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Hydrogen Production in Polyvinyl-Immobilized *Anabaena azollae* Cells

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Polyvinyl에 고정화된 *Anabaena azollae*에서의 수소생성

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ABSTRACT

Physiological and morphological characteristics of *Anabaena azollae* cells immobilized in a synthetic polymer, polyvinyl(PV), were investigated. The cell density of the non-immersed PV foam reached 4.4mg Chl/g dry wt. PV foam. This is 8 times higher than that of PV-immobiliz ation in immersed batch system. And MSX-induced ammonia productivity and the photosynt-hetic oxygen evolution activity are higher than that of free cells after short-term dark storage. Nitrogenase activity and thermostability of photosynthetic activity are also higher than that of free *Anabaena* cells after immobilization. Total hydrogen production reached to 1.6ml H_2 per reactor(total 4mg Chl) after 6 days.

INTRODUCTION

Under the impetus of recent energy shortage, much research effort has been spent on alternative energy pro-duction, with hydrogen being one of desired future fuels. Demonstrated systems for biological hydrogen pro-duction include; cell-free mixtures of isolated plant chlo-roplasts and bacterial hydrogenase; organisms capable of biophotolytic conversion of water to hydrogen and oxygen; and organisms that produce hydrogen through fermentation of organic carbonic substrates(1-3).

Cyanobacteria are good sunlight harvesters and are easy and economical to grow. So there have been many app-roaches on a potential use of cyanobacteria for the prod-uction of ammonia, pharmaceuticals, single cell proteins and hydrogen. Cyanobacteria are able to fix atmospheric nitrogen via ATP-dependent nitrogenase activity. Electrons, reductant and ATP are derived from photosynthesis. The nitrogenase enzyme is oxygen-sensitive. However, in most N_2-fixers, this enzyme is localized in specialized cells, hetercysts, which lack photosystem II and have an enve-lope impermeable to oxygen. Many species of cyanobac-teria, heterocystous as well as nonheterocystous, can evolve hydrogen when grown under specified condition. Hydrogen evolution in cyanobacteria is catalysed mainly by nitroge-nase and is ATP-dependent.

A large number of studies have been done in recent years on the immobilization of microorganisms for the production of pharmaceuticals, chemicals and fuels. And there is some evidence that immobilization of cells may lead to increased yields in the biotransformation of natural products(4-5). For immobilization several matrices were

used: such as, alginate, carrageenan, polyurethane(PU), PV, and polyvinylalcohol(PVA) etc. Among these matrices, synthetic polymer is a best candidate for immobilization because the price is lower than that of natural polymer and diffusion of nutrient and gas is better. So there is a good potential for the use of synthetic polymer in the production of some chemicals using symbiotic filamentous cyanobacteria, as the separation of products from the cells is easy and there is less chance of contamination in practical reactors. However, some polymers are slightly toxic to the cell. Another problem encountered with polymer foams is that the cell entrapment or adsorption is very loose(although mucilage was excreted and attached to the surface of polymers). So the cells can easily leak out from the foam when the reactor is bubbled or stirred during reaction.

This work reports some physiological and morphological characteristics of immobilized *Anabaena* cells in synthetic polymer and continuous photoproduction of hydrogen in a "trickling-medium" bioreactor using immobilized *Anabaena* cells.

MATERIALS AND METHODS

Culture

Anabaena azollae, a presumtive isolated from *Azolla filiculoides*, obtained from Dr. Tel-Or(Hebrew University, Rehovot, Isdrael), was grown in medium BG-11(6) without combined nitrogen at 27℃ under cool white fluorescent lamps at a photon flux density of 100μE · m^{-2} sec^{-1}. The cells were grown in a 5% CO_2/air mixture in 250ml Elernmeyer flask kept agitated on a rotary shaker at 125−140 rpm. Cell growth was determined by the chlorophyll concentration in the culture suspension.

Immobilization

Hydrophilic polyvinyl(PV: PR22/60, Caligen Foam Ltd., Accrington, Lancs., U.K.) foams were cut into 5㎜ cubes and washed 3−5 times in distilled water for a few days with the bubbles being removed from the foam after each wash by squeezing. After washing, the foams were autoclaved and then used to immobilize *Anabaena* cells by adsorption metod(7)

Nitrogenase Activity

Nitrogenase activity was determined as acethylene reducton(8). Ethylene formation was followed with a gas chromatograph equipped with a flame ionization detector and a Poropak S column operated at 45℃ with nitrogen as carrier gas. Incubation were in 7.5ml growth medium. The gas phase was 10% C_2H_2 in argon.

Hydrogen production

Hydrogen was measured using a gas chromatograph(Taylor Servomex, Crowborough, U.K.) fitted with a thermal conductivity detector and Porapak Q column(9).

Photosynthetic oxygen evolution

Photosynthetic oxygen evolution activity was detrermined with Clark type oxygen electrode (Rank Brothers, U.K.) at 27℃ with illumination of incandescent light(2000μE · m^{-2} sec^{-1}) through an orange filter(10).

Determination of Ammonia

For chemical determination of ammonia, *Anabaena* cells were suspended in fresh culture medium with 100uM L-methioninesulfoximine (MSX). After incubation of 3 h, the cell suspension was centrifuged at 2500xg for 10 min and the content of ammonia in the supernatant was determined according to Solorzano(11).

Chlorophyll determination

Chlorophyll content was calculated from the absorbance at 665nm after extraction with 95% methanol(12).

Bioreactor

"Trickling-medium" column bioreactor using PV-immobilized *Anabaena* cells(Fig. 1) was made of water jacketed Pyrex glass column(18×280㎜). The column was filled with 100 pieces of sterilized PV-foam(5㎜ cubes). Immobilization was initiated by flushing with free cell culture suspension. During immobilization, air(10L/min) and the medium (6.25ml/h) trickling through the foams were supplied from the top of the column. Illumination was with fluorescent lamps(100μE · m^{-2}sec^{-1}) and temperature was 27℃.

Scanning Electron Microscopy

Immobilized cyanobacterial cells were viewed by critical point dry method. Cells were fixed in 2.5% glutaraldehyde

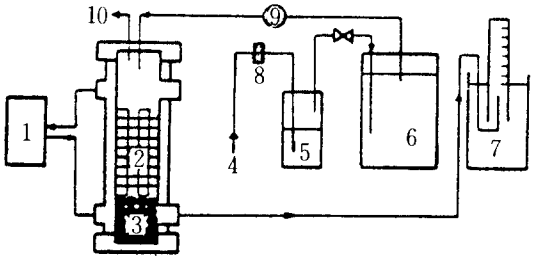

Fig. 1. Schematic diagram of "trickling-medium" reactor for hydrogen production using PV-immobilized *A. azollae* cells. 1, water circulator; 2, PV-immobilized cell; 3, glass bead; 4, argon gas; 5, humidifier; 6, medium; 7, gas trap; 8, air filter; 9, peristaltic pump; 10, gas sampling port.

in 0.1 M phosphate buffer(pH7.2) containing 6% sucrose (w/v) for 2 hr at room temperature. The samples were washed in the same buffer, transferred to 1.0% osmium tetroxide in buffer under the same condition. The speciemen were washed and dehydrated in graded ethanol(50−100%) and dried in a Samdri−780 Critical Point Drying Apparatus(Tousimis Research Corp., U.S.A.) using liquid carbon dioxide. Dried speciemen were coated in a Sputter Coater (EM-Scope, U.K.) and examined in a model S−510 Scanning Electron Microscope(Hitachi Scientific Instruments Co., Japan) at accelerating voltage of 15 or 25 KV(13).

RESULTS AND DISCUSSION

Immobilization of cyanobacteria

Table 1 shows the cell density of the several immobilized and free batch culture systems. It is clear from the table that the cell density in both immobilized and "trickling-medium" reactor using immobilized cells was higher than that of free cells. And the cell density of the non immersed "trickling-medium" reactor reached 4.4mg Chl/g dry wt PV foam. This is 8 times than that of PV-immobilization in immersed batch system.

Physiological Characteristics of immobilized cells

Table 1. Composition of cell density in free batch culture and PV-immobilized systems of *Anabaena azollae* cells

Type of culture	Cell density
Free batch culture	45μg Chl / ml medium
PV-foam in batch(immersed)	0.43mg Chl / g dry wt PV foam
PV-foam in reactor (Non-immersed)	1.6−4.4mg Chl / g dry wt PV foam
	129−380. 7μg Chl / ml medium

Immobilized *Anabaena* cells showed continued stabilities on long storage. And Sometimes immobilized cells exhibit higher physiological activity than that of free cells(14). We tested the short-term stabilities of some physiological activities after short-term dark storage(Fig. 2). When the ammonia productivity(it reflects the nitrogen metabolism) in the presence of MSX(100μM) was measured after dark storage, the rate rapidly decreased in free cells but in PV-immobilized cells, the activity was almost same or even increased after dark storage. And also the photosynthetic oxygen evolution activity of PV-immobiliz-

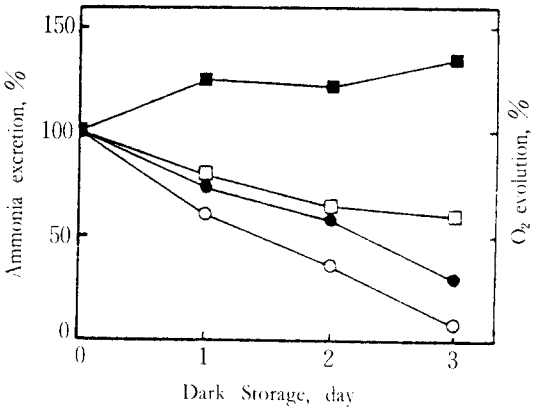

Fig. 2. Photosynthetic oxygen evolution activity (○) and MSX-induced ammonia production rate(□) in free (open) and immobilized(closed) *A. azollae* cells after dark storage.

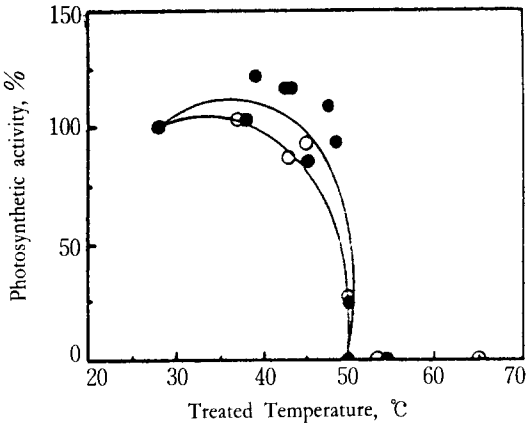

Fig. 3. Thermostability of photosynthetic oxygen evolution activity in free(open) and immobilized(closed) *A. azollae* cells after heat treatment. the cells were treated for 5 minuters and then O_2 evolution activity was measured.

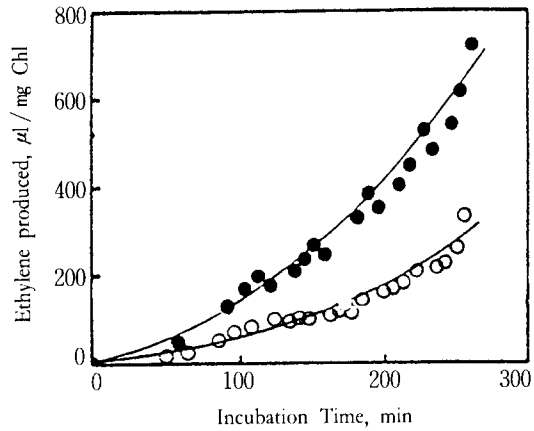

Fig. 4. Nitrogenase activity in free(open) and immobilized(closed) *A. azollae* cells.

ed *Anabaena* cells is also higher than that of free *Anabaena* cells after dark storage. It shows higher stability of PV-immobilized cells compared to that of the free cells. Fig. 3 shows the photosynthetic activity after heat treatment for 5min at various temperatures. Thermostability of immobilized cells is also higher than free *Anabaena* cells. Yamamoto *et al.* (14–15) reported the increased yields of metabolite production after immobilization of bacteria in polyacrylamide metrices. And Brouers *et al.*(16) showed that immobilization increase and stabilized the nitrogenase activity in *Anabaena* cells. We compared nitrogenase activity of free and immobilized *Anabaena* cells. The nitrogenase activity of *Anabaena* cell increased after immobilization(Fig. 4).

This increase of nitrogenase activity may be due to the increase of heterocyst increasement(17). With regard to the changes in metabolic behavior, Mattiason and Hahn-Hargerdal(18) proposed a model in which decreased water activity results in the changed metabolic activity and product formation of immobilized cells. The changed water activity would be due to the high polymer concentration of the microenvironment; these macromolecules "organize" water and thereby decrease the amount of water available to the cells. The work of Holeberg and Margalith (19) and Kraube *et al.*(20) is in agreement with this hypothesis and shows that the presence of polymer even at a low level has substantial effects on biochemical reactions which are water-dependent. And Shie *et. al.*(17) suggested that the increased yield of ammonium upon immobilizing of cyanobacteria in polymer was at least partly related to the changes in membrane permeability by the immobilization process itself. So it can be inferred that in our case the PV polymer has an effect on water activity and cell membrane permeability and as a consequence on the metabolism of the cells.

Continuous production of hydrogen by nitrogenase

There are several approaches on the photoproduction of hydrogen using immobilized cyanobacteria(12, 21, 22). And a few investigators tried to make a bioreactor for the hydrogen production using immobilized cyanobacteria(13). But all the experiments were performed in fluid-bed bioreactor in which the immobilized matrix and cells were fully immersed to medium. So there are some problems such as gas diffusion or nutrient diffusion. So in most cases, the reactors were bubbled with gas. In this case, many cells leaked out into the medium from the matrix. Therefore the packed bed reactor has been found unsuitable for algal cell reactor immobilized by adsorption methods. So we tested the hydrogen evolution from "trickling-medium" reactor in which the medium

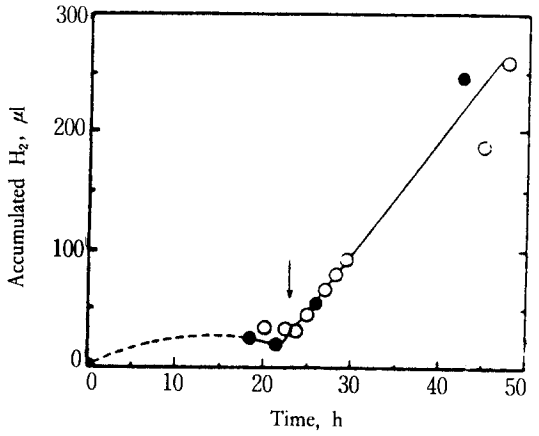

Fig. 5. Effect of CO injection into the bioreactor on the production of H_2 by nitrogenase in "trickling-medium" reactor using PV-immobilized *A. azollae* cells, Arrow shows CO(4%) point.

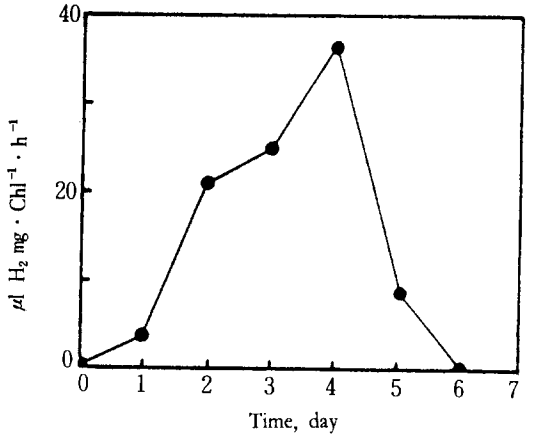

Fig. 6. H_2 production rate of the "trickling-medium" reactor using PV-immobilized *A. azollae* cells.

was trickled from the top of the bed of PV foam of the column reactor. In the column reactor, the immobilized cells were not fully immersed but soaked only. So there is no cell leakage from the PV foam. In the beginning, hydrogen production was observed in the absence of uptake hydrogenase inhibitor(carbon monooxide), however, after the first day the hydrogen concentration gradually

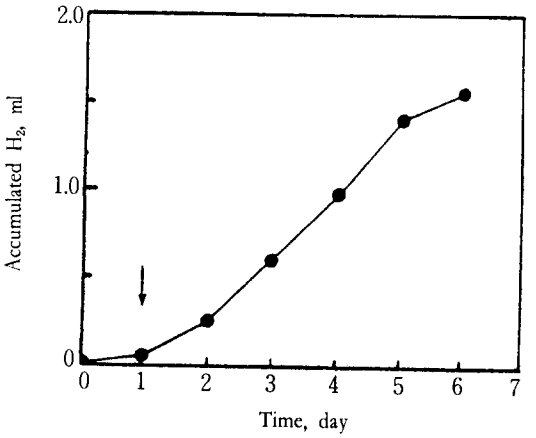

Fig. 7. Cummulative H_2 production in "trickling-medium" reactor. The reactor contained PV-immobilized *A. azollae*(4 mg Chl) cells. Arrow shows CO(4%) point.

decreased. When carbon monooxide was injected to the column reactor, hydrogen evolution increased significantly(Fig. 5). This means the decrease is due to the uptake of hydrogen by uptake hydrogenase enzyme. In the presence of carbon monooxide, hydrogen evolving rate increased for 4 days and thereafter the rate gradually decreased(Fig. 6). Total hydrogen production reached to 1.6ml H_2 per reactor(total 4mg Chl) after 6 days(Fig.7).

Electron microscopy of PV-immobilized *Anabaena* cells

Fig. 8 shows the electron microscopy of *Anabaena* cells in PV foam. It shows good adsorptive immobilization and growth. The mechanisms of adhesion and retention of cyanobacteria to the polymer foam surface is not yet well understood. From studies on mammalian cell adsorption, it was concluded that a primary reversible interaction between the cell and the solid surface must somehow induce a secondary irreversible interaction(23). And Shi et al.(17) suggested that a primary reversible charge interaction between cells and charged groups of the matrix surface induced a secondary interaction related to the formation of the mucilagionus envelope.

Naturally *A. azollae* live in the leaf cavity of *Azolla* symbiotically(17). So there is a possibility that the sym-

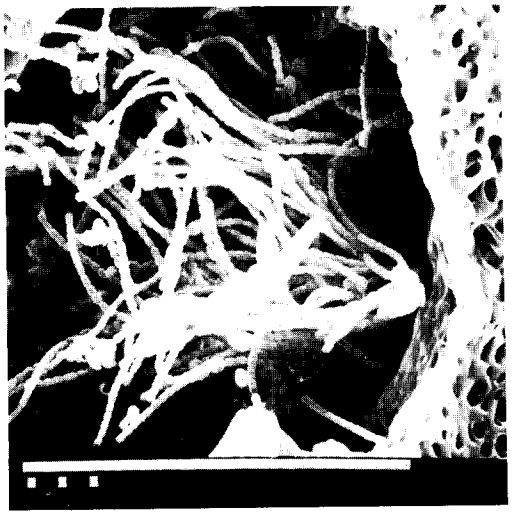

Fig. 8. Scanning Electron Microscopy of *A. azollae* cells immobilized in PV foam. Bar indicates 100μm.

biotic cyanobacteria may be more suitable than free living organism for immobilization. Because the matrix may act like as a host tissue and provide mimic natural environments.

요 약

본 연구에서는 PV에 아나베나세포를 흡착법에 의하여 고정화하였다. 진탕배양하면서 고정화한 경우에 비하여 반응기속에 배양액을 trickling 시키면서 배양하고 고정한 경우 높은 세포밀도를 보였다. PV에 고정화한 세포들의 생리적 활성을 조사한 결과 광합성능이 암처보관시에 free cell에 비하여 안정됨을 보였고, msx처리시의 암모니아의 방출도 증가하였다. 뿐만 아니라 고정화한 세포에서 광합성활성의 온도에 대한 내성이 증가하였으며 nitrogenase 효소 활성도 free cell에 비하여 크게 증가하였다. nitrogenase효소에 의한 연속적인 광수소발생을 조사한 결과 혐기적 조건하에서 연속적으로 수소가 생성됨을 볼 수 있었는데 그 발생양은 4mg의 엽록소에 해당하는 아나베나를 충진한 trickling-medium 반응기에서 6일동안 1.6ml의 수소가 생성되었다.

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