Isolation and Characterization of a Unicellular Marine Green Alga Exhibiting High Activity in Dark Hydrogen Production

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Dark hydrogen production by a newly isolated marine green alga, Chlamydomonas MGA 161 was studied. This alga was a halotolerant, not a halophilic type, and grew well in both natural and artificial seawater media. From an experiment with cycloheximide addition, it was found that the hydrogenase reaction in this alga was not a rate-limiting step of dark hydrogen evolution. Starch accumulation increased at a low NH₄Cl concentration (0.5 mM), at a low temperature (20°C), or at a high NaCl concentration (7%). Hydrogen evolution was correlated with starch degradation rather than starch accumulation, and the molar yield of hydrogen from starch-glucose was very high, at about 2 (mol H₂/mol glucose). A comparison of the distribution pattern of fermentation products in various green algae, showed a unique fermentation pattern for Chlamydomonas MGA 161, with high hydrogen evolution but almost no formate.

Hydrogen production through the biophotolysis of water by green plants or microalgae is an interesting use of solar energy, and a variety of photosynthetic organisms have been proposed as the candidate catalysts in biophotolysis systems. Green algae are capable of producing hydrogen either by photoreactions or by fermentation. Light-dependent hydrogen evolution was soon abolished owing to the inhibitory effect of photosynthetically produced oxygen. Oxygen defense systems must be provided to ensure a prolonged hydrogen evolution. Pow and Krasna added to a green algal culture an oxygen scavenging reagent such as sodium dithionite and glucose-glucose oxidase. Greenbaum continuously introduced an inert gas to the reaction cuvette in order to sweep out oxygen produced. The longest sustained hydrogen photoevolution was observed for 100 to 200 hours, which was, however, shorter than the hydrogen production periods of heterocystous blue-green algal biophotolysis.

Green algae also produce hydrogen under dark anaerobic conditions, as first observed by Gaffron and Rubin. The whole process is mediated through fermentative degradation of accumulated starch. By making use of this property, a stable biophotolysis system is possible, in which hydrogen production is temporally separated from oxygen production. Our previous studies with a freshwater alga, Chlamydomonas reinhardtii, demonstrated an intermittent but stable O₂/H₂ production system in an alternating light/dark cycle. The dark-period evolution of hydrogen by C. reinhardtii was comparable to that of other species of freshwater green algae, but was much less than its light-dependent rate.

Hydrogen production could be increased by screening for more active algal strains from unexplored marine environments. Few studies deal specifically with the hydrogen metabolism of marine green algae. Gibbs measured the hydrogen evolution in the macro algae available in the North Atlantic Ocean. In succession to his work, Ben-Amotz et al. studied hydrogen uptake and evolution in marine red algae in the dark. No macro al-
gae were found which exhibited higher activities than freshwater species of unicellular green algae. Hearley\textsuperscript{13}) first compared hydrogen evolution in a marine Chlorella and freshwater microalgae. Greenbaum et al.\textsuperscript{14}) demonstrated the simultaneous photoproduction of hydrogen and oxygen from marine green algae such as Chlamydomonas sp. and Chlorella sp. Marine microalgae were shown to evolve hydrogen in the light and dark at a rate similar to that of freshwater species, but further characterization of hydrogen metabolism in these marine species has never been investigated.

The aim of the present study was to isolate highly active strains of unicellular marine green algae for our hydrogen production system. An isolate, Chlamydomonas MGA 161, was characterized with respect to growth, starch accumulation, and hydrogen evolution in an artificial seawater medium, and its fermentation metabolism was compared with that of other green algae.

MATERIALS AND METHODS

Algal strains. A marine green alga, Chlamydomonas MGA 161, was isolated from an enrichment culture of marine samples (seawater, seaweeds, drifts, and soil) collected in coastal environments of the Kinki area of Japan, and used throughout this study. This alga was purified by an agar-plate method. Stock cultures of freshwater green algae, Chlamydomonas reinhardtii C-238, Chlorella pyrenoidosa C-212 and Chlorococcum minutum C-138, were obtained from the culture collection of the Institute of Applied Microbiology, University of Tokyo.

Growth conditions. The medium for Chlamydomonas MGA 161 which is a modification of Okamoto medium, contained the following components: NH\textsubscript{4}Cl 268 mg, CaCl\textsubscript{2} \cdot 2H\textsubscript{2}O 200 mg, MgSO\textsubscript{4} \cdot 7H\textsubscript{2}O 250 mg, FeSO\textsubscript{4} \cdot 7H\textsubscript{2}O 20 mg, vitamin B\textsubscript{1} 100 \mu g, vitamin B\textsubscript{12} 1 \mu g, KH\textsubscript{2}PO\textsubscript{4} 41 mg, K\textsubscript{2}HPO\textsubscript{4} 500 mg: trace-metal mixture A\textsubscript{5} 1 ml; in 1000 ml of deionized water. Trace-metal mixture A\textsubscript{5} contained the following components: H\textsubscript{3}BO\textsubscript{3} 2.86 g, MnCl\textsubscript{2} \cdot 4H\textsubscript{2}O 1.81 g, ZnSO\textsubscript{4} \cdot 7H\textsubscript{2}O 220 mg, CuSO\textsubscript{4} \cdot 5H\textsubscript{2}O 80 mg, Na\textsubscript{2}MoO\textsubscript{4} 20 mg, CoCl\textsubscript{2} \cdot 6H\textsubscript{2}O 10 mg, EDTA-2Na 50 g; in 1000 ml of deionized water. The pH of the medium was adjusted to 8.0 by the addition of NaOH. The algae were grown at 30°C in 1.5-liter Roux flasks containing 1.0 liter of the medium. The algal cultures were continuously illuminated by a bank of fluorescent lamps at a light intensity of 25 W/m\textsuperscript{2}. The cultures were continuously sparged with air containing 5% CO\textsubscript{2} for purposes of agitation and CO\textsubscript{2}-supply at a flow rate of about 300 ml/min. In some experimental runs, culture conditions were modified as indicated.

Dark-period hydrogen evolution. Algal cells in the late logarithmic growth phase were harvested by centrifugation (1500 \times g, 5 min), and washed with the growth medium. The cells were resuspended in 10 ml of the same medium at a final density of 0.5~1.0 mg dry wt/ml in a light-shielded test tube (34 ml, 18 mm dia.) fitted with a rubber stopper. The tube was then flushed for 20 min with O\textsubscript{2}-free N\textsubscript{2} gas (99.99%) and incubated at 30°C on a reciprocal shaker (100 rpm).

Analysis of algal metabolites. Hydrogen and carbon dioxide released from algal suspensions by perchloric acid were measured by gas chromatography. The column was fitted with Molecular Sieve 13X, 30/60 mesh (for H\textsubscript{2} assay) or Porapak Q, 50/80 mesh (for CO\textsubscript{2} assay).

Starch was assayed by a modification of the method of Hirokawa et al.\textsuperscript{15}) Algal suspension was centrifuged (4000 \times g, 10 min) at 0°C, and the pellet was mixed with 4 ml of 40% perchloric acid, and allowed to stand at 25°C for 2 hours. The mixture was slowly neutralized by the addition of NaOH in an ice-water bath. Then, the sample was heated in a boiling water bath for 5 min to solubilize the starch, and centrifuged (15000 \times g, 20 min) at 0°C. After this starch solution was appropriately diluted, starch was assayed by an iodo-starch reaction method. One ml of 0.02% iodine and 0.03 M potassium iodide reagent (containing 1 drop of conc. H\textsubscript{2}SO\textsubscript{4}) was added to 9 ml of the starch solution and mixed, then absorption of the starch solution was read at 550 nm; background due to eluted pigments was corrected on all measurements. Further, in some experimental runs, starch was acid-hydrolyzed to glucose by heating the extract on a boiling water bath for one hour, and the amount of glucose in the acid-hydrolysate was assayed by a hexokinase method.\textsuperscript{16)} Comparison of values from the iodo-starch and enzyme methods gave good agreement. Sucrose was assayed by an invertase method.\textsuperscript{17)}

Ethanol and acetate were assayed by a gas chromatograph fitted with a column with Adsorb P-1 (Nishio Kagyo), 60/80 mesh. Glycerol was assayed with glycerokinase, pyruvate kinase, and lactate dehydrogenase (a test kit for glycerol, Boehringer-Mannheim); and formate with formate dehydrogenase.\textsuperscript{18} D-Lactate was assayed with d-lactate dehydrogenase, and L-lactate with l-lactate dehydrogenase.\textsuperscript{18}

Other analyses
Activity of hydrogenase was measured by a modifi-
carnation of the method of Kakuno et al.20) Under anaerobic conditions, methyl viologen (final concn. 2.5 mM) and Triton X-100 (final concn. 0.1%) were added to an algal suspension, and the reaction was started by syringe injection of fresh sodium dithionite solution to the final concentration of 10 mM. The activity of hydrogenase was calculated from the volume of hydrogen evolved in 10 min at 30°C.

To measure dry weight, 10 ml of cell suspension was centrifuged at 4000 × g. The cells were washed twice with deionized water, and dried on an aluminum cup of known weight in an oven at 110°C until constant weight was reached. Total chlorophyll was measured by the method of Arnon.21) A conversion factor of 0.026 mg chl/mg dry wt was obtained.

RESULTS AND DISCUSSION

Isolation of highly active strains

Unicellular microalgae were isolated on an enriched seawater medium from marine sources collected in the Kinki area of Japan. Hydrogen evolution by all 167 isolates was evaluated under dark anaerobic conditions. Four strains exhibited the significantly higher production activity of 1.91 ± 0.45 μmol/mg dry wt/9 hr than the majority of isolates, which had a mean activity of 0.30 ± 0.16 μmol/mg dry wt/9 hr. We chose one strain MGA 161 because of its high evolution and low uptake activity of hydrogen. This strain was a green alga belonging to the Chlamydomonas genus.

Growth of Chlamydomonas MGA 161

The optimum growth conditions for Chlamydomonas MGA 161 were first determined. Although this alga grew well in natural seawater media enriched with soil extract, we developed for reproducible laboratory studies an artificially defined medium on the basis of the Okamoto medium originally prepared for halotolerant Chlamydomonas species. Chlamydomonas MGA 161 required vitamin B₃ and B₁₂. Both NH₄⁺ (as NH₄Cl or (NH₄)₂SO₄) and NO₃⁻ (as KNO₃ or NaNO₃) were well used as an N-source; NH₄Cl at 5.0 mM was used for routine cultures. As shown in Fig. 1 (A), this alga grew in the range of 15°C to 35°C with an optimum temperature of 30°C. The upper limit of NaCl concentration for maximum growth of this alga was 3.0% (Fig. 1 (B)); further addition of NaCl resulted in inhibited growth, indicating that this alga is a halotolerant, not a halophilic type. The optimum pH for growth was 8.0, similar to the pH value of natural seawater. A maximum specific growth rate of 0.1 hr⁻¹ was observed under these experimental conditions in the defined medium with an artificial seawater base.

Starch accumulation was studied under var-

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**Table I. Relationship between Starch Accumulation and Dark Hydrogen Evolution**

<table>
<thead>
<tr>
<th>Growth conditions</th>
<th>Starch accumulation (A)</th>
<th>Hydrogen evolution (B)</th>
<th>B/A</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(μmol glucose/mg dry wt)</td>
<td>(μmol/mg dry wt/6 hr)</td>
<td></td>
</tr>
<tr>
<td>Standard</td>
<td>1.1</td>
<td>1.1</td>
<td>1.0</td>
</tr>
<tr>
<td>5% NaCl</td>
<td>1.6</td>
<td>0.83</td>
<td>0.52</td>
</tr>
<tr>
<td>7% NaCl</td>
<td>1.8</td>
<td>1.1</td>
<td>0.61</td>
</tr>
<tr>
<td>0.5 mM NH₄Cl</td>
<td>1.8</td>
<td>0.88</td>
<td>0.49</td>
</tr>
<tr>
<td>20°C</td>
<td>2.4</td>
<td>1.3</td>
<td>0.54</td>
</tr>
</tbody>
</table>

Algal cells were normally grown at 3% NaCl, 5 mM NH₄Cl and 30°C (Standard conditions). In this series of experiments, cells were also grown under the conditions modified as indicated, but dark-period anaerobic incubation was carried out under the standard conditions of 3% NaCl, 5 mM NH₄Cl and 30°C. Data are taken from the mean of three or more experiments.
ious growth conditions. As shown in Table I, the alga accumulated 18% of cell dry weight (1.1 μmol glucose/mg dry wt) as starch under the standard growth conditions. Elevating the NaCl concentration or lowering the NH₄Cl concentration resulted in a high accumulation of starch; a more than 2-fold increase was observed at a lower growth temperature (20°C). Starch accumulation under these conditions would be due to growth limitation in the presence of sufficient CO₂. However, hydrogen evolution was not proportional to starch content.

Expression of hydrogenase activity in Chlamydomonas MGA 161

A period of dark anaerobic incubation is required to express hydrogenase activity in green algae. The duration of dark incubation and the expressed activity vary widely from strain to strain. Thus, hydrogenase activity may limit the overall rate of dark hydrogen evolution. In the cells of Chlamydomonas MGA 161, hydrogenase activity was observed immediately after the onset of dark anaerobic incubation, and reached a maximum 2 hr later (Fig. 2(A)). The corresponding dark hydrogen evolution, which depended on endogenous electron donation through starch degradation, was observed without appreciable lag time (Fig. 2(B)). Dark hydrogen evolution was not inhibited by cycloheximide, which was added 30 min before or 1 hr after the initiation of dark anaerobic incubation, in spite of its inhibitory effect on hydrogenase expression (Fig. 2). A similar inhibition study was done with Chlamydomonas reinhardtii for comparison; the dark hydrogen evolution in this alga was inhibited by more than 50% upon addition of cycloheximide at the beginning of dark incubation (data not shown).

Rosseler and Lien suggested the existence of cycloheximide-insensitive hydrogenase activity in Chlamydomonas reinhardtii, confirming the effect of cycloheximide with the incorporation of 14C-arginine. In spite of cycloheximide (50 μg/ml) addition 30 min before the onset of the dark incubation, hydrogenase activity was partially observed in Chlamydomonas MGA 161. It is not clear at this time whether this activity is due to a cycloheximide-insensitive enzyme or to the uninhibited part of sensitive hydrogenase. The hydrogenase reaction in this alga in any event was not a rate-limiting step of dark hydrogen evolution, since the partial activity expressed was fully capable of supporting dark hydrogen evolution.

Correlation between starch degradation and hydrogen evolution

Green algae are known to evolve hydrogen evolution

\[ \text{Fig. 2. Effect of Cycloheximide on Hydrogenase Induction (A) and Dark Hydrogen Evolution (B) of Chlamydomonas MGA 161.} \]

Anaerobic incubations were performed under an N₂ atmosphere in the dark: control was carried out without cycloheximide addition ( ). Cycloheximide (50 μg/ml) was added 30 min before ( ) or 1 hr after ( ) the initiation of dark anaerobiosis. Arrows indicate additions.
Isolation and Characterization of High H₂-evolving Marine Green Alga

Fig. 3. Relationship between Dark Hydrogen Evolution and Starch Degradation of *Chlamydomonas* MGA 161 under Various Conditions of Dark Hydrogen Evolution.

Hydrogen evolution (○) and starch degradation (▲, determined in glucose units) were measured, and therefrom the hydrogen molar yield (■) was calculated as mol H₂/mol starch-glucose.

**Fig. 3(A).** Effect of NH₄Cl Concentration in the Dark Incubation Medium.

Algal cells grown under N-deficient (0.5 mM) conditions (starch accumulation: 46%) were incubated at indicated NH₄Cl concentrations.

**Fig. 3(B).** Effect of Dark Incubation Temperature.

Algal cells grown at 30°C (starch accumulation: 18%) were incubated at indicated temperatures.

**Fig. 3(C).** Effect of NaCl Concentration in the Dark Incubation Medium.

Algal cells grown at 3% NaCl (starch accumulation: 14%) were incubated at indicated NaCl concentrations.

Under light or dark anaerobic conditions by use of cellular starch via the Embden–Meyerhof pathway. Based on the quantitative analysis of starch, glucose and sucrose in the normally grown cells of *Chlamydomonas* MGA 161, it was found that starch was the main reserve (96%) of fermentation substrates. Therefore, the relation between starch degradation and hydrogen evolution was investigated under various conditions for dark-period anaerobic incubation.

The effect of NH₄Cl concentration on hydrogen evolution is compared in Fig. 3(A). Algal cells were grown under NH₄Cl deficient (0.5 mM) conditions, and then hydrogen evolution was measured at various concentrations of NH₄Cl in the dark incubation medium. Hydrogen evolution of NH₄Cl deficient cells was restored by the addition of NH₄Cl (at above 5 mM) to the level (0.99 ± 0.25 μmol H₂/mg dry wt/6 hr) of hydrogen evolution of NH₄Cl sufficient cells. Although Miyachi and Miyachi observed that starch in *Chlorella* cells was greatly degraded by the addition of NH₄Cl, KNO₃, or NaNO₂, the effect of NH₄Cl addition on starch degradation in *Chlamydomonas* MGA 161 was restorative rather than stimulative. The molar yield of hydrogen, which was calculated from hydrogen evolution rates and starch-glucose degradation rates, was constant at around 2 (mol H₂/mol glucose), in spite of the variations in these rates with the NH₄Cl concentration.

The effect of temperature on the relationship between starch degradation and hydrogen evolution is shown in Fig. 3 (B). Algal cells were grown at 30°C, then incubated anaerobically at 25, 30, or 37°C. With the elevation of the dark incubation temperature, both starch degradation and hydrogen evolution increased proportionally, resulting in a constant molar yield of hydrogen in this series of experiments.

Figure 3 (C) shows the effect of the NaCl concentration in the dark incubation medium.
on the starch degradation and hydrogen evolution of 3% NaCl-grown cells. Although starch degradation was independent of NaCl concentration, hydrogen evolution was drastically changed with increasing NaCl concentration. Maximum hydrogen evolution was usually observed at an NaCl concentration between 1 and 3%, and the hydrogen evolution rate decreased both at lower and higher concentrations of NaCl, presumably due to metabolic alteration in the algal cells.

As a result of limited growth, starch accumulation increased at a low NH₄Cl concentration, at a low temperature or at a high NaCl concentration. However, increments of hydrogen in proportion to starch content were not observed (Table 1). Hydrogen evolution under the conditions studied here was correlated with starch degradation, and the molar yield of hydrogen was constant at about 2 (mol H₂/mol glucose), except at non-optimal NaCl concentrations.

**Characterization of Chlamydomonas MGA 161 with respect to fermentation products**

Figure 4 demonstrates the distribution of the end-products of starch fermentation in various green algae. *Chlamydomonas* MGA 161 showed a unique distribution of fermentation products; this alga excreted acetate, ethanol in addition to the gaseous products, hydrogen and carbon dioxide, while formate, lactate and glycerol were produced in only negligible amounts. This alga evolved a particularly high amount of hydrogen compared with the other three strains investigated. Among formate non-producing green algae, *Chlamydomonas moewusii 11-5/10*(24,26) produced ethanol or glycerol as a main reducing product, in contrast to hydrogen for our isolate *Chlamydomonas MGA 161*.

*Chlamydomonas reinhardtii* and *Chlorella pyrenoidosa* exhibited a very similar anaerobic metabolism, producing formate, acetate and ethanol in a ratio of approximately 2:2:1. Gfeller and Gibbs,(27) and Kreuzberg(28) reported that formate, acetate and ethanol were in a ratio of 2:1:1 in the dark incubation of *Chlamydomonas reinhardtii*. This discrepancy in the ratio between our results and theirs would be attributable to differences of incubation conditions, because we observed that the amounts of hydrogen and formate were variable depending upon shaking or static conditions of the incubation vessels.9) Furthermore, we observed the fact that the ratio of formate, acetate and ethanol was easily altered by adding hydrogen or carbon monoxide to the incubation vessels (data not shown). *Chlorococcum minutum* was found to be another unique strain; this alga excreted acetate and glycerol as main products, whereas hydrogen, formate and ethanol were produced in only negligible amounts. The results ob-

### Table 1: Starch degradation or product formation

<table>
<thead>
<tr>
<th>Product</th>
<th><em>Chlamydomonas MGA 161</em></th>
<th><em>Chlamydomonas reinhardtii</em></th>
<th><em>Chlorella pyrenoidosa</em></th>
<th><em>Chlorococcum minutum</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Starch</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>H₂</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>CO₂</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>Acetate</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>Ethanol</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Formate</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>Glycerol</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>Lactate</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
</tbody>
</table>

Fig. 4. Comparison of Product Distribution of Fermentative Starch Degradation in Various Green Algae.

Cells grown under the standard growth conditions were incubated in the dark as shown in Materials and Methods. Data are taken from the mean of three or more experiments.
tained with *C. minutum* is a first demonstration of a new type of fermentation metabolism in unicellular green algae.

Unicellular green algae ferment endogenous starch to multiple end-products such as hydrogen, carbon dioxide, acetate, ethanol and formate. Klein *et al.*[^26] distinguished two types of green algal fermentation on the basis of hydrogen and formate formation. From the data obtained so far, the new isolate *Chlamydomonas* MGA 161 belongs to the first type of Klein's classification e.g. fermentation with “relatively high hydrogen evolution but almost no formate”. However, this alga was further characterized by its high molar yield of hydrogen from starch-glucose, compared to even the highest reported yield of 0.43 ± 0.07 for *C. reinhardtii*.[^27] In addition, it was shown that hydrogenase reaction in this alga was not a rate-limiting step of fermentative hydrogen evolution. These characteristics contribute to its high rates of hydrogen evolution (6 μmol/mg chl/hr) which are comparable to the rates of light-driven hydrogen evolution in other green algae.[^4] [^14]

Hydrogen evolution in this alga was correlated with starch degradation rather than starch accumulation. For further improvement of hydrogen production by this alga, starch degradation should be enhanced to completely use up the stored starch within the dark incubation period. It is well documented in bacterial fermentations that the ratio of end products can be altered by changing the environmental factors.[^29] [^30] The hydrogen yield in algal fermentation would also be improved by regulating the environmental conditions, and thereby altering the reducing equivalent flux in algal cells.

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**REFERENCES**
