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Hydrogen production by immobilized cells of unicellular halotolerant cyanobacterium *Aphanothece halophytica* in alginate beads

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Abstract

Hydrogen is an interesting alternative energy carrier that can be produced by various kinds of cyanobacteria. The unicellular halotolerant cyanobacterium *Aphanothece halophytica* is one of the potential cyanobacterial candidates for H_2 production. Its dark fermentative H_2 production is catalyzed by bidirectional hydrogenase activity through the catabolism of storage glycogen. This work aimed to study H_2 production by *A. halophytica* cells immobilized in alginate beads. The result showed that under nitrogen deprivation H_2 production by the immobilized cells of *A. halophytica* in alginate cells was obviously higher than that of free cells. The highest H_2 production was found in immobilized cells prepared from 4.5 % (w/v) sodium alginate in 100 mM calcium chloride. Finally, H_2 production yield of 50 immobilized cell beads per 20 mL glass vial was higher than of 100 and 150 immobilized cell beads.

Keywords : *H_2 production, Aphanothece halophytica, Immobilization, Alginate bead*

1. Introduction

Nowadays, fossil fuel is mostly used as an energy source in all countries over the world. It is going to be diminished in the next future. The combustion of petrol derived from fossil fuels usually releases carbon dioxide, carbon monoxide, methane, and other toxic gases that causes an environmental pollution. Therefore, the alternative energy has been intensively attractive. Hydrogen is one of alternative potential energy sources because it provides a high heating value of 141.6 MJ kg^{-1} (1) and its combustion generates the environmental-friendly products.

H_2 can be produced from several kinds of microorganism, such as fermentative bacteria, photosynthetic bacteria, green algae and cyanobacteria. Cyanobacteria produce H_2 via a nitrogen fixation process by nitrogenase activity and/or through a photosynthetic process by a bidirectional hydrogenase activity (2,3). Bidirectional hydrogenase can receive electrons from two processes. First, solar energy is captured at photosystem II and then water-splitting occurs. This process mediates protons, electrons and oxygen. The electrons are transferred via photosystem to reduce ferredoxin. Finally, hydrogen atoms will accept electrons from ferredoxin and produce hydrogen via bidirectional hydrogenase. Second, storage glycogen can be degraded under dark anaerobic condition, resulting in gaining the electrons in the cells. These electrons are shuttled to bidirectional hydrogenase for H_2 production (4).

The unicellular halotolerant cyanobacterium *Aphanothece halophytica* has been reported to be one of the potential H_2 producing cyanobacteria (5,6). It mainly produces H_2 under dark anaerobic fermentation. To enhance the efficiency of H_2 production in *A. halophytica*, cell immobilization in alginate beads was investigated in this study. The advantages of cell immobilization are that it can protect enzyme activity and/or cells from an external environment by separating cells from a liquid phase (7). Furthermore, immobilization avoids cells from forming clumps. The cell entrapment in alginate is widely used for immobilization. The immobilized cells are free in solution but limited in a movement by the lattice structure of a gel. The pore size of gel allows medium to freely flow-through. Alginate is an anionic polysaccharide composed of α-(1,4)-linked L-guluronic and β-(1,4)-linked D-mannuronic acid residues. The gel formation occurring at room temperature causes an electrostatic interaction between the carboxylic groups on the guluronic acid residues and the divalent ions (Ca^{2+}). The polymerization process of alginate solution between 3-6% (w/v) is stable (8). In addition, alginate is a low-cost material and can be produced from a renewable resource in a large scale. In this research, we focused on H_2 production by alginate immobilized cells of the halotolerant cyanobacterium *A.halophytica* under dark anaerobic fermentation.

2. Materials and methods

2.1 Growth condition

The cyanobacterium *A. halophytica* was grown in a 250-mL Erlenmeyer flask containing 100 mL of BG11 medium supplemented with Turk Island salt solution (pH 7.6) (9). The medium consisted of 17.6 mM $NaNO_3$, 30 mM $MgSO_4.7H_2O$, 0.189 mmol C L^{-1} Na_2CO_3 and 0.5 M NaCl as a main component. For $BG11_0$ medium, $NaNO_3$ is removed from BG11 medium. The cells were cultivated in an incubator shaker with a shaking speed of 120 rpm at 30 °C under the light intensity of 30 μmol photons m^{-2} s^{-1} for 7 days.

2.2 Cell immobilization

A. halophytica was grown as previously described for 7 days. The cells were harvested by centrifugation at 5,000×g at 4 °C for 10 min, washed twice and resuspended in 10 mL of $BG11_0$ (Nitrate-free BG11) medium. The cell culture containing approximately 1 mg dry

cell weight per mL was mixed with 3% (w/v) sodium alginate. The mixture was dropped into 100 mM calcium chloride through a 10-mL syringe (10). The immobilized cells were immersed in calcium chloride solution for at least 2 h and washed twice with $BG11_0$ medium before use. To investigate the effect of aginate gel concentrations on H_2 production, cells were entrapped with 3%, 3.5%, 4%, 4.5% and 5% (w/v) sodium alginate.

2.3 Measurement of H_2 production

One hundred immobilized cell beads were added in a 20-mL glass vial containing 10 mL of $BG11_0$ medium. The glass vial was tightly sealed with a rubber stopper carrying an aluminum rim and incubated at 30 °C under the light intensity of 30 μmol photons m^{-2} s^{-1} for 24 h in order to induce H_2 production under nitrogen–free condition. After incubation, air in glass vial was removed from a vial by purging argon gas for 10 min. H_2 measurement in the head space was performed in the dark. To study the effect of immobilized beads amount on H_2 production, 50, 100 and 150 immobilized beads were transferred to a 20-mL glass vial. H_2 in the gas phase was determined by Gas Chromatograph (GC) (Shimadzu 15-A, Japan) using a molecular sieve 5°A (60/80 mesh) packed column and a thermal conductivity detector. The GC condition was shown as previously described (5).

2.4 Chlorophyll a determination

Chlorophyll a in the immobilized cells was extracted with 90 % (v/v) methanol at 70°C for 15 min. The chlorophyll extract was determined by measuring an absorbance at 665 nm (11).

3. Results

3.1 H_2 production of immobilized cells under nitrogen deprivation

A. halophytica cells grown in medium for 7 days were harvested by centrifugation and resuspended in BG11 and $BG11_0$ medium. The immobilized cells containing a dry cell weight of 1 mg mL^{-1} were prepared by entrapment cells with 3% (w/v) sodium alginate in 100 mM calcium chloride. One hundred immobilized cell beads were transferred to a-20 mL glass vial containing the indicated medium and further incubated in the light for 24 h. After incubation, H_2 production of immobilized cells was measured. It was found that H_2 could not be detected in the free and immobilized cells incubated in complete BG11 medium (data not shown). In addition, H_2 production of the immobilized cells incubated in N-deprived medium were significantly 2-3 folds higher than that of free cells (Fig. 1). The highest H_2 production with 4.637±0.326 μmolH_2 mg chl a^{-1} was found in immobilized cells incubated in $BG11_0$ for 3 days (Fig. 1).

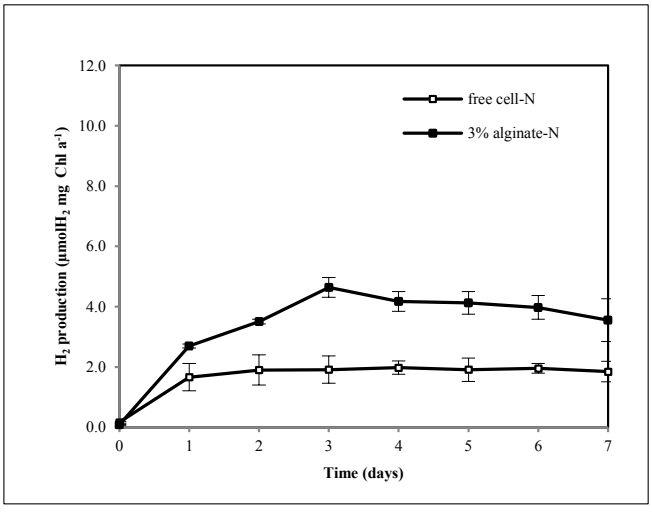

Figure 1. H_2 production by the free and immobilized cells of *A. halophytica* in nitrogen-deprived BG11 medium ($BG11_0$) supplemented with Turk Island salt solution

3.2 Effect of alginate concentrations on H_2 production of immobilized cells

A. halophytica cells were immobilized with 3%, 3.5%, 4%, 4.5% and 5% (w/v) sodium alginate in 100 mM calcium chloride and subsequently transferred to a 20-ml glass vial containing 10 mL of $BG11_0$ and further incubated in the light for 24 h prior to H_2 measurement under darkness. The result revealed that the immobilized cells in 4.5% (w/v) alginate gel gave the highest H_2 yield with 10.459±1.239 µmolH_2 mg chl a^{-1} whereas those in 5% and 4% (w/v) alginate gel gave the H_2 production with 9.626±0.089 and 9.385±0.335 µmolH_2 mg $chla^{-1}$, respectively (Fig. 2).

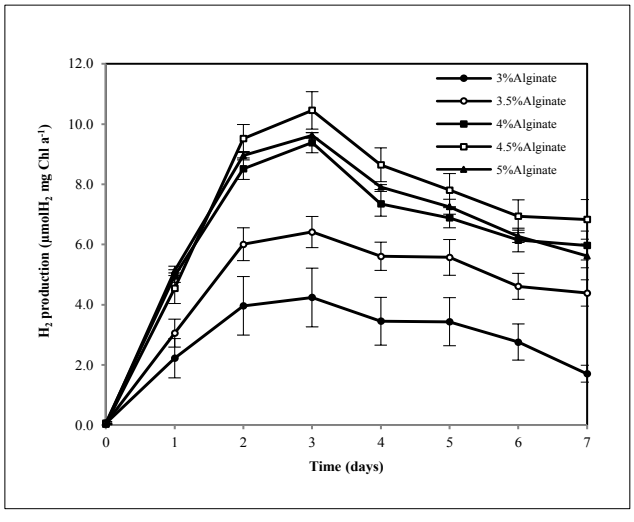

Figure 2. Effect of alginate gel concentrations on H_2 production by immobilized cells of *A. halophytica.* Cells were entrapped with 3%, 3.5%, 4%, 4.5%, and 5% (w/v) sodium alginate in 100 mM calcium chloride

3.3 Effect of cell bead number on H_2 production in immobilized cells

H_2 production of the 50, 100 and 150 immobilized cell beads per a 20-mL glass vial, prepared by mixing cells with 4.5% (w/v) sodium alginate solution and dropping in 100 mM calcium chloride solution, was investigated. The result showed that the highest H_2 production with 19.241±0.507 μmolH_2 mg chl a^{-1} was obtained in a glass vial containing 50 immobilized cell beads. It was approximately 2 folds higher than H_2 produced by 100 and 150 immobilized cell beads (Fig. 3).

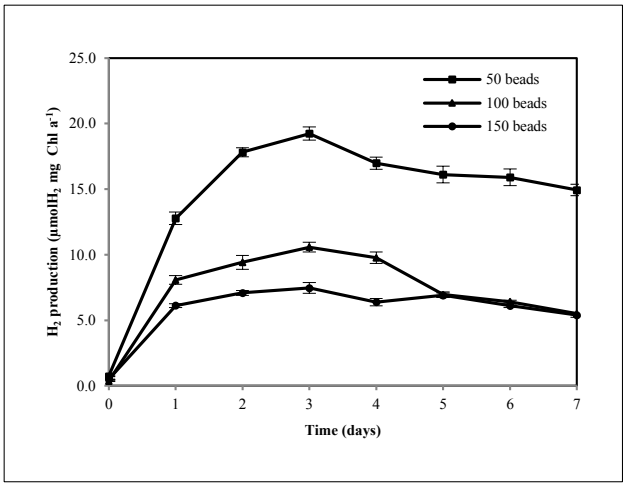

Figure 3. Effect of immobilized cell bead number (50, 100, and 150 beads per 20 mL vial) on H_2 production in *A. halophytica*

4. Discussion

Aphanothece halophytica is a halotolerant cyanobacterium which can grow in extreme NaCl concentrations (upto 3.0 M NaCl) (12). It can produce H_2 by a bidirectional hydrogenase activity under a dark anaerobic fermentation. Under this condition, glycogen accumulated in cells is consumed and the released electrons are transferred to bidirectional hydrogenase enzyme for generating H_2 (5,6). In this study, H_2 production of *A. halophytica* incubated under complete BG11 medium could not be detected (data not shown). It was suggested that under enriched nitrogen condition, cyanobacteria use nitrogen atoms, ATP and NAD(P)H for protein synthesis, resulting that NAD(P)H is not sufficient for being an electron donor for H_2 production (13).

In our result, H_2 production of free and immobilized cells of *A. halophytica* incubated in nitrogen-free medium was detectable (Fig. 1). Under N-deprivation, cyanobacteria decrease protein synthesis but accumulate glycogen within cells instead. When cells are transferred to the anoxic condition, storage glycogen will be consumed as electron donor. The released electrons are used to generate H_2 molecules by a function of the bidirectional hydrogenase enzyme. This result was similar to the previous study investigated in the same microorganism *A. halophytica* that in $NaNO_3$-free medium cells produced the highest H_2 production rate, an approximately fourfold increase compared to that of cells grown in the normal BG11 medium containing $NaNO_3$ (5). In addition,

cyanobacterium *Arthrospira maxima* stimutated 4.2-fold H_2 production yield after transferring cells to the NO_3 depletion medium for 24 h (4).

Immobilization in alginate gel is one of the popular entrapment methods. Alginate is a linear polymer composed of D-mannuronic and L-guluronic acid. The divalent Ca^{2+} cations bind preferentially to the monomer of L-guluronic acid. Figure 1 showed that under nitrogen deprivation H_2 production by the immobilized cells of *A. halophytica* in alginate beads was higher than that of free cells, suggeating that cell immobilization with the alginate entrapment can protect bidirectional hydrogenase from the external environments including O_2, a cyanobacterial strong hydrogenase inhibitor. The immobilized cells reduce the growth and other metabolisms, balance the intracellular energy and excessive electrons by generating H_2. This result was in agreement with the previous study in immobilized cells of cyanobacterium *Lyngbya perelegans*. H_2 production of *L. perelegans* immobilized in alginate and agar was 2-4 times higher than that in free cells during 24 h (14).

For studying the effect of alginate concentrations on H_2 production by immobilized cells of *A. halophytica*, it was shown that immobilized cells of *A. halophytica* in 4.5% (w/v) alginate gel showed the highest H_2 production (Fig. 2). Gel concentration affects the cellular survival and enzyme activity. An increase of gel concentration in immobilized cells can enhance efficacy of cell survival and enzyme activity. The previous study in filamentous cyanobacterium *Calothrix* 336/3 showed that immobilization cells with 4% (w/v) sodium alginate in 50 mM $CaCl_2$ solution caused the improvement of H_2 production and H_2 was prolong produced over several cycles (15). However, a higher concentration of alginate might decrease H_2 production. In our result, immobilized cells in 5% (w/v) alginate gel produced less H_2 than those in 4.5% (w/v) alginate gel. It was due to the high density of gel influencing the passing of substance and medium, as well as the release of H_2 from the cells. Figure 3 showed that 50 immobilized cell beads per a 20-mL glass vial produced H_2 yield higher than 100 and 150 immobilized cell beads. It was indicated that 50 immobilized cell beads is the suitable bead number for a 20-mL glass vial. An increase of immobilized cell beads causes the decrease of surface area, and the low gas and substance exchange in a vial.

By comparison of H_2 production rate with other immobilized cyanobacterial cells, H_2 production rate with 0.532 $\mu molH_2$ mg chl a^{-1} h^{-1} by the immobilized *A. halophytica* cells prepared from 4.5% (w/v) sodium alginate in 100 mM $CaCl_2$ was higher than that by filamentous cyanobacteria *L. perelegans* and *Oscillatoria subbrevis* strain 111 which produced H_2 at a rate of 0.024 $\mu molH_2$ mg dry wt^{-1} h^{-1} and 0.019 $\mu molH_2$ mg chl a^{-1} h^{-1}, respectively (14,16) but it was too low to compare with that of a diazotrophic cyanobacterium *Calothrix* sp.336/3 immobilized with 4% (w/v) sodium alginate in 50 mM $CaCl_2$ which showed the maximum H_2 production rate of 3,500 μmol H_2 mg chl^{-1} h^{-1} (15). However *A. halophytica* has some advantages for cultivation and H_2 production because it could grow in sea water supplemented with only 1.76 mM $NaNO_3$ and showed a long-term H_2 accumulation (6).

5. Acknowledgements

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6. References

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