Effect of nitrogen and extraction method on algae lipid yield

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Abstract: Effects of nitrogen source and concentration as well as lipid extraction method on the lipid yield of autotrophic *Scenedesmus dimorphus* and heterotrophic *Chlorella protothecoides* were studied. Three concentration levels of nitrate, urea and glycine/yeast extract as the nitrogen source were investigated. The highest lipid yield of *S. dimorphus* in the 17-d autotrophic culture was 0.40 g/L from the 1.8 g/L urea medium, and the maximum lipid yield of *C. protothecoides* in the nine-day heterotrophic culture was 5.89 g/L from the 2.4 g/L nitrate medium. Four different cell disruption methods—bead-beater, French press, sonication and wet milling—were studied for their effectiveness in solvent extraction of algal lipids from *S. dimorphus* and *C. protothecoides*. Wet milling followed by hexane extraction was most effective for *S. dimorphus* lipid extraction, whereas bead-beater disruption followed by hexane extraction was best for *C. protothecoides*.

Key words: algae, nitrogen source, lipid content, lipid extraction, *Scenedesmus dimorphus*, *Chlorella protothecoides*
DOI: 10.3965/j.issn.1934-6344.2009.01.051-057

Citation: Ying Shen, Zhijian Pei, Wenqiao Yuan, Enrong Mao. Effect of nitrogen and extraction method on algae lipid yield. Int J Agric & Biol Eng, 2009; 2(1): 51－57.

1 Introduction

Because of diminishing petroleum reserves and increasing environmental concerns associated with increasing demand of fossil energy, renewable and cleaner biofuels from microalgae have attracted widespread attention in recent years[1-3]. Microalgae have been regarded as the only potential and the most promising renewable source of biodiesel that could completely replace fossil diesel [1] and the most promising renewable energy source [1, 3, 4, 5]. Unlike other oil crops, algae can be grown in the desert or on marginal lands and, therefore, will not compete for arable lands currently used for producing human food and animal feed. Algae can also grow in salty water, so competition for valuable fresh water can be avoided. Currently, however, algae lipid production is still too costly. Given the estimated algae dry biomass production cost of 3000 $/ton[2] and 30% lipid content, algae lipid cost would be as high as $11/kg without considering lipid extraction costs. It must be noted that current U.S. soybean oil price is around 80 ¢/kg. The high cost of algae lipid is largely due to the lower-than-expected lipid yield (LY) of algae culture.

Nitrogen source and concentration in the growth media greatly influence algae lipid yield. In nitrogen-limited situations, algae lipid content usually increases because lipid-synthesizing enzymes are less susceptible to disorganization than carbohydrate-synthesizing enzymes due to nitrogen deprivation; thus, the major proportion of carbon can be bound in lipids[6]. However, biomass growth is often inhibited in nitrogen-lacking situations, so there is usually a lipid

Received date: 2009-02-04 Accepted date: 2009-03-03
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yield peak for each algal strain at certain nitrogen types and concentrations. Miyamoto[7] reported the effect of nitrate concentration on *Nannochlolis*. When nitrate concentration increased from 0.9 mMol/L to 9.9 mMol/L, lipid content dropped about 22%, but lipid yield increased approximately fivefold as a result of the increased biomass productivity. It is also well known that each algal species or strain prefers different kinds of nitrogen sources[6,8,9]. Shi et al.[9] investigated the effects of nitrate, ammonium and urea nitrogen sources on heterotrophic growth of *Chlorella protothecoides* and found that urea was best for the strain CS-4, whereas ammonium was the worst. However, ammonium, a main nitrogen source in wastewater, is preferred by other algal strains such as *Chlorella* sp.[10, 11].

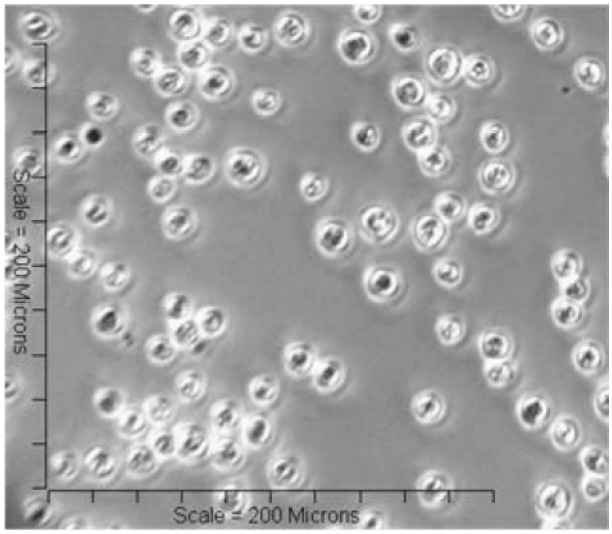
In addition to nitrogen source and concentration, lipid extraction method can also significantly affect lipid yield[12-14]. Microalgae lipid extraction usually follows two steps: cell disruption and solvent extraction. Because of large variations in algal cell shape, size, cell wall structure and characteristics of algal lipids, various lipid extraction methods work differently on various algae species. Lee[13] investigated the effects of five cell disruption methods (sonication, homogenization, French press, bead-beater and lyophilization) followed by five solvent systems (chloroform/methanol, hexane/ isopropanol, dichloroethane/methanol, dichloroethane/ ethnol and acetone/dichloromethane) on lipid recovery of *Botryococcus braunii* and found that disrupting cells with a bead-beater followed by solvent system of chloroform/methanol (2:1, V/V) was the most effective (46%－96% higher in lipid recovery than other methods). The dichloroethane/methanol and dichloroethane/ethanol systems that were effective on lipid extraction of green alga *Cladofora*[15] showed minimum recovery rate for *B. braunii.*

The objectives of this study were to (1) identify the effect of various nitrogen sources and concentrations on the lipid yield of algae and (2) investigate the effects of several cell disruption methods and solvent systems on the lipid recovery of algae. Although similar research results have been reported for other algae species, no such endeavor can be found in literature for the two algal strains (*C. protothecoides* and *S. dimorphus*) that were selected in this study.

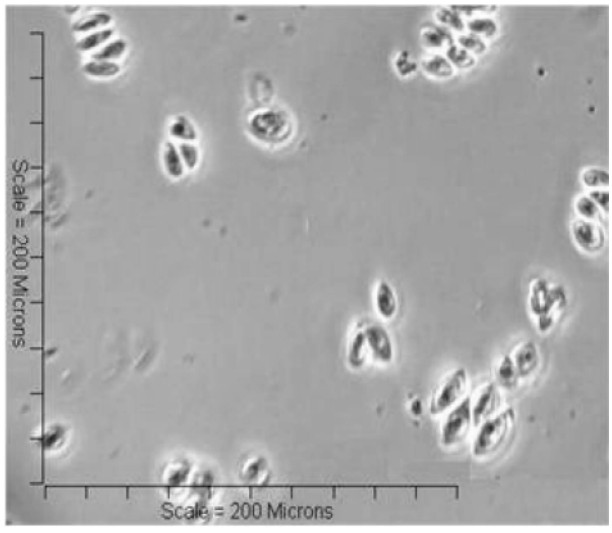
2 Material and methods

2.1 Microalgal strains

C. protothecoides (UTEX 255) and *S. dimorphus* (UTEX 417) were obtained from the Culture Collection of Algae at the University of Texas at Austin, USA. These two strains were selected because they have been well studied and identified as good candidates for lipid production[8,16] and also show very different cell characteristics. As shown in Figure 1, cells of *C. protothecoides* are round, small (diameters between 5－10 μm) and normally individually disassociated. Cells of *S. dimorphus* are bean shaped, big (long axis between



a. *C. protothecoides*



b. *S. dimorphus*

Figure 1 *C. protothecoides and S. dimorphus* cells

10 − 20 μm) and normally clustered as groups. *C. protothecoides* and *S. dimorphus* were grown heterotrophically and autotrophically, respectively. *C. protothecoides* and *S. dimorphus* on agar were inoculated into modified Basal (MB) medium (described in Section 2.2). Cultures were carried out for 5−7 d in 250-mL Erlenmeyer flasks containing 125 mL of growth media at (30±1)℃ under continuous shaking (150 r/min). Light (60−70 μmol photons (m^2 • s） $^{-1}$) was provided by cool white fluorescence lamps with 12h:12h light: dark.

2.2 Growth media

Basal medium[17] has been found effective for *C. protothecoides* growth[18] and was slightly modified for use in this study. Each liter of the MB medium contained 1250 mg KNO_3, 1250 mg KH_2PO_4, 1000 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 culture of autotrophic inoculums (*C. protothecoides* and *S. dimorphus*). For autotrophic cultivation of *S. dimorphus*, KNO_3 in the medium was replaced by urea, nitrate or glycine at concentrations listed in Table 1. For heterotrophic cultivations of *C. protothecoides*, the MB medium was supplemented with 40 g/L glucose, and KNO_3 in the medium was replaced by urea, nitrate or yeast extract at concentrations listed in Table 1. The pH value of the media was adjusted to 6.8. Three stock solutions for heterotrophic cultivation containing 200 g/L glucose and one of the following nitrogen sources were separately prepared: 7 g/L urea, 7 g/L KNO_3 and 12 g/L yeast extract. The stock solutions were used to daily feed algae with the same type of nitrogen source to keep glucose concentration at 20 g/L whenever it was below 15 g/L in the media.

Table 1 Growth media for *S. dimorphus* and *C. protothecoides*

S. dimorphus				*C. protothecoides*[a]			
Medium type	Medium code	Supplements	Nitrogen concentration	Medium type	Medium code	Supplements	Nitrogen concentration
Nitrate media	A-1 (low)	1.25 g/L nitrate	0.01 mol	Nitrate media	H-1 (low)	2.4 g/L nitrate	0.02 mol
	A-2 (medium)	3.75g/L nitrate	0.04 mol		H-2 (medium)	4.2 g/L nitrate	0.04 mol
	A-3 (high)	6 g/L nitrate	0.06 mol		H-3 (high)	6 g/L nitrate	0.06 mol
Urea media	A-4 (low)	0.4 g/L urea	0.01 mol	Urea media	H-4 (low)	1.8 g/L urea	0.06 mol
	A-5 (medium)	1.2 g/L urea	0.04 mol		H-5 (medium)	2.7 g/L urea	0.09 mol
	A-6 (high)	1.8 g/L urea	0.06 mol		H-6 (high)	3.6 g/L urea	0.12 mol
Glycine media	A-7 (low)	1.0 g/L glycine	0.01 mol	Yeast extract media	H-7 (low)	2.4 g/L yeast extract	−
	A-8 (medium)	3.0 g/L glycine	0.04 mol		H-8 (medium)	4.2 g/L yeast extract	−
	A-9 (high)	4.5 g/L glycine	0.06 mol		H-9 (high)	6 g/L yeast extract	−

Note: [a]40 g/L glucose was also added in all media for *C. protothecoides* cultivation.

2.3 Cultivation

For autotrophic culture of *S. dimorphus*, 10% inoculums were added to the nine media listed in Table 1. Cultures were carried out in 250-mL Erlenmeyer flasks containing 125 mL of growth media at (30±1)℃ under continuous shaking of 150 r/min. Light with 60 − 70 μmol photons/m^2/s was provided by cool white fluorescence lamps with 12h:12h light and dark periods. For heterotrophic cultivation of *C. protothecoides*, 5% heterotrophic inoculums were added to the nine media listed in Table 1. Cultivations were carried out in 500-mL Erlenmeyer flasks containing 300 mL of growth media at (28±1)℃ under continuous shaking of 250 r/min in the dark. Glucose concentration was determined daily by using a Megazyme D-Glucose (GOPOD Format) assay kit (Megazyme, Co. Wicklow, Ireland) following the manufacturer's standards. When glucose concentration in the medium was less than 15 g/L, stock solution with the same type of nitrogen was fed to increase the concentration to 20 g/L.

2.4 Biomass dry weight (DW) measurement

At the end of the culture period, 10 mL of the culture was filtered through a pre-dried (75℃ for five hours in an oven) and weighed (w_0) glass-fiber filter paper (55 mm,

nominal pore size 1.2 μm) under 4.4 to 7.3 kPa vacuum pressure. The filter paper was dried again in the same oven (75℃ for five hours) and kept in a vacuum desiccator overnight before weighing (w_1). Algae biomass DW was obtained by subtracting w_0 from w_1.

2.5 Cell disruption

S. dimorphus in medium A-1 and *C. protothecoides* in medium H-8 were randomly selected for comparison of cell disruption methods on lipid recovery. Each 100-mL algae culture was washed twice with distilled water and then centrifuged at 5000 g for 15 minutes. After supernatant was carefully removed, 15 mL of concentrated algae biomass was obtained for lipid extraction by using the following cell disruption methods: (1) direct extraction with a Soxhlet apparatus (KIMAX, Pittsburgh, PA) without cell disruption; (2) sonication with a Sonics, Model 100-watt ultrasonic processor (Sonics, Newtown, CT) for 2 min, twice; (3) French press at 1500 psi for five times by a Thermo, Model FA-081A120 VAC French Press (Thermo, Waltham, MA); (4) bead-beater, Model 3110 BX with 1 mm glass beads (Biospec, Bartlesville, OK), running for 2 min, twice; and (5) wet milling with a Straub, 100-g (Straub Co., Hatboro, PA) for three times. After disruption of algal cells, the suspensions were transferred to 50-mLcentrifuge tubes, and 45 mL of ethanol/hexane (1:1, V/V) mixture was added to each sample. The tube containing disrupted algal cells and solvent was shaken on a reciprocating shaker (150 r/min) overnight. After that, the tube was centrifuged at 4000 g for 15 minutes to remove the glass beads and algal solids. The supernatant was carefully collected and vaporized and then dried in an oven at 95℃ for 1.5 h. Lipids left in the flask without solvent were weighed to calculate lipid content.

2.6 Solvent systems

After disruption of algal cell with the bead-beater, two different solvent systems that are widely used for lipid extraction were selected for comparative tests: ethanol/ hexane (1:1, V/V) and hexane only. In the solvent system of ethanol/ hexane (1:1, V/V), the concentration of ethanol was 1.5 times as that of water to make a 40% V/V hydroalcoholic phase, which showed the best results[19]. The ratio of hexane/water was 1:1, V/V.

3 Results and discussion

3.1 Effects of nitrogen on biomass DW and LY

All results presented in this article are the average of two replications. The effects of nitrogen on biomass DW and lipid yield of autotrophic *S. dimorphus* are shown in Table 2. Maximum biomass DW achieved by *S. dimorphus* in the three types of nitrogen sources in 17 days were (1.2 ± 0.1) g/L in medium A-3 (6 g/L KNO_3), (1.3 ± 0.1) g/L in medium A-6 (1.8 g/L urea) and (1.2 ± 0.1) g/L in medium A-7 (1 g/L glycine). *S. dimorphus* did not survive in the higher-concentration glycine media. The highest LY values achieved were (0.29 ± 0.01) g/L in medium A-3 (6 g/L KNO_3), (0.40 ± 0.01) g/L in mediumA-6 (1.8 g/L urea) and (0.27 ± 0.01) g/L in medium A-7 (1 g/L glycine). *S. dimorphus* produced more biomass and lipids in high-nitrogen-concentration media (about 0.06 mol N in nitrate and urea media), except for glycine, which was found not suitable for *S. dimorphus* growth. As a strain isolated from wastewater, *S. dimorphus* may prefer high nitrogen concentration. Urea was a better nitrogen source than nitrate and glycine for lipid production of *S. dimorphus*. The highest lipid yield in urea medium was at least 38% higher than in nitrate media and 48% higher than in glycine media under the same culture conditions.

The effects of nitrogen on biomass DW and lipid yield of heterotrophic *C. protothecoides* are shown in Table 3. Maximum biomass DW values achieved by *C. protothecoides* in the three nitrogen sources in nine days were (12.8 ± 1.2) g/L in medium H-3 (6 g/L KNO_3), (11.8 ± 0.6) g/L in medium H-4 (1.8 g/L urea) and (14.2 ± 0.4) g/L in medium H-8 (4.2 g/L yeast extract). The highest LY values achieved were (5.89 ± 0.03) g/L in medium H-1 (2.4 g/L KNO_3), (2.90 ± 0.01) g/L in medium H-4 (1.8 g/L urea) and (4.27 ± 0.02) g/L in medium H-7 (2.4 g/L yeast extract). Unlike *S. dimorphus*, *C. protothecoides* produced more lipids in low-nitrogen-concentration media. It is also evident that nitrate was the best among the three nitrogen sources for lipid production of *C. protothecoides*. Maximum lipid yield in nitrate media was at least 103% higher than that in urea media and 38% higher than that in yeast extract media.

Table 2 Effects of nitrogen on biomass dry weight (DW) and LY of *S. dimorphus*

Nitrogen source	Nitrogen concentration	Biomass DW /g • L^{-1}	Lipid yield* /g • L^{-1}
Nitrate	Low (A-1)	0.9 ± 0.1	0.21 ± 0.01
	Medium (A-2)	1.0 ± 0.1	0.22 ± 0.01
	High (A-3)	1.2 ± 0.1	0.29 ± 0.01
Urea	Low (A-4)	1.0 ± 0.1	0.32 ± 0.01
	Medium (A-5)	1.1 ± 0.1	0.31 ± 0.01
	High (A-6)	1.3 ± 0.1	0.40 ± 0.01
Glycine	Low (A-7)	1.2 ± 0.1	0.27 ± 0.01
	Medium (A-8)	NS[b]	NS[b]
	High (A-9)	NS[b]	NS[b]

Note: *lipids were obtained by bead-beater disruption followed by hexane extraction; NS[b]- algae did not survive.

Table 3 Effects of nitrogen on biomass DW and LY of *C. prototheocoides*

Nitrogen source	Nitrogen concentration	Biomass DW /g • L^{-1}	Lipid yield* /g • L^{-1}
Nitrate	Low (H-1)	11.7 ± 0.5	5.89 ± 0.03
	Medium (H-2)	11.2 ± 0.4	4.57 ± 0.02
	High (H-3)	12.8 ± 1.2	4.46 ± 0.02
Urea	Low (H-4)	11.8 ± 0.6	2.90 ± 0.01
	Medium (H-5)	2.5 ± 0.4	0.60 ± 0.01
	High (H-6)	6.9 ± 0.3	1.66 ± 0.01
Yeast extract	Low (H-7)	12.2 ± 0.2	4.27 ± 0.02
	Medium (H-8)	14.2 ± 0.4	3.56 ± 0.02
	High (H-9)	8.3 ± 0.5	1.07 ± 0.01

Note: *lipids were obtained by bead-beater disruption followed by hexane extraction.

3.2 Effect of nitrogen on lipid content

Lipid content of algae is an important parameter that determines the economy of algae biodiesel production[2]. Higher lipid content reduces lipid production costs. The lipid contents of *S. dimorphus* and *C. prototheocoides* in all media are shown in Figure 2. It appears that concentration of urea and nitrate did not significantly affect lipid content of *S. dimorphus*, although urea media achieved higher lipid content than nitrate media. The lipid content of *C. prototheocoides* was, however, sensitive to nitrogen concentration, except in urea. As shown in Figure 2, lipid content achieved by *C. prototheocoides* in low-nitrate-concentration medium was at least 23% higher than that in media with a higher nitrogen concentration, but the biomass DW was only 9% lower than the maximum value. Similarly, lipid content achieved by *C. prototheocoides* in low-yeast-extract medium was at least 40% higher than that in media with a higher yeast concentration, but biomass DW was only 16% lower than the maximum value. Urea concentration seemed to have no effect on lipid content of *C. prototheocoides*. These results indicate that nitrate was the best in terms of lipid content of *C. prototheocoides*.

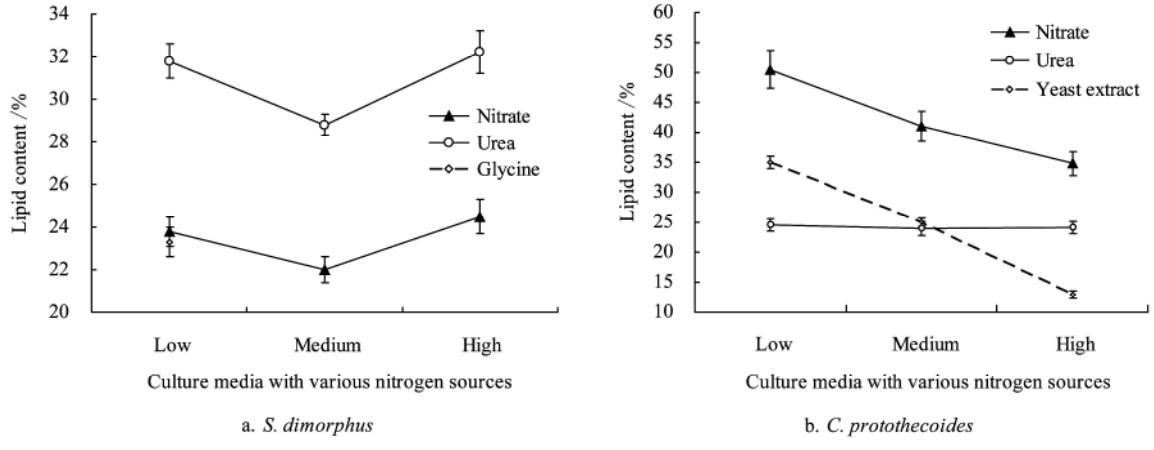


Figure 2 Effects of nitrogen on lipid contents of *S. dimorphus* and *C. prototheocoides*

3.3 Effects of cell disruption methods on lipid recovery

The lipid contents of *S. dimorphus* and *C. prototheocoides* recovered through five different cell disruption methods with the solvent system of ethanol/hexane (1:1, V/V) is shown in Figure 3. The highest recovered lipid content of *S. dimorphus* was achieved by using wet milling (25.3%), followed by French press (21.2%), sonication (21.0%), bead-beater (20.5%) and direct soxhlet extraction (6.3%). Unlike *S. dimorphus*, the highest recovered lipid content of *C. prototheocoides* was achieved by using bead-beater

(18.8%), followed by French press (14.9%), wet milling (14.4%), sonication (10.7%) and direct soxhlet extraction (5.6%). As shown in Figure 1, the cells of *S. dimorphus* are big, bean-shaped and clustered together, whereas cells of *C. protothecoides* are round and small. Differences in cell size, shape and structure may have caused the difference in effectiveness of lipid recovery between these two strains. Among the five methods, wet milling and bead-beater showed the highest lipid recovery rate for *S. dimorphus* and *C. protothecoides*, respectively. Efficiency achieved by using wet milling was at least 19% higher than that achieved with other methods for *S. dimorphus*, and efficiency achieved by using bead-beater was at least 26% higher than that achieved with other methods for *C. protothecoides.*

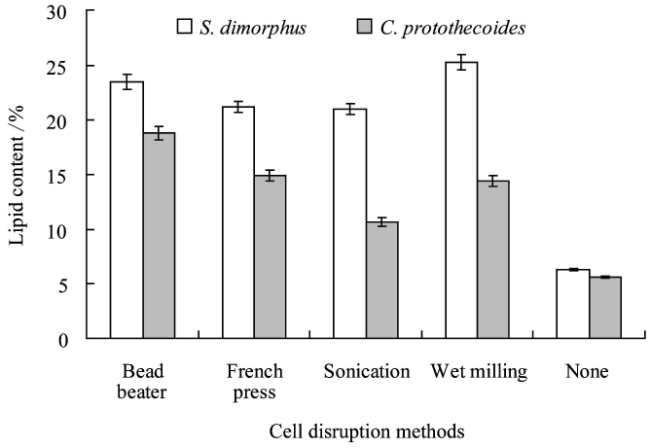


Figure 3 Effects of cell disruption methods on recovered lipid contents of *S. dimorphus* and *C. protothecoides*

Wet milling is a well-developed technology used commercially in the starch processing industry that could be applied to algal lipid extraction, as demonstrated in this study. Advantages of using wet milling as a cell disruption method may include high lipid recovery rate, easy and low-cost operation and maintenance and availability of large-scale commercial equipment. Although bead-beater is also efficient at cell disruption for some species, it is limited to small-scale use. Other methods tested are either too expensive or low in lipid recovery rate. According to the results, however, no single method can apply to all algae species because of large variations in cell characteristics.

3.3 Effect of solvent systems

Lipid contents of *S. dimorphus* and *C. protothecoides* recovered by using bead-beater as the cell disruption method followed by two solvent systems (hexane only and hexane/ethanol (1:1, v:v)) are shown in Figure 4. For both strains, lipid recovery rates were higher in the hexane-only system. Lipid content of *S. dimorphus* was 29.7% with hexane, which was 16% higher than that with hexane/ethanol, and lipid content of *C. protothecoides* was 23.5% with hexane, which was 25% higher than that with hexane/ ethanol. A possible cause for the lower lipid recovery efficiency in hexane/ethanol solvent may be that a small quantity of lipids are bound in ethanol and water solvent that cannot be extracted by hexane. However, the hexane/ethanol solvent was proven effective in lipid recovery of *Phaeodactylum tricornutum*[19], suggesting that efficiencies of solvent systems are affected by algal strains and cell disruption methods.

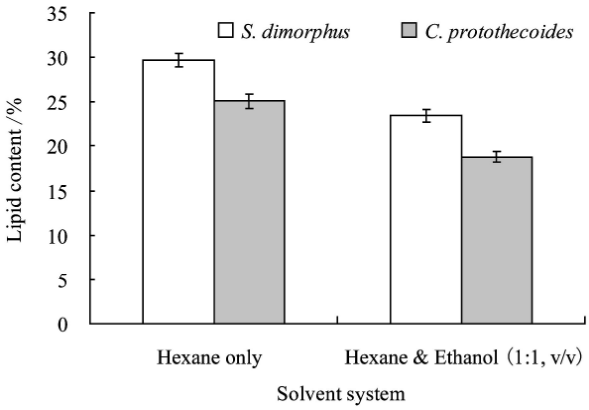


Figure 4 Effects of solvent systems on recovered lipid contents of *S. dimorphus* and *C. protothecoides*

4 Conclusions

This study investigated the effects of nitrogen source and concentration as well as lipid extraction method on lipid yields of autotrophic *S. dimorphus* and heterotrophic *C. protothecoides*. Maximum biomass DW and LY achieved by *S. dimorphus* in the seventeen-day autotrophic culture were 1.3 g/L and 0.4 g/L, respectively, both in the 1.8 g/L urea medium (medium A-6). Urea was the best nitrogen source for *S. dimorphus*. In the nine-day heterotrophic culture of *C. protothecoides*, maximum biomass DW and lipid yield achieved were 14.2 g/L in the 4.2 g/L yeast extract medium (medium H-8) and 5.89 g/L in the 2.4 g/L nitrate medium (medium

H-1), respectively. Nitrate was the best nitrogen source for *C. protothecoides* lipid production. Of the five cell disruption methods (direct extraction, sonication, French press, bead-beater and wet milling) and two commonly used solvent systems (ethanol/hexane and hexane only) tested, wet milling followed by hexane extraction was the best for *S. dimorphus*, whereas bead-beater followed by hexane extraction was most effective for *S. dimorphus* lipid recovery.

Acknowledgments:

The authors acknowledge Dr. Donghai Wang at Kansas State University for allowing the use of some equipment. This research is funded by NSF (Award CMMI-0836610) and Kansas Agricultural Experiment Station (Contribution No. 09-237-J from the Kansas Agricultural Experiment Station).

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