Single-Step Conversion of Wet *Nannochloropsis gaditana* to Biodiesel under Subcritical Methanol Conditions

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ABSTRACT

A single-step process for a direct conversion of wet algal biomass containing about 80% of water into biodiesel under subcritical methanol conditions is developed and presented in this work. This process may have energetic advantages with high free fatty acid (FFA) and water contents as the feedstock. At elevated temperatures, water hydrolysis and FFA esterification can simultaneously occur in a subcritical state. The influences of process parameters including reaction temperature (150-225 °C), reaction time (15-120 min) and methanol to wet biomass (vol./wt.) ratio (3:1-8:1) were investigated. The optimal conditions for this process are reported as: methanol to wet biomass (vol./wt.) ratio of around 6:1, temperature and time of about 225 °C, and reaction time of 90 min. The fatty acid methyl esters (FAMES) were analyzed with GC-MS, FT-IR and thermogravimetric (TGA). This process does not require a catalyst and any drying pre-treatment steps. Thus, the process is cost-saving and environmental friendly for renewable biodiesel production.

Key words: Wet algae, Biodiesel, Fatty acid methyl ester, Subcritical methanol condition, Nannochloropsis gaditana.

1. Introduction

Biodiesel, as a renewable, economically viable alternative to petroleum-derived diesel, has received much attention as the world seeks to prevent the future oil crisis and the unpredictable consequences of climate change [1]. It is generally produced from the oils of palm, soybeans, canola, jatropha, etc [2]. However, the great requirement of unsustainable large cultivation area of these oil crops has limited the wide application of biodiesel in supplementing petroleum derived diesel [3].

In recent years, microalgae have been suggested as a potential feed stock for biodiesel production. They have numerous advantages, including higher photosynthetic efficiency, higher biomass production, and higher grow rates, as compared to other food crops [4]. In addition, microalgae can grow in fresh water, marine non-potable water, or wastelands where nothing else grows without using arable land and competing with food production [5].

The conventional biodiesel production from microalgae usually requires dry algal biomass with water content no more than 10%. Microalgae have high moisture content after harvesting by centrifugal dewatering more than 60% of water [6]. Algal oil is extracted with organic solvent and then converted into biodiesel using a catalyst. The energy consumption in drying and solvent extraction processes accounted for up to 90% of the total process energy [7]. Moreover, the conventional production uses organic solvent is not environmentally friendly. Direct transesterification is promising technique that generates fatty acid methyl esters (FAMES) from wet algal biomass. It eliminates the need to isolate and purify lipid before converting it to biodiesel, which could be reduced the cost of biodiesel production [8]. However, this method has a problem that moisture content is a limiting factor for conversion efficiency of triglyceride [9, 10].

Acid catalysts are less sensitive to the presence of free fatty acids (FFA) and can simultaneously conduct esterification and transesterification [11]. The esterification of free fatty acid is more resistant to the inhibition by water and enabled the higher FAME yield. Thus, acid hydrolysis of lipid from microalgae followed by esterification was carried out by Takisawa et al. 2013 [12]. In their report, wet algal biomass reacted with sulphuric acid to hydrolyzed intracellular lipid and subsequently FFA underwent esterification with methanol to produce FAME. The excess sulphuric acid in a hydrolysis process causes the degradation of FFA. Therefore, time, temperature and acid concentration must be controlled more carefully. Hence, supercritical alcohol reaction is of great interest to carry out such as no catalyst is required and a high conversion can be obtained in a short time [13, 14]. A single-step extraction and conversion of wet algae to fatty acid methyl esters under supercritical methanol (SCM) has been demonstrated [15]. This process was performed at a high ratio of wet algae/ methanol (1:9 wt/vol.), a high reaction temperature (255 °C) and a high reaction pressure (1200 psi) within 25 min. Under the supercritical condition, methanol acts as both an acid catalyst and a reactant to accelerate the reaction [16, 17]. An excessive amount of methanol is still required to suppress the backward reaction of FAME to TAG and acts as an acid catalyst. [18]. In addition, the reaction requires temperatures and pressures are much higher than the critical point of the reaction mixture led to high production costs and energy consumption. The addition of co-solvent such as hexane, propane, with supercritical conditions seems to be an efficient means to reduce can decrease the operating temperature, pressure and the amount of alcohol required [19,20]. However, the use of co-solvent has negative effects on environment and the purity of biodiesel produced.

Subcritical water (SCW) is considered as a cost-saving and environmentally friendly. At high temperature, SCW acts as a nonpolar solvent like organic solvent. Moreover, SCW can also act as an effective catalyst for a hydrolysis [21]. In a previous work, it reported that triglyceride (TAG) was hydrolyzed to FA under SCW without adding catalyst [22-23]. H^+ (from dissociation of water and FA) and FA serve as a catalyst for hydrolysis. Additionally, phospholipid is known to be a polar lipid and was also hydrolyzed to FA and phosphorus-containing products [24]. Thus biodiesel can be produced from phospholipids, free fatty acids, and triglycerides under subcritical conditions. Hydrolysis of dewatered algae (80–90% water) under SCW followed by esterification with no added catalyst was also investigated [25]. They suggested a two-step non-catalyst biodiesel production process through intracellular lipid hydrolysis and supercritical esterification. First, cellular lipids were converted to fatty acids under subcritical water conditions. Second, fatty acids were esterified with supercritical ethanol to produce biodiesel in the form of fatty acid ethyl esters (FAEEs). They reported a maximum FAEE yield of 66% (w/w). It indicated that esterification was inhibited from the biomass with high water content. Tsigie and co-workers have developed a single-step non-catalytic conversion process for producing fatty acid methyl esters (FAMES) from wet *Chlorella vulgaris* under subcritical water and methanol conditions, and reported a FAME conversion yield of 89.71% at 175 °C after 4 h of reaction time [26].

The objective of this study is to investigate the direct biodiesel production of wet algal biomass under subcritical water and methanol in the presence of nitrogen. The influence of parameters on the process including reaction temperature, reaction time and ratio of wet biomass to methanol was investigated. Gas Chromatography-Mass Spectroscopy (GC-MS) was used to determine the FAME yield.

2. Experimental

2.1. Materials and methods

Nannochloropsis gaditana (CCMP - 1775) culture used in this study was obtained from the Provasoli-Guillard National Center for Culture of Marine Phytoplankton (CCMP). The *N. gaditana* was grown in the f/2 growth medium [27]. Methyl Tricosanoate (C23:0) from Sigma Aldrich was used as an internal standard for the GC–MS analysis. Hexane and methanol was purchased from PHARMCO-AAPER (Shelbyville, KY) were used in this study. The CDCl₃ [99.8% D with 0.03% (v/v) tetramethylsilane (TMS)] used as the NMR solvent was purchased from Aldrich Chemical Co. (Milwaukee, WI). SPE silica columns for the purification of crude FAME's were purchased from Thermo Scientific (Waltham, MA). The subcritical methanol esterification was performed in a PARR 4593 stainless steel bench top reactor accompanied by a 4843 controller unit and were manufactured by Parr Instrument Company (Moline, Illinois, USA). Thermogravimetric analysis (TGA) of wet algal biomass and biodiesel were performed using Perkin Elmer Pyris 1 TGA (Perkin Elmer Inc., USA) instrument. A VARIAN^{unity} INOVA, nuclear magnetic resonance spectrometer and Attenuated total reflection-Fourier transform-infrared Instrument (ATR-FTIR) (Bruker, Equinox 55, Germany) was used to determine the chemical structure of algae biodiesel.

2.2 Properties of *Nannochloropsis gaditana* algal biomass

Composition of algal biomass samples were characterized with crude carbohydrate - 56.97%, crude protein -14.26%, lipid content -21.76% and ash content -7.0%. The high heating value was determined to be 25.0 MJ/kg. Analyses of algal biomass with TGA were also performed.

2.3 Reaction of wet *N. gaditana* with methanol under subcritical condition

Five grams of wet algae containing 80% water and methanol were loaded in a 100 mL PARR micro-reactor. To analyze the effects of conversion process parameters, experiments were conducted in three batches by varying one of the parameters and keeping the remaining two parameters constant. The reaction was carried out at reaction temperature from 150 - 225 °C, reaction times from 15–120 min, wet biomass to methanol (vol./wt.) ratio was 3:1–8:1 and the pressure was maintained (600–900 psi) in all experiments. At the end of the reaction, the reactor was cooled to room temperature before collecting the product mixture. This mixture was processed under a vacuum in a rotary evaporator to separate the unreacted methanol from the product mixture. About 10 mL of n-hexane was added to the remaining product to separate the biodiesel and then centrifuged (3200 rpm) for 5 min. The hexane layer containing biodiesel was purified in a short SPE silica column to elute only neutral components that were analyzed in GC–MS.

2.4 FAME analysis

Analysis of FAME content was performed using gas chromatography–mass spectrometry (GC–MS) system composed of an Agilent 5975 C MSD and an Agilent 7890 A GC equipped with a capillary column (DB-23, 60 m x 250 mm x 0.15 mm nominal). 10 mg of samples were mixed with 1 mL of an external standard solution (C23:0, 0.1 mg/mL in heptane) in 2 mL vials. The content of the FAME in the final product was calculated quantitatively by comparing the peak areas of FAME to the peak area of the internal standard obtained from GC–MS. 1 µL sample was injected into the GC. Helium was used as the carrier gas. The injection was performed in split mode (50:1). The analysis conditions were used with the following temperature program: 50 °C for 2 min, then increasing with 25 °C /min intervals up to 175 °C and

rinse up to 235 with 4 °C /min intervals (5 min). The temperatures of the injector and detector were set at 250 °C.

$$\text{FAME content in crude biodiesel (\%)} = \frac{(\sum A) - A_s}{m} \times \frac{V_s \times C_s}{1} \times 100$$

Where $\sum A$ is the total peak area of FAMES;

A_s is the peak area of internal standard;

C_s is the concentration of internal standard (mg/L);

V_s is the volume of internal standard (mL);

m is the weight of crude biodiesel (g)

3. Results and discussions

3.1 Influence of reaction temperature

The critical temperature of the methanol is 240 °C, the experiments were carried out between 150 and 225 °C at a constant time of 60 min and methanol/wet biomass ratio of 4:1. From the Fig. 1a, it is clear that the increase in reaction temperature favors a higher FAME. The optimum temperature for maximum biodiesel yield is found to be 225 °C. With the increase in temperature the conversion takes place at a faster rate. Due to high temperature can increase the solubility of methanol and oil phase. Thus the time for the mass transfer between oil and methanol is shortened [28, 29].

3.2 Influence of algae to methanol (wt./vol.) ratio (wet basis)

The algae to methanol ratio also is one of the most important variables influencing the conversion into methyl ester. A high molar ratio of methanol is required for driving the reaction towards completion and to get a maximum conversion [30,31]. In the one-step conversion, methanol acts as both a solvent and a reactant for the extractive-conversion process. An excess methanol increased contact between methanol and oil, improved in more efficient methanolysis reaction [14]. In order to study the effects of the amount of methanol on the FAME yield, the methanol to wet biomass ratio was varied from 3:1 to 8:1 while reaction temperature was kept constant at 225 °C and reaction time at 60 min. The results are shown in Figure 1b, it was observed that the conversion of FAME increased as the methanol to algae ratio rose, reached a maximum at the methanol to algae ratio of 6:1, then slightly decreased with the algae to methanol ratio. The separation of ester and glycerin phase becomes increasingly difficult. This might be due to the excess methanol increased solubility of the glycerin, which resulted in lower biodiesel yields [32].

3.3 Influence of reaction time

We also studied the effect of varying time in the range 15 – 120 min on the formation of FAMES. The reactions were carried out with a fixed methanol/wet biomass ratio of 6:1 and temperature at 225 °C. The resulting experimental data are shown in Fig. 1c. with the increase in reaction time, the reaction rate was increased. The maximum yield was achieved for reaction time of 90 min. The reaction of methanolysis reached to equilibrium after 90 min, increasing reaction time had no effect on the FAME. So a reaction time of 90 min was taken as the optimum reaction time for maximum FAME yield. As expected, a longer reaction time allows the hydrolysis-esterification reaction to proceed to completion and results in a higher yield.

The above experimental results show that the optimum condition of FAME were obtained at 225 °C, with 90 min of reaction time and 6:1 methanol to wet algae (vol./wt.) ratio. The maximum of FAME content in crude biodiesel is 59.28% or FAME yield (based on the dried weight of biomass) is 12.27%. When compared to Simultaneous hydrolysis-esterification of wet microalgal using acid [33], the optimum reaction temperature was 150 °C, methanol to wet biomass ratio 2.67:1, sulfuric acid 200 mL/kg dry algae and 180 min of reaction time. Acid hydrolysis was performed at lower temperature and methanol to wet biomass. Thus, the reaction time will longer than subcritical condition. The addition of excess sulphuric acid can occur the degradation of FFA. Moreover, an excess methanol causes the esterification inhibition. From work of Patil [15] an excessive amount of methanol 9:1 methanol to wet algae (vol /wt.) ratio were added to suppress the backward reaction of FAME to TAG. However, the result was not the case. The higher FA concentration from hydrolysis as a result of a small amount of methanol used in the reaction system can enhance the methyl esterification reaction [18]. On the other hand, the maximum FAME yield from non-catalytic transesterification at supercritical conditions can be achieved in a lower reaction time (25 min).

3.4. TGA analysis of algal biomass and biodiesel

The thermogravimetric curves measured on wet algal biomass and algae biodiesel are shown in Fig. 2. Samples of algal cells were heated at a rate of 10 °C/min from 30 to 950 °C in a nitrogen atmosphere at a constant purge rate of 20 mL/min at the pan. The thermogravimetric plots in linear and differential form corresponding to the biomass has undergone three phases. First stage begins at room temperature and ends at about 120 °C. During this phase, *N. gaditana* attains a weight loss of about 80% caused by moisture is released from the biomass sample. In second at 320 °C corresponding to the volatilization of organic materials. The third phase goes

from 480 °C to the final temperature (950 °C), the weight loss curve of the carbonaceous matters in the solid residuals continuously decomposed at a very slow rate [34-36]. The TGA plot for algae biodiesel consists of three steps. In the first phase, the weight loss of biodiesel starts to decrease at approximately after 120 °C. The rate of weight loss increased to 250 °C and it was attributed to vaporization and/or pyrolysis of biodiesel. The second step started to decrease at 270 °C, and it may be due to some algae oil that was not converted to biodiesel [20,37]. The TGA curve for FAME's in oxygen atmosphere is similar to that of the algal FAMEs in the nitrogen atmosphere. It indicated a good oxidation stability of the FAMEs [38].

3.5. Analysis of fatty acid methyl esters with GC-MS, ¹H-NMR, FT-IR and bomb calorie meter

FAME profile of the experimental run carried at 225 °C for 90 min with a 6:1 methanol to wet algae (vol./wt.) ratio shown in Table 1. Hexadecanoic acid, methyl ester (C16:0) is the major constituents present in biodiesel. 9-Hexadecenoic acid, methyl ester (C16:1) and 9-Octadecenoic acid, methyl ester (C18:1n9c) were the next major FAMEs. Most of the major FAMEs were saturated (40.57-47.47%) and monounsaturated (41.68-44.33%). The presence of high mono-saturated fatty acids will decrease the viscosity of biodiesel. Moreover, small quantities of poly-unsaturated FAMEs (1.44-5.26%) will have positive effect on oxidative stability [39].

The IR spectra were obtained using Attenuated total reflection-Fourier transform-infrared Instrument (ATR-FTIR) (Perkin Elmer). ATR-FTIR spectra of algal biodiesel and palm biodiesel are shown in Fig 4. The C=O stretching of methyl ester appears at 1741 cm⁻¹, whereas asymmetric axial stretching vibrations of O-C-C bonds was observed around 1170 cm⁻¹. The result showed that main components of biodiesel are mono-alkyl ester. It is clear that algae

biodiesel was in the same fingerprints as palm biodiesel, which indicating that algae biodiesel same chemical properties as palm biodiesel [40].

Algae biodiesel were analyzed by qualitative ^1H -NMR spectroscopy (400 MHz for proton). Deuterated chloroform (CDCl_3) was used as solvent (0.05 ml of sample dissolved in 0.5 ml of solvent) and tetramethylsilane as internal standard. Fig. 5 shows the spectrum of biodiesel obtained by ^1H -NMR spectroscopy. The presence of FAME can be readily detected by the appearance of singlet peak at 3.67 ppm from the methoxy group. It was revealed high conversion of FAME as the temperature rose from 175, 200 and 225 $^\circ\text{C}$, respectively. In addition, some peaks also appeared at 4.05–4.20 ppm which are doublets of doublets from the H-1 and H-3 on the glycerol carbon [41]. From Fig. 6. the signal at 4.05–4.20 ppm will decrease when increasing temperature from 175 to 225 $^\circ\text{C}$. It was also observed that high temperature became a much higher conversion than low temperature. The calorific values of FAME were measured by a bomb calorie meter. This analysis revealed that FAME has a higher heating value (HHV) of 39.68 MJ/kg.

4. Conclusion

The investigation of one-step non-catalytic FAME production from wet microalgae under subcritical methanol conditions was carried out. The method is achieved without drying and extraction of algal biomass. The high water content in the reactants showed that the hydrolysis process reduced the inhibition of water in FAME production. It can be achieved in short reaction times and does not require catalysts. The FAME profiles of *Nannochloropsis gaditana* were

shown as a potential feedstock for biodiesel production. The single-step esterification without a catalyst is highly recommended for algal biodiesel production in a large scale process design.

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365 **Table 1 Fatty acid profile of *Nannochloropsis gaditana* (as% of methyl esters).**

Type of FAME	%Area
Dodecanoic acid, methyl ester (C12:0)	0.23 - 0.26
Tetradecanoate acid (C14:0)	3.84 - 4.55
Hexadecanoic acid, methyl ester (C16:0)	35.09 - 41.00
9-Hexadecenoic acid, methyl ester (C16:1)	29.9 - 32.0
Octadecanoic acid, methyl ester (C18:0)	1.41 - 1.66
9-Octadecenoic acid, methyl ester (C18:1n9c)	11.27 - 11.74
11-Octadecenoic acid, methyl ester (C18:1n11t)	0.51 - 0.59
9,12-Octadecadienoic acid , methyl ester (C18:2n6c)	0.60 - 1.36
9,12,15-Octadecatrienoic acid, methyl ester (C18:3n6)	0.68 - 0.93
8,11,14- Eicosatrienoic acid, methyl ester (C20:3n3)	0.06 - 0.25
5,8,11,14,17-Eicosapentaenoic acid methyl ester (C20:5n3)	0.10 - 2.72
Saturated fatty acid	40.57 - 47.47
Monounsaturated fatty acid	41.68 - 44.33
Polyunsaturated fatty acid	1.44 - 5.26

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368 Fig. 1. FAME (%) against (a) reaction temperature,(b) methanol to algae biomass (vol. /wt.) ratio
 369 and (c) reaction time

Fig. 2. Thermogravimetric (TGA) curves of wet algal biomass and algae biodiesel

Fig. 3. A typical GC chromatogram of algae biodiesel.

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Fig.4. FT-IR spectra of algae biodisel and palm biodiesel

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430 Fig. 5. ^1H -NMR spectra of algae biodiesel after methanolysis at different temperature.

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Fig. 6. Expansion of 4.0–4.3 ppm region in the ^1H NMR spectra of algae biodiesel after the methanolysis reaction at different temperature