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Kinetic and transcriptional exploration of *Chlorella sorokiniana* in heterotrophic cultivation for nutrients removal from wastewaters

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ABSTRACT

Wastewater effluents containing nutrients would lead to eutrophication in natural water bodies, whereas microalgae based treatment could effectively allow their safe disposal. In this study, a green microalga *Chlorella sorokiniana*, able to grow under both light and lightless conditions, was investigated for its ability to remediate wastewater under heterotrophic conditions. The kinetics of the removal process was modeled and the cellular transcriptional responses in critical metabolic steps were assessed to unravel the nutrients assimilation puzzle. The experimental results demonstrated that *C. sorokiniana* had substantial tolerance to high nutrients concentrations. Meanwhile, the nutrients removal efficiency and rate were largely influenced by the initial concentration of nutrients. The highest removal efficiency up to 99% was achieved in the culture containing 123.6 mg N L^{-1} and 26.8 mg P L^{-1}. The decline of nutrients in waters was found following the first-order kinetics. The transcriptional expressions of *nrt2*, *nr* and *ppk* genes were directly affected by the external concentration of N and P, which indicates the correlation between the nutrients assimilation process by *C. sorokiniana* and its intracellular metabolic activity rather than the change of biomass quantity. In all, comparing to previous reports, this study validates that heterotrophic cultivation of this microalga is a promising technology for advanced wastewater treatment, with reasonable treatment performance and profound mechanism analysis.

1. Introduction

The extensive wastewater generation is an unavoidable consequence of nowadays human activities. In most cases, the primary treatment (to eliminate the settleable materials) and the secondary treatment (to oxidize the organic materials) are the only steps performed on wastewaters before their disposal to the natural aquatic environment. Consequently, a large quantity of nutrients predominantly composed of nitrates and phosphates, are discharged with secondary effluents from wastewater treatment plants, which in turn results in eutrophication in waters and the upset of the ecosystem balance [1].

The conventional technologies to remove nutrients, such as nitrification-denitrification, chemical precipitation, bacterial phosphorus removal, etc., require significant amounts of energy, chemicals, carbon source and even produce high volume of excessive sludge wastes [2]. Fortunately, microalgae, a feedstock for food, feed, chemicals and biofuels, appear as a strategy in recent decades, as they can uptake nutrients and hence have the potential to remediate wastewaters during the advanced treatment. But the obstacles for the scale-up application of microalgae in wastewater treatment lie on the large working area required for an efficient light harvesting and the low biomass growth restricted to the shading effect among microalgae, as these treatments are commonly conducted photoautotrophically. In this sense, heterotrophic cultivation of microalgae in which the organic substrates are used as the sole carbon and energy sources so as to eliminate the requirement for light, provides the possibility to achieve a dual goal of the fast growth and the efficient removal of nutrients from wastewaters [3]. Meanwhile, there are only few studies on this topic till now, thus leading to a scarce of experimental data to make the sound evaluation and model the process for the practical use of this approach.

Besides, the metabolic processes of microalgae during the nutrient removal are still far from our knowledge. Nevertheless, the biological assimilation has been widely recognized as the dominant reason why nutrients can be removed by algal biomass. To assimilate nitrate, these anions are imported into the cell by a specific nitrate transporter (NRT), and reduced to nitrite by the NAD(P)H-dependent nitrate

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reductase (NR) in the cytosol. Nitrite is subsequently reduced to ammonium by the ferredoxin-dependent nitrite reductase (NiR) in plastids. Finally, ammonium is incorporated in amino acids through the glutamine synthetase (GS)-glutamate synthase (GOGAT) cycle. Nitrate transport is thereafter the preliminary step in controlling the efficiency of the whole nitrogen assimilation. Moreover, NR performs a key role in the nitrogen metabolic pathway and the reduction of nitrate is considered to be the rate limiting step for the nitrate assimilation [4]. With respect to the phosphorus removal, under certain conditions (e.g., high phosphorus supply), microalgae can be triggered to take up much more phosphorus than necessary for survival and store it as polyphosphate for use as an internal resource when the external concentration of phosphorus is limiting [5]. The synthesis of polyphosphates is catalyzed by polyphosphate kinase (PPK) and their amount may reach high in some organisms. In *Chlorella* cells for instance, the amount of polyphosphate is thereby in the range of 30–60% of total phosphates content. Based on the above theory, activities of NRT, NR and PPK are critical to get some insights on the nutrient removal mechanism by microalgae. And the genes encoding these enzymes, namely *nrt2*, *nr* and *ppk*, are demanded to investigate the genetic regulation basis of the nitrate uptake and assimilation, as well as the polyphosphate accumulation in microalgae during the nutrients removal from wastewaters.

Therefore, in this present work, the green microalga *Chlorella sorokiniana*, thermotolerant, able to grow at high growth rates in dense cultures, under either light or dark condition, and with a high capacity of accumulating carbohydrates, was studied for the purpose of the nitrate and phosphorous removal. By varying the initial nutrient contents in the culture, its potential as an agent for wastewater treatment under heterotrophic conditions was explored. Further, the algal growth characteristics were assessed, and the kinetic description on the removal process of organic compounds and nutrients was carried out as well. The changes of the genetic systems were also analyzed to look into the major metabolic mechanism for the nutrients uptake and assimilation during the cultivation.

2. Materials and methods

2.1. Microalgae strain, maintenance and cultivation conditions

The strain of *Chlorella sorokiniana* FACHB-275 was obtained from the Freshwater Algae Culture Collection at the Institute of Hydrobiology (FACHB-Collection) (Chinese Academy of Sciences, China), and maintained on agar plates containing BG-11 medium. BG 11 medium was comprised of (in mM): $NaNO_3$ 2.94, $MgSO4 \cdot 7H_2O$ 0.3, K_2HPO_4 1.29, KH_2PO_4 0.43, $CaCl_2$ 0.17, NaCl 0.43, Na-EDTA 0.171, KOH 0.553, H_3BO_3 0.185 and trace elements (in μM): $FeSO_4 \cdot 7H_2O$ 18, $ZnSO_4 \cdot 7H_2O$ 30.7, $MnCl_2 \cdot 4H_2O$ 7.3, $CuSO_4 \cdot 5H_2O$ 6.3, MoO_3 4.9 and $Co(NO_3)_2 \cdot 6H_2O$ 1.7, at pH 7. The medium used to pre-culture and culture the strain was Modified Bold 3 N medium consisting of (in mM): $NaNO_3$ 8.8, K_2HPO_4 0.22, $MgSO_4 \cdot 7H_2O$ 0.3, $CaCl_2 \cdot 2H_2O$ 0.17, KH_2PO_4 0.43, NaCl 0.43 and trace metals according to Berges et al. [6], and the medium was supplemented with glucose (10 g L^{-1}) to support the heterotrophic growth as the simulated wastewater. The microalga was pre-cultured and cultured in a 250 mL flask containing 100 mL medium on a rotary shaker at 30 °C and the agitation rate of 150 rpm, pH 6.8–7, in the dark for 4 and 7 days respectively. To assess the ability of *C. sorokiniana* FACHB-275 to remediate wastewater, the initial concentration of $NaNO_3$, K_2HPO_4, and KH_2PO_4 in the culture medium (formulated to simulate wastewater) were adjusted. Seven different concentrations for each of nutrients, i.e. 15.45, 30.90, 61.80, 123.60, 247.19, 494.38 and 988.77 mg N L^{-1} for nitrogen, and 3.35, 6.70, 13.40, 26.80, 53.60, 107.20 and 214.40 mg P L^{-1} for phosphorous, were studied. All media were prepared with distilled water and heat-sterilized at 121 °C for 20 min. Samples were collected every day to be analyzed for the biomass concentration, residual concentrations of glucose, nitrogen and phosphorus.

2.2. Analytical methods

The biomass concentration was determined using a calibration curve correlating to the optical density (measured at $OD_{685\ nm}$) values to the biomass dry weight (DW). The optical density values were read from a UV/Vis spectrophotometer (U-1800, Shimadzu, Japan) after a proper dilution with deionized water. The nitrate concentration in samples was measured by the UV/Vis spectrometric method at the wavelength of 220 nm. The calibration curve was established using sodium nitrate ($NaNO_3$) as the standard. The concentration of phosphate was determined using the ascorbic acid method. The absorbance at the wavelength of 880 nm for each sample was read after the color development. The calibration curve was established using potassium phosphates monobasic (KH_2PO_4) as the standard. The concentration of glucose was estimated spectrometrically via the dinitrosalicylic acid method.

To carry out gene transcription analysis, RNA was extracted from the frozen cell pellet using the Takara MiniBEST Universal RNA Extraction kit (Takara Bio Inc., Japan). The RNA concentration and purity were determined spectrophotometrically (Quawell Nanodrop Q6000, USA). The RNA's integrity was tested by electrophoresis in 1% agarose gel. The reverse transcription to synthesize cDNA was performed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA). Primers for *nrt2*, *nr* and *ppk* genes were designed based on the sequences published in GenBank using Vector NTI® Express Designer and synthesised by Sangon Biotech Co., Ltd. (Shanghai, China). Primer sequences were: *nrt2* forward: CTCCGTCATGTTCAACGCCAAG; *nrt2* reverse: GATGTAGGGCATGATGAGGTGGG; *nr* forward: ACCTCATCCTGGCCTACAAGCAG; *nr* reverse: CCACTTGATCATGCGGCCAC; *ppk* forward: TTTGGTCGGAGACCCTAGTCGTG; and *ppk* reverse: ACATACTCCGCATCCTCCTCCTG. Polymerase reaction chain (PCR) was run to amplify the cDNA product to prepare the standard. The single amplified PCR product was verified based on size in a 1% agarose gel under UV illumination. The gel band containing the DNA target was excised and digested to recover and purify the amplified product using E.Z.N.A.® Gel Extraction Kit (Omega Bio-tek, USA). The concentration of the amplified product was measured with a spectrophotometer (Quawell Nanodrop Q6000, USA). Using the average molecular weight of the product and Avogadro's constant, the number of copies per unit volume was calculated. And finally, Quantitative real-time PCR (QRT-PCR) was run on samples and standards using iTaq™ universal SYBR® Green supermix reagent (Bio-Rad, China) and a thermocycler (StepOne Real-Time PCR System, Applied Biosystems, USA) under standard cycling conditions (95 °C for 10 min, (95 °C for 15 s, 60 °C for 30 s, 72 °C for 40 s) × 40 cycles, 95 °C for 60 s, followed by dissociation curve analysis). All measurements were conducted in triplicate.

2.3. Calculations

Biomass productivity (P, in g L^{-1} d^{-1}) was estimated based on the variation in biomass concentrations (X, in g L^{-1}) along with the cultivation time (t, in d) (Eq. (1)), and the specific growth rate (μ, in d^{-1}) was computed using Eq. (2).

$$P = \frac{X_t - X_0}{t_t - t_0} \tag{1}$$

where X_0 and X_t are the biomass concentrations (g L^{-1}) at the initial and final sampling time (t_0 and t_t), respectively.

$$\mu = \left.\frac{d \ln X}{dt}\right|_{\max} \tag{2}$$

where X and t indicate the biomass concentrations (g L^{-1}) and the sampling time (d), respectively.

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