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Characterization of Extracted Lipid Obtained from Subcritical Water Extraction of Wet Algae for Biodiesel Production

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In this research, subcritical water at 200 °C for 0.5 h was shown to improve the efficiency for lipid extraction from wet algae *Nannochloropsis gaditana* (80 % moisture content), compared to the conventional solvent extraction method. Scanning electron microscopy was used to confirm the algae cell collapsed after extraction. The composition of extracted lipid was analyzed by gas chromatography. Fourier transform infrared spectroscopy, ^{1}H NMR and ^{13}C NMR spectroscopy were also conducted to study the properties of extracted lipid. The predominant fatty acids of algae lipid were shown to be palmitic acid (37.35-38.48 %), palmitoleic acid (28.54-32.85 %) and oleic acid (14.24-11.18 %). The 56.61 % wt. of free fatty acid was also detected. These properties indicated the possibility of extracted lipid as raw material for biodiesel production *via* two steps of reaction (esterification and transesterification).

Keywords: Subcritical water extraction, Wet microalgae, Biodiesel, Lipid, Characterization.

INTRODUCTION

Biodiesel from microalgae is a promising solution for the alternative fuel requirements. Microalgae have higher photosynthetic efficiency, higher biomass production and higher growth rates, as compared to other food crops[1]. In addition, they can be cultivated in waste water or salt water and require less cultivation land[2,3]. Microalgae are also used to produce feed or fertilizer, or can be fermented to produce ethanol and methane[4].

Traditional process for biodiesel production from microalgae usually requires dry algal biomass with water content no more than 10 %. Microalgae contain high moisture content (more than 60 %) after harvesting by centrifugal dewatering[5]. The energy consumption in drying and solvent extraction processes accounted for up to 90 % of the total process energy[6]. Moreover, the conventional solvent extraction is not environmentally friendly. Water is identified as an environmentally benign, non-toxic, readily available, inexpensive and available green solvent.

Recently, subcritical water (SCW) extraction or hydrothermal liquefaction (HTL) has been successfully employed to improve the lipid extraction from wet biomass. The process of converting biomass in hydrothermal liquefaction typically takes place within 200-370 °C and high pressure. The solubility of organic matter begins to increase rapidly at about 200 °C and this enhanced solubility for organic compounds is provided by a homogeneous single-phase medium for organic synthesis in subcritical water[7]. The reduction of dielectric constant makes water a suitable solvent for small organic compounds, as its dielectric constant drops from 80 at 25 °C to 40 at 200 °C[8]. Moreover, Nguyen *et al.*[9] reported that pH of water decreased from 7.4 at 0 °C to 5.5 at 250 °C. Hence subcritical water can also act as an effective acid catalyst for a hydrolysis. Subcritical water treatment was able to disrupt cell wall, then allowing easier extraction of neutral lipids followed the work of Huynh *et al.*[10]. They extracted the neutral lipid from activated sludge using subcritical water pretreatment. Reddy *et al.*[11] developed subcritical water extraction of lipid from wet algae using microwave (MW-SCW) heating compared with the conventional heating (C-SCW). The result revealed that both extractions were highly efficient for lipid extraction (70 % in C-SCW and 100 % in MW-SCW) at 205- 220 °C and 25 min of extraction time. The advantage of these both subcritical water were more superior than the conventional solvent extraction process in term of energy saving.

Nannochloropsis sp. and *Chlorella sp.* are green microalgae which are popular for biodiesel production due to their

high lipid contents, 31-68 % and 28-32 %, respectively[3]. From our literature review, no information was obtained about *Nannochloropsis gaditana* lipid. Thus in this study, the properties of extracted lipid obtained from subcritical water of *N. gaditana* was analyzed by different advance analytical techniques. The known lipid properties will be beneficial for biodiesel production process followed subcritical water pretreatment step.

EXPERIMENTAL

The marine microalgae *Nannochloropsis gaditana* (CCMP-1775) culture used in this study was obtained from the Provasoli-Guillard National Center for Culture of Marine Phytoplankton (CCMP) USA. The *N. gaditana* was grown in the f/2 growth medium[12] modified to contain 5 mM NO_3^- L^{-1} and 0.287 mM PO_4^{3-} at a salinity of 20 g L^{-1} in a 4000 L outdoor Solix photo bioreactor. The algae was grown with 0.8 % CO_2 enrichment and maintained at 25 °C with ambient outdoor lighting. Composition of algal biomass samples were characterized with crude carbohydrate -56.97 %, crude protein -14.26 %, lipid content -21.76 %[13] and ash content -7 %. The high heating value was determined to be 25 MJ/kg. Methyl tricosanoate (C23:0) from Sigma Aldrich was used as an internal standard for gas chromatography-flame ionization detector (GC-FID) analysis. Methanol, *n*-hexane and *n*-heptane were purchased from Q Rëc Chemical Co. Ltd (Selangor, Malaysia) were used. The $CDCl_3$ [99.8 % D with 0.03 % (v/v) tetramethylsilane (TMS)] used as the NMR solvent was purchased from Aldrich Chemical Co. (Milwaukee, WI). The subcritical water (SCW) extraction was performed in a homemade high-pressure reactor with the size of 600 cm. The reactor could resist an estimated maximum pressure of 50 MPa. The reactor was equipped with an external electric heater (T), cooling coil, pressure gauge (P) and magnetic stirrer. The reactor was made of Type 316 stainless steel with ten M8 screws for tighten the reactor with its cap as shown in Fig. 1. The required pressure was achieved through the insertion of nitrogen gas in to the reactor. The reactor was heated through electric heater, of which the temperature was controlled by temperature controller.

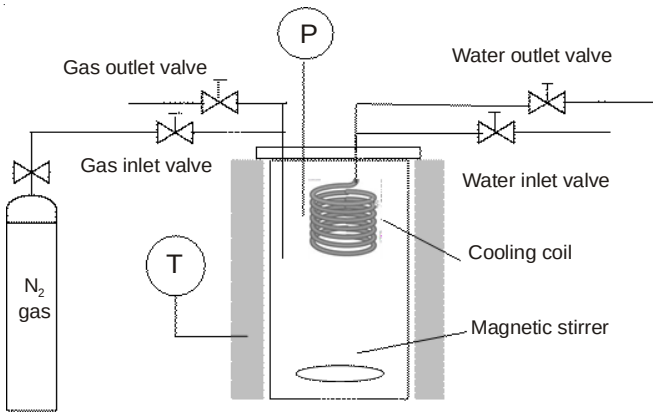

Fig. 1. Schematic diagram of a high pressure reactor system

Lipid extraction using Soxhlet: The wet microalgae pellets were dried in hot air oven at 60 °C prior to lipid extraction. The 10 g of dry microalgae were settled in a cellulose thimber. The extraction was performed for 6 h in a Soxhlet apparatus containing 150 mL of solvent. After extraction, the solvent was evaporated under reduced pressure and the lipid fraction was dried until constant weight in an oven at 60 °C. Then, the yield of extracted oil was calculated.

Lipid extraction using subcritical water treatment: 100 g of wet microalgae (80 % moisture content) was loaded into a reactor. Temperature in a reactor was controlled at 200 °C for 0.5 h, whereas the pressure was maintained at 1.38 MPa throughout the experiments. At the end of the reaction, the reactor was cooled to room temperature and the product mixture was transferred into a separation funnel. About 20 mL of *n*-hexane was used to wash the reactor to free any product adhered to it and then the product was transferred into the separation funnel. The hexane phase was further evaporated under vacuum pressure at 60 °C by rotary evaporator. The obtained lipid was weighed using an analytical balance for lipid content analysis. The extraction process using subcritical water was shown in Fig. 2.

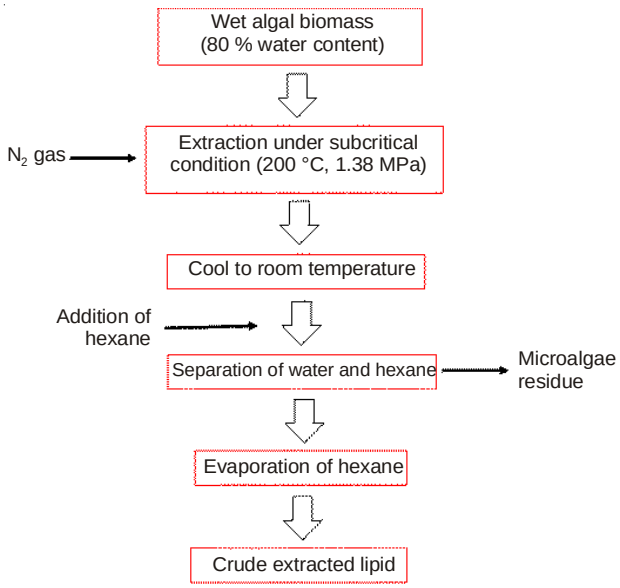

Fig. 2. Subcritical water extraction process of lipid from wet microalgae

Characterization of microalgae biomass: The morphology of dry algae biomass after subcritical water treatment was conducted using a scanning electron microscope (SEM) (FEI Quanta 450, Czech). The biomass sample was amounted on alumina scanning electron microscope stub and then coated with gold.

Analysis of extracted lipid

Chemical structure: Attenuated total reflection-fourier transform-infrared instrument (ATR-FTIR) (Bruker, Equinox 55, Germany) and VARIANunity INOVA, nuclear magnetic resonance spectrometer were used to determine the chemical structure of algae lipid from subcritical water extraction.

Free fatty acid content: Free fatty acid (FFA) content was determined according to the AOCS official method 5a-40. Lipid and esterified lipid sample were introduced into flask, then ethanol solution and phenolphthalein were added. The mixed solution was titrated with 0.1 N sodium hydroxide solution. Free fatty acid content was calculated in accordance with eqn. 1.

$$\text{FFA}\ (\%) = \frac{[(A-B)\times N\times 20.03]}{W} \quad (1)$$

where A = milliter of sodium hydroxide solution titrated with sample; B = milliter of sodium hydroxide solution titrated with blank; N = concentration of sodium hydroxide solution in normality unit; W = weight in gram of sample.

Fatty acid composition: Fatty acid composition of algae lipid was analyzed by gas chromatography (GC). Briefly explained, the lipid was methylated with boron trifluoride in methanol followed with sodium hydroxide/ methanol treatment to form methyl ester, which was then subjected to GC analysis. A DB-WAX capillary column (30 m, 0.32 mm, 0.25 µm, Agilent J&W, Agilent Technologies) was used with flame ionization detector. The temperature of injector and detector block was maintained at 275 and 300 °C, respectively. The column temperature was programmed as initial temperature at 50 °C for 2 min, then temperature was increased to 220 °C with 4 °C/ min and hold for 15 min. Helium was used as carrier gas at 30 mL/min flow rate. Peaks were identified by comparison with retention time of 37 component fatty acid methyl ester mix standard (Supelco Co., USA) and quantification was performed by internal normalization method.

RESULTS AND DISCUSSION

The effectiveness of extraction method in enhancing the lipid extraction process was proven by scanning electron microscope analysis of extracted algae biomass. The surface of dried algae before and after samples extraction was illustrated in Fig. 3. The cell wall of dried algae (Fig. 3a) was densely packed, which could be significantly resistant for mass transfer during lipid extraction process. After Soxhlet extraction, the pore sizes and number of pores on the flat sheet of algae were clearly increased (Fig. 3b), while subcritical water extraction resulted in the breakdown of cell walls into small particle thus loosened and made the algae cell wall surface rougher (Fig. 3c). Certainly, such a loose structure and small particle could promote mass transport and improve the overall process rate compared to the dense structure. It proved that subcritical water extraction effectively improved lipid extraction since the disruption and destruction of cell membrane.

Characterization of extracted lipid: In this work, lipid composition in extracted lipid obtained from subcritical water and Soxhlet extraction was investigated by FT-IR spectrum as shown in Fig. 4. The individual lipid spectrums showed the following general characteristic features: (i) the presence of ester (C-O-C) characterized by the absorption band at 1168

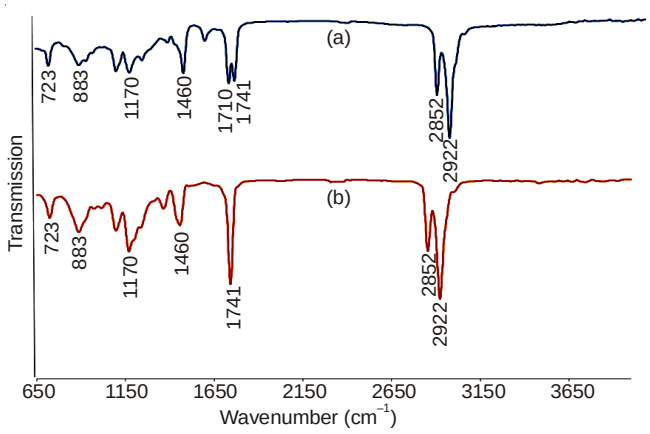

Fig. 4. FT-IR analysis of algae lipid; (a) from subcritical water (b) from Soxhlet extraction

cm^{-1} (ii) the presence of carbonyl groups (C=O) characterized by the absorption at 1741 cm^{-1} (iii) the strong bands close to 2922, 2852 cm^{-1} were assigned to stretching vibrations of -CH_2 and -CH_3 whereas the band recorded at 1461 cm^{-1} was assigned to bending vibration-CH aliphatic groups from the alkyl group[14]. The spectrum of extracted lipid obtained from subcritical water extraction clearly showed the band at 1710 cm^{-1} which revealed the presence of free fatty acid (COO-) in sample[15,16].

Fig. 5a shows ^{13}C NMR spectrum of extracted lipid from subcritical water extraction. In the region of the carbonyl carbons (173-180 ppm), the presence of free fatty acid was detected at δ 179.79 ppm while the acylglycerols composition did not appear in this region. In the region of the aliphatic carbons (22-35 ppm), the peaks of acyl groups in both triglyceride and free fatty acid were observed. The peak for C-3 of free fatty acid was δ24.63 ppm while the peak for C-3 of 1,3-acyl chains of the triglyceride was δ24.83 ppm. From Fig. 5b, the signal at δ173.19 ppm in ^{13}C NMR spectrum represented the carbonyl carbon of triglyceride obtained after Soxhlet extraction[17,18]. Moreover, it shows the signals at δ 62.07 and δ 68.88 of glycerol carbon of triglyceride, thus triglyceride was the most lipid composition of algae lipid from Soxhlet extraction.

The ^{1}H NMR spectra of algae lipid from subcritical water extraction shows the resonances of protons attached to glycerol carbons of 1,2-diglycerides, 1,3-diglycerides and triglycerides detected approximately at 3.64, 4.01 and 4.1-4.3 ppm (Fig. 6a). While, the ^{1}H NMR spectra of algae lipid from Soxhlet extraction shows high signal at 4.0-4.3 ppm assigned as H-1 and H-3 on the glycerol carbon of triglyceride (Fig. 6b)[18,19].

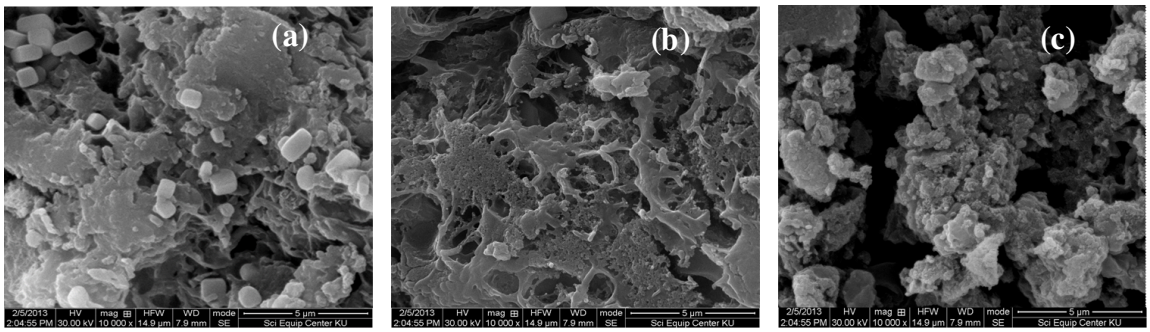

Fig. 3. Scanning electron microscope of algae biomass before (a) and after soxhlet extraction (b), subcritical water extraction (c)

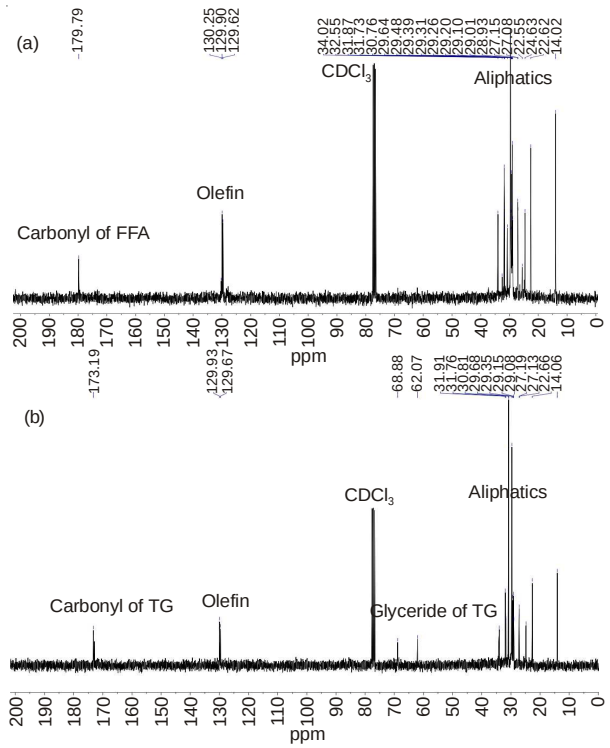

Fig. 5. ^{13}C NMR spectra of algae lipid from subcritical water extraction (a) and Soxhlet extraction (b)

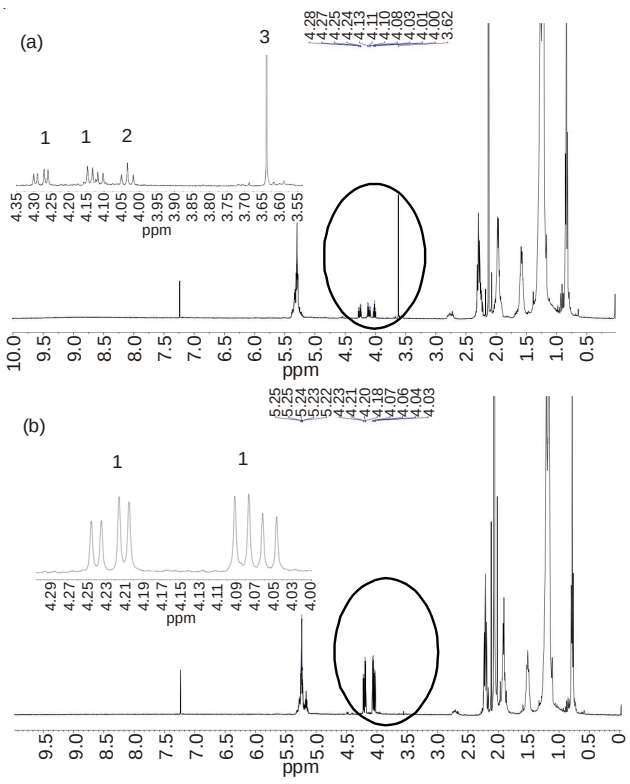

Fig. 6. ^{1}H NMR spectra of algae lipid from SCW extraction (a) and Soxhlet extraction (b) assigned as (1) H-1(H-3) of TGs (2) H-2 of sn-1,3-DGs; (3) H-3 of sn-1,2-GDs

This result confirmed that triglyceride was hydrolyzed to free fatty acid during subcritical water extraction.

Fatty acid composition of extracted lipid is very important in identifying the carbon chains and its properties. Fig. 7 and Table-1 show high similarity of the fatty acid compositions

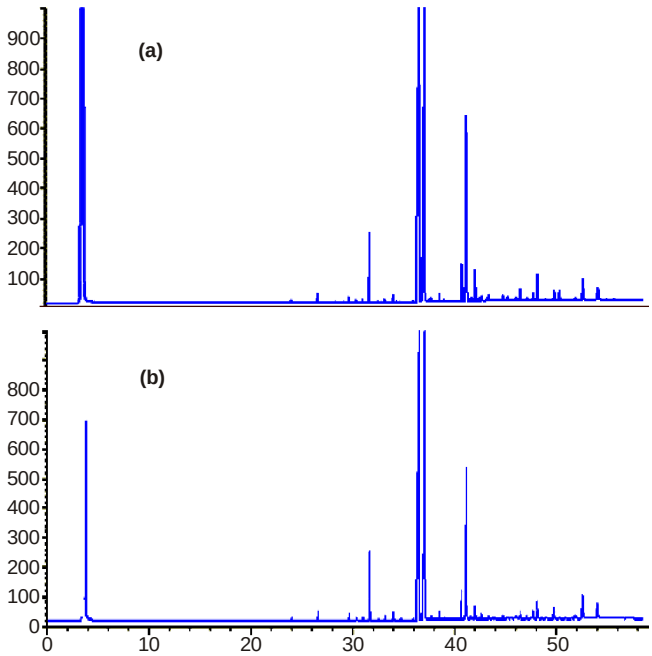

Fig. 7. Chromatogram of extracted lipid obtained from subcritical water extraction (a) and Soxhlet extraction (b)

from the Soxhlet- and subcritical water-extracted algae lipid. The results also showed that 47.31-46.99 % was saturated fatty acids whereas 41.68-44.03 % was unsaturated fatty acids, with the major fatty acids as palmitic acid (37.35-38.48 %), palmitoleic acid (28.54-32.85 %) and oleic acid (14.24-11.18 %). High proportion of monounsaturated fatty acids was reported to decrease the viscosity of biodiesel[20]. Mono unsaturated fatty acids such as palmitoleic acid (C16:1) and oleic acid (C18:1) were capable of giving the finest compromise between oxidative stability and cold flow[20]. Consistently, both extracted algae lipid samples displayed low polyunsaturated fatty acid (8.72-8.15 %), which would have a positive effect on their oxidative stability. After extraction, the extracted lipid content was determined gravimetrically. The subcritical water extraction resulted in 14.89 ± 1.41 % lipid, whereas the Soxhlet extraction using hexane gave 12.77 ± 0.95 % lipid. The weight fraction free fatty acid in the extracted lipid was as high as 56.61 ± 1.54 % wt while that obtained from Soxhlet extraction was 1.55 ± 0.07 %. This could be due to that fatty acid which bound to triglyceride (TG) and phospholipid (PL) were broken down to free fatty acid under subcritical water extraction[9]. Changi and co-worker studied the hydrolysis pathway of 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) in high temperature water (175-350 °C)[21], in which DOPC was hydrolyzed to oleic acid. Thus, Soxhlet extraction with hexane extracted triglyceride and free fatty acid, whereas subcritical water extraction with hexane could extract neutral lipid such as free fatty acid and bound fatty acid from triglyceride and phospholipid. Consequently, the gravimetric lipid content as determined by Soxhlet extraction with hexane solvent was less than that obtained from subcritical water extraction.

Advantages of subcritical water extraction: From the experimental results, the advantages of subcritical water extraction could be summarized as follows: (i) Microalgae cell walls were disrupted as observed by scanning electron microscope

TABLE-1
FATTY ACID COMPOSITION OF EXTRACTED LIPID

Fatty acid	Carbon	Retention time (min)	Composition (%)	
			SCW	Soxhlet
Lauric acid	12:0	26.50	0.45	0.47
Myristic acid	14:0	31.63	3.69	3.85
Palmitic acid	16:0	36.32	37.35	38.48
Palmitoleic acid	16:1	36.87	28.54	32.85
Stearic acid	18:0	40.64	2.26	1.67
Oleic acid	18:1n9c	41.06	14.24	11.18
Linoleic acid	18:2nc	41.98	1.78	0.80
γ-Linolenic acid	18:3n6	42.60	0.45	0.45
α-Linolenic acid	18:3n3	43.30	0.44	0.00
Eicosanoic acid	20:0	44.74	0.48	0.32
11-Eicosenoic acid	20:1	45.18	0.38	0.00
cis-11,14-Eicosadienoic acid	20:2	46.34	1.05	0.59
cis-11,14,17-Eicosatrienoic acid	20:3n3	47.06	0.51	0.25
cis-8,11,14-Eicosatrienoic acid	20:3n6	47.70	0.87	0.70
5,8,11,14- Eicosatetraenoic acid	20:4n6	48.15	2.42	1.42
5,8,11,14,17-Eicosapentaenoic acid	20:5n3	49.74	1.19	0.91
Behenic acid	22:0	50.26	1.13	0.00
cis-13,16-Docosadienoic acid	22:2	52.86	0.00	3.03
Lignoceric acid	24:0	55.52	1.94	2.19
Total saturated fatty acid	-	-	47.31	46.99
Total monosaturated fatty acid	-	-	43.16	44.03
Total polyunsaturated fatty acid	-	-	8.72	8.15

analysis, then allowing easier extraction of lipid. (ii) Triglycerides and other structural lipids were hydrolyzed during subcritical water extraction, thus the free fatty acid content was increased as observed from FT-IR, ^{13}C NMR, ^{1}H NMR and free fatty acid content analysis.

Disdvantages of subcritical water extraction: (i) Poor lipid properties due to extracted lipid consisted of free fatty acid content higher than triglyceride content. (ii) Longer reaction time in subsequent biodiesel production due to that the reaction consists of two steps (esterification and transesterification reaction) according to Chen *et al.*[22].

Conclusion

This study demonstrated that the lipid extraction from wet *N. gaditana* microalgae by subcritical water treatment was able to maximize not only the release of neutral lipid but also free fatty acid through the disruption of cell wall and hydrolysis of triglyceride and other structural lipids. The obtained extracted lipid contained high free fatty acid content in the same level as that in sludge oil, so this feedstock could be converted into biodiesel by acid catalyzed esterification followed by alkali catalyzed transesterification.

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