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PRODUCTION OF HYDROGEN FROM WATER USING BIOPHOTOLYTIC METHODS (*Anacystis nidulans*/Biophotolysis/*Rhodospirillum rubrum*/NADP/Immobilized Microorganisms)

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Hydrogen gas has been produced on a continuous basis using two immobilized microorganisms. One organism, the cyanobacteria *Anacystis nidulans*, oxidizes water, producing molecular oxygen, and reduces exogenous NADP. The second organism, *Rhodospirillum rubrum*, reoxidizes NADPH and produces molecular hydrogen. Thus the complete system oxidizes water and produces oxygen and hydrogen while recycling NADP.

INTRODUCTION

Hydrogen is attracting a great deal of attention as a possible fuel source. A variety of bacteria and algae are capable of hydrogen production under anaerobic conditions (1,2). Photosynthetic bacteria can produce hydrogen from oxidizable organic compounds. The organism most studied in this respect has been *Rhodospirillum rubrum* (3). There have been several reports in the literature concerning H_2 production and biophotolysis (4–7). In our laboratory, we have previously shown that this organism when immobilized by gel entrapment will continuously produce hydrogen from malate for several days with an operational half life of approximately 180 h (8).

Photosynthetic bacteria, because they do not have photosystem II, are incapable of oxidizing water and evolving O_2. These organisms do utilize substitute hydrogen donors. Blue-green algae or cyanobacteria, on the other hand, have photosystem II and consequently do evolve O_2. According to the Hill and Nendall model (9), the photosystem II oxidizes water to free

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oxygen, while at photosystem I, the enzyme cofactor nicotinamide adenine dinucleotide phosphate (NADP) is reduced to NADPH.

Most photosynthetic bacteria have the ability to fix carbon dioxide by the Calvin cycle. *R. rubrum* can obtain the necessary reducing equivalents anaerobically by oxidation of organic acids via the photosynthetic apparatus. The reducing equivalents produced are used in the reduction of carbon compounds. Under some conditions the reducing equivalents can be utilized for the production of molecular hydrogen. The NADPH, formed photosynthetically, is normally oxidized within the Calvin cycle in a dark reaction. In the case of *R. rubrum*, if the bacterium were capable of accepting an externally supplied NADPH over and above the quantity required for necessary functions, it might convert the excess to molecular hydrogen photosynthetically.

It is known that the cyanobacteria *Anacystis nidulans*, if previously freeze-dried, can produce NADPH from exogenously offered NADP by the oxidation of water in a light reaction. The reduced cofactor is released into the surrounding medium (10). Since *R. rubrum* is capable of oxidizing NADPH to produce H_2, it is logical to attempt to combine these two photosynthetic organisms into a single system capable of producing reduced NADP from the oxidation of water followed by the oxidation of the NADPH with the production of molecular hydrogen. Thus, the overall reaction is the oxidation of water with the production of H_2 plus O_2 (Fig. 1).

MATERIALS AND METHODS

R. rubrum grown in a malate–glutamate medium (11) was stored at 4°C until use. Illumination of the flasks during incubation was provided by eight standard 100-W incandescent light bulbs. Aliquots of gas (usually between 0.2 and 1.0 ml) were removed from the flask and assayed for H_2, N_2, and O_2 production on a Tracor 550 gas chromatograph using an 8-ft × $\frac{1}{8}$-in. column of 5-Å molecular sieve. The blue-green algae were grown in a chemically

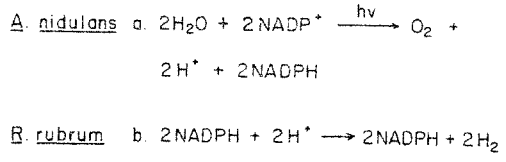

FIG. 1. The proposed mechanism for hydrogen production from water by biophotolysis using *A. nidulans* and *R. rubrum*.

defined medium with 1.5% $NaNO_3$ (12). Cells were harvested, lyophilized, and stored at −20°C under P_2O_5.

Immobilization of R. rubrum

Samples of *R. rubrum* were initially immobilized in various concentrations of acrylamide-bisacrylamide. No active preparations could be prepared. It was found that the monomers destroyed the ability of the organism to produce hydrogen. Previous studies (8) had shown that agar could be successfully used to immobilized these organisms, and it was therefore decided to continue all studies using agar for immobilization.

Various quantities of *R. rubrum* were added to 15.0 ml of a 5% solution of a Noble agar. This solution was spread evenly on both sides of a plastic slab approximately 5 × 8 in. in size, which was composed of Lucite ® plastic. The slab was put into an airtight system and the substrate, purged with argon, was passed through the system at 8.0 ml/h. The system was immersed in a water bath that was maintained between 18 and 19°C. The gas produced was channeled to a glass tube that was kept airtight with rubber septums (Fig. 2). The substrate solution was pulled through the system with a syringe until all the gas in the collection tube was displaced. A sample of gas was taken after the flow rate was reestablished and a reasonable quantity of gas

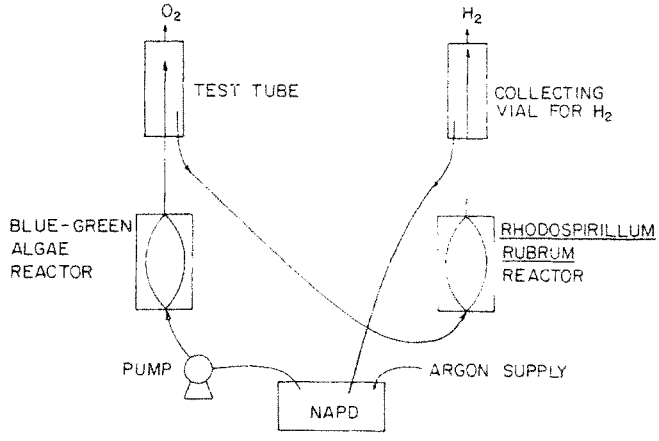

FIG. 2. Schematic diagram of the reactor system used for the production of hydrogen from water. The two reactors shown were of clear Lucite and could be illuminated with a bank of incandescent bulbs. The substrate was 0.001 M NADP in 0.01 M Tris-HCl buffer, pH 7.6, containing 100 mg of glucose oxidase 15,000 NBC U/g, and 1.0% dextrose as an oxygen scavenger.

was trapped. A sample of the produced gas was withdrawn from the collector and assayed by gas chromatography. The H_2 produced was calculated from an H_2 standard curve.

Effect of Metals and Exogenous NADP on Immobilized R. rubrum

The cells were immobilized as described above. Substrates tested were 0.01 M malate containing one of the following: 0.001 M $MgCl_2$, 0.001 M $CuCl_2$, 0.001 M $MnCl_2$, or 0.001 M NADP in 0.1 M Tris-HCl, pH 7.6. In addition NADP with no malate was examined as a substrate.

Effect of Photosynthesis Inhibitor

Two known inhibitors of photosynthesis were tested for their ability to prevent the oxidation of malate in the *R. rubrum*, production of NADPH in the algae, the H_2 production in the complete system. The inhibitors Diuron ® and Monuron ® were used at μM concentrations in all three systems.

Cyanobacteria (Blue-Green Algae)

Algae were immobilized in agar as described for *R. rubrum*. To determine the reducing efficiency of the algae, a 250-mg sample of agar-entrapped algae was incubated with 5 ml of 0.001 M $MgCl_2$ and 0.001 M NADP dissolved in 0.1 M Tris-HCl, pH 7.6. The samples were incubated for 60 min over a 100-W spotlight, and the contents centrifuged to remove the immobilized cells. The supernatant was assayed at 340 nm against a control for NADPH production.

Reaction with both R. rubrum and A. nidulans

The blue-green algae and the *R. rubrum* were immobilized in a flow type reactor system as previously described. the concentration was 50 mg of lyophilized cells/ml in 15 ml of 5% Noble agar. Seven and one-half g wet weight *R. rubrum* was also entrapped in 15 ml of 5% Noble agar. The substrate that was recycled was 0.015 M Tris-HCl buffer, pH 7.6, with 0.001 M NADP. The substrate also contained 0.001 M $MgCl_2$ and 0.001 M $MnCl_2$ as noted. The substrate was continually purged with argon. A schematic diagram can be seen in Fig. 2.

RESULTS

Before combining the bacteria and the blue-green algae reactors together, a series of experiments were carried out on each independent system to determine that system's characteristics. The effect of trace metals on the immobilized *R. rubrum* is summarized in Table 1. It is obvious from this table that higher productivity is observed in the presence of Mn^{2+} ion than in its absence. However, with the higher productivity rate shorter halflife values were also observed. A typical halflife observed for the *R. rubrum* in the absence of added Mn^{2+} ion is plotted in Fig. 3. The observed halflife is 114.5 h. The addition of $MnCl_2$ decreased the halflife to 23 h (Fig. 4). However, the initial rate of H_2 production is greater by one order of magnitude. the total H_2 produced during one halflife was also greater.

Table 2 gives some typical results for *R. rubrum* test systems using two weights of microorganisms. The total hydrogen produced in the presence of Mn^{2+} is in all cases greater than in the ion's absence. Also the halflife values are shorter. The total quantities of H_2 produced are related to the total cells used, although not proportionally.

TABLE 1. **Effective Transition Metals on *R. rubrum***

	Time H	H_2 production[a] (μl/min)
Reactor no. 1:	21	13.30
0.001 M $MgCl_2$	27	19.60
	46	59.20
	50	49.20
Reactor no. 2:	4	130.00
0.001 M $MnCl_2$	25	320.00
	31	128.50
	50	211.30
	54	128.50
	70	99.90
	75	70.50
	90	25.00
Reactor no. 3:	18	69.07
0.001 M $CuCl_2$	23	61.40
	43	43.20
	46	33.30

[a]The results of column 2 were plotted to determine operation halflife (Fig. 4).

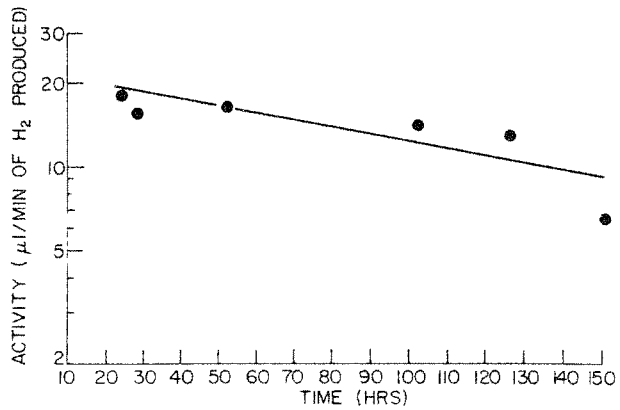

FIG. 3. Semilog regression analysis for H_2 production of immobilized *R. rubrum*, using 0.01 M malate with 0.001 M $MgCl_2$ in 0.1 M Tris-HCl, pH 7.6. The halflife and the upper and lower confidence values at the 95% level are: upper, 641.8; lower, 62.84. The halflife is 114.5, the slope is −0.006, the standard error is 0.232, and the correlation is −0.8136.

Controls were run using Tris-HCl buffer, Monuron, or Diuron plus malate. The blue-green algae system, as expected, show inhibition of NADP reduction both in the dark and in the presence of μM concentrations of Monuron® and Diuron®. The *R. rubrum* at the same inhibitor concentrations was unaffected. Addition of the photosynthetic poisons to the

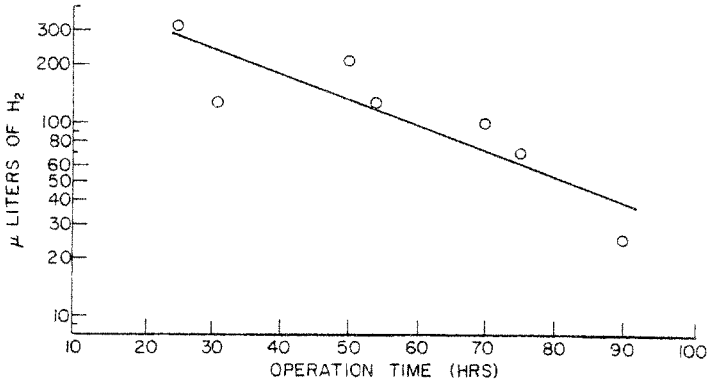

FIG. 4. Semilog regression analysis for H_2 production of immobilized *R. rubrum*, using 0.01 M malate with 0.001 M $MgCl_2$, 0.001 M $MnCl_2$ in 0.1 M Tris-HCl, pH 7.6. The slope is −0.030, the standard error is 0.4328, the correlation is −0.8757, and the halflife is 22.8. The halflife and the upper and lower confidence values at the 95% level are: upper, 62.34; lower, 13.9.

TABLE 2. ***R. rubrum*** **Systems Tested**

Reactor no.	Weight of bacteria (mg)	Halflife (H)	Total H_2 produced over 2 × halflife (μl)	H_2 produced (mol)
1[a]	600	25	0.525×10^6	0.023
2[b]	600	180	0.405×10^6	0.018
3[c]	300	21	0.225×10^6	0.01
4[d]	300	200	0.120×10^6	0.005

[a] Substrate was 0.01 M malate, 0.001 M $MgCl_2$, 0.001 M $MnCl_2$, 0.1 M Tris-HCl, pH 7.6.
[b] Substrate was 0.01 M malate, 0.1 M Tris-HCl, pH 7.6.
[c] Substrate was 0.01 M malate, 0.1 M MgCl, 0.001 M $MnCl_2$, 0.1 M Tris-HCl, pH 7.6.
[d] Substrate was 0.01 M malate, 0.1 M Tris-HCl, pH 7.6.
Note. Several control reactors were run in Tris-HCl buffer with 1 μM Monuron and 1 μM Diuron, and in the absence of light. In the absence of light, no H_2 was produced. In μM Monuron and Diuron, H_2 production was observed.

complete system containing both the *Anacystis* and the *R. rubrum* prevented all H_2 production. The addition of exogenous NADP and malate to the immobilized *R. rubrum* had a major effect on H_2 production, causing a five- to eight-fold increase in total productivity and operational halflife values (Table 3). Elimination of malate from the *R. rubrum* system prevented H_2 production after a short initial production observed during the first 6 h of incubation.

Two observations are immediately apparent upon addition of the exogenous NADP to the malate system (Fig. 5). First, the halflife values and total H_2 productivity are increased. Second, the apparent time required to reach maximum production rates is decreased and the observed exponential decay of the NADP system begins sooner. it is also interesting to note that the maximum H_2 production rate was increased to even higher values than observed by addition of the Mn^{2+} alone to the *R. rubrum* system.

TABLE 3. ***R. rubrum*** **Systems Tested**[a]

Reactor no.	Weight of bacteria (mg)	Calculated Halflife (h)	H_2 produced over two halflives (mol)
5	600	185	0.075
7	300	155	0.04
9	300	180	0.05

[a] All systems tested contained 0.001 M $MgCl_2$, 0.001 M $MnCl_2$, 0.01 M malate, and 0.001 M NADP in 0.1 M Tris-HCl, at pH 7.6. Reactors 6 and 8 were controls containing NADP in Tris-HCL. These systems showed no H_2 production.

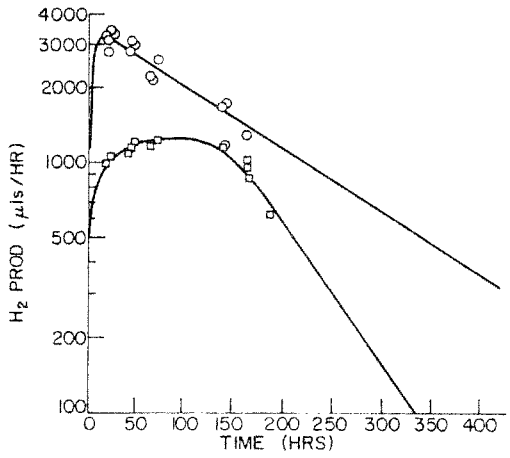

FIG. 5. Semilog plots of H_2 production with 4.0 g wet weight immobilized *R. rubrum*, using 0.01 M malate with 0.001 M $MgCl_2$ and $MnCl_2$ in 0.1 M Tris-HCl, pH 7.6. However, in the curve designated by circles, 0.001 M NADP was present. The squares represent the control system lacking the added NADP.

The blue-green algae system was examined for the production of NADPH using 0.001 M NADP in 0.1 M Tris-HCl, pH 7.6, containing 0.001 M $MgCl_2$ and 0.001 M $MnCl_2$. The observed halflife did not exceed 12 h. The total system was operated in the presence and absence of Mn^{2+} ion using 0.001 M NADP in 0.015 M Tris, pH 7.6. The observed operational halflife for the complete system was approximately 12 h. Total H_2 production did not exceed 0.002 mol during that 12-h period. No hydrogen production in the complete system was observed in the absence of the exogenously added NADP or in the absence of light.

DISCUSSION

Several interesting observations were made during the course of these studies that were rather unexpected. The increased activity of the *R. rubrum* observed by the addition of the exogenous NADP leads to several interesting hypotheses. First, the NADP apparently can get into the *R. rubrum* across the cell membrane in the immobilized system, whereas in freshly harvested cells the membranes remain impermeable to the cofactor. Second, the cofactor is apparently a limiting factor in the production of H_2, since we observed a several-fold increase in the production rate when it was added

exogenously. And third, if the cells do accept NADP, they most likely are accepting the NADPH, the product of a biophotolytic reaction observed in the lyophilized blue-green algae, *A. nidulans.*

The combined system of *R. rubrum* and *A. nidulans* did indeed produce H_2 gas as expected. However, the addition of the bivalent transition metals to the system increased H_2 production. The cause of this increased activity is not known. It has been shown by others that when these metals are presented in small quantities, the permeability of some bacterial cell membranes is increased (13). Therefore, when increased amounts of substrate are available, the activity of the enzyme system could be increased considerably.

It is interesting to note that the lag time required before H_2 production is greatly decreased on addition of the $MnCl_2$. It is obvious that some changes in membrane permeability of the *R. rubrum* must occur after immobilization and before H_2 production begins. Addition of the transition metal may decrease the lag time by increasing the permeability.

In a recent publication by Adams and Hall (14), they described the properties of hydrogenase purified from *R. rubrum.* This enzyme was neither capable of reducing NAD^+ or $NADP^+$ with H_2 nor oxidizing NADH or NADPH, forming H_2. The results of Adam's and Hall's work would confirm our belief that the hydrogenase is not the enzyme system involved in H_2 production using NADPH produced by the *Anacystis.* We do not believe that NADPH is the direct precursor providing electrons for H_2 formation. The precursor could be malate produced by the reduction of oxalacetate by NADPH through some mechanism, this is a more reasonable hypothesis. In addition, it is well known that *R. rubrum* will produce H_2 using its nitrogenase system.

In summary, we have shown that a biophotolytic system consisting of two immobilized organisms in series can produce H_2 from water. Present production rates are relatively low. Operational halflives are short. However, the system does merit further study and optimization.

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