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Dark Hydrogen Production by a Green Microalga, *Chlamydomonas reinhardtii* UTEX 90

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Abstract The production of hydrogen by *Chlamydomonas reinhardtii* UTEX 90, a marine green alga, was performed under dark fermentation. The effects of initial nitrogen and phosphorus concentration on the cell growth and the production of hydrogen and organic substances were investigated. In the growth stage, the maximum dry cell weight (DCW) was 3 g/l when the initial ammonium concentration was 15 mM. In the dark fermentation, the maximum hydrogen production was 3.5 μmol/ mg DCW when the initial nitrogen concentration was 7.5 mM. The nitrogen concentration had a greater effect on organic compound and hydrogen production than the phosphorus concentration during the dark fermentation. An investigation of the duration of dark fermentation showed that, at least until three days, dark fermentation should be prolonged for maximum hydrogen production.

Key words: *Chlamydomonas reinhardtii*, dark fermentation, hydrogen, organic compound

Hydrogen has many advantages as an energy source. It is the simplest, naturally occurring element, and is found in numerous materials. It is hoped that hydrogen, an energy carrier, will join electricity as a basis for a globally sustainable energy system using renewable energy. Moreover, hydrogen use does not produce CO_2 or other greenhouse gases [2, 11].

The methods used to produce hydrogen depend on the quantity and purity of hydrogen desired. It is believed that biological production is the most important method, using photosynthetic microbes to produce H_2 from sunlight energy [7, 13]. However, the photosynthetic production of oxygen and hydrogen must be separated, because hydrogenase is sensitive to oxygen. Consequently, in a two-stage process, CO_2 is first fixed during normal oxygenic photosynthesis, and then molecular hydrogen is generated when microalgae are incubated under anaerobic conditions [1, 8, 10]. In the two-stage concept, a photobioreactor is used to allow microalgal CO_2 fixation as storage carbohydrates; then the culture is transferred to a dark anaerobic fermentation vessel, in which the hydrogenase is activated and induced, enabling H_2 production [3].

Microalgae can produce various compounds via photosynthesis, and the catabolism of endogenous carbohydrates occurs via respiration under dark aerobic conditions and via fermentation under dark anaerobic conditions. The latter process seems to be more useful for cell viability than for growth. Although endogenous storage compounds are completely decomposed to carbon dioxide by aerobic respiration, various end products are formed by anaerobic fermentation. The light period is the carbohydrate accumulation phase in photosynthetic organisms. When cells are transferred to dark anaerobic condition, carbohydrates are degraded via the so-called fermentation pathway into lower-molecular-weight compounds such as ethanol and acetate. These organic compounds are important for hydrogen production. Photosynthetic bacteria that produce large amounts of hydrogen use ethanol, acetate, and other organic compounds as a carbon source for growth. Therefore, these organic compounds are the basic material for hydrogen production using photosynthetic bacteria [5, 9].

The aim of the present study was to optimize the dark fermentation conditions for the production of hydrogen and other organic compounds. The effects of initial concentrations of ammonium and phosphate on the cell growth and hydrogen production were investigated. In addition, the production of organic compounds such as acetic acid and ethanol were monitored under different initial nutrient conditions. These organic compounds can be good nutrients

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for the further production of hydrogen using photosynthetic bacteria.

MATERIALS AND METHODS

Strain and Culture Conditions

Chlamydomonas reinhardtii UTEX 90 was obtained from UTEX, The Culture Collection of Algae at the University of Texas at Austin, in the U.S.A. *C. reinhardtii* UTEX 90 was grown in 250-ml Erlenmeyer flasks containing 100 ml of culture medium, at 25°C with alternating light/dark incubation (12 hr/12 hr) repeatedly operated. The culture medium was TAP (Tris-acetate-phosphate) medium.

Light Condition

C. reinhardtii was grown in photobubble bioreactors [12]. The algal cultures were continuously illuminated by a fluorescent lamp at a light intensity of 100 $\mu E/m^2sec$. The cultures were continuously purged with air containing 3% CO_2 for the purpose of agitation and CO_2 supply at a flow rate of about 100 ml/min. The mixing gas used in this experiment was made by mixing air and CO_2 with a gas mixer.

Dark Fermentation

C. reinhardtii in the logarithmic growth phase was harvested. Then, the cells were transferred to a 140-ml fermentation bottle fitted with a rubber stopper. The bottle was bubbled for 30 min with O_2-free N_2 gas (99.99%). Then, it was capped with the rubber stopper and the fermentation bottle sealed with aluminum foil to prevent light transmission. When this dark anaerobic condition was settled, the algal cells were incubated at 25°C on a shaking incubator operating at 120 rpm.

Analytical Methods

Turbidometric measurements standardized (optical density) to a cell count curve are satisfactory for the estimation of cell density. Cell growth was monitored by measuring the optical density (OD) at 660 nm with a spectrophotometer (HITACHI, U-2000). To find the correlation between dry cell weight and optical density, 10 ml of cell suspension was centrifuged at 4,000 ×*g*. Then, the cells were washed twice with deionized water, and dried on filter paper in an oven at 80°C until the filter paper with cells reached a constant weight. Starch was measured by a modified iodo-starch reaction method. Algal suspension was centrifuged (4,000 ×*g*, 10 min) at 0°C, and the pellet was mixed with 4 ml of 40% perchloric acid, and then it was left at 25°C for 6 h. The mixture was slowly neutralized by adding NaOH in an ice-water bath. Then, the sample was heated in a boiling water bath for 30 min to dissolve the starch, and centrifuged (15,000 ×*g*, 20 min) at 0°C. After this starch solution was appropriately diluted, starch was assayed by an iodo-starch reaction method [6]. Ethanol and acetate were assayed by gas chromatography (GC) using an FID detector of which the temperature was 230°C. The oven temperature was 200°C and the injector temperature was 215°C. The column-packing material was Porapak Q. Each concentration was obtained through a standard curve, which indicated the correlation between concentration and GC area. Hydrogen was assayed by a GC using a TCD detector in which the temperature was 180°C. The oven temperature was 80°C and the injector temperature was 200°C.

The results were the mean value of three experiments.

RESULTS AND DICUSSION

Growth Aspects of *C. reinhardtii*

C. reinhardtii uses CO_2 as its carbon source during the growth stage. The rate of cell growth decreases severely in 5% CO_2, whereas the rates in 1% and 3% CO_2 are similar [4]. During dark fermentation, *C. reinhardtii* grown in 3% CO_2 produced more hydrogen than that grown in 1% CO_2. Therefore, the optimal CO_2 concentration was 3% (data not shown).

TAP medium with nitrogen concentrations of 0.8, 3.7, 7.5, and 15.0 mM NH_4^+ was tested. The cell density at the four nitrogen concentrations differed. The dry cell weight (DCW) increased with nitrogen concentration, and was 3 g/l in cells grown in 15 mM NH_4^+. Nevertheless, the early growth was similar in all four cases (Table 1). The growth of UTEX 90 at different phosphorus concentrations was also examined. Unlike the effects of nitrogen concentration, the cells grew well at all phosphorus concentrations tested, although growth was lowest in 0.1 mM PO_4^{3-}. Nevertheless, the final cell density was 1.5 to 2.5 g/l, and phosphorus concentration did not have a major effect on cell growth.

Table 1. Effects of initial ammonium and phosphate concentrations on maximum dry cell weight in *Chlamydomonas reinhardtii* UTEX 90.

Initial ammonium concentration (mM)	Maximum dry cell weight (g/l)	Initial phosphate concentration (mM)	Maximum dry cell weight (g/l)
0.8	0.3	0.1	1.5
3.7	1.0	0.5	2.5
7.5	1.7	1.0	2.2
15.0	3.0	2.0	1.8

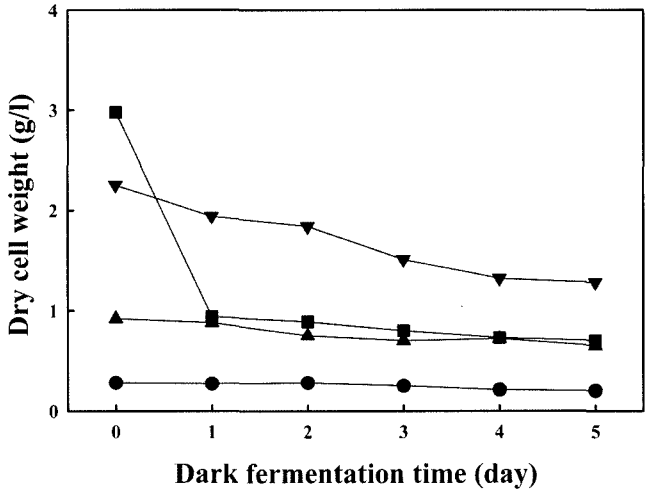

Fig. 1. Time course of dry cell weight of cells grown in different nitrogen concentrations during dark fermentation.
Ammonium concentration: 0.8 mM (●), 3.7 mM (▲), 7.5 mM (▼), 15.0 mM (■).

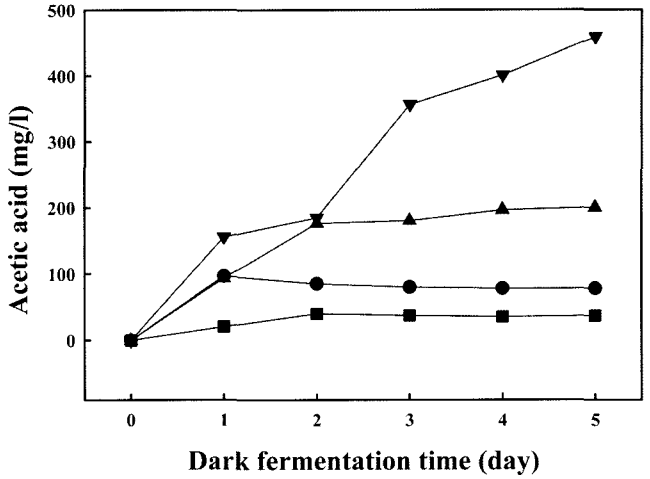

Fig. 3. Time course of acetic acid produced in different nitrogen concentrations during dark fermentation.
Ammonium concentration: 0.8 mM (●), 3.7 mM (▲), 7.5 mM (▼), 15.0 mM (■).

Time Courses of Dark Anaerobic Fermentation

Dry cell weight decreased with elapsed time (Figs. 1 and 2). Therefore, once dark fermentation occurs, cells do not grow further, but degrade intracellular materials, such as starch. As a result, the DCW did not change much in the nitrogen experiment, whereas it changed significantly in the phosphorus experiment. Cell density was high for cells grown in 15 mM NH_4^+, but these cells did not perform dark fermentation actively. The starch content was also low (<5%) (data not shown). The starch content decreased from about 40% to 30%, although the decrease differed with growth conditions. DCW decreased steeply with active dark fermentation.

Cells produced larger amounts of organic compounds with time (Figs. 3, 4, 5, and 6). Cells grown in different phosphorus concentrations and fermented for 5 days produced about twice as many organic compounds as cells fermented for 2 days. Cells grown in 2.0 mM PO_4^{3-}, which produced small amounts of organic compounds initially, subsequently produced slightly larger amounts. Acetic acid production stopped on the second day at 0.5 and 2.0 mM PO_4^{3-}, while production continued for more than 4 days at the other phosphorus concentrations. In 0.1 mM PO_4^{3-}, cells produced the largest amounts of organic compounds. This was related to the decrease in DCW shown in Fig. 2, in

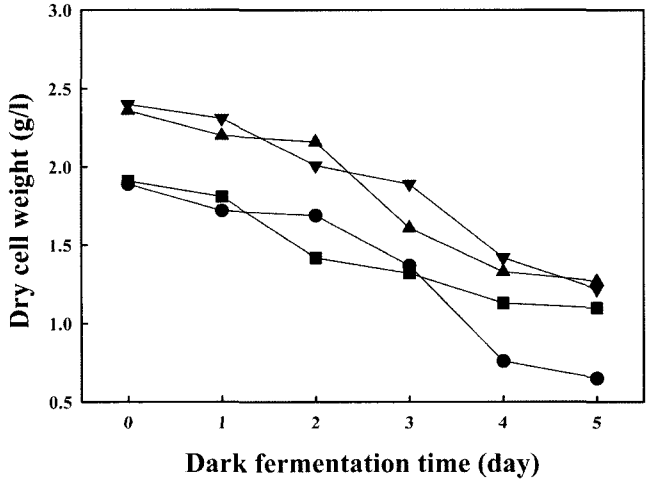

Fig. 2. Time course of dry cell weight of cells grown in different phosphorus concentrations during dark fermentation.
Phosphate concentration: 0.1 mM (●), 0.5 mM (▲), 1.0 mM (▼), 2.0 mM (■).

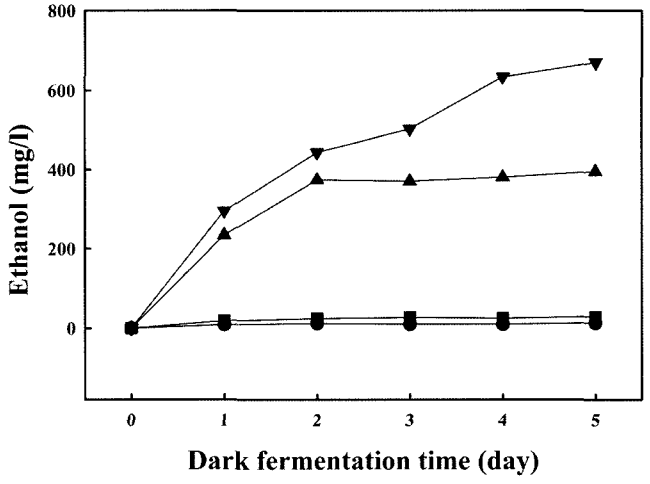

Fig. 4. Time course of ethanol produced in different nitrogen concentrations during dark fermentation.
Ammonium concentration: 0.8 mM (●), 3.7 mM (▲), 7.5 mM (▼), 15.0 mM (■).

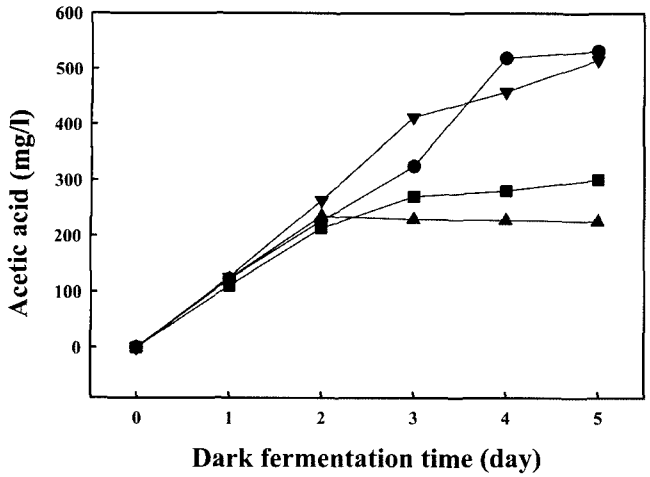

Fig. 5. Time course of acetic acid produced in different phosphorus concentrations during dark fermentation.
Phosphate concentration: 0.1 mM (●), 0.5 mM (▲), 1.0 mM (▼), 2.0 mM (■).

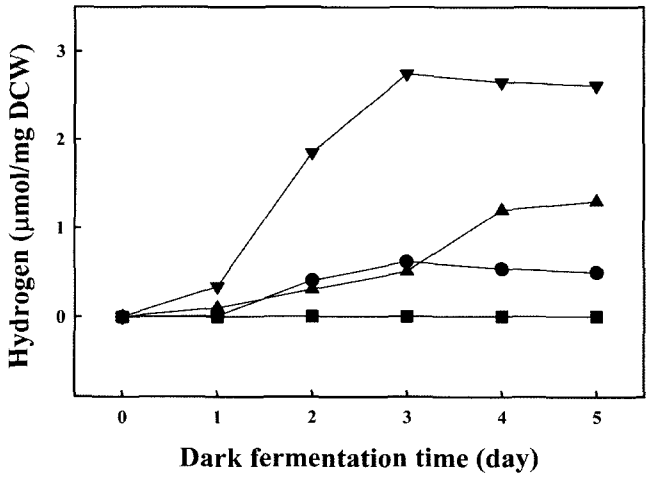

Fig. 7. Time course of hydrogen produced in different nitrogen concentrations during dark fermentation.
Ammonium concentration:0.8 mM (●), 3.7 mM (▲), 7.5 mM (▼), 15.0 mM (■).

which DCW decreased continuously until Day 5, indicating that the cells grown in 0.1 mM PO_4^{3-} performed dark fermentation actively during that period.

The difference was more pronounced with different nitrogen concentrations. Cells grown in 3.7 and 7.5 mM NH_4^+ produced larger amounts of organic compounds with time. Larger quantities of organic compounds were produced with 7.5 mM NH_4^+, whereas cells grown in 0.8 mM NH_4^+ produced less acetic acid, and cells grown in 15.0 mM NH_4^+, which had a very low starch content, produced extremely small amounts of organic compounds. In 3.7 mM NH_4^+, cells stopped organic compounds production on Day 2, which paralleled the halt in pH change at Day 2 (data not shown.). By contrast, cells grown in 7.5 mM NH_4^+ produced organic compounds until Day 5, and the pH decreased concomitantly until Day 5. Cells undergoing dark fermentation produced roughly twice as much ethanol as acetic acid.

Hydrogen Production in Dark Anaerobic Fermentation

The production of hydrogen differed from the production of organic compounds in that it increased with time (Figs. 7 and 8). Cells grown in different nitrogen and phosphorus concentrations produced much hydrogen if dark fermentation progressed sufficiently. However, the time of maximum hydrogen production depended on the experimental conditions. Cells grown in 1.0 mM PO_4^{3-} showed a sharp increase in

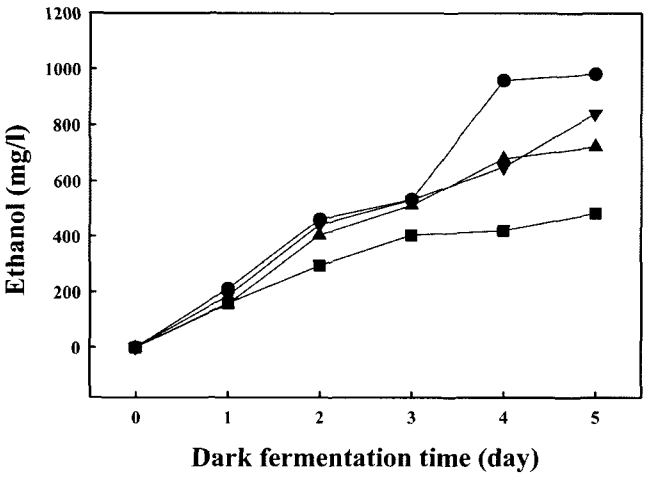

Fig. 6. Time course of ethanol produced in different phosphorus concentrations during dark fermentation.
Phosphate concentration: 0.1 mM (●), 0.5 mM (▲), 1.0 mM (▼), 2.0 mM (■).

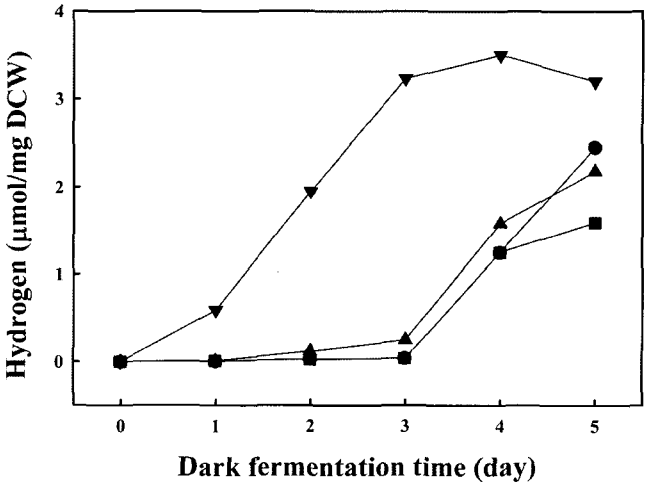

Fig. 8. Time course of hydrogen produced in different phosphorus concentrations during dark fermentation.
Phosphate concentration: 0.1 mM (●), 0.5 mM (▲), 1.0 mM (▼), 2.0 mM (■).

hydrogen production until the third day. By contrast, cells grown in 0.1, 0.5, and 2.0 mM PO_4^{3-} produced increased amounts of hydrogen until Day 5. Little hydrogen was produced with 15.0 and 0.8 mM NH_4^+ because the initial starch content was low; the maximum production was on Day 4, with 3.7 mM NH_4^+ and on Day 3 with 7.5 mM NH_4^+. As the growth curves show, when there was sufficient cell growth, cells produced more hydrogen for a longer time than otherwise. When conditions were not suitable, cells produced small amounts of organic compounds and hydrogen.

In this study, the time profile of dark fermentation, during which hydrogen and organic compounds are produced, was observed. During dark fermentation, the dry cell weight decreased with time, while the amounts of organic compounds and hydrogen produced increased with time. The pH should decrease with time as dark fermentation uses starch to produce organic compounds (data not shown). In fact, the pH of cell broth after dark fermentation for four days was 0.5 to 1.7 lower than the initial pH. The nitrogen concentration had a greater effect on organic compound and hydrogen production than the phosphorus concentration. Moreover, cells grown in TAP medium with standard nitrogen and phosphorus concentrations produced the most hydrogen and larger amounts of organic compounds. While the production of organic compounds increased with time, the production of hydrogen peaked on the third day. Therefore, from the perspective of hydrogen production, a longer period might be needed for dark fermentation than reported in a previous report [10].

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