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Biodiesel production from algae oil high in free fatty acids by two-step catalytic conversion

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ABSTRACT

The effect of storage temperature and time on lipid composition of *Scenedesmus* sp. was studied. When stored at 4 °C or higher, the free fatty acid content in the wet biomass increased from a trace to 62.0% by day 4. Using two-step catalytic conversion, algae oil with a high free fatty acid content was converted to biodiesel by pre-esterification and transesterification. The conversion rate of triacylglycerols reached 100% under the methanol to oil molar ratio of 12:1 during catalysis with 2% potassium hydroxide at 65 °C for 30 min. This process was scaled up to produce biodiesel from *Scenedesmus* sp. and *Nannochloropsis* sp. oil. The crude biodiesel was purified using bleaching earth. Except for moisture content, the biodiesel conformed to Chinese National Standards.

1. Introduction

Biodiesel is composed of fatty acid alkyl esters produced from triacylglycerols (TAG), diacylglycerols (DAG), free fatty acids (FFA) and phospholipids (PL), traditionally derived from vegetable oils or animal fats (Leung et al., 2010; Vyas et al., 2010). Compared to conventional diesel, biodiesel generally contains a higher level of oxygen and lower levels of sulfur and nitrogen and therefore, less SO_x, NO_x, CO, benzene and toluene are released upon combustion (Tica et al., 2010). A major bottleneck limiting the development of the biodiesel industry is supply and price of feedstocks (Greenwell et al., 2010; Naik et al., 2010). A promising source of biodiesel is microalgae, which can grow in fresh water or marine environments, without using arable land and competing with food production (Singh et al., 2011a). Some microalgae have high biomass and oil productivity (Hu et al., 2008; Williams and Laurens, 2010).

Biodiesel production from algae is generally done by one of three methods. The first is a two-step protocol in which algae oil is extracted with organic solvent and then converted to biodiesel using a catalyst, such as an acid (Krohn et al., 2011; Nagle and Lemke, 1990), a base (Umdu et al., 2009; Vijayaraghavan and Hemanathan, 2009), or an enzyme (Li et al., 2007). The second method directly produces biodiesel from algae biomass using an acid catalyst at atmospheric pressure and ambient temperature (Ehimen et al., 2010; Johnson and Wen, 2009; Wahlen et al., 2011). The third method is one-step conversion to biodiesel at high pressure and high temperature in the absence of a catalyst (Huang et al., 2011; Patil et al., 2011). Each method has innate advantages and disadvantages. Method 2 requires high concentrations of sulfuric acid since moisture in the biomass is a limiting factor for conversion efficiency (Ehimen et al., 2010; Johnson and Wen, 2009). In contrast, moisture can be ignored under the subcritical or supercritical conditions of method 3 (Patil et al., 2011); however, side reactions happen at subcritical or supercritical conditions that produce organic acids and heterocyclic nitrogen compounds from the degradation of proteins and carbohydrates (Huang et al., 2011). These contaminants lower the quality of biodiesel or interfere with the purification process.

From an economics and energy cost point-of-view, oil extraction directly from wet algal slurry is thought to be preferable (Xu et al., 2011), but issues regarding stability of the oils in harvested wet algae still have to be addressed. Cellular lipids in wet algae biomass may be enzymatically degraded by internal enzymes (Singh et al., 2011b). During long-term storage, cellular lipids can be degraded to volatile organic acids (Foree and Mccarty, 1970) or free fatty acid (Alencar et al., 2010). Krohn et al. (2011) reported that free fatty acid in oil extracted from algae biomass can reach as high as 84% (oil weight). Such high levels of FFAs are unlikely to have been present in the algae during growth since they would have had a cytotoxic effect on the cells (Wu et al., 2006). In the current study, changes in FFA and TAG in wet algae biomass stored under various conditions were investigated. Algae oil from the

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fresh water species *Scenedesmus* sp., the marine species *Nannochloropsis* sp. and a heterotrophic *Dinoflagellate*, containing different free fatty acid levels were converted to biodiesel using a two-step process under optimum conditions. The biodiesel yield and fuel properties were analyzed.

2. Methods

2.1. Algae cultivation and harvesting

Scenedesmus sp. was obtained from Dr. Hu Qiang at Arizona State University. It was grown in a 500-L panel bioreactor, with modified BG11 medium containing 0.375 g L^{-1} of sodium nitrate. Cultures were grown under 200 μmol m^{-2} s^{-1} of artificial light and bubbled with compressed air (1% CO_2) for 14 days. *Nannochloropsis* sp., isolated from the coast of Qingdao, China, was cultivated under the same conditions as the *Scenedesmus* sp., but in modified f/2 medium containing 0.375 g L^{-1} of sodium nitrate for 10 days. The cultures were concentrated to around 100 g L^{-1} using a hollow fiber PVDF microfiltration membrane (Motimo Co., Tianjin, China), followed by centrifugation at 2632*g* for 5 min. This process resulted in algae pastes with about 75% moisture. *Dinoflagellate* oil was purchased from Hubei Fuxing Biotechnology Co., Ltd., China, and was a hexane extract of a heterotrophic culture.

2.2. Storage of algae paste

The harvested algae pastes of *Scenedesmus* sp. were divided into two parts. One part was stored at −80 °C for long-term storage. The other part was divided into several smaller portions and stored at various temperatures (−80, −20, 4, 20 and 37 °C). After 24, 48 and 72 h, the samples were lyophilized at −80 °C and their lipid content was analyzed.

2.3. Gravimetric analysis of lipid content

The total lipid content was determined by gravimetric analysis using a modified Bligh & Dyer's method (Bligh and Dyer, 1959). Approximately 100 mg of dried algae powder was mixed with 5 ml chloroform/methanol (1:2, v/v) at 65 °C for 1 h. The mixture was then centrifuged at 948*g* for 5 min. The supernatant was collected and residual biomass was extracted twice more. The supernatants were combined, and chloroform and 1% sodium chloride solution were added to a final volume ratio of 1:1:0.9 (chloroform/methanol/water). The solution was allowed to settle, and carefully transferred to a vial and dried to constant weight at 60 °C under nitrogen flow. The total lipid content was calculated as a percentage of the dry weight of the algae.

2.4. Lipid composition analysis

Lipid components were analyzed using a thin-layer chromatography (TLC) system (TLC-FID, MK-6, Iatron Laboratories, Inc., Japan) (Fedosov et al., 2011). Samples were dissolved in chloroform to a concentration of 5 mg ml^{-1}. They were then spotted onto Chromarod S-III silica coated quartz rods held in a frame. The rods were developed in a solvent system of benzene:chloroform:methanol (150:60:2, v/v/v) for the first migration to 7 cm, followed by a solution of benzene:hexane (50:50, v/v) for the second migration to 10 cm. The rods were oven-dried at 70 °C for 1 min before they were scanned in the Iatroscan analyzer, which was operated at a flow rate of 160 ml min^{-1} for hydrogen and 2 L min^{-1} for air. The individual lipid components were identified by co-chromatography with pure standards (sterol ester, SE; fatty acid methyl ester, FAME; FFA; TAG; DAG; monoacylglycerol, MAG; PL, purchased from Sigma, St. Louis, MO, USA). The quantities of individual components were estimated from the peak areas of pure standards.

To determine the acid value, samples were dissolved in a mixture of anhydrous ethanol and diethyl ether (1:1), and titrated with 0.1 mol L^{-1} KOH, according to Chinese National Standard (GB/T 264-1983). Results are expressed as mg KOH per gram oil.

2.5. Extraction of oil from wet algae paste

The method described by Chen et al. (2011) was followed for extractions of oil from wet algae paste. Briefly, algae paste (75.4% of moisture) was thawed at room temperature, and mixed with ethanol before it was loaded into the chamber of a high pressure extractor. Nitrogen gas was driven into the chamber to maintain a pressure of 1.5 MPa. The temperature of the extractor was maintained at 120 °C for 50 min. Samples were cooled to room temperature before the pressure was decreased. The extraction mixture was centrifuged at 2632*g* for 5 min to separate the oil solution and residual algae. Finally, the solvent was evaporated using a rotary evaporator to recover algae oil.

2.6. Biodiesel production from algae oil

Dinoflagellate oil was degummed by stirring with 1% phosphoric acid and 10% water at 85 °C for 1 h to remove most of phospholipids and non-lipid impurities. A mixture of various ratios of *Dinoflagellate* oil and FFAs, which were produced by hydrolysis of *Dinoflagellate* oil in our laboratory, was prepared to achieve oil samples with FFA concentrations of 8.5%, 23% and 96%.

The degummed oil or mixtures of degummed oil and FFA were first pretreated with acid catalyst to lower FFA level. A 5-g sample was mixed with 2.0 ml methanol containing 3.3% (g/100 mL) sulfuric acid. The mixtures were stirred at 65 °C for 120–180 min. For the sample with highest FFA level, 2.0 ml methanol was added to the mixture and the esterification was repeated. The acid value of each oil sample was determined every 15 min. The treated oils (5 g) were mixed with 2 ml of methanol containing one of following catalysts (potassium hydroxide, KOH; sodium hydroxide, NaOH; or potassium methoxide, $KOCH_3$) (10%, g/100 mL) at 65 °C for 60 min under continuous stirring at 100 revolutions/min for 1 h. Samples, collected at 10 min intervals, were washed with deionized water to remove un-reacted methanol and catalysts. Relative content of TAG and FAME were determined.

Once the optimum conversion conditions were determined, 100 g degummed algae oil from *Scenedesmus* sp. and *Nannochloropsis* sp. were used to prepare crude biodiesel.

2.7. Purification of crude algae biodiesel

At 80 °C and under vacuum, 10 g of bleaching earth (Sinopharm Chemical Reagent Co., Ltd., China) was added to 50 g crude algae biodiesel for 1 h in rotary evaporator to remove pigments and other impurities. The bleaching earth was separated using centrifugation at 3790*g* for 10 min. Both the purified biodiesel and crude biodiesel were diluted with heptane to a concentration of 2.74 g L^{-1}. The diluted sample was scanned from 400 to 800 nm using a Varian 50 Bio UV–Visible spectrophotometer (Varion Inc., US). The chlorophyll and total carotenoids content was calculated as described by Wellburn (1994).

$$\begin{aligned}
&\text{Chlorophyll } a\ (C_a)(\mu g\ mL^{-1}) = 10.05(OD_{662}) - 0.77(OD_{644})\\
&\text{Chlorophyll } b\ (C_b)(\mu g\ mL^{-1}) = 16.37(OD_{644}) - 3.14(OD_{662})\\
&\text{Total carotenoids}(C_{x+c})(\mu g\ mL^{-1}) = (1000OD_{470} - 1.28C_a - 56.7C_b)/205
\end{aligned} \quad (1)$$

2.8. Fatty acid analysis

A 0.5-mg sample of purified biodiesel was dissolved in 1 ml heptane containing 50 μg heptadecanoic acid methyl ester ($C_{18}H_{37}COOCH_3$) as internal standard for FAME analysis on a Varian 450GC (Varian Inc., USA) equipped with a flame ionization detector (FID) and Agilent HP-5 GC Capillary Column (30 m × 0.25 mm × 0.25 μm). Nitrogen was used as carrier gas. The injector temperature was set at 280 °C with an injection volume of 2 μL under split mode (10:1). The detector temperature was set at 280 °C. The individual FAMEs were identified by chromatographic comparison with authentic standards (Sigma). The quantities of individual FAMEs were calculated using Eq. (2), where W is the relative content of each fatty acid, presented in a percentage of total fatty acid; m_s is the mass of internal standard, f_i is the coefficient value of section i, A_i is the peak area of section i, m is the weight of sample and A_s is the area of standard.

$$W = \frac{m_s \times f_i \times A_i}{m \times A_s} \times 100\% \quad (2)$$

2.9. Characterization of algae biodiesel

Density, kinematic viscosity, oxidative stability, moisture content, sulfur content, sulfated ash, free glycerol and distillation temperature (atmospheric equivalent temperature, 90% recovered) were tested according to the procedures recommended by Chinese National Standard GB/T 20828-2007. Phosphorus content was determined according to ASTM D4951, and the gross heating value was determined using an IKA Calorimeters C2000 (Germany) according to Chinese National Standard GB/T 384-81. The hydrogenation of FAME was carried out using Pd/C as catalyst and hydrogen as hydrogen donor. Briefly, 0.1 g of Pd/C (5 wt.%, Aladdin Chemistry Co., Ltd., China) was dispersed in 25 ml absolute ethanol under 0.2 MPa of hydrogen and a 20 ml min^{-1} outflow. Thereafter a solution of 5 g FAME and 25 ml absolute ethanol was fed inside the reactor, kept at 45 °C for 2 h and cooled down. Finally, the catalyst was separated using a filter (0.45 μm) and the solvent was evaporated using a rotary evaporator to recover biodiesel for analysis.

2.10. Statistical analysis

Statistical analysis was carried out using SPSS 11.0 software (SPSS Inc., Chicago, USA). ANOVA was performed to evaluate significance of individual differences with a probability threshold of 0.05, followed by a Post-Hoc Tukey test.

3. Results and discussion

3.1. Effect of storage condition on the composition of algae oil

The changes in the lipid composition of wet algae pastes over a 1-day period are listed in Table 1. The lipid contents were 30–36% of dry weight biomass regardless of storage temperature. The differences among lipid contents was not significant ($p > 0.05$); however, the composition of the lipids changed under different storage temperatures. The TAG content (% of oil weight) decreased significantly ($p < 0.01$) from 72.1% to 3.3% when the storage temperature was 37 °C instead of −80 °C; meanwhile, the FFA content increased significantly ($p < 0.01$) from a negligible level to 70.3% with increasing storage temperatures, except that the difference between −80 and −20 °C was not statistically significant. The DAG content also increased slightly when the storage temperature increased from −80 °C to 4 °C. The content of SE and PL remained nearly constant.

Changes in lipid composition of samples stored at 4 °C for 24, 48 and 72 h are listed in Table 2. Generally, the longer the biomass was stored, the more FFA were detected. Conversely, TAG decreased in samples stored for longer periods of time, but the relative content of total lipid and polar lipid did not vary.

As shown in Tables 1 and 2, a decrease in TAG was accompanied by a proportional increase in FFAs, which was confirmed by the fact that the net content of TAG and FFA remained constant. Moreover, the increase in DAG content can be offered as further proof for the break-down of TAG, as DAG is a product of TAG degradation. Thus, it is reasonable to assume that the FFA was produced by hydrolysis from TAG.

The lipid components in algae cells (TAG, DAG, phospholipids, etc.) can be degraded to FFAs by lipases, peroxidases and phospholipases present in algae cells or produced by microorganisms present in the algae paste (Singh et al., 2011b). Enzymatic hydrolysis increases with time and temperature up to about 40 °C (Alencar et al., 2010).

From a process point-of-view, alkaline catalysis is more efficient and less expensive than an acid catalyzed process (Leung et al., 2010); however, the alkali-catalyzed process can be inhibited by a level of FFA greater than 3%, due to the consumption of alkali catalyst by FFAs (Vyas et al., 2010). Unfortunately, the content of FFAs of algae oil can reach as high as 70.3%, which is much higher than the allowable limits (Leung et al., 2010).

3.2. Optimization of algae biodiesel production from high FFA content algae oil

3.2.1. Acid esterification

During incubation, the acid value of each oil sample decreased (Fig. 1). After 120 min, the FFA content of samples with initial FFA concentrations of 8.5%, 23% and 96% leveled off at 0.73%, 2.35% and 5.7%, respectively. After increasing the reaction time to 3 h, the FFA content of the sample with the highest initial FFA content (96%) only decreased to 4.35%, which is still higher than the threshold content (2.5%) for further alkali-catalyzed transesterification (Leung et al., 2010). However, when the 96% FFA oil was treated with an additional acid esterification, the FFA content decreased to 0.415%, a level within the requirements for alkali transesterification. Thereby, all three oil samples with widely varying FFA

Table 1
Chemical composition of lipid extracted from algae feedstock stored in different temperature.

Storage temperature (°C)	Lipid class content (% oil)					Total lipid (% biomass)	Acid value (mg KOH/g)
	SE & HC	TAG	FFA	DAG	PL		
−80	1.7	72.1	ND	1.7	24.3	33.4	9.1
−20	1.8	68.4	ND	2.0	28.1	30.6	8.2
4	2.3	40.5	26.9	6.3	24.2	35.7	66.3
25	2.5	4.4	70.3	2.8	19.5	36.6	117.1
37	3.1	3.3	66.8	4.3	22.6	35.4	115.8

ND: not detected.

Table 2
Chemical composition of lipid extracted from algae feedstock stored at 4 °C for different time.

Storage time (day)	Lipid class content (% oil)					Total lipid (% biomass)	Acid value (mg KOH/g)
	SE & HC	TAG	FFA	DAG	PL		
0	1.7	72.2	ND	1.7	24.4	33.4	9.1
1	2.3	51.7	19.2	6.1	20.6	35.5	63.1
2	2.5	27.2	43.8	4.2	22.3	35.4	95.3
3	2.6	19.8	55.6	2.3	19.8	36.0	108.3
4	2.5	11.2	62.0	2.4	22.0	35.6	116.9

ND: not detected.

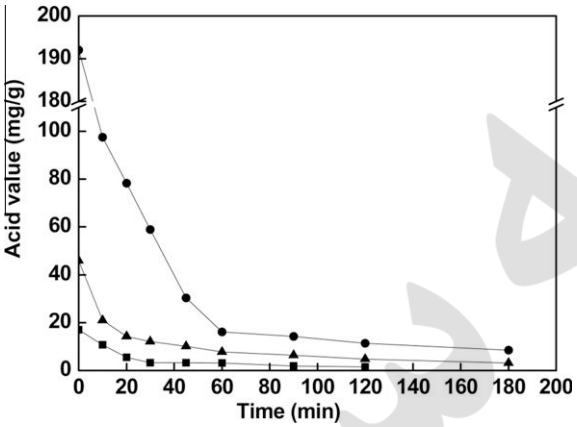

Fig. 1. Acid values of algae oil with different initial FFA content following pre-esterification. Levels of FFA tested were ●, 192 mg/g; ▲, 46 mg/g; ■, 17 mg/g.

contents were successfully pretreated to be suitable for the alkali-catalyzed process.

3.2.2. Alkali transesterification

The three catalysts efficiently catalyzed TAG conversion to FAME (Fig. 2). The relative contents of FAME catalyzed with NaOH, KOH and $KOCH_3$ increased to 88.3%, 91.6% and 87.6%, respectively. This increase in FAME was accompanied by a decrease in TAG contents of 6.1%, 3.3% and 4.2%. KOH performed better than the other catalysts, and was chosen for subsequent experiments.

After the first 5 min of transesterification, the relative contents of FAME ranged from 66.6% to 87.6% when the catalyst amount increased from 0.6% to 2.0% (Fig. 3). The highest FAME content was 91.7% using 2.0% KOH (oil weight). Since prior studies reported that high levels of base catalyst (beyond 2%) resulted in serious saponification of the reaction mixture and loss of TAG, which interferes in the downstream separation process (Veljkovic et al., 2006), 2% of catalyst additive was reasonable.

To identify the optimal molar ratio of methanol to oil, three levels of methanol/oil were tested at 65 °C and catalyzed by 2% KOH (basis of oil weight). As shown in Fig. 4, after a reaction time of 20 min, the relative contents of FAME were 85.7%, 92.6% and 96.6% on the basis of crude biodiesel weight, for methanol/oil ratios of 6:1, 9:1 and 12:1, respectively. The difference among the FAME contents was significant ($p < 0.05$), which indicated a positive correlation between methanol amount and oil conversion rate. No TAG was detected at each of the methanol/oil ratios, indicating an almost 100% conversion rate.

Therefore, the optimum conditions for the conversion of algae oil extracted from the *Dinoflagellate* were, a methanol dosage of 30%, a sulfuric acid concentration of 1%, and a reaction time of 2 h. The acid value of oil was reduced from the initial acid value of 17–46 mg KOH/g to below 2 mg KOH/g. The molar ratio of alcohol to oil was 12:1 during catalysis with 2% KOH at 65 °C for 30 min.

3.3. Application of two-step conversion of algae oil from three sources

Once the optimum conversion conditions were determined, algae oil from *Scenedesmus* sp. and *Nannochloropsis* sp. were tested. *Dinoflagellate* oil had the highest TAG content of 82.9% (Table 3). The other two oil feedstocks had a lower TAG content, ranging from 42.1% to 70.0%, but contained more polar lipids.

The FAME yield achieved with *Dinoflagellate* oil was the highest, at 90.1%, and this value is only slightly lower than the theoretical yield. The FAME yields for *Scenedesmus* sp. and *Nannochloropsis* sp. were 78.3% and 70.4%, respectively. Both TAGs and FFAs were

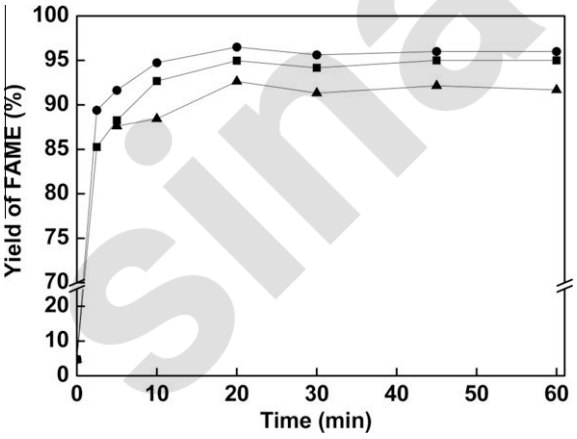

Fig. 2. Transesterification of pre-esterificated algae oil using different catalyst. Catalysts tested were ●, KOH; ▲, $KOCH_3$; ■, NaOH.

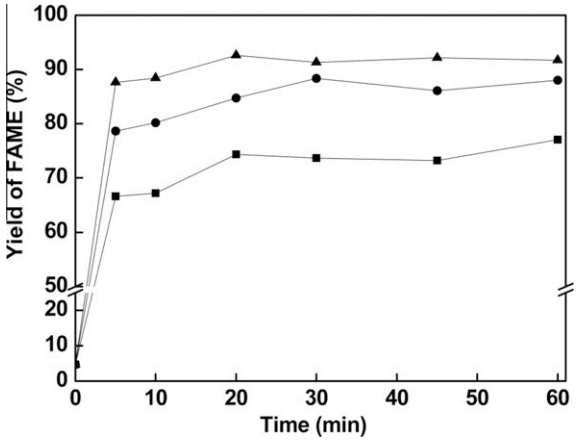

Fig. 3. Transesterification of pre-esterified algae oil using different amounts of KOH. Catalysts dosage tested were ■, 0.6%; ●, 1.2%; ▲, 2.0%.

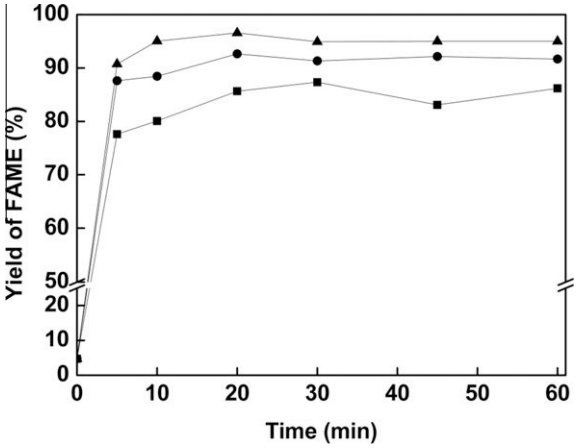

Fig. 4. Effect of molar ratio of methanol/oil on pre-esterificated algae oil. Ratios were ■, 6:1; ●, 9:1; ▲, 12:1.

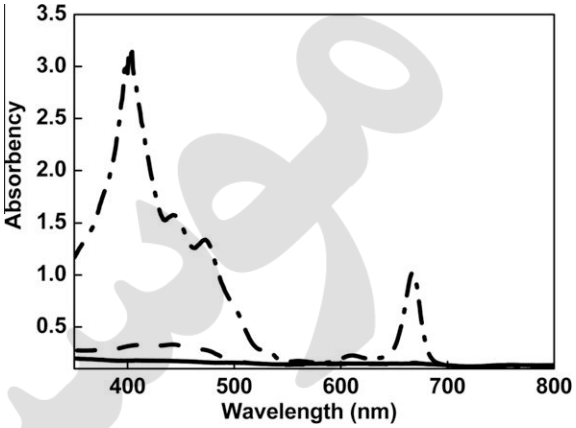

Fig. 5. Absorbance of crude biodiesel from *Scenedesmus* sp. oil before and after treatment with 20% (oil weight) of bleaching earth at 80 °C for 1 h. _, heptane; _._, crude biodiesel; _ _ _, refined biodiesel.

not detected in crude biodiesel, and the biodiesel content was nearly equals to or slightly higher than the sum of TAGs and FFAs.

Besides of FFAs and TAGs, phospholipids were also present in algae oil (Table 3). The phospholipids could be converted to FAME under appropriate conditions; however, the incomplete reaction of phospholipids can result in loss of the product by as much as 45% due to precipitation and saponification (Balasubramanian and Obbard, 2011). In the present study, a gel-like precipitation was observed between the FAME and water phases. The precipitate was collected, dissolved using chloroform and analyzed using TLC-FID. The result revealed that lysophosphatidylcholine (LPC) and other phospholipids (PL) were present in the precipitation (data not shown). Balasubramanian and Obbard (2011) reported that some LPC can be found in the crude biodiesel resulting in a high level of residual phosphorus. The phosphorus concentrations in crude biodiesel from *Scenedesmus* sp. was as high as 295.6 ppm (data not shown), which is higher than the ASTM limit of 10 ppm. This observation is in agreement with that of Krohn et al. (2011), who concluded that only FFA and TAG can be converted to pure ASTM grade biodiesel.

3.4. Purification of crude biodiesel

The crude biodiesel contained impurities, such as chlorophyll, soap and phospholipids which diminish the qualities of biodiesel (Balasubramanian and Obbard, 2011; Kulkarni et al., 2006). Using bleaching earth, these impurities were removed. The crude biodiesel had strong absorbance peaks at 667 and 470 nm, due to chlorophyll and carotene, respectively (Kulkarni et al., 2006). After treatment with bleaching earth, the peak at 667 nm disappeared and the peak at 470 nm significantly decreased (Fig. 5). The content of chlorophyll and total carotene decreased from 4296.7 and 1918.9 ppm to 40.3 and 199.0 ppm, respectively, which is a removal efficiency 99.1% for chlorophyll and 89.6% for total carotene. The refined biodiesel had a red or orange color. This could offer additional benefits in enhancing oil oxidative stability due to antioxygenation of carotene (Goulson and Warthesen, 1999). After the purification, the phosphorus concentrations in biodiesel from *Scenedesmus* sp. decreased to 2.4 ppm (Table 5), satisfying the ASTM limit.

3.5. Fatty acid analysis of algae biodiesel

The fatty acid composition of the biodiesels produced by three microalgae oils is represented in Table 4. Each source resulted in a distinct predominating fatty acid. *Scenedesmus* sp. oil mainly contained C18:1 (49.6%); *Dinoflagellate* oil C22:6 (44.98%), and *Nannochloropsis* sp. oil C16:1 (32.9%). Medium-chain fatty acids (⩽C18) comprised 96.6% and 91.6% of the biodiesel from *Scenedesmus* sp. and *Nannochloropsis* sp., respectively. The content of medium-chain fatty acids in *Dinoflagellate* biodiesel was lower than that of the biodiesels from the other algae. Table 4 also shows that mono- and polyunsaturated fatty acid were the dominant components, comprising 62.6–71.6% of the total fatty acids in the biodiesels from the three algae. Particularly, the biodiesel produced from the *Dinoflagellate* contained 66.14% polyunsaturated fatty acids, which was quite remarkable compared to the 22.82% and 8.57% of the *Scenedesmus* sp. and *Nannochloropsis* sp., respectively.

3.6. Characterization of biodiesel

Density, kinematic viscosity, acid value, sulfated ash, sulfur and phosphorous satisfied the criteria set by Chinese National

Table 3
Results of biodiesel preparation with different algal lipid oil.

Microalgae	Lipid component					Acid value	FAME yield	Predicted yield [a]
	SE	TAG	FFA	DAG	PL			
Scenedesmus sp. [b]	2.2	70.0	1.6	1.3	25.0	8.7	78.3	82.9
Scenedesmus sp. [c]	1.8	42.1	14.7	4.1	37.3	32.2	56.2	76.4
Nannochloropsis sp.	2.3	67.3	0.0	12.0	18.3	6.5	70.4	86.1
Dinoflagellate [d]	2.8	82.9	7.2	0.8	6.4	17.0	90.1	93.8

[a] Calculated from chemical reaction equation, FAME yield for 100 g of SE, TAG, FFA, DAG and PL at completely conversion are 0, 100, 105, 96 and 40 g.
[b] Cultured for 14 days.
[c] Cultured for 7 days.
[d] After refined by degumming.

Table 4
Fatty acids composition of biodiesels converted from three microalgae oil.

	Scenedesmus sp. FAME	*Nannochloropsis* sp. FAME	*Dinoflagellate* FAME
C14:0	ND	5.37	6.01
C16:0	18.42	28.83	16.65
C16:1	2.31	32.93	3.35
C16:2	3.26	ND	ND
C18:0	3.43	0.98	ND
C18:1	49.64	21.16	2.10
C18:2	11.30	2.24	ND
C18:3	8.26	ND	ND
C20:5	ND	6.33	2.89
C22:5	ND	ND	18.27
C22:6	ND	ND	44.98
C24:0	ND	ND	2.65
Others	3.38	2.14	3.10
Saturated	21.85	35.18	25.31
Mono-unsaturated	51.95	54.09	5.45
Poly-unsaturated	22.82	8.57	66.14

ND: not detected.

Table 5
Properties of algae biodiesel.

Items	*Scenedesmus* sp.	*Nannochloropsis* sp.	*Dinoflagellate*	Limitation	Test methods
Density at 15 °C (kg/L)	0.852	0.854	0.878	0.82–0.90	GB/T 2540
Acid value (mg KOH/g oil)	0.52	0.46	0.44	0.80	GB/T 264
Kinematic viscosity at 40 °C (mm^2/s)	4.15	5.76	3.74	1.9–6.0	GB/T 265
Oxidative stability at 110 °C (h)	5.42	1.93	1.02	>6	EN 14112
After hydrogenation	60.3	42.4	11.2		
Moisture content (%)	0.04	ND	0.07	0.05	SH/T 0246
Sulfur content (%)	0.02	0.06	0.04	<0.05	SH/T 0689
Sulfated ash (%)	ND	ND	0.01	<0.02	GB/T 2433-2001
Free glycerol (%)	ND	ND	ND	<0.02	SHT 0796-2007
Phosphorus content (ppm)	2.4	4.5	2.8	10.0	ASTM D4951
Methyl ester content (%)	91.0	92.2	96.6	>96.5	EN 14103
Distillation temperature (°C)	266	300	368	<360	GB/T 6536
Gross heating value (MJ/kg)	39.76	39.81	39.84	>35	GB/T 384-81

ND: not detected.

Standards (Table 5). The heating values of the biodiesel samples were 39.84, 39.76 and 39.81 MJ kg^{-1} for *Dinoflagellate*, *Scenedesmus* sp. and *Nannochloropsis* sp., respectively, which is comparable to that of fossil oil of 42 MJ kg^{-1}. An exception were the methyl ester contents of *Scenedesmus* sp. (91.0%) and *Nannochloropsis* sp. (92.2%), which were slightly lower than the required value of 96.5%. The *Dinoflagellate* biodiesel had a moisture content that was slightly higher than the limit of 0.7%. The oxidative stability of biodiesels from *Scenedesmus* sp., *Nannochloropsis* sp. and *Dinoflagellate* was 5.42, 1.93 and 1.02 h, respectively. This is much shorter than the required 6 h recommended by the Chinese National Standard. As expected, after the hydrogenation process catalyzed by Pd/C, the oxidative stability was significantly improved to 11.2–60.3 h (Table 5).

Knothe (2005) reported that the molecular characteristic of the fatty acids, such as length of carbon chain and degree of unsaturation, greatly affected the properties of biodiesel, such as oxidative stability and ignition quality. As shown in Tables 4 and 5, regardless of algae resource, a positive correlation between the relative content of long-chain fatty acids ($\geqslant$C20) (0, 6.3% and 68%, Table 4) and the distillation temperature (266 °C, 300 °C and 368 °C, Table 5) was observed. The distillation temperature was slightly higher than the limit of 365 °C and therefore, an additional process might be required to decrease the distillation temperature.

4. Conclusion

It was demonstrated that the lipid components change during storage of wet algae biomass. High amounts of free fatty acid are produced by the hydrolysis of TAG at temperatures above freezing. After optimized esterification–transesterification procedures, the conversion rate of TAG and FFA reached up to 100%. The resultant biodiesel satisfied most, but not all Chinese National Standards (GB/T 20828-2007). The proposed two-step catalytic conversion has thus shown good potential for production of biodiesel from algae oil high in free fatty acid.

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