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Photoproduction of Hydrogen from Glucose by a Co-Culture of a Photosynthetic Bacterium and *Clostridium butyricum*

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Clostridium butyricum IFO-13949 and a photosynthetic bacterium, *Rhodopseudomonas* sp. RV were co-cultured to produce hydrogen from glucose with light irradiation.

C. butyricum converts glucose mainly to butyrate with evolution of hydrogen by hydrogenase. Butyrate was then converted to hydrogen by nitrogenase of *Rhodopseudomonas* sp. RV, newly isolated for the combination. Agar-immobilized co-culture of both bacteria evolved hydrogen at 7.0 mol per mol of glucose consumed in the light, and 1.1 mol in the dark. We estimate the contributions of hydrogenase and nitrogenase to hydrogen production were 16 and 84%, respectively.

Clostridium butyricum evolves hydrogen from carbohydrates at a high rate.[1,2] However, the yield is limited because it produces organic acids besides hydrogen. The following equations show the major reactions of hydrogen production from glucose in *C. butyricum*.[3]

$$\text{glucose} \longrightarrow 2\,H_2 + 2\,CO_2 + \text{butyrate} \quad (1)$$

$$\text{glucose} + 2\,H_2O \longrightarrow 4\,H_2 + 2\,CO_2 + 2\,\text{acetate} \quad (2)$$

Because photosynthetic bacteria produce hydrogen from organic acids with high yields under illumination,[4-6] a combination of *C. butyricum* and photosynthetic bacteria makes possible the efficient conversion of carbohydrates to hydrogen.

The combination is also interesting from the viewpoint of energy for hydrogen production. The hydrogenase of *C. butyricum* and nitrogenase of photosynthetic bacteria work cooperatively through the degradation of carbohydrates.

In this study, a photosynthetic bacterium capable of hydrogen production from butyrate was newly isolated for the combination with *C. butyricum*. An immobilized co-culture of the two kinds of bacteria produced hydrogen of 7.0 mol per mol glucose, 16% of which was mediated by hydrogenase.

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Materials and Methods

Bacterial strains and growth conditions A photosynthetic bacterium isolated from muddy water in the Tsukuba area was used. The bacterium was tentatively named *Rhodopseudomonas* sp. RV; it was a rod-like ellipsoid without a spiral and grew on propionate, mannitol, and sorbitol, but not on thiosulfate. Details of the isolation method will be reported elsewhere. *Clostridium butyricum* (IFO-13949) was supplied from the Institute for Fermentation Osaka.

Basal medium of 1 *l* contained 866 mg of KH_2PO_4, 733 mg of K_2HPO_4, 200 mg of $MgSO_4 \cdot 7H_2O$, 75 mg of $CaCl_2$, 20 mg of EDTA (disodium salt), 11.8 mg of $FeSO_4 \cdot 7H_2O$, 2.8 mg of H_3BO_3, 2.1 mg of $MnSO_4 \cdot 4H_2O$, 750 μg of $Na_2MoO_4 \cdot 2H_2O$, 240 μg of $ZnSO_4 \cdot 7H_2O$, 40 μg of $Cu(NO_3)_2 \cdot 3H_2O$, and 0.15 μg each of biotin, thiamin, *p*-aminobenzoate, nicotinate, and nicotinamide. All culture media were prepared by adding carbon and nitrogen sources to the basal medium. Culture medium for *Rhodopseudomonas* sp. RV contained the basal medium, 10 mM ammonium sulfate, 50 mM butyrate, 0.1% $NaHCO_3$, and 0.1% yeast extract (pH 7). Culture medium for *C. butyricum* contained the basal medium, 50 mM glucose, 0.2% meat extract, 0.4% peptone, and 0.4% yeast extract (pH 7). Pre-culture medium contained the basal medium, 10 mM sodium glutamate, 20-50 mM glucose, 0.01% meat extract, 0.02% peptone, 0.02% yeast extract, and 0.1 M sodium phosphate buffer (pH 8). Co-culture medium contained the basal medium, 2.5 mM sodium glutamate, 28.8 mM glucose, and

0.1 M sodium phosphate buffer (pH 8). *Rhodopseudomonas* sp. RV was cultured anaerobically under illumination of 1 klux by tungsten lamps at 30°C. *C. butyricum* was cultured in an anaerobic box (Forma Scientific Co.) at 32°C.

Hydrogen evolution by *Rhodopseudomonas* sp. RV from several substrates Hydrogen evolution by *Rhodopseudomonas* sp. RV in suspension culture was examined by the method previously described.[6,7] For hydrogen production by immobilized cells, a cell suspension of 4 ml (cells of *ca.* 4 mg in dry weight) was mixed with agar solution of 6 ml at 45°C (final agar concentration was 2% and a gel volume of 10 ml), and poured into a flat tissue culture flask of 24 cm^2 (flask volume 70 ml). Cells were grown in the gel for about 4 days under an illumination of 1 klux on the medium containing the basal medium, *ca.* 50 mM carbon source, 10 mM ammonium sulfate, and 0.1% yeast extract. For the hydrogen evolution experiment, the medium was changed to remove ammonia and yeast extract, *i.e.* the new medium contained the basal medium, 16–53 mM carbon source and 10 mM sodium glutamate. The same carbon source was used both for the cell growth and the hydrogen evolution experiment. Exceptions were the tests of glucose and glutamate, in which cases cells were grown on lactate.

Co-immobilization and hydrogen production by *Rhodopseudomonas* sp. RV and *C. butyricum* Suspension of the cells of *Rhodopseudomonas* sp. RV of 10 ml (dry weight *ca.* 10 mg) and that of *C. butyricum* of 3 ml (dry weight *ca.* 2 mg) were mixed with 27 ml of agar solution of 3% at 45°C (Agar was dissolved in the pre-culture medium.). A gel was made in a flat tissue culture flask of 200 ml (gel size; irradiated area of 76 cm^2 and thickness of 0.53 cm). Cells in the gel were grown for 48 h using the pre-culture medium with an illumination of 1 klux at 30°C. For the hydrogen production experiment, the medium was changed to the co-culture medium and the gel was illuminated at 10 klux.

Assay methods Nitrogenase activity was measured by the acetylene reduction method as described previously.[7] Protein was assayed by Lowry's method[8] using bovine serum albumin as standard. Malate, lactate, and butyrate were measured by liquid chromatography using a Shimadzu LC-5A Liquid Chromatograph equipped with a Zorbax ODS column of 4.6×150 mm. Glucose and acetate were assayed enzymatically using kits from Boehringer Mannheim. Other organic acids, alcohols, and acetone were measured by gas chromatography using a Shimadzu Gas Chromatograph GC-8A with a 2 m Tenax GC column at 180°C.

Results

Nitrogenase activity The relationships between hydrogen evolution and nitrogenase activities of *Rhodopseudomonas* sp. RV and *C. butyricum* are shown in Table 1. No significant activity of nitrogenase was observed in *C. butyricum* in contrast to the high one of *Rhodopseudomonas* sp. RV. As hydrogen production was completely repressed by ammonium ion in *Rhodopseudomonas* sp. RV, hydrogen production of the bacteria is thought to be mediated by nitrogenase alone.

Hydrogen evolution by *Rhodopseudomonas* sp. RV *C. butyricum,* strain IFO-

Table 1. Relationship between hydrogen evolution and nitrogenase activity of *Rhodopseudomonas* sp. RV and *C. butyricum.*

Bacteria	Electron donor	Nitrogen source*	H_2 evolution (μ mol/h/mg cells)	Ethylene formation (μ mol/h/mg cells)
C. butyricum	Glucose	Glutamate	7.0	0.03
Rhodopseudomonas sp. RV	Malate	Glutamate	3.8	2.8
		Glutamate+NH_4^+**	0	nm

Hydrogen production and ethylene formation were measured separately at the same time using the aliquots of the same culture. Tests were done at 40 h after inoculation in *Rhodopseudomonas* sp. RV and 7 h in *C. butyricum.*

* 10 mM each.

** Ammonium sulfate was added to the culture which contained glutamate and was producing hydrogen. Hydrogen production ceased completely within 2.5 h after the addition of ammonia.

nm, not measured.

Table 2. Anaerobic conversion of glucose by immobilized cells of *C. butyricum*.

Product	mol of product per mol of glucose consumed
Formate	n
Acetate	0.38
Lactate	0.16
Propionate	n
Butyrate	0.59
Acetone	n
Alcohols*	n

17.5 mmol of glucose in a 750 ml flask was converted by immobilized *C. butyricum* (Agar volume of 40 cm^3). Products were assayed after glucose was completely consumed.
* methanol, ethanol, propanol, and butanol.
n, trace or not detectable.

13949, converted glucose to organic acids such as acetate, lactate, and butyrate (Table 2). Of the acids, butyrate was the major product.

Hydrogen production by photosynthetic bacteria from butyrate has not been well investigated except for the works of Stevens *et al.*[9] and Sigers *et al.*[10] In this study, we newly isolated a photosynthetic bacterium that produces hydrogen from butyrate. The isolated bacterium, *Rhodopseudomonas* sp. RV, evolved hydrogen from organic acids but little from glucose (Table 3). Yields of hydrogen production was examined from lactate, acetate, and butyrate with immobilized cells (Table 4).

Table 3. Hydrogen evolution by *Rhodopseudomonas* sp. RV from several substrates.

Substrate	Maximum H_2 evolution rate ($\mu l/cm^2/h$)*	Cell concn.** (mg/ml)
D,L-Malate	188	1.6
Acetate	42	1.5
D,L-Lactate	259	2.8
Butyrate	57	3.4
D-Glucose	2.6***	0.6
L-Glutamate	0	nm

Light intensity was 10 klux. Initial cell concentration was *ca.* 0.5 mg(dry)/ml. Maximum hydrogen evolution rates were observed between 16 and 21 h after the inoculation.
* For the comparison with the hydrogen evolution rate by immobilized cells, the rate was measured per irradiated area.
** Cell concentration was measured 21 h after the inoculation except for the case of hydrogen evolution from glucose, in which it was measured at 45 h.
*** The value is the average rate (0 to 45 h) because the rate was too low to measure its maximum.
nm, not measured.

Hydrogen production by a co-culture of *C. butyricum* and *Rhodopseudomonas* sp. RV *C. butyricum* and *Rhodopseudomonas* sp. RV were cultured in an agar gel to protect the bacteria from the attack of oxygen. They were pre-cultured for two days because the photosynthetic bacterium did not evolve hydrogen just after the immobilization probably due to the effects of the exposure to

Table 4. Hydrogen evolution by agar-immobilized cells of *Rhodopseudomonas* sp. RV.

Substrate		H_2 evolution		Yield* (%)	Cell density** (mg/g gel)
	initial concn.*** (mM)	max. rate ($\mu l/cm^2/h$)	total amount (ml/bottle)		
Acetate	16.3	37	117	40.2	7.3
D,L-Lactate	52.5	145	251	45.6	6.4
Butyrate	46.2	205	604	75.1	9.4

Bottles had 70 ml capacity (irradiated area of 24 cm^2) illuminated at 10 klux.
* Yields were calculated according to the following equations.
Acetate ; $C_2H_4O_2+2H_2O=4H_2+2CO_2$
Lactate ; $C_3H_6O_3+3H_2O=6H_2+3CO_2$
Butyrate ; $C_4H_8O_2+6H_2O=10H_2+4CO_2$
** Final cell densities were measured by protein concentration using a ratio of protein/dry weight=0.64.
*** Every substrate was consumed completely at the end of the experiment (285 h).

oxygen or high temperature in the process of immobilization. The concentrations of yeast extract, peptone, and meat extract were set low in the pre-culture medium to prevent the fast growth of *C. butyricum* and the repression of nitrogenase synthesis in *Rhodopseudomonas* sp. RV. When the pre-culture was finished, glucose in the medium was completely consumed and organic acids were formed by *C. butyricum*. For the experiment of hydrogen production, the medium in the bottle was changed to the co-culture medium, which contained no carbon source except for glucose. However, the medium in the gel was not exchanged and some organic acids remained in the gel as shown in Table 5.

Time courses of hydrogen production and glucose consumption by the co-culture are shown in Fig. 1. Per bottle, 958 ml (38.5 mmol) of hydrogen was obtained by the co-culture in 324 h. Although hydrogen was produced mostly from glucose, organic acids remaining in the gel (See Table 5) were also substrates of hydrogen production by *Rhodopseudomonas* sp. RV. To know the net amount of hydrogen produced from glucose, the contribution of organic acids should be eliminated. The calculation is as follows.

Substrate (mmol)		mol H_2/mol substrate		Yield		H_2 (mmol)
Acetate:	0.51	× 4	×	0.40	=	0.82
Lactate:	0.22	× 6	×	0.46	=	0.61
Butyrate:	0.64	× 10	×	0.75	=	4.8
total						6.23

Hydrogen from glucose: 38.5–6.23=32.3 mmol (803 ml)

It was found that 32.3 mmol of hydrogen was produced from 4.6 mmol of glucose by the co-culture, *i.e.* 7.0 mol of hydrogen per mol of glucose.

The contribution of hydrogenase to the production of hydrogen of the co-culture was examined. *C. butyricum* and *Rhodopseudomonas* sp. RV were co-immobilized and pre-cultured under illumination in the manner described above. But the co-culture was kept in the dark after the exchange of the medium to the co-culture medium. In the dark 1.1 mol of hydrogen were produced per mol of glucose by a co-culture. Immobilized

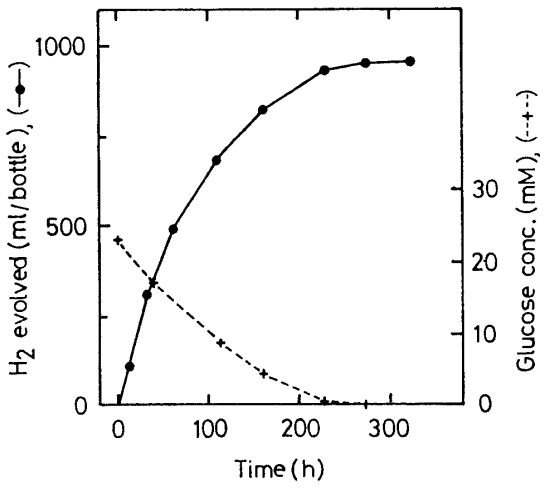

Fig. 1. Hydrogen production from glucose by the co-culture of *Rhodopseudomonas* sp. RV and *C. butyricum*.
The co-immobilized cells in a gel were pre-grown for 2 days under illumination and the medium was changed to the co-culture medium. Hydrogen evolution was measured under illumination of 10 klux. Initial glucose amount was 4.6 mol per bottle of 200 ml. Initial and final pH values were 8.0 and 6.95, respectively. Protein concentration in the gel was unchanged during the experiment (2.2 mg/g gel).

Table 5. Conversion of glucose and organic acids to hydrogen by the co-culture of *C. butyricum* and *Rhodopseudomonas* sp. RV under illumination.

Substrate/ Product	Amount per bottle* (mmol) initial	final**	
Substrates			
Glucose	4.6	0	
Acetate	0.51	0	
Lactate	0.22	0	
Butyrate	0.64	0	
Product			
H_2	0	38.5	(958 ml)

Of the substrates, organic acids were produced during the pre-culture. After the pre-culture, organic acids in the pre-culture medium were assayed to estimate the amount in the gel assuming that the concentrations in and out of the gel were equal. The volume of hydrogen converted from the organic acids were presumed to be 154 ml (See "Results").

* A 200-ml bottle was used.

** After the culture of 324 h.

C. butyricum also produced 1.1 mol hydrogen per mol of glucose. Therefore, of the hydrogen evolved by the co-culture under illumination, 16% was produced by hydrogenase of *C. butyricum*.

Discussion

We presented here a system for the hydrogen photoproduction from glucose by a co-culture of *C. butyricum* and *Rhodopseudomonas* sp. RV. The two-step hydrogen production *via* butyrate showed a high efficiency, *i.e.*, 7.0 mol of hydrogen from a mole of glucose.

This combination of bacteria was unique because of the combination of hydrogenase and nitrogenase. So far, co-culture of *Klebsiella pneumoniae* and *Rhodospirillum rubrum*[11] and that of *Cellulomonas* and *Rhodopseudomonas capsulata*[12] have been reported for the conversion of carbohydrates to hydrogen. Hydrogen production is mediated by nitrogenase in *K. pneumoniae*[13] and photosynthetic bacteria.[14] *Cellulomonas* did not evolve hydrogen.[12] Hence, hydrogen was produced by nitrogenase in the combinations in the literatures.

The high energy cost of nitrogenase limits the rate and the yield in hydrogen production.[15] However, in photosynthetic bacteria, as the reaction of nitrogenase is driven by light energy, energetically unfavorable reactions, such as butyrate or acetate to hydrogen, could be performed ($C_4H_8O_{2\ liq}+6H_2O_{\ liq}\rightarrow 4CO_{2\ gas}+10H_{2\ gas}$ $\Delta G^\circ=223.3$ kJ, $C_2H_4O_{2\ liq}+2H_2O_{\ liq}\rightarrow 2CO_{2\ gas}+4H_{2\ gas}$ $\Delta G^\circ=75.2$ kJ).[16] On the other hand, hydrogenase catalyzes hydrogen formation in the reaction of *C. butyricum* (Eqs. (1) and (2)) without any energy supply. Therefore, the combination of *C. butyricum* and *Rhodopseudomonas* sp. RV enabled a degradation of glucose to hydrogen with a rational use of hydrogenase and nitrogenase from the viewpoint of energy. In this study, hydrogenase of *C. butyricum* mediated 16% of the hydrogen production from glucose. Enhancement of the contribution of hydrogenase could be expected if the degradation of glucose proceeded *via* acetate.

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