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Article

Economical Wet Extraction of Lipid from labyrinthula *Aurantiochytrium limacinum* by Using Liquefied Ddimethyl Ether

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Abstract. Recently, a simple method for the extraction of lipids from wet biomass using liquefied dimethyl ether (DME) without drying, cell disruption, or heating was proposed. Here, the versatility of this method was evaluated for labyrinthula *Aurantiochytrium limacinum* (*A. limacinum*). The liquefied DME was passed through the extractor that filled by *A. limacinum* at different time intervals. The extraction of lipids from *A. limacinum* of moisture-rich microorganism was successfully achieved, the yield of lipid was 46.1 wt% of the dry weight of the sample. In comparison, the yields of lipid were 21.3 wt%, 43.6 wt% and 50.7 wt% when supercritical carbon dioxide ($SCCO_2$), hexane-Soxhlet and Bligh-Dyer (BD) extraction methods were applied as extractants, respectively. However, the drying and cell-disruption process were required in $SCCO_2$, hexane-Soxhlet, and BD extraction methods.

Keywords: *Aurantiochytrium limacinum*, liquefied dimethyl ether, supercritical carbon dioxide, fatty acid, bio fuel, dewatering.

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1. Introduction

In recent year, the depletion of fossil fuels such as coal oil are feared, and the technology for making biomass fuel from labyrinthula attracts the world's attention. Labyrinthula *Aurantiochytrium limacinum* (*A. limacinum*) as heterotrophic microorganisms is capable of growing in the presence of organic substances of brackish water region [1]. *A. limacinum* have a potential to rapidly accumulate lipids important for biofuel production [2]. The growth rate of *A. limacinum* is very fast, production efficiency of hydrocarbons production is higher than *Botryococcus braunii* [3]. In general, the lipids content in *A. limacinum* is around 30-60wt% on a dry weight basis [4]. Commonly, chloroform, methanol, ethanol, and n-hexane were used as medium to extract lipids from marine tissue such as microalgae. In particular, solvent mixtures containing a polar and no-polar solvent could extract a total lipids [5]. For example, a combination of chloroform (non-polar), methanol (polar) and water, known as Bligh-Dyer (BD) method, has been used for total lipid extraction from biological materials [6]. However they were toxic organic solvents and costly. [7–9]. Several step processes were also required when these solvents were employed as extraction media. They are as follows: drying of starting materials, cell disruption as a pre-treatment, evaporation of extraction solvents by heating, and condensation of them by cooling [10–12]. Fig. 1(A, B) describes the procedures of conventional organic solvent extraction and BD method to extract biofuel from wet microalgae. Therefore, it could be said that these extraction processes consume a lot of energy and is environmentally-unfriendly.

Instead of organic solvents as extractants, liquefied DME would be used as an extractant to solve their drawbacks. Li *et al.* 2014 [13] proposed the energy-saving extraction of bio-solid fuel and bio-crude from vegetal biomass without pre-treatments such as drying and cell-disruption by using liquefied DME. Fig. 1(C) describes the procedures of proposed liquefied DME extraction. They used common vegetal biomasses such as spent coffee ground, tea leaf waste, orange peel and gramineous weed as selected materials. They informed that the liquefied DME could efficiently extract lipids, implying its utilization to produce bio-liquid fuel from lipid-rich biomass. Kanda *et al.* 2014 [14] also extracted carotenoid by using liquefied DME in semi-continuous flow-type system from microalgae. Compared with ethanol Soxhlet and supercritical CO_2 ($SCCO_2$) extraction, which includes drying and cell disruption, the yield of carotenoid from *Undaria pinnatifida* was quite high. Moreover, DME as an extractant is the simplest form of ether with the following characteristics: (i) standard boiling point is -24.8 °C [15], (ii) high affinity to oily compositions and partial miscibility with water [16], (iii) safe extraction solvent for the production of foodstuff and food ingredients [17], (iv) resistance to autoxidation unlike other alkyl ethers [18]. In this work, the efficacy of DME extraction was evaluated using labyrinthula *A. limacinum* at 0.51 MPa and 20 °C to verify its versatility. The results would be compared with $SCCO_2$ extraction, hexane extraction and BD extraction as other methods. Furthermore, the extracted lipids and the extracted residues would be analyzed by elemental analysis, molecular weight distribution (MWD), fatty acids analysis and functional substances.

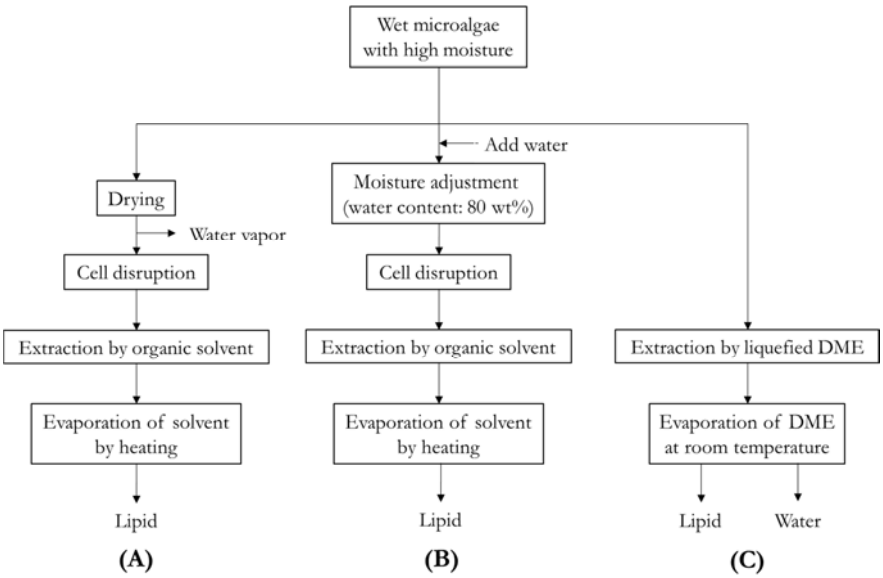

Fig. 1. Comparison of the liquefied DME and conventional organic solvent extraction procedures. (A) Conventional organic solvent extraction. (B) Bligh-Dyer method. (C) Proposed method.

2. Experiments

2.1. Material and Methods

A. limacinum strain was provided by Nippon Suisan Kaisya, Ltd, Tokyo, Japan and used as a starting material. The moisture content was determined by continual drying at 107 °C until constant weights. It was around 67.9 wt%. HPLC grade tetrahydrofuran (THF), n-hexane, chloroform, and methanol were purchased from Wako Pure Chemical Industries, Ltd., Japan. CO_2 (99.9 wt%) was obtained from Sogo Co., Aichi, Japan. For GPC analysis, the series of polystyrene calibration standards (Tosoh Co., Tokyo, Japan) were used.

2.2. Lipid Extraction

Four methods of lipid extraction were performed: liquefied DME method; $SCCO_2$ method; hexane-Soxhlet extraction method; and BD extraction method. Schematic diagrams of the apparatus used for liquefied DME and $SCCO_2$ extraction are shown in Fig. 2 and 3. In the liquefied DME method, wet *A. limacinum* was fed directly without drying and cell disruption as pre-treatments. However, in the case of $SCCO_2$, hexane and BD methods, the *A. limacinum* cells were disrupted using a pestle and mortar for 10 min. The liquefied DME extraction apparatus and procedure has been described in detail in previous studies [14,19,20]. A storage tank of liquefied DME (volume: 100 cm^3) an extractor (diameter: 11.6 mm, length: 190 mm, volume: 10 cm^3) and a collector, were connected in series. 2.51 g (dry weight, 0.81 g) of *A. limacinum* slurry was loaded into the lower half of the extractor, and glass beads were placed at upper half of the extractor. A filter paper (DURAPORE MEMBRANE FILTERS, pore size: 0.65 μm, material: hydrophilic polyvinylidene difluoride, Merck Millipore, Darmstadt, Germany) was placed at the outlet of the extraction column. The flowrate of liquefied DME was 10 cm^3 min^{-1}, and the temperature was kept at 20 °C. In the $SCCO_2$ extraction method, the main apparatus consists of a high-pressure pump for CO_2, a heating chamber, a extractor (volume: 25 cm^3), a CO_2 chiller, back-pressure regulator, and a wet gas meter. In order to determine the effect of temperature and pressure on the yield of extracted components, lipids was extracted from *A. limacinum* at temperatures between 60, 80, 100, 120 °C, pressures of 40 MPa and CO_2 flow rate of 5 cm^3 min^{-1}. In each experiment, 2.0 g of dried *A. limacinum* was loaded into the extraction column, and the remaining volume was filled with glass beads at the bottom and top of the extraction column. The cell was placed in a heating chamber maintained at the set operating temperature. The extract collection was collected at 15, 45, 75 min. In order to investigate the extracted components using the hexane-Soxhlet extraction method, 1.0 g of pre-treated *A. limacinum* were loaded in a Soxhlet apparatus with 200 cm^3 of n-hexane for 15 h at 70 °C. In this work, the total lipid content in *A. limacinum* was determined by BD method [6]. 1.0 g of pre-treated *A. limacinum* were extracted using a Soxhlet apparatus using 200 cm^3 of mixture of chloroform and methanol (1:1, v/v) for 15 h at 70 °C.

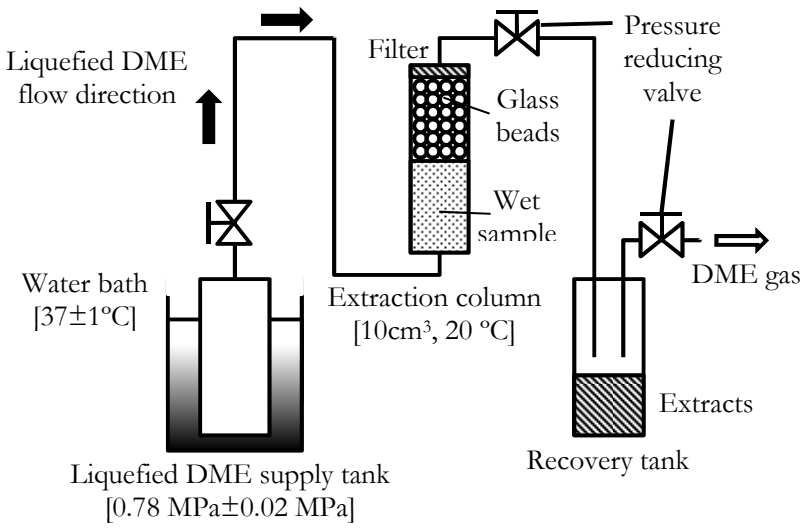

Fig. 2. Schematic diagram of liquefied DME extraction apparatus.

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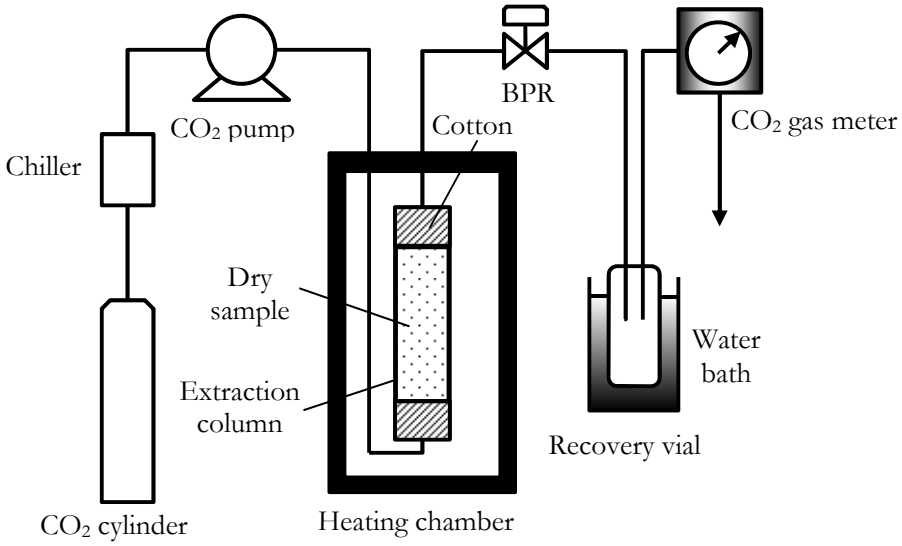

Fig. 3. Schematic diagram of $SCCO_2$ extraction apparatus.

2.3. Product Analysis

The extracted lipids and the extracted residues were analyzed by elemental analysis, molecular weight distribution (MWD), fatty acids analysis and functional substances.

2.3.1. Elemental Analysis

Elemental analysis was performed for original *A. limacinum*, extracted lipids and the extracted residues. The original *A. limacinum* and the extracted residues were conditioned at 107 °C until constant weights. The carbon, hydrogen, and nitrogen content were determined by a CHN analyzer (Yanaco, CORDER MT-6) based on flash combustion, which converts all organic substances into combustion gases. The oxygen content was calculated using the differences.

2.3.2. Molecular Weight Distribution

The molecular weight distributions (MWD) of the extracted lipids was determined by gel permeation chromatography (GPC) performed at 40 °C by diluting the lipids in THF. The MWDs were compared with that of a polystyrene standard. A lipid concentration of 1 mg/mL was used for each measurement and the injection amount was 20 μL. The analytical equipment and condition used for GPC were HPLC system controller and UV/Vis detector with chromatographic columns. The wavelength was set at 250 nm [21]. The MWDs of lipids obtained by liquefied DME extraction were compared with those obtained by $SCCO_2$, hexane and BD extraction methods.

2.3.3. Fatty Acid Profile

The major lipid components were identified by gas chromatograph mass spectrometer (GC/MS) with an Agilent 7890A GC system connected to an Agilent 5975C mass spectrometer, and a phenyl arylene capillary column (HP-5MS; 30 m × 0.25 mm i.d.) Agilent Technologies Tokyo Ltd., Japan). The temperature program was as a follow: initially at 60 °C for 5 min, then ramped to 320 °C with 4 °C min^{-1}. The injector and detector temperatures were set at 250 °C. The split ratio was 1:1; with a helium gas flow rate of 24 mL min^{-1} and the injection volume was 1.0 μL. Qualitative analysis of the detected fatty acids methyl esters (FAME) were carried out by comparison of their retention time and mass spectra with FAME standard (Supelco 37 Component. FAME Mix. Sigma–Aldrich St. Louis, MO, USA). The esterification of extracted lipids was carried out by using a fatty acid methylation kit and fatty acid methyl ester purification kit, which purchased from Nakalai Tesque, Inc., Kyoto, Japan.

3. Results and Discussion

3.1. Extraction Yield of Lipids

Figure 4 described the yield of lipid that extracted by liquefied DME. The increasing amount of liquefied DME consumed was followed by increasing the amount of lipid extracted. It seems to increase rapidly when the liquefied DME consumed was around 300 g then remained constant after 335 g. The yield of lipid could approach up to 46 wt% (g/g dry weight of *A. limacinum*). Compared with other methods, such as $SCCO_2$, hexane Soxhlet and BD extraction methods, the yield of lipid by liquefied DME was quite high. The maximum yield of lipids were 22.5, 43.6, and 50.7 wt% when $SCCO_2$, hexane Soxhlet and BD extraction methods were employed as extraction medium, respectively. As informed above, that liquefied DME could dissolve a wide range of polar and non-polar substances. DME is also good solvents for many hydrogen-bonded substances. To dissolve hydrogen bonded substances, high solvation energy was needed to break the hydrogen bonds. Due to DME has ability to act hydrogen bond acceptors, forming hydrogen bonds with hydrogen-bonding solutes, liquefied DME can penetrate into *A. limacinum* and flow out together with *A. limacinum* components include lipid. In addition, liquefied DME could also produce a lower viscosity of the analytes in the matrix and, accordingly, a better diffusion rate of the solute from the solid phase to the solvent. Consequently, the lipid in *A. limacinum* might be extracted easily. Fig. 5 shows the yield of lipid extracted from *A. limacinum* by $SCCO_2$ at various extraction temperatures. Generally, the yield of extract influenced by the increase of pressure or temperature at constant temperature or pressure when $SCCO_2$ was applied as an extraction medium. This phenomena occurred due to direct change of density and hence solubility of $SCCO_2$ [22,23]. In this work, it should be noted that the lipid extraction by $SCCO_2$ was performed at pressure of 40 MPa with various extraction temperatures. As shown in Fig. 5, the yield of lipid increased with increasing extraction temperature. Except at 60 °C, the yield of lipid increased rapidly till 520 g CO_2 consumed then remained constant after 1000 g CO_2 consumed in each extraction temperature. The highest extraction rate of lipid was reached when the extraction temperature is 100 °C. It could approach to 22 wt% (g/g dry weight of *A. limacinum*). This extraction condition likely had a stronger effect on the solubility, consequently the yield of lipid is lower than at 100 °C when the extraction temperature was performed at lower or higher 100 °C. As explained before, in the case of hexane Soxhlet extraction, the main drawbacks of this extraction methods are the long time needed and the large amount of solvent wasted, which is costly and cause environmental problems. Like hexane Soxhlet extraction, the toxic solvents also were used in large amount when using BD extraction technique. Judging the results, it could be said that the lipid extraction by DME is very simple and versatile technique. In addition, the extraction process not required the pre-treatments such as drying and cell-disruption. Therefore, DME method is an economical method that can significantly reduce the energy required for lipid extraction from wet materials.

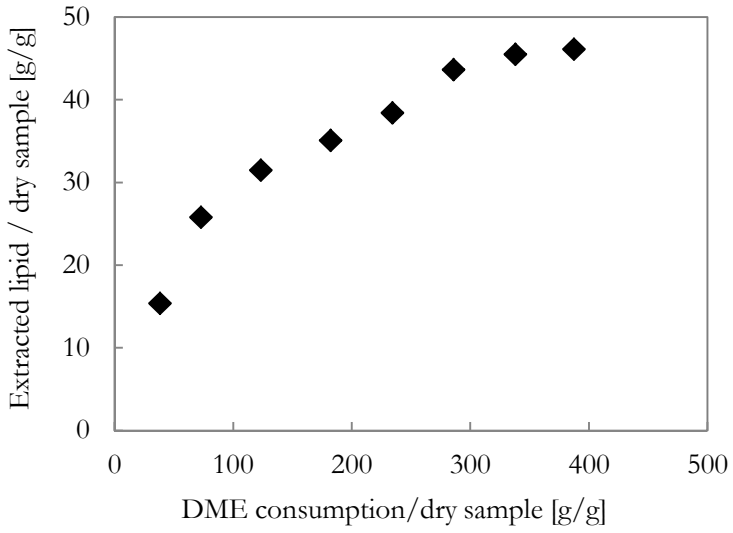

Fig. 4. Extraction behavior of the water from *A. limacinum* by using liquefied DME.

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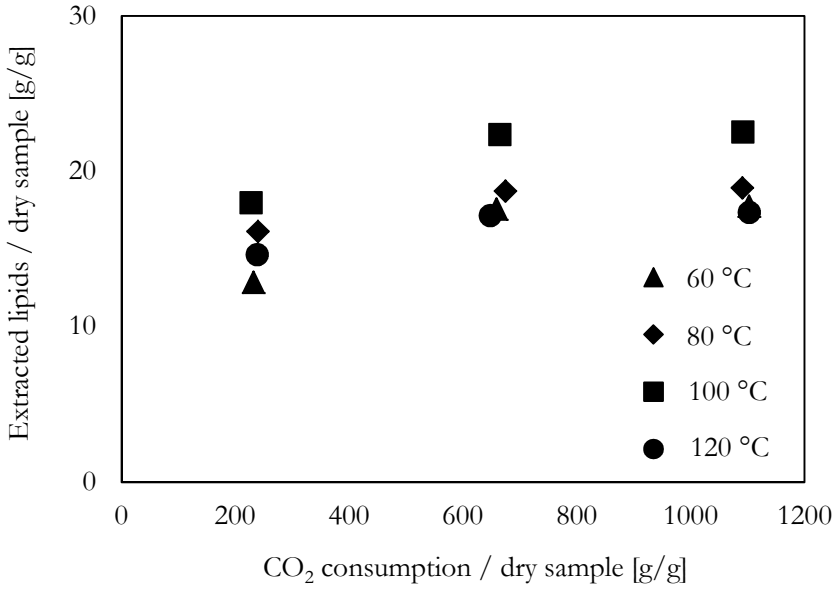

Fig. 5. Extraction behavior of the lipid from *A. limacinum* by using supercritical carbon dioxide.

3.2. Elemental Analysis

Table 1. Elemental analysis of extracted lipids and the residues.

Analysis [dry ash free]	Liquefied dimethyl ether		$SCCO_2$		Hexane		Bligh-Dyer		Original
[%]	Lipid	Residue	Lipid	Residue	Lipid	Residue	Lipid	Residue	Feed
C	68.7	47.2	67.2	48.8	75.2	44.6	64.7	51.1	55.9
H	10.4	7.3	10.0	7.4	10.8	6.8	9.4	7.7	8.3
N	0.2	5.4	0.1	5.4	0.7	5.0	0.2	4.7	4.4
O*	20.8	40.1	22.7	38.5	13.4	43.6	25.7	36.5	31.5

*By difference

It was well known that the elemental analysis for carbon (C), hydrogen (H), and nitrogen (N) content is one of the typical ways to express the components in the organic matter as fuel resources. The three concentrations obtained these components were expressed in % daf. The C/N ratio can be referenced as an indicator to identify the most suitable technique for the biomass conversion. When C/N is lower than 30, the conversion processes of biomass appropriate to biochemical processes, while when the C/N ratio is higher than 30, the thermochemical conversion process could be applied. Table 1 showed the elemental analysis of extracted lipids from *A. limacinum* and their residues when liquefied DME, $SCCO_2$, hexane Soxhlet and BD were applied as extraction method. As shown in Table 1, the elemental content of C, H, N, and O in the *A. limacinum* as a starting material was 55.9, 8.3, 4.4, and 31.5%, respectively. After liquefied DME was applied as an extractant, the content of these elements in the residue of *A. limacinum* material decreased significanty to 47.2, 7.3, 5.4, and 40.1%, respectively. This indicated that liquefied DME could extract *A. limacinum* consituents successfully. The same phenomenon was also occurred when $SCCO_2$, hexane Soxhlet and BD were used as extraction media. However, the lipids extracted using liquefied DME composed of C (68.7%), H (10.4%), N (0.2%), and O (20.8%), respectively. Compared with other extraction methods, except hexane Soxhlet, the C and H contents of the extracted lipids are higher than those extracted lipids by other methods. In general, high nitrogen content is one of the drawbacks in the terms of fuel quality [24]. In this work, the extracted lipids that shown in Table 1 had low nitrogen content. Accordingly, these extracted lipids from *A. limacinum* was suitable as bio-fuel.

3.3. Molecular Weight Distribution

In principle, the different extraction methods provide the different molecular weight extracted constituents of matrix which may give the different properties in chemically and physically. Hence, the properties of extracted lipids from *A. limacinum* by liquefied DME, $SCCO_2$, hexane Soxhlet and BD extraction methods were analyzed by GPC. This technique is simple and has become a powerful method for the determination of the MWD of extracted compounds. Fig. 6 showed the MWD of extracted lipids relative to the polystyrene standards. The MWD of extracted lipids by liquefied DME were compared with those obtained by $SCCO_2$, hexane Soxhlet and BD extraction methods using GPC with THF as a solvent. As illustrated in Fig. 5, the MWD of extracted lipids are almost the same. They seemed to have molecular weights ranging from 1 to 100 kDa. This indicated that liquefied DME that employed as a solvent media allowed to extract lipid components from *A. limacinum* matrix. The result also showed that liquefied DME may have high polarity property which is suitable for extracting higher-molecular-weight polar compounds.

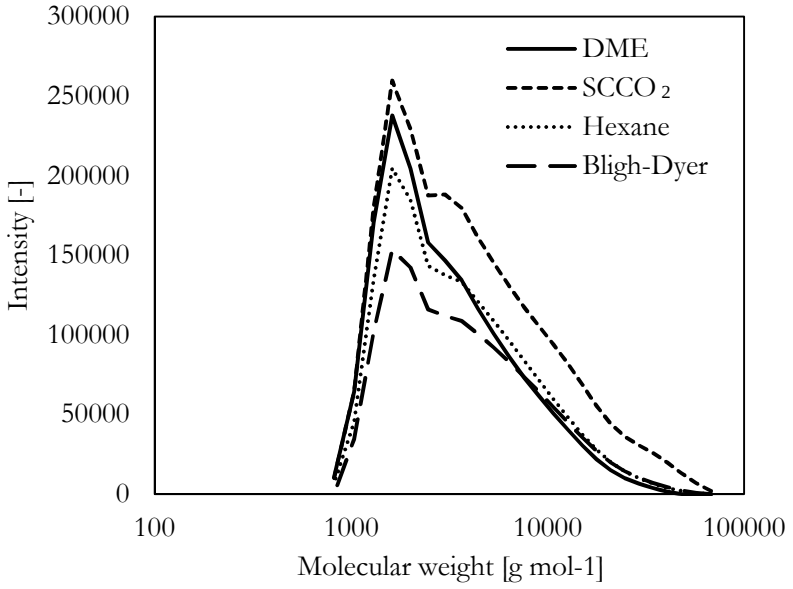

Fig. 6. Molecular weight distribution curve of the extracted lipids.

3.4. Fatty Acid Profile

Table 2. Percentage fatty acid components of the lipids from *A. limacinum*.

Fatty acids	Components [Peak area %]			
	Liquefied DME	$SCCO_2$	Hexane	Bligh-Dyer
C14:0	3.7	1.5	1.6	2.9
C16:0	89.4	63.7	57.9	76.9
C18:0	2.5	0.8	1.0	1.4
C22:5n-6	1.1	7.1	7.8	4.0
C22:6n-3	3.4	27.0	31.7	14.8
Saturated fatty acid	95.5	66.0	60.5	81.2
Unsaturated fatty acid	4.5	34.0	39.5	18.8

In order to understand the fuel properties, the systematic analysis of the fatty acid composition is very important for species selection for biodiesel production. The most common components of fatty acid are Palmitic-(hexadecanoic-C16:0), Stearic-(octadecanoic-C18:0), Oleic (octadecenoic-C18:1), Linoleic-(octadecadienoic-C18:2) and Linolenic-(octadecatrienoic-C18:3) acids [25]. However, the small amounts of eicosapentaenoic acid (EPA) (C20:5) and docosahexaenoic acid (DHA) (C22:6) were also found in several biodiesel resources, including microalgae. The fatty acid composition of lipids from *A. limacinum* was shown

in Table 2. Their compositions varied significantly with the different methods. In the liquefied DME extraction, the amount of saturated fatty acid was 95.5 % with palmitic acid (C16:0) as main components of extracted lipids from *A. limacinum*. The amount of palmitic acid was 89.4 %, followed by 3.7 % myristic acid (C14:0) and 2.5 % stearic acid (C18:0). The small amounts of docosapentaenoic acid (DPA; C22:5n-6) and docosahexaenoic acid (DHA; C22:6n-3) were obtained. This is, of course, a beneficial result in terms of biodiesel fuel where the high content of polyunsaturated fatty acids are not suitable for use as biodiesel oil [26]. Compared with other methods, liquefied DME could extract lipids containing more saturated fatty acid than unsaturated fatty acids. Thus, extraction by using liquefied DME appears to be a good method for lipids recovery from *A. limacinum* with improved yields.

4. Conclusion

This work has clearly verified that the liquefied DME method can be used to directly extract lipids from the wet *A. limacinum* at room temperature. Comparison of the extraction yields, elemental analysis, the MWD curves, and the GC/MS spectra, the liquefied DME extraction technique indicated that this technique performs as well as hexane or BD extraction techniques despite the steps of drying, cell disruption, and heating at high temperature. Moreover, the *A. limacinum* lipids were consisted of saturated fatty acids, implying that *A. limacinum* is suitable as a fine fuel source and easily modified into a biodiesel fuel. Thus, liquefied DME method, which can skip drying and cell disruption, has the advantage of being energy-saving for lipid extraction.

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