0273-2289/05/121–124/403–412/$30.00

Continuous Hydrogen Photoproduction by *Chlamydomonas reinhardtii*

Using a Novel Two-Stage, Sulfate-Limited Chemostat System

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Abstract

This study demonstrates, for the first time, that it is possible to couple sulfate-limited *Chlamydomonas reinhardtii* growth to continuous H_2 photoproduction for more than 4000 h. A two-stage chemostat system physically separates photosynthetic growth from H_2 production, and it incorporates two automated photobioreactors (PhBRs). In the first PhBR, the algal cultures are grown aerobically in chemostat mode under limited sulfate to obtain photosynthetically competent cells. Active cells are then continuously delivered to the second PhBR, where H_2 production occurs under anaerobic conditions. The dependence of the H_2 production rate on sulfate concentration in the medium, dilution rates in the PhBRs, and incident light intensity is reported.

Index Entries: Green algae; sulfur deprivation; photobioreactor; chemostat; H_2 production; *Chlamydomonas reinhardtii.*

Introduction

When *Chlamydomonas reinhardtii* cultures are deprived of sulfate, photosynthetic O_2- evolution activity is substantially inhibited *(1)*, the cultures become anaerobic *(2,3)*, the [FeFe]-hydrogenase is induced *(4,5)*, and the algae utilize their residual water-oxidation activity to photoproduce H_2 gas *(3,6,7)*. This process has been studied extensively in *C. reinhardtii* cultures, where the O_2-evolution and H_2-production phases are separated temporally in the same photobioreactor (PhBR) *(2,4,6–11)*. In the batch system, sulfur deprivation and H_2 photoproduction last 100–150 h, at which point the effects of sulfur deprivation on other metabolic activities

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lead to more global inhibition of cellular function *(2,10)*. It is possible to reinitiate H_2 photoproduction by re-adding sulfate to the exhausted cultures by allowing a 2-d recovery period before a second round of sulfur deprivation *(3)*.

The addition of small concentrations of sulfate either at the start of sulfur deprivation *(4)* or during the H_2- production phase *(12)* has an enhancing effect on residual photosystem II (PSII) activity by inducing a temporary recovery of PSII-catalyzed water-oxidation capacity. Once the re-added sulfate is consumed by the cultures, increased H_2-photoproduction rates are observed after a short delay. Based on this observation, we hypothesized that it might be possible to prolong the H_2-production phase of sulfur-deprived cultures by continuously replacing the cells during the H_2-production phase with sulfur-deprived but photosynthetically active cells. To implement this idea, we devised a method to separate photosynthetic, sulfate-limited growth physically from H_2-production by applying continuous cultivation techniques. Microalgae have been cultivated in chemostat mode under heterotrophic *(13)*, photoautotrophic *(14)*, and outdoor *(15)* conditions for biomass production, when all nutrients are available at excess concentrations. We demonstrate here that, under appropriate conditions, long-term cultivation of *C. reinhardtii* under limited-sulfate concentrations can be coupled with simultaneous H_2-production for a total of at least 4000 h.

Materials and Methods

Strain and Media

C. reinhardtii strain cc124 *(16)* was grown photoheterotrophically in a 1-L Erlenmeyer flask (800 mL of liquid phase) on Tris-acetate-phosphate-minus-sulfur (TAP-S) medium (pH 7.2) with 90 μ*M* sulfate added, at 25°C. The cell suspension was sparged with 3% CO_2 in air, mixed by magnetic stirring (PC-131; Corning, NY), and illuminated continuously with cool-white fluorescent light (~200 μE/ [$m^2 \cdot s$] of photosynthetically active radiation on the outer surface of the culture flasks). The gas mixture was sterilized using autoclavable membrane filters with a 1-μm pore size (Bacterial Air Vent; Pall, Ann Arbor, MI). The cell culture was grown under these conditions for 3 to 4 d to a final concentration of 24–28 mg of chlorophyl (Chl)/L. At this point, all sulfate was consumed from the medium, and the culture was used as an inoculum to start the two PhBRs (see next section). The cells were resuspended in TAP-S at a final concentration of 10 mg of Chl/L.

TAP-S medium is a modification of standard TAP medium *(16)*, in which all sulfate salts were replaced with chloride salts at the same concentrations. A stock solution of 100 μ*M* $MgSO_4 \cdot 7H_2O$ (Sigma, St. Louis, MO)

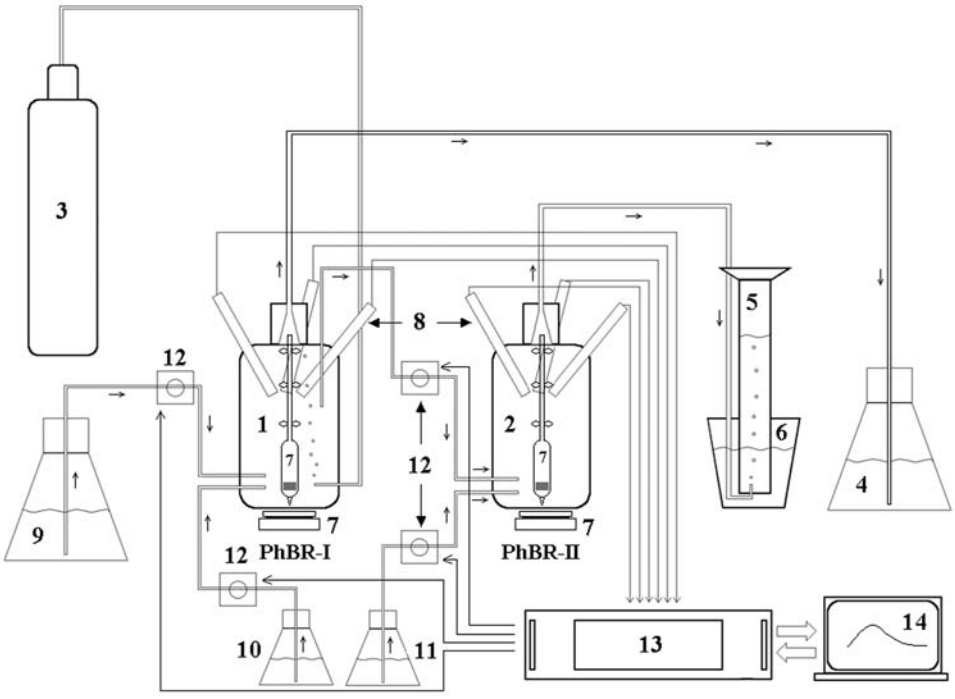

Fig. 1. Schematic diagram of the computer-controlled, two-stage chemostat PhBR system for continuous H_2 production by *C. reinhardtii*. [1, 2]: PhBR-I and PhBR-II respectively; [3]: tank supply for CO_2/air gas input; [4]: harvest bottle for cell suspension from PhBR-I; [5]: graduated reservoir for gas collection from PhBR-II; [6]: collector reservoir for excess cell suspension from PhBR-II; [7]: magnetic stirring rods and stirrers; [8]: built-in ports for electrodes (pH, pO_2, E_h); [9]: media bottle; [10, 11]: acid and base bottles (for pH titration), respectively; [12]: peristaltic pumps; [13, 14]: microprocessor/laptop computer control and recording system. The tubing connecting [1] to [2] was 1.5 m long and had a 1.14 mm inner diameter.

was used to prepare TAP-limited sulfate media (with concentrations as indicated in the text).

Design and Operation of PhBR System

Figure 1 shows a schematic diagram of the computer-controlled two-PhBR system. The system consists of two identical, specially fabricated glass PhBRs (5-cm optical path; 1050-mL culture volume). The first PhBR (PhBR-I) [1] is used to grow algal biomass under aerobic, sulfate-limiting conditions, and it is operated in a chemostat mode at specified dilution rates with fresh medium [9] indicated by D_1 (h^{-1}). The cell suspension is sparged with sterile 3% CO_2 in air at 1.5 mL/min [3] to obtain photosynthetically competent cells. The cells from PhBR-I are continuously delivered to the second PhBR (PhBR-II) [2] at a dilution rate of $D_{1\text{-}2}$ (h^{-1}). If $D_1 > D_{1\text{-}2}$, the excess cell suspension is collected in a 12-L harvest bottle [4]. PhBR-II is used to maintain algal cells under sulfur-deprived conditions,

which are favorable for establishing culture anaerobiosis and subsequent H_2-production activity. The gas produced in PhBR-II is collected in a graduated cylinder [5] by displacement of liquid 1 *M* NaOH solution [6].

The algal cells were cultivated at 28°C under continuous two-sided illumination of ~100 μE/($m^2 \cdot s$) at each surface of PhBR-I, provided by fourteen 34-W cool-white fluorescent lamps (Philips). Similar conditions were used for PhBR-II, except that the incident light intensity was varied with neutral density filters, as specified in the text. Specially designed vertical glass stirring rods with a magnetic bar located at the bottom of the rod were used to mix the algal cultures continuously at 350 rpm (magnetic stirrer from Isotemp, Fisher, Pittsburgh, PA) [7] to provide homogeneity of the suspension density along the vertical axes of the PhBRs. Each PhBR has ports [8] for, respectively, a pH electrode with built-in temperature sensor, a dissolved O_2 (pO_2) electrode, and a redox (E_h) electrode. Furthermore, each PhBR has additional ports for gas/liquid output, culture sampling, gas bubbling (only PhBR-I), and pH titrants. Fresh medium, cell suspension, and titrants (0.2 *M* CH_3COOH; 0.2 *M* NaOH) [9,11] are delivered to each photobioreactor, as required, by peristaltic pumps and microbore Tygon® tubing (MasterFlex C/L, Cole-Parmer, Vernon Hills, IL) [12]. The pH in PhBR-I is set at 7.5 (only acid is added) and that in PhBR-II at 7.8 (only base is added) *(5)*. Parameters such as pH, pO_2, E_h, D_1, and $D_{1\text{-}2}$ are monitored and/or controlled automatically by an industrial microprocessor and computer system [13,14] as described in ref. *4*. The redox potential was measured against a standard Ag/AgCl electrode. Autoclavable electrodes were used, including an Inpro 3100 (pH and temperature) and a Pt4805-DPAS-SC-K8S/120 (redox potential), both from Mettler-Toledo (Woburn, MA), as well as glass, DO polarographic probe from Cole-Parmer. PhBR-I is started in a chemostat mode at the beginning of the process. PhBR-II is started in the batch mode. At 24–28 h, when H_2 gas production in PhBR-II commences, we initiated cell replacement from PhBR-I.

Other Analytical Procedures

Chlorophyll (total *a* and *b)* content was assayed spectrophotometrically in 95% ethanol extracts by the method of Spreitzer *(16)*, and the optical density (OD) of the cell suspensions was measured at 750 nm in a 1-cm glass cuvet. Both assays were done using an ultraviolet–visible spectrophotometer (Model UV1610PC, Shimadzu, Kyoto, Japan).

Sulfate concentration in the supernatants was measured by ion chromatography (according to method 300.1 of the US Environmental Protection Agency, Cincinnati, OH). The retention time for sulfate is 13.5 min and the detection limit of this method is 1 μ*M* SO_4^{2-}.

Incident light intensity I_o was measured with an Li-COR quantum photometer (Model LI-250; Lincoln, NE) at six points along the height of the PhBR on each side, and the values in the text represent the average light intensity at each surface of the PhBR.

Flash-probe Chl *a* fluorescence and *in vitro* hydrogenase activity measurements were conducted as described previously *(5)*.

A conversion factor of 1 mL:33 μmol was used to calculate the quantity of H_2 produced at the 1609-m altitude of the experiments. All measurements were made with steady-state cultures after at least five periods of culture doubling. Mean values ± SE in the figures and tables were calculated from three to eight independent measurements made during the steady-state phase of each experiment.

Results and Discussion

When algal cells are cultivated photoheterotrophically in a chemostat mode, it is necessary to continuously deliver fresh medium, light, and CO_2 to the bioreactor to maintain uniform culture growth. However, it is possible to control the growth rate of the chemostat cultures by limiting one or more of the nutrients (such as sulfate) in the medium. Nevertheless, even under sulfate-limiting growth, the cultures will store some residual intracellular sulfate, which prevents substantial inhibition of O_2-evolution activity in PhBR-II. On the other hand, while inducing anaerobicity and H_2- production activity, total sulfate deprivation could not sustain cell growth in PhBR-II. The two-stage chemostat system used in the present study ensures that the sulfur-deprived cultures in PhBR-II are continuously diluted with sulfur-limited cells from PhBR-I, which (a) quickly become sulfur deprived when cultivated anaerobically in PhBR-II, (b) are competent in H_2 production, and (c) can sustain long-term H_2 production in PhBR-II.

Chemostat Culture Growth in PhBR-I Under Sulfate-Limiting Conditions

We studied the effect of sulfate limitation on the growth *C. reinhardtii* cultures in PhBR-I by monitoring their cell densities and Chl concentrations (Fig. 2). When the cultures were grown at an incident light intensity of 100 $\mu E/(m^2 \cdot s)$ and at a medium dilution rate of $D_1 = 0.04\ h^{-1}$ (except $0.02\ h^{-1}$ for the 50 μ*M* SO_4^{2-} experiment), the steady-state Chl concentration increased linearly as the sulfate concentration increased from 50 to 150 μ*M*. On the other hand, the OD increased linearly only up to 100 μM sulfate. We suggest that at above 100 μ*M* sulfate, cell growth becomes light-limited owing to the high pigment concentration achieved.

Small amounts of extracellular residual sulfate, on the order of 2–4 μ*M* (Fig. 2) were detected in all cultures. We consider these levels to be negligible because they are near the minimum detection limit of the assay method (about 1 μ*M*). Moreover, the *C. reinhardtii* high-affinity sulfate transporter system is characterized by a $K_{1/2}$ of $2.2 \pm 0.9 \times 10^{-6}$ *M* SO_4^{2-} and a V_{max} of 206 ± 32 fmol of SO_4^{2-}/s per 10^5 cells *(17)*, demonstrating that residual 4 μ*M* sulfate should be entirely consumed in seconds during the time that the

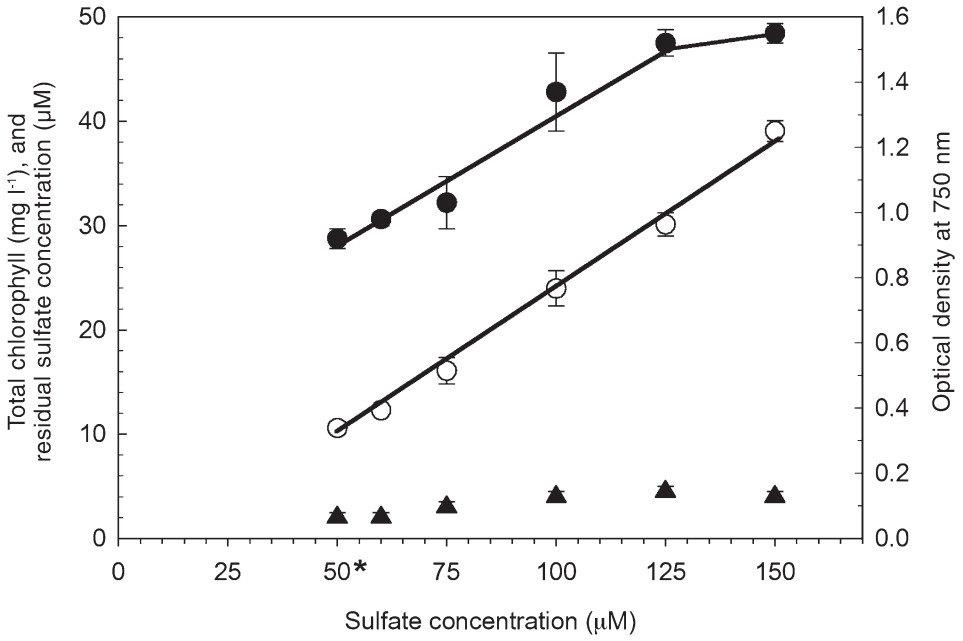

Fig. 2. Effect of input medium sulfate concentration on steady-state Chl concentration (○), OD (●), and residual extracellular sulfate concentration (▲) in chemostat cultures of *C. reinhardtii*. The cells were grown in PhBR-I at $D_1 = 0.04\ h^{-1}$ and an I_0 of 100 μE/($m^2 \cdot s$). *$D_1 = 0.02\ h^{-1}$.

cells are transported from PhBR-I to sulfur-deprived PhBR-II if there were no additional sulfate input.

H_2 Photoproduction in PhBR-II

All of the PhBR-I cultures tested in the previous section were aerobic (pO_2 100–350% of air, E_h + 80 to +200 mV; data not shown) when cultivated at the indicated sulfate concentrations. As shown in Fig. 3A, the photochemical capacity of the PhBR-I culture was almost equal ($F_{max} = 0.5$) to the F_{max} of fully sulfur-replete algal cultures ($F_{max} = 0.6$; *[5,10]*). Furthermore, the photochemical capacity of the culture from the corresponding PhBR-II was close ($F_{max} = 0.3$; Fig. 3B) to that of the cells producing H_2 in batch culture ($F_{max} = 0.2$; *[5,10]*). However, H_2 production in PhBR-II only occurred when the cultures in PhBR-I were cultivated with TAP medium containing about 50-75 μ*M* sulfate. The observed maximum H_2-production rate under these conditions was 0.58 mL/(h · L_{PhBR}) at 60 μ*M* SO_4^{2-} and at dilution rates $D_1/D_{1\text{-}2} = 0.025\ h^{-1}/0.025\ h^{-1}$ (Table 1). When the dilution rates were changed to $D_1/D_{1\text{-}2} = 0.04\ h^{-1}/0.02\ h^{-1}$, the H_2-production rate decreased to 0.42 mL/h · L_{PhBR}. However, the total volume of H_2 gas photoproduced would double, because under these conditions one PhBR-I could support two PhBR-IIs. At sulfate concentrations of 50 ($D_1/D_{1\text{-}2} = 0.02\ h^{-1}/0.01\ h^{-1}$)

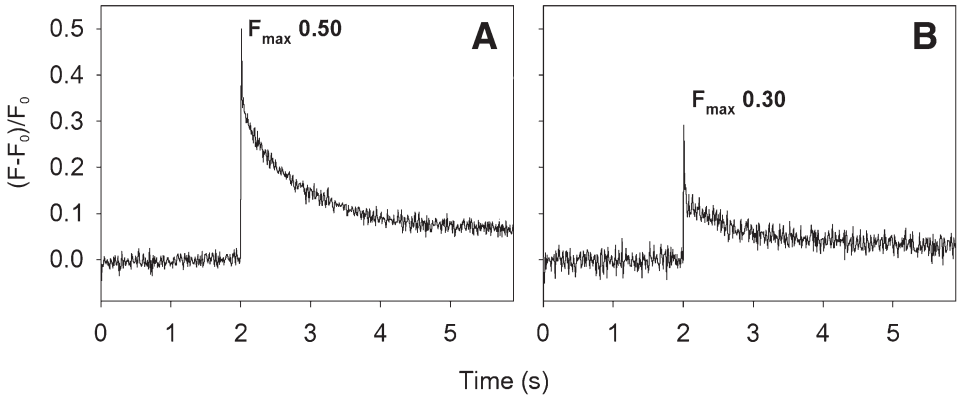

Fig. 3. Kinetics of Chl *a* fluorescence yield of DCMU-treated *C. reinhardtii* cells. Fluorescence was induced by a single actinic flash, measured in the presence of background 100-Hz LED probes and normalized to the background F_0 level. The cells were cultivated on TAP (60 μM SO_4^{2-}) at $D_1/D_{1-2} = 0.04\ h^{-1}/0.02\ h^{-1}$, and at an I_0 of 100 and 80 $\mu E/(m^2 \cdot s)$ in (**A**) PhBR-I and (**B**) PhBR-II, respectively. Samples were taken directly from the PhBRs operating under steady-state conditions and treated as described in Materials and Methods.

and 75 μ*M* ($D_1/D_{1\text{-}2} = 0.04\ h^{-1}/0.02\ h^{-1}$), the H_2-production rates were one half and one-fifth of that at 60 μ*M*, respectively (Table 1). Furthermore, maximum H_2 photoproduction seems to be observed at a redox potential (detected by the electrode directly in contact with the cell suspension of PhBR-II) of about – 450 mV vs Ag/AgCl. We surmise that a faster D_1 dilution rate must result in higher intracellular levels of residual sulfate in the PhBR-I cells that are transferred to PhBR-II, thus resulting in lower H_2 productivity.

It is important to note that, although the H_2 production varied widely, the *in vitro* hydrogenase activity of samples taken from PhBR-II under all conditions was always high (data not shown). This demonstrates that H_2 photoproduction was limited by factors other than hydrogenase enzyme activity in these experiments.

Despite the fact that any residual sulfate in the PhBR-I extracellular medium is probably consumed before reaching PhBR-II, the amount of intracellular sulfur reaching PhBR-II could be different under different cultivation conditions in PhBR-I. For example, the higher the sulfate concentration in the input TAP medium and the higher the dilution rate, the higher the amount of intracellular sulfur that would be expected in the algal cells being transferred to PhBR-II. If the sulfur content of the cells is too high, residual PSII activity could be high enough that anaerobicity could not be maintained in PhBR-II, and H_2 production would stop. This conjecture is based on the observation that PSII activity decreases as a function of time under sulfur-deprived conditions *(1–3,11)*. Consequently,

Table 1
Extracellular Redox Potential, Chl Concentration, and H_2-Photoproduction Rates in Cultures of *C. reinhardtii* Maintained in PhBR-II at an I_o of 50 μE/(m² · s) at Each Surface[a]

Parameter measured	Sulfate concentration in TAP, (μ*M*) (D_1 h^{-1}/$D_{1\text{-}2}$, h^{-1})			
	50	60		75
	(0.02/0.01)	(0.025/0.025)	(0.04/0.02)	(0.04/0.02)
E_h (mV)	−502 ± 13	−473 ± 6	−448 ± 7	−389 ± 20
Chl (mg/L)	8.2 ± 0.4	11.2 ± 0.2	11.8 ± 0.2	16.1 ± 1.3
H_{2av} (mL/[L_{PhBR} · h])	0.27	0.58	0.42	0.11
H_{2av} (μmol/[mg_{Chl} · h])	1.08	1.71	1.17	0.23

[a] The cells were grown in PhBR-I on TAP media containing the indicated sulfate concentrations at an I_o of 100 μE/(m² · s). The values in parentheses represent, respectively, the dilution rate of the TAP medium into PhBR-I (D_1, h^{-1}) and of the cell suspension from PhBR-I into PhBR-II ($D_{1\text{-}2}$, h^{-1}).

Table 2
Effect of I_0 on Chl Concentration and Average H_2-Photoproduction Rates of *C. reinhardtii* Cultures in PhBR-II[a]

Parameter measured	D_1 h^{-1}/$D_{1\text{-}2}$ h^{-1}					
	0.025/0.025			0.04/0.02		
	I_0 (μE/[m² · s])					
	25	50	80	25	50	150
E_h (mV)	−392 ± 9	−473 ± 6	−487 ± 18	−454 ± 4	−448 ± 7	−371 ± 27
Chl (mg/L)	11.9 ± 0.2	11.2 ± 0.2	10.9 ± 0.2	14.0 ± 0.2	11.8 ± 0.2	11.2 ± 0.2
H_{2av} (mL/[L_{PhBR} · h])	0.33	0.58	0.23	0.28	0.42	0.24
H_{2av} (μmol/[mg_{Chl} · h])	0.91	1.71	0.70	0.66	1.17	0.71

[a]The cells were grown in PhBR-I on TAP medium containing 60 μ*M* sulfate at an I_o of 100 μE/(m² · s) and at the dilution rates indicated.

there should be an optimal sulfate input concentration to PhBR-I at which H_2 photoproduction in PhBR-II would be maximal.

Finally, cultivation of *C. reinhardtii* on TAP-S with 60 μ*M* SO_4^{2-} with simultaneous H_2-photoproduction in the two-stage chemostat system was maintained for more than 4000 h (data not shown), and the system was used in our investigations of the effects of light intensity described next.

Effect of Incident Light Intensity on H_2 Photoproduction

Because H_2 production by sulfur-deprived *C. reinhardtii* is a light-dependent process, light intensity is expected to have a large effect on H_2-production rates *(18)*. These effects were measured with cultures cultivated in the presence of 60 μ*M* sulfate (input to PhBR-I) at the two dilution rates $D_1/D_{1-2} = 0.025\ h^{-1}/0.025\ h^{-1}$ and $0.04\ h^{-1}/0.02\ h^{-1}$. As shown in Table 2, H_2- photoproduction rates were maximal (0.58 and 0.42 mL/[h · L_{PhBR}]) under a light intensity of 50 μE/[m^2 · s]. Higher intensities (80–150 μE/[m^2 · s]) inhibited H_2 production, perhaps because of increased, residual PSII activity. Low (25 μE/[m^2 · s]) light intensity also decreased H_2-production rates, perhaps owing to limited photosynthetic electron transport. These results are consistent with the observations made previously by Laurinavichene et al. *(18)* using a batch system.

Conclusion

This work is the first report of continuous H_2 photoproduction by green algae obtained without neutral gas purging or addition of reductant. Future studies will be performed to further optimize the system by increasing the biomass concentration in the system without a parallel increase in intracellular sulfate levels. Furthermore, greater understanding of the biochemistry of the process will aid in making this system more attractive for future application in the biotechnology industry.

Acknowledgment

This work was supported by the Hydrogen, Fuel Cells, and Infrastructure Technologies Program, EERE, US Department of Energy.

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