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Production of primary metabolites in *Microcystis aeruginosa* in regulation of nitrogen limitation

Zhaojiang Zuo[a,*], Binbin Ni[a], Lin Yang[b]

[a] School of Forestry and Biotechnology, Zhejiang A & F University, Hangzhou 311300, China
[b] Tianjin Key Laboratory of Animal and Plant Resistance, College of Life Sciences, Tianjin Normal University, Tianjin, 300387, China

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ABSTRACT

The aim of this work was to study the regulatory effect of nitrogen (N) deficiency on primary metabolites in *Microcystis aeruginosa*, and promote the utilization of the alga. Low-N and Non-N conditions, especially Non-N, reduced the cell growth and photosynthetic abilities compared to Normal-N, as N deficiency triggered the down-regulation of genes involving in the photosynthetic process. Non-N not changed lipid content, due to no up-regulation of genes that promoted lipid synthesis. Soluble protein content significantly decreased under Non-N, which may result from the declined expression of genes relating to amino acid and histidyl-transfer RNA synthesis. Soluble and insoluble carbohydrate content significantly increased under Non-N, as the expression variation of genes blocked sugar degradation and promoted lipopolysaccharide synthesis. Therefore, *M. aeruginosa* can be used as the feedstock to produce carbohydrates under N deficiency for bioethanol production, and the remainder lipids after carbohydrate extraction can be used to produce biodiesel.

1. Introduction

Fossil fuels are limited non-renewable resources, and account for about 85% of the global energy consumption. Their over-consumption will lead to a series of issues in the incoming decades, such as energy crisis, raise in oil price, depletion of natural resources and climate change (Daroch et al., 2013). To address these issues, sustainable biofuels have attracted much attention, and microalgae are considered as the promising alternative feedstocks for the biofuel production, due to their great photosynthetic efficiency, fast growth rate, high carbohydrate and neutral lipid yields and other numerous environmental advantages relative to terrestrial plants (Ho et al., 2012).

In microalgae, carbohydrates and neutral lipids are their primary carbon and energy reserves, which can be used to produce bioethanol and biodiesel (Ho et al., 2012), respectively. Lots of studies have focused on increasing the accumulation of the 2 kinds of compounds in microalgal cells to lower the production cost (Wang et al., 2014; de Farias Silva and Sforza, 2016; Shang et al., 2017). The accumulation of carbohydrates and lipids can protect microalgal cells to tolerate environmental stresses, so several stressful conditions has been detected to improve their content, including element (nitrogen (N), phosphorus (P) or sulfur (S)) limitation (Moussa et al., 2017), high light intensity (Ho et al., 2012), H_2O_2 (Burch and Franz, 2016) and salinity (Yao et al., 2013). Among them, N deficiency is perhaps the most effective and widely explored strategy, which can cause carbohydrate accumulation in *Chlorella vulgaris* (Dragone et al., 2011; de Farias Silva and Sforza, 2016), *Scenedesmus obliquus* (Ho et al., 2012) and *Isochrysis zhangjiangensis* (Wang et al., 2014), lipid accumulation in *Chlamydomonas reinhardtii* (Li et al., 2012), *Dunaliella parva* (Shang et al., 2016a) and *Phaeodactylum tricornutum* (Burch and Franz, 2016; Longworth et al., 2016), and both accumulation in *Neochloris oleoabundans* (Morales-Sánchez et al., 2014; Sun et al., 2014) and *C. zofingiensis* (Zhu et al., 2015).

N deficiency can improve carbohydrate and/or lipid content in microalgal cells, but is not beneficial to the cell growth and biomass increase (de Farias Silva and Sforza, 2016). Then, a two-stage cultivation strategy has been proposed, which involves growth under N sufficient condition to high biomass levels then shifting to N deficiency for carbohydrate and/or lipid accumulation, e.g., this strategy improved starch content in *C. vulgaris* (Dragone et al., 2011), lipid content in *Chlorella* sp. (Zhu et al., 2018) and *Tetraselmis marina* (Moussa et al., 2017), and both content in *N. oleoabundans* (Sun et al., 2014), indicating that N deficiency is a signal in triggering the accumulation.

Although many studies focused on the accumulation of carbohydrates and lipids in different microalgae, limited reports uncovered the regulating mechanism of N deficiency. ADP-glucose pyrophosphorylase and starch phosphorylase were involved in starch synthesis, whose encoded genes improved expression in *C. vulgaris* var L3 under N

* Corresponding author.
E-mail address: zuozhaojiang@126.com (Z. Zuo).

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starvation (Ikaran et al., 2015). In *C. reinhardtii*, galactoglycerolipid lipase gene played an important regulatory role in triacylglycerol (TAG) accumulation in response to N deprivation (Li et al., 2012), while acyl-CoA: diacylglycerol acyltransferase gene served as the regulatory function in TAG synthesis in *C. vulgaris* var L3 (Ikaran et al., 2015). Meanwhile, the transcriptome and proteome were analyzed in *C. reinhardtii* (Schmollinger et al., 2014), *P. tricornutum* (Yang et al., 2013; Longworth et al., 2016), *C. vulgaris* (Liu et al., 2016) and *D. parva* (Shang et al., 2016a, 2017) under N deprivation. These results provided much-valued help in studying the regulatory mechanism of carbohydrate and lipid synthesis under N limitation.

Cyanobacteria are prokaryotic microalgae, whose blooms impact drinking water supplies, poison aquatic organism, and cause a series of environmental and ecological problems, due to the production of algal toxins and volatile organic compounds (Zuo et al., 2018a,b). It has been reported that cyanobacteria produced simple lipids and fatty acids which can be easily transesterified into biodiesel (Murata and Nishida, 1987). *Microcystis aeruginosa* is a typical species for cyanobacteria blooms, and widely exists in eutrophic waters. Nitrogen-limited cultivation efficiently promoted carbohydrate accumulation in the cells (Huang et al., 2018). Palmitic and lauric acids were the main components of the saturated fatty acids in *M. aeruginosa*, while oleic and linoleic acids were the main components of the unsaturated fatty acids (Da Rós et al., 2012). After transesterification, the biodiesel produced from the microalgal lipids was within the limits of American ASTM D6751 (Ashokkumar et al., 2014). These results suggest that *M. aeruginosa* has potential as the feedstock for biofuel production. To promote the development and utilization of *M. aeruginosa*, we investigated the growth, photosynthetic abilities, and the content of carbohydrates, lipids as well as proteins in the cells under different N levels, and analyzed the transcriptome to uncover the response mechanism to N deficiency.

2. Material and methods

2.1. Cell cultures

M. aeruginosa FACHB-912 was cultured in liquid BG11 medium with 17.6 mM $NaNO_3$ as N source, and kept at a regime of 16 h light (30 μmol $m^{-2} s^{-1}$) and 8 h dark, with temperature at 23 °C. The cell cultures were used for the experiments when their density reached to mid-logarithmic phase. The cell density was determined by using a 25 × 16 hemocytometer, with each value being the means of 6 repeats.

2.2. Treatments with N supply

According to our previous method (Zuo et al., 2018a), *M. aeruginosa* cells collected by centrifugation were washed twice with BG11–N (BG11 minus nitrogen) medium to remove the residual N. Then, the cells were transferred into the media with 0 mM, 8.8 mM and 17.6 mM $NaNO_3$, respectively, for Non-N, Low-N and Normal-N treatment, with the density of 1.6×10^7 cells mL^{-1}. The pH of these media was adjusted to 7.1. There were 4 conical flasks of cell cultures for each treatment, and each conical flask contained 300 mL of cell suspensions and was considered as a repeat. The cell density, photosynthetic pigment content, Chl fluorescence were measured every day for 5 days. The content of carbohydrates, proteins and lipids was determined after 1, 3 and 5 days. The transcriptomes in Non-N and Normal-N treatments were analyzed after 1 day.

2.3. Measurement of photosynthetic pigment content

Cell pellets were collected from 3 mL *M. aeruginosa* cultures by centrifugation at 6000 rpm for 8 min. Their chlorophylls (Chls) and carotenoids (Car) were extracted using 3 mL 80% acetone, and determined according to our previous method (Zuo et al., 2018b).

2.4. Analysis of Chl fluorescence

Following our previous method (Xu et al., 2017), a certain *M. aeruginosa* cultures about 1×10^7 cells were harvested by centrifugation, resuspended in the same culture medium of 10 μL, pipetted on a piece of filter paper, and incubated in darkness for 15 min. Their Chl fluorescence was measured by using a non-modulation Chl fluorescence analyzer (YZQ-500, YZQ Technology Co., Beijing, China). The Chl fluorescence parameter maximum quantum yield of photosystem II (PSII) photochemistry (Fv/Fm) was evaluated using the equation of Fv/Fm = Fm − Fo/Fm.

2.5. Determination of carbohydrate content

The harvested *M. aeruginosa* cells were dried by using a freezer dryer (LGJ-10C, Four-Ring Science Instrument Plant, Beijing, China). The dried alga of 50 mg was ground with a mortar, added in 5 mL distilled water to dissolve the soluble carbohydrates at 40 °C for 30 min. After centrifugation at 4000 rpm for 10 min, the supernatant and sediment were used to determine the soluble carbohydrate and insoluble carbohydrate content, respectively. They were added in a certain distilled water to 15 mL, and then added in 6 M HCl to decompose the polysaccharides to monosaccharides in boiling water bath for 1 h. Then the solution was neutralized with NaOH, and set to 100 mL with addition of distilled water. After centrifugation at 12,000 rpm, 1 mL supernatant was added into 1 mL 6% phenol and 5 mL H_2SO_4, and determined the absorbance at 490 nm. The carbohydrate content (%) was calculated according to the standard curve of glucose (Zhang et al., 2014).

2.6. Measurement of protein content

M. aeruginosa cells centrifuged from 25 mL cultures were resuspended with 1 mL of 50 mM buffer solution (4.6 mM KH_2PO_4 and 45.4 mM K_2HPO_4), crushed with liquid N_2, and centrifuged at 12,000 rpm for 25 min at 4 °C. The supernatant was used to determine the soluble protein content using coomassie brilliant blue G-250 method described by Bradford (1976).

2.7. Measurement of lipid content

The dried *M. aeruginosa* cells of approximately 70 mg (m_0) were added in 4 mL chloroform: methanol (2:1 v/v) solution and stirred for 1 h in a shaker. After centrifugation, the lipids were extracted again, and the deposited algae were dried and weighed (m_1). The photosynthetic pigment content (m_2) in dried *M. aeruginosa* cells was determined according to the method Section 2.3. The quantification of the lipids (M_L) was evaluated using the equation of $M_L = (m_0 - m_1 - m_2)/m_0 \times 100\%$, according to the method of Ramluckan et al. (2014).

2.8. RNA extraction and illumina sequencing

Following our previous method (Zuo et al., 2018a), total RNA in *M. aeruginosa* cells was extracted using a Total RNA Extraction System (Takara, Japan), and mRNA was purified and fragmented to about 200 nt. These fragments were used as templates to synthesize cDNA. After purification and addition of a terminal A at the 3′ ends, they were ligated with sequencing adaptors and performed polymerase chain reaction (PCR) with addition of Phusion high-fidelity DNA polymerase, universal PCR primers and index (X) primer. After purification, their sequencing was performed in Novogene Bioinformatics Technology Co. (Beijing, China).

2.9. Sequence annotation

Clean reads obtained from editing raw reads were mapped onto unigene sequences using Bowtie2-2.2.3. The method of expected