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Oil Accumulation via Heterotrophic/Mixotrophic *Chlorella protothecoides*

Tamarys Heredia-Arroyo · Wei Wei · Bo Hu

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Abstract Microalgal oil is a potential energy source because it can be easily converted to fatty acid methyl ester or hydrocarbon type of diesel, and it is produced with relatively higher productivity compared with oil from plants and animals. Heterotrophic growth of microalgae is superior due to its high final product concentration; however, the cost of the raw materials is unacceptable if sugar is utilized as the carbon source. The aim of this study is to optimize the lipid accumulation of *Chlorella protothecoides* by using carbon sources other than glucose in heterotrophic and mixotrophic cultures. Different factors such as different carbon sources, carbon to nitrogen ratio, initial pH level, salinity, and rotational speed are studied in affecting the cell growth and the oil accumulation. Our experiments revealed that the heterotrophic and mixotrophic cultures of *C. protothecoides* grew better than autotrophic cultures. *C. protothecoides* can grow on glycerol or acetate, as well as on glucose. Several stress factors were confirmed or discovered to significantly increase the lipid content of microalgae cells. The replacement of glycerol and acetate as carbon sources for microalgae cultivations provides potential for waste utilization: glycerol from biodiesel industry and acetate from biohydrogen production.

Keywords Microalgae oil accumulation · Chlorella · Glycerol · Acetate · Bioenergy

Introduction

One of the worldwide concerns is the depletion of fossil fuels primordially because none of the existent substitute technologies can handle the current energy demand [1–4]. As

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consequences of the many applications of crude oil, with transportation as the most consuming sector, fossil fuel prices are in general increasing and so are the environmental concerns due to the greenhouse gases emissions [2]. Biofuel produced from agricultural materials offers an alternative to fossil fuels. It brings several benefits such as alleviation from foreign oil dependence, carbon neutral process without excessive greenhouse emission, and profits to local farmers and local community due to its scattered production nature [5]. However, the use of grain or soybean for biofuel production might compete with human food, which is in an intense debate recently [6, 7]. New approaches need to be developed to use an alternative source for biofuel production, such as the biomass left after harvest or the biomass produced in non-traditional agricultural land as the source for biofuel and bioproducts.

Lipid accumulation with microalgae to provide alternative oil resources is an exciting research area and is obtaining increasing attention for the biodiesel production due to its high production efficiency and less demand of agricultural land although it is still not economically feasible [4, 8–10]. Biodiesel, fatty acid methyl ester, obtained by trans-esterification of microalgae lipid with methanol or ethanol, is showing similar combustion performance as the biodiesel produced with vegetable oil or other plants oils [11]. Biodiesel is an ideal bioenergy product, which can be used in pure form (B100) or may be blended with fossil diesel at any rate. Biodiesel is around 5–8% less efficient than conventional fossil diesel; other than that, its application actually brings beneficial to the environment (except the NO_x emission). Biodiesel industry suffered for a long time due to the limited resources of raw materials such as soybean oil or vegetable oil. Different raw materials are proposed to produce biodiesel, including waste cooking oil, plant oil, etc. [12, 13], but they are still too limited to replace current fossil diesel. Oil accumulated with microalgae may have the potential to be the only final solution to the raw material shortage for the biodiesel industry [14]. Besides biodiesel, hydrocarbon type of biofuel, developed recently with direct decarboxylation of fatty acid or lipid, showed its superior characteristics to be the right candidate for the jet fuel due to its resistance of water contamination [15–17]. Microalgae can provide raw materials for this type of the fuel development.

Microalgae cells can be cultured under autotrophic, heterotrophic, and mixotrophic conditions. Autotrophic cells harvest light as the energy source and assimilate CO_2 as carbon source, heterotrophic cells utilize organic substrates as carbon and energy sources, and mixotrophic cells harvest light and use inorganic and organic substrates as energy and carbon sources [18]. The areas of current interest in microalgae research are primarily dominated in the selection of the autotrophic strains and cultivation conditions that lead to the highest lipid yields in less time [18, 19]. Heterotrophic and mixotrophic microalgae are relatively less studied due to their requirement of organic carbons, which may bring competition with human diet.

Chlorella protothecoides is a commercially important green microalga because it has the potential to serve as a source of food and energy due to its high photosynthetic efficiency, which can, in theory, reach 8% [20]. It can grow with both autotrophic and heterotrophic modes [21]. Previous studies have mentioned that *Chlorella* species are capable to grow using glucose ($C_6H_{12}O_6$), acetate ($C_2H_3O_2^-$), glycerol ($C_3H_5(OH)_3$), and other carbon sources [22–27], but to our knowledge, there is only insufficient data about their lipid productivities when cultured on these carbon sources with different culture conditions. *Chlorella* species are robust microorganisms that can grow in many conditions around the world; they can serve as an example for heterotrophic and mixotrophic growths supplied with glucose, glycerol, acetate, or other organic compounds from waste resources with zero or negative costs as carbon source to accumulate lipids for biodiesel production.

Lipid accumulation occurs within the microalgae cells, and it varies with strain and growth conditions. There are many nutritional and environmental factors controlling the cell growth and its lipid content, such as organic and inorganic carbon sources [28, 29], nitrogen source [30–32], and other essential macro- and micro-nutrients like magnesium and copper [33, 34], temperature, pH level, salinity, agitation speed (dissolved oxygen), etc. [35–37]. Since C/N ratio is generally considered to influence the cellular lipid content in the cells [38–40], carbon source and concentration, and nitrogen concentration were examined in this study, along with the salinity, pH level, and agitation speed of the cultures.

Materials and Methods

Microalgal Strain

C. protothecoides 249, obtained from the Culture Collection of Algae at the University of Texas at Austin (UTEX), was selected as the unicellular green microalgae for this investigation.

Culture Medium

The culture medium was the modified basal medium [41], containing carbon sources, yeast extract (Acros Organics, Fisher Cat No. AC611801000) as nitrogen source, and sufficient amounts of inorganic supplements, including KH_2PO_4 (0.7 g/L), K_2HPO_4 (0.3 g/L), $MgSO_4{\cdot}7H_2O$ (0.3 g/L), $FeSO_4{\cdot}7H_2O$ (0.003 g/L), H_3BO_3 (0.00286 g/L), $MnCl_2.4H_2O$ (0.00181 g/L), $ZnCl_2$ (0.000105 g/L), Na_2MoO_4 $2H_2O$ (0.000039 g/L), $CuSO_4$ $5H_2O$ (0.000079 g/L), and $CoCl_2$ (0.000030 g/L) [41]. The carbon sources (glucose, glycerol, or acetate) and concentrations in each experiment were specified in each cultural condition together with other important growth factors like nitrogen concentrations, initial pH level, rotational speed, etc.

Cultivation Methods

Autotrophic, heterotrophic, and mixotrophic flask cultures of *C. protothecoides* were carried out in 250-mL Erlenmeyer flasks containing 100 mL of the culture medium. Autotrophic and mixotrophic cells were cultured under 16 h light and 8 h dark regime while heterotrophic cells were kept 24 h under the dark. No extra carbon dioxide was provided except naturally existing in the atmosphere to all the cultures, and light was provided with 8-W fluorescent light within the shaker (INNOVA 42R). The culture medium was always sterilized before the inoculation and then was inoculated with 5% exponentially growing microalgae from glucose based cultures.

Analytical Methods

C. protothecoides cell concentrations were relatively estimated at OD 540 nm by using an UV/Visible spectrophotometer [41] and were precisely determined with the dry cell weight method by centrifuging the cultural broth at 3,000 rpm for 15 min, washing twice with double distilled water and then drying the cell pellet at 105 °C till constant weight. Glucose concentration was estimated by using dinitrosalicylic acid assay [1, 41].

The lipid contents of *C. protothecoides* were determined using Soxhlet extraction with hexane [41].

Statistical Analysis

The statistical analysis was performed by using SAS 9.1. One-way ANOVA, Tukey's method, and non-parametric Kruskal–Wallis tests were chosen for the treatment comparisons for each data set. All used statistics were based on a confidence level of 95%, and p values <0.05 were considered statistically significant.

Results

Effect of Autotrophic, Heterotrophic, and Mixotrophic Growth Modes

Our experiments confirmed that *C. protothecoides* has the capacity to grow under autotrophic, heterotrophic, and mixotrophic conditions (Fig. 1a). There is a possibility that the autotrophic cultures still had some glucose from the inoculums, but it can be generally be ignored since the inoculums were taken at the end of log phase where glucose was mostly consumed. Colors of the cultivation broth were significantly different, with autotrophic broth at green and heterotrophic at yellow, due to different chlorophyll concentrations each growth mode has. After almost the same time of lag phase, cell concentrations reached different peaks via log growth phase (Fig. 1b). The mixotrophic culture had a similar specific growth rate, $\mu_{\text{mixotrophic}}$ at 0.04 h^{-1}, as the heterotrophic cultures, at 0.04 h^{-1} too; but, the one obtained from autotrophic cultures was dramatically lower, 0.005 h^{-1}. There were no significant differences on the final biomass concentrations and productivities between heterotrophic and mixotrophic cultivations, but they were almost five times over the ones obtained in autotrophic mode (Fig. 1b, c). Figure 1c showed that there were no significant differences in the cells' lipid contents between different growth modes ($p>0.05$).

The difference in cell density between autotrophic and heterotrophic as well as mixotrophic microalgae fermentations may be attributed to the light limitation on autotrophic cells [18]. Since autotrophic cells obtain all the energy they require for growth from inorganic compounds only, insufficient CO_2 concentration in the culture would not sustain optimal growth [42]. The effects of CO_2 supply have been studied for *Scenedesmus acutus* where higher concentrations of CO_2 produced higher biomass and lipid contents [43]. Although *C. protothecoides* has the capacity to grow on carbon sources and/or light, autotrophic culture received significantly lower growth rate and lower final cell concentration, which makes this process quite challenging to be economically feasible. In addition, the almost same specific growth rates of the heterotrophic and mixotrophic cultures imply that the growth-stimulating effects of light and CO_2 utilization in mixotrophic cultures were not as strong as the effects of glucose. The above observation might be an indication that *C. protothecoides* 249 cannot utilize light in the presence of carbon sources, like an amphitrophic organism, in which the term is used to describe organisms able to live either auto- or heterotrophically [44]. Our studies showed the lipid contents were not significantly different between different growth mode ($p>0.05$) while higher lipid contents in heterotrophic *C. protothecoides* was reported than in cells cultured under autotrophic conditions [2]. This dissimilar oil accumulation behavior may be attributed to the variations in the culture conditions used such as glucose concentration, nitrogen source and concentration, light intensity, CO_2 concentration, etc.

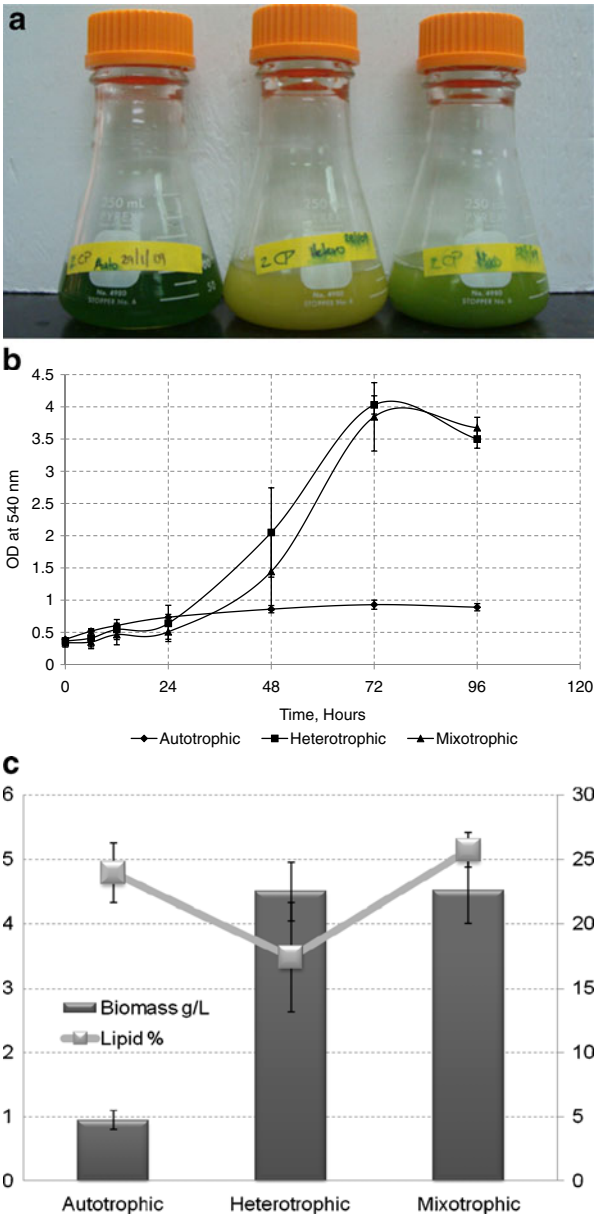

Fig. 1 **a** Autotrophic, heterotrophic, and mixotrophic *Chlorella protothecoides* cultures (15 g glucose/L for heterotrophic and mixotrophic cultures, 4 g YE/L, 160 rpm, initial pH 6.5, 26 °C). **b** Growth curves comparison (15 g glucose/L for heterotrophic and mixotrophic cultures, 4 g YE/L, 160 rpm, initial pH 6.5, 26 °C). **c** Biomass concentration and lipid content (15 g glucose/L for heterotrophic and mixotrophic cultures, 4 g YE/L, rotational speed 160 rpm, initial pH 6.5, 26 °C)

Effect of Initial Glucose Concentration

As shown in Fig. 2a, higher initial glucose concentration significantly extended the log phase of growth although no significant difference was found for the lag phase. Biomass concentrations started to decline after they reached their maximum at the end of the log phase. The final cell density increased as the initial glucose concentration increased in the culture. However, the specific growth rates for 5, 15, and 30 g/L of initial glucose cultures

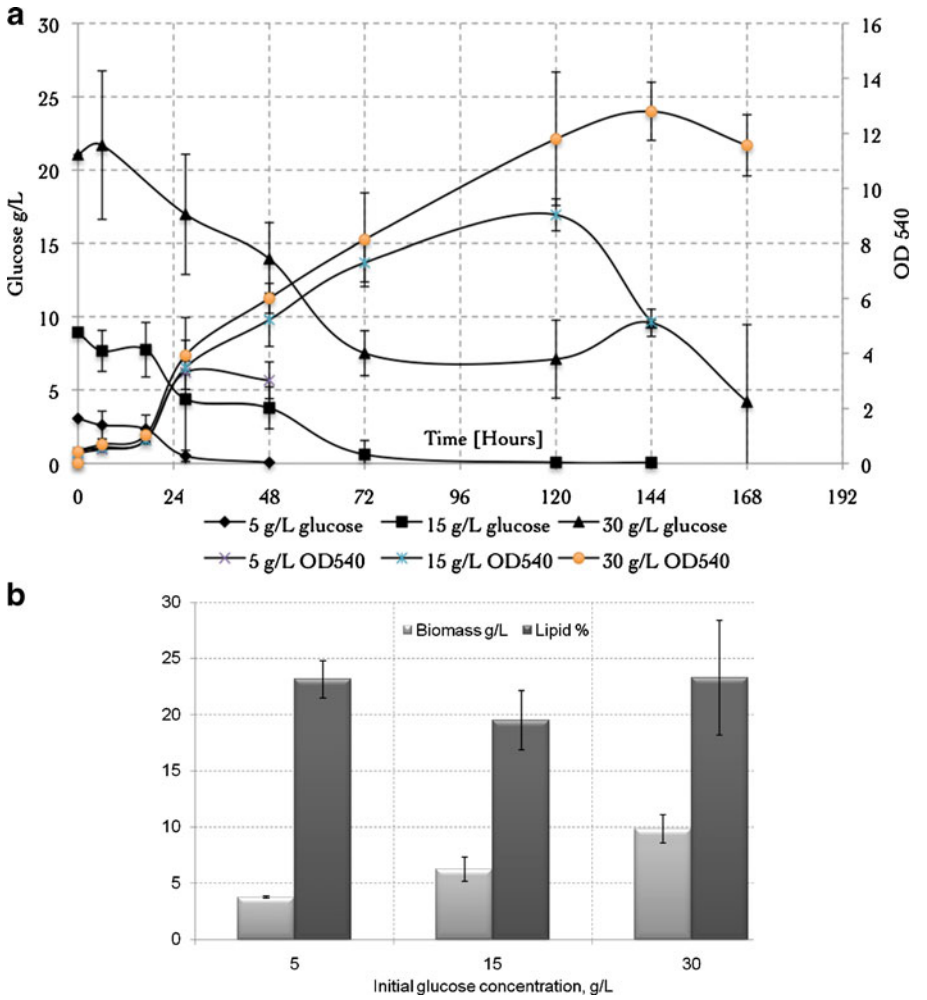

Fig. 2 **a** Mixotrophic *Chlorella protothecoides* at different initial glucose concentrations (4 g YE/L, 160 rpm, initial pH 6.5, 26 °C). **b** Mixotrophic *Chlorella protothecoides* at different initial glucose concentrations (4 g YE/L, 160 rpm, initial pH 6.5, 26 °C)

0.14, 0.02, and 0.02 h^{-1}, respectively, show that cell growth was negatively influenced at high glucose concentrations. The initial glucose concentration did not have significant effects ($p>0.05$) in the lipid contents (Fig. 2b). Glucose was completely utilized when the initial glucose concentration was 5 and 15 g/L, but, as shown in Fig. 2a, there was still an excess of substrate in the cultures containing 30 g/L of initial glucose even after they reached the peak biomass concentrations at approximately 144 h (Fig. 2a). Other than cell and lipid productivity, the final cell biomass concentration is always the key bottleneck parameter for the process to be commercially available considering the costly harvest and separation. Substrate inhibition, which caused low cell growth rate and low final biomass concentration, has also been reported in other studies. Continuous feeding of glucose at low concentrations might be the right strategy for the culture in a bioreactor [41].

Effect of Different Nitrogen Concentrations

The considered yeast extract concentrations did not cause significant differences ($p>0.05$) among the biomass concentrations and lipid contents for and glucose cultures at chosen levels (Fig. 3). Complex nitrogen sources like yeast extract are considered superior for heterotrophic microalgal growth because they also provide amino acids, vitamins, and other growth factors [45–48]. Nitrogen sources have been reported as a stress factor for the algae growth where limited supply would change significantly the composition of the organism [49], causing higher lipid content of the algae biomass. *C. protothecoides* has been cultured with different concentrations of yeast extract resulting in higher biomass concentrations at high nitrogen concentrations while in higher lipid contents at low nitrogen concentrations [41]. There is considerable evidence indicating that *Chlorella* species when grown on nitrogen-poor media but with sufficient light and/or organic carbon sources accumulate fat or starch within the cells, like the case of *Chlorella ellipsoidea* SK that accumulated 2.3% of lipids when grown with nitrogen and 26.8% when cultured without; *Chlorella pyrenoidosa* 82 accumulated 16.7% with nitrogen in the medium and 47.1% without [43]. Our results did not confirm these reports, probably because the nitrogen levels we were choosing at the lowest level (1 g/L for 15 g/L glucose) were still too high to cause the metabolic change, and the real need for the yeast extract can be even lower. In the same time, no strong inhibition for high nitrogen carbon ratio on the cell growth and lipid accumulation was found either; even the yeast extract reached 10 g/L for a 15-g/L glucose culture. This alleviates our concerns when the strain is applied to the agricultural and industrial waste materials, which usually have a very high organic nitrogen level.

Effect of Agitation Speed

There is a trend of increasing both biomass concentration and lipid content with the agitation speed: The biomass concentrations increased as the rotational speed was increased, as well as their lipid contents as the rotational speed was decreased (Fig. 4). The level of rotational speed is one of the key factors for the high production of cell density and lipid content in heterotrophic *C. protothecoides* cultures. The requirement for oxygen may be very high during the rapid growth phase of a batch culture, and oxygen limitation may result in inadequate growth and incomplete oxidation of the primary energy source [50]. Agitation is considered one of the most important requirements in microalgae

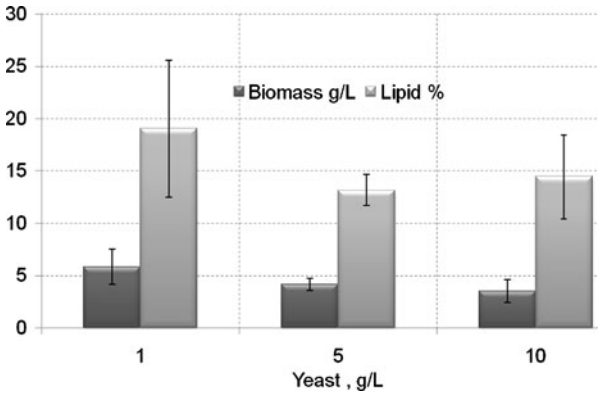

Fig. 3 Heterotrophic *Chlorella protothecoides* at different nitrogen concentrations (15 g glucose/L, 160 rpm, 100 h, 26 °C, initial pH 7.1)

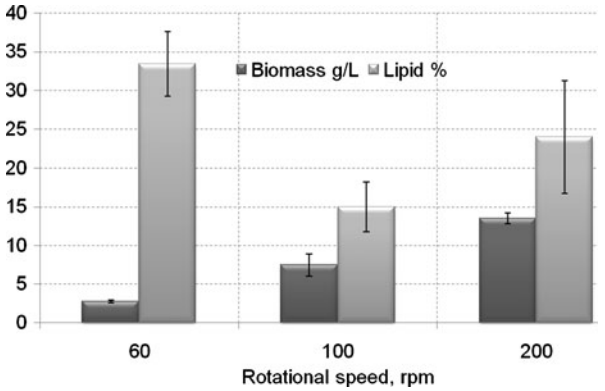

Fig. 4 Heterotrophic *Chlorella protothecoides* at different rotational speeds (15 g glucose/L, 1 g YE/L, 86 h, 26 °C, initial pH 7.1)

cultivation because higher biomass concentrations are produced as a result of better homogeneous distribution of nutrients and increase of air bubbles in the cultures [44]. The dissolved oxygen in *C. protothecoides* growth in fermentors has been generally controlled by coupling agitation speed with airflow to keep it at 20–50% air saturation [41]. In flask cultures, the oxygen supply is generally provided by high agitation speeds (180 to 200 rpm) [9].

Effect of Salinity

Although Fig. 5 shows that the highest cell density was obtained in cultures containing 17.5 g/L of initial salinity, no significant differences ($p>0.05$) were found between the biomass concentrations for the three NaCl levels. Statistical analysis also indicated that there were no significant differences between the lipid contents at the different salinity concentrations; however, the minor trend observed in Fig. 5 might indicate that increasing salinity concentration increases the lipid contents. The effects of salinity have been previously studied, specifically for marine microalgae, where a similar behavior has been attained. The lipid contents of *Botryococcus braunii* increased from 36% to 51% as the salinity levels increased from 0% to 6%, respectively [43]. The biomass productivity of *Muriellopsis* species did not significantly change in response to the increase of the NaCl concentration from 2 mM (0.12 g/L, standard culture medium) up to 200 mM (11.69 g/L) [51]. Our experiments showed that *C. protothecoides* has a strong tolerance to the salinity

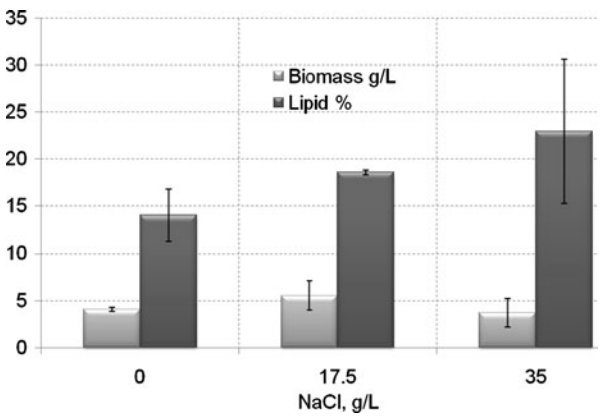

Fig. 5 Mixotrophic *Chlorella protothecoides* at different salinity (15 g glucose/L, 4 g YE/L, 160 rpm, initial pH 6.5, 72 h, 26 °C)

as high as the seawater (35 g/L NaCl) although we did not consider other salts such as magnesium in the seawater here in the study.

Effect of Glycerol as a Carbon Source

As indicated in Fig. 6, the highest biomass concentration was produced using 100% glucose as carbon source, showing that glucose is still the preferred carbon source for the cell growth. Mixture of glucose and glycerol based on the ratio at 80:20%, 60:40%, 40:60%, 20:80%, and 0:100% as carbon source received lower final cell concentration at 72-h cultivation, compared with pure glucose, but the there is no significant difference for the final cell concentration and productivity between different mixture of glucose and glycerol, even pure glycerol (Table 1). Although not the favorite carbon source, glycerol can be utilized as the substrate for the microalgae growth. As with the lipid contents, there are no significant differences ($p>0.05$) between different mixtures of carbon sources (Fig. 6). Biomass concentrations from glycerol cultures could be enhanced by changing the state of the inoculi used, i.e., to adapt the inoculi to the supplemented carbon sources in the cultures before its use. Aside from the possible effects of the inoculum, the nature of the nitrogen source, pH level, and other culture conditions may affect the ability of the microalgae to utilize glycerol [43].

Effect of Sodium Acetate as Carbon Source Without pH Adjustment

As shown in Fig. 7, the cell growth was not significantly changed by the addition of sodium acetate in the cultures without adjustment of initial pH if compared with the 100% of glucose cultures. A similar behavior was observed in Fig. 7 where comparable ($p>0.05$) lipid contents were obtained from different glucose/acetate mixture without pH adjustment. Table 2 shows the biomass concentrations, oil contents, and biomass and oil productivities obtained at 72 h. The biomass and lipid productivities of the cultures were very similar between the different treatments, but since the initial pH level of these cultures was unknown, that might bring some complications to the system.

Effect of Acetate as Carbon Source With pH adjustment

The culture conditions in this experiment were similar to the conditions used in the previous paragraph except for the initial pH level, which this time was adjusted to 6.5. These

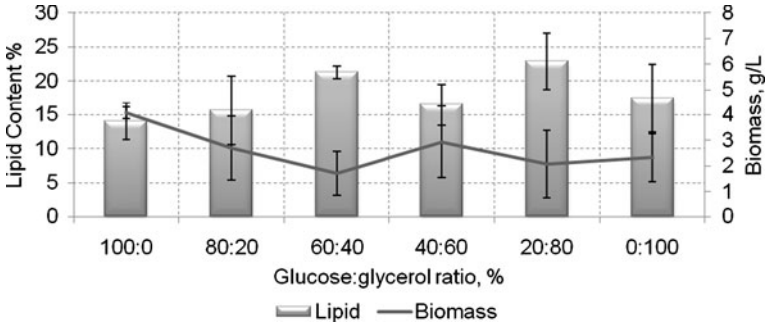

Fig. 6 Glycerol partial replacement as carbon source for mixotrophic *Chlorella protothecoides* cultivation (4 g YE/L, 160 rpm, initial pH 6.5, 72 h, 26 °C; glucose/glycerol 100:0%→15:0 g/L; 80:20%→12:4.1 g/L; 60:40%→9:8.2 g/L; 40:60%→6:12.2 g/L; 20:80%→3:16.3 g/L; 0:100%→0:20.4 g/L)

Table 1 Biomass and lipid productivities of mixotrophic *Chlorella protothecoides* grown under different initial glucose/glycerol concentrations (4 g YE/L, 160 rpm, initial pH 6.5, 72 h, 26 °C; glucose/glycerol 100:0%→15:0 g/L; 80:20%→12:4.1 g/L; 60:40%→9:8.2 g/L; 40:60%→6:12.2 g/L; 20:80%→3:16.3 g/L; 0:100%→0:20.4 g/L).

Culture conditions glucose/glycerol ratio, %	Biomass concentration, g L^{-1}	Biomass productivity, g L^{-1} day^{-1}	Lipid content, %	Lipid productivity, g L^{-1} day^{-1}
100:0	4.07±0.23	1.36±0.08	14.06±2.73	0.19
80:20	2.70±1.24	0.90±0.41	15.66±5.10	0.14±0.02
60:40	1.72±0.85	0.57±0.28	21.29±0.98	0.12
40:60	2.94±1.39	0.98±0.46	16.47±2.97	0.16±0.01
20:80	2.08±1.32	0.69±0.44	22.91±4.11	0.16±0.02
0:100	2.33±0.93	0.78±0.31	17.50±4.98	0.14±0.02

variations in the culture conditions altered significantly the results of these two sets of data. For example, the biomass concentration and productivity of the cultures with 100% of acetate without pH adjustment were approximately three times as the one with initial pH adjustment at 6.5 (Fig. 8). In the same time, the pure acetate cultures with pH adjustment were able to accumulate higher lipid contents than the other cultures in both experiments.

Table 3 shows the biomass concentrations, oil contents, and biomass and oil productivities obtained at 72 h. A trend can be observed in Table 3 and Fig. 8: The cell growth decreases as the initial acetate concentration in the culture increases. An opposite behavior can be observed that the lipid contents increased at higher concentrations of acetate. Significant differences ($p<0.05$) were found in the biomass and lipid contents at the different glucose/acetate mixing ratio. Lipid accumulation elevated with the increase of the acetate ratio while the lipid productivity relatively stayed stagnant due to the decrease of biomass accumulation. However, it is undetermined whether the acetate served as a stress factor for the growth, because the variation of pH during the cultures was not measured in these batch tests.

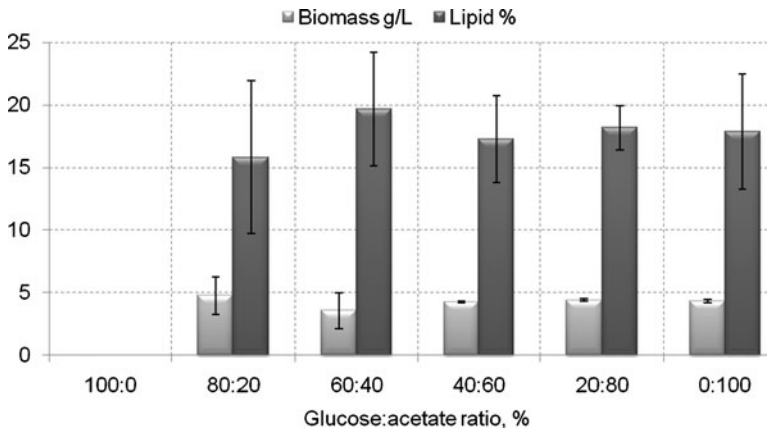

Fig. 7 Mixotrophic *Chlorella protothecoides* at different glucose/acetate concentrations (4 g YE/L, 160 rpm, initial pH unknown, 72 h, 26 °C, glucose/acetate 100:0%→15:0 g/L; 80:20%→12:4.1 g/L; 60:40%→9:8.2 g/L; 40:60%→6:12.3 g/L; 20:80%→3:16.4 g/L; 0:100%→0:20.5 g/L)

Table 2 Biomass and lipid productivities of mixotrophic *Chlorella protothecoides* grown under different initial glucose/acetate concentrations without pH adjustment (4 g YE/L, 160 rpm, initial pH unknown, 72 h, 26 °C, glucose/acetate 100:0%→15:0 g/L; 80:20%→12:4.1 g/L; 60:40%→9:8.2 g/L; 40:60%→6:12.3 g/L; 20:80%→3:16.4 g/L; 0:100%→0:20.5 g/L).

Culture conditions glucose/acetate ratio, %	Biomass concentration, g L^{-1}	Biomass productivity, g L^{-1} day^{-1}	Lipid content, %	Lipid productivity, g L^{-1} day^{-1}
100:0	–	–	–	–
80:20	4.76±1.50	1.59±0.50	15.84±6.11	0.25±0.03
60:40	3.57±1.46	1.19±0.49	19.66±4.55	0.23±0.02
40:60	4.24±0.06	1.41±0.02	17.30±3.49	0.24
20:80	4.41±0.09	1.47±0.03	18.20±1.79	0.27
0:100	4.34±0.13	1.45±0.04	17.89±4.60	0.26

Acetate has been previously considered as carbon source for microalgae [22, 24, 25, 45]. Several *Scenedesmus* species have been successfully cultured in the dark using acetate [43]. *Crypthecodinium cohnii* can directly activate acetate to acetyl-CoA, the basic building block of fatty acids synthesis [48]. Also, it has been remarked that the heterotrophic growth of *C. protothecoides* supplied with acetate, glucose, or other organic compounds as carbon source results in high biomass and high content of lipid in cells [52]. To improve acetate or any other carbon source utilization, actively dividing microalgal cells from the exponential phase should be used as inoculums [2, 41]. Our results imply that the initial pH of the acetate culture medium may have a strong effect on the cell growth and oil accumulation.

Effect of Different pH Levels with Different Carbon Sources

Table 4 shows the biomass concentrations, oil contents, and biomass and oil productivities obtained at 103 h from cultures using glycerol, sodium acetate, and glucose as carbon sources. For the glycerol cultures (Fig. 9a, b), there were no significant differences ($p>$ 0.05) in biomass concentration or lipid content among the different initial pH level from 6.3

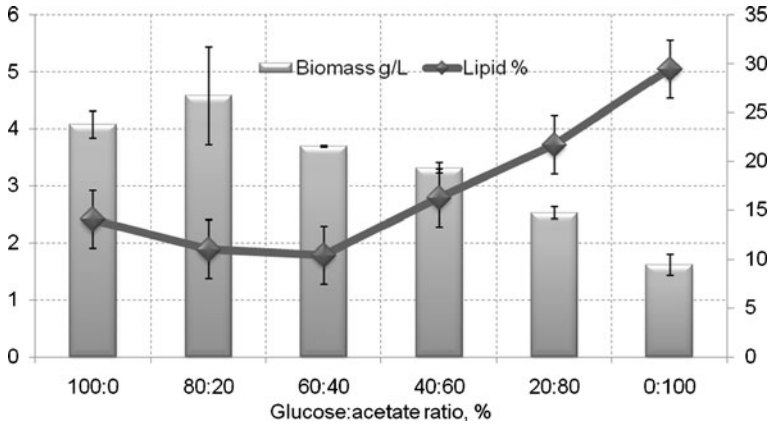

Fig. 8 Mixotrophic *Chlorella protothecoides* at different glucose/acetate concentrations (4 g YE/L, 160 rpm, initial pH 6.5, 72 h, 26 °C, glucose/acetate 100:0%→15:0 g/L; 80:20%→12:4.1 g/L; 60:40%→9:8.2 g/L; 40:60%→6:12.3 g/L; 20:80%→3:16.4 g/L; 0:100%→0:20.5 g/L)

Table 3 Biomass and lipid productivities of mixotrophic *Chlorella protothecoides* grown under different initial glucose/acetate concentrations with pH adjustment (4 g YE/L, 160 rpm, initial pH 6.5, 72 h, 26 °C, glucose/acetate 100:0%→15:0 g/L; 80:20%→12:4.1 g/L; 60:40%→9:8.2 g/L; 40:60%→6:12.3 g/L; 20:80%→3:16.4 g/L; 0:100%→0:20.5 g/L).

Culture conditions glucose/acetate ratio, %	Biomass concentration, g L^{-1}	Biomass productivity, g L^{-1} day^{-1}	Lipid content, %	Lipid productivity, g L^{-1} day^{-1}
100:0	4.07±0.23	1.36±0.08	14.06±2.73	0.19
80:20	4.59±0.86	1.53±0.29	11.02±3.40	0.17±0.01
60:40	3. 70±0.01	1.23	10.44±0.90	0.13
40:60	3.31±0.10	1.10±0.03	16.28±3.42	0.18
20:80	2.53±0.11	0.84±0.04	21.70±8.75	0.18
0:100	1.62±0.19	0.54±0.06	29.45±0.84	0.16

to 7.1. For the glucose cultures, there were also no significant differences in biomass concentration or lipid content among the different initial pH level from 6.3 to 7.1. However, for the acetate cultures, significant differences were found among biomass concentrations; specifically, the final biomass concentration at initial pH 6.3 was significantly lower than at other neutral conditions. In terms of lipid content, cultures using acetate reached dramatically higher lipid content at initial pH 6.3, which showed that pH serves as a stress factor at acidic conditions interestingly only when acetate serves as the carbon source. No such phenomena were found when comparing glycerol and glucose cultures.

Table 4 Biomass and lipid productivities of heterotrophic *Chlorella protothecoides* grown under different initial pH values using glycerol, acetate, or glucose as carbon source (20.4 g glycerol, 15 g glucose/L, or 20.5 g acetate/L, 4 g YE/L, 160 rpm, 103 h, 26 °C).

Carbon source and pH level		Culture conditions			
		Biomass concentration, g L^{-1}	Biomass productivity, g L^{-1} day^{-1}	Lipid content, %	Lipid productivity, g L^{-1} day^{-1}
Glycerol	6.3	3.46±0.31	0.81±0.07	16.21±1.67	0.13
	6.5	3.38±0.16	0.79±0.04	16.14±0.40	0.13
	6.7	3.59±0.18	0.84±0.04	18.73±1.69	0.16
	6.9	3.88±0.24	0.90±0.06	17.38±1.87	0.16
	7.1	3.97±0.29	0.93±0.07	20.33±5.13	0.19
Acetate	6.3	0.97±0.41	0.23±0.10	52.38±25.77	0.12±0.02
	6.5	3.24±0.29	0.75±0.07	21.81±2.57	0.16
	6.7	3.11±0.86	0.72±0.20	23.08±3.18	0.17±0.01
	6.9	2.66±1.24	0.62±0.29	21.42±3.11	0.13±0.01
	7.1	3.62±0.12	0.84±0.03	19.74±2.10	0.17
Glucose	6.3	3.46±0.14	0.81±0.03	17.74±3.09	0.14
	6.5	3.88±0.34	0.90±0.08	20.74±0.52	0.19
	6.7	4.25±1.43	0.99±0.33	13.02±2.26	0.13±0.01
	6.9	2.24±0.76	0.99±0.17	25.25±0.07	0.25
	7.1	4.00±1.33	0.93±0.31	15.73±3.98	0.15±0.01

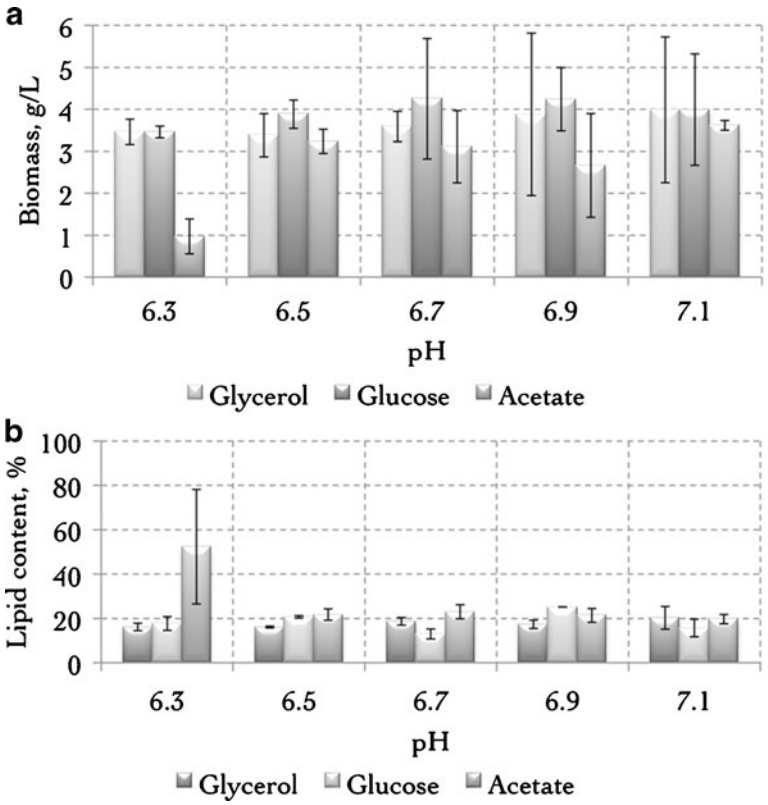

Fig. 9 **a** Heterotrophic *Chlorella protothecoides* cultivation at different initial pH levels and different carbon source (20.4 g glycerol/15 g glucose/L/20.5 g acetate/L, 4 g YE/L, 160 rpm, 103 h, 26 °C). **b** Heterotrophic *Chlorella protothecoides* cultivation at different initial pH levels and different carbon source (20.4 g glycerol/15 g glucose/L/20.5 g acetate/L, 4 g YE/L, 160 rpm, 103 h, 26 °C)

Discussion

Comparison of Our Results with the Literature

Table 5 contains experiment results found in the literature for *C. protothecoides*. Flask cultures report smaller biomass concentrations and lipid contents than cultures in fermentors under similar growth conditions, and this is due to the precise conditions control that fermentors could provide. The data are yet again consistent in that heterotrophic and mixotrophic cultures provide higher lipid contents and biomass concentrations than autotrophic cultures. As Tables 5 and 6 show, *C. protothecoides* has been mostly cultured under heterotrophic conditions and with glucose as carbon source. The highest lipid concentration in *C. protothecoides* flask cultures was 8.28 g/L using 30 g/L of glucose and 4 g/L of yeast [41]. Our experiments of glucose cultures showed the intermediate levels among the references for the biomass concentration; however, the lipid contents were relatively lower. It is confirmed in our study that heterotrophic growth could result in higher biomass final concentration than the autotrophic cultures, but the lipid content showed no significant difference between heterotrophic cultures and autotrophic cultures, which is not surprising considering many factors influencing the lipid accumulation.

Table 5 Biomass concentrations and lipid contents of *Chlorella protothecoides*.

Type of culture	Biomass concentration, g/L	Lipid content, %		Reference
	Heterotrophic	Autotrophic	Heterotrophic	
Batch culture	–	14.57	55.20	[2]
With urea and glucose: flask culture	18.8 (μ=0.0467/h)	–	–	[45]
Fermentor	19.6 (at 142 h)			
Flask culture	–	14.3	–	[1]
3.7 L fermentor	4.6 (w/glucose)	–	–	[58]
30 L Fermentor	16.4 (w/glucose)			
With 30 g/L glucose: flask culture	9.05 (w/1 g/L yeast)	–	53.2	[41]
	17.99 (w/4 g/L yeast)		46.0	
	18.6 (w/7 g/L yeast)		21.5	
	19.81 (w/10 g/L yeast)		18.9	
With 4 g/l of yeast: flask culture	10.38 (w/15 g/L glucose)		-	
	16.35 (w/30 g/L glucose)			
	18.25 (w/45 g/L glucose)			
	21.15 (w/60 g/L glucose)			
5-L fermentor	3.2		57.8	
	16.8 (at 184 h)		55.2	
	51.2 (at 168 h)		50.3	
Flask culture at 144 h	3.92 (w/corn powder hydrolysate)	–	55.3	[9]
	3.74 (w/glucose)		54.7	
5-L fermentor	15.5 (at 184 h, w/corn powder hydrolysate)		46.1	
Batch culture	19.6 (w/glucose)	–	–	[59]
3.7-L fermentor	48 (w/glucose)			
30-L fermentor	45.8 (w/glucose)			
3.7-L fermentor	4.6 (w/glucose)	–	–	[58, 60]
30-L fermentor	16.4 (w/glucose)			
14-L fermentor	3.4–10.8	–	0.03–0.17	[49]

Compared with other heavily researched microalgae species (Table 6), *C. protothecoides* is one of the excellent candidate strains for the future commercialization. It is very similar with *Chlorella vulgaris* in many aspects. *C. protothecoides* grows significantly slower than *C. cohnii*, the commercially used strain that produces omega-3 fatty acid. *C. cohnii* can grow on glycerol and acetic acid, but it behaves more like a yeast strain because it does not utilize sunlight. *Chlamydomonas reinhardtii* is an excellent strain to produce hydrogen; however, its oil content is relatively lower. On the other hand, *B. braunii* and *Dunaliella salina* can generate relatively higher content of oil, but they only grow on the autotrophic growth mode.

Waste Materials as Raw Materials for the Microalgae Oil Accumulation

There are very few recent publications about the lipid accumulations of *Chlorella* sp. under heterotrophic and mixotrophic conditions with carbon sources other than glucose [52].

Table 6 Comparison among some microalgae species.

Microalgae	Culture			Substrates	Lipid Content	Reference
	AC	MC	HC			
Chlorella protothecoides	x		x	glucose, acetate/CO_2	55.2%	[61]
Chlorella vulgaris	x	x	x	glucose, acetate, lactate and glutamate/CO_2	11.8–57.9%	[62]
Crypthecodinium cohnii			x	Glucose/acetate	15-70%	[63]
Scenedesmus obliquus	x		x	glucose/CO_2	14-22%	[62]
Chlamydomonas reinhardtii	x	x	x	acetate/CO_2	21%	[44]
Schizochytrium sp.	x		x	glycerol/CO_2	50-77%	[4]
Spirulina platensis	x		x	glucose/CO_2	4.2-6.2%	[62]
Botryococcus braunii			x	CO_2	25 - 75%	[4]
Dunaliella salina	x	x		CO2	15-55%	[64]

AC autotrophic culture, *MC* mixotrophic culture, *HC* heterotrophic culture

Waste materials are proposed in this study to replace glucose, the major cost of the process. A typical waste material that can be produced at a large amount in current biodiesel plants is glycerol. For every 10 tons of biodiesel produced, around 1 ton of impure glycerol is produced with high sodium concentration as the by-product. Microalgal species with tolerance to salt, such as *C. protothecoides* as shown in this study, are good candidates. Another typical waste material is the acetate from the modified anaerobic digestion system. Acetate represents the major intermediate chemical in the anaerobic food chain. Within this food chain, acetogenic bacteria degrade all the organic materials to produce acetic acid and hydrogen, and methanogenic archaea utilize these intermediates to generate methane. Several reports have already proposed to disrupt this food chain to harvest hydrogen as the final product, commonly referred to as the fermentative hydrogen production, and acetate as the by-product [53]. Our results showed that *C. protothecoides* can utilize both glycerol and acetate as their substrate to the accumulation of oil, which is opening future opportunity to develop oil accumulation cultures from industrial waste materials. Glycerol, a by-product from current biodiesel industry, can be utilized to accumulate oil, which eventually goes back to the biodiesel production. Acetate is the by-product from the fermentative hydrogen production and can be fed to cultivate microalgae, so that both hydrogen and biodiesel can be harvested from the system. For these two applications, extra salt in the solution is quite a concern. Waste glycerol has a high sodium concentration, which inhibits its industrial utilization. Similarly, to maintain the fermentative hydrogen fermentation at neutral pH, alkalinity decrease will cause the system to release ions such as calcium and sodium, which elevates the salinity of system. The microalgae chosen for this process showed strong tolerance to certain range of salinity (Fig. 5), one of the superior characteristics of this microalgae species that alleviate the concerns for the salinity.

Stress Conditions for the Cell Growth and Oil Accumulation

It has already been proved that several stress conditions can have a significant impact not only on the cell growth but also on the lipid content of microalgae cells. Many microalgae respond to these stress conditions by significantly increasing the lipid content capacities, commonly ranging from 30% to 60% of the dry cell weight. Nitrogen deprivation or

limitation was commonly thought as the default stress condition to improve the lipid content [47, 49] due to its lower cost and ease in manipulation. Sunlight availability has played another important role for the lipid content of microalgae cells. It was revealed that high light intensity [54], acting as a stress factor, can also cause the higher lipid content. Some species, especially *C. protothecoides*, tend to accumulate higher lipid content in the heterotrophic growth mode than the autotrophic one. Actually, this behavior primarily is due to the increased carbon nitrogen ratio that the heterotrophic growth mode can reach. Other factors, such as low temperature [55], high salt concentration [49], and high iron concentration [56], are proved to be the stress factors that inhibit the cell growth while increasing the lipid content. Our experiments in Fig. 5 also confirmed the effectiveness of higher salt concentration as a stress factor. Initial pH was revealed in our study as a stress factor as long as acetate is utilized as carbon source and the lower initial pH drastically increased the lipid content up to 60% although the cell growth was inhibited. The detailed mechanism still needs some investigation; however, compared with other stress factors, including the nitrogen limitation [57], pH is even a much easier factor to be controlled and manipulated. Future investigations that will take advantage of this discovery are needed to develop a two-stage engineering system for the optimized lipid accumulation with microalgae cultivation system.

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