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Increased hydrogen production in co-culture of *Chlamydomonas reinhardtii* and *Bradyrhizobium japonicum*

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HIGHLIGHTS

- Decreasing the oxygen content in the cultures of *Chlamydomonas reinhardtii* is essential for its hydrogen productivity.
- Co-cultivation of *Bradyrhizobium japonicum* and *C. reinhardtii* promoted oxygen consumption and improved hydrogen yield.
- *B. japonicum* promoted H_2 yield and growth of the transgenic alga, lba, by 14 times and 26% respectively.

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ABSTRACT

Co-cultivation of *Bradyrhizobium japonicum* with *Chlamydomonas reinhardtii* strain cc849 or the transgenic strain lba, which was hetero-expressed the gene of the soybean leghemoglobin apoprotein Lba in chloroplasts of the strain cc849, in Tris–acetate-phosphate (TAP) or TAP-sulfur free media, improved H_2 yield. H_2 production was 14 times and growth was 26% higher when strain lba and *B. japonicum* were co-cultured, as compared with cultivation of the algal strain alone under the same conditions. The increase in respiration rate or fast O_2 consumption by about 8 times in the co-cultures was the major reason for the improvement.

1. Introduction

The renewable and environment-friendly generation of H_2 in large quantities is a major challenge for the utilization of H_2 as an energy resource because, at present, most H_2 production depends on effective thermochemical and photoelectrochemical processes that may be highly energy-consuming and damaging to the environment (Sen et al., 2008). Therefore, photobiological H_2 production by green microalgae is of interest (Esper et al., 2006).

H_2 production in photosynthetic bacteria is mediated primarily by the nitrogenase system that requires high-energy input in the form of ATP (Taygankov et al., 1999). In contrast, H_2 production in green algae is mediated primarily by the Fe-hydrogenase system in the absence of ATP-input by accepting electrons from ferredoxin (Fd), a terminal acceptor in the photosynthetic electron transport chain in chloroplast thyllakoids, to reduce protons (Kosourov and Seibert, 2008). *Chlamydomonas reinhardtii* has been chosen as model species for studying photohydrogen production because of its high Fe-hydrogenase activity, easy cultivation, and clear genetics (Melis, 2007). However, Fe-hydrogenase is highly oxygen sensitive (Forestier et al., 2003), while O_2 is also the principal product of algal photosynthesis. Therefore, O_2 generation by photosynthesis must be controlled upon illumination. The current control method relies on the use of a sulfur-deficient medium for the inactivation of O_2 evolution at algal PSII (Photosystem II) under illumination and creation of anaerobic conditions (Melis et al., 2000) to prolong H_2 production. However, the algal H_2 productivity in such a system has not yet qualified for commercial viability since the stress of sulfur deprivation eventually leads to reduced ferredoxins oxidization, degradation of photosynthetic complexes and accumulation of toxic metabolites (Melis, 2007; Terauchi et al., 2009; Matthew et al., 2009).

Attempts to enhance H_2 productivity in algae have included repeated cycles of light restriction and oxygen depletion with unaffected photosynthesis (Melis et al., 2000), control of photosynthesis by both restricted illumination, and genetic engineering to produce algae that have limited light harvesting mechanisms (Polle et al., 2003). Increasing starch reserves and inhibiting the

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cyclic electron flow around PSI as well as its respiration metabolism were also investigated (Kruse et al., 2005), as were the introduction of a glucose transporter (Rühle et al., 2008), diminishing sulfur uptake (Chen et al., 2005) and reducing oxygen sensitivity of hydrogenase (King et al., 2006). Co-cultivation of algae and bacteria has also been employed. For instance, Kawaguchi et al. (2001) grew the photosynthetic bacterium *Rhodobium marinum* along with *Lactobacillus amylovorus* and algal biomass for metabolizing the algal starch into lactate as an electron donor for hydrogen production by the bacteria. Another example is the co-culture of photosynthetic, hydrogen producing algae (wild type and genetically engineered for reduced sulfate utilization) with the hydrogen-producing bacterium, *Rhodospirillium rubrum* (Melis and Melnicki, 2006). Kim et al. (2006) also found that cultivation of algal biomass of *C. reinhardtii* with *Clostridium butyricum* and *Rhodobacter sphaeroides* KD131 under anaerobic and photosynthetic fermentation conditions improved H_2 production. Similar results have been reported by Miura et al. (1992) and Ohmiya et al. (2003).

Bradyrhizobium japonicum is a symbiotic rhizobium of the soybean, *Glycine max*. Naturally, it infects the root of host plants and forms root nodules, which contain abundant leghemoglobins (lbs) that have high affinity for O_2 and carry O_2 to the respiration metabolism, resulting in a low O_2 concentration and protection of the oxygen-sensitive nitrogenase activity in the nodules (Appleby, 1984; Kundu et al., 2003). When the *lba* gene from *G. max*, encoding the lb apoprotein, was introduced into *C. reinhardtii* and expressed in its chloroplasts (Wu et al., 2010), rapid O_2 consumption and about 1.5-time H_2 yield of the wild type, strain cc849 (stain 849) in sulfur-deficient medium were observed. In the present study, *B. japonicum* was co-cultured with the transgenic alga, lba, (transgenic lba) as well as strain 849 of *C. reinhardtii* at various inoculation ratios and H_2 yields of the co-cultures were monitored.

2. Methods

2.1. Cultures and culture conditions

C. reinhardtii strain 849 is a cell wall-deficient mutant (a gift from Professor Dr. Madeline Wu in Hong Kong University of Science and Technology). The transgenic algal strain lba was engineered to express the soybean *lba* gene in its chloroplasts (Wu et al., 2010). Both algae were grown photoheterotrophically on TAP (Tris–acetate-phosphate, pH 7.0) agar plates or in TAP liquid media under continuous illumination (100 μmol photons m^{-2} s^{-1}) with cool-white fluorescent light at 25 ± 1 °C. Batch cultures were maintained by either standing or shaking at 100–130 rpm and subcultured in the liquid medium once every five days. The solid medium (containing 2% agar) was inoculated once every two weeks (Harris, 2009). For sulfur-free TAP medium (TAP-S), sulfate compounds were replaced with their chloride counterparts (Harris, 2009). *B. japonicum* was purchased from the Chinese Academy of Agricultural Sciences and grown on YEM (yeast extract–mannitol) medium (pH 7.2) in the dark at 28 ± 1 °C (Regensburger et al., 1986). Batch cultures were maintained in liquid YEM medium and shaken at 200 rpm until the OD_{600} reached 0.6–0.8 (logarithmic phase).

2.2. Co-culturing algae and B. japonicum

Algal and bacterial cultures in their liquid media were collected at the late exponential phase, washed with TAP medium three times, and resuspended in a fresh TAP medium to a cell density of 1×10^7 cells ml^{-1} (for algae) and OD_{600} of 1.0 (for rhizobia). Then, 30 ml of algal and 2 ml of bacterial suspensions were mixed in a 100 ml-flask. Single algal and bacterial cultures were used as controls. All cultures were grown at 25 ± 1 °C under a light intensity of 60 μmol m^{-2} s^{-1} for 7 days. For H_2 production, algal and bacterial culture were washed with TAP-S medium (3×) and resuspended in fresh medium at a final chlorophyll (Chl) concentration of 12.5 μg ml^{-1}. The concentration of the bacteria was adjusted to an OD_{600} of 1.0 with TAP-S medium and added into algal cultures at a volume ratio of 1:4, 1:8, 1:20, 1:80, 1:200, 1:400 and 1:4000 (alga to bacteria). The final volumes of the co-cultures were adjusted to 40 ml with fresh TAP-S medium, sealed with a rubber gas-tight septum in 60 ml cylindrical glass bottles and incubated in the dark for 24 h to induce anaerobic conditions, followed by culturing under continuous illumination with an intensity of 60 μmol m^{-2} s^{-1} for three weeks. H_2 and O_2 contents in the headspace of the bottles were detected by high pressure gas chromatography (GC). The O_2 consumption in liquid media was measured every day. Single cultures of strain 849 and the transgenic lba as well as of *B. japonicum* were used as controls. Experiments were carried out in triplicates.

2.3. Algal and bacterial growth

Algal cell numbers were counted with a blood counting chamber. Total algal chlorophyll was extracted with 95% ethanol and its content was assayed spectrophotometrically according to the Spreitzer method (Harris, 2009).

2.4. O_2 evolution and consumption

O_2 evolution and consumption rates in algal cultures were measured with a Clark-type oxygen electrode (Lab-2, Hansatech, UK) at 25 °C according to Makarova et al. (2007). Briefly, 2 ml of algal samples were taken from the growing cultures every day, placed in an O_2-electrode chamber, dark adapted for 5 min to measure the respiration rate, and illuminated at a light intensity of 60 μmol m^{-2} s^{-1} for 5 min to measure photosynthetic rate. The initial level of O_2 consumption or O_2 evolution was recorded for the calculation of dark respiration and photosynthetic rates, respectively, according to the Hansatech manual.

2.5. H_2 and O_2 content detection

Under hydrogen production conditions, the evolved gas was collected from the headspace of the bottles using a gas-tight lockable syringe and injected into a gas chromatograph (Agilent™ 7890, USA) with a thermal conductivity detector in order to monitor concentrations of H_2, O_2, and N_2 simultaneously. A 5 Å molecular sieve column (2 m × 1/8 mm) was used and argon was the carrier gas. The injection volume was 0.5 ml. The volume of H_2 and O_2 was calculated using the external standard method (Wu et al., 2010).

3. Results and discussion

3.1. Co-culture of algae and B. japonicum

Co-cultivation of bacteria and algae with a starting ratio of 1:15 (bacteria to algae) led to algal cell density and chlorophyll content of co-cultures of *B. japonicum* and the transgenic lba reached values of 3.9×10^7 ml^{-1} and 50 mg l^{-1}., respectively, nearly 26% and 50% higher than those of single transgenic algal cultures which reached about 3.06×10^7 ml^{-1} and 33.4 mg l^{-1}, respectively (Fig. 1A and B). Although the maximal chlorophyll content of the co-cultures of the strain 849 and *B. japonicum* was improved by about 14% (Fig. 1B), the cell number of the strain 849 was not enhanced by its