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Lipid Production by a CO_2-Tolerant Green Microalga, *Chlorella* sp. MRA-1

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Since CO_2 concentrations in industrial flue gases are usually 10%–20%, one of the prerequisites for efficient CO_2 removal by algae is the level of tolerance of microalgal species to exposure to high concentrations of CO_2. A newly isolated microalgal strain, *Chlorella* sp. MRA-1, could retain growth with high concentrations of CO_2 up to 15%. The highest lipid productivity for *Chlorella* sp. MRA-1 was 0.118 g/l/day with a 5% CO_2 concentration. Octadecenoic acid and hexadecanoic acid, the main components of biodiesel, accounted for 70% of the total fatty acids. A lipid content of 52% of dry cell weight was achieved with limited amounts of nitrogen. *Chlorella* sp. MRA-1 seems to be an ideal candidate for biodiesel production when cultured with high concentrations of CO_2.

Keywords: Biodiesel, CO_2 tolerance, fatty acid composition, lipid production, microalgae

Introduction

Biodiesel derived from soybean, windmill palm, rapeseed, and other oil plants has proved eminently suitable as a transport fuel and, because of its nontoxicity, biodegradability, and renewability, continues to generate widespread interest [20, 22]. Nevertheless, a major obstacle to increasing biodiesel production using these oil plants is their relatively high cost. In addition, growing these plants requires the allocation of large amounts of land and energy resources. Although oil plants already provide a small fraction of the liquid fuel demanded by our transportation systems, biodiesel production from oil plants still faces stiff competition from entrenched fossil fuel industries [16, 22].

Because of its higher lipid productivity and photosynthetic efficiencies in comparison with oil plants, microalgal lipids are currently regarded as the third-generation feedstock for sustainable biodiesel production [6, 7]. Furthermore, microalgae cultivation does not compete with food sources and can generate valuable co-products [31, 33]. Generally, phototrophic microalgal growth requires CO_2 as a carbon source. In recent years, bioregenerative methods, particularly those using microalgae photosynthesis, have been implemented to reduce atmospheric CO_2 to ensure a safe and reliable living environment [11, 24]. Absorption of CO_2 using microalgae could convert CO_2 into microalgae biomass [24, 30], and producing 100 tons of algal biomass fixes roughly 183 tons of CO_2 [6]. At the same time, flue gases from power plants contain 10%–20% (v/v) CO_2, which may provide a carbon source for large-scale microalgal cultivation [36]. Direct utilization of flue gas reduces the cost of pretreatment, but imposes extreme conditions on microalgae–such as high concentrations of CO_2. Although most microalgae grow well under conditions of CO_2 concentrations from 1% to 5%, only a few highly CO_2-tolerant species able to withstand high CO_2 concentrations (> 5%) have been found [1, 8, 15, 33].

For biodiesel production, the lipid content is another important factor in selecting microalgae species. Many microalgae can accumulate lipids due to excess photosynthesis, and some species can accumulate large amounts of lipids from heterotrophic or environmental stress factors, such as

nutrient deficiency [28]. In this study, the newly isolated microalgal strain *Chlorella* sp. MRA-1 was evaluated for its CO_2 tolerance, and the effects of CO_2 and nitrogen concentrations on growth and lipid accumulation were investigated.

Materials and Methods

Strain and Culturing Conditions

Chlorella sp. MRA-1 was isolated from a wastewater pond in Qingdao, China, and conserved at the China General Microbiological Culture Collection Center (CGMCC 4652; China). The strain was cultivated in a 500 ml glass bubble column (30 mm in diameter) containing 300 ml of BG-11 medium with 1.5 g/l $NaNO_3$. The columns were maintained at 25 ± 2°C, bubbled with sterile gas composed of air supplemented with varying CO_2 concentrations (5%–30%). Trichromatic lamps (Y228-T5; NVC Lighting Technology Corporation, China), with spectra ranging from 380 to 780 nm, were used as light sources. Microalgal cells were grown under low illumination (50 μmol photons m^{-2} s^{-1}) for 3 days, and then subjected to high illumination (220 μmol photons m^{-2} s^{-1}) for another 10 days [19].

To assess the effects of different nitrogen concentrations on the growth and lipid accumulation of microalgae, we prepared four samples with an initial $NaNO_3$ concentration of 0.375, 2.5, 5, and 10 g/l; and blowing air supplemented with a 5% CO_2 concentration. The growth of microalgae was monitored using a UV/VIS spectrophotometer at 750 nm (UNICO 2600, Shanghai, China), and the final biomass was determined by measuring the dry cell weight (DCW). After 13 days, a 5 ml microalgae sample was taken and filtered through preweighted 0.8 μm microporous filter paper. The filter paper was oven-dried for 6 h at 105°C. The difference between the final weight and the weight of the paper before filtration was taken as the DCW.

Lipid Extraction

Microalgal cells were harvested by centrifugation at 3,000 rpm for 10 min. A freeze-drying procedure was performed using a vacuum freeze dryer (Alpha1-2LD plus; Martin Christ GmbH, Osterode, Germany) to get dry algal powder. The lipids were extracted as previously described [3], with modifications. Four milliliters of chloroform and 2 ml of methanol were added to a glass tube containing 50 mg of dry algal powder. The mixture was shaken at 180 rpm at 30°C for 12 h, and then centrifuged at 3,000 rpm for 10 min. The supernatant was transferred to a new glass tube, and 2 ml of methanol and 3.6 ml of water were added to make the final ratio of chloroform:methanol:water to be 10:10:9 (v/v/v). Further centrifugation of this mixture at 3,000 rpm for 10 min produced two distinct layers. The chloroform layer was transferred into a preweighted glass tube and removed at 60°C under the protection of N_2. The lipid was dried in a vacuum-drying oven (Liantian DZF-6050, Hangzhou, China) at 0.09 Mpa, 60°C, for 2 h. The final weight was measured using an electronic balance (0.01 mg: XS105 DualRange; Mettler Toledo, Zurich, Switzerland). Extraction was performed in duplicate for each sample.

Analysis of Fatty Acid Composition

Transesterification was performed by incubating the total lipids sample in 2 ml of methanol containing 2% (v/v) H_2SO_4 for 2.5 h at 85°C. After cooling to room temperature, 1 ml of HPLC-grade hexane and 1 ml of saturated NaCl solution were added. The mixture was centrifuged for 10 min at 3,000 rpm, and the upper layer containing fatty acid methyl esters (FAMEs) was obtained for further analysis. FAME analysis was performed with a gas chromatograph (Varian 450-GC; Varian Inc., USA) using a fused CP-WAX 58 column (25 m × 0.25 mm). The carried gas was helium, and 1 μl of methyl ester sample solution was injected for analysis. The split ratio was 1:30. The temperature program was as follows: column temperature was maintained at 100°C for 2 min and then elevated to 250°C at a rate of 10°C/min and maintained for a further 3 min. The injector temperature was set at 250°C. The flame-ionization detector temperature was set at 280°C. Fatty acids were identified by comparing the retention time obtained with the analytical standard FAME mixture C8–C24 (Sigma-Aldrich, Shanghai, China).

Elemental Analysis

The elemental composition (C, H, N, and S) of microalgae biomass under different conditions was analyzed using Vario EL (Elementar Analysensysteme GmbH, Hanau, Germany) with helium as the carrier gas. The combustion temperature and oxygen pressure were 1,150°C and 0.25 MPa, respectively.

Carbon Dioxide Fixation Rate

The carbon dioxide fixation rate of microalgae can be calculated by the following equation [35]:

$$\text{Carbon dioxide fixation rate} = \frac{X_{max} - X_0}{t} \times C \times \frac{M_{co_2}}{M_c} \quad (1)$$

where X_{max} and X_o represent maximum dry cell weight and initial inoculated dry cell weight (mg/l); t stands for the time required to reach the maximum dry cell weight; and C is the carbon content of the dry cell biomass per the element analyzer found in Table 2. M_{CO_2} and M_C represent the molecular weight of CO_2 and elemental carbon, respectively.

Results

Cultivation and Lipid Production of *Chlorella* sp. MRA-1 with Different Concentrations of CO_2

Chlorella sp. MRA-1 could grow in the presence of 5% to 20% CO_2 and reached a stationary stage after 10 days (Fig. 1). The maximum biomass concentration of 4.48 ± 0.30 g/l was achieved with 5% CO_2. With 10% and 20%

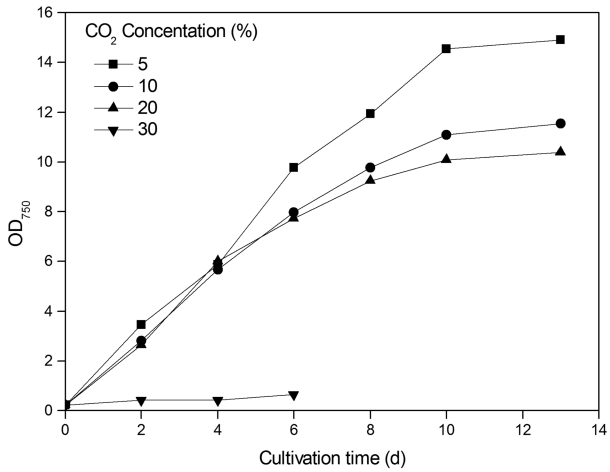

Fig. 1. Growth of *Chlorella* sp. MRA-1 under different CO_2 concentrations in BG-11 medium with 1.5 g/l $NaNO_3$.

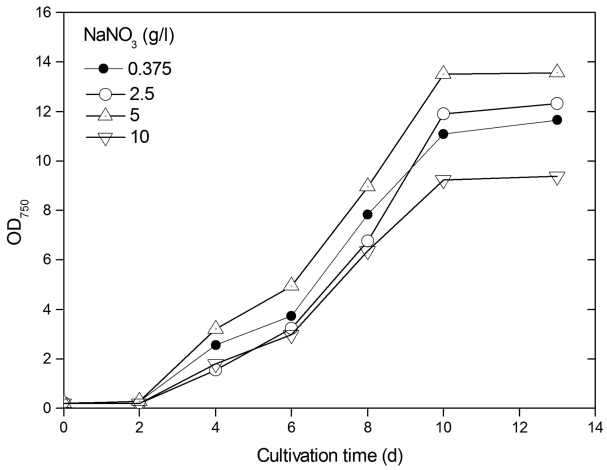

Fig. 3. Growth of *Chlorella* sp. MRA-1 with different nitrogen concentrations in BG-11 medium with 5% CO_2.

CO_2, biomasses of 3.52 ± 0.17 and 3.20 ± 0.29 g/l were obtained, respectively. After 13 days, the lipid contents and lipid yields of strain MRA-1 (grown with different CO_2 concentrations) were compared (Fig. 2). The highest lipid yield of 1.53 g/l was obtained when cultured with 5% CO_2. When the CO_2 concentration was increased, the lipid yield decreased greatly (Fig. 2). Lipid yields of the cells grown with CO_2 concentrations of 10% and 20% were 1.26 and 0.79 g/l, respectively.

Effects of Different Nitrogen Concentrations on the Growth and Lipid Accumulation of *Chlorella* sp. MRA-1

To improve the lipid productivity, the effects of different nitrogen concentrations on the growth and lipid accumulation of *Chlorella* sp. MRA-1 were investigated (Fig. 3). The maximum biomass concentration of 4.14 ± 0.31 g/l was achieved in 13 days when the alga was grown in BG-11 medium with 5 g/l $NaNO_3$. Microalgal cells were collected, and extractable lipid contents (% DCW) were determined by the gravimetric method. The lipid contents and productivities of *Chlorella* sp. MRA-1, grown with different concentrations of $NaNO_3$, are shown in Fig. 4. Strain MRA-1 showed the highest lipid content of 52.1% DCW with 0.375 g/l $NaNO_3$. The lipid contents of the cells grown with 2.5, 5, and 10 g/l $NaNO_3$ were significantly lower than with 0.375 g/l $NaNO_3$. As has been shown, nitrogen limitation

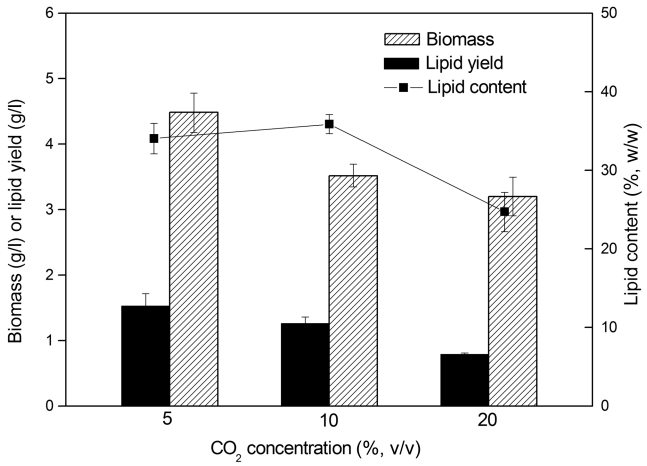

Fig. 2. Algae biomass, lipid yield, and lipid content of *Chlorella* sp. MRA-1 in BG-11 medium with 1.5 g/l $NaNO_3$ under different CO_2 concentrations.

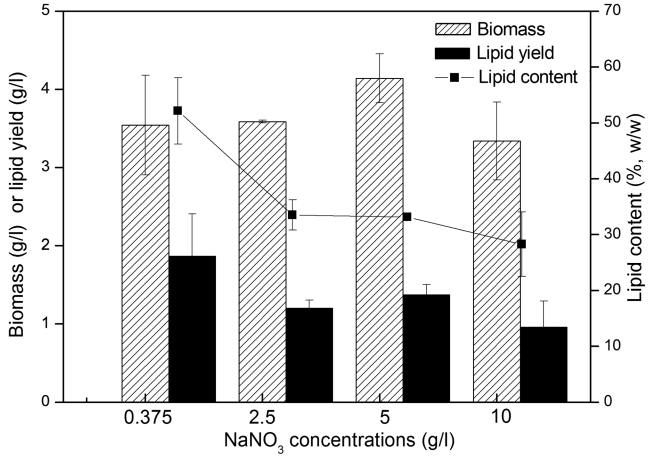

Fig. 4. Algae biomass, lipid yield, and lipid content of *Chlorella* sp. MRA-1 grown in BG-11 medium with different concentrations of $NaNO_3$ with 5% CO_2.

Table 1. Fatty acid composition of *Chlorella* sp. MRA-1 grown with different CO_2 and $NaNO_3$ concentrations.

Fatty acid	$NaNO_3$ concentration (g/l)				CO_2 concentration (%)		
	0.375	2.5	5	10	5	10	20
Saturated fatty acids							
C16:0	18.36	18.14	19.83	17.43	16.09	18.57	17.96
C18:0	2.01	2.29	3.07	0.99	0.77	1.97	1.54
C20:0	0.10	0.22	0.25	0.12	0.16	0.11	0.08
C22:0	0.18	0.17	0.24	0.09	0.23	0.19	0.17
C24:0	0.83	0.51	1.12	0.91	0.37	0.85	0.70
Sum	21.48	21.32	24.51	19.53	17.61	21.69	20.45
Monounsaturated fatty acids							
C16:1	0.79	0.82	0.79	3.25	1.07	0.82	0.83
C18:1	52.04	51.91	53.49	56.98	57.77	54.31	55.46
C20:1	0.22	0.09	0.20	0.21	0.22	0.14	0.12
Sum	53.04	52.82	54.48	60.43	59.07	55.27	56.41
Polyunsaturated fatty acids							
C16:2	3.63	3.99	3.04	2.95	2.47	2.20	2.26
C16:3	3.08	2.97	2.42	1.00	4.12	3.99	3.82
C18:2	9.87	10.44	8.37	7.80	5.82	6.66	7.20
C18:3	8.83	8.39	7.13	8.20	10.86	10.13	9.87
C18:4	0.07	0.07	0.05	0.07	0.05	0.07	0.00
Sum	25.48	25.86	21.01	20.03	23.31	23.05	23.14

can stimulate lipid accumulation in *Chlorella* sp. MRA-1. In this study, the maximum lipid yield of 1.87 g/l was achieved with a nitrogen limitation of 0.375 g/l $NaNO_3$.

Fatty Acid Composition of *Chlorella* sp. MRA-1 Grown with Different CO_2 and $NaNO_3$ Concentrations

The fatty acid composition of *Chlorella* sp. MRA-1 grown with different CO_2 and $NaNO_3$ concentrations was analyzed using GC (Table 1). The major fatty acid components for all cells grown under different conditions were octadecenoic acid and hexadecanoic acid, constituting 70% of the total fatty acid content. The fatty acid profiles of total lipids were similar for *Chlorella* sp. MRA-1 grown with different concentrations of $NaNO_3$. However, the proportion of monounsaturated fatty acids that included C16:1, C18:1, and C20:1 reached 60.43% of the total lipid composition when strain MRA-1 was cultured with 10 g/l $NaNO_3$. This amount was 11%–14% higher than for the cells grown with 0.375, 2.5, and 5 g/l $NaNO_3$.

Element Analysis and Carbon Dioxide Fixation Rate

The levels of carbon, nitrogen, hydrogen, and sulfur in

Table 2. Elemental analysis of *Chlorella* sp. MRA-1 grown with different CO_2 and $NaNO_3$ concentrations.

Element	$NaNO_3$ concentration (g/l)				CO_2 concentration (%)		
	0.375	2.5	5	10	5	10	20
N%	2.48 ± 0.14	6.03 ± 1.69	6.23 ± 0.13	6.02 ± 0.41	6.00 ± 0.18	5.82 ± 0.12	4.98 ± 0.05
C%	53.72 ± 1.30	51.57 ± 1.72	51.48 ± 1.17	50.04 ± 1.67	52.18 ± 2.80	56.11 ± 4.13	56.69 ± 5.65
H%	7.46 ± 0.14	7.22 ± 0.25	7.03 ± 0.15	6.94 ± 0.17	7.32 ± 0.29	7.27 ± 0.25	7.13 ± 0.34
S%	2.33 ± 1.00	1.31 ± 0.23	1.09 ± 0.16	1.21 ± 0.20	1.20 ± 0.29	1.18 ± 0.23	0.98 ± 0.18
C/N	21.78 ± 1.51	9.74 ± 5.12	8.28 ± 0.36	8.36 ± 0.78	8.71 ± 0.59	9.64 ± 0.74	11.39 ± 1.22
C/H	7.20 ± 0.07	7.14 ± 0.08	7.32 ± 0.04	7.21 ± 0.11	7.12 ± 0.13	7.71 ± 0.35	7.94 ± 0.46

Data are reported as means ± standard deviation of triplicates.

Table 3. Tolerable concentration of CO_2 and biomass yield by microalgae.

Species	CO_2 concentration (%)	Biomass yield (g/l/day)	Lipid productivity (mg/l/day)	Cultivation condition	References
Botryococcus braunii SAG-30.81	5	0.207	30.81	BioFlo Fermentor	[27]
Botryococcus raunii	10	0.026	5.51	Bioreactor	[30]
Chlorella sp.	9–10	0.15	NA	Erlenmeyer flasks	[18]
Chlorella vulgaris LEB-104	5	0.31	11.54	BioFlo Fermentor	[27]
Spirulina platensis LEB-52	5	0.73	14.3	BioFlo Fermentor	[26]
Chlorella kessleri	6	0.206	NA	Vertical tubular photobioreactors	[9]
Chlorella sp.	6–8	0.323–0.38	NA	Thin-layer photobioreactor	[10]
Chlorella sp. MRA-1	5	0.344	117.6	Column	This research
	10	0.271	96.92	Column	This research
	20	0.246	60.76	Column	This research
Scenedesmus sp.	10	0.217	39.44	Bioreactor	[30]
Scenedesmus obliquus	12	0.106	NA	Vertical tubular photobioreactors	[9]
Synechocystis aquatilis	10	0.15	NA	Vertical flat-plate photobioreactor	[37]

NA: not available.

Chlorella sp. MRA-1 grown with different CO_2 and $NaNO_3$ concentrations were analyzed (Table 2). The carbon content was 53.72% ± 1.30% when grown with 0.375 g/l $NaNO_3$, which was higher than that found in cells grown with sufficient nitrogen. Cultivation with high concentrations of CO_2 (10% and 20%) also led to microalgae cells exhibiting a carbon content greater than 56%. However, the highest C/N ratio of 21.78 ± 1.51 was obtained when the cells were cultured with 0.375 g/l $NaNO_3$. This indicated that nitrogen deficiency could induce *Chlorella* sp. MRA-1 to produce more lipid than CO_2 stress.

The carbon dioxide fixation rate was compared when this strain was cultured with different CO_2 concentrations (Table 4). The maximum CO_2 fixation rate of *Chlorella* sp. MRA-1 was 710.7 mg/l/day when cultured with 5% CO_2. The CO_2 fixation rate declined when higher levels of CO_2 were supplied.

Discussion

Waste gases from thermal power stations contain 10%–20% (v/v) CO_2, which may provide a carbon source for microalgae cultivation. Several microalgal species have been tested with high CO_2 concentrations (Table 3). *Chlorococcum littorale* could grow with 60% CO_2 using the stepwise adaptation technique [5]. Another high CO_2-tolerant species is *Euglena gracilis* whose growth was enhanced under 5%–45% of CO_2. *Scenedesmus* sp. could grow with 80% CO_2, but maximum cell mass was observed with 10%–20% CO_2 [13]. It is also reported that some species such as *Chlorella* sp. and *Cyanidium* sp. could even grow with 100% CO_2 [2, 21].

Hu *et al.* [14] summarized the total lipid contents of oleaginous green algae reported in the literature, which showed an average total lipid content of 25.5% DCW. Mata *et al.* [23] suggested *Chlorella* sp. to be a good option for biodiesel production, according to a wide range of literature. In a previous study reported by Demorais and Costa [9], *Chlorella kessleri* isolated from a coal-fired thermoelectric power plant showed a maximum biomass productivity of 0.087 g/l/day with 6% CO_2, even growing in a medium containing up to 18% CO_2. Tang *et al.* [29] reported that a

Table 4. Carbon dioxide fixation rate of *Chlorella* sp. MRA-1 in this work.

CO_2 concentration (%)	Carbon dioxide fixation rate (mg CO_2/l/day)	Biomass yield (mg/l/day)	Culture medium
5	710.7	344	BG-11
10	599.7	271	BG-11
20	548.6	246	BG-11

lipid content of 20.65% DCW was achieved by a CO_2-tolerant microalga, *Chlorella sorokiniana* CS-01, when cultured with 5% CO_2 [29]. The lipid content of *Chlorella vulgaris* grown with 10% CO_2 was found to be less than 11.92% DCW [32]. In the present study, it was shown that *Chlorella* sp. MRA-1 could grow with 5%–20% CO_2, with the best growth (0.34 g/l/day) achieved at 5% CO_2. The highest CO_2 fixation rate was achieved when the strain was grown with 5% CO_2. Lipid content and lipid productivity of *Chlorella* sp. MRA-1, when cultured with 5% CO_2, were 34.1% DCW and 116 mg/l/day, respectively.

A high content of algal lipid increases the process yield coefficient and reduces the cost of extraction and purification per unit product [4]. Algal lipids respond to nitrogen deprivation stress and CO_2 enrichment in a heterogeneous fashion. Pratt and Johnson [25] proposed that lipid synthesis was promoted at high CO_2 levels in *Chlorella*. In this study, we also found that a higher lipid content was achieved with higher CO_2 concentrations. On the other hand, it is known that nitrogen deprivation promotes lipid accumulation [17]. In the present study, *Chlorella* sp. MRA-1 accumulated more lipid through nitrogen limitation; the highest lipid content of 52.1% DCW was obtained when cells were grown in BG-11 medium with 0.375 g/l $NaNO_3$-one-fourth the normal nitrogen concentration.

The unsaturation degree of fatty acids in *Chlorella vulgaris* was inversely related to the CO_2 concentration. Tsuzuki *et al.* [30] reported that the unsaturation levels of fatty acids in *C. vulgaris* were greater in low-CO_2 cells than in high-CO_2 cells, indicating a shift of the relative content of saturated and unsaturated fatty acids with a change in CO_2 concentrations. In this study, we also found that the contents of unsaturated fatty acid (monounsaturated fatty acids and polyunsaturated fatty acids) were greater in low-CO_2 cells than in high-CO_2 cells in *Chlorella* sp. MRA-1. Moreover, the contents of monounsaturated fatty acids in lipids were lower under nitrogen starvation conditions, which is similar to *Tetraselmis subcordiformis* in previous reports where the major increase in fatty acids under nitrogen deprivation conditions was of the monounsaturated type [26]. An additional characteristic of oleaginous microalgae is the suitability of lipids for biodiesel in terms of the type and amount produced by a microalgal species (*e.g.*, chain length, degree of saturation) [12]. This study confirms that the major components of the lipids from *Chlorella* sp. MRA-1 are suitable for biodiesel production.

In conclusion, the newly isolated strain *Chlorella* sp. MRA-1, with its tolerance to high concentrations of CO_2 and high lipid productivity, could efficiently exploit flue gases for biodiesel production. We also found that nitrogen deficiency effectively induced the strain to produce more lipid than CO_2 stress. *Chlorella* sp. MRA-1 seems to be an ideal candidate for biodiesel production when cultured with high concentrations of CO_2.

Acknowledgments

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