid content of 64.9 wt%. The lipid droplet morphology differences during different culture time intervals also proved the high lipid accumulation potential of 4# following NP11, and we could observe that the cell size of the haploid strain is nearly half that of the diploid strain from microscope photos in Figure 3. The larger cell volume of the diploid strain is always the main reason for increasing storage space for intracellular production and the diploid strain also has advantages of high lipid production and robustness using different complex carbon feedstocks [24,25]. The lipid and cell mass yield as well as the productivity of 4# all were significantly two-fold higher than those of NP11(Figure 2b). Therefore, the preparation of the diploid strain is an effective strategy to improve its production capabilities through metabolic engineering.

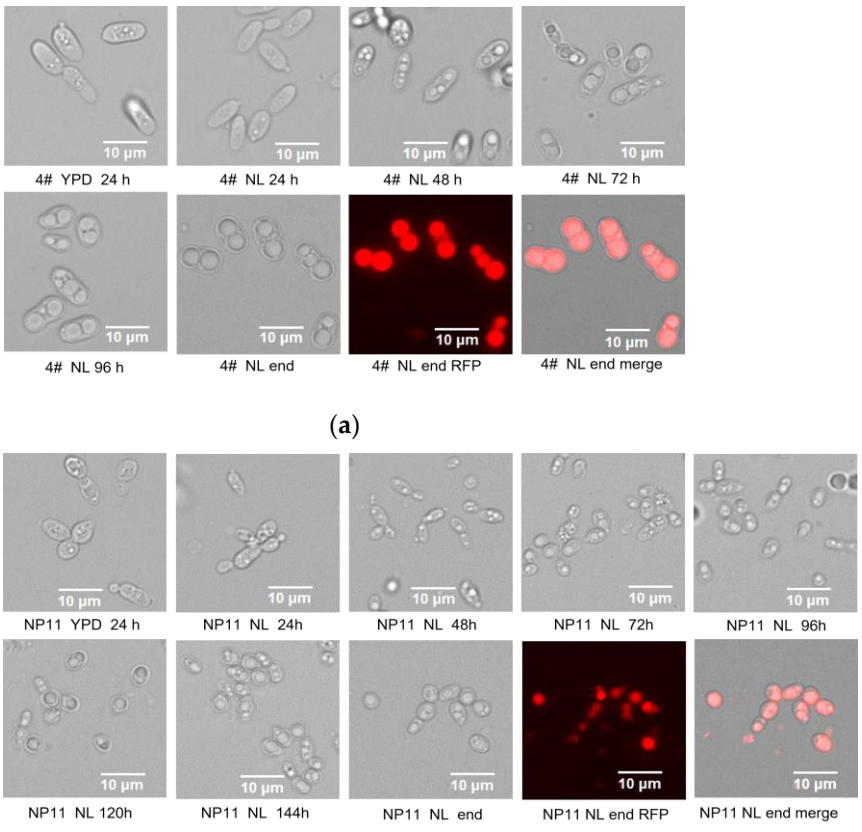
It is noteworthy to mention that there was an obvious color difference in the lipid extracts, e.g., the lipid extracts of 4# and NP11 were saffron yellow and deep red, respectively (Figure S2). Meanwhile, the culture broth colors of the two strains also showed differences after incubation for 24 h until the end of the culture (Figure S2). As is known, the red color of *R. toruloides* is mainly due to the presence of carotenoids, torularhodin and lycopene [26]. Therefore, we speculated that these two strains produced different pigments, and the reason

for the change in the phenotype of carotenoids might be verified by analyzing the genome or metabolic flow of the mevalonate (MVA) pathway in further research. This was based on the difference between the MVA pathway flux and terpenoids compounds content in these strains that could be used as the starting material for the synthesis of different terpenoids.



(**a**) (**b**)

Figure 2. Lipid production results of *R. toruloides* NP11 and 4#. (**a**) Lipid and cell mass titer, and lipid content of strain NP11 and 4#. (**b**) The comparison of lipid and cell mass yield and productivity of the two strains. *** $p < 0.001$ (p-value); ** $p < 0.01$; of the one-tailed Student's t-test. All data are presented as mean ± SD of biological triplicates.



(**a**)

(**b**)

Figure 3. Microscope photos of *R. toruloides* NP11 and 4# cells during lipid production culture. (**a**) The diploid strain *R. toruloides* 4# cells during the culture and those at the end of the culture being treated by Nile Red. (**b**) The haploid strain *R. toruloides* NP11 cells during the culture and those at the end of the culture being treated by Nile Red. NL, nitrogen-limited culture. RFP, red fluorescence module of the microscope.

3.3. Analysis of Fatty Acid Compositional Difference of Haploid and Diploid Strains

As is known, the very long-chain FAs (VLCFAs) biosynthesis enzymes are evolutionarily conserved [27], and VLCFAs such as C20 and C22 can be synthesized by *R. toruloides* [17]. Although the complete FAs compositional profile of the *R. toruloides* using glucose as a carbon feedstock is still missing, and the fatty acid compositional difference between *R. toruloides* diploid and haploid strains has never been revealed. We compared the differences of FAs profile incorporated short-chain FAs (SCFAs) C6–C8, medium-chain FAs (MCFAs) C9–C12, long-chain FAs (LCFAs) C14–C18, and unexplored VLCFAs C20–C24, including the odd-chain FAs (OCFAs, C9, C15 and C17) shown in Table 1. The results showed that both strains produced oleic acid (C18:1) most abundantly followed by palmitic acid (C16:0) and stearic acid (C18:0), which was consistent with previous reports [15,16]. The content ratio of SCFAs and MCFAs was very low; the content of these two kinds of FAs in any strain was no more than 1 wt%. These two strains produced VLCFAs including C20:0, C22:0, C23:0 and C24:0, the percentage content of VLCFAs was 2.40 wt% for the haploid strain NP11 and 1.63 wt% for the diploid strain 4#. It was found that a much longer carbon chain VLCFAs such as C22:0 and C24:0 was accumulated in the haploid strain, which might take place during a longer culture time where more substrates were converted to VLCFAs by elongation enzymes. In conclusion, *R. toruloides* tend to produce long-chain fatty acids, and the proportion of LCFAs in both strains is above 96 wt%–97 wt%.

Table 1. Fatty acid compositional profiles of haploid strain *R. toruloides* NP11 and diploid strain 4#.

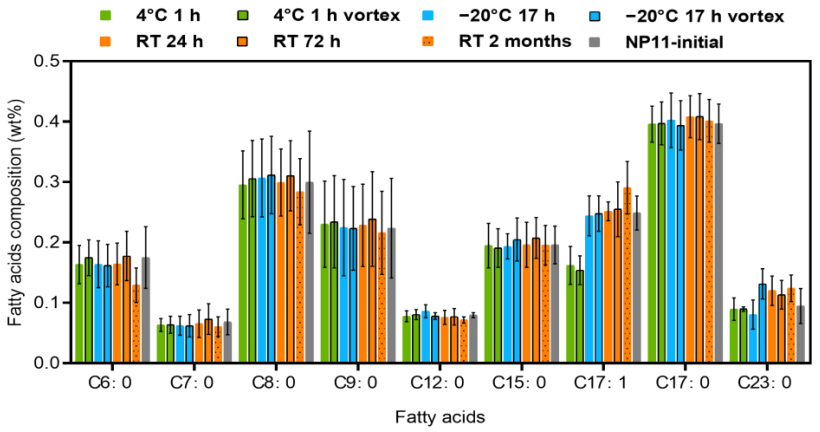
Fatty Acid Profiles (wt%)	Haploid-NP11	Diploid-4#	*p*-Value	FDR
C6:0	0.18 ± 0.05	0.06 ± 0.01	0.0609	0.1676
C7:0	0.07 ± 0.02	0.04 ± 0.00	0.1276	0.2160
C8:0	0.30 ± 0.08	0.16 ± 0.01	0.0963	0.1925
Short-chain FAs (SCFAs)	0.54 ± 0.16	0.26 ± 0.02	0.0861	0.1894
C9:0	0.22 ± 0.08	0.17 ± 0.02	0.3161	0.4636
C12:0	0.08 ± 0.00	0.13 ± 0.01	0.0001 ***	0.0029 *
Medium-chain FAs (MCFAs)	0.30 ± 0.09	0.30 ± 0.02	0.9984	0.9984
C14:0	1.85 ± 0.16	2.32 ± 0.02	0.0359 *	0.1315
C15:0	0.20 ± 0.03	0.21 ± 0.01	0.5810	0.7102
C16:1	0.56 ± 0.05	0.57 ± 0.01	0.8364	0.9200
C16:0	37.32 ± 2.53	38.11 ± 0.42	0.6231	0.7215
C17:1	0.25 ± 0.03	0.10 ± 0.01	0.0009 ***	0.0094 *
C17:0	0.40 ± 0.03	0.35 ± 0.00	0.1211	0.2219
C18:2	1.30 ± 0.73	0.42 ± 0.12	0.1679	0.2638
C18:1	40.99 ± 3.38	41.43 ± 0.62	0.8365	0.8763
C18:0	13.88 ± 0.93	14.32 ± 0.26	0.4770	0.6173
Long-chain FAs (LCFAs)	96.75 ± 7.87	97.81 ± 1.47	0.0526	0.1653
C20:0	0.50 ± 0.05	0.53 ± 0.00	0.4068	0.5594
C22:0	0.76 ± 0.04	0.54 ± 0.01	0.0010 **	0.0075 *
C23:0	0.09 ± 0.03	0.03 ± 0.00	0.0617	0.1509
C24:0	1.05 ± 0.09	0.53 ± 0.01	0.0094 **	0.0517
Verylong-chain FAs (VLCFAs)	2.40 ± 0.21	1.63 ± 0.02	0.0224 *	0.0987

p value, *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$ of two-tailed Student's *t*-test. All data are presented as mean ± SD of biological triplicates. FDR (False Discovery Rate) was the correction of *p*-value after multiple comparison; * FDR < 0.05.

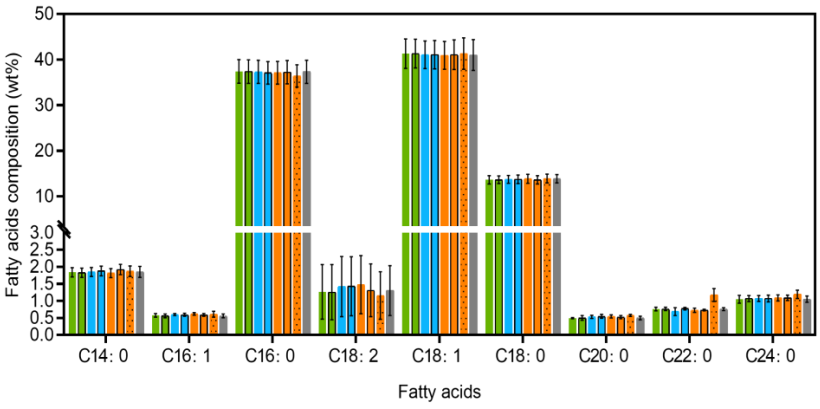
In addition, we confirmed the OCFAs, i.e., C7:0, C9:0, C15:0, C17:0 and C17:1 with secondary ion mass spectrometry by using commercial OCFAs standards (data not shown). OCFAs hold a very small proportion of *R. toruloides* lipids and are often neglected in the detection and analysis process, which was not conducive for the related genes mining and modifying the metabolic pathway. In this work, we concluded that NP11 produced 1.14% OCFAs of total fatty acids which was 0.86 wt% in 4#. The highest content of OCFAs was C17:0 in both strains, followed by C17:1 in NP11, but the diploid strain 4# had less C17:1 than that of NP11. It was assumed that the culture time of 4# would be insufficient to accumulate some special unsaturated OCFAs such as C17:1 and elongate the fatty acid chains.

3.4. Stability of FAMEs under Different Preservation Conditions

To explore the stability of fatty acid methyl esters (FAMEs) under various preservation conditions, we selected FAMEs samples prepared from lipids by *R. toruloides* NP11 as the representative for verification. Samples in triplicates were stored in three different temperature conditions, i.e., 4 °C, −20 °C, and room temperature (RT, 25 ± 1 °C), respectively. One sample in each duplicate was vortexed well before detection to confirm the effects of low-temperature crystallization of FAMEs on compositional profiles, while the other was detected directly. There was little change in the fatty acids compositional profiles of the FAMEs samples; those were stored at 4 °C for 1 h and no significant effect was observed of the vortex treatment group (Figure 4). Although C17:1 of these two groups decreased a little compared to that of NP11, it was not significant (Table S3). This suggests that storage at 4 °C for a short time does not cause FA crystallization and change but may not be good for FAMEs with C17:1. Interestingly, all the samples showed good stability at −20 °C for 17 h with no change in FAs profile regardless of samples with vortex treatment or without vortex treatment prior analysis (Figure 4). The constant appearance suggested no crystallization effects at −20 °C on FAMEs for 17 h. Moreover, we concluded that there was no change in the FAs profile after storing the samples at −20 °C for up to 26 h and two months with vortex treatment before (data not shown). As most of the suppliers of standard FAMEs generally suggest −20 °C storage, we used to keep samples at −20 °C. We were also surprised by the fact that nearly no significant change in terms of FAs composition was found after we stored the samples at room temperature for up to two months (Figure 4 and Table S3). FAMEs were stable when stored at room temperature for a long time, and thus it is unnecessary to store them at 4 °C or −20 °C.



(**a**)



(**b**)

Figure 4. Effect of storage conditions on fatty acid compositions of lipids produced by *R. toruloides* NP11. (**a**) Profiles of the trace fatty acids. (**b**) Profiles of the main fatty acids. The green column for

samples being held at 4 °C for 1 h and those with a black line box for samples with vortex treatment; the blue column for samples being kept at −20 °C for 17 h and those with a black line box for samples with vortex treatment; the orange for samples being stored at RT for 24 h, the orange with a black line box for samples being held at RT for 72 h, and those with black point background for samples being held at RT for 2 months; the grey column for as-prepared samples without storage. RT, room temperature (25 ± 1 °C). All data are presented as a mean ± SD of biological triplicates. *p*-value of two-tailed Student's *t*-test and FDR were shown in Table S3.

4. Discussion

Many studies demonstrated *R. toruloides* as a promising platform for lipid production owing to its diverse substrate appetites, robust stress resistance, and other favorable features. In recent years, significant progress has been made in genome sequencing [12,13], multi-omics analysis [14], and genome-scale modeling [28]. Moreover, several molecular and genetic tools for engineering *R. toruloides* were previously developed, including gene transformation [29,30], and an RNA interference method as well as a CRISPR Cas9 genome editing system [23,31]. Meanwhile, the development of genetic components (promoters, markers, terminators, etc.) and transformation methods have paved the way towards refined genomic and metabolic engineering regulation.

In addition, it is necessary to understand the endogenous metabolic pathways and related metabolites. There have been numerous basic studies on lipid synthesis and fatty acid metabolisms, such as lipid droplets and fatty acid synthases (FASs) of *R. toruloides* [32,33]. However, we did not know exactly the fatty acid composition of *R. toruloides* as a potential chassis microorganism, especially the differences between the haploid and diploid strains, which made it difficult to choose the starting strains for engineering transformation. So, in this work, we compared the difference in fatty acid compositions between the haploid strain NP11 and the diploid strain 4#, by incorporation of the usual LCFAs C14 to C18, and unusual VLCFAs C20 to C24 and OCFAs C15 to C17, as well as SCFAs C6 to C8. Our results showed the possibility of using *R. toruloides* NP11 as the parent strain for metabolic engineering to produce special fatty acids (OCFAs and VLCFAs) and high-value fatty acids. Moreover, it is possible to hybridize it with a mating A2 strain into the diploid strain to improve production without affecting the composition of the fatty acids. Hence, the advantages of the diploid strain in growth and lipid production in large-scale culture could be utilized well, while the difficulty of genetic editing of diploid strains was also avoided.

Firstly, different cell growth and sugar consumption rates were achieved for both strains during the lipid production process (Figure 1). The diploid strain 4# grew faster with a comparatively short lag phase, and its cell density rapidly increased after 24 h during the culture. Nevertheless, the haploid strain NP11 needs more time to achieve the same level of cell density. Although the cell density of NP11 was a little higher than that of 4# in the end, its dry cell mass weight was lower than that of 4#. Similarly, significant differences were found in the growth and lipid production capacity between both strains, which could be caused by genes involved in fatty acid synthesis being formed of two copies of the diploid strain, where it has only one copy in the haploid strain. Nevertheless, NP11 is a promising oil-producing, broadly studied and well-annotated *R. toruloides* haploid strain; it showed potential applications in the genetic manipulation of biosynthesis.

Secondly, there were no significant differences in major fatty acid compositional profiles between the diploid and haploid strains. Prolonged culture time may be beneficial to the accumulation of special fatty acids such as VLCFAs (C22:0 and C24:0) and OCFAs (C17:1), or the enzymes involved in the synthesis of VLCFAs and OCFAs in *R. toruloides* were less active and thus need longer culture time; these assumptions could also be demonstrated in future work.

Lastly, we demonstrated that FAMEs are in fact very stable at room temperature. Thus, FAMEs samples can be stored normally and problems in terms of quantitative

analysis associated with cryopreservation may be avoided. These findings provide sample preservation guidelines for FAs analysis as well as a method to decipher the properties of FAMEs of oleaginous yeasts. Moreover, this study showed comparison data in terms of cell growth, lipid production, and the FA composition of *R. toruloides* haploid and diploid strains. The information is valuable to explore *R. toruloides* as a host for the construction of an advanced cell factory.

5. Conclusions

In summary, the diploid strain *R. toruloides* 4# could afford higher yields of cell mass and lipid than the haploid strain NP11. There were noticeable differences in the proportion of very few fatty acids in lipids from *R. toruloides* haploid and diploid strains; however, overall FAs compositions were quite similar. Importantly, FAMEs were found to be stable under varied storage conditions to ensure the reliable estimation of fatty acid compositions of microbial lipids.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fermentation8090467/s1, Figure S1. The growth curves fitting of *R. toruloides* NP11 and *R. toruloides* 4# in nitrogen-limited cultivation. Figure S2. The lipid extract and culture broth of the haploid strain *R. toruloides* NP11 and the diploid strain 4#. Table S1. *p*-value of OD_{600} and residual glucose comparation between the haploid strain *R. toruloides* NP11 and the diploid strain 4# at different time by statistical analysis. Table S2. *p*-value of some strain parameters comparison between the haploid strain *R. toruloides* NP11 and the diploid strain *R. toruloides* 4# by statistical analysis. Table S3. *p*-value and FDR of FAMEs composition of NP11 under different preservation conditions compared by statistical analysis. Reference [34] is cited in the supplementary materials.

Author Contributions: Z.K.Z. conceived the research; Q.W. and Y.Z. designed and conducted experiments; Q.L. contributed to the GC-MS method in analyzing fatty acids; R.K. proposed and contributed to the fatty acid samples stability part; Y.Z., Q.W. and X.Y. analyzed data; Y.Z., R.K., Q.W. and Z.K.Z. wrote and revised the manuscript. All authors have read and agreed to the published version of the manuscript.

Funding: This study was funded by the National Natural Science Foundation of China (31870042).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

Acknowledgments: We thank the National Natural Science Foundation of China (31870042) for the financial support. We thank Qi Wang for helpful discussion in writing the paper.

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

Nomenclature

FAs	Fatty acids
SCFAs	Short-chain fatty acids
MCFAs	Medium-chain fatty acids
LCFAs	Long-chain fatty acids
VLCFAs	Very long-chain fatty acids
OCFAs	Odd-chain fatty acids
FAMEs	Fatty acid methyl esters

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