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Assessment of photosynthesis regulation in mixotrophically cultured microalga *Chlorella sorokiniana*

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ABSTRACT

Mixotrophic growth of microalgae offers great potential as an efficient strategy for biofuel production. In this study, photosynthetic regulation of mixotrophically cultured *Chlorella sorokiniana* cells was systematically evaluated. Mixotrophic cells in the exponential growth phase showed the highest photosynthetic activity, where maximum photosynthetic O_2 evolution was approximately 3- and 4-fold higher than cells in the same phase grown photoautotrophically in 1% CO_2 (in air) and air, respectively. Additionally, characteristic chlorophyll fluorescence parameters demonstrated that no limitation in electron transport downstream of PSII was detected in mixotrophic cells. Up-regulation of photosynthetic activity was associated with high total ribulose-1, 5-bisphosphate carboxylase/oxygenase (Rubisco) carboxylase activity and expression level of phosphoribulokinase (PRK). After 3 days, photosynthetic O_2 evolution of mixotrophic cells that went to the stationary phase, was strongly reduced, with reduced photochemical efficiency and reorganization of the PSII complex. Simultaneously, enzymatic activity for Rubisco carboxylase and mRNA levels of Rubisco and PRK diminished. Importantly, there was almost no non-photochemical quenching for mixotrophic cells, whether grown in log or stationary phase. A decline in the quantum efficiency of PSII and an oxidized plastoquinone pool (PQ pool) was observed under N-depleted conditions during mixotrophic growth. These results demonstrate that photosynthesis is regulated differently in mixotrophically cultured *C. sorokiniana* cells than in cells grown under photoautotrophic conditions, with a particularly strong impact by nitrogen levels in the cells.

1. Introduction

Mixotrophic growth of microalgae is gaining increasing interest, largely because it offers the promise of an efficient strategy for commercial production of lipids and other value-added products. The combination of organic carbon assimilation and simultaneous CO_2 fixation in the light leads to higher growth rates and biomass accumulation in algae cultured mixotrophically than the same strains cultivated photoautotrophically or heterotrophically [1,2]. Mixotrophic growth also offers augmented lipid productivity [3], which is relevant for biofuel production from microalgae.

Our previous study demonstrated that the green alga *Chlorella sorokiniana* shows the best growth performance under mixotrophic conditions [1]. The mixotrophic growth of algal cells is based on CO_2 and organic carbon, thus both photosynthetic and organic substrate metabolic pathways have to operate concurrently. However, how this occurs, how it is regulated, and what the exact mechanism is that the mixotrophically grown cells use to accumulate biomass are largely unanswered questions. Of particular interest is determining how the photosynthetic and organic substrate metabolic pathways interact with each other. In photosynthetic pathway, light energy gathered as ATP and NADPH in light reactions is used to fuel cellular metabolism, in particular the reduction of CO_2 to three-carbon sugars. For the substrate metabolic pathway, glucose (as a model substrate) is metabolized to three carbon sugars following ATP-dependent entry into glycolysis. Thus, concurrent glucose metabolism may influence the utilization of carbon compounds arising from CO_2 fixation, and alter electron flux of

Abbreviations: Rubisco, ribulose-1, 5-bisphosphate carboxylase/oxygenase; PRK, phosphoribulokinase; PAM, pulse-amplitude modulation; rbcL, Rubisco large subunit; DCW, dry cell weigh; RuBP, ribulose-1, 5-bisphosphate; I_k, the saturation light intensity; α-slope, the slope of the light-limited part of the *P-I* curve; P_{max}, the light-saturated rate of photosynthesis; R_d, respiration rate; F_v/F_m, Potential maximum quantum efficiency; Φ_{PSII}, quantum yield of PSII photochemistry in the light; NPQ, non-photochemical quenching; qE, pH-dependent energy quenching; qL, the fraction of open PSII reaction centers; Q_A, quinone A; LHCSR3, light-harvesting complex stress-related protein 3; PQ pool, plastoquinone pool.

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photosynthesis by modulating the consumption rate of ATP and NADPH [4,5].

It has been reported that acetate inhibits photosynthesis in *Chlamydomonas reinhardtii*, and high acetate virtually abolishes photosynthetic carbon gain [6,7]. Numerous groups have observed the influence of exogenous organic carbon on the photosynthetic reaction centers of algal cells. Lewitus et al. showed that glycerol supply in photoautotrophically grown Pyrenomonas salina led to a reduction of light-harvesting related photosynthetic components in algal cells [8]. The addition of acetate to C. reinhardtii grown photoautotrophically significantly reduced the fraction of carbon biomass derived from photosynthesis, with dramatically declined net O2 evolution rate and CO2 fixation rate [9]. Similarly, the mixotrophically cultured diatom, Phaeodactylum tricornutum, supplemented with different kinds of organic substrate, showed a decline in O2 evolution accompanied by decreased PSII activity and reduced electron transport rate, which demonstrated that organic carbon metabolism can have an adverse impact on photosynthesis beyond the green algae [10]. However, glucose was shown to enhance the net photosynthesis rate in the cyanobacterium Synechococcus sp., indicating that this is not a universal situation [11].

Furthermore, exogenous organic substrate metabolism also affected the capacities of photosynthetic enzymes. Previously studies have reported that organic carbon represses the expression of some proteins encoded in nucleus, such as chlorophyll a/b-binding proteins evolved in photosystem II, and ribulose-1, 5-bisphosphate carboxylase/oxygenase (Rubisco) in Calvin cycle [12–14]. The addition of glucose lowered abundance of Rubisco and levels of the PSII reaction center protein D1 in alga Galdieria sulphuraria [15]. Thus, exogenous organic carbon exerts diverse influence on photosynthetic activity of algal cells in mixotrophic growth.

In this study, we investigated the effect of glucose metabolism on photosynthesis and related processes, and how microalgal cells respond under mixotrophic conditions compared to photoautotrophic conditions. In particular, attention was paid to determine how photosynthetic activity is regulated as the algae grow mixotrophically.

2. Materials and methods

2.1. Organism and medium

The green microalga *Chlorella sorokiniana* (UTEX 1602) was purchased from the Culture Collection of Algae (UTEX). For algal seeds preparation, *C. sorokiniana* were cultured in Kuhl medium at 25 °C bubbled with 1% CO_2 in 250-mL Erlenmeyer flasks, and illuminated with fluorescent light to give constant light intensity of 100 μmol m^{-2} s^{-1}.

Except where indicated, the medium for photoautotrophic culture was Kuhl medium, agitated with 1% CO_2 in air (1% CO_2-conditioned) or supplied with only air (air-conditioned). For heterotrophic and mixotrophic culture, the Kuhl medium was supplemented with 6 g L^{-1} glucose and the cells were grown with orbital shaking.

2.2. Growth analysis

Dry cell weight (DCW) was determined according to our previous methods [1]. Nitrate-nitrogen was determined with the H_2SO_4-salicylic acid method, as described by Cataldo et al. [16].

2.3. Photosynthetic oxygen evolution

Measurement of photosynthetic O_2 evolution was conducted by a Clark-type oxygen electrode (Hansatech, UK), and 1.2 mL of cell suspension with the same chlorophyll concentration (20 μM) was loaded in the oxygen electrode chamber. Prior to the O_2 evolution measurements, 20 μL of a 0.5 M sodium bicarbonate solution (pH 7.4) was supplied to guarantee enough carbon source available to the cells. Chlorophyll extracted with 100% methanol from algal cells was quantified according to Porra et al. [17]. The parameters for the photosynthetic responses to irradiance curves were analyzed according to Henley [18]:

$$P = P\,max\left(\alpha I/\sqrt{P\,max^2 + (\alpha I)^2}\right) + R_{d.}$$

$$Ik = P\,max/\alpha$$

where I (μmol photons m^{-2} s^{-1}) represents irradiance, P (mmol O^2 mmol Chl^{-1} h^{-1}) the photosynthetic rate at irradiance I, P_{max} (mmol O^2 mmol Chl^{-1} h^{-1}) the light-saturated rate of photosynthesis, I_k (μmol photons m^{-2} s^{-1}) the saturation light intensity, α ((mmol O_2 (mmol Chl)$^{-1}$ h^{-1}) (μmol m^{-2} s^{-1}) $^{-1}$) the slope of the light-limited part of the *P-I* curve, and $R_{d.}$ the dark respiration rate (mmol O^2 mmol Chl^{-1} h^{-1}).

2.4. Photosynthetic activity measurements

Photosynthetic activities were measured by pulse-amplitude-modulation (PAM) fluorometry with a Hansatech system. Algal cell suspensions were loaded with chlorophyll concentration of 10 μM and kept in dark for 15 min before measurement. The minimal fluorescence yield (F_0) was measured by activating the modulated light which was too weak to close reaction center. A saturating light pulse (about 3000 μmol m^{-2} s^{-1}) was then applied to determine the maximal fluorescence yield (F_m) of the dark-adapted samples. The actinic light from light-emitting diode was then turned on to provide continuous actinic light, and a steady fluorescence yield (F_s) could be obtained. Saturating pulses were applied to the sample at different intervals to obtain a stationary level of maximum fluorescence (F_m'). The fluorescence parameters mentioned in the following study were calculated according to Baker [19].

2.5. 77 K fluorescence spectroscopy

Fluorescence spectra at 77 K were measured to analyze stoichiometry and composition of PSI and PSII. Low temperature fluorescence spectra were obtained as described by Kirchhoff et al. [20] using a Horiba Jobin Yvon FluoroMax 4 spectrofluorimeter. Cells were adjusted to a low chlorophyll concentration of about 2 μM to avoid fluorescence reabsorption, shock-frozen in liquid nitrogen, and excited at 435 nm (slit width 2 nm). Emission spectra were recorded in the spectral range of 650–800 nm.

2.6. Measurements of Rubisco activity

Rubisco is the initial enzyme of CO_2 fixation in the Calvin-Benson cycle, catalyzing the addition of CO_2 to ribulose-1, 5-bisphosphate (RuBP). Microalgae were grown photoautotrophically (1% CO_2, air), heterotrophically, and mixotrophically. After 1 and 3 days, the cells pellets after centrifugation were frozen immediately in liquid nitrogen, and subsequently ground on ice in 1 mL of extraction buffer with a mortar and pestle. The extraction buffer was modified based on Kodama et al. [21]. Briefly, it includes 50 mM Hepes, pH 7.8, 10 mM dithiothreitol (DTT), 1% polyvinylpolypyrrolidone (PVPP), 1 mM ethylenediaminetetraacetic acid (EDTA), 0.1% Triton with 10 μL of protease inhibitor cocktail (Sigma, St. Louis, MO, USA). The supernatant of the extract was added with 15 mM $MgCl_2$ and 15 mM $NaHCO_3$ and incubated for 10 min at room temperature to activate enzymes. Total Rubisco activity was determined in activated supernatant in 1 mL of assay buffer (100 mM EPPS-NaOH pH 8.0, 20 mM $MgCl_2$, 1 mM EDTA, 0.2 mM NADH, coupling enzymes, 20 mM $NaHCO_3$) as described previously [22]. The activity was determined by monitoring NADH consumption, following initiation with 0.5 mM ribulose bisphosphate (RuBP).