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In situ biodiesel production from wet *Chlorella vulgaris* under subcritical condition

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HIGHLIGHTS

- The conventional biodiesel production process is not environmentally friendly.
- Biodiesel can be made directly from wet algal biomass and subcritical methanol.
- Stirring shortens the reaction time to achieve high conversion yield of FAMEs.

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ABSTRACT

The conventional base catalyzed biodiesel production process uses refined vegetable oil as feedstock oil and is not environmentally friendly. The supercritical methanol technology does not require the use of catalyst but it is energy intensive due to the high temperature and pressure required in the process. In this work, a process was developed for producing biodiesel directly from wet *Chlorella vulgaris* biomass (80% moisture content) using subcritical water as catalyst. Under the following conditions: The ratio of wet biomass to methanol is 1/4 (g/mL), the reaction temperature is 175 °C and after 4 h, the reaction product contained 89.71% fatty acid methyl esters (FAMEs). The yield is 0.29 g FAME per g dry biomass. This is considerably higher than the yield of 0.20 g FAME per g dry biomass obtained when the neutral lipid of *C. vulgaris* biomass was extracted and converted into FAME.

1. Introduction

In order to satisfy the world's energy demand for fuel and decrease the dependence on fossil fuels, research has been directed towards finding renewable, clean and environmentally-friendly alternative energy sources. Biofuel, especially biodiesel, is one such source that is receiving special attention. Oleaginous microalgae are being considered as potential feedstock for biodiesel production. Their rapid growth rate and high intracellular lipid content [1,2] make them a potential candidate for feedstock. *Chlorella* strains have been considered as promising candidates for commercial lipid production due to their fast growth and easy cultivation. In addition *Chlorella* strains are not contaminated by other strains of microalgae when cultivated in open ponds [3].

Although high biomass productivity, rapid lipid accumulation and ability to survive in saline water make microalgae a promising feedstock for industrial-scale biodiesel production. The high cost of producing microalgae biomass and conventional biodiesel production processes make biodiesel production from microalgae biomass uneconomical [4]. The conventional method used for biodiesel preparation from microalgae is to first extract lipids. The lipids are then converted into fatty acid alkyl esters. The extraction efficiency depends on factors such as microalgae species, method of cell wall disruption and solvent used for extraction [5–8]. Cell disruption prior to extraction can increase the amount of extractable oil. The most commonly used physicochemical techniques for microalgal cell disruption include grinding followed by ultrasonication, microwave treatment, autoclaving, bead-beating and sonication [9,10]. However, the oil extraction step is considered uneconomical. Attention is now being focused on direct or *in situ* production of biodiesel from microalgae biomass.

The conventional production of biodiesel uses refined oil (with free fatty acid (FFA) content less than 0.5%). This refined oil reacts with methanol and is catalyzed by alkali. The biodiesel production from microalgae uses alkali as a catalyst. This would not be suitable due to the high FFA content of microalgae lipids. The high FFA concentration leads to soap formation and difficulties in biodiesel purification [11].

Most studies on biodiesel production from microalgae were based on dry algal biomass. It was necessary to remove the water after harvesting biomass. Drying the biomass is energy intensive

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and efforts are currently devoted to develop process for producing biodiesel directly from wet algal biomass. A two-step process to produce biodiesel from wet biomass of *C. vulgaris* was proposed by Levine et al. [12]. In the first step, wet algal biomass (80% moisture) was hydrolyzed in subcritical water to release intracellular lipids. In the second step, the fatty acid-rich wet biomass was reacted with ethanol under supercritical condition to produce biodiesel. They reported a maximum fatty acid ethyl ester yield of 66% (w/w). During acid catalyzed *in situ* production of biodiesel from biomass, inhibition occurred when the biomass water content was greater than 115% [13].

One step *in situ* biodiesel production eliminates unnecessary and complex steps such as oil extraction. Velasquez-Orta et al. [14] reported that *in situ* alkali catalyzed transesterification of dry algal biomass can achieve high conversion (77.6%) in less time than that when using an acid catalyst. Xu and Mi [15] showed that addition of co-solvents such as mixture of toluene and methanol (2:1, v/v) resulted in the highest efficiency. They achieved a 86% yield from the *in situ* transesterification. It was found that in the *in situ* transesterification of the wet algae biomass, neutral lipids such as triacylglycerols (TAGs), free fatty acids (FFAs) and phospholipids all contributed to the formation of fatty acid methyl esters (FAMEs) [16].

It is still a challenge to minimize the cost in biodiesel production related to the use of acid, base and biological catalysts. The catalyst can be eliminated if production is done under supercritical conditions. The supercritical methanol method is simpler, more environmentally friendly and can reach high conversion (>95%) in a very short time. The presence of high contents of FFAs and water has no effect on the efficiency of this method [17–20]. In this reaction, a single homogenous phase is formed between methanol and oil. This accelerates the reaction by eliminating mass transfer resistance between phases. Pretreatment of feedstock is not required [21]. A high alcohol to oil molar ratio (usually >40:1), a high temperature (300–350 °C) and a high pressure (20–50 MPa) makes this process energy intensive [22,23].

Reaction involving subcritical water (SCW) is considered as environmentally friendly. SCW can be used for extraction, hydrolysis, and wet oxidation of organic compounds. SCW is water at temperatures between 100 and 374 °C under high pressure to maintain it in liquid state. The dielectric constant is the most important factor when using water as solvent for extraction. It decreases from 80 at room temperature to 27 at 250 °C [24–26]. In a previous work, it was reported that SCW pretreatment of the biomass of the oleaginous yeast *Yarrowia lipolytica* Po1 g can increase its extractable neutral lipids two folds [27]. SCW can also act as an effective catalyst for hydrolysis or biodegradation reactions and to increase the extractable neutral lipids from activated sludge [28,29]. Base catalyzed methanolysis of soybean oil under a subcritical condition of 160 °C was reported by Yin et al. [30]. A 98% yield of methyl esters can be obtained in 10 min. Without using a catalyst, only a 6% yield of methyl ester was obtained at 260 °C.

The present work was focused on investigating the *in situ* preparation of fatty acid methyl esters (FAMEs) from wet *C. vulgaris* biomass without the need of traditional acid or base catalyst. The effects of reaction time, amount of methanol and stirring on the FAME yield were systematically investigated.

2. Materials and methods

2.1. Apparatus and chemicals

Solvents and reagents used in the experiments are either gas chromatography (GC) or analytical reagent grade obtained from different suppliers. For GC analysis, all standards of fatty acids and FAMEs were purchased from Acros Organics (New Jersey, USA) and Sigma–Aldrich (St. Louis, MO 63103, USA), respectively. Qualitative filter paper (grade No. 2, 0.26 mm thickness, 80% collection efficiency and grade No. 5C) was obtained from Advantec (Tokyo, Japan). A 37 components FAME mixture was supplied by Sigma–Aldrich (Bellefonte, USA). GC-2010 gas chromatograph equipped with a flame ionization detector (Shimadzu, Japan) and a polar column Rtx-2330 composed of 10% cyanopropylphenyl–90% biscyanopropyl polysiloxane (30 m × 0.25 mm, Restek, Bellefonte, PA) were used for analyzing FAMEs in the reaction product. For analysis of lipid contents and fatty acid profile, a gas chromatograph (GC-17A, Shimadzu, Japan) with a flame ionization detector and a DB5-HT capillary column (30 m × 0.32 mm) were used. Magnetic stirring was provided by using a Corning PC 320 hot plate magnetic stirrer model PC-320 (Lowell, USA).

2.2. Experimental setup

All reactions were conducted in a stainless steel autoclave reactor. The reactor has a working volume of about 175 mL. The reactor is 2 cm thick and can withstand an estimated maximum operation pressure of 20 MPa. Temperature and pressure in the reactor was monitored by a thermocouple and a pressure gauge, respectively. Nitrogen gas (99.9% purity) was used to maintain pressure in the reactor required to keep water and methanol in liquid state. The experimental setup is shown in Fig. 1.

2.3. Production of biodiesel from C. vulgaris biomass

The objective of this study was to produce biodiesel from wet microalgae biomass. The biomass was cultivated according to Yeh et al. [31]. Typically, 5 g of wet *C. vulgaris* biomass (moisture content adjusted to 80% by adding 4 mL deionized water to 1 g dry biomass) and a pre-determined amount of methanol were put into the reactor. Temperature in the reactor was raised to 175 °C, with a corresponding vapor pressure of about 22 bar, based on results from our previous works [27,28]. After a pre-determined time, reaction was stopped by releasing vapor in the reactor to reduce the pressure to about 2 bar. The reactor was cooled to room temperature and its content was transferred to a separatory funnel. Hexane was then added and after shaking, the aqueous and organic phases were separated. After removing hexane in the organic phase, its composition was analyzed. Fig. 2 is the flowchart showing the producing of biodiesel from wet microalgae.

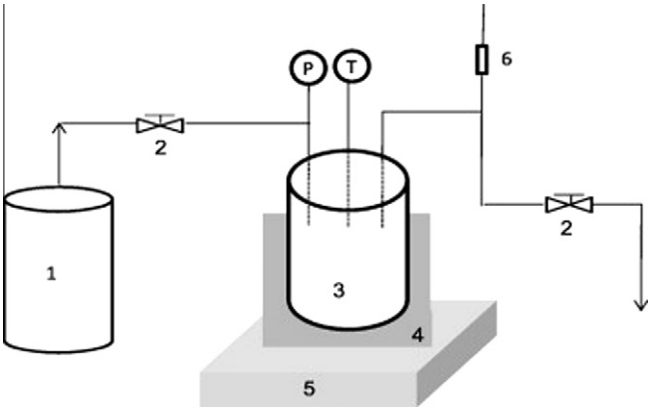

Fig. 1. Schematic diagram of reactor set-up. (1) Nitrogen cylinder. (2) Needle valve. (3) Reactor. (4) Electric heater. (5) Magnetic stirrer plate. (6) Safety valve. (*P*) Pressure gauge. (*T*) Thermocouple.

2.4. Analysis of reaction product

FFA and acylglycerols (AGs) contents of the reaction products were analyzed by using a GC-17A gas chromatograph (Shimadzu, Japan) with a flame ionization detector, as described elsewhere [32]. Separations were carried out on a DB5-HT capillary column (30 m × 0.32 mm; Agilent Technologies, USA). Temperatures of the injector and the detector were both set at 370 °C. Temperature of the column was started at 80 °C, then was increased to 365 °C at a rate of 15 °C/min and maintained at 365 °C for 10 min. The total run time was 29 min. The split ratio was 1:50 using nitrogen as the carrier gas with a linear velocity of 30 cm/s at 80 °C. A 20 mg sample was dissolved in 1 mL ethyl acetate, and 0.5 μL sample was taken and injected into the GC. To identify the types of fatty acids in the products, 20 mg of a standard of fatty acid was dissolved in 1 mL ethyl acetate and 0.5 μL was injected into the GC.

2.5. Determination of FAME profile

Chromatographic analysis of FAME profile in the product was performed using a GC-2010 gas chromatograph (Shimadzu, Japan) equipped with a flame ionization detector. The column used was Rtx-2330 10% cyanopropylphenyl–90% biscyanopropyl polysiloxane column 30 m × 0.25 mm i.d., (Restek, Bellefonte, PA). The operating conditions were as follows. The injector and detector temperatures were set at 250 °C. The column temperature was held at 150 °C for 2 min, and then raised to 250 °C at 5 °C/min and held for 8 min. Hydrogen flow, air flow and make up flow were set at 50.0 mL/min, 500.0 mL/min and 30 mL/min, respectively. The linear velocity and purge flow were 8.0 cm/s and 3.0 mL/min, respectively. Individual FAME was identified by comparing its retention time with the retention times of a 37-component FAME mix (Sigma–Aldrich, Bellefonte, USA). Total biodiesel yield was calculated by using the equation:

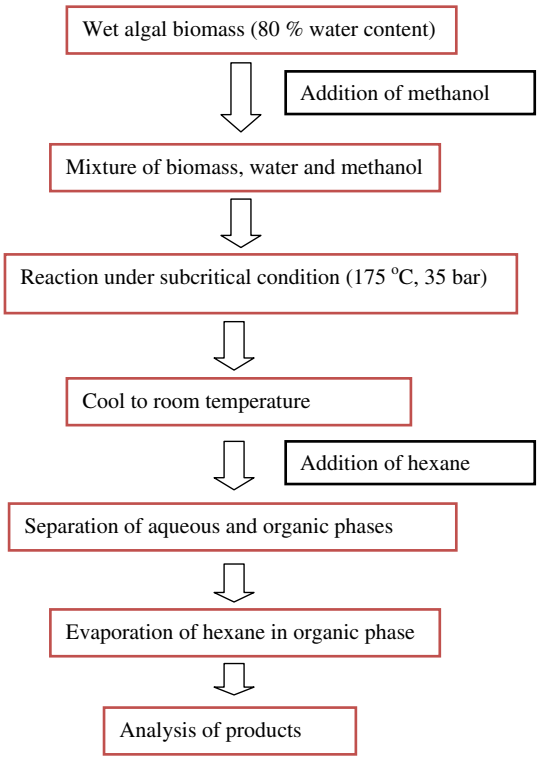

Fig. 2. Flow chart for the preparation of biodiesel from wet microalgae.

$$\text{Total FAMEs yield } (\%, \text{w/w}) = \frac{\text{Total weight of FAMEs(g)}}{\text{Dry biomass (g)}} \times 100\%$$

FAME conversion (%, peak area) was calculated based on peak area of the GC chromatogram of the reaction product.

$$\text{FAMEs conversion yield } (\%, \text{peak area}) = \frac{\text{Total peak area of FAMEs}}{\text{Total peak area of the products}} \times 100\%$$

3. Results and discussion

3.1. Effect of reaction time and stirring

Variables such as reaction time, type and amount of alcohol, catalysts, temperature and method of preparation play important roles in determining biodiesel yield from different feedstocks [13]. Tsigie et al. [27] showed that after SCW pretreatment, the extractable lipid of *Yarrowia lipolytica* biomass increased two times. It has been suggested that SCW hydrolysis of vegetable oils is predominately a homogeneous reaction in the oil phase, consisting of three reversible stepwise reactions that convert triacylglycerol (TAG) into diacylglycerol (DAG), monoacylglycerol (MAG), and finally glycerol [33].

To study the effect of reaction time and stirring on FAMEs yield, the reaction mixture was allowed to react for a predetermined time (0, 2, 4, 6 or 8 h) with or without stirring. The results are shown in Fig. 3. A general trend was observed. An increase in reaction time has a positive effect on the amount of FAMEs that can be produced from the microalge biomass.

According to Ehimen et al. [13], during *in situ* acid catalyzed transesterification of *Chlorella* biomass, the FAME yield increased from 70% to 92% when reaction time was increased from 15 min to 1 h. These results are better than the results of this study. The maximum FAME yields were obtained at 4 h (88.65%, with stirring) and at 8 h (89.12%, without stirring). An acid catalyzed *in situ* FAMEs synthesis from *Chaetoceros gracilis* biomass indicated that increasing time from 25 min to 2.5 h using 2.5 mL methanol per 100 mg biomass, increased the yield of FAME from 7.4% to 22.6% [16]. A longer time (⩾4 h) is necessary for high FAME yield during *in situ*, uncatalyzed FAMEs synthesis from wet *C. vulgaris*. A longer time is necessary to break down cell wall, expose lipids in the cells

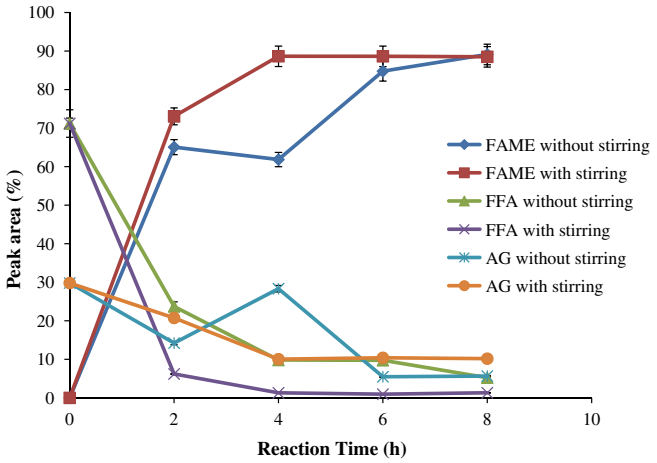

Fig. 3. Effect of reaction time and stirring on product composition. Reaction conditions: 5 g wet microalgae (80% moisture content), 30 mL methanol, 175 °C and 22 bar. Data are average of at least two independent experiments.

and react lipid with methanol under subcritical condition without using acid catalyst.

Stirring significantly increases the rate of biodiesel formation in the acid-catalyzed *in situ* production of biodiesel from microalgae lipids [13]. In our study, the effect of stirring on the *in situ* process was investigated and the results are shown in Fig. 3. During the first 2 h of reaction, no significant difference was observed in the FAME yields between reaction with and without stirring. The maximum achievable FAME yield at 2 h was 69.07% without stirring and 71.05% with stirring. A maximum FAME yield of 88.65% can be obtained in 4 h with stirring. It took 8 h to reach a maximum FAME content of 89.12% without stirring. The effect of stirring on FAME content is most pronounced at 4 h. At 6 h and 8 h, the differences in FAME contents between reactions with stirring and without stirring are insignificant.

The purpose of stirring was to prevent clumping and ensure that biomass was adequately exposed to methanol. When the *in situ* transesterification was conducted without stirring, the conversion of microalgae oil to FAME was significantly lower than that with stirring. This phenomenon was also observed in the *in situ* acid catalyzed transesterification of microalgal biomass [13]. Ma et al. [39] reported that stirring had a significant effect on the transesterification of beef tallow and methanol.

A catalyst free, two-step biodiesel production from wet *C. vulgaris* was developed by Levine et al. [15]. Without stirring the reaction mixture, they showed that hydrolysis of wet algal biomass by SCW at 250 °C followed by supercritical *in situ* transesterification with ethanol at 275 °C for 2 h produces crude biodiesel of which 79% (w/w) is fatty acid ethyl esters [15]. Our work suggested a maximum FAME yield (88.65%) through a simple one step SCW assisted reaction at 175 °C with continuous stirring. Better results can be achieved with *in situ* transesterification of wet microalgae biomass with stirring at considerably lower temperature.

3.2. Effect of methanol amount

During *in situ* transesterification, alcohol acts both as solvent for extracting lipids from biomass and as the reactant for converting lipids to fatty acid esters [16]. In transesterification of lipids and alcohol, alcohol amounts higher than the theoretical amount was used to favor the formation of fatty acid esters [34,35]. The effect of methanol amount on FAME content in the *in situ* methanolysis of wet *C. vulgaris* biomass is shown in Fig. 4.

Fig. 4 shows that as the amount of methanol (mL) per gram of wet microalgae was raised from 2 to 4, the FAME content in the product increased from 79.45% to 89.71%. Further increase in methanol amount (6 or 8 mL) caused a slight decrease in FAME content. It has been suggested that the presence of excess methanol in transesterification process is essential since it is responsible for breaking the glycerin–fatty acid linkages [36]. A study on biodiesel preparation from *Chlorella protothecoides* showed that excess methanol in large quantities reduced the amount of products and slowed down the separation of glycerol and FAME [6]. The conversion of crude microalgal oil to biodiesel increased with increasing methanol to oil ratio. This reached a maximum and started to decrease when methanol to oil ratio was further increased [8]. A similar trend was observed in this study (Fig. 4).

According to Yin et al. [30], the advantage of using excess MeOH is that the reaction can be carried out in one phase because oil becomes soluble in subcritical methanol. Both yield of FAME and reaction rate are enhanced due to homogeneous mixture of reactants as well as higher concentration of methanol which favors FAME formation. We found that a wet microalgae biomass to methanol ratio of 1:4 (w/w) was necessary to achieve maximum FAME yield because the presence of excess methanol is conducive to extracting microalgae oil and transforming the oil into FAMEs.

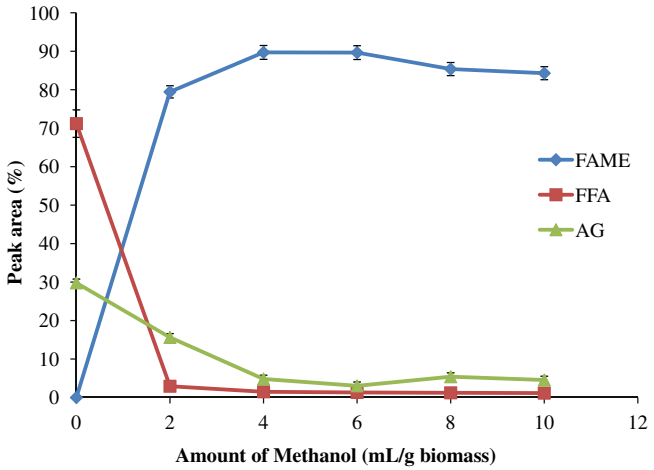

Fig. 4. Effect of methanol amount on product composition. Reaction conditions: 5 g wet microalgae (80% moisture content), 175 °C and 22 bar, reaction time 4 h.

The water amount was fixed at 4 g water per g dry microalgae to simulate the moisture content of wet algae after harvesting. In our previous study on producing FAMEs from soybean oil under subcritical condition, it was found that water amount did have significant effect on the yield of FAMEs [40].

3.3. Composition of FAMEs

FAME profile of the product was analyzed by using gas chromatography and the result is shown in Table 1.

Palmitoleic acid methyl ester (C16:1) is the most abundant FAME present in the biodiesel produced from the *in situ* methanolysis of *C. vulgaris* under subcritical condition. Other FAMEs present in substantial amounts (~15%) are palmitic acid methyl ester (C16:0), linolenic acid methyl ester (C18:3n3), and linoleic acid methyl ester (C18:2n6c). The fatty acids composition of lipids from *C. vulgaris* was studied by Petkov and Garcia [37] and their results are similar to ours. Small quantities of methyl esters from fatty acids with less than 15 carbons and more than 19 carbons were also observed in this study, and was confirmed by a previous study [38]. The presence of substantial amounts of linolenic acid methyl ester (15.19%), and linoleic acid methyl ester (13.84%) will have negative effect on the oxidative stability of the biodiesel produced. Modifying fatty acid profile of *C. vulgaris* through genetic

Table 1
Composition of FAMEs produced from wet biomass of *Chlorella vulgaris* at 175 °C and 22 bar. Each value is the average of two independent experiments.

Type of FAME	Amount (peak area (%))
Capric acid methyl ester (C10:0)	0.85
Lauric acid methyl ester (C12:0)	3.32
Tridecanoic acid methyl ester (C13:0)	1.41
Myristic acid methyl ester (C14:0)	6.5
cis-10-Pentadecenoic acid methyl ester (C15:1)	3.17
Palmitic acid methyl ester (C16:0)	15.66
Palmitoleic acid methyl ester (C16:1)	27.73
cis-10-Heptadecenoic acid methyl ester (C17:1)	3.86
Stearic acid methyl ester (C18:0)	0.21
Elaidic acid methyl ester (C18:1n9t)	6.08
Oleic acid methyl ester (C18:1n9c)	1.32
Linoleic acid methyl ester (C18:2n6c)	13.84
cis-11-Eicosenoic acid methyl ester (C20:1n9)	0.33
Linolenic acid methyl ester (C18:3n3)	15.19
cis-5,8,11,14,17-Eicosapentaenoic acid methyl ester (C20:5n3)	0.32
Nervonic acid methyl ester (C24:1n9)	0.21

manipulation and/or optimizing of culturing conditions on this species is required if it is to be considered as a potential candidate as feedstock for biodiesel production.

4. Conclusion

A catalyst free method to produce biodiesel directly from wet biomass of *C. vulgaris* under subcritical condition was studied. Stirring has positive effect on the reaction by shortening reaction time required to achieve high conversion. The maximum biodiesel yield from *C. vulgaris* using this method was 0.29 g/g dry biomass. This was obtained under the following conditions: 5 g wet biomass (80% moisture content), 20 mL methanol, 175 °C, with stirring for 4 h. This compares favorably with the theoretically yield of 0.20 g FAME/g dry biomass which can be obtained by firstly extracting all neutral lipids from dry biomass of *C. vulgaris* and then transform all neutral lipids into FAMEs.

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