



Carbon dioxide fixation and lipid storage by *Scenedesmus obtusiusculus*



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HIGHLIGHTS

- ▶ CO₂ fixation by *Scenedesmus obtusiusculus* is among the highest values for Scenedesmaceae.
- ▶ *S. obtusiusculus* tolerates gas stream with 10% of CO₂ v/v.
- ▶ The microalga stores a high lipid content (55.7%) under nitrogen starvation.
- ▶ High lipid productivities (200 g m⁻³ d⁻¹) are achieved with *S. obtusiusculus*.

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ABSTRACT

An indigenous microalga was isolated from the springs in Cuatro Ciénegas, México. It was morphologically identified as *Scenedesmus obtusiusculus* and cultivated in bubble-column photobioreactors in batch operation mode. This microalga grows at 10% of carbon dioxide (CO₂) showing a maximum CO₂ fixation rate of 970 g m⁻³ d⁻¹. The microalga, without any nutrient limitation, contained 20% of nonpolar lipids with a biomass productivity of 500 g m⁻³ d⁻¹ and a maximum biomass concentration of around 6,000 g m⁻³ at 5% CO₂ and irradiance of 134 μmol m⁻² s⁻¹. Furthermore, it was observed that the microalga stored 55.7% of nonpolar lipids when 5% CO₂ was fed at 0.8 vvm and 54.7 μmol m⁻² s⁻¹ under nitrogen starvation. The lipid profile included C16:0, C18:0, C18:1n9t, C18:1n9c, C18:3n6 with a productivity of 200 g lipid m⁻³ d⁻¹. Therefore, the microalga may have biotechnological potential producing lipids for biodiesel.

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1. Introduction

The increase of carbon dioxide concentration in the atmosphere is considered the main cause of global warming. CO₂ emissions have grown exponentially at a rate of 6.5% per year (CDIAC, 2011) and the increase in deforestation has limited the capacity of the environment to absorb this extra CO₂ load. Emissions sources are mostly from the combustion of fossil fuels used in power generation, transport and industry. Also, gaseous discharges from power plants are responsible for more than 7% of the global release and contain about 15% v/v of CO₂ concentration (Matsumoto et al., 1997).

Some techniques have been developed for CO₂ mitigation including sequestration and storage, mineral carbonation and biological fixation by plants and algae. Biological CO₂ fixation with

microalgae is 10 times more efficient than terrestrial plants and microalgae can grow about 10 to 50 times faster (Chen et al., 2011a). In addition, microalgae are a source of high-value products such as polyunsaturated fatty acids, natural colorants, biopolymers, therapeutic substances and biofuels. It is currently accepted that a combination of CO₂ fixation with biofuel production could yield a sustainable process for CO₂ mitigation.

Biodiesel obtained by transesterification of lipids from microalgae may provide a renewable and environmentally friendly fuel source. Despite the massive investments currently being made into algal biofuels research, the scalability, sustainability and cost-efficiency of full-scale algae cultivation for energy production remain unproven and controversial.

CO₂ fixation by microalgae has been positively correlated with its cell growth rate and light utilization efficiency, since it involves photoautotrophic growth (Jacob-Lopes et al., 2009). Some of the physicochemical parameters that affect CO₂ fixation include: temperature, medium composition, pH, light intensity, and CO₂ concentration (Ho et al., 2011). Hence, efficient CO₂ uptake coupled to the production of lipids requires extensive exploration for

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microalgae strains with high growth rate, lipid content, tolerance to high CO₂ levels and temperature (Yoo et al., 2010) as well as tolerance to NO_x and SO_x (Matsumoto et al., 1997).

Therefore, in this study, a microalga with high potential for application on CO₂ fixation was isolated. First, the effect of three key factors influencing cell growth: CO₂, aeration and light intensity were evaluated and then the accumulation of lipids assessed under nitrogen limitation.

2. Methods

2.1. Microorganisms and culture medium

The microalga was harvested from Poza Churince located 19.5 km southwest from the town of Cuatro Ciénegas, Coahuila, México which is located in the Chihuahuan Desert of Coahuila in north central Mexico. The basin is surrounded by high mountains and is a system of springs, streams, and pools. These ecosystems support several endemic species, living stromatolites and other microbial communities representing a desert oasis of high biodiversity (Souza et al., 2006). Endemic species are adapted to live in extreme conditions, such as lack of phosphorus and high incidence of solar radiation.

Water samples were distributed in 500 mL flasks capped with cotton plugs and with 100 mL of sterile BG11 medium containing in g L⁻¹: NaNO₃, 1.5; K₂HPO₄, 0.04; MgSO₄·7H₂O, 0.075; magnesium disodium EDTA, 0.001; CaCl₂·2H₂O, 0.036; citric acid, 0.006; ferric ammonium citrate, 0.006; Na₂CO₃, 0.02; and the following salts in mg L⁻¹: H₃BO₃, 2.86; MnCl₂·4H₂O, 1.81; ZnSO₄·7H₂O, 0.222; NaMoO₄·2H₂O, 0.39; CuSO₄·5H₂O, 0.079; Co(NO₃)₂·6H₂O, 0.494; at pH 7.5 and incubation temperature of 30 °C under continuous illumination of 60 μmol m⁻² s⁻¹.

The microalgal culture was then enriched in illuminated flasks and samples were taken for morphological identification. Single cells were separated into several 5 mL sterile tubes with modified Bristol medium containing in g L⁻¹: NaNO₃, 1.5; K₂HPO₄, 0.075; KH₂PO₄, 0.175; MgSO₄·7H₂O, 0.075; CaCl₂·2H₂O, 0.025; NaCl, 0.025; at pH 7.5, and cultivated at 20 °C under 12/12 h (light/dark) illumination of 42.85 μmol m⁻² s⁻¹. Clonal cultures of microalgae growth with atmospheric CO₂ after 20 days. Then, morphological characteristics of the microalgae were observed with a microscope with differential interference contrast (Nikon Optiphot 1990, Japan).

2.2. Photobioreactor

Bubble column photo-bioreactors (BCR) were located inside a chamber equipped with sixteen 40W-fluorescent tube lamps (OSRAM universal 40W day-light type, Brazil). The photobioreactors were built using 4 mm thick glass with an inner diameter of 0.105 m, height of 0.705 m and a nominal volume of 0.003 m³. Air or CO₂ enriched air was supplied through a 0.105 m diameter sintered glass plate located at the bottom of the column.

2.3. Cultivation conditions

The microalga was cultivated in the BCR maintained at 30 °C with a working volume of 0.0025 m³ and an inoculum of 20% v/v of a concentrated microalgal suspension. Various operating conditions were assayed including non-sterile gas flow rates of 0.4 or 0.8 vvm (volume of air per volume of reactor per minute), inlet CO₂ concentrations of 5% or 10% v/v and light intensities of 54.7, 94.4 or 134 μmol m⁻² s⁻¹ these were adjusted by switching on/off some of the lamps. CO₂ and air flow rates were measured with rotameters (Cole Parmer, USA) and the light intensity with a light

meter (Extech Instruments 407026sp model 2.2, USA). Temperature and pH of the culture medium were monitored daily. Table 1 shows the conditions for each experiment. They were performed only once and lasted 13 days.

Microalgal cultivation with limited nitrogen source was performed to promote lipid accumulation. In these assays, two CO₂ concentrations were tested: 0.04% (air without CO₂-enrichment) and 5% CO₂. Also a two-stage process was implemented. In the first stage, the microalga was grown on complete medium to allow cell growth. In the second stage, the biomass was transferred to a nitrogen-limited medium (10% and 28% of the original nitrogen in mineral medium) to enhance the lipid content. In both cases, the microalga was cultivated in the BCR with BG11 medium, a light input of 54.7 μmol m⁻² s⁻¹ and a gas flow rate of 0.8 vvm.

2.4. Analytic determinations

2.4.1. Biomass, carbon content and nitrate concentration

Twenty mL of culture medium were collected daily and replaced with non-sterile distilled water. Biomass was quantified by filtering a known volume of culture medium through a 0.45 micron membrane and dried at 50 °C for 24 h. The carbon content of the biomass was determined using a CHNS analyzer (Series II 2400 CHNS/O Perkin Elmer, Boston, USA).

For nitrate determination, 2 mL of a sample previously filtered through 0.22 microns pore size membrane, were mixed with 1 mL of HCl 1 N and read at wavelength of 220 nm. A calibration curve was prepared using NaNO₃.

Total biomass after each run was recovered by flocculation. The pH of the culture medium was first adjusted to 7.5 with HCl or CaCO₃. Then, mixed in a 4:1 proportion with a solution containing chitosan/HCl/H₂O (100 mg of chitosan were dissolved into 10 mL of 0.1 M HCl solution and diluted with distilled water to 100 mL) and stirred by 30 min. The flocculated biomass solution was vacuum filtered with Whatman filter paper (no. 50). Recovered biomass was dried at 60 °C for 48 h. Dry biomass was pulverized in a mortar and sieved at 1 mm mesh. Hexane was used to extract the nonpolar lipids by the Soxhlet method.

Nile red (7-diethylamino-3,4-benzophenoxazine-2-one) (N3013 Sigma Aldrich) was used to determine daily the nonpolar lipid content. A 20 μL sample of the culture medium was mixed with 100 μL of dimethyl sulfoxide and shaken for 1 min. It was then heated in a microwave oven (100% power 1650 W) for 50 s, then 20 μL of Nile Red (0.25 mg L⁻¹ in acetone) were added and diluted to 1 mL with distilled water. The mixture was shaken for 1 min and heated again for 60 s. The sample was maintained for 10 min in the dark and then transferred to a 2 mL vial with 1 mL of distilled water (total volume of the mixture was 2 mL) and analyzed in a fluorometer (Turner Designs Instrument model 7200-000, Sunnyvale CA, USA) (Chen et al., 2011b). The excitation and emission band on the equipment were 485 and 585 nm, respectively. The reading units given as Reference Fluorescent Units (RFU) were transformed to grams of lipid per liter of culture medium using a reference curve

Table 1

Parameters obtained at different light intensities and CO₂ inlet concentrations. gas inlet flow was 0.4 vvm.

CO ₂ concentration (% v/v)	5	5	5	10	10	10
CO ₂ load (kg m ⁻³ d ⁻¹)	40	40	40	80	80	80
Irradiance (μmol m ⁻² s ⁻¹)	54.7	94.4	134	54.7	94.4	134
X _{max} (g m ⁻³)	3,370	3,120 ^a	6,000	3,100 ^a	2,700	5,700
P _{max} (g m ⁻³ d ⁻¹)	250	320	500	260	310	520
μ _{max} (d ⁻¹)	0.18	0.23	0.37	0.18	0.34	0.38
P _{CO₂} (g m ⁻³ d ⁻¹)	470	600	950	490	570	970

^a The means are statistically equal, ANOVA *P* < 0.0001.

constructed with RFU versus grams of lipids extracted by the Soxhlet method.

Total protein was measured by a modified Lowry method (Lowry et al., 1951) for microalgal biomass. Five milliliters of NaOH 0.5 N were added to 1 ml of culture medium. The mixture was sonicated for 20 min and then boiled to 100 °C for 60 min and then centrifuged for 20 min at 4000 rpm. The supernatant was analyzed with the Bio-Rad protein assay kit and measured at 750 nm wavelength. A calibration curve was prepared, using bovine serum albumin standard.

Total carbohydrates were analyzed by a modified phenol-sulfuric acid method (Dubois et al., 1956) adapted for microalgal biomass. Five mL of 1 M H₂SO₄ were added to 1 mL of culture medium. The mixture was sonicated for 20 min and then boiled to 100 °C for 60 min and then centrifuged for 20 min at 4000 rpm. The supernatant was mixed with 1 mL of a 5% phenol solution and then allowed to stand for 40 min, 5 mL of concentrated H₂SO₄ were then added. The optical density was read at 485 nm and compared to a calibration curve prepared with D + glucose.

A spectrophotometric method was used to determine the chlorophyll content in the cells using the equation: Chlorophyll (µg mL⁻¹) = 16.5 (A₆₆₅) – 8.3 (A₆₅₀) (Becker, 1994). Absorbencies at 650 and 665 nm were corrected for turbidity by subtracting absorbencies at 750 nm. One milliliter of culture medium was suspended in 5 mL of 90% methanol solution for pigment extraction. The mixture was sonicated for 20 min, warmed at 60 °C for 15 min and then centrifuged at 4000 rpm for 20 min. The supernatant was recovered and read at 650, 665 and 750 nm. The inorganic matter (ashes) was determined gravimetrically after incineration at 490 °C for 6 h.

2.4.2. Determination of the lipid profile

The lipid composition was determined as fatty acid methyl esters (FAMES) through direct transesterification. Two milliliters of culture medium were centrifuged (4000 rpm for 15 min) and re-suspended in 1 mL of dichloromethane. Subsequently, 2 mL of a methanol/HCl solution (4:1, v/v) were added and the mixture heated to 110 °C for 6 h in a digester (HACH model DRB200, USA). Later, 1 mL of deionized water was added to separate the phases. The mixture of organic phases from replicates (10 mL) were purified using a packed silica gel column (17 g, 47 mL, high purity grade, pore size 60 Å, 70–230 mesh, for column chromatography), by repeated washes with dichloromethane (4–5 column volumes) and finally clean FAMES were concentrated on a rotary evaporator.

FAMES were analyzed in a gas chromatograph with a flame ionization detector (FID) (HP6890). Samples were injected into a 25 m long capillary column (AT-WAX) with an internal diameter of 0.25 mm and 0.2 µm film thickness. Helium was used as carrier gas (20 cm s⁻¹). The injection volume was 2 µL, Split 100:1. The temperature of the injector and detector were set to 250 °C. The oven temperature was initially set at 140 °C for 5 min, increased from 140 to 240 °C at a rate of 1.5 °C min⁻¹, and held for 10 min.

2.5. Kinetic parameters and CO₂ fixation rate

The specific growth rate (µ, d⁻¹) is calculated as:

$$\mu = \frac{\ln X_t - \ln X_0}{t - t_0} \quad (1)$$

The biomass productivity (P, g m⁻³ d⁻¹) is defined as:

$$P = \frac{X_t - X_0}{t - t_0} \quad (2)$$

where X₀ is the initial biomass concentration (g m⁻³) at time t₀ (d) and X_t is the biomass concentration at any time. P_{max} (g m⁻³ d⁻¹)

and µ_{max} (d⁻¹) are referred to as the maximum values obtained for each experiment.

Lipid productivity was determined by:

$$P_{\text{Lip}} = X\mu Y_{\text{Lip/bio}} \quad (3)$$

where X is the biomass concentration (g m⁻³) and Y_{Lip/bio} the lipid content per gram of biomass (g lipid g biomass⁻¹).

The CO₂ fixation rate (P_{CO₂}) was calculated by:

$$P_{\text{CO}_2} = C_C P \left(\frac{M_{\text{CO}_2}}{M_C} \right) \quad (4)$$

where M_C is the molecular weight of carbon, M_{CO₂} is the molecular weight of CO₂ and C_C is the carbon content in the biomass (g_C g_{biomass}⁻¹).

3. Results and discussion

3.1. Isolation and morphological identification of the microalga

Clonal cultures propagated from a single cell were obtained and identified to the genus and species level using morphological characteristics. All cells belonged to *Scenedesmus* genus, grouped in four cell cenobium and sometimes solitary. The cells were fusiform, with an apex rounded and an empty extension of the cell wall. They have a well-defined pyrenoid in the center of the cell and the chloroplasts were parietal. The size of the solitary cells varied, but the average value was 4.8 microns in width × 13.2 microns length. It was observed that cell division occurs longitudinally and transversally by autospore formation of the larger and solitary cells. Those characteristics were consistent with *S. obtusiusculus* described by Chodat (1913) and reviewed by Uherkovich (1966) who stated that this microalga is commonly confused with *Scenedesmus acutus* for their morphology, because it was not clearly described. Micrographs showing morphology of *S. obtusiusculus* can be found in Supplementary material.

3.2. Effect of CO₂, irradiance and flow

Table 1 shows the effect of the CO₂ concentration at 0.4 vvm and irradiance on CO₂ fixation and growth rate of the microalga. The highest biomass values (final content of 6,000 g m⁻³, growth rate of 0.37 d⁻¹ and productivity of 500 g m⁻³ d⁻¹) and the maximum CO₂ fixation rate were obtained at the highest irradiance tested and did not depend on the inlet CO₂ concentration. Statistical data analysis showed that biomass productivity has no difference (P < 0.0001) when CO₂ concentrations of 5% or 10% were supplied at the same light intensity. These results suggest that, under the conditions studied, available luminous energy may be the limiting factor. Also, no inhibition was observed with 10% CO₂ at a loading rate up to 160 kg CO₂ m⁻³ d⁻¹ (data not shown). Previous studies showed that CO₂ above 5% could be harmful to microalgal cells and inhibit the growth of *Nannochloropsis oculata* (Chiu et al., 2009). In contrast, microalgae like *S. obliquus* can grow beyond 6% and reach the maximum biomass at 18% CO₂ (Morais and Costa, 2007b). In this study *S. obtusiusculus* was able to grow at 10% CO₂.

Growth and CO₂ fixation by *S. obtusiusculus* was further studied at 94.4 µmol m⁻² s⁻¹ and a fixed CO₂ load rate of 80 kg m⁻³ d⁻¹ and varying the inlet flow at 0.4 or 0.8 vvm. To maintain the CO₂ load, the inlet concentration was set at 5% or 10% for the 0.8 or 0.4 vvm, respectively.

Fig. 1 shows that, when the same CO₂ load, 80 kg m⁻³ d⁻¹ was fed, the maximum biomass contents were 2,700 g m⁻³ with an inlet flow of 0.4 vvm and 3,800 g m⁻³ with 0.8 vvm. An increment in the specific growth rates from 0.34 to 0.51 d⁻¹ was registered when airflow increased twice; and the maximum P_{CO₂} was also

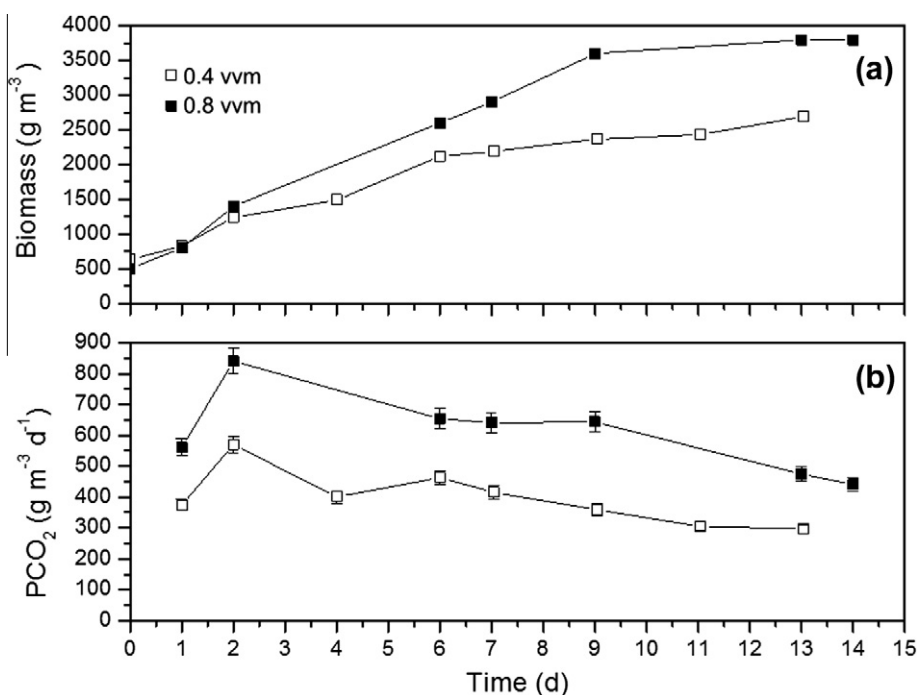


Fig. 1. (a) Growth curves and (b) CO₂ fixation rates with a CO₂ load rate of 80 kg m⁻³ d⁻¹ and 94.4 μmol m⁻² s⁻¹. □, denotes 0.4 vvm or ■, denotes 0.8 vvm.

obtained at 0.8 vvm (Fig. 1). Statistical data analysis showed that biomass, productivity, CO₂ fixation rate and specific growth rate increased when the CO₂-air flux was augmented ($P < 0.0001$).

On the other hand, the growth rates obtained at two different CO₂ load rates (40 and 80 kg m⁻³ d⁻¹) and 94 μmol m⁻² s⁻¹ also suggest that hydrodynamics is a relevant issue. A slight increase on the growth rate from 0.23 to 0.34 d⁻¹ was observed when CO₂ concentration doubled from 5% to 10% (see Table 1) at the same airflow, 0.4 vvm; there was no effect of these two assayed CO₂ loads on the growth rates, at 54.7 and 134 μmol m⁻² s⁻¹ (Table 1); but when air flow increased from 0.4 vvm (Table 1) to 0.8 vvm (Fig. 1) with the same CO₂ concentration (5%), a noticeable increase on growth rate, from 0.23 to 0.51 d⁻¹, was achieved. In general, the amount of gas supplied to reactors strongly influences the mixing of medium, the gas holdup, the distribution of cells, the nutrient availability to cells, and absorption of carbon dioxide as well as reduce the mutual microalgae shading (Ugwu et al., 2008). However, by increasing the gas flow rate above a critical value, the bubbles could coalesce reducing the contact area between the liquid and the gas, thereby resulting in poor mass transfer rates. In our case, the highest gas velocity used was 0.00384 m s⁻¹ (at 0.8 vvm) which induced homogeneous bubbles and no coalescence was observed. With this gas velocity a $k_{L}a$ of 0.0065 s⁻¹ can be estimated (Doran, 2000). These values are similar to the superficial velocities (0.00054–0.0082 m s⁻¹) and $k_{L}a$ (0.002–0.005 s⁻¹) reported by Merchuk et al. (2000) for a 13 L bubble-column for *Porphyridium* sp. cultivation. They observed the maximum biomass content (4,000 g m⁻³) at 0.0032 m s⁻¹ and lower biomass contents at other different gas velocities.

Morais and Costa (2007a) obtained μ_{max} of 0.22 d⁻¹, X_{max} of 1,800 g m⁻³ and P_{max} of 140 g m⁻³ d⁻¹ with *S. obliquus*. The photobioreactors used received an irradiance of 54.7 μmol m⁻² s⁻¹ under a 12 h dark/light photoperiod and with 12% CO₂ enriched air fed at 0.3 vvm for 15 min every 2 h during the 12 h light period. The biomass concentration and productivity obtained with *S. obliquus* at 10% CO₂ (Table 1) were higher than those reported by *S. obliquus* with similar specific growth rates.

3.3. CO₂ fixation rate

The maximum fixation rate and biomass productivity are important parameters used to evaluate the potential for CO₂ removal. Table 2 shows a comparison of our results and some taxonomic related microalgae grown in photobioreactors for CO₂ removal from air streams. Reported P_{max} values were between 70 and 840 g m⁻³ d⁻¹ and the range of CO₂ consumption rates 131–1,420 g m⁻³ d⁻¹. In general, the P_{CO_2} and P_{max} values of *S. obliquus* (Table 2) strains which suggest the potential of our microalga for capturing CO₂ from gas stream and for producing biomass or biofuel. However, the carbon dioxide fixation would vary with operational factors, such as light intensity, air flow, pH, working volume of the photobioreactor and CO₂ feeding rate.

3.4. Nitrogen limitation to enhance lipid production

S. obliquus was not only studied for its ability to fix CO₂ but also for its capacity to produce lipids under nitrogen stress. In the first strategy, the microalga was cultivated at 0.8 vvm with 0.04% or 5% of CO₂ v/v and irradiance of 54.7 μmol m⁻² s⁻¹ during 22 days until nitrogen was below 20 g m⁻³. As shown in Fig. 2, a significant increase on lipid accumulation was only observed at 5% CO₂. For the inlet CO₂ concentrations of 0.04% and 5% CO₂, the maximum lipid contents were 15% and 49% and the maximum lipid productivities were 51 and 200 g lipid m⁻³ d⁻¹, respectively.

In the second strategy, the microalga was initially grown on complete mineral medium and then the biomass was washed out and re-suspended in BG11 medium containing only 69 g Nm⁻³, corresponding to 28% of the original N-NO₃ concentration. The initial biomass concentration was 1,400 g m⁻³. Fig. 3 shows that the initial lipid content increased by 6-fold to 55.7% after 15 days of cultivation in the reduced nitrogen medium but diminished thereafter to 16% at the end of the experiment.

The lipid productivity (80 g lipid m⁻³ d⁻¹) was lower than that obtained in the first strategy as the biomass content was lower

Table 2
CO₂ fixation rate and productivity of some microalgae used in CO₂ removal from air stream.

Microalgae	% CO ₂	P _{max} (g m ⁻³ d ⁻¹)	P _{CO₂} (g CO ₂ m ⁻³ d ⁻¹)	Light intensity (μmol m ⁻² s ⁻¹)	References
<i>Scenedesmus obliquus</i>	12	140	263 ^a	55 ^b	Morais and Costa (2007a)
<i>S. obliquus</i> CNW-N	20	280	527 ^a	60	Ho et al. 2010a.
<i>S. obliquus</i>	12–18	70	131	43 ^b	Morais and Costa (2007b)
<i>S. obliquus</i> AS-6-1	20	380	719 ^a	60	Ho et al. (2010a).
<i>Scenedesmus</i> sp. KCTC AG20831	10	217	408 ^a	150	Yoo et al., 2010.
<i>S. obliquus</i> CNW-N	10	201	390	60	Ho et al. (2010b).
<i>S. obliquus</i> CNW-N	2.5	840	1,420	140	Ho et al. (2012)
<i>S. obtusiusculus</i>	5	500	950 ^a	134	This study
<i>S. obtusiusculus</i>	10	520	970 ^a	134	This study

^a Calculated from the biomass productivity according to the equation: CO₂ fixation rate (P_{CO₂})=1.88 × biomass productivity (g m⁻³ d⁻¹), which is derived from the molecular formula of microalgal biomass, CO_{0.48}H_{1.83}N_{0.11}P_{0.01}.

^b Calculated from the conversion factor for daylight reported by Hershey (1991).

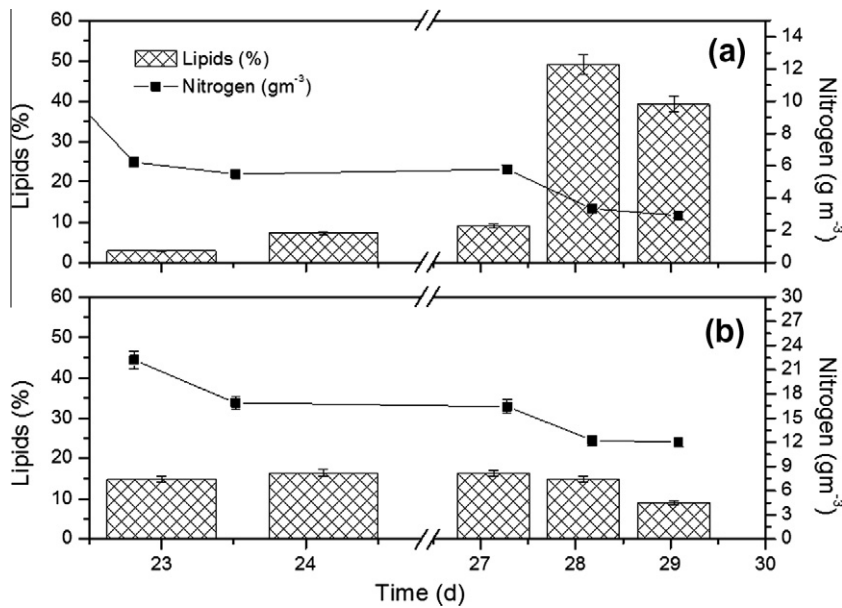


Fig. 2. ■, Denotes nitrogen concentration and vertical bars denote lipid storage supplying (a) 5% CO₂ v/v and (b) 0.04% CO₂ v/v at 0.8 vvm and 54.7 μmol m⁻² s⁻¹.

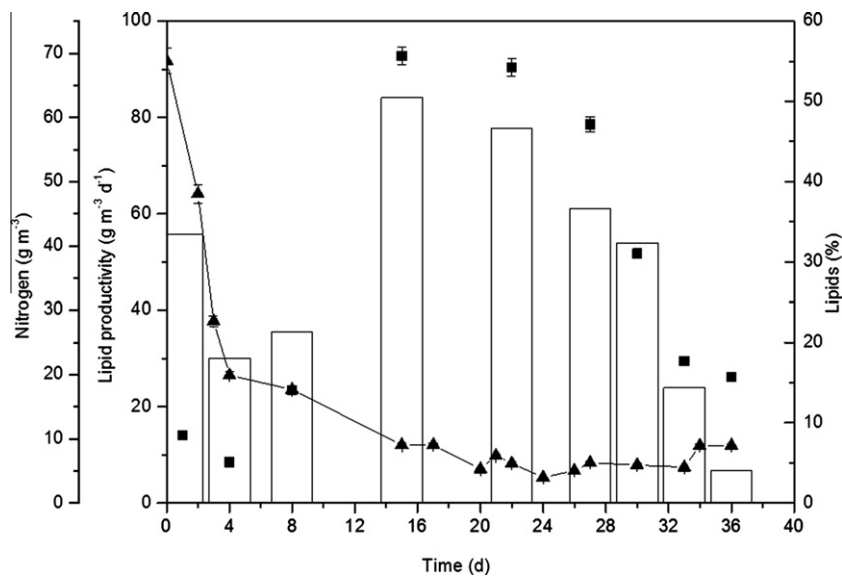


Fig. 3. ▲, Denotes nitrogen concentration, vertical bars denote lipid productivity and ■, denotes lipid storage (in %) under nitrogen starvation supplying 5% CO₂ v/v at 0.8 vvm.

(2,900 g m⁻³) when the maximum lipid accumulation (55.7%) was reached. It can be also observed in Fig. 3 that, from day 15, the

amount of nitrogen did not change (9 g m⁻³ residual nitrogen). The final biomass content in these experiments was 4,400 g m⁻³.

The global biomass increase reflects lipid accumulation considering that the other cell materials remain constant during N-limitation. Li et al. (2008) proposed the consumption of intracellular nitrogen pools, such as chlorophyll molecules, when they observed that *Neochloris oleoabundans* continued growing after the exhaustion of nitrogen. The chlorophyll cell content in *N. oleoabundans* increased from 3 mg g⁻¹ dry weight (DW) to 5.6 mg g⁻¹ DW at day 1 and then decreased drastically to approximately 2.7 mg g⁻¹ DW at day 3. This observation allowed them to suggest that microalgae accumulated large quantities of chlorophyll molecules when nitrogen was available. But, when the nitrogen was exhausted, microalgae started to utilize chlorophylls as an intracellular nitrogen source. This was also observed by Wu et al. (2012) who reported in *Scenedesmus* sp. cells that the intracellular nitrogen and phosphorus per cell reached maximum values in the exponential growth phase, and decreased in the stationary phase.

Our experiment with 5% CO₂ allowed a lipid accumulation up to 55.7% DW, this CO₂ concentration was higher than the 2.5% used for the growth of *S. obliquus* CNW-N (Ho et al., 2012) who reported 22.4% of accumulated lipid with a productivity of 140 g lipid m⁻³ d⁻¹ under nitrogen starvation. This value was lower than the 200 g lipid m⁻³ d⁻¹ obtained with *S. obtusiusculus*. Moreover, according to the lipid productivities reported in that work for different *Scenedesmus* species (22–140 g m⁻³ d⁻¹), it is possible to rank our strain as one of the most productive.

Besides our results also indicate that there was a preferential harvesting time (Fig. 3) where the cells attain their maximum lipid content. Most of the reports in the literature present only the maximum value of lipid content but do not show the complete cycle on the lipid production.

The maximum accumulation of lipid has been reported in the range of 2–20 days (Khozin-Goldberg et al., 2002; Solovchenko et al., 2007; Recht et al., 2012; Praveenkumar et al., 2012 and Yeh and Chang, 2011). Khozin-Goldberg et al. (2002) cultivated *Parietochloris incisa* under nitrogen starvation for 14 days showing that the fatty acid content increased significantly in the N-free culture from 16.5% to 35.8% DW in comparison with 24.7% in the control. Jiang et al. (2012) studied the variation on lipid content in response to N limitation in *Dunaliella tertiolecta* and *Thalassiosira pseudonana*, reporting a maximum lipid content at day 10, and afterwards the lipid content was reduced. Praveenkumar et al. (2012) reported that after 20 days, *Chlorella* sp. BUM11008 accumulated more lipids in the stationary phase. Ho et al. (2012) indicated that the length of nitrogen starvation period is an important factor influencing the lipid/carbohydrates accumulation. During their experiments, the protein content decreased whereas the lipid and carbohydrate contents increased as the starvation time increased.

On the other hand, Yeh and Chang (2011) reported that initial nitrogen source concentration might be the key factor influencing the lipid accumulation. In the case of *Chlorella vulgaris* a mineral medium with low initial nitrogen source concentration (i.e., 313 g m⁻³ KNO₃ or 43.4 g m⁻³ N-NO₃) was the most effective approach to enhance microalgal lipid production, attaining a lipid productivity of 78 g lipid m⁻³ d⁻¹ and a lipid content of 55.9%. Li et al. (2008) cultivated *N. oleoabundans* in different nitrogen sources observing that with an initial low nitrate concentration, the lipid productivity increased. The maximum value obtained, with 275 g m⁻³ of NaNO₃, was 125 g lipid m⁻³ d⁻¹ with low biomass productivity (310 g m⁻³ d⁻¹) and a biomass concentration of 1,850 g m⁻³. Xin et al. (2010) have reported that in conditions of nitrogen limitation (2.5 g m⁻³), *Scenedesmus* sp. LX1 could accumulate lipids up to 53% DW.

Among the different nutrient limitations tested by Praveenkumar et al. (2012), nitrogen deprivation yielded the highest lipid productivity of 54 g lipid m⁻³ d⁻¹, followed by the combined deprivation

condition of nitrogen, potassium-phosphate, and iron free treatment. After 16 days of normal growth, the microalgae *Chlorella* sp. BUM11008 was exposed to a second phase of nutrient deprivation for 4 days, which affected the biomass but allowed an increase of the lipid content up to 42%.

Microalgal cultures under nitrogen starvation for lipid accumulation require adequate energy source (light) and carbon source (CO₂) availability (Praveenkumar et al., 2012). Different authors have used a CO₂ supply in the range of 1–5% under nitrogen starvation (Zhekisheva et al., 2002; Solovchenko et al., 2007; Li et al., 2008; Recht et al., 2012). It has been also reported that gas streams with high CO₂ levels (30–50%) were favorable for the accumulation of total lipids and polyunsaturated fatty acids (Tang et al., 2011). Consequently, to obtain an appropriate level of lipids for biodiesel production it is necessary to supply the adequate carbon quantity. According to all the above-mentioned results, three aspects seem particularly relevant to promote the lipid accumulation: (a) a moderated to high CO₂ concentration in a range from 1–50%, (b) the appropriate harvesting time and (c) the nitrogen content which promote the lipid accumulation.

3.5. Biochemical and lipid profiles

The biochemical profile of *S. obtusiusculus* obtained at growth conditions (complete nitrogen content) or nitrogen limitation under different CO₂ concentrations are shown in Table 3. As it can be observed, the lowest total lipid content (25.4%) obtained under nitrogen limitation and 0.04% of CO₂ concurs with the highest carbohydrate proportion (63.4%). The protein content decreased 48% under nitrogen limitation regarding to the value obtained at growth conditions and chlorophylls were below detection. The biomass composition obtained by elemental analysis was 51.57% C, 8.13% H, and 3.91% N.

Table 3 also shows the profiles of fatty acids obtained; the results indicated the presence of mainly saturated or monounsaturated fatty acids from C16 to C18. The proportion of palmitic acid (C16:0) in neutral lipids remained unchanged (30%) independently of the tested condition. The C18 compounds constituted 58.5% of the total neutral lipids at growth conditions and, under nitrogen limitation, an averaged value of 69.6% was obtained. It was also observed an increment in oleic acid (C18:1n9c) in comparison with the profile obtained under growth condition.

Table 3
Biochemical profile and lipid profile (in %DW) obtained under growth condition and nitrogen starvation at different CO₂ concentrations and 94.4 μmol m⁻² s⁻¹.

% CO ₂	Growth condition		Nitrogen limitation	
	2	0.04	5	
<i>Biochemical profile</i>				
Carbohydrates (%)	28	63.4	27.9	
Proteins (%)	25	11.2	12.7	
Chlorophylls (%)	0.8	BD	BD	
Ashes (%)	8.2	ND	ND	
Neutral lipids (%)	20	15	55.7	
Polar lipids (%) ^a	18	10.4	3.7	
<i>Fatty acid composition</i>				
C16:0 (%)	30.2	30.2	30.6	
C16:1n (%)	7.3	0	0	
C17:1 (%)	4.0	0	0	
C18:0 (%)	0	4.2	4.6	
C18:1n9t (%)	19.5	9.9	11.3	
C18:1n9c (%)	26.2	44.4	47	
C18:3n6 (%)	12.8	11.3	6.5	

ND, no determined; BD, below detection.

Fatty acids: C16:0 (palmitic acid), C16:1n (palmitoleic acid), C17:1 (heptadecanoic acid), C18:0 (stearic acid), C18:1n9t (elaidic acid), C18:1n9c (oleic acid), C18:3n6 (γ-linolenic acid).

^a Calculated to attain 100%.

Our results suggested that under nitrogen starvation with an adequate carbon supply the microalgae accumulates lipids with an appropriate profile for biodiesel production.

Biodiesel are fatty acid methyl esters commonly produced by transesterification of vegetable oils, which have similar lipid profile to microalgae, mainly C16 and C18 fatty acids. Common fatty acids in biodiesel include palmitic, stearic, oleic, linoleic and linolenic acids (Knothe, 2008).

4. Conclusions

S. obtusiusculus exhibited CO₂ fixation rates and productivities higher than the reported by other species of microalgae used for CO₂ removal. This microalga may have the potential to be grown in gas exhaust from thermoelectric industry since no inhibition at 10% CO₂ was observed. The recovery of lipids with an appropriate profile for biodiesel production is also possible, since productivity of around 500 g m⁻³ d⁻¹, a maximum biomass of 6,000 g m⁻³ and a lipid productivity 200 g m⁻³ d⁻¹ were obtained and it was able to store a high lipid content (55.7% DW) under nitrogen starvation.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.biortech.2012.12.081>.

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