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Enhanced Production of Biomass and Lipids by Supplying CO_2 in Marine Microalga *Dunaliella* sp.

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Non-food-based biofuel feedstocks are in high demand worldwide. Among the various feedstocks, microalgae are the most promising feedstock for mitigating atmospheric CO_2 and producing biodiesel. In this study, various concentrations of CO_2, from 0.03 to 12%, were used to investigate their effect on the cell growth, biomass and lipid production and fatty acid composition of *Dunaliella* sp. in a closed photobioreactor. The results showed that the highest biomass and total lipids, 521 mg/L/d and 40 mg/L/d, respectively, were produced with 5% CO_2 aeration during the logarithmic growth phase. The oleic acid (18:1n9c) and elaidic acid (18:1n9t) contents were increased approximately two fold. The physiological responses of *Dunaliella* sp. at 10% CO_2 were similar to those at 5% CO_2. Therefore, the present results suggest that 5–10% is a suitable CO_2 concentration range for *Dunaliella* sp. growth to mitigate atmospheric CO_2 and increase biofuel production.

Keywords: carbon dioxide, photobioreactor, CO_2 sequestration, *Dunaliella*, biodiesel

Introduction

Recently, algae have emerged as a third-generation feedstock for biodiesel and hydrocarbon production (Scott *et al.*, 2010; Wijffels and Barbosa, 2010). Photosynthetic organisms fix CO_2 and produce O_2, and the organic materials that form biomass can be used for biodiesel production via transesterification (Shay, 1993; Lestari *et al.*, 2009). Therefore, these organisms, which mitigate atmospheric CO_2, can be utilized for the production of alternative energy. Microalgal lipids are an appropriate candidate for biodiesel feedstock because their fatty acid composition is similar to that of vegetable oils (Posten and Schaub, 2009). In general, microalgal lipid accumulation is induced and biomass productivity is reduced under stress conditions. The production of biomass and lipids by microalgae is influenced by various environmental factors, such as temperature and the concentrations of atmospheric CO_2 and salt (Sosik and Mitchell, 1994; Fu *et al.*, 2007). Therefore, one of the important characteristics desired in a feedstock for biodiesel production is high biomass productivity with enhanced lipid productivity.

Dunaliella sp., one of the microalgae most studied under laboratory and mass culture conditions, is a unicellular green alga that thrives in hyper-saline environments, enabling large-scale outdoor cultivation without contamination (Borowitzka, 1981). *Dunaliella* sp. also has a high tolerance to high temperature and high light conditions, so it is relatively easy to cultivate. To investigate the potential application of *Dunaliella* for biodiesel production, various studies have been performed. One of the important factors that affect biomass and lipid productivity is the concentration of carbon dioxide in closed photobioreactors. Using CO_2 as a carbon source significantly enhances biomass productivity. However, excessive CO_2 retards the growth of *Dunaliella*. For this reason, enhanced growth, even at high concentrations of CO_2, is required for the direct use of the CO_2-rich gas emitted by power plants or industrial exhausts in which the CO_2 concentration is 15%.

In the present study, we investigated the effect of the CO_2 concentration (0.03–12% v/v) on the production of biomass and lipids by the marine microalga *Dunaliella* sp. in a closed photobioreactor. To find the best culture condition for enhancing microalgal carbon fixation and biofuel production, the cell growth, the amounts of biomass and lipids produced, and the fatty acid profile were investigated under each CO_2 aeration condition.

Materials and Methods

Strain, medium, and culture

The green microalga *Dunaliella* sp. was originally isolated by Mordhay Avron (Park *et al.*, 2006). This microorganism was formerly named *D. salina*, but a recent molecular phylogenetic re-characterization showed that it is similar to *D. tertiolecta* (Kim *et al.*, 2010). The *Dunaliella* sp. cells were cultured in D medium with 1.0 M NaCl at 23°C. D medium [40 mM Tris-HCl (pH 7.4), 5 mM KNO_3, 4.5 mM $MgCl_2 \cdot 6H_2O$, 0.5 mM $MgSO_4 \cdot 7H_2O$, 0.3 mM $CaCl_2$, 0.1 mM K_2HPO_4, 2 μM $FeCl_3$, 20 μM EDTA, and 1 ml (per L) of micronutrient solution] is modified artificial seawater (Pick *et al.*, 1986). The micronutrient solution is composed of 50 mM H_3BO_3, 10 mM $MnCl_2 \cdot 4H_2O$, 0.8 mM $ZnSO_4 \cdot 7H_2O$, 0.4 mM $CuSO_4 \cdot 5H_2O$, 2 mM $NaMoO_4 \cdot 2H_2O$, 0.2 mM $CoCl_2 \cdot 6H_2O$, and 1.5 mM $NaVO_3$.

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Cultivation of microalgae in a photobioreactor with CO_2 aeration

Dunaliella sp. cultures were grown in a 400-ml bubble column photobioreactor (40 mm in diameter and 500 mm in height) illuminated with 120 μmol photons/m^2/s of light with a 12:12 light/dark cycle. This culture was aerated with various concentrations of CO_2, ranging from 0.03 to 12% at a feed velocity of 80 ml/min. The initial cell concentration for each condition was 1.0×10^6 cells/ml.

Growth curve and biomass

The cell growth was determined from cell numbers, and the biomass was determined from dry weight. The cells were counted using a Neubauer hemocytometer (Marienfeld, Germany) and a light microscope (CX21, Olympus Imaging Corp., Japan). To estimate the dry weight of *Dunaliella* sp., 5 ml of microalgal culture was filtered through a mixed cellulose ester membrane (Advantec, Japan). The membrane was dried in an electric oven at 65°C for 24 h and then weighed.

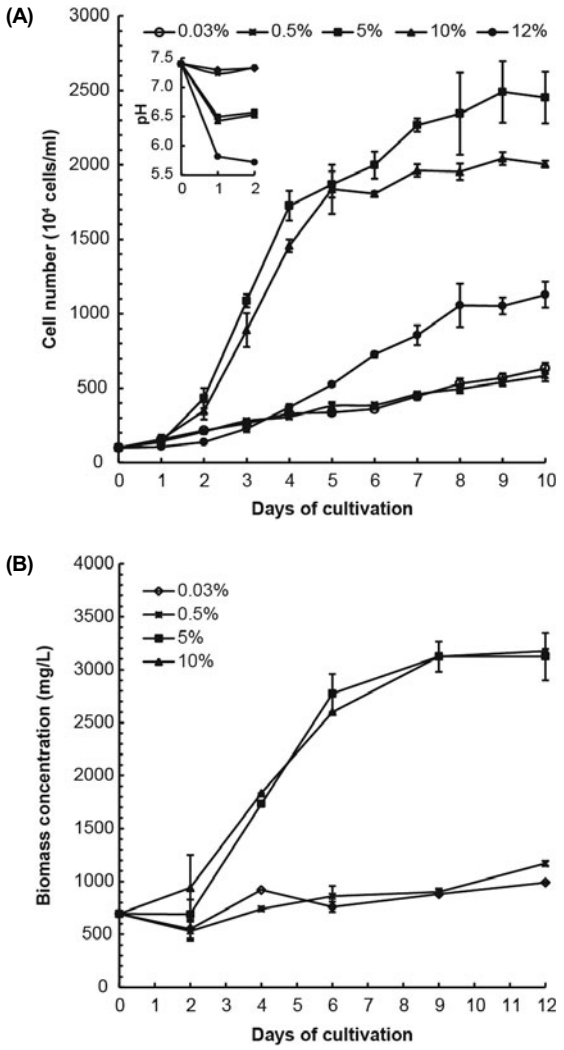

Fig. 1. Cell growth (A) and biomass production (B) in *Dunaliella* sp. cultures aerated with different concentrations of CO_2. The inset shows the changes in pH of cultures aerated with different concentrations of CO_2.

Extraction of total microalgal lipids

Lipid extraction from *Dunaliella* sp. was performed using the method adapted from Bligh and Dyer (1959). Lipids were extracted from the algal cells using methanol, chloroform, and water. Ten milliliters of culture were harvested by centrifugation at 2,000×g for 10 min. The pellets were suspended with 4 ml of dH_2O. After that, 10 ml of methanol and 5 ml of chloroform were added in sequence. After an overnight incubation in a shaker at room temperature, 5 ml of water and 5 ml of chloroform were added. The samples were centrifuged for 10 min at 2,000×g to obtain two layers. The lipid-containing lower layer of chloroform was removed by pipetting and placed in a pre-weighed aluminum dish. The chloroform was evaporated by heating on a hot plate. After evaporation, the dish was weighed again. The difference between the weight before and after evaporation was recorded as the weight of the microalgal lipids from the cultured sample.

Fatty acid methyl ester (FAME) analysis

For gas chromatography of the fatty acid methyl esters of *Dunaliella* sp., the samples were freeze-dried and weighed. Two milliliters of a mixture of acetyl chloride and methanol at the ratio of 5:100 (v/v) were added to the samples. As an internal standard, one mg of tricosanoic acid was also added. Under pure nitrogen and dark conditions, the reagents were incubated at 100°C for 1 h in a Teflon-capped Pyrex container, and then cooled to 30–40°C. Next, 1 ml of the hexane was added, and the solution was mixed by vortexing for 30 sec. For purification, the solution was salted out by adding 1 ml of saturated NaCl solution, which caused the formation of two separated liquid phases, and then the upper phase was transferred to another tube. The hexanic phase was dried under anhydrous Na_2SO_4 and injected into an HP 6890 gas chromatograph (Hewlett-Packard, USA).

Results and Discussion

Growth and biomass production of *Dunaliella* sp. in a photobioreactor

To investigate the effect of the CO_2 concentration on the growth of *Dunaliella* sp., cultured cells were incubated at 23°C under 120 μmol/m^2/s of light and aerated with different CO_2 concentrations. Interestingly, the maximal algal concentration (2.5×10^7 cells/ml) was observed on day 9 with 5% CO_2. In addition, 5% and 10% CO_2-aerated cultures exhibited similar growth patterns. In contrast, low concentrations (0.03% and 0.5%) of CO_2 had little effect on the microalgal growth compared to the high concentrations (5% and 10%) of CO_2. The tolerance of microalgae against CO_2 concentration is varied, however. *Chlorella* and *Spirulina* showed optical growth in the range of 5–10% CO_2 concentration (Kumar *et al.*, 2010). Cell growth was significantly inhibited by 12% CO_2 aeration. This effect may have been due to the high concentration of CO_2 reducing the pH of the medium (Mercado *et al.*, 1999). A pH value lower than that of the optimal culture condition inhibits microalgal growth (Kim *et al.*, 2012). With 12% CO_2 aeration, the low pH of

Table 1. Fatty acid methyl ester (FAME) composition and productivity of maximum biomass and lipid of *Dunaliella* sp. cultured with various concentration of CO_2. Data indicates the Means±SE.

	Dunaliella sp. based FAME composition (%)			
	0.03% CO_2	0.5% CO_2	5% CO_2	10% CO_2
C14:0	1.81 ± 0.44	1.20 ± 0.22	0.66 ± 0.02	0.42 ± 0.21
C14:1	0.88 ± 0.03	0.78 ± 0.07	0.38 ± 0.03	0.39 ± 0.01
C16:0	20.71 ± 0.37	21.30 ± 0.44	24.34 ± 0.07	23.39 ± 0.23
C16:1	1.74 ± 0.18	2.08 ± 0.30	1.28 ± 0.36	1.97 ± 0.19
C18:0	20.81 ± 0.52	20.85 ± 0.64	18.28 ± 0.28	18.36 ± 0.12
C18:1n9c	2.32 ± 0.10	2.17 ± 0.10	9.14 ± 0.10	8.98 ± 0.09
C18:1n9t	5.67 ± 0.27	5.51 ± 0.42	7.39 ± 0.55	7.92 ± 0.11
C18:3	46.05 ± 0.22	46.11 ± 0.31	38.52 ± 0.27	38.57 ± 0.34
∑saturated FAME	43.33 ± 0.54	43.35 ± 0.49	43.28 ± 0.27	42.17 ± 0.54
∑unsaturated FAME	56.67 ± 0.54	56.65 ± 0.49	56.72 ± 0.27	57.83 ± 0.54
Maximum biomass productivity (mg/L/d)	52.50 ± 12.50	82.50 ± 2.50	521.25 ± 13.75	415.00 ± 95.00
Maximum total lipid productivity (mg/L/d)	6.25 ± 3.75	6.25 ± 1.25	40.00 ± 7.50	35.00 ± 2.50

the medium may have inhibited cell growth.

In a previous study, *D. tertiolecta* showed the highest growth rate in the range of 2–6% CO_2 aeration, but the growth rate was remarkably reduced by 10% CO_2 aeration (Suzuki *et al*., 1995; Tang *et al*., 2011). Other studies on *D. salina* and *Dunaliella* sp. demonstrated that the optimum CO_2 concentrations at 27°C were 3.0% and 1.0% CO_2 aeration, respectively (Kim *et al*., 2012). In this study, *Dunaliella* sp. exhibited high growth and high biomass production with 10% CO_2 (Fig. 1). The differences in growth capacity at the high CO_2 concentrations found in several studies, including our present study, might be due to the species used and variations in the parameters of the growth conditions, such as the light intensity, the temperature, the bioreactor used and the feed velocity of CO_2. For these reasons, many scientists are interested in mutant microalgae that tolerate low pH and exhibit a high growth rate with a high concentration of CO_2 in the culture. A mutant *Chlorella* that tolerated high CO_2 conditions was identified, but its growth rate was slow (Chang and Yang, 2003). However, *Dunaliella* sp., which was used in this study, showed the highest growth in 5% CO_2 aerated cultures and exhibited enhanced growth, even with 10% CO_2 aeration. Therefore, *Dunaliella* sp. might have a much higher tolerance of low pH and a greater ability to utilize CO_2 than other species.

The *Dunaliella* sp. biomass was determined from the dry weight. During cultivation with 5% or 10% CO_2, the biomass of *Dunaliella* sp. dramatically increased (to 3.12 g/L), which correlated with the increase in the number of cells (Fig. 1A and 1B). The cells grown at a 10% CO_2 concentration grew slightly slower than the cells grown at a 5% CO_2 concentration. This implies that the biomass of the cells grown at a 10% CO_2 concentration is higher than that of the cells grown at a 5% CO_2 concentration. Indeed, the cells grown at a 10% CO_2 concentration were larger than the cells grown at a 5% CO_2 concentration (data not shown). As biomass production is a function of the biomass concentration and culturing period (Fig. 1B), biomass productivity is calculated based on increased amount of biomass during unit period. The maximum biomass productivity was calculated from the data during exponential periods of culture (from 2 to 6 days of culture periods), therefore, the maximum biomass productivities for 5% CO_2 and 10% CO_2 were presented as 521 mg/L/day and 415 mg/L/day, respectively (Table 1). However, at other CO_2 concentrations, the biomass barely increased (Fig. 1B). These results demonstrate that the 5% and 10% CO_2 culture conditions are appropriate for growing *Dunaliella* sp. In addition, the maximal biomass (with 5% and 10% CO_2 aeration) was similar to that of *Chlorella* or *Nannochloropsis*, which are known to be robust, industrial fresh-water algae (Griffiths and Harrison, 2009). To our knowledge, *Dunaliella* sp. has achieved the highest biomass production among *Dunaliella* species.

Effect of the CO_2 concentration on the total lipid concentration at different growth stages

The microalgal cells were harvested at different stages of growth to measure the amount of total lipid present. The results revealed that lipid production was also affected by the CO_2 concentration of the culture. Total lipid production was dramatically increased with 5% CO_2 and 10% CO_2 aeration. The maximal lipid productivity was 40.0 mg/day under the 5% CO_2 condition (Table 1). This result indicated that the 5% and 10% CO_2 culture conditions enhanced the production of both lipids and biomass by *Dunaliella* sp.

According to our study, the maximal lipid concentration (315 mg/L) was not much higher than that reported in other studies (Griffiths and Harrison, 2009). In addition, the lipid

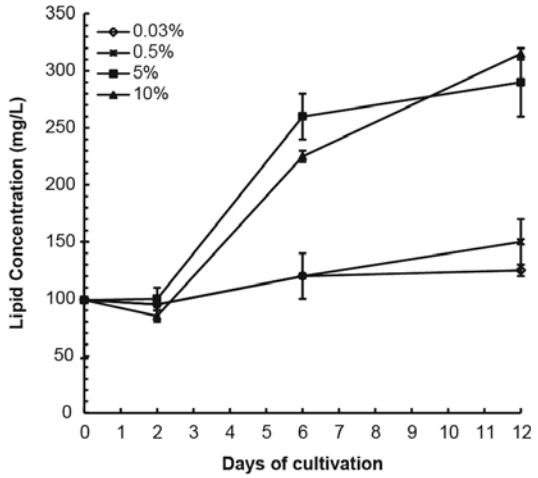

Fig. 2. Lipid production in *Dunaliella* sp. cultures aerated with different concentrations of CO_2.

content attained under the 5% and 10% CO_2 conditions were also slightly lower than those attained with lower CO_2 concentrations (Fig. 2). For example, the lipid content on day 6 under 0.03% and 0.5% CO_2 conditions was 16.10±3.90% and 14.23±1.99%, respectively. Under 5% and 10% CO_2 conditions, the lipid content decreased to 9.37±0.04% and 8.65± 0.19%, respectively. Although the lipid content per cell had decreased, the total lipid concentration had increased because of the dramatic increase in the cell number. The lipid content of microalgae is an important consideration for biodiesel production, but currently, lipid and biomass productivity are also regarded as important key indicators of biodiesel production (Griffiths and Harrison, 2009). The most well-known microalga with high lipid content is *Botryococcus braunii*, which accumulates lipids to approximately 70% of the biomass. However, its doubling time is 5 to 7 days. Therefore, its lipid productivity is lower than those of other candidate species (Banerjee *et al*., 2002). In this respect, the enhanced biomass and lipid productivity of *Dunaliella* sp. in response to high CO_2 aeration suggests that *Dunaliella* sp. has the high potential to become a feedstock for biodiesel production.

Fatty acid methyl ester (FAME) analysis

The microalgal fatty acid composition was determined using gas chromatography. The common fatty acids of biodiesel are palmitic, stearic, oleic, and linolenic acids (Knothe, 2008). In this study, the fatty acid profile included C14:0, C14:1, C16:0, C16:1, C18:0, C18:1n9c, C18:1n9t, and C18:3. The main component was linolenic acid, followed by palmitic acid and stearic acid. Interestingly, with high CO_2 aeration (5% and 10% CO_2), the contents of oleic acid (18:1n9c) and elaidic acid (18:1n9t) were increased two fold (Table 1). Although the high content of unsaturated fatty acids (such as, C18:3) may impart poor oxidative stability to the total lipids, the enhanced biomass and lipid productivity that results from CO_2 aeration may make *Dunaliella* sp. a good candidate for biodiesel production.

Conclusion

The effects of CO_2 aeration on the growth and the lipid production of *Dunaliella* sp. were investigated with respect to its potential application in biodiesel production. This microalga can tolerate high concentrations of CO_2 (5% and 10% CO_2). Moreover, the biomass production and the lipid production of *Dunaliella* sp. were both enhanced by high CO_2 aeration. Based on these results, the high growth and the increased biomass and lipid productivity of *Dunaliella* sp. obtained from aeration with a high concentration of CO_2 deem *Dunaliella* sp. a potential candidate for carbon capturing and biodiesel production.

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