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Heterotrophic Culture of *Chlorella protothecoides* in Various Nitrogen Sources for Lipid Production

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Abstract The influences of urea, yeast extract, and nitrate as the nitrogen source on heterotrophic growth of four strains of *Chlorella protothecoides* were investigated in 9-day feed-batch cultures. Biomass dry weight concentration (DWC) and lipid yield (LY) of the four strains in all media were compared. The highest LY in 9 days was 654 mg/L/day by UTEX 255 in 2.4 g/L KNO_3 medium with a biomass DWC of 11.7 g/L and lipid content of 50.5%. Using green autotrophic seeds instead of yellow heterotrophic seeds improved the biomass DWC (13.1 vs. 11.7 g/L), LY (850 vs. 654 mg/L/day), and lipid to glucose consumption ratio (0.607 vs. 0.162). Moreover, 17.0 g/L DWC and 489 mg/L/day LY were obtained from the sequentially mixed-nitrogen medium, and the lipid to glucose consumption ratio was improved to 0.197 from 0.162 in 2.4 g/L nitrate medium and from 0.108 in 4.2 g/L yeast extract medium in the first batch.

Keywords Algae · Biofuel · Nitrogen · *Chlorella protothecoides* · Lipid

Introduction

As energy prices reach historical highs and environmental concerns continue to grow, broad interest exists in producing and utilizing biofuels from domestic biomass resources. Algae, a group of organisms that can grow, autotrophically or heterotrophically, in freshwater or saltwater are one such resource. The potential for producing biofuels (especially biodiesel) from algae was demonstrated and extensively studied about 20 years ago [1]; on the basis

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of this research, it was concluded that raceway ponds were the most promising means of algae production. However, large-scale open-pond algae cultivation for biofuel production is still facing some technical and economical challenges, largely due to difficult algal species control [1], low-biomass productivity [2–5], and high harvesting costs [6, 7].

Heterotrophic algal cultivation has the potential to solve the problems associated with open-pond algae production. First, contamination from wild algae can be prevented because target algae are usually cultivated in enclosed sterile reactors. Second, biomass productivity can be significantly higher than that of autotrophic cultivation. It is common to reach a biomass growth rate of 4–20 g/L/day by using heterotrophic microalgae culture with different scales from flasks to large fermentor [8–11]. Wu and Shi [11] optimized the heterotrophic cultivation of *Chlorella* on the basis of a hybrid neural network model and achieved a biomass dry weight concentration (DWC) of 116.2 g/L in 5 days. However, biomass productivities of autotrophic microalgae culture are much lower. Open-pond systems usually have biomass productivities less than 60–100 mg/L/day (or 10–25 g/m^2/day) [12], and closed photobioreactors have biomass productivities lower than 200–360 mg/L/day (or 30 g/m^2/day) [13].

Two important aspects in heterotrophic algal cultivation are (1) finding the right algal species and (2) selecting the right type and concentration of nitrogen in the culture medium. Among the few algae species appropriate for heterotrophic cultivation, *Chlorella* have been extensively studied [14–16] and showed great potential for lipid production [17]. However, no single strain of *Chlorella protothecoides* has been proved to be the best candidate for lipid production so far. It is also well known that each algal species or strain favors different nitrogen sources [15, 18, 19]. This study aimed to find a strain of *C. protothecoides* and its optimal nitrogen source and concentration for lipid production by (1) comparing biomass productivity and oil content of four strains of *C. protothecoides* that have never been studied or reported elsewhere, (2) identifying the optimum nitrogen source and concentration in fed-batch heterotrophic cultivation for each strain, and (3) enhancing growth of selected strains for biomass and/or lipid production.

Materials and Methods

Microalgal Strains and Growth Media

Four strains of *C. protothecoides* were chosen in this study. The four strains (UTEX 25, 31, 249, and 255) were obtained from the Culture Collection of Algae at University of Texas at Austin, Texas, USA. Basal medium [20] has been found effective for *C. protothecoides* growth [21] and was slightly modified for use in this study. Each liter of the modified Basal (MB) medium contained 1,250 mg KNO_3, 1,250 mg KH_2PO_4, 1,000 mg $MgSO_4$.7H2O, 500 mg EDTA, 114.2 mg H_3BO_3, 111 mg $CaCl_2.2H_2O$, 49.8 mg $FeSO_4.7H_2O$, 88.2 mg $ZnSO_4.7H_2O$, 14.2 mg $MnCl_2.4H_2O$, 15.7 mg $CuSO_4.5H_2O$, and 4.9 mg $Co(NO_3)_2.6H_2O$. The sterilized MB medium was directly used in autotrophic seed inoculation. In heterotrophic cultivations, the MB medium was supplemented with 40 g/L glucose, and KNO_3 in the medium was replaced by urea, KNO_3, or yeast extract at concentrations listed in Table 1. The low, medium, and high concentrations of each nitrogen source are listed in Table 1, and 40 g/L glucose supplement was used. The 40 g/L glucose was chosen because a study showed that higher initial glucose concentration promoted biomass accumulation but if too high (e.g., ≥50 g/L), it may cause long lag-phases [21]. The pH of the media was adjusted to 6.8 after sterilization. Three types of stock solutions containing 200 g/L glucose

Table 1 Glucose- and nitrogen-supplemented Basal media.

Medium type	Medium code	Supplements[a]
Nitrate media	1 (low)	2.4 g/L (24 mM) nitrate
	2 (medium)	4.2 g/L (42 mM) nitrate
	3 (high)	6.0 g/L (60 mM) nitrate
Urea media	4 (low)	1.8 g/L (60 mM) urea
	5 (medium)	2.7 g/L (90 mM) urea
	6 (high)	3.6 g/L (120 mM) urea
Yeast extract media	7 (low)	2.4 g/L yeast extract
	8 (medium)	4.2 g/L yeast extract
	9 (high)	6 g/L yeast extract

[a] All media also contained 40 g/L glucose

and one of the following nitrogen sources were separately prepared: 7 g/L urea, 7 g/L KNO_3, or 12 g/L yeast extract. The sterilized stock solutions were used to feed algae whenever glucose concentration was below 15 g/L in the media with the same type of nitrogen source.

Inoculation

Two types of seeds of each strain were prepared: green, obtained from autotrophic inoculation, and yellow, grown from heterotrophic inoculation of the green seeds. *C. protothecoides* colonies on agar plates were first inoculated into 250-mL Erlenmeyer flasks containing 150 mL sterilized MB media. The flasks were placed on a reciprocating shaker (120 rpm) under 100 to 120 μmol photons/m^2/s fluorescence light in a constant temperature room (22 °C) for 5 to 7 days until dense green (autotrophic) seeds were observed in the flasks. To obtain yellow seeds, 5% exponentially growing green seeds were inoculated into sterilized MB medium supplemented with 20 g/L glucose. The heterotrophic seed cultivations were carried out in 500-mL Erlenmeyer flasks containing 300 mL growth media at 28±1°C under continuous shaking (250 rpm) in the dark for 9 days.

Cultivation of the Four Strains in All Media

The first step of cultivation was to compare biomass DWC and lipid yield (LY) of the four *C. protothecoides* strains and identify the best nitrogen type and concentration for each strain. Fifteen milliliters of yellow seeds from each strain was inoculated into the nine media listed in Table 1. Cultivations were carried out in 500-mL Erlenmeyer flasks containing 300 mL growth media at 28±1 °C under continuous shaking (250 rpm) in the dark. When glucose concentration in the medium was less than 15 g/L, stock solution with the same type of nitrogen was fed to increase glucose to 20 g/L. Biomass DWC and glucose concentration were tested daily. All cultivations and measurements were carried out in two separate replications.

Enhanced Cultivation of UTEX 255

The second step of cultivation was to improve growth of UTEX 255. UTEX 255 was chosen from the four strains as the best candidate for lipid production because it showed more than 50% lipid content (LC) and reasonably high biomass productivity in the first step of cultivation. Two strategies were used for the enhancement. Strategy one was to use a

sequentially mixed-nitrogen medium to improve biomass production. Because medium no. 8 (4.2 g/L yeast extract) achieved the fastest biomass accumulation and medium no. 1 (2.4 g/L nitrate) yielded the highest LC (50.5%) in the first step of cultivation, these two types of nitrogen were sequentially used for UTEX 255: (1) medium no. 8 (4.2 g/L yeast extract) was used in the first 2 days, and (2) 2.4 g/L nitrate was added at the end of day 2. Strategy 2 was to use green autotrophic seeds instead of yellow heterotrophic seeds in the cultivation. Although dense yellow seeds are more easily obtained for larger scale cultivation, our previous studies indicated that green seeds behave differently than yellow seeds, and using green seeds has the potential to improve the biomass to glucose consumption ratio. The enhanced cultivations were carried out in 500-mL Erlenmeyer flasks containing 300 mL medium at 28±1 °C in the dark for 9 days under the same growth conditions as in step 1. Two replications were studied.

Analytical Methods

Ten milliliter of the culture was filtered through a pre-dried (75 °C for 5 h in an oven) and weighed (w_0) glass-fiber filter paper (55 mm, nominal pore size 1.2 μm) under 35 to 55 mm Hg vacuum pressure. The filter paper was dried again in the same oven (75 °C for 5 h) and kept in a vacuum desiccator overnight before weighing (w_1). Algae biomass dry weight was obtained by subtracting w_0 from w_1 [22]. For faster and more convenient measurement, DWC was correlated to OD measured with a spectrophotometer at 540-nm wavelength similarly to a previous study [22]. In the calibration, 90 mL of samples from each of the culture was concentrated by a centrifuge at 4,000×g for 15 min. Sixty milliliter of the supernatant medium was carefully removed to leave three-fold concentrated algal culture in the centrifuge tubes. The three-fold algal culture was diluted with distilled water to prepare 2.5, 2, 1.5, 1, 0.6, 0.3, 0.15, and 0.09-fold calibration samples. The following regression equations between biomass DWC and OD values were developed: $y_{25}=1.0947\ x$, $y_{31}=0.9494\ x$, $y_{249}=1.5812\ x$, and $y_{255}=1.2061\ x$ ($R^2>0.96$, $P<0.05$) where y (g/L) is the biomass DWC, x is the OD of the suspension at 540 nm, and the subscript indicates the strain number. The same regression equation was used for green and yellow seed cultures of UTEX 255. OD values at 540 nm were measured daily in the period of culture to be used in the regression equations to calculate biomass DWC. At the end of the cultures, lipid extraction was carried out using a BioSpec Model 3110 BX bead-beater (Bartlesville, OK, USA) for cell disruption (3 min) followed by solvent extraction with *n*-hexane. Lipid yield was obtained by multiplying biomass DWC with lipid content. The oils collected after evaporation was dried at 95 °C for 1.5 h before weighing. Glucose concentration was determined by using a Megazyme D-Glucose (GOPOD Format) assay kit following the standard method provided by the manufacturer (Megazyme International Ireland, Wicklow, Ireland).

Results and Discussion

Growth Comparison of the Four Strains

All results presented in this article are based on the average of the two replications. Maximum biomass DWC and the highest LY of the four strains achieved in the three types of nitrogen-supplemented media are shown in Table 2. Maximum biomass DWC achieved were: UTEX 25–26.9±1.2 g/L in medium no. 1 (2.4 g/L KNO_3); UTEX 31–14.9±0.8 g/L

Table 2 Maximum biomass dry weight and lipid yield of the four strains.

Strain number	Nitrogen source	Max biomass DWC (g/L)	Max lipid yield (mg/L/day)
UTEX 25	Urea	12.7±0.8	303±1
	Yeast extract	19.8±0.9	292±1
	Nitrate	26.9±1.2	407±2
UTEX 31	Urea	14.9±0.8	412±2
	Yeast extract	8.6±0.4	228±1
	Nitrate	12.0±1.2	410±2
UTEX 249	Urea	11.9±0.8	286±1
	Yeast extract	9.8±0.6	168±1
	Nitrate	31.3±1.6	542±2
UTEX 255	Urea	11.8±0.8	322±1
	Yeast extract	14.2±0.8	474±1
	Nitrate	12.8±1.2	654±3

in medium no. 4 (1.8 g/L urea); UTEX 249–31.3±1.6 g/L in medium no. 2 (4.2 g/L KNO_3); and UTEX 255–12.8±1.2 g/L in medium no. 3 (6 g/L KNO_3). The highest LY achieved were: UTEX 25–407±2 mg/L/day in medium no. 1 (2.4 g/L nitrate); UTEX 31–412±2 g/L in medium no. 4 (1.8 g/L urea); UTEX 249–542±2 g/L in medium no. 2 (4.2 g/L nitrate); and UTEX 255–654±3 mg/L/day in medium no. 1 (2.4 g/L KNO_3). It is evident that the nitrogen source preference of each strain is different. UTEX 25 and 249 produced maximum biomass DWC and LY in nitrate media. UTEX 31 favored urea media for biomass production, but KNO_3 media yielded the highest LY. For UTEX 255, the three types of nitrogen yielded similar biomass DWC; however, KNO_3 was the best nitrogen source for lipid accumulation. Among the four strains in all media, the highest LY was 654±3 mg/L/day (LC 50.5%) by UTEX 255 in 2.4 g/L KNO_3 medium, followed by 542±2 mg/L/day (LC 15.6%) by UTEX 249 in 4.2 g/L KNO_3 medium, and 508±2 mg/L/day (LC 40.8%) by UTEX 255 in 4.2 g/L KNO_3 medium.

Glucose consumption is another important factor in evaluating the commercial value of algal strains. The ratio of LY to glucose consumption (L/G) of the top nine lipid producers are shown in Table 3. The highest L/G of 0.162±0.001 g/g was obtained by UTEX 255 in

Table 3 Lipid yield and lipid to glucose consumption ratio of the top nine lipid producers.

Rank	Strains	Nitrogen sources	Nitrogen concentration (g/L)	Lipid yield (mg/L/day)	L/G (g/g)
1	255	Nitrate	2.4	654±3	0.162±0.001
2	249	Nitrate	4.2	542±2	0.136±0.001
3	255	Nitrate	4.2	508±2	0.125±0.001
4	255	Nitrate	6	496±2	0.127±0.001
5	255	Yeast extract	2.4	474±2	0.144±0.001
6	31	Urea	1.8	412±2	0.122±0.001
7	31	Nitrate	6	410±2	0.095±0.001
8	25	Nitrate	2.4	407±2	0.068±0.001
9	31	Nitrate	2.4	398±2	0.093±0.001

2.4 g/L KNO_3 medium. Data in Table 3 indicated that the KNO_3 media are favored for lipid production by all four strains. Also, UTEX 255 has the potential to produce more lipids than the other strains in the same cultivation conditions and consumed relatively less glucose. Because of its higher LY and L/G ratio, UTEX 255 was chosen as the best candidate among the four strains for lipid production, and its growth was further analyzed.

Growth Analysis of UTEX 255 in Step 1

The biomass DWC and LY of UTEX 255 in all media are shown in Table 4. Generally, under lower nitrogen concentrations, UTEX 255 achieved higher LY and LC. Among the three nitrogen sources, nitrate was preferred by UTEX 255. The average LY and biomass DWC of UTEX 255 in nitrate media were 552 mg/L/day and 11.9 g/L, respectively, which were even higher than the maximum values obtained in the yeast extract and urea media. The final biomass concentration in the "medium" urea treatment was 2.5 g/L, whereas the two extremes, i.e., the "low" and "high" nitrate cultures yielded much higher biomass concentrations (11.8 and 6.9 g L^{-1}, respectively). This phenomenon does not seem to be reasonable and could be caused by some unexpected factors, such as contamination to the 2.7 g/L urea medium by bacteria or other organisms.

Daily biomass dry weight and glucose concentration of UTEX 255 in the nitrate and yeast extract media are shown in Fig. 1. On day 1 of cultivation, growth rates of UTEX 255 in yeast extract media were positively proportional to the nitrogen concentration but opposite in KNO_3 media, suggesting that UTEX 255 is more adaptable to higher concentrations of yeast extract but lower concentrations of nitrate in the early phase. Growths of UTEX 255 in the yeast extract media were also significantly faster than in the nitrate media in this phase. Starting on day 2, UTEX 255 in the nitrate media (Fig. 1a) entered the accelerating growth phase. Higher nitrogen concentrations promoted biomass accumulation in this period. Feeding was provided on day 5 to the 6 g/L nitrate medium because of fast glucose consumption, and biomass growth seemed improved after day 6. Feedings to all these media after day 7 did not improve biomass growth. The biomass DWC of UTEX 255 in the yeast extract media (Fig. 1b) increased slowly after day 1, and glucose consumptions were slow, too. Only one feeding was provided (on day 8 to the 6 g/L yeast extract medium). By day 9, when the cultivation ended, the moderate level (4.2 g/L) yeast extract medium performed better than the other two media in terms of biomass DWC. It is important to note that the media that generated the highest growth rates in the lag phase

Table 4 Biomass DWC and lipid yield of UTEX 255 in all growth media.

Nitrogen sources	Nitrogen concentration	Biomass DWC (g/L)	Lipid yield (mg/L/day)
Nitrate	Low (2.4 g/L)	11.7±0.5	654±3
	Medium (4.2 g/L)	11.2±0.4	508±2
	High (6 g/L)	12.8±1.2	496±2
Urea	Low (1.8 g/L)	11.8±0.6	322±1
	Medium (2.7 g/L)	2.5±0.4	67±1
	High (3.6 g/L)	6.9±0.3	184±1
Yeast extract	Low (2.4 g/L)	12.2±0.2	474±2
	Medium (4.2 g/L)	14.2±0.4	509±2
	High (6 g/L)	8.3±0.5	119±1

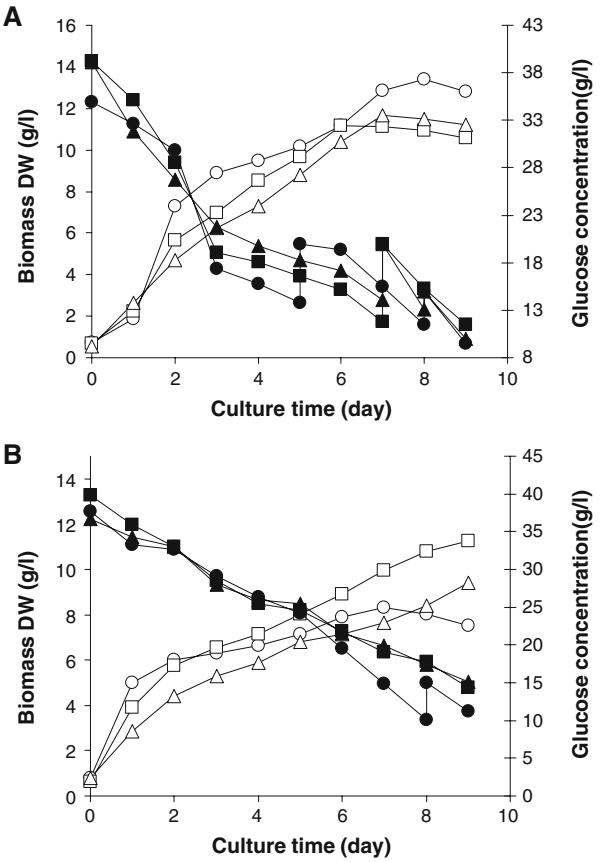

Fig. 1 Biomass dry weight (DWC) and glucose concentration in **a** nitrate media and **b** yeast extract media. *Open circles* biomass DWC in 6 g/L nitrogen media, *closed circles* glucose concentration in 6 g/L nitrogen media, *open squares* biomass DWC in 4.2 g/L nitrogen media, *closed squares* glucose concentration in 4.2 g/L nitrogen media, *open triangles* biomass DWC in 2.4 g/L nitrogen media, *closed triangles* glucose concentration in 2.4 g/L nitrogen media

(media no. 1 and no. 9) yielded almost the lowest biomass DWC on day 9 in both types of nitrogen sources. Glucose consumptions throughout the growth period were generally proportional to biomass accumulation.

Growth Analysis of UTEX 255 in Enhanced Cultivation

The biomass DWC and glucose consumption of green autotrophic seeds in the 2.4 g/L KNO_3 medium (medium no. 1) and the sequentially mixed-nitrogen medium of step two are shown in Fig. 2. For comparison, the DWC and glucose consumption curves of yellow heterotrophic seeds in medium no. 1 are also shown in the figure. As can be seen from Fig. 2, the yellow seeds accumulated biomass much faster in the first 5 days, during which the green seeds were still in the lag phase. However, after day 5, the green seeds started to grow exponentially and exceeded the yellow seeds on day 9. Meanwhile, glucose of the green seeds was consumed much slower than glucose of the yellow seeds during the entire

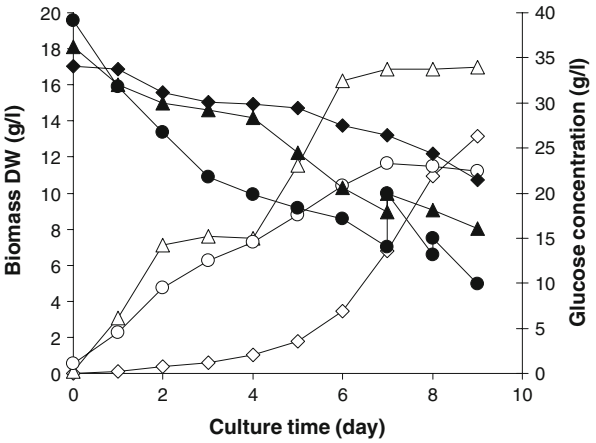

Fig. 2 Biomass dry weight (DWC) and glucose consumption in the enhanced cultivation. *Open squares* biomass DWC in 2.4 g/L nitrate medium with green seeds, *closed squares* glucose concentration in 2.4 g/L nitrate medium with green seeds, *open triangles* biomass DWC in sequentially mixed-nitrogen medium with green seeds, *closed triangles* glucose concentration in sequentially mixed-nitrogen medium with green seeds, *open circles* biomass DWC in 2.4 g/L nitrate medium with yellow seeds, *closed circles* glucose concentration in 2.4 g/L nitrate medium with yellow seeds

period. The green seeds in the sequentially mixed-nitrogen medium behaved differently. In the first 2 days, only 4.2 g/L yeast extract was provided as the nitrogen source. As expected, the biomass dry weight accumulation was the highest among the three cultures. At the end of day 2, 2.4 g/L nitrate was added to the medium; consequently, the algae showed a lag period of 2 days followed by an exponential growth period of 2 days. The stationary phase started on day 6 when the glucose concentration was 20.6±0.3 g/L. Feeding was provided on day 7, but the growth rate was not improved, indicating that glucose was not the limiting factor.

Table 5 shows the performance of green autotrophic seeds in the enhanced cultivation. For comparison, the LY, L/G, and DWC of yellow heterotrophic seeds in medium no. 1 are also shown in the table. The total biomass DWC of UTEX 255 (green seeds) produced in medium no. 1 in 9 days was 13.1±0.9 g/L, and the LY was 850±2 mg/L/day; these values are slightly higher than those obtained from the previous step using yellow seeds (11.7 g/L DWC and 654±3 mg/L/day LC). A more significant improvement in the enhanced cultivation was the higher L/G ratio, which increased from 0.162±0.001 in the first batch to 0.607±0.002. The much higher lipid to glucose consumption ratio from the green seeds was probably because the green seeds already contained biomass and lipid from photosynthesis that did not consume glucose. For the mixed-nitrogen medium, 17.0±0.5 g/L DWC was obtained but the LY (489±3 mg/L/day) was lower than in medium no. 1, although the L/G ratio was improved to 0.197±0.001 from 0.162±0.001 in medium no. 1 and from 0.108±

Table 5 Lipid yield, biomass dry weight, and lipid to glucose consumption ratio of the enhanced cultivation.

Growth media	Biomass DWC (g/L)	LY (mg/L/day)	L/G (g/g)
2.4 g/L KNO_3 (y)	11.7±0.5	654±3	0.162±0.001
Y.E and KNO_3 (g)	17.0±0.5	489±3	0.197±0.001
2.4 g/L KNO_3 (g)	13.1±0.9	850±2	0.607±0.002

y yellow seeds, *g* green seeds

0.002 in medium no. 8 in the first batch. On the basis of results from the two batches, it seems that using green or yellow seeds can slightly affect LC and biomass productivity of UTEX 255 but significantly improve the B/G ratio. More investigation is needed to understand this phenomenon.

Justification of Using *C. protothecoides* for Lipid Production

Although it is beyond the scope of this study to investigate the economic feasibility of growing *C. protothecoides* for lipid production, it is still worthwhile to compare heterotrophic cultivation of *C. protothecoides* with autotrophic cultivation and ethanol fermentation when production cost and sugar dependence are concerned. The overall lipid production costs of the two methods are comparably high (estimated at about $9–10/gal) [23]. However, heterotrophic cultivation has many advantages over autotrophic cultivation, such as small land requirement, no climate limitation, and ease of scaling up and commercialization. One major issue with heterotrophic algae cultivation is its dependence on sugars, which usually come from other crops. The sugar dependence of algae cultivation is similar to ethanol fermentation, so these two pathways are compared as follows.

When sugar dependence is concerned, one has to keep in mind that ethanol cannot replace diesel fuel or vegetable oil derived fuels, such as biodiesel, green diesel, or jet fuels. Therefore, co-existence of algae cultivation and ethanol fermentation will be likely in the future because they have totally different end-products. Competition for sugars will exist in the long term, and any breakthrough in sugar production, such as cellulose-based sugars, will promote both pathways. With currently limited supply of sugars, which pathway will be dominant depends on the conversion cost and efficiency. It is recently demonstrated that microalgal biodiesel is a better alternative than bioethanol from sugarcane [24]. Table 6 shows the comparison of the two processes. First of all, heterotrophic algae cultivation is less selective on sugar sources. Various carbon sources such as glycerol and acetate in addition to monosaccharides and disaccharides (e.g., glucose, fructose, and sucrose) have been reported useable by algae [25–27]. However, for yeast fermentation, glucose, fructose, and sucrose are almost exclusively used. In terms of sugar to end product conversion rate, 30% has been reported for heterotrophic algae [23]. For yeast fermentation, the transfer rate from sugar to bioethanol is about 40% [28], while the theoretical high is about 50%. Meanwhile, potentially valuable proteins and carbohydrates are produced by algae as the leftover of oil extraction, while in yeast fermentation, 49% glucose is wasted as CO_2. In the perspective of energy conversion, lipids usually have higher heating values than ethanol, and the input energy in processing algae to lipid is expected to be less than ethanol

Table 6 The comparison of lipid from heterotrophic algae and ethanol from yeast fermentation.

Factors	Lipid from heterotrophic algae	Ethanol from yeast fermentation
Sugar source	Less selective (various types of carbon sources are usable)	Very selective (mainly glucose)
Sugar to product conversion rate	~30% [9, 19]	~40–50%
Byproduct	Algae cake (proteins and carbohydrates)	CO_2
Heating value	High (35 MJ/kg)[a]	Low (29.7 MJ/kg)
Energy input in processing	Low (50% lipid in cell)	High (~10% ethanol in medium)

[a] Data for rapeseed oil

distillation [29]. However, with currently available technologies, the cost of lipids from heterotrophic algae is still higher than that of fuel ethanol from yeast fermentation mainly because of low algae LY and expensive culture medium and maintenance. Considering that it is still a very new industry as compared to fuel ethanol, algae biofuel will have tremendous space for improvement to reduce the costs of production and processing.

Conclusions

Feed-batch experiments were conducted to understand the effects of urea, yeast extract, and nitrate as nitrogen sources on heterotrophic growth of four strains of *C. protothecoides*. The biomass DWC productivity and lipid yield of the four strains in all media were measured and compared. Among the four strains, UTEX 255 was found to be the best candidate for lipid production. The highest LY with heterotrophic seeds were 654 mg/L/day in 2.4 g/L KNO_3 medium. Using green autotrophic seeds instead of yellow heterotrophic seeds improved the biomass DWC (13.1 vs. 11.7 g/L), LY (850 vs. 654 mg/L/day), and L/G ratio (0.607 vs. 0.162). From the sequentially mixed-nitrogen medium with green seeds, 17.0 g/L DWC was obtained, and the L/G ratio also improved to 0.197 from 0.162 in 2.4 g/L nitrate medium and from 0.108 in 4.2 g/L yeast extract medium in the first batch. However, the LY of UTEX 255 in the mixed medium (489 mg/L/day) was lower than in 2.4 g/L nitrate medium. It is believed that UTEX 255 with green seeds can be a good candidate for lipid production in its optimal medium of 2.4 g/L KNO_3 MB medium because of the high lipid yield and low glucose consumption demonstrated in this study.

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