

## Semi-continuous cultivation of microalgal consortium using low CO<sub>2</sub> concentration for large-scale biofuel production

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In this study, the microalgal consortium (MC) was cultivated with low CO<sub>2</sub> concentration (1% v/v) in the semi-continuous outdoor open system in order to apply for large-scale biofuel production. The results found that the recorded levels of dry weight, lipid content of MC cultured with 1% CO<sub>2</sub> supplementation in all cycle were higher than of those with ambient air (0.03% CO<sub>2</sub>) supplementation. The means of biomass concentration and lipid content of the MC with 1% CO<sub>2</sub> supplementation were 0.41±0.04 g/l and 17.3±0.77% of dry weight, respectively. CO<sub>2</sub> supplementation also had strongly affected on biovolume ratio of microalgae. The results of characteristics and chemical compositions analysis found that cultivating algae with a low concentration of CO<sub>2</sub> supplement (1% v/v) enhanced the properties that make MCB suitable as a raw material for biofuel production. MCB cultivated with CO<sub>2</sub> supplement showed high volatile matter content, carbon content, and HHV (78.01 wt%, 45.68 wt%, and 16.37 MJ/Kg, respectively). The major fatty acids from MCB under 1% CO<sub>2</sub> concentration were palmitic acid and oleic acid. Therefore, cultivating microalgae with low CO<sub>2</sub> concentration was considered suitable for applications in large scale biofuel production.

**Keywords:** microalgal consortium; semi-continuous cultivation; carbon dioxide; characteristic; biofuel.

**Abbreviations:** MC: microalgal consortium; MCB: microalgal consortium biomass; HHV: higher heating value; TG: thermal gravimetric; DTG: differential thermal gravimetric; FTIR: fourier transform infrared spectroscopy; GC-MS: gas chromatograph-mass spectrometer.

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### Introduction

Microalgae are photosynthetic microorganisms that fix atmospheric CO<sub>2</sub> and converted it into chemical energy such as protein, carbohydrates, and lipids [1]. Many reports have suggested that several species of microalgae can produce and accumulate high lipid content in their cells and

their lipid production rate of microalgae is normally 45-220 times higher than terrestrial plant [2]. *Botryococcus* may attain up to 80% in lipid content while *Chlorella* and *Acutodesmus* (*Scenedesmus*) species have up to 50% and 60%, respectively [3, 4]. The algal lipids could be used as a feedstock to produce biodiesel by transesterification reaction and have a potential

to replace 'fossil diesel'. When compared with other types of oil crops, microalgae possess higher oil yield and they do not require a large land area for cultivation. Microalgae also have more advantages in terms of photosynthetic efficiency, CO<sub>2</sub> fixation ability, and growth rate than oilseed crops [5-8].

Besides biodiesel production, microalgae have high potential to CO<sub>2</sub> capture. There are several species of microalgae can tolerate and grow well in high CO<sub>2</sub> concentrations of up to 10-100% v/v [9]. Many researchers reported that CO<sub>2</sub> supplementation had strongly effect on the growth characteristics, lipid production, and CO<sub>2</sub> fixation of microalgae [10-12]. Many research studies conducted on the lab-scale suggested that high CO<sub>2</sub> concentrations (above 10% v/v) could promote microalgal growth and lipid accumulation [6, 11, 13]. However, when using the high concentration of CO<sub>2</sub> for large-scale cultivation, the cost of the cultivation process will increase because pure CO<sub>2</sub> is expensive. In our previous study, we found that cultivating of the microalgal consortium (MC) with high CO<sub>2</sub> concentrations (10-30% v/v) in lab-scale could enhance microalgal biomass and lipid, and also help to regulate population of microalgae. On the other hand, the low CO<sub>2</sub> fixation rate of the MC was observed. It can be seen that microalgae could not uptake all of supplied CO<sub>2</sub>, resulted in high amount of CO<sub>2</sub> remain in the culture system. Thus, a low concentration of CO<sub>2</sub> (1% v/v) was used for microalgae cultivation in this study in order to apply in large scale microalgae cultivation. The MC was cultivated with 1% CO<sub>2</sub> and ambient air (0.03% CO<sub>2</sub>) supplementations in a semi-continuous outdoor open system for biomass and lipid production. Moreover, some characteristics and chemical compositions of microalgal consortium biomass (MCB) cultivated with and without CO<sub>2</sub> supplementations were investigated in order to use as raw materials for biofuel production. Several methods were implemented to determine the algal biomass characteristics, including proximate, ultimate and higher heating value (HHV) analyses. The thermal profile and functional groups of MCB

were examined by using Thermogravimetric analyzer and Fourier transform infrared spectroscopy (FTIR), respectively. Also, the fatty acids composition of the extracted lipids from the MCB were analyzed by using Gas chromatograph-mass spectrometer (GC-MS).

## Materials and methods

### Microalgae samples

The MC used for this study were obtained from the algal collection of Applied Algal Research Laboratory (AARL), Department of Biology, Faculty of Science, Chiang Mai University. The MC was incubated in 10 L of CMU03 medium [14] under continuous light of 24.3 μmol/m<sup>2</sup>/s at room temperature for two weeks. The algal culture was observed by a light microscope (Olympus C011) to contain primarily *Acutodesmus* sp., *Carteria* sp., and *Chlorella* sp.

### Microalgae cultivation

The MC was cultivated in the semi-continuous outdoor open system. The cultures were inoculated to 50 L of CMU03 medium in a 60 L plastic tank and aerated with two different conditions; ambient air (0.03% v/v CO<sub>2</sub>) and 1% v/v CO<sub>2</sub> (pure CO<sub>2</sub> balanced with ambient air) at a flow rate of 0.2 volume-to-volume per minute (vvm). Both treatments were conducted in triplicate. The algal growth (determined by dry cell weight) was monitored every day using a modified method of Yoo *et al.* [15]. When the growth reached to the early stationary phase, 90% of the cultures were harvested. Then, the culture tank was refilled up to its initial volume with the culture medium. The semi-continuous cultures were repeatedly operated for three cycles. The harvested sample was dried with solar heat, then stored in an auto desiccator until use.

### Microscopic observation

The populations of MC were examined on the initial and final day of each cultivation using light microscope (Olympus C011) and identified

according to relevant keys, i.e. John *et al.* [16], Huber-Pestalozzi [17], and Komarek and Anagnostidis [18]. Three hundred of cells were counted under a light microscope and the biovolume of microalgal species were estimated following the method of Hillebrand *et al.* [19]. The biovolume of each species were calculated in terms of percentage of total microalgal biovolume.

#### **Characterizations of microalgal biomass**

The characteristics of microalgal consortium biomass (MCB) were analyzed according to ASTM methods. Proximate analysis of MCB with and without CO<sub>2</sub> supplementation was conducted using a Leco TGA 701 analyzer. The moisture, volatile matter, and ash content were determined at 107°C in 60 min, 900°C in 30 min, and 815°C in 30 min, respectively. Ultimate analysis (carbon, hydrogen, nitrogen, and oxygen contents) and sulfur content were conducted with a Leco CHN-2000 elemental analyzer and a Leco SC-432 sulfur analyzer, respectively. The higher heating value (HHV) was determined according to ASTM D2015 [20].

#### **Thermal profile analysis**

The thermal profile of the algal samples was determined using a differential thermal analyzer (TGD 9600S, ULVAC-RIKO) [21]. About 20 mg of MCB was placed in a pottery crucible and heated from ambient temperature to 1,000°C at a rate of 10°C/min under an N<sub>2</sub> atmosphere at a flow rate of 100 ml/min.

#### **FT-IR analysis**

The functional groups of MCB were identified using the method of Cao *et al.* [21]. The FT-IR spectra were recorded by collecting 128 scans at a resolution of 4/cm in the reflectance mode with measuring regions of 4000-400/cm using a Nicolet MAGNA II 550 spectrometer.

#### **Lipid extraction**

The lipid of MCB samples were extracted using chloroform:methanol (2:1, v/v) followed method of Bligh and Dyer [3, 22]. The liquid mixture was placed in an ultrasonic bath (40 kHz, 50°C for 30

min), and then separated into the solvent and cell debris layer by centrifugation at 6,000 rpm for 10 min. Finally, the solvent layer containing the lipids was evaporated to dry. Lipid contents were measured gravimetrically. The lipid productivity was calculated following the way of Yadavalli *et al.* [23].

#### **Lipid profile analysis**

Lipid extracted from MCB samples was analyzed by GC-MS. Each lipid sample was dissolved in chloroform:methanol (2:1). The solution (0.2 µl) was analyzed with Agilent 7890A/5975C (EI) GC/MSD equipped with a DB5-MS column (30 x 0.25 mm ID x 0.25 µm film thickness). Helium was used as the carrier gas at a constant flow rate of 1.0 ml/min. The compounds were then identified by comparing the mass spectra with the NIST08 library data.

#### **Statistical analysis**

The results are expressed as mean ± SD (standard deviation) of the three replicates. All data were performed by SPSS (version 16.0 for Windows). One-way analysis of variance (ANOVA) and the least significant difference (LSD) test were used to evaluate the differences between the MC cultivated with 1% CO<sub>2</sub> and ambient air supplementation. A value of  $p < 0.05$  was considered statistically significant.

## **Results and discussion**

#### **Growth of microalgae**

The MC were cultivated with two different aerations; 1% v/v CO<sub>2</sub> and ambient air. Figure 1 shows the growth curve of the microalgae under three cycles. The microalgae growth rate of the first cycle under 1% CO<sub>2</sub> was slightly higher than that of MC under ambient air condition while the growth rate at the second and third cycles were considerably high. This happened because aeration with CO<sub>2</sub> could help to control the culture pH during algae cultivation, resulted in an enhancement of the microalgae growth. The pH of culture medium with 1% CO<sub>2</sub> (7.5-8.2) was lower than that of ambient air condition (7.5-9.3)

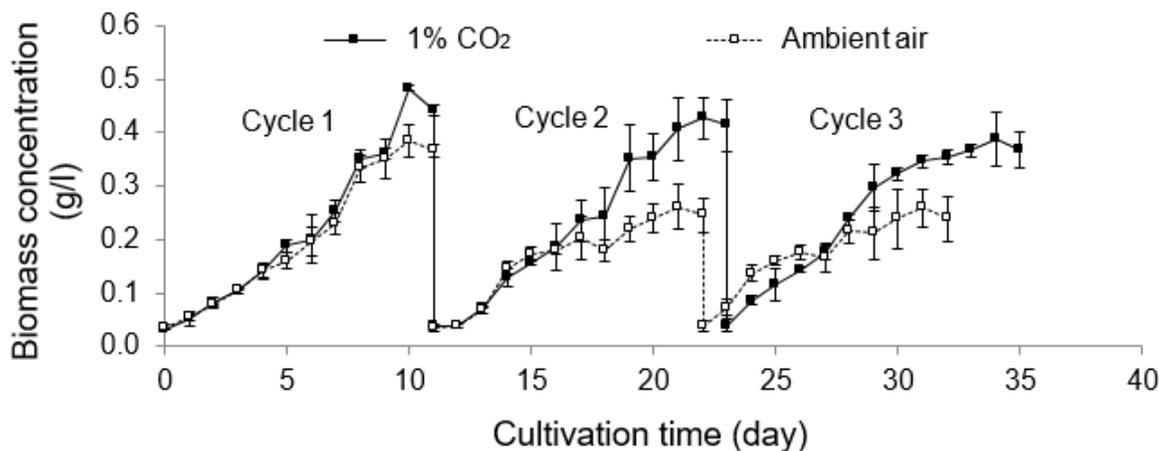


Figure 1. Biomass concentration of MC under 1% CO<sub>2</sub> and ambient supplementations.

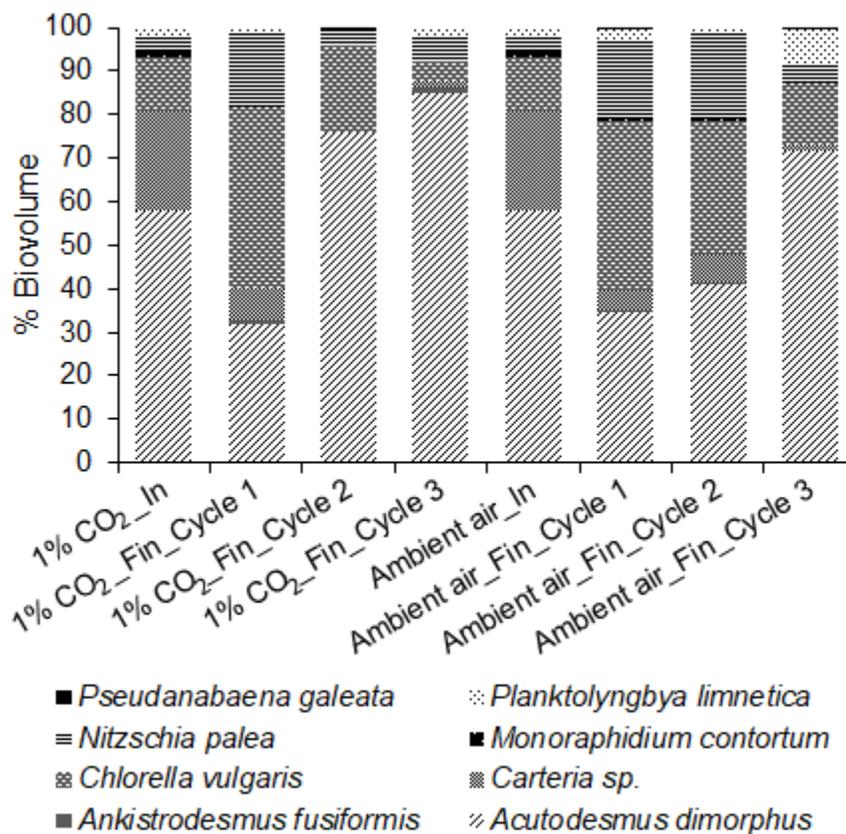
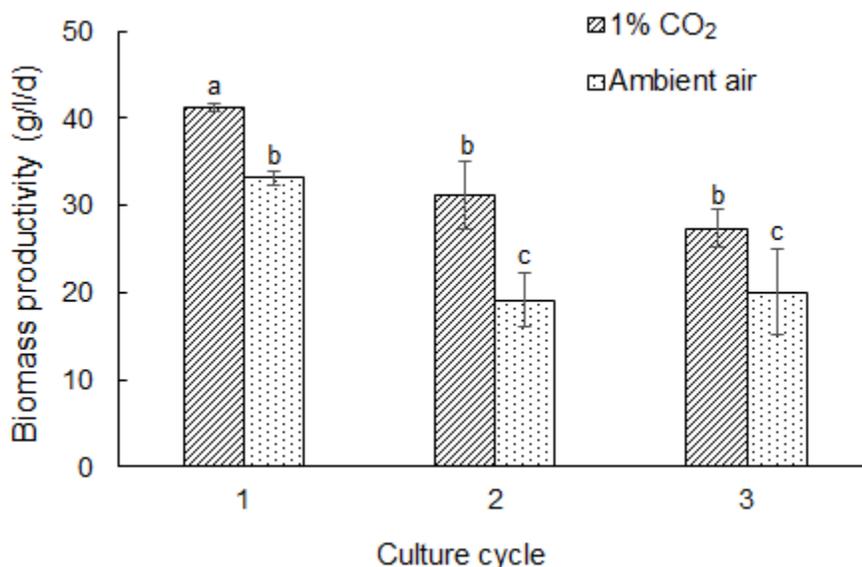


Figure 2. % Biovolume of microalgal strains in MC under 1%CO<sub>2</sub> and ambient supplementations. In = Initial day, Fin = Final day.

(data not shown). Addition of CO<sub>2</sub> to the culture medium is an alternative method that can be used to decrease the pH of the medium and prevent rapid changes. When CO<sub>2</sub> dissolves in

the medium, it will form the carbonate (HCO<sub>3</sub><sup>-</sup>) and prevents an increase in pH during the growth of the microalgae [24]. Also, microalgae can utilize HCO<sub>3</sub><sup>-</sup> and CO<sub>2</sub> as a carbon source for



**Figure 3.** Biomass productivity of the MC under 1%CO<sub>2</sub> and ambient supplementations. Letters a, b, and c indicate a significant difference ( $p < 0.05$ ) of biomass productivity between each condition.

their growth and metabolism [3, 11]. In this study, the highest dry cell weight of  $0.44 + 0.01^a$  g/l was observed in the MC cultured with 1% CO<sub>2</sub> in the first cycle, while the lowest dry weight of  $0.24 + 0.042^c$  g/l was observed in the MC cultured with ambient air in the third cycle. These results indicate that using CO<sub>2</sub> for MC cultivation could promote the growth of microalgae. Similar results were also found with *Scenedesmus quadricauda*. The alga grew faster and had a higher growth rate with 1% CO<sub>2</sub> than that with pure air [25]. Furthermore, Tanadul *et al.* [26] found that the growth rate of *Chlorella sorokiniana* was higher when cultures were grown in 2% CO<sub>2</sub> aeration compared to that in ambient air.

The biomass productivity of the MC with and without CO<sub>2</sub> supplementation are shown in figure 2. The biomass productivity of MC with CO<sub>2</sub> supplementation ranged from 27.39-41.3 g/l/d. During the cultivation process, the biomass productivity levels of the MC with 1% CO<sub>2</sub> supplementation in first cycle were significantly higher than that of all cycles with ambient air supplementation. These results are in agreement with Kin-Chung *et al.* [27], who

found that the biomass concentration of *Chlorella vulgaris* could grow well under CO<sub>2</sub> levels ranging from 1% to 5% when compared with ambient conditions. Microalgae can grow well under the CO<sub>2</sub> aerated conditions because CO<sub>2</sub> can be utilized as a carbon source, and carbon can be converted into biomass and lipids via photosynthesis [28]. Meanwhile, feeding of CO<sub>2</sub> in algal medium is helpful for controlling the pH value. Increasing the level of CO<sub>2</sub> leads to the change of pH value and dissolution of inorganic carbon and has an impact on microalgae growth [25].

#### Microalgae populations

The populations of MC were observed under light microscope. The percentage of biovolume of each microalgal species was calculated and shown in figure 3. At the initial day of MC cultivation, 5 species of green algae (57.8% *Acutodesmus dimorphus*, 0.2% *Ankistodesmus fusiformis*, 23% *Carteria sp.*, 12.2% *Chlorella vulgaris*, and 1.8% *Monoraphidium contortum*), 2 species of cyanobacteria (1.9% *Planktolyngbya limnetica* and 0.1% *Pseudanabaena galeata*) and 1 species of diatom (3% *Nitzschia palea*) were found in both culture conditions (with and

without CO<sub>2</sub> supplementations). At the end of the first cycle, the MC under 1% CO<sub>2</sub> and ambient air conditions had equal amount of biovolume ratio and the most dominant species observed were *A. dimorphus* (32-34.6%) followed by *C. vulgaris* (39-41.6%) and *N. palea* (16.7-18.4%). During the semi-continuous cultivation using 1% CO<sub>2</sub>, it was clearly seen that the % biovolume change of *A. dimorphus* between the end of the third and the first cycles of cultivation period was dramatically increased (2.7-fold) and followed by *A. fusiformis* (2.3-fold). Nevertheless, a significant negative % change was found for *C. vulgaris* (9.0-fold loss). Interestingly, % biovolume of *A. dimorphus* at 1% CO<sub>2</sub> supplementation in the third cycle of cultivation was 1.2-fold higher than that at the ambient air supplementation while the biovolume ratio of *P. limnetica* was 5.3-fold lower. These results indicated that CO<sub>2</sub> supplementation has positively impacted on population dynamics control in mixed microalgae cultivation. Moreover, many researchers reported that *A. dimorphus* is the one of the oleaginous microalgae which might be appropriate strain for biofuel production [29-31]. Therefore, this study suggested that using CO<sub>2</sub> in MC cultivation could help to control the microalgae population and enhance the suitable species for biofuel production.

**Table 1.** Characteristics of MCB samples.

Characteristics	Aeration	
	1% CO <sub>2</sub>	Ambient air
<b>Proximate analysis (wt%)</b>		
Moisture (adb)	6.84	6.52
Volatile Matter (db)	78.10	75.49
Ash (db)	14.83	20.68
Fixed Carbon (db)	7.07	3.84
<b>Ultimate analysis (wt%)</b>		
Carbon	45.68	40.60
Hydrogen	9.10	6.55
Nitrogen	3.86	4.00
Oxygen*	41.37	48.85
Sulfur content (wt%)	0.33	0.29
HHV analysis (MJ/Kg)	16.37	13.40

Adb: air-dried basis; db: dried basis; \*: calculated by difference.

### MCB Characterizations

The biomass of MC in all cycles was pooled in order to investigate the characteristic. Table 1 showed some characteristics of MCB samples. According to TG analysis, the moisture content of MCB under 1% CO<sub>2</sub> and ambient air supplementations were 6.84 wt% and 6.52 wt%, respectively. In algal lipid extraction process, the moisture content of algal biomass influenced the lipid yield. Balasubramanian *et al.* [32] found that the biomass moisture content up to 5% had no impact lipid extraction efficiency but higher moisture content (above 20.6%) reduced the lipid extraction yield. In this study, the moisture content of MCB was approximately 7%, which seemed to be suitable for lipid extraction. The volatile matter and fixed carbon represent the combustible fraction of the fuel biomass and high value of them could induce the highly reaction in the thermochemical conversion process [2]. The MCB with 1% CO<sub>2</sub> contained more volatile matter (78.01 wt%) and fixed carbon (7.07 wt%) than MCB with ambient air condition. Generally, microalgae contain 2-30% ash content. However, ash content of microalgae varies among different species, cultivation conditions, harvesting processes, and so on [33, 34]. The ash content of MCB with 1% CO<sub>2</sub> supplementation (14.83 wt%) was lower than that of ambient air condition (20.68 wt%). This result indicated that the addition of CO<sub>2</sub> could enhance the volatile matter and fixed carbon, and also reduce the ash content of microalgal biomass.

Carbon, hydrogen, nitrogen, oxygen, and sulfur are the major elements in microorganism, up to 96% on a dry weight basis, especially carbon. Microalgal biomass consists of approximately 50% carbon of the dry cell weight and all of this carbon is usually received from CO<sub>2</sub> [5]. In this study, the results found that CO<sub>2</sub> had a strong effect on carbon content. The carbon content of MCB with 1% CO<sub>2</sub> supplementation (45.68 wt%) was higher than that with ambient air supplementation (40.6 wt%). This result is similar to that of Tang *et al.* [11] who reported that the carbon contents of *Scenedesmus*

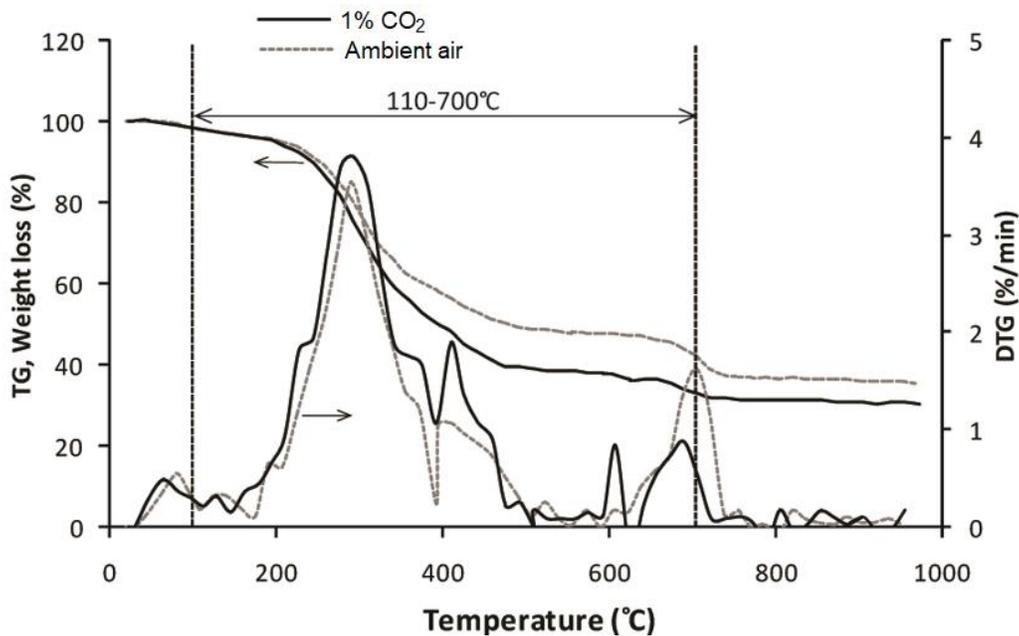


Figure 4. TG and DTG curves of MCB under 1% CO<sub>2</sub> and ambient supplementations.

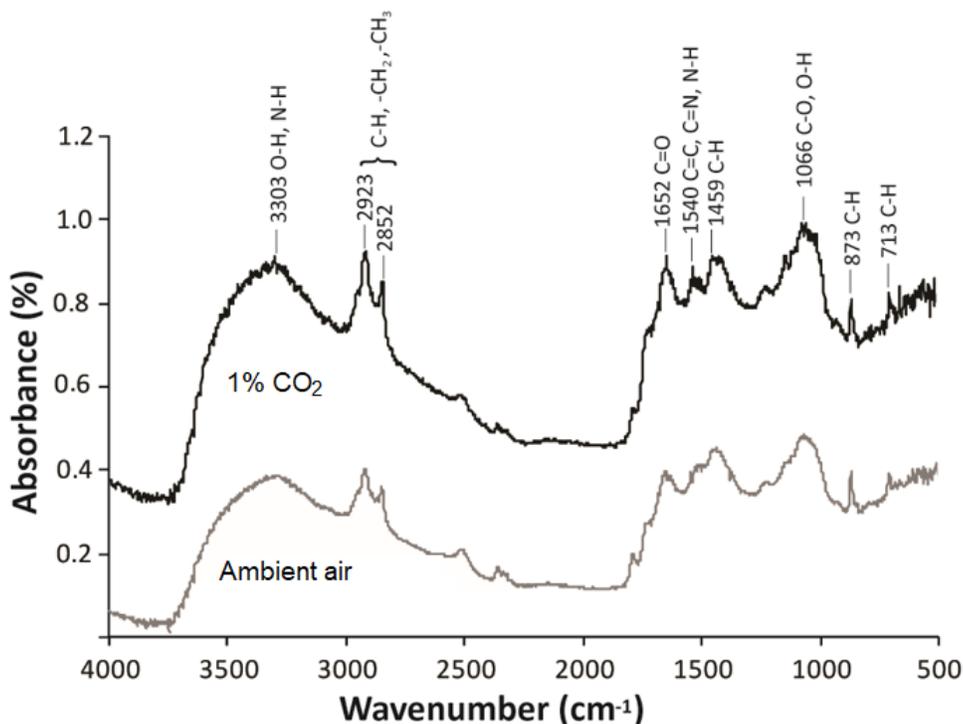


Figure 5. FTIR spectrum of MCB under 1% CO<sub>2</sub> and ambient supplementations.

*obliquus* SJTU-3 cultivated with CO<sub>2</sub> supplement (5-50% CO<sub>2</sub>, v/v) were higher than it without CO<sub>2</sub>

supplement. Moreover, we found that the HHV of the MCB with 1% CO<sub>2</sub> supplementation was

1.22 times higher than it with ambient air supplementation. These results indicated that cultivating MC with a low CO<sub>2</sub> concentration (1% v/v) enhanced the properties that make MCB suitable as a raw material for biofuel production. During cultivation, increasing the carbon source, even with just a low concentration of CO<sub>2</sub> supplement, increased the carbon content in the biomass.

### Thermal profile analysis

Thermal gravimetric (TG) and differential thermal gravimetric (DTG) curves verified the thermal profile of MCB, as shown in figure 4. The first weight loss, occurring before 110°C, was moisture loss. During the next stage of weight loss, from about 110°C to 700°C, the volatile matter decomposed. During this stage, the MCB under 1% CO<sub>2</sub> had lost 60% more weight than it in the MCB with ambient air supplementation. The DTG peaks moved to a higher temperature range as well. During the last stage (700-1,000°C), solids decomposed including both ash and fixed carbon. This thermal profile showed that MCB with CO<sub>2</sub> supplementation was a suitable feedstock as substrate in the thermal process (e.g., pyrolysis and gasification).

### FTIR analysis

Figure 5 shows the FTIR spectrum of MCB with CO<sub>2</sub> and ambient air supplementation. The major intense peak around 3,303, 2,923, 2,852, 1,652, 1,540, 1,459, 1,066, 873, and 713 /cm. The O-H stretching and amides group were observed at 3,303 /cm [35]. Peaks at 2,852 and 2,923 /cm were caused by aliphatic groups [35, 36]. C=C stretching was observed at peak 1,540 /cm that could be attributed to unsaturated aliphatics, aromatics, and alkenes [35]. Oxygen groups were found at peaks 1,652 /cm (C=O vibrations in Carboxylic acid, ketone, aldehydes, and esters) and 1,066 /cm (C-O stretching vibrations in ethers, alcohols, polysaccharides, and O-H bending vibrations) [35-37]. Peaks at 873 and 713 /cm were attributed to aromatics and alkenes C-H bending vibrations [36, 37]. The FTIR analysis results showed the presence of aliphatic, carboxylic acid, alkenes, alcohols, and

esters, which indicated that MC has potential suitability as a biofuel feedstock (e.g., biodiesel and bioethanol).

### Lipid production of microalgae

The lipid contents of the MC samples were analyzed. As shown in table 2, the lipid content levels of the MC cultivated with 1% CO<sub>2</sub> supplementation of all cycles (16.75-18.18%) were higher than that with ambient air supplementation (7.92-11.39%). These results are similar to that reported for *Spirulina* sp. and *Chlorella fusca* [24]. According to the results of lipid productivity, it was clearly seen that the supplementation of CO<sub>2</sub> in the MC cultivation could enhance the lipid productivity of microalgae. The means of lipid productivity levels of the MC cultivated with CO<sub>2</sub> supplementation (6.26 + 0.97 g/l/d) were higher than that with ambient air supplementation (2.76 + 1.01 g/l/d).

**Table 2.** Lipid content and lipid productivity of the MC under 1% CO<sub>2</sub> and ambient air supplementation.

Aeration	Culture cycle	Lipid content (%)	Lipid productivity (g/l/d)
1% CO <sub>2</sub>	1	16.75±0.96 <sup>a</sup>	7.37±0.42 <sup>a</sup>
	2	16.98±1.66 <sup>a</sup>	5.85±0.57 <sup>b</sup>
	3	18.18±1.53 <sup>a</sup>	5.57±0.47 <sup>b</sup>
	Mean	17.30±0.77	6.26±0.97
Ambient air	1	10.34±0.95 <sup>b</sup>	3.79±0.35 <sup>c</sup>
	2	7.92±0.33 <sup>b</sup>	1.76±0.07 <sup>d</sup>
	3	11.39±4.47 <sup>b</sup>	2.72±1.07 <sup>c</sup>
	Mean	9.88±1.78	2.76±1.01

Letters a, b, and c indicated significant differences ( $p < 0.05$ ) between the MC under 1% CO<sub>2</sub> and ambient air supplementations.

### Fatty acids composition

The fatty acids composition of extracted lipid from MCB samples were analyzed using GC-MS, as shown in table 3. The major fatty acids observed under both conditions with 1% CO<sub>2</sub> and ambient air were palmitic acid (C16:0) and oleic acid (C18:1), which were the common compounds that can be found in plant oils and animal fats, and serve as the substrate for

biodiesel production [38]. In addition, it was demonstrated that CO<sub>2</sub> supplementation in the microalgae culture had positively effect on fatty acid profile of algal lipid. The percentage of fatty acids, for example, palmitic acid and oleic acid from the MCB cultured with 1% CO<sub>2</sub> supplementation were higher than those without CO<sub>2</sub> supplement. This result was in good agreement in literatures [15, 39, 40].

**Table 3.** Fatty acids composition of extracted lipid from the MC under 1% CO<sub>2</sub> and ambient air supplementation.

Fatty acid	Composition %	
	1% CO <sub>2</sub>	Ambient air
Myristic acid (C14:0)	1.68	1.13
Palmitic acid (C16:0)	39.78	34.51
Palmitoleic acid (C16:1)	4.68	3.60
Stearic acid (C18:0)	3.77	3.02
Oleic acid (C18:1)	46.25	42.70
Linoleic acid (C18:2)	2.07	0.93
Others	1.76	14.10

### Conclusion

Supplementation of 1% v/v CO<sub>2</sub> in the semi-continuous cultivation of MC could support the biomass and lipid productivity of microalgae as well as help to control the culture pH during microalgae cultivation. CO<sub>2</sub> addition also could help to control the microalgae population and enhance the suitable species for biofuel production. Therefore, cultivating microalgae with low CO<sub>2</sub> concentration is highly suitable for applications in large scale biofuel production.

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