CHAPTER 3

Effect of CO_2 concentrations on growth, lipid production and CO_2 fixation of microalgal consortium

3.1 Introduction

Microalgal biomass contains approximately 0.5 g carbon of g dry weight. All of this carbon is typically derived from atmospheric CO_2. Theoretically, microalgae could fix about 183 tons of CO_2 in producing 100 tons of biomass (Chisti, 2007). Thus, biological CO_2 sequestration using photosynthetic microalgae for CO_2 mitigation has received widespread attention. Moreover, microalgae possess a number of advantages in terms of CO_2 sequestration and bio-oil production when compared to other forms of plant feedstock, including (i) photosynthesizing more efficiently, (ii) higher biomass and lipid yield in terms of land area requirement, (iii) faster growth, (iv) the ability to fix CO_2 faster, (v) the potential be grown in a liquid medium, which is easy to handle (vi) the ability to be grown in diverse ecosystems and (vii) the fact that it is non-competitiveness with other food sources (Chisti, 2007; Zeng *et al*., 2011; Gupta *et al*., 2012).

A number of microalgal species have been reported to be able to tolerate relatively high CO_2 levels. Some species of microalgae can grow in 10-100% v/v CO_2 but the maximum biomass was observed under conditions of lower CO_2 concentrations (below 20% v/v CO_2) (Ono and Cuello, 2003; Ge *et al*., 2010; Rosa *et al*., 2011;Tang *et al*., 2011). Therefore, this study was aimed at investigating the effect of high CO_2 levels on microalgae growth in the lab scale. In the first experiment, the microalgal consortium (MC) was treated with different levels of aeration: non aeration (control), ambient air (0.03% CO_2) and 10% CO_2. Next, the effect of CO_2 concentrations (0.03%-30% v/v CO_2) on algal biomass, lipid production, and CO_2 fixation was investigated.

3.2 Materials and methods

The methods used to study the effect of CO_2 concentrations on growth, lipid production and CO_2 fixation of MC is presented in Figure 3.1

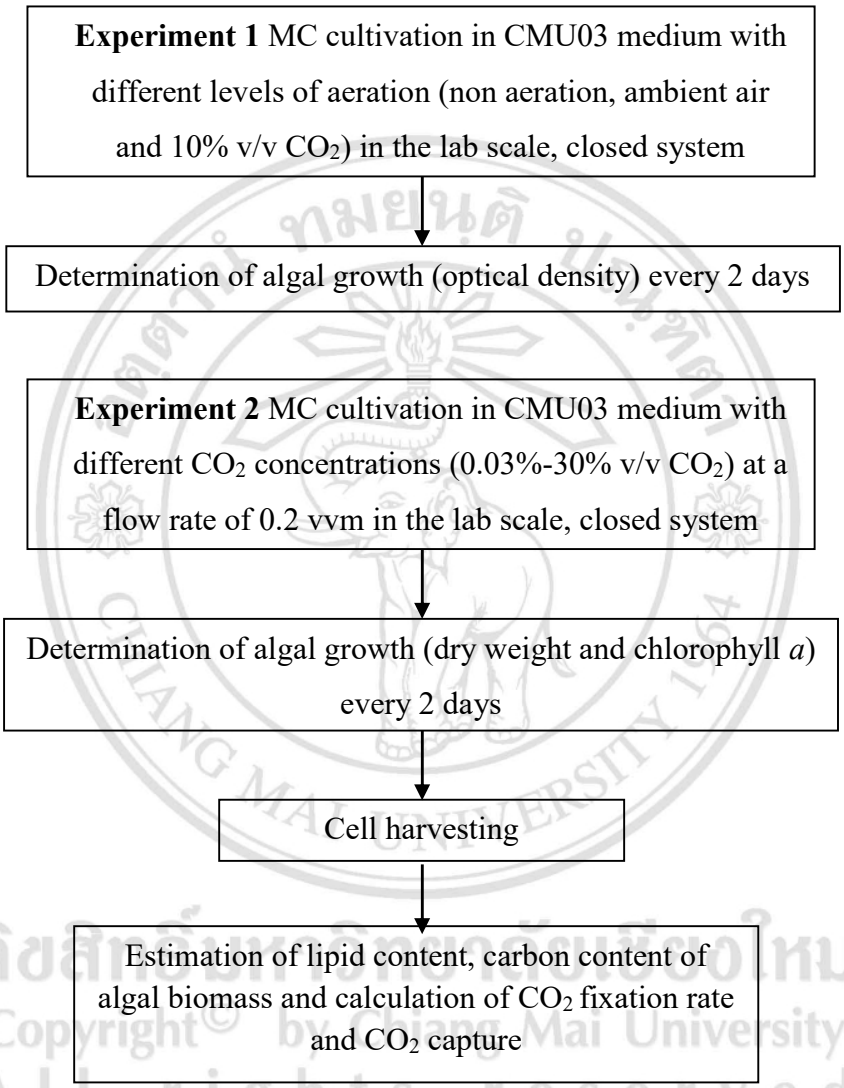


Figure 3.1 Flowchart diagram of microalgal cultivation to study the effect of different aeration levels and CO_2 concentrations on microalgal growth

3.2.1 Algal and culture

MC was prepared by succession of air borne cultures enriched with CMU03 medium (Sriphuttra *et al*., 2013) and maintained in the same medium at the algal collection facility of the Applied Algal Research Laboratory (AARL),

Department of Biology, Faculty of Science, Chiang Mai University. The MC specimens (composed of 23% *Chlorella vulgaris*, 20% *Monoraphidium contortum*, 19% *Acutodesmus obliquus*, 13% *Dictyosphaerium granulatum*, 5% *Chlamydomonas crassa*, 5% *Pediastrum duplex*, 4% *Coelastrum astroideum*, 3% *Closterium gracile*, 2% *Actinastrum hantzschii*, 1% *Ankistrodesmus fusiformis*, 1% *Pandorina* sp., 1% *Pinnularia* sp. and 1% *Pseudanabaena galeata*) were incubated at ambient temperatures under continuous illumination for two weeks.

3.2.2 Cultivation of MC

In the first experiment, the MC was cultivated in a 1 L modified laboratory glass bottle with 500 mL working volume of CMU03 medium in a closed system. The cultures were incubated with different levels of aeration: non aeration (control), ambient air (0.03% v/v CO_2) and 10% v/v CO_2 (balanced with N_2) at a flow rate of 0.2 volume-to-volume per minute (vvm), under ambient temperatures and continuous illumination with a fluorescent lamp at 24.3 µmol m^{-2} s^{-1}. The microalgal growth was determined by optical density measurement at 665 nm using a Thermo Spectronic Genesys 20 spectrophotometer (Devi Prasad, 1983). The optical density measurement was performed every 2 days throughout the growth cycle.

In a second experiment, the effects of high CO_2 on growth, lipid content and CO_2 fixation of microalgae were investigated. The MC was cultivated in a 1 L modified laboratory glass bottle (Figure 3.2) with 500 mL working volume of CMU03 medium in a closed system. The cultures were aerated with different CO_2 concentrations: ambient air (0.03% CO_2), 10% CO_2 and 30% v/v CO_2, balanced with N_2 and a gas flow rate in all experiments of 0.2 vvm. The cultures were illuminated by a fluorescent lamp providing continuous light of 24.3 µ mol m^{-2} s^{-1} at ambient temperatures. Figure 3.3 shows the setup of the cultivation systems. Each treatment was conducted in triplicate. The growth of the microalgae, dry weight and chlorophyll *a*, was determined every 2 days until the early stationary phase was reached.

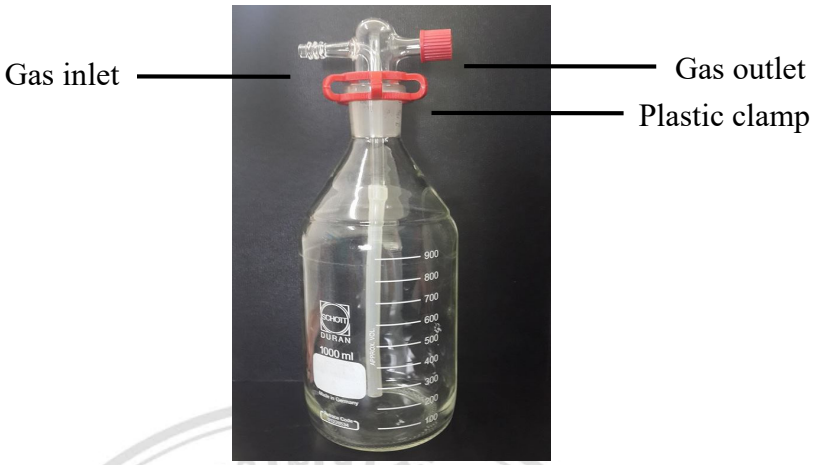


Figure 3.2 Modified laboratory glass bottle

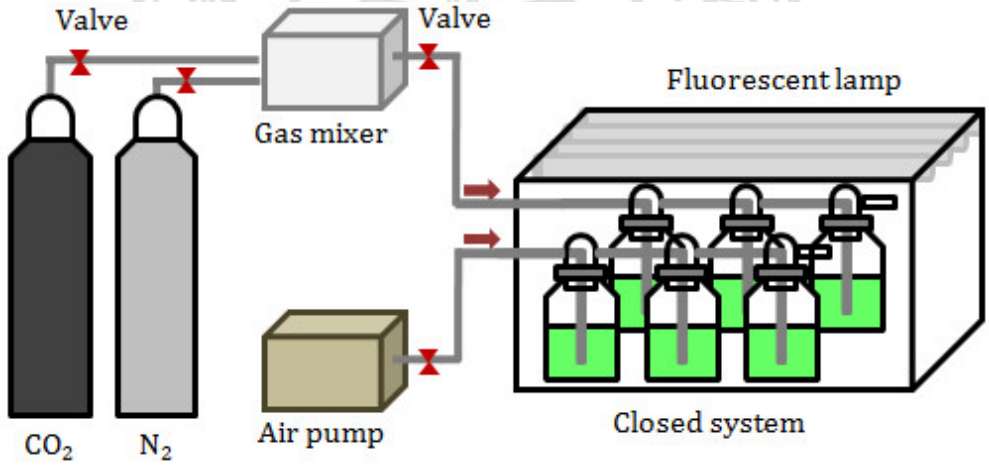


Figure 3.3 Schematic diagram of cultivation of MC under different CO_2 concentrations

3.2.3 Microalgal dry weight

The dry weight of the microalgae was measured using a modified method of Yoo *et al*. (2010). A known volume of MC suspension was filtered through pre-weighted Whatman's GF/C glass microfiber filters (pore size 0.45 µm, 47 mm diameters) using vacuum filtration equipment. The filtered algal samples were washed by 20 mL distilled water. Then, filters were dried at 60°C for 48 h. The microalgal biomass was calculated in terms of dry weight (g L^{-1}), which was determined gravimetrically.

3.2.4 Chlorophyll *a* determination

Chlorophyll *a* content was determined according to Wintermans and De Mots (1965) and Saijo (1975). A known volume of microalgal suspension was filtered through a glass microfiber filter. The 10 mL of 90% methanol was added to the algal sample and incubated at 70°C for 20 min. The mixer was centrifuged (3,000 rmp, 10 min, 4°C) and the pooled extract was spectrophotometrically measured at 630, 645, 665 and 750 nm. Calculations were made using the following equation:

$$\text{Chl } a = \frac{(11.6(\text{A}_{665} - \text{A}_{750}) - 1.31(\text{A}_{645} - \text{A}_{750}) - 0.14(\text{A}_{630} - \text{A}_{750}))v}{V \times l}$$

Where Chl a represents chlorophyll *a* content (μg L^{-1}), v is the volume of the extract (mL), V is the volume of the sample filtered (L) and l is the path length of the spectrophotometer cuvette (cm).

3.2.5 Lipid estimation

The lipid content of MC was estimated using a procedure adapted from Bligh and Dyer (1959). 15 mL chloroform: methanol (2:1, v/v) was added to 0.5 g of dry microalgal biomass and then it was placed in an ultrasonic bath (40 kHz, 40°Cfor 1 h). The solvent layer was separated by centrifugation at 6000 rpm for 10 min. The solvent layer was evaporated to dryness at room temperature. Lipid contents were measured gravimetrically.

3.2.6 Biomass and lipid productivity

The biomass productivity was calculated according to the following equation (Tang *et al*., 2011):

$$P_{biomass} = \frac{(X_1 - X_0)}{(t_1 - t_0)}$$

Where $P_{biomass}$ represents biomass productivity (mg $L^{-1}d^{-1}$), X_0 is the initial biomass (mg L^{-1}) at time t_0 (d) and X_1 represents the final biomass (mg L^{-1}) at any time t_1 (d)

The lipid productivitywas calculated according to the following equation (Yadavalli *et al*., 2013):

$$P_{lipid} = \frac{C_{lipid} \times \text{DCW}}{\text{Time}}$$

Where P_{lipid} represents lipid productivity (mg $L^{-1}d^{-1}$), C_{lipid} is the lipid content of the cells (mg g^{-1}) and DCW is the dry cell weight of microalgal biomass (mg L^{-1}), and Time represents the cultivation period in days.

3.2.7 Estimation of CO_2 fixation rate and CO_2 capture

The CO_2 biofixation rate of MC was calculated according the method of Yun *et al*. (1997), as shown in the following equation:

$$R_{CO_2} = C_C \times P \times \left(\frac{M_{CO_2}}{M_C}\right)$$

Where R_{CO_2} represents the CO_2 fixation rate (g CO_2 L^{-1} d^{-1}), P is the algal biomass productivity (g L^{-1} d^{-1}), respectively. M_{CO_2} is the molecular weight of CO_2, M_C is the molecular weight of elemental carbon and C_C is the carbon content (g dw^{-1}) measured by an CHNS/O elemental analyzer (PE2400 SeriesII, Perkin Elmer).

CO_2 capture (g CO_2 dw^{-1}) was calculated from the ratio of fixation rate to biomass productivity (Taher *et al*., 2015).

3.2.8 Microscopic observation

Microalgal species of MC were observed under a 20X and 40X light microscope (Olympus C011) and photographed using an Olympus Normaski microscope. The microalgal species identification was performed according

to relevant keys, *i.e.* Huber-Pestalozzi (1983), Hindák (2008), and John *et al.* (2011). 300 cells in number were counted under a light microscope and the biovolume of dominant algal species were estimated after counting and identification were complete by using the method of Hillebrand *et al.* (1999). The biovolume of each species were calculated in terms of percentage of total algal biovolume.

3.2.9 Statistical analysis

The results are expressed as mean±SD (standard deviation) of three replicates. All data were performed by SPSS version 16.0 for Windows using one-way analysis of variance (ANOVA) and least significant difference (LSD) test in order to evaluate the differences among the three CO_2 concentration levels. Differences were considered statistically significant at $p<0.05$.

3.3 Results and discussions

3.3.1 Effect of different aerations on growth of microalgae

In the first experiment, the MC was cultivated at different aeration levels: non aeration (control), ambient air (0.03% CO_2) and 10% CO_2. During the cultivation period, the ambient temperature ranged from 26.5 to 32.5°C. Figure 3.4 shows the growth curve of MC under different aeration levels. The growth curve showed that the MC aerated with 10% CO_2 grows rapidly at the beginning of cultivation, and was followed by a stationary phase. The cell concentration reached the maximum on the 22nd day and decreased thereafter. The maximum cell concentration of the MC under 10% CO_2aeration was about 0.43, while the maximum cell concentration of the control, ambient air (0.03% CO_2) were 0.047 and 0.07, respectively. These results indicate that using CO_2 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_2 than with pure air (Xiao *et al*., 2011). Furthermore, Tanadol *et al.* (2010) found that the growth

rate of *Chlorella sorokiniana* was higher when cultures were grown in 2% CO_2 aeration compared to ambient air. Microalgae can grow well under the CO_2 aerated conditions because CO_2 can be utilized as a carbon source and carbon can be converted into biomass and lipids via photosynthesis (Jajesniak *et al*., 2014).

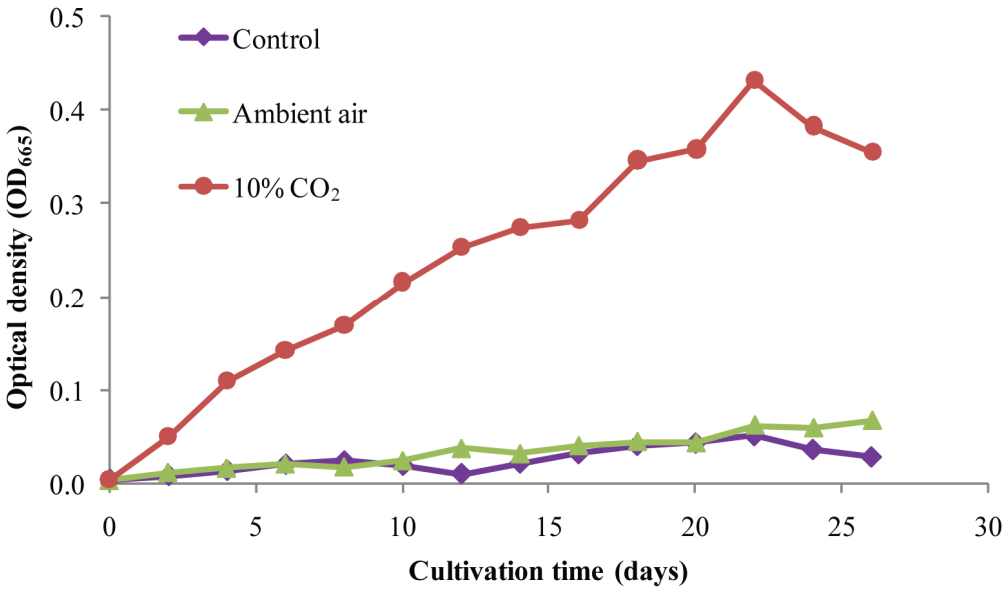


Figure 3.4 Growth curve of MC under different aeration levels

3.3.1 Effect of different CO_2 concentrations on growth of microalgae

The concentration of CO_2 supplied to the algal cultivation is one of the main factors affecting microalgal growth (Yue *et al*., 2005). In a second experiment, three parallel samples were cultivated. One was conducted with ambient air and the other with 10% and 30% (v/v) CO_2 to investigate the effects of CO_2 on microalgal growth, lipid production, and CO_2 fixation. The CO_2 levels in the range of 10-30% was selected for this study because it approximated the level of CO_2 in a typical industrial exhaust gas. The effects of different CO_2 concentrations on the growth of MC are shown in Figure 3.5.

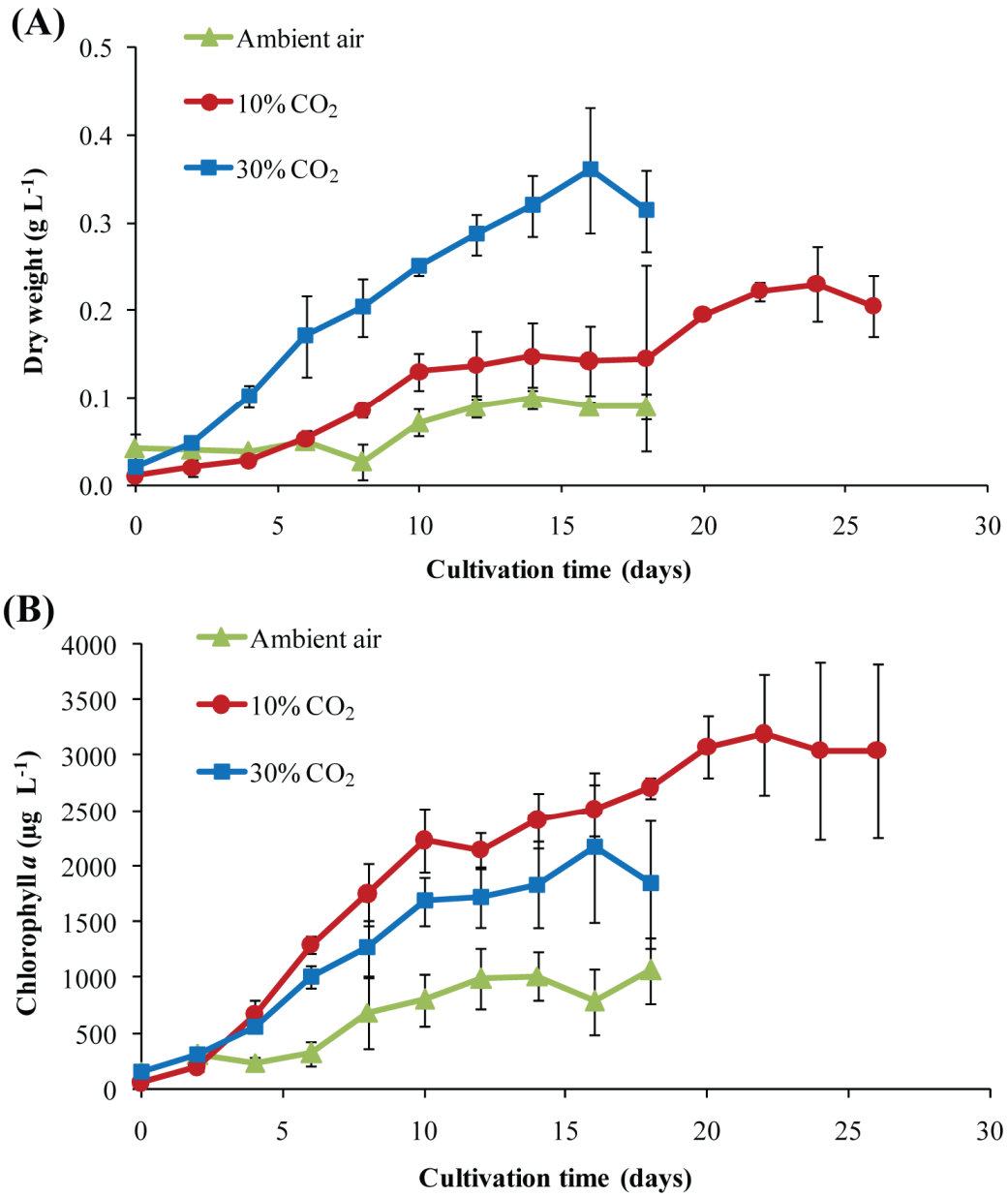


Figure 3.5 Dry weight (A) and Chlorophyll *a* contents (B) of the MC under different CO_2 concentrations

From Figure 3.5A, it was found that the maximum microalgal dry weight was 0.36±0.07 g L^{-1} on day 18 with 30% CO_2, while under ambient air conditions and (0.03% CO_2) and 10% CO_2 aeration level, maximum dry biomass readings were 0.1±0.03 and 0.23±0.06 g L^{-1}, respectively. These results were similar to those of Jeong *et al*. (2003), who studied the effects of CO_2 on the growth of *Chlorella vulgaris*. They found that *C. vulgaris* grows very slowly

and the growth rate was lowest when only utilizing 0.035% v/v CO_2 in the air. With 30% (v/v) CO_2 concentration, the growth rate was higher than under the other conditions. Tang *et al*. (2011) found the two green algal strains *Scenedesmus obliquus* SJTU-3 and *Chlorella pyrenoidosa* SJTU-2 could grow in CO_2 concentrations ranging from 0.03% to 50% but the maximum yield of algal biomass was observed at 10% CO_2 supplement with 1.8 and 1.5 g L^{-1} of *S. obliquus* SJTU-3 and *C. pyrenoidosa* SJTU-2, respectively. Rendón *et al.* (2013) reported that the biomass production of *C.vulgaris* UTEX 26 increased with increasing CO_2 concentration levels under all light sources (white, blue, red and red+blue). The highest biomass reading of 1.59 was obtained from the algae with 8.5% CO_2 under white light. The lowest biomass reading of 0.148 g L^{-1} was found in ambient air (0.035% CO_2) under red light. In this study, the maximum dry weight of MC was observed at 30% CO_2 supplement. It was noted that the most CO_2 tolerant microalgae in the mixed microalgal community were enriched and determined by high CO_2 concentrations.

In this experiment, under ambient air conditions of (0.03% CO_2), 10% and 30% CO_2 aeration levels, chlorophyll *a* content reached 1073.04$\pm$294.14, 3193.32$\pm$795.14 and 2179.67$\pm$674.55 µg L^{-1} on day 18, 22 and 16, respectively (Figure 3.5B).

From all of the results on microalgal growth, the findings associated with the chlorophyll *a* contents show a difference from the dry biomass concentration findings. The chlorophyll *a* content of the 10% CO_2 concentration level was higher than the 30% CO_2 concentration level. These results indicate that the chlorophyll *a* content was not related to the algal biomass. Similar results were found in *Botryococcus bruunii* 765. The chlorophyll *a* content at 2% CO_2 aeration was higher than 20% CO_2 aeration while the maximum biomass of *B. bruunii* 765 was found in 20% CO_2 aeration. (Ge *et al*., 2010). Ramaraj *et al.* (2013) studied the relationship between chlorophyll (*a*, *b*, and *a*+*b*) and the algal biomass of the mixed algal culture (the genera *Anabaena*, *Chlorella*, *Oedogonium* and *Oscillatoria*). After statistical analysis, they found that there

was no relationship found between chlorophyll *a* and the algal biomass. The chlorophyll content was dependent on various parameters such as light intensity, temperature, nutrient availability and species composition (Felip and Catalan, 2000; Boyer *et al*., 2009).

3.3.3 Lipid content of MC

According to the results of lipid content of MC (Table 3.1), this experiment seemed to indicate that the lipid contents increased with an increase of the CO_2 concentration level. The lipid content of MC with 30% CO_2 aeration showed the highest value (27.6%) followed by 10% CO_2 and ambient air (0.03% CO_2) with 24.55% and 12.96% of the dry weight, respectively. This was similar to the results of Abd El Baky *et al.* (2014), in which it was reported that the lipid accumulation of *Dunaliella salina* increased with an increase in the CO_2 concentration. The lipid content of *D. salina* was 2.33%, 5.62%, 10.28%, 28.36% and 40.65% of the dry weight with 0.01%, 0.03%, 3.0%, 9.0% and 12% CO_2 levels, respectively. Ge *et al*. (2010) studied the effects of CO_2 on the lipid contents of *Botryococcus bruunii* 765. They found that the levels of CO_2 in the culture medium had significant effects on the lipid production of the algae. The lipid content of *B. bruunii* 765 cultivated with different levels of 2%, 5%, 10% and 20% CO_2 were 10.41%, 11.21%, 12.44% and 12.71% of the dry weight, respectively.

Table 3.1 Lipid content of MC under different CO_2 concentrations

Aeration	Lipid content	
	mg L^{-1}	% of dry weight
Ambient air	13.17±0.53^{c}	12.96±0.52^{c}
10% CO_2	50.95±1.03^{b}	24.55±0.5^{b}
30% CO_2	86.49±2.11^{a}	27.6±0.68^{a}

Different letters indicate statistical differences ($p< 0.05$)

3.3.4 Biomass and lipid productivity of MC

Biomass and lipid productivity of MC under different CO_2 concentrations are shown in Figure 3.6. After 18 days of cultivation, the biomass productivity of MC under ambient air, 10% CO_2 and 30% CO_2 conditions were 2.69±0.32, 7.46±0.78 and 16.3±2.26 mg L^{-1} d^{-1}, respectively. The lipid productivity of MC under ambient air, 10% CO_2 and 30% CO_2 conditions were 0.73±0.03, 1.94±0.04 and 4.8±0.12 mg L^{-1} d^{-1}, respectively.

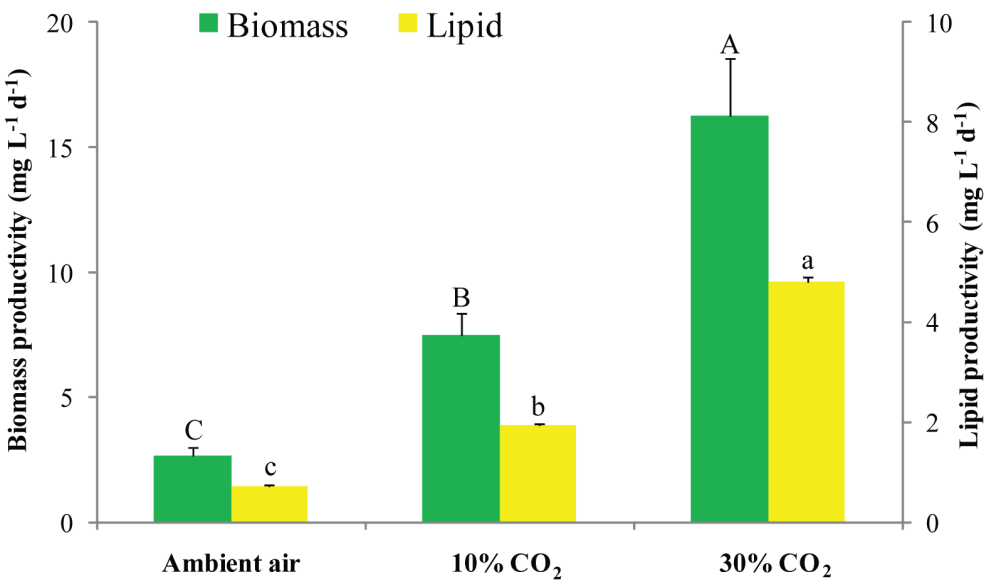


Figure 3.6 Biomass and lipid productivity of the MC under different CO_2 concentrations Letters (A, B and C) and (a, b and c) indicate a significant difference ($p<0.05$) of biomass and lipid productivity between each condition, respectively.

According to the results of biomass and lipid productivity, the MC with 30% CO_2 supplement showed the highest biomass and lipid productivity with 16.3±2.26 and 4.8±0.12 mg L^{-1} d^{-1}, respectively. Furthermore, biomass and lipid productivity at 10% CO_2 supplement was also found to be higher than at ambient air conditions. These results indicated that CO_2 concentration levels were higher than in the ambient air and this could enhance the biomass and lipid productivity of microalgae. These results were similar to Olivieri *et al.* (2012), who studied the effects of CO_2 on the lipid production of *Stichococcus bacillaris*. Indoor cultures of *S. bacillaris* were aerated with

ambient air (0.035% CO_2), 5% and 15% CO_2. They reported that high CO_2 levels improved the process performances in terms of productivity and concentration levels of biomass and lipids. The cultures in inclined bubble columns with 5% CO_2 resulted in significantly higher levels of biomass and lipid productivity (256 and 80 mg L^{-1} d^{-1}). de Morais *et al*. (2007b) found that the maximum biomass productivity of *Scenedesmus obliquus* increased with an increase in CO_2 levels (0-12% CO_2).

3.3.5 Carbon content, CO_2 fixation rate and CO_2 capture of MC

Table 3.2 shows an increase in the carbon content in algal biomass, which enhanced microalgal CO_2 fixation. Among all the conditions, the maximum carbon content was found to be 0.45g dw^{-1} with 30% CO_2 supplement followed by 10% CO_2, the ambient air readings were 0.43 and 0.4 g dw^{-1}. The carbon content of 30% CO_2 supplement coincided approximately with the carbon content of *S. obliquus* SJTU-3 and *C. pyrenoidosa* SJTU-2 (about 0.5 g dw^{-1}) when cultivated with 30% (v/v) of CO_2 concentration (Tang *et al*., 2011).

Table 3.2 Carbon content and CO_2 fixation rate of the MC under different CO_2 concentrations

Aeration	Carbon content (g dw^{-1})	CO_2 fixation rate (g CO_2 $L^{-1}d^{-1}$)	CO_2 capture (g CO_2 dw^{-1})
Ambient air	0.4000	0.0045	1.4667
10% CO_2	0.4303	0.0107	1.5778
30% CO_2	0.4529	0.0271	1.6606

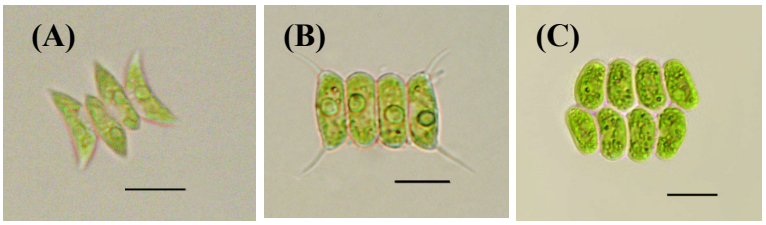
As shown in Table 3.2, the CO_2 fixation rate of the MC with 10% and 30% CO_2 supplements were higher than of those under ambient air conditions (0.03% CO_2). The CO_2 fixation rate of MC with 30% CO_2 supplement was 0.0271 g CO_2 L^{-1} d^{-1} and was 6-fold higher than at the CO_2 fixation rate (0.0045 g CO_2 L^{-1} d^{-1}) of MC cultivated with the ambient air. Hirata *et al.* (1996) obtained a similar result with *Chlorella* sp. UK001, where in a maximum CO_2 fixation rate of 0.0318 g CO_2 L^{-1} d^{-1} was obtained at 10% CO_2

concentration. Furthermore, *C. vulgaris* ARC 1 cultured with 6% CO_2 supplement could fix 0.0384 g CO_2 L^{-1} d^{-1}, which was higher than in the ambient air (0.018 g CO_2 L^{-1} d^{-1}) (Chinnasamy *et al*., 2009). Theologically, microalgae can be captured about 1.83 g of CO_2 for 1 g of produced microalgal dry biomass (Bernnan and Owende, 2010). In this study, MC with 30% CO_2 supplement could capture CO_2 at about 1.66 g dw^{-1}, it was higher than with the cultures examined in ambient air (1.47 g dw^{-1}) and 10% CO_2 (1.58 g dw^{-1}). All results in Table 3.2 indicated that high CO_2 fixation rates were observed among the MC at 30%, where CO_2 concentration levels present a good potential for use in biodiesel production with CO_2 mitigation.

3.3.6 Species composition of MC

The dominant species of MC was investigated on the first day and the last day of cultivation. The most common microalgal species found under all conditions were green microalgae such as *Acutodesmus (Scenedesmus) obliquus*[1], *Dictyosphaerium granulatum, Monoraphidium contortum* and *Chlorella vulgaris* (Figure 3.7).

[1]Currently, the genus *Desmodesmus* and *Acutodesmus* were separated from *Scenedesmus*. The genus *Acutodesmus* (A) is characterized by acute cell poles and having no mucilage surrounding the coenobia, while the genus *Desmodesmus* (B) and *Scenedesmus* (C) have ellipsoidal *cell* shape with *obtuse or truncate cell poles* (differentiated by the presence or absence of spines respectively) (An *et al*., 1999; Tsarenko and Petlovanny, 2001).



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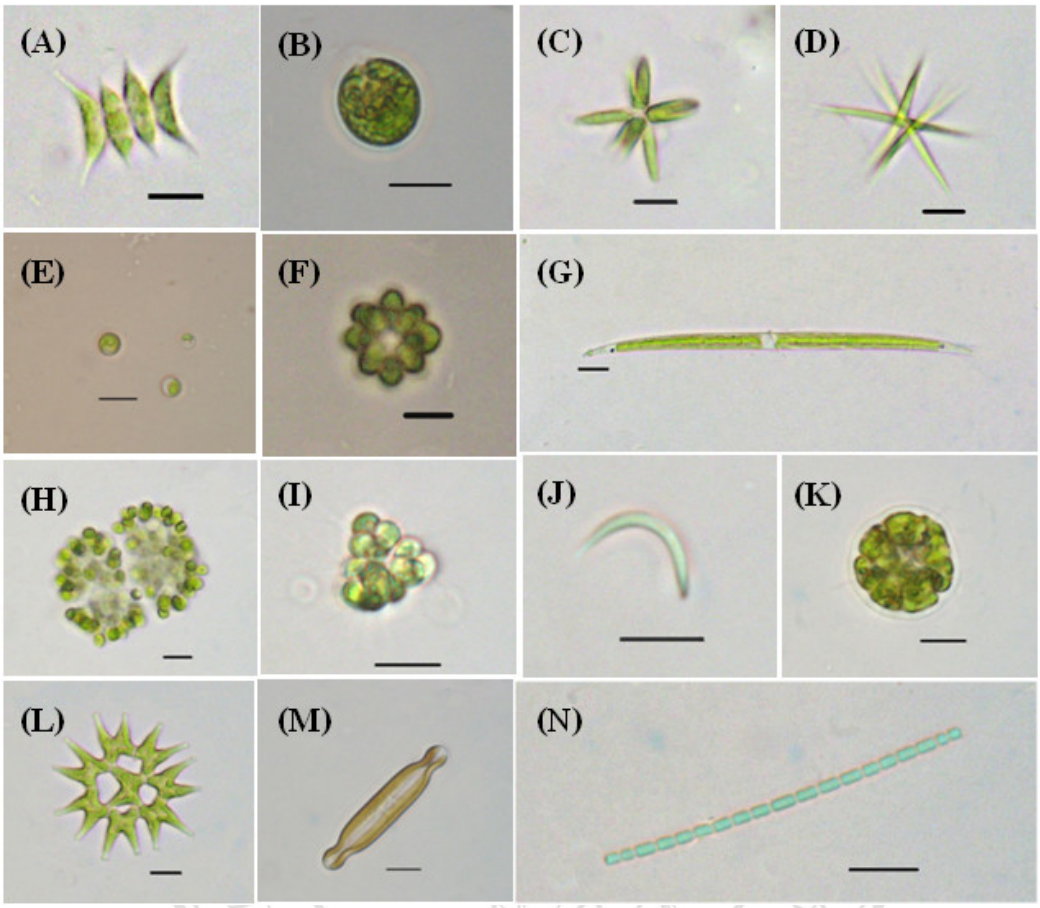

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Figure 3.7 Dominant species of MC under different CO_2 concentrations; (A) *Acutodesmus obliquus,* (B) *Chlamydomonass crassa*, (C) *Actinastrum hantzschii,* (D) *Ankistrodesmus fusiformis,* (E) *Chlorella vulgaris*, (F) *Coelastrum astroideum*, (G) *Closterium gracile*, (H) *Dictyosphaerium granulatum*, (I) *Micractinium pusillum,* (J) *Monoraphidium contortum*, (K) *Pandorina* sp., (L) *Pediastrum duplex,* (M) *Pinnularia* sp. and (N) *Pseudanabaena galeata*

The percentage of biovolume of microalgal species in MC also estimated (Figure 3.8). It was observed that the dominant population of the algal community changed during cultivation. At the early of the cultivation, the inoculum of MC in all conditions had equal amount of biovolume ratio while at the end of the cultivation, the biovolume show the change in ratio and the most dominant microalgal species observed were *Acutodesmus obliquus*. This result indicated that the simple medium, CMU03 had strongly effect on the

variations in the algal community. Previous research reported that CMU03 medium seemed to be a good selective medium and it could control the algal population of MC to be in the group of green microalgae, especially *Scenedesmus* sp. (Thurakit, 2012; Boonkhot, 2013; Lomakool, 2014).

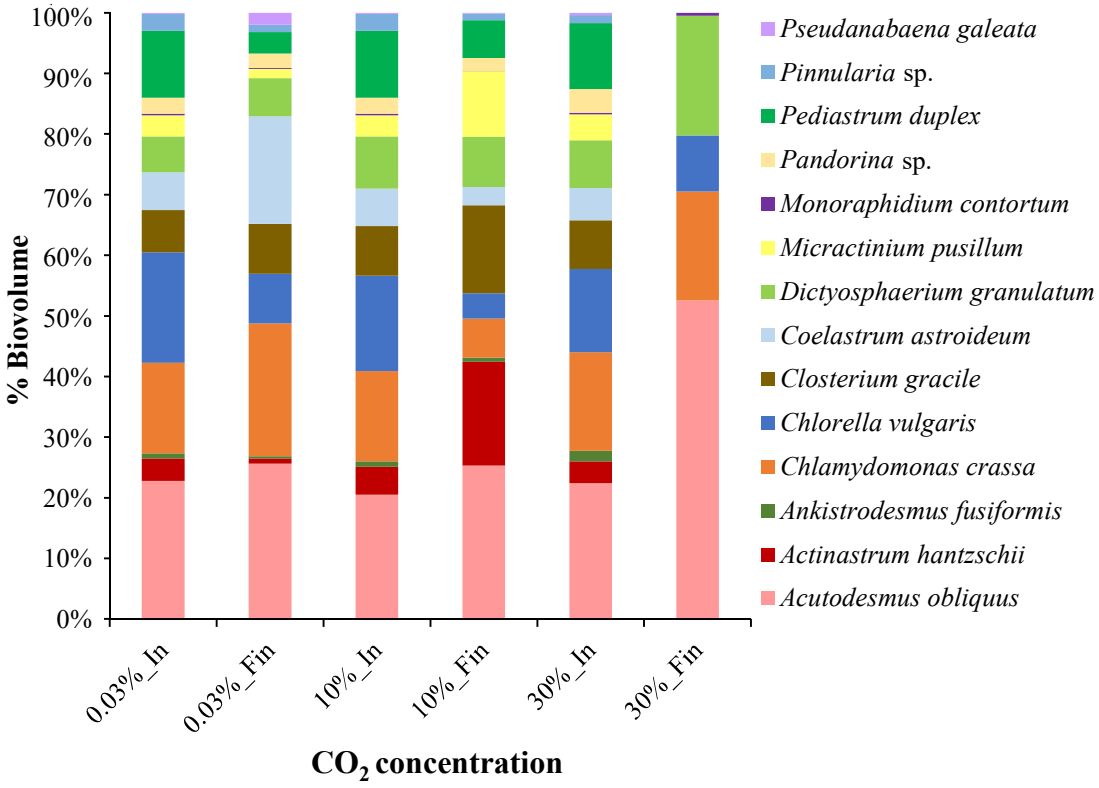

Figure 3.8 % Biovolume of microalgal species in MC cultured under different CO_2 concentrations; In = initial (first day), Fin = Final (last day)

Interestingly, % biovolume of *A. obliquus* at 30% CO_2 supplement in this study was 2.1-fold higher than the other conditions. It indicated that the variations of *A. obliquus* in the microalgal community was also affected by CO_2 cultivation. Similar to the results of Sriputthra *et al*. (2013), it was found that the dominant species were *Scenedesmus* spp. (>80% of total microalgal community) when the mixed algal cultures were cultivated under high CO_2 concentration levels. Hena *et al.* (2015) found that the biovolume ratio of *Scenedesmus* sp. increased by 50.2% than inoculum when cultured in treated

wastewater with 10% CO_2 aeration. Many researchers have reported that *Scenedesmus* sp. is one of the algal species that is most highly tolerant to CO_2. This microalga could grow under 80% CO_2 (Kumar *et al*., 2011; Salih, 2011). Moreover, it has also been reported that *Scenedesmus* sp. is suitable for biodiesel production due to the fact that it displayed a very high biomass and lipid content (Yoo *et al*., 2010; Goswami *et al*., 2011; Ren *et al*., 2013).