0273-2289/98/70–72—0301$10.50

Improvement of Substrate Conversion to Molecular Hydrogen by Three-Stage Cultivation of a Photosynthetic Bacterium, *Rhodovulum sulfidophilum*

ISAMU MAEDA,[1] WASIMUL Q. CHOWDHURY,[1] KENJI IDEHARA,[1] KIYOHITO YAGI,*[,1] TADASHI MIZOGUCHI,[1] TORU AKANO,[2] HITOSHI MIYASAKA,[2] TOSHIO FURUTANI,[2] YOSHIAKI IKUTA,[3] NORIO SHIOJI,[3] AND YOSHIHARU MIURA[2]

[1]*Faculty of Pharmaceutical Sciences, Osaka University, Suita, Osaka 565;* [2]*Kansai Electric Power Company, Amagasaki, Hyogo 661; and* [3]*Mitsubishi Heavy Industries, Takasago, Hyogo 676, Japan*

ABSTRACT

In photosynthetic bacteria, after transition to light-anaerobic and nitrogen-deficient conditions, hydrogen evolution starts with expression of nitrogenase activity. Until the expression of enough activity, *Rhodovulum sulfidophilum* consumed substrates and converted them to poly(3-hydroxybutyrate) (PHB), resulting in a decrease in the proportion of substrate converted into hydrogen gas. To prevent conversion to PHB during the period when nitrogenase activity is derepressed, the authors employed a cultivation method consisting of three stages: cell growth, nitrogenase derepression, and hydrogen production. Cells cultivated by this method exhibited no lag time before the commencement of hydrogen evolution and gave an improved yield of hydrogen from the algal fermentative products.

Index Entries: Hydrogen production; poly(3-hydroxybutyrate) (PHB) accumulation; nitrogenase derepression; ethanol; algal fermentative products.

INTRODUCTION

Phototrophic purple bacteria have been studied extensively because of their metabolic variety and their possible biotechnological applications.

* Author to whom all correspondence and reprint requests should be addressed. E-mail yagi@phs.osaka-u.ac.jp.

They cannot utilize water as an electron donor, but, instead, utilize low-mol-wt organic compounds, and reduced sulfur under light illumination *(1)*. They also have the potential to evolve hydrogen hydrogen gas under the catalysis of nitrogenase in the light. Many scientists have proposed applying this property of purple bacteria to the production of hydrogen gas for use as a clean energy source *(2)*.

The authors have proposed a method in which photosynthetic bacteria produce hydrogen gas from organic substrates photosynthetically produced from carbon dioxide and water by algae *(3)*. Acetic acid, ethanol, glycerol, and trace amounts of organic acids were excreted into a minimal medium after anaerobic treatment of the green alga *Chlamydomonas* sp. MGA161, which accumulated starch *(4,5)*. A photosynthetic bacterium, *Rhodovulum sulfidophilum* strain W-1S, continuously produced molecular hydrogen for at least 7 d from an algal fermentative broth, used as a culture supernatant after anaerobic incubation of the green alga in the dark *(3)*. However, in a daily cycle of cell resuspension into newly prepared algal fermentative broth, the hydrogen evolution rate in the first few days was less than that in the subsequent days, resulting in a drop in the efficiency of substrate conversion to hydrogen gas.

The present study examines hydrogen evolution by the phototroph *R. sulfidophilum* strain W-1S from an algal fermentative broth and the individual organic compounds that constituted the fermentative product. The results indicate that the decrease in the efficiency of hydrogen evolution from the substrates was caused by an accumulation of poly(3-hydroxybutyrate) (PHB) that promptly occurred until the beginning of hydrogen evolution. Improvement of the method of cultivating strain W-1S resolved this problem, and enhanced the production of hydrogen gas.

METHODS

Strain Origin

Strain W-1S was isolated by enrichment culture of marine samples collected near the coast in the Kinki region of Japan *(3)*. Although W-1S has previously been characterized as a strain of the genus *Rhodopseudomonas (6–8)*, nucleotide sequence data of its 16S rRNA amplified by the PCR (LA PCR kit; Takara Shuzo, Shiga, Japan) have established that the isolate is actually a strain of *R. sulfidophilum*. The strain has been deposited at FERM (Fermentation Research Institute, Ibaraki, Japan) under accession number FERM P-15320.

Cultivation Conditions

Strain W-1S was cultured in modified Okamoto medium (MOM) *(4)*, with the omission of vitamin B_{12} and the addition of 3% NaCl, 400 μg vitamin B_1/L, 500 μg nicotinic acid/L, 300 μg *p*-aminobenzoic acid/L, and

50 μg biotin/L. Pyruvate, succinate, malate, and acetate were simultaneously added as substrates for photoheterotrophic growth, each at a concentration of 0.1%. Cells were grown at 30°C under illumination from incandescent lamps at 140 W m^{-2}. Cultivation was done in a 1.6-L Roux bottle containing 1.0 L of the medium, with gentle agitation by a magnetic stirrer.

Experimental Procedures

Cells in the logarithmic growth phase were harvested and washed once or twice with the carbon- and nitrogen-free (CN-free) medium used for cultivation. The cells were then resuspended in the CN-free medium or an algal fermentation broth. After resuspension into the CN-free medium, the organic substrate used for each experiment was added. To prepare the algal fermentation broth, cells of *Chlamydomonas* sp. MGA161 were incubated under dark-anaerobic conditions in the CN-free medium *(9)*. Incubation for hydrogen evolution was carried out in vessels sparged with argon at 30°C under 55-W m^{-2} illumination.

Assay of Hydrogen Evolution, and Measurement of Nitrogenase Activity and Polysaccharide Content

The hydrogen produced was measured by a gas chromatograph (model-164; Hitachi, Tokyo). The column was filled with a molecular sieve 13X, 30/60 mesh (Gasukuro Kogyo, Tokyo). Nitrogenase activity was measured by the acetylene reduction method, as described previously *(6)*.

PHB Extraction and Analysis

PHB extracted by chloroform, as described previously *(8)*. Extracts were identified by the 200-MHz ^1H NMR spectra (VXR-200; Varian, Palo Alto, CA). The polymer extracted from strain W-1S was shown to be the homopolymer PHB by the NMR analysis *(8)*.

Conversion of the substrate into PHB was quantitated by the radioactivity of the chloroform extract from cells incubated in the presence of [1-^{14}C]acetate (Du Pont/NEN, Wilmington, DE). The radioactivities of the chloroform extract and [^{14}C]acetate remaining in the medium were determined by scintillation counting (Tri-Carb 2100TR; Packard, Meriden, CT). After chloroform was completely evaporated, the polymer in the extract was hydrolyzed by boiling in 10 *N* NaOH for 1 h for HPLC analysis. After adequate dilution, the hydrolysate was subjected to HPLC (model 302; Gilson, France) on a TSK-GEL column (Tosoh, Tokyo), and the radioactivities of the elutes were determined by scintillation counting (LS 3801, Beckman, Palo Alto, CA). A peak of 3-hydroxybutyric acid was identified by using the alkaline-hydrolysate of natural-origin PHB (Aldrich, Milwaukee, WI) as a standard.

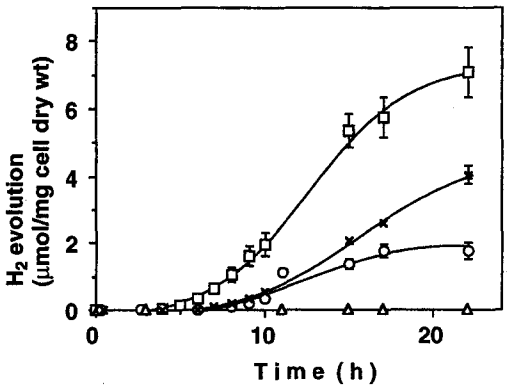

Fig. 1. Hydrogen evolution from the main organic compounds excreted during the algal fermentation. Succinate was used as a control. Each substrate was added at a concentration of 1 mM to carbon- and nitrogen-free minimal medium. △, ethanol; ○, acetate; ×, glycerol; □, succinate.

RESULTS

Hydrogen Evolution from Algal Fermentative Products

Because acetic acid, ethanol, and glycerol are the principal products excreted into the medium during anaerobic cultivation in the dark of the green alga *Chlamydomonas* sp. MGA161, the characteristics of each of these organic compounds as a substrate for hydrogen evolution in strain W-1S were assessed (Fig. 1). Hydrogen evolution was investigated during incubation of W-1S suspended in the CN-free minimal medium. Hydrogen evolution from succinate was employed as a positive control. Slower rates of hydrogen evolution and longer lag times were observed during the incubations with acetate and glycerol, compared to that with succinate; in the presence of ethanol, no hydrogen gas was detected during the incubation period. The conversion efficiencies of succinate, acetate, and glycerol to hydrogen gas were 44.1, 19.3, and 25.1%, respectively at 22 h. The uptake rates of these organic compounds in hydrogen evolving incubation were next investigated (Fig. 2). The culture of strain W-1S could consume acetate, ethanol, and glycerol immediately after transition to the hydrogen evolving condition. The uptake of acetate added to the medium was almost completed within 1 h. There was no proportional relationship between the conversion efficiency and the uptake rate. The rates of uptake of ethanol and glycerol were much lower than that of acetate.

PHB Accumulation Under the Condition for Hydrogen Evolution

The effect of incubation under the hydrogen evolving condition on the PHB content was investigated in the presence of ethanol as an external substrate (Table 1). After sufficient growth, cells accumulated PHB to the

Table 1
Conversion of Ethanol to PHB During Incubation for Hydrogen Evolution

Condition	Initial PHB mg	Initial PHB % of cell dry wt	Final PHB mg	Final PHB % of cell dry wt	PHB accumulation[a] mg	H_2 evolution[a] μmol/mg cell dry wt
Succinate[b]	52.7	44.7	29.0	29.1	−23.7	34.3
Ethanol[b]	52.7	44.7	61.5	51.5	8.8	0.46
No substrate[c]	52.7	44.7	13.0	15.4	−39.7	23.8

[a] Incubation was continued for 43 h.
[b] Each substrate was added at a concentration of 1 m*M*.
[c] No external substrate was added.

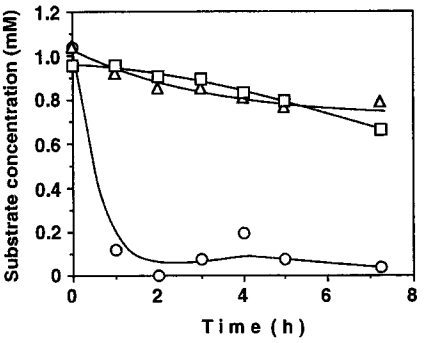

Fig. 2. Consumption of substrates in hydrogen evolving incubation in the presence of each product of algal fermentation. ○, acetate; △, ethanol; □, glycerol.

extent of 44.7% of the cell dry wt. The PHB content increased to 51.5% after the incubation for hydrogen evolution ended, as consequences of ethanol uptake and the absence of hydrogen evolution. On the other hand, extensive PHB degradation and hydrogen evolution occurred during the incubation with succinate, which is one of the substrates suitable for hydrogen evolution with strain W-1S. Similar results were obtained in the absence of an external substrate. A reciprocal relationship was observed between PHB accumulation and hydrogen evolution. To analyze this relationship further, the uptake of [^{14}C]acetate into PHB was examined under the hydrogen evolving condition. Cells were incubated anaerobically in the presence of [^{14}C]acetate, and PHB was extracted by chloroform, as described in Methods (Table 2). Cells having no nitrogenase activity incorporated about 70% of the count into the PHB fraction within 3 h. The labeled PHB fraction was hydrolyzed by alkaline-heat treatment, and the hydrolysate was analyzed by HPLC with scintillation counting, to confirm that the [^{14}C]acetate was actually incorporated into PHB. About 84% radioactivity was detected in a 3-hydroxybutyrate fraction, indicating that al-

Table 2
Effect of Cellular Condition Regarding Nitrogenase on Hydrogen Evolution and PHB Accumulation from Acetate

	0 h	1 h	3 h	6 h
Before derepression of nitrogenase[a]				
H_2 evolution (μmol/200 mL batch)	0	N.T.[d]	0	Trace
PHB[c] (× 10^6 CPM/200 mL batch)	1.0	N.T.[d]	18.7 ± 8.8	18.2 ± 3.9
Supernatant[c] (× 10^6 CPM/200 mL batch)	26.8 ± 0.6	N.T.[d]	4.3 ± 0.5	4.1 ± 0.3
After derepression of nitrogenase[a,b]				
H_2 evolution (μmol/200 mL batch)	0	54.4	87.5	N.T.[d]
PHB[c] (× 10^6 CPM/200 mL batch)	1.0	1.3 ± 0.1	1.2 ± 0.2	N.T.[d]
Supernatant[c] (× 10^6 CPM/200 mL batch)	26.8 ± 0.6	1.8 ± 0.1	7.1 ± 0.1	N.T.[d]

[a] Nitrogenase activity was measured in a sample anaerobically taken at 0 h before addition of [^{14}C]acetate. The enzyme activities of cells before and after derepression were 0 and 1087 ± 126 nmol/mg cell dry wt/h, respectively.

[b] Cells after growth were incubated in the carbon- and nitrogen-free minimal medium supplemented with 1 m*M* succinate, and, 1 d later, [^{14}C]acetate was injected anaerobically.

[c] Radioactivity values are means ± SD.

[d] N.T., not tested.

most all the count incorporated was derived from PHB. Cells incubated anaerobically with succinate for 1 d had high nitrogenase activity. After the addition of [^{14}C]acetate, hydrogen evolution was observed, but no significant incorporation of the count into PHB occurred, in spite of an immediate decrease of [^{14}C]acetate in the supernatant. Therefore, the fate of the incorporated acetate could be dependent on the nitrogenase activity.

Improvement of Conversion of Substrates to Hydrogen Gas

When a three-stage cultivation method, composed of cell growth, nitrogenase derepression, and hydrogen production from the substrate, was applied to a culture for hydrogen evolution from the algal fermentative broth (Fig. 3), no lag time was observed before hydrogen evolution began, regardless of contact with oxygen during the resuspension of cells into the broth (Fig. 4). The conversion efficiency of the substrate to hydrogen gas over a period of 1 d was improved to 60.8% in this three-stage cultivation method, compared with 29.4% in a culture that lacked a stage for nitrogenase derepression. An anaerobic atmosphere in the nitrogenase derepres-

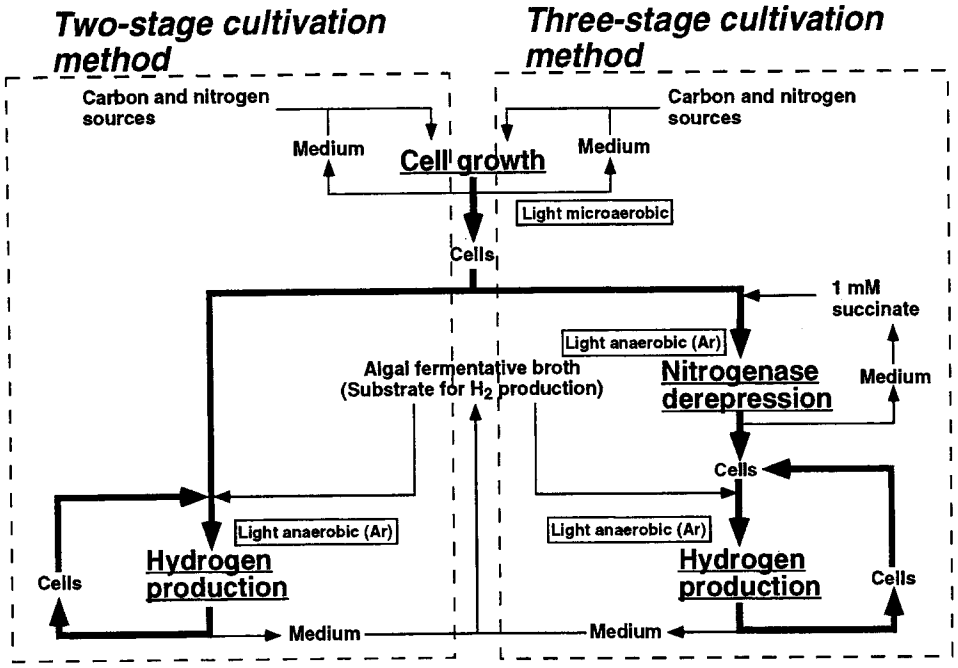

Fig. 3. Schematic diagram of the three-stage cultivation method for hydrogen evolution.

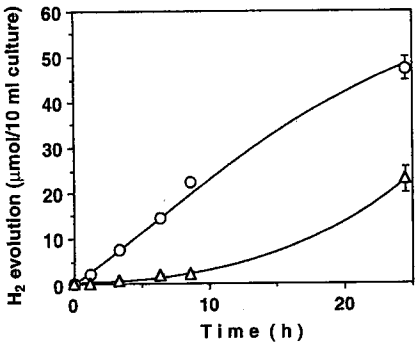

Fig. 4. Effect of preincubation for nitrogenase derepression on hydrogen evolution from the algal fermentative broth. Cells were suspended in the broth after growth (△) or after the derepression of nitrogenase by preincubation with 1 mM succinate (○).

sion and hydrogen evolution stages was achieved by filling the incubation vessel with argon gas. The uptake of substrates during incubation for hydrogen evolution was accelerated in a culture employing three-stage cultivation, compared to that in a culture in which the substrates were supplied after cell growth (Table 3). When the strain W-1S was used for a hydrogen evolution test after growth, it could not convert ethanol to hydrogen gas because of PHB accumulation (Table 1). To establish whether or not the

Table 3
Effect of Preincubation for Nitrogenase Derepression on Uptake of Substrates

		Substrate concentration (m*M*)	
		Before depression of nitrogenase[a]	After depression of nitrogenase[a]
Acetate	0 h	5.64	5.64
	21 h	1.85 ± 0.03	0.45 ± 0.18
	Consumption	3.79	5.19
Ethanol	0 h	4.58	4.58
	21 h	4.23 ± 0.18	3.86 ± 0.17
	Consumption	0.35	0.72
Glycerol	0 h	5.05	5.05
	21 h	4.63 ± 0.11	3.87 ± 0.09
	Consumption	0.42	1.18

[a] Cells were suspended in the medium containing acetate, ethanol, and glycerol after growth or after the derepression of nitrogenase by preincubation with 1 m*M* succinate.

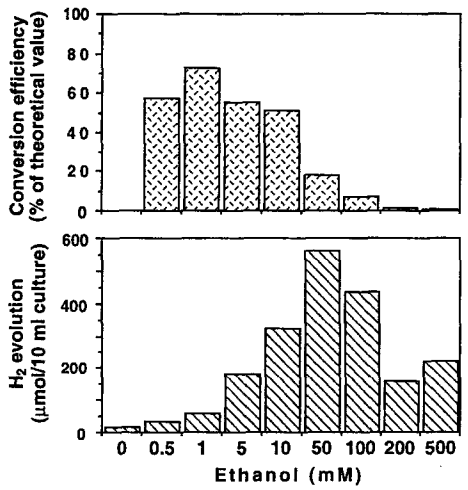

Fig. 5. Conversion of ethanol at various concentrations to hydrogen gas when supplied to the culture after the nitrogenase derepression stage.

three-stage cultivation method for hydrogen evolution was effective with ethanol as a substrate, the conversion of ethanol at various concentrations to hydrogen gas was investigated in cultures to which adequate quantities of ethanol were anaerobically added after the nitrogenase derepression stage (Fig. 5). Although the largest quantity of hydrogen gas was evolved at an ethanol concentration of 50 m*M*, the conversion efficiency deteriorated markedly at concentrations of 50 m*M* or more. Using the three-stage cultivation method, more than 50% of ethanol at a concentration of 10 m*M* could be converted into hydrogen gas.

DISCUSSION

The cultivation of photosynthetic bacteria for H production can be categorized into two basic methods; in one, the cell growth and H production are carried out concomitantly; in the other, the H production period is independent of cell growth. Although the former method is advantageous because of its simplicity, glutamate must be added to the cultivation medium as a nitrogen source *(10,11)*. Hence, the latter method is more commonly employed in H production by photosynthetic bacteria *(12,13)*, including the culture of immobilized cells *(14,15)*. The authors have investigated hydrogen production, using the fermentative broth of a marine green alga as a H-donor, of the marine photosynthetic bacterium *R. sulfidophilum* strain W-1S *(3)*. Using the above cultivation methods, however, strain W-1S could not evolve H gas with high efficiency from the fermentative products represented by acetate, ethanol, and glycerol. The rate of acetate uptake was much greater than that of ethanol or glycerol, and uptake of acetate, along with the conversion of PHB, started promptly before occurrence of H evolution. On the other hand, H evolution from fermentative products was improved in cells exposed to a nitrogenase derepression stage. This suggests that the rate-limiting stage in H evolution from fermentative products is the derepression of enzyme activity. In a period of cell growth, nitrogenase activity is repressed by ammonium ion and dissolved oxygen in the medium *(1,2)*. The purpose of the three-stage cultivation is to derepress nitrogenase sufficiently to allow H to be produced promptly after cell growth.

These results showed that the introduction of a period of nitrogenase derepression after cell growth is essential for the efficient evolution of H gas from fermentative products. Without such a period, a lag time and a low conversion efficiency to H was observed in the case of acetate, and only a small amount of H was evolved from ethanol. Competition between H evolution and PHB accumulation for the consumption of reducing equivalents has been suggested in *Rhodopseudomonas palustris (16)*, *Rhodobacter sphaeroides*, and *Rhodospirillum rubrum (17)*. As suggested previously for photosynthetic bacteria, with strain W-1S, the absence of nitrogenase activity and the occurrence of substrate uptake immediately after cell growth facilitated PHB accumulation from the substrates, resulting in a decrease of the ratio of conversion into H gas. Improvement of the conversion efficiency by the three-stage cultivation method is not only the result of a reduction of the substrates left in the medium, but also because of prevention of the conversion of substrates into PHB. This cultivation method for photosynthetic bacteria described here should enable the use of a broader range of H substrates that are not suitable at present for conversion into H gas by the bacteria.

REFERENCES

1. Vignais, P. M., Colbeau, A., Willison, J. C., and Jouanneau, Y. (1985), *Adv. Microb. Physiol.* **26**, 155–234.

2. Sasikara, K., Ramana, C. V., Raghuveer Rao, P., and Kovacs, K. L. (1993), *Advan. Appl. Microbiol.* **38,** 211–295.
3. Miura, Y., Saitoh, C., Matsuoka, S., and Miyamoto, K. (1992), *Biosci. Biotech. Biochem.* **56,** 751–754.
4. Miura, Y., Ohta, S., Mano, M., and Miyamoto, K. (1986). *Agric. Biol. Chem.* **50,** 2837–2844.
5. Maeda, I., Miyashiro, M., Hikawa, H., Yagi, K., Miura, Y., and Mizoguchi, T. (1996), *Biosci. Biotech. Biochem.* **60,** 975–978.
6. Yagi, K., Maeda, I., Idehara, K., Miura, Y., Akano, T., Fukatu, K., Ikuta, Y., and Nakamura, H. K. (1994), *Appl. Biochem. Biotechnol.* **45/46,** 429–436.
7. Miura, Y. (1995), *Process Biochem.* **30,** 1–7.
8. Chowdhury, W. Q., Idehara, K., Maeda, I., Umeda, F., Yagi, K., Miura, Y., and Mizoguchi, T. (1996), *Appl. Biochem. Biotechnol.* **57/58,** 361–366.
9. Ohta, S., Miyamoto, K., and Miura, Y. (1987), *Plant Physiol.* **83,** 1022–1026.
10. Kim, J. S., Ito, K., and Takahashi, H. (1982), *Agric. Biol. Chem.* **46,** 937–941.
11. Stevens, P., Plovie, N., De Vos, P., and De Ley, J. (1986), *Syst. Appl. Microbiol.* **8,** 19–23.
12. Nakada, E., Asada, Y., Arai, T., and Miyake, J. (1995), *J. Ferment. Bioeng.* **80,** 53–57.
13. Zürer, H. and Bachofen, R. (1979), *Appl. Environ. Microbiol.* **37,** 789–793.
14. Planchard, A., Mignot, L., Jouenne, T., and Junter, G. A. (1989), *Appl. Microbiol. Biotechnol.* **31,** 49–54.
15. Fißler, J., Kohring, G. W., and Giffhorn, F. (1995), *Appl. Microbiol. Biotechnol.* **44,** 43–46.
16. De Philippis, R., Ena, A., Guastini, M., Sili, C., and Vincenzini, M. (1992), *FEMS Microbiol. Rev* **103,** 187–194.
17. Hustede, E., Steinbühel, A., and Schlegel, H. G. (1993), *Appl. Microbiol. Biotechnol.* **39,** 87–93.