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Evolution of Molecular Hydrogen from Glucose by *Rhodopseudomonas* sp. KCTC 1437

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Rhodopseudomonas sp. KCTC 1437에 의한 포도당으로부터의 수소 생성

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Rhodopseudomonas sp. KCTC 1437 evolved molecular hydrogen efficiently under light illuminated anaerobic culture conditon in the presence of organic acids and various sugars, especially glucose when low concentration of NH_4 + or L-glutamate was added to cultures. It was revealed that hydrogen formation from *Rhodopseudomonas* sp. KCTC 1437 was mediated by two different enzyme systems. Under the nitrogen limiting condition, hydrogen evolution from glucose was catalyzed by nitrogenase. For the nitrogenase activation *in vivo*. the precultured cells grown on limiting concentration of NH_4^+ as a sole nitrogen source showed more capacity of hydrogen evolution from glucose in the presence of L-glutamate than any other cells grown on sufficient concentration of NH_4^+, L-glutamate, NH_4^+, or both of L-glutamate and N_2. A significant volume of molecular hydrogen was evolved from glucose even in the presence of excess NH_4^+ either in the light or dark anaerobic condition, presumably due to the mediation of hydrogen evolution by fromic hydrogenlyase.

Recently, hydrogen evolution by photosynthetic bacteria has received much more attention with the possibility that solar energy could be utilized as a new source of fuel to substitute conventional fossil hydrocarbons. The photoevolution of molecular hydrogen has been suspected to be mediated by nitrogenase, while hydrogen utilization through its uptake by hydrogenase on the bases of many indirect evidences.[1-5] But more detailed studies on the nature of hydrogen evolution are required to increase the rate of hydrogen evolution for possible utilizations of the bacteria with practical purposes.

A long-range goal of this research is the utilization of the photosynthetic bacteria for large-scale production of molecular hydrogen either as a primary fuel source or as a necessary component in synthetic fuel processes. Therefore, it is much necessary to obtain photosynthetic bacteria capable of efficient production of molecular hydrogen from various kinds of reduced carbon compounds such as sucrose or glucose which might be readily available from agricultural or sewage wastes.

We isolated a purple non-sulfur photosynthetic bacteria which evolved molecular hydrogen efficiently from glucose

in the presence of low concentrations of NH_4^+ under light illuminated anaerobic condition and even excess of NH_4^+ under light or dark anaerobic condition. It is generally known that hydrogen evolution from photosynthetic bacteria is catalyzed by nitrogenase. This was proved by many investigators that the rate of hydrogen evolution was almost proportional to nitrogenase activity from the result that both hydrogen evolution and nitrogenase activity were completely inhibited by the addition of excess ammonium salts as well as under dark anaerobic condition.[1-5]

It is very interesting and unique properties for practical use that a photosynthetic bacterium evolves molecular hydrogen in the presence of excess NH_4^+ under dark or light anaerobic condition.

In this report, the physiological significances and regulatory effects of NH_4^+ on hydrogen evolution are discussed.

MATERIALS AND METHODS

Bacterial Strain

As previously reported,[6] we reisolated about 640 strains from soil and water samples in Korea. Among them, one strain with good capacity of hydrogen evolution from glucose was selected and identified to be a similar species to those of genus *Rhodopseudomonas*. However, no correct coincidence with any species in the genus was observed. Therefore, we thought this strain to be a new strain or a variant of *Rhodopseudomonas* and named as *Rhodopseudomonas* sp. KCTC (Korean Collection of Type Cultures) 1437.

Growth media and conditions

The medium of Ormerod *et al.*[7] was used with slight modification. Yeast extract was omitted. To the above medium, glucose (30mM) was added as a carbon source. In addition, ammonium sulfate was replaced by L-glutamate (7mM) as a nitrogen source. In plate culture, 1.5% agar was added to the medium.

Photosynthetic anaerobic cultures were grown in Roux bottles filled with the medium nearly to neck and incubated at 30°C under light intensity of about 6,000-10,000 lux. The intensity of illumination was measured by Radiometer/Photometer Model 550-1 (EG & G, Electro-Optics Div., Salem, Mass.) Aerobic cultures were described previously.[8]

Preparation of precultured cell suspension.

Precultured cells were prepared as previously reported.[5,8] The cell suspensions were stored under argon at 0°C until required.

Measurement of hydrogen evolution

Hydrogen evolution was carried out in serum bottles (40ml) containing 5ml mixtures of the precultured cell suspension (A_{660}, 0.6) and 5 ml of medium supplemented with glucose (30mM) as a electron donor. After capping the reaction vessels with gas-tight rubber stoppers, the mixtures were gassed with argon for 3min and incubated in a shaker bath at 30°C under illuminated condition of about 6000 lux by 200-w incandescent lamps. After a given time passed, samples (300μl) of the gas phase were withdrawn with a gas syringe (Precision Sampling Co.).

It was analyzed for hydrogen with a Varian 3700 gas chromatograph equipped with a thermal conductivity detector as previously reported.[5,8]

Nitorgenase assay

Nitrogenase activity was measured by the reduction of acetylene to ethylene.[9] All reaction procedures were the same as those of hydrogen evolution except that 10% of gas phase was replaced by acetylene after gassing with argon. The analysis for ethylene reduced by nitrogenase from acetylene was performed with a hydrogen flame ionization detector of Varian 3700 gas chromatograph as previously reported.[5,8] Nitrogenase activity was expressed by the rate of ethylene formation.

Cell mass determinations

Bacterial concentrations were measured either by deter-

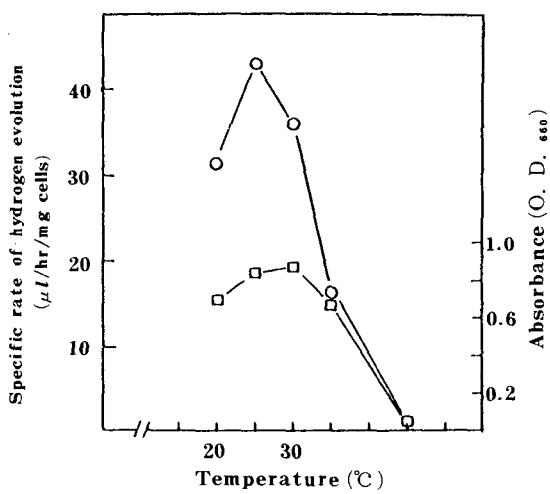

Fig. 1. Effect of temperature on cell growth and hydrogen evolution.

○ : The specific rate of hydrogen evolution,

□ : Difference of cultural absorbance between initial and final one of 40 hrs-culture.

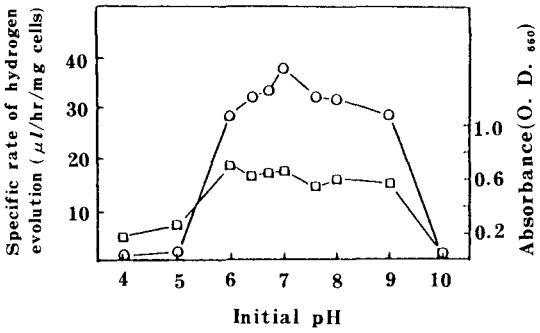

Fig. 2. Effect of pH on cell growth and hydrogen evolution.

○ : The rate of hydrogen evolution,
□ : Difference of cultural absorbance between initial and final one of 40hrs-culture.

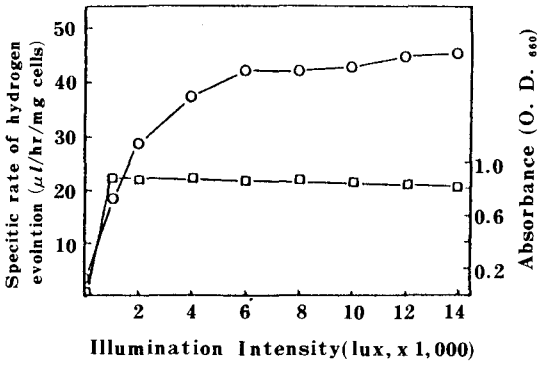

Fig. 3. Effect of illumination intensity on cell growth and hydrogen evolution.

○ : The specific rate of hydrogen evolution,
□ : Difference of cultural absorbance between initial and final one of 40 hrs-culture.

mining culture absorbancy at 660nm or dry weight of centrifuged cells (washed once with distilled water and dried in preweighed aluminum cups at 90°C o/n). Numerous parallel determinations of the absorbancy at 660nm and dry weight in different media and during complete growth cycles showed that these two parameters (absorbancy at 660nm: milligrams of dry weight per milliliter) were consistently in the ratio of 10:4.9 within less than 10% error.

Results and Discussion

Effect of temperature and pH on hvdroqen evolution

Fig. 1. shows effect of temperature on cell growth and hydrogen evolution from glucose. At optimum temperatures of 30-35°C for cell growth. hydrogen was also evolved more largely. At 40°C, cell growth occurred in the nearly same extent as at 25°C but hydrogen evolution was decreased largely. This indicated that hydrogen-evolving system in *Rhodopseudomonas* sp. KCTC 1437 might be rather heat-labile. In Fig. 2, cell growth and hydrogen evolution at different pHs are shown. Optimum pH for hydrogen evolution was around 7.0 although optimum growth was observed at pH 6.0-9.0. In two other sides of pH 6.0-9.0, both cell growth and hydrogen evolution decreased markedly. After measurement for hydrogen evolution, final pHs were checked. These values were somewhat different from initial ones presumably due to accumulation of metabolites.

Effect of light on hydrogen evolution

In Fig. 3, effect of illumination intensity on cell growth and hydrogen evolution is shown. The specific rate of hydrogen evolution became to be saturated to form a plateau from the illumination intensity of 6000 lux, although cell growth began to form plateau from the low intensity of 1000 lux. In addition, Fig. 4 shows the effect of light "on" and "off" on hydrogen evolution and nitrogenase activity. Under dark condition, both of these did not increase, while there were consistent increases under light condition. Both of these also increased with normal rates when illuminated again. Thus, hydrogen-evolving system in this bacterium was also dependent both on light and nitrogenase activity as previously reported.[5,8]

Hydrogen evolution from various electron donors

It has been known that purple nonsulfur photosynthetic bacteria require various organic acids to supply additional energy needed for hydrogen evolution/nitrogen fixation although a signigicant portion comes from light through photophosphorylation.[3,10] We examined the efficiency of various sugars and organic acids on hydrogen evolution as electron donors (Table 1). Among them, higher rates of hydrogen evolution were observed with the sugars such as glucose or fructose and organic acids such as DL-malate or pyruvate. The specific rates of hydrogen evolution varied to a certain extent with different electron donors, presumably because of differences in their reduction states and patterns of metabolism. From the result, it was evident that *Rhodopseudomonas* sp. KCTC 1437 had great capacity of utilizing various kinds of reduced carbon compounds as electron donors.

Hydrogen evolution in fermentor-scale

As a preliminary work for outdoor production of hydro-

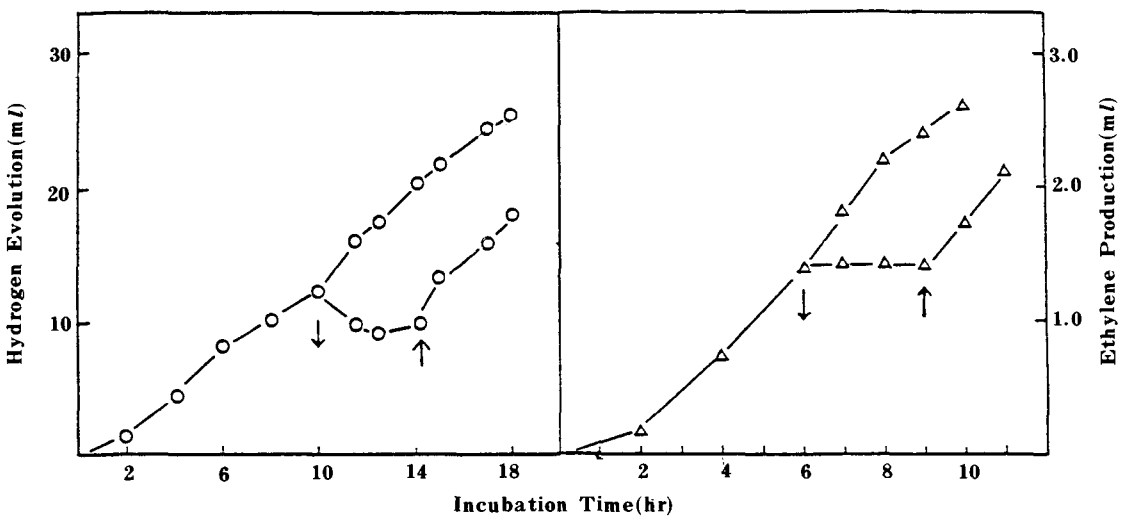

Fig. 4. Effect of light "on" and "off" on hydrogen evolution and nitrogenase activity.

↓ : Light off, ↑ : Light on, ○ : Hydrogen evolution, △ : Nitrogenase activity.
Cell growth occurred from initial A 660 of 0.6 to 1.0 and 0.8 after 18 and 10 hrs respectively.

Table 1. Hydrogen evolution[a] from various electron donors.

Substrate	Specific rate of hydrogen evolution[b] (μl/hr/mg cells)	Substrate	Specific rate of hydrogen evolution (μl/hr/mg cells)
Dextrin	3.9	DL-Malate	40.0
Starch	0.8	Pyruvate	31.9
CMC	0	Fumarate	31.7
		Succinate	31.6
Melizitose	7.7	Mannitol	29.7
Raffinose	1.9	Gluconate	27.4
		α-Ketoglutarate	27.3
Sucrose	16.0	Sorbitol	26.3
Maltose	8.8	Lactate	24.7
Cellobiose	6.6	Acetate	13.2
Lactose	6.2	Control[b]	0
Mellobiose	6.0		
Trehalose	3.0		
Glucose	40.5		
Fructose	31.0		
Mannose	19.3		
Galactose	3.4		

a. Incubation was performed for 40 hrs in mixtures containing the indicated electron donors (30mM) of D-form and L-glutamate (7mM).
b. Mixture containing only L-glutamate (7mM) was used.

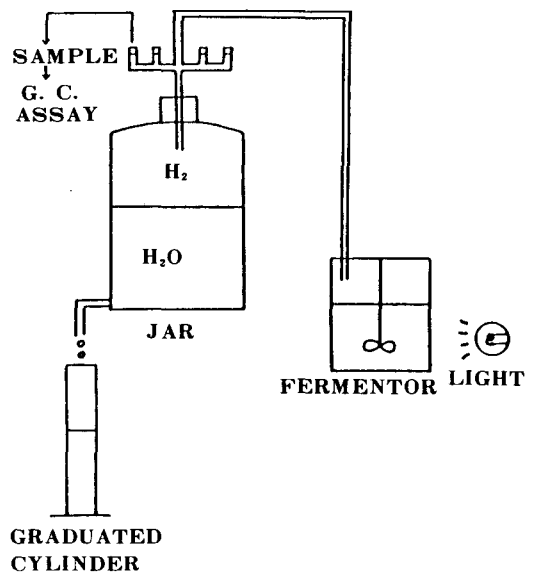

Fig. 5. The appratus used for hydrogen evolution in fermentor-scale under continuous light illumination.

gen, we devised the reactor of culture apparatus and gas reservoir to trap the hydrogen evolved from glucose (Fig 5). Light (6000 lux) was irradiated continuously throughout the whole culture time by incandescent lamps. Hydrogen evolution was carried out in 2.5*l* jar fermentor (Marubishi Co. Japan, MD-250) containing 1*l* of production medium con-

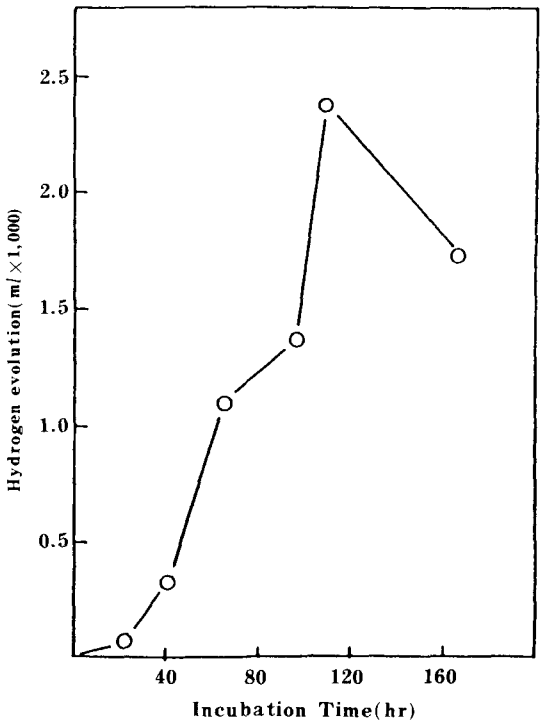

Fig. 6. Hydrogen evolution in fermentor-scale.

sisted of glucose (30 mM) and L-glutamate (7 mM) with gas phase of argon. Time-course evolution of hydrogen is illustrated in Fig. 6. Hydrogen was evolved to the amount of 2370 ml at 110hr. Thereafter, a rapid decrease in hydrogen evolution was observe. It might be due to the utilization of hydrogen through its uptake by anaerobic hydrogenase.

Equation below shows the stoichiometry of the photoconversion process for glucose.[11]

$$C_6H_{12}O_6 + 6H_2O \xrightarrow{\text{light}} 12H_2 + 6CO_2$$

According to this equation, about 8064ml of hydrogen should be evolved from 30mM of glucose in 1 *l*-reaction mixture. Therefore, conversion efficiency based on the theoretical value of 29.4% ($\frac{2370}{8064} \times 100$) was calculated.

Hydrogen evolution by the precultured cells prepared from cultures of different nitrogen sources

Precultured cells were prepared from cultures of different nitrogen sources and were used for hydrogen evolution in medium of glucose and L-glutamate as shown in Table 2. The rate of hydrogen evolution was largely enhanced when resting cells from limiting concentration (0.5mM) of NH_4^+ were used. This indicated that the precultured cells from 0.5mM of NH_4^+ had their introgenase as almost "Nitrogenase

Table 2. Hydrogen evolution[a] by the precultured cells prepared differently.

Growth media[b]	Specific rate of hydrogen evolution[c] (μl/hr/mg/ cells)
Glutamate (7mM)	42.4
NH_4^+ (0.5mM)	79.6
NH_4^+ (7 mM)	66.7
N_2 gas (100 m*l*/min)	51.5
N_2 gas (100 m*l*/min) and Glutamate (7mM)	68.9

a. Incubation was stopped after 40 hrs.
b. Gulcose (30mM) was used as carbon source.
c. Production medium was consisted of glucose (30mM) and glutamate (7mM) in Ormerod mineral soln.

A" forms as proposed by Sweet and Burris.[12] Therefore, in order to increase the rate of hydrogen evolution, it could be one way to transfer cultures from limiting concentration of NH_4^+ to glutamate.

Effect of NH_4^+ on hydrogen evolution

NH_4^+, product of nitrogen fixation, is well known as an inhibitor of nitrogenase activity.[3-5,10,12] Effect of NH_4^+ on hydrogen evolution was inhibited as nitrogenase activity in a

Table 3. Effect of NH_4^+ on hydrogen evolution and nitrogenase activity[a].

NH_4^+ cone. (mM)	Specific rate of hydrogen evolution[b] (μl/hr/mg cells)	Nitrogenase activity (μl-ethylene/hr/mg cells)
0	23.9	13.8
0.5	15.6	10.7
1.0	2.3	3.1
7.0	52.9	0
25.0	52.1	0
50.0	45.4	0
100.0	37.6	0

a. Incubation was performed for 26 hrs in glucose (30mM) medium in which L-glutamate was replaced by NH_4^+ as indicated.
b. If NH_4^+ was present at all, lag time of 16 hrs was observed in both of hydrogen evolution and ethylene production. So, the rate was calculated from data obtained after the lag period.

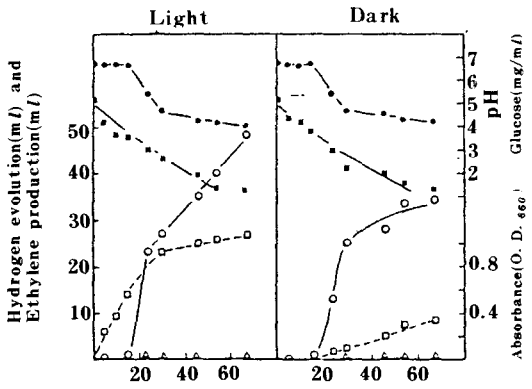

Fig. 7. Hydrogen evolution in glucose(30mM) medium supplemented with NH_4^+(50mM).

- ● : pH,
- ■ : Glucose,
- ○ : Hydrogen evolution,
- △ : Nitrogenase activity,
- □ : Difference of cultural absorbance between initial and final one.

Incubation was performed in 700 m*l* bottle containing 150 m*l* of the reaction mixtures.

Table 4. Effect of CO and hypophosphite on the rate of hydrogen evolution[a]

		Specific rate of hydrogen evolution[b] (μl/hr/mg cells)	
		Light	Dark
Ar (%)	100	46.7	58.5
CO (%)	5	35.1	50.9
	10	25.4	5.3
	30	0.8	4.8
Hypophosphite[c] (mM)	1	0	0
	2	0	0
	5	0	0
	10	0	0

a. Incubation was performed for 40hrs in glucose (30mM) medium in which L-glutamate was replaced by NH_4^+ (50mM).
b. Lag time of about 16 hrs was observed in all cases, therefore the rate was calculated from data obtained after the lag period.
c. Sodium hypophosphite was used.

similar fashion in the low concentrations of NH_4^+. But hydrogen was evolved largely at relatively high concentrations of NH_4^+, where nitrogenase activity still remained to be zero. The discrepancy between hydrogen evolution and nitrogenase activity in NH_4^+ -rich conditions might be due to activity of some anaerobic hydrogenase which is universally present in most photosynthetic bacteria.[1-5] To elucidate the nature of hydrogen evolution in NH_4^+ -rich conditions, we studied the time-course evolution of hydrogen in 50mM of NH_4^+ under light and dark conditions. As shown in Fig. 7, hydrogen was evolved largely even under dark condition as light condition, while nitrogenase activity was total zero under both of conditions. Marked drops in pH were observed in the beginning of hydrogen evolution under light and dark conditions, which indicated that accumulation of some organic acids might occur in broth and be responsible for the hydrogen evolution. Interestingly, no hydrogen evolution was detected in the presence of hypophosphite which is known as formate analog and potent inhibitor of pyruvate formate-lyase[13] (Table 4). In addition, CO gas, inhibitor of hydrogenase,[1,11,13,14] exerted its inhibitory effect on hydrogen evolution with the increasing percents in gas phase. From the result, pyruvate formate-lyase and formic hydrogen-lyase which are known as hypophosphite and CO-sensitive respectively[13] might play an important role in the hydrogen evolution from glucose in NH_4^+ -rich conditions by *Rhodopseudomonas* sp. KCTC 1437.

Thus, hydrogen evolution by this bacterium must be mediated by nitrogenase in NH_4^+ -free or limiting conditions, while it might be mediated by hydrogenase in NH_4^+ -rich conditions. This implies significant advantages in outdoor production of hydrogen because hydrogen could be evolved even under dark and NH_4^+ -rich conditions.

적 요

Rhodopseudomonas sp. KCTC 1437은 글루타민산이 질소원으로 존재할 때, 포도당으로부터 질소고정효소 (nitrogenase)에 의해 효율적으로 수소를 생성하였다. ***in vivo***에서 질소고정 효소의 활성도를 조사해 본 결과, 암모니아 이온의 제한 농도에서 키운 균체가 다른 질소원에서 키운 균체보다 더 큰 정도로 수소를 글루타민산 존재하에서 포도당으로부터 생성하였다. 이 균주는 또한 수소 생성의 억제물질 및 조건으로 알려져 있는 암모니아 이온의 높은 농도와 암조건에서 높은 정도로 수소를 생성했는데 이것은 수소효소 (hydrogenase)의 일종인 포르믹산 수소분해효소 (formic hydrogenlyase)에 의한 것이라 고려된다.

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