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Hydrogen Production by the Immobilized Cells of *Rhodopseudomonas* sp. E15-1

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Rhodopseudomonas sp. E 15-1의 균체 고정화에 의한 수소생성

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For improvement of photobiological hydrogen production, *Rhodopsedomonas* E15-1, a photosynthetic becterium capable of producing a high yield of hydrogen, was immobilized and conditions for hydrogen production by immobilized cells were examined. The optimum concentration for the combined matrix was obtained when sodium alginate was used at final concentration of 4%. The immobilized cells may reduce the inhibitory effects of nitrogen or oxygen. To minimize the diffusion resistance of the nutrients in alginate gel, the bead size less than 2 mm in diameter was desirable. The immobilized cells were also able to utilize a wide range of organic substrates for the production of hydrogen. The hydrogen producing activity of the immobilized cells was maintained for 20 days without loss of activity during semi-continuous operation of the reactor by feeding of new medium periodically and continuous production of hydrogen could be successfully performed for 30 days.

In recent years, considerable worldwide interest has arisen in development of new energy production system. Hydrogen is considered to be an ideal and pollution-free fuel for the future. Many microbial species have been reported to produce hydrogen. Among these microorganisms, the photosynthetic bacteria have possible advantages that they can produce large quantities of hydrogen from many organic compounds in the light(1,2,3,4) and can be effectively used for the treatment of polluted organic waste water (5). Therefore, the concerns with energy production have initiated an amount of researches into the use of photosynthetic bacteria in solar energy conversion system to produce hydrogen. For research along such lines, it is known that photosynthetic bacteria is immobilized to allow continuous operation of the reaction. But, more detailed studies on the nature of hydrogen evolution are required to increase the rate of hydrogen production for possible utilizations of the bacteria with practical purpose.

A long-range goal of this research is the utilization of the photosynthetic bacteria for large-scale production of hydrogen. Therefore, the studies were focused to increase the amount of hydrogen gas produced by photosynthetic bacteria under proper conditions. This report describes the properties and functions of immobilized cell of Rhodopseudamonas E15-1 and the effects of various factors on the hydrogen production from organic compounds by the immobilized cells. The semi-continuous culture by the immobilized cells was also carried out.

Materials and Methods

Organisms

The strains used throughout this work were *Rhodopseudomonas sphaeroides* K-7 and *Rhodo-*

Key words: Hydrogen production, immobilized cell, *Rhodopseudomonas*
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pseudomonas E15-1. *Rhodopseudomonas sphaeroides* K-7 (8,9) was previously isolated and identified by one of authors at KAIST. *Rhodopseudomonas* sp. E15-1 was newly isolated and used because of its excellent capability of hydrogen evolution.

Culture media and conditions

The medium of Ormerod *et al.*(6) was used with slight modification(7) (MOM). To test the utilization of various organic substrates, 30 mM of organic subbstrate and 7 mM of glutamate were added to the basal medium(7).

Cells were grown at 30 °C under anaerobic condition illuminated laterally with two 200-W, incandesent lamps (ca. 10,000-15,000 Lux).

Preparation of resting cell suspension

Resting cells were prepared and stored as previously described[7].

Measurement of hydrogen evolution

Hydrogen evolution was carried out in serum bottles (25 m*l*) containing the medium (MOM) to which resting cell suspension was added. The optical density of the medium was adjusted to 0.5-0.6 at 660 nm and its total volume was 10 m*l*. Capping, gassing with argon and incubation were followed as described[7].

After incubation for a given time, the amount of hydrogen produced was analyzed with a Shimadzu model GC-9A gas-chromatograph equipped with a thermal conductivity detector which was set at 80 °C according to the method previously described(7).

Immobilization of photosynthetic bacterial cells

Carrageenan, alginate or agar was examined as the material for immobilizing the cells. (1) *k*-Carrageenan method: 5 m*l* of a cell suspension containing 5 g wet weight cells in saline solution was mixed with 25 m*l* of a given concentration of *k*-Carrageenan solution at 50 °C. The mixture was rapidly cooled to 10 °C. After 10 mins, the resulting gel was cut into 2×2×3mm. To increase gel-strength, cubic immobilized cells were soaked in 0.3 KCl solution.[4,10] (2) Alginate method; 5 g (wet weight) of whole cells was suspended in 5 m*l* of saline solution and mixed with a given concentration of sodium alginate dissolved in 25 m*l* of saline solution. The mixture was extruded through a syringe (needle size; 19-25G) into cold 0.1 M $CaCl_2$ solution. Then, bead immobilized cells were formed and soaked in cold 0.1 M $CaCl_2$ solution for 12 hr. to stabilize the gel. The bead immobilized cells were washed with distilled water and stored in 0.025 M $CaCl_2$ solution at 4 °C.[11,12] (3) Agar method: 5 m*l* of a cell suspension containing 5 g wet weight cells in saline solution was fully vortexed with 15 m*l* of a given concentration of agar solution which kept at 45 °C. The mixture was rapidly cooled to 0 °C. The resulting gel was cut into 2×2×3 mm and soaked in 0.3 M KCl.[3,13]

For hydrogen evolution of immobilized cells, immobilized cells containing 50 mg wet weight cells per 0.25 m*l* gel were places in a 25 m*l* serum bottle and 10 m*l* of the medium (MOM) was added.

Results and Discussion

Optimum condition of immobilization

There have been several reports (3-5) in which whole cells of photosynthetic bacteria were immobilized by entrapment in matrices such as polyacrylamide, *k*-carrageenan, alginate, cellulose and

Table 1. Effect of various matrix on cell immobilization

Matrix		Total H_2 production (μl/m*l* medium)	
		Strain K-7	Strain E15-1
Carrageenan			
type I	1%	1017	927
	4%	724	859
type II	1%	1005	768
	4%	684	742
type III	1%	982	742
	4%	675	786
Alginate			
	1%	620	628
	4%	975	1100
Agar			
	1.5%	828	793
	2.0%	812	811
	4.0%	913	724

Hydrogen produced was determined by GC, after incubation for 190 hr.

agar. *k*-Carrageenan is chemically stable, but has limits concerning its applicable temperature ranges. Alginate forms gel rapidly in very mild conditions and provides suitable matrix for immobilization by entrapment of whole cell. Accordingly, in order to obtain immobilized cells with large hydrogen productivity and good operational stability, various concentrations of *k*-Carrageenan, alginate and agar were employed for immobilization of *Rhodopseudomonas Sphaeroides* K-7 and *Rhodopseudomonas* E15-1. As shown in Table 1, the optimum concentration for the combined matrix was obtained when *Rhodopseudomonas sphaeroides* K-7 was immobilized in 1% of *k*-Carrageenan and *Rhodopseudomonas* E15-1 in 4% of alginate.

There are various barometers to express the amount of hydrogen evolved. The most used one is μl of hydrogen per hr. mg of cell dry weight, but it is not proper in certain case such that high concentration of immobilized cells produced a large amount of hydrogen. Therefore, milliliter of produced hydrogen per volume of medium was able to be used as a barometer because the ultimate goal of this work is to increase hydrogen productivity by immobilization methods.

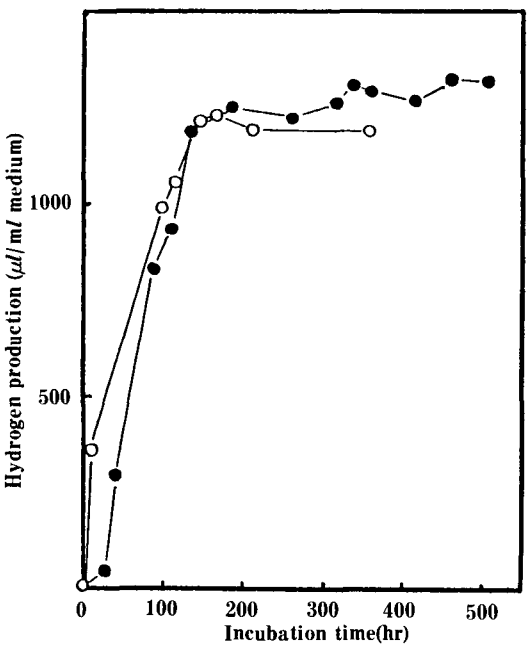

Fig. 1. Hydrogen production from intact cells and the immobilized cells of strain E15-1.
○ - ○ ; Intact cells
● - ● ; Immobilized cells

Comparision in hydrogen production between immobilized and intact cells

The activity of immobilized cells in hydrogen production was compared with that of intact cells (Fig. 1). The results have shown that the hydrogen production by immobilized and intact cell was similar. After immobilized cells were incubated for 14 days, the medium was replaced with new one. And, after further incubation for 140 hr., the amount of hydrogen production was 64% of that of the previous immobilized cells, while intact cells formed trace amount (Table 2). Therefore, immobilization of cells well stabilized their hydrogen-evolving activity for a long time.

Table 3 shows the effects of gas phase on the hydrogen production by alginate-immobilized cells and suspended intact cells. When the gas phase was argon, the largest amounts of hydrogen were evolved. Replacing the gas phase with air, nitrogen, or oxygen resulted in inhibition of hydrogen production. But, immobilized cells had a less inhibitory

Table 2. The stability of immobilization

	Hydrogen production (μl/hr)	
	Intact cells	Immobilized cells
1	86.5	85.5
2	.	54.3

1; Hydrogen produced was determined after incubation for 140 hr.
2; The serum bottle was incubated at 30 °C for 14 days since first assay.
The medium was replaced with new one and then after further incubation for 140 hr, hydrogen produced was determined.

Table 3. Effect of gas phase on hydrogen evolution

	Intact cell		Immobilized cell	
Atmosphere	Total H_2 production (μl/ml medium)	%	Total H_2 production (μl/ml medium)	%
Ar	677	100	745	110
N_2	530	46	657	97
O_2	173	26	320	47
Air	486	72	736	109

Hydrogen produced was determined by GC, after incubation for 48 hr.

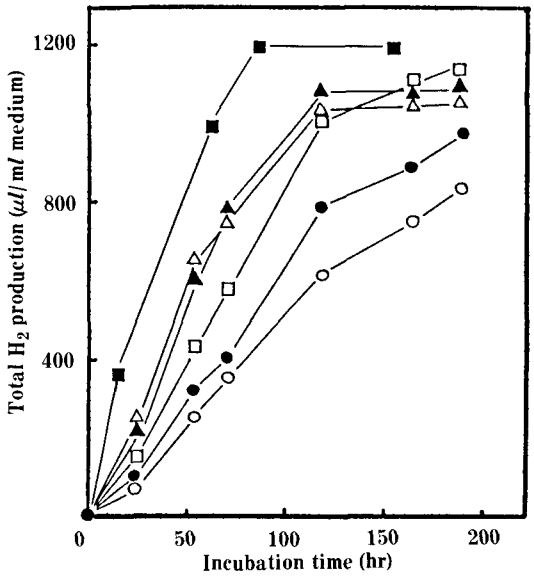

Fig. 2. Effect of bead size on hydrogen production by alginate immobilized cells.

○-○; 3.0mm ●-●; 2.5mm
□-□; 2.2mm △-△; 2.0mm
▲-▲; 1.5mm ■-■; Intact cells

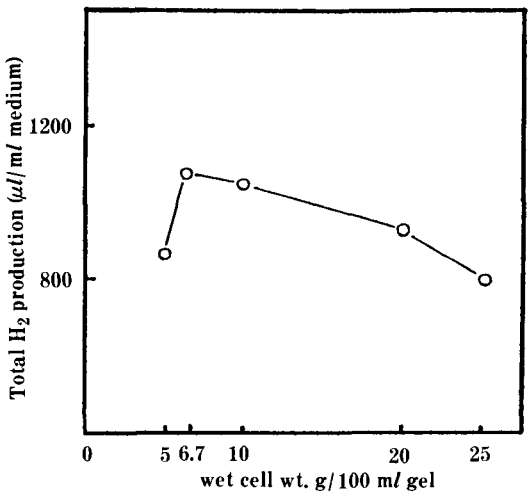

Fig. 3. Effect of cell mass loading in matrix on hydrogen production.

Hydrogen produced was determined after incubation for 200 hr.

effects by nitrogen or oxygen because the enzyme molecules were protected by a diffusion barrior.

Effect of bead size on alginate-immobilized cells

As shown Fig. 2, the productivity of hydrogen was also influenced by bead size of cell immobilization when whole beads of immobilized cell contained approximately same number of cells, the hydrogen production was increased as the bead size was decreased. Because the ratio of surface to volume is increased and the resistance of diffusion of the gel is reduced.

Table 4. Effect of substrates as electron donor on hydrogen production by the immobilized strain E15-1

Substrate (30mM)	H_2 production (μl/ml medium)
Organic acid	
sodium acetate	12
sodium citrate	1196
fumarate	1032
sodium lactate	317
DL-malate	927
pyruvate	339
sodium succinate	296
Saccharides	
fructose	620
glucose	428
galactose	402
mannose	458
xylose	556
cellobiose	491
lactose	430
maltose	656
saccharose	566
cellulose	31
starch	489

Hydrogen produced was measured after incubation for 72 hr.

Effect of cell concentration in alginate gel

To estimate effect of cell mass loading in alginate gel on hydrogen production, each immobilized cells containing 5, 6.7, 10, 20 and 25 g wet weight cells per 100 ml gel respectively was made and incubated for 200 hr (Fig. 3). When immobilized cells of 6.7 g wet cell/100 ml gel was used, the largest amounts of hydrogen were evolved.

Utilization of organic compounds by immobilized cells

It has been known that purple nonsulfar photosynthetic bacteria require various organic acids to

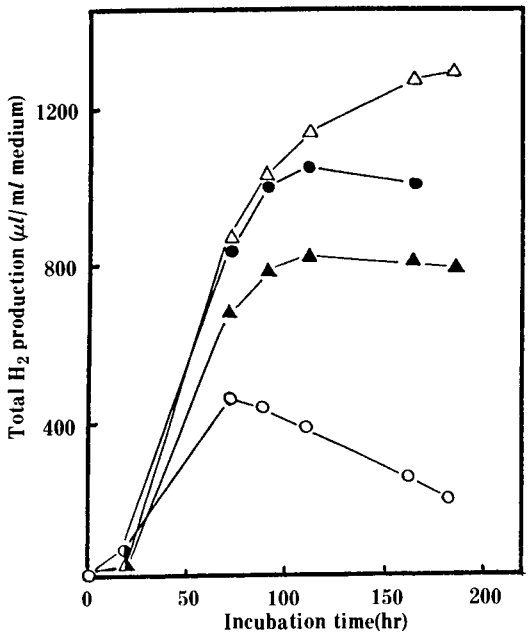

Fig. 4. Effect of citrate concentration on hydrogen production.

○-○; 5mM ●-●; 15mM
△-△; 30mM ▲-▲; 50mM

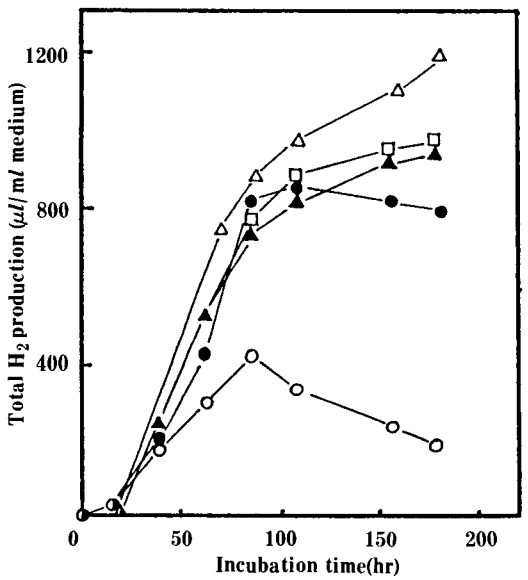

Fig. 5. Effect of fumarate concentration on hydrogen production.

○-○; 5mM ●-●; 15mM
△-△; 30mM ▲-▲; 50mM
□-□; 80mM

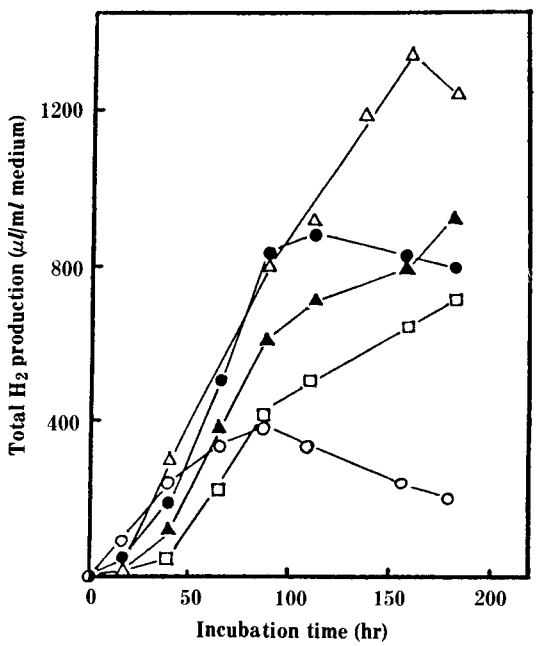

Fig. 6. Effect of malate concentration on hydrogen production.

○-○; 5mM ●-●; 15mM
△-△; 30mM ▲-▲; 50mM
□-□; 80mM

supply additional energy needed for hydrogen evolution.[1,4] The efficiency of various organic acids and sugars as a electron donor was examined on hydrogen evolution with L-glutamate as the source of nitrogen. Among the carbon sources tested, the organic acids such as citrate, fumarate and malate were found to be good substrates for the hydrogen production. This immobilized cells did utilize various saccharides but cellulose as a electron donor (Table 4). Thus, the immobilized cells could produced hydrogen from a wide range of carbon sources as electron donor under the light.

Also, Fig. 4,5, and 6 show the effect of the substrate concentration on the hydrogen production by immobilized cell. The appropriate concentration of malate, fumarate and citrate in the medium was 30 mM on the hydrogen production.

Hydrogen evolution of the immobilized cells by semi-continuous feeding of medium

For semi-continuous production of hydrogen, the bacterial cells immobilized by the alginate were used. The reaction bottle (650 m*l*) was loaded with

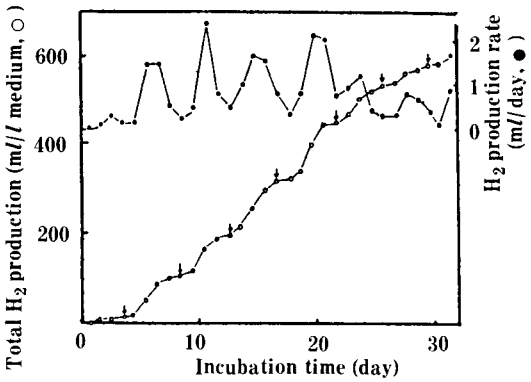

Fig. 7. Hydrogen production of the immobilized cells by semi-continuous feeding of medium.
←; Added new medium

the immobilized cells (containing 1 g wet cells) and 500 m*l* of medium. Once growth was initiated, fresh medium was supplied on every five day at a rate of 10 m*l* per day. The volume of liquid in the growth chamber was maintained constant by allowing a siphon overflow. The hydrogen production was measured at 24 hr. intervals. As shown in Fig. 7, the rate of hydrogen production increased by adding of fresh medium. But, after 20 days the rate decreased very slowly and semi-continuous production of hydrogen could be successfully performed for 30 days.

When continuous hydrogen production of immobilized cell was campared with that of intact cells by experiment of Kim *et al.*[15] total hydrogen production of the former is slightly inferior to of the latter. It implied that amounts of immobilized cells used were inappropriate to the volume of the medium, because wet weight of intact cells amounts to 4 grams per liter of medium at late exponential phase. Therefore, the concerns with consumption of substrate solution may result in a large amount of hydrogen evolution.

For the continuous hydrogen production with immobilized cells, there are two main problems to be solved. The first is prolonged maintenance of the cell functions by means of an improved method of cell immobilization. The second is how to supply the feeding substrate so as to maintain their concentrations and the pH at the optimum values. Thus, more detailed researches are required for the efficient hydrogen production with immobilized cells.

요 약

광합성 세균의 수소생성 향상을 위한 노력의 일환으로 수소생성능이 좋은 *Rhodopseudomonas* E 15-1을 고정화하여 수소생성에 적당한 조건을 조사하였다. 담체로 4%의 alginate를 사용하였을 때 수소생성량이 많았으며 고정화함에 따라 산소, 질소에 대한 억제효과를 덜 받았다. Alginate 고정화 세포는 그 지름을 2 mm 이하로 하는 것이 겔내로의 확산저항이 적으므로 적당했다. 여러가지 유기물을 활용하여 수소를 생성할 수 있었으며 특히 citrate, fumarate와 malate 같은 유기산의 경우 30 mM의 농도가 수소생성에 있어서 적당하였다. 또한 고정화 세포를 이용한 계속적인 수소생성에 있어서 20일부터 활성이 감소하기는 하나 겔의 구조적 안정성은 30일까지 유지되었다.

사 사

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