Hydrogen Production by a Mixed Culture of a Green Alga, *Chlamydomonas reinhardtii* and a Photosynthetic Bacterium, *Rhodospirillum rubrum*

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An about 4-fold H_2 evolution rate and a 5-fold H_2 molar yield (mol H_2/mol glucose) were obtained with a mixed culture of *Chlamydomonas reinhardtii* and *Rhodosprillum rubrum*, compared with in the case of an algal culture of *C. reinhardtii* alone. This increasing effect was due to the consumption of formate formed by *C. reinhardtii*; *R. rubrum* evolved hydrogen from formate *via* the formate hydrogen-lyase system under dark anaerobic (N_2) conditions. Maximum H_2 evolution by the mixed culture was observed with a ratio of 8 : 2 (alga : bacterium) at a total cell concentration of above 0.6 mg dry wt/ml. Sustained H_2 production with an alternating light/dark cycle in a membrane reactor, in which this alga and bacterium were cultured in separate compartments, was performed for one week.

Green algae grown autotrophically can evolve hydrogen under dark anaerobic conditions using the cellular starch reserve as a substrate.[1] Our previous studies on a freshwater alga, *Chlamydomonas reinhardtii*, demonstrated alternating but stable O_2/H_2 production with a light/dark cycle.[2,3] However, the rates of hydrogen production by green algae are low, both in the light and the dark, compared to the rate of photosynthesis. Hydrogen production in the dark is accompanied by the excretion of organic compounds such as formate, acetate and ethanol.[5,8,11,12] In contrast to green algae, many bacteria can evolve hydrogen from organic compounds as exogenous substrates.[6,7,14,18,19] Photosynthetic bacteria exhibit H_2-evolving activity in the dark, and more efficiently, in the light. Therefore the use of a mixed culture of a green alga and a photosynthetic bacterium, capable of utilizing the fermentation products of the green alga, may improve overall hydrogen production rates.

Few studies on H_2 production by mixed cultures of microorganisms have been reported. Miyake *et al.*[7] reported that *Clostridium butyricum* and *Rhodopseudomonas* sp. were co-cultured to produce hydrogen from glucose, and they observed that the mixed culture evolved hydrogen in the light, the molar yield being 7.0 mol per mol of glucose consumed. Odom and Wall[15] reported that on anaerobic co-culturing of a *Cellulomonas* strain and *Rhodopseudomonas capsulata* hydrogen was photoevolved with cellulose as the sole exogenous substrate. However, H_2 production by mixed cultures of green algae and photosynthetic bacteria has not yet been reported. Here we report increased hydrogen production rates with a mixed culture of *C. reinhardtii* and *Rhodospirillum rubrum*, and sustained H_2 production with an alternating light/dark cycle in a membrane reactor, in which *C. reinhardtii* and *R. rubrum* were cultured in separate compartments.

MATERIALS AND METHODS

Strains and growth conditions. Chlamydomonas reinhardtii C-238 was obtained from the culture collection of

the Institute of Applied Microbiology, University of Tokyo, Japan. *Rhodospirillum rubrum* NCIB 8255 was obtained from the National Collection of Internation Bacteria, Great Britain.

The alga and bacterium were grown at 30°C in 1.5 l-Roux flasks containing 1.0 l of a modified Bristol medium (MBM, pH 7.0).[3] The algal cultures were continuously illuminated, with a bank of fluorescent lamps, at a light intensity of 25 W/m^2. The cultures were continuously sparged with air containing 5% CO_2, for agitation and as a CO_2-supply, at a flow rate of about 300 ml/min. The medium for *R. rubrum* contained 680 mg HCOONa, 820 mg CH_3 COONa, 460 mg C_2H_5OH and 60 μg biotin in 1000 ml of the modified Bristol medium. The bacterial cultures were grown without agitation and continuously illuminated, with a bank of tungsten lamps, at a light intensity of 200 W/m^2.

Dark anaerobic conditions for H_2 evolution. Cultures of algae or bacteria in the mid-logarithmic growth phase were harvested by centrifugation (4000 × *g*, 10 min), and then washed and resuspended in the modified Bristol medium, in a total volume of 10 ml at a final density of 0.5 ~ 1.0 mg dry wt/ml in a test tube (34 ml, 18 mm dia.) fitted with a rubber stopper. The tube was flushed for 20 min with O_2-free N_2 gas (99.99%) and then incubated at 30°C on a reciprocal shaker (100 rpm). No exogenous substrate was added to the pure algal cultures or the mixed cultures, but a substrate was added for the pure bacterial cultures at a concentration of 10 mM.

Sustained H_2 production with an alternating light/dark cycle. The light/dark cycle cultures were grown at 30°C in 0.1 l of the modified Bristol medium supplemented with 6 μg of biotin, illuminated, with a bank of tungsten lamps, at a light intensity of 200 W/m^2 with a 12 hr light/12 hr dark cycle, and continuously sparged with air containing 5% CO_2 during the light period. The ratio of the alga to the bacterium was 8 : 2 during this experimental run: 64 mg dry weight of algal cells was suspended in 0.1 l of MBM in a glass tube (0.3 l, 45 mm dia.), and 16 mg dry weight of bacterial cells was suspended in the permeable cellulose membrane tube (15 ml, 16 mm dia.) that separated the cultures. During the dark-period, the cultures were sparged with N_2 for the initial 20 min, and incubated at 30°C for 12 hr on a reciprocal shaker (100 rpm). The alternating light/dark incubation was continued for over one week.

Analysis of metabolites. Hydrogen and carbon dioxide released from a cell suspension with perchloric acid were measured by gas chromatography (model-164, Hitachi). The column was filled with Molecular Sieve 13X, 30/60 mesh (Gasukuro Kogyo, Inc.), for the H_2 assay, or Porapak Q, 50/80 mesh (Water Associates Inc.), for the CO_2 assay.

Starch was extracted from cells as described previously,[8] then the sample was acid-hydrolyzed to glucose by heating the extract in a boiling water bath for one hour, and after neutralization, the amount of glucose in the acid-hydrolysate was determined by the hexokinase method.[9]

Ethanol was assayed with alcohol dehydrogenase, acetate with acetyl-CoA synthetase, citrate synthase and malate dehydrogenase, and glycerol with glycerolkinase, pyruvate kinase and lactate dehydrogenase. These enzymes were obtained as test kits from Boehringer-Mannheim. Formate was assayed with formate dehydrogenase.[10]

Other tests. To determine the dry weight, 10 ml of a cell suspension was centrifuged at 4000 × *g*. The cells were washed twice with deionized water and then dried on an aluminum cup of known weight in an oven at 110°C until a constant weight was reached.

RESULTS AND DISCUSSION

Dark H_2 evolution in R. rubrum

Green algae form fermentation products under anaerobic conditions through the use of cellular starch as an endogenous substrate *via* the Embden-Meyerhof pathway.[11,12] The starch degradation rate of *Chlamydomonas reinhardtii* was very high; two or three times that of other green algal strains.[8] This alga produced several organic compounds (Table I), and the ability of these organic compounds to support H_2 production by *Rhodospirillum rubrum* was tested (Table I). *R. rubrum* only evolved hydrogen from formate among the five substrates tested under dark anaerobic conditions. It was suggested that there would be an increasing effect on the dark H_2 evolution by a mixed culture of *C. reinhardtii* and *R. rubrum*.

Figure 1 shows the effects of chloramphenicol (50 μg/ml) and Na-hypophosphite (5 mM) on the hydrogen evolution by *R. rubrum*. The dark H_2 evolution of *R. rubrum* was found to be an inducible reaction, as 3 hr-dark anaerobic incubation was required for hydrogen evolution to start, which was completely inhibited by the addition of chloramphenicol. Photosynthetic bacteria can evolve hydrogen from formate *via* the formate hydrogen-lyase system under dark anaerobic conditions,[16,17] and the formate hydrogen-lyase system is composed of a soluble type formate dehydrogenase

TABLE I. FERMENTATION PRODUCTS OF *C. reinhardtii* AND THEIR AVAILABILITY AS SUBSTRATES FOR H_2 EVOLUTION BY *R. rubrum*

Product or substrate	*Chlamydomonas reinhardtii*		*Rhodospirillum rubrum*
	Molar production (μmol/mg dry wt/6 hr)	Molar yield of product (mol product/mol glucose)	Hydrogen evolution* (μmol/mg dry wt/12 hr)
No addition	—	—	0.07
H_2	0.45	0.51	—
CO_2	0.57	0.64	0
Acetate	1.09	1.22	0.05
Ethanol	0.60	0.74	0.05
Formate	0.95	1.07	2.04
Glycerol	0.10	0.11	0.05
Starch	−0.89	—	—
Carbon recovery (%)	98		

* Dark anaerobic assays were performed with added substrate (10 mM). The data are the means for five experiments.

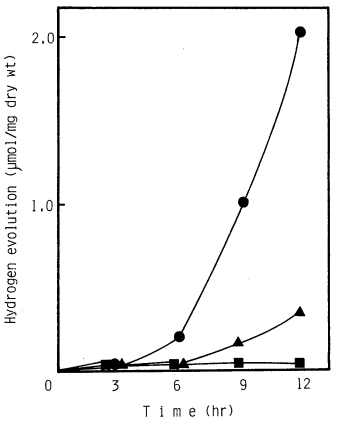

FIG. 1. Effects of Chloramphenicol and Na–Hypophosphite on Hydrogen Evolution by *R. rubrum*.

Anaerobic incubations were performed with added formate (10 mM) under an N_2 atmosphere in the dark: a control (●) incubation was carried out without chloramphenicol or Na–hypophosphite. Chloramphenicol (■, 50 μg/ml) or Na–hypophosphite (▲, 5 mM) was added immediately before the anaerobic incubation.

TABLE II. DARK H_2 EVOLUTION AS A FUNCTION OF THE MIXING RATIO OF THE ALGA AND THE BACTERIUM

C. reinhardtii : *R. rubrum*	Hydrogen evolution*	
	Total mass base (μmol/mg dry wt/12 hr)	Algal mass base (μmol/mg dry wt/12 hr)
10 : 0	0.60	0.60
8 : 2	2.39	2.99
5 : 5	1.50	3.00
2 : 8	1.09	5.45
0 : 10	0	0

* H_2 evolution was measured with a total cell concentration of 0.64 mg dry wt/ml. The data are the means for five experiments.

and a membrane-bound type hydrogenase in *Rhodopseudomonas pulustris*.[13] *R. rubrum* also made use of the formate hydrogen-lyase system, because hydrogen evolution by this strain was inhibited by more than 80% upon addition of Na–hypophosphite, an inhibitor of formate dehydrogenase.[4] Nitrogenase activity, as assayed by an acetylene reduction method, was not observed (data not shown). Therefore, it was concluded that *R. rubrum* evolve hydrogen *via* the formate hydrogen–lyase system under these conditions.

H_2 evolution by the mixed culture

Table II shows the dark H_2 evolution with various ratios of *C. reinhardtii* and *R. rubrum*. Dark H_2 evolution increased when *R. rubrum* was mixed with *C. reinhardtii*. With increasing cell concentration of *C. reinnhardtii*, H_2 evolution increased. Maximum H_2 evolution by

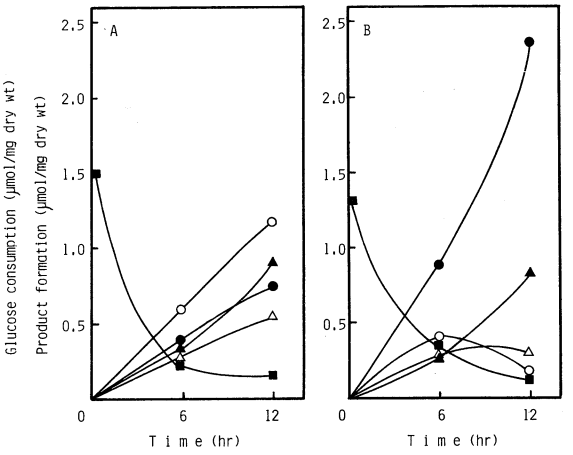

FIG. 2. Time Courses of Fermentation in the Algal Culture (A) and the Mixed Culture of an Alga and a Bacterium (B).

Glucose consumption (■) and the formation of hydrogen (●), acetate (▲), ethanol (△) and formate (○) were measured. *C. reinhardtii* and *R. rubrum* were mixed in the ratio of 8 : 2. Fermentation was performed with a total cell concentration of 0.66 mg dry wt/ml. The points are the means for five experiments.

the mixed population of *C. reinhardtii* and *R. rubrum* was observed at the ratio of 8 : 2, and the highest amount of hydrogen per algal dry weight was observed at the ratio of 2 : 8, when the total cell concentration was fixed at 0.64 mg dry wt/ml. However, more detailed understanding of the relationship between the algal cell concentration and the mixing ratio will be necessary to optimize the H_2 evolution by the mixed culture.

The time courses of fermentation in the *C. reinhardtii* and the mixed cultures were investigated (Fig. 2). *C. reinhardtii* alone accumulated H_2, acetate, ethanol and formate, while degrading starch (Fig. 2A). In the case of the mixed culture (ratio of algae–bacteria, 8 : 2), fermentation products were accumulated in the medium for the first 6 hr dark incubation. Thereafter the formate concentration decreased, ethanol remained constant, and hydrogen and acetate continued to increase (Fig. 2B). Although it is evident that *R. rubrum* evolved hydrogen by using the formate formed by *C. reinhardtii*, this bacterium could not evolve hydrogen from acetate or ethanol (Table I). Furthermore, *R. rubrum* itself accumulated acetate in the medium, using the cellular glycogen-like reserve under dark anaerobic conditions (data not shown). H_2 evolution by the mixed culture increased about four times compared with that in the case of *C. reinhardtii* alone, apparently due to the consumption of formate by the mixed culture. Although *R. rubrum* could not evolve hydrogen from the added ethanol, the bacterium consumed ethanol under dark anaerobic conditions (data not shown).

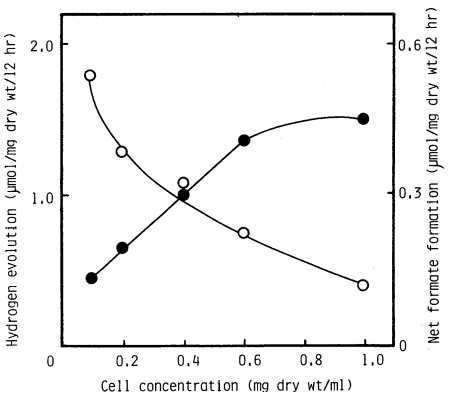

FIG. 3. Effect of the Total Cell Concentration of the Hydrogen Evolution by the Mixed Culture.

Hydrogen evolution (●) and formate formation (○) were measured. *C. reinhardtii* and *R. rubrum* were mixed in the ratio of 8 : 2.

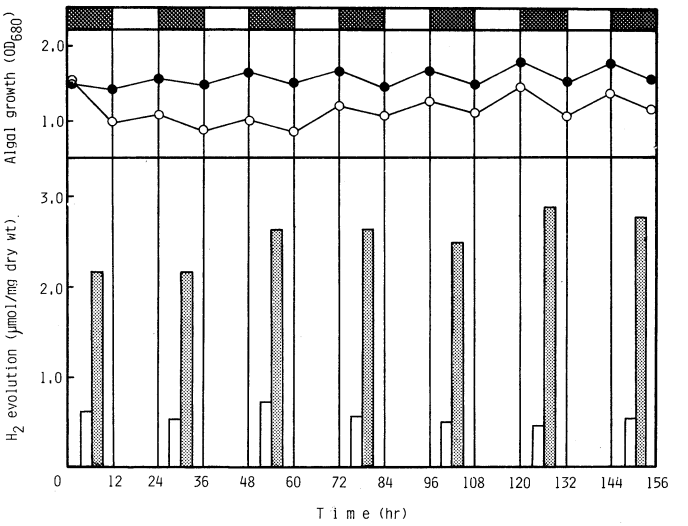

FIG. 4. Sustained Hydrogen Production by the Algal Culture and the Mixed Culture with a Light/Dark Cycle.

An algal culture (○, □) and a mixed culture (●, ▩), synchronized with an alternating 12 hr light/12 hr dark cycle, were performed in a membrane reactor as described under MATERIALS AND METHODS. Algal growth (○, ●; expressed as OD_{680}) and hydrogen evolution (□, ▩) were measured.

Effect of the total cell concentration

The effects of the total cell concentration on H_2 evolution and formate formation, when the total cell concentrations of *C. reinhardtii* and *R. rubrum* were 0.1 and 1.0 mg dry wt/ml with a ratio of 8:2, were investigated. The dark anaerobic H_2 evolution at 1.0 mg dry wt/ml drastically increased after 6 hr, with concomitant formate uptake. In contrast, with only 0.1 mg dry wt/ml, no increase in H_2 evolution was observed. Formate hydrogen–lyase activity was found to be inductive, and it is suggested from our data that the induction of formate hydrogen–lyase activity would be dependent on the concentration of formate which is produced by the algal cells. Therefore, the minimum cell concentration for the induction of the full activity of this enzyme system and for maximizing the H_2 evolution by the mixed culture was determined to be about 0.6 mg dry wt/ml (Fig. 3).

Hydrogen production with a light/dark cycle in a membrane reactor

Figure 4 shows that the H_2 production was synchronized with an alternating 12 hr light/12 hr dark cycle in a membrane reactor, in which the alga and the bacterium were separated (see MATERIALS AND METHODS). The algal cell concentration (expressed as OD_{680}) decreased during the dark period, and increased to the initial level during the subsequent light period. The culture maintained the orginal algal : bacterial ratio of 8 : 2 during one week of operation. The observed dark H_2 evolution by the mixed culture was over 4-fold greater than that in the case of *C. reinhardtii* alone. Even after *R. rubrum* had been subjected to several light periods with aeration and photosynthetic oxygen evolution, it exhibited the full H_2-evolving activity when assayed with added formate (data not shown).

The membrane reactor system was evaluated from the standpoints of the H_2 evolution rate and the H_2 molar yield (mol H_2/mol glucose); an about 4-fold H_2 evolution rate and a 5-fold H_2 molar yield were obtained. This mixed culture accumulated acetate under dark anaerobic conditions (Fig. 2). Therefore, in order to further improve the H_2 evolution rate and the H_2 molar yield with this system, light-dependent H_2 evolution by the photo-

synthetic bacterium from acetate is required.

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