Biotechnol Lett
DOI 10.1007/s10529-011-0672-y

ORIGINAL RESEARCH PAPER

Biomass and lipid production of heterotrophic microalgae *Chlorella protothecoides* by using biodiesel-derived crude glycerol

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Received: 15 April 2011 / Accepted: 1 June 2011

Abstract Microalgal lipids may be a more sustainable biodiesel feedstock than crop oils. We have investigated the potential for using the crude glycerol as a carbon substrate. In batch mode, the biomass and lipid concentration of *Chlorella protothecoides* cultivated in a crude glycerol medium were, respectively, 23.5 and 14.6 g/l in a 6-day cultivation. In the fed-batch mode, the biomass and lipid concentration improved to 45.2 and 24.6 g/l after 8.2 days of cultivation, respectively. The maximum lipid productivity of 3 g/l day in the fed-batch mode was higher than that produced by batch cultivation. This work demonstrates the feasibility of crude biodiesel glycerol as an alternative carbon substrate to glucose for microalgal cultivation and a cost reduction of carbon substrate feed in microalgal lipid production may be expected.

Keywords Biodiesel · *Chlorella protothecoides* · Crude glycerol · Heterotrophic growth · Lipid productivity

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Introduction

Biodiesel production using microbial lipid, which is also known as single cell oil (SCO), has attracted attention during the past decade as a sustainable and biodegradable fuel (Li et al. 2008; Meng et al. 2009). During the biodiesel production process, triacylglycerols are mixed with alcohol and catalysts to produce fatty acids esters, with crude glycerol as a primary by-product. In general, for 10 lbs (~4.5 kg) of biodiesel produced, approx. 1 lb (~0.45 kg) crude glycerol is produced as a co-product. As more crude glycerol was continuously generated over the past decade, a dramatic decrease in crude glycerol (80% purity) price, from $US 0.25/lb to $US 0.025/lb between 2004 and 2005, has resulted (Yazdani and Gonzalez 2007). Besides, the cost to purify this product to commercial pharmaceutical-grade is high, approx. $US 0.2/lb (Chi et al. 2007). This refining process is cost-prohibitive for both small and medium-scale biodiesel plants (Haas et al. 2006). However, at lower prices of crude glycerol (approx. $US 0.025/lb), the cost becomes very competitive with sugars, such as glucose, that are used in the production of biomass and lipid production by oleaginous microorganisms. In addition, converting crude glycerol to value-added products provides an alternative for crude glycerol disposal for its surplus problems.

Before the crude glycerol can be considered as a potential carbon substrate, characterization of its physical, chemical and nutritional properties is necessary. Thompson and He 2006 reported the distinguishing features crude glycerol obtained from different feedstocks, including rapeseed, canola, crambe, soybean, and recycled cooking oil. The composition of crude glycerol varies depending on two factors: feedstock and biodiesel production conditions. Most biodiesel plants utilize a 6:1 molar ratio of alcohol to oil, which is an excess of 100% alcohol to efficiently drive the reaction to completion (Ma and Hanna 1999). Most of the excess alcohol (up to 80%) will end up in the glycerol layer after the reaction. Several publications have reported the utilization of crude glycerol for microbial lipid production through microalgae such as *Schizochytrium limacinum SR21* (Chi et al. 2007; Liang et al. 2010b), fungi such as *Pythium irregulare* (Athalye et al. 2009; Dong and Walker 2008), and *Aspergillus niger* (André et al. 2010), and yeast such as *Yarrowia lipolytica* (André et al. 2009) and *Cryptococcus curvatus* (Liang et al. 2010a).

Microalgal biodiesel is a second-generation biofuel. It has the distinct advantage of avoiding threatening food supplies and biodiversity (Cheng et al. 2009b). Microalgal lipids are good candidates for biodiesel production because of their higher lipid content, shorter time growth cycle, and need for less land compared to other energy crops (Milne et al. 1990). High biomass and lipid production under heterotrophic conditions have been achieved with *Chlorella protothecoides* by using different carbon sources (Miao and Wu 2006; Xu et al. 2006). Xu et al. (2006) reported that *C. protothecoides* could accumulate lipid as high as 55% of the cell dry weight after six days of cultivation with feeding of corn powder hydrolysate in fermentors. Through nitrogen limitation, the lipid content of the heterotrophic *C. protothecoides* was about four times higher than that in photoautotrophic *C. protothecoides* (Miao and Wu 2004; Xu et al. 2006).

The feedstock charge and operation expense are two major components of biodiesel production cost. The cost of feedstock accounts for 60–70% of the total cost of the biodiesel (Huang et al. 2010). Furthermore, microalgal biodiesel production is restricted mostly due to the high cost of the fermentation substrate, while the cost of glucose accounted for 80% of the total medium cost (Li et al. 2007). For realizing commercial production of biodiesel from heterotrophic *C. protothecoides* lipids, lower cost and effective alternatives to glucose are desirable. In this study, we have evaluated the growth of *Chlorella protothecoides* on three carbon substrates: glucose, pure glycerol, and crude glycerol from biodiesel production, and also to perform fed-batch fermentations to improve biomass and lipid production by using a lower-cost carbon substrate, namely crude glycerol. This is also the first study to investigate the cultivation of *C. protothecoides* using crude glycerol for lipid production.

Materials and methods

Materials

All chemicals were obtained commercially and of analytical grade. Commercial crude glycerol was obtained from Southeast Biodiesel-North Charleston plant (N. Charleston, SC, USA).

Microorganism and inoculum preparation

Chlorella protothecoides UTEX 256 was from the culture collection of algae at the University of Texas (Austin, TX). The components of basal culture medium are as follows (per liter): 0.7 g KH_2PO_4, 0.3 g K_2HPO_4, 0.3 g $MgSO_4 \cdot 7H2O$, 25 mg $CaCl_2 \cdot H_2O$, 25 mg NaCl, 3 mg $FeSO_4 \cdot 7H_2O$, 0.01 mg vitamin B_1, and 1 ml A5 solution. For the preparation of inoculum, microalgal cells were suspended in liquid basal medium supplemented with glucose at 30 g/l and yeast extract at 4 g/l. The initial pH of the medium was adjusted to 6.8. The cultures were incubated at 28°C with shaking at 200 rpm in the dark. After 4 days, heterotrophic cells were used for further experiments.

Crude glycerol characterization

Crude glycerol from Southeast Biodiesel (SE crude glycerol) was derived from poultry fat by using alkali-catalyzed trans esterification of oil (sodium methylate as catalyst) with methanol to produce the biodiesel. The characteristics of SE crude glycerol are shown in Tables 1 and 2. Glycerol and methanol concentrations were determined by HPLC described below. Samples were dried at 105°C for 48 h to

Table 1 South Ester crude glycerol composition

Composition	% (w/w)
Glycerol	62 ± 0.62
Methanol	22.6 ± 0.22
Ash	2.4 ± 0.0
Total nitrogen	0.24 ± 0.01
Water	8 ± 0.54
Other impurities	4.8 ± 0.51

Data are reported as means of three replicates ± standard deviation

Table 2 Elemental composition of South Easter crude glycerol by ICP analysis

Elements	Parts per million (ppm)
Aluminum	14.7 ± 8.81
Arsenic	ND[b]
Boron	6.4 ± 1.64
Calcium	123 ± 12.2
Cadmium	ND[b]
Chromium	0.22 ± 0.11
Copper	0.74 ± 0.2
Iron	13 ± 3.1
Potassium	607 ± 72.8
Magnesium	10.4 ± 0.92
Manganese	0.12 ± 0.03
Molybdenum	1.3 ± 0.27
Sodium	8573 ± 960
Nickel	ND[b]
Phosphorus	ND[c]
Lead	1.2 ± 0.66
Sulfur	217 ± 29.4
Selenium	ND[b]
Zinc	1.8 ± 0.21

[a] Data are means of three replicates ± standard deviations

[b] Below detection limit

[c] Above detection limit

evaluate the moisture content. The water content was determined by subtracting methanol content from the moisture content. Total nitrogen was determined according to the Kjehldahl method. The ash content was determined by heating the sample at 600°C for 2 h. The elemental analysis was performed with an inductively coupled plasma (ICP) method according to wet ash digestion procedure from the Agricultural Service Laboratory of Clemson University (Clemson, USA).

Batch fermentations

The cultures were carried out in 500 ml shake-flasks containing 200 ml basal medium supplemented with 30 g carbon substrate/l (glucose, pure glycerol or SE real glycerol) and 4 g yeast extract/l. The initial pH value of medium was adjusted to 6.8. The cultures were incubated at 28°C in the dark with shaking at 200 rpm. Inocula were at 10% (v/v).

Fed-batch fermentations with pH control

Heterotrophic, fed-batch fermentation was performed in a 5.5 l working volume bioreactor (BioFlo 310, New Brunswick Scientific, USA) containing 2 l basal medium with 30 g carbon substrate/l (glucose, pure glycerol or real glycerol) and 4 g yeast extract/l. The stock solution containing 150 g carbon substrate/l (glucose, pure glycerol, SE glycerol separately) and 15 g yeast extract/l was added to maintain the concentrations of yeast extract and carbon substrate at desired levels. The pH was maintained at 6.8 by automatic addition of 0.5 M KOH and 0.5 M H_2SO_4. Temperature was maintained at 28°C. The dissolved O_2 concentration was maintained at 20–50% air saturation by airflow and agitation speed. Aeration rate and the agitation speed were variable and initially set at 30 l/h and 100 rpm, respectively. Samples were taken at intervals for determination of biomass, carbon substrate.

Analytical procedures

Cell growth was monitored from the OD_{540} values and correlated with cell dry weight (CDW) determined directly. The specific growth rate was calculated during the logarithmic phase. Glucose, glycerol and methanol concentrations were determined by HPLC equipped with a pulsed refractive index detector. Analytes were separated using an Aminex HPX-87H column at 60°C with 50 mM H_2SO_4 as the mobile phase at 0.8 ml/min. The lipid content was determined by extracting the biomass with hexane. The method was modified from the previous studies (Cantrell and Walker 2009; Dong and Walker 2008). Briefly, the microalgal cells were harvested by centrifugation and washed with distilled water and the biomass then lyophilized to a constant weight. The dried biomass was extracted in a 50 ml centrifuge tube using 20 ml hexane, homogenized with a homogenizer for 5 min, kept at 55°C for 5 min, and then homogenized for 5 min. The slurry was centrifuged and supernatant then transferred to another centrifuge tube. This extraction procedure was repeated twice using 5 ml hexane separately for the residue until no lipid was left in the biomass. Supernatants were filtered and then evaporated using rotory evaporator. Lipid left in the tube without solvent was weighed to an accuracy of 0.1 mg.

Experimental data was subjected to analysis of least significant difference test (LSD) of multiple comparisons at 95% confidence ($\alpha = 0.05$) by Statistical Analysis System (SAS, SAS Institute, USA).

Results and discussion

SE crude glycerol characterization

The characteristics and compositions of crude glycerol vary mainly depending on the different lipid feedstocks and the biodiesel production process conditions (Thompson and He 2006). The SE crude glycerol had a density of 1.103 g/ml, and was dark brown. Its analysis is shown in Table 1. Glycerol purity of crude glycerol substrates used for microbial fermentations range from 42.3% (Liang et al. 2010b) to 85% (Mu et al. 2006), which probably results from different glycerol purification procedures and biodiesel production conditions applied by biodiesel plants. Table 2 shows ICP elemental analysis results of the SE crude glycerol. Sodium was the major element in SE crude glycerol, which is attributed to Southeast Biodiesel using sodium methylate as a catalyst. Trace amounts of iron were also found in the SE crude glycerol, and the effect of iron on growth and lipid accumulation in marine microalgae *Chlorella vulgaris* has been investigated (Liu et al. 2008). Total lipid content (% dry wt) of *C. vulgaris* in media supplemented with 1.2×10^{-8} mol $FeCl_3$/l was higher (51% increase) than that in media without $FeCl_3$. The standard deviations of sodium, potassium, aluminum and sulfur were relatively high, indicating wide fluctuations of concentrations of these elements in each individual sample.

Effect of different carbon substrates on batch fermentation of *Chlorella protothecoides*

Previous studies have shown microalgae would increase cellular lipid level from 20–30 to 60–70% through nitrogen deficiency but at the expense of greatly reduced growth rates, resulting in small increase of overall lipid productivity (Hsieh and Wu 2009; Xiong et al. 2008). Lipid productivity has been regarded as the primary concern for microalgal biodiesel production. To study the potential use of the crude glycerol as a carbon substrate for production of biomass and lipid by the microalgae *C. protothecoides* batch fermentation experiments were performed without pH control. Fig. 1(a–c) shows the time course of biomass, substrate consumption and pH condition of the microalgal cultures with 30 g glucose/l, pure glycerol and real glycerol (SE crude glycerol) being used as substrates in the medium. As shown in Fig. 1(b, c), the cells grew well in pure and crude glycerol and the biomass reached peak levels in 5–6 days. Crude glycerol consumption was low during first two days (0.85–0.46 g substrate consumed/g cells), but after that the cells became acclimated and then the substrate uptake increased to 0.91–1.18 g substrate consumed/g cells during days three through six. Glucose was completely consumed in four days which resulted in termination of growth due to lack of substrate (Fig. 1 a). The results suggested that microalgae *C. protothecoides* could consume glucose much faster than other substrates. The pH value of both glucose and pure glycerol cultures decreased after day one, suggesting biosynthesis and accumulation of organic acids or acidic wastes in the medium. The pH was stable in the SE glycerol culture, probably because of the buffering capacity of this medium.

The specific cell growth rates in glucose, pure glycerol and SE crude glycerol media were 0.83, 0.7, and 0.74 day^{-1}, respectively. Maximum biomass concentration and biomass productivity of the crude glycerol culture were higher than those of the pure glycerol culture (Table 3). Higher biomass concentration and biomass productivity probably were the direct result of residual nitrogen source and other nutrients presents in the crude glycerol medium. Beneficial effects of small amounts of impurities on microalgae growth have been reported (Chi et al. 2007).

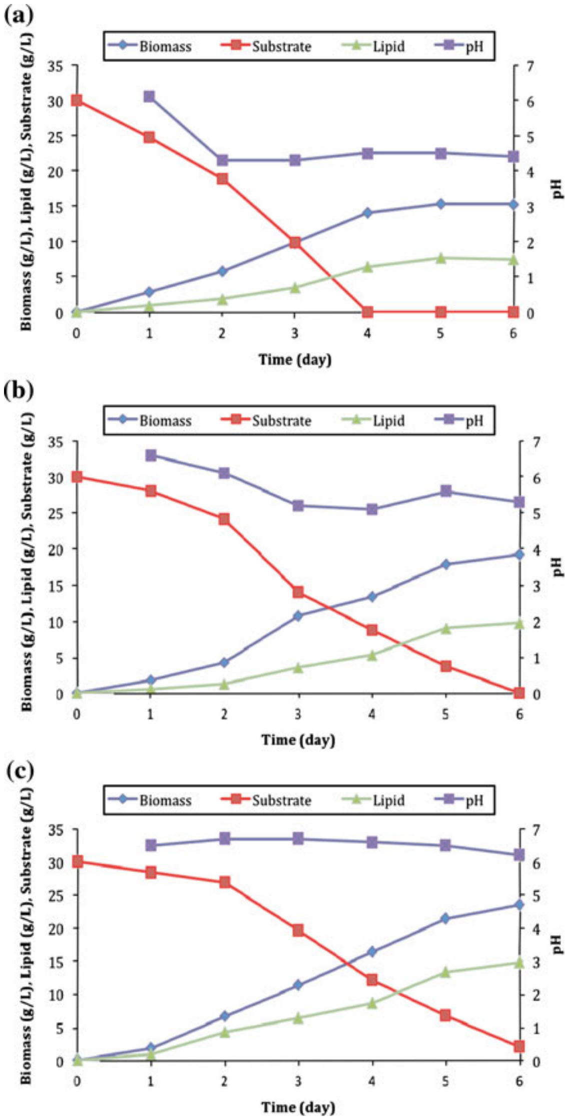

Fig. 1 Cell growth, substrate consumption and pH condition of *Chlorella protothecoides* **a** glucose, **b** pure glycerol and **c** crude glycerol in batch fermentation. Each point is the mean value of duplicate of two independent measurements

Chlorella protothecoides has a strong tolerance to the salinity as high as seawater (35 g NaCl/l) (Heredia-Arroyo et al. 2010). Thus, the presence of the impurities in the crude glycerol medium should not be detrimental to cell growth and lipid production. The lipid productivity of crude glycerol culture was also higher than that of pure glycerol culture because of both higher biomass and lipid content in crude glycerol culture. The lipid concentration and productivity of crude glycerol culture reached 14.6 and 2.4 g/l day, respectively. Figure 2 shows comparison of biomass and lipid productivity in batch fermentations by *C. protothecoides* with different carbon substrates after four days of cultivation. No significant difference of biomass concentration and lipid productivity obtained for glucose culture and pure glycerol culture was observed ($\alpha = 0.05$), but those obtained from the crude glycerol culture were significantly greater. This result indicated that growth of *C. protothecoides* on pure glycerol had similar effects on biomass and lipid productivity as those from glucose in the batch fermentation. Another study also found that growth of *Chlorella vulgaris* on pure glycerol had similar dose effects as those grown on glucose (Liang et al. 2009).

Chlorella protothecoides can grow on a variety of carbon substrates such as glucose (Shen et al. 2010; Xiong et al. 2008; Xu et al. 2006), fructose (Gao et al. 2009), sucrose (Gao et al. 2009), glycerol (Heredia-Arroyo et al. 2010), acetate (Heredia-Arroyo et al. 2010) and reducing sugars from Jerusalem artichoke and sugar cane (Cheng et al. 2009a, b). Crude glycerol from the biodiesel production was used for lipid production by *Schizochytrium limacinum* SR21 (Liang et al. 2010b), *Yarrowia lipolytica* (André et al. 2009) and *Mortierella isabellina* ATHUM 2935 (Papanikolaou et al. 2008). The results obtained in this work indicated that crude glycerol was a potentially good carbon substrate for microalgal *C. protothecoides* cultivation. Crude glycerol was used directly without any pretreatment, which would simplify the process and reduce the operating costs. Further improvements were attempted by using fed-batch fermentation for approaching higher biomass and lipid production. These results are discussed in the next section.

Effect of different carbon substrates on fed-batch fermentations of *Chlorella protothecoides*

Fed-batch cultures of *C. protothecoides* were cultivated with glucose, pure glycerol and crude glycerol as substrates in the medium. Cell growth and substrate consumption are shown in Fig. 3. In the culture with glucose as substrate (Fig. 3 a), the biomass CDW was 11.3 g/l at two days, which was higher than that from glucose batch experiment after three days cultivation. During days five and six, the cell growth rate was lower, indicating waste inhibition on cell growth. Cell growth continued due to continuous feeding of stock

Table 3 Biomass and lipid productivities of *Chlorella protothecoides* grown on different carbon substrate

Different carbon substrates	Max biomass concentration (g/l, CDW)	Biomass productivity (g/l day, CDW)	Max lipid concentration (g/l)	Lipid productivity (g/l day)
Glucose[a]	15.3 ± 0.25	3.1 ± 0.05	7.7 ± 0.31	1.5 ± 0.06
Pure glycerol[b]	19.2 ± 0.47	3.2 ± 0.07	9.8 ± 0.02	1.6 ± 0.01
Crude glycerol[b]	23.5 ± 0.74	3.9 ± 0.12	14.6 ± 0.11	2.4 ± 0.02

[a] Cell growth data from fifth day were used for calculation

[b] Cell growth data from sixth day were used for calculation

Results shown are the mean values of duplicate of two independent measurements ± standard deviations

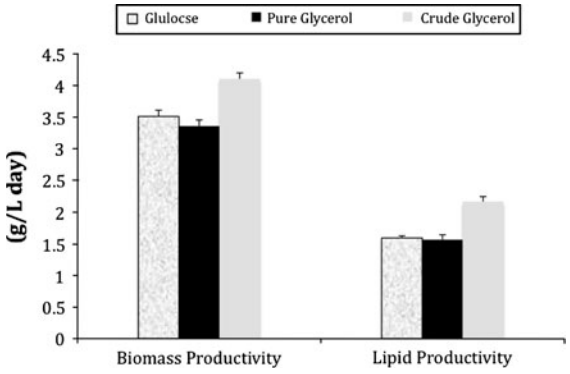

Fig. 2 Comparison of biomass and lipid productivity in shake flask fermentations by *Chlorella protothecoides* with different carbon substrate at 4 days cultivation. Data is the mean value of duplicate of two independent measurements ± standard deviation

medium to the culture. The highest biomass CDW reached was 46 g/l and lipid content was 0.53 g/g CDW over eight days (192 h). Thus, our fed-batch approach was proven effective in improving biomass production similar to the results obtained by Xiong et al. (2008) with glucose and yeast extract in the medium.

As shown in Fig. 3b, the biomass CDW reached 43.3 g/l and lipid content is 0.53 g/g CDW (Table 5) in pure glycerol fed-batch at 8.2 days (197 h). During days four and five, the cell growth rate was lower in the pure glycerol fed-batch culture than that during day one through four. Although more feedstock was added to increase glycerol concentration to 32.9 g/l at day five, the cell growth rate was still lower than that during days two through four. This result indicated the possibility of inhibition from byproducts on cell growth and not substrate limitation after day five. The dissolved O_2 level, which was controlled by airflow and agitation speed, is important in microalgal culture (Heredia-Arroyo et al. 2010; Xiong et al. 2008). The requirement of O_2 was higher during the exponential phase of all fed-batch fermentation. Operating in glucose and pure glycerol fed-batch mode, the lipid content was higher than that of shake-flask batch mode. This lower lipid content obtained in the shake-flask batch experiments may have occurred due to insufficient aeration where insufficient O_2 levels could alter gene expression, resulting in back-regulation of proteins affecting lipid accumulation and thus decreasing of its production (Ratledge and Wynn 2002). Two critical regulatory enzymes, ATP:citrate lyase (ACL) and NADPH$^+$-malic enzyme, affect lipid accumulation where strong correlation between the presence of ACL activity and the ability to accumulate lipid in yeast, fungi and other oleaginous microorganisms has been reported (Ratledge 2002, 2004). The other major factor affecting lipid accumulation and biomass production is the ratio of carbon and nitrogen (C/N) source (Cheng et al. 2009a; Shi et al. 2000). With nitrogen deprivation or limitation the microalgal cell proliferation is prevented, but lipid accumulation of oleaginous microalgae begins when an excess carbon substrate is still assimilated by the cells and is converted to triacylglycerols (Meng et al. 2009). The lipid content in *Chlorella* species can, however, be increased to 53–66% by nitrogen deprivation (Hsieh and Wu 2009; Xiong et al. 2008).

The biomass CDW reached 45.2 g/l and lipid content is 0.54 g/g CDW in crude glycerol fed-batch at 8.2 days (197 h) (Fig. 3c; Table 5). Comparing the pure glycerol and crude glycerol experiments, the results related to biomass and lipid production are similar (Table 4). However, the maximum biomass concentration and lipid productivity of the crude glycerol was higher than those of pure glycerol culture in batch mode as discussed previously (Table 3). This indicated that one of the major issues related to usage of crude glycerol could be methanol inhibition. Although methanol was partially removed from culture medium by evaporation through autoclaving, it was added back to the culture medium through the addition of feedstock in the fed batch mode (Fig. 3c). Chi et al. (2007) and Pyle et al. (2008) have reported negative effects of methanol on growth and DHA production of microalgae *Schizochytrium limacinum*. The maximum cell dry weight, DHA productivity, and cell yield of *S. limacinum* decreased as methanol concentration was increased from 0 to 20 g/l.

Comparison of results with the literature

There have been several publications on lipid accumulation in *C. protothecoides* using glucose as the sole carbon substrate for lipid production producing, which produced microalgal cultures high in both lipid content and biomass density (Li et al. 2007; Miao and Wu 2006; Xiong et al. 2008; Xu et al. 2006). Heterotrophic microalgal cultivation has received much attention due to several advantages over photosynthetic cultures. These include: (1) higher biomass concentration and lipid content could be achieved in shorter time (Xiong et al. 2008); (2) it is easier to scale-up to industrial scale because the culture of interest could be grown in traditional stirred-tank bioreactors without light limitation problems (Li et al. 2007); (3) the contamination problem from other microorganisms associated with open-pond systems could easily be solved by using enclosed bioreactors with sterilization-in-place capability. Although *C. protothecoides* grows on pure glycerol (Heredia-Arroyo et al. 2010), no study has investigated a fed-batch strategy for enhancement of

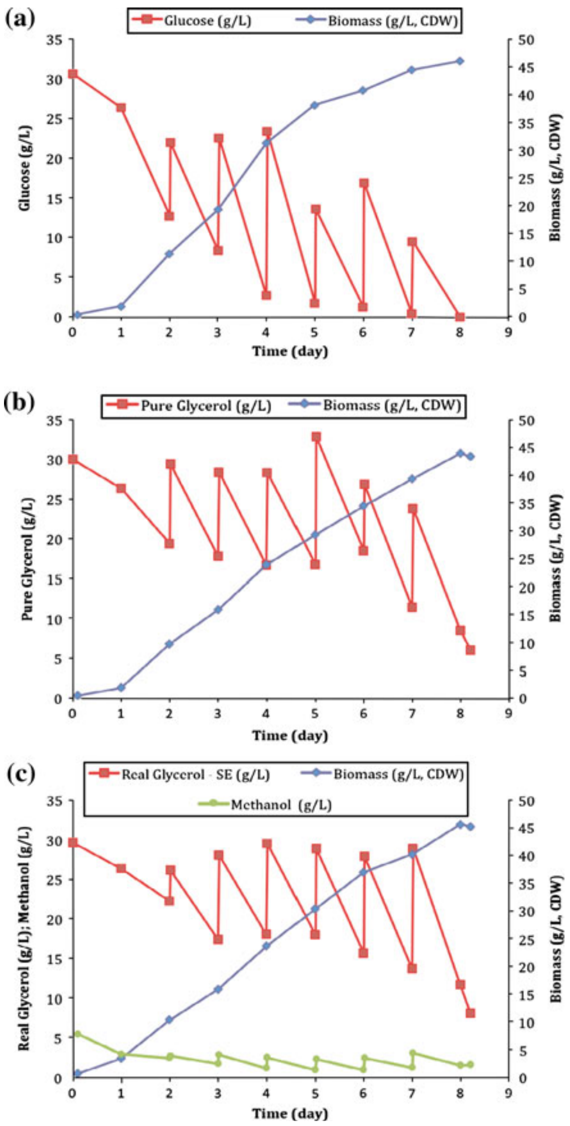

Fig. 3 Cell growth and substrate consumption of *Chlorella protothecoides* **a** glucose, **b** pure glycerol and **c** crude glycerol in fed-batch fermentation. Each point is the mean value of triplicate measurements

Table 4 Comparison of biomass lipid productivities and residual glycerol on fed-batch fermentation by *Chlorella protothecoides* using pure and crude glycerol

Different carbon substrates	Biomass concentration (g/l, CDW)	Lipid concentration (g/l)	Lipid productivity (g/l day)
Pure glycerol	43.3 ± 0.6	23 ± 0.32	2.8 ± 0.03
Crude glycerol	45.2 ± 0.85	24.6 ± 0.46	2.99 ± 0.05

Data from 8.2 days (197 h) cell growth were used for calculation

Data is the mean value of triplicate ± standard deviation

Table 5 Biomass and lipid production of heterotrophic *Chlorella protothecoides* with various substrates and culture strategies

Culture strategies	Carbon substrates (g/l)	Nitrogen Sources (g/l)	Biomass concentration (g/l, CDW)	Lipid content (g/g, CDW)	Lipid productivity (g/l-day)	Strain sources	Reference
Batch							
250 ml	40 [Glucose]	1.7 [Urea]	19.6	–	–	CSIRO CS-41	(Shi et al. 2000)
250 ml	15 [Glucose]	4 [YE]	4.2	0.13	0.13	UTEX 249	(Heredia-Arroyo et al. 2010)
	20.5 [Acetate]	4 [YE]	3.1	0.23	0.16	UTEX 249	(Heredia-Arroyo et al. 2010)
	20.4 [Glycerol]	4 [YE]	3.6	0.19	0.17	UTEX 249	(Heredia-Arroyo et al. 2010)
500 ml	10 [Glucose]	4 [YE]	3.7	0.53	–	UTEX	(Gao et al. 2009)
500 ml	10 [Glucose]	–	3.7	0.55	–	UTEX	(Xu et al. 2006)
	30 [Glucose]	1 [YE]	9.1	0.53	–	UTEX	(Xiong et al. 2008)
		4 [YE]	18	0.46	–	UTEX	(Xiong et al. 2008)
		7 [YE]	18.6	0.22	–	UTEX	(Xiong et al. 2008)
		10 [YE]	19.8	0.19	–	UTEX	(Xiong et al. 2008)
	15 [Glucose]	4 [YE]	10.4	–	–	UTEX	(Xiong et al. 2008)
	30 [Glucose]		16.3	–	–	UTEX	(Xiong et al. 2008)
	45 [Glucose]		18.2	–	–	UTEX	(Xiong et al. 2008)
	60 [Glucose]		21.1	–	–	UTEX	(Xiong et al. 2008)
500 ml	40 [Glucose]	4.2 [YE]	14.2	–	0.51	UTEX 255	(Shen et al. 2010)
500 ml	30 [Glucose]	4 [YE]	15.3	0.5	1.53	UTEX 256	This study
	30 [Pure Glycerol]	4 [YE]	19.2	0.51	1.62	UTEX 256	This study
	30 [Real Glycerol][a]	4 [YE]	23.5	0.62	2.46	UTEX 256	This study
Fed Batch							
3.7 l	[Glucose]	[Urea]	48	–	–	CSIRO CS-41	(Shi et al. 2002)
30 l			45.8	–	–	CSIRO CS-41	(Shi et al. 2002)
5 l	[Glucose]	[YE]	16	0.46	–	UTEX	(Li et al. 2007)
750 l			12.8	0.49	–	UTEX	(Li et al. 2007)
11000 l			14.2	0.44	–	UTEX	(Li et al. 2007)
5 l	[Glucose]	[YE]	3.2	0.58	–	UTEX	(Xiong et al. 2008)
			16.8	0.55	–	UTEX	(Xiong et al. 2008)
			51.2	0.50	–	UTEX	(Xiong et al. 2008)
5.5 l	[Glucose]	[YE]	46	0.53	3.04	UTEX 256	This study
	[Pure Glycerol]		43.3	0.53	2.80	UTEX 256	This study
	[Real Glycerol]*		45.1	0.54	2.99	UTEX 256	This study

[a] Real glycerol from SE crude glycerol

CSIRO CSIRO Marine Laboratory (Hobart, Australia), *UTEX* The culture collection of algae at the University of Texas at Austin (USA), *YE* Yeast extract

Table 6 Biomass and lipid production among different oleaginous species grown on crude glycerol

Strain	Culture strategies	Biomass concentration (g/l, CDW)	Biomass productivity (g/l-day)	Lipid content (g/g CDW)	Lipid productivity (g/l-day)	Reference
Chlorella protothecoides	Batch	23.5	3.9	0.62	2.4	This study
	Fed batch	45.1	5.5	0.54	2.9	This study
Cryptococcus curvatus	Fed batch	31.2	2.6	0.45	1.2	(Liang et al. 2010a)
	Improved fed batch	32.9	2.7	0.53	1.5	(Liang et al. 2010a)
Schizochytrium limacinum SR21	Batch	18	3.1	0.51	1.5	(Chi et al. 2007)
	Batch	11.5	1.9	0.5	1	(Pyle et al. 2008)
	Batch	7	1.13	–	0.74	(Liang et al. 2010b)
Yarrowia lipolytica	Continuous	8.1	–	0.43	2.6	(Papanikolaou and Aggelis 2002)

biomass and lipid production by *C. protothecoides*. To the best of our knowledge, our study is the first to investigate crude glycerol utilization for biomass and lipid production by *Chlorella* species.

Table 5 compares biomass and lipid production of heterotrophic *C. protothecoides* with various substrates and culture strategies from the literature and this study. *C. protothecoides* has been mostly cultured with glucose as carbon source and yeast extract as nitrogen source. Xiong et al. (2008) reported that yeast extract was the best nitrogen source for biomass production of *C. protothecoides* among three inorganic nitrogen sources (urea, potassium nitrate, and ammonium nitrate) and two organic nitrogen sources (glycine and yeast extract). With glucose at 30 g/l and yeast extract at 4 g/l, the biomass concentration from our results is slightly lower than that reported in the literature, but the lipid content from our results was higher. This may be due to the different *C. protothecoides* microalgal strain and inoculum conditions. Shen et al. (2010) investigated four strains of heterotrophic *Chlorella protothecoides* (UTEX 25, 31, 249 and 255) for lipid production, and UTEX 255 produced higher lipid yield and lipid/glucose ratio. Therefore, UTEX 255 was chosen as the best candidate among the four strains for lipid production. They also reported the difference of biomass concentration and lipid/glucose ratio between green-seed inoculation, obtained from autotrophic cultivation, and yellow-seed inoculation, grown from heterotrophic cultivation. Furthermore, the difference of biomass and lipid production from other literature may due to variances in basal media composition, inoculum size and cultivate conditions. As shown in Table 6, compared with other oleaginous microorganism (microalgae and yeast) grown on crude glycerol, *C. protothecoides* produces the higher biomass and lipid production in both batch and fed-batch modes. In future research, the effects of C/N ratio and optimization of the fed-batch strategy to improve higher biomass and lipid production using crude glycerol will be investigated.

Conclusion

This study indicated that (1) *Chlorella protothecoides* can use crude glycerol as a carbon substrate; (2) fed-batch mode was a better culture strategy than batch for improving biomass concentration, lipid production and crude glycerol consumption; (3) in fed-batch mode, crude glycerol (62% purity) from the biodiesel production processes could be used directly with *C. protothecoides* to obtain results similar to those with pure glycerol. Considering the high biomass and lipid production rates, a crude glycerol-to-lipid fermentation model potentially provides additional feedstock for production of biodiesel while offering a lower-cost carbon substrate and eliminating crude glycerol disposal. These criteria are important for the bioconversion of industrial byproducts into valuable products.

Acknowledgments This research was supported by Clemson University Public Service Activities and in part by the US of

Department of Energy. We also appreciate Southeast Biodiesel-North Charleston Plant (N. Charleston, SC, USA) for providing the crude glycerol samples.

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