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Effect of co-cultivation of *Chlamydomonas reinhardtii* with *Azotobacter chroococcum* on hydrogen production

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ABSTRACT

Chlamydomonas reinhardtii cc124 and *Azotobacter chroococcum* bacteria were co-cultured with a series of volume ratios and under a variety of light densities to determine the optimal culture conditions and to investigate the mechanism by which co-cultivation improves H_2 yield. The results demonstrated that the optimal culture conditions for the highest H_2 production of the combined system were a 1:40 vol ratio of bacterial cultures to algal cultures under 200 μE m^{-2} s^{-1}. Under these conditions, the maximal H_2 yield was 255 μmol mg^{-1} Chl, which was approximately 15.9-fold of the control. The reasons for the improvement in H_2 yield included decreased O_2 content, enhanced algal growth, and increased H_2ase activity and starch content of the combined system.

Introduction

Environmental pollution is becoming increasingly severe, and traditional energy reserves are limited [1]. Hydrogen is considered an ideal, clean and renewable energy carrier; however, it is primarily derived from the dissociation of fossil fuels that release a considerable amount of pollution into the air [2]. Through exploitation of some photosynthetic microorganisms for hydrogen generation, particularly cyanobacteria and microalgae, hydrogen may be a promising alternative for renewable as well as clean energy production [3]. *Chlamydomonas reinhardtii*, a unicellular green algae, is widely considered a model system for researching bio-hydrogen production given its high H_2ase activity, which is 10- to 100-fold of cyanobacterial H_2ase. Its genetic background and rapid growth also make it an ideal model system [4,5]. The H_2ase of *C. reinhardtii* uses a hydrogen proton and electron from either the photosynthetic electron transfer chain or consumption of endogenous substrates (mainly starch) to produce H_2 [5,6]. The H_2ase exhibits sensitivity to O_2 that is simultaneously generated upon H_2O oxidation, as performed in PSII [5,6]. This step is the bottleneck, limiting H_2 yield. Thus, decreasing O_2 levels in these cells may be an effective way to enhance H_2 yield. In 2000, Melis et al. reported a "two-step" method of photobiological H_2 production by removing sulfur from the medium of *C. reinhardtii* [6]. This method increases

Abbreviations: sulfur, S; Chl, chlorophyll; photosystem, PS; hydrogenase, H_2ase; hour, h; dissolved oxygen, DO; minute, min.

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the output of H_2 and extends the duration of H_2 production by hindering the repair of the D1 subunit of PSII. However, the activity of PSII and the electron resources are reduced in medium without sulfur, thus resulting in an H_2 yield lower than its theoretical capacity [6]. An ideal mode of photohydrogen production in *C. reinhardtii* involves maintaining high H_2ase activity and a sufficient electron supply. To achieve this goal, timely removal of intracellular O_2 generated from photosynthesis is necessary. In nature, some bacterial species form mutualistic relationships with algae and secrete organic nutrients and vitamins to algae [7–9]. The growth of algae is improved by bacteria in algal-bacterial co-cultures [10]. In addition, some researchers have found that some bacteria enhance H_2 yield in algal-bacterial co-cultures by consuming O_2 generated from algal photosynthesis [11–13].

Azotobacter chroococcum, a nitrogen-fixing aerobic bacteria, fixes nitrogen in the air, thereby forming nitrate and generating hydrogen in media without nitrogen [14]. These bacteria have been widely used in research on biochemical processes, electron transport, and iron storage [15]. Great progress has been made in the understanding of the biochemistry and genetics of H_2 metabolism and nitrogen fixation through studies on *A. chroococcum*. These studies have determined the potential of *A. chroococcum* to stimulate plant growth through the production of plant growth substances, fixed nitrogen, and other factors [16].

In this work, we co-cultured *C. reinhardtii* cc124 with *A. chroococcum* to enhance H_2 yield under a variety of light densities and inoculation ratios and preliminarily investigated the mechanism underlying the increased H_2 yield. Decreases in O_2 concentration and increases in H_2 yields were monitored. Moreover, the growth, H_2ase activity and starch contents of pure algae and its co-culture with *A. chroococcum* were detected. These data provide new insights into methods to improve the H_2 yield of green algae *C. reinhardtii* through treatment with bacteria.

Materials and methods

Algal and bacterial strains

Wild type *C. reinhardtii* strain cc124 was used in our experiment. The algal cells were cultured at (25 ± 1)°C in TAP liquid medium adjusted to pH 7.0 under 0–80 μE m^{-2} s^{-1}. TAP medium without sulfur (TAP-S) was modified from TAP medium by replacing sulfates with chloride salts [17]. *C. reinhardtii* growth was indicated by the algal density at OD_{750} and the total chlorophyll (Chl) content (Chl content = OD665 × 6.1 + OD649 × 20.04) [18].

A. chroococcum strain 1.0233 was used in our research and cultured in *Azotobacter* medium (pH 7.0) at 30 °C. The growth of *A. chroococcum* was determined on the basis of the OD_{600} [19].

Detection of H_2 yield and O_2 content

Algal (OD_{750} = 3.0) and bacterial (OD_{600} = 2.0) cells in the saturation period were centrifuged at 4000 × g for 5 min and resuspended in TAP-S culture. Then, TAP-S medium was added to the samples, such that the OD_{750} of algae and OD_{600} of *A. chroococcum* reached 1.0 [11,19]. Bacterial cultures were then placed in the algal cultures at various volumetric ratios (1:80, 1:40, 3:80 and 1:20), and the final total volume was 40 ml. The samples were cultivated in darkness for 24 h to decrease the concentration of O_2 and then incubated under a variety of light intensities: 30 μE m^{-2} s^{-1}, 60 μE m^{-2} s^{-1}, 100 μE m^{-2} s^{-1} and 200 μE m^{-2} s^{-1} [20,21].

Samples were extracted from the culture tube and detected with a gas chromatograph (Agilent™ 7890, USA) [11,21]. H_2 and O_2 were monitored using a thermal conductivity detector. The carrier gas was argon.

DO and respiration rate measurements

The concentration of DO in the culture was monitored with an oxygen analyzer. O_2-exchange activity of the cultures was detected with a Clark-type O_2 electrode at 26 °C. Then, 2 ml of the cultures was placed in an O_2-electrode chamber and allowed to adapt to the dark for 5 min to detect the respiratory rates [22,23].

Detection of H_2ase activity

H_2ase activity both *in vivo* and *in vitro* was detected using a previously described method [24] with some modifications. Briefly, sodium dithionite, 0.2% Triton X-100, and methyl viologen were added into a 50-ml glass pipe. Then, 1 ml of culture was placed into the glass pipe. The vial was then shaken sharply to separate the cells and placed into a shaking water-bath at 37 °C for 60 min. Then, 5 ml of the culture was transferred to the other 50-ml glass pipe to measure the H_2ase activity *in vivo*. All solution and glass vials were flushed with argon for 5 min to produce anaerobic conditions. Gas chromatography was used to detect the H_2 yield above the reaction mixture. The activity of H_2ase was represented by the H_2 yield per h and μg Chl content of total algae in the cultured tube.

Measurements of starch contents

1 ml of sample was collected from the culture flask and centrifuged at 9000×*g* for 5 min. The pellet was resuspended with 0.1 ml methanol, centrifuged at 12000×*g* for 3 min and washed with 2 ml of Na-acetate (100 mM, pH 4.7) twice. The pellet was resuspended with 2 ml acetate buffer (pH 4.7) and then lysed by sonication (for 30 s, 15 Hz). The crude sample was placed in an autoclave (120 °C) for 15 min and then incubated in 55 °C water for 14 h with 2.5 units of amyloglucosidase (Sigma-Aldrich, Germany). Finally, the sample was centrifuged to obtain the supernatant fraction, which was used to assay glucose content [25].

Significance test

Differences between the experimental and control groups were detected with independent sample t-tests. All statistical analyses were conducted by SPSS19.0, and $P < 0.05$ was considered to be statistically significant. "*" indicates a significant differences between the experimental and control groups. Values are shown as Mean ± SE.