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The roles of starch and lipid in *Chlorella* sp. during cell recovery from nitrogen starvation

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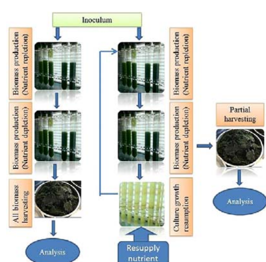
GRAPHICAL ABSTRACT



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ABSTRACT

The influence of N-replenishment on cell growth, chlorophyll content, nitrogen uptake, and accumulation of starch and lipid was studied in *Chlorella* sp. N-starved algal cells were fully recovered within 2 days after N-replenishment. Stored starch in N-deficient culture was degraded immediately during recovery process. However, lipid response had a period of delay when suffered from long starvation. During the recovery process, neutral lipid was reduced accompanied by an increase in membrane lipid. It was speculated that starch served as a source of carbon and energy catabolized to support quick recovery of the culture, while lipid preferred to play a structural role that specific fatty acid species were released from storage lipid and as building blocks for quick synthesis of membrane lipid. In light of rapid growth recovery and no net degradation in total fatty acids, a semi-continuous cultivation process might be a potential way to enhance lipid productivity.

1. Introduction

Nowadays, microalgae have emerged as a promising alternative feedstock for biofuel production due to their great photosynthetic efficiency, fast growth rate, and high energy yields. When exposure to environmental stresses, especially nutrient starvation or high irradiation, many eukaryotic green microalgae are capable of switching photosynthetic carbon partitioning toward energy-rich molecules, such as starch and/or lipid, which can be further converted into biofuels, including bioethanol, biodiesel, green gasoline, kerosene, etc. (Breuer et al., 2015; Li et al., 2015; Msanne et al., 2012). It has been generally accepted that starch and lipid in algal cells serve as protective mechanisms in response to environmental stimuli, which possibly exert important roles during the rejuvenation of microalgal cells when conditions become favorable (de Jaeger et al., 2014; Zhu et al., 2014). Thus, it is of significance to understand the regulatory mechanisms of starch and lipid in green microalgae.

Among all the environmental stresses, nitrogen starvation is one of the most commonly adopted methods to stimulate starch and lipid accumulation (Dragone et al., 2011; Mujtaba et al., 2012). Extensive

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studies have done by various groups and illustrated the metabolic changes in starch and lipid triggered by N-deprivation and their temporal relationship in various microalgae (Fernandes et al., 2013; Li et al., 2015; Msanne et al., 2012; Zhu et al., 2014). Basically, N-starvation leads to a severe inhibition of cell growth and preferentially triggers starch accumulation followed by lipid synthesis. Apart from being as carbon and energy storage compounds under N-depleted conditions, starch and lipid have their respective roles. For example, starch is considered as an energetically low-cost primary storage compound, and predicted as essential to provide carbon sources partially for lipid production (de Jaeger et al., 2014; Li et al., 2011). Lipid, however, is deemed as a secondary storage compound and an electron sink which could prevent the over-production of reactive oxygen species induced by excess electrons derived from photosynthesis (Hu et al., 2008). Besides, it is noted that lipid accumulation occurred only when carbon availability surpasses the demand for starch synthesis (Fan et al., 2012). Our previous study also demonstrated that starch accumulates in *Chlorella* cells as a quick response to environmental stress, while lipid is accumulated for long-term energy storage (Zhu et al., 2014). Despite that starch and lipid accumulation under N-starvation has been studied widely, negligible attention has been paid to their degradation or conversion under N-resupply condition. It was reported that stored starch and lipid in microalgal cells may be reused and play pivotal roles during culture growth recovery after N-replenishment (Fernandes et al., 2013; Siaut et al., 2011), nevertheless our understanding of their physiological roles and relationship during this process is still limited and not well studied. Hence, a systematic survey of variations of starch and lipid upon N-resupply is greatly needed.

So far, there have been a few reports on the dynamics of both starch and lipid after N-resupply in *Chlamydomonas reinhardtii*, *Parachlorella kessleri* and *Chromochloris zofingiensis* (Siaut et al., 2011; Fernandes et al., 2013; Mulders et al., 2015). Besides, some other investigators only focused on lipid change after N-resupply in microalgal species such as *Chlorella vulgaris*, *Phaeodactylum tricornutum* and *Nannochloropsis oceanica* (Dong et al., 2013; Pribyl et al., 2013; Valenzuela et al., 2013). However, all those studies ignored the influence of N-starvation timescales even though N-starvation duration strongly affected starch and lipid accumulation in microalgal cells (Zhu et al., 2014), which might further influence the dynamics of these two storage compounds upon N-resupply as well.

To gain more insights into physiological roles of starch and lipid in microalgae during cell recovery upon N-resupply, it is essential to investigate the cell recovery process in different timescale N-deficient cultures. In this study, the changes of starch and lipid in N-resupplied *Chlorella* sp. after exposure to three N-starvation timescales (short, medium and long starvation) were studied. Fatty acid profiles and lipid classes were also characterized. This work provided valuable implications into the physiology and culture recovery, and would benefit the optimization of semi-continuous cultivation process for this microalga.

2. Materials and methods

2.1. Microorganism and culture conditions

Chlorella sp. was isolated from native ponds (Guangzhou, China) and stored in the Microalgae Culture Collection of Guangzhou Institute of Energy Conversion, Chinese Academy of Sciences (China). The strain was maintained in BG11 medium. The algal cultivation device was designed as described in our previous study (Zhu et al., 2014). BG11 medium and nitrogen-depleted BG11-N medium were prepared and autoclaved at 121 °C for 30 min to cultivate *Chlorella* sp. Cultures were incubated under continuous illumination (150 μmol $m^{-2} s^{-1}$ photosynthetically active radiation) at 25 ± 2 °C and stirred by bubbling with a mixture of 2% CO_2 in air.

2.2. Experimental design

Algal cells were previously grown in BG11 medium for 4 days to reach the logarithmic phase, and then collected by centrifugation and re-suspended at an initial concentration of 0.12 g L^{-1} in BG11-N medium without $NaNO_3$. After a certain period of N-deficiency, $NaNO_3$ was replenished directly to the culture to supply a concentration of 1.5 g/L, which was a value of the regular BG11 medium, to study the recovery process under N-replenishment. The timescales of N-starvation were set at 1, 4, and 10 days in this work, representing a short, medium, and long period of N-starvation respectively. Unless stated otherwise, the moment when culture was inoculated in BG11-N medium was considered the start of the experiment and was referred to as day 0. In all experiments, distilled water was replenished before each treatment or sampling to compensate for the lost by evaporation. Each experiment was performed in triplicate.

2.3. Growth parameters

Cell numbers were counted using a hemocytometer after appropriate dilution under a light microscope (CX31, Olympus). The microalgal biomass was determined gravimetrically by filtering and drying of an aliquot of culture as described previously (Zhu et al., 2014). Nitrogen concentration was measured using a water quality analyzer (DR 2700, Hach) following the manufacturer's instructions.

2.4. Determination of chlorophyll

The contents of chlorophyll *a* and *b* were determined by a modified method described by Wellburn (1994). An aliquot of culture was centrifuged and the pellet resuspended in DMSO and placed in a water bath at 55 °C to extract pigments. The absorption of the extract was measured at 649 and 665 nm using a spectrophotometer. The contents of chlorophyll *a* and *b* were calculated using the following equations:

$$\text{Chl}\,a\,(\text{mg L}^{-1}) = 12.47 \times OD_{665} - 3.62 \times OD_{649} \quad (1)$$

$$\text{Chl}\,b\,(\text{mg L}^{-1}) = 25.06 \times OD_{649} - 6.5 \times OD_{665} \quad (2)$$

$$\text{Total chlorophyll content}\,(\text{mg L}^{-1}) = \text{Chl}\,a + \text{Chl}\,b \quad (3)$$

2.5. Determination of starch

Aliquots of lyophilized microalgal biomass were used to determine the starch content following the method described in a previous study (Zhu et al., 2014), based on the total hydrolysis of starch by 30% perchloric acid and quantification of liberated glucose by colorimetry.

2.6. Lipid and fatty acid analyses

For fatty acid analysis, fatty acid methyl esters (FAME) were prepared by incubating lyophilized biomass in methanol containing 2% (v/v) H_2SO_4 at 80 °C for 2.5 h. FAME were analyzed using a gas chromatograph (GC-2010, Shimadzu) according to the previous report (Zhu et al., 2014).

Total lipids (TL) were extracted following the method described by Bigogno et al. (2002), and determined gravimetrically. Neutral lipid (NL) and polar lipid (PL) were determined according to the previous work (Zhu et al., 2015) with a minor modification. A 500 mg Sep-pak cartridge of silica gel (Waters) was initially equilibrated with 5 mL of chloroform. Subsequently, 1 mL chloroform solution containing about 50 mg of lipid was applied to it. NL was collected by eluting with 10 mL of chloroform, followed by evaporation and weighing. PL content was the difference between the content of TL and NL.

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