0273-2289/93/3940—0753$03.00

Stimulation of Hydrogen Production in Algal Cells Grown Under High CO_2 Concentration and Low Temperature

Y. MIURA,[1,*] W. YAMADA,[1] K. HIRATA,[1]
K. MIYAMOTO,[1] AND M. KIYOHARA[2]

[1]*Faculty of Pharmaceutical Sciences, Osaka University, Suita, Osaka 565 Japan; and* [2]*The Kansai Electric Power Co., Inc., Amagasaki, Hyogo 661 Japan*

ABSTRACT

When cells of *Chlamydomonas* sp. MGA 161, a marine green alga, were cultivated at a high CO_2 concentration (15% CO_2) and low temperature (15°C), the growth lag time was much longer, but the starch accumulated was two times higher than under the basal conditions (5% CO_2 30°C). When the cells grown in the high-CO_2/low-temperature conditions were incubated under dark anaerobic conditions, the degradation of starch and production of hydrogen and ethanol were remarkably higher than those grown under the basal conditions. The lag time of cell growth was shortened, whereas the high capacity of starch accumulation and hydrogen production was maintained, by cultivating the cells alternately every 12 h under the basal and high-CO_2/low-temperature conditions. Using this dual system, in which the cultivation was alternated between the two conditions, the total productivity was significantly improved.

Index Entries: Hydrogen production; marine green alga; CO_2 fixation.

INTRODUCTION

Power plants burn enormous amounts of fossil fuels, such as coal, oil, and LNG, for the generation of steam. The amount of CO_2 discharged from these plants accounts for more than 16% of all CO_2 emissions (*1*)

*Author to whom all correspondence and reprint requests should be addressed.

and, thus, contributes considerably to raising the concentration of CO_2 in the atmosphere. Since increased atmospheric levels of CO_2 are implicated in global warming, the so-called Greenhouse Effect, possible engineering improvements and changes in policy that might decrease the rising CO_2 concentration have been widely discussed.

A reduction of CO_2 in the flue gas can be achieved by physicochemical methods, as in wet absorption, dry adsorption, and membrane-separation techniques (*2*). Alternatively, biological CO_2 fixation by microalgal mass cultivation has also been proposed (*3*). Algae are grown photoautotrophically and fix CO_2. The percentage of CO_2 in flue gas is about 13%,and a process model in which marine microalgae fix CO_2 in the gas from a power plant has already been proposed (*1*). In addition, certain substances are produced that are available as alternative energy sources to fossil fuels. In particular, hydrogen is well known as a clean energy source, because its combustion product is water.

The production of hydrogen by green algae under dark anaerobic conditions was observed by Gaffron and Rubin (*4*). The whole process of hydrogen evolution in the fermentation stage is mediated through the degradation of accumulated starch. In addition, acetate, ethanol, formate, glycerol, lactate, butandiol, and gaseous CO_2 are known to be fermentation products of green algae (*5–7*). The fermentation process is regulated by ATP and reducing equivalents generated during starch degradation.

In our previous studies, a halotolerant green alga, *Chlamydomonas* sp. MGA 161, isolated from sea water, produced high levels of hydrogen, ethanol, and acetate, but formate formation was scarcely observed. Ethanol and acetate were used as substrates for hydrogen production by a marine photosynthetic bacterium sp. W1-S, under light anaerobic conditions (*12*). The levels of starch accumulated in the growth stage and starch degradation in the fermentation stage are very important for hydrogen evolution, because the reducing equivalents supplied by starch degradation are mostly consumed for production of hydrogen, ethanol, and acetic acid (*9,10*). Starch accumulation was studied under various growth conditions, and was found to increase more than twofold at a lower growth temperature (20°C). After fermentation, the level of starch was still high, and hydrogen evolution was not proportional to the initial starch content (*9*). In the present study, the effects of cultivation conditions (high CO_2 concentration and low temperature) on growth and fermentation in *Chlamydomonas* sp. MGA 161 were investigated in order to improve the fixation of CO_2 and the production of hydrogen.

MATERIALS AND METHODS

Algal Strain

A marine green alga, *Chlamydomonas* sp. MGA 161, was isolated and purified as described in Miura et al. (*9*).

Growth Conditions

For the cultivation of *Chlamydomonas* sp. MGA 161, a modified Okamoto medium (pH 8.0), was used (*9*). The algal cells were cultivated at 30°C under fluorescent lamp illumination (25 W/m^2) with aeration of 5% CO_2 in air at a rate of 300 mL/min (basal conditions). The CO_2 concentration was monitored by infrared gas analysis with a CO_2 analyzer (Shimadzu, URA-107). Cell growth was monitored by measuring the optical density at 680 nm (OD_{680}) with a spectrophotometer (Shimadzu, UV-2100). Unit OD_{680} was equal to a cell concentration of 2.1×10^6 cells/mL. To examine the effect of a high CO_2 concentration and low temperature on growth and fermentation, cells cultivated under the basal conditions were incubated with 15% CO_2 and at 15°C.

Fermentation Conditions

Algal cells cultivated under various conditions in the late-logarithmic growth phase, in which the cell concentration was about 2.1×10^6 cells/mL, were harvested by centrifugation (6000 rpm, 10 min). The cells were resuspended in 10 mL of the growth medium to adjust the cell concentration to about 4.2×10^6 cells/mL in a light-shielded test tube (34 mL, 18-mm diameter) fitted with a rubber stopper. The cells were then flushed with O_2-free N_2 gas for 20 min and incubated at 30°C with reciprocal shaking at 100 rpm.

Analysis of Algal Fermentation Products

Hydrogen in the gas phase of the test tube, including the culture broth, was measured by gas chromatography (HITACHI, Gas Chromatograph 164). The column was fitted with a molecular sieve 13×, 30/60 mesh (Gasukuro Kogyo Inc.). Starch content was assayed by a modification of the method of Hirokawa et al. (*11*), as follows. The algal suspension was centrifuged (12,000 rpm, 10 min) at 0°C, and the pellet was mixed with 4 mL of 40% (v/v) $HClO_4$ and kept at room temperature for 2 h. The mixture was then heated at 100°C for 20 min to solubilize the starch. After neutralization of the mixture with 10*N* NaOH in an ice-water bath, it was centrifuged (12,000 rpm, 20 min) at 0°C. Glucose, which was formed in the supernatant, was assayed by an enzymatic method with an F-kit D-glucose (Boehringer-Mannheim, Germany, 716251). The starch content is indicated as glucose units. Ethanol and acetate in the fermentation broth were also assayed by enzymatic methods with F-kit ethanol and F-kit acetic acid (Boehringer-Mannheim, Germany, 176290, 148261).

Measurement of Ash-Free Dry Weight

To measure the dry weight of cells, 5 mL of cell suspension were centrifuged at 3000 rpm. The cells were dried in a glass test tube in an oven at 110°C until a constant weight was reached. After the cell dry weight was

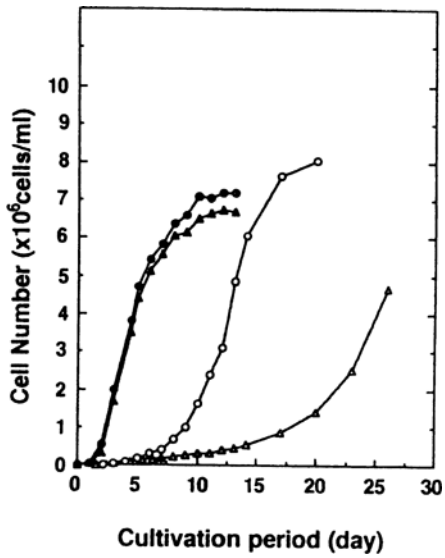

Fig. 1. Effects of temperature and CO_2 level on growth of *Chlamydomonas* sp. MGA 161. Cultivation temperature was regulated at 30 or 15°C, and CO_2 level of aeration gas was regulated at 5 or 15% CO_2 in air. —●— 30°C, 5% CO_2; —▲— 30°C, 15% CO_2; —○— 15°C, 5% CO_2; —△— 15°C, 15% CO_2.

measured, the test tube was burned to measure the weight of inorganic compounds contained in the cell body. The ash-free dry weight (AFDW) is the cell dry weight subtracting the weight of inorganic compounds. One milligram of AFDW was equal to 6.9×10^6 cells/mL.

RESULTS AND DISCUSSION

Effect of High CO_2 and/or Low Temperature on Growth in *Chlamydomonas* sp. MGA 161

In our previous studies, the optimal concentration of CO_2 in air for the growth of *Chlamydomonas* sp. MGA 161 was found to be 5%, and the cells could grow in the range from 15 to 35°C (*9*). Figure 1 shows the growth curves of cells under high CO_2 and/or low temperature conditions. The cells grew well under the high CO_2 condition (15%) at 30°C, in a similar manner as under the basal conditions, whereas the growth lag time at 15°C was longer than that under the basal condition; growth was especially delayed under high-CO_2 and low-temperature (15°C) conditions.

Starch Accumulation and Dark Anaerobic Fermentation

Table 1 shows the starch content and fermentation product formation in MGA 161 cells. In the growth stage, the amount of starch accumulated

Table 1
Effects of Growth Conditions on Starch Content, Starch Degradation, and Fermentation Product Formation in *Chlamydomonas* sp. MGA 161[a]

Cultivation period	Basal conditions,[d] 5 d	High-CO_2/low-temperature conditions[e], 22 d
Starch content[b]	0.88	2.21
Starch degradation[c]	0.35	0.90
Hydrogen evolution[c]	0.90	1.25
Ethanol production[c]	0.18	1.12
Acetate production[c]	0.48	0.64

[a]The cells were harvested at 2.1×10^6 cells/mL.
[b]μmol/mg ash-free dry weight (AFDW).
[c]μmol/mg AFDW/12 h.
[d]Basal conditions, 30°C/5% CO_2.
[e]High-CO_2/low-temperature conditions, 15°C/15% CO_2.

in the cells under high-CO_2/low-temperature conditions was twice that under the basal conditions. In the fermentation stage, a higher amount of starch degradation was obtained in the cells grown under high-CO_2/low-temperature conditions than in those cells grown under the basal conditions. Cells grown under the former conditions produced about 1.5 times the amount of hydrogen and six times the amount of ethanol than those grown under the basal conditions. These results indicated that stimulation of starch degradation resulted in stimulation of hydrogen and ethanol production, because reducing equivalents formed by starch degradation were supplied for their production.

Improvement of Algal Growth

Although cells grown under high-CO_2/low-temperature conditions could produce significantly higher levels of hydrogen and ethanol than those grown under the basal conditions, the growth lag time under the former conditions was much longer than that under the latter. Shortening the lag time while maintaining high levels of hydrogen and ethanol production would be very beneficial for the improvement of total hydrogen production. The growth was not improved by regulating the pH (by NaOH or ammonium hydroxide, pH 7.5–8.0) or by discontinuing the CO_2 supplement every 12 h (data not shown). As shown in Fig. 2, the growth lag time was shortened when the cultivation conditions were alternately changed between high-CO_2/low-temperature conditions and the basal conditions every 12 h. The results shown in Fig. 1 suggested that the main factor in growth suppression under high-CO_2/low-temperature conditions may be the low-temperature condition and that the high-CO_2 condition enhanced this effect.

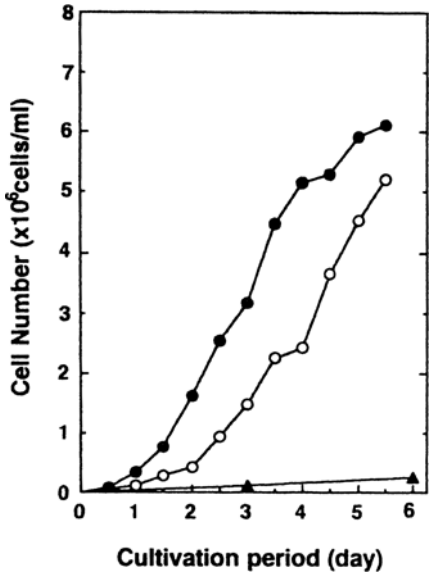

Fig. 2. Growth improvement of *Chlamydomonas* sp. MGA 161 under high-CO_2 and low-temperature conditions. The cultivation conditions were continuously regulated at 30°C/5% CO_2 (—●—), continuously regulated at 15°C/15% CO_2 (—▲—), or alternately changed between 30°C/5% CO_2 and 15°C/15% CO_2 every 12 h (—○—).

Table 2 shows the effects of cultivation conditions on starch content, starch degradation, and fermentation product formation. When cells cultivated under the basal conditions were incubated for a subsequent 12 h under high-CO_2/low-temperature conditions, the starch content increased remarkably, and the amounts of starch degraded, and of hydrogen and ethanol produced, increased (Table 2, column A). By contrast, when the cells were transferred from high-CO_2/low-temperature conditions to the basal conditions, the starch content did not change, and the starch degradation and production of hydrogen and ethanol also showed little change from the results obtained under the basal conditions (Table 1 and Table 2 column B).

Batch Culture with Two Stages

The results shown in Table 2 suggested that the effects of incubation under high-CO_2/low-temperature conditions on fermentation occurred within 12 h. Figure 3 shows the effects of the incubation period on growth and starch accumulation under high-CO_2/low-temperature conditions after switching from the basal conditions. When cells were transferred to high-CO_2/low-temperature conditions from the basal conditions, the accumulation of starch immediately increased (Fig. 3B), although growth

Table 2
Effects of Growth Conditions on Starch Content, Starch Degradation, and Fermentation Product Formation in *Chlamydomonas* sp. MGA 161[a]

	A	B
Starch content[b]	2.23	0.80
Starch degradation[c]	1.56	0.50
Hydrogen evolution[c]	1.51	0.92
Ethanol production[c]	1.30	0.22

[a]The cultivation conditions were alternately changed between 30°C/5% CO_2 and 15°C/15% CO_2 every 12 h. The cells were collected at the end of 15°C/15% CO_2 (A) or 30°C/5% CO_2 (B) cultivation, and fermented under dark anaerobic conditions at 30°C.
[b]μmol/mg AFDW.
[c]μmol/mg AFDW/12 h.

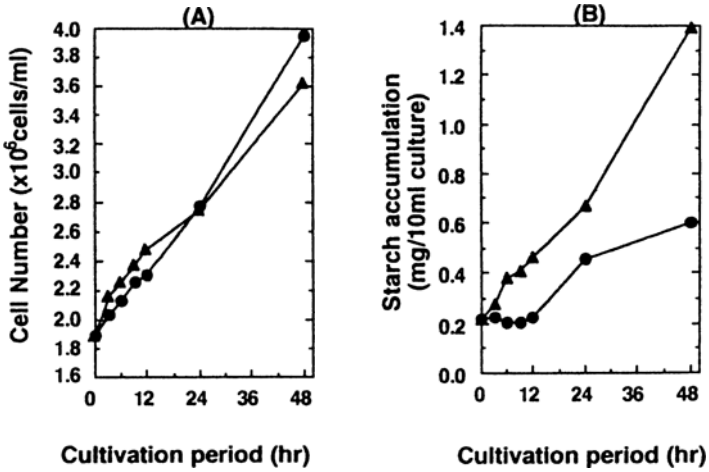

Fig. 3. Effects of switchover to high-CO_2 and low-temperature conditions on growth (A) and starch accumulation (B) in *Chlamydomonas* sp. MGA 161. The cells were cultivated throughout under 30°C/5% CO_2 conditions (—●—) or transferred at time 0 h to 15°C/15% CO_2 conditions (—▲—).

suppression was not observed (Fig. 3A). Figure 4 shows the profile of starch degradation, hydrogen evolution, and ethanol production after switchover to high-CO_2/low-temperature conditions from the basal conditions. The production of hydrogen was stimulated about 1.3 times within 3 h and thereafter reached saturation under high-CO_2/low-temperature conditions. The stimulation of ethanol production was dependent on cultivation period, and the level reached after 12 h was about 3.5 times the initial amount. Starch degradation immediately increased to about two

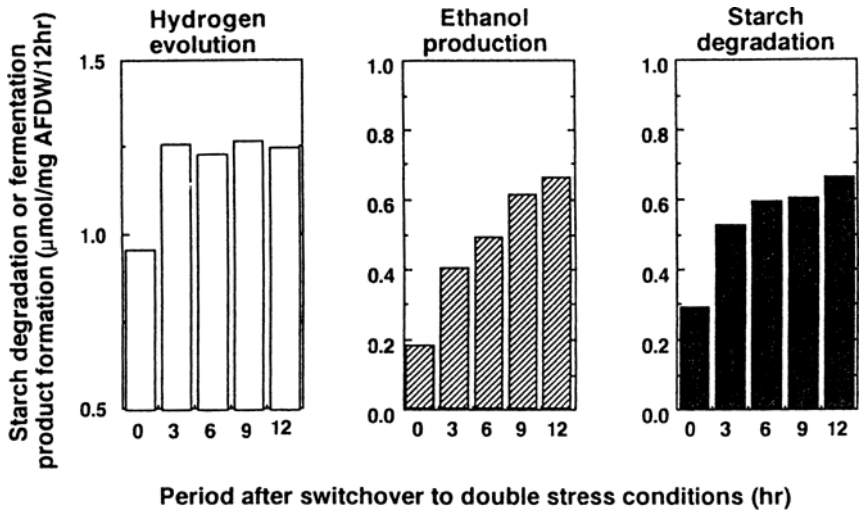

Fig. 4. Effects of switchover to high-CO_2 and low-temperature conditions on fermentation in *Chlamydomonas* sp. MGA 161. The cells were cultivated under 30°C/5% CO_2 conditions and transferred to 15°C/15% CO_2 conditions at time 0 h. After that, the cells were collected every 3 h and fermented for 12 h under dark anaerobic condition at 30°C.

times the initial value within 3 h, after which it increased gradually (Fig. 4). These results suggested that the enzymes that mediate hydrogen evolution and ethanol production may be activated immediately by the incubation of cells under high-CO_2/low-temperature conditions.

In this study, the production of hydrogen and ethanol in *Chlamydomonas* sp. MGA 161 could be improved by cultivating cells in the growth stage under the high-CO_2/low-temperature conditions favorable for starch accumulation and degradation, and for the production of fermentation products, combined with the basal conditions favorable for growth. This suggests the possibility of designing a CO_2 fixation and hydrogen production system using the marine green alga *Chlamydomonas* sp. MGA 161 in a dual batch-culture system.

REFERENCES

1. Negoro, M., Shioji, N., Miyamoto, K., and Miura, Y. (1991), *Appl. Biochem. Biotech.* **28/29,** 877.
2. Uchiyama, Y. (1989), *Energy and Resources* **10,** 380.
3. Benemann, J. R., Goebal, R. P., Weissman, J. C., and Augenstein, D. C. (1982), Microalgae as a Source of Lipid Fuels. Final Technical Report Produced by Enbio, Inc., for U.S. Department of Energy, Office of Energy Research.

4. Gaffron, H. and Rubin, J. (1942), *J. Gen. Physiol.* **26,** 219.
5. Gfeller, R. P. and Gibbs, M. (1984), *Plant Physiol.* **75,** 212.
6. Klein, U., Kreuzberg, K., and Betz, A. (1981), in *Advances in Biotechnology,* vol. 2, Moo-Young, M. and Robinson, C. W., eds., Pergamon, New York, p. 97.
7. Kreuzberg, K. (1984), *Physiol. Plant* **61,** 87.
8. Mahro, B., Kusel, A. C., and Grimme, L. H. (1986), *Arch. Microbiol.* **14,** 91.
9. Miura, Y., Ohta, S., Mano, M., and Miyamoto, K. (1986), *Agric, Biol. Chem.* **50,** 2837.
10. Ohta, S., Miyamoto, K., and Miura, Y. (1987), *Plant Physiol.* **83,** 1022.
11. Hirokawa, T., Hata, M., and Takeda, H. (1982), *Plant Cell Physiol.* **23,** 813.
12. Miura, Y., Saitoh, C., Matsuoka, S., and Miyamoto, K. (1992), *Biosci. Biotech. Biochem.* **56,** 751.