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Hydrogen photoproduction by the synchronously grown marine unicellular cyanobacterium *Synechococcus* sp. Miami BG 043511 under extremely high oxygen concentration

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The effect of exogenous oxygen on hydrogen photoproduction was examined in the synchronously grown cells of marine *Synechococcus* sp. Miami BG 043511 under conditions of high cell density (0.6-0.8 mg chl-*a* ml^{-1}) and high light intensity (1000 $\mu E\ m^{-2}\ s^{-1}$). Hydrogen evolution after 20-h incubation did not decline under the initial oxygen concentrations up to 20%, but declined by half under 34% oxygen. 50% and 100% oxygen gas phase did not completely inhibit the hydrogen photoproduction during 40-h incubations. After 2-day pretreatment under 100% exogenous oxygen the hydrogen photoproduction capabilities were not irreversibly inhibited, which was demonstrated in the subsequent 9-day incubation under initial 0, 50 and even under 100% oxygen gas phase. This strain could be useful for developing a hydrogen photoproduction system under atmospheric oxygen concentration.

INTRODUCTION

Oxygen is known to be a potent inhibitor of hydrogen photoproduction in green algae (Gaffron and Rubin, 1942; Spruit, 1958) and cyanobacteria (Benemann and Weare, 1974; Phlips and Mitsui, 1986). Photochemically produced H_2 was partially absorbed in the dark by *Scenedesmus* D3 during the first 15 minutes after the introduction of external oxygen (Gaffron and Rubin, 1942). In a strain of *Chlorella* hydrogen photoproduction was stopped through the poisoning of hydrogenase by simultaneously evolved oxygen during the first few minutes of incubation. Hydrogen evolution was only slightly inhibited by 18% oxygen in a heterocystous cyanobacterium, *Anabaena cylindrica* (Benemann and Weare, 1974), while that in a non-heterocystous filamentous cyanobacterium *Oscillatoria* sp. Miami BG7 was severely inhibited under 1% oxygen (Phlips and Mitsui, 1986). Inhibition of hydrogen production under less than 10% oxygen in argon was reported even in more oxygen tolerant unicellular cyanobacteria such as *Gloeothece* sp. PCC6090, *Synechococcus* sp. PCC 7425, *Cyanothece* sp PCC7822 and *Chroococcidiopsis* thermalis ATCC29380 (Van der Oost *et al.*, 1987; Almon and Böger, 1988). Hence, nitrogen-fixing unicellular cyanobacteria capable of hydrogen photoproduction under atmospheric oxygen tension may be more suitable for the practical applications (Lambert and Smith, 1981).

Random batch culture of a marine unicellular cyanobacterium *Synechococcus* sp. Miami BG 043511 was reported to photoproduce hydrogen and oxygen simultaneously for several days (Mitsui *et al.*, 1983). Later studies on the synchronous culture of this strain revealed that hydrogen production is temporally separated from oxygen evolution (Suda and Mitsui, 1995). Thus, the lack of inhibition of hydrogen photoproduction by the photoproduced en-

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dogenous oxygen has been clarified. However, the effect of exogenous oxygen on hydrogen photoproduction was not investigated in this strain. Here, we report the effect of exogenous oxygen on the hydrogen photoproduction by synchronously grown cells of a marine unicellular cyanobacterium, *Synechococcus* sp. Miami BG 043511.

MATERIALS AND METHODS

Organisms and synchronous cultures

Aerobically nitrogen-fixing marine unicellular cyanobacterium, *Synechococcus* sp. Miami BG 043511, was used (Suda and Mitsui, 1995). To obtain synchronous growth, cells were pretreated with 16-h dark, 16-h light and 16-h dark periods in 3.3-l glass columns containing inorganic medium lacking combined nitrogen at 30°C, 200 μE m^{-2} s^{-1}, 4% CO_2 (in air) and pH 7.6 (Mitsui and Cao, 1988). The synchronous culture was then subjected to continuous illumination for 10 hours before harvesting the cells.

Assays of photoproduced hydrogen and oxygen

The harvested cells were suspended in A-N medium (pH 7.5) without $NaHCO_3$ (Suda *et al.*, 1992) to make 0.6-0.8 mg chl-*a* ml^{-1} suspension. The 25-ml flasks with 2-3 ml cell suspension each were vacuum ventilated, and then filled with argon. This procedure was repeated 10 times. The flasks were further treated to prepare predetermined oxygen concentrations in argon gas phase at one atmospheric pressure. Using a 60-ml plastic syringe the calculated amount of oxygen was injected into each flask. Another empty syringe was attached to the vessel through the butyl rubber stopper, and the two syringes were used to mix the gas by pushing down and pull out the two pistons alternately. After the treatment, a 60-ml plastic syringe was fitted to each flask, and used to observe gas accumulation at one atmospheric pressure. The flasks were continuously illuminated (1000 μE m^{-2} sec^{-1}), and incubated at constant temperature (30°C) in a shaker (65 strokes per minute). For the measurement of the photoproduced hydrogen and oxygen, gas samples (0.1 ml) taken from the 25-ml flasks were assayed in a Fisher model 25V Gas Partitioner (Kumazawa and Mitsui, 1981).

RESULTS AND DISCUSSION

Effect of exogenous oxygen on the hydrogen photoproduction

The capability of hydrogen photoproduction by synchronously grown *Synechococcus* sp. Miami BG 043511 was reported to change significantly during the cell cycle (Suda and Mitsui, 1995; Suda *et al.*, 1992). The cells 10 hours after the onset of the synchronous culture under continuous illumination (= 10-hr LL cells) have shown maximum capability of hydrogen photoproduction, and 22-hr LL cells (= the cells 22 hours after the onset of the synchronous culture under continuous illumination) had a minimum capability (Suda *et al.*, 1992). Thus, the 10-hr LL cells were chosen as suitable experimental material for examining the effect of exogenous oxygen on the hydrogen photoproduction by this strain. Under argon gas phase the 10-hr LL cells exhibited highest hydrogen accumulations (up to 9.27 ml H_2 (ml cells)$^{-1}$ (12 hrs)$^{-1}$) with the 2-3 ml cell suspension of 0.8 mg chl-*a* ml^{-1} (data not shown here).

Figure 1 shows the time course of the hydrogen photoproduction by 10-hr LL cells under the initial oxygen concentrations of 0, 50, and 100%. Although the stepwise manner of hydrogen accumulation (Mitsui *et al.*, 1983) is not so typical in the time course of 0% argon flask, approximately 6 ml of H_2 (ml cells)$^{-1}$ were photoproduced during the first 12 hours. It was noted that even under very high initial oxygen concentrations such as 50% and 100% the amounts of photoproduced hydrogen were not negligible. This result is remarkably different from other previous reports on some unicellular cyanobacterial strains (Van der Oost *et al.*, 1987; Almon and Böger, 1988). Unicellular cyanobacteria, *Gloeothece* sp. PCC6090 (Van der Oost *et*

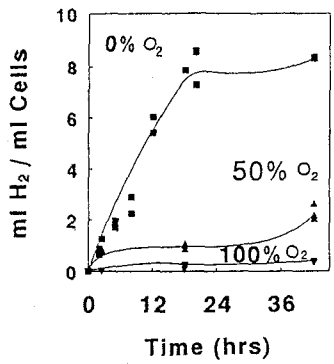

Fig. 1. H_2 accumulation under the initial O_2 concentration in Ar of 0, 50, and 100% by cells harvested after 10-h of continuous illumination during synchronous culture. Two ml of 0.8 mg chl-*a* ml^{-1} cell suspension in 25 ml Fernbach flask were incubated in a shaker.

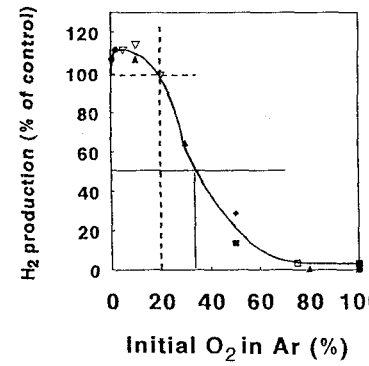

Fig. 2. H_2 accumulation after 20-h incubation of cells harvested after 10-h of continuous illumination during synchronous culture under different initial O_2 concentrations in Ar expressed as the % of the ml H_2 accumulated under Ar gas (<▲>: 3 ml of 0.6 mg chl-*a* ml^{-1} c.s. (cellsuspension), <●, ■, ◆>: 2 ml of 0.8 mg chl-α ml^{-1} c.s., <▽, □>: 3 ml of 0.8 mg chl-α ml^{-1} c.s.).

al., 1987), *Synechococcus* sp. PCC7425 (Van der Oost *et al.*, 1987), *Cyanothece* sp PCC7822 (Van der Oost *et al.*, 1987) and *Chroococcidiopsis thermalis* ATCC29380 (Almon and Böger, 1988) appeared to lose hydrogen producing activities under 1, 2, 9 and 2% oxygen, respectively.

Effect of the concentration of exogenous oxygen on the hydrogen photoproduction by 10-hr LL cells was studied in more details by measuring hydrogen accumulation after 20-h incubation in the light (Fig. 2). At the oxygen concentrations less than 20% the hydrogen photoproduction was slightly enhanced in comparison with the control vessel without any added oxygen. Figure 2 demonstrats that hydrogen photoproduction was slightly stimulated in the presence of small amounts of oxygen (0.5 to 10 % oxygen added). This stimulative effect of the oxygen can be partially explained by the existence of the light-limiting layer of the cell suspension in the vessel. In this light-limiting layer the added oxygen could stimulate the cells to readily mobilize the intracellular carbohydrates through respiration, which consume up the oxygen providing additional ATP and reductants for hydrogen production (Kumazawa and Mitsui, 1994). As oxygen concentration increased over 20%, the hydrogen photoproduction declined gradually. 50% inhibition of hydrogen photoproduction was found at as high as 34% oxygen. 50% inhibitions of hydrogen photoperoduction were reported to occur at 0.5% oxygen in a non-heterocystous cyanobacterium, *Oscillatoria* sp. Miami BG 7 (Phlips and Mitsui, 1986), and at 0.5, 0.7, 3.4 and less than 1.0% oxygen in unicellular cyanobacteria, *Gloeothece* sp. PCC6090 (Van der Oost *et al.*, 1987), *Synechococcus* sp. PCC7425 (Van der Oost *et al.*, 1987), *Cyanothece* sp PCC7822 (Van der Oost *et al.*, 1987) and *Chroococcidiopsis thermalis* ATCC29380 (Almon and Böger, 1988), respectively.

Figure 3 shows the changes in actual oxygen concentrations in the reaction vessels during the incubation for hydrogen photoproduction. During the incubation initial oxygen concentrations decreased and increased once, and then remained at approximately the same level as the initial concentrations. The notable fluctuations of oxygen concentrations in the closed vessel during the first 20 hours are unexplained yet.

Effect of the preincubation in oxygen on hydrogen photoproduction

Figure 4 shows the effect of 100% oxygen pretreatment of the 10-hr LL cells for the first 48 hours on the subsequent hydrogen photoproduction under 0, 50, and 100% oxygen during the following 9

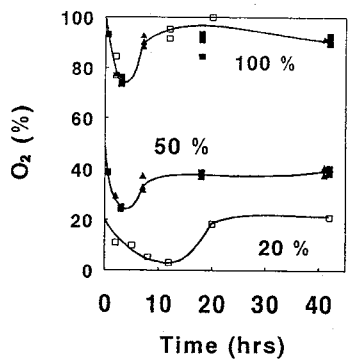

Fig. 3. O_2 concentration changes during the incubation of cells harvested after 10 h of continuous illumination during synchronous culture under different initial O_2% in Ar. Two ml of 0.8 mg chl-*a* ml^{-1} cell suspension in 25 ml Fernbach flask were incubated in a shaker.

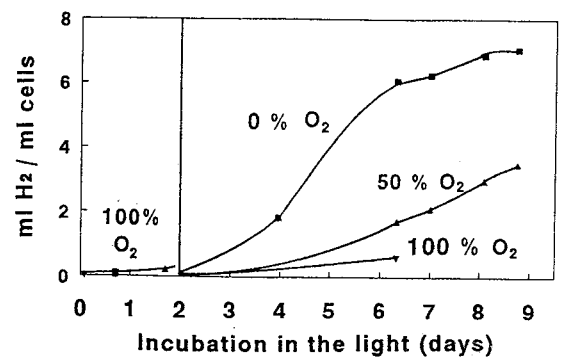

Fig. 4. H_2 accumulation in 2 consecutive incubations of cells harvested after 10 h of continuous illumination during synchronous culture. After the first incubation under 100% O_2, samples were incubated successively under 0, 50, 100% O_2 in Ar in the second incubation. Two ml of 0.8 mg chl-*a* ml^{-1} cell suspension in 25 ml Fernbach flask were incubated in a shaker.

days. The hydrogen productions were also observed under 0, 50 and even under 100% oxygen during the subsequent incubations at slower rates than those in Figure 1. These data indicate that even under the extremely high oxygen concentration (initial oxygen concentration of 100%) for 2 days the capability of hydrogen photoproduction were never irreversibly inhibited.

Present results are important for the applied study of hydrogen photoproduction, because our experimental strain can keep producing high level of hydrogen under oxygen partial pressure equivalent to the atmosphere (Lambert and Smith, 1981). Mechanisms of oxygen resistance of this unique strain is not known at present. Cell cycle events such as cyclic formation and disintegration of the glycogen granules (Nemoto et al., 1994), temporal separation of oxygen evolution from the hydrogen photoproduction (Suda *et al.*, 1992) and cyclic new synthesis of nitrogenase (Campbell *et al.*, 1995) may be closely related to the protection of its nitrogenase and/or hydrogenase enzyme system against exogenous oxygen. Lack of the metabolism for the uptake of extracellular hydrogen (Mitsui *et al.*, 1983) is another advantage of this strain for the aerobic hydrogen photoproduction. Using the information obtained through the present and previous studies, the elucidation of the actual mechanisms involved in the anti-oxygen resistance would be made in the near future.

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