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Stimulatory Effect of Red Light on Starch Accumulation in a Marine Green Alga, *Chlamydomonas* sp. Strain MGA161

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ABSTRACT

A marine green alga, *Chlamydomonas* sp. strain MGA161 was cultivated under illumination of red and white lights. The growth rate under red light illumination was almost the same as that in the basic conditions under white light illumination, but red light-grown cells accumulated almost twice as much starch as white light-grown cells. Although there was a slight decrease in carbonic anhydrase activity, red light-illuminated cells had almost 2.3 times the fructose-1,6-diphosphatase activity of white light-illuminated cells. Red light might stimulate starch accumulation by increasing the amounts of enzymes related to carbon fixation through the phytochrome system. Cells grown under red light degraded 1.6 times as much starch and produced 1.7 times as much hydrogen and 1.6 times as much ethanol compared with cells grown under white light during 12 h of dark anaerobic fermentation.

Index Entries: Red light; starch; hydrogen; green algae; *Chlamydomonas*.

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INTRODUCTION

Hydrogen production by the biophotolysis of water based on microalgal photosynthesis would be an ideal solar energy conversion system. Green algae evolve hydrogen through a hydrogenase-dependent reaction under light (*1,2*). Light-dependent hydrogen evolution cannot be maintained for long, however, because concomitantly evolved oxygen has an inhibitory effect on hydrogenase. Although many attempts have been made to scavenge oxygen photosynthetically evolved to sustain hydrogen photoevolution, satisfactory results have not yet been obtained (*3,4*). Gaffron and Rubin first observed that green algae can also evolve hydrogen under dark anaerobic conditions (*5*). Starch accumulated in the light is degraded to produce hydrogen in the absence of a terminal electron acceptor other than H^+. In our laboratory, a stably sustained hydrogen production sytem was constructed with *Chlamydomonas reinhardtii* by using this property; dark hydrogen evolution and oxygen photoevolution were separated in an alternating dark/light cycle (*6*). However, the rate of dark anaerobic hydrogen evolution of *C. reinhardtii* was much less than that of light-dependent evolution. We then tried to isolate from marine environment an algal strain with high dark hydrogen evolution activity. One of the isolates, *Chlamydomonas* sp. strain MGA161, is a marine green alga that was found to actively produce hydrogen under dark anaerobic conditions with a high yield of conversion from starch-glucose (2 mol H_2/mol glucose) (*7*). The alga also excreted ethanol and acetate into the medium during the dark fermentation period. In addition, we isolated a marine photosynthetic bacterium, *Rhodopseudomonas* sp. strain W-1S, which could evolve hydrogen from acetate and ethanol (*8*). A significantly high conversion yield of 8 mol H_2/mol glucose was achieved by a combination of the marine green alga and the photosynthetic bacterium. As well as the conversion yield the increase of starch content in the green alga is significant for this alga-bacterial hydrogen production system. In the present study, we examined the effect of various light conditions on starch accumulation in *Chlamydomonas* sp. strain MGA161, and found that red light illumination has a stimulatory effect.

MATERIALS AND METHODS

Cultivation

Chlamydomonas sp. strain MGA161 was cultivated in a modified Okamoto medium at pH 8.0. The alga was grown at 30°C in a 1.6-L Roux bottle containing 1.0 L of the medium. The cultures were continuously sparged with air containing 5% CO_2 and illuminated with incandescent lamps at a light intensity of 25 W/m^2. Beside white light, near ultraviolet

(300–400 nm), blue (400–500 nm), green (500–600 nm), and red (600–700 nm) lights were used at 25 W/m^2.

Starch Assay

Starch accumulated in the alga was extracted by the method of Hirokawa et al. (*9*). Cells were harvested by centrifugation at 3,000*g* for 10 min and treated with 4 mL of 40% perchloric acid for 2 h at room temperature. After heat treatment at 100°C for 20 min, the pH was adjusted to 7.0–7.5 with NaOH. The amount of glucose in the supernatant obtained by centrifugation at 12,000*g* for 20 min was measured with hexokinase and glucose-6-phosphate dehydrogenase (test kit for glucose, Boehringer-Mannheim, Mannheim, Germany).

Carbonic Anhydrase (CA) Assay

Cells were harvested by centrifugation at 3000*g* for 5 min, washed with 50 m*M* Veronal buffer (pH 8.3, Wako Pure Chemical, Osaka, Japan), and resuspended in the same buffer. The cells were disrupted by ultrasonic treatment performed three times for 1 A and 1 min. The supernatant obtained by centrifugation of the homogenate at 12,000*g* for 20 min was used for the determination of CA activity. Three milliliters of the sample was mixed with 2 mL of CO_2-saturated water and incubated at 2°C until the pH decreased to 7.3. The activity was calculated from the time required for decreasing of pH from 8.3 to 7.3 by the method described by Rickli et al. (*10*).

Fructose-1,6-Diphosphatase (FDPase) Assay

Cells were harvested by centrifugation at 3,000*g* for 10 min, washed twice with 1*M* Tris-HCl buffer (pH 7.5) containing 0.1 m*M* EDTA, and resuspended in the same buffer. The cells were disrupted by three times of ultrasonic treatment at 1 A for 1 min. The supernatant obtained by centrifugation of the homogenate at 12,000*g* for 20 min was used for the determination of FDPase. The activity was measured by the increase of NADPH using fructose-1,6-diphosphate as a substrate, phosphoglucose isomerase, and glucose-6-phosphate dehydrogenase.

Protein Assay

Protein was measured by the method of Lowry et al. (*11*).

Dark Anaerobic Fermentation

Algal cells were harvested at the late logarithmic phase of growth by centrifugation (1500*g*, 5 min), and washed with the growth medium. The

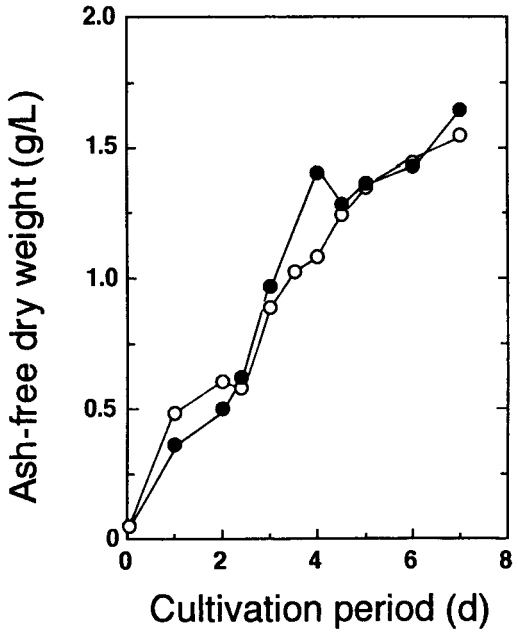

Fig. 1. Effect of red light illumination on growth of *Chlamydomonas* sp. strain MGA161. Cells were cultivated under white (○) and red (●) light, and the ash-free dry weight was measured at the times indicated.

cells were resuspended in 10 mL of the same medium in a light-shielded test tube (34 mL, 18 mm in diameter) fitted with a rubber stopper. The tube was flushed with nitrogen gas and incubated at 30°C on a reciprocal shaker (100 rpm). Evolved hydrogen was measured by gas chromatography (model-164, Hitachi). Acetic acid was assayed with acetyl-CoA synthetase, citrate synthetase, and malate dehydrogenase (test kit for acetate, Boehringer-Mannheim). Ethanol was assayed with alcohol dehydrogenase and aldehyde dehydrogenase (test kit for ethanol, Boehringer-Mannheim).

RESULTS

The effect of red light on the growth of *Chlamydomonas* sp. strain MGA161 was examined by cultivating cells under white or red light illumination. As shown in Fig. 1, the growth rate under red light illumination was almost same as that in the basic conditions (under white light illumination). When the amount of accumulated starch in cells cultivated under red and white light was measured (Table 1), red light-grown cells were found to have accumulated almost twice as much starch as white light-grown cells. Although the same amount of starch was accumulated in cells cultivated under white light at 15°C, the total amount of starch

Table 1
Stimulation of Starch Accumulation by Red Light Illumination in *Chlamydomonas* sp. Strain MGA161

Culture conditions	Starch content, μmol glucose/mg dry wt
White light, 30°C	1.1
Red light, 30°C	2.1
White light, 15°C	2.2

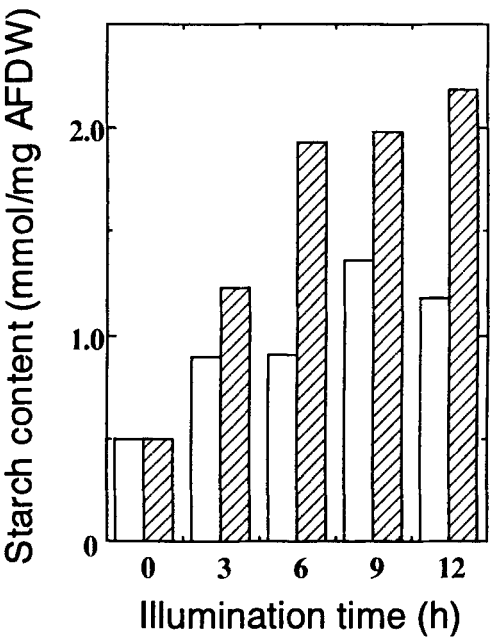

Fig. 2. Time-courses of starch accumulation under red and white light illumination in *Chlamydomonas* sp. strain MGA161. At the logarithmic phase, cells grown under white light were incubated under red (▨) and white (□) light. Starch content was measured at the incubation times indicated.

was the same as in the basic conditions because the growth rate at 15°C was almost half that in the basic conditions at 30°C. Thus, red light-illumination seems to be very effective in increasing the substrate for dark anaerobic hydrogen evolution in *Chlamydomonas* sp. strain MGA161.

We then examined the time required for the effect of red light to be manifested. Cells were cultivated in the basic conditions under white light, and at the logarithmic phase of growth the illumination lamps were changed from white to red. As shown in Fig. 2, the 6 h of red light illumination, cells accumulated almost twice as much starch as white light-grown cells. Since the addition of 6-methylpurine, an inhibitor of transcription, reversed the stimulatory effect (data not shown), red light might

Table 2
Effect of Red Light Illumination on Activities on Carbonic Anhydrase and Fructose-1,6-Diphosphatase in *Chlamydomonas* sp. Strain MGA161

Light	CA activity, U/mL pcv[a]	FDPase activity, U/mg protein
White	77.5	1.4
Red	59.2	3.2

[a]Packed cell volume.

Table 3
Metabolites in Dark Anaerobic Fermentation in *Chlamydomonas* sp. Strain MGA161 Cultivated Under Red and White Light

Metabolites, μmol/mg dry wt	Growth conditions	
	White light	Red light
Hydrogen	0.60	1.02
Ethanol	0.71	1.17
Acetate	0.60	0.31
Starch degraded[a]	0.42	0.67

[a]The amount was calculated as glucose units.

activate the expression of some genes related to the transport of inorganic carbon and starch metabolism. We measured the activities of carbonic anhydrase (CA) and fructose-1,6-diphosphatase (FDPase), which are key enzymes of inorganic carbon transport and starch metabolism, respectively, in red light- and white light-illuminated cells. Although there was a slight decrease in CA activity, red light-illuminated cells had almost 2.3 times as much FDPase activity as white light-illuminated cells (Table 2).

Dark anaerobic fermentation was conducted for 12 h in cells cultivated under red and white light. The amounts of the fermentation products are shown in Table 3. Cells grown under red light degraded 1.6 times as much starch and produced 1.7 times as much hydrogen, 1.6 times as much ethanol, and 0.5 times as much acetate as cells grown under white light. The molar yield of hydrogen by starch degradation was about 1.5 H_2/mol glucose under both conditions. Thus red light illumination during growth might not affect dark anaerobic metabolism.

DISCUSSION

Light quality affects growth and metabolism in plant and photosynthetic microalgae. The effects of various types of light on growth and starch accumulation in *Chlamydomonas* sp. strain MGA161 was examined

with the aim of increasing dark anaerobic hydrogen evolution. Near ultraviolet and green light inhibited both growth and starch accumulation in comparison with the basic conditions under white light, while blue light did not affect these parameters at all (data not shown). Red light, however, stimulated starch accumulation without changing the growth. Nomarski differential interference microscopic observation confirmed that the stroma starch was increased by red light illumination. It has been reported that red light stimulates some forms of metabolism in plants and algae through a mechanism involving phytochrome system (*12–14*). These metabolisms are regulated at the transcriptional level. In this study, the stimulation of starch accumulation in *Chlamydomonas* sp. strain MGA161 was also dependent on the transcription of certain genes. We found that the activity of FDPase increased 2.3 times during growth under red light. The enzyme irreversibly catalyzes the reaction to produce fructose-6-phosphate, which is a starting material of starch synthesis, from fructose-1,6-diphosphate in the Calvin cycle. Gene expression of riburose-1,5-bisphosphate carboxylase (RuBisCO), which catalyzes the first step of carbon dioxide fixation in the Calvin cycle, is also reported to be stimulated by red light through phytochrome system in radish seedling (*12*). Thus, red light might stimulate starch accumulation by increasing the amounts of enzymes related to carbon fixation.

The amounts of hydrogen produced during dark anaerobic fermentation were increased by cultivating *Chlamydomonas* sp. strain MGA161 under red light. Moreover, when the photosynthetic bacterium *Rhodopseudomonas* sp. strain W-1S was incubated anaerobically in the fermentation broth, about 6 times more hydrogen was recovered than in the fermentation broth prepared from white light-grown algal cells in our preliminary experiment. Red light-grown cells might secrete a certain substance that stimulates bacterial hydrogen evolution during dark anaerobic fermentation. In our proposed biophotolysis system consisting of microalga and photosynthetic bacterium, red light illumination will improve the yield of hydrogen production. On a practical scale the use of filter that can cut unfavorable wavelengths of sunlight might be preferable to red light illumination. Further investigation on the stimulative effect of red light would clarify the limiting step in starch accumulation. The alga could accumulate a large amount of starch under white light by amplifying the genes coding the enzymes responsible for the limiting steps.

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