



## Full Length Article

# A combined fermentation and ethanol-assisted liquefaction process to produce biofuel from *Nannochloropsis* sp.

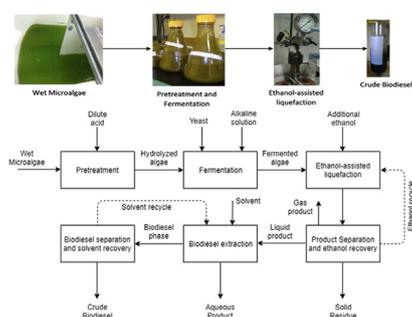
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## GRAPHICAL ABSTRACT



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## ABSTRACT

A combined pretreatment, fermentation and ethanol-assisted liquefaction process was studied to produce bio-fuels and chemicals from marine microalga *Nannochloropsis* sp. Wet (~80% moisture) and dry microalgal biomass were initially pretreated with dilute acid (3% H<sub>2</sub>SO<sub>4</sub>) and subsequently fermented with yeast *Saccharomyces cerevisiae*. By pretreatment and fermentation, about 10% of required ethanol in liquefaction was produced and the lipid content of fermented microalgal biomass was increased by 40%. Following fermentation, liquefaction assisted with 15% (v/v) ethanol (2:1 ethanol to algae ratio) at 265 °C converted fermented microalgae to crude biodiesel, aqueous products and solid residues. This combined algae to liquid process could increase the crude biodiesel yield by three-fold compared to liquefaction of microalgae. The main advantage of the process is the utilization of wet algae in essential ethanol production within the process to enhance the crude biodiesel production by ethanol-assisted liquefaction.

## 1. Introduction

Microalgae have been considered as one of the most promising feedstocks for biodiesel production due to their higher growth rate and high lipid/oil content [1]. Depending on the species and growth condition, microalgae possess a significant amount of carbohydrate, lipid and protein. A range of 20–50% lipid of dry cell weight has been

reported in the literature [2–4]. Different methods have been explored to produce biodiesel from microalgae [5]. The general approach involves extraction of algal oil from dried algae followed by transesterification of the oil to biodiesel using an alcohol in presence of a catalyst [4,6]. While the conventional extraction process is effective for analysis purpose, it is inefficient as it requires a dried feedstock which is energy intensive [7–9]. It has been reported that, in biodiesel production

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process from dried microalgae, 90% of the energy produced can be consumed in the drying and extraction processes [10,11]. To avoid the drying step, direct extraction of lipids from wet algal biomass was demonstrated through catalytic (acid/base) hydrolysis [12,13]. But this approach experiences the inhibition by free fatty acids (FFAs) and excess water due to catalyst deactivation and saponification [14–17].

Non-catalytic lipid hydrolysis and esterification were also achieved under supercritical or subcritical condition with or without using organic solvents [13,18,19]. Organic solvents can assist the thermal treatment of microalgae for multiple purposes, such as extraction of algal lipids, in situ transesterification of lipids as well as assisting liquefaction yield [20]. Increased bio-oil quality and biodiesel yield have been reported in the literature for organic solvent (examples-ethanol and methanol) assisted wet microalgae thermal treatment [6,11,21,22]. Because methanol is readily available at a low price, it has been widely used in supercritical transesterification [14,23]; however, the toxic properties of methanol and its production from petroleum-based resources restrict the development of byproducts from residual biomass as livestock feed for cattle and aqua culture [11,24]. The use of ethanol is advantageous as ethanol can be produced exclusively from carbohydrate rich renewable sources which can make the process more sustainable and renewable [11,25,26]. In fact, some microalgal species have higher carbohydrate contents with the absence of lignin which makes them excellent substrates for bioethanol production [27–30]. Therefore, making full use of lipid and carbohydrate in microalgae biomass for joint production of biodiesel and bioethanol has been described as an economic method for biofuel production from microalgae [31,32]. Wang et al. [32] reported joint production of biodiesel and bioethanol via acid hydrolysis of *Tribonema* sp. utilizing both lipid and carbohydrate. But the process involved dried algae hydrolysis, separation of supernatant, lipid extraction and catalytic transesterification which requires additional solvents, catalyst and energy. To eliminate the algae drying process, liquefaction is a promising technique as it uses wet biomass to produce liquid biocrude oil [24,33]. Other advantages of liquefaction of wet microalgae include conversion of lipid, protein and carbohydrate fractions into liquid bio-oil with or without catalyst [34]. As liquefaction can use wet microalgae, it can be incorporated in green biorefinery concept.

Green biorefinery represents an appropriate approach to utilize the fresh aquatic biomass, eliminating the drying process of conventional bioenergy-converting system [35]. Biorefinery concept with wet microalgae *Chlorella* and *Scenedesmus* for integrated lipid and carbohydrate-based biofuels production was demonstrated by Lauren et al. [36]. But the direct use of in-situ ethanol in the fermented hydrolysate through combined fermentation and liquefaction has not been explored so far. This study explores a biorefinery approach combining fermentation and ethanol assisted liquefaction to produce biofuels from marine microalga *Nannochloropsis* sp. The main concept of this study is to develop a combined algae to liquid process (combined ATL) for wet microalgae utilizing all three major biochemical components, i.e., carbohydrate, protein and lipid.

## 2. Methods

### 2.1. Microalgae biomass preparation

*Nannochloropsis* sp. (Item # 153220) was purchased from Carolina Biological (Burlington, North Carolina, USA) and initially cultured in Alga-Gro® Seawater medium (Item # 153751, Carolina Biological). To scale up the culture, F/2 medium (0.884 mM NaNO<sub>3</sub>, 0.362 mM NaH<sub>2</sub>PO<sub>4</sub>·H<sub>2</sub>O, 0.106 mM Na<sub>2</sub>CO<sub>3</sub>, 0.0117 mM FeCl<sub>3</sub>·6(H<sub>2</sub>O), 0.017 mM Na<sub>2</sub>(EDTA)<sub>2</sub>(H<sub>2</sub>O), 0.0393 μM CuSO<sub>4</sub>·5(H<sub>2</sub>O), 0.026 μM Na<sub>2</sub>MoO<sub>4</sub>·2(H<sub>2</sub>O), 0.0765 μM ZnSO<sub>4</sub>·7(H<sub>2</sub>O), 0.042 μM CoCl<sub>2</sub>·6(H<sub>2</sub>O), 0.91 μM MnCl<sub>2</sub>·4(H<sub>2</sub>O), 0.296 μM thiamine HCl, 0.00205 μM Biotin, 0.000369 nM Cyanocobalamin) was prepared in sea water (Item #163390, Carolina Biological). This microalga was grown in open

raceway ponds with a constant agitation and 24 h constant light intensity (400 μmol·m<sup>-2</sup>·s<sup>-1</sup>) in the greenhouse for three weeks. Microalgal biomass was harvested by using a centra-GP8R Centrifuge (Model 120, Thermo International Equipment Company (IEC), Tennessee, USA) at 2300 × G for 20 min. The liquid fraction was recycled, and the biomass was kept in a refrigerator at –20 °C for wet biomass experiments. The harvested wet alga was referred to as original wet biomass during experiments. A portion of harvested microalgae was dried at 60 °C for 3 days, ground by mortar and pestle and stored in the air tight container for analysis and experiments. This dried alga was referred to as original dry biomass during experiments.

### 2.2. Microalgae biomass characterization

Microalgal biomass were analyzed for the ash content, the solid content and carbohydrates using the laboratory analytical procedures developed by the National Renewable Energy Laboratory (NREL) [37,38]. The lipid content of microalgal biomass was also determined according to NREL's protocol, where the total lipids are expressed as fatty acid methyl esters (FAME) [39]. This procedure involves a whole biomass transesterification of lipids to FAME, which eliminates the need for extraction and therefore is able to access all fatty acids in the biomass and represent an accurate reflection of the biofuels potential.

Elemental analyses for carbon (C), hydrogen (H), and nitrogen (N) contents were determined using a Perkin–Elmer 2400 CHN/S analyzer (Waltham, MA). The contents of C, H, N and S were calculated on a dry basis. Protein content was determined by using 4.78 as nitrogen to protein conversion factor [40]. All experiments and analyses were performed in duplicate.

#### 2.2.1. Estimation of biodiesel fuel properties based on FAME profile

Biodiesel is a mixture of fatty acid methyl/ethyl esters, whose profiles, size distribution and the degree of unsaturation could significantly influence the physical and chemical properties of biodiesel [41], such as cetane number (CN), iodine value (IV), cloud point (CP), cold filter Plugging Point (CFPP), the oxidation stability [42], kinematic viscosity (ν), specific gravity (ρ), higher heating value (HHV), and sulfur content [42,43].

To predict biodiesel properties, many equations have been used in the literature. The equations developed by Hoekman et al. [41] are widely accepted due to the calculated values were closer to the measured values [44]. In this study, the equations of Hoekman et al. [41] were selected to predict important biodiesel properties.

Average degree of unsaturation (ADU) can be computed as-

$$ADU = \sum M \times Y_i \quad (1)$$

where M is the number of carbon-carbon double bonds in each fatty acid constituents and Y<sub>i</sub> is the mass fraction of each fatty acid constituents.

The correlation between the average degree of unsaturation (x) and biodiesel properties (y) including kinematic viscosity, specific gravity, cloud point, cetane number, iodine value and higher heating value are shown in Eqs. ((2)–(7)), respectively [44].

$$y = -0.6326x + 5.2065 \quad (2)$$

$$y = 0.0055x + 0.8726 \quad (3)$$

$$y = -13.356x + 19.994 \quad (4)$$

$$y = -6.6684x + 62.876 \quad (5)$$

$$y = 74.373x + 12.71 \quad (6)$$

$$y = 1.7601x + 38.534 \quad (7)$$

### 2.3. Bioethanol production by yeast fermentation

#### 2.3.1. Yeast preparation

*Saccharomyces cerevisiae* (ATCC 245858) was cultured in yeast mold (YM) broth medium (Dickinson and Company, Sparks, Maryland, USA). The initial culture was prepared in test tube with a volume ratio of 1:4 seed inoculation to culture volume. The yeast cultures were scaled up by 10 times on a volumetric basis by transferring 0.5 ml of previously cultured yeast to 5 ml YM broth solution and grown at 30 °C for 24 h [45]. After culturing, the yeast cells were harvested by centrifuging the cultures and washed twice with peptone water. The centrifuged and washed yeast cells were used for fermentation.

#### 2.3.2. Pretreatment and fermentation

Pretreatment was carried out in a 250 ml shake flask with 8% (w/v) biomass concentration and 3% (w/w) sulfuric acid. Weight of algae used was 4 g dry weight in total 50 ml volume. An autoclave was used to maintain the pretreatment temperature at 121 °C for 60 min. Loss of solvent during autoclaving was measured and compensated during pH adjustment step. The acid pretreated algae slurry was adjusted to pH 5 by adding 4 N ammonium hydroxide and directly used as the fermentation medium for bioethanol production. Centrifuged and washed yeast cells prepared as section 2.3.1 (5 mg dry basis) were added to hydrolysate and anaerobically fermented for 48 h in the shaker at 30 °C and 150 rpm. The liquid samples were obtained and analyzed in a Waters high-performance liquid chromatography (HPLC, Milford, MA, USA) for residual sugar and ethanol concentrations. A small amount of fermented sample was dried for the determination of lipid content and characterization. Yeast was also grown in YM medium separately as a control.

### 2.4. Crude biodiesel production from fermented algae

Fermented algal broth obtained with initial dry algae weight of 4 g was thermally treated by using ethanol assisted liquefaction to produce crude biodiesel. The experiments were performed in a 75 ml stainless steel bench top reactor (50 ml working volume) accompanied by a controller unit (Parr Instrument Company, Moline, Illinois, USA). Fermented algal broth and required amount of ethanol (Sigma-Aldrich, Ethyl alcohol, Pure 200 proof, anhydrous) was added to obtain a final ethanol concentration of 15% (by volume) in the reactor. For liquefaction of non-fermented original *Nannochloropsis* sp., ethanol and deionized water were added to obtain the same ethanol concentration of 15% (by volume). The reactor was heated to 265 °C at a heating rate of 15 °C/min and held at the final temperature for 30 min. The temperature was measured by a thermocouple inserted into the slurry, and the reactor pressure was monitored by a pressure gauge connected to the reactor.

The liquefaction temperature and time used in this study were chosen based on the findings from the literature [11] and our previous study [20]. The ethanol concentration of 15% was chosen based on the fact that the commercial starch-to-bioethanol fermentation process could yield a 10–15% ethanol concentration [46,47].

After 30 min, the reactor was cooled down to the room temperature by using an electric fan. Gaseous products were then released through a control valve, and the content in the reactor was collected. The solid product was separated from the liquid by vacuum filtration and dried at 105 °C overnight for elemental analysis. The liquid fraction was further extracted with 50 ml hexane in a separatory funnel, and the hexane phase (upper layer) and the aqueous products (bottom layer) were separated. Hexane was evaporated under nitrogen to yield crude biodiesel, and the weight remained was recorded to calculate the biodiesel yield.

The product yield was expressed in wt.% and calculated by following equations-

$$\text{Solid residue yield \%} = \frac{\text{Weight of solid residue}}{\text{Initial weight of microalgae}} \times 100 \quad (8)$$

$$\text{Crude Biodiesel yield \%} = \frac{\text{Weight of crude biodiesel}}{\text{Initial weight of microalgae}} \times 100 \quad (9)$$

$$\text{Aqueous product yield\%} = 100 - \text{Solid residue yield\%} - \text{Crude Biodiesel yield\%} \quad (10)$$

The gas yields were calculated from the final pressure after reactions, the volume of free space in the reactor and the gas composition using the ideal gas law, and was considered negligible (< 2 wt%) [24,48].

Both the crude biodiesel and the aqueous products were analyzed by using an Agilent 7890 GC/5975 MS equipped with a DB-1 nonpolar capillary column (30 m × 0.25 mm × 0.25 μm). The injection temperature was set at 250 °C. The oven temperature was set at 40 °C and held for 2 min, followed by a ramp at 10 °C/min to 250 °C and then held for 10 min. The components in the samples were identified by comparing to the library of the National Institute of Standards and Technology (NIST, USA).

Fatty acid ethyl esters (FAEE) content in the crude biodiesel was quantified using the FAEE standard (Supelco, #49454-U). FAEE yield was calculated as the weight percentage (wt%) of total lipid content and was defined as biodiesel yield. The crude biodiesel and solid residue were analyzed by a Perkin Elmer 2400 series II CHNS/O Analyzer (Maryland, USA) to determine the elemental composition.

## 3. Results and discussion

### 3.1. Characterization of microalgae biomass

*Nannochloropsis* sp. has been considered as a promising feedstock for microalgal biodiesel production due to its high biomass accumulation rate and high lipid content [44]. The proximate and ultimate analysis of *Nannochloropsis* sp. are listed in Table 1.

Total carbohydrates (glucose, xylose and arabinose) obtained were 13.36% of dry weight; and the total lipids (as FAME content) obtained were 49.58% of dry weight. The lipid content is within the range reported in the literature 37–60% (of dry weight) for *Nannochloropsis* sp. [44,50,51]. Corresponding fatty acids profile of algal biomass is presented in Table 2.

### 3.2. Ethanol production by microalgae pretreatment and fermentation

Both wet and dried *Nannochloropsis* sp were pretreated using 3% sulfuric acid at 121 °C for 60 min with a biomass concentration of 80 g/L. HPLC analysis of hydrolysates revealed that the glucose concentrations reached 3.30 g/L and 2.29 g/L for wet and dry biomass,

**Table 1**  
Proximate and ultimate analysis of algae biomass.

Proximate Analysis, wt.% (dry basis)	
Moisture content	1.57
Volatile organic content	68.28
Fixed carbon content	8.08
Ash content	22.07
Ultimate Analysis, wt.%	
Carbon	53.15
Hydrogen	9.32
Nitrogen	7.95
Sulfur	2.27
Oxygen <sup>a</sup>	27.31
algae HHV <sup>b</sup> (MJ/Kg)	26.99

<sup>a</sup> Calculated by difference as O = 100-(C + H + N + S).

<sup>b</sup> Calculated by Boie's formula HHV = 0.3516 C + 1.16225 H + 0.0628 N - 0.1109 O [49].

**Table 2**  
Fatty acid profile of non-fermented and fermented *Nannochloropsis* sp.

Total lipid content (as total FAME) dry basis	Original	Fermented
Fatty acid composition	% of total methyl esters	
	Original	Fermented
C14:0	0.30	0.62
C16:0	10.25	13.98
C16:1	5.10	14.00
C16:2	6.87	3.26
C16:3	14.70	5.98
C18:0	1.09	1.62
C18:1	14.10	15.82
C18:2	26.16	17.94
C18:3	19.82	24.30
C20:0	1.52	2.42
ΣSFA*	13.16	18.64
ΣMFA*	19.20	29.82
ΣPFA*	67.55	51.48

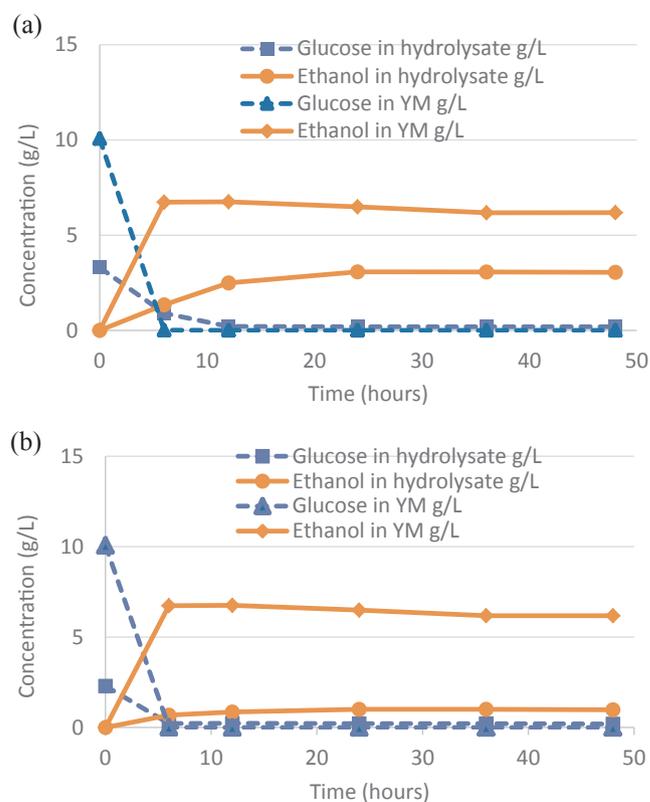
\* SFA – Saturated Fatty Acid, MFA – Mono-unsaturated Fatty Acid, PFA – Poly-unsaturated Fatty Acid.

respectively. Total glucose yield (g/g glucose available in algae) reached 98.21% and 68.15% for the pretreatment of wet and dry microalgae, respectively.

Total carbohydrate (glucose, xylose, and arabinose) yields (g/g available in dry algae) for wet and dry microalgae reached 96.36% and 44.40%, respectively. The results are consistent with the literature [32], which reported as 81.6% sugar yield for a 30 min hydrolysis with 3% (v/v) or higher  $H_2SO_4$  at 121 °C. Drying algal biomass may adversely affect the pretreatment and fermentation processes. Compared to dry microalgae, the higher carbohydrate yield from acid pretreatment of wet *Nannochloropsis* can be due to easy accessibility of acid to microalgae cell wall in original wet biomass. It is well-known that the complex carbohydrates are entrapped in the microalgae cell wall [52]. Drying process aggregates the cells into chunks which can hinder the accessibility of acids to cell wall resulting lower carbohydrate yield during acid pretreatment.

After acid pretreatment, microalgal hydrolysates containing released sugars were adjusted to pH 5 and directly used as fermentation media for bioethanol production. The profiles of residual glucose and ethanol concentrations during the bioethanol fermentation of pretreated algae are shown in Fig. 1.

It was observed that, the glucose concentration dropped rapidly at the beginning, which were accompanied by a sharp increase in ethanol concentration in both pretreated algae and YM medium as a control. The glucose concentration in the YM medium decreased from 10.07 g/L to 0.01 g/L while bioethanol concentration reached 6.74 g/L after 6 h of fermentation. For pretreated wet microalgae (Fig. 1a), the glucose concentration decreased from 3.07 g/L to 0.9 g/L, while the bioethanol concentration reached 1.43 g/L after 6 h of fermentation. Glucose and ethanol profiles for pretreated dry algae followed the similar trend. Glucose utilization was faster in the YM medium compared to those of pretreated algal broths of both wet and dry *Nannochloropsis* sp. Faster glucose utilization in the YM medium is understandable as it contains additional nutrients for yeast growth. After 24 h of fermentation, ethanol concentration of 3.07 g/L and 1.00 g/L were obtained for pretreated wet and dry microalgae, respectively. An ethanol yield of 0.286 g/g total carbohydrates in algae was obtained from wet microalgae fermentation, compared to 0.093 g/g from dry microalgae fermentation. Similar ethanol yield was reported in the literature for *Nannochloropsis oculata* yeast fermentation [53]. After 24 h no change in ethanol concentration was observed. These results indicated that pretreatment and fermentation of wet microalgae is advantageous than using dry microalgae as feedstock which can also save energy for the drying process.



**Fig. 1.** a) Glucose and ethanol concentrations during the bioethanol fermentation in pretreated wet *Nannochloropsis* sp. b) in pretreated dry *Nannochloropsis* sp.

### 3.3. Effect of pretreatment and fermentation on the total lipid, fatty acid profiles and biodiesel properties

The total lipid contents of feedstocks were measured before and after pretreatment and fermentation. It was observed that the total lipid content increased from 49.58% to 70.89% of dry weight after pretreatment and fermentation. This increase in lipid content indicated that the dilute sulfuric acid pretreatment and fermentation facilitate the lipid release by breaking the cell membrane and similar results have been reported in the literature from acid hydrolysis [32]. Ideal microalgal candidates for biodiesel production should have the suitable fatty acid composition in addition to the high lipid content. Fatty acid profiles of lipid eventually affects the quality of the biodiesel product [25], since the carbon chain length of saturated and unsaturated fatty acids affects biodiesel properties such as heat of combustion, lubricity, viscosity, low-temperature properties and oxidative stability [32,41,50]. Hence, the fatty acid profiles of both original and fermented feedstock were obtained, and some important biodiesel properties were estimated to compare.

The consensus view is that the most common feedstocks suitable for biodiesel production were enriched in the five most common C16–C18 fatty acids, including C16:0 (palmitic acid), C18:0 (stearic acid), C18:1 (oleic acid), C18:2 (linoleic acid), and C18:3 (linolenic acid) [44]. Majority of fatty acids present in the chosen *Nannochloropsis* sp. were C16 and C18 carbon chain (Table 2) and a very small quantity of C14:0 is present which is considered good because the ideal mix of fatty acids in good quality biodiesel has been suggested to be 16:1, 18:1, 14:0 in the ratio of 5:4:1 [42,54]. Although there was no significant difference in carbon number and contents between original and fermented microalgae, the amount of saturated and mono-unsaturated fatty acids increased where some polyunsaturated fatty acids decreased in fermented algae. The saturated and mono unsaturated fatty acids are also considered to be better than polyunsaturated fatty acids for improving

**Table 3**  
Estimated biodiesel properties from original and fermented *Nannochloropsis* sp. FAME profile.

Property	Original <i>Nannochloropsis</i> sp.	Fermented <i>Nannochloropsis</i> sp.	EN 14214 [41,55,56]	ASTM D6751-08 [41,57]
Kinematic viscosity 40 °C (mm <sup>2</sup> .s <sup>-1</sup> )	4.05	4.26	3.5–5	1.9–6
Specific gravity (Kg L <sup>-1</sup> )	0.88	0.88	–	0.85–0.9
Cloud point (°C)	–4.54	0.086	–	–
Cetane number (CN)	50.62	52.94	Min 51	min 47
Iodine value	149.34	123.57	max 120	–
HHV (MJ/kg)	41.77	41.16	–	–
ADU (Average Degree of Unsaturation)	1.84	1.49	–	–

oxidative stability without any associated adverse effect on the cold properties of biodiesel [32]. Table 3 compares seven estimated properties of biodiesels produced from original and fermented *Nannochloropsis* sp. according to ASTM D6751-08 and EN14214.

The properties of kinematic viscosity, specific gravity and cetane number satisfied the specifications of ASTM and EN standards. The iodine value was much higher for original non-fermented *Nannochloropsis* sp. due to higher degree of unsaturation, while the iodine value for fermented *Nannochloropsis* sp. decreased to 124 which is close to acceptable limit of 120. The biodiesel property of average degree of unsaturation (ADU) was proved to have high correlation with several other properties. Higher ADU leads to lower CN and poorer oxidation stability but improves low temperature performance [41]. The degree of unsaturation ADU was higher for original *Nannochloropsis* sp. whereas the ADU value was less than 1.5 for fermented algae which is within average range for common biodiesel feedstock [41]. This may have occurred due to positive change of saturated and unsaturated fatty acid through pretreatment and fermentation. Comparison of the estimated properties indicated that the acid pretreatment and fermentation process can enhance the fatty acid profile of microalgae which makes the fermented algal biomass a potential feedstock for biodiesel production via thermal treatment.

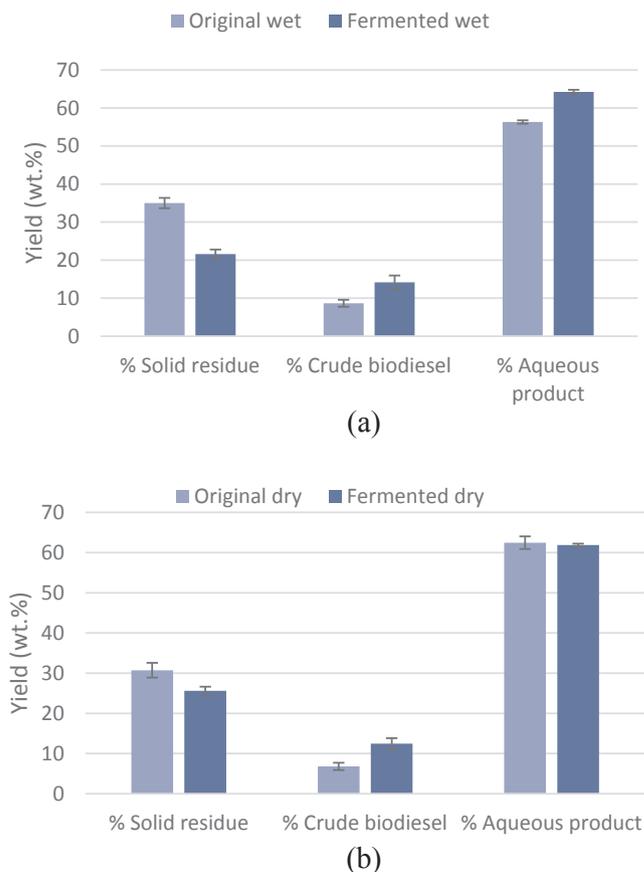
### 3.4. Ethanol-assisted liquefaction of fermented algal biomass

#### 3.4.1. Effect of acid pretreatment and fermentation on product yields

Product yields (wt% of microalgae) (Eqs. (8)–(10)) after ethanol-assisted liquefaction of fermented and original algal biomass are shown in Fig. 2. Compared to original microalgae, liquefaction of fermented wet and dry microalgae resulted in a lower solid residue yield which indicates a higher conversion. Application of pretreatment and fermentation prior to liquefaction of microalgae increased crude biodiesel production from 8.67% to 14.18% and 6.8% to 12.48% for wet and dry microalgae, respectively. The increase of total lipid in fermented algae may have attributed to this increase in the crude biodiesel yields.

Fig. 3 shows FAEE yields (wt. %, i.e., biodiesel yield) obtained via ethanol-assisted liquefaction of fermented and non-fermented microalgae. The FAEE yields of fermented wet and dry biomass were higher, compared to original biomass liquefaction. The highest FAEE yield obtained was 14.16% of the total lipid for fermented wet microalgae. FAEE yield of fermented wet *Nannochloropsis* sp. increased by three-fold of original biomass liquefaction with a 2:1 ethanol-to-algae ratio, which indicated that pretreatment and fermentation can improve the FAEE yield at lower ethanol concentration in reaction.

The biodiesel yields obtained in this study are relatively low, which can be due to the low ethanol-to-algae ratio (2:1) or high biomass concentration in the reaction mixture. Necessity of higher ethanol-to-algae ratios is mentioned to drive the ethyl ester production reaction at a faster rate and shift the equilibrium towards the product side in non-catalytic supercritical alcohol processes [11,22]. Levine et al. [18] reported 34–66% FAEE yields on the basis of lipid in hydrolyzed solids from high temperature hydrolysis (250 °C) and ethanol assisted (2:1–8:1 ethanol-to-algae) supercritical transesterification process. Reddy et al. [11] reported a 25–67% FAEE yield for liquefaction of wet



**Fig. 2.** a) Comparison of product yield (wt.%) from ethanol assisted liquefaction of fermented and original wet *Nannochloropsis* sp. b) Comparison of product yield (wt.%) from ethanol assisted liquefaction of fermented and original dry *Nannochloropsis* sp.

*Nannochloropsis salina* with a 6:1–9:1 ethanol-to-algae ratio at similar temperature and reaction time, but the moisture content of algae used in both process are much less-46% [18] and 60% [11], respectively. Thus, the ethanol concentration in their reaction mixtures was very high, which can lead to higher energy requirement for downstream processing.

#### 3.4.2. Biochemical composition of crude biodiesel

Major components found in crude biodiesel obtained from wet fermented algae were fatty acids, fatty acid ethyl esters (FAEE) and nitrogenated compounds (Supplementary Table A1). The major FAEE components (up to 48.9 wt% of crude biodiesel) found in crude biodiesel were palmitic acid ethyl ester (C16:0), oleic acid ethyl ester (C18:1), linoleic acid ethyl ester (C18:2) and linolenic acid ethyl ester (C18:3) (Table 4).

Special attention should be paid to high levels of polyunsaturated fatty acids which are susceptible to oxidation. It was observed that no

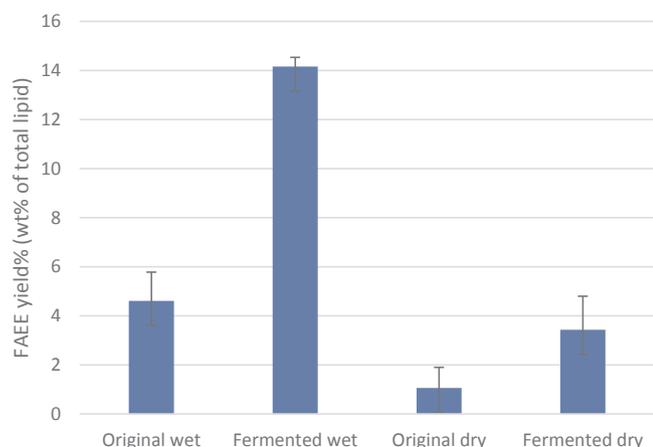


Fig. 3. FAEE yields obtained by ethanol-assisted liquefaction at 265 °C and 30 min.

**Table 4**  
FAEE profile of crude biodiesel from fermented wet *Nannochloropsis* liquefaction.

Fatty acid ethyl ester (FAEE)	% of total FAEE
C14:0	1.42
C16:0	30.06
C 16:1	6.10
C18:0	4.91
C18:1	10.04
C18:2	27.87
C18:3	19.13

**Table 5**  
Relative percentage of biochemical components available in crude biodiesel from fermented wet *Nannochloropsis* liquefaction.

Chemical Components	Area % of total
Hydrocarbons	7.2%
Fatty acids	49.9%
Saturated Fatty acid ethyl esters	14.5%
Unsaturated fatty acid ethyl esters	21.6%
Nitrogenated compound	4.4%
Acetate compounds	1.7%
Sulphur phosphorus	0.2%
Other esters	0.6%

fatty acid ester with more than three double bonds is present in the crude biodiesel, which is good in terms of fuel properties [32]. Among other components in crude biodiesel, higher amounts of free fatty acids were observed (Table 5). Higher amount of fatty acids in the crude biodiesel indicates incomplete esterification reaction in the process [18]. The lower liquefaction temperature and lower ethanol concentration used can attribute to incomplete esterification during liquefaction. Fatty acids and glycerides recovered from the crude biodiesel could be recycled back to the supercritical reactor, generating additional FAEE [18].

Nitrogenated and acetate components were also observed. Higher protein content can contribute to these nitrogenated components present in crude biodiesel phase, which have been considered as an important drawback in view of fuel application [58]. A positive way to improve the quality of the fuel could be hydrotreating of the crude biodiesel. It has been reported that free fatty acids would not cause difficulties if hydrotreating of the oil for upgrading purposes is preferred [58]. Clearly, further work is required to explore the process parameters and identify optimal conditions for producing fuel-grade biodiesel from ethanol-assisted liquefaction of fermented wet

**Table 6**  
Elemental analysis of fermented algae and residual solid obtained after ethanol assisted liquefaction, wt.%.

Elemental Analysis, wt.%	Fermented algae	Solid residue
Carbon	49.7	10.29
Hydrogen	8.61	4.45
Nitrogen	7.31	6.98
Sulfur	5.20	11.87
Oxygen <sup>a</sup>	29.18	66.41

<sup>a</sup> Obtained by difference.

microalgae [18].

### 3.4.3. Analysis of solid and aqueous products

Solid residue obtained was analyzed for elemental composition (Table 6). The nitrogen content of the solid residue was 6.98%, which was similar to fermented algae used for liquefaction. This nitrogen rich residue can be a potential candidate for soil application.

The aqueous product was analyzed by GC–MS to identify the organic chemicals present (Supplementary Table A2). The analysis showed that majority of the components were nitrogenated compounds such as pyrrole, pyrazine and amides. Among other components, alcoholic, phenolic compounds, oxygenated hydrocarbons and acids were also present in the aqueous phase (Table A2). This nitrogen rich aqueous phase can be utilized to supplement algal growth step. Several researchers reported microalgae cultivation using recovered or diluted aqueous product from thermochemical liquefaction which can contribute to the sustainability of the whole process [59,60].

Aqueous products were also analyzed in HPLC to determine unreacted ethanol concentration. It was observed that about 75–85% (v/v) of ethanol remains unreacted in the aqueous product which can be separated and recycled back to the liquefaction step. Based on this preliminary fermentation study, the pretreatment and fermentation of the microalgae (8% wt.% biomass concentration) could provide ~10% of ethanol required for ethanol-assisted liquefaction reactions. Higher bioethanol concentration from high carbohydrate content species has been reported in the literature [52,61]. Theoretically, a microalgal slurry of ~20 wt% might be used for this process and provide at least 30% of ethanol required. The result from the proposed study is advantageous as it can save additional solvent for the liquefaction process from wet algae.

## 4. Conclusion

The feasibility of an integrated process combining pretreatment, fermentation and liquefaction for production of biofuel and chemicals from marine microalga *Nannochloropsis* sp. was confirmed. Pretreatment and fermentation of wet microalgae increased the total lipid by 40% and produced 10% of required solvent for subsequent biodiesel production step. Maximum ethanol yield of 0.286 g ethanol/g total carbohydrate was obtained from wet algae. This approach increased FAEE yield by three-fold of non-fermented liquefaction yield at 265 °C and 30 min with a 2:1 ethanol to algae ratio. Both residual solid and aqueous phase contains high nitrogen which can be used for nutrient recycling.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.fuel.2018.10.116>.

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