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Effect of crude glycerol on heterotrophic growth of *Chlorella pyrenoidosa* and *Coccomyxa subellipsoidea* C-169

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Abstract

The feasibility of utilizing crude glycerol as carbon source for heterotrophic growth of the green microalgae *Chlorella pyrenoidosa* and *Coccomyxa subellipsoidea* C-169 was investigated. The highest biomass concentration of *C. pyrenoidosa* (6.25 g L^{-1}) and *C. subellipsoidea* C-169 (7.62 g L^{-1}) was achieved in basal medium containing 5 and 10 g L^{-1} crude glycerol. Compared to pure glycerol and glucose, the algal cells grown in crude glycerol media obtained a higher intracellular protein content, while the microalgal lipid consists of a large amount of unsaturated fatty acids. The most abundant fatty acids in *C. pyrenoidosa* and *C. subellipsoidea* C-169 were linolenic acid (C18:3) and palmitic acid (16:0). The cetane number (49.0) of both investigated algal species corresponds to the requirements of the Standard B100. Our results indicate that crude glycerol could provide a promising alternative feedstock for heterotrophic growth of these two microalgae.

Keywords Chlorophyceae · Microalgae · Crude glycerol · Heterotrophic growth · Biodiesel

Introduction

Due to the short shortage of fossil fuel and increasing global concerns about environmental issues, the biodiesel industry has become increasingly important for its sustainability. During the manufacturing process of biodiesel, crude glycerol is generated as a main by-product in considerable quantities. Generally, approximately 1 kg of crude glycerol will be generated for every 10 kg of biodiesel produced (Johnson and Taconi 2010). In the USA and EU, 6.97 million tonnes of biodiesel are being produced each year (Dasari et al. 2005), which results in a considerable amount of crude glycerol as waste and its associated disposal costs. Crude glycerol typically consists of 70–75% glycerol along with a few contaminants in small quantities such as soap, oils, methanol, salts, and solid organic materials (Kumar et al. 2015). However, the refining process of this product to commercial pharmaceutical grade is time-consuming and not economically feasible (Zhang et al. 2013). In the current stage, crude glycerol is mostly considered as an industrial waste that causes severe environmental problems. Therefore, developing a sustainable and efficient approach to utilize crude glycerol is crucial.

Microalgae, which have the ability to grow autotrophically, mixotrophically, or heterotrophically, are considered as prolific producers of value-added products including pigments, proteins, polysaccharides, and fatty acids (Lu et al. 2016). Moreover, a variety of biofuels (e.g., biodiesel, bioethanol, and bio-hydrogen) have been generated from microalgal biomass in lab scale. Several limitations, such as low biomass yields and low photosynthetic efficiency, have largely prevented the autotrophic algal cultivation from achieving commercial success (Cheirsilp and Torpee 2012). Hence, heterotrophic cultivation has received increasing attention because it is easier to maintain and does not depend on sunlight (Brennan and Owende 2010). However, the industrialization of microalgal fermentation is restricted mostly due to the high price of glucose feedstock which accounts up to 80% of the total substrate cost. Renewable, affordable, and effective alternatives to glucose for heterotrophic cultivation of microalgae are desirable.

Recently, extensive research has been conducted on the production of value-added metabolites from microalgae using crude glycerol as feedstocks. Bioactive compounds extracted

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from microalgae, like the polyunsaturated fatty acids, pigments, and functional polysaccharides, could be used as green and natural alternatives for chemicals in health care and cosmetics. Protein is an important component in microalgal biomass and has been recommended by the World Health Organization as a well-balanced protein source for humans and animals (Joseph et al. 1960). Heterotrophic cultivation of *C. pyrenoidosa* based on straw substrate was favorable in composition. The highest protein in microalgal biomass reached to 62% under nitrogen-excessive conditions. Over 40% of amino acids in biomass belonged to essential amino acids (EAA) (Zhang et al. 2017). *Schizochytrium limacinum* has been reported to convert crude glycerol to docosahexaenoic acid (DHA) with a yield of as high as 4.91 g L^{-1} (Chi et al. 2007). Crude glycerol with lower price (approx. \$US 0.055 kg^{-1}) has become more competitive than sugars (Chen and Walker 2011). Furthermore, the abundant macro-elements (e.g., calcium, potassium, sulfur, and magnesium) in crude glycerol may be important supplementary nutrients for microalgal growth. Crude glycerol also contains surfactants which have been reported to exhibit growth-promoting effects on microorganisms. Applying crude glycerol as a carbon source for heterotrophic culture of microalgae might be a win-win strategy for both waste disposal and valuable biomass production.

Chlorella pyrenoidosa, a well-studied green microalga, has been approved as a new food source by the Food Safety Law of the People's Republic of China in 2012. Besides that, it has potential applications such as feedstock for biofuels, synthesis of bioactive compounds, and feedstocks for animal feed and live feed in aquaculture (Tu et al. 2016; Wells et al. 2017). *Coccomyxa subellipsoidea* C-169 is the first sequenced eukaryotic microalgae from the polar environment (Blanc et al. 2012). Due to its fragile cell wall and high lipid content, *C. subellipsoidea* C-169 is an attractive and promising candidate for biodiesel production (Peng et al. 2016). However, no studies have investigated the growth of these two green microalgae on crude glycerol. In the current study, the feasibility of using crude glycerol as a carbon source for heterotrophic cultivation of these two strains is examined. This study will provide a potential route to integrate the microalgal biomass production with economical crude glycerol disposal.

Materials and methods

Crude glycerol characterization

The trace elements in crude glycerol were determined by inductively coupled plasma (ICP) (Linge 2010). The parameters were as follows: radio frequency power 1050 kW, atomized gas pressure 4.25×10^6 Pa, the auxiliary airflow speed 1 L min^{-1}, the sample flow rate 1.70 mL min^{-1}, the sample washed for 1 min, the high wave scanning 5 s, and the low wave scanning 30 s. Glycerol and methanol content were determined by colorimetric methods (Anthon and Barrett 2004).

Microalgae strains and seed culture conditions

The green microalga *Chlorella pyrenoidosa* SJTU-2 was provided by Professor Feng Chen of Peking University; *Coccomyxa subellipsoidea* C-169 was from the Japan National Institute of Environmental Research (NIES), Japan. Crude glycerol was collected from Guangdong Wuzhou Pharmaceutical Co., Ltd.

For cultivation, 250-mL Erlenmeyer flasks with a working volume of 100 mL were used. *Chlorella pyrenoidosa* and *C. subellipsoidea* were selected from single colonies and cultured in basal medium with 10 g L^{-1} glucose. The initial concentrations of inoculated microalgae 0.21 g L^{-1}. After inoculation, the culture was placed in a 150-rpm, 28 ± 1 °C dark shaking chamber for 8 days. All treatments were carried out in triplicate.

The dry biomass concentration was calculated by gravimetric analysis. Two milliliter of individual culture liquid was centrifuged for 3 min at 8000 rpm, then washed three times with distilled water and dried in an oven at 60 °C to a constant weight.

Heterotrophic culture

Heterotrophic growth was carried out by grown algae species in modified basal medium (Ogbonna et al. 1997) containing 1250 mg L^{-1} $NaNO_3$, 1250 mg L^{-1} KH_2PO_4, 1000 mg L^{-1} $MgSO_4 \cdot 7H_2O$, 500 mg L^{-1} EDTA (disodium salt), 114.2 mg L^{-1} H_3BO_3, 111 mg L^{-1} $CaCl_2$, 4.98 mg L^{-1} $FeSO4 \cdot 7H_2O$, 8.82 mg L^{-1} $ZnSO_4 \cdot 7H_2O$, 1.42 mg L^{-1} $MnCl_2 \cdot 4H_2O$, 1.19 mg L^{-1} $NaMoO_4 \cdot 2H_2O$, 1.57 mg L^{-1} $CuSO_4 \cdot 5H_2O$, and 0.49 mg L^{-1} $Co(NO_3)_2 \cdot 6H_2O$. The pH was adjusted to 6.1 ± 0.1. Different carbon sources (glucose, pure glycerol, and crude glycerol) were added to the medium.

Analysis of protein, polysaccharide, and fatty acid profiles

Lyophilized biomass (20 mg) was prepared in a 2-mL centrifuge tube and then completely disrupted by a bead beater with 50-mg ceramic beads. After that, the tube was cooled in liquid nitrogen. The supernatant extraction solvent-containing protein was collected by centrifugation. Protein was determined by the Bradford method (Bradford 1976).

The content of intracellular polysaccharides was measured with phenol sulfuric acid (Xi et al. 2010); the preprocessing method was the same as for intracellular protein. The supernatant was collected for polysaccharide determination.

Table 1	The chemical elements and components of crude glycerol		
Element	ppm	Components	% (W/W)
Phosphorus	647.35 ± 45.28	Glycerol	75 ± 0.83
Calcium	84.11 ± 10.47	Methanol	8.9 ± 0.66
Potassium	1044 ± 127.22	Ash	4.1 ± 0.09
Magnesium	5.35 ± 0.78	Moisture	11.27 ± 1.77
Sulfur	423 ± 17.36		
Sodium	6359.12 ± 473.05		
Copper	1.28 ± 0.05		
Iron	9.88 ± 0.86		
Lead	0.9 ± 0.04		
Zinc	2.1 ± 0.33		

Values are means $\pm$ SD ($n = 3$)

The determination of fatty acid composition was carried out by GC-MS. Fatty acid methyl esters (FAMEs) were prepared according to the previous procedure (Lu et al. 2012). Nonadecanoic acid (C19:0) (Sigma, USA) was added in each sample as internal standard. The chromatographic parameters were as follows: DB-5 column (30 m × 0.25 mm), inlet temperature at 260 °C, programmed temperature rise, 2 min at 60 °C, 30 min^{-1} rise to 120 °C, 1.5 min^{-1} increased to 250 °C retained for 2 min, high-purity helium gas, and column flow rate was 1.2 mL min^{-1}. Mass spectrum conditions: the ionization mode was EI, and the ionization voltage was 70 EV.

Statistics

All values in figures and tables are shown as mean ± standard deviation (SD). One-way ANOVA was used to evaluate the mean difference between groups. A value of $p < 0.05$ was considered statistically significant.

Results

Characteristics of crude glycerol

Table 1 shows the elements and components of crude glycerol determined by inductively coupled plasma. As expected, sodium and potassium were the main elements in crude glycerol, accounting for 6359 and 1044 ppm, respectively. Additionally, it is worth noting the high levels of elements such as phosphorus (647 ppm) and sulfur (423 ppm) in the crude glycerol, which are essential nutrients in cell proliferation. The crude glycerol obtained during biodiesel production from soybean oil had the following chemical composition (w/w): glycerol 75%; methanol 8.9%; water 11.27%; other impurities in ash such as non-glycerol organic matter and salts, about 3–4%.

Growth of *C. pyrenoidosa* and *C. subellipsoidea*

Different concentrations of crude glycerol affected the growth of *C. pyrenoidosa* and *C. subellipsoidea* (Fig. 1). A lag phase in *C. pyrenoidosa* growth was observed for all crude glycerol concentrations in the first 3 days. There was a sharp increase in biomass from day 3 to day 6 in the medium with an initial glycerol concentration of 5 g L^{-1} and this increase was significantly higher than that at 10 g L^{-1} ($P < 0.05$). Maximum biomass concentrations of *C. pyrenoidosa* (6.25 g L^{-1}) and *C. subellipsoidea* C-169 (4.97 g L^{-1}) were obtained in the stationary growth phase in the media containing 5 and 10 g L^{-1} crude glycerol, respectively. Rapid initial growth was observed for *C. subellipsoidea* at all crude glycerol concentrations. During the first 5 days, the growth curves of *C. subellipsoidea* were very similar in all media with different concentrations of crude glycerol. The maximum biomass concentration of *C. subellipsoidea* C-169 (7.62 g L^{-1}) in the
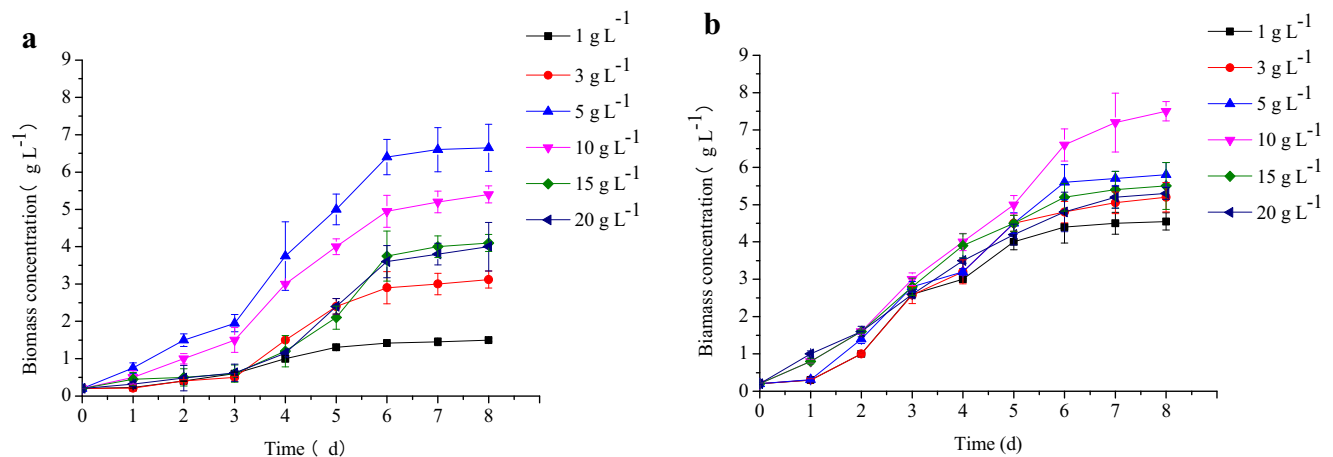

Fig. 1 Growth curves of *C. pyrenoidosa* (**a**) and *C. subellipsoidea* C-169 (**b**) with different concentrations of crude glycerol. Error bars are shown as ± SD ($n = 3$)
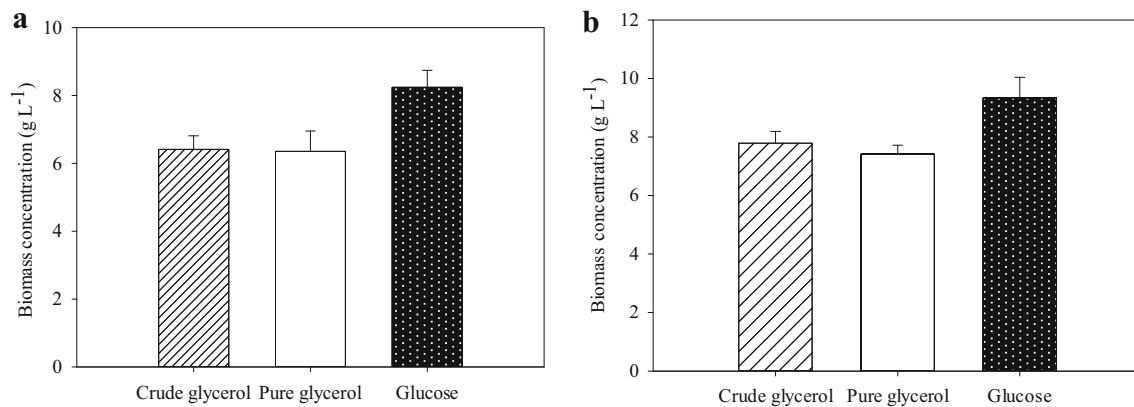
Fig. 2 Biomass concentration of *C. pyrenoidosa* (**a**) and *C. subellipsoidea* C-169 (**b**) under heterotrophic cultivation with different carbon sources. Error bars are shown as ± SD (*n* = 3)

medium containing 10 g L^{-1} crude glycerol was 34.38% higher than that in the medium with a crude glycerol concentration of 20 g L^{-1}.

The biomass concentration obtained in the culture grown with crude glycerol was less than that in glucose, whereas there is no significant difference in biomass concentration of *C. pyrenoidosa* between crude glycerol and pure glycerol (Fig. 2). Likewise, the highest biomass concentration of *C. subellipsoidea* C-169 was in basal medium with 10 g L^{-1} glucose.

Proximate composition of *C. pyrenoidosa* and *C. subellipsoidea*

The highest protein content of *C. pyrenoidosa* was 19.37% in the group with 5 g L^{-1} crude glycerol, which was significantly higher than that of glucose (*P* < 0.05) (Fig. 3). A similar result was obtained in *C. subellipsoidea* where the highest protein content was 15.61% obtained in the basal medium with 10 g L^{-1} crude glycerol, whereas for the other two carbon sources the protein contents were 12.44 and 13.11%.
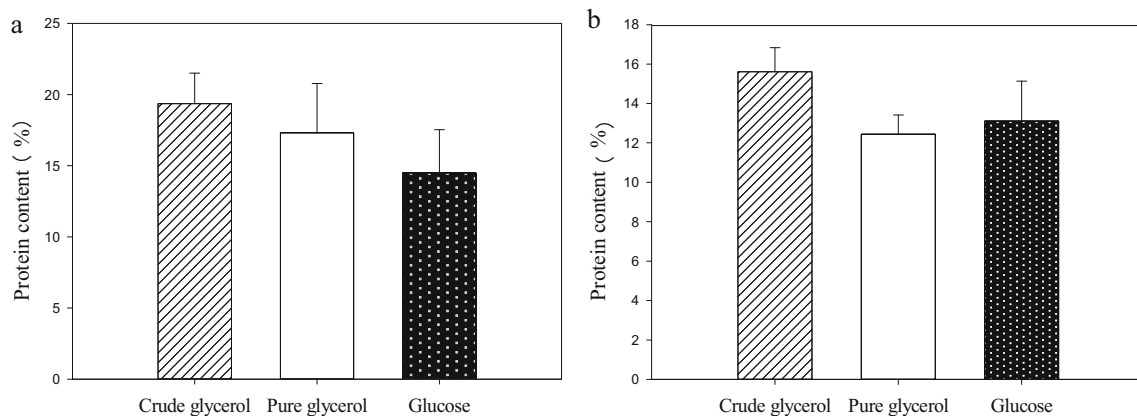
The intracellular polysaccharide contents of *C. pyrenoidosa* and *C. subellipsoidea* under heterotrophic cultures with different carbon sources are shown in Fig. 4. *Chlorella pyrenoidosa* grown in pure glycerol contained more intracellular polysaccharide (24.31%) than *C. subellipsoidea* (15.13%). Generally, there was no significant effect of glucose and crude glycerol on the accumulation of polysaccharide in the two microalgae.

The total lipid contents of *C. pyrenoidosa* cultured in crude glycerol, pure glycerol, and glucose were 28.66, 26.37, and 30.24%, respectively. The total lipid content in *C. pyrenoidosa* was not significantly affected by the addition of 5.0 g L^{-1} crude glycerol or glucose (*p* > 0.05). The total lipid contents of *C. subellipsoidea* cultivated in the three different carbon sources were 20.41, 18.87, and 22.37%, respectively. The total lipid content did not differ statistically between the crude glycerol and glucose groups.

Fatty acid composition

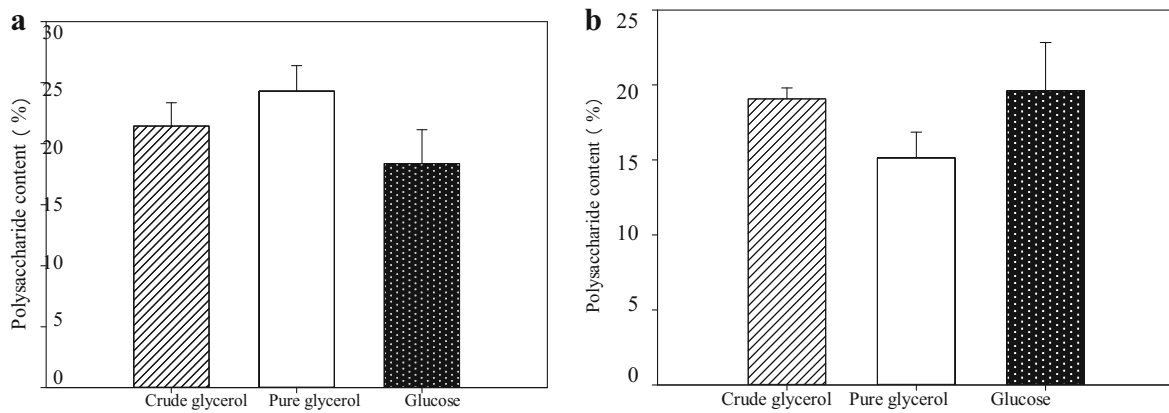The fatty acid composition of algae under heterotrophic condition with crude glycerol is shown in Table 2. *Chlorella*

Fig. 3 Intracellular protein production of *C. pyrenoidosa* (**a**) and *C. subellipsoidea* C-169 (**b**) under heterotrophic cultivation with different carbon sources. Error bars are shown as ± SD (*n* = 3)

Fig. 4 Intracellular polysaccharide production of *C. pyrenoidosa* (**a**) and *C. subellipsoidea* C-169 (**b**) under heterotrophic cultivation with different carbon sources. Error bars are shown as ± SD ($n = 3$)

pyrenoidosa and *C. subellipsoidea* grown in 5 and 10 g L^{-1} crude glycerol, respectively, were used for analysis due to the maximum biomass obtained in the different conditions. The most abundant fatty acids in the two microalgae were palmitic acid (16:0), linoleic acid (18:2), and linolenic acid (18:3), accounting for over 85% of the total fatty acids. The proportions of total unsaturated fatty acids in *C. pyrenoidosa* and *C. subellipsoidea* accounted for 75.59 and 69.57%, respectively. Palmitic acid (16:0) predominates the fatty acid profiles in the two microalgae.

Discussion

There is a slight variation in the composition of crude glycerol depending on the different industrial processes. Generally, biodiesel-generated crude glycerol with various physical properties contains many impurities such as alcohol, spent catalyst, ash, and fatty acids in different proportions. The color of crude glycerol ranges from yellow to dark brown, which contains salt and free fatty acid. According to Table 1, sodium and potassium occupied a large proportion of chemical elements in the crude glycerol. Hu et al. (2012) reported that the glycerol

content in the crude glycerol generated from different types of feedstock ranged from 60 to 70%. In the current study, glycerol accounted for 76% in crude glycerol, which could be considered as a high content. According to the literature, high concentrations of crude glycerol (over 15 g L^{-1}) showed inhibitory effects on microalgal growth, which might be caused by the presence of methanol, ash, and salinity. For example, high concentration of methanol has been reported to have unfavorable effects on the growth of algae (Nakai et al. 1999). Salt stress can interfere with physiological processes of microalgae inhibiting algal growth and photosynthesis (Xia et al. 2004). On the other hand, trace elements such as iron, magnesium, and zinc may promote cell growth. Magnesium is an important component of chlorophyll and an activator of many enzymes (Kaplan et al. 1986). Iron is a component of photosystem II and a co-factor for some enzymatic reactions (Quigg 2016). Liu et al. (2008) found that supplementation of chelated Fe^{3+} in the late exponential growth phase increased the final cell density and induced lipid accumulation in *C. vulgaris*.

In the current study, the highest biomass concentration of *C. pyrenoidosa* was 6.25 g L^{-1}. Similar results were obtained of *C. vulgaris* under mixotrophic condition by Kong et al.

Table 2 Fatty acid profiles of *C. pyrenoidosa* and *C. subellipsoidea* C-169 cultured in crude glycerol medium

Fatty acid	Composition of total fatty acids (mg g⁻¹)		Fatty acid content (%)	
	C. pyrenoidosa	*C. subellipsoidea*	*C. pyrenoidosa*	*C. subellipsoidea*
C16:0	17.35 ± 0.31	18.12 ± 0.08	24.41 ± 2.01	30.43 ± 2.76
C16:2	6.34 ± 0.09	5.68 ± 0.22	8.92 ± 0.41	9.54 ± 0.32
C18:1	–	2.31 ± 0.07	–	3.88 ± 0.54
C18:2	12.37 ± 1.02	17.13 ± 1.33	17.4 ± 0.99	28.78 ± 1.97
C18:3	35.02 ± 0.73	16.3 ± 0.89	49.27 ± 2.33	27.38 ± 0.88
CN			49.14	50.29

CN cetane numbers
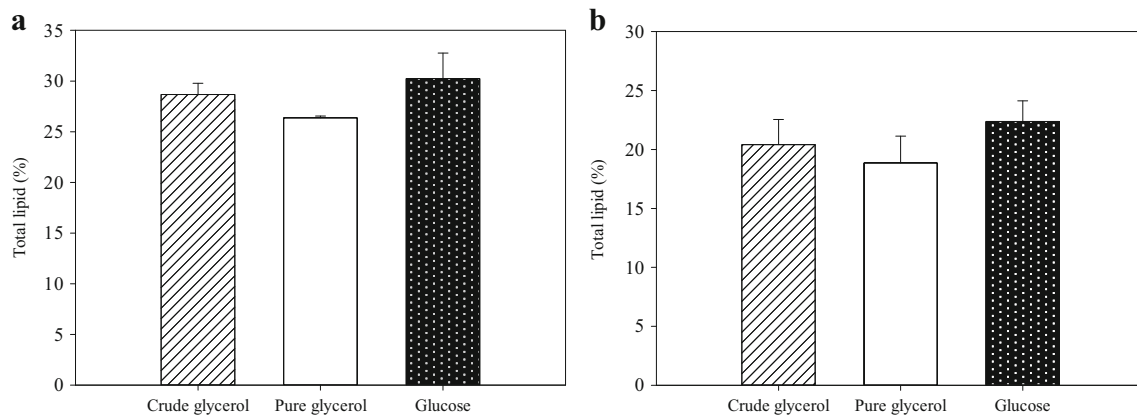
Values are means ± SD ($n = 3$)

Fig. 5 Total lipid production of *C. pyrenoidosa* (**a**) and *C. subellipsoidea* C-169 (**b**) under heterotrophic cultivation with different carbon sources. Error bars are shown as ± SD ($n = 3$)

(2013). Compared to autotrophic condition, the addition of glycerol at lower concentration to the culture has a positive effect on biomass production. There is limited information on the feasibility of *C. subellipsoidea* C-169 as a potential strain for lipid production. Our results show that *C. subellipsoidea* C-169 has similar growth rates as *Chlorella* sp.

Many algal species can grow mixotrophically and heterotrophically with the addition of various carbon sources (Neilson et al. 1973). Glucose is the most widely used organic carbon source to cultivate microalgae in heterotrophic conditions (Mohan et al. 2015). As shown in Fig. 5, compared to pure glycerol and waste glycerol, glucose was advantageous for total lipid accumulation in algal cell. Nevertheless, in comparison to pure glycerol and glucose, a significantly higher protein content was achieved ($p < 0.05$) in cultures utilizing crude glycerol. Therefore, in order to obtain higher intracellular protein in *C. pyrenoidosa* and *C. subellipsoidea* C-169 in heterotrophic culture, basal medium supplemented with 5 and 10 g L^{-1} crude glycerol is recommended.

Numerous heterotrophically grown algae have been reported as potential sources of biodiesel. For example, *Chlorella vulgaris* (Sharma et al. 2016) and *Chlorococcum* sp. (Sabeela Beevi and Sukumaran 2015) can accumulate large amount of lipids when the culture medium is supplemented with glycerol as carbon source. The properties of biodiesel are highly related to the fatty acid profile. The property of biodiesel can be greatly affected by the composition of fatty acid methyl esters, such as the carbon chain length and the unsaturation degree. For instance, the presence of unsaturated bonds will reduce the melting point of biodiesel and increase its low-temperature fluidity. Excess double bonds also affect its stability and combustion property (Knothe 2013). According to the European standards (Han et al. 2016), unsaturated fatty acids with four or more double bonds are easily to be oxidized during storage, which is unfavorable for biodiesel production. The fatty acid composition of the heterotrophic growth of *C. pyrenoidosa* and *C. subellipsoidea* C-169 using crude glycerol is shown

in Table 2. In addition, the quality of biodiesel is highly related to the cetane number, which influences the ignition quality in engines. In the current study, the cetane numbers of *C. pyrenoidosa* and *C. subellipsoidea* C-169 were 49.14 and 50.29, which suggested that the fatty acids of these two microalgae might be suitable for biodiesel production. It is also noteworthy that the proportion of linolenic acid (18:3) content of *C. pyrenoidosa* is above (49.27% of total fatty acids), which is higher than other algae cultivated with crude glycerol (Liang et al. 2009). Linolenic acid is an essential fatty acid needed for human health and has been reported to have anti-cancer, neuro-protective, cardiovascular-protective, anti-osteoporotic, and antioxidative, anti-inflammatory effects (Callaway et al. 2005; Moranis et al. 2012; Kim et al. 2014). Palmitic acid has been utilized as cosmetics, food additives, and lubricant, which have wide broad application prospects in various areas (Rose-Monde and Sébastien 2015; Hurtado-Benavides et al. 2016).

In conclusion, biodiesel-derived crude glycerol was demonstrated as an effective, low-cost, and renewable carbon substrate for heterotrophic growth of microalgae and value-added metabolite production. Compared with pure glycerol and glucose, crude glycerol is more beneficial to protein accumulation in the algae cells. The lipids from the microalgae growing on crude glycerol contained C16 and C18 as the main fatty acid components. Additionally, he cetane numbers of the two investigated algae species complied with the requirements of the Standard B100 (Lu et al. 2016) (> 49.0). This study provides new insights into the integration of economical cultivation of heterotrophic microalgae with industrial waste disposal.

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