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Research Article

Bioconversion of glycerol into lipids by *Rhodospiridium toruloides* in a two-stage process and characterization of lipid properties

Rhodospiridium toruloides AS 2.1389 has been considered a promising oleaginous strain due to its flexible substrate adaptability, high lipid content, and co-production of some pigments. In previous work, *R. toruloides* has shown good potential to directly convert crude glycerol into intracellular lipids. However, the difference in nutritional demand between cell growth and lipid accumulation was found to be a dilemma. Therefore, a glycerol-based two-stage process was proposed in the present work to separately meet the nutritional demand of both the cell proliferation phase and lipid accumulation phase. Factors that affect microbial conversion of glycerol into lipid were investigated, statistically analyzed, and optimized. As a result, 26.5 g L⁻¹ biomass with 10 g L⁻¹ lipid was obtained in the two-stage process. Lipid yield (0.20 g g⁻¹) and productivity (0.083 g L⁻¹ h⁻¹) achieved were significantly higher than the previously optimized batch culture. In *R. toruloides* lipids, the dominant fatty acid compositions are palmitic acid (28.5%), stearic acid (12.9%), oleic acid (41.3%), and linoleic acid (12.8%). Phospholipids accounts for 0.63% in total lipid. Lipase-catalyzed methanolysis could achieve up to 95% biodiesel yield. The characterization of *R. toruloides* lipid suggests its great application potential for biodiesel and specialty-type lipid products.

Keywords: Biodiesel / Fermentation / Glycerol / Microbial lipid / *Rhodospiridium toruloides*

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1 Introduction

Due to the energy crisis and environmental concerns, biodiesel has garnered much interest in recent decades as a substitution for petroleum diesel. Many countries have mandates to increase main rates of biodiesel blends in transportation fuels most notably USA, Brazil, and EU, which becomes a key policy driver of biodiesel consumption. It was estimated that the consumption of first-generation biodiesel, made from agricultural feedstocks, will have a significant increase in the next decade. The implication of first-generation biodiesel for the environment and food prices is indeed worrisome [1]. Microbial oil, as an

alternative nonfood oil feedstock, has thus received much attention, which can be synthesized and accumulated intracellularly with similar fatty acid compositions to that of vegetable oils. Microbial oil production has several advantages such as short life cycle, less need of land use, and adaptabilities to various renewable resources including but not limited to lignocellulosic biomass as well as industrial, municipal, and agricultural wastes [2].

Rhodospiridium toruloides is considered a potential lipid producer due to its excellent capabilities of lipid accumulation, broad adaptabilities to various carbon substrates, and coproduction of some carotenoids [2,3]. According to our previous study, it can convert a number of substrates, such as glucose, xylose, glycerol, lignocellulosic hydrolysate, and even excess sludge hydrolysate (excess sludge is generated from the activated sludge process, the widely applied waste-water treatment [4]) into triacylglycerols [3, 5, 6]. *Rhodospiridium toruloides* can

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Abbreviations: SL, sodium lignosulphonate

well convert glycerol into lipids, suggesting a great potential to utilize biodiesel byproduct glycerol, a sustainable and inexpensive carbon source, to produce microbial oil, which, in turn, can be used as the oil feedstock for biodiesel preparation.

In order to produce microbial oils in a more effective way, much effort has been focused on directing carbon fluxes toward lipid synthesis either through process optimization or metabolic engineering. Some general conclusions have been drawn from microbial conversion of glucose into lipids. It showed that lipid accumulation in oleaginous microorganisms does not occur simultaneously with cell growth/proliferation due to their differences in nutrition requirements [7]. Normally, lipid accumulation in *R. toruloides* was achieved by over-feed of glucose. Once the nitrogen concentration is reduced to certain extent, the majority of glucose flow into lipid synthesis pathway with lipid bodies formed intracellularly [8]. To achieve higher lipid productivity, separating cell proliferation and lipid accumulation by applying a two-stage process was studied by some researchers. Cells were cultivated in nutrient-rich medium for proliferation, and resuspended in carbon-rich medium without auxiliary nutrients for lipid accumulation. The usefulness of two-stage process for conversion of glucose and lignocellulose-derived sugars into lipids were demonstrated in *R. toruloides* Y4 [9], *Lipomyces starkeyi* [10], and *Cryptococcus curvatus* [11]. However, there is a lack of research on the behavior of *R. toruloides* in converting glycerol into lipids. It is known that glycerol goes through glycolysis pathway where it is converted into glyceraldehyde-3-phosphate, transformed into phosphoenolpyruvate, and finally into pyruvate. Pyruvate may enter the mitochondria and is converted into acetyl-CoA, which enters the citric acid cycle. Acetyl-CoA is used as the precursor of fatty acid and carotenoid synthesis pathway [12]. In silico analysis of triacylglycerol production on different substrates (glucose, glycerol, xylose and arabinose) indicated that glycerol could be a desirable substrate in terms of carbon utilization efficiency [13], which implied its great potential for lipid production. Therefore, the objective of the present work was to investigate the behaviors of *R. toruloides* in cell growth and lipid accumulation by using glycerol as the sole carbon source, and establish a two-stage process accordingly. Effects of some key factors in lipid accumulation were investigated and optimized. Finally, the lipid obtained was characterized for its potential utilizations.

2 Materials and methods

2.1 Microorganism and chemicals

Rhodospiridium toruloides AS 2.1389 was obtained from General Microbiological Culture Collection Center (Beijing, China). For preparing inocula, it was precultured overnight in 250 mL Erlenmeyer flasks loaded with 50 mL yeast extract-peptone-glycerol (YPG) medium at 30°C and 200 rpm in an air-bath shaker. The YPG medium contains 15 g L⁻¹ glycerol, 10 g L⁻¹ yeast extract, and 10 g L⁻¹ peptone.

Lipases Novozyme 435 and Lipozyme TL were provided by Novozymes Co. Ltd. (Beijing, China), whose activities are 4217 and 760 U, respectively. (1 unit (U) is the amount of enzyme that catalyzes the hydrolysis of glycerol tributyrates and pro-

duces 1 μ mol butyric acid at 40°C.) Reference standards for chromatographic analysis were chromatographically pure and were purchased from Sigma-Aldrich Corporation (Shanghai, China). Sodium lignosulphonate (SL) was purchased from Tokyo Chemical Industry Co., Ltd. (Shanghai, China), containing 5.0–7.0% methoxyl group. The soybean oils were purchased from COFCO Co. Ltd. (Beijing, China). All other chemicals were purchased locally and were analytically pure.

2.2 Fermentation conditions

The behavior of *R. toruloides* was investigated under different conditions. Under nitrogen abundant condition, seed cells were precultured overnight and inoculated into YPG medium with a starting biomass of 0.1 g L⁻¹. Under nitrogen limited condition, seed cells were precultured overnight and inoculated into the medium containing 50 g L⁻¹ glycerol, 0.1 g L⁻¹ (NH₄)₂SO₄, and 0.75 g L⁻¹ yeast extract, with a starting biomass of 1.0 g L⁻¹.

For the two-stage fermentation, in the first stage, yeast was cultivated 36–60 h in 500 mL Erlenmeyer flasks with 100 mL YPG medium at 30°C and 200 rpm in an air-bath shaker. For the second stage culture, cell inocula were collected from the first-stage by spinning down in centrifuges. Extra media was discarded. The biomass was transferred to 500 mL Erlenmeyer flasks and resuspended in 100 mL glycerol solution. Concentrations of glycerol and other supplements were decided according to the specific experiment design as described in Section 3. Each culture was performed by duplicate test. SDs were used for data descriptions.

2.3 Analytical methods

Glycerol concentration was determined by a Shimadzu 10AVP HPLC system (Shimadzu Corp., Japan) equipped with a RID-10A refractive index detector, Aminex HPX-87H column (300 $\times$ 7.8 mm, Bio-Rad, USA) at 65°C with 5 mM H₂SO₄ as the eluent at a flow rate of 0.8 mL/min. Samples of fermentation broth were centrifuged, washed with deionized water, and dried at 105°C to a constant weight for biomass measurement.

Intracellular lipid was extracted by acid-heating procedure with mixture of chloroform and methanol as extractants [14]. Methanolysis was carried out following the procedures described by Li et al. [15]. Fatty acid composition of the methyl ester was analyzed with a GC-14B GC (Shimadzu, Japan) equipped with a CP-FFAP CB capillary column (25 m $\times$ 0.32 mm $\times$ 0.30 μ m) produced by Agilent. Heptadecanoic acid methyl ester was used as internal standard. The column temperature was kept at 180°C for 0.5 min, heated to 250°C at 10°C/min, and maintained for 6 min. The temperatures of the injector and detector were set at 245 and 260°C, respectively. The phospholipid content was determined by molybdenum blue colorimetric method following the procedures of National Standard GB/T 5537-2008 [16].

Pigments were qualitatively analyzed by an ultraviolet-visible spectrophotometer. Cell biomass was spinned down, loaded with 4 mol L⁻¹ hydrochloric acid (hydrochloric acid solution:fermentation broth = 1:10, v/v), mixed, and heated in a boiling water bath for 4 min. The hydrolyzed cell pellets were rapidly cooled down in room temperature, centrifuged to discard

the supernatant, and washed with distilled water. Centrifugation and washing were repeated twice. Afterwards, pigment components were extracted from the cell debris in 10 mL acetone and used for spectrophotometry analysis.

The immobilized lipase-catalyzed transesterification [17] was carried out as follows. 0.8 g lipid and 6 g of *tert*-butanol are loaded into 50 mL Erlenmeyer flask, shaking to fully mix. A total of 0.5 gram Novozyme 435 and 0.5 gram Lipozyme TL were added. Methanol was added for 1 mL at the beginning and 0.5 mL at 8 h, utilized as acyl acceptor. The flasks were kept in the 45°C incubator for 24 h with a shaking speed of 190 rpm.

Cell biomass (C_B), lipid concentration (C_L), lipid yield (Y_L), nonlipid biomass yield (Y_B), lipid content (Cn_L), lipid productivity (P_L) and biodiesel yield (Y_{BD}) are defined as follows:

$$C_B \text{ (g L}^{-1}\text{)} = \frac{\text{dry weight of cell biomass (g)}}{\text{volume of fermentation broth (L)}}$$

$$C_L \text{ (g L}^{-1}\text{)} = \frac{\text{weight of lipid (g)}}{\text{volume of fermentation broth (L)}}$$

$$Y_L \text{ (g/100g carbon substrate)} = \frac{\text{weight of lipid (g)}}{\text{weight of carbon substrate consumed (g)}} \times 100$$

$$Y_B \text{ (g/100g carbon substrate)} = \frac{\text{weight of nonlipid biomass (g)}}{\text{weight of carbon substrate consumed (g)}} \times 100$$

$$Cn_L \text{ (%) } = \frac{\text{weight of lipid (g)}}{\text{weight of cell biomass (g)}} \times 100$$

$$P_L \text{ (g L}^{-1}\text{h}^{-1}\text{)} = \frac{\text{Weight of lipid (g)}}{\text{volume of fermentation broth (L)} \times \text{fermentation time (h)}}$$

$$Y_{BD} \text{ (%) } = \frac{\text{weight of FAMES (g)}}{\text{weight of microbial lipid or soybean oil (g)}} \times 100$$

For the second-stage culture, calculations of Y_L , Y_B , and P_L were based on data of lipid/nonlipid biomass generated and glycerol consumed in the second stage.

2.4 Statistical analysis

Design Expert 8.0.5b (State Ease Inc., Minneapolis, MN, USA) was used to facilitate experiment design and data analysis. Central composite design was used to analyze the effects of four variables, including the initial glycerol concentration (X_1), fermentation time (X_2), initial biomass concentration (X_3), and concentration of SL (X_4). In the experiment design, five levels (−2, −1, 0, 1, 2) for each variable were X_1 (50, 80, 110, 140, 170), X_2 (5, 6, 7, 8, 9), X_3 (2.0, 4.5, 7.0, 9.5, 12.0), and X_4 (0.5,

1.5, 2.5, 3.5, 4.5), respectively. The range of each variable was set according to results of the single-factor experiment in Section 3.2, and former results regarding the kinetics of cell growth and lipid production. Nonlinear multivariable regression and linear multivariable regression as follows were used to model the experimental data:

$$Y = \beta_0 + \sum_{i=1}^4 \beta_i x_i + \sum_{i=1}^4 \beta_{ii} x_i^2 + \sum_{i,j=1}^4 \beta_{ij} x_i x_j$$

$$Y = \beta_0 + \sum_{i=1}^4 \beta_i x_i$$

Each experimental condition was performed by duplicate test.

3 Results and discussion

3.1 Behavior of *R. toruloides* in converting glycerol into lipids under different nutrition conditions

Microbial conversion of glucose into lipids has been widely investigated. It was found that in most oleaginous microorganisms lipid accumulation does not occur simultaneously with the cell growth and proliferation due to their differences in nutrition requirements, where the ratio of carbon to nitrogen (C/N ratio) in the culture medium is key to lipid production [7]. However, there is a lack of research on the behavior of *R. toruloides* in converting glycerol into microbial lipids. Herein, first we compared the lipid accumulation activity of *R. toruloides* under different nutrition conditions when glycerol was used as the sole carbon substrate.

As shown in Fig. 1, in the YPG medium with a low C/N ratio (C/N ratio = 3), biomass increased quickly from 0.108 to 7.24 g L^{−1} in 60 h, but the intracellular lipid content did not exceed 20%. While in the nitrogen-limited medium (C/N ratio = 300), although it took about 75 h to reach a biomass concentration of 7.96 g L^{−1}, lipid synthesis was quickly activated. Intracellular lipid content reached approximately 43% in 60 h, and further reached 47.7% at 75 h. It suggested that when growing on glycerol, a high C/N ratio is good for lipid accumulation, while a comparatively low C/N ratio is imperative for rapid harvest of cell biomass.

A desired lipid production relies on achieving both high biomass concentration and high lipid content. Therefore, it is important to well balance cell proliferation and lipid synthesis. According to the behavior of *R. toruloides* as described above, it is possible to promote lipid production efficiency by separately optimizing the cell proliferation and lipid accumulation stage. In the first stage, we can simply use YPG medium, which provides sufficient nutrients for cell proliferation. Meanwhile, cultivation time can be easily adjusted to obtain a specific biomass concentration. By contrast, more efforts are needed to explore the lipid accumulation behavior in the second stage. In this stage, inoculum age, initial glycerol concentration and biomass concentration should play important roles [18]. Some inorganic salts may also affect lipid accumulation by influencing the activities of certain key enzymes. In the present work, the effects of these key factors were investigated and optimized.

Figure 1. Lipid accumulation under different culture conditions: (A) YPG medium and (B) nitrogen-limited medium.

Table 1. Effect of inoculum age on lipid accumulation

Inoculum age (h)	C_B (g L ⁻¹)	C_L (g L ⁻¹)	C_{NL} (%)	Y_L (%)
12	28.6 ± 0.5	13.3 ± 0.6	46.5 ± 1.6	21.3 ± 1.4
24	26.8 ± 0.6	13.2 ± 0.6	49.1 ± 1.5	22.5 ± 1.7
36	25.0 ± 0.3	13.2 ± 0.6	52.8 ± 1.8	21.4 ± 1.3
48	25.1 ± 0.3	13.8 ± 0.7	55.0 ± 2.5	23.8 ± 1.8
60	24.5 ± 0.4	14.6 ± 0.7	59.6 ± 3.5	24.3 ± 1.5

3.2 Exploration of the influential factors in microbial conversion of glycerol into lipids

3.2.1 Effect of inoculum age on lipid accumulation

In the first stage, *R. toruloides* grew and proliferated rapidly in YPG medium, and successively underwent the lag phase (0–12 h), logarithmic phase (12–50 h), and stationary phase (50 h later) (Supporting information Fig. S1). Cells of different inoculum ages may have different physiological statuses. To investigate their influences on lipid accumulation, biomass cultivated for different length of time was collected and inoculated into the second stage medium. Their lipid contents were 0% (12 h), 9.9% (24 h), 15.4% (36 h), 15.5% (48 h), 15.5% (60 h), respectively, all of which were less than 20% of cell dry weight, suggesting no significant lipid accumulation in the first stage.

The final biomass and lipid concentrations of the second stage cultivation were summarized in Table 1. It indicated that inoculum age had a significant impact on lipid accumulation. Within the inoculum age of 12–60 h, an aged inocula led to less biomass increase, but higher lipid content and lipid yield. When the inoculum age was up to 60 h, the final intracellular lipid content reached 60%. The lipid yield was up to 24.3%, which was much closer to the theoretical yield of converting glycerol into triacylglycerols (around 32%) than most reported data [5, 19–22]. It was very likely that TAG biosynthesis in *R. toruloides* cells had been fully activated at the middle/late period of the logistical phase, the majority of glycerol could be directly fluxed to lipid synthesis pathways.

3.2.2 Effect of initial glycerol concentration on lipid accumulation

The initial glycerol concentration also constitutes a key factor for lipid production. Different initial concentrations (20 ~ 160 g L⁻¹) have been investigated for their effects on lipid accumulation. The initial biomass was 5.2 ± 0.2 g L⁻¹ with lipid content less than 15%, harvested from 40 h first-stage culture. Table 2 shows result obtained from the second-stage culture. When the initial glycerol concentration was as low as 20 or 40 g L⁻¹, the final biomass was comparatively low due to the lack of a desired glycerol gradient after the first 24 h. The final biomass, lipid concentration, and lipid content increased with increment in initial glycerol concentration from 20 to 160 g L⁻¹.

The increase of biomass in the second-stage culture could be largely attributed to the significant lipid accumulation. Some trace nitrogen residual remained on the cell surface from the first stage culture might also, to some extent, contribute to the cell growth. Besides, it has been reported that during nitrogen limitation, protein turnover and autophagy occur to provide alternative sources of nitrogen [23]. Such kind of cellular regulation may contribute to the lipid biosynthesis and maintenance of cell activities in the second stage culture.

However, growth lag has been observed when initial glycerol concentration was as high as 120 and 160 g L⁻¹. Moreover, within the same time scale, yield of nonlipid biomass shows a trend of decrease with increments in glycerol concentration, suggesting the negative influence of a high initial glycerol on cell growth, whereas no significant inhibition is observed for lipid accumulation, and the final lipid content actually increases due to the decrease in nonlipid biomass. It suggests that in the second-stage culture, initial glycerol concentration may have more impact on cell growth than lipid production.

From those reported studies, we can see that performance of glycerol assimilation is species dependent. Decreases in biomass yield/biomass productivity have also been reported in *Yarrowia lipolytica* LFMB 19 [24], *Y. lipolytica* ACA-DC 50109 [20], and *Mortierella isabellina* ATHUM 2935 [25], while no decrease in lipid yield was observed. On the other hand, significant substrate inhibition has been observed in the batch culture of *R. toruloides* NRRL Y-27012 and *L. starkeyi* DSM 70296. The yields of lipid

Table 2. Effect of initial glycerol concentration on lipid accumulation

Glycerol (g L ⁻¹)	C _B (g L ⁻¹)	C _L (g L ⁻¹)	C _{NL} (%)	Y _L (%)	Y _B (%)	P _L (g L ⁻¹ h ⁻¹)	Consumed glycerol (g L ⁻¹)
20	12.2 ± 0.1	3.83 ± 0.19	31.3 ± 0.4	23.4 ± 1.5	26.4 ± 1.4	0.016 ± 0.001	13.0 ± 0.4
40	16.0 ± 0.4	5.83 ± 0.28	36.3 ± 0.0	19.0 ± 1.1	18.2 ± 1.2	0.026 ± 0.001	26.2 ± 1.6
80	25.4 ± 0.6	12.4 ± 0.60	48.8 ± 3.0	20.0 ± 1.1	13.2 ± 0.7	0.061 ± 0.003	57.8 ± 0.9
120	25.6 ± 1.2	14.5 ± 0.72	56.6 ± 1.6	20.4 ± 1.1	8.9 ± 0.8	0.072 ± 0.004	66.9 ± 3.7
160	26.2 ± 0.5	14.7 ± 0.74	56.1 ± 3.4	24.2 ± 1.4	11.0 ± 0.6	0.073 ± 0.004	57.2 ± 0.9

and biomass both decreased when initial glycerol is higher than 120 g L⁻¹ [26]. These reported data suggest that having an excessively high glycerol concentration may cause an effect of substrate inhibition, which usually results in a limited cell growth, and might cause a decrease in lipid yield in some cases. Based on these considerations, exploring a proper initial glycerol concentration is needed to enhance biomass titer while achieving satisfied lipid content.

3.2.3 Effect of inoculation size on lipid accumulation

A proper inoculum size is essential to lipid production [27]. In the two-stage process, cell growth can be maximized in the first stage, followed by harvest of high density biomass and transfer of biomass into the second-stage media for lipid accumulation. However, few studies reported the effect of initial biomass concentration in a two-stage culture. In present study, *R. toruloides* cells were cultivated for 36 h in the first stage until the optical density reached approximately 12 OD_u (optical density units) at 620 nm. Afterwards, they were collected and transferred to the second-stage culture. The initial biomass concentration was regulated to different levels by applying different inoculation sizes ranging from 50 to 200%.

In the 190 h fermentation, when the initial OD_u of the second-stage culture was 6.3, which is equivalent to a 52% inoculum size, final biomass concentration was up to 18.8 g L⁻¹ with an intracellular lipid content of 47.8%. When the initial OD_u was 12.0 (100% inoculum size), biomass concentration reached 25.8 g L⁻¹ with 42.7% of lipid. And when the initial OD_u was 22.5 (188% inoculum size), biomass concentration reached 36.3 g L⁻¹ with 32.0% lipid. It indicated that lipid concentration could be enhanced with the increase of initial biomass concentration, while the intracellular lipid content decreased with the initial biomass concentration.

3.2.4 Effect of some inorganic salts on lipid accumulation

Some literatures have reported the influences of some inorganic salts on microbial lipid production [28–31]. According to the reported data and our previous work, which was mainly focused on bioconversion of glucose into lipids though, inorganic salts of MgSO₄ and KH₂PO₄ might exert some influences on lipid accumulation of *R. toruloides*. Here, we investigated their effects for glycerol-based fermentation.

The result indicated that addition of 0.4–1.0 g L⁻¹ KH₂PO₄ could exert a positive impact on cell growth, although an excessively high concentration showed adverse effect to lipid accumulation. Addition of 1.5–3.0 g L⁻¹ MgSO₄ resulted in a positive

effect on lipid accumulation, but an excessively high concentration led to a trend of decrease in biomass. No significant variation in fatty acid compositions was observed. The result was in accordance with other reported results. It is likely that certain trace elements have beneficial effects on cell physiology and play a role in lipid synthesis related enzymes. For instance, ATP-citrate lyase activity was reported to be dependent on the presence of Mg²⁺ in *L. starkeyi* [29]. Mg²⁺ is also a catalytically essential cofactor of malic enzyme in *Aspergillus niger* [28]. NADP⁺-dependent isocitrate dehydrogenase was divalent cation dependent and Mg²⁺ was found to be the most favorable cofactor in *Y. lipolytica* [30]. Moreover, some elements even act as the activator/inhibitor of desaturases, thus could influence the biosynthesis of long-chain fatty acids [28,29]. However, the response of cells to metal ions seems to be a strain-related mechanism, and molecular study of their role on fatty acid synthesis has been limited [31].

3.3 Optimization of the lipid production

The above results showed how each factor affected the bioconversion of glycerol into lipids, and suggested the ranges of local optimum for these individual factors. Inoculation cells at the middle/late exponential phase could achieve a high lipid yield. Proper addition of KH₂PO₄/MgSO₄ could enhance lipid production. Other influential factors included initial biomass, initial glycerol concentration, fermentation time, and supplementation of SL, which might have interactive effects. For instance, the tolerance to initial glycerol concentration grew with the increase of biomass concentration. Lipid synthesis rate varied with time. Besides, the positive effect of SL on consumption of cellulose sugars [6] and glycerol [32] in *R. toruloides* has been previously reported. In a two-stage process, addition of 1–2 g L⁻¹ SL at the starting of second-stage media could result in a 10% increase of lipid accumulation (Supporting information Table S1). Since the positive effect might be related to oxygen transfer [32], it is likely to have a correlative effect to the factor of biomass concentration.

Although data shown in Section 3.2 have indicated that bioconversion of glycerol into lipids by *R. toruloides* could achieve a similar lipid yield to that of glucose (19–24%) [7,21], the lipid productivity (0.02–0.07 g L⁻¹ h⁻¹) was inferior to that of glucose (0.1 g L⁻¹ h⁻¹) under comparable culture modes [33]. Exploration of better cultivation conditions is needed. In the present work, we used the response surface methodology to investigate the interactive effects of various factors, optimized the lipid accumulation process, and evaluated the potential of our two-stage process by applying optimized conditions.

Table 3. Experimental and modeled lipid production

Level of variables ^{a)}				C_L (g L ⁻¹)	C_B (g L ⁻¹)	C_{NL} (%)	P_L (g L ⁻¹ h ⁻¹)	
X_1	X_2	X_3	X_4	Data	Data	Data	Data	Model
0	0	0	0	11.7	19.7	59.4	0.070	0.066
1	1	-1	1	10.8	15.7	68.8	0.056	0.058
-1	-1	1	-1	10.9	25.1	43.4	0.076	0.074
-1	1	1	1	13.1	28.4	46.1	0.068	0.068
-1	-1	-1	1	9.9	20.2	49.0	0.069	0.066
1	-1	1	1	9.3	20.2	46.0	0.064	0.075
1	1	1	-1	13.2	24.4	54.1	0.069	0.067
0	0	0	0	10.8	19.1	56.5	0.064	0.066
1	-1	-1	-1	9.7	16.6	58.4	0.068	0.065
-1	1	-1	-1	11.4	20.1	56.7	0.060	0.058
1	1	-1	-1	9.7	15.0	64.7	0.050	0.058
1	1	1	1	12.1	22.3	54.3	0.063	0.068
-1	1	-1	1	11.8	17.8	66.3	0.061	0.059
-1	-1	1	1	11.4	21.1	54.0	0.079	0.076
-1	1	1	-1	13.4	24.7	54.3	0.070	0.068
0	0	0	0	11.1	19.1	58.1	0.066	0.067
1	-1	-1	1	9.3	13.8	67.4	0.064	0.066
0	0	0	0	12.2	20.5	59.5	0.073	0.067
-1	-1	-1	-1	9.4	15.4	61.0	0.065	0.065
1	-1	1	-1	10.9	20.4	53.4	0.076	0.075
2	0	0	0	11.6	21.9	53.0	0.069	0.062
0	0	0	2	4.5	11.41	39.4	0.068	0.063
0	0	0	0	10.6	21.8	48.6	0.063	0.062
0	0	-2	0	7.0	9.5	73.7	0.042	0.053
-2	0	0	0	8.6	20.3	42.4	0.051	0.062
0	0	0	0	11.0	22.2	49.5	0.066	0.062
0	2	0	0	14.1	22.7	62.1	0.065	0.055
0	0	2	0	10.8	26.9	40.1	0.064	0.072
0	0	0	-2	9.7	21.5	45.0	0.058	0.061
0	-2	0	0	9.1	17.9	50.8	0.076	0.069

a) X_1 – X_4 are symbols that stand for four variables, namely glycerol concentration, fermentation time, biomass concentration, SL concentration, respectively.

Experimental conditions and results are shown in Table 3. By applying nonlinear multiple regression analysis, coefficients for the regression model were determined (Supporting information Table S2). Analysis of variance (ANOVA) data (Supporting information Table S3) revealed that the model well described the experimental data. p -value was lower than 0.05, suggesting the statistical significance. Effects of different variables on lipid productivity were shown in the three-dimensional response surface plots (Fig. 2). The effect of initial glycerol concentration was significant at the first few hours of lipid production, whose interactive effect with biomass could also be observed (Fig. 2B). For a specific level of initial biomass, an optimal glycerol concentration could be found to achieve a maximal lipid productivity. The effect of SL was less significant than variables of initial biomass and fermentation time. We have reported the effect of SL on *R. toruloides* and identified a mechanism related to oxygen transfer [32]. At the initial stage of lipid production, oxygen transfer could constitute a limiting factor especially when the biomass concentration reached a high level. Under such a circumstance, the influence of SL on lipid production could be observed (Fig. 2C).

The aim of the present work was to maximize the lipid productivity. Since most quadratic terms of the quadratic regression model were not significant, this model could be further simplified to a linear polynomial, which can provide applicable instructions to the fermentation process. By applying linear regression analysis, coefficients for the regression model were determined (Supporting information Table S4). ANOVA data (Supporting information Table S5) revealed that the model well described the experimental data. p -value was lower than 0.01, suggesting the statistical significance. Experimental results and predictive data matched well (Supporting information Table S6). Lipid productivity can be expressed as the follows:

$$Y_{\text{productivity}} = 0.065 - 8.333 \times 10^{-5} X_1 - 3.583 \times 10^{-3} X_2 + 4.833 \times 10^{-3} X_3 + 4.167 \times 10^{-4} X_4$$

Taking into account the decrease of lipid productivity with time, a minimum lipid concentration was prescribed at 9.0 g L⁻¹. As a result, a predicted maximum of lipid productivity was 0.082 g L⁻¹ h⁻¹. The optimized conditions were testified in our two-stage process. First, yeast cells were cultivated in YPG

Figure 2. Response surfaces with regard to lipid productivity. (A) glycerol concentration and time (biomass: 7.0 g L⁻¹, SL: 2.5 g L⁻¹); (B) glycerol concentration and biomass concentration (time: 6 h, SL: 2.5 g L⁻¹); (C) glycerol concentration and SL concentration (time: 6 h, biomass: 12.0 g L⁻¹); (D) time and biomass concentration (glycerol: 110 g L⁻¹, SL: 2.5 g L⁻¹); (E) time and SL concentration (glycerol: 110 g L⁻¹, biomass: 7.0 g L⁻¹); (F) biomass concentration and SL concentration (glycerol: 110 g L⁻¹, time: 7 h).

Table 4. Lipid production in the two-stage culture and comparisons with reported results of red yeasts growing on glycerol

Strain	Glycerol type	Glycerol (g L ⁻¹)	Time (h)	C _B (g L ⁻¹)	C _L (g L ⁻¹)	Y _L (%)	P _L (g L ⁻¹ h ⁻¹)	Ref.
<i>R. toruloides</i> AS 2.1389	Pure	52	120	26.5	10.0	20.4	0.083	Present work
<i>R. toruloides</i> AS 2.1389	Pure	50	168	12.8	5.6	13.9	0.033	Previous work [3]
<i>R. diobovatum</i>	Crude	60	120	14.1	7.1	NA	0.059	[52]
<i>R. toruloides</i> DSM 4444	Crude	100	150	~19	7.6	NA	0.051	[34]
<i>R. toruloides</i> NRRL Y-27012	Crude	120	395	30.1	12.0	10.0	0.030	[26]
<i>R. toruloides</i> Y4	Pure	50	120	17.7	6.2	19.3	0.052	[38]
<i>R. toruloides</i> Y4	Synthetic crude	50	120	24.9	12.2	22.0	0.102	[38]
<i>R. glutinis</i> TISTR 5159	Crude	100	72	8.17	4.3	6.7	0.058	[53]

medium for 36 h. A certain quantity of fermentation broth from the first-stage was collected to reach a biomass equivalent to 12 g L⁻¹ for the second-stage culture. The biomass was inoculated into the second-stage medium, which contained 52 g L⁻¹ glycerol and was supplemented with 0.4 g L⁻¹ KH₂PO₄, 1.5 g L⁻¹ MgSO₄, and 4.5 g L⁻¹ SL. Culture time was set to 120 h. As a result, the lipid productivity achieved 0.083 g L⁻¹ h⁻¹, which matched well to the prediction. Comparing with our previous result obtained from the optimized batch culture [3], the lipid titer, yield, and productivity are greatly improved by applying the two-stage process (Table 4). The lipid productivity has even been close to the reported data of converting glucose into lipids under comparable culture conditions [33].

Moreover, the current result is among the highest for shake-flask fermentations of oleaginous microorganism growing on glycerol [5, 25, 26, 34, 35], and compare favorably with many other results achieved in bioreactor fermentations [5, 21, 36, 37]. Specifically, when compared with those rarely reported results of cultivating oleaginous red yeasts on pure/crude glycerol, our result shows a particular merit (Table 4) in that it achieved a high lipid yield while implementing improvement of lipid productivity. This merit could be attributed to the two-stage fermentation mode. Cells were cultivated in nutrient-rich medium in the first-stage for proliferation, and resuspended in carbon-rich medium of the second stage for lipid accumulation. It is possible that lipid biosynthesis pathways were fully activated with coenzymes accessible at the middle/late phase of the growth phase, thus majority of glycerol could efficiently flow into lipid synthesis in the second-stage culture.

There is one reported result that outcompetes our result [38], when cells growing on the synthetic crude glycerol. Their pure glycerol-based fermentation actually showed an inferior lipid titer, but the synthetic crude glycerol-based fermentation had a significantly improved lipid titer and yield. It was proposed that the presence of methanol (6 wt %) and K₂SO₄ (6 wt %) favored lipid production. Similar conclusions have also been obtained in our previous study [5] that some impurity compositions of crude glycerol can promote lipid production. In view of this advantage, it is possible to achieve desired lipid titer in the future using the industrial waste derived glycerol.

In order to further improve microbial lipid production for industrial application, several issues including but not limited to oxygen supply, glycerol assimilation, and high-density fermentation need to be studied in the future. As reported before, oxygen supply affects the cell growth and lipid synthesis [8, 32].

In high-density culture of oleaginous microorganisms, oxygen transfer can be a limiting factor. Second, inefficient glycerol assimilation could limit downstream metabolic processes. An optimized glycerol feeding strategy is needed. Moreover, since glycerol assimilation is presumably limited by insufficient coordination of some enzymes [24, 25, 39, 40], genetic engineering toward the primary metabolic steps, for example, the glycerol kinase and glycerol-3-phosphate dehydrogenase, can be taken into account. Another concern is nitrogen source. Cheaper nitrogen source, and those derived from agriculture/food/municipal waste, should substitute the YPG medium for the first-stage fermentation. Also, fermentation mode should be properly chosen and optimized. Some strategies, such as fed-batch and repeated batch, can be exploited. Considering the fact that even if waste streams such as crude glycerol could provide a cheap resource, the cost of fermentation based on current technology contributes the most important part to the manufacture cost, it is imperative to develop a cost efficient process. Isolation and adaptation of robust oleaginous yeast strains, innovative fermenter designs, and optimization of scale-up process are needed in order to further promote lipid productivity and reduce the cost of fermentation [41] to make commercialization of microbial oil production possible.

3.4 Characterization of *R. toruloides* lipids

Microbial lipids obtained from growing *R. toruloides* on glycerol was characterized. Figure 3 shows the fatty acid profiles in glycerides, the majority of which were C16~C18 fatty acids, including palmitic acid (28.5%), stearic acid (12.9%), oleic acid (41.3%), and linoleic acid (12.8%). Saturated fatty acids constitute 43.1% of the total lipids. The similarity in fatty acid composition of *R. toruloides* lipid to that of soybean oil suggests its potential application for biodiesel production. Moreover, we noticed that *R. toruloides* lipid shows comparable fatty acid profiles with that of cocoa butters [42]. Cocoa butter is a high value added exotic fat that is principally composed of saturated fatty acid (55~67%, mainly palmitic acid and stearic acid) and monounsaturated acid (30~37%, oleic acid) [43, 44]. By contrast, oleaginous yeasts/fungi generally produce lipid containing more unsaturated fatty acids (~50%). In some species, such as *Y. lipolytica*, *T. elegans*, etc., this value can reach over 70% [2]. Over years many efforts have been made to isolate or adapt oleaginous microorganisms to produce specialty fats, which are considered

Figure 3. Comparison of fatty acid profiles.

more cost competitive than microbial lipid based biodiesel production [41]. From this point of view, in addition to the potential of being utilized as biodiesel feedstock, *R. toruloides* lipids may also be exploited as a specialty-type lipid for certain value added applications.

Besides glycerides, phospholipids also exist in *R. toruloides* lipid as a minor composition. We compared its content with that of vegetable oils and other microbial lipids. Crude vegetable oils normally contain phospholipid at a range of 0.1–0.6% [45]. In our results, the phospholipid contents of crude soybean oil and rapeseed oil were 0.56 and 0.25%, respectively. For microbial lipids, phospholipid content exhibits a species-dependent feature, which was reported to be 6.6% in *Y. lipolytica* ACA-YC 5033 [46], 5.9% in *Y. lipolytica* LFMB 19, 4.5% in *Pichia membranifaciens* LFMB 8, and 9.2% in *Thamnidium elegans* CCF 1465 [24]. By contrast, lipids of *R. toruloides* AS 2.1389 contain 0.63% phospholipid, which was very close to that of vegetable oils. The effects of phospholipids on biodiesel production have been evaluated previously. Compared with refined soybean oils, reaction rate significantly decreased in immobilized lipase-catalyzed reactions when phospholipid content exceeded 1.0% [45]. While for the free lipase-mediated process, phospholipid content within 2% was proved to be noninhibitive, and even showed some beneficial effects [47]. Our result indicated that the phospholipid composition remaining in *R. toruloides* lipids was lower than the threshold for an inhibitive effect. Additionally, phospholipids could be removed by degumming, which are widely used for vegetable oil treatment. Therefore, it can be anticipated that phospholipid composition in *R. toruloides* lipids will not affect the biodiesel preparation.

Furthermore, the yield of transforming *R. toruloides* lipids into biodiesel was investigated. There have been extensive studies using various approaches such as homogeneous alkali/acid catalysis, heterogeneous alkali/acid catalysis, enzyme catalysis, and supercritical fluid system for biodiesel preparation. Among these approaches, enzymatic method has been widely adopted [15,47] due to advantages of moderate reaction conditions, low alcohol to oil ratio, easy product recovery, environmental friendliness, and the capability of completely converting free fatty acids of

low-grade oils into alkyl esters. Therefore, immobilized lipase catalysis was chosen for transformation of *R. toruloides* lipids. It is already known that the rate of lipid-to-biodiesel for microbial lipid was comparable to that of soybean oil [6]. To explore its application potential for biodiesel production, in the present work excess amount of enzyme was used. Thus, the present biodiesel yield indicated the convertibility of microbial lipid into biodiesel. As a result, the biodiesel yield of *R. toruloides* lipids was up to 95%, which was very close to that of soybean oil (96%), suggesting that *R. toruloides* lipids can be effectively transformed into biodiesel.

Furthermore, we qualitatively identified the coproduction of carotenoids in *R. toruloides*. Carotenoids production has been reported in several yeast genres such as *Rhodospiridium*, *Rhodotorula*, and *Phaffia* [48,49]. The main carotenoid compositions include β -carotene, γ -carotene, torulene, and torularhodin, which can be analyzed by spectrophotometry method due to their absorbance at 453, 463, 487, and 496 nm, respectively [48]. In this work, we found that the acetone extract from *R. toruloides* had a significant absorbance at the range of 450–500 nm, indicating the existence of carotenoids. As some carotenoids can be converted into vitamin A, and possesses antioxidant properties, they are considered as valuable compounds for nutritional supplement, food colorant, and pharmaceutical use [12]. For this reason, coproduction of carotenoids might be a potential strategy to further increase the economic viability of microbial lipid production. Also, it will be an interesting topic to study the effect of carotenoid composition on biodiesel preparation. Some vegetable oils, such as palm oil, also contain carotenoids. It was reported that carotenoid has no significant influence on lipase-catalyzed transesterification [50], while Liang et al. even found the positive effect. Biodiesel derived from carotenoid-containing palm oil showed better oxidative stability [51]. Since there is limited knowledge about carotenoids biosynthesis in oleaginous yeasts, further studies on carotenoid separation, analysis, and utilization are needed to fully understand their effects on lipid properties and biodiesel preparation.

4 Concluding remarks

An efficient lipid production process was established by maximizing biomass production and lipid accumulation in separate stages. It was found that inoculation of *R. toruloides* cells at the middle/late exponential phase (36–60 h) into the second stage culture facilitated the achievement of a high lipid yield. A high initial biomass concentration and proper glycerol concentration was in favor of rapid lipid accumulation. Proper addition of KH_2PO_4 , MgSO_4 , and SL could further promote lipid production. By applying an optimized condition suggested by the statistical model, the lipid productivity was up to 2.5 times of the batch process. Furthermore, the *R. toruloides* lipids were demonstrated to be a competent oil feedstock, which could be successfully transformed into biodiesel. The conclusions obtained in the present work provide valuable hints for further promoting the bioconversion of glycerol into lipids by *R. toruloides* in scale-up processes.

Practical application

The feasibility of direct utilization of crude glycerol without purification for lipid production has been previously demonstrated. In the present work, the behavior of *R. toruloides* in converting glycerol into lipids was further investigated and improved in the two-stage process, and the lipid obtained was characterized for its potential applications. With the fast growing of biodiesel industry, glycerol will be largely generated as a by-product from local plants, and thus can be a promising carbon source for biofuel production. Results of the present study provide valuable hints for further promoting the bioconversion of glycerol into lipids by *R. toruloides* in scale-up processes.

Nomenclature

C_B	[g L ⁻¹]	Biomass concentration
C_L	[g L ⁻¹]	Lipid concentration
Y_L	[%]	Lipid yield
Y_B	[%]	Nonlipid biomass yield
C_{nL}	[%]	Lipid content
P_L	[g L ⁻¹ h ⁻¹]	Average lipid productivity
Y_{BD}	[%]	Biodiesel yield

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5 References

- [1] Wise, T., Cole, E., Mandating food insecurity: The global impacts of rising biofuel mandates and targets. *Global Development and Environmental Institute Working Paper*, Tufts University: Medford, MA 2015, pp. 15–01.
- [2] Xu, J., Du, W., Zhao, X., Zhang, G. et al., Microbial oil production from various carbon sources and its use for biodiesel preparation. *Biofuels Bioprod. Biorefin.* 2013, 7, 65–77.
- [3] Xu, J., Oura, T., Liu, D., Kajiwar, S., Heat-alkaline treatment of excess sludge and the potential use of hydrolysate as nitrogen source for microbial lipid production. *Chin. J. Biotechnol.* 2011, 27, 482–488.
- [4] Wei, Y., Houten, R., Rorger, A., Eikelboom, D. et al., Minimization of excess sludge production for biological wastewater treatment. *Water Res.* 2003, 37, 4453–4467.
- [5] Xu, J., Zhao, X., Wang, W., Du, W. et al., Microbial conversion of biodiesel byproduct glycerol to triacylglycerols by oleaginous yeast *Rhodospiridium toruloides* and the individual effect of some impurities on lipid production. *Biochem. Eng. J.* 2012, 65, 30–36.
- [6] Zhao, X., Peng, F., Du, W., Liu, C. et al., Effects of some inhibitors on the growth and lipid accumulation of oleaginous yeast *Rhodospiridium toruloides* and preparation of biodiesel by enzymatic transesterification of the lipid. *Bioprocess Biosyst. Eng.* 2012, 35, 993–1004.
- [7] Ratledge, C., Wynn, J., The biochemistry and molecular biology of lipid accumulation in oleaginous microorganisms. *Adv. Appl. Microbiol.* 2002, 51, 1–51.
- [8] Zhao, X., Hu, C., Wu, S., Shen, H. et al., Lipid production by *Rhodospiridium toruloides* Y4 using different substrate feeding strategies. *J. Ind. Microbiol. Biotechnol.* 2011, 38, 627–632.
- [9] Li, Y., Zhao, Z., Bai, F., High-density cultivation of oleaginous yeast *Rhodospiridium toruloides* Y4 in fed-batch culture. *Enzyme Microb. Technol.* 2007, 41, 312–317.
- [10] Lin, J., Shen, H., Tan, H., Zhao, X. et al., Lipid production by *Lipomyces starkeyi* cells in glucose solution without auxiliary nutrients. *J. Biotechnol.* 2011, 152, 184–188.
- [11] Gong, Z., Shen, H., Wang, Q., Yang, X. et al., Efficient conversion of biomass into lipids by using the simultaneous saccharification and enhanced lipid production process. *Biotechnol. Biofuels* 2013, 6, 36.
- [12] Lee, J., Chen, L., Shi, J., Trzcinski, A. et al., Metabolomic profiling of *Rhodospiridium toruloides* grown on glycerol for carotenoid production during different growth phases. *J. Agric. Food Chem.* 2014, 62, 10203–10209.
- [13] Bommarreddy, R., Sabra, W., Maheshwari, G., Zeng, A., Metabolic network analysis and experimental study of lipid production in *Rhodospiridium toruloides* grown on single and mixed substrates. *Microb. Cell Fact.* 2015, 14, 36.
- [14] Li, Z., Zhang, L., Shen, X., Lai, B. et al., A comparative study on four methods of fungi lipid extraction. *Microbiology* 2001, 28, 72–75.
- [15] Li, L., Du, W., Liu, D., Wang, L. et al., Lipase-catalyzed transesterification of rapeseed oils for biodiesel production with a novel organic solvent as the reaction medium. *J. Mol. Catal. B Enzym.* 2006, 43, 58–62.
- [16] COFCO Beihai Industry (Tianjin) Co. Ltd., GB/T 5537-2008 Inspection of grain and oils—Determination of phospholipid content. Standards Press of China, Beijing 2008.
- [17] Li, W., Du, W., Li, Y., Liu, D. et al., Enzymatic transesterification of yeast oil for biodiesel fuel production. *Chin. J. Process Eng.* 2007, 1, 137–140.
- [18] Ageitos, J., Vallejo, J., Veiga-Crespo, P., Villa, T.G., Oily yeasts as oleaginous cell factories. *Appl. Microbiol. Biotechnol.* 2011, 90, 1219–1227.
- [19] Meesters, P., Huijberts, G., Eggink, G., High cell density cultivation of the lipid accumulating yeast *Cryptococcus curvatus* using glycerol as a carbon source. *Appl. Microbiol. Biotechnol.* 1996, 45, 575–579.
- [20] Papanikolaou, S., Aggelis, G., Biotechnological valorization of biodiesel derived glycerol waste through production of single cell oil and citric acid by *Yarrowia lipolytica*. *Lipid Technol.* 2009, 21, 83–87.
- [21] Raimondi, S., Rossi, M., Leonardi, A., Bianchi, M. et al., Getting lipids from glycerol—new perspectives on biotechnological exploitation of *Candida freyschussii*. *Microb. Cell Fact.* 2014, 13, 83.
- [22] Makri, A., Fakas, S., Aggelis, G., Metabolic activities of biotechnological interest in *Yarrowia lipolytica* grown on glycerol in repeated batch cultures. *Bioresour. Technol.* 2010, 101, 2351–2358.

- [23] Kerkhoven E., Pomraning K., Baker S., Nielsen J., Regulation of amino-acid metabolism controls flux to lipid accumulation in *Yarrowia lipolytica*. *Syst. Biol. Appl.* 2016, 2, 16005.
- [24] Chatzifragkou, A., Makri, A., Belka, A., Bellou, S. et al., Biotechnological conversions of biodiesel derived waste glycerol by yeast and fungal species. *Energy* 2011, 36, 1097–1108.
- [25] Fakas, S., Papanikolaou, S., Batsos, A., Galiotou-Panayotou, M. et al., Evaluating renewable carbon sources as substrates for single cell oil production by *Cunninghamella echinulata* and *Mortierella isabellina*. *Biomass Bioenergy* 2009, 33, 573–580.
- [26] Tchakouteu S., Kalantzi O., Gardeli C., Koutinas A. et al., Lipid production by yeasts growing on biodiesel-derived crude glycerol: Strain selection and impact of substrate concentration on the fermentation efficiency. *J. Appl. Microbiol.* 2015, 118, 911–927.
- [27] Kurosawa K., Boccazzi P., Almeida N., Sinskey A., High-cell-density batch fermentation of *Rhodococcus opacus* PD630 using a high glucose concentration for triacylglycerol production. *J. Biotechnol.* 2010, 147, 212–218.
- [28] Jernejc, K., Legisa, M., The influence of metal ions on malic enzyme activity and lipid synthesis in *Aspergillus Niger*. *FEMS Microbiol. Lett.* 2002, 217, 185–190.
- [29] Boulton, C.A., Ratledge, C., Partial-purification and some properties of ATP-citrate lyase from the oleaginous yeast *Lipomyces starkeyi*. *J. Gen. Microbiol.* 1983, 129, 2863–2869.
- [30] Li X., Wang P., Ge Y., Wang W. et al., NADP⁺-specific isocitrate dehydrogenase from oleaginous yeast *Yarrowia lipolytica* CLIB122: Biochemical characterization and coenzyme sites evaluation. *Appl. Biochem. Biotechnol.* 2013, 171, 403–416.
- [31] Laoteng, K., Certik, M., Cheevadhanark, S., Mechanisms controlling lipid accumulation and polyunsaturated fatty acid synthesis in oleaginous fungi. *Chem. Pap.* 2011, 65, 97–103.
- [32] Xu, J., Du, W., Zhao, X., Liu, D., Exploration of sodium lignosulphonate's effects on lipid production by *Rhodospiridium toruloides*. *Process Biochem.* 2015, 50, 424–431.
- [33] Lin, J., Sheng, H., Zhang, Z., Hu, C. et al., Microbial lipid production by *Rhodospiridium toruloides* in a two-stage culture mode. *Chin. J. Biotech.* 2010, 26, 997–1002.
- [34] Leiva-Candia, D., Tsakona, S., Kopsahelis, N., Garcia, I. et al., Biorefining of by-product streams from sunflower-based biodiesel production plants for integrated synthesis of microbial oil and value-added co-products. *Bioresour. Technol.* 2015, 190, 57–65.
- [35] Sestric, R., Munch, G., Cicek, N., Sparling, R. et al., Growth and neutral lipid synthesis by *Yarrowia lipolytica* on various carbon substrates under nutrient sufficient and nutrient-limited conditions. *Bioresour. Technol.* 2014, 164, 41–46.
- [36] Cui, Y., Blackburn, J., Liang, Y., Fermentation optimization for the production of lipid by *Cryptococcus curvatus*: Use of response surface methodology. *Biomass Bioenergy* 2012, 47, 410–417.
- [37] Celinska, E., Grajek, W., A novel multigene expression construct for modification of glycerol metabolism in *Yarrowia lipolytica*. *Microb. Cell Fact.* 2013, 12, 102.
- [38] Yang X., Jin G., Gong Z., Shen H. et al., Recycling biodiesel-derived glycerol by the oleaginous yeast *Rhodospiridium toruloides* Y4 through the two-stage lipid production process. *Biochem. Eng. J.* 2014, 91, 86–91.
- [39] Hao, G., Chen, H., Gu, Z., Hao, Z. et al., Metabolic engineering of *Mortierella alpine* for arachidonic acid production with glycerol as carbon source. *Microb. Cell Fact.* 2015, 14, 205.
- [40] Papanikolaou, S., Fakas, S., Fick, M., Chevalot, I. et al., Biotechnological valorisation of raw glycerol discharged after biodiesel (fatty acid methyl esters) manufacturing process: Production of 1, 3-propanediol, citric acid and single cell oil. *Biomass Bioenergy* 2008, 32, 60–71.
- [41] Koutinas A., Chatzifragkou A., Kopsahelis N., Papanikolaou S. et al., Design and techno-economic evaluation of microbial oil production as a renewable resource for biodiesel and oleochemical production. *Fuel* 2014, 116, 566–577.
- [42] Lipp M., Simoneau C., Ulberth F., Anklam E. et al., Composition of genuine cocoa butter and cocoa butter equivalents. *J. Food Comp. Anal.* 2001, 14, 399–408.
- [43] Papanikolaou S., Aggelis G., *Yarrowia lipolytica*: A model microorganism used for the production of tailor-made lipids. *Eur. J. Lipid Sci. Technol.* 2010, 112, 639–654.
- [44] Ratledge C., Single cell oils-have they a biotechnology future. *Trends Biotechnol.* 1993, 11, 278–284.
- [45] Xu, Y., Study on lipase-catalyzed biodiesel production and characterization of the enzyme catalytic properties. Doctoral Thesis, Tsinghua University, Beijing 2005.
- [46] Andre A., Chatzifragkou A., Diamantopoulou P., Sarris D. et al., Biotechnological conversions of biodiesel-derived crude glycerol by *Yarrowia lipolytica* strains. *Eng. Life Sci.* 2009, 6, 468–478.
- [47] Li, Y., Du, W., Liu, D., Exploration on the effect of phospholipids on free lipase-mediated biodiesel production. *J. Mol. Catal. B Enzym.* 2014, 102, 88–93.
- [48] Sperstad, S., Lutnaes, B., Stormo, S., Jensen, S. et al., Torularhodin and torulene are the major contributors to the carotenoid pool of marine *Rhodospiridium babievae* (Golubev). *J. Ind. Microbiol. Biotechnol.* 2006, 33, 269–273.
- [49] Frengova, G., Beshkova, D., Carotenoids from *Rhodotorula* and *Phaffia*: Yeasts of biotechnological importance. *J. Ind. Microbiol. Biotechnol.* 2009, 36, 163–180.
- [50] Talukder, M., Wu, J., Nguyen, T., Fen, N. et al., Novozym 435 for production of biodiesel from unrefined palm oil: Comparison of methanolysis methods. *J. Mol. Catal. B Enzym.* 2009, 60, 106–112.
- [51] Liang, Y., May, C., Foon, C., Ngan, M. et al., The effect of natural and synthetic antioxidants on the oxidative stability of palm diesel. *Fuel* 2006, 85, 867–870.
- [52] Munch, G., Sestric, R., Sparling, R., Levin, D., Cicek, N., Lipid production in the under-characterized oleaginous yeasts *Rhodospiridium babievae* and *Rhodospiridium diobovatum* from biodiesel-derived waste glycerol. *Bioresour. Technol.* 2015, 185, 49–55.
- [53] Saenge, C., Cheirsilp, B., Suksaroge, T., Bourtoom, T., Potential use of oleaginous red yeast *Rhodotorula glutinis* for the bioconversion of crude glycerol from biodiesel plant to lipids and carotenoids. *Process Biochem.* 2011, 46, 210–218.