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Mechanisms of ammonium assimilation by *Chlorella vulgaris* F1068: Isotope fractionation and proteomic approaches

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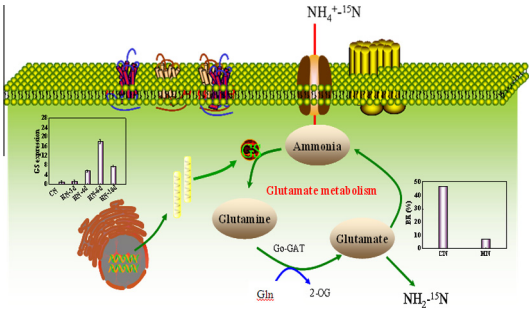
HIGHLIGHTS

- The NH_4^+-N concentrations affect the removal efficiency of NH_4^+-N by algae.
- The NH_4^+-N concentrations affect the biotransformation efficiency by algae.
- ^{15}N isotope fractionation effect depends on the NH_4^+-N concentration.
- Glutamine synthase was a key enzyme for ammonium assimilation.

GRAPHICAL ABSTRACT



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ABSTRACT

Removal of ammonium (NH_4^+-N) by microalgae has evoked interest in wastewater treatment, however, the detailed mechanisms of ammonium assimilation remain mysterious. This study investigated the effects of NH_4^+-N concentration on the removal and biotransformation efficiency by *Chlorella vulgaris* F1068, and explored the mechanisms by ^{15}N isotope fractionation and proteome approaches. The results showed NH_4^+-N was efficiently removed (84.8%) by F1068 at 10 mg L^{-1} of NH_4^+-N. The isotope enrichment factor ($\varepsilon = -2.37 \pm 0.08‰$) of ^{15}N isotope fractionation revealed 47.6% biotransformation at above condition, while 7.0% biotransformation at 4 mg L^{-1} of NH_4^+-N ($\varepsilon = -1.63 \pm 0.06‰$). This was due to the different expression of glutamine synthetase, a key enzyme in ammonium assimilation, which was up-regulated 6.4-fold at proteome level and 18.0-fold at transcription level. The results will provide a better mechanistic understanding of ammonium assimilation by microalgae and this green technology is expected to reduce the burden of NH_4^+-N removal for municipal sewage treatment plants.

1. Introduction

Ammonium is one of the most common nitrogen contaminants in the water environment. This is due to the discharges of wastewater treatment plants (WWTPs) and industrial plants, and leaching of fertilizers from agricultural activities into water bodies, contributing to the accelerated incidence of eutrophication in lakes and rivers (Paerl et al., 2011). Various efforts such as air stripping (Chai et al., 2015), chemical precipitation (Huang et al., 2011), adsorption and biological treatment (Bassin et al., 2011), etc., have been carried out to remove NH_4^+-N. Among these techniques, biological treatment, especially the use of microalgae such as *Chlorella* sp. (He et al., 2013), *Desmodesmus* sp. (Ji et al., 2014), *Scenedesmus* sp. (Park et al., 2010) and *Chlamydomonas* sp. (Escudero et al., 2014) have gained a lot of attention due to their

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ability to efficiently remove NH_4^+-N. As microalgae can assimilate inorganic carbon through photosynthesis, thus reduce energy requirement for wastewater purification compared with the conventional treatment technologies (Menger-Krug et al., 2012). Moreover, microalgae are applied in aquaculture as a food source for aquatic life because of the high yield of protein-rich algal biomass. They are also used in the production of biofuel due to their high lipid content, rapid growth and high biomass (Selvaratnam et al., 2015). Therefore, the removal of NH_4^+-N by microalgae has been regarded as a green and environment-friendly technology in wastewater treatment.

Previous studies showed that the assimilation of NH_4^+-N by some bacteria, microalgae and even higher plants into biomass can be indicated by the decrease in the isotope enrichment factor (ε) using the ^{15}N isotopic fractionation technique (Vo et al., 2013). It was noted that the differences in ε depended on the concentration of NH_4^+-N, for instance, the values of ε significantly decreased (from $-7.8 \pm 3.0‰$ to $-27.2 \pm 1.6‰$) when *Skeletonema costatum* was grown in increasing concentrations of NH_4^+ from 5 to 100 μmol L^{-1} (Pennock et al., 1996). Additionally, the isotope fractionation effect might be related to the changes in the ammonium assimilation pathway that are regulated by glutamine synthetase (GS) (Evans, 2001; Vo et al., 2013). However, the mechanisms that control fractionation of different concentrations of ammonium by algae are poorly understood, and the biotransformation efficiency of NH_4^+-N in microalgae is rarely presented. Proteomic approaches, such as the two-dimensional gel electrophoresis (2-DE) and the matrix assisted laser desorption ionization/time of flight MS (MALDI-TOF-MS), can reveal the different expression proteins involved in the changes of ambience (Li et al., 2013). Previous studies revealed that GS *in vivo* exquisitely regulated the process of NH_4^+-N assimilation into glutamine, an organic precursor of most cellular nitrogen (Hakeem et al., 2012). However some studies found that the increase in GS activity at protein level was inconsistent with the increase in the expression of the corresponding gene at the transcription level through reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR) (Huang et al., 2005). Thus, the combination of proteomics and RT-qPCR was needed and would further elucidate the effects of NH_4^+-N concentrations on the ammonium assimilation cycle and, thereby, the regulation of related genes and enzymes in microalgae cells.

Therefore, this study was attempted to exploit ^{15}N isotope fractionation, the proteomics coupled with RT-qPCR to reveal how the concentrations of NH_4^+-N regulate the related genes and enzymes to assimilate NH_4^+-N in *Chlorella vulgaris* F1068, a dominant unicellular green alga in the water environment. Such an integrated approach is expected to reveal a better and detailed mechanistic understanding of ammonium assimilation by microalgae and in turn provide theoretical bases for nitrogen elimination technique by microalgae, the result of which would reduce the large burden of NH_4^+-N removal in municipal sewage treatment plants, and in turn reduce the risk of NH_4^+-N to the aquatic environment.

2. Methods

2.1. Algal, medium and growth conditions

C. vulgaris F1068 used in this study was obtained from the Freshwater Algae Culture Collection of the Institute of Hydrobiology (FACHB, Chinese Academy of Sciences). The F1068 cells were cultured at pH 7.5 in sterile OECD medium followed Organization for Economic Co-operation and Development guideline (OECD, 2006). NH_4Cl was added as sole nitrogen resource and approximately equivalent to 4 mg L^{-1} NH_4^+-N. Culturing was carried out in a shaking incubator (HZ-200LG, Wuhan Ruihua Instrument and Experiment Co. Ltd., China) with 120 rpm at 25 °C. External illumination was provided by fluorescent lamps (Led-T5, Ningbo Yizhou Lumilife Lighting Co. Ltd., China) with a light intensity of approximately 2500 lux and 12 h:12 h light:dark cycle.

In order to obtain the optimal conditions for algal growth and ammonium removal, the cells density (cells L^{-1}) and the residual concentration of NH_4^+-N (mg L^{-1}) in the cultures were measured and then expressed in growth rate (cells L^{-1} d^{-1}) and average single-cell uptake rate (mg $cell^{-1}$ d^{-1}). The F1068 cells were harvested in the late logarithmic phase (4000*g*, 10 min), and then inoculated to the OECD medium containing various concentrations of NH_4^+-N: 4, 8, 10, 20, 30, 40 and 50 mg L^{-1}. The F1068 cell density was measured every 24 h at 680 nm using the spectrophotometer (722S, Shanghai Precision and Scientific Instrument, China) and calculated by the method described by Xu et al. (2011). The absorbed NH_4^+-N dose was measured with standard methods established by Ministry of Environmental Protection of the People's Republic of China (MEPPRC, 2002).

2.2. Nitrogen isotope analysis

The F1068 cells cultured for 6 days under different concentrations of NH_4^+-N were harvested by centrifugation, then washed twice with the sterile medium without NH_4^+-N. The freeze-dried F1068 cells were transferred into pre-weighed tin capsules for nitrogen isotope analysis. In addition, capsules containing solid NH_4Cl and others containing freeze-dried initial OECD medium were also prepared.

Nitrogen isotope abundance ($\delta^{15}N$) of all samples was determined using Elemental Analyzer coupled with Isotope Ratio Mass Spectrometer (Thermo Scientific Flash 2000, Thermo Fisher Scientific, USA). The instrument was calibrated for measurement of the nitrogen isotope and converted to $\delta^{15}N$-values reported in permille related to atmospheric nitrogen (Coplen et al., 2002). The nitrogen isotopic composition is presented in the delta notation:

$$\delta^{15}N = 10^3\{R_{sample}/R_{standard} - 1\}(\text{in } ‰) \quad (1)$$

where $^{15}R_{sample} \equiv {}^{15}N/{}^{14}N$. The isotope enrichment factor (ε) associated with the assimilation of nitrogen was estimated based on the above measurement and expressed with the following equation (Vo et al., 2013):

$$\varepsilon_b \equiv 10^3\{({}^{15}R_{e0}/{}^{14}R_{b0}) - 1\} \quad (2)$$

The $^{15}R_{e0}$ is the ratio of ^{15}N to ^{14}N in the initial medium and $^{14}R_{b0}$ is the same ratio in the first increment of biomass, respectively. According to the equilibrium nitrogen equation:

$$f\delta^{15}N_e + (1-f)\ \delta^{15}N_i = \delta^{15}N_{e,0} \quad (3)$$

where $\delta^{15}N_{e,0}$ is the measured $\delta^{15}N$ of the initial medium. $\delta^{15}N_e$ is the measured $\delta^{15}N$ of the final medium and the $\delta^{15}N_i$ was expressed to the biotransformation efficiency of NH_4^+-N. In addition, $f = {}^{14}N_e/({}^{14}N_{e,0} + {}^{15}N_e)$ is the fraction of ammonium remaining. Therefore, the $\delta^{15}N_i$ on $f\{\ln f/(1-f)\}$ fits the observations to a linear equation of the form:

$$\delta^{15}N_i = \delta^{15}N_{e,0} - \varepsilon f\{\ln f/(1-f)\} \quad (4)$$

The isotope enrichment factor (ε) indicates the degree of fractionation of nitrogen. For example, if $\varepsilon = -19.8‰$, it indicates that ^{15}N is assimilated and transformed into other materials containing nitrogen 19.8 parts per thousand more slowly than ^{14}N.