

Alpha-Linolenic Acid Production by the Mutant *Rhodospodium toruloides*

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Abstract— α -Linolenic Acid (ALA, C18:3) that is an essential Polyunsaturated Fatty Acid (PUFA) plays a vital role in many metabolic processes. The oleaginous yeast *Rhodospodium toruloides* has generally been used for triacylglycerol production, including PUFAs. In this work, α -linolenic acid, not its γ - isomer, is firstly identified to be synthesized by *R. toruloides* and the mutant M18 that was obtained through plasma mutagenesis in the previous studies. The effects of five different nitrogen sources and four C/N ratios on the biomass, total lipid and ALA production from *R. toruloides* and M18 were studied. It was found that the highest biomass and lipid production were achieved when tryptone used as nitrogen with C/N ratio 100:1. In fed-batch cultivation, M18 has the highest ALA accumulation of 5.33% of total lipid, compared to 3.67% in the wild type strain. M18 has a promising potential in ALA production and commercial application of microbial PUFAs.

Index Terms— α -Linolenic Acid (ALA), C/N ratio, nitrogen sources, *R. toruloides*

I. INTRODUCTION

Alpha-Linolenic Acid (ALA, C18:3) is an essential ω -3 (cis Δ 9, 12, 15) Polyunsaturated Fatty Acid (PUFA). It is needed for normal human growth and development as well as beneficial effects and functions on human health and immunity regarding cardiovascular disease, arrhythmia, systemic immune modulation, anti-inflammatory and neuro-protective properties [1]. Acting as the precursor of eicosapentaenoic acid (EPA, C20:5) and Docosahexaenoic Acid (DHA, C22:6), ALA can be transformed into EPA and DHA through a series of reactions including elongation and desaturation in mammals. EPA and DHA have been identified as crucial compounds in the structure and function of cellular membranes and wide range of physiological and clinical effects [2], [3]. ALA must be obtained from food sources due to it cannot be synthesized in the human body. It is found in walnuts, meat and dairy products as well as vegetable oils such as flaxseed oil, rapeseed oil or soybean oil [3]. The rising demand and high cost of ALA extraction from edible plant oils have encouraged rapid development of PUFAs from other resources such as

microbial oil. Oleaginous microbes have attracted increasing attention because they can convert various substrates such as carbon dioxide, sugars, and organic acids to triacylglycerol (TAG), and multiple advantages including its high intracellular value-added fatty acids (e.g. PUFAs) accumulation, less labor required, reduced sensitivity to season, climate, and scaling-up facilitation [4], [5].

The oleaginous yeast *Rhodospodium sp.* has generally been used for triacylglycerol production, among the species, *Rhodospodium toruloides* is one of the high-yield TAG producers and considered for potential applications in the biodiesel production [6]. In our previous work, a hydrolyzate-resistant mutant strain M18 was obtained using Atmospheric Room Temperature Plasma (ARTP) mutagenesis [5]. This mutant M18 strain exhibited strong tolerance for almost all the inhibitory compounds in lignocellulosic hydrolyzate and had considerable fatty acid productivity, which make it of promising potential use in the biodiesel industry [5].

Actually PUFAs (i.e. oleic acid, linoleic acid and linolenic acid) of *R. toruloides* account for more than 50% of total fatty acids [6], and it is significant for these PUFAs to function as nutrition rather than serving as the substrates for biodiesel production. Among the lipids derived from *R. toruloides*, linolenic acid (C18:3) was the most valuable PUFA. However, to the best of the knowledge, there is few report to make it clear that which isomer (α - or γ -) of the linolenic acid in *R. toruloides* is. γ -linolenate (C18: 6,9,12) differs from α -linolenate (C18: 9,12,15) in the positions of the double bonds. In the present study, it is firstly identified that α -linolenic acid was synthesized by *R. toruloides*. Then the two *R. toruloides* strains of wild type and the mutant M18 have been utilized for total lipid and ALA production under different culture conditions, and optimized in fed-batch fermentation.

II. MATERIALS AND METHODS

A. Strain and Culture Conditions

The oleaginous *R. toruloides* mutant M18 strain obtained through the Atmospheric Room Temperature Plasma (ARTP) mutagenesis method has high tolerance to the inhibitors in lignocellulosic hydrolyzates [5]. *R.*

toruloides ACCC 20341 was used as the wild type strain and both of the two strains are haploid strains. *R. toruloides* were grown in YEPD liquid medium (yeast extract 10g/L, glucose 20g/L, peptone 20g/L) at pH 6.0, 28-30 °C, and 200 rpm in flasks.

B. Lipid Production

A two-stage culture strategy was utilized for studying the biomass and lipid production of the mutant M18 under different culture conditions in 500mL flask. Firstly, inoculum was grown in YEPD medium at pH 6.4, 30 °C, and 200 rpm in an air-bath shaker until the cell density (OD_{600}) was higher than 10. Then the yeast cells were centrifuged and harvested for lipid production in the nitrogen-limited liquid medium. Effects of nitrogen source and C/N ratio on the lipid and ALA production from *R. toruloides* have been studied. Organic nitrogen compounds (glutamic acid, tryptone and urea) and inorganic nitrogen compounds (NH_4Cl and $(NH_4)_2SO_4$) were used as the nitrogen source at four different initial C/N ratios: 50:1, 100:1, 200:1 and 400:1 for lipid production. 40g/L glucose was added as the initial concentration of carbon sources in the nitrogen-limited liquid medium.

C. Crude Lipid Extraction and Fatty Acid Analysis

The crude lipid was obtained using an acid-heating procedure [7]. After cultured in the nitrogen-limited medium for 5 days, the mutant M18 and wild type cells under different conditions were harvested, washed with deionized water, re-suspended 10mL/g of wet cell weight in 4moL/L HCl solution, and then heated at 78 °C for 2h in water bath. The mixed solvent (methanol: chloroform = 1: 1) was supplemented, and centrifuged at 5000 rpm for 20 min. After that, the chloroform layer was separated, and equivalent mass of 0.1% NaCl was added and centrifuged at 5000 rpm for 3min. Then chloroform layer was collected again and chloroform was removed by rotary evaporation. The lipid productivity was calculated as weight of triacylglycerol per Dry Cell Weight (DCW). To determine the fatty acid compositions, the free fatty acid sodium salts have to be converted to fatty acid methyl esters (FAME) by incubation with sulfuric acid, methanol and methanol (2:20:1) at 35 °C for 30 min, and examined by gas chromatograph mass spectrometer (GC-MS). The methyl α - and γ -linolenic acids used as the linolenic acid standards for identification were bought from Sigma-Aldrich (St. Louis, US).

D. Fed-Batch Fermentation

Fed-batch fermentation was performed at 30 °C in a 15-L bioreactor (Bailun Bio-Tech, Shanghai, China) with an initial culture volume of 8 L. 5% (v/v) of the inoculation was carried out in the initial medium including 3% glucose, 0.5% yeast extract, 1% tryptone, $(NH_4)_2SO_4$ 0.01%, KH_2PO_4 0.04% and $MgSO_4$ 0.15%. Dissolved oxygen level was maintained above 30% of saturated dissolved oxygen concentration by automatically control of agitation rate at 200-400 rpm (aeration rate 3-6 L/min). The pH value of medium was adjusted and maintained at 6.2 with addition of 25% NH_4OH . The feeding medium of 40% glucose and 0.33%

tryptone was pumped into the bioreactor to maintain the C/N ratio about 200:1 after 20h culture with the initial medium. The chemicals used in this study are all analytically pure grade and purchased from Beijing Chemical Works (Beijing, China), while the reagents including glutamic acid and urea were from Amresco (Solon, OH, US).

E. Analysis Methods

The Agilent GC-MS (5975C series of mass selective detector) equipped with a CP-FFAP CB capillary column (25m \times 0.32m \times 0.30 μ m) was employed for analysis of the FAME obtained from *R. toruloides* M18 and the wild type strains as described in the previous works.⁵ Heptadecanoic acid methyl ester was used as an internal standard. The column temperature was maintained at 180 °C for 0.5 min and heated to 290 °C at 10 °C/min for 6min. Mass spectra were set in full scan mode (m/z 40-440) for selecting the universal fragment ions of m/z 74, 79, 81 and 87 with the ion source temperature set at 230 °C. The results of spectra were recorded 5 scans per second with the ionization energy at 70 eV after a solvent delay for 4 min. After that, the results of FAMES were obtained and identified with the Agilent GC/MSD ChemStation software.

III. RESULTS AND DISCUSSIONS

A. Identification of ALA from the *R. toruloides* and M18

The total FAME extracted from *R. toruloides* and the mutant strain M18 were analyzed by GC/MS (Fig. 1). The essential composition of FAME from *R. toruloides* including palmitic acid, stearic acid, oleic acid and linoleic acid has been reported before [5], [6], [8], [9]. The isomer type of linolenic acid was not classified probably because linolenic acid has always been treated as minor composition for biodiesel production in the crude lipids. In this work, it is proved that α -linolenic acid, not the other isomer (γ -), could be produced by both of *R. toruloides* and the mutant M18. However the content of ALA in the both strains was less than 4%, thus the improvement of ALA accumulation was carried out using optimization of cultivation conditions such as nitrogen sources and C/N ratios, coupled with fed-batch fermentation.

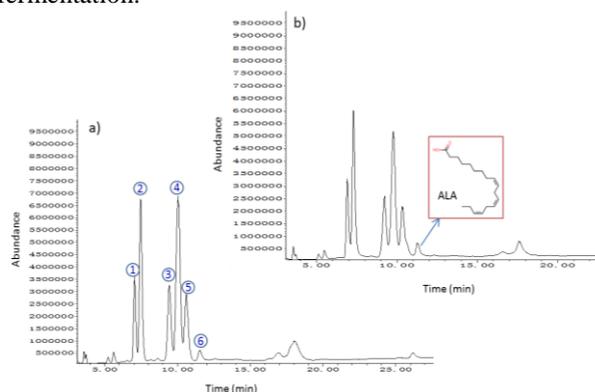


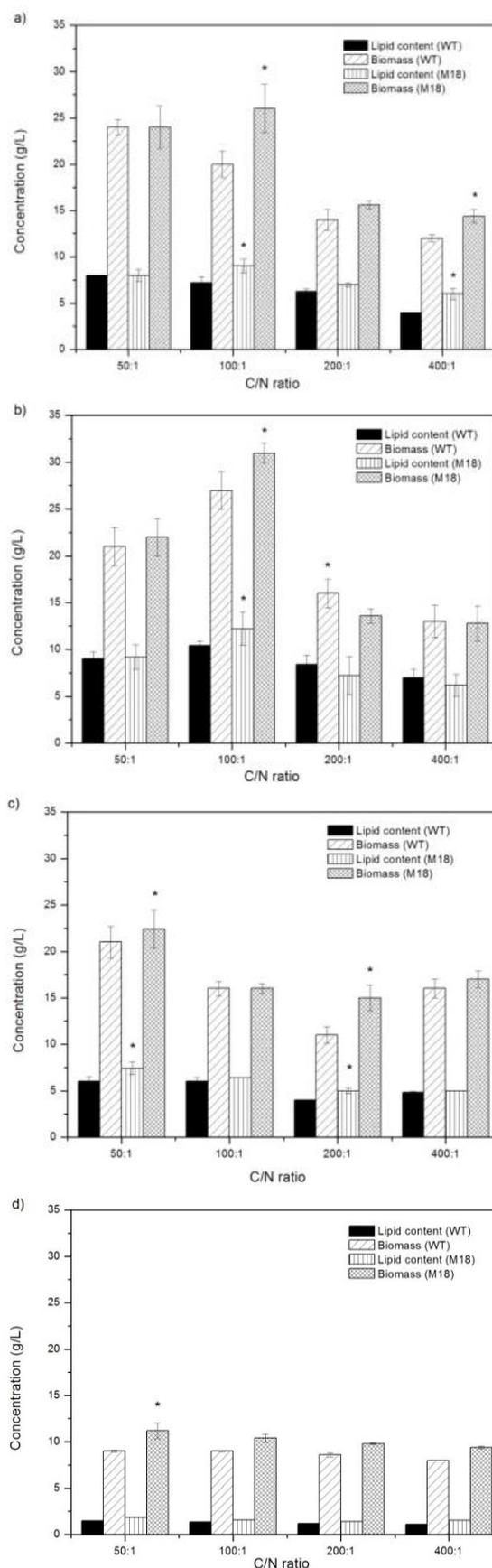
Figure 1. Identification of the total FAME contents obtained from *R. toruloides* (a) and the mutant strain M18 (b) by GC/MS. The FAME peaks: 1, 2, palmitic acid; 3, stearic acid; 4, oleic acid; 5, linoleic acid; 6, α -linolenic acid.

B. Biomass and Lipid Accumulation of the *R. toruloides* and M18

Effects of five different nitrogen sources (i.e. glutamic acid, tryptone, urea, NH_4Cl and $(\text{NH}_4)_2\text{SO}_4$) and four C/N ratios (i.e. 50:1, 100:1, 200:1 and 400:1) on the biomass and total lipid production from *R. toruloides* and the mutant M18 have been studied (Fig. 2). M18 and the wild type could accumulate much higher (2-3 times) biomass and total lipid as grown on the organic nitrogen than inorganic nitrogen medium. Compared to the wild type, M18 have the highest biomass (27.4g/L) and lipid (12.2g/L) accumulation using tryptone as the sole nitrogen source. Furthermore, it is found that tryptone is better for biomass and lipid production than the other nitrogen sources. The effects of nitrogen source on biomass and lipid accumulation profiles had been studied and the results varied with the yeast species [10], [11], but for the most yeast species in the research of Evans and Ratledge [10], little difference was found in lipid content among those yeasts grown with nitrogen source of asparagine, glutamate and ammonium chloride, respectively. In addition, they also observed that *Rhodospiridium toruloides* had much higher lipid content when grown on organic nitrogen than inorganic nitrogen, which was consistent with the results of this work. It is also indicated that tryptone could be the optimal nitrogen for biomass and lipid accumulation in yeast species of *R. toruloides*.

For many oleaginous yeasts species, the C/N ratio of the growth medium plays a crucial role on lipid metabolic pathway and production [4], [12]. In this work, the effects of four different initial C/N ratios (i.e. 50:1, 100:1, 200:1 and 400:1) on biomass and lipid production of *R. toruloides* wild type strain and the mutant M18 had been determined using a two-stage culture strategy. Fig. 2 shows that C/N ratio clearly influenced biomass and the total lipid yield of the both strains when grown in the medium containing organic nitrogen. For the mutant M18 strain, the relative highest lipid production could be achieved with C/N ratio of 100:1 in the presence of glutamic acid or tryptone, while for the wild type strain, the optimal C/N ratio is 50:1 when glutamic acid or urea used as nitrogen source. The biomass and lipid yield shared the similar patterns at different C/N ratios when the ammonium sulphate and ammonium chloride were used as nitrogen source (Fig. 2). C/N ratio about 100 has been considered as ideal for lipid production in the oleaginous yeast species and recommended for fed-batch process [12]-[14]. Thus the results of lipid accumulation of both strains were in accordance with the typical reports. However the optimal C/N ratio of the *R. toruloides* and M18 strains was very different when grown on urea (50:1), indicating that some kinds of key enzymes of the two *R. toruloides* strains related to lipid synthesis regulation or trigger triglyceride accumulation probably could be inactive or down-regulated at transcriptional or expressional levels when urea used as the sole nitrogen source. In fact, a minimum C/N ratio of 20 is proved to be sufficient for triggering lipid synthesis [15] and too high C/N was detrimental to biomass and lipid

accumulation [9], which explained the significant reduced biomass and lipid production as the C/N ratio increased to 200:1 and 400:1.



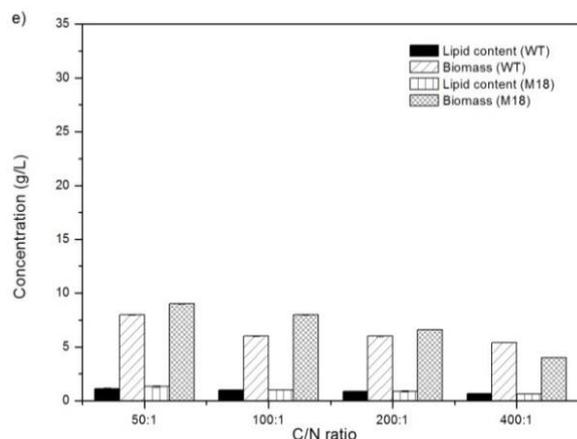


Figure 2. Effects of five different nitrogen sources (a. glutamic acid, b. tryptone, c. urea, d. $(\text{NH}_4)_2\text{SO}_4$, e. NH_4Cl) and four C/N ratios (i.e. 50:1, 100:1, 200:1 and 400:1) on the biomass and total lipid production from wild type *R. toruloides* and the mutant M18.

C. ALA Production of the *R. toruloides* and M18

When the two strains grew on inorganic nitrogen, biomass and lipid yield were much less and little ALA could be found in the total lipid. Therefore glutamic acid, tryptone and urea were employed for studying the specific ALA production. Fig. 3 illustrated the ALA content in the total lipid in the presence of three organic nitrogen sources at four C/N ratios. From the results, it is found that M18 has higher ALA content than the wild type at different C/N ratios and using different nitrogen sources. When tryptone was used as the sole nitrogen source, C/N ratio of 200:1 is optimal for ALA yield (wild type: 2.92% and M18: 4.55%). However the highest ALA content of 5.05% was obtained by M18 as glutamic acid used as nitrogen source at C/N ratio of 400:1. It seems that M18 had a promising potential in ALA production for industrial application.

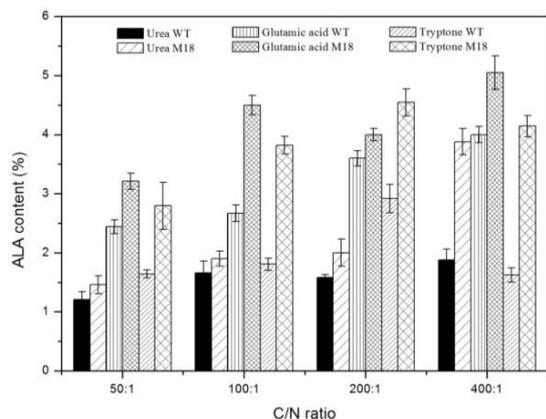


Figure 3. ALA content in the total lipid from both wild type *R. toruloides* and the mutant M18 in the presence of three organic nitrogen sources (glutamic acid, tryptone, and urea) with four C/N ratios (50:1, 100:1, 200:1 and 400:1).

It is interesting to notice that glutamic acid was favorable for ALA synthesis, although tryptone was proved better for biomass and lipid production than the other nitrogen sources. In fact, glutamic acid metabolic pathway was not only involved in nitrogen metabolic process but a key molecule in fatty acid synthesis and

cellular metabolism [16]. The result indicated that glutamic acid or glutamate metabolic process might promote formation of unsaturated fatty acids. On the other hand, C/N ratio is another important factor that causes more ALA accumulation in both *R. toruloides* strains. Mondala *et al.* found that increasing levels of the saturated fatty acids, such as C16:0 and C18:0, were achieved with increasing initial C/N ratios [17]. And it was considered that low C/N ratios would lead to low saturated fatty acids accumulation and an increased content of unsaturated fatty acids [12], [17]. However, these reports are not in agreements with the results of Fig. 3. In this study, both of the two strains *R. toruloides* and M18 had enhanced production of ALA as C/N ratios increased from 50:1 to 400:1, especially glutamic acid was used as nitrogen source. Thus it seemed that the *R. toruloides* is an exception since the effects of nitrogen source and C/N ratio on the synthesis of unsaturated fatty acid are much different with other lipid-producing microorganisms. Actually it is still difficult to give the accurate connections between C/N conditions and the proportions of saturated and unsaturated fatty acid [12].

D. Fed-Batch Fermentation for ALA Production

Fed-batch strategy was employed for enhancing total lipid and ALA production with the *R. toruloides* and M18 strains using tryptone as nitrogen source with C/N ratio of 200:1. After a batch phase of 20h, fed-batch cultivation was conducted using constant feeding of glucose and tryptone. The biomass and lipid yield of wild type *R. toruloides* have reached 49.8 and 21.3g/L, respectively, while the mutant M18 has higher production of 53.4 and 25.6g/L after 160h cultivation (Fig. 4). ALA from the total lipid could be detected after 80h and 72h in the wild type *R. toruloides* and M18, respectively. M18 has the higher ALA accumulation of 5.33% of total lipid, compared to 3.67% of lipid in the wild type strain. Table I shows the comparison of lipid and linolenic acid production by several oleaginous *Rhodospiridium sp.* and *Rhodotorula sp.* strains.

The final yields of biomass and lipid from the two strains were not very high compared with some similar studies. For example, Zhao's group utilized fed-batch culture strategy with high-density cultivation of *Rhodospiridium toruloides* Y4, and they obtained 106.5g/L of dry cell biomass and 67.5% (w/w) of lipid content [6]. Since this work was designed to improve the production of ALA using fed-batch fermentation, the high-density strategy for increasing biomass and lipid accumulation hadn't been utilized in this study. When grew with C/N ratio of 100:1 in flask culture, M18 has two times more biomass than that of C/N ratio 200:1 according to Fig. 2, but 200:1 has been proved to be the optimal C/N ratio for ALA accumulation with tryptone used as the sole nitrogen resource. In addition, lipid content in *R. toruloides* could also be changed by different carbon sources. Wiebe *et al.* found that arabinose and/or xylose in the medium would increase the proportion of palmitic and linoleic acid, while reduced the proportion of oleic acid in the fatty acids compared to the cells grew in medium containing glucose

[18]. Thus the further study for optimization of ALA accumulation might focus on carbon sources, although lipid content has been considered more dependent on nitrogen source.

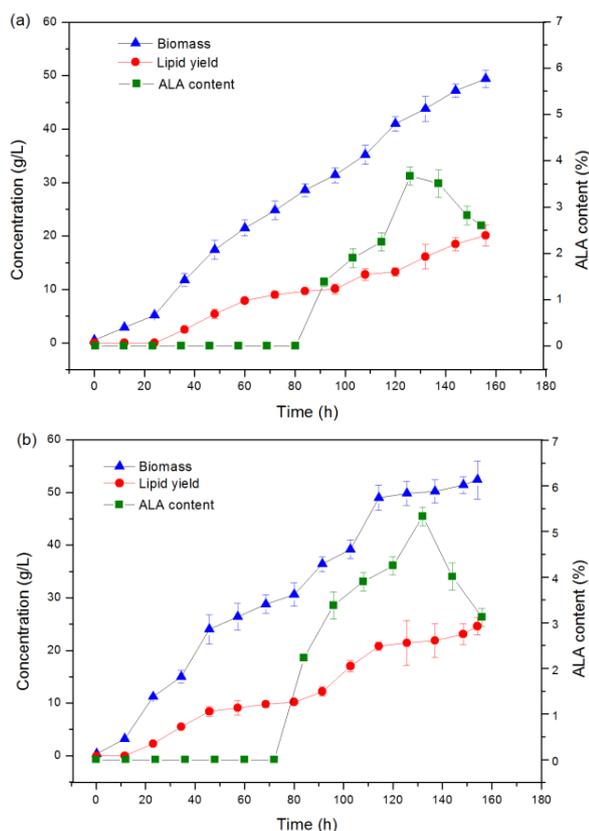


Figure 4. Biomass, total lipid and ALA production of wild type *R. toruloides* (a) and the mutant M18 (b) in fed-batch cultivation using tryptone as the sole nitrogen source with the optimal C/N ratio of 200:1.

ALA can be detected after 70h in fed-batch process, which is similar with Gema's studies [19]. The results confirmed the hypothesis that the rate of linolenic acid biosynthesis was much lower than the rate of total lipid accumulation [19]. ALA accumulation further increased as lipid synthesis had been completed, indicating that the $\Delta 12$ and $\Delta 15$ -dehydrogenase were activated late. Furthermore it was observed that ALA production began to decrease from 120h and 132h of wild type strain and

the mutant M18, respectively. From the Fig. 5, yield of linoleic acid rapidly increased from 108 h, suggesting that part of synthesized ALA was transformed into linoleic acid by reduction in the process due to the triggered or improved reverse reaction or "side-reactions" of the $\Delta 15$ -desaturase [20]. Another explanation could be that ALA biosynthetic pathway from the saturated stearic acid seemed to be inhibited at the level of $\Delta 15$ -dehydrogenase after ALA accumulation reached a certain concentration, otherwise high ALA concentration would also suppressed enzymatic activity of $\Delta 12$, $\Delta 15$ -dehydrogenase because it was found the oleic acid concentration was considerably increased near the end of fermentation (Fig. 5).

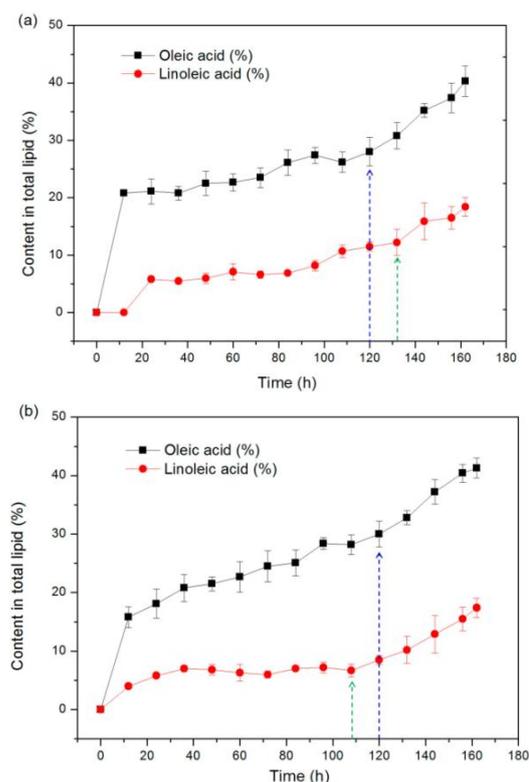


Figure 5. The oleic acid and linoleic acid production of wild type *R. toruloides* (a) and the mutant M18 (b) in fed-batch cultivation using tryptone as the sole nitrogen source with the optimal C/N ratio of 200:1. Accumulation of oleic acid and linoleic acid yield were considerably increased from 108-132 h near the end of fermentation.

TABLE I. COMPARISON OF LIPID AND LINOLENIC ACID PRODUCTION BY SEVERAL OLEAGINOUS RHODOSPORIDIUM SP.

<i>Rhodospiridium sp.</i>	Lipid content (%)	ALA content (%)	Culture methods	References
<i>Rhodospiridium toruloides</i> Y4	67.5	3.5	Fed-batch cultivation	Li <i>et al.</i> 2007 ^[6]
<i>Rhodospiridium toruloides</i> Y4	61.8	2.6	Fed-batch cultivation	Zhao <i>et al.</i> 2011 ^[21]
<i>Rhodotorula minuta</i>	48.0	nd*	Batch & fed- batch cultivation	Saxena <i>et al.</i> 2008 ^[22]
<i>Rhodotorula glutinis</i>	40.0	nd	Fed-batch cultivation	Pan <i>et al.</i> 1986 ^[23]
<i>Rhodotorula gracilis</i> CFR-1	58.5-65.0	3.1	Batch cultivation	Jacob <i>et al.</i> 1990 ^[24]
<i>Rhodospiridium toruloides</i> ACCC 20341	50.1	5.3	Fed-batch cultivation	This work

nd*: not detected

IV. CONCLUSION

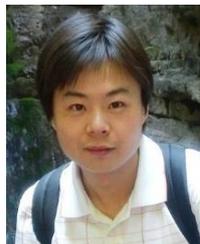
In this work, α -linolenic acid, not its γ - isomer, is firstly identified to be synthesized by *R. toruloides* and the mutant M18. Nitrogen sources and C/N ratios have crucial influences on the biomass and lipid production in both *R. toruloides* strains, and the optimal culture condition for ALA accumulation was studied. Then fed-batch fermentation was employed for improving ALA production with the *R. toruloides* wild type and M18 strains in the optimal culture conditions. The results indicated that the mutant M18 has a promising potential in ALA production and commercial application of microbial PUFAs.

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