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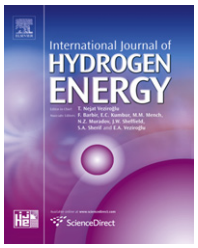

Maximizing the hydrogen photoproduction yields in *Chlamydomonas reinhardtii* cultures: The effect of the H_2 partial pressure

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ABSTRACT

Photoproduction of H_2 gas has been examined in sulfur/phosphorus-deprived *Chalmydomonas reinhardtii* cultures, placed in photobioreactors (PhBRs) with different gas phase to liquid phase ratios ($V_{g.p}/V_{l.p}$). The results demonstrate that an increase in the ratio stimulates H_2 photoproduction activity in both algal suspension cultures and in algae entrapped in thin alginate films. In suspension cultures, a 4× increase (from ~0.5 to ~2) in $V_{g.p}/V_{l.p}$ results in a 2× increase (from 10.8 to 23.1 mmol l^{-1} or 264–565 ml l^{-1}) in the total yield of H_2 gas. Remarkably, 565 ml of H_2 gas per liter of the suspension culture is the highest yield ever reported for a wild-type strain in a time period of less than 190 h. In immobilized algae, where diffusion of H_2 from the medium to the PhBR gas phase is not affected by mixing, the maximum rate and yield of H_2 photoproduction occur in PhBRs with $V_{g.p}/V_{l.p}$ above 7 or in a PhBR with smaller headspace, if the H_2 is effectively removed from the medium by continuous flushing of the headspace with argon. These experiments in combination with studies of the direct inhibitory effect of high H_2 concentrations in the PhBR headspace on H_2 photoproduction activity in algal cultures clearly show that H_2 photoproduction in algae depends significantly on the partial pressure of H_2 (not O_2 as previously thought) in the PhBR gas phase.

1. Introduction

Some cyanobacteria and eukaryotic algae but not higher plants are capable of water biophotolysis, the light-dependent, photosynthetic process that results in the simultaneous production of molecular hydrogen and oxygen [1]. In green algae, this occurs (*i*) after a period of dark, anaerobic induction [2], (*ii*) in sulfur-deprived cultures when anaerobiosis is established spontaneously as the result of the partial inactivation of photosystem II (PSII)-mediated, water-splitting activity in the

Abbreviations: Chl, chlorophyll; Fd, ferredoxin; GC, gas chromatography; PAR, photosynthetic active radiation; PhBR(s), photobioreactor(s); PSII, photosystem II; RuBisCo, ribulose-1,5-bisphosphate carboxylase-oxygenase; TAP, Tris-Acetate-Phosphate; TA-S-P, Tris-Acetate minus sulfur and phosphorus; WT, wild-type.

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cells [3] or (iii) in engineered algae with a depleted level of PSII core proteins [4]. Under anaerobic conditions, algal cells express [FeFe]-hydrogenase enzymes [5,6] that catalyze the following ferredoxin (Fd)-linked reaction [7]:

$$2H^+ + 2Fd_{red} \leftrightarrow H_2 + 2Fd_{ox}. \quad (1)$$

The initial rate of H_2 photoproduction in dark-adapted algal cells is very high, but the reaction cannot be sustained due to the rapid (within seconds) inactivation of [FeFe]-hydrogenase enzyme by O_2, co-evolved by photosynthesis [8]. As a result, significant efforts to surmount the O_2-sensitivity issue have been made [8,9]. Continuous H_2 photoproduction is possible under normal photosynthetic conditions, if the algae are continuous sparged with inert gas to rapidly remove the O_2, or in sulfur-deprived, wild-type (WT) cultures and in certain mutants with decreased levels of PSII activity, when the O_2 produced by the residual PSII centers is lowered sufficiently by respiration. However, H_2 photoproduction under these conditions usually proceeds at lower rates as compared to the initial rates in dark-adapted algae [10–12]. Among other factors known to decrease H_2-production yields in algal cultures are low light saturation levels of photosynthesis [10], competition for reductant from alternative metabolic pathways [13], and the reversible nature of the hydrogenase-driven reaction. The latter has not been well studied in green algae. In general, the hydrogenase enzyme accelerates the establishment of equilibrium between the concentration of H_2 and the level of reduced Fd. The midpoint redox potential of the major photosynthetic Fd (encoded by *PETF*) from *Chlamydomonas reinhardtii* is close to −0.398 V [14], whereas the redox potential of H_2 at pH 7 and 1 atm (100% H_2 in the gas phase) is equal to −0.413 V. This means that under standard conditions, when 50% of the Fd molecules are reduced, the system is close to equilibrium, and the rate of the reverse reaction in Eq. (1) is significant. In contrast, the redox potential of H_2 at pH 7 and 0.01 atm H_2 is equal to −0.354 V, which favors the forward reaction. In terms of H_2 production by microalgae, this means that the presence of high levels of gaseous H_2 in a photobioreactor (PhBR) inhibits the H_2-production reaction. This phenomenon has been known for a long time and has been well studied in some anaerobic bacteria that perform dark fermentation [15]. The high partial pressure of H_2 not only inhibits H_2 production in anaerobic bacteria but often shifts metabolic pathways toward the production of more reduced substrates such as lactate, ethanol, acetone, butanol, or alanine [15]. Under certain conditions *Clostridium* can tolerate up to 2.5 atm of H_2 before responding by shifting metabolic flux to other products [16]. However, in most other microorganisms metabolic changes occur at much lower H_2 partial pressures (0.016–0.75 atm [17,18]).

In green algae, an increased rate of H_2 photoproduction was detected in a photobioreactor with a large headspace volume after extensively purging the culture with inert Ar gas in the dark [19]. The authors explained the effect by the dilution of photosynthetically-produced O_2 in the photobioreactor headspace, which accelerated the removal of O_2 from algal cells and, hence, decreased its inhibitory effect on the hydrogenase enzyme. Assuming high O_2-evolving activity in algal cells, the accumulation of O_2 in the cells can indeed be a major issue for hydrogenase-driven H_2 production process. Similar results were obtained with sulfur-deprived algae, when continuous purging of cell suspensions with Ar resulted in higher rates of H_2 photoproduction than those measured in control cultures [20]. Although the authors again explained the results by dilution of the O_2 evolved by residual PSII activity, an improvement in the rate of H_2 photoproduction under Ar flow could also be due to a decrease in the partial pressure of H_2 in the medium. Sulfur-deprived algal cells have significantly lower PSII activity than sulfur-replete algae [10,21], and the inhibitory effect of O_2 evolved by PSII on H_2 photoproduction should be less pronounced. However, when sulfur-deprived algae were purged, residual amounts of O_2 were indeed detected in the effluent gas mixture, at least in the beginning of the H_2 photoproduction phase [22,23]. For this reason, it was difficult to determine conclusively whether the dilution of H_2 or the dilution of O_2 was causing the increase in the rate of H_2 photoproduction under continuous Ar flow.

An increase in the rate of H_2 photoproduction was also observed in short-term experiments when photoautotrophic sulfur-deprived cultures were withdrawn from experimental PhBRs and placed in anaerobic vials with a large headspace volume [24]. In these experiments, the headspace was almost seven times greater than the volume of the algal cell suspension. The cells in these vials produced H_2 gas at noticeably higher rates than the algae in the photobioreactors, which contained minimal headspace. Since O_2 was not detected in the vials by gas chromatography (due to high respiratory activity in sulfur-deprived cells) [3], the inhibition of the hydrogenase enzyme by O_2 in this system was not a viable explanation as was the case with the Ar-purging experiments, and we will argue that the increase in the rate of H_2 photoproduction is most probably caused by the effective dilution of the H_2 in the headspace (from nearly 100% to near 0.05%). This dilution effect, however, has never been demonstrated in long-term studies.

In the current work, we have investigated the effect of H_2 partial pressure in the photobioreactor headspace on the H_2 photoproduction activity in green algal cultures and demonstrated conclusively that H_2 photoproduction yields can be improved significantly if H_2 gas is effectively removed from the culture medium.

2. Materials and methods

2.1. Strain and growth conditions

Stock cultures of *C. reinhardtii*, strain 137c *mt*$^+$, were maintained in 250 ml Erlenmeyer flasks containing 50 ml of a standard Tris-Acetate-Phosphate (TAP) medium. The flasks were placed on an orbital shaker (~100 RPM) and illuminated from the top with cool-white fluorescent lamps (~20 μE m^{-2} s^{-1} PAR). The cultures were grown at room temperature and diluted weekly with fresh TAP medium. For each experiment, a 10-ml aliquot of stock culture was inoculated into 1.5-l flat glass bottles containing 700 ml of TAP medium. The algae were then grown mixotrophically for three days at 28 ± 1 °C under continuous illumination from one side (~100 μE m^{-2} s^{-1} PAR from cool white fluorescent lamps). The

cultures were bubbled with 2 ± 0.5% CO_2 in air, filtered through 0.2 μm pore-size membrane filters (Acro 37 TF, Gelman Sciences, Inc., Ann Arbor, MI). The CO_2 content in the airflow was analyzed with a DX6100-01 gas analyzer (RMT Ltd., Russia) and maintained using a TRM1 microprocessor system (Oven, Russia).

Algal cultures were grown to the late logarithmic phase (~25–28 mg Chl l^{-1}), harvested by centrifugation at 3000×*g* for 3 min, washed once in TAP-minus-sulfur-minus-phosphorus (TA-S-P) medium to remove sulfates and phosphates, and re-suspended in the same medium as required. The TA-S-P medium is a modification of standard TAP medium, in which all sulfates were replaced with chloride salts at the same concentrations, and the phosphate buffer was excluded from the composition [25]. Sulfur/phosphorus-deprived cells were used in all experiments with suspension and immobilized cultures. Phosphorus exclusion in addition to sulfur deprivation was necessary for stabilization of alginate films, as discussed by Kosourov and Seibert [25], but it was also used in suspension cultures as well, because the absence of phosphate did not affect the rates of H_2 photoproduction under the experimental conditions.

2.2. Design and operation of photobioreactors

To determine how the volume of the PhBR gas phase affects H_2 photoproduction in algal cultures, we fabricated three glass PhBRs with different gas-to-liquid ratios. All three PhBRs had the same illuminated surface area (190 cm^2) but different total volumes. The PhBRs were constructed with a glass base plate at the bottom (Fig. 1). A silicon rubber gasket sealed the base plate to a glass ledge attached to the glass walls that constituted the sides of the PhBR, and the structure was held in place with ten binder clips. Each PhBR was equipped with two ports for replacing the air in the gas phase with Ar and for sampling and collecting gas for analysis. The PhBRs were designed to hold up to a 500 ml of culture medium each, and they had dimensions of 190 × 100 (length × width) mm and 35, 55, and 75 mm deep, respectively. Since all PhBRs used in this work were hand-made, the dimensions varied slightly in experiments with the suspension cultures as compared to immobilized cultures. In the case of the immobilized cultures, a different rubber gasket was used that slightly increased the headspace volumes.

At the beginning of each experiment (Fig. 2), the PhBRs were filled with 500 ml of sulfur/phosphorus-depleted cell suspension containing about 165, 545, or 925 ml of headspace, respectively, and then bubbled with Ar through the liquid phase for 20 min. The PhBRs were placed in a growth chamber and illuminated from the top with cool-white fluorescent lamps (~70 μE m^{-2} s^{-1} PAR at the surface of the liquid). Since the gas volume inside each PhBR had significant temperature dependence, the temperature inside the growth chamber was set at 25 ± 0.2 °C using a TRM1 microprocessor system (Oven, Russia). Furthermore, the algal cultures inside the PhBRs were mixed thoroughly with magnetic stirrers. The quantity of gas produced by the cultures was measured by the displacement of water from a graduated syringe connected to the PhBR through gas-tight tubing. The gas content inside the PhBRs was monitored twice per day with a gas chromatograph (GC).

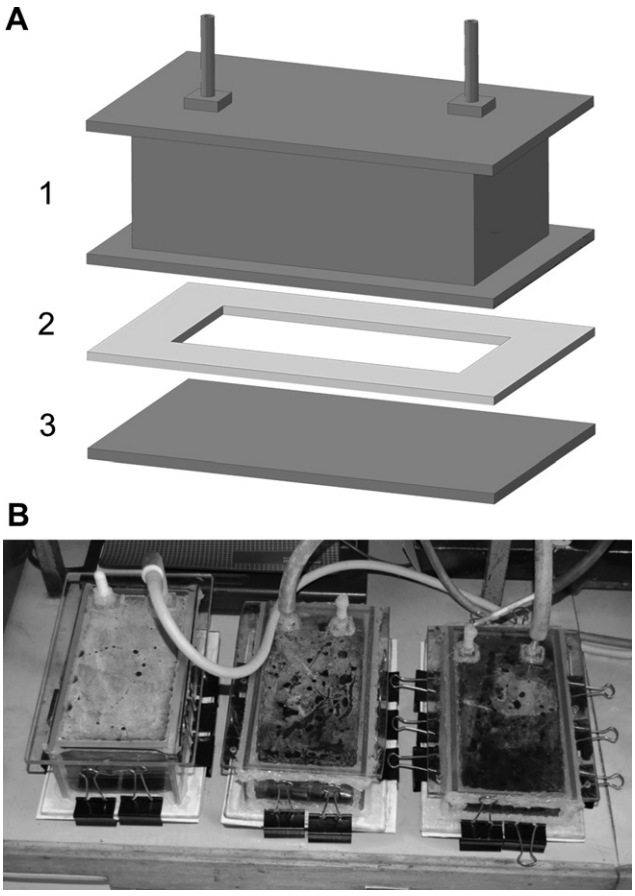

Fig. 1 – (A) Schematic diagram of the photobioreactors (PhBRs) fabricated for this study. (1) A glass PhBR with a glass ledge at the bottom, (2) a silicon rubber gasket, and (3) a glass base plate. The glass base plate with the rubber gasket was attached to the glass frame with ten binder clips. The tubes indicated at the top were for gas access to and from the PhBR. Three PhBRs were used, and each was constructed with a different gas-to-liquid phase ratio (see text). All PhBRs contained 500 ml of sulfur/phosphorus-deprived *C. reinhardtii* cell suspension or 171 cm^2 algae/alginate film in 500 ml TA-S-P medium, but different headspace volumes. (B) Photograph of three PhBRs taken during the Ar-sparging process.

2.3. Hydrogen photoproduction in immobilized cultures

Sulfur/phosphorus-deprived algae were immobilized in Ca^{2+}-alginate films according to the procedure developed by Kosourov and Seibert [25]. The films were prepared using a formulation ratio of 1 g of wet algal cell biomass, 0.5 ml water, and 1 ml 4% sodium alginate. Polymerization of the film was initiated by spraying the surface with a 50 mM $CaCl_2$ solution. The films strips were cut into 3 × 1-cm strips and transferred into vials with different total volumes ranging from 14 ml to 124 ml. The vials were filled with 5 ml of TA-S-P medium and sealed with gas-tight, butyl-rubber stoppers (Belco Glass Inc., NJ). At the beginning of each experiment (Fig. 3), the gas phases in the vials were evacuated several times by applying a vacuum and then refilled with Ar. Finally,

the vials were placed into a growth chamber at 25 °C and exposed to an optimal light intensity of about 30 μE m^{-2} s^{-1} PAR. Hydrogen photoproduction in the vials was monitored once a day by GC.

For the experiments with PhBRs (Figs. 3 and 4), 171 cm^2 of algae/alginate films were produced as described above and placed in the PhBRs with different headspace volumes (Fig. 1). The PhBRs were carefully filled with 500 ml of anaerobic TA-S-P medium and purged with Ar for 20 min. Then, the PhBRs were placed into a growth chamber at 25 °C and exposed to 30 μE m^{-2} s^{-1} PAR light from the top. In contrast to the suspension cultures, the media inside photobioreactors was mixed once a day just before taking the gas samples for GC analysis. Continuous mixing damaged the alginate films.

2.4. *Effects of imposing different H_2 partial pressures on H_2 photoproduction in suspension cultures*

In these experiments (Fig. 5), H_2 photoproduction by sulfur/phosphorus-deprived algae was measured in 75 ml vials. Sulfate was removed from the algal cells as described above. Aliquots of algae (15 ml) were transferred to sealed vials, which were then evacuated several times and refilled with Ar each time. Pure H_2 gas was injected into the vials at the indicated concentrations, and the pressure inside the vials was equilibrated to atmospheric pressure. The vials were then placed on an orbital shaker (~100 RPM) and illuminated from the top with cool-white fluorescent light (~70 μE m^{-2} s^{-1} PAR). The gas phase in the vials was analyzed once a day by GC.

2.5. *Chlorophyll determination*

Chlorophyll (Chl) a + b content was assayed spectrophotometrically in 95% ethanol algal extracts by the method of Spreitzer [26]. The Chl content in the alginate films was assayed in randomly chosen strips after solubilization of the alginate matrices in 50 mM Na-EDTA solution (pH = 7.0). The Chl content was between 21 and 24 mg l^{-1} in all experiments with suspension cultures, 78–90 μg per strip in all experiments with immobilized cultures in vials and 7300–10,500 μg per film in the experiments with immobilized cultures in PhBRs.

3. Results and discussion

3.1. *Suspension cultures — the effect of gas phase volume of the photobioreactor on H_2 photoproduction*

According to the Henry's law, at equilibrium the amount of H_2 dissolved in water is directly proportional to the partial pressure in the gas phase above the water. Therefore, if H_2 photoproduction in algae depends significantly on the concentration of H_2 dissolved in the cell suspension, the rate of H_2 production can be increased by increasing the gas-to-liquid volume ratio of the PhBR, at least at the beginning of an experiment. The dependence of H_2 photoproduction on the gas-to-liquid ratio of the PhBR can only be precisely quantified (and compared to theoretical predictions) when the diffusion of H_2 is not affected by other factors, such as mixing, changes in the diffusion area, and changes in the temperature and pressure. Taking into account these considerations, we constructed three PhBRs with different gas-to-liquid volume ratios (see the Materials and Methods). All three PhBRs were filled with exactly the same amount of algae (500 ml, 23–26 mg Chl l^{-1}), but each had different total PhBR volumes. As shown in Fig. 2, an increase in the volume of the PhBR headspace above the cell suspension increases the H_2 photoproduction yield. Indeed, 500 ml of sulfur/phosphorus-deprived culture of *C. reinhardtii* placed in the 1425 ml PhBR produced ~1.4-times more H_2 gas than the culture placed in the 1045 ml PhBR and ~2-times more H_2 gas than the culture placed in the 665 ml PhBR. The maximum specific rates of H_2 production were also improved significantly in the PhBRs with higher gas-to-liquid ratios (12.5, 7.3 and 4.8 μmol H_2 mg Chl^{-1} h^{-1}, respectively).

In contrast, a control PhBR with a historically normal (small) gas phase volume (~5–10 ml) and a limited area for gas diffusion only produced up to 120 ml l^{-1} of H_2 (compare in Fig. 2, where the cultures produced 10.8–23.1 mmol l^{-1} or 264–565 ml l^{-1} of H_2 at 25 °C in about 190 h) under approximately the same experimental conditions (137c mt^+ strain, ~65 μE m^{-2} s^{-1} PAR, 22 mg Chl l^{-1}, 28 °C [20]). Under optimal pH (7.7) and high light (200 μE m^{-2} s^{-1}) conditions, unsynchronized, sulfur-deprived cultures of *C. reinhardtii*, strain CC124, produced up to 195 ml l^{-1} H_2 gas [27], which is still significantly lower than the yields observed in the current experiment. At the same time, the maximum specific rate of H_2 photoproduction in previous experiments was around 9.4 μmol H_2 mg Chl^{-1} h^{-1}. It should be noted here that these previous experiments were performed at significantly lower Chl concentrations (9–12 mg l^{-1}) and higher light intensities (200 μE m^{-2} s^{-1}), which increase the apparent maximum specific rates. Note that the maximum specific rate is normally calculated during the short period of maximum H_2 photoproduction activity and, thus, demonstrates only the

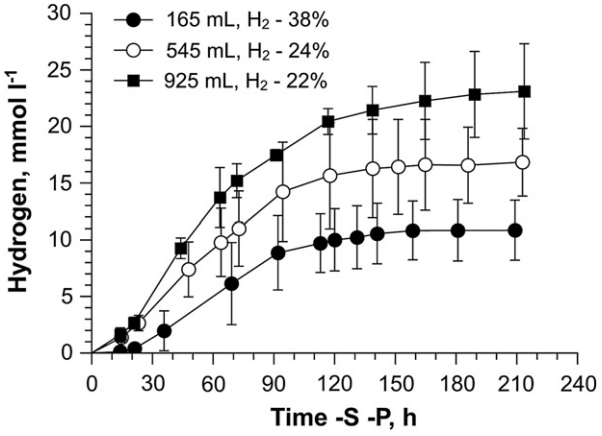

Fig. 2 – Hydrogen photoproduction by 500 ml of sulfur/phosphorus-deprived algal cultures placed in photobioreactors with different headspace volumes (165–925 ml). The final percentages of H_2 gas in the gas phase of the photobioreactors are indicated in the figure, but the ordinate reports actual volumes of H_2 produced. Each experimental point represents 3–5 replicates, and the error bars are one standard deviation.

potential productivity of the system. In most other reports, the maximum specific rate of H_2 photoproduction by sulfur-deprived suspension cultures placed in standard TAP-minus-sulfur medium under $\leq$100 μE m^{-2} s^{-1} PAR light did not exceed 5–8 μmol H_2 mg Chl^{-1} h^{-1} for WT algae [12,28–30]. In addition, as shown in Fig. 2, the duration of H_2 photoproduction decreases with decreasing headspace volume, as does the maximum H_2 photoproduction rate. Notably, the dead-time after sulfur/phosphorus-deprivation in our cultures also increases as the headspace volume decreases.

Improvements in H_2 photoproduction activity have been reported previously in dark-adapted suspension cultures in PhBRs with large headspace volumes after sparging the algae with Ar [19] and in sulfur-deprived autotrophic cultures (short-term experiments) after replacing the headspace in the culture vials with pure Ar [24]. In addition, the H_2 photoproduction rate in sulfur-deprived algae was improved after an increase in mixing efficiency [29]. All these results support the idea that there is a direct inhibitory effect of H_2 over-accumulation in a PhBR system on the H_2 photoproduction activity of algae.

3.2. *Immobilized algae — the effect of gas phase volume in the photobioreactor on H_2 photoproduction*

In the next series of experiments, we examined how the gas-to-liquid ratio of the photobioreactor affects H_2 production in algal cultures entrapped in alginate matrices. Any exchange between the gas phase and the liquid volume is determined by the rate of diffusion, the intensity of cell movement, and the degree of liquid mixing in the suspension cultures. However, alginate immobilization prevents the movement of microalgae and creates a layer where only diffusion is the physical driving force for gas to liquid exchange. In addition, the diffusion coefficient in alginate and in aqueous liquid is different. Indeed, alginate polymer significantly restricts gas permeability, especially for O_2 [31]. As a result, immobilized algal cells produce H_2 gas even in the presence of atmospheric levels of O_2 in the headspace [25]. If H_2 diffusion is also limited in the alginate matrix, the inhibitory effect of H_2 on H_2 photoproduction should be more pronounced in immobilized algae than in suspension cultures since the diffusion of H_2 gas from the film will be restricted by the matrix. We found that H_2 gas produced by immobilized algae indeed forms bubbles inside the film (visual observation; data not shown), especially during the period of the experiment when the cells produce H_2 at the highest rate.

We first checked on how the gas phase to liquid phase ratio affects H_2 photoproduction by algae/alginate films placed in small vials with 14–124 ml total volume (Fig. 3A, solid symbols). As shown in the figure, the kinetics of H_2 photoproduction and the total yields of H_2 gas did not change significantly in vials with 5 ml of liquid phase and above 35 ml of gas phase (gas-to-liquid ratio above 7). The maximum specific rate of H_2 photoproduction was approximately the same in all vials (9.5 μmol H_2 mg Chl^{-1} h^{-1}). However, the vials with 9 and 20 ml gas phases (gas-to-liquid ratios of 1.8 and 4, respectively) produced ~40% and 22% less H_2 gas than the vials with 35–119 ml gas phase, while the maximum specific rate of H_2 photoproduction was only 5.7 μmol H_2 mg Chl^{-1} h^{-1}

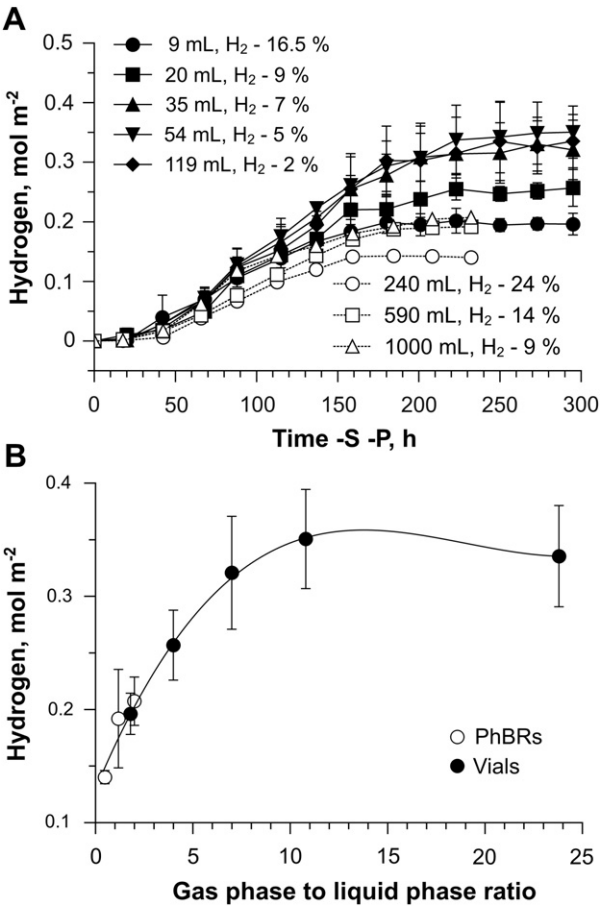

Fig. 3 – The effect of photobioreactor gas phase volume on H_2 photoproduction by immobilized *C. reinhardtii* cultures under sulfur/phosphorus deprivation. (A) The kinetics of H_2 photoproduction was measured with either 3 cm^2 algae/alginate films placed in 14–124 ml vials (solid symbols) filled with 5 ml TA-S-P medium or 171 cm^2 algae/alginate films placed in 740–1500 ml PhBRs (open symbols) filled with 500 ml of the same medium. The final percentages of H_2 gas in the gas phase of the vials are indicated on the figure. (B) The total yields of H_2 gas from panel A were plotted against the gas-to-liquid ratio in vials (solid symbols) and PhBRs (open symbols). Each experimental point represents 6 replicates, and the error bars are one standard deviation.

in vials with 9 ml of gas phase. It is important to note here that the gas-to-liquid ratio in these vials was around 1.8, and this was very close to the gas-to-liquid ratio in the PhBR with the largest headspace volume (~1000 ml), which was designed to hold 171 cm^2 alginate films in 500 ml of medium (gas-to-liquid ratio of around 2). As shown in Fig. 3B, the vials with a ratio of around 1.8 and the PhBR with the ratio of around 2 produced almost the same amount of H_2 gas per m^2 of film surface (~0.19 and 0.21 mol, respectively). The kinetics of H_2 photoproduction was also similar in the PhBR and vials (Fig. 3A), indicating that bioreactor scale-up does not decrease the performance of algae/alginate films, at least in this volume

range. However, a decrease in the gas-to-liquid ratio did decrease the H_2 photoproduction yields (Fig. 3B). As expected, the PhBR with the lowest gas-to-liquid ratio (0.48) produced the least amount of H_2 gas (0.14 mol m^{-2}).

Interestingly, the maximum specific rates of H_2 photoproduction in suspension cultures were approximately twice those observed for immobilized culture films placed in the same PhBRs. For example, the maximum specific rates in immobilized and suspension cultures were 2.6 and 4.8 μmol H_2 mg Chl^{-1} h^{-1} in the PhBR with the gas-to-liquid ratio of around 0.5, and 5.4 and 12.5 μmol H_2 mg Chl^{-1} h^{-1} in the PhBR with the ratio of around 2, respectively. Since the cell density per ml of medium was approximately the same in both cultures (18–19 mg Chl l^{-1} in immobilized cultures vs. 21–24 mg Chl l^{-1} in suspension cultures), we surmise that the rate of H_2 photoproduction in the immobilized cells is limited by the diffusion of H_2 through the alginate matrix. If the alginate matrix indeed limits H_2 diffusion, the output of H_2 gas in immobilized cultures might be improved by decreasing the thickness of the layer without mixing, by increasing the porosity of alginate polymer, or by decreasing the partial pressure of H_2 in the PhBR gas phase. To check the latter possibility, we purged the medium-sized PhBR (1090 ml total volume) continuously with 100% Ar through the gas phase at a rate of about 4 ml per min. In this case, the content of H_2 in the PhBR gas phase did not exceed 0.5% throughout the experiment. The result was that the maximum H_2 photoproduction rate increased by more than 60% (Fig. 4).

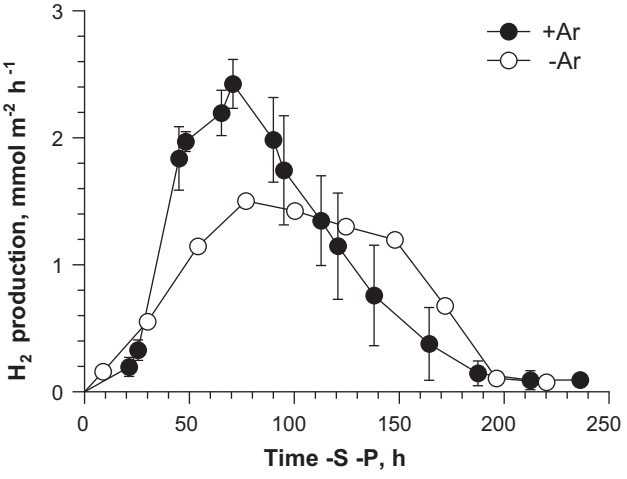

Fig. 4 – The effect of continuous argon purging on the H_2 photoproduction rate in immobilized green algal cultures. Sulfur/phosphorus-deprived algae were entrapped in 171 cm^2 alginate films and placed in a 1090 ml PhBR containing 500 ml of anaerobic TA-S-P medium. PhBRs were continuously purged with argon (+Ar) at the rate of about 4 ml min^{-1}. Control PhBRs (−Ar) were only sparged with Ar for 20 min at t = 0. The H_2 photoproduction rates in the PhBRs with continuous Ar flow were determined by sampling the outlet gas with a GC. The H_2 photoproduction rates in the control PhBRs were calculated based on H_2-photoproduction yields (see Fig. 3A).

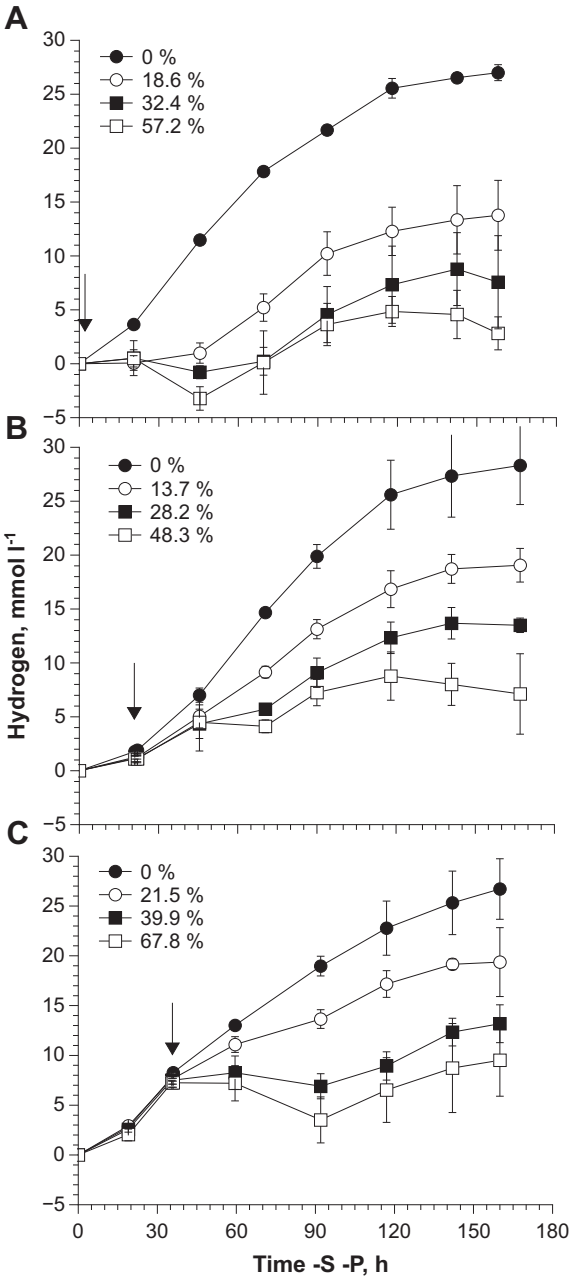

Fig. 5 – The effect of the partial pressure of H_2 in the photobioreactor headspace on the kinetics of H_2 photoproduction by algal suspension cultures. The arrows indicate different times at which H_2 gas was injected into the vials: 0 h (A), 22 h (B) 36 h (C) from the beginning of nutrient deprivation. The added H_2 concentrations in the headspace right after H_2 injection are also indicated in the figure. Each experimental point represents 5 replicates, and the error bars are one standard deviation.

3.3. *Suspension cultures — the effect of H_2 partial pressure on H_2 photoproduction*

The results above demonstrate that H_2 photoproduction in algal cultures depends on the partial pressure of H_2 in the PhBR gas phase. However, in these experiments the

concentration of the H_2 in the PhBR gas phase changed significantly with time, and therefore, did not allow us to make any specific conclusions on how H_2 concentrations affect the rates of H_2 photoproduction in the PhBR. To study this dependence, we injected different amounts of pure H_2 directly into vials containing algal cultures at different times after sulfur/phosphorus-deprivation. As shown in Fig. 5, the kinetics of algal H_2 photoproduction changed dramatically after injection of H_2 gas into vials. The effective rate of H_2 photoproduction in the cultures not only decreased soon after injection, but in some cases the cultures also started to consume H_2 on the second day after H_2 addition. H_2 uptake was most pronounced in the vials with the most injected H_2. The reason(s) for the delay in H_2 uptake are not clear at this point. According to the Le Chatelier's principle, the shift in a chemical equilibrium should occur almost immediately after a change in product concentration. However, our cultures adapted to the change over a period of about two days, and again started to produce more H_2 gas by the third day after injection. In the end, the final H_2 photoproduction yields in the algal cultures were always positive even under highest H_2 partial pressures (Fig. 5).

The final yields of photoproduced H_2 gas in algae always decreased exponentially with increasing H_2 partial pressure in the vial (Fig. 6). As expected, the most significant inhibition of H_2 photoproduction activity was observed in photobioreactors containing above 60% H_2 in the headspace. Such high concentrations of H_2 are typical for photobioreactors with little gas headspace [3,27]. Interestingly, the H_2 photoproduction yields also decreased with increasing time of exposure to high H_2 partial pressure. Indeed, cells exposed to high H_2 levels at the beginning of the experiment (0 h curve) always produced less H_2 gas as compared to algae exposed to high H_2 levels thereafter (under the same initial H_2 levels, see Fig. 6). Although such behavior can be a result of the induction of the H_2 uptake pathway, it also can be caused by reasons not directly related to H_2 metabolism in cells, such as adaptation of the photosynthetic apparatus to over-reducing conditions and changes in fermentation metabolism pathways. It is clear that more study will be required to completely understand all of the mechanisms underlying this effect.

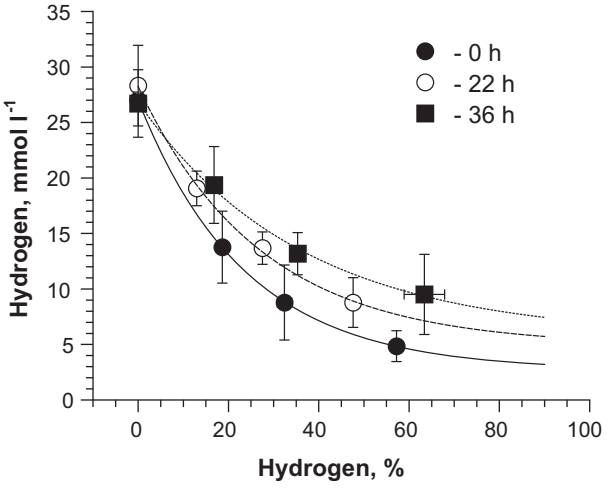

Fig. 6 – The effect of partial pressure of H_2 on the total yield of H_2 gas in the photobioreactors. Each point represents the maximum H_2 photoproduction yield for a kinetic curve shown in Fig. 5. The times indicated are the times at which H_2 gas was injected into the vials. Each experimental point represents 5 replicates, and the error bars are one standard deviation.

4. Conclusions

The results presented in this work clearly demonstrate that H_2 photoproduction by algal cultures depends directly on the H_2 partial pressure in the gas phase above the culture. The inhibitory effect of high H_2 concentrations in algae is significant and comparable to the effect observed in anaerobic bacteria, where H_2 gas over-accumulation in a bioreactor leads to the inhibition of H_2 production and changes in fermentation metabolism directed toward the production of other reduced products [15]. It is possible that similar adaptive mechanisms to H_2 gas over-accumulation exist in green algae.

The results from this study may also have direct practical consequences. First, much higher H_2 photoproduction yields in the PhBR with H_2-producing algae might be observed, if H_2 is efficiently removed from cell suspensions. In practice, the H_2 content in the PhBR headspace should not exceed at most 5%, as can be seen from Fig. 6. Second, extended exposure of algal cultures to high H_2 levels increases the reverse, uptake reaction (see Eq. (1)) and decreases the final yield of H_2 gas photoproduction. The mechanism of the H_2 uptake reaction in nutrient-deprived algae is not very clear at this point since it may involve additional inducible component(s) (Fig. 5). The occurrence of H_2 uptake in the presence of CO_2 was first demonstrated in another green alga, *Scenedesmus* sp., by Gaffron [32] more than 70 years ago, but since that time little follow-up progress in resolving the metabolic pathways participating in the H_2 uptake reaction has been made. Two mechanisms of H_2 utilization in green algae must be considered: photoreduction of CO_2 and the oxy-hydrogen reaction [32–34]. Photoreduction usually occurs in dark-adapted algae placed in low light under high H_2 partial pressures [32,34], while the oxy-hydrogen reaction can proceed under microaerobic conditions (mostly in the dark) due to the high sensitivity of [Fe–Fe]-hydrogenase enzymes to oxygen [34]. The possibility of photoreduction in nutrient-deprived algae is questionable because of the significant degradation of the RuBisCo enzyme by the time H_2 photoproduction begins [35]. Therefore, it is most likely that nutrient-deprived algae utilize H_2 gas through the oxy-hydrogen reaction involving the chlororespiration pathway from [Fe–Fe]-hydrogenase(s) to O_2 through Fd, $NADP^+$/NADPH and the PQ pool.

In cultures entrapped in thin alginate films, the decreased rates of H_2 photoproduction may be caused by the decreased diffusion properties of alginate matrix. To overcome this barrier, a more porous material should be developed and investigated. This material should be stable for at least several months and renewable, if possible. Otherwise, immobilization of algal cells on a cheap substrate like a glass textile material [36] might always be used. In the context of this work, immobilization may have another advantage; thin matrices with entrapped algae can easily be attached to a fuel cell

electrode for electricity generation. This may help decrease the cost of algal H_2 production due to low H_2 pressure in the output gas.

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