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INTERNATIONAL JOURNAL OF HYDROGEN ENERGY ▮ (▮▮▮▮) ▮▮▮–▮▮▮

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Investigation of H_2 production using the green microalga *Chlamydomonas reinhardtii* in a fully controlled photobioreactor fitted with on-line gas analysis

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ARTICLE INFO

Article history:
Received 18 July 2007
Received in revised form
14 January 2008
Accepted 30 March 2008

Keywords:
Photobioreactor
Hydrogen
Chlamydomonas reinhardtii
Sulfur deprivation
Energetic yields
Mass spectrometer

ABSTRACT

Chlamydomonas reinhardtii is able to produce molecular hydrogen in a clean way. Overall H_2 release is the result of complex, interacting and transient intracellular mechanisms. To relate the dynamic coupling between culture conditions and biological responses, an original lab-scale set-up has been developed. Such device enables culture conditions to be highly controlled and provides on-line mass-spectrometric measurement of gas production. A first validation was conducted with the well-known protocol of sulfur deprivation. Biochemical analysis combined with gas flow control enabled instantaneous productivities to be calculated, and kinetic evolutions of successive physiological states to be obtained. An energetic study was also conducted. A maximal energetic yield of light conversion to H_2 energy of 0.125% was achieved, far from the photosynthesis potential and usual photobioreactor efficiencies reported for biomass application (around 10%). However, the designed photobioreactor connected with data acquisition system is an innovative tool for future methodical optimization of H_2 production using photosynthetic microorganisms, integrating both bioprocess and physiological aspects.

1. Introduction

The development of alternative clean and renewable energies is of primary interest considering the current environmental and energetic situation in the world. Hydrogen appears as one of the most attractive solutions as a potential energy vector for the future. It is well known for many decades that photosynthetic microorganisms are able to produce H_2 under given conditions. For example, *Chlamydomonas reinhardtii*, an unicellular green microalga, uses light and CO_2 as only carbon source to produce H_2 in anoxic conditions [1–3]. This is the result of complex metabolic mechanisms, implying a Fe-hydrogenase enzyme coupled with the photosynthetic chain that enables a reduction of protons given by water splitting to produce molecular hydrogen [4]. The main limitation is that H_2 production in natural conditions is only a transient process. The Fe-hydrogenase being highly sensitive to oxygen, a rapid inhibition occurs as soon as oxygen is released by water biophotolysis, and H_2 production is stopped [5–7]. Because of this transient nature, the physiological responses are difficult to analyze.

This makes experiments under well-defined conditions in devoted lab-scale photobioreactors of primary interest, especially in order to in-depth investigate the high coupling of interacting transient intracellular mechanisms with extracellular conditions (medium composition, light, etc.). Examples of such kind of processes can be found in the literature, either in the general context of biomass growth [8–11], or

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doi:10.1016/j.ijhydene.2008.03.067

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dedicated to the particular case of H_2 production [12–17]. The latter systems have confirmed the high dependence of the biological response on the cells environment under H_2 producing conditions, with a marked dynamic behavior evolving all along the process.

In this study, an original laboratory-scale photobioreactor system is proposed, dedicated to H_2 production under fully controlled and monitored conditions. This set-up combines a photobioreactor that enables a high control of the light received by the culture to be obtained, with an on-line analysis of gas injected in the reactor or produced by cells using a mass spectrometer. Kinetic information on culture evolution during H_2 production can thus be obtained, including gas released or consumed (O_2, CO_2, H_2), and changes in biotic (total biomass and biomass composition in sugars, proteins, lipids, pigments) and abiotic phases (carbon and mineral compounds consumption). The relevance of this set-up has been investigated by using the well-known protocol of sulfur deprivation for *C. reinhardtii*, which is actually considered as a reference for H_2 production with green algae [18]. However, although the feasibility of this protocol has been stated, the metabolic pathways and the influence of culture conditions are not well identified. As demonstrated in this study, experiments under fully controlled and monitored conditions will appear as an interesting tool to provide a better insight in the dynamic behavior of the hydrogen-producing microalgae.

2. Design of the photobioreactor

2.1. General description of the reactor

The set-up is based on a torus-shaped photobioreactor (Fig. 1). This geometry of loop configuration generates efficient mixing conditions without dead volumes, while keeping shear-stress in a reasonable range. This has been demonstrated in a previous study by using computational fluid dynamics [19]. Because of the high dependence of the photosynthetic metabolism on light, the radiative-field inside the reactor has to be known, especially in a perspective of modeling. The cross-section being square, the light attenuation can be considered to occur along only the culture depth (one-dimensional hypothesis). A complete description of light transfer modeling in our photobioreactor is presented in Pottier et al. [20].

2.2. Culture parameter regulation

The light source was provided by fluorescent tubes (OSRAM L13W/12-950) emitting a white light in the visible domain. The agitator shaft was magnetically coupled with a motor (APPLIKON). Such system guaranteed a total tightness of the drive shaft, thus removing risk of H_2 leak.

Temperature was measured using a Pt100 sensor integrated to the pH probe. An automatic cooling of the reactor by means of ambient air blowing was operated when the requested temperature was exceeded (fan placed on the backside of the reactor). If air-cooling was not sufficient, a circulation of thermostated water was applied, the reactor being double-wall jacketed (Fig. 1a).

pH measurement was used as an indirect regulation of the dissolved carbon, by automatically adjusting pH with CO_2 bubbling and dissolution. The pH of the medium was measured with a pH sensor InPro 3200 (METTLER TOLEDO) (Fig. 1b) and the CO_2 injection was performed with a mass flow controller.

Because of the Fe-hydrogenase sensitivity to O_2, the control of this parameter during H_2 production was needed. It was measured with an oxymetric sensor InPro 6000 (METTLER TOLEDO).

Data acquisition and storage, setting of regulation parameters and automation of the process were performed via a data acquisition and command system (DAQ 6023E-National Instruments) (Fig. 2). The "Labview" software was used as an interface.

2.3. Gas analysis system

A system for gas control and analysis has been developed and implemented on the reactor for an on-line measurement of the gas produced by the culture (Fig. 2). The mass flow rate of gas injected (N_2/CO_2) was regulated and measured using two mass flow rate controllers. The composition and the total mass flow rate of the outlet gas mixture were measured with a mass flow-meter (EL-FLOW devices—Bronkhorst High-Tech) associated with a quadrupole mass spectrometer (PFEIFFER VACUUM). It should be noticed that a gas condenser was used at the reactor outlet to prevent from water evaporation.

The combination of mass spectrometry with mass flow-meters enabled the flow rates for O_2, H_2 and CO_2 (and N_2 if needed) produced by the algal culture to be obtained, and thus the instantaneous kinetics of gases released or consumed by the culture to be followed. A general overview of the system is presented in Fig. 2.

3. Validation of the set-up for hydrogen production

3.1. Materials and methods

For a first validation of the developed set-up under H_2 production conditions, the protocol of sulfur deprivation has been applied. By reducing photosynthetic activity, this protocol enables the problem of the high sensitivity of the Fe-hydrogenase to O_2 to be get round. The transition to anoxic conditions is then realized as the O_2 consumption by respiration process becomes higher than the O_2 released during photosynthesis. H_2 is then produced under light conditions [18]. This protocol has been applied using the common tris-acetate-phosphate medium (TAP) pH 7.2, with the *C. reinhardtii* strain wt 137c (from the *Chlamydomonas* Genetic Center, Duke University, Durham, USA). The sulfur deprivation protocol generates a temporal separation between the growth phase (with O_2 release) and the H_2 production phase. To apply this protocol in the torus reactor, the cells were previously grown in flasks under photosynthetic conditions. The pre-cultures were maintained at 25 °C,

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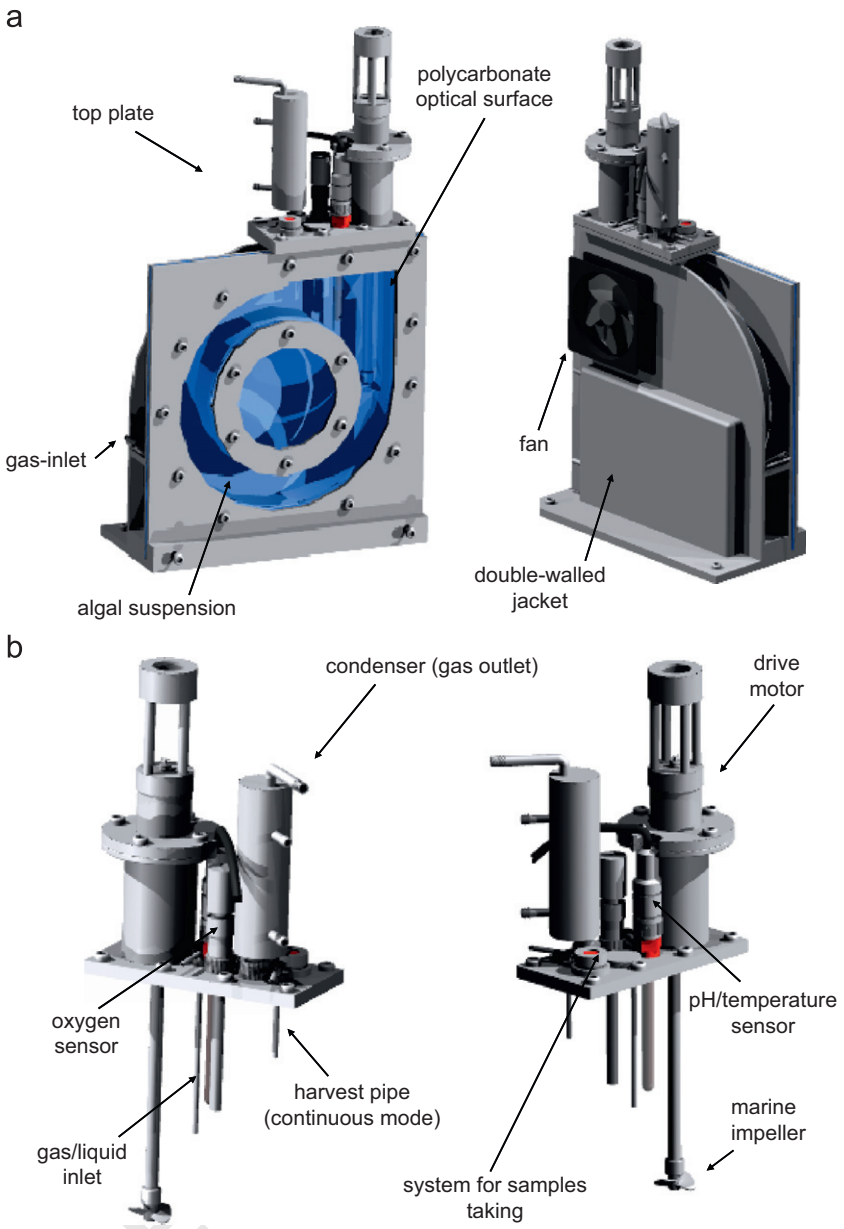

Fig. 1 – Schematic representation of the torus photobioreactor (a. left: front view; right: back view; b. details of the top plate). It was made of stainless steel (type 316L), polycarbonate being used for the transparent optic surface. EPDM seals (ethylene propylene diene) have been used, thereby making the reactor tight to the very diffusive H_2 gas.

under constant agitation and continuous illumination (110 μmol photons m^{-2} s^{-1}). When a density of about 5×10^6 cells ml^{-1} was reached (corresponding to a late logarithmic growth state) the cells were placed under sulfur-deprived conditions to induce the H_2 production (second phase). The algal culture was centrifuged at 2.000*g* during 5 min, washed, resuspended in a sulfur-deprived medium (TAP-S) and injected in the photobioreactor with a peristaltic pump. During all the experiments, a constant agitation of 300 rpm was applied in the reactor with an incident light of 110 μmol photons m^{-2} s^{-1}. In this case, only the temperature was regulated (25 °C), TAP medium being pH buffered. After 8.3 days of sulfur deprivation, 200 ml of TAP-S medium supplemented with 10 mg l^{-1} of S under ($CuSO_4$, $5H_2O$) form were injected in the reactor to test the effect of replacing cells in non-sulfur limited conditions after hydrogen production phase. The photobioreactor was operated in batch mode.

The objectives of the present study being to relate time-variation of the different parameters characterizing gas, liquid (culture medium, dissolved gas) and solid (biomass) phases, a continuous bubbling of the culture with a gas vector (N_2) was applied. The gas–liquid equilibrium is rapidly obtained thanks to the N_2 bubbling and the related gas desorption. The drawback associated with this method was the potential effect of the bubbling on the biological process, especially on the establishment of anoxia by oxygen removal. Nevertheless, as the sensitivity of mass flow rate instruments and mass spectrometer is high, a very small N_2 gas flow rate

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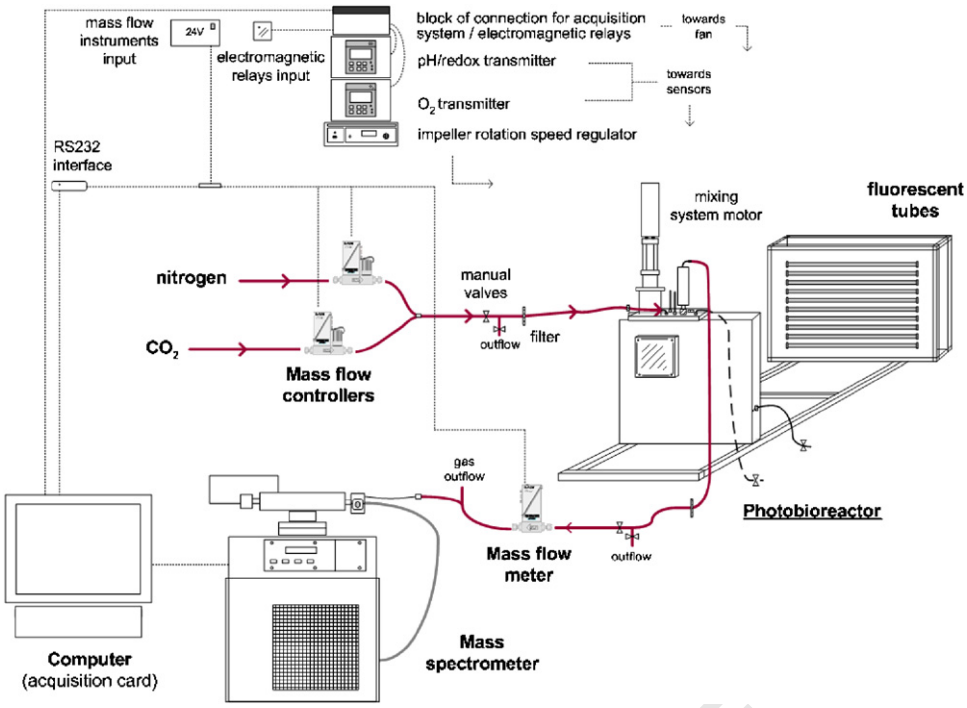

Fig. 2 – General overview of the set-up used for experimental studies of H_2 production.

($5.25\,ml\,min^{-1}$) can be used, which tend to reduce such influence. Moreover, some preliminary experiments under classical growth conditions have shown that small N_2 bubbling was not able to induce anoxia and did not influence growth kinetics (data not shown). It should also be noticed that this gas flow rate was greater than the one induced by the mass spectrometer continuous analysis ($3\,ml\,min^{-1}$), thus avoiding effect of gas sampling on the process. Obviously, the reactor could also be operated without gas bubbling, which would give additional information (like the real kinetics of anoxic establishment in closed system). But without a neutral gas injection, the on-line monitoring of gas released by the culture was only possible when the net biological production was positive. This was especially the case at the beginning of the H_2 production phase, where gas consumption (O_2, CO_2) was observed (data not shown). Although a significant evolution of the metabolism occurs during this period, no information about the biological behavior could be thus obtained from gas release analysis. Otherwise, it would be necessary to analyze the gas phase inside the photobioreactor.

In addition to gas analysis, the culture samples were regularly taken from the reactor through a septum with a syringe. Except for dry weight and quantum yield of PSII photochemistry, samples were centrifuged at 20.000*g* for 2 min to separate aqueous phase from cells for medium or cellular analysis.

Dry weight was determined by filtration through a pre-dried (110 °C) and pre-weighed glass-fiber filter (Whatman GF/F). The filter was then dried, cooled in a desiccator, and weighed again.

Pigments content was determined using a spectrophotometric method. Previously chlorophyll-*a*, chlorophyll-*b* and carotenoids were extracted with a 90% acetone solvent (24 h in the dark at 4 °C). Samples were then centrifuged and the absorption spectrum was collected on the supernatant in the range of 400–750 nm. Concentrations were then calculated according to the equations of Parsons and Strickland [21].

Algae were acclimated to the dark for 10 min and the quantum yield of PSII photochemistry [$\Delta F/Fm' = (Fm' - F)/Fm'$] [22] was determined using a Water-Pam Chlorophyll Fluorometer (Walz). (F) the steady-state fluorescence was measured in the dark and (Fm') the maximal fluorescence in the light-adapted state was measured for a saturating light pulse of 583 μmol photons $m^{-2}\,s^{-1}$.

Proteins were quantified with the Lowry et al. method [23] that used the Folin phenol reagent. Total sugar content was determined by the phenol–sulfuric acid method of Dubois et al. [24]. Starch analysis was performed using a method slightly modified from Klein and Betz [25] described by Fouchard et al. [26].

The determination of the TOC (total organic carbon) and the TIC (total inorganic carbon) was carried out with a TOC Analyzer (Shimadzu TOC-5000A) based on the combustion/non-dispersive infrared gas analysis method. In this system, TC (total carbon) was measured by heating sample to 680 °C. For TIC measurement, sample was decomposed using a phosphoric acid solution. CO_2 evolved during each treatment was successively detected by an infrared analyzer. Finally, TOC content was calculated by subtracting TIC from TC.

Ion chromatography (Dionex DX-120, IonPac AS12A anionic column) was used for analysis of sulfate, phosphate, acetate and formate medium content. Eluent was a solution of Na_2CO_3 2.7 mM and $NaHCO_3$ 0.3 mM.

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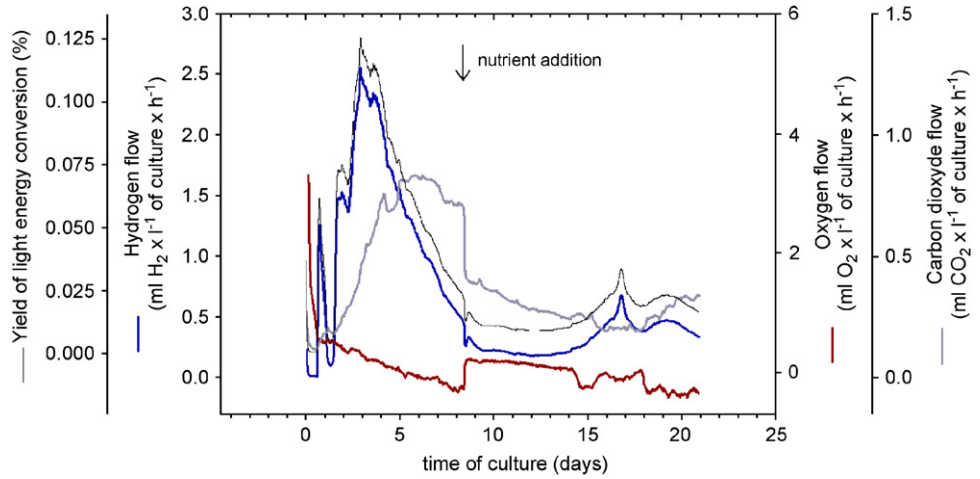

Fig. 3 – Gas flow rates variation (H_2, O_2 and CO_2) in a *Chlamydomonas reinhardtii* culture under sulfur deprived conditions. The instantaneous yields of light energy conversion are also reported here. The arrow (8.3 days) indicates the addition of 200 ml of culture medium supplemented with 10 mg l^{-1} of S, in the reactor.

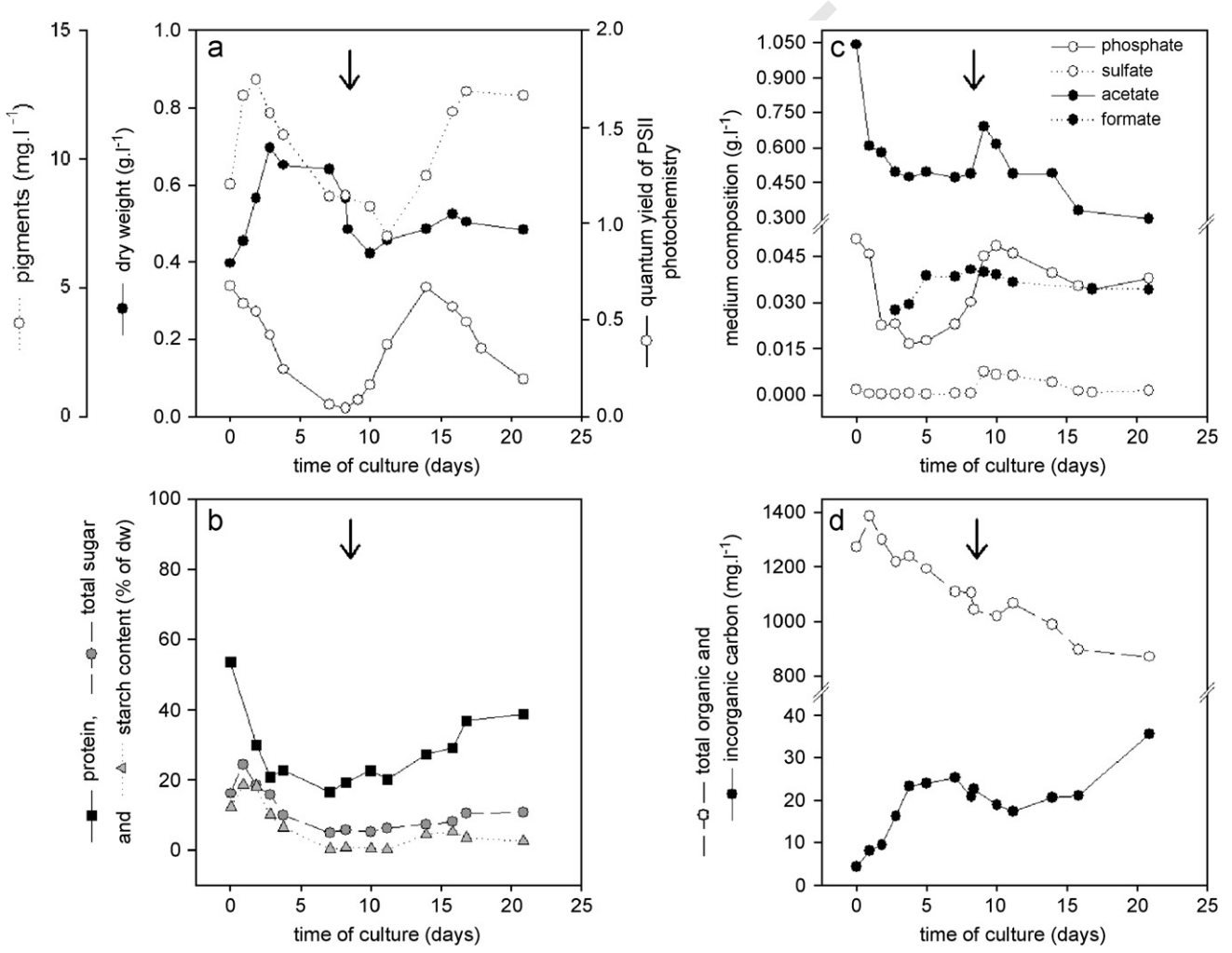

Fig. 4 – Kinetic variations of various metabolites and nutrients: (a) dry weight, pigments concentration and quantum yield of PSII photochemistry; (b) protein, total sugar and starch cells content; (c) phosphate, sulfate, acetate and formate concentrations in the medium; (d) total organic and total inorganic carbon content of the culture medium. The arrow (8.3 days) indicates the addition of 200 ml of culture medium supplemented with 10 mg l^{-1} of S, in the reactor.

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3.2. *Results and discussion*

The results related to gas and biochemical analysis are presented in Figs. 3 and 4, respectively. They qualitatively agree with previous studies dealing with sulfur deprivation effects at the metabolic level [18,27]. Structural and functional properties of the H_2-producing photosynthetic apparatus were modified. After the second day of sulfur deprivation, pigments content of the culture declined over the duration of the deprivation (Fig. 4a) as well as photosynthetic capacity of the cells due to the inactivation of the PSII (photolysis site). The quantum yield of PSII photochemistry decreased from 0.68, a standard value for classical growth conditions [28,29], to 0.04 in 8 days (Fig. 4a). The comparison between this result and the net O_2 gas production measured at the reactor outlet, resulting from both photosynthesis and respiration, demonstrates that the progressive decrease of O_2 production (Fig. 3) was directly correlated to the decrease of PSII activity. This is the main feature, and a well-known result of sulfur deprivation protocol [30]. As the mitochondrial respiration is nearly constant [18], the decrease in photosynthetic activity creates an imbalance between the two processes leading to a progressive decrease of O_2 concentration. Finally, anoxic conditions are obtained enabling the synthesis of the Fe-hydrogenase and the H_2 release. For previous studies in closed bottles, this was achieved after 2–3 days [18,26]. In this experiment, a first peak of H_2 was observed at the beginning of the sulfur deprivation (Fig. 3). This can be explained by the bubbling of nitrogen used as a gas vector. If the bubbling accelerates probably the transition to anoxia, the low flow rate applied is not able to induce anoxic conditions when photosynthetic activity is unaffected (under classical growth conditions). So, the effect of N_2 bubbling on dissolved O_2 flushing cannot be sufficient to explain the H_2 production release after only few hours, the PSII activity being not yet greatly affected. The main reason should be the N_2 bubbling influence on gas phase. Gas circulation preventing from gas accumulation, various exchanges that appear at the beginning of the sulfur deprivation protocol (alternative biological production and gas consumption, especially O_2 and CO_2) cannot occur. Indeed, without a gas vector, O_2 produced with remaining PSII activity and released in the gas phase has to be consumed by cells. The anoxia state is then a function of both biological activities and gas–liquid mass transfer rates in the culture vessel. When using a gas vector, and thus a continuous renewal of the gas phase with an inert gas, gas is not accumulated and thus the kinetics of establishment occurs faster.

The observed variations of nutrients and metabolites (Fig. 4) during H_2 production process are in agreement with the literature [15,18,31–33]. An important starch accumulation occurred at the beginning of the deprivation (Fig. 4b). This was already observed in a previous study and explained as a second effect of sulfur deprivation, with the PSII activity reduction [26]. The starch catabolism sustained after the indirect biophotolysis process, as shown in Fig. 4b by the progressive decrease of starch content after 1 day. At this stage, the indirect pathway of H_2 production and water photolysis were coupled. CO_2 release was also observed, due to the carbonaceous reserve degradation (Fig. 3). This is confirmed by the level of formate in the medium, which is a product of the starch catabolism via the glycolytic pathway [34], and transiently increased after the second day of deprivation (Fig. 4c). It should be noticed that on the third day of deprivation, starch represented almost the total sugar content of the cells (Fig. 4b). The amount of acetate in the culture medium decreased about 50% during the first 2 days after the sulfur deprivation and its assimilation then stopped (Fig. 4c). This indicates that acetate was probably used in the starch accumulation process, but was also implied in the consumption of a part of the residual photosynthetic O_2 released as long as the PSII was not affected by the deprivation (other part being removed by the gas vector flow rate).

After 8.3 days of deprivation, the physiological state of cells was affected, as shown by the degradation of pigments, proteins and total sugars. When sulfur was re-introduced in the medium in a limited concentration (Fig. 4c) an almost complete recovery of pigments biosynthesis and PSII activity was obtained (Fig. 4a). However, biomass, proteins and sugars increased in less important proportions, but reflected also the reactivation of the photosynthetic process (Fig. 4a and b). This was accompanied by a progressive consumption of nutrients and, when sulfur deprivation was again obtained, a new period of H_2 production was induced, but with a smaller magnitude (Fig. 3). This shows that the effects of sulfur deprivation on the photosynthetic apparatus are (almost partially) reversible and that the cells are able to produce H_2 in a cyclic way. Nevertheless, as described by other previous studies, the duration of sulfur deprivation is crucial because of its large effect on cell metabolism [33]. This was clearly revealed with the variation of the different intracellular compounds during the experiment.

To emphasize the interest of using a fully controlled photobioreactor, and to validate the analyses conducted, a carbon balance, based on biochemical and gas flow measurements, has been realized. The initial and final carbon contents were calculated from initial and final concentrations of dry weight (Fig. 4a), TOC and TIC contents (Fig. 4d), related to the initial and final culture volumes. Under standard conditions, carbon was considered to represent about 50% of the microalgal biomass [35]. This value was used for the calculation of both initial and final biomass carbon contents, as the initial biomass was in the same physiological state than under standard conditions, and as the deprivation effects on algae composition were mainly implied in the transient modification of carbonaceous reserves (accumulation and consecutive degradation), having thus only a slight influence on the final biomass composition. Even if the value of 50% is an estimation, the biomass represented only a small part of the TC of the system, and thus possible error related to this percentage can be considered negligible in the final balance results. The difference between the initial and final carbon contents in the culture volume corresponds to the carbon collected by sampling for various liquid analyses, the carbon supplied when medium was re-introduced after 8.3 days, and the carbon released under CO_2 gas form. These values were estimated, respectively, from the dry weight, TOC and TIC concentrations of each sampling reported to the sample volumes (20 ml), from the amount of acetate re-

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Table 1 – Data obtained for carbon balance calculation (initial, final and sampled carbon contents of biomass and culture medium)

	Biomass	Organic carbon	Inorganic carbon	Total
$C_{initial}$	0.27388	1.73399	0.00579	2.01367
C_{final}	0.31844	1.13352	0.04620	1.49816
$C_{sampled}$	0.06938	0.28618	0.00529	0.36085
C added under acetate from		0.0008		
C released under CO_2 gas		0.13075		

Acetate supply and CO_2 release during the experiment are also given. All values are expressed in C mass (g).

introduced and from the outlet CO_2 gas flow. The carbon composition of the last two components has been taken into account for the calculation. The data obtained are summarized in Table 1 in terms of grams of carbon. A recovery of 98.8% of the initial carbon content present in the system was observed. This almost complete carbon balance verifies the tightness of the system and validates the method. This also gives global information on grown algal metabolism during H_2 production. For example, 99.5% of the carbon contained initially in the TAP medium was under organic form (mainly acetate), thus representing the main source of carbon available for cells. Thirty-four percent (0.6 g) of this organic carbon was assimilated during the experiment. A part of the assimilated carbon (considered to be mainly under organic form) was next released under dissolved inorganic form in the medium. This corresponds to the net increase in inorganic carbon content of the medium throughout the experiment (from 0.006 g to 0.046 g). Another part was obviously released in the gas phase (0.13 g under CO_2 form).

In conclusion, the time-variations of the measured parameters (gas, metabolites and nutrients) and their intercorrelations confirm the complexity of the H_2 production process with photosynthetic microorganisms. Under sulfur deprivation, a reorganization of the cells metabolism occurs. The H_2 production stage appears as a fully dynamic process, reinforcing the interest of conducting investigations under well-known conditions with on-line monitoring of the pertinent parameters, such as gas production.

4. Energetic yields for hydrogen production

As the H_2 production is monitored during all the process, an instantaneous energetic yield of biological conversion can be defined as

$$\text{energetic yield (\%)} = \frac{H_2 \text{ production rate} \times H_2 \text{ energy content}}{\sum \text{energy consumption rates}}$$

The energy sources in this case can be light used by the photosynthetic chain, but also acetate or endogenous organic compounds degradation. The consumption rates of these last two energy sources could be calculated from a deeper insight in cellular composition variation and knowing the implication of acetate degradation in the process. As it still remains unclear, the energetic yield was reduced in this study to the light conversion efficiency, neglecting thus the other potential sources of chemical energy. One of the objectives for mass-scale production being to achieve acetate-free conditions to obtain fully autotrophic H_2 production, the energetic yield based only on light conversion is of primary relevance. It is defined as the energy stored as H_2 produced per unit of light energy absorbed [1]:

$$\text{yield of light energy conversion (\%)} = \frac{H_2 \text{ production rate} \times H_2 \text{ energy content}}{\text{light energy absorption rate}}$$

The light conversion efficiency is rarely referred in literature, mainly because the light energy absorbed by the culture is difficult to determine accurately. This is not the case in the torus geometry as this parameter can be controlled. The H_2 volumetric flow rates have been converted to molar flow rates using the molar volume of H_2 at the atmospheric pressure ($24.41\,mol^{-1}$ for 25 °C). The H_2 energy content is $290\,kJ\,mol\,H_2^{-1}$. The light energy received by the culture (6.7 W) has been calculated from the amount of energy provided to the system (mean incident flux $q_0 = 110\,\mu mol$ of photons $m^{-2}\,s^{-1}$) and the illuminated surface of the reactor ($0.28\,m^2$). The energy of a photon being wavelength dependent ($E = hc/\lambda$), the light energy received is based on the PAR (photosynthetically active radiations, range from 400 nm to 700 nm), which corresponds to the daylight emission spectrum of the fluorescent tubes used. An amount of 4.6 μmol of photons $m^{-2}\,s^{-1}$ are equivalent to $1\,W\,m^{-2}$ (considering the solar spectrum, $1.8\,\mu mol$ of photons $m^{-2}\,s^{-1} = 1\,W\,m^{-2}$).

The yields of light energy conversion obtained enable the performances of the studied photosynthetic microorganism to produce H_2 in the described experimental conditions to be evaluated. The associated results are presented in Fig. 3. A maximal energetic yield of light conversion to H_2 energy of 0.125% was achieved (0.049% considering the solar spectrum). It would be interesting to compare the obtained values to an optimal one. Nevertheless, it seems difficult to give a reasonable theoretical maximal energetic conversion yield of solar energy to H_2 by photosynthetic microorganisms. One reason is that H_2 is not only produced directly from water photolysis but also from starch degradation, implying thus that the energetic conversion yield would ideally be defined for each pathway. Indeed, if only the release of H_2 by direct photolysis is considered, the instantaneous conversion yield will not be relevant, due to the possible electron input in Z-scheme from starch degradation. It should be noted that four photons are theoretically required for the photosynthetic electron trans-

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port chain to reduce one molecule of H_2 from water photolysis. In this case, this would give an energetic conversion yield of about 10%, a probably unrealistic value. When considering only the photosynthetic conversion efficiency, it is well known that a non-negligible part of absorbed photons can be lost in antennae before entering the electron transport chain, depending on the irradiance received. In photobioreactor, the concentrations achieved are usually high, making irradiance-field very heterogeneous. The cells are thus far from their optimal photosynthetic conversion yield. It is evident that a further understanding of cell physiology under H_2 production conditions would give a more accurate estimation of the optimal energetic conversion yield (especially when both direct and indirect biophotolysis pathways) with their interconnection with photosynthesis apparatus, are totally elucidated. Such an overview on the cell metabolism remains difficult to obtain with classical macroscopic approach, even in fully controlled conditions. An interesting perspective lies in the metabolic flux computation-based modeling of *C. reinhardtii* behavior. Such a method has been successfully applied to photosynthetic microorganisms [36]. Metabolic engineering investigations have been performed to compute the metabolic fluxes for *Arthrospira platensis* and to analyze the detailed light energy conversion in terms of electron transport chain efficiency. A transposition to H_2 production with eukaryotic cell will certainly give interesting information, such as for example the optimal energetic conversion yield that can be reasonably considered. Such a method has, however, never been applied for this application. The prerequisite is a deep experimental characterization of the cell response in bioreactor including the variation of intracellular composition, in fully controlled conditions. In this context, the presented set-up is of great interest, as well as additional analytical measurements for a further modeling of *C. reinhardtii* metabolism under H_2 production conditions.

5. Conclusion and prospects

An experimental set-up combining a lab-scale fully controlled photobioreactor with an on-line system for gas production analysis has been used to characterize the behavior of *C. reinhardtii* under the sulfur deprivation protocol. The different phases leading to the H_2 production have been observed. The time-variations of relevant parameters, like gas released or metabolites and nutrients, have been measured. Such a set-up appears as a pertinent tool for further investigations linked to the specific and transient biological responses involved in the H_2 production with green microalgae, as well as for the optimization of the cultivation parameters and protocols for this specific application. The system and analytical methods proposed enable a carbon balance ($\pm 0.2\%$) to be established, demonstrating the high relevance of the designed set-up. It was also observed that the earlier anoxia (induced by the inert gas bubbling) results in a sooner H_2 production. Even if the rate of H_2 production reached a maximum of about $2.5\,ml\,l^{-1}\,h^{-1}$, which is comparable to the one found in the literature [18], the H_2 was produced during a longer period and then released in a more significant amount.

Thanks to the control of light received by the culture, it was also possible to determine the instantaneous and mean energetic conversion yields achieved. Although the results obtained in this study are close to the ones reported in the literature, it seems unrealistic to propose mass-scale production with such low energetic conversion yields (0.125%) and productivities ($2.5\,ml\,l^{-1}\,h^{-1}$). At least, the energetic conversion yields should be 20 or 30 times higher. This implies important breakthroughs in the understanding of H_2 metabolism in green algae, as well as in photobioreactor development, to optimize their light conversion efficiency. In addition, it appears interesting to develop adapted control procedure to be able, for example, to sustain H_2 production by dynamic modification of environmental conditions in fully controlled production systems.

Acknowledgments

This work was supported by the Interdisciplinary CNRS Energy Program (2002–2005), by the CPER-Etat-Region of Pays de la Loire (France) and by the "PhotobioH_2" ANR Program (French National Program, 2006–2009).

REFERENCES

[1] Akkerman I, Janssen M, Rocha J, Wijffels R. Photobiological hydrogen production: photochemical efficiency and bioreactor design. Int J Hydrogen Energy 2002;27:1195–208.
[2] Asada Y, Miyake J. Photobiological hydrogen production. J Biosci Bioeng 1999;88:1–6.
[3] Das D, Veziroglu NT. Hydrogen production by biological processes: a survey of literature. Int J Hydrogen Energy 2001;26:13–28.
[4] Florin L, Tsokoglou A, Happe T. A novel type of iron hydrogenase in the green alga *Scenedesmus obliquus* is linked to the photosynthetic electron transport chain. J Biol Chem 2001;276:6125–32.
[5] Benemann JR, Berenson J, Kaplan N, Kauren M. Hydrogen evolution by a chloroplast–ferredoxin–hydrogenase system. P Natl Acad Sci USA 1973;70:2317–20.
[6] Ghirardi ML, Togasaki RK, Seibert M. Oxygen-sensitivity of algal H_2 production. Appl Biochem Biotechnol 1997;63:141–51.
[7] Ghirardi ML, Zhang L, Lee JW, Flynn T, Seibert M, Greenbaum E, et al. Microalgae: a green source of renewable H_2. Trends Biotechnol 2000;18:506–11.
[8] Spolaore P, Joannis-Cassan C, Duran E, Isambert A. Commercial applications of microalgae. J Biosci Bioeng 2006;101(2):87–96.
[9] Lee YK. Microalgal mass culture systems and methods: their limitation and potential. J Appl Phycol 2001;13:307–15.
[10] Richmond A. Handbook of microalgal culture: biotechnology and applied phycology. Blackwell Sciences Ltd; 2004.
[11] Pulz O. Photobioreactors: production systems for autotrophic microorganisms. Appl Microbiol Biotechnol 2001;57(3):287–93.
[12] Ghirardi ML, Kosourov S, Tsygankov A, Seibert M. Two-phase photobiological algal H_2-production system. In: Proceedings of the 2000 DOE Hydrogen Program Review. NREL/CP-570-28890.
[13] Guan Y, Deng M, Yu X, Zhang W. Two-stage photo-biological production of hydrogen by marine green alga *Platymonas subcordiformis*. Biochem Eng J 2004;19:69–73.

Please cite this article as: Fouchard S, et al. Investigation of H_2 production using the green microalga *Chlamydomonas reinhardtii* in a fully controlled photobioreactor fitted with on-line gas analysis. Int J Hydrogen Energy (2008), doi:10.1016/j.ijhydene.2008.03.067

[14] Kojima E, Lin B. Effect of partial shading on photoproduction of hydrogen by *Chlorella*. J Biosci Bioeng 2004;97:317–21.
[15] Tsygankov A, Kosourov S, Seibert M, Ghirardi ML. Hydrogen photoproduction under continuous illumination by sulfur-deprived, synchronous *Chlamydomonas reinhardtii* cultures. Int J Hydrogen Energy 2002;27:1239–44.
[16] Kosourov S, Tsygankov A, Seibert M, Ghirardi ML. Sustained hydrogen photoproduction by *Chlamydomonas reinhardtii*: effects of culture parameters. Biotechnol Bioeng 2002;78:731–40.
[17] Tsygankov AA, Kosourov SN, Tolstygina IV, Ghirardi ML, Seibert M. Hydrogen production by sulfur-deprived *Chlamydomonas reinhardtii* under photoautotrophic conditions. Int J Hydrogen Energy 2006;31:1574–84.
[18] Melis A, Zhang L, Forestier M, Ghirardi ML, Seibert M. Sustained photobiological hydrogen gas upon reversible inactivation of oxygen evolution in the green alga *Chlamydomonas reinhardtii*. Plant Physiol 2000;122:127–35.
[19] Pruvost J, Pottier L, Legrand J. Numerical investigation of hydrodynamic and mixing conditions in a torus photobioreactor. Chem Eng Sci 2006;61(14):4476–89.
[20] Pottier L, Pruvost J, Deremetz J, Cornet JF, Legrand J, Dussap CG. A fully predictive model for one-dimensional light attenuation by *Chlamydomonas reinhardtii* in a torus reactor. Biotechnol Bioeng 2005;91(5):569–82.
[21] Strickland JDH, Parsons TR. A practical handbook of seawater analysis. Bull Fish Res Bd Canada 1968;167:49–80.
[22] Genty B, Briantais JM, Baker NR. The relationship between the quantum yield of photosynthetic electron transport and quenching of chlorophyll fluorescence. Biochim Biophys Acta 1989;990:87–92.
[23] Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem 1951;193:265–75.
[24] DuBois M, Gilles KA, Hamilton JK, Rebers PA, Smith F. Colorimetric method for determination of sugars and related substances. Anal Chem 1956;28(3):350–6.
[25] Klein U, Betz A. Fermentative metabolism of hydrogen-evolving *Chlamydomonas moewusii*. Plant Physiol 1978;61:953–6.
[26] Fouchard S, Hemschemeier A, Caruana A, Pruvost J, Legrand J, Happe T, et al. Autotrophic and mixotrophic hydrogen photoproduction in sulfur-deprived *Chlamydomonas* cells. Appl Environ Microbiol 2005;71:6199–205.
[27] Antal TK, Kredeleva TE, Laurinavichene TV, Makarova VV, Ghirardi ML, Rubin AB, et al. The dependence of algal H_2 production on photosystem II and O_2 consumption activities in sulfur-deprived *Chlamydomonas reinhardtii* cells. Biochim Biophys Acta 2003;1607:153–60.
[28] Xiong J, Hutchison R, Sayre R, Govindjee. Modification of the photosystem II acceptor side function in a D1 mutant (arginine-269-glycine) of *Chlamydomonas reinhardtii*. Biochim Biophys Acta Bioenerg 1997;1322:60–76.
[29] Ekelund N, Aronsson A. Changes in chlorophyll *a* fluorescence in *Euglena gracilis* and *Chlamydomonas reinhardtii* after exposure to wood-ash. Environ Exp Bot 2007;59:92–8.
[30] Wykoff DD, Davies JP, Melis A, Grossman AR. The regulation of photosynthetic electron transport during nutrient deprivation in *Chlamydomonas reinhardtii*. Plant Physiol 1998;117:129–39.
[31] Kosourov S, Patrusheva E, Ghirardi ML, Seibert M, Tsygankov A. A comparison of hydrogen photoproduction by sulfur-deprived *Chlamydomonas reinhardtii* under different growth conditions. J Biotechnol 2007;128:776–87.
[32] White AL, Melis A. Biochemistry of hydrogen metabolism in *Chlamydomonas reinhardtii* wild type and a Rubisco-less mutant. Int J Hydrogen Energy 2006;31:455–64.
[33] Ghirardi ML, Kosourov S, Tsygankov A, Rubin A, Seibert M. Cyclic photobiological algal H_2-production. In: Proceedings of the 2002 DOE Hydrogen Program Review. NREL/CP-610-32405.
[34] Gfeller RP, Gibbs M. Fermentative metabolism of *Chlamydomonas reinhardtii*. I. Analysis of fermentative products from starch in dark and light. Plant Physiol 1984;75:212–8.
[35] Weers PMM, Ramesh DG. Growth and reproduction of *Daphnia galeata* in response to changes in fatty acids, phosphorus, and nitrogen in *Chlamydomonas reinhardtii*. Limnol Oceanogr 1997;42(7):1584–9.
[36] Cogne G, Gros JB, Dussap CG. Identification of a metabolic network structure of *Arthrospira (Spirulina) platensis* metabolism. Biotechnol Bioeng 2003;84(6):667–76.

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