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RESEARCH PAPER

Lipid Extraction from *Tetraselmis* sp. Microalgae for Biodiesel Production Using Hexane-based Solvent Mixtures

Hee-Yong Shin, Sang-Hyeok Shim, Young-Jin Ryu, Ji-Hyun Yang, Sang-Min Lim, and Choul-Gyun Lee

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Abstract Lipid extraction is a critical step in the downstream processing of biodiesel production from microalgae. Solvent extraction using mixtures of non-polar and polar solvents is one of the most well-known processes for this purpose. Hexane is the most common solvent of choice for large-scale lipid extractions due to its technical and economic advantages, especially its high selectivity toward lipids and low cost. In this study, extractions using mixtures of hexane and polar solvents were evaluated for their performance in order to develop a more efficient method for large-scale lipid extraction from microalgae. The combination of hexane and methanol resulted in the highest fatty acid methyl ester (FAME) yield for lipids from *Tetraselmis* sp. The effects of extraction conditions, including proportions of methanol to hexane, ratios of total solvent volume to dry biomass, and extraction time, on extraction yields were evaluated to determine optimum conditions providing higher lipid and FAME yields. The optimal conditions were as follows: proportion of hexane to methanol of 1:1, ratio of total solvent volume to dry biomass of 10 mL/g, and extraction time of 120 min. Finally, the selected solvent mixture and optimal conditions were applied to larger scale extraction experiments with scale-up factors of 10, 50, and 100. FAME yields of large-scale extractions were almost completely consistent with increasing scale-up factors. The results of this study suggest that a hexane and methanol mixture is a promising solvent for large-scale lipid extraction from microalgae.

Hee-Yong Shin†, Sang-Hyeok Shim†, Young-Jin Ryu, Ji-Hyun Yang, Sang-Min Lim, Choul-Gyun Lee*
National Marine Bioenergy R&D Consortium & Department of Biological Engineering, Inha University, Incheon 22212, Korea
Tel: +82-32-872-7518; Fax: +82-32-873-7518
E-mail: leecg@inha.ac.kr

†The first two authors contributed equally to this work.

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1. Introduction

Fossil fuels are non-renewable sources of energy that generate pollutants and are linked to global warming, climate change, and even some incurable diseases. The impending challenges and environmental implications of fossil fuels have been reviewed widely in the literature [1-3]. It was previously reported that 98% of carbon emissions are the result of fossil fuel combustion [4]. Therefore, development of a sustainable and renewable energy pathway to satisfy the energy needs of the future is desirable.

Biodiesel is defined as mono-alkyl esters of long chain fatty acids originating from natural oils and fats of plants and animals, and it is a kind of alternative to fossil fuels. Biodiesel has attracted wide attention worldwide due to its renewability, biodegradability, non-toxicity, and environmentally friendly benefits [5]. Additionally, U.S. Department of Energy life cycle analysis of biodiesel has shown that biodiesel produces 78.5% less net carbon dioxide emissions compared to petroleum diesel [6].

Liquid biofuels (such as bioethanol, biodiesel, *etc*.) are categorized into different generations of biofuels based on their type of feedstock. First-generation biofuels are derived from edible feedstocks such as corn, soybean, sugarcane, and rapeseed while second-generation biofuels are from non-edible feedstocks such as jatropha, miscanthus, and switch grass. However, escalating demand for an edible feedstock as a food source coupled with the finite availability of arable land for cultivation of edible and non-edible feedstocks makes first and second generation biofuels unsustainable. Thus, third-generation biofuels, which are derived from microalgae, have an edge over the previous

two categories [7].

Microalgae are photosynthetic unicellular microorganisms capable of converting sunlight, water, and carbon dioxide into algal biomass. High photosynthetic rates enable microalgae to serve as an effective carbon capture platform while rapidly accumulating lipids in their biomass. Even in a conservative scenario, microalgae are predicted to produce about 10 times more biodiesel per unit area of land than a typical terrestrial oleaginous crop [8-11]. Furthermore, production of algal biomass does not place additional strain on food production since microalgal species can be cultured on non-arable land [12]. For these reasons, microalgae are currently considered as some of the most promising alternative and renewable sources of biodiesel feedstocks.

Lipid extraction is a critical step in the downstream processing of biodiesel production from microalgae [13]. Organic solvent extraction is one of the most well-known processes used for this purpose due to its economic and technical advantages, especially its high selectivity and solubility toward lipids, low cost, and relatively easy scale-up based on its equipment [14].

Hexane is the most common solvent of choice for large-scale lipid extractions due to its cost-effectiveness. When extracting lipids from microalgae, hexane has minimal affinity for non-lipid contaminants as well as higher selectivity towards neutral lipid fractions that can be converted into biodiesel [15,16]. Despite its great advantages, use of hexane alone has been reported to be less efficient for microalgal lipid extractions [17-20].

Lipids in microalgae exist in various forms such as neutral lipids in complex with polar lipids. Addition of a polar solvent to non-polar solvent can be used to extract both non-polar lipids as well as lipids associated with polar lipids, such as membrane-associated lipids [13]. These procedures have proven effective for the majority of lipid extractions from microalgae performed on a laboratory-scale for lipid analyses [15,21-25].

Based on these considerations, in this work, various polar solvents with hexane were used to extract lipids from microalgal biomass. The abilities of solvent mixtures to extract lipids were compared in terms of yields of lipids and fatty acids (as fatty acid methyl ester: FAME) in an effort to select the most efficient hexane-polar solvent mixture. In the results, a mixture of hexane (non-polar) and methanol (polar) was selected as the most suitable solvent mixture for extracting lipids from *Tetraselmis* sp. In addition, the effects of extraction parameters, including proportions of methanol to hexane, ratios of total solvent volume to dry biomass, and extraction time, on extraction yields were investigated to determine optimum conditions providing higher lipid and FAME yields. Finally, larger scale experiments were performed under the optimum extraction conditions to evaluate extraction performance with increasing scale-up factors.

2. Materials and Methods

2.1. Strain and culture conditions

Tetraselmis sp. KCTC12429BP was isolated from natural seawater at Young-Heung Island, Incheon, Korea. Base culture medium used in this experiment was MBL (artificial seawater; ASW), consisting of 24.7 g/L NaCl, 0.66 g/L KCl, 8.48 g/L $MgCl_2 \cdot 6H_2O$, 1.9 g/L $CaCl_2 \cdot 2H_2O$, 6.318 g/L $MgSO_4 \cdot 7H_2O$, and 0.18 g/L $NaHCO_3$. For the nutrients, f/2-Si medium, consisting of 75 mg/L $NaNO_3$, 5 mg/L $NaH_2PO_4 \cdot H_2O$, 3.15 mg/L $FeCl_3 \cdot 6H_2O$, 4.36 mg/L $Na_2EDTA \cdot 2H_2O$, 0.18 mg/L $MnCl_2 \cdot 4H_2O$, 0.022 mg/L $ZnSO_4 \cdot 7H_2O$, 0.01 mg/L $CoCl_2 \cdot 6H_2O$, 0.01 mg/L $CuSO_4 \cdot 5H_2O$, and 0.006 mg/L $Na_2MoO \cdot 2H_2O$, was additionally added to base medium. Microalgae were cultured in three open raceway ponds with a working volume of 10 kL at 20 ~ 25°C for 7 days. Cells were harvested after the 7-day culture. After centrifugation, the harvested cells (19 ~ 21% solids) were freeze-dried and used for lipid extraction.

2.2. Materials and extraction methods using mixtures of hexane and polar solvents

Algal biomass from a single harvest (8.7 kg dry weight) was used for all experiments. The lyophilized algae were ground up until the algae particles were less than 150 μm. After grinding and before all extraction procedures, algae were heated to 100°C for 1 h to remove residual water. All organic solvents were reagent grade and were used without further purification. Acetyl chloride (97.0% pure, DAEJUNG Corp.) was used for the transesterification of lipid extracts. A fatty acid methyl ester (FAME) mixture (99.9% pure, Sigma-Aldrich Corp.) was used as a standard, and methyl nonadecanoate (C19:0) was used as an internal standard for analyzing FAME content.

After placing 10 g of algae (3.7% biodiesel convertible lipid fraction) in an Erlenmeyer flask, prescribed amounts of hexane and polar solvent mixtures were poured into the flask. The mixture was then shaken (250 rpm) for a certain period of time set in advance. When the extraction was finished, the mixture was immediately filtered to remove algae and avoid further lipid extraction. The mixture was then transferred to a separatory funnel, and a certain amount of water was added to allow separation of the organic and aqueous layers. The lipid and hexane layer was then separated from the polar solvents and water layer. The hexane was evaporated using a rotary evaporator to leave behind the extracted lipids. The weight of the extracted lipids was then recorded. Lipid yield was calculated by

dividing the weight of the crude lipids by the weight of dry algae. All extractions were replicated three times, and mean average values were used.

$$\text{Lipid yield (wt.\%)} = \frac{Weight\ of\ extracted\ lipids}{Weight\ of\ dry\ biomass}$$

2.3. Microalgal lipid analysis

The obtained lipids (10 ~ 20 mg) were transesterified at 80ºC for 1 h using 1 mL of an acetyl chloride and methanol mixture (1:10, v/v) to determine FAME content of the extracted lipids. After the reaction was completed, 1 mL of internal standard solution was added and centrifugation was performed. A solution of methyl nonadecanoate in heptane (3 mg/mL) was used as an internal standard for FAME analysis. The upper phase was collected and analyzed by a gas chromatograph equipped with flame ionization detector (YL6500GC, Younglin, Anyang, Korea) with an HP-INNOWAX column (30 m length, 0.53 mm I.D., 1.0 μm film thickness). Each sample (1 μL) was injected at an initial oven temperature of 140ºC. After injection, the oven was heated at 8ºC/min to 180ºC, and at 5ºC/min to 230ºC, after which it was held for 20 min. The flow rate of the carrier gas (He) was 3 mL/min. The injector and detector temperatures were set at 250ºC. FAMEs in samples were identified by comparing the retention times of FAME peaks with those of authentic standards. FAME yield was calculated as follows:

$$\text{FAME yield (wt.\%)} = \frac{Lipid\ yield\ (wt.\%) \times FAME\ content\ (\%)}{100}$$

2.4. Large-scale hexane/methanol extraction

Fig. 1 shows the flow diagram of the large-scale microalgal lipid extraction using a mixture of hexane and methanol. The extraction system consists of a 50 L jacketed glass reactor (DL-50L, HCS, Singapore) with a stirrer, PID temperature controller, filter, and evaporator with a condenser. Large-scale extractions with 100 g, 500 g, and 1 kg of dry algae were carried out under optimum conditions obtained in a laboratory-scale experiment. Briefly, after placing dry algae in the 50 L jacketed glass reactor, prescribed amounts of hexane and methanol were pumped into the reactor. The proportion of methanol to hexane and total solvent volume to dry biomass were fixed at 1 (v/v) and 10 mL/g, respectively. The mixture was then shaken at 100 rpm for 120 min. When the extraction was finished, algae residue was removed from the mixture using a glass Nutsche filter system (50 L volume, 8 μm pore size, Buchi, Switzerland). The mixture was then transferred to the reactor again, and a certain amount of water was added to allow separation of the organic and aqueous layers. The aqueous layer was then removed from the reactor. The organic layer was concentrated under reduced pressure and finally hexane was removed from the concentrate using a rotary evaporator to leave behind the extracted lipids. Lipid yields and FAME yields of extracted lipids were compared with those obtained on a laboratory scale.

3. Results and Discussion

3.1. Selection of hexane/polar solvent mixtures for microalgal lipid extraction

In this study, FAME yield as well as crude lipid yield were used as important indices to evaluate extraction efficiency, as the purpose of extraction is biodiesel production. To select the most efficient hexane-based solvent mixtures, various equivolume mixtures of hexane as well as two groups of polar solvents were tested. A polar protic group is composed of methanol, ethanol, and isopropanol, whereas a polar aprotic group consists of acetone, acetonitrile, and tetrahydrofuran. Table 1 shows the solvent properties [26] of hexane and both groups, and these solvents were selected by considering their boiling points and dielectric constants, which generally provide a rough measure of a solvent's polarity. The total solvent volume to dry biomass and extraction time were fixed at 10 mL/g and 120 min, respectively, and a chloroform-methanol mixture was used as a benchmark [27,28] for microalgal lipid extractions. The crude lipid and FAME yields are shown in Fig. 2.

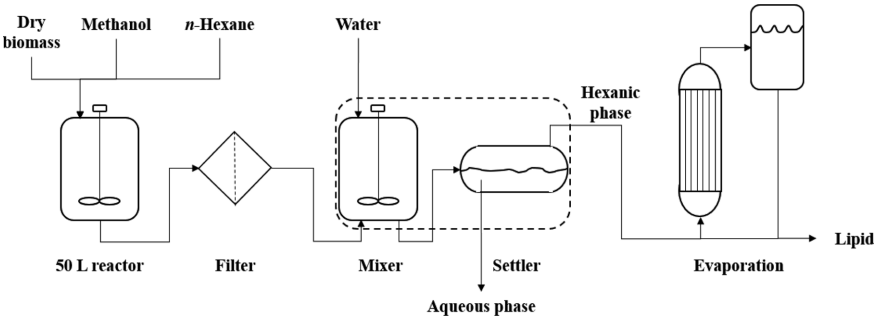

Fig. 1. Flow diagram of large-scale microalgal lipid extraction.

Table 1. Properties of hexane and polar solvents used in this study

Solvent		Boiling point (°C)	Dielectric constant
Non-polar solvent	Hexane	69	1.88
Polar protic solvent	Isopropanol	82	18
	Ethanol	79	24.55
	Methanol	65	33
Polar aprotic solvent	Tetrahydrofuran	66	7.5
	Acetone	56	21
	Acetonitrile	82	37.5

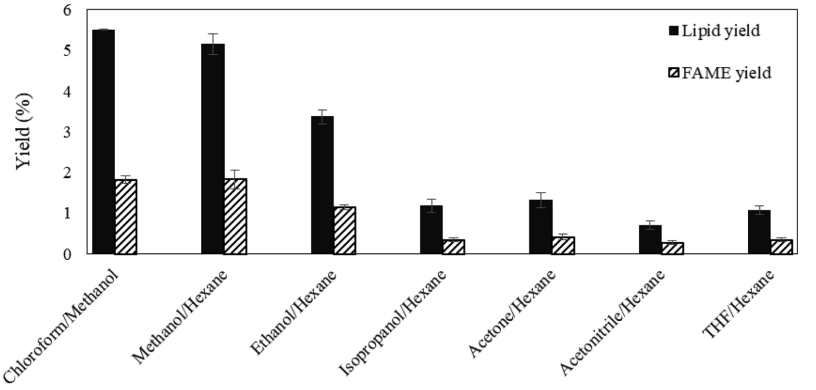

Fig. 2. Crude lipid and FAME yields from lyophilized biomass by using different equivolume mixtures of hexane and polar solvents.

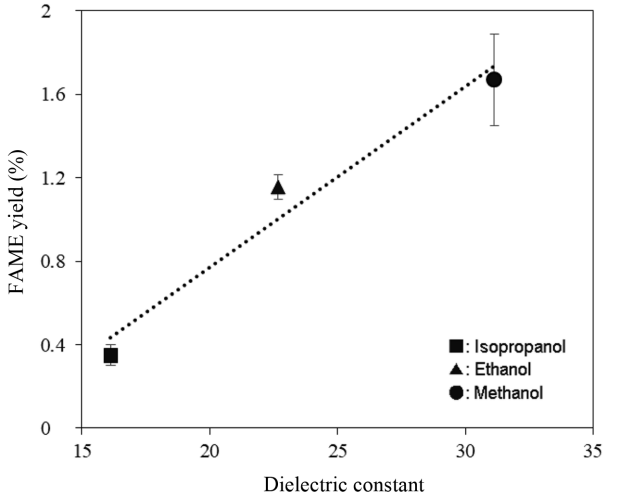

Fig. 3. Linear relationships between FAME yield and dielectric constants of protic solvents in hexane-based solvent mixtures.

Mixtures of hexane and polar protic solvents resulted in higher lipid and FAME yields than those of hexane and polar aprotic solvents. A mixture of hexane and methanol showed higher efficiency compared to mixtures of hexane with other polar protic solvents such as ethanol and isopropanol. Interestingly, as shown in Fig. 3, linear relationships between FAME yield and dielectric constants of protic solvents were observed with a determination coefficient (R^2) of 0.9603, which means that the higher polarities of the protic solvents in the hexane-based solvent mixtures provided higher FAME yields. Additionally, the combination of hexane and methanol showed high efficiency that is comparable to the chloroform-methanol mixture. Thus, a mixture of hexane and methanol was selected as a suitable solvent mixture for extracting lipids from *Tetraselmis* sp.

The fatty acid compositions as well as extraction performances of the described solvent mixtures are shown in Table 2, and it is evident that the fatty acid compositions of lipid extracts were similar regardless of extraction method. The main fatty acids in the lipid extracts were methyl esters of palmitic acid (C16:0), palmitoleic acid (C16:1), hexadecadienoic acid (C16:2), hexadecatrienoic acid (C16:3), hexadecatetraenoic acid (C16:4), stearic acid (C18:0), oleic acid (C18:1), linoleic acid (C18:2), linolenic acid (C18:3), octadecatetraenoic acid (C18:4), and eicosapentaenoic acid (C20:5).

3.2. Optimization of lipid extraction using a mixture of hexane and methanol

Based on the results of section 3.1, a mixture of hexane (non-polar) and methanol (polar) was selected as a suitable solvent mixture for extracting lipids from *Tetraselmis* sp. The effects of various extraction parameters, including proportions of methanol to hexane, ratios of total solvent volume to dry biomass, and extraction time, on extraction yields were investigated to determine optimum conditions providing higher lipid and FAME yields.

Table 2. Comparison of fatty acid profile and extraction performance for the different extraction methods

	Extraction methods						
	Methanol /Chloroform	Methanol /Hexane	Ethanol /Hexane	Isopropanol /Hexane	Acetone /Hexane	Acetonitrile /Hexane	THF /Hexane
Crude lipid yield (wt.%)	5.51 (± 0.02)	5.16 (± 0.25)	3.36 (± 0.17)	1.06 (± 0.16)	1.18 (± 0.19)	0.71 (± 0.10)	1.66 (± 0.10)
FAME composition (%)							
Palmitic acid (C16:0)	23.8	23.4	23.8	21.8	21.6	22.5	22.3
Palmitoleic acid (C16:1)	6.7	6.2	8.1	8.7	9.3	10.6	7.8
Hexadecadienoic acid (C16:2)	1.9	2.0	1.5	0.8	0.8	0.9	1.2
Hexadecatrienoic acid (C16:3)	3.1	2.6	2.8	3.3	2.9	2.4	3.1
Hexadecatetraenoic acid (C16:4)	6.3	5.7	5.4	8.1	7.6	3.8	6.7
Stearic acid (C18:0)	2.4	2.6	2.5	2.9	2.6	2.7	2.5
Oleic acid (C18:1)	17.1	17.2	17.4	13.9	14.2	15.7	16.5
Linoleic acid (C18:2)	3.4	3.8	3.0	3.2	3.4	3.0	3.4
Linolenic acid (C18:3)	10.5	11.3	9.4	9.8	10.1	9.1	10.3
Octadecatetraenoic acid (C18:4)	5.3	5.1	4.2	5.4	5.4	4.2	5.3
Eicosapentaenoic acid (C20:5)	3.7	3.9	4.8	4.6	4.3	4.9	4.2
FAME content (%)	33.1	32.3	34.5	33.0	34.8	38.0	32.4
FAME yield (wt.%)	1.82 (± 0.09)	1.67 (± 0.22)	1.15 (± 0.06)	0.35 (± 0.05)	0.41 (± 0.07)	0.27 (± 0.04)	0.34 (± 0.05)

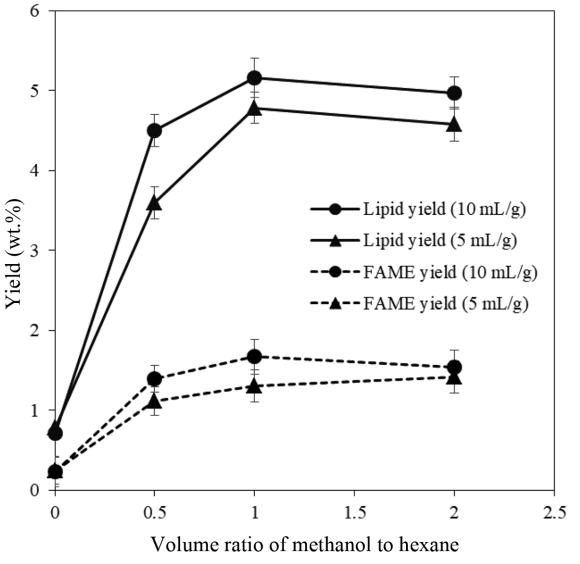

Fig. 4. Effect of proportion of methanol to hexane (v/v) on lipid and FAME yields.

Lipid extractions were carried out using various hexane/methanol ratios to obtain optimum extraction conditions. Fig. 4 shows the effects of hexane/methanol ratios (v/v) from 0 to 2 on lipid and FAME yields at fixed total solvent volumes (50 and 100 mL) with an extraction time of 120 min. The results show that adding a small amount of methanol provided significantly higher lipid and FAME yields compared to using hexane alone to extract lipids from microalgae. At a solvent extraction volume of 100 mL, lipid and FAME yields improved from 0.72 and 0.25 to 5.16 and 1.67% (w/w), respectively, when volume ratios of methanol to hexane increased from 0 to 1 but decreased slightly above a hexane/methanol ratio of 1. At a solvent extraction volume of 50 mL, lipid and FAME yields were quite lower than those obtained at 100 mL, although a similar tendency was observed for the volume ratio effect. With respect to solvent recovery after layer separation, recovery of methanol from the aqueous phase requires more energy than removing hexane from organic phase due to the relatively higher heat of vaporization of methanol, indicating that a hexane/methanol mixture containing a high ratio of methanol needs more energy to recover extraction solvents. Thus, 1:1 was chosen as a suitable proportion of methanol to hexane.

It is important to determine the minimum solvent volume providing higher lipid yields since organic solvents are costly to recycle when used on a large scale. Therefore, the effects of different ratios of total solvent volume to dry biomass (v/w) on lipid and FAME yields were investigated, as shown in Fig. 5. The proportion of methanol to hexane and extraction time were fixed at 1 (v/v) and 120 min, respectively. As evident in Fig. 5, lipid and FAME yields were considerably affected until the total solvent volume/dry algae was increased to 5 mL/g. Lipid and FAME yields slightly increased up to 10 mL/g, after which they remained almost constant at higher solvent volumes. As a result, 10 mL/g was selected as an appropriate ratio of total solvent volume to dry biomass. This value is significantly lower than those reported in previous research, and the data are summarized in Table 3.

Time is another important parameter influencing the overall costs of extraction processes. Therefore, the effect of extraction time (min) on lipid and FAME yields was investigated as shown in Fig. 6. The proportion of methanol to hexane and total solvent volume to dry biomass were fixed at 1 (v/v) and 10 mL/g, respectively. Lipid and FAME

Table 3. Total solvent volumes to biomass from the literature for microalgal lipid extractions

Reference	Solvent	Ratio of total solvent volume to dry biomass (mL/g)	Extraction scale (g biomass/batch)
This study	Hexane/methanol (1:1, v/v)	10	10 ~ 1,000
Lee *et al*. [15]	Chloroform/methanol (2:1, v/v)	250	0.12
Halim *et al*. [17]	Hexane/isopropanol (3:2, v/v)	75	4
Molina Grima *et al.* [24]	Chloroform/methanol/water (1:2:0.8, v/v/v)	76	5
Folch *et al*. [27]	Chloroform/methanol/water (8:4:3, v/v/v)	20	1
Bligh and Dyer [28]	Chloroform/methanol/water (1:1:0.9, v/v/v)	29	20

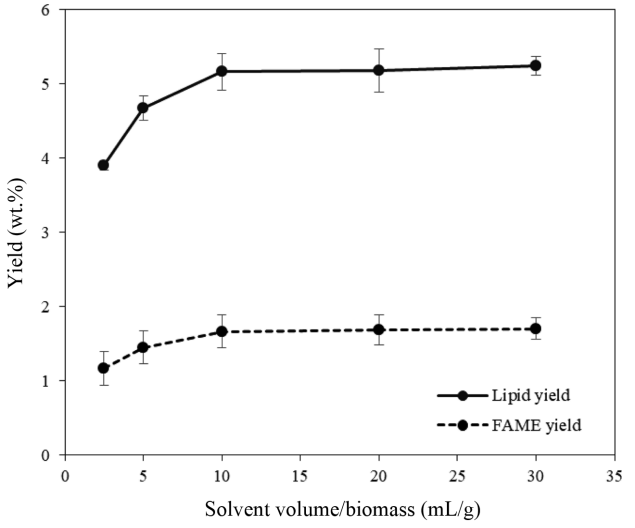

Fig. 5. Effect of ratio of total solvent volume to dry biomass (v/w) on lipid and FAME yields.

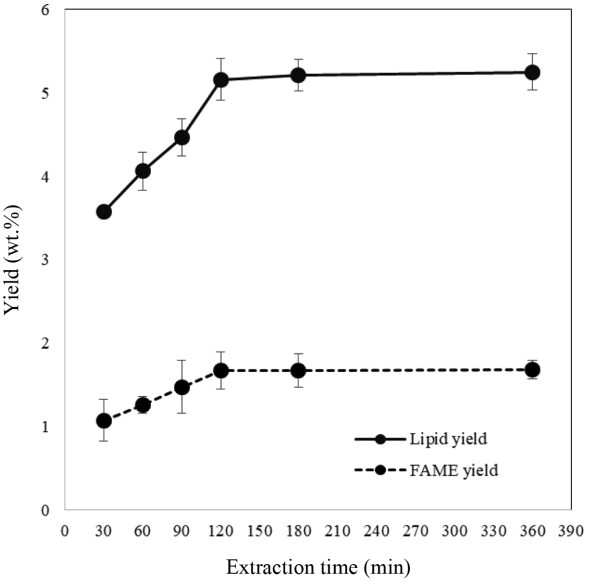

Fig. 6. Effect of extraction time on lipid and FAME yields.

yields improved from 3.58 and 1.07 to 5.16 and 1.67% (w/w) as extraction time increased from 30 to 120 min, and no significant increase was observed after 120 min. Extending time after the extraction reached equilibrium unavoidably leads to extra energy costs. As a result, 120 min was selected as the optimum extraction time.

3.3. Large-scale hexane/methanol extraction

Larger scale experiments were conducted with scale factors of 10, 50, and 100 under the optimum conditions obtained in the laboratory-scale experiment. Lipid yields and FAME yields of extracted lipids were compared with those obtained on a laboratory scale. Table 4 shows the extraction conditions and results obtained on a laboratory scale and large scale. As the scale-up factor increased, lipid yields showed a tendency to decrease slightly, whereas FAME yields were almost constant. Overall, extraction performances of large-scale extractions were almost entirely consistent with those obtained on a laboratory scale.

4. Conclusion

Lipid extraction from *Tetraselmis* sp. biomass was performed using hexane-based solvent mixtures. Two groups of polar solvents were mixed with hexane and tested to select the most efficient solvent mixtures. A mixture of hexane and methanol provided the highest crude lipid yield and FAME yield compared to the mixtures of hexane with other polar

Table 4. Comparison between laboratory-scale and large-scale extraction conditions and results

Scale	Dry biomass (g)	Hexane (mL)	Methanol (mL)	Agitation speed (rpm)	Extraction time (min)	Lipid yield (wt.%)	FAME yield (wt.%)
Lab-scale	10	50	50	250	120	5.16 (± 0.25)	1.67 (± 0.22)
× 10	100	500	500	250	120	5.21 (± 0.24)	1.70 (± 0.18)
× 50	500	2,500	2,500	100	120	4.98 (± 0.22)	1.69 (± 0.12)
× 100	1,000	5,000	5,000	100	120	4.79 (± 0.19)	1.69 (± 0.11)

solvents, and its extraction yield was even comparable to that of the chloroform-methanol mixture. The effects of the extraction parameters, including (i) proportions of methanol to hexane, (ii) ratios of total solvent volume to biomass, and (iii) extraction time, on extraction yields were evaluated to obtain optimum conditions providing higher lipid and FAME yields. The optimum conditions were as follows: proportion of hexane to methanol of 1:1, ratio of total solvent volume to dry biomass of 10 mL/g, and extraction time of 120 min. Finally, large-scale experiments were performed under the optimum extraction conditions with scale factors of 10, 50, and 100. Extraction performances of large-scale experiments were almost consistent with that obtained on a laboratory scale. Based on these results, a mixture of hexane and methanol is a promising solvent medium for lipid extraction from microalgae. This work provides useful information for the efficient extraction of microalgal lipids on a large scale.

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