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Immobilized Cells of a Unicellular Green Alga and a Photosynthetic Bacterium for Use in a Biophotolysis System

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ABSTRACT

Immobilization of algal and bacterial cells was investigated, and found applicable to our hydrogen production system. Both a unicellular green alga, *Chlamydomonas reinhardtii*, and a photosynthetic bacterium, *Rhodospirillum rubrum*, were separately entrapped in calcium alginate gel. The photosynthetic starch accumulation and subsequent dark fermentation of *C. reinhardtii* were not affected by cell immobilization in Ca-alginate gel. Immobilized cells of *R. rubrum* retained their ability to utilize various electron donors for hydrogen evolution. Immobilized *R. rubrum* was stable, at least for a week, in a light and dark cycle. These and other observations suggest that the immobilization of cells could facilitate the broth-recycle between an algal culture system and a bacterial hydrogen production unit.

Index Entries: Cell immobilization; hydrogen production; green alga, photosynthetic bacterium.

INTRODUCTION

A new biophotolysis system based on photosynthesis and fermentation in green algae followed by light-driven hydrogen production by photosynthetic bacteria is currently being studied in our laboratory (*1–4*).

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The system consists of three steps of energy conversion: (first step) photosynthetic starch accumulation in algal cells; (second step) fermentation of cellular starch for the production of hydrogen and organic compounds, such as formate, acetate, and ethanol; (third step) further conversion of the organic compounds for the production of hydrogen by photosynthetic bacteria. The sequential use of algae and photosynthetic bacteria increases the molar yield of hydrogen from starch-glucose.

However, since the nitrogenase that catalyzes hydrogen production in the cells of photosynthetic bacteria is very sensitive to oxygen, the bacterial production unit must be separated from the oxygenic photosynthetic unit of green algae, i.e., only the fermentation broth must be supplied to the bacterial hydrogen production unit. Because of the microscopic nature of algae and photosynthetic bacteria, it is very difficult to separate the cells from the culture broth. Cell immobilization would facilitate their separation, and allow the broth-recycle between an algal culture/fermentation unit and a bacterial hydrogen production unit.

Cell immobilization techniques for biological hydrogen production were intensively investigated in recent years because of the ardent need for the development of alternative energy sources (*5,6*). The possible advantages of cell immobilization in this field are well documented, which include among others (a) high operational stability needed for the production of low-cost materials and (b) maintenance of whole-cell activities necessary for multienzyme reactions in solar energy conversion to hydrogen. Although ease of hydrogen separation is not a major problem in single-stage hydrogen production systems, cell separation from algal culture broth would be a necessity for our three-stage system.

In a previous study (*7*), it was demonstrated that hydrogen production could be sustained in a bioreactor with an alternating 12 h light/12 h dark cycle, wherein the alga and the bacterium were separated by a permeable cellulose membrane. However, only the formate produced by *C. reinhardtii* was used by *R. rubrum* for hydrogen evolution, which in turn was catalyzed by its formate hydrogen lyase system. In order to further increase the hydrogen molar yield (mol H_2/mol glucose) and production rate of the system, the light-driven hydrogen evolution of photosynthetic bacteria is required. In the present study, both *C. reinhardtii* and *R. rubrum* were separately immobilized in alginate gel. Also, the effects of entrapment on algal photosynthetic starch accumulation and its degradation during dark anaerobic fermentation were investigated, in addition to the effect on hydrogen production of *R. rubrum*.

MATERIALS AND METHODS

Microorganisms

A green alga, *Chlamydomonas reinhardtii* C-238, and a photosynthetic bacterium, *Rhodospirillum rubrum*, were obtained from the culture collec-

tions of the Institute of Applied Microbiology, University of Tokyo, Japan, and from the National Collection of International Bacteria, Great Britain, respectively.

Culture Methods

The alga and the 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) (2). 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-source, at a flow rate of about 300 mL/min. Algal cells grown under nitrogen-limitation (*N*-deficient cells) were compared with normally grown, *N*-sufficient cells.

The medium for *R. rubrum* contained 680 mg HCOONa, 820 mg CH_3COONa, 460 mg C_2H_5OH, and 60 μg biotin in 1000 mL of the modified Bristol medium containing 5 m*M* NH_4Cl. 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.

Immobilization

Algal or bacterial cell suspension was mixed with 2% (w/v) sodium alginate solution. The mixture was added dropwise to 50 m*M* $CaCl_2$ solution agitated gently with a magnetic stirrer. Calcium alginate beads formed within 5 min were washed with the fresh medium and then used for activity assays.

Hydrogen Evolution

Algal cells in mid-logarithmic growth phase were harvested by centrifugation and washed with growth medium. The cells, free or immobilized, were suspended in 10 mL of the same medium in a light-shielded test tube fitted with a rubber stopper. The tube was then flushed for 20 min with O_2-free N_2 gas and incubated at 30°C on a reciprocal shaker.

Photosynthetic bacterial cells in the mid-logarithmic growth phase were harvested, washed, and incubated in the dark or in the light. For the photohydrogen evolution, free or immobilized cells were flushed for 30 min with argon gas and then incubated with malate at a final concentration of 10 m*M*. Glutamate (final conc. 5 m*M*) was used as a nitrogen source, unless otherwise specified. A light intensity of 25 W/m^2 was found insufficient to cause hydrogen evolution of bacterial cells; thus, 200W/m^2 was employed for the present study.

Analysis

Evolved hydrogen was measured by a gas chromatograph equipped with a column filled with Molecular Sieve 13X. Starch and algal metabo-

Table 1
Effect of Entrapment and Polylysine-Coating
on Hydrogen Evolution and Formate Formation of *C. reinhardtii*

Cell conditions	Coating	Product formation* (μmol/mg dry wt/12 h) Hydrogen	Formate
Free		0.40	1.35
Ca-alginate entrapped	–	0.38	1.26
	+	0.36	0.72
κ-carrageenan entrapped	–	0.15	1.10
	+	0.20	0.54

*Product formation was measured under dark anaerobic conditions.

lites were assayed enzymatically with commercial test kits. To determine the dry wt, 10 mL of a cell suspension were first centrifuged at 4000×*g*. The cells were washed twice with deionized water and then dried on a tared-aluminum cup in an oven at 110°C until a constant weight was reached.

RESULTS AND DISCUSSION

Effects of Immobilization on Algal Activities

Cells of *Chlamydomonas reinhardtii* were immobilized by entrapment in various gel matrices. Ca-alginate, κ-carrageenan, agar, and agarose were evaluated for their ease of preparation and mechanical stability. Ca-alginate and κ-carrageenan gel beads could be made in smaller diameter, and the former showed the highest mechanical stability in a shaking flask. Table 1 shows the effect of cell entrapment and polylysine coating on the fermentation activity of *C. reinhardtii*. The product formation in fermentation was not affected by Ca-alginate entrapment, but hydrogen evolution was decreased by κ-carrageenen entrapment. Polylysine coating decreased formate formation, though it increased mechanical stability. Fermentation activity decreased slightly with increasing Ca-alginate concentration up to 3% (w/v), and 1.0% was chosen for routine experiments by considering gel strength.

As shown in Table 2, Ca-alginate did not affect starch degradation and product formation of nitrogen-sufficient or normal cells of *C. reinhardtii*, but those of nitrogen-deficient cells were lowered by entrapment. Repeated exchange of liquid medium, which was an easy treatment for immobilized cells, overcame this detrimental effect and increased the fermentation activities of entrapped *N*-deficient cells (Fig. 1).

Table 2
Effect of Entrapment on Starch Degradation and Product Formation of *C. reinhardtii* *

Cell conditions	Starch degradation, μmol glucose / mg dry wt·12 h	Product formation (μmol/mg dry wt/12 h) Hydrogen	Formate	Acetate	Ethanol
N-sufficient					
Free	0.85	0.42	1.45	0.71	0.96
Entrapped**	0.81	0.38	1.33	0.65	0.92
N-deficient					
Free	1.05	0.19	1.38	0.71	1.29
Entrapped**	0.77	0.12	1.04	0.50	0.86

*Starch degradation and product formation were measured under dark anaerobic conditions.
**Algal cells were entrapped in 1% Ca-alginate gel.

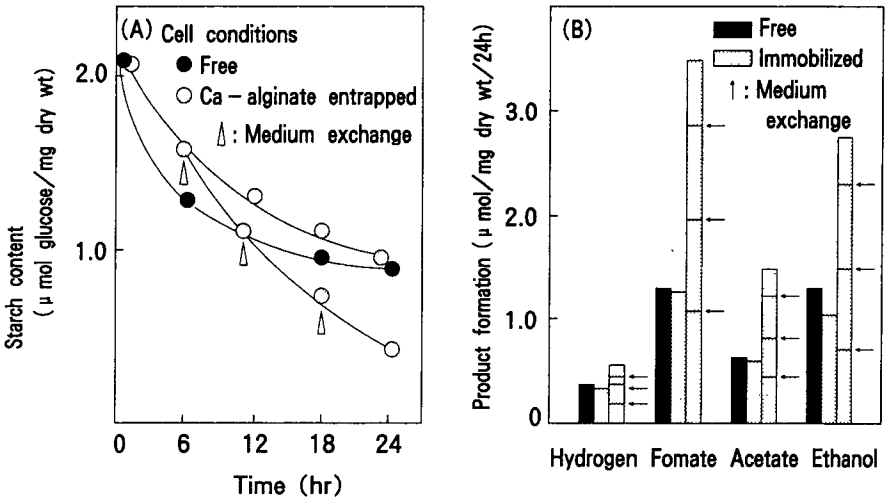

Fig. 1. Enhancement of starch degradation (A) and fermentation product formation (B) by medium exchange of entrapped N-deficient cells of *C. reinhardtii*.

Stabilities of Immobilized Algal Cells

The stability of immobilized preparations was compared: 1% Ca-alginate entrapped cells with and without $CaCl_2$ treatment, and 2% Ca-alginate entrapped cells were tested for their hydrogen evolution activity by incubating them for a week in a repeated light/dark cycle. Change in diameter of gel beads was also measured as an indication of mechanical stability. The results of such study with *C. reinhardtii* are shown in Fig. 2. Both 1%

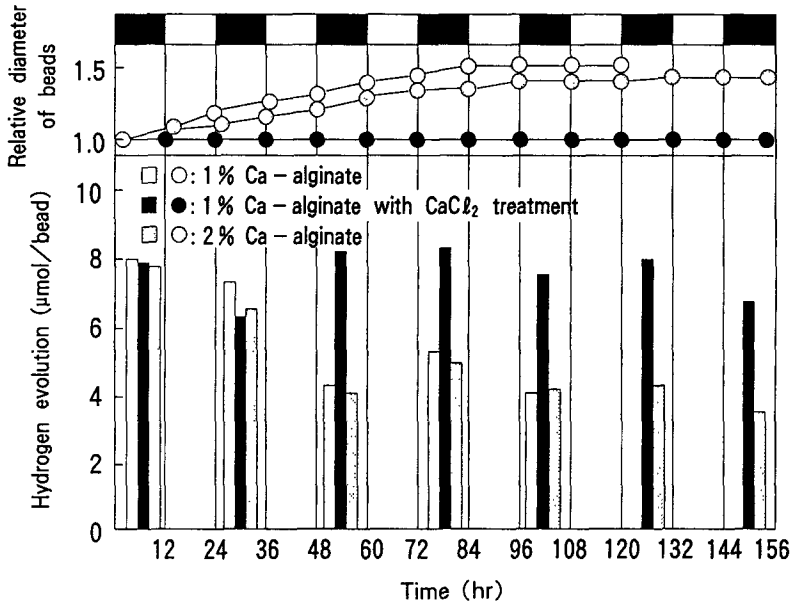

Fig. 2. Sustained hydrogen production by $CaCl_2$ treatment of entrapped cells of *C. reinhardtii* in a light/dark cycle.

Table 3
Utilization of Organic Compound for Hydrogen Production of Free and Entrapped Cells of *R. rubrum*

Organic compound*	Light conditions	Hydrogen production (μmol/mg dry wt/12 h)	
		Free	Entrapped
Malate	Light	2.0	2.1
Acetate	Light	0.15	0.18
Ethanol	Light	0.0	0.0
Formate	Light	1.9	2.0
Formate	Dark	1.8	1.8

*Concentration was 1 mM.

and 2% alginate gels swelled up to 1.5-fold, and a concomitant decrease was observed in hydrogen evolution activity (1% gel beads were broken up at 120 h). However, when washed every 24 h with 50 m*M* $CaCl_2$, 1% alginate gels showed stable activity in hydrogen evolution while keeping the bead diameter constant. Equally good results have been obtained by incubating 1% alginate gels in 50 m*M* $CaCl_2$-containing medium.

Effects of Immobilization on Bacterial Activities

Actively growing cultures of *R. rubrum* were harvested in their mid-logarithmic growth phase, and cells were entrapped in Ca-alginate gel in a similar manner as *C. reinhardtii*. Table 3 compares the utilization of vari-

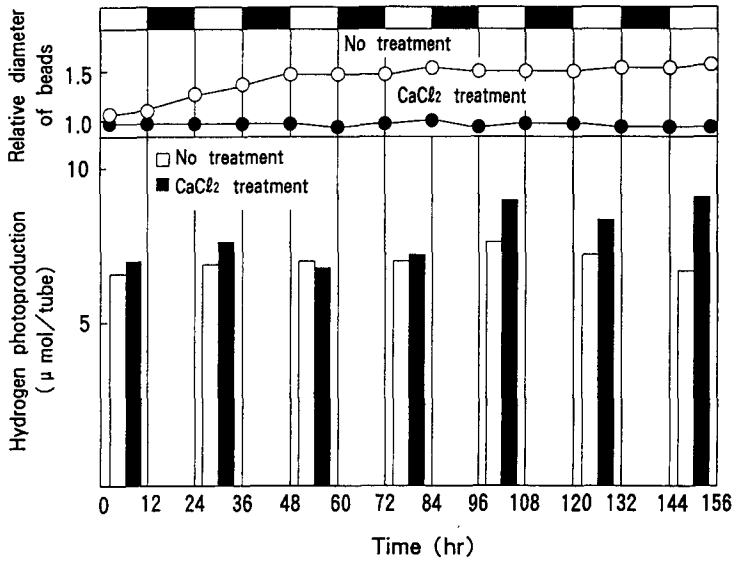

Fig. 3. Sustained hydrogen photoproduction by $CaCl_2$ treatment of entrapped cells of *R. rubrum* in a light/dark cycle.

ous organic substrates by free and immobilized cells of *R. rubrum*. Photoproduction of hydrogen in this bacterium was driven by nitrogenase, whereas hydrogen evolution in the dark was catalyzed by a formate hydrogenlyase system. Hydrogen-evolving activities of *R. rubrum* were not influenced by the immobilization.

The immobilized cell preparation of *R. rubrum* produced hydrogen stably, at least for a week, in the light period when added with glutamate in the medium (Fig. 3). The gel swelling observed here was prevented by washing with 50 m*M* $CaCl_2$ solution. A similar experiment was run with *N*-free algal fermentation broth of *C. reinhardtii* (data not shown), because hydrogen evolution of *R. rubrum* was inhibited by NH_4Cl, a nitrogen source in the algal growth medium. The broth, after fermentation in the dark, contained formate, acetate, and ethanol in a ratio of about 2:2:1. With this fermentation broth as a substrate, repeated but unstable hydrogen production by the immobilized cells of *R. rubrum* was observed. Bacterial growth in the gel beads occurred both in $CaCl_2$-treated and non-treated preparation, and a gradual decrease was observed in photoproduction of hydrogen. However, a nitrogen gas supply (Fig. 4) or a controlled supply of NH_4Cl (data not shown) could enhance the hydrogen-evolving activity of the immobilized cells of *R. rubrum*.

CONCLUSIONS

It has been shown that immobilization of algal and bacterial cells could give active and stable biocatalysts for use in biophotolytic hydrogen production. The fermentation broth could be easily separated from immo-

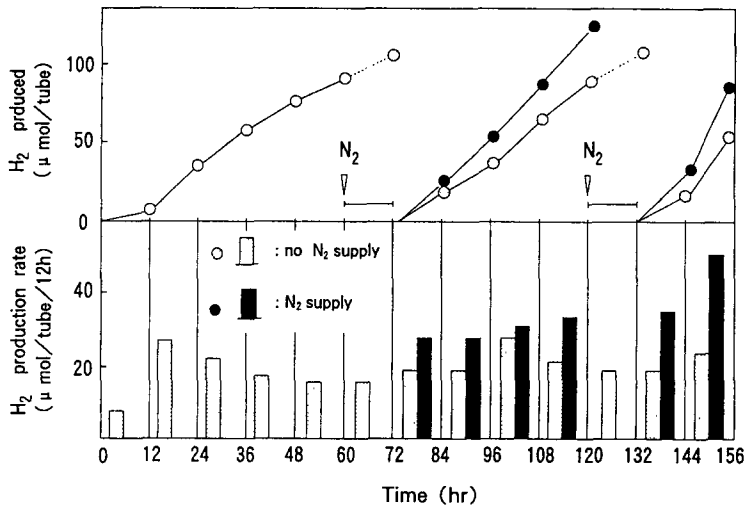

Fig. 4. Effect of N_2 supply on hydrogen photoproduction of entrapped cells of *R. rubrum*. Entrapped cells with and without nitrogen supply were incubated under continuous light. Shaded symbols: nitrogen was not supplied. Closed symbols: nitrogen gas was supplied for 12 h at the times indicated.

bilized cells of *C. reinhardtii* and used as substrate for hydrogen evolution by the immobilized cells of *R. rubrum*. These observations suggest that the immobilization of microscopic cells could facilitate the operation of our biophotolysis system. The design of reactors for the system depends on the development of efficient biocatalysts. Studies on simple and inexpensive methods of immobilization using marine strains of microalgae and photosynthetic bacteria are in progress.

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