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Temperature Effect on Production of Hydrogen and Oxygen by *Chlamydomonas* Cold Strain CCMP1619 and Wild-Type 137c†

Scientific Note

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INTRODUCTION

Photosynthetic water splitting for hydrogen and oxygen production is a promising biological process that converts sunlight into useful chemical energy (*1,2*). In green algae, this process becomes active when hydrogenase is induced (*3,4*). In this process, water is split into molecular oxygen, protons, and electrons by photosystem II (PSII). The electrons acquired from water splitting are transferred through PSII to photosystem I (PSI) via a series of intermediate carriers, plastoquinone (PQ), cytochrome b/f, and plastocyanin (PC). At PSI, these electrons are further energized by the PSI photochemical reaction. The energized electrons emergent from the reducing side of PSI are transferred to hydrogenase via ferredoxin (Fd), and thereby utilized in a hydrogenase-catalyzed reaction, the reduction of protons and production of molecular hydrogen.

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The protons consumed in the reduction reaction are derived ultimately from water splitting. The net result of this process is cleavage of water to molecular hydrogen and oxygen (*2,4–6*).

Hydrogenase is a key enzyme in the photoproduction of hydrogen. In multicellular algae and higher plants, this enzyme is lost or no longer inducible for photoproduction of hydrogen (*2,7*). This enzyme is, however, inducible for photoevolution of hydrogen in certain microscopic algae such as *Chlamydomonas*. However, not all species of *Chlamydomonas* have an inducible enzyme to produce hydrogen in the light (*8*). In the work described in this article, a *Chlamydomonas* cold strain, CCMP1619, was assayed for its potential hydrogenase activity by measuring anaerobically induced production of dark- and light-dependent hydrogen. This cold strain was originally isolated from Lake Bonney (ice-covered), Antarctica, and known to grow at low temperatures. The effect of temperature on hydrogen production by CCMP1619 was compared with the wild-type *Chlamydomonas* st rain 137c. The results indicated that 137c and CCMP1619 contain inducible hydrogenase, and that temperature had a significant effect on the rates of hydrogenase induction and on the kinetics of hydrogen production. Simultaneous photoevolution of hydrogen and oxygen was observed in the cold strain at 4°C. Since this type of algae can grow at low temperatures, it is a potential alga for hydrogen production in cold seasons or areas where wild-type *Chlamydomonas* strains cannot grow.

MATERIALS AND METHODS

Chylamydomonas cold strain CCMP1619 was obtained from the Center For Culture of Marine Phytoplankton, Bigelow Laboratory For Ocean Science (West Boothbay Harbor, ME 04575). It was grown photoautotrophically at 4°C using CO_2 from air in 125-L culture flasks containing about 75 mL of minimal media. To allow air exchange, the culture flasks were covered with sterile foam stoppers and shaken constantly at 120 rpm by a mechanical shaker (New Brunswick Scientific Co. Inc., Edison, NJ). A wild-type *Chlamydomonas* strain 137c, obtained from the culture collection of Duke University, was grown using the same method, except that the culture was maintained at room temperature (20°C).

The algal cells were harvested by centrifugation at 500*g* for 5 min, and the chlorophyll content of samples was determined in methanol extract (*9*). The algae were resuspended in minimal medium, and the chlorophyll concentration of the samples was adjusted to 5 μg chl/mL for hydrogen and oxygen assay.

A laboratory-built dual-reactor flow system (Fig. 1) was used for the detection of hydrogen and oxygen. This dual-reactor flow system is essentially a doublet of the flow-detection system described previously

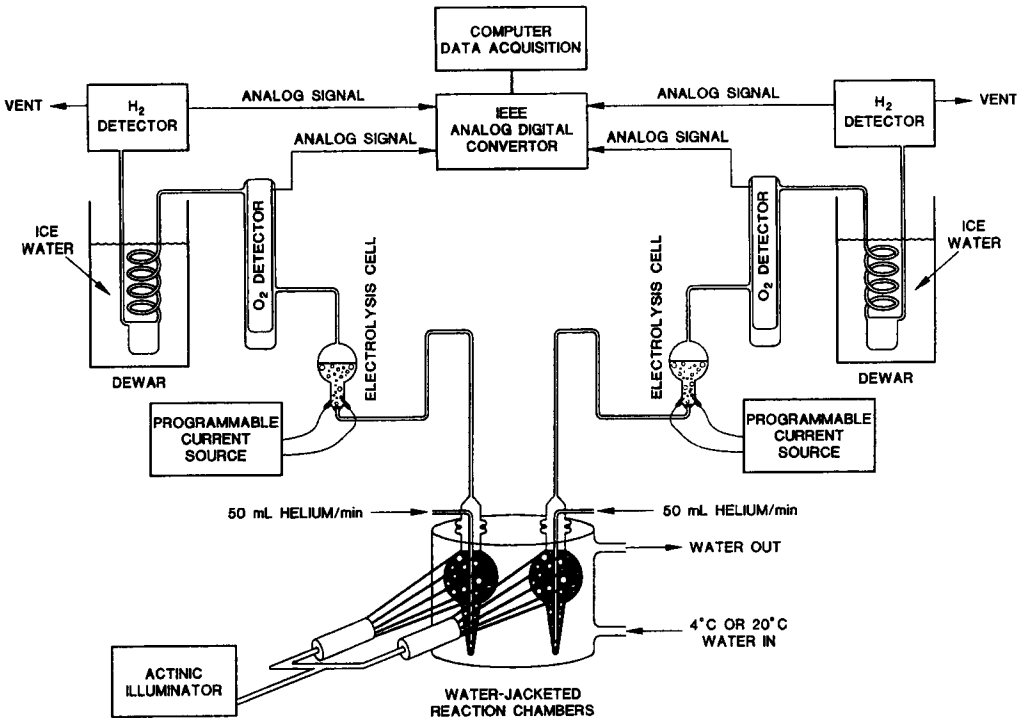

Fig. 1. Schematic illustration of a dual-reactor flow system for hydrogen and oxgyen detection.

(*10*). The advantage of the dual-reactor flow system is that it allows assay of two different samples simultaneously and at virtually identical conditions. Any systematic error of the dual-reactor system can be eliminated by interchanging two samples between the two reactors for each replication of assays. For each assay, 40 mL CCMP1619 sample (5 μg chl/mL) was loaded into one of the two water-jacketed reaction chamber, while the other reaction chamber was loaded 40 mL of 137c alga (5 μg chl/mL) as a control. The assay temperature was maintained either at 4 or 20°C by a temperature-regulated water bath (Lauda RMS6, Brinkmann Instruments, Inc., Westbury, NY). Hydrogenase was induced by removing oxygen gas, with a pure helium flow (50 mL/min) purging through each of the two liquid samples simultaneously. The helium flow served as a carrier to remove hydrogen and oxygen produced by each sample for transport to the detectors. Photosynthetic water splitting for molecular hydrogen and oxygen production was driven by actinic illumination (1.5×10^{16} quanta·s^{-1}·cm^{-2}) provided through two identical fiber-optic cables by an illuminator (Model A3200, Dolnan-Jenner Industries, Inc., Woburn, MA). These fiber cables shared the same light source, so that the actinic outputs from the two cables were essentially identical. As previously described (*10*), oxygen was detected by a galvanic cell (*11,12*), whereas hydrogen

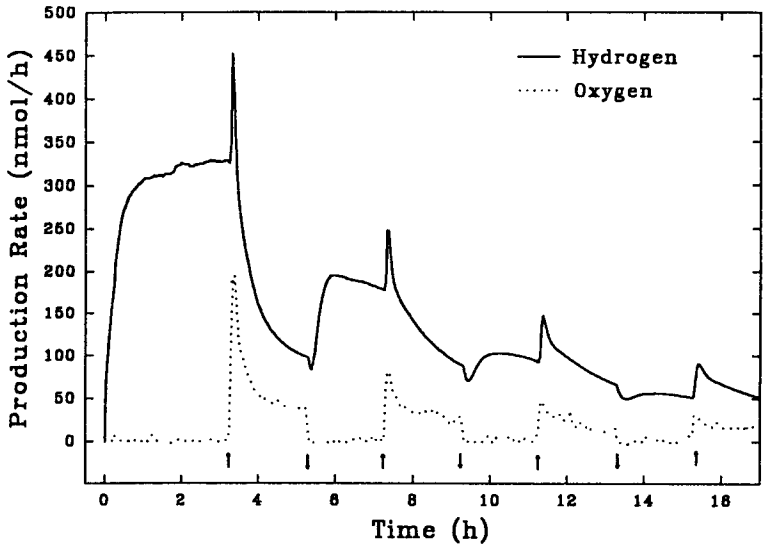

Fig. 2. Production of hydrogen and oxygen by *Chlamydomonas* cold strain CCMP1619 at 20°C under CO_2-free helium atmosphere.

was detected by a tin oxide semiconductor. Data were recorded by a PC microcomputer after analog-to-digital conversion of both hydrogen and oxygen signals. Each assay was repeated four times, with two samples swapped between the two reaction chambers in each replicate to minimize experimental error. The hydrogen and oxygen detectors were calibrated by electrolysis of water using known currents (5, 10, 15, 25, 50, 100, and 200 μA) through programmable current source and Faraday's law of electrochemical equivalence.

RESULTS AND DISCUSSION

The experimental results demonstrated that hydrogenase activity can be induced in the cold strain CCMP1619. Figures 2 and 3 illustrate the production of hydrogen and oxygen in CCMP1619 at 20 and 4°C. For 3.3 h after removing oxygen initially, the samples were kept in darkness and under anaerobic conditions for hydrogenase induction. It is known that hydrogen can be produced fermentatively with carbohydrates and/or proteins as the source of reductant in addition to photosynthetic water splitting. The hydrogen production in this dark period is also known as thermally activated hydrogen production, which reflects fermentative and hydrogenase activity. The rate of thermally activated hydrogen production indicated that the metabolism and hydrogenase induction at 20°C was over 30 times faster than its rate at 4°C and was six times faster than that of the wild-type 137c strain at 20°C (Fig. 4). These results demonstrated that CCMP1619 is highly sensitive to warm temperature.

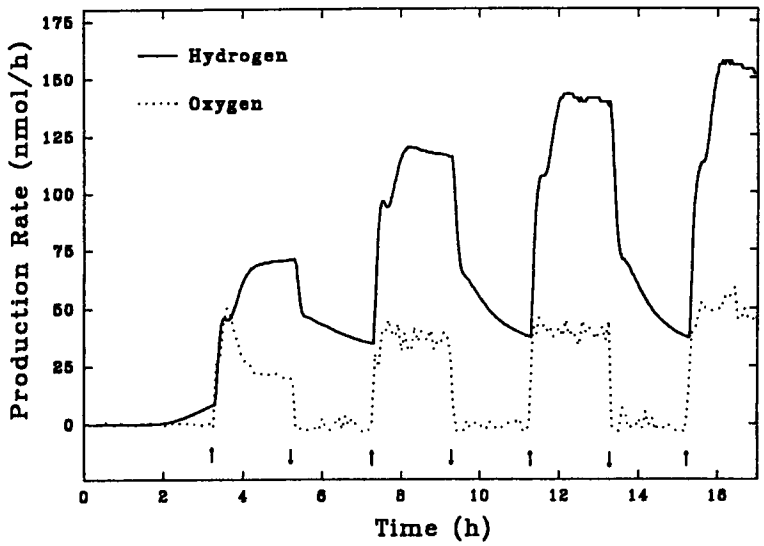

Fig. 3. Production of hydrogen and oxygen by *Chlamydomonas* cold strain CCMP1619 at 4°C under CO_2-free helium atmosphere.

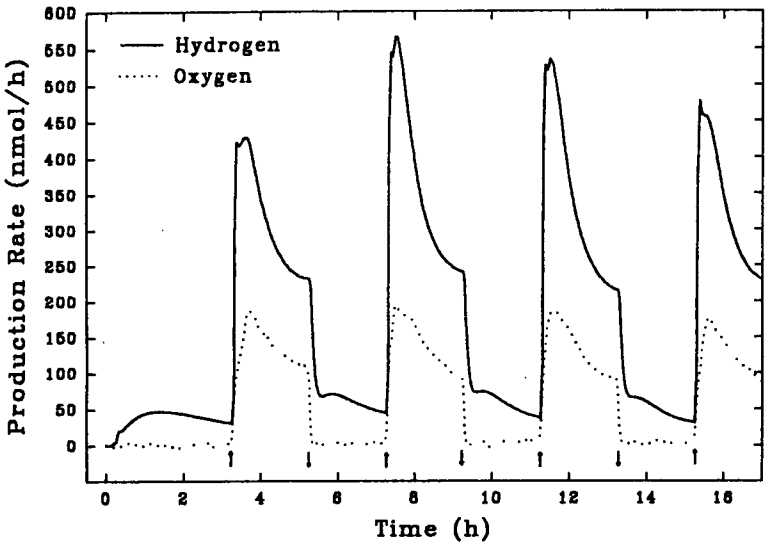

Fig. 4. Production of hydrogen and oxygen by *Chlamydomonas* strain 137c at 20°C under CO_2-free helium atmosphere.

Simultaneous photoproduction of hydrogen and oxygen by photosynthetic water splitting was observed at both temperatures during 2-h intervals of actinic illumination (1.5×10^{16} quanta·s^{-1}·cm^{-2}). The kinetics of the photodependent hydrogen and oxygen production at 20°C (Fig. 2) differed dramatically from those at 4°C (Fig. 3). At 20°C, onset of actinic light (upward arrow) led to a sharp burst of hydrogen followed by a quick

and large decrease in the hydrogen production. At the end of the first illumination period, the total rate of hydrogen production was even slower than that in the first dark period prior to illumination. When actinic light turned off (downward arrow), hydrogen production dropped only for a very short while and then rose quickly again apparently because of onset of "dark" hydrogen production. The dark activation and light inhibition of hydrogen production clearly indicated that light can switch off the thermally activated hydrogen pathway. These kinetic characteristics of hydrogen production can also be observed repeatedly in subsequent light–dark cycles. The maximum rate of hydrogen production decreased somewhat in later illumination cycles. This decrease in the maximum rate of hydrogen production demonstrated the instability of the cold strain CCMP1619 at 20°C.

At 4°C, however, photosynthetic water-splitting activity is fairly stable in CCMP1619. In contrast to the kinetics at 20°C, the first onset of actinic illumination at 4°C led to a hydrogen burst followed by a dip, and then by a continuous increase in production rate (Fig. 3). This small dip, which occurred soon after onset of illumination, may have resulted from the light inhibition of thermally activated hydrogen production that was apparently limited by enzymatic activity at 4°C. The increase in photoproduction of hydrogen after this dip is more interesting. It indicates that some light-dependent physiological process appears to enhance hydrogen production. The maximum rate of hydrogen production increased significantly in successive illumination cycles (Fig. 3). As expected, oxygen was produced only in the light periods. The rate of oxygen production reflects the activity of photosynthetic water splitting. The oxygen production curves at the two temperatures (Figs. 2 and 3) show that the water-splitting activity of CCMP1619 strain increased with time at 4°C, whereas it decreased with time at 20°C. This result is consistent with the hydrogen production data. It again indicated that the activity of CCMP1619 is more stable at 4°C than at 20°C. At 4°C, photoevolution of hydrogen and oxygen was sustained for over 50 h (data not shown). CCMP1619 may be useful in the development of this new hydrogen production system.

Chlamydomonas 137c responded to temperature changes also. Figures 4 and 5 contain hydrogen and oxygen data at two different temperatures. At 20°C, dark hydrogen production was greater than at 4°C. Maximum rate (50 nmol/h) of dark hydrogen production during the first 3.3 h at 20°C was about three times faster than that (16 nmol/h) at 4°C. On illumination (upward arrow) at 20°C, both hydrogen and oxygen production quickly rose to their peak rates and then decayed dramatically (to about 50% of maximum). this type of hydrogen production decay kinetics has been observed previously in a study with carbon dioxide (CO_2) (*13*). In that study, the decay of photodependent hydrogen production was explained by kinetic competition with the Calvin cycle for reductant generated from water splitting. In the current work, the samples were con-

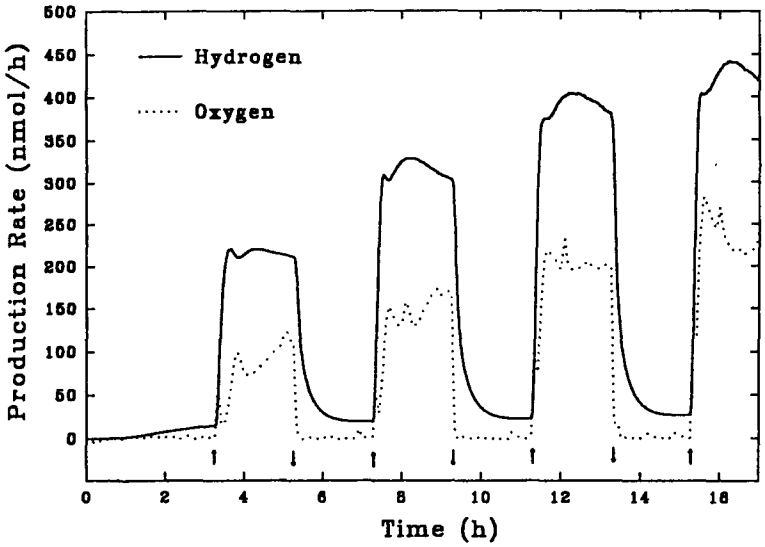

Fig. 5. Production of hydrogen and oxygen by *Chlamydomonas* strain 137c at 4°C under CO_2-free helium atmosphere.

stantly purged with CO_2-free helium gas, and the Calvin cycle was most likely suppressed owing to the depletion of CO_2 in the reaction medium. Therefore, the decay of light-activated hydrogen production observed in this study cannot be explained by competition with the Calvin cycle. The effective purge by helium maintained each liquid sample almost free of oxygen and hydrogen (< 10 ppm), so that the oxy-hydrogen reaction (*14*) was essentially prevented from occurring. We have previously performed experiments with oxygen supplied upstream from the reactor and found no effect on hydrogen production by the low level of titrated oxygen (data not shown). Something else other than the oxy-hydrogen reaction and Calvin cycle limited hydrogen production during illumination. Interestingly, very little of such a decay was observed at 4°C (Fig. 5). Both hydrogen and oxygen production at 4°C were fairly constant in each illumination period. This result indicated that the decay of photoactivated hydrogen production can be largely prevented with cold (4°C) temperature. Photoevolution of both hydrogen and oxygen sustained for over 50 h was similar to that in CCMP1619 at 4°C (data not shown). Furthermore, the maximum production rate at 4°C increased in subsequent illumination cycles and was about two times faster than those of CCMP1619 at the same temperature. According to data analysis, the integrated yield of oxygen and hydrogen photoproduction by 137c in later cycles at 4°C was similar to that at 20°C. Photoevolution of hydrogen and oxygen can be performed well by strain 137c at 4°C. Unlike CCMP1619, however, 137c was not able to grow at 4°C. Therefore, at low temperature, CCMP1619 has an advantage over 137c.

CONCLUSIONS

Although photoproduction of hydrogen and oxygen by *Chlamydomonas* CCMP1619 can be achieved stably at 4°C, it is highly sensitive to warmer (20°C) temperature. Within 3.3 h at 20°C, dark hydrogen production can reach 300 nmol/h, 30 times as fast as its rate (about 10 nmol/h) at 4°C and six times faster than that (50 nmol/h) of strain 137c at 20°C. Hydrogen production can be controlled by light and temperature. Warm temperature stimulates dark hydrogen production, whereas cold temperature (4°C) can largely prevent decay of hydrogen and oxygen photoproduction in both strains CCMP1619 and 137c.

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