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Effects of inorganic carbon concentration on carbon formation, nitrate utilization, biomass and oil accumulation of *Nannochloropsis oculata* CS 179

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ABSTRACT

This investigation examined the effects of the inorganic carbon concentration (4, 0.8 and 0 g/L $NaHCO_3$) on the carbon formation, nitrate utilization, growth and fatty acids compositions of *Nannochloropsis oculata*. The dissolved inorganic carbon (DIC) concentration in the three treatments decreased sharply during the first 6 days, and the percentage of dissolved organic carbon (DOC) (% of total organic carbon (TOC)) decreased with the depletion of the DIC. The NO_3^- assimilation of the algae was correlated with the DIC concentration. The algae in the highest DIC treatment had the highest specific grow rate (0.0843 d^{-1}) ($P < 0.0001$), and their biomass and fatty acid methyl esters (FAME) productivity were 84.00 and 9.69 mg/L/d, respectively ($P < 0.0001$). Contents of C16 and C18 series (% of FAME) were high and the C16:0 increased with the decrease of C18:1 during the cultivation. The iodine value (IV) of the algae was low at the low DIC media.

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1. Introduction

Combining the mass culture of microalgae for renewable biofuel production with the bio-fixation of CO_2 to decrease the greenhouse effect is an attractive idea, although the problem of the cost effectiveness needs to be addressed (Lee and Lee, 2002). Moreover, the shortage and unstable supply of exhaust gas from power plants, cement plants or other manufacture plants is seriously limited for the carbon demand of the mass culture of microalgae (Kadam, 1997; McGinn et al., 2011). In contrast, soluble carbonate (e.g., CO_3^- or CO_3^{2-}) is a stable and ample carbon source for the commercial culture of microalgae (Merrett et al., 1996; Huertas et al., 2000). Many studies have reported the mechanism of inorganic carbon utilization in microalgae, and generally the carbonate can be converted into CO_2 through the internal carbonic anhydras and extracellular carboanhydrase before being assimilated by microalgae (Ginzburg, 1993; Huertas and Lubian, 1998). The carbon fixation by microalgal photosynthesis and biomass conversion into biofuels is considered a simple and appropriate process for CO_2 circulation on earth (Takagi et al., 2000).

For the mass cultivation of microalgae, nitrogen and phosphorus utilization is essential as the key nutrient source to affect the growth, lipid content and fatty acid profiles in algal cells, and the efficiency of nitrogen and phosphorus utilization in many microalgae has been widely reported (Li et al., 2011). Under the integrative effects of the culture conditions, such as temperature, light intensity, salinity and nutrient deficiency, the composition of fatty acid methyl esters (FAMEs) in most microalgae can be varied to supply the biofuel production (Takagi et al., 2000; Converti et al., 2009; Lin and Lin, 2011). Among the FAMEs, the C16 and C18 series are often used to evaluate the biofuel productivity from algae (Converti et al., 2009) as the diesel fuel mainly being consisted of C16 and C18 compounds (Hu et al., 2008).

As an essential substrate for photosynthesis and structural composition of algal cells, the carbon element has been reported to dramatically affect the growth and composition of fatty acids in certain strains of microalgae (Ota et al., 2009). Fulke et al. (2010) reports that the lipid productivity of *Chlorella* sp. is significantly different under different carbon concentrations, and the biomass and lipid accumulations of *Nannochloropsis oculata* also varies under different concentrations of CO_2 aeration (Chiu et al., 2009). Generally, microalgae can release some photosynthetically fixed organic carbon into the surrounding water through the leaky algal cells (Puddu et al., 2003), which are resulted from physical stress, and this should be the cause of the organic compounds in water (Myklestad, 2000). However, no study has been reported on the dynamic form of carbon and the combined study of carbon and nitrogen utilization during the microalgae cultivation for biofuel production.

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The present study aimed to investigate: (a) the dynamics of the dissolved inorganic carbon (DIC) and dissolved organic carbon (DOC) in the media, (b) the utilization efficiency of nitrogen and (c) the biomass, FAME composition and oil accumulation under the different bicarbonate concentrations during the 16-day cultivation of *N. oculata*.

2. Methods

2.1. Microalgae and culture conditions

The microalgae *N. oculata* CS 179 used in this study was purchased from the Key Laboratory of Aquaculture of Ocean University of China (obtained from Australian CSIRO Collection of Living Microalgae). Seawater used in the experiment was pumped directly from the Daya Bay (near Hong Kong) of the South China Sea and treated with double sand filtration, and the DIC, NO_3-N, NH_4-N and PO_4-P concentration in the seawater was approximately 19.73, 0.42, 0.07 and 0.06 mg/L. $NaHCO_3$ was used as the inorganic carbon source, and the algae were grown in the media including 2 mL Guillard's F/2 trace metal solution and 6 mL modified Guillard's F/2 formula (85 g $NaNO_3$, 6 g $NaH_2PO_4{\cdot}H_2O$, 0.05 g vitamin B_1, and 0.0001 g vitamin B_{12}) per liter of the sterilized fresh seawater.

Roux bottles (Pyrex, Sigma–Aldrich, USA) (25 cm height, 11.5 cm width and 5.5 cm depth) with 900 mL of media were used to cultivate the *N. oculata.* Magnetic stir plates (Scholar 171, Corning. Co., Ltd, Germany) were used to continuously mix the cultivation medium in each bottle. The light source was composed of the fluorescent tubes (Speethalux^{TM} T5HO54W 6500 K, China), which were placed horizontally and parallel to the front side of bottles. Air, which was filtered by the 1 M NaOH solution, was equably injected into the bottles. During the cultivation, the light was on continuously and the temperature, salinity, pH and light intensity were 26 ± 1 °C, 31 ± 0.5‰, 7.7 ± 0.2 and 160 ± 5 $\mu E\,m^{-2}\,s^{-1}$, respectively.

2.2. Experimental design

Three treatments (Tr-1: 400 mg/L $NaHCO_3$; Tr-2: 80 mg/L $NaHCO_3$ and Tr-3: 0 mg/L $NaHCO_3$) was carried out, each with four replicates to investigate the effects of carbon concentration on the dynamics of nutrient composition in media, growth and oil accumulation of *N. oculata*. To keep an sufficient nutrient for the growth of *N. oculata*, 2 mL Guillard's F/2 trace metal solution and 6 mL modified Guillard's F/2 formula were added to the cultivation media for the nutrient supplement at day 5 and day 9. Three phases (days 1–5, days 6–9 and days 10–16, respectively) were compared on the utilization of nitrate in the algae.

Thirty milliliter of the culture media from each bottle was collected to measure the biomass concentration, carbon forms (TOC, DIC and DOC), nitrate (NO_3^{-1}) and orthophosphate (PO_4^{3-}) concentration at 8:00 a.m. every day, and then the media was replaced by 30 mL sterile seawater. The algae were sampled every other day to measure and analyze the fatty acid compositions.

2.3. Measurement

2.3.1. Determination of TOC, DIC and DOC

The measurements of the TOC, DIC and DOC in cultivation media were performed using a high temperature TOC/TNb analyzer (liquiTOC II, Elementar, German) coupled with automatic sampling instrument. To prepare for the measurement, 3 mL algal media was diluted 10 times with the distilled water and transferred into a 30 mL brown glass reagent bottle. Another 3 mL media filtered by pre-combusted GF/F filter (Whatman, 0.7 μm pore size, 25 mm) was diluted 10 times with distilled water, and then transferred to a 30 mL brown glass reagent bottle. Both samples were acidified with 100 μL nitric acid and then stored at −20 °C for the measurement.

2.3.2. Nitrate determination

The collection and preparation of each sample for nitrate determination were similar to the process for TOC. Nitrate (NO_3^-) concentration was measured with a nutrients-autoanalyzer (Quickchem 8500, Lachat Instruments, USA) as described by Kirkwood et al. (1996). This equipment had analytical errors of less than 10% and detection limits of 0.014, 0.05, 0.005 and 0.075 $\mu mol\,L^{-1}$ for NO_3^-, respectively, and was regularly calibrated against CSK standard solutions.

2.3.3. Dry biomass and specific grow rate

A certain volume (depends on the concentration of the sample, normally less than 10 mL) of algal media was filtered through a pre-dried (105 °C, 3 h) and weighted glass microfiber filter disks (d = 47 nm, 0.7 μm nominal pore size) (Whatman CF/F), and the filter with algae were dried in the oven at 105 °C for over 3 h. The specific grow rate (μ) was calculated as the formula: $\mu = \ln(W_f/W_0)/\Delta t$. W_f and W_0 were the final and initial dry biomass, respectively. Δt was the cultivation time in days (Ono and Cuello, 2007).

2.3.4. Fatty acid compositions and iodine value (IV) analysis

FAME and fatty acid composition were determined following the method of Lin and Lin (2011). Direct transesterification for determination of free and bound fatty acid content of the algae was conducted. Approximately 5 mg algae were used for FAME analysis. 90 μL heptadecanoic acid (2 μg/μL, Fluka, puriss: ⩾99 (GC)) and 1 mL methanol (0.5 M) solution were added to each sample. Heptadecanoic acid was used as the internal standard. The sample was being shaken vigorously under the water bath (75 °C) for 10 min, and then cooled to the room temperature. Then 2 mL boron trifluoride methanol complex solution (RS-Aldrich) was added with the vigorous shaking at water bath (75 °C, 10 min). One hundred microliter saturated salt water and 2 mL hexane was injected to the sample that cooled to room temperature, and then the sample was mixed well and centrifuged at 1000 r/m for 5 min. Finally, the FAME was combined and evaporated with N_2 gas to dryness on the Reacti-Vap III. Samples were re-dissolved in 300 μL of gas chromatography (GC) grade *n*-decane, and later transferred to a GC vial with poly spring insert and run using the FAST FAME method on the GC.

A GC-MS (Agilent 6890GC-5975MSD) equipped with a split/splitless injector and a capillary column (30 m, 0.25 mm and 0.25 μm) (Agilent 122-2332 DB-23) was used for the rapid GC analysis according to the modified method of Lin and Lin (2011). The carrier gas was high purity helium at 36 cm/s. Temperature program was as follows: initially, 70 °C; ramp at 10 °C/min to 140, 5 °C/min to 200 °C with 1 min hold; final temperature was 230 °C. Instrument conditions were as follows: FID set at 250 °C; air flow of 450 mL/min and makeup flow of 45 mL/min; split ratio of 3:1 with a 2.9 mL/min flow; sampling frequency of 20 Hz; auto sampler injections of 2 μL volume. Run time for a sample was 21 min. Duplicates of each FAME analysis were done and FAMEs identification was run by comparison with standard certificate, Supelco FAME10 mix 37 (Bellefonte, PA, USA).

The iodine value (IV) of *N. oculata* was calculated according to AOCS recommended practice CD 1c-85. This method estimates the iodine value from the fatty acid compositions and the grams of halogen absorbed by 100 g of fat (Damiani et al., 2010).

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