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Direct Transesterification of Microalga *Botryococcus braunii* Biomass for Biodiesel Production

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Abstract

Microalgae biodiesel are reported to be better than fossil fuels in terms of life-cycle energy performance. Green alga, *Botryococcus braunii* symbolizes one of the most favorable resources of biodiesel due to relatively high lipid content. The present study focused on the cultivation of *Botryococcus braunii* with the fed-batch in 4 L lab-scale continuously stirred tank reactors (CSTRs) through inexpensive red Nile tilapia effluent medium (RNTEM), biomass growth, protein, carbohydrate, lipid, hydrocarbon production and fatty acids profiles. Additionally, in this study we have evaluated the feasibility of biodiesel production directly from *B. braunii* biomass at laboratory scale achieved through direct transesterification process. *B. braunii* growth confirmed the highest biomass yield (8.57 g L^{-1}) and 35.32% hydrocarbon content was observed. Further, 47.59% lipids, 16.39% proteins and 38.21% carbohydrates were observed under laboratory conditions. Fatty acid methyl esters (FAME) synthesis by direct conversion of *B. braunii* biomass was carried out using sulfuric acid as a catalyst and methanol as solvent. The experimental results obtained in the present study proved that the production of *B. braunii* by RNTEM is potentially feasible.

Keywords biodiesel, biomass, direct transesterification, microalgae, red tilapia effluent medium

Introduction

Biodiesel fuels, derived from a variety of animal, plant and microbial feedstocks are promising renewable alternative diesel fuels in view of energy security and environmental protection with great potential of carbon dioxide (CO_2) reduction from the entire cycle of biodiesel production [1]. It is one of the most promising renewable fuels that is biodegradable, less toxic and can be directly applicable for vehicles. Biodiesel is an alternative biofuel obtained from the transesterification of microbial biomass, vegetable oil or an animal fat and a short-chain alcohol. Over a decade, its usage has been steadily increasing in many countries.

Biodiesel, i.e., fatty acids methyl ester (FAME), is mainly produced from edible vegetable oils, animal fats and microbial biomass. In general, biodiesel could be produced from any type of oil such as soybean, corn oil, palm or algal oil (especially microalgal oil). Biodiesel is routinely produced by the extraction of algal oil followed by transesterification [2]. Microalgae are the dominant primary producers in most of the aquatic habitats [3-4]. They are fast-growing beasts with a voracious appetite for carbon dioxide. In comparison to any other feedstock, microalgae are more productive in oil yield for biodiesel production as it can be grown on land that is inappropriate for food crops. High production cost of biodiesel from microalgae is because of several downstream steps including harvesting, dewatering, drying, extraction, separation and transesterification processes. To solve these issues, a one step process called green technology, is introduced where lipids are directly extracted and trans-esterified from wet/dry biomass and processed using transesterification. Algal oil is more attractive because of the algal capacity to yield more oil without requiring large area of arable lands, scope for better strain improvement and the capacity to enhance the value through co-products.

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Samori et al. [5] stated that *B. braunii* is a potential renewable energy resource identified through hydrocarbons production. It is also known to produce large amounts of fatty acids. The quantity and composition of fatty acids varies with both species and races [6-7].

B. braunii is widespread in freshwater reservoirs, ponds and sometime can be found in brackish lakes. It is a unique colonial green alga that synthesizes unusually high level of hydrocarbons in a range of 15–76% dry weight [8-9]. Largeau et al. [10] showed that *B. braunii* had two distinct sites for lipid accumulation, intracellular and extracellular pools, and the hydrocarbons are predominantly located in the extracellular pool. Although *B. braunii* is usually cultivated in modified Chu 13 medium [11], Bold basal medium, BG-11 medium [12], modified BG-11 medium [13] or Prat medium [14], several studies reported that *B. braunii* can be successfully grown on wastewater medium [15-17]. The objective of this research article is to present the applicability of *B. braunii* culture grown in inexpensive red Nile tilapia effluent medium in biomass production for biodiesel synthesis through direct transesterification process.

Methodology

Algal culture and biomass preparation

In present study, microalga *Botryococcus braunii* was used for the experiment. The algae were cultivated in the plant physiology and technology laboratory, Maejo University, Chiang Mai, Thailand. The experimental procedures for algal cell culture were followed as previously described [18]. *B. braunii* were cultivated in 4 L lab-scale continuously stirred tank reactors (CSTRs) system. Fed-batch method was used with 10 day detention time, for 28 days growth period. Triplicate reactors were used under room temperature with light illumination through fluorescent lamps and light intensity was 51 $\mu mol^{-1}m^2sec^{-1}$. The pH was 7.8. All the reactors were feed and other operational parameters were same as our previous study [18]. Green microalga *B. braunii* structure has been illustrated in Figure 1.

Red Nile tilapia culture effluent was collected from the faculty of Fisheries Technology and Aquatic Resources, Maejo University and was used as a substrate to cultivate. Effluent went under pre-treatment process for macro particles and non-soluble particulate solids. Subsequently, the substrate was autoclaved for 20 min at 121°C. Then liquid was stored at 4 °C for 2 days, after that the supernatant was utilized for *B. braunii* production. Algae were filtered with glass microfiber filter paper (GFC, Whatmann) and washed with distilled water. The paper with the deposited algal cells was dried at 105 °C. The dry weight of the biomass was measured. The cell pellet was washed twice with double distilled water. The cell pellets were dried for direct transesterification processes.

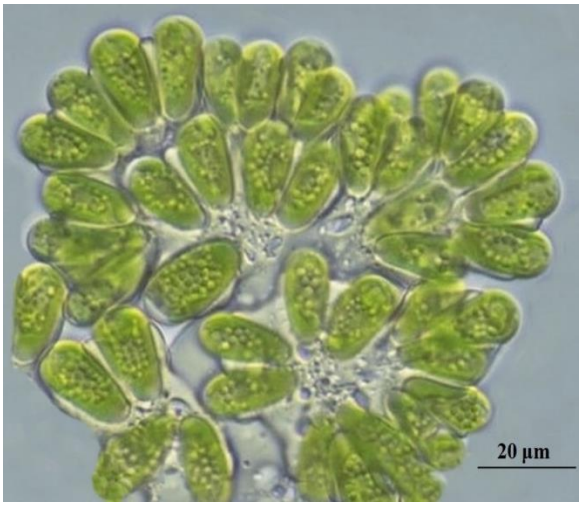

Figure 1. ***Botryococcus braunii***

Direct transesterification

One gram of dried algal biomass or wet algal biomass (1 g dry weight equivalent) was placed in a glass test tube and mixed with 3.4 mL of methanol and 0.6 mL of sulfuric acid. Chloroform solvent system was applied. The methodology of direct transesterification processes were adopted from Johnson and Wen [19]. Analytical grade organic solvents including chloroform, hexane and petroleum ether were used and 4 mL of solvent was added to the tube.

The reaction mixture was heated at 90°C for 40 min and the samples were well-mixed during heating. After the tubes were cooled to room temperature, 2 mL distilled water was added to the tube and mixed for 45 s. Further, tubes were allowed to separate into two phases. The solvent layer that contained biodiesel (FAME) was collected and transferred to a pre-weighed glass vial. Solvent was evaporated using N_2, and the mass of biodiesel was determined gravimetrically.

Analytical procedure

According to Tipnee et al. [20], the spectrophotometric method was performed for pigment determination of macroalgae extract. Weighed samples, having been put separately in 96% methanol (50 ml for each gram), were homogenized at 1000 rpm for one minute. Further, homogenate was filtered and centrifuged at 2500 rpm for ten minutes. Then, supernatant was separated and the absorbance was read at 400-700 nm on Schimadzu spectrophotometer (Spectronic Genesys 20, Thermo Fisher Scientific). Chlorophyll contents were recorded. Chlorophyll-a showed maximum absorbance at 666 nm, chlorophyll-b at 653 nm and total carotene at 470 nm. After extraction, pigments concentrations were spectrophotometrically determined; total chlorophyll content and total carotene was calculated according to the formulae mentioned by Lichtenthaler and Wellburn [21].

Elemental compositions (carbon, hydrogen, and nitrogen) were analyzed through element analyzer (Perkin−Elmer Mode1 2400). Oxygen content was calculated using formula; O =100-(C+H+N). Nitrogen content was determined by Kjeldahl method and analytical procedure was adopted from the standard method [22]. The findings were expressed in percent of dry weight. Protein content was measured by multiplying the nitrogen content by a conversion factor 6.25 [23]. According to Dubois et al. [24], the total carbohydrate was estimated by phenol sulphuric acid method with glucose as standard. According to Bligh and Dyer [25], the lipids were extracted and total lipid content was determined gravimetrically. Hydrocarbon extraction and analysis process was adopted from Dayananda et al. [26].

Fatty acid composition biodiesel yield evaluation

Fifty milligram samples were placed into capped test tubes, saponified with 1 ml of a saturated KOH–CH_3 OH solution at 75°C for 10 min, and then submitted to methanolysis with 5% HCl in methanol at 75°C for another 10 min. Thereafter, the phase containing the fatty acids was separated by adding 2 ml of distilled water and then recovered. A fatty acid composition analysis was performed using GC-MS (Agilent 6890-HP5973 model, Australia). The biodiesel yield was evaluated by its weight relative to the weight of algal biomass. FAME compositions were analyzed via gas chromatography (GC). The operation procedure was adopted from Chi et al. [27]. FAME analysis was performed using GC-MS (Agilent 6890-HP5973 model, Australia).

The physicochemical properties of *B. braunii* biodiesel including calorific value, acid value, total glycerine, cetane number, flash point, pour point, viscosity, density and copper strip correction were evaluated using the standard methods and compared to ASTM standards.

Statistical analyses

All the values or readings are the result of mean of three replicates. Data was reported as mean ± standard deviation (SD). Statistical analyses were performed using the SPSS statistical package (SPSS Inc., Chicago, IL, USA). The statistical significances were achieved when $p<0.05$.

Table 1 Chemical characteristics of microalgae *B. braunii*

Parameters	Values
Proximate composition (%)	
Moisture	02.16± 0.04
Ash	01.55± 0.04
Volatiles	66.57± 0.01
Fixed carbon	11.78± 0.11
HHV (MJ kg^{-1})	42.41± 0.08
Ultimate composition (%)	
Carbon	73.43± 0.15
Hydrogen	10.87± 0.07
Nitrogen	02.34± 0.13
Oxygen	13.28± 0.00
Sulphur	00.17± 0.50
Pigments (mgg^{-1})	
Total chlorophyll	09.76±0.29
Total carotenoids	01.78±0.82
Biochemical composition	
Total protein (%)	16.39±3.43
Total carbohydrate (%)	38.21±4.34
Total lipid (%)	47.59±0.58
Hydrocarbon (%)	35.32±4.10
Biomass Max (gL^{-1})	08.57±0.21

Results and Discussion

Characteristics of Botryococcus braunii

In this study, *B. braunii* was grown under fluorescent lamps using CSTR up flow photobioreactor. Biomass productivities, proximate and ultimate analysis, and biochemical composition of *B. braunii* are presented in Table 1. The moisture content was relatively lower (14.94%); elemental composition of carbon, hydrogen, nitrogen, oxygen

and sulfur contents were 73.4%, 10.8%, 2.3%, 13.2% and 0.17% respectively. It showed that richest ultimate compositions were available in the *B. braunii* biomass.

The highest biomass of 8.57±0.21 gL^{-1} with the highest lipid content of 47.59±0.58% was achieved that was comparable to the previously available literature [14-16, 26, 28]. Protein and carbohydrate contents are among the most important biochemical constituents and their levels may differ in various algae [3, 29]. Present results showed that *B. braunii* were successfully grown under lab conditions although they differed in biomass yields, protein, carbohydrate, lipid and hydrocarbon content.

Fatty acid composition

In this study, we found that the dominant component was oleic acid, followed by palmitic acid, linolenic acid, and linoleic acid from the *B. braunii* extract. Ahlgren et al. [30] stated that these compounds mainly contribute in fatty acid composition in *B. braunii*. Besides free fatty acids, the algal extract also contained hydrocarbons, carotenoids and chlorophyll [31]. The major fatty acid composition of the tested microalgae was determined using the GC analysis. The fatty acid profile is shown in Table 2, which indicated the presence of C 16:0, C 16:1, C 18:0, C 18:1, C 18:2 and C 22:0 fatty acids with variation in their relative proportion. Fang et al. [32] reported palmitic acid and oleic acids as major components in the *Botryococcus* sp. The most common fatty acids contained in biodiesel are palmitic, stearic, oleic, and linolenic acid [33]. Kumar and Rengasamy [34] reported that oleic, linolenic and palmitic fatty acids are the major fatty acids in *B. braunii* Kutz (AP-103).

Oils containing high oleic acid content have been reported to have a reasonable balance of fuel properties [35]. Higher oleic acid content can increase the oxidative stability for longer storage [33]. Fatty acids of microalgae can be either saturated or unsaturated and array from 12 to 22 carbons in length that can be converted into biodiesel [36]. The fatty acids profile of *B. braunii* lipids is mainly composed of oleic acid (C18:1, 34.74± 0.07%), followed by palmitic acid (C16:0, 19.32± 0.08%), palmitelaidic acid (C 16:1, 11.67± 0.80), linolenic acid (C18:3, 6.32± 0.11%), stearic acid (C18:0, 6.10± 0.13%) and linoleic acid (C18:2, 5.56± 0.07%). Consequently, in this study *B. braunii* showed the highest oleic acid content emphasizing on its utility for the good quality biodiesel production.

Biodiesel production from wet and dry B. braunii biomass

Algal biodiesel production is typically performed by the transesterification process utilizing algal oil

Table 2 Fatty acid profile of *B. braunii*

Fatty acid	(%)
Cyclohexane (C 12)	2.63 ± 0.01
Lauric acid (C 12:0)	0.41 ± 0.34
Myristic acid (C 14:0)	1.38 ± 0.29
Pentadecanoic acid (C 15:0)	0.84 ± 0.10
Palmitic acid (C 16:0)	19.32± 0.08
Palmitelaidic acid (C 16:1)	11.67± 0.80
Heptadecane (C 17)	1.44 ± 0.30
Margaric acid (17:0)	0.60 ± 0.12
Stearic acid (C 18:0)	06.10± 0.13
Oleic acid (C 18:1)	34.74± 0.07
Elaidic acid (9-18:1)	0.56 ± 0.06
Linoleic acid (18:2)	05.56± 0.07
Linolenic acid (C18:3)	06.32± 0.11
Homo-ɣ- linolenic acid (20:3)	00.36 ± 0.11
Eicosatrienoic acid(20:4)	01.24 ± 0.05
Docosanoic acid (C22:0)	03.09± 0.09
Erucic acid (C22:1)	05.05± 0.01
Lignoceric acid (24:0)	01.87 ± 0.09
2-Pentadecanone,6,10,14-trimethyl	00.92 ± 0.31
6-Octen-1-ol, 3,7-dimethyl acetate	00.31 ± 1.73
1,3-Cyclohexanediamine	00.17 ± 0.55
(1R,4S)-1,7,7-Trimethylbicycloheptan-2-yl 3-hlorobenzoate	00.79 ± 0.63
2-Methylthio-5-nitro anisole	00.25 ± 0.36
Ethyl 4,8,12-trimethyl-tridecanoat	00.22± 0.28

[2, 19]. Direct transesterification of the raw biomass has also been reported in some algal and fungal species [37, 38]. Table 3 illustrated the biodiesel production from wet and dry biomass of *B. braunii***.** As cost of algal biodiesel production is increased due to dewatering and drying of the biomass, the chances of employing freshly harvested wet algae as biodiesel feedstock was examined. As shown in Table 3, the wet and dry biomass biodiesel yield was comparable to each other. Both the wet and dry biomasses had similar FAME compositions; however, the total FAME yield from the wet biomass was much less than that from the dry biomass.

The FAME content in the wet-biomass derived biodiesel was also lower. In the direct transesterification method, the wet and dry biomass resulted in a similar crude biodiesel yield. However, the wet biomass resulted in a high portion of unknown FAME. The FAME yield and FAME content from the wet biomass was also significantly lower than that from the dry biomass. Previous literature on direct transesterification using dried biomass has been reported [2, 18, 19, 37]. Our study confirmed that the biodiesel yield and FAME content was higher using dry biomass than wet biomass. Almost 36% of FAME content was higher through dry biomass process. Consequently, drying the algae is a necessary step for direct transesterification.

Scaled-up production and characterization of B. braunii biodiesel

The aforementioned results indicated that direct transesterification (with dry biomass) had the best performance for producing biodiesel from *B. braunii*, in terms of biodiesel yield, FAME yield, and FAME content. Therefore, this method was used to prepare a larger batch of biodiesel for ASTM standard tests. The scaled-up biodiesel production resulted in ~ 100 mL of liquid fuel from 200 g of algal biomass. The ASTM standard tests of this liquid fuel indicated that the acid number, corrosiveness to copper, flash point, particulate matter check, viscosity, and soap check meet the standards. According to Miao and Wu [2] and Francisco et al. [39], the long chain fatty acids (C16–(C16–18) are more preferable as a biodiesel fuel. FAME compositions are presented in Table 3. It is clearly demonstrated that in FAME containing high content of fatty acids, range was found between C16 and C18. Therefore, this fatty acid range from *B. braunii* strain is appropriate for biodiesel production.

Table 3 Biodiesel production from wet and dry biomass of *B. braunii*

Characteristic feature	Wet	Dry
FAME composition		
C12:0	-	2.8± 0.1
C12:1	0.22± 0.2	0.45± 0.2
C14:0	0.1± 0.1	0.2± 0.1
C14:1	1.03± 0.2	1.1± 0.1
C16:0	5.43± 0.5	11.66± 0.2
C16:1	2.25± 0.8	3.96± 0.4
C18:0	3.91± 0.4	5.85± 0.1
C18:1	24.12± 0.6	45.4± 0.2
C18:2	2.75± 0.2	3.05± 0.4
C18:3	3.82± 0.1	5.21± 0.1
C20:0	2.88± 0.1	2.1± 0.4
C20:1	2.15± 0.2	1.1± 0.3
C20:4	1.73± 0.4	2.20 ± 0.8
C20:5	0.07 ± 0.43	1.07± 0.2
C22:2	0.15± 0.22	0.97 ± 0.2
Unidentified	6.45± 0.5	8.85± 1.2
Unsaturation index	0.87± 2.1	0.94± 0.1
FAME content in biodiesel (%)	57.78± 0.1	93.74

Conclusion

B. braunii grew and accumulated better lipid under the photoautotrophic conditions using red Nile tilapia effluent medium. The cultivation effectively enhanced the biomass, lipid production and other biochemical compositions. This study also showed that it is possible to economically produce a high cell density of microalgae using RNTEM medium without adding carbon dioxide. In addition, a fed-batch algal cultivation mode was shown to be an effective method for achieving high biomass quantity with rich lipid content. The FAME produced from direct transesterification of dry biomass was suggested for further large scale biodiesel production in the future.

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