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HYDROGEN EVOLUTION BY CO-IMMOBILIZED *CHLORELLA VULGARIS* AND *CLOSTRIDIUM BUTYRICUM* CELLS

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Whole cells of *Chlorella vulgaris* and *Clostridium butyricum* were co-immobilized in 2% agar gel. NADP was suitable as an electron carrier. The rate of hydrogen evolution increased with increasing NADP concentration. The optimum conditions for hydrogen evolution were pH 7.0 and 37°C. The immobilized *C. vulgaris*-NADP-immobilized *Cl. butyricum* system continuously evolved hydrogen at a rate of 0.29–1.34 μmol/h per mg Chl for 6 days. On the other hand, the system without NADP evolved only a trace amount of hydrogen.

Introduction

Biophotolysis of water is now attracting attention as one of the hydrogen production systems. Various approaches are now under investigation. Coupling of the photosynthetic system in plant chloroplasts with a hydrogenase could result in light-driven splitting of water into hydrogen and oxygen [1]. However, the lifetime of the isolated chloroplasts is very short, and hydrogenase is very unstable. Therefore, the continuous production of hydrogen by the isolated chloroplast-isolated hydrogenase system is difficult.

Recently, immobilization techniques for living microorganisms and organelles have been developed. The authors succeeded in stabilizing the hydrogenase system by immobilization of living hydrogen-producing bacteria [2,3]. Hydrogenase in immobilized cells was protected from the deleterious effects of oxygen. Furthermore, the immobilized spinach chloroplast-whole bacteria (containing hydrogenase) system was applied to the biophotolysis of water [4]. The lifetime of immobilized chloroplasts was longer than that of isolated intact chloroplasts. The system could evolve hydrogen for only 6 h. However, immobilized chloroplasts were still unstable. Moreover, electron carriers such as ferredoxin and benzyl viologen were oxidized by the oxygen evolved by the chloroplasts.

The green alga, *Chlorella vulgaris*, is known to reduce NADP under anaerobic conditions. In this study, *C. vulgaris* was employed for the light-induced water-splitting hydrogen evolution in place of chloroplasts. *C. vulgaris* and *Clostridium butyricum* were co-immobilized in agar gel, and hydrogen evolution by photolysis of water was attempted by the use of the immobilized *C. vulgaris*-NADP-*Cl. butyricum* system.

Materials and Methods

Materials. Yeast extract was obtained from Difco Laboratories. Peptone (from casein) and agar were purchased from Kyokuto Pharmaceutical Co. NADP, NAD and NADPH were purchased from Oriental Yeast Co. Other reagents were commercially available reagents or laboratory grade materials. Distilled water was used in all procedures.

Culture of microorganisms. C. vulgaris was grown under aerobic conditions at 30°C for 10 days under illumination in 5 l of tap water medium (pH 6.0) [5].

Cl. butyricum (IFO 3847) was grown under anaerobic conditions at 37°C for 9 h in 1 l of tap water

Abbreviation: Chl, chlorophyll.

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medium (pH 7.0) [4]. *Escherichia coli* (IFO 12173) was grown under aerobic conditions, without shaking, at 37°C for 11 h in 1 l of tap water medium (pH 7.0) containing 10 g yeast extract, 10 g glucose, 10 g peptone, 5 g meat extract and 5 g NaCl. *Desulfovibrio salexigens* (NCIB 8308) was grown under anaerobic conditions at 37°C for 20 h in 1 l of tap water medium (pH 7.0) containing 5 g peptone, 5 g calcium lactate, 1 g meat extract, 0.2 g yeast extract, 0.002 g $FeSO_4 \cdot 7\ H_2O$, 2.3 g Na_2SO_4 and 35 g NaCl. Microbial cells were isolated by centrifugation at 8000 × *g* for 10 min. The cells were washed twice with 0.1 M Tris-HCl buffer (pH 8.0, 4°C) at 8000 × *g* for 10 min before use.

Chlorophyll content and the dry weight of microbial cells were measured according to the procedure described in the previous paper [6].

Immobilization of microorganisms. For the immobilization of *Cl. butyricum, E. coli* and *D. salexigens*, 4 g of agar were dissolved in 100 ml Tris-HCl buffer (0.1 M, pH 8.0) in a flask and cooled to 50°C. Then 1 ml of the agar solution at 50°C in a flask was mixed with 1 ml of Tris-HCl buffer (pH 8.0) containing *Cl. butyricum* (2% wet cells) and NADPH (1600 μM) at room temperature. As soon as both solutions were mixed, the flask was cooled in a water bath at 0°C. The gel of immobilized *Cl. butyricum* was cut into a small blocks (1 mm^3). *E. coli* and *D. salexigens* were also immobilized using the same procedure described above.

For the immobilization of *C. vulgaris*, 0.25 ml of Tris-HCl buffer (0.1 M, pH 8.0, 50°C) containing 4% agar, and 0.25 ml Tris-HCl buffer containing *C. vulgaris* (0.125 mg Chl, 7.1 mg dry cells) and 1600 μM NADP were mixed in a test tube and immediately cooled to 30°C. The immobilized *C. vulgaris* gel was cut into small blocks (1 mm^3).

Measurement of hydrogen-evolution rate by immobilized cells. The activity of hydrogenase in immobilized cells was determined by measuring the initial hydrogen-evolution rate. The reaction mixture contained 0.5 g immobilized bacteria (10% wet cells) gel and 4.5 ml Tris-HCl buffer (0.1 M, pH 8.0, 800 μM NADPH). The reaction mixture was placed in a flask which was then exhausted with a vacuum pump. The gas in the flask was replaced with argon. The reaction mixture was incubated at 37°C for 12 h. Hydrogen gas evolved was determined by gas chromatography (Shimadzu Seisakujo, model GC-4BT) under the conditions described previously [6].

Measurement of NADP-reduction rate by immobilized C. vulgaris. The activity of Photosystems I and II in immobilized algae was determined by measuring the initial rate of NADP reduction in the Hill reaction. The reaction mixture, which consisted of 0.5 g immobilized *C. vulgaris* gel and 4.5 ml Tris-HCl buffer containing 800 μM NADP, was placed in a shrenk flask and mixed. The gas in the flask was replaced by argon. The reaction mixture was incubated at 37°C for 12 h.

After incubation, 1 ml of reaction mixture was filtered with a porous acetylcellulose membrane (Millipore, type TM-4, 0.2 μm pore size). The extent of NADPH reduction in the Hill reaction was measured by a spectrometric method (the increase in absorbance at 340 nm).

Hydrogen evolution by the immobilized C. vulgaris-Cl. butyricum system. Hydrogen evolution was assayed in a shrenk flask. In the shrenk flask, 0.5 g of gel containing *C. vulgaris* (0.125 mg Chl, 7.1 mg dry cells) and *Cl. butyricum* (5 mg wet cells), and 4.5 ml of Tris-HCl buffer (0.1 M, pH 8.0) containing NADP (800 μM) and $NaHCO_3$ (10 mM) were mixed. The gas in the flask was replaced by argon. The reaction mixture was incubated at 37°C under illumination provided by a fluorescent lamp (3000 lx). Hydrogen evolved was determined by gas chromatography.

Results

Screening of bacteria for hydrogen evolution from NADPH

The rate of hydrogen evolution from NADPH was examined by using various immobilized bacteria containing hydrogenase. The rates of hydrogen evolution from NADPH by immobilized *Cl. butyricum, E. coli* and *D. salexigens* were 5.72, 0.55 and 0.16 μmol/h per g wet cells, respectively. *Cl. butyricum* uses ferredoxin and *E. coli* and *D. salexigens* use cytochrome *c* as electron carriers. *Cl. butyricum* and *D. salexigens* are strict anaerobes and *E. coli* is a facultative anaerobe. *Cl. butyricum* evolved the largest amount of hydrogen. *E. coli* and *D. salexigens* evolved only a trace amount of hydrogen. Electron transfer from NADPH to cytochrome *c* may be difficult. Therefore, immobilized *Cl. butyricum* was employed for further experiments.

NADP reduction by immobilized C. vulgaris

The rate of NADP reduction by immobilized *C. vulgaris* was examined. Immobilized *C. vulgaris* reduced NADP under illumination when it was preincubated in the dark. The rate of NADP reduction was 4.3 μmol/h per mg Chl.

Immobilized *C. vulgaris* can reduce NADP under illumination, and immobilized *Cl. butyricum* evolves hydrogen from NADPH. Consequently, light-driven splitting of water into hydrogen is possible by coupling immobilized *C. vulgaris* and *Cl. butyricum*.

Effect of electron carriers

As described above, an electron carrier is needed for hydrogen evolution using immobilized *C. vulgaris* and *Cl. butyricum*. The effect of electron carriers on hydrogen evolution was examined. NADP, NAD and FAD were employed as electron carriers. The rates of hydrogen evolution were 0.322, 0.174 and 0.048 μmol/h per g wet cells, respectively. On the other hand without an electron carrier, the rate of hydrogen evolution by the system was 0.046 μmol/h per g wet cells. The largest amount of hydrogen was evolved when NADP was used as an electron carrier. Preliminary experiments show that immobilized *Cl. butyricum* can evolve hydrogen from NADH and NADPH. However, the rate of NAD reduction by immobilized *C. vulgaris* was slower than that of NADP reduction. Therefore, the rate of hydrogen evolution was slow when NAD was used as an electron carrier. In contrast, immobilized *C. vulgaris* cannot reduce FAD. Thus, the immobilized *C. vulgaris*-FAD-*Cl. butyricum* system evolves only a trace amount of hydrogen like the system without electron carriers.

Fig. 1 shows the effect of NADP concentration on the rate of hydrogen evolution. The rate of hydrogen evolution increased with increasing NADP concentration.

Optimum reaction conditions for the immobilized C. vulgaris-Cl. butyricum system

The optimum temperature for hydrogen evolution from NADPH by immobilized *Cl. butyricum* was 37°C, and that for NADP reduction by immobilized *C. vulgaris* 30°C. Fig. 2 shows the effect of temperature on the rate of hydrogen evolution by the immobilized *C. vulgaris-Cl. butyricum* system. The rate of hydrogen evolution increased with increasing temperature up to 37°C.

Fig. 3 shows the effect of pH on hydrogen evolution by the immobilized *C. vulgaris-Cl. butyricum* system. The rate of hydrogen evolution was decreased below pH 7.0 and above pH 8.0. The optimum pH for hydrogen evolution was 7.0. Therefore, further experiments were performed at 37°C and pH 7.0.

The rate of hydrogen evolution increased with increasing $NaHCO_3$ concentration. A high concentration of $NaHCO_3$ accelated the NADP reduction by immobilized *C. vulgaris*. The optimal $NaHCO_3$ concentration was 10 mM.

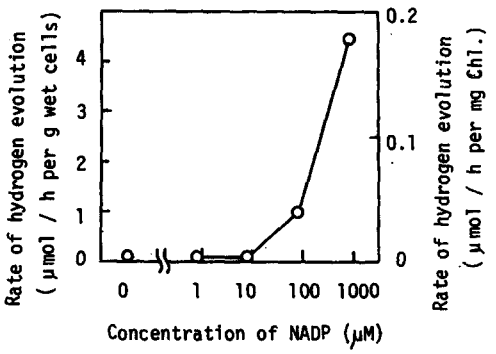

Fig. 1. Effect of NADP concentration on the hydrogen-evolution rate by the *C. vulgaris-Cl. butyricum* system. The *Cl. butyricum* content was 10 mg wet cells per g wet gel, and the *C. vulgaris* content 250 μg Chl per g wet gel. The reactions were carried out at pH 8.0 and 37°C under illumination (3000 lx).

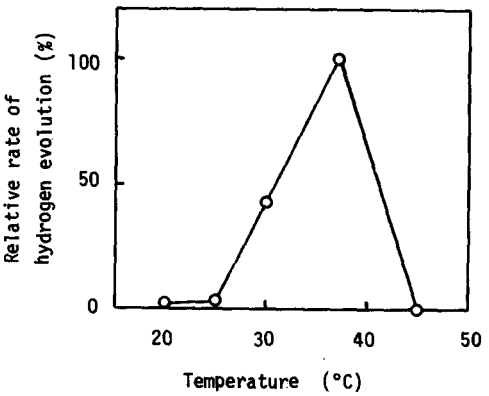

Fig. 2. Effect of temperature on the hydrogen-evolution rate. A reaction medium containing 800 μM NADP was employed. The reactions were carried out under the same conditions as those in Fig. 1 except for temperature.

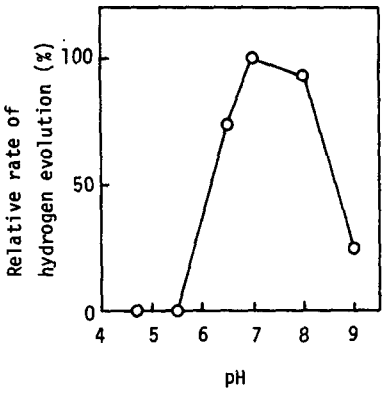

Fig. 3. Effect of pH on the hydrogen-evolution rate. The reactions were carried out at 37°C. Other conditions as in Fig. 2 except for pH.

Effect of cell concentrations

Fig. 4 depicts the relationship between the hydrogen-evolution rate and the cell concentration of *Cl. butyricum* when *C. vulgaris* (125 μg Chl) was immobilized in agar gel. The rate of hydrogen evolution increased with increasing cell concentration. Therefore, 200 mg wet cells per g wet gel of *Cl. butyricum* were immobilized in agar gel.

Fig. 5 shows the relationship between the hydrogen-evolution rate and the cell concentration of *C. vulgaris* when 200 mg wet cells per g wet gel of *Cl. butyricum* were immobilized in agar gel. The rate of hydrogen evolution also increased with increasing cell concentrations below 125 mg Chl per g wet gel. How-

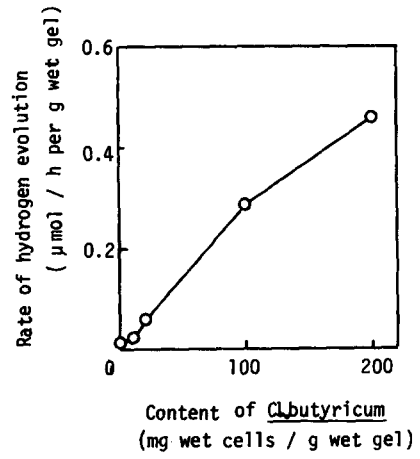

Fig. 4. Relationship between the *Cl. butyricum* content and hydrogen-evolution rate. The *C. vulgaris* content was 250 μg Chl per g wet gel. Hydrogen was evolved under illumination at the optimal conditions.

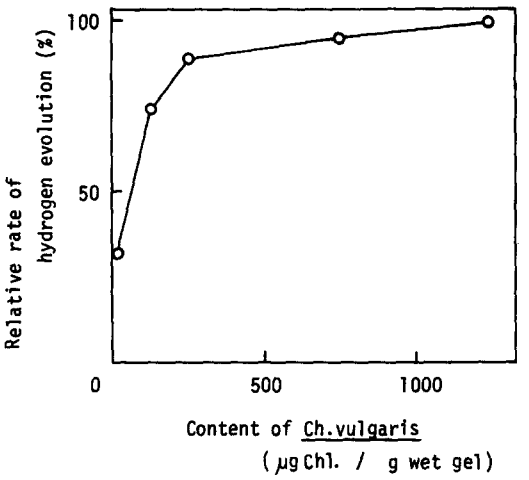

Fig. 5. Relationship between the *C. vulgaris* content and hydrogen-evolution rate. The *Cl. butyricum* content was 10 mg wet cells per g wet gel. The hydrogen-evolution reactions were carried out under the optimal conditions.

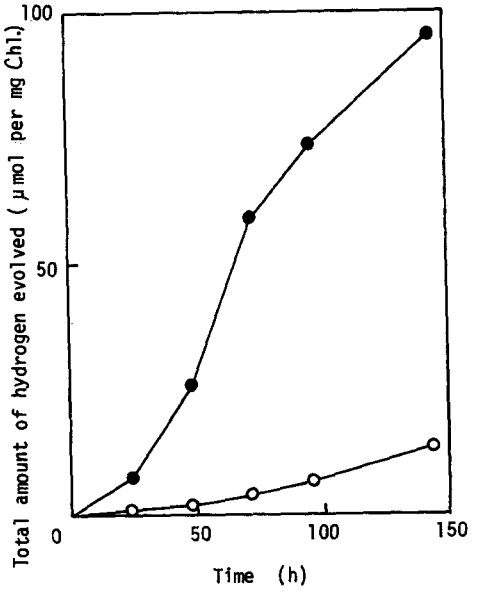

Fig. 6. Continual hydrogen evolution by the immobilized *C. vulgaris-Cl. butyricum* system under optimal conditions with 800 μM NADP (●——●) or without NADP (○——○). The *Cl. butyricum* content was 10 mg wet cells per g wet gel, and the *C. vulgaris* content 250 μg Chl per g wet gel.

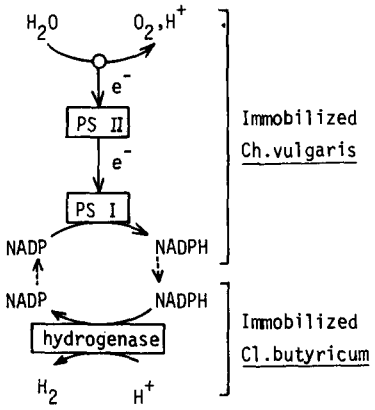

Fig. 7. Principle of hydrogen evolution by the immobilized *C. vulgaris-Cl. butyricum* system.

ever, it became constant above 250 mg Chl per g wet gel.

Continuous hydrogen evolution by the system

Fig. 6 shows the time course of hydrogen evolution by the immobilized *C. vulgaris*-NADP-immobilized *Cl. butyricum* and immobilized *C. vulgaris*-immobilized *Cl. butyricum* systems. The complete system continuously evolved hydrogen at a rate of 0.29–1.34 μmol/h per mg Chl for 6 days. On the other hand, the system without NADP evolved only a trace amount of hydrogen. Immobilized *C. vulgaris* also evolved only a trace amount of hydrogen. Hydrogen was not produced by immobilized *Cl. butyricum* because the reaction mixture did not contain any organic compounds.

Discussion

One problem of the coupling system using chloroplasts and hydrogenase is the instability of hydrogenase in the presence of oxygen. Benemann et al. [1] employed glucose and glucose oxidase as an oxygen scavenger system, and the amount of hydrogen evolved increased 7-fold. However, glucose is consumed with oxygen consumption. Rao et al. [7] linked ferredoxin to AH-Sepharose 4B and then bound hydrogenase on the immobilized ferredoxin. But this immobilized ferredoxin-hydrogenase system was not effective for water-splitting hydrogen evolution. The hydrogenase immobilized on glass beads was also inactivated by oxygen [8]. Recently, we found that hydrogenase in immobilized living cells was protected from the deleterious effects of oxygen [2,3]. Therefore, immobilized cells were employed for hydrogen evolution from NADPH. As a result, hydrogenase was not inactivated by the oxygen evolved by *C. vulgaris*.

Immobilized cells retained the initial hydrogenase activity after repeated usage for continual light-induced hydrogen evolution from water.

Another main problem of the water-splitting hydrogen-evolution system is the stability of the photosystem. Isolated chloroplasts lost 50% of the photoreduction activity after 15 min. The photoreduction activity of immobilized chloroplasts decreased to 30% of the initial activity after 2 h reaction [9]. In this study, immobilized whole cells of *C. vulgaris* were used for the photoreduction of NADP. Immobilized *C. vulgaris* retained the photoreduction activity of NADP for 6 days.

Fig. 7 shows the principle of the hydrogen-evolution system using immobilized *C. vulgaris* and *Cl. butyricum*. NADP is reduced by Photosystems I and II in *C. vulgaris* under irradiation with light. Hydrogen is evolved through oxidation of NADPH. Organic compounds were not added to the system as an electron donor. Furthermore, oxygen was evolved from immobilized cells of *C. vulgaris*. Therefore, most hydrogen was produced by splitting water with light irradiation.

The immobilized *C. vulgaris-Cl. butyricum* system evolved hydrogen at a rate of about 5 μmol/h per g wet cells (0.2 μmol/h per mg Chl). This value is comparable to that (from NADPH) of immobilized *Cl. butyricum*, and is less than the rate of NADP reduction by immobilized *C. vulgaris*. The rate of hydrogen evolution depends on the hydrogen-evolution rate from NADPH by immobilized *Cl. butyricum*. This is supported by the fact that the optimal temperature of the system is the same as that of immobilized *Cl. butyricum*. Furthermore, the rate of water-splitting hydrogen evolution by the system increased with increasing NADP concentration, and a high concentration of NADP was need for the system. Therefore, the hydrogen-evolution rate may be limited by the diffusion of NADPH into the hydrogenase system in *Cl. butyricum*.

References

1 Benemann, J.R., Berenson, J.A., Kaplan, N.O. and Kamen, M.D. (1973) Proc. Natl. Acad. Sci. U.S.A. 70, 2317–2320

2 Karube, I., Matsunaga, T., Tsuru, S. and Suzuki, S. (1977) Biotechnol. Bioeng. 19, 1727–1737

3 Suzuki, S., Karube, I. and Matsunaga, T. (1978) Biotechnol. Bioeng. Symp. No. 8, 501–511

4 Karube, I., Matsunaga, T., Otsuka, T., Kayano, H. and Suzuki, S. (1981) Biochim. Biophys. Acta 637, 490–495

5 Kanazawa, T., Fujita, C., Yuhara, T. and Sasa, T. (1958) J. Gen. Appl. Microbiol. 4, 135–152

6 Kayano, H., Karube, I., Matsunaga, T., Suzuki, S. and Nakayama, O. (1981) Eur. J. Appl. Microbiol. Biotechnol. 12, 1–5

7 Rao, K.K., Gogotov, I.N. and Hall, D.O. (1978) Biochimie 60, 291–296

8 Lappi, D.A., Stolzenbach, F.E., Caplan, N.O. and Kamen, M.D. (1976) Biochem. Biophys. Res. Commun. 69, 878–884

9 Karube, I., Otsuka, T., Kayano, H., Matsunaga, T. and Suzuki, S. (1980) Biotechnol. Bioeng. 22, 2655–2665