Hydrogen Production by the Green Alga *Scenedesmus* sp. KMITL-O1 under Heterotrophic Conditions

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Abstract

Biohydrogen is a clean alternative energy produced by a number of different organisms. Green microalgae can produce hydrogen via the photobiological-H₂ production process. Hydrogen production of a unicellular green alga *Scenedesmus* sp. KMITL-O1 isolated from a natural pond occurred photoheterotrophically in medium containing acetate. The highest hydrogen was produced when cultivated cells in TAP medium for 18 hours under light and then incubated under anaerobic adaptation for 2 hours.

Keywords: H₂ production, Green algae, Heterotrophic condition, *Scenedesmus* sp.

1. Introduction

Hydrogen is considered to be the most promising energy for the future. Nowadays in industry it is produced by a thermo-chemical transformation of fossil fuels or by electrolysis of water [1], however it can be produced by various kinds of microorganisms such as microalgae, photosynthetic bacteria, cyanobacteria and other bacteria. Among these organisms hydrogen metabolism of photosynthetic eukaryotic algae has been intensively studied. It was firstly reported in the 1940s that the green alga *Scenedesmus obliquus* can use hydrogen as an electron donor to fix CO₂ under anaerobic condition in the dark [2-3]. *S. obliquus* in particular can utilize the energy of sunlight in photosynthesis to produce hydrogen [4]. Besides this strain, hydrogen evolution has been also investigated in other kinds of green algae such as *Chlamydomonas reinhardtii* [5-6] and *Chlorella vulgaris* [7]. One of the advantages of green algae for producing hydrogen is their ability to grow under photoautotrophic and photoheterotrophic condition [8]. In this study the green alga dominating a natural pond was isolated to study the hydrogen production under the

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photoheterotrophic cultivation and to identify to species by 18S rDNA nucleotide sequence analysis. Factors affecting hydrogen production such as the growth period, anaerobic adaptation time and dark-light condition were also studied.

2. Materials and Methods

2.1 Green algal isolation and growth conditions
The water samples were taken from a natural green pond in Podpittayapayat School, Ladkrabang district, Bangkok. The unicellular green algae were isolated from the water samples by single cell isolation technique [9] and grown in 24-wells culture plate containing 3 ml of BG11 medium per well [10]. The culture plate was incubated at room temperature for 2 weeks under fluorescent light illumination of 30 \( \mu \text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1} \) for 18 hours per day. A single colony was obtained by streaking the cell culture on BG11 agar.

The isolated green alga was grown in 250 ml Erlenmeyer flasks each containing 100 ml of Tris-acetate-phosphate (TAP) medium [11] for the heterotrophic growth condition. It was cultivated at room temperature for 3 days under fluorescent light illumination of 30 \( \mu \text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1} \) for 18 hours per day with a shaking speed of 120 rpm.

2.2 Green algal identification by 18S rDNA nucleotide sequencing
Genomic DNA of the purified green alga was isolated by using DNA Wizard® SV Genomic DNA Purification System (Promega, USA). PCR product of 18S rDNA was amplified using primers 18S rDNA F1 (5'-CTGCGAATGGCTCATTAAATC-3') and 18S rDNA R1 (5'-AAGGCCAGGG ACGTAATCAA-3') [12] in the DNA thermal cycler (Perkin Elmer 480, USA). The nucleotide sequence of PCR product was analyzed with ABI PRISM® 3700 DNA Analyzer and obtained nucleotide sequence was compared with GenBank nucleotide sequence databases.

2.3 Growth and chlorophyll determination
Growth of the algal culture was measured every 3 hours of cultivation by optical density measurement at wavelength 750 nm. The chlorophyll of cell suspension was extracted with 90% methanol and the total chlorophyll concentration was calculated by the method of Raymond [13]. Triplicate measurements were done in each experiment.

2.4 Determination of hydrogen evolution
One hundred ml of cell culture was harvested by centrifugation at 5,000xg for 10 min at 4\(^{\circ}\)C. The cell pellet was washed with fresh TAP medium and the cell resuspension was transferred into a 10 ml glass vessel and sealed with a rubber stopper. The anaerobic adaptation was performed by purging Argon gas to the cell suspension for 5 min under dark condition and the cells were incubated at room temperature for 2 h. Hydrogen evolution was determined by analyzing gas phase by a Gas Chromatograph Shimadza 15-A (Japan) with a molecular sieve 5A 60/80 mesh packed column using a thermal conductivity detector. The injector and detector temperatures were kept at 100\(^{\circ}\)C whereas the oven temperature was maintained at 50\(^{\circ}\)C. Argon gas was used as a carrier gas during hydrogen analysis. Hydrogen production was calculated as a term of hydrogen evolved per chlorophyll content per time (nmolH\(_2\)/\(\mu\)g chl/h). Each analysis was performed in triplicate.

2.5 Effect of growth period on growth and hydrogen production
The isolated green alga was grown in 100 ml of TAP medium under illumination as described above with the shaking speed of 120 rpm at room temperature for 36 hours. Growth was
determined every 3 hours of cultivation by optical density at 750 nm. Hydrogen evolution of cells at 12, 18, 24 and 36 hours of cultivation was measured.

2.6 Effect of an anaerobic adaptation time on hydrogen production
The isolated green alga was heterotrophically grown in 100 ml of TAP medium in the light (see above) with the shaking speed of 120 rpm at room temperature for 18 hours. Cells were harvested and incubated under anaerobic condition for 2, 4, 6, 8 and 24 hours in darkness before measuring hydrogen evolution.

2.7 Effect of light on growth and hydrogen production
The isolated green alga was grown in 100 ml of TAP medium with the shaking speed of 120 rpm at room temperature for 18 hours either under light illumination of 30 µEm⁻²s⁻¹ or under dark condition. Growth by optical density at 750 nm was determined every 3 hours of cultivation. Hydrogen evolution of cells under light and dark condition was measured.

3. Results and Discussion

3.1 Isolation and identification of a unicellular green alga
Only a single typical unicellular green alga obtained from water samples was found under the light microscope. These green algal cells were oval shaped, with small knobby protrusions at the apices. For strain identification, approximately 1,500 bp of 18S rDNA PCR product was amplified by using genomic DNA of the isolated green alga as a template and sequenced. Nucleotide sequence analysis using BlastN program of 18S rDNA gene revealed that the isolated green alga showed the highest homology to Scenedesmus deserticola, S. pectinatus, S. acutus, S. obliquus, S. littoralis and S. acuminatus (Table 1). From the observation of morphological characteristics under microscope and the result of 18S rDNA analysis, it could be concluded that the isolated green alga belongs to the genus Scenedesmus. Therefore the isolated green alga was called Scenedesmus sp. KMITL-O1, as the available information was insufficient for an assignment to species.

Table 1. Nucleotide sequence comparison of isolated green algae with other nucleotide sequences database of GenBank using BlastN

<table>
<thead>
<tr>
<th>No.</th>
<th>Name</th>
<th>Homology (%)</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>Scenedesmus deserticola isolate BCP-SNI-2</td>
<td>99.67</td>
</tr>
<tr>
<td>2</td>
<td>Scenedesmus deserticola isolate BCP-HAF2-VF10</td>
<td>99.67</td>
</tr>
<tr>
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<td>Scenedesmus deserticola isolate BCP-YPGChar</td>
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</tr>
<tr>
<td>4</td>
<td>Scenedesmus deserticola isolate BCP-EM2-VF3</td>
<td>99.67</td>
</tr>
<tr>
<td>5</td>
<td>Scenedesmaceae sp. Tow 9/22 P-1w</td>
<td>99.60</td>
</tr>
<tr>
<td>6</td>
<td>Scenedesmus pectinatus</td>
<td>99.60</td>
</tr>
<tr>
<td>7</td>
<td>Scenedesmus acutus</td>
<td>99.60</td>
</tr>
<tr>
<td>8</td>
<td>Scenedesmus obliquus strain UTEX 1450</td>
<td>99.60</td>
</tr>
<tr>
<td>9</td>
<td>Scenedesmus littoralis</td>
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</tr>
<tr>
<td>10</td>
<td>Scenedesmus acuminatus</td>
<td>99.47</td>
</tr>
</tbody>
</table>
3.2 Growth and hydrogen production of Scenedesmus sp. KMITL-O1 during the growth period

*Scenedesmus* sp. KMITL-O1 was grown in 100 ml of TAP medium at room temperature with the shaking speed of 120 rpm for 36 hours. Growth by optical density at 750 nm was determined every 3 hours of cultivation. It was found that the cells stayed in the lag phase period for the first 9 hours of cultivation. After that cells grew rapidly and entered the log phase period until reaching the stationary phase at about 15-24 hours of growth (Fig. 1). Hydrogen evolution of *Scenedesmus* sp. KMITL-O1 was measured in cells at different time (12, 18, 24 and 36 hours of cultivation). The result showed that hydrogen production was highest with 1.356 nmolH₂/µg chl/h in cells grown for 18 hours (late-log phase cells) in TAP medium (Fig. 2). After 18 hours of cultivation, hydrogen production of cells decreased sharply (Fig. 2).

![Figure 1. Growth of Scenedesmus sp. KMITL-O1 cultivated under photoheterotrophic condition in TAP medium](image)

![Figure 2. Hydrogen production of Scenedesmus sp. KMITL-O1 during different cultivation periods in TAP medium](image)

It was found that the late-log phase cells or 18-hours old cells could produce the highest hydrogen yield due to the enough accumulation of glycogen from the fermentation process with the carbon source (acetic acid or acetate) in the medium. Accumulated glycogen was utilized as electron and proton donors for producing hydrogen. In the lag-phase cells or 12-hours old cells,
under photoheterotrophic condition they need acetic acid for generating energy utilized in the cellular metabolism and for dividing cells. The generated energy and reducing powers are necessarily used for cell growth instead of producing hydrogen. In case of stationary phase cells (24-and 36-hours old cells), they were not fit and began to die because of carbon source starvation. No more reducing powers providing electron and proton donors was produced, therefore, hydrogen production is lower than in the late-log phase cells.

3.3 Hydrogen production of Scenedesmus sp. KMITL-O1 during the period of anaerobic adaptation time

*Scenedesmus* sp. KMITL-O1 was heterotrophically grown in 100 ml of TAP medium at room temperature with the shaking speed of 120 rpm for 18 hours. Cells were incubated under anaerobic condition for 2, 4, 6, 8 and 24 hours in darkness before measuring hydrogen evolution. It was found that hydrogen production was highest, with 0.121 nmolH$_2$/µg chl/h, in cells incubated under anaerobic adaptation for 2 hours (Fig. 3). After that hydrogen production of cells was obviously decreased (Fig. 3). It might be explained that during anaerobic adaptation, oxygen, an inhibitor of hydrogenase enzyme, was decreased resulting in an increase of hydrogen production in the first 2 hours after anaerobic adaptation. Incubation under anaerobic condition for more than 2 hours did not promote the higher hydrogen production because of the decrease of electron and proton donors in cells as well as the limitation of hydrogenase enzyme.

![Figure 3](image-url)  
*Figure 3.* Hydrogen production of *Scenedesmus* sp. KMITL-O1 in TAP medium under different anaerobic adaptation time

3.4 Hydrogen production of Scenedesmus sp. KMITL-O1 under light and dark condition

*Scenedesmus* sp. KMITL-O1 cells were separately grown in 100 ml of TAP medium either providing light illumination of 30 µEm$^{-2}$s$^{-1}$ or under dark condition. The result showed that cells grown under dark condition have higher optical density than those under light condition (Fig. 4). It might be explained that cells grown under dark condition used only acetic acid as carbon source for growing and dividing cells whereas cells grown under light condition could fix CO$_2$ from the atmosphere via photosynthetic process, therefore requiring more time for the initial growth. After 18 hours of cultivation hydrogen production of both cells were determined. The result showed that cells grown under light produced hydrogen about 2 fold higher than cells grown under dark condition (1.356 and 0.771 nmolH$_2$/µg chl/h, respectively) (Fig. 5). It was suggested that under light condition the energy in form of ATP and the reducing powers NADPH or NADH were obtained from the light reaction of photosynthesis, giving their electrons to excess protons for
hydrogen production. Under dark condition cells produced less ATP and reducing powers, resulting in less hydrogen production.

Figure 4. Growth of Scenedesmus sp. KMITL-O1 in TAP medium under light (■) and dark (♦) condition

Figure 5. Hydrogen production of Scenedesmus sp. KMITL-O1 in TAP medium under light and dark condition

4. Conclusions

The isolated green alga Scenedesmus sp. KMITL-O1 produced the highest hydrogen when cultivated cells for 18 hours in TAP medium under light and then incubated cells under anaerobic adaptation for 2 hours.

5. Acknowledgements

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References

Under such conditions, it was possible to photoproduce and to accumulate significant volumes of H2 gas, using the green alga C. reinhardtii, in a sustainable process that could be employed continuously for several days. Thus, progress was achieved by circumventing the sensitivity of the Fe hydrogenase to O2 through a temporal separation of the reactions of O2 and H2 photoproduction, i.e. by the so-called two-stage photosynthesis and H2 production process (Melis et al., 2000). The reverse reaction, e.g. hydrogen production in the light, was first reported with the green alga Scenedesmus obliquus (Gaffron and Rubin, 1942). High rates of H2 evolution could be measured in the light for short periods of time (from several seconds to a few minutes). Dark CO2 production was stimulated by Cl-CCP. H2 evolution in the light was stimulated by adding glucose to autotrophically grown cells or by growing the cells heterotrophically with glucose; starvation had an opposite effect. Adapted cells released 14CO2 from the 3 and/or 4 position of specifically labeled glucose, indicating that degradation occurred via the Embden-Meyerhof pathway. The amount of H2 released by autotrophically grown cells was the same either with continuous illumination or with short periods of light, followed by darkness. Bio-hydrogen production research using Scenedesmus is actively spurred by its applications to wastewater treatment. (See subsequent section on waste management by Scenedesmus). Bio-diesel production (see biodiesel production). Scenedesmus is known to have high biomass productivity among green algae, and has been actively researched for its use for bio-diesel production. A Novel Type of Iron Hydrogenase in the Green Alga Scenedesmus obliquus is Linked to the Photosynthetic Electron Transport Chain." Journal of Biological Chemistry276.9 (2001): 6125-6132. ^ Winkler M, Hemscheier A, Gotor C, Melis A, Happer T. [Fe]-hydrogenases in green algae: photo-fermentation and hydrogen evolution under sulfur deprivation. Keywords : Hydrogen production, marine green algae, heterotrophic cultivation. 1. Introduction. Biohydrogen is one of alternative. Scenedesmus sp. KMITL-O1 (9) and. Pediastrum duplex Meyen (10). Therefore, this work focused on studying H2 production. by marine green algae isolated from, seawater in Thailand. Marine green algae. are one type of green algae that are widespread and abundant in the ocean. They can tolerate and survive under the extreme. salt concentrations resulting that some. marine green algae can grow in the natural. seawater. In green algae, several environmental. strains under aerobic condition (Table 1). Among them, the green alga Chlorella sp. LSD-W2 gave the highest H2 production. with 66.47±5.44 and 67.64±1.77 nmolH2.