APPLIED AND ENVIRONMENTAL MICROBIOLOGY, May 1979, p. 789-793
0099-2240/79/05-0789/05$02.00/0
Vol. 37, No. 5

Hydrogen Production by the Photosynthetic Bacterium *Rhodospirillum rubrum*

HANS ZÜRRER AND REINHARD BACHOFEN*
Institut für Pflanzenbiologie, Universität Zürich, CH-8008 Zürich, Switzerland

Received for publication 5 February 1979

Continuous photosynthetic production of hydrogen by *Rhodospirillum rubrum* in batch cultures was observed up to 80 days with the hydrogen donor, pure lactate or lactic acid-containing wastes, supplied periodically. Hydrogen was produced at an average rate of 6 ml/h per g (dry weight) of cells with whey as a hydrogen donor. In continuous cultures with glutamate as a growth-limiting nitrogen source and lactate as a hydrogen donor, hydrogen was evolved at a rate of 20 ml/h per g (dry weight). The composition of the gas evolved remained practically constant (70 to 75% H_2, 25 to 30% CO_2). Photosynthetic bacteria processing specific organic wastes could be an advantage in large-scale production of hydrogen together with food protein of high value, compared to other biological systems.

Research has been intensified to find new fuel sources as a replacement for fossil hydrocarbons. Hydrogen is considered as an ideal and pollution-free fuel for the future (11). Biological production of hydrogen has been observed in a great number of microbial species (6, 12, 21). For large-scale production, photosynthetic organisms that can use solar energy offer several advantages over heterotrophs.

In 1942, Gaffron and Rubin (3) discovered the formation of hydrogen by the green algae *Scenedesmus*. This ability was observed subsequently with several other species of unicellular green algae (7). Further investigations, however, showed that the hydrogen production was small and decreased after a short period, due to the coproduction of O_2. Heterocystous, filamentous blue-green algae (cyanobacteria) seemed to be less sensitive to oxygen (1), but high rates of hydrogen evolution were only observed in an anaerobic environment. Weissman and Benemann (20) described continuous hydrogen evolution by *Anabaena cylindrica* for 18 days and Jeffries et al. (10) observed it for 30 days, both under limited light conditions.

The light-dependent production of hydrogen by photosynthetic bacteria was first observed with cultures of *Rhodospirillum rubrum* (4). Purple non-sulfur bacteria produce hydrogen and CO_2 by a light-dependent decomposition of several organic compounds (5, 16). Since the evolution of hydrogen by photosynthetic bacteria as well as by blue-green algae is inhibited by ammonium salts and molecular nitrogen, hydrogen production is catalyzed in both groups of organisms by nitrogenase.

Besides hydrogen, algae also produce oxygen. Before the hydrogen can be used as fuel, it has to be separated from oxygen and possibly other gases in an energy-consuming process. However, in photosynthetic bacteria, CO_2 is the only gas evolved besides hydrogen. This is a great advantage, because the gas liberated can be used without any further treatment. Algae can use water as a hydrogen donor, whereas purple non-sulfur bacteria need an organic substrate. High yields of hydrogen can be achieved, e.g. with lactic acid as a hydrogen donor (8). There also exist lactic acid-containing wastes, available in large amounts. In this paper we tested several lactic acid-containing substrates for their ability to serve as hydrogen donor and carbon source, and we studied the possibility of their continuous conversion into hydrogen and cell mass over a long period of time.

MATERIALS AND METHODS

Organisms and culture methods. *R. rubrum* S-1 was cultivated in the mineral salt medium of Ormerod et al. (16) with L-glutamate (25 mM) and L-(+)-lactate (50 mM) (pH of the free acids was adjusted to 6.8 with NaOH). Yeast extract and peptone were omitted. Besides sodium lactate and calcium lactate, the following wastes were tested as hydrogen donors.

(i) Waste from the production of lactic acid bacteria (Bioferment SA, Barbengo-Lugano). Lactic acid is formed during growth of *Streptococcus faecalis* in a complex medium with dextrose as C source. The lactic acid was continuously neutralized with NaOH during fermentation. After removal of the cells by centrifugation, the supernatant (lactic acid concentration, 0.35 M), which was wasted by the factory, was used for the experiments.

(ii) Yogurt waste (Toni-Molkerei, Zürich). Yogurt waste was repeatedly neutralized until the lactic acid fermentation was terminated. The neutral supernatant after centrifugation was used (lactic acid concentration, 0.45 M).

(iii) Whey (Toni-Molkerei, Zürich). Whey was treated in the same manner as yogurt waste. Albumin, precipitated by heating, was removed by centrifugation, and the supernatant was used (lactic acid concentration, 0.47 M).

The waste substrates were given to cultures primarily grown with the synthetic medium of Ormerod et al. (16). The additions are indicated in the results described. The cultures were incubated anaerobically in closed vessels at 30°C under continuous illumination. For hydrogen production over longer periods, two systems were used, as follows. (i) Rubber-stoppered serum bottles (500 ml; diameter, 7 cm) were stirred with a magnetic stirrer in a water bath of constant temperature. Hypodermic needles were inserted into the rubber stopper for gas outlet, sampling, and addition of substrate. The cultures were illuminated from one side by a 100-W spot-light tungsten lamp. Light intensity was, on the average, 20 mW/cm^2, measured with a YSI-Kettering model 65 radiometer. (ii) To obtain better illumination (short light path combined with large volume), we constructed a fermentor with a rectangular cross section and a volume of 1,000 ml (Fig. 1). The fermentor was illuminated from the large side (light path, 4 cm) by two 100-W spot-light tungsten lamps (light intensity, on the average, 30 mW/cm^2). The culture was kept at 30°C and mixed with a stirrer, magnetically coupled to a magnetic stirring motor. This fermentor was used to investigate the hydrogen production from whey as well as for the chemostatic growth with synthetic medium limited by L-glutamate as the sole nitrogen source. Each fermentation vessel was inoculated with 5% of exponentially growing cultures grown on synthetic medium.

Analytical methods. Densities of cell suspensions were estimated by measuring the absorbency at 660 nm in a Hitachi 101 spectrophotometer. For the determination of the dry weight, a sample of cells was centrifuged, washed once with distilled water, and dried to constant weight at 105°C. L-Glutamate was determined enzymatically with glutamic dehydrogenase (2), L-lactate with lactic dehydrogenase (9). The gas produced was trapped in a calibrated cylinder, and the measured volume was converted to the volume at standard conditions. Gas samples taken directly out of the culture vessels were injected into a Gow-mac 550 gas chromatograph with a thermal conductivity detector. The column was packed with molecular sieve 5A for the determination of hydrogen and with Porapack for CO_2. Argon was used as the carrier.

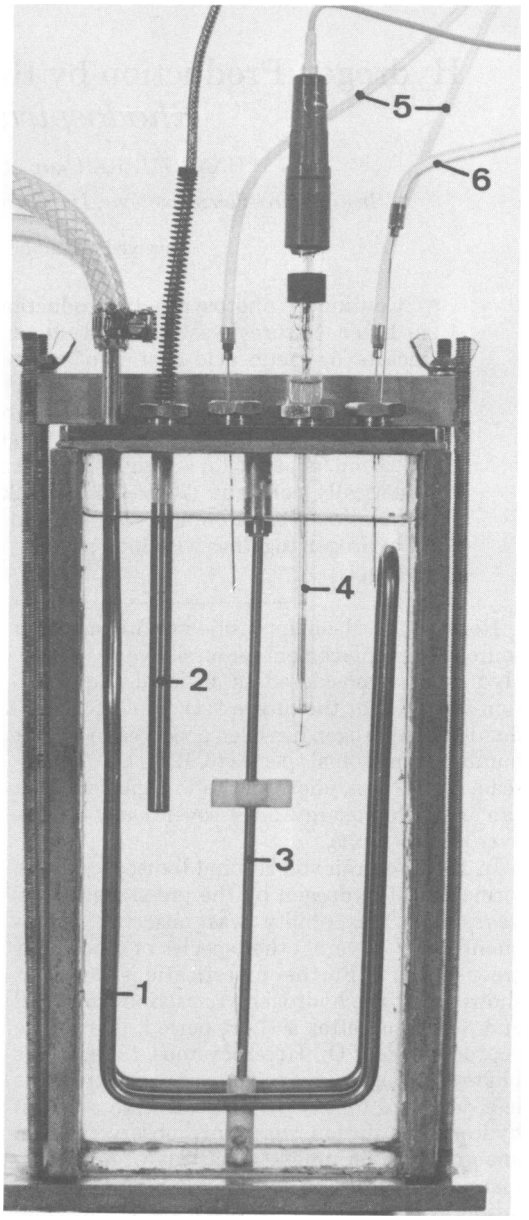

FIG. 1. *Fermentor (1,000 ml) with rectangular cross section (4 by 12 cm). Light path, 4 cm. (1) Cooling coil. (2) Pt-100 temperature sensor. (3) Stirrer. (4) pH electrode. (5) Inlet and outlet for growth medium and cells. (6) Gas outlet.*

RESULTS

Hydrogen production of 500-ml cultures with sodium lactate or calcium lactate as the substrate was followed over several weeks (Fig. 2A). Lactate was supplied at the intervals indicated by addition of 3.75 ml of a 2 M solution. Although no N source or mineral salts were supplied, the rate of hydrogen evolution remained almost constant. When added as calcium lactate, the cheapest form of lactate, Ca precipitations were observed in the culture medium. However, this did not affect gas evolution. The yield of conversion into hydrogen was approximately 75% (100% = complete decomposition of the

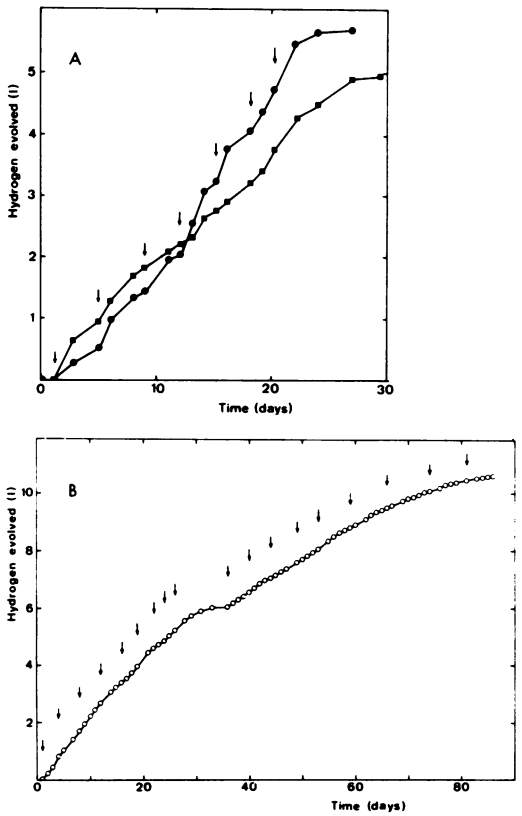

FIG. 2. *Total hydrogen production by 500-ml cultures of R. rubrum. (A) Lactate as substrate. Culture density (absorbency at 660 nm), 7.5 to 8 optical density units; dry weight, 4 to 5 mg/ml. Arrows indicate the addition of 7.5 mmol of lactate (3.75 ml of 2 M solution without mineral salts or nitrogen source) and the removal of an equal volume of the cell suspension. Symbols: (■) sodium lactate; (●) calcium lactate. (B) Yogurt waste or whey as substrate. Culture density, 11.5 to 17.5 optical density units. Arrows indicate the addition of 10-ml samples of yogurt waste from days 1 to 26 or 10-ml samples of whey from days 36 to 81.*

substrate to H_2 and CO_2: $C_3H_6O_3 + 3\ H_2O \rightarrow 6\ H_2 + 3\ CO_2$). The pH fluctuated between 7.0 and 7.3. The density of the cell suspension and the composition of the evolved gas (70 to 75% H_2, 25 to 30% CO_2) remained constant with these nongrowing cultures. During exponential growth, however, the pH rose to an extent that large quantities of CO_2 were bound by the growth medium. The percentage of hydrogen in the collected gas was 90 to 95%. Similar observations were made by Hillmer and Gest (8).

Figure 2B represents the hydrogen production with yogurt waste or whey by a 500-ml batch culture. We succeeded in maintaining hydrogen production over a period of more than 12 weeks with the same culture. The lactic acid-containing wastes were supplemented periodically as indicated. As described for the experiments before with lactate, culture density, pH, and gas composition remained nearly constant. The shoulder after 30 days is the result of a break in feeding of the substrate.

Table 1 compares the hydrogen production from lactate with its production from waste substrates. Since it was not possible to grow *R. rubrum* from the beginning with the wastes tested as the only substrate, cells were first cultivated with a synthetic substrate. After the hydrogen evolution started, the culture was adapted to the waste substrate. Addition of larger amounts of wastes at one time, especially waste from the production of lactic acid bacteria, inhibited the gas production, probably due to inhibition by bound nitrogen. With limited addition, complete decomposition of the lactic acid without inhibition of the gas evolution was achieved. The three waste substrates tested showed about the same concentrations of lactic acid, and the quantity of hydrogen produced from equal amounts of waste was in the same order of magnitude. The higher yield with waste from the production of lactic acid bacteria indicates that, besides lactic acid, other substrates gave rise to hydrogen production (Table 1). Figure 3 illustrates the hydrogen production from whey in a 1,000-ml culture grown in the fermentor described. The higher rates as compared to those of the 500-ml cultures can be explained by the higher light intensity. The volume was kept constant by removing the same amount of cell suspension during the addition of substrate. By means of supplementing the culture with mineral nutrients, a decrease of the average rate of

TABLE 1. *Photoproduction of H_2 from various substrates by R. rubrum in 500-ml cultures*

Substrate	Hydrogen production[a]		Hydrogen yield[b] (%)
	ml of H_2 per h per liter culture	ml of H_2 per ml of substrate	
L-(+)-Lactate	16–36		
Waste from lactic acid bacteria production	16–24	52	99
Yogurt waste	12–20	45	67
Whey	8–20	47	67

[a] Hydrogen production was measured over a period of 10 days after adaptation to the substrate. Initial cell concentration (absorbency at 660 nm) was 8 optical density units; dry weight was 4 mg/ml.

[b] With respect to lactic acid concentration; 100% = complete decomposition of the original lactic acid to H_2 and CO_2.

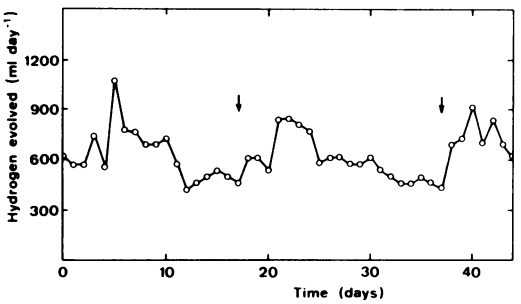

FIG. 3. *Hydrogen production by a 1,000-ml culture of R. rubrum with whey as substrate. Culture density (absorbency at 660 nm), 11.0 to 13.0 optical density units. Arrows indicate the addition of 20 ml of mineral salt solution without nitrogen source (Ormerod et al. [16]). Whey was added at a rate of 18 ml/day. Equal amounts of cell suspension were removed to keep the culture volume constant.*

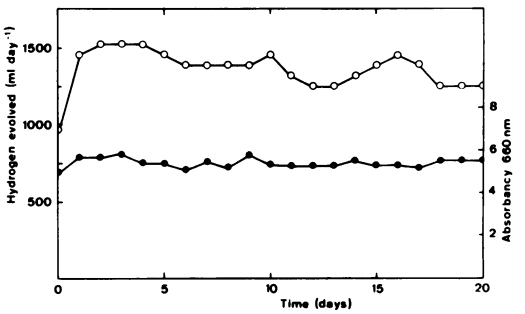

FIG. 4. *Hydrogen production by a 1,000-ml continuous culture of R. rubrum with lactate (50 mM) and glutamate (15 mM) as growth-limiting N source. Dilution rate, 0.0135 h^{-1}. Symbols: (○) hydrogen production; (●) culture density.*

hydrogen production was avoided.

Hydrogen evolution of *R. rubrum* was also measured in continuous cultures. As in the previous experiments, our main interest was a high yield of hydrogen relative to the carbon source and not maximal rates of gas evolution or cell production. The standard conditions for these cultures were as follows: 0.015 M glutamate was used as a limiting N source and 0.05 M lactate as a hydrogen donor. The culture attained a steady-state absorbency of 5.2 to 5.8 at 660 nm and 7.1 to 7.7 at 885 nm with a dilution rate of 0.0135 h^{-1}. Dry weight was 3 to 3.5 mg/ml (Fig. 4). The ratio of the absorbencies at 660 and 885 nm remained constant, indicating that the bacteriochlorophyll concentration of the cells did not change. After removal of the cells by centrifugation, the supernatant was assayed for L-glutamate and L-lactate. Glutamate was beyond the detection limits of our method. The concentration of lactate decreased to 4.1–0.6 mM, indicating that 92 to 99% of the supplied lactate was used for gas production and cell growth. Hydrogen evolution varied between 1,250 and 1,550 ml/day per liter of culture volume, which is significantly higher than the production observed in batch cultures during a longer period. The average yield was 65%. In cultures with high rates of gas evolution the pH remained constant at 7.2; hence pH regulation was not necessary.

DISCUSSION

Continuous hydrogen production by *R. rubrum* during a longer period with relatively simple equipment is possible. The observed rate, 65 ml of H_2 per h per liter of culture volume (equal to 20 ml of H_2 per h per g [dry weight]), is comparable to the results of Weissman and Benemann (20) with the blue-green alga *Anabaena* (30 ml of H_2 per h per liter of culture or 32 ml of H_2 per h per g [dry weight]). Optimizing experimental conditions with *R. rubrum*, it should be possible to reach rates as high as 130 ml of H_2 per h per liter of culture (equal to 230 ml of H_2 per h per g [dry weight]) (8). These are the minimal rates necessary for large-scale production of hydrogen using a photosynthetic process as calculated by Neil et al. (15). So far, continuous production of hydrogen with blue-green algae is only successful when the cultures are gassed with an inert gas, e.g. argon (10, 20). Therefore, technical production of hydrogen with photosynthetic bacteria using organic wastes seems to be more realistic.

Wastes are produced in industrial countries in large quantities. Waste water, for example, has been successfully treated with photosynthetic bacteria (13, 17, 18). Furthermore, agricultural by-products may serve as a source of nutrients (19). Cells of photosynthetic bacteria are composed of about 65% protein, containing large quantities of essential amino acids and vitamins. In view of economic considerations, it is important that cultures of *R. rubrum* not only evolve hydrogen, but also eliminate wastes (17, 18) and produce single-cell protein. Calculated for 1 m^2 of surface, in a culture of 40 liters with whey as a substrate, 25 liters of hydrogen was produced simultaneously with about 4.5 g of bacterial dry substance per day. With lactate in a continuous culture, about 40 g of dry substance per day was obtained, but much larger quantities may be obtained after optimization of the process. *R. rubrum* can grow in continuous culture with molecular nitrogen as the sole nitrogen source (14). Therefore cultures could be grown also on waste essentially lacking nitrogen.

ACKNOWLEDGMENTS

This work was supported by the Swiss National Foundation for Scientific Research, grant 4.048.0.76.04, National Research Program "Energy."

LITERATURE CITED

1. **Benemann, J. R., and N. M. Weare.** 1974. Hydrogen evolution by nitrogen-fixing *Anabaena cylindrica* cultures. Science **184**:174–175.
2. **Bernt, E., and H. U. Bergmeyer.** 1970. L-Glutamat, Bestimmung mit Glutamat-Dehydrogenase und NAD, p. 1659–1663. *In* H. U. Bergmeyer (ed.), Methoden der enzymatischen Analyse. Verlag Chemie, Weinheim.
3. **Gaffron, H., and J. Rubin.** 1942. Fermentative and photochemical production of hydrogen in algae. J. Gen. Physiol. **26**:219–240.
4. **Gest, H., and M. D. Kamen.** 1949. Photoproduction of molecular hydrogen by *Rhodospirillum rubrum*. Science **109**:558–559.
5. **Gest, H., J. G. Ormerod, and K. S. Ormerod.** 1962. Photometabolism of *Rhodospirillum rubrum*: light dependent dissimilation of organic compounds to carbon dioxide and molecular hydrogen by an anaerobic citric acid cycle. Arch. Biochem. Biophys. **97**:21–33.
6. **Gray, C. T., and H. Gest.** 1965. Biological formation of molecular hydrogen. Science **148**:186–192.
7. **Healey, F. P.** 1970. Hydrogen evolution by several algae. Planta **91**:220–226.
8. **Hillmer, P., and H. Gest.** 1977. H_2 metabolism in the photosynthetic bacterium *Rhodopseudomonas capsulata*: H_2 production by growing cultures. J. Bacteriol. **129**:724–731.
9. **Hohorst, H. J.** 1970. L-(+)-Lactat, Bestimmung mit Lactat-Dehydrogenase und NAD, p. 1425–1429. *In* H. U. Bergmeyer (ed.), Methoden der enzymatischen Analyse. Verlag Chemie, Weinheim.
10. **Jeffries, T. W., H. Timourian, and R. L. Ward.** 1978. Hydrogen production by *Anabaena cylindrica*: effects of varying ammonium and ferric ions, pH, and light. Appl. Environ. Microbiol. **35**:704–710.
11. **Jones, L. W.** 1971. Liquid hydrogen as a fuel for the future. Science **174**:367–370.
12. **Kessler, E.** 1974. Hydrogenase, photoreduction, and anaerobic growth, p. 456–473. *In* W. D. P. Stewart (ed.), Algal physiology and biochemistry. Blackwell Scientific Publications, London.
13. **Kobayashi, M., M. Kobayashi, and H. Nakanishi.** 1971. Construction of a purification plant for polluted water using photosynthetic bacteria. J. Ferment. Technol. **49**:817–825.
14. **Munson, T. O., and R. H. Burris.** 1969. Nitrogen fixation by *Rhodospirillum rubrum* grown in nitrogen-limited continuous culture. J. Bacteriol. **97**:1093–1098.
15. **Neil, G., D. J. D. Nicholas, J. O'M. Bockris, and J. F. McCann.** 1976. The photosynthetic production of hydrogen. Int. J. Hydrogen Energy **1**:45–48.
16. **Ormerod, J. G., K. S. Ormerod, and H. Gest.** 1961. Light dependent utilization of organic compounds and photoproduction of molecular hydrogen by photosynthetic bacteria; relationship with nitrogen metabolism. Arch. Biochem. Biophys. **94**:449–463.
17. **Sawada, H., R. C. Parr, and P. L. Rogers.** 1977. Photosynthetic bacteria in waste treatment: role of *Rhodopseudomonas capsulata* with agricultural/industrial effluents. J. Ferment. Technol. **55**:326–336.
18. **Sawada, H., and P. L. Rogers.** 1977. Photosynthetic bacteria in waste treatment: pure culture studies with *Rhodopseudomonas capsulata*. J. Ferment. Technol. **55**:297–310.
19. **Shipman, R. H., I. C. Kao, and L. T. Fan.** 1975. Single-cell protein production by photosynthetic bacteria cultivation in agricultural by-products. Biotechnol. Bioeng. **17**:1561–1570.
20. **Weissman, J. C., and J. R. Benemann.** 1977. Hydrogen production by nitrogen-starved cultures of *Anabaena cylindrica*. Appl. Environ. Microbiol. **33**:123–131.
21. **Zajic, J. E., N. Kosaric, and J. D. Brosseau.** 1978. Microbial production of hydrogen, p. 57–109. *In* T. K. Ghose, A. Fiechter, and N. Blakebrough (ed.), Advances in biochemical engineering, vol. 9. Springer, Berlin.