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Cell Age Optimization for Hydrogen Production Induced by Sulfur Deprivation Using a Green Alga *Chlamydomonas reinhardtii* UTEX 90

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Abstract Under sulfur deprived conditions, PS II and photosynthetic O_2 evolution by *Chlamydomonas reinhardtii* UTEX 90 are inactivated, resulting in shift from aerobic to anaerobic condition. This is followed by hydrogen production catalyzed by hydrogenase. We hypothesized that the photosynthetic capacity and the accumulation of endogenous substrates such as starch for hydrogen production might be different according to cell age. Accordingly, we investigated (a) the relationships between hydrogen production, induction time of sulfur deprivation, increase of chlorophyll after sulfur deprivation, and residual PS II activity, and (b) the effect of initial cell density upon sulfur deprivation. The maximum production volume of hydrogen was 151 ml H_2/l with 0.91 g/l of cell density in the late-exponential phase. We suggest that the effects of induction time and initial cell density at sulfur deprivation on hydrogen production, up to an optimal concentration, are due to an increase of chlorophyll under sulfur deprivation.

Key words: Hydrogen production, *Chlamydomonas reinhardtii*, sulfur deprivation, cell growth stage

Hydrogen (H_2) has the highest gravimetric energy density among thus far known fuels, and is compatible with electrochemical and combustion processes for energy conversion without evolving carbon-based emissions that lead to environmental pollution and the greenhouse effect [11, 15]. Numerous methodologies have been developed for effective hydrogen production. Among them, the biological hydrogen production has gained attention, because hydrogen can be produced by cellular metabolism under the presence of water and sunlight.

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Green algae is one of the microorganisms that can produce hydrogen [1, 2, 12]. Green algae has photosynthetic system like that of plants [14], therefore, they can produce hydrogen by using carbon dioxide as the carbon source [10], sunlight as the energy source, and water as the electron donor. Hydrogen production by the green algae is catalyzed by hydrogenase, an enzyme that exhibits activity only under anaerobic condition. Hydrogenase activation is a prerequisite to produce hydrogen by the green algae, however, the enzyme is severely oxygen sensitive [8] and easily inactivated by photosynthetic oxygen evolution. This problem can be overcome by separating photosynthetic oxygen evolution and anaerobic hydrogen production by using sulfur deprived media [3].

Sulfur is one of the six macronutrients required by plants, microorganisms, animals, and human for growth and development. When algal cells are cultured under the sulfur deprived medium, the cells cease their division [5], change their morphology [18], and degrade endogenous substrates such as protein and starch [9, 13, 16]. One of the most noticeable adaptive responses of algae under the sulfur deprived condition is to stop water splitting, which is mediated by photosystem II (PS II) breakdown in the light. The rate of oxygen evolution drops below the rate of respiratory oxygen uptake, and the environment of algal culture is converted from aerobic to anaerobic condition [13, 17]. The prompt conversion to anaerobic condition activates the hydrogenase and hydrogen production by the activated hydrogenase subsequently.

In this study, photobiological hydrogen was produced, using a green alga *Chlamydomonas reinhardtii* UTEX 90 under sulfur deprived condition, by the two-stage process, which consisted of aerobic cell growth stage and anaerobic hydrogen production stage. The *C. reinhardtii* UTEX 90 was photoheterotrophically cultivated, and the anaerobic

condition for hydrogen production was prepared by sulfur deprivation in the medium. To find out the important induction parameters for hydrogen production, the effect of cell growth stage on hydrogen production was investigated under various cell densities.

MATERIALS AND METHODS

Algal Cell Culture

C. reinhardtii UTEX 90 was photoheterotrophically cultivated on Tris-acetate-phosphate (TAP) medium [10] at pH 7.2 and 25°C in 250-ml Erlenmeyer flasks, shaken at 150 rpm. The light was provided by alternating light (12 h) and dark (12 h), using fluorescent light with 60 $\mu Em^{-2}s^{-1}$ of intensity [16].

Hydrogen Production Under Sulfur Deprived Condition

The *Chlamydomonas* cells were harvested at different growth stages, such as early-exponential phase, mid-exponential phase, late-exponential phase, and stationary phase (Fig. 1), by centrifugation at 2,000 ×*g* for 5 min. To remove residual sulfur in the cell culture, the harvested cells were washed 5 times with sulfur-omitted TAP medium (TAP-S medium) [4]. The washed cells in 40 ml volume were placed in the 100-ml serum bottles, and argon (Ar) gas was purged to remove oxygen in the headspace of each bottle. The cells were cultivated under continuous fluorescent light (~200 $\mu Em^{-2}s^{-1}$) at 25°C and 150 rpm for 5 days.

Analytic Methods

Cell growth was monitored by measuring the dry cell weight (DCW), using the dried filter paper method at 80°C. The HP 5890 gas chromatograph system (Hewlett-Packard, U.S.A.) with a carboxen-1000 column (Supelco, U.S.A.) and thermal conductivity detector was used to determine the levels of hydrogen in the headspace of the serum bottle. Argon was used as a carrier gas. For chlorophyll analysis, the cells were harvested by centrifugation at 3,000 ×*g* for 5 min, and the chlorophyll in the cell pellet was extracted with 90% ethanol. Total chlorophyll content was spectrophotometrically determined by the Spreitzer's method [4]. In starch analysis, cell pellets collected by centrifugation were treated successively with 80% ethanol and acetone-acetone ether (1:1) to remove lipid components. Starch was extracted from these defatted cells with 25% perchloric acid at 45°C for 1 h. The concentration of starch was assayed by an iodo-starch reaction method, measuring the absorbance of the starch-iodine complex at 550 nm [6]. Light intensity in the photo incubator was measured with a LI-250 Li-Cor quantum photometer (Lambda Instrument Corp., U.S.A.).

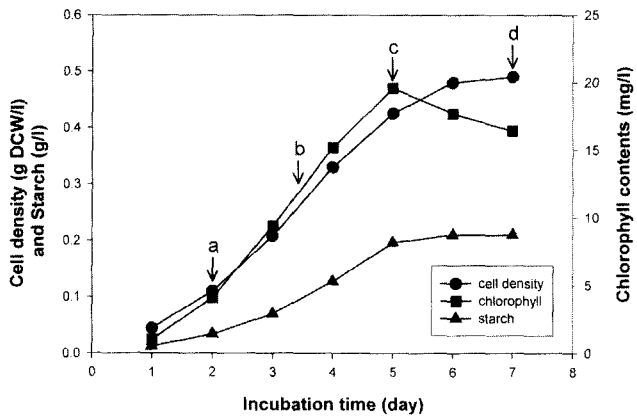

Fig. 1. Contents of chlorophyll and starch depend on growth of *Chlamydomonas reinhardtii* UTEX 90.
The arrows represent the harvest points of the cells for the hydrogen production under the sulfur deprived condition (a. early-exponential phase, b. mid-exponential phase, c. late-exponential phase, d. stationary phase).

RESULTS AND DISCUSSION

Cultivation of the Green Alga *C. reinhardtii*

Hydrogen production was conducted by the photosynthetic microalga *C. reinhardtii* UTEX 90 under anaerobic sulfur deprived environment. Thus, the *Chlamydomonas* cells were heterotrophically cultured on the TAP medium under synchronized illumination (light 12 h: dark 12 h), and the contents of chlorophyll and starch in the cells at different growth stages were measured. As illustrated in Fig. 1, the chlorophyll content increased during the cell growth and reached the maximum level (19.8 mg/l) at the late-exponential phase. The maximum starch content (0.2 g/l) was also obtained at the late-exponential phase, and this maximum value was maintained during the stationary phase. The starch concentration did not appear to increase any more after the exponential growth phase, because the photosynthetic capacity of the cells was reduced as the decreased chlorophyll content [7]. Therefore, the photosynthetic capacity and the accumulation of endogenous substrates, such as starch, seemed to reach the maximum values at the late-exponential phase.

Effect of Cell Growth Stage on Hydrogen Production

In order to investigate the effect of cell growth stage on hydrogen production, anaerobic hydrogen production was carried out with the cells, which were harvested from the culture at the early-exponential phase, mid-exponential phase, late-exponential phase, and stationary phase, as shown in Fig. 1, and the harvested cells were washed and resuspended in TAP-S medium for anaerobic condition. As shown in Fig. 2, the amount of hydrogen production based per cell increased as the cell growth progressed under the exponential growth phase. The hydrogen production in the stationary phase was lower than that in the late-exponential

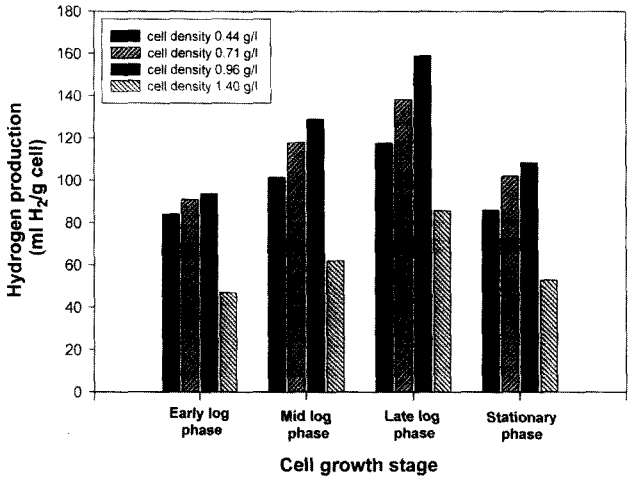

Fig. 2. Production volume of hydrogen after 5 days of sulfur deprivation depends on cell growth stage and initial cell density.

phase. The maximum amount of hydrogen production was 159 ml H_2/g cell, which was obtained by the cells at the late-exponential phase with 0.96 g/l of cell density.

From Kosourov's study [9] on hydrogen production, using sulfur deprived algal cells, in the presence of PS II electron transport inhibitors, it is known that at least 80% of the electrons required for hydrogen production originate from the residual water oxidation activity in PS II. In green algae, chlorophyll is the pigment primarily responsible for harvesting light energy used in photosynthesis. When light is absorbed by the antenna chlorophyll located in the photosynthetic membranes, the electrons are released from the oxygen-evolving complex (OEC) by the water splitting reaction, and travel down an electron transport chain in the thylakoid membrane. Therefore, as the intracellular chlorophyll content increases, the light energy transported to PS II also increases. Then, in the PS II system, more electrons of OEC can be obtained by water splitting, and these electrons can be used for hydrogen production, mediated by hydrogenase, under sulfur deprived condition. Figure 3 shows the change of chlorophyll content in the hydrogen production stage under various cell growth stages and cell densities. In all cases, the chlorophyll content increased sharply for a day and decreased gradually thereafter. The increase of chlorophyll content and maximum chlorophyll content in the cells from the late-exponential phase were higher in all cases of the cell density than those of the cells from other cell growth phases. From these results, it seems that the high content of chlorophyll in these cells from the late-exponential phase brought about the increase of residual water oxidation activity in PS II, which in turn increased the electrons required for hydrogen production. Therefore, the cells harvested from the late-exponential phase, having

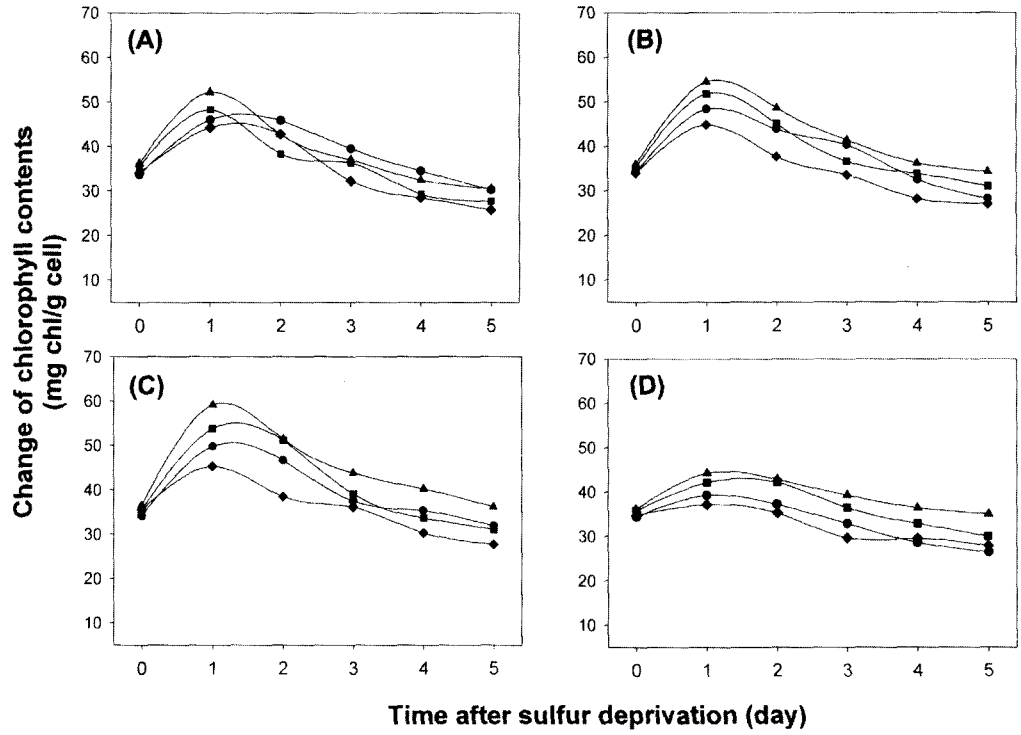

Fig. 3. Profiles of chlorophyll contents after sulfur deprivation.
(A) Cell density 0.44 g/l, (B) Cell density 0.71 g/l, (C) Cell density 0.96 g/l, (D) Cell density 1.4 g/l. Symbols: -●-, early-exponential phase; -■-, mid-exponential phase; -▲-, late-exponential phase; -◆-, stationary phase.

Table 1. Initial chlorophyll contents and initial cell density at the start of the sulfur deprivation stage (only the initial chlorophyll contents on the late-exponential phase are shown).

Case	Initial cell density (g/l)	Initial chlorophyll contents (mg/l culture)
(A)	0.44	16.70
(B)	0.71	26.75
(C)	0.96	36.29
(D)	1.40	52.56

high chlorophyll content, produced the most hydrogen under anaerobic hydrogen production stage induced by sulfur deprivation.

Effect of Cell Density on Hydrogen Production

The algal cultures were diluted at the beginning of the experiment to have various initial cell densities (0.44 to 1.4 g/l culture) at the start of the hydrogen production stage. As shown in Fig. 2, the hydrogen production volume increased, depending on the cell density up to 0.96 g/l, in all cases of cell growth phases, because initial chlorophyll content increased as the cell density increased, as listed in Table 1. There are four steps for hydrogen production under sulfur deprivation: aerobic, oxygen consumption, anaerobic, and hydrogen production stage [9]. When the cell density is higher under sulfur deprivation, it takes less time for oxygen consumption due to their high chlorophyll content, resulting in quicker conversion to the anaerobic environment. The fast conversion to the anaerobic stage quickly activates hydrogenase, which has strong oxygen sensitivity. The anaerobic stage was promptly switched to the hydrogen production stage. Consequently, the period of hydrogen production was prolonged, and the *C. reinhardtii* cell culture with higher cell density produced more hydrogen gas.

On the other hand, in the case where the cell density was 1.4 g/l, the hydrogen production volume and the increasing rate of chlorophyll content were lower than those of 0.96 g/l cell density, although the cell density was higher (Figs. 2 and 3). The low increase of chlorophyll content by light limitation due to high cell density seems to hinder not oxygen consumption rate, but the water splitting rate in PS II (Fig. 3). Therefore, the electrons available for the hydrogen production decreased, and subsequently, the amount of hydrogen production decreased. If the stronger light were provided in the culture with higher cell density, the light limitation due to shade effect decreases and the hydrogen production subsequently increased.

In conclusion, the optimization of hydrogen production in this system was achieved by carefully controlling several parameters. First, the growth phase of the cells was one of the important factors affecting hydrogen production. The highest hydrogen production was obtained by the cells from the late-exponential phase, which had the highest increase of chlorophyll concentration during 24 h under the sulfur deprivation. The water oxidation activity in PS II increased in proportion to the chlorophyll content, increasing the electrons used for hydrogen production. Second, the hydrogen production was dependant on the switching time from the aerobic to the anaerobic stage after sulfur deprivation. As the cell density and initial chlorophyll content at sulfur deprivation increased, the period of aerobic stage shortened, resulting in high activation of the hydrogenase and high hydrogen production. However, it is important to keep the cell density below the critical cell concentration in order not to limit light absorption. It might be possible to maintain the cell age in the real continuous process for hydrogen production by controlling HRT (hydraulic retention time) of the cell growth stage photoreactor. We expect that the results presented from this study would be useful in the system design for algal hydrogen production.

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