Investigation of H₂ production by microalgae in a fully-controlled photobioreactor

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ABSTRACT:
Green microalgae are able to produce molecular hydrogen in a clean way. H₂ release is the result of complex, interacting, and transient intracellular mechanisms, influenced by extracellular conditions. To relate the dynamic coupling between culture conditions and biological responses, an original lab-scale photobioreactor has been developed. Such device allows to conduct cells culture under well-defined conditions, with on-line measurement of gas production. A first validation is conducted with a well-known protocol, the H₂ production by Chlamydomonas reinhardtii under sulphur deprivation conditions. Instantaneous productivities were measured, and various successive physiological states were analysed and quantified. The proposed photobioreactor appears thus as an innovative tool for methodical optimisation of H₂ production using photosynthetic microorganisms, including both bioprocess and physiological aspects in an integrated approach.

KEYWORDS: photobioreactor, hydrogen, Chlamydomonas reinhardtii

INTRODUCTION
Since the middle of the XIX century, the increase of anthropic activities is accompanied by parallel massive greenhouse gas emissions. These emissions, essentially explained by the use of fossil fuel (CO₂), are still increasing to support the worldwide energetic demand. In this context, development of alternative clean and renewable energies are of primary interest. Hydrogen appears as one of the most attractive solution as a potential energy vector for the future. But even if the use of H₂ energy is totally clean (its combustion in air generates only water), its production is not well-established. Short-term methods induce greenhouse gas release (use of methane or organic products derived from oil) or electricity use (electrolysis of water). Ideally, H₂ would be produced with renewable energies, without CO₂ release. This can be achieved by different ways, photovoltaic electrolysis, thermochemical cycles (solar energy) or biological production. Different microorganisms are able to produce H₂: bacteria by fermentation, photosynthetic bacteria by organic compounds degradation and autotrophic organisms (microalgae and cyanobacteria). Because H₂ is the result of the water splitting by photosynthesis (with a parallel release of O₂), photosynthetic microorganisms have the ability to produce H₂ from the two most abundant renewable energies of our planet, water and sun, without parallel greenhouse gas release (with CO₂ assimilation during photosynthetic growth).
It is well-known since many decades that photosynthetic organisms are able to produce hydrogen under given conditions. This is the case of Chlamydomonas reinhardtii, a unicellular green microalga. Under illumination and in anoxic conditions, H₂ is released [1-3]. This is the result of complex metabolic mechanisms, implying a Fe-hydrogenase enzyme coupled to the photosynthetic chain that enables reduction of protons given by the water splitting to produce molecular hydrogen [4]. Electrons are given by the photosynthetic process, even directly by water photooxydation (direct photoclysis) or by the catabolism of carbohydrates compounds previously accumulated in the cell (indirect biophotolysis).
The main limitation is that hydrogen production is only transient. Fe-hydrogenase being highly sensitive to oxygen, a rapid inhibition occurs as soon as oxygen is released by water biophotolysis, and H₂ production stops [5-7]. H₂ production by photosynthetic microorganisms can thus be considered as an “emergency” biological way, with the purpose to re-oxidize the photosynthetic apparatus after an anoxic period. This makes metabolic pathways difficult to characterize. Because they are only activated during short periods, yields of conversion are thus not optimised, in contrary of the photosynthetic conversion of light in biomass.
However, a protocol based on the sulphur deprivation of algal cells is known to induce a sustained H₂ production (four days) [8]. It creates anoxic conditions, resulting in Fe-hydrogenase induction, and thus H₂ release.
Despite feasibility of this protocol has been shown, productivity remains too low for industrial application (around 2.5 ml of H₂ per liter and hour of culture). Metabolic pathways are indeed not well identified. To optimise them, it is necessary to improve the knowledge on the H₂ metabolism, especially bioenergetic mechanisms involved and the relation between photosynthesis and biohydrogen uptake (coupling with respiration, or electrons transport in the photosynthetic apparatus for examples). In this context, and because of the high coupling of the interacting transient intracellular mechanisms to extracellular conditions (medium composition, light...), experiments under well-defined conditions in devoted lab-scale photobioreactors are of primary interest.

Specificity of H₂ production by photosynthetic microorganisms makes difficult the development of such a set-up. Even for classical application (like biomass production), photobioreactors are highly dependent of the light supply and reactor geometry, that determines radiative-field in the culture, which is heterogeneous due to light absorption and scattering by cells. Ratio of illuminated surface to culture volume, and hydrodynamic conditions applied to optimise the light conversion by photosynthetic cells are for example to be considered with attention. In the particular application of H₂ photobioproduction, complexity is increased (tightness to H₂ for example). Examples are available with photosynthetic bacteria and cyanobacteria cultures [9-11]. Concerning H₂ photobioproduction with microalgae, systems described in literature are simple, limited in the use of bottles (type Schott or Roux), Erlenmeyer or other kind of glassware [12-15]. Because of the complex and dynamic coupling between culture conditions and biological responses, such devices permit only primary investigations of H₂ production.

An original laboratory scale photobioreactor is thus proposed, dedicated to H₂ production under fully controlled conditions. It is based on a torus geometry enabling culture parameters and especially light received by cells to be accurately determined. To obtain information on the dynamic evolution of the culture during H₂ production, the set-up is made up with an on-line measurement of gas injected and collected at the reactor outlet. This allows to quantify productivities and energetic yields of the system. Primary results obtained with the well-known sulphur deprivation protocol, leading to H₂ production in Chlamydomonas reinhardtii cells, are presented.

**DEVELOPMENT OF THE PHOTOBIOREACTOR**

**General description of the reactor**

The photobioreactor is presented in Figure 1. The light supply is placed in front of the geometry, light emission direction being perpendicular to the illuminated surface of the reactor. This illuminated surface is of torus shape. The reactor presents thus a large illumination surface for a given culture volume. To prevent from optical distortion and to avoid curved surface perpendicular to the emitting direction of the light source, the front surface is plane, and the torus channel is square-sectioned with a gap width of 4 cm, leading to a reactor volume of 1.3 liter. By this way, light attenuation in the depth of culture can be considered monodirectionnal, allowing simple light transfer models to be applied to characterize the radiative-field in the photobioreactor [16]. To allow cultivation under axenic conditions, the reactor is autoclavable and can be sterilized in place by steam injection (120°C). It is thus managed in stainless steel (type 316L), the transparent optic surface being in polycarbonate. Because of its low molecular mass, H₂ is very diffusive. EPDM seals (Ethylen Propylen Diene) have been used for their tightness to H₂ gas and also for their resistance to the sterilization conditions.

Interest of the torus shape is that it allows a simple and accurate determination of the light received by the culture, while providing good mixing conditions. The culture is indeed circulated by means of a marine impeller. This generates a three-dimensional swirling motion in the geometry that interacts with Dean vortices generated by the reactor bends [17]. The combination of these two effects leads to an efficient mixing along the light gradient, homogenising the light received per cell [18], and an absence of dead volumes in the reactor.

The photobioreactor receives a complete loop of common sensors and automations for microalgae culture (pH, temperature, nutrients, dissolved O₂), and allows a high control of the injected and collected gas (O₂, H₂, N₂, CO₂). The reactor can be conducted in batch modes, or continuous modes. For the last ones, continuous cultures in chemostat as well as turbidostat are permitted. It must be noticed that for
photosynthetic microorganisms studies, turbidostat mode can be relevant because it allows to maintain a

given biomass concentration in the reactor (dilution rate is regulated as a function of the biomass

centration), that is interesting to control with accuracy the light received per cell (with low biomass
cultivations for example). A specific biomass sensor has been developed for this purpose, based on optical

measurement of culture absorption.

**Cultivation parameters regulation**

**Light and mixing system**

The light source of the system is provided by fluorescent tubes (OSRAM L13W/12-950) emitting a white light

in the visible domain. These tubes are placed parallel to the front side of the reactor, with the same total

height as the reactor to have an homogeneous supply of light on reactor illuminated surface. Tubes and

reactor are fixed on a framework to keep them parallel. The light source can be moved parallel to the

photobioreactor, allowing to adjust the incident light by modifying the distance between the two elements (up

to 800µE.m⁻².s⁻¹).

The marine impeller and agitator shaft are magnetically coupled to the motor (APPLIKON). Such system

guarantees a total tightness of the drive shaft, eliminating risk of bacterial contamination and H₂ leak.

Rotation speed of the impeller can be controlled from 0 to 600rpm.

**Temperature, pH and dissolved oxygen**

Temperature has an important role on metabolic cellular reactions and thus on the photosynthetic growth

and H₂ production in microalgae. To prevent form thermal variations (due for example to heat generated by

fluorescents tubes), temperature is thus regulated. Temperature is measured using a Pt100 integrated to the

pH probe. Automatic cooling of the reactor by ambient air blowing is induced when the requested

temperature is exceeded (with a fan placed on the backside of the reactor). If air cooling is not sufficient,
circulation of a thermostated water is applied, the reactor being double-jacketed.

pH is also implied in many biological cellular reactions. This is thus also important to regulate it during

cultivation. But, because of the carbon uptake during photosynthesis, growth induces an imbalance between

the carbonated species in the culture medium, resulting in medium basification. pH measurement is thus

also used in photobioreactor as an indirect regulation of the dissolved carbon, by automatically adjusting pH

with CO₂ bubbling and dissolution. By this way, pH regulation prevents from a metabolic limitation by

adjusting the supply of inorganic carbon in accordance with its consumption by the culture. The pH of the

medium is measured with a pH sensor InPro 3200 (METTLER TOLEDO) and the CO₂ injection is done with

a mass flow controller. This allows measurement of CO₂ injected in the reactor.

The O₂ release is linked to the net photosynthetic activity of the cells. It is also an important parameter during

H₂ production, because of Fe-hydrogenase sensitivity. Even for classical growth conditions, high dissolved

O₂ concentration can have adverse effects on photosynthesis. Regulation of this parameter is thus also

necessary. It is measured with an oxymetric sensor InPro 6000 (METTLER TOLEDO). When dissolved O₂

coloration is too high, N₂ or air is injected for oxygen flushing. For convenience, it is also possible to

apply a constant bubbling to maintain O₂ at a weak concentration.

All of the sensors for culture parameters regulation (temperature, pH and dissolved O₂) are connected to a
data acquisition and command system (DAQ 6023E - National Instruments). The "Labview" software is used

as an interface (data acquisition and saving, setting of regulation parameters).

**Gas analysis system**

Because of the H₂ application, gas released by the culture are measured on-line (fig. 2). Mass flow of gas

injected (N₂/CO₂) are regulated and measured using two mass flow controllers. To determine the mass flow
rate for each one of the gas produced by the culture, the composition and the total flow rate of the gas
mixture are measured. A mass flow meter (EL-FLOW devices - Bronkhorst High-Tech) is thus associated

with a quadrupole mass spectrometer (PFEIFFER VACUUM). It must be noticed that a gas condenser is

used in the reactor outlet to prevent from water evaporation and keep constant the culture volume in the
reactor.
The developed gas analysis system allows measurement of flow rates for O₂, H₂ and CO₂ (and N₂ if needed) produced by the algal culture, and thus to follow the instantaneous kinetics of gases released or consumed by the culture.

**HYDROGEN PRODUCTION IN PHOTOBIOREACTOR**

**Method**

So as to validate the experimental set-up before further investigation, the well-known protocol of sulphur deprivation has been applied. *Chlamydomonas reinhardtii* strain wt 137c (from the Chlamydomonas Genetic Center, Duke University, Durham, USA) was used in this experiment. This protocol allows to get rid of the high sensibility of Fe-hydrogenase to O₂, by reducing photosynthetic activity, and thus enabling a transition to anoxic conditions, O₂ consumption by respiration process being higher than the O₂ released during photosynthesis. H₂ is then produced under light conditions [8]. This protocol generates a temporal separation of the growth phase (with O₂ release) and H₂ production phase.

To apply this protocol in the torus reactor, cells were previously grown under normal conditions in flasks containing TAP medium (Tris-acetate-phosphate - pH 7.2). Cultures were maintained at 25°C, under constant agitation and continuous illumination (110μmol photons.m⁻².s⁻¹). When a density around 5x10⁶ cells/ml was reached, corresponding to a late logarithmic growth state, cells were placed under sulphur-deprived conditions to induce H₂ production (second phase). Algal culture were centrifuged at 1,000 g during 5 min, washed, re-suspended in a sulphur-deprived medium (TAP-S) and injected in the photobioreactor with a peristaltic pump. This corresponds to the beginning of the H₂ production phase (i.e. second phase).

During all this second phase, a constant agitation 300tr.min⁻¹ is applied in the reactor with an incident light of 110μmol photons.m⁻².s⁻¹. In this test case, only temperature is regulated (25°C), the TAP medium being buffered. The photobioreactor is operated in batch mode. A constant bubbling of the culture with a neutral gas (nitrogen gas) is performed so as to obtain an accurate measurement of gases released, that remain low (few ml/min) and to avoid gas accumulation in the reactor. Gases are continuously monitored, and culture samples are taken regularly to measure physiological parameters. In addition to cells counting obtained using a Malassez hemacytometer, starch accumulated by the biomass is measured, because of its importance in the indirect biophotolysis process [19].

**Results and discussion**

Results of the experiment are shown in figure 3. H₂ is produced at the beginning of the sulphur deprivation, N₂ bubbling inducing a transition of the culture to anoxic conditions after only few hours (fig. 3-A). A fast decrease of dissolved O₂ until zero is also observed (data not shown), allowing the synthesis of the Fe-hydrogenase. This agrees with previous studies dealing with the sulphur deprivation effects at the metabolic
level, which have shown a progressive reduction of photosynthetic capacity of the cells due to the inactivation of the PSII (photolysis site) [20], resulting in a decrease of $O_2$ production (fig. 3-B). If the sulphur deprivation induces a decrease in photosynthetic activity, mitochondrial respiration is maintained. From this imbalance between the two processes, a progressive decrease of the $O_2$ concentration is induced, and anoxic conditions are obtained [21].

In our experiment, the rate of $H_2$ production reaches a maximum of 1.9ml.l$^{-1}$.h$^{-1}$ (fig. 3-A) after 1.75 day of sulphur deprivation. This rate is comparable to the ones found in the literature (2ml.l$^{-1}$.h$^{-1}$ [8]). In our case the $H_2$ flow rate is maintained only few days and decreases then regularly due to the negative effect of sulphur deprivation that progressively affects cells metabolism.

**Figure 3**: Kinetics of the gases flows produced by a *Chlamydomonas reinhardtii* culture incubated in the torus photobioreactor in sulphur deprivation conditions. (A): hydrogen, (B): oxygen and (C): CO$_2$. Intracellular starch evolution (D) was also measured during the experiment.

Kinetics of starch accumulation (fig. 3-D) confirms that sulphur deprivation has not only an influence on the photosynthesis reduction, but induces also during the first day a mobilisation of the carbon source available in the culture medium [19]. This results in an over-accumulation of intracellular starch, which degradation next sustains with water photolysis the $H_2$ production process (direct and indirect biophotolysis are thus coupled under sulphur deprivation). This is linked to the CO$_2$ release due to carboneous reserves degradation (fig. 3-C).

**CONCLUSION AND PROSPECTS**

The protocol of sulphur deprivation applied for this study has been applied to evaluate in a lab-scale and fully controlled photobioreactor the behaviour of *Chlamydomonas reinhardtii* when producing $H_2$. The different phases conducting to hydrogen production were observed. Kinetics of relevant data, like gas released, or starch accumulated, were measured. This shows interest of such a set-up as a new tool for further investigation in the understanding of specific biologic transient responses implied during $H_2$ production with green microalgae, as well as in the optimisation of the cultivation parameters and protocols for this specific application.
References:


