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Identification of carbohydrates as the major carbon sink of the marine microalga *Isochrysis zhangjiangensis* (Haptophyta) and optimization of its productivity by nitrogen manipulation

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HIGHLIGHTS

- Carbohydrates were the major carbon sink in *Isochrysis zhangjiangensis*.
- Carbohydrate production was optimized by nitrogen manipulation.
- The parameter F_v/F_m can serve as an indicator of carbohydrate accumulation.

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ABSTRACT

Microalgae represent a potential feedstock for biofuel production. During cultivation under nitrogen-depleted conditions, carbohydrates, rather than neutral lipids, were the major carbon sink of the marine microalga *Isochrysis zhangjiangensis* (Haptophyta). Carbohydrates reached maximum levels of 21.2 pg cell^{-1} on day 5, which was an increase of more than 7-fold from day 1, while neutral lipids simultaneously increased 1.9-fold from 4.0 to 7.6 pg cell^{-1} during the ten-day nitrogen-depleted cultivation. The carbohydrate productivity of *I. zhangjiangensis* was improved by optimization of the nitrate supply mode. The maximum carbohydrate concentration was 0.95 g L^{-1} under batch cultivation, with an initial nitrogen concentration of 31.0 mg L^{-1}, which was 2.4-fold greater than that achieved under nitrogen-depleted conditions. High performance liquid chromatography (HPLC) analysis showed that the accumulated carbohydrate in *I. zhangjiangensis* was composed of glucose. These results show that *I. zhangjiangensis* represents an ideal carbohydrate-enriched bioresource for biofuel production.

1. Introduction

Microalgae are considered to be a third-generation feedstock for biofuel production (Nigam and Singh, 2011). Carbohydrates and neutral lipids are the primary carbon and energy reserves in microalgae, and neutral lipids in the microalgae biomass are important precursors for microalgae-based biodiesel production, which has drawn considerable attention in recent years. Recently, research has focused on bioethanol converted from carbohydrates (John et al., 2011), because the production of carbohydrates in cells is more economical than that of neutral lipids (Johnson and Alric, 2013). Thus, the production of carbohydrate-enriched microalgae biomass represents a practicable method to produce a sustainable biofuel resource.

Microalgae species accumulate considerable amounts of carbohydrates under stressful conditions, such as macroelement (nitrogen, sulfur, or phosphorus) limitation (Ball et al., 1990; Gao et al., 2013; Yao et al., 2012), high light intensity (Sakamoto et al., 2012), and salinity (Takagi et al., 2006; Yao et al., 2013), among which nitrogen removal is perhaps the most effective and widely explored strategy to trigger carbohydrate accumulation (Dragone et al., 2011). However, nitrogen depletion reduces biomass productivity and therefore affects overall carbohydrate production (Ho et al., 2013a).

High cellular carbohydrate content and high biomass productivity enhance carbohydrate production. Considering the mutually exclusive nature of microalgae growth and efficient energy storage compound production, a two-stage cultivation strategy has been implemented to enhance compound productivity, which involved

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increasing biomass density in the first stage and inducing energy storage compound accumulation in the second stage. Considerable information has been reported with regard to the photoautotrophic two-stage cultivation strategy of transferring algal biomass from nutrient-rich to nitrogen-starvation conditions (Ho et al., 2013b). However, high cost and inconvenient operation are drawbacks of the two-stage cultivation strategy (Xia et al., 2013). In two-stage cultivation, the addition of limited nutrition could serve as an alternative to the transference of the microalgae from favorable conditions to stressful conditions. This nutrient limitation strategy has been successfully applied to enhance starch accumulation in *Tetraselmis subcordiformis* (Yao et al., 2012).

Isochrysis zhangjiangensis (Haptophyta), a marine golden-brown flagellated microalga, has been reported to accumulate lipids under nitrogen-replete conditions (Feng et al., 2011). However, the effects of nitrogen deprivation on the accumulation of carbohydrates and neutral lipids in *I. zhangjiangensis* have not been reported. In this study, we assessed the effects of different levels of nitrogen deprivation on the production of energy-rich compounds (carbohydrates or neutral lipids) in *I. zhangjiangensis*. To maximize the production of energy-rich compounds, a limited nitrogen strategy was introduced and studied for its ability to enhance the production of carbohydrates. The parameter F_v/F_m was determined as an indicator of the levels of stressful conditions endured during the carbohydrate accumulation process.

2. Methods

2.1. Strains and culture conditions

I. zhangjiangensis FACHB-1750, a marine microalga, was maintained by the Freshwater Algae Culture Collection of the Institute of Hydrobiology (FACHB collection), Chinese Academy of Sciences. The microalgae had been previously cultivated in f/2 medium without silica (Feng et al., 2011). Algal cells were harvested during the late exponential phase and resuspended in nitrate-free f/2 medium. The cultures were inoculated in a 600-mL glass air bubble column photobioreactor with a 500-mL working volume. Membrane-filtered aeration (0.22-μm membrane) was maintained at 0.32 vvm with 2% CO_2 enriched air, and CO_2 was supplemented only during illumination. The temperature was maintained at 25 ± 2 °C. The cultures were illuminated from one side with cool white fluorescent lamps under a 14:10 h light:dark cycle. The light intensity was increased gradually; an average irradiance of 80 μmol $m^{-2}s^{-1}$ was provided on the first day, and then increased to 180 μmol $m^{-2}s^{-1}$ on the second day. The light intensity was measured by a photosynthetically active radiation (PAR) detector (Optometer P9710 with PAR detector 3701, Gigahertz Optik Corporation, Türkenfeld, Germany). The initial cell density was 8.0×10^6 cells mL^{-1}. In the nitrogen-replete experiment, sodium nitrate was added to a final nitrogen concentration of 12.4 mg L^{-1} every 24 h. Different amounts of sodium nitrate were added on the initial day for the limited nitrogen experiment; nitrogen-starvation conditions were reached when the nitrate in the medium was exhausted. To investigate the influence of nitrate supply modes on carbohydrate production, nitrate was supplied at an interval of 48 h, with 6.2 mg L^{-1} nitrogen supplied in nitrogen-free f/2 medium (termed "feeding cultivation"), thus the total nitrate added to the medium was the same during a 10-day cultivation under the two cultivation modes. At last results were compared to those obtained with batch cultivations.

2.2. Growth measurements

Cell density was determined using a spectrophotometer (Jasco, Japan) at 680 nm. For biomass, 3–5 mL of culture was filtered onto pre-weighed Whatman GF/C filter (47 mm diameter), washed twice with 0.5 mol L^{-1} NH_4HCO_3 solution, and dried to a constant weight at 60 °C.

2.3. Photosystem II (PS II) activity measurement

PS II activity of algal cells was measured by a chlorophyll fluorometer (Water-PAM Heinz Walz GmbH, Effeltrich, Germany). Cells were adapted to the dark for 10 min before a saturating pulse was applied to measure the maximal PS II quantum yield. Maximal PS II quantum yield was calculated as F_v/F_m, where F_v was the variation in chlorophyll fluorescence between the maximal fluorescence (F_m) induced by the saturating pulse and the initial fluorescence (F_0).

2.4. Nitrate assimilation analysis

Nitrate analysis was conducted with a SEAL Analytical AutoAnalyzer 3 (SEAL Analytical, Mequon, WI, USA) following the manufacturer's instructions. Briefly, nitrate was reduced to nitrite by hydrazine in alkaline solution with a copper catalyst, and the nitrite was reacted with sulfanilamide and *N*-(1-naphthyl)ethylenediamine dihydrochloride (NEDD) to form a pink compound that was measured at 550 nm.

2.5. Biochemical composition and morphology analysis

The cell pellet centrifuged from 2 to 4 mL of culture was used for carbohydrate and protein determination. For carbohydrate analysis, the sulfuric acid-anthrone method was used (Somani et al., 1987). The proteins were extracted according to the method described by Yao et al. (2012) and protein content was analyzed using the Bradford method (Sedmak and Grossberg, 1977). Neutral lipids were determined by the Nile red method. 0.5–1 mL culture was diluted to a cell density of 1×10^6 cells mL^{-1} by sea water. Nile red dissolved in dimethyl sulfoxide (50 μg mL^{-1}) was added at a final concentration of 1 μg mL^{-1}. After staining for 10 min, the intensity of fluorescence was determined by a fluorescence spectrophotometer (Agilent Technologies, Santa Clara, CA, USA). The excitation and emission wavelengths were 480 and 580 nm, respectively. Neutral lipid content was calculated based on fluorescence intensity as follows:

$$I = 103.99C - 38.021 \quad (1)$$

where I and C are fluorescence intensity and neutral lipid content (pg $cell^{-1}$), respectively.

Carbohydrate productivity (P_s, mg L^{-1} d^{-1}) was calculated by the following equation:

$$P_s = \frac{DW_t C_{st} - DW_0 C_{s0}}{t} \quad (2)$$

where C_{st} and C_{s0} (mg $cell^{-1}$) are the carbohydrate contents at culture times t and 0 (d), respectively, and DW_t and DW_0 (cell L^{-1}) are the cell density at culture times t and 0, respectively. Observations of *I. zhangjiangensis* were conducted with a TE2000-U microscope (Nikon Corporation, Japan). The cell diameter was measured by using Image-pro plus 5.1 (Media Cybernetics, Warrendale, USA).

2.6. Monosaccharide composition determination

Storage carbohydrates were extracted from the algae biomass at 90 °C for 1 h after sonication. Protein was removed by adding 3% (v/v, final concentration) trichloroacetic acid and the solution was incubated for 3 h at 4 °C. After centrifugation to remove the solids, 4 volumes of ethanol were added and the solution was placed at 4 °C for 12 h, after which storage carbohydrates were