Improved hydrogen production and biomass through the co-cultivation of

Chlamydomonas reinhardtii* and *Bradyrhizobium japonicum

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Abstract: Three algae strains—*Chlamydomonas reinhardtii* hemHc-lbac (transgenic), cc124 and cc503—were co-cultured with *Bradyrhizobium japonicum* to improve H₂ production. The maximum H₂ productions of the three different co-cultures were 3.5-fold, 17-fold and 4.4-fold of the pure algal cultures. Meanwhile, the biomass of each of the algae strains was increased by *B. japonicum*. Furthermore, the respiratory rates of the co-cultures were 1.4-fold, 1.2-fold and 2.6-fold higher than the controls, respectively; the in vitro maximum hydrogenase activities of the co-cultures were 1.1-fold higher, equal to 2.4-fold higher than those of the controls, respectively, and the in vivo maximum hydrogenase activities of the co-cultures were 1.5-fold, 3.8-fold and 2.1-fold higher than the controls, respectively. The maximum starch content of the co-cultures were 8.3-fold, 8.4-fold and 4.4-fold higher than the content of the controls, respectively. Therefore, *B. japonicum* improved the H₂ production of the co-cultures by increasing the biomass, respiratory rates, hydrogen activity and starch content of the co-cultures relative to those of pure algae cultures.

Key words: *Chlamydomonas reinhardtii*; *Bradyrhizobium japonicum*; Co-culture; Hydrogen production; Biomass

1. Introduction

With the development of industry, traditional fossil fuels are becoming limited and environmental pollution is increasingly severe. Thus, identifying alternative and clean energy resources is an urgent task [1]. Hydrogen is considered one of the cleanest energy sources because of its high energy efficiency and because its only combustion product is water [2]. Approximately 60 years ago, Hans Fraenkel and his coworkers reported the existence of hydrogen metabolism in green algae [3]. *Chlamydomonas reinhardtii* has been used as a model species for studying photo-hydrogen production because of its high Fe-hydrogenase activity, easy cultivation, and clear genetic background [4]. There are two hydrogenases in *C. reinhardtii* that can accept electrons from ferredoxin and donate them to H^+ to generate H_2 [5]. The hydrogenase in *C. reinhardtii* has high activity, at approximately 10- to 100-fold that of cyanobacteria, but it is sensitive to O_2 [6, 7]. O_2 is produced by photosynthesis, which results in hydrogenase inactivation and reduces H_2 production [7]. In 2000, Melis and coworkers proposed that the depletion of sulfur in the culture could significantly improve hydrogen activity and prolong the H_2 production time by reducing the activity of PSII; however, when the sulfur in the culture was depleted, the H_2 productivity under this method was lower than its theoretical capacity because of the simultaneous depletion of electron resources [5]. An ideal commercial H_2 production system using green algae would be based on a continuous process in which the PSII and hydrogen function were simultaneously maintained at high efficiency to drive H_2 production from water and solar energy [5].

Some reports have described the use of photosynthetic bacteria for biological hydrogen production and the co-cultivation of algae and photosynthetic bacteria to improve hydrogen production. For example, Miura grew the marine green alga *Chlamydomonas* sp. strain MGA 161 along with marine photosynthetic bacterium strain W-1S in acetic acid, ethanol and broth with the MGA 161 strain as an electron donor for hydrogen photoevolution [8]. Kawaguchi et al. improved hydrogen production by mixing a culture of the photosynthetic bacteria *Rhodobium marinum* A-501 with *Lactobacillus amylovorus* and algal biomass to metabolize the algal starch into lactate as an electron donor for use in hydrogen production by the bacteria [9].

In our previous work, we hetero-expressed the codon-optimized hemH and lba genes, which from *B. japonicum*, and *Glycine max.*, respectively, in the chloroplast of *C. reinhardtii* cc849, and demonstrated rapid O₂ consumption and about 5.5-fold of H₂ yield in a sulfur-free medium when compared with its wild type strain cc849 [10].

Like hydrogenase, nitrogenase is sensitive to O₂, but it has high nitrogen fixation efficiency under normal conditions in soybean nodule cells because of the existence of abundant leghemoglobins (Lbs), which can combine with O₂ quickly and transport it to take part in respiration, resulting in a low O₂ concentration and the activation of nitrogenase activities in the soybean nodules [11, 12]. Meanwhile, *Bradyrhizobium japonicum*, which is a type of aerobic bacteria, can fix nitrogen to form nitrate and combine with soybean roots to form root nodules. In this study, the transgenic algae hemHc-lba, *C. reinhardtii* cc124 and cc503 were co-cultured with *B. japonicum* to improve hydrogen production, which would provide a new way of using *B. japonicum*

to improve green algal H₂ productivity and pointed out a novel strategy to genetically engineering *C. reinhardtii* for the improvement of H₂ production.

2. Material and methods

2.1 Strains and culture condition

The transgenic algal strain hemHc-lbac was engineered from *C. reinhardtii* strain cc 849 (a gift from Professor Dr. Madeline Wu in Hong Kong University of Science and Technology), which is a cell wall-deficient mutant, to express the codon-optimized lba gene from soybeans and the codon-optimized hemH gene derived from the rhizobia in its chloroplasts. *C. reinhardtii* strain cc124 is the wild type (purchased from *Chlamydomonas* Resource Center, <http://chlamycollection.org/>), and it has a cell wall. *C. reinhardtii* strain cc503 is a cell wall-deficient mutant (purchased from *Chlamydomonas* Resource Center, <http://chlamycollection.org/>). Strain cc 849, cc124 and cc503 originate from *C. reinhardtii* strain cc137 [13].

All the algae were grown photoheterotrophically on TAP (Tris-acetate-phosphate, pH 7.0) agar plates or in TAP liquid media under continuous illumination (120 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$) with cool-white fluorescent light at $25 \pm 1^\circ\text{C}$. For sulfur-free TAP medium (TAP-S), sulfate compounds were replaced with their chloride counterparts [14].

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 $28 \pm 1^\circ\text{C}$ [15]. Batch cultures were maintained in liquid YEM medium and shaken at 200 rpm

until the OD₆₀₀ reached 1.0.

2.2 Algal and bacterial growth

The algal density was checked by using a hemocytometer-type counting chamber or by measuring the absorbance at 750 nm (OD₇₅₀). The total algal chlorophyll was extracted with 95% ethanol, and the content was spectrophotometrically assayed according to the method by Spreitzer [16]. The bacterial density was checked by using a hemocytometer-type counting chamber or by measuring the absorbance at 600 nm (OD₆₀₀).

2.3 Co-culturing algae and *B. japonicum*

Algal and bacterial cultures were harvested at the late exponential phase, washed with TAP-S medium three times, and resuspended in fresh TAP-S medium to cell densities at an OD₇₅₀ of 1.0 and an OD₆₀₀ of 1.0 for the algae and rhizobia, respectively [10, 15]. 0.5ml, 0.5ml and 2ml bacterial suspensions were then added to the algal strain culture (hemHc-lbac 39.5ml, cc124 39.5ml and cc503 38ml), respectively. Single algal and bacterial cultures were used as controls. The mix was sealed with a rubber gas-tight septum, incubated in the dark for 24 h to induce anaerobic conditions and then cultured under continuous illumination.

2.4 Measurements of H₂ production and O₂ co-consumption

Under H₂ production conditions, the evolved gas was collected from the headspace of the bottles with a gas-tight lockable syringe and injected into a gas chromatograph (Agilent™ 7890, USA) with a thermal conductivity detector to monitor the concentrations of H₂, O₂, and N₂ simultaneously. A 5A molecular sieve column (2 m

× 1/8 mm) was used, and argon was the carrier gas. The injection volume was 0.5 ml. The H₂ and O₂ volumes were calculated by using the external standard method [10, 14].

2.5 H₂ase activity assay

To measure in vitro H₂ase activity, a 1-ml preparation of co-cultured samples was added to a 55-ml glass vial sealed with a rubber stopper, and then 1 ml of 10 mM oxidized methyl viologen in 50 mM Tris buffer (for pH 7.1-9.0) with 0.2% (w/v) Triton X-100 was injected into the vial. Finally, 100 µl of 100 mM sodium dithionite in 0.03 M NaOH was mixed into the glass vial. The sample turned from green to purple as the reaction began. The assay was performed at 37°C in the dark for 60 min [17]. To measure in vivo H₂ase activity, 5-ml cell suspension samples were withdrawn from the 55-ml culture bottles at designated time points and then injected into other 55-ml glass vials. All reagents and reaction glass vials were made anaerobic by purging them with argon gas for 2 min to eliminate the inhibitory effect of O₂ on H₂ase. The amount of evolved H₂ in the headspace of the glass vial was determined by gas chromatography, and the rate of H₂ production was calculated on the basis of the total Chl content in the glass vial, unless otherwise indicated [17].

2.6 Starch determination

The starch assay samples were taken directly from the glass bottles at the appointed times and centrifuged for 5 min at 2000 × g. The pellets were stored frozen at -80°C until all the samples were ready for processing. The starch levels inside the cells were determined in the pellet of each ml of culture according to the method of Gfeller and

Gibbs with some modifications [18]. The pellet was resuspended in 1 ml of 95% methanol twice, then in 1.7 ml of 100 mM Na-acetate (pH 4.5) (Sigma-Aldrich, Germany), and resuspended in 1.5 ml of the acetate buffer. The samples were then sonicated for 10 s. After reacting for 10 min at 121°C, 2.2 units of amyloglucosidase (Sigma-Aldrich, Germany) was added, the samples were incubated for 14 h in a water bath at 55°C and then centrifuged to obtain a clear supernatant fraction, and the glucose was assayed by glucose oxidase-peroxidase method (Sigma GAHK-20).

3. Results and Discussion

3.1 Algal and bacterial growth under H₂ production conditions

The growth of algae and bacteria under H₂ production conditions is shown in Figure 1. The cell density and chlorophyll content of the algae when mixed with *B. japonicum* were clearly higher than in samples that were not mixed with *B. japonicum*. The co-cultivation of bacteria and algae with a starting ratio of 1:80 (bacteria to algae) led to an algal cell density and an algal chlorophyll content in hemHc-lbac reaching values of 1.32 and 19.39 mg/l, respectively, which were nearly 164% and 113% higher than those of single algal cultures, which reached approximately 0.52 and 9.11 mg/l, respectively (Fig. 1 A, B). The results for the two other algal strains were similar to those of hemHc-lbac. The cell density and chlorophyll content of co-cultures containing *B. japonicum* and cc124 algae reached values of 1.47 and 17.44 mg/l, respectively, which were nearly 145% and 424% higher than those of single algal cultures, which reached approximately 0.60 and 3.33 mg/l, respectively (Fig. 1 C, D). The co-cultivation of bacteria and algae with a starting ratio of 1:20

(bacteria to algae) led to algal and bacterial cell densities and chlorophyll content of co-cultures in cc503 algae that reached values of 2.28 and 37.47 mg/l, respectively, which were nearly 407% and 455% higher than those of single algal and bacterial cultures, which reached approximately 0.46 and 6.75 mg/l, respectively (Fig. 1 E, F). The results showed that combining *B. japonicum* with *C. reinhardtii* improves the cell density and chlorophyll content of algal co-cultures. *B. japonicum* increases the biomass of *C. reinhardtii*, which is one reason for the improved hydrogen production. In natural environments, there is a special relation between bacteria and algae related to the release of extracellular substances [19]. Algae use nitrogen and inorganic phosphorous salts that are released by bacteria, and they release organics such as amino acids and enzymes that can be used by bacteria [20, 21]. Bell reported that there is syntrophism between micro-algae and bacteria via extracellular substances [22]. Zheng et al. found that the activity of extracellular co-cultivation enzymes from *Alexandrium tamarense* and *Bacillus halmapalus* was higher than that of independent cultures, which indicated that the biomass of co-cultures was increased [23]. Ietswaart revealed that *Pseudomonas diminuta* and *P. vesicularis* could enhance the growth of *Scenedesmus bicellularis* and *Chlorella* [24]. Gyurjan et al. observed that nitrogen-fixing bacteria were always near the surface of the micro-algae because of their exosymbiotic action via complementary metabolism [25]. In the present research, we also observed that *B. japonicum* gathered around *C. reinhardtii* and formed a special “algae-bacteria aggregate” under a microscope, which was similar to Gyurjan’s result.

3.2 H₂ production and O₂ content detection

The hydrogen yields and **O₂ content** of different systems are shown in Figure 2. The results showed that *B. japonicum* alone and freeze-thawed algae mixed with *B. japonicum* did not produce H₂, the single algae and co-cultures produce H₂, and the H₂ yields of the co-cultures were higher than the yields of the single alga culture. These results demonstrate that the algae produced H₂ but *B. japonicum* did not produce H₂; the latter only promoted H₂ production by algae (Fig. 2 A, C, E). Meanwhile, O₂ content of *B. japonicum* alone, freeze-thawed algae mixed with *B. japonicum* and co-cultures decreased more quickly than that of the single algae cultures (Fig. 2 B, D, F). A maximum H₂ production of 280 $\mu\text{mol}\cdot\text{mg}^{-1}$ Chl was measured during the co-cultivation of transgenic algae hemHc-lbac and *B. japonicum* when their inoculation volume ratio was 80:1 (algae to bacteria) under 30 $\mu\text{E m}^{-2} \text{ s}^{-1}$ of light intensity (Fig. 2 A). This production level was 2.8 times that of the single culture of strain hemHc-lbac (99 $\mu\text{mol}\cdot\text{mg}^{-1}$ Chl). O₂ content of *B. japonicum* alone, freeze-thawed transgenic algae hemHc-lbac mixed with *B. japonicum* and co-cultures reached 3.2-4.4% after 24h incubation in the dark, but O₂ content of the single algae culture reached 14.1% (Fig. 2 B). The maximum H₂ production of 280 $\mu\text{mol}\cdot\text{mg}^{-1}$ Chl was detected in the co-culture of strain 124 and *B. japonicum* when their inoculation volume ratio was 80:1 (algae to bacteria) under 200 $\mu\text{E m}^{-2} \text{ s}^{-1}$ of light intensity, and this production level was 14.0 times that of the single culture value of strain cc124 (20 $\mu\text{mol}\cdot\text{mg}^{-1}$ Chl) (Fig. 2 C). Be similar with transgenic algae hemHc-lbac, O₂ content of the single cc124 strain culture reached 15.1% and other O₂ contents of three

cultures reached 2.5-2.8% after 24h incubation in the dark (Fig. 2 D). A maximum H₂ production of 310 $\mu\text{mol}\cdot\text{mg}^{-1}$ Chl was obtained in the co-culture of strain cc503 with *B. japonicum* when their inoculation volume ratio was 20:1 (algae to bacteria) under 200 $\mu\text{E m}^{-2} \text{ s}^{-1}$ of light intensity(Fig. 2 E). This production level was 3.6 times the level for the single culture of strain 503 (70 $\mu\text{mol}\cdot\text{mg}^{-1}$ Chl). O₂ content of the cc503 strain culture reached 16.1% and other O₂ contents of three cultures reached 2.7-3.0% after 24h incubation in the dark (Fig. 2 F).

The H₂ production of co-cultures was influenced by the light density [26], the ratio of algae to bacteria, and the growth periods of the algae and bacteria [27]. The H₂ production of *C. reinhardtii* was related to the light density and its biomass [26]. To investigate the optimal hydrogen production condition, hydrogen production was detected under four different light intensities (30 $\mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, 60 $\mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, 100 $\mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, and 200 $\mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) and with six different ratios of algae and bacteria (200:1, 80:1, 40:1, 20:1, only algae, and only bacteria) [28]. Therefore, in this research, the bacteria and algae grew to the late exponential phase and the maximum H₂ production of three strains was similar, with approximately 300 $\mu\text{mol}\cdot\text{mg}^{-1}$ Chl, the results show that it is effective to co-cultivate *C. reinhardtii* with *B. japonicum* to improve H₂ production[28, 29]. In Miura's research, *C. reinhardtii* MGA161 was co-cultured with the photosynthetic bacterium W-1S, which could use the acetic acid and ethanol excreted by the algae as electron donors for hydrogen photoproduction [8]. Ike et al. used *Lactobacillus amylovorus* to hydrolyze the microalgae *C. reinhardtii*, *Chlorella pyrenoidosa* and *Dunaliella tertiolecta* to obtain substrates for hydrogen

production by using the photosynthetic bacterium *Rhodobacter sphaeroides* RV [30]. Kawaguchi et al. combined the lactic acid bacterium *Lactobacillus amylovorus* and the photosynthetic bacterium *Rhodobium marinum* A-501 with the green marine alga *D. tertiolecta* and the freshwater green alga *C. reinhardtii* to produce hydrogen from the starch that accumulated in the algal biomass [9]. Kim et al. grew *Rhodobacter sphaeroides* KD131 and *Clostridium butyricum* with *C. reinhardtii* UTEX90 to produce hydrogen [31]. Melis and Melnicki created an integrated biological system (*Rhodospirillum rubrum*, *Clostridium* sp. and *C. reinhardtii*) to produce hydrogen [32]. All these studies are based on the fact that hydrogen is primarily produced by photosynthetic or fermentative bacteria, whereas *C. reinhardtii* only serves as a biomass resource. In our research, the hydrogen is produced by *C. reinhardtii* but not *B. japonicum*, which acts as an accelerator, and our work is thus clearly different from the above reports. In summary, using *B. japonicum* to increase H₂ production provides new insights into how to improve H₂ production of *C. reinhardtii*.

The co-cultivation of *B. japonicum* with *C. reinhardtii* transgenic algae hemHc-lbac, cc124 and cc503 improved H₂ production by 2.8-fold, 14.0-fold and 3.6-fold that of the controls, respectively. These results show that adding *B. japonicum* to *C. reinhardtii* improved H₂ production, but the effects of the algae strains as influenced by *B. japonicum* differed. *B. japonicum* is a species of *Rhizobium*, which has four genera. It is unclear whether the interactions between other *Rhizobium* species and *C. reinhardtii* resemble those between *B. japonicum* and *C. reinhardtii*.

3.3 O₂ consumption in co-cultures of algae and *B. japonicum*

The respiratory rates and oxygen consumption of the algae-bacteria co-culture system were higher than the values of the controls (Fig. 3), which is likely the direct reason for the enhanced hydrogen yields. The respiratory rate of the co-culture system of the transgenic algae hemHc-lbac with *B. japonicum* was higher than that of the pure algal culture, and in particular, the respiratory rate of the co-culture was $5.04 \mu\text{mol O}_2 \cdot \text{mg Chl}^{-1} \cdot \text{h}^{-1}$ on the 4th day, which was 1.4 times that of the control, at $3.03 \mu\text{mol O}_2 \cdot \text{mg Chl}^{-1} \cdot \text{h}^{-1}$ (Fig. 3 A). Similar to the above results, the respiratory rates of the co-culture system with *C. reinhardtii* cc124, cc503 and *B. japonicum* were higher than those of the controls, which were $6.05 \mu\text{mol O}_2 \cdot \text{mg Chl}^{-1} \cdot \text{h}^{-1}$ and $3.34 \mu\text{mol O}_2 \cdot \text{mg Chl}^{-1} \cdot \text{h}^{-1}$ at the 2nd day and the 5th day after co-cultivation, respectively, values 1.2 times and 2.6 times the control values of $5.12 \mu\text{mol O}_2 \cdot \text{mg Chl}^{-1} \cdot \text{h}^{-1}$ and $1.26 \mu\text{mol O}_2 \cdot \text{mg Chl}^{-1} \cdot \text{h}^{-1}$, respectively (Fig. 3 B, C).

The O₂ content is important for hydrogen production because hydrogenase is sensitive to O₂ [5]. The O₂ content of the headspace and the DO (dissolved oxygen) of the culture were measured, and the results show that the O₂ content and DO in the co-culture were lower the values for pure algal cultures (Fig 2 B, D, F). Higher respiratory rates result in lower O₂ content and dissolved oxygen in culture, which provides a direct explanation for the improved hydrogen production. High DO value usually leads to increased photorespiration, which decreases the algal biomass of algae [33]. In our study, a low DO value was one reason for the rapid growth of algae in the co-cultures.

3.4 H₂ase activity of algae

After adding *B. japonicum* to *C. reinhardtii*, both the in vitro and vivo hydrogenase activity of the algae strains were higher than the controls (Fig. 4). The in vitro and in vivo maximum hydrogenase activity of the transgenic alga hemHc-lbac system and its co-culture system with *B. japonicum* were 57.9 nmol H₂ µg Chl⁻¹·h⁻¹ and 25.3 nmol H₂ µg Chl⁻¹·h⁻¹, which were 2.1-fold and 2.5-fold of the controls, respectively (Fig. 4 A, B). Similar to hemHc-lbac, the in vitro and in vivo maximum hydrogenase activity of *C. reinhardtii* cc124 and its co-culturing system with *B. japonicum* were 51.0 nmol H₂ µg Chl⁻¹·h⁻¹ and 13.0 nmol H₂ µg Chl⁻¹·h⁻¹, which were 2.0-fold and 4.8-fold of the controls, respectively (Fig. 4 C, D). The in vitro and in vivo maximum hydrogenase activity of *C. reinhardtii* cc503 and a co-culturing system with cc503 and *B. japonicum* were 62.8 nmol H₂ µg Chl⁻¹·h⁻¹ and 21.9 nmol H₂ µg Chl⁻¹·h⁻¹, which were 3.4-fold and 3.1-fold of the controls, respectively (Fig. 4 E, F). The hydrogen production of *C. reinhardtii* was catalyzed by hydrogenase [5,6,7], and the hydrogenase activity greatly improved in the algae-bacteria co-culture system, showing that rapid oxygen consumption was the most important reason for the enhanced activity of hydrogenase and hydrogen production [6,7].

3.5 Starch content in co-cultures of algae and *B. japonicum*

The starch content of the co-cultures greatly increased (Fig. 5), which was another primary reason for the improvement in hydrogen production. The maximum starch content of the co-culture system with transgenic hemHc-lbac algae and *B. japonicum* was 7.23 µg/ml, which was 8.3 times that of the control, at 0.87 µg/ml. Similar to the hemHc-lbac, the maximum starch content of the co-culture system with *C. reinhardtii*

cc124 and *B. japonicum* was 13.01 µg/ml, which was 8.4 times that of the control, at 1.55 µg/ml, and the maximum starch content of the co-culture system with *C. reinhardtii* cc503 and *B. japonicum* was 13.99 µg/ml, which was 4.4 times that of the control, at 3.18 µg/ml. There are two sources of electrons for H₂ photo-evolution, one of which is residual, that is, the H₂O oxidation in photosynthesis, and the other of which is an endogenous substrate (primarily starch) [5, 34]. Zhang et al. believed that in the course of such H₂ production cells consumed substantial amounts of internal starch and protein. Posewitz et al. reported that STA7 isoamylase gene and starch metabolism played an important role in *C. reinhardtii* H₂ photoproduction, STA7 isoamylase gene deletion mutant displayed low H₂ production [35]. The enhancement of starch content in the co-cultures may lead to the donation of more electrons to the hydrogenase, which was another reason for the higher H₂ production than in pure *C. reinhardtii* culture.

Conclusions

The co-cultivation of *C. reinhardtii* with *B. japonicum* has high H₂ yields than pure *C. reinhardtii* culture. Such improvement was possible due to the higher biomass and respiration rate, resulting in the improved starch content and lower oxygen concentration in co-culture system, finally up regulating the activity of hydrogenase and blooming up the hydrogen production.

Abbreviation: PSII: photosystem II; Chl: chlorophyll; h: hour; S: sulfur; DO: dissolved oxygen

Acknowledgements

This work was funded by grants from National Science Foundation of China (NSFC No. 31271397), the Shanghai Normal University Program (No. SK201517).

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Fig.1 Growth of *C. reinhardtii* and its co-cultivation with *B. japonicum* in TAP-S medium. The OD₇₅₀ and chlorophyll content of hemHc-lbac transgenic algae (A, B); the OD₇₅₀ and chlorophyll content of *C. reinhardtii* cc124 (C, D); the OD₇₅₀ and the chlorophyll content of *C. reinhardtii* cc503 (E, F). Standard errors were calculated from at least three independent experiments.

Fig.2 H₂ yields (A, C, E) and O₂ contents (B, D, F) from different incubation combination systems of *C. reinhardtii* and *Rhizobium*. The diamond indicates *C. reinhardtii* co-cultured with *Rhizobium* at an optimal ratio of algae and bacteria; the straight line indicates that *C. reinhardtii* was fractured by liquid nitrogen and then co-cultured with *Rhizobium* at an optimal ratio of algae and bacteria; the dotted diamond and the triangle indicate pure *C. reinhardtii* culture and *Rhizobium* culture, respectively. All the data are the averages of at least three independent experiments.

Fig.3 Respiratory rates of *C. reinhardtii* and its co-cultivation with *B. japonicum* in TAP medium. The transgenic algae hemHc-lbac (A), *C. reinhardtii* cc124 (B), and *C. reinhardtii* cc503 (C). All the data are averages of at least three independent experiments.

Fig.4 In vitro and in vivo H_2ase activity of *C. reinhardtii* and its co-cultivation with *B. japonicum*, the in vitro (A) and in vivo (B) H_2ase activity of *hemHc-lbac* transgenic alga, the in vitro (C) and in vivo (D) H_2ase activity of *C. reinhardtii* cc124, and the in vitro (E) and in vivo (F) H_2ase activity of *C. reinhardtii* cc503.

Fig.5 Starch content of *C. reinhardtii* and its co-cultivation with *B. japonicum* under hydrogen production conditions. The transgenic algae hemHc-lbac (A), *C. reinhardtii* cc124 (B), and *C. reinhardtii* cc503 (C).