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Enhancing lipid productivity by co-cultivation of *Chlorella* sp. U4341 and *Monoraphidium* sp. FXY-10

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To improve lipid productivity, co-cultivation of *Chlorella* sp. U4341 and *Monoraphidium* sp. FXY-10 for lipid production was studied. Compared with mono-cultivations, co-cultivation of the two microalgae significantly increased the accumulation of total biomass and total lipid yield, and enhanced the lipid productivity (29.52 mg L^{-1} d^{-1}). Fatty acid compositions significantly varied in different cultivations. The content of C18 fatty acids in co-cultivation significantly increased, especially for oleic acid (32.45%) and linolenic acid (10.03%) compared with that in mono-cultivation. Moreover, high saturated and monounsaturated fatty acids (55.85%) were obtained in co-cultivation, which suggests their potential as a biodiesel feedstock.

[**Key words:** Lipid productivity; Co-cultivation; *Chlorella*; *Monoraphidium*; Fatty acids; Biodiesel]

The scarcity of known petroleum reserves makes renewable energy resources highly attractive. The most feasible technique to meet the growing energy demand is the use of alternative fuels. An alternative fuel to petrodiesel must be technically feasible, economically competitive, environmentally acceptable, and easily available (1). One such fuel that exhibits great potential is biofuel, particularly biodiesel. Biodiesel (monoalkyl esters of fatty acids) is produced from vegetable oils, animal fats, restaurant waste and microalgal oils by transesterification or esterification with short chain alcohols. Although vegetable oils have been used as a diesel fuel source since the early 1930s, they may also be used for human consumption, thereby increasing the demand and cost for this resource because large areas of land, capital, and manpower are required for cultivation, which makes biodiesel production an economically challenging process (2,3). Although the use of animal fats and restaurant waste can reduce costs, their saturated compounds and crystallization at high temperatures are the disadvantages of using animal oils as a feedstock (4). Microalgae appear to be the only source of biodiesel with the potential to displace fossil diesel completely, and grow extremely rapidly, many are exceedingly rich in oil (3).

However, production of biodiesel from microalgae is technically, but not yet economically, feasible (3). For the reduction of the production cost of biodiesel to compete with petrodiesel, high lipid productivity is a key desirable characteristic of species for biodiesel production, Microalgae should be simultaneously cultivated in low-cost cultivation systems, such as co-cultivations. Co-cultivations are similar to mixed cultivations of microorganisms which are common in ecosystems, but with a unique difference in cultivation. In co-cultivations the quantity and type of organisms in the cultivation are all defined at inoculation whereas in naturally occurring mixed cultivations, different organisms, depending on cultivation conditions, may become dominant during the cultivation period (5).

The exploration of co-cultivation has become highly critical in many key biochemical processes. The benefits of this growth and lipid production strategy can potentially be exploited for high lipid productivity for biofuel. However, the reports on co-cultivation have mainly concentrated on the two different nutritional growth modes by addition of organic carbon source, and heterotrophic and mixotrophic nutrition. For example, co-cultivation of microalgae and yeast under heterotrophic condition showed improvements in cellular biomass and oil accumulation (6). A Louisiana native co-cultivation of microalgae and cyanobacteria under mixotrophic condition using sodium acetate as carbon source resulted in high mean biomass productivity (7).

In this study, microalgae *Chlorella* sp. U4341 and *Monoraphidium* sp. FXY-10 were isolated from a local lake, and used in co-cultivation under photoautotrophic condition without an organic carbon source to reduce the cost of raw materials for biofuel production. Lipid productivity and fatty acid composition were compared with those of the mono-cultivations. Both *Chlorella* and *Monoraphidium* are the most promising feedstock for biodiesel (8,9). *Chlorella* sp. U4341 has a relatively high growth rate and quick nutrient consumption ability. *Monoraphidium* sp. FXY-10 is an oleaginous microalga that can accumulate high amount of lipids especially when nutrition is low (10). Compared with other lipid-producing microalgae, *Chlorella* sp. U4341 and *Monoraphidium* sp. FXY-10 in a

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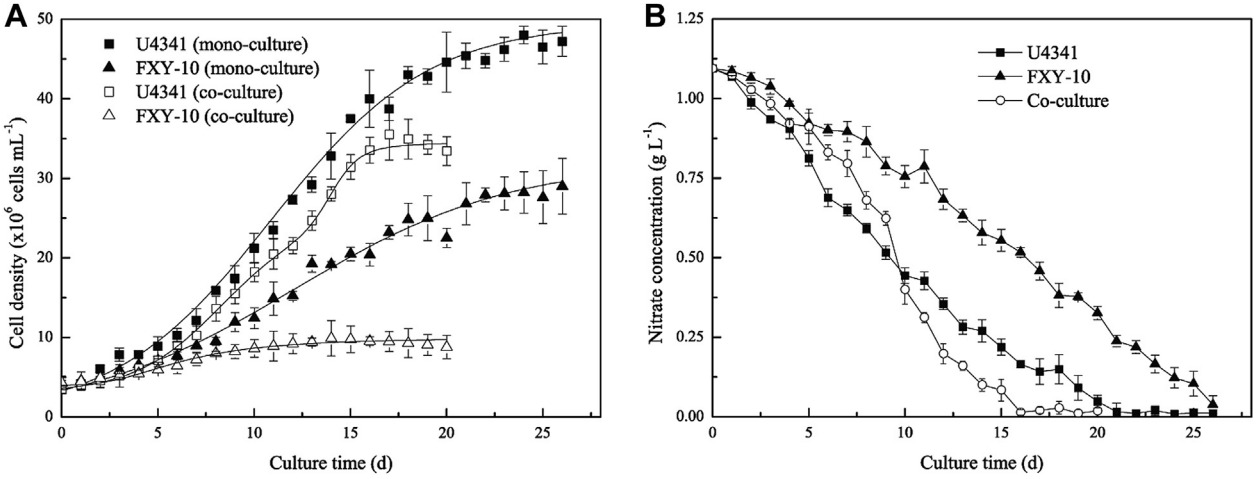

FIG. 1. Growth curves (A) and nitrate consumption profiles (B) of *Chlorella* sp. U4341 and *Monoraphidium* sp. FXY-10 in mono- and co-cultivation conditions. Bars are means of three replications ± SD.

co-cultivation system have the potential to accumulate more total lipids in a relatively short time. To our knowledge this study is the first to report on the lipid-accumulating properties of *Chlorella* and *Monoraphidium* sp. cells in co-cultivation. This study aimed to investigate a novel method for increasing lipid productivity and decreasing the cost of raw materials for biofuels production in a co-culturing system.

MATERIALS AND METHODS

Microorganisms and cultivation conditions *Chlorella* sp. U4341 and *Monoraphidium* sp. FXY-10 were isolated from water samples collected from Lake Fuxian (24°20′67″ N and 102°32′9″ E), a plateau freshwater lake in Yunnan Province in China. U4341 and FXY-10 were cultivated using modified Bold's Basal medium with the following composition: $NaNO_3$ (1.5 g L^{-1}), KH_2PO_4 (0.7 g L^{-1}), $K_2HPO_4 \cdot 3H_2O$ (0.45 g L^{-1}), $MgSO_4 \cdot 7H_2O$ (0.3 g L^{-1}), $FeSO_4 \cdot 7H_2O$ (3 mg L^{-1}) and trace metal mix A5 (1 mL L^{-1}). All media were adjusted to pH 6.3 prior to autoclaving.

Photoautotrophic flask cultivations of U4341, FXY-10, or a mixture of both were carried out in 200 mL Erlenmeyer flasks containing 500 mL of the cultivation medium autoclaved for 20 min at 121°C, and inoculated with exponentially growing microalgae. Thus, initial cell count of U4341 and FXY-10 were 3.41×10^6 and 4.26×10^6 cells mL^{-1}, respectively. The microalgae were continuously illuminated at 70 μ Em^{-2} s^{-1} intensity, with white fluorescent light within the shaker and orbital shaking at 150 rpm at 25 ± 1°C. No extra carbon dioxide was provided except what was naturally existing in the atmosphere to all the cultivations.

Determination of algal growth and biomass concentrations To evaluate the growth of microalgae in mono- and co-cultivations, cell numbers were counted using a hemacytometer microscope (Nikon eclipse 50i). The specific growth rate (μ) was calculated according to the following equation:

$$\mu = \left(\ln N_f - \ln N_i\right) \Big/ \left(t_f - t_i\right) \quad (1)$$

where N is the cell density (cells mL^{-1}) at final (f) or initial (i) at time (t).

Cells were harvested by centrifugation at 13,000 ×g for 5 min after cultivation. The pellets were washed twice with deionized water, frozen overnight at −70°C and freeze-dried at −80°C under vacuum conditions for 24 h. The pellets were weighed and considered as dry biomass weight (DBW). Biomass productivity was calculated by dividing the DBW with cultivation time (d). Experiments were performed in triplicate, and data are expressed as mean standard deviation (±SD).

Nitrate determination Nitrate concentration in medium was determined by a colorimetric method (11). In brief, 1 mL algae culture was collected and centrifuged, and 100 μL of the supernatant was mixed with 400 uL of 5% (w/v) salicylic acid in concentrated H_2SO_4. After incubation at room temperature for 20 min, 9.5 mL of 2 M NaOH was slowly added. Samples were cooled to room temperature, and absorbance was measured at 410 nm.

Lipid extraction and determination Total lipid extraction from dry biomass was performed according to the procedure of Bligh and Dyer (12). The dry biomass was ground into a fine powder, 1 g of power was blended with 3 mL of chloroform/methanol (2:1). The mixture was agitated for 20 min in an orbital shaker at 100 rpm at room temperature. The solvent phase was recovered by centrifugation at 2000 ×g for 10 min. The pellet was re-extracted in 3 mL of chloroform/methanol solution twice. The collected extract was evaporated at 40°C, dried at 70°C for 2 h, and subsequently weighed after cooling to room temperature. The lipid content was calculated using the following equation:

$$\text{Lipid content}(\%) = W_L/W_A \times 100\% \quad (2)$$

where W_L (g) is the weight of the extracted lipids and W_A (g) is the dry algae biomass.

The lipid productivity was calculated as follows:

$$P_{\text{Lipid}}\left(\text{mg } l^{-1} \text{ d}^{-1}\right) = W_A(g) \times C_{\text{Lipid}}(\%)/V(l) \times T(d) \quad (3)$$

where P_{Lipid} is the lipid productivity, C_{Lipid} is the lipid content, V is the working volume, and T is the cultivation time.

Fatty acid composition analysis Fatty acid methyl esters (FAMEs) were prepared by *in situ* transesterification on lyophilized cells (13). The lipids were solubilized in 0.2 mL of chloroform/methanol (2:1, v/v), and simultaneously transesterified *in situ* with 0.3 mL of HCl/MeOH (5%, v/v) for 1 h at 85°C. The resulting FAMEs were extracted with 1 mL of *n*-hexane at room temperature for at least 1 h, and the top *n*-hexane layer was analyzed by gas chromatography/mass spectrometry (Agilent 7890A/5975C) with an HP-5MS capillary column (5% phenyl methyl silox, 30 m × 0.25 mm × 0.25 μm). The injector was as 250°C with an injection of 1 μL under split mode (1:40). The temperature was programmed to 170–190°C at a rate of 10°C min^{-1}, 190°C (1 min) to 207°C at a rate of 0.8°C min^{-1}, and held at 207°C for 1 min. The carrier gas was helium, with a flow rate of 1.0 mL min^{-1}. The mass spectrometer was operated in EI (Electron Ionization) mode at 70 eV, and the scanned mass ranged from 15 amu to 650 amu. Peak identification was accomplished by comparing the mass spectra with the National Institute of Standards and Technology (NIST) mass spectral library (NIST08.L). Experiments were performed in triplicate, and data are expressed as mean standard deviation (±SD).

The degree of unsaturation (DU) was calculated as follows (14):

$$\text{DU} = (\text{monounsaturated, wt.\%}) + 2 \times (\text{polyunsaturated, wt.\%}) \quad (4)$$

The long chain saturated factor (LCSF) was calculated as follows (14):

$$\text{LCSF} = 0.1 \times \text{C16(wt.\%)} + 0.5 \times \text{C18(wt.\%)} + 1 \times \text{C20(wt.\%)} + 1.5 \times \text{C22(wt.\%)} + 2 \times \text{C24(wt.\%)} \quad (5)$$

The cold filter plugging point (CFPP) was calculated as follows (14):

$$\text{CFPP} = 3.1417 \times \text{LCSF} - 16.477 \quad (6)$$

RESULTS

Growth characteristics of the microalgae in mono- and co-cultivations The dynamics of the growth curves of the three cultivations are shown in Fig. 1A. The logistic growth model gives the best fitting degree to experimental data, but U4341 in co-cultivation use BiDoseResp function to show high degree of