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Characterization of *Chlorococcum pamirum* as a potential biodiesel feedstock

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HIGHLIGHTS

- *Chlorococcum pamirum* can grow fast with μ_{max} of 1.888 d^{-1}.
- The lipid content can reach 64.9% of dry weight under nitrogen-deficient condition.
- Initial cell concentrations influenced the lipid accumulation evidently.
- NaCl enhanced the lipid accumulation and saturated fatty acids content outdoors.
- *Chlorococcum pamirum* is a promising organism for biofuel feedstock production.

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ABSTRACT

To evaluate the potential of *Chlorococcum pamirum* for producing biodiesel, the effects of nitrogen, phosphate, initial cell concentrations and NaCl on lipid accumulation and growth were studied. The highest specific growth rate (μ_{max}), biomass productivity and lipid content achieved was 1.888 d^{-1}, 350.1 mg L^{-1} day^{-1}, and 64.9%, respectively. Under nitrogen-deficient condition, the cells accumulated lipids faster at low initial cell concentration. Additional NaCl to nitrogen-deficient media accelerated the lipid accumulation. When adding 10 g L^{-1} NaCl to nitrogen-deficient media, the lipid content and productivity of cells cultured outdoors with high initial cell concentration increased from 38.2% and 153 mg L^{-1} day^{-1} to 54.3% and 192 mg L^{-1} day^{-1} respectively. Moreover, NaCl enhanced the saturated fatty acids content from 56.40% to 73.41% of total fatty acids. The results show that *C. pamirum* is a promising organism for biofuel production.

1. Introduction

With increasing air pollution and global warming caused by burning fossil fuel, the world is paying more and more attention on developing renewable, biodegradable and environment-friendly alternative energy sources to alleviate the dependence on the crude oil (Farrell et al., 2006; Lin and Lin, 2011). In recent years, the potential value of microalgae to produce biodiesel has been widely recognized (Rodolfi et al., 2009). Compared with other oil crops, microalgae possess distinct advantages, such as higher growth rate, lipid content, photosynthetic efficiency, and much less land required for cultivation. (Chisti, 2007; Rodolfi et al., 2009). Therefore, microalgae are regarded as a promising feedstock for sustainable biodiesel production (Chisti, 2007; Hu et al., 2008).

However, microalgal biodiesel has not been successfully commercialized mainly due to high production costs. Low efficiency of lipid accumulation (e.g. lipid content and lipid productivity) by microalgae is one of the important reasons causing high production costs (Sheehan et al., 1998; Chisti, 2007; Feng et al., 2011a). It is now generally recognized that high cellular lipid contents are usually produced by cells under stress, typically nutrient limitation, which is often associated with low growth rate and low overall lipid productivity (Li et al., 2008, 2010; Feng et al., 2011a). In order to enhance the lipid accumulation of microalgae, a two-phase cultivation process was suggested: a nutrient-sufficient phase to cultivate high biomass concentration followed by

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a nutrient limitation phase to induce lipid accumulation (Rodolfi et al., 2009). Therefore, according to the two-phase cultivation process, microalgal strains that can be potentially employed for biodiesel production should be capable of growing fast and accumulating large amount of lipids.

Many factors could influence the lipid accumulation and cell growth of microalgae, such as CO_2 concentration (Lv et al., 2010), light intensity (Ho et al., 2012; Yeesang and Cheirsilp, 2011), temperature (Xin et al., 2011), nutrition (Rodolfi et al., 2009; Li et al., 2008; Feng et al., 2011a). Among those factors, nitrogen and phosphate are the most crucial factors affecting the growth and lipid accumulation of microalgae. Nitrogen deficiency is a stress condition, and normally alters carbon partitioning from carbohydrate or protein into lipid (Harwood and Jones, 1989). Lv et al. (2010) reported that the lipid productivity of *Chlorella vulgaris* reached 40 mg L^{-1} day^{-1} by adjusting cultivation condition. The lipid content of 40% and lipid productivity of 133 mg L^{-1} day^{-1} were obtained in *Neochloris oleoabundans* cells by Li et al. (2008). Our previous study also showed the lipid content and productivity of *Chlorella zofingiensis* could reach to 65.1% and 87.1 mg L^{-1} day^{-1} by nitrogen deficiency, respectively (Feng et al., 2012).

A freshwater unicellular green microalga, *Chlorococcum pamirum*, was isolated from Yadkin River at Forsyth county of North Carolina of USA. In our preliminary experiments, we found this strain can grow with a specific growth rate above 1.5 d^{-1} and possess the ability of accumulating large amount of lipids (lipid content >55% of dry cell weight). But, there are no systematic studies regarding the application of this strain for biodiesel production.

In this study, we aimed to evaluate the potential of *C. pamirum* to produce lipids for biodiesel. First, the effects of nitrogen and phosphate on growth rate and lipid accumulation were studied. Furthermore, we investigated the effects of initial cell concentration and NaCl concentration on lipid accumulation of *C. pamirum*. Finally, to enhance the lipid production and improve fatty acids composition in *C. pamirum* cells cultured with high initial cell concentration, we also investigated the effects of NaCl stress on culture outdoors. The results from this research are important for improving the two-phase cultivation process.

2. Methods

2.1. Algal strain and cultivation conditions

C. pamirum was collected from Yadkin River at Forsyth county of North Carolina, USA, and was maintained in BG-11 medium.

Indoor cultivation conditions: cells at exponential phase were inoculated into column photobioreactors (clear glass; diameter: 3.2 cm; culture volume: 400 ml) with constant aeration at about 0.6 L min^{-1} and cultivated under continuous fluorescent light (fluorescent tube) at 25 °C. The light intensity on the surface of columns was about 100 ± 5 μmol m^{-2} s^{-1}. Constant aeration was supplied by air pump.

Outdoor cultivation conditions: cells had been cultured outdoors for at least one generation to adapt the outdoor conditions before the inoculation. The photobioreactor used outdoors was 40 L column with diameter of 16 cm. Aeration was provided for each photobioreactor from 6:00 am to 6:00 pm. The light intensity and medium temperature were measured every hour from 8:00 am to 5:00 pm during the experiments.

Photometer (maker: Li-COR; model type: Li-190SA) was used to measured the light intensity (the surface of photobioreactors).

2.2. Dry weight determination

Cells were collected onto preweighed Whatman GF/C filter paper (0.45 μm pore size; diameter 47 mm) by filtering algal culture, and washed with distilled water. The cells on the filter paper discs were dried in oven at 105 °C over night and left in cooling chamber until the weight was constant (Zhu and Lee, 1997).

2.3. OD_{550} (the optical density at 550 nm) determination

The OD_{550} of culture was measured by spectrophotometer. Samples were diluted with appropriate ratios to ensure that the measured OD_{550} values were in the range of 0.200–0.300 if applicable (Lu et al., 1994).

2.4. Total lipids measurement

Total lipids were extracted according to the modified protocol of Bligh and Dye (1959). The cells (V ml algal culture) were collected by centrifuging at 4000*g* for 5 min. 2 ml chloroform, 4 ml methanol and 1.6 ml distilled water (1:2:0.8, v:v:v) was added into the centrifuge tube. The mixture was agitated on a vortex for 10 min followed by centrifugation at 4000*g* for 5 min. The supernatant was transferred to a pre-weighted glass tube (M_1 g). Then, 2 ml methanol and 1 ml chloroform were added into the centrifuge tube, agitated on a vortex for 3 min and kept in dark for 24 h again. After that, the extracts were centrifuged at 4000*g* for 5 min, the supernatant was collected in the same pre-weighed glass tube, then 3 ml chloroform was added, followed by 3.8 ml distilled water (1:1:0.9, v:v:v). When complete phase separation occurred, the top layer was removed, and the bottom layer was dried with N_2. The residue and glass tube were further dried under oven at 105 °C until the weight was constant (M_2 g). Lipid content was calculated by subtracting M_1 from M_2, and was expressed as% of dry weight.

2.5. Fatty acid profiles analysis

The fatty acid profiles were determined as fatty acid methyl esters (FAMEs) following the method by Indarti et al. (2005). FAMEs were extracted from 50 mg freeze-dried cells by 2.5 ml 2% H_2SO_4–methanol solution in water bath with stirring at 80 °C for 2.5 h. After the mixtures cooled to room temperature, 1 ml hexane (high performance liquid chromatography grade) and 1 ml saturated NaCl solution were added. When complete phase separation occurred, the upper layer containing FAMEs was collected for FAMEs composition analysis.

FAMEs compositions were analyzed by using a gas chromatograph (GC-2010, Shimadzu, Japan) with a flame ionization detector (FID). Nitrogen was used as carrier gas with a flow rate of 1.2 ml min^{-1}. The injector and detector temperature were set at 250 °C and 280 °C, respectively. The initial temperature of column was set at 140 °C, and increased to 180 °C at a rate of 10 °C min^{-1} followed by a rise to 230 °C with 1 °C min^{-1}, and then the temperature was kept constant at 230 °C for 10 min. Ultimately, the fatty acids were recorded as percentages of total fatty acids.

2.6. Calculations on specific growth rate and biomass productivity

The max specific growth rate (μ_{max}) at the exponential phase was calculated according to $\mu_{max} = (\ln x_2 - \ln x_1)/(t_2 - t_1)$, where x_2 and x_1 is the dry weight (g L^{-1}) at time t_2 and t_1, respectively. The biomass productivity (P) was calculated according to $P = (DW_x - DW_1)/(T_x - T_1)$. DW_x and DW_1 is the dry weight (g L^{-1}) at time T_x and T_1 (the first day), respectively.