Highlights:

Lipids of microalgae with biomineralized cell walls were extracted by liquefied DME;

Hexane Soxhlet and Bligh–Dyer extraction methods were performed for comparison;

Liquefied DME extraction did not require pretreatments like drying or cell disruption;

Liquefied DME extraction has a similar or better performance than other two methods;

Lipids extracted by liquefied DME were suitable for further biodiesel production.

Lipid Extraction from Microalgae Covered with Biomineralized Cell Walls using Liquefied Dimethyl Ether

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Abstract

Cell disruption is regarded as an indispensable pretreatment step before the extraction of microalgae with biomineralized cell walls. Here, two typical microalgae—diatom *Chaetoceros gracilis* (*C. gracilis*) and coccolithophore *Pleurochrysis carterae* (*P. carterae*)—covered by "hard" biomineralized cell walls were used as starting materials for lipid extraction using liquefied dimethyl ether (DME) without any pretreatment such as drying or cell disruption. The liquefied DME extraction experiments were performed at 25 °C and 0.59 MPa using a semi-continuous, flow-type system. The results of the yield, elemental composition, molecular weight distribution, fatty acid composition, and trace element composition indicated that the performance of liquefied DME extraction was similar to that of Bligh–Dyer extraction and better than that of hexane Soxhlet extraction, despite the latter two methods requiring pre-drying and cell disruption processes. It was also proven that the cell wall of microalgae would not affect lipid extraction of liquefied DME, thereby the liquefied DME extraction method is suitable for extracting lipids from microalgae with biomineralized cell walls. Besides, the lipids extracted by liquefied DME can be further used for biodiesel production.

Keywords: Lipid extraction; biomineralized cell wall; cell disruption; dimethyl ether; diatom; coccolithophore.

1. Introduction

Biofuel is a renewable energy resource produced from biomass by means of biological (e.g., fermentation and anaerobic digestion), physico-chemical (e.g., extraction and transesterification), and thermochemical (e.g., gasification and liquefaction) approaches [1-3]. Owing to the capacity to lower greenhouse gas emissions compared to fossil fuels

and low sulfur contents, biofuel has considerable potential as an environmentally friendly fuel [4, 5]. Recently, microalgae have been considered one of the most promising alternative sources for biofuels due to a number of advantages, such as high photosynthetic efficiency, high biomass productivity, fast growth rate, and the best performance of CO_2 fixation and O_2 production [6-9]. Microalgae do not need arable land or freshwater for cultivation, and they can satisfy the large demands of biofuels using limited land resources without causing potential biomass deficits [7-9]. Another critical advantage of microalgae is high lipid content. In general, the content of lipids in microalgae is in the range of 15% to 50% in the dry base, but under certain circumstances, the lipid content in some microalgae such as *Botyococcus braunii* can go up to 80% [10]. Furthermore, microalgae are particularly suitable for biodiesel production [7, 8].

Microalgae have various species and strains [11]. Some microalgae have high lipid contents, but their "hard" cell walls make them stable and thus difficult for extraction. The most typical examples are microalgae with biomineralized cell walls such as diatom and coccolithophore. The cell walls of the diatom are composed of silica (SiO_2), and the lipid content of the diatom, such as *Chaetoceros gracilis* (*C. gracilis*), is around 60% per dry weight [12]. Coccolithophore has a high potential for fixing carbon dioxide from the atmosphere because of coccoliths, which are calcium carbonate ($CaCO_3$) plates arranged outside the cell walls of coccospheres. Before extracting lipids from these two microalgae, a pretreatment step of disrupting or damaging the "hard" cell walls was regarded as indispensable and expected to increase extraction rates and yields, thereby reducing overall costs and time [13]. There have been many cell disruption methods for various microalgae, such as mechanical (e.g., pressing, bead milling, and

63 homogenization), thermal (e.g., microwave), physical (e.g., osmotic shock and
64 ultrasound), enzymatic, and chemical (e.g., acid, base, ionic liquid, and deep eutectic
65 solvent) methods [14]. The cell disruption step exerts positive advantages during the
66 extraction process using traditional organic solvents; however, it can increase the total
67 time and energy cost and cause contamination from external sources. Therefore, a better
68 solution is to find an extraction method that can not only avoid the pretreatment step of
69 cell disruption but also maintain the efficiency as high as those of other extraction
70 methods, even for microalgae with biomineralized cell walls.
71 Dimethyl ether (DME) is the simplest form of ether and well known as a synthesized
72 fuel. DME has a medium polarity, which makes it suitable for the extraction of a broad
73 range of compounds. Owing to a high affinity to oily substances, liquefied DME can be
74 used as an efficient solvent for neutral and complex lipids [15]. Another major
75 advantage of DME is the partial miscibility with water; even at room temperature (25
76 °C and 0.59 MPa), the solubility of water in DME is around 7.8 wt%. This property
77 allows that liquefied DME can be applied to wet feedstocks directly and leads to a
78 considerable saving of energy because the pre-drying step can be avoided [16]. Besides,
79 DME is easy to compress and liquefy and can evaporate from the final products at
80 normal temperatures due to a low normal boiling point of −24.8 °C [17-19].
81 DME extraction method has been successfully implemented to recover lipids or organic
82 components from various feedstocks such as microalgae [20-25], sludge [20, 21], and
83 vegetal biomass (e.g., pulverized coffee grounds, tea-leaf, orange peel, and gramineous
84 weed) [24]. In 2013, Kanda *et al*. [22] conducted liquefied DME extraction of
85 hydrocarbons and lipids from wet *B. braunii*, which showed the same yield as that
86 obtained from dried algae biomass by hexane Soxhlet extraction; meanwhile, the cell

disruption step was not involved. Even though the cell wall of *B. braunii* is much thinner and weaker than that of diatom or coccolithophore, its successful extraction suggested that liquefied DME extraction might be a potential method for extracting lipids from microalgae with biomineralized cell walls efficiently without the need for cell disruption.

Here, we employed liquefied DME as a solvent to extract lipids from diatom *Chaetoceros gracilis* (*C. gracilis*) and coccolithophore *Pleurochrysis carterae* (*P. carterae*) without a cell disruption step. The performance of the liquefied DME extraction method would be compared with those of the Bligh–Dyer and hexane Soxhlet extraction methods in terms of the yield, elemental composition, molecular weight, the composition of fatty acids methyl esters (FAME) or fatty acids, and trace element composition of the extracted lipids.

2. Materials and Methods

2.1 Materials

C. gracilis was purchased from ISC Corporation (Nagasaki, Japan), and *P. carterae* was purchased from MAC Research Institute of MicroAlgae Corporation (Gifu, Japan). **Table 1** lists the ultimate and proximate analyses of microalgae samples. *C. gracilis* and *P. carterae* samples have high moisture contents of 88.5 wt% and 62.0 wt%, respectively, as well as high ash contents of 19.4 wt% and 40.0 wt%, respectively. In the experiments, both wet and dry microalgae samples were used as the starting materials.

The chemical reagents used included *n*-hexane, chloroform, and methanol (HPLC grade), purchased from Wako Pure Chemicals Industries Ltd. (Osaka, Japan). A series

of polystyrene calibration standards were purchased from Tosoh Co. (Tokyo, Japan). All chemicals were used as received. The liquefied DME was purchased from Tamiya, Inc. (Japan) and used without further purification.

2.2 Liquefied DME extraction

Figure 1 illustrates the apparatus employed to evaluate the efficiency of liquefied DME extraction, as described in previous reports [20, 21, 23, 24]. The apparatus comprised a DME supply tank, an extraction column (HPG-10-5, Taiatsu Techno Corp., Saitama, Japan; volume: 10 or 100 mL), and a recovery tank for the extract (HPG-96-3, Taiatsu Techno Corp., Saitama, Japan; volume: 96 mL). The extraction column and recovery tank consisted of pressure-resistant glasses coated with polycarbonate. These parts were connected by a 1/16-inch tube (SUS 316) equipped with needle valves to control the liquefied DME flow rate. Before the extraction process, the cell walls of the microalgae samples were not disrupted.

In a typical experiment, a wet microalgae sample (10.41 g of *C. gracilis* sample or 1.79 g of *P. carterae* sample) was loaded into the extraction column (volume: 100 mL for *C. gracilis* sample or 10 mL for *P. carterae* sample), and then both ends of the extraction column were filled with colorless glass beads. A filter (pore size: 0.65 μm) was placed at the outlet of the extraction column. Liquefied DME in the DME supply tank was maintained at 37 ± 1 °C in a water bath, with a saturated vapor pressure at 0.82 ± 0.02 MPa. Then the liquefied DME flowed to the extraction column owing to a pressure difference between the DME supply tank and extraction column. The flow rate of liquefied DME was adjusted to 10 mL min^{-1}; the extraction was conducted at 25 °C and 0.59 MPa. Liquefied DME passed through the extraction column at different time

intervals and was then released by controlling the reducing valve of the recovery tank. The total extraction time was around 45 min. After the extraction process, the residues and lipids were collected from the extraction column and the recovery tank, respectively, and were stored in a refrigerator until further analysis.

2.3 Hexane Soxhlet extraction

Hexane Soxhlet extraction is a widely used method for extracting lipids from microalgae in the mass production of biofuels, in which hexane is used as a solvent owing to its nonpolarity and high selectivity to fats and oil. In a typical experiment, 8 g of microalgae sample (dry *C. gracilis* or *P. carterae*) and 200 mL of hexane were loaded into a flask. Before the extraction process, the microalgae sample was disrupted using a homogenizer equipped with a sawtooth generator probe (Dremel 300 Series, Robert Bosch Tool Corp., Illinois, USA; 10 mm for outside diameter) for 5 min at 10,000 rpm. Then, the hexane Soxhlet extraction was conducted using the Soxhlet apparatus (Vidrolabor®). The refluxing started at 69 °C. The hexane solvent was vaporized, condensed, and percolated through the dry microalgae samples repeatedly until the extraction limit was reached. After the extraction reaction proceeded for 8 h, the solvent was separated from the extract under reduced pressure using a rotary evaporator. The residues and lipids were collected and stored in a refrigerator until further analysis.

2.4 Bligh–Dyer extraction

Bligh–Dyer extraction is a classical and reliable method for the quantitative extraction of lipids from biological materials and usually considered as a benchmark for total lipid

recovery from microalgae [26, 27]. In general, Bligh–Dyer method was applied using a combination of chloroform-methanol-water as a solvent; chloroform as a non-polar solvent was expected to dissolve the neutral lipids while methanol as a polar solvent may dissolve the polar lipids by disrupting the hydrogen bonding and electrostatic forces between the lipids and proteins [28]. Here, different solvent mixtures were applied for different microalgae samples based on the experimental data. In the case of *C. gracilis*, a mixture of chloroform-methanol-water with a ratio of 1:2:0.8 in volume was used as the solvent. In the case of *P. carterae*, a mixture of chloroform-methanol with a ratio of 1:1 in volume was used as the solvent. In a typical experiment, the solvent and microalgae sample (dry *C. gracilis* or *P. carterae*) were mixed and loaded slightly into a flask. The ratio of solvent to sample was 1:1. The microalgae sample was disrupted in the solvent by a hand-held homogenizer equipped with a sawtooth generator probe (Dremel 300 Series, Robert Bosch Tool Corp., Illinois, USA; 10 mm for outside diameter). Then, the mixture of the solvent and microalgae sample was transferred into a separatory funnel and shaken for 5 min. Finally, the solvent was evaporated by a rotary evaporator, and the residues and lipids were collected and stored in a refrigerator until further analysis.

2.5 Characterizations

The elemental analysis was conducted for original microalgae samples and the extracted lipids using a CHN analyzer (CORDER MT-6, Yanaco Group, Kyoto, Japan). The morphologies of the extracted lipids were observed using a scanning electron microscope (SEM; JSM–6390LV, JEOL Ltd., Akishima, Japan). The molecular weight distributions (MWD) of the extracted lipids were analyzed using gel permeation

chromatography (GPC) at 40 °C with tetrahydrofuran (THF) as a solvent. GPC analysis is a simple, quick, and powerful technique for determining molecular weights of the compounds extracted from plant matters.

During an acid-catalyzed transesterification, the fatty-acid-containing lipid fractions in the extracted lipids were converted to FAME; further, the compositions of FAME were identified using gas chromatography-mass spectrometry (GC-MS; 7890A GC system and 5975C inert XL MSD with a triple-axis detector, Agilent Technologies Japan, Ltd., Hachioji, Japan) according to NIST mass spectral database and quantified by gas chromatography-flame ionization detection (GC-FID; GC-2014, Shimadzu Corporation, Kyoto, Japan), based on a FAME standard (Supelco 37 Component FAME Mix, Sigma-Aldrich St. Louis, MO, USA).

The contents of trace elements (Na, K, Mg, Ca, P, S, and Si) in the extracted lipids were analyzed by inductively coupled plasma-atomic emission spectrometry (ICP-AES; ICPS-8100, Shimadzu Corporation, Kyoto, Japan). Prior to ICP-AES analysis, the lipids were carbonized by adding sulfuric acid and then combusted at 500 °C for 4 h. The ashes obtained from the lipids were dissolved in a mixture of nitric acid and perchloric acid, and the diluted solution was supplied to the ICP-AES analyzer. Sulfur concentration was analyzed by combustion-ion chromatography (C-IC), which was conducted by an ion chromatography analyzer (HIC-20Asp, Shimadzu Corporation, Kyoto, Japan) coupled with an ion chromatograph pretreatment unit (AQF-2100H, Mitsubishi Chemical Analytech Co., Ltd., Chigasaki, Japan).

The yield of the lipids or extraction yield was defined as the percentage of the mass of the lipids against the total mass of the raw microalgae sample, excluding the moisture. The calculation equation (Eq. 1) is shown as follows.

$$Extraction\ yield\ = \frac{Mass\ of\ lipid\ (g)}{Mass\ of\ raw\ microalgae\ (g)} \times 100\% \quad (1)$$

3. Results and Discussion

3.1 Yield

Figure 2 shows the photos of wet *C. gracilis* and *P. carterae* and their residues after liquefied DME extraction. Different from the feedstocks, the residues seemed like dry powders with a light color, indicating that the water in the microalgae was removed simultaneously with the lipids during the extraction process. The changes in the amount of removed water during the liquefied DME extraction of wet *C. gracilis* were shown in **Figure 3**. The accumulation of removed water from *C. gracilis* increased proportionally with the consumption of liquefied DME. When the amount of consumed liquefied DME reached 584 g, the dewatering step was terminated, where the ratio of removed water to the intimal water amount was about 81%. Liquefied DME can work on wet microalgae samples directly because liquefied DME is partially miscible with water when mixed with water [19].

Figure 4 shows the yields of the lipids extracted from *C. gracilis* and *P. carterae* by liquefied DME. Similar to the progress of water removal, the yield of the extracted lipids from *C. gracilis* increased with the consumption of liquefied DME. Initially, the yield of the extracted lipids and the consumption amount of liquefied DME were around 6% and 38 g, respectively; then, they increased proportionally as the extraction proceeded. Finally, when the consumption amount of liquefied DME increased to 584 g, the yield of the lipids extracted from *C. gracilis* reached the maximum and maintained at 22%. Similar results were observed for *P. carterae.* As shown in **Figure 4**, the amount of the extracted lipids from *P. carterae* increased rapidly and proportionally by

following the increase of liquefied DME consumption until 280 g of liquefied DME was consumed. Finally, the lipid yield was around 11.6%, and it did not increase even if the consumption amount of the liquefied DME increased to 383 g, indicating that the maximum amount of extracted lipids was achieved.

On the other hand, the maximum yields of the lipids extracted from dry *C. gracilis* and *P. carterae* using hexane Soxhlet extraction were 12.3% and 7.5%, respectively, and those using Bligh-Dyer method were 21.5% and 9.0%, respectively. As summarized in **Table 2**, even without any pretreatment such as drying or cell disruption, the yield of the lipids extracted by liquefied DME was close to that by Bligh–Dyer method and much higher than that by hexane Soxhlet extraction. Furthermore, compared with the other two methods, the application of liquefied DME extraction saved considerable energy consumed in the pre-drying process and avoided the errors caused by the contamination during the cell disruption step. Here, a simple mechanism for explaining the outstanding performance of liquefied DME extraction was proposed. Besides a high dissolving ability, liquefied DME may also generate a lower viscosity of the analyte, as well as a better diffusion rate of the solute from the solid phase to the solvent [25]. Therefore, liquefied DME can enter into microalgae cells and flow out with the components inside the cells, which allowed it to extract substances such as lipids easily. Furthermore, liquefied DME extraction can work well on two microalgae samples covered by different biomineralized cell walls, indicating that the cell wall of the microalgae would not influence the effect of liquefied DME on lipid extraction.

3.2 Elemental composition

Table 1 presents the elemental compositions and ash contents of raw microalgae samples and the lipids extracted from them using different extraction methods. The contents of C, H, N, and O were calculated on a dry ash-free (daf) basis. C/N ratio is regarded as an important index when choosing a suitable technique for biomass conversion [29]. When the C/N ratio is higher than 30, the biomass conversion suits a thermochemical conversion process, while when the C/N ratio is lower than 30, a biochemical process is preferable. The C/N ratios of *C. gracilis* and *P. carterae* were 6.6 and 9.6, respectively; therefore, three methods used in this work were suitable. Compared with the feedstocks, all extracted lipids had considerably higher contents of C and H and lower contents of N and O. Because low N content is in favor of combustion [30], the extracted lipids would be used as qualified biofuels. Despite the different elemental compositions of two raw microalgae samples, the lipids extracted from them using liquefied DME and hexane Soxhlet method had very similar elemental compositions. The carbon contents of the lipids extracted from two microalgae samples by the Bligh–Dyer method were lower than those extracted using other methods; this result can be attributed to the isolation of a wide range of oily components from microalgae during the extraction process.

As shown in **Table 1**, each extracted lipid had a much lower ash content than the feedstock. Compared with other extracted lipids that had ash contents less than 1.4 wt%, the lipid extracted from *P. carterae* by hexane had an especially high ash content of 5.2 wt%. **Figure 5** shows the SEM images of the lipids extracted from *P. carterae* by hexane and liquefied DME. Broken coccoliths were observed in the lipids extracted from *P. carterae* by hexane, which can be used to explain its high ash content.

Meanwhile, no coccoliths were observed in the lipids extracted by liquefied DME, indicating that liquefied DME extraction did not cause cell disruption.

3.3 Molecular weight analysis

For microalgae with thin or without cell walls such as *Euglena gracilis* [25], all nonpolar and polar components can be extracted by either liquefied DME or hexane Soxhlet extraction, which indicates that the MWD of the extracted lipids are independent of the solvent polarity. However, *C. gracilis* and *P. carterae* are different because their "hard" cell walls probably hinder lipid extraction. In this case, the efficiency of extracting lipids from two microalgae samples depends on the polarity of the organic solvent or solvent mixture used, even with the application of appropriate cell disruption techniques. Therefore, the lipids extracted by different methods have different MWD, as well as different chemical or physical properties.

Figure 6 shows the MWD of the lipids extracted from *C. gracilis* and *P. carterae* using the three extraction methods. The molecular weights of compounds in all extracted lipids primarily ranged from 100 Da to 10 kDa. The MWD of the lipids extracted from *C. gracilis* by both liquefied DME and Bligh–Dyer methods had bimodal distributions including a sharp peak around 1000 Da and a small peak around 400 Da, while that of the lipids extracted by the hexane Soxhlet method had a unimodal distribution centering on a peak above 1000 Da. Different MWD of the lipids extracted by the hexane Soxhlet method and the other two methods coincided with the significant difference in yields. It was speculated that the sealing tightness of diatom cell walls possibly limits the extraction of low-molecular compounds by hexane. On the contrary, the lipids extracted from *P. carterae* using three extraction methods had similar bimodal MWD, including

two peaks below and above 1000 Da separately. The MWD results supported that liquefied DME could extract lipids from *P. carterae* the same as the other two methods, and it had a better performance than the hexane Soxhlet method when extracting lipids from *C. gracilis*. High molecular weights may cause high viscosity in the lipids or crude oils extracted from *C. gracilis* and *P. carterae*. Jain and Sharma [31] pointed out that it is challenging to introduce vegetable oils with high viscosity directly into engines owing to the risk of piston ring sticking, gum generation, and problems in the automation of fuel. Therefore, a modification process is required to convert microalgae-extracted lipids or crude oils to constituents with lower molecular weights, such as FAME or fatty acid alkyl esters, which are the main components in biodiesel, thereby decreasing the viscosity.

3.4 FAME or fatty acid composition

Most lipids contain fatty acids and can roughly be divided into neutral and polar lipids based on the polarity of the molecular head group [32]. When the carboxylate end of the fatty acid molecule is bonded to an uncharged or charged head group, a neutral or polar lipid molecule is formed, respectively. Fatty acids can be converted to FAME or biodiesel during a transesterification process. According to the FAME results, fatty acid compositions of the extracted lipids could also be obtained.

Tables 3 and 4 present FAME or fatty acid compositions of the lipids extracted from *C. gracilis* and *P. carterae*. The fatty acid components of the lipids extracted by liquefied DME, were almost the same as those by hexane Soxhlet and Bligh-Dyer extraction methods, primarily consisting of C_{12}-C_{22}. As shown in **Table 3**, the prominent fatty acid components of the lipids extracted from *C. gracilis* are palmitoleic acid ($C_{16:1}$),

hexadecatrienoic acid ($C_{16:3}$), and eicosapentaenoic acid ($C_{20:5}$), with the percentages at 24.8–27.3%, 21.4–24.4%, and 18.8–19.8%, respectively. This result is outstanding because palmitoleic acid is a strong candidate for improving fuel properties besides methyl oleate [33]. In the lipids extracted from *P. carterae* as shown in **Table 4**, the prominent fatty acid components were palmitic acid ($C_{16:0}$), oleic acid ($C_{18:1}$), and stearidonic acid ($C_{18:4}$), with the percentages at 25.3–30.1%, 26.5–30.5%, and 13.5–15.7%, respectively. Knothe [33] reported that the most common fatty esters contained in biodiesel are those of palmitic acid ($C_{16:0}$), oleic acid ($C_{18:1}$), linoleic acid ($C_{18:2}$), linolenic acid ($C_{18:3}$), and stearic acid ($C_{18:4}$). The total percentages of these four fatty acids in the lipids extracted from *C. gracilis* and *P. carterae* are 6.6–7.2% and 73.7–82.1%, respectively. These results indicated that the lipids extracted from either *C. gracilis* or *P. carterae* contained ideal biodiesel components, and the lipids extracted by liquefied DME were suitable for biodiesel production.

3.5 Trace element composition

As a fuel generated from renewable sources, biodiesel—also recognized as vegetable diesel—may reduce the emission of air pollutants and has emerged as a promising and green alternative to petroleum. Biodiesel usually contains elements such as Na, K, Mg, Ca, P, and Si, which originate from the catalyst applied and/or the raw materials. Even at low levels, these trace elements can cause ash build-up in engines, lead to engine deterioration, increase air pollution, and reduce the oxidative stability of the biodiesel [34]. **Table 5** lists the contents of trace elements (Na, K, Mg, Ca, P, S, and Si) in the lipids extracted from two microalgae using liquefied DME. Even though the cell walls of *C. gracilis* and *P. carterae* are rich in Si and Ca, respectively, the contents of Si and Ca in the lipids extracted from *C. gracilis* and *P. carterae* were as low as 0.006% and

0.041%, respectively, suggesting that the liquefied DME extraction method is suitable for extracting lipids from microalgae with biomineralized cell walls, without needing any pretreatment process.

In the lipids extracted from *C. gracilis* and *P. carterae*, the contents of alkali metals (Na and K) were 0.065% and 0.009%, respectively; the contents of alkaline earth metals (Mg and Ca) were 0.335% and 0.057%, respectively; and the contents of P were 0.089% and 0.100%, respectively. According to the Japan Standard (JIS K 2390:2008) and European Standard (EN 14214:2009), as shown in **Table 5**, the microalgae-extracted lipids or crude oils obtained here would require a further purification in a subsequent refining process to reduce the contents of trace elements before practical application as automotive fuels.

4. Conclusions

As a simple and environment-friendly extraction method, liquefied DME was applied to extract lipids from two microalgae with biomineralized cell walls—*C. gracilis* and *P. carterae*. For comparison, hexane Soxhlet and Bligh–Dyer extraction methods were employed, in which the raw microalgae samples were pre-dried, and cell walls were disrupted before the extraction process. Even without any pretreatment such as drying and cell disruption, liquefied DME extracted lipids from two microalgae samples with the yields close to that obtained using the Bligh–Dyer method and considerably higher than that obtained using hexane Soxhlet extraction. The outstanding performance of liquefied DME extraction on two microalgae samples covered by different biomineralized cell walls indicated that the cell wall of microalgae would not affect lipid extraction of liquefied DME. The elemental compositions and ash contents of the

lipids extracted by three extraction methods did not vary significantly. The MWD results supported that liquefied DME extraction could work on *P. carterae* as did the other two methods, and it was better than the hexane Soxhlet method when extracting lipids from *C. gracilis*. Based on the compositions of FAME or fatty acids, the lipids extracted from *C. gracilis* and *P. carterae* by liquefied DME had low N contents and contained ideal biodiesel components, suggesting that the extracted lipids were suitable for biodiesel production. The contents of trace elements in the lipids extracted by liquefied DME were much lower than those in feedstocks, but further purification will still be required in a subsequent refining process before the lipids are applied as automotive fuels.

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References

[1] Li P, Sakuragi K, Makino H. Extraction techniques in sustainable biofuel production: A concise review. Fuel Process Technol 2019;193:295-303.

[2] Wang J-X, Cao J-P, Zhao X-Y, Liu S-N, Ren X-Y, Zhao M, et al. Enhancement of light aromatics from catalytic fast pyrolysis of cellulose over bifunctional hierarchical HZSM-5 modified by hydrogen fluoride and nickel/hydrogen fluoride.

Bioresource Technol 2019;278:116-23.

[3] Ren J, Cao J-P, Zhao X-Y, Yang F-L, Wei X-Y. Recent advances in syngas production from biomass catalytic gasification: A critical review on reactors, catalysts, catalytic mechanisms and mathematical models. Renewable and Sustainable Energy Reviews 2019;116:109426.

[4] Antolín G, Tinaut FV, Briceño Y, Castaño V, Pérez C, Ramírez AI. Optimisation of biodiesel production by sunflower oil transesterification. Bioresource Technol 2002;83(2):111-4.

[5] Demirbas A. Biofuels sources, biofuel policy, biofuel economy and global biofuel projections. Energ Convers Manage 2008;49(8):2106-16.

[6] Adams C, Bugbee B. Enhancing lipid production of the marine diatom *Chaetoceros gracilis*: synergistic interactions of sodium chloride and silicon. J Appl Phycol 2014;26(3):1351-7.

[7] Gouveia L. Microalgae as a feedstock for biofuels. Springer-Verlag Berlin Heidelberg; 2011.

[8] Huang G, Chen F, Wei D, Zhang X, Chen G. Biodiesel production by microalgal biotechnology. Applied Energy 2010;87(1):38-46.

[9] Raheem A, Azlina WAKGW, Yap YHT, Danquah MK, Harun R. Thermochemical conversion of microalgal biomass for biofuel production. Renew Sust Energ Rev 2015;49:990-9.

[10] Singh A, Nigam PS, Murphy JD. Renewable fuels from algae: An answer to debatable land based fuels. Bioresource Technol 2011;102(1):10-6.

[11] Geada P, Vasconcelos V, Vicente A, Fernandes B. Chapter 13 - Microalgal biomass cultivation. In: Rastogi RP, Madamwar D, Pandey A, editors. Algal Green

421 Chemistry. Amsterdam: Elsevier; 2017, p. 257-84.
422 [12] Levering J, Broddrick J, Zengler K. Engineering of oleaginous organisms for
423 lipid production. Curr Opin Biotech 2015;36:32-9.
424 [13] Tommasi E, Cravotto G, Galletti P, Grillo G, Mazzotti M, Sacchetti G, et al.
425 Enhanced and selective lipid extraction from the microalga p. tricornutum by dimethyl
426 carbonate and supercritical CO_2 using deep eutectic solvents and microwaves as
427 pretreatment. ACS Sustain Chem Eng 2017;5(9):8316-22.
428 [14] Alhattab M, Kermanshahi-Pour A, Brooks MSL. Microalgae disruption
429 techniques for product recovery: influence of cell wall composition. J Appl Phycol
430 2019;31(1):61-88.
431 [15] Bauer MC, Kruse A. The use of dimethyl ether as an organic extraction solvent
432 for biomass applications in future biorefineries: A user-oriented review. Fuel
433 2019;254:115703.
434 [16] Goto M, Kanda H, Wahyudiono, Machmudah S. Extraction of carotenoids and
435 lipids from algae by supercritical CO_2 and subcritical dimethyl ether. The Journal of
436 Supercritical Fluids 2015;96:245-51.
437 [17] Holldorff H, Knapp H. Binary vapor-liquid-liquid equilibrium of dimethyl
438 ether - water and mutual solubilities of methyl-chloride and water: Experimental results
439 and data reduction. Fluid Phase Equilibr 1988;44(2):195-209.
440 [18] Pozo ME, Streett WB. Fluid phase-equilibria for the system dimethyl ether
441 water from 50 degree to 220 degree and pressures to 50.9 MPa. J Chem Eng Data
442 1984;29(3):324-9.
443 [19] Tallon S, Fenton K. The solubility of water in mixtures of dimethyl ether and
444 carbon dioxide. Fluid Phase Equilibr 2010;298(1):60-6.

445 [20] Kanda H, Li P. Simple extraction method of green crude from natural
446 blue-green microalgae by dimethyl ether. Fuel 2011;90(3):1264-6.
447 [21] Kanda H, Morita M, Makino H, Takegami K, Yoshikoshi A, Oshita K, et al.
448 Deodorization and dewatering of biosolids by using dimethyl ether. Water Environment
449 Research 2011;83(1):23-5.
450 [22] Kanda H, Li P, Yoshimura T, Okada S. Wet extraction of hydrocarbons from
451 Botryococcus braunii by dimethyl ether as compared with dry extraction by hexane.
452 Fuel 2013;105:535-9.
453 [23] Kanda H, Kamo Y, Machmudah S, Wahyudiono, Goto M. Extraction of
454 fucoxanthin from raw macroalgae excluding drying and cell wall disruption by liquefied
455 dimethyl ether. Mar Drugs 2014;12(5):2383-96.
456 [24] Li P, Kanda H, Makino H. Simultaneous production of bio-solid fuel and
457 bio-crude from vegetal biomass using liquefied dimethyl ether. Fuel 2014;116:370-6.
458 [25] Oshita K, Toda S, Takaoka M, Kanda H, Fujimori T, Matsukawa K, et al. Solid
459 fuel production from cattle manure by dewatering using liquefied dimethyl ether. Fuel
460 2015;159:7-14.
461 [26] Bligh EG, Dyer WJ. A rapid method of total lipid extraction and purification.
462 Can J Biochem Phys 1959;37(8):911-7.
463 [27] Silva APFD, Costa MC, Lopes AC, Neto EF, Leitao RC, Mota CR, et al.
464 Comparison of pretreatment methods for total lipids extraction from mixed microalgae.
465 Renew Energ 2014;63:762-6.
466 [28] Kates M, Burdon RHRH. Techniques of lipidology: Isolation, analysis, and
467 identification of lipids. 2nd Revised Edition ed.: Elsevier; 1986.
468 [29] Dahlquist E. Technologies for converting biomass to useful energy:

Combustion, gasification, pyrolysis, torrefaction and fermentation. London: CRC Press Inc.; 2013.

[30] Pimentel D. Biofuels, solar and wind as renewable energy systems: Benefits and risks. Springer Science & Business Media; 2008.

[31] Jain S, Sharma MP. Prospects of biodiesel from Jatropha in India: A review. Renew Sust Energ Rev 2010;14(2):763-71.

[32] Halim R, Danquah MK, Webley PA. Extraction of oil from microalgae for biodiesel production: A review. Biotechnol Adv 2012;30(3):709-32.

[33] Knothe G. "Designer" biodiesel: Optimizing fatty ester composition to improve fuel properties. Energ Fuel 2008;22(2):1358-64.

[34] Machmudah S, Winardi S, Kanda H, Goto M. Sub- and supercritical fluids extraction of phytochemical compounds from Eucheuma cottonii and Gracilaria sp. Chemical Engineering Transactions 2017;56:1291-6.

Figure Captions:

Figure 1 Schematic of liquefied DME extraction.

Figure 2 Photo images of wet microalgae samples and their residues after liquefied DME extraction. (a) *C. gracilis*, (b) the residue obtained from *C. gracilis*, (c) *P. carterae*, and (d) the residue obtained from *P. carterae*.

Figure 3 The relationship between removed water and DME consumption during the liquefied DME extraction of wet *C. gracilis*.

Figure 4 Yields of the lipids extracted from *C. gracilis* and *P. carterae* using liquefied DME.

Figure 5 SEM images of the lipids extracted from *P. carterae* by (a) hexane Soxhlet method and (b) liquefied DME method.

Figure 6 Molecular weight distributions of the lipids extracted from *C. gracilis* (left) and *P. carterae* (right) using different extraction methods: (a) Liquefied DME method (solid line), (b) hexane Soxhlet method (dotted line), and (c) Bligh–Dyer method (dashed line).

Tables and Figures:

Table 1. Ultimate and proximate analyses of microalgae samples and the extracted lipids.

Sample name	Extraction methods	Ultimate analysis (wt%, daf)[a]				Atomic ratio (-)	Ash content (wt%)
		C	H	N	O[b]	C/N	
C. gracilis	--	35.0	5.5	6.2	53.3	6.6	19.4
Lipid from *C. gracilis*	Liquefied DME	71.9	9.9	1.5	16.7	55.9	0.4
	Hexane Soxhlet	71.7	10.0	1.1	17.2	76.0	0.8
	Bligh-Dyer	67.5	9.6	1.7	21.2	46.3	0.5
P. carterae	--	31.3	4.2	3.8	60.7	9.6	40.0
Lipid from *P. carterae*	Liquefied DME	71.0	10.0	1.3	17.7	63.7	0.2
	Hexane Soxhlet	71.9	9.7	1.5	16.9	55.9	5.2
	Bligh-Dyer	57.0	7.7	3.6	31.7	18.5	1.4

[a:] Daf refers to dry ash-free.

[b]: Oxygen content was calculated by the difference.

504 **Table 2**. Yields of the lipids extracted from microalgae samples using different
505 extraction methods and pre-treatments.

Microalgae sample	Extraction methods	Drying	Cell disruption	Yield (wt%)
C. gracilis	Liquefied DME	No	No	22.0
	Hexane Soxhlet	Yes	Yes	12.3
	Bligh-Dyer	Yes	Yes	21.5
P. carterae	Liquefied DME	No	No	11.6
	Hexane Soxhlet	Yes	Yes	7.5
	Bligh-Dyer	Yes	Yes	9.0

506

507 **Table 3.** FAME or fatty acid compositions of the lipids extracted from *C. gracilis*.

FAME (% of total fatty acids)	Extraction method		
	Liquefied DME	Hexane Soxhlet	Bligh-Dyer
$C_{12:0}$	0.2	<0.1	<0.1
$C_{14:0}$	11.6	13.2	12.1
$C_{14:1}$	0.2	0.3	0.2
$C_{15:0}$	0.3	0.3	0.3
$C_{16:0}$	5.1	5.1	5.1
$C_{16:1}$	24.8	27.3	26.3
$C_{16:2}$	9.4	9.7	9.4
$C_{16:3}$	24.4	21.4	24.0
$C_{16:4}$	0.4	0.5	0.4
$C_{17:1}$	0.1	0.1	0.1
$C_{18:0}$	0.8	0.4	0.5
$C_{18:1(cis-9)}$	0.5	0.4	0.5
$C_{18:1(trans-9)}$	0.7	0.3	0.4
$C_{18:2}$	0.7	0.6	0.7
$C_{18:3}$	0.2	0.2	0.2
$C_{20:4}$	0.4	0.4	0.3
$C_{20:5}$	19.8	19.4	18.8
$C_{22:0}$	0.1	<0.1	0.5
$C_{22:1}$	--	<0.1	--
$C_{22:6}$	0.3	0.4	--
$C_{24:0}$	--	<0.1	--
Others	0.0	0.0	<0.2
Saturated fatty acids	18.1	19.2	18.6
Unsaturated fatty acids	81.9	80.8	81.4

508

509 **Table 4.** FAME or fatty acid compositions of the lipids extracted from *P. carterae*.

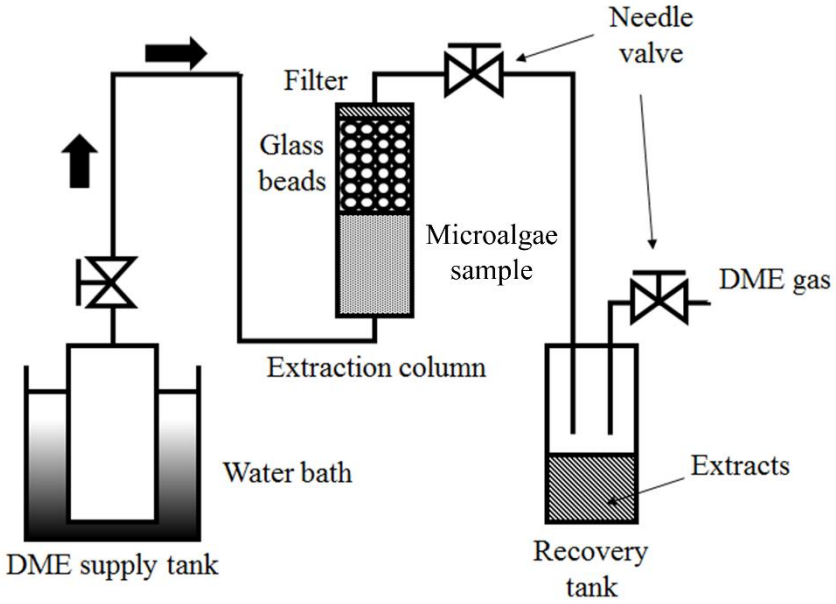
FAME (% of total fatty acids)	Extraction method		
	Liquefied DME	Hexane Soxhlet	Bligh-Dyer
$C_{12:0}$	0.4	--	--
$C_{14:0}$	0.5	0.4	0.3
$C_{15:0}$	0.1	--	0.1
$C_{16:0}$	26.2	30.1	25.3
$C_{16:1}$	0.2	0.2	--
$C_{16:3}$	0.4	--	0.3
$C_{16:4}$	3.2	2.9	1.5
$C_{17:0}$	0.5	0.4	0.4
$C_{17:1}$	1.4	1.4	1.4
$C_{17:2}$	1.7	1.5	1.9
$C_{18:0}$	5.1	4.0	3.7
$C_{18:1}$	26.5	30.5	27.0
$C_{18:2}$	7.2	8.0	6.9
$C_{18:3}$	--	--	--
$C_{18:4}$	15.7	13.5	14.5
$C_{20:0}$	0.5	--	--
$C_{20:1}$	2.8	3.1	2.8
$C_{20:2}$	0.1	0.1	0.2
$C_{20:3}$	--	--	0.1
$C_{20:4}$	2.1	--	5.4
$C_{20:5}$	1.1	0.1	1.7
$C_{21:5}$	0.1	0.2	0.2
$C_{22:0}$	0.1	--	--
$C_{22:5}$	--	--	--
$C_{22:6}$	1.6	3.2	6.3
Others	2.5	0.4	0.0
Saturated fatty acids	33.2	34.9	29.7
Unsaturated fatty acids	64.2	64.9	70.3

510

Table 5. Contents of trace elements in the lipids extracted from *C. gracilis* and *P. carterae* by liquefied DME.

Element	Content (wt%)			
	C. gracilis	*P. carterae*	Japan standard[a]	European standard[b]
Na	0.05	--	⩽0.0005	⩽0.0005
K	0.015	0.009		
Mg	0.31	0.016	⩽0.0005	⩽0.0005
Ca	0.025	0.041		
P	0.089	0.10	⩽0.001	⩽0.0004
S	0.11	--	⩽0.001	⩽0.001
Si	0.006	--	--	--

[a]: JIS K 2390:2008;
[b]: EN 14214:2009.



515

516 **Figure 1** Schematic of liquefied DME extraction.

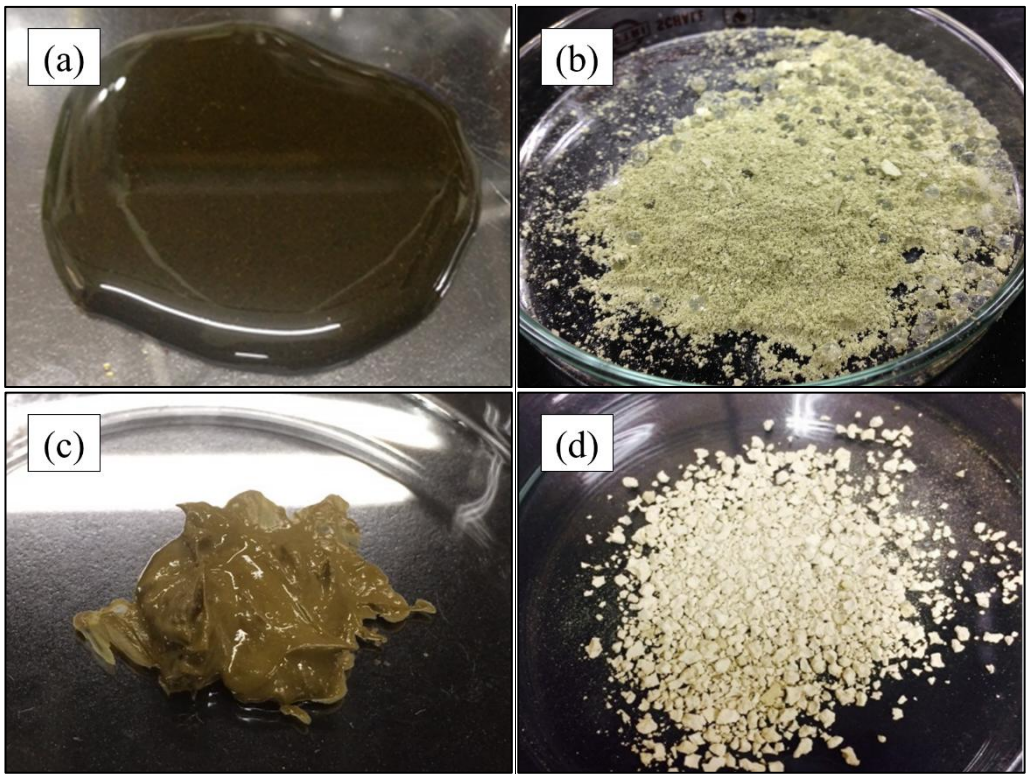
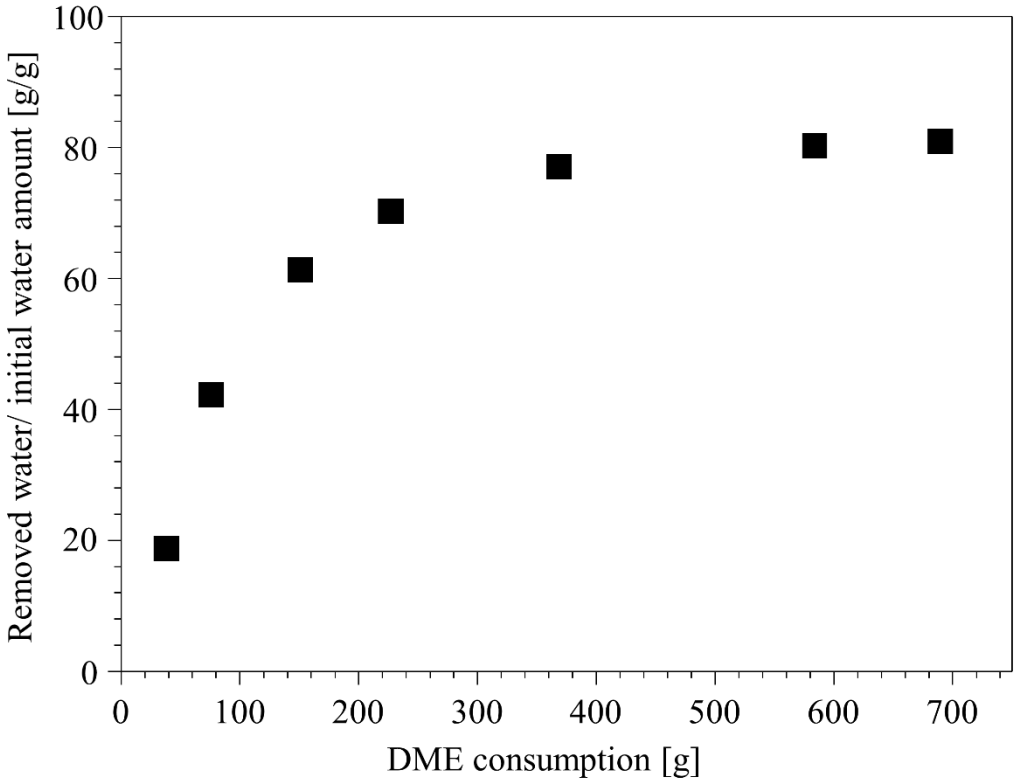


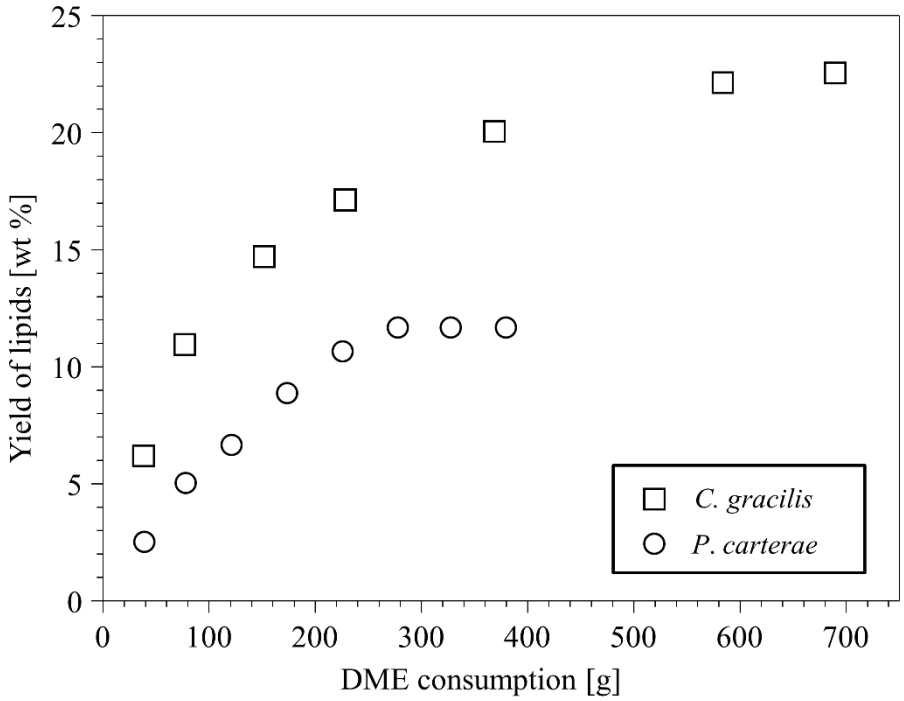
Figure 2 Photo images of wet microalgae samples and their residues after liquefied DME extraction. (a) *C. gracilis*, (b) the residue obtained from *C. gracilis*, (c) *P. carterae*, and (d) the residue obtained from *P. carterae*.



522

523 **Figure 3** The relationship between removed water and DME consumption during the

524 liquefied DME extraction of wet *C. gracilis*.

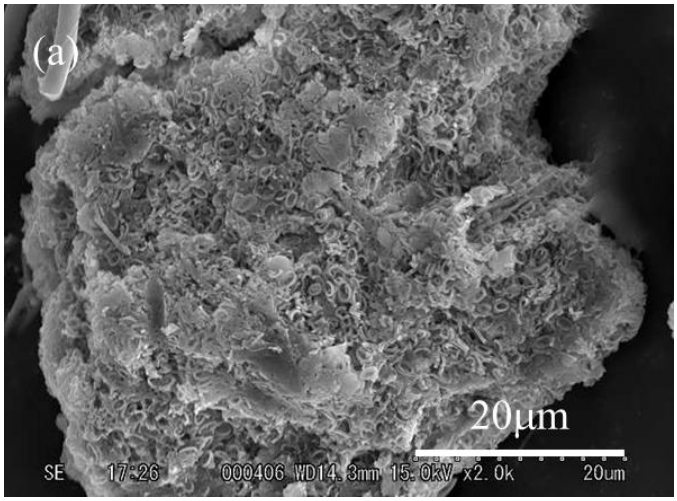


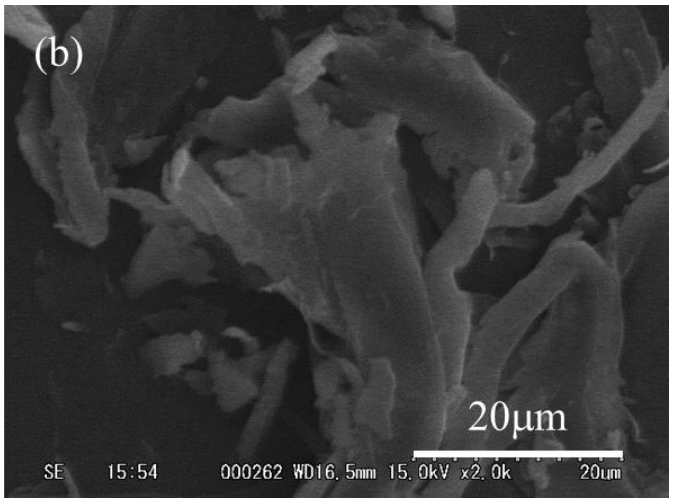
525

526 **Figure 4** Yields of the lipids extracted from *C. gracilis* and *P. carterae* using liquefied

527 DME.

528

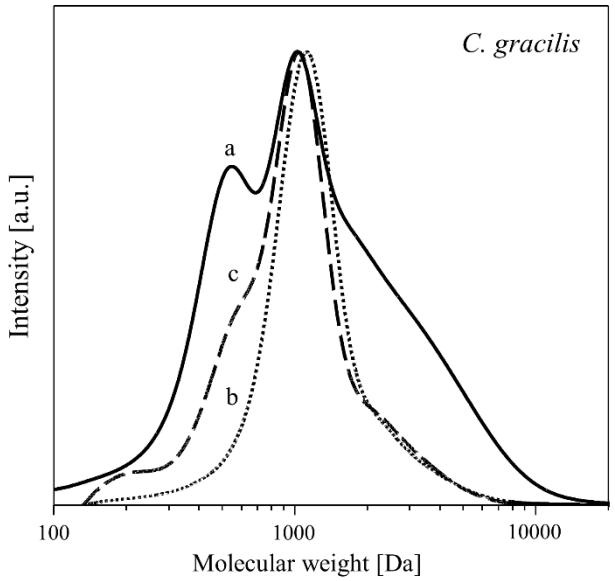




529

530 **Figure 5** SEM images of the lipids extracted from *P. carterae* by (a) hexane Soxhlet

531 method and (b) liquefied DME method.



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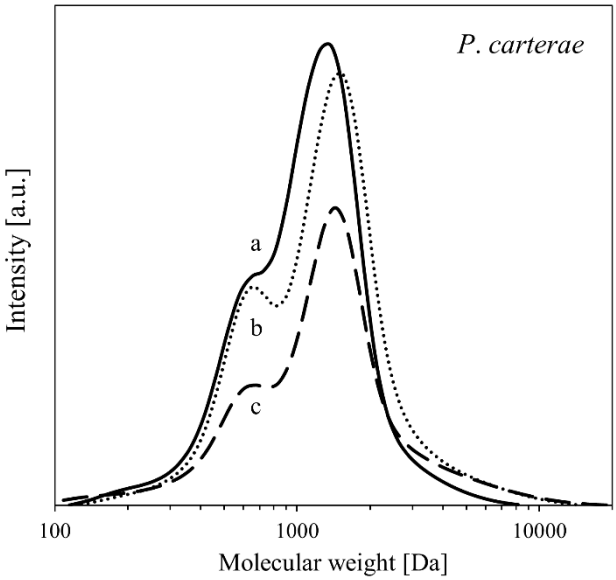

533

534 **Figure 6** Molecular weight distributions of the lipids extracted from *C. gracilis* (left)
535 and *P. carterae* (right) using different extraction methods: (a) Liquefied DME method
536 (solid line), (b) hexane Soxhlet method (dotted line), and (c) Bligh–Dyer method
537 (dashed line).