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Physiology and Biotechnology of the Hydrogen Production with the Green Microalga *Chlamydomonas reinhardtii*

Fisiologia e Biotecnologia della Produzione di Idrogeno con la Microalga Verde *Chlamydomonas reinhardtii*

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A problem is a chance for you to do your best
(*Un problema è un'occasione per fare del tuo meglio*)
Duke Ellington (1899-1974)

A hundred times a day I remind myself that my inner and outer lives are based on the labors of other people, living and dead, and that I must exert myself in order to give in the same measure as I have received and am still receiving
(*Cento volte al giorno ricordo a me stesso che le mie vite, interiore ed esteriore, sono basate sulle fatiche di altri uomini, vivi e morti, e che io devo fare il massimo sforzo per dare nella stessa misura in cui ho ricevuto e sto ancora ricevendo*)
Albert Einstein (1879-1955)

Since we will never think the same way and we will see the truth for fragments and from different points of view, the rule of our behavior is the mutual tolerance
(*Dato che non penseremo mai nello stesso modo e vedremo la verità per frammenti e da diversi angoli di visuale, la regola della nostra condotta è la tolleranza reciproca*)
Mahatma Gandhi (1869-1948)

Nothing in life is to be feared, it is only to be understood. Now is the time to understand more, so that we may fear less.
(*Niente nella vita deve essere temuto, deve essere solo compreso. Ora è il momento di capire di più, così che potremo avere meno paura*)
Marie Curie (1867-1934)

Theory like mist on eyeglasses: obscures facts
(*Teoria è come appannamento su occhiali: oscura fatti*)
Charlie Chan in Egypt, 1935

Chi sono io? Cosa posso io fare? Cosa posso io sperare? Chi me l'ha fatto fare?
Who am I? What can I do? What can I hope? Who made me do this?
Damiano Eletti (1980-*living*)
(estrapolazione sulle domande basilari dell'Uomo, come tramandateci dalla nostra insegnante di Filosofia, Prof.ssa Piccardi)
(deduction out of Man's fundamental questions, as handed down to us by our teacher of Phylosophy, Mrs. Piccardi)

Executive summary

The hydrogen (H_2) production in the green microalga *Chlamydomonas reinhardtii* was evaluated by means of a detailed physiological and biotechnological study. First, a wide screening of the H_2 productivity was done on 22 strains of *C. reinhardtii*, most of which mutated at the level of the D1 protein. The screening revealed for the first time that mutations upon the D1 protein may result on an increased H_2 production. Indeed, productions ranged between 0 and more than 500 mL H_2 L^{-1} of culture (Torzillo, Scoma et al., 2007a), the highest producer (L159I-N230Y) being up to 5 times more performant than the strain cc124 widely adopted in literature (Torzillo, Scoma, et al., 2007b). Improved productivities by D1 protein mutants were generally a result of high photosynthetic capabilities counteracted by high respiration rates.

Optimization of culture conditions were addressed according to the results of the physiological study of selected strains. In a first step, the photobioreactor (PBR) was provided with a multiple-impeller stirring system designed, developed and tested by us, using the strain cc124. It was found that the impeller system was effectively able to induce regular and turbulent mixing, which led to improved photosynthetic yields by means of light/dark cycles. Moreover, improved mixing regime sustained higher respiration rates, compared to what obtained with the commonly used stir bar mixing system. As far as the results of the initial screening phase are considered, both these factors are relevant to the H_2 production. Indeed, very high energy conversion efficiencies (light to H_2) were obtained with the impeller device, prooving that our PBR was a good tool to both improve and study photosynthetic processes (Giannelli, Scoma et al., 2009).

In the second part of the optimization, an accurate analysis of all the positive features of the high performance strain L159I-N230Y pointed out, respect to the WT, it has: (1) a larger chlorophyll optical cross-section; (2) a higher electron transfer rate by PSII; (3) a higher respiration rate; (4) a higher efficiency of utilization of the hydrogenase; (5) a higher starch synthesis capability; (6) a higher *per cell* D1 protein amount; (7) a higher zeaxanthin synthesis capability (Torzillo, Scoma et al., 2009).

These information were gathered with those obtained with the impeller mixing device to find out the best culture conditions to optimize productivity with strain L159I-N230Y. The main aim was to sustain as long as possible the direct PSII contribution, which leads to H_2 production without net CO_2 release. Finally, an outstanding maximum rate of 11.1 ± 1.0 mL L^{-1} h^{-1} was reached and maintained for 21.8 ± 7.7 hours, when the effective photochemical efficiency of PSII ($\Delta F/F'_m$)

underwent a last drop to zero. If expressed in terms of chl (24.0 ± 2.2 μmoles mg^{-1} chl h^{-1}), these rates of production are 4 times higher than what reported in literature to date (Scoma et al., 2010a *submitted*). DCMU addition experiments confirmed the key role played by PSII in sustaining such rates. On the other hand, experiments carried out in similar conditions with the control strain cc124 showed an improved final productivity, but no constant PSII direct contribution. These results showed that, aside from fermentation processes, if proper conditions are supplied to selected strains, H_2 production can be substantially enhanced by means of biophotolysis.

A last study on the physiology of the process was carried out with the mutant IL. Although able to express and very efficiently utilize the hydrogenase enzyme, this strain was unable to produce H_2 when sulfur deprived. However, in a specific set of experiments this goal was finally reached, pointing out that other than (1) a state 1-2 transition of the photosynthetic apparatus, (2) starch storage and (3) anaerobiosis establishment, a *timely* transition to the H_2 production is also needed in sulfur deprivation to induce the process before energy reserves are driven towards other processes necessary for the survival of the cell.

This information turned out to be crucial when moving outdoor for the H_2 production in a tubular horizontal 50-liter PBR under sunlight radiation. First attempts with laboratory grown cultures showed that no H_2 production under sulfur starvation can be induced if a previous adaptation of the culture is not pursued outdoor. Indeed, in these conditions the H_2 production under direct sunlight radiation with *C. reinhardtii* was finally achieved for the first time in literature (Scoma et al., 2010b *submitted*). Experiments were also made to optimize productivity in outdoor conditions, with respect to the light dilution within the culture layers.

Finally, a brief study of the anaerobic metabolism of *C. reinhardtii* during H_2 oxidation has been carried out. This study represents a good integration to the understanding of the complex interplay of pathways that operate concomitantly in this microalga.

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GENERAL INDEX

ABBREVIATIONS

Photosystem I/II: PSI/PSII

Photobioreactor: PBR

Photosynthesis-light response curve: P/I curve

Light/dark cycle: L/D cycle

Oxygen evolving complex: OEC

Chlorophyll: chl

Carotenoid: car

Ferredoxin: FDX

Maximum rate of photosynthesis: P_{max}

Photoinhibition irradiance: I_h

Saturation irradiance: I_k

Light-saturated rate: I_s

Compensation point: I_c

Relative apparent quantum yield of photosynthesis: $Ø_{rel}$

Chlorophyll-specific optical absorption cross-sections: a*

Photon flux density: PFD

Plastoquinone pool: PQ pool

3-(3,4-dichlorophenyl)-1,1-dimethylurea: DCMU

2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone: DBMIB

Carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone: FCCP

Glycolaldehyde: GA

Steady-state fluorescence emission: F'_s

Maximal fluorescence in light-adapted algae: F'_m

Effective quantum yield of the photosystem II photochemistry: $\Delta F/F'_m$

Maximum quantum yield of the photosystem II photochemistry: F_v/F_m

Nonphotechemical quencing: NPQ

Electron transfer rate: ETR

Pulse amplitude modulating: PAM

Primary and secondary electron acceptor of P680 of the photosystem II: Q_a and Q_b

Anaerobic induction: AI

Pyruvate formate lyase: Pfl

Pyruvate decarboxylase: Pdc

Pyruvate ferredox/flavodoxin-oxidoreductase: PFO

Ribulosebisphosphate-carboxylase/oxygenase: Rubisco

Proteins-carbohydrates ratio: p/c ratio

Tris-acetate-phosphate: TAP

Tris-acetate-phosphate without inorganic sulfur: TAP-S

INTRODUCTION

Photosynthesis is considered the most significant chemical process in the evolution of life on Earth (Govindjee, 2008; Blankenship, 2002; Ranger, 2008). The process concerns carbon turnover in the biosphere powered by the continuous supply of sunlight energy. This energy is stored as reduced carbon compounds in plant cells and is coupled to the production of oxygen from water. Oxygen availability, in turn, allows for effective recycling of the stored energy by means of cellular respiration. The most used energy resources of the last centuries, fossil fuels, are nothing but geologically stored products of ancient photosynthesis, collected over the last 2.5 billion years. However, these energy reserves will encounter a more rapid depletion in the next future. The economical and socio-political consequences of the shortage of fossil fuels are already felt today. With the increase of world population and economic growth the global energy demand will continue to increase in the coming decades. Based on recent reports (International Energy Agency, 2001), reserves of coal, oil and natural gas are expected to provide about 2150 TW $year^{-1}$ (actual consumption being 13 TW $year^{-1}$). On the other hand, global energy consumption is predicted to face a 3-fold increase by 2100 (Hoffert et al., 1998). Most of all, the impact of CO_2 emissions on sea levels, global climate patterns, and on many living organisms has been indicated as particularly severe if overpassing the atmospheric level of 450 ppm (O'Neill and Oppenheimer 2002; Thomas et al., 2004), which is not that far away.

One of the most obvious ideas to solve or mitigate these problems would be to use sunlight energy, which overwhelm the Earth with renewable energy at a rate of 178,000 TW $year^{-1}$ (more than 13,500 times the actual energy demand). In this respect, hydrogen (H_2) has been considered as one of the most suitable energy vectors for sunlight energy (Bockris, 1976; Benemann, 1996; Abraham, 2002), its reliability being discussed also with respect to its generation, storage and transport (Cammack et al., 2001; Hoffmann, 2002; Sperling, 2004). Its combustion coupled with O_2 produces only heat and water, and generates electricity if the reaction is conducted in a fuel cell. However, its viability is completely dependent on production processes, as it is not naturally found in the environment.

The capacity of certain microorganisms to metabolize molecular H_2 was first noted at the end of the 19^{th} century (Hoppe-Seyler, 1887). To date, a wide variety of microbial processes are known to be able to generate H_2 gas (Figure 1), many of which using light as the energy source, an electron-donating substrate, and a catalyst that combines electrons and protons. Light energy allows photosynthetic organisms to extract electrons from high-redox-potential compounds such as water

(in oxygenic photosynthesis) or organic acids (in non-oxygenic photosynthesis), yielding energy (in the form of ATP) and low-redox-potential reductants that can be utilized as substrates for H_2 production by either hydrogenases or nitrogenases (Fig. 1).

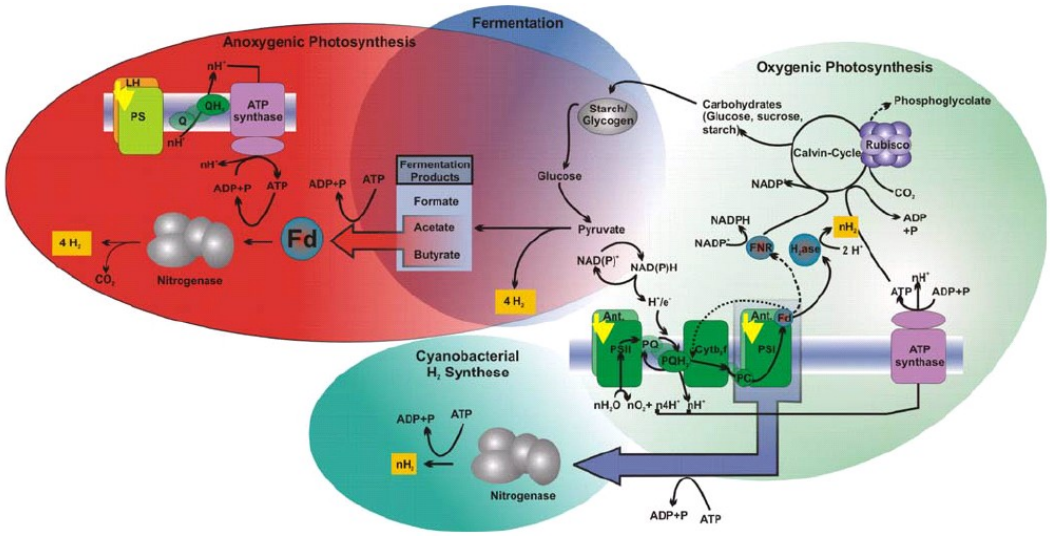


Figure 1. Hydrogen production pathways in nature are found in green algae, cyanobacteria and bacteria (found in Rupprecht et al., 2006)

First claims of solar-driven biophotolytic systems for H_2 production from water were given in the early '70 (Hollaender et al., 1972; Gibbs et al., 1973). The idea was based on the discovery of Gaffron (1939; 1940; 1942) and his coworker Rubin (1942) of the reversible activity of a hydrogenase in some green algae, which were able to photoproduce as well as uptake molecular H_2. This fundamental discovery was important evidence in support of van Niel's general scheme relating plant photosynthesis and more primitive bacterial photosynthesis:

$$CO_2 + 2H_2A \rightarrow [CH_2O] + 2A + H_2O$$

where $[CH_2O]$ represents carbohydrate, H_2A is an oxidizable molecule (e.g. an organic acid or water) and A is the oxidized product. Particularly, Gaffron's research demonstrated that $2H_2$ could substitute for $2H_2A$ in some algae (Boichenko et al., 2004).

The key difference between oxygenic and anoxygenic photosynthesis is that the former can use water as the source of reductant (electrons) for carbon reduction (or H_2 evolution), whereas the latter cannot. This is an important distinction since the simultaneous photoevolution of O_2 and fixation of carbon dioxide (or H_2 evolution) using water as the substrate is an energetically uphill reaction, whereas the dehydrogenation of energy-rich organic substrates by bacteria is not (Boichenko et al., 2004).

Hydrogen production in oxygenic photosynthetic microorganisms

Green algae and cyanobacteria are photoautotrophic organisms: they can grow under sunlight and CO_2, without organic sources of carbon. They perform photosynthesis, which converts light energy into chemical energy according to the Z-scheme (Figure 2).

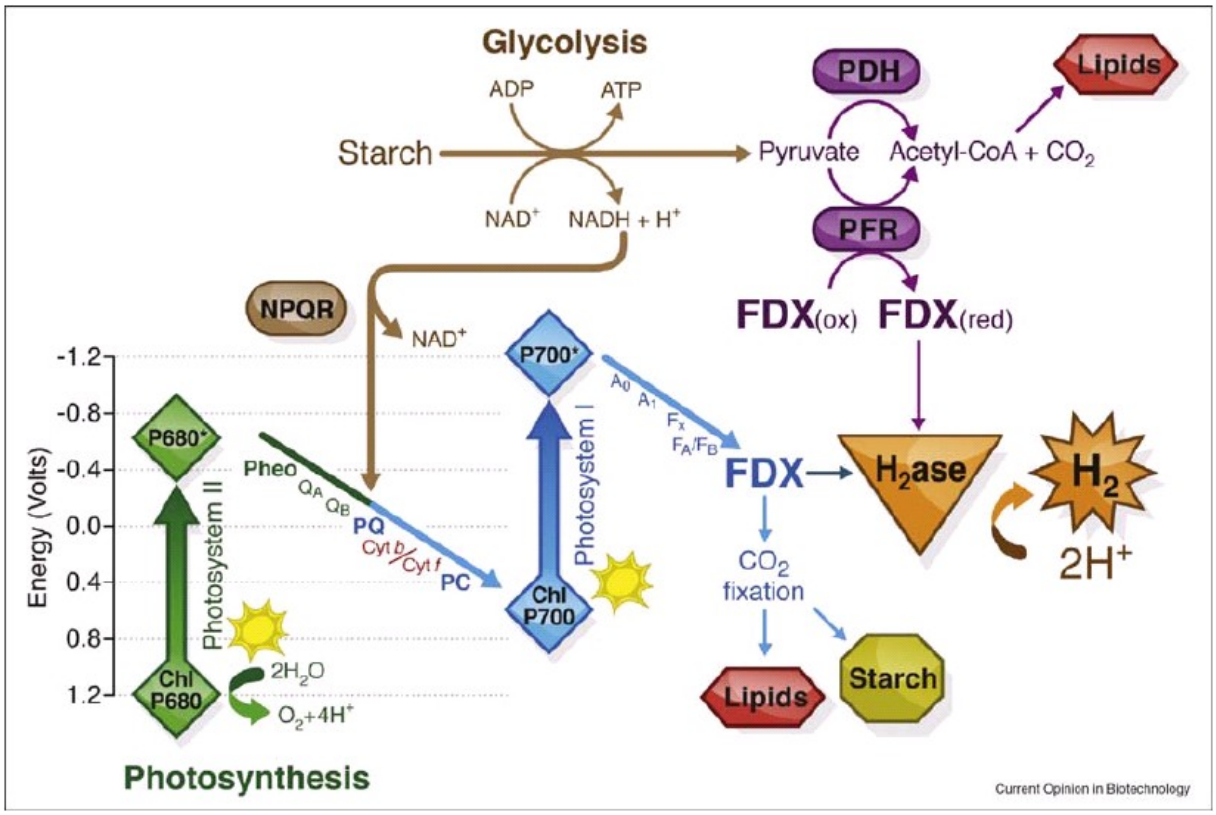


Figure 2. Photosynthetic and glycolytic pathways in green algae related to biofuel and biohydrogen production (found in Beer et al., 2009)

This process, which takes place in the thylakoid membranes, starts with light absorption by pigment molecules (chlorophylls, phycobilins, and carotenoids) bound to light-harvesting protein complexes associated with two multi-membrane protein complexes, Photosystem I (PSI) and Photosystem II (PSII). The absorbed light energy is then transferred to the reaction center pigments of PSII and PSI where charge separation occurs. These two photosystems cooperate to span the potential difference between water oxidation and H^+ reduction. This normally results in the photoevolution of molecular O_2 (PSII) and the reduction of CO_2 (PSI) by electrons from reduced ferredoxin (FDX) and NADPH in the Calvin cycle, which leads to the biosynthesis of organic compounds. The distribution of reductants for the synthesis of proteins, nucleic acids, carbohydrates, and/or lipids is achieved by an integration of metabolic pathways coordinated through complex mechanisms that regulate photosynthetic output.

This is true also for H_2 evolution, which historically has been observed (in the light) only on a transient basis, lasting from several seconds to a few minutes. As a matter of fact, photosynthetically generated O_2, which is concurrently produced upon H_2O-oxidation in the course of illumination, acts as a powerful inhibitor of the hydrogenase enzyme (Ghirardi et al. 1997; 2000b) and a positive suppressor of *HydA* gene expression (Florin et al. 2001; Happe and Kaminski 2002; Forestier et al. 2003). This incompatibility in the simultaneous O_2 and H_2 photoevolution has impeded efforts to accurately investigate the underlying biochemistry and to further develop and enhance the process. Nevertheless, the ability of green algae to operate in two distinctly different environments (aerobic and anaerobic), and to photosynthetically generate molecular H_2, has captivated the fascination and interest of the scientific community because of the fundamental and practical importance of the process (Melis, 2007).

Hydrogen production with *Chlamydomonas reinhardtii*

Under anaerobic conditions, *Chlamydomonas reinhardtii* is able to release H_2 in the light as a result of the enzymatic activity of a chloroplast [FeFe]-hydrogenase, which catalyzes the reversible reduction of protons to H_2, using FDX as an electron donor. Anaerobiosis is realized by down-regulating the PSII activity by means of sulfur starvation (Melis et al., 2000). The PSII activity is further reduced by the effect of the transition of the photosynthetic apparatus from state 1 to state 2, which takes place as soon as the culture is exposed to the anaerobic conditions (Antal et al., 2001; 2003). This phenomenon is clearly evidenced by a sudden drop of the effective quantum yield of PSII (Antal et al., 2001; 2003).

The electron sources for the H_2 production achieved in these conditions is a result mainly of two pathways, the so-called direct and indirect contribution of the PSII. The former is represented by the electrons driven through the electron transport chain to the [FeFe]-hydrogenase thanks to the remaining PSII activity after the transition of the photosynthetic apparatus from state 1 to state 2. This pathway thus concerns electrons coming directly from the water splitting activity of the PSII. On the contrary, the indirect PSII contribution is represented by endogenous substrates (mainly starch) which are stored during the initial aerobic phase after sulfur starvation (Melis et al., 2000; Tsygankov et al., 2002; Zhang et al., 2002) and are subsequently fermented, entering the electron transport chain at the level of the PQ pool (for a Review, see Posewitz et al., 2009). Finally, after several days of starvation, H_2 production ceases as soon as endogenous substrates are completely consumed and/or PSII activity is definitely brought down.

Notwithstanding this simplified scheme, since Melis discover of the surprising effects of sulfur

deprivation on the metabolism of this microalga, the exact mechanisms and relative relevance of the [FeFe]-hydrogenase electron feeding pathways were a matter of debate. According to the different culture conditions, PSII direct contribution was found to vary from 0 to 85% (Ghirardi et al., 2000a, Antal et al., 2001; 2003; Kosourov et al., 2003, Lee and Greenbaum, 2003; Fouchard et al., 2005; Kruse et al., 2005; Hemschemeier et al., 2008b). Indeed, recent findings by Chochois and coworkers (2009) as well as by Antal and coworkers (2009) revealed by different inhibitors addition that starch fermentation (PSII indirect contribution) may be minority, even as concerns the induction of the hydrogenase enzyme itself (Chochois et al., 2009). Rather than utilizing the light-driven reduction of the FDX (Fig. 2), a dark fermentative pathway may also be involved through a pyruvate ferredoxin/flavodoxin oxidoreductase (PFO) enzyme. Although PFO-catalyzed pyruvate oxidation/FDX reduction in the *Chlamydomonas reinhardtii* fermentative H_2-production pathway is not absolutely proven under dark H_2-producing conditions, a *Pfr* gene is up-regulated in *C. reinhardtii* (Mus et al., 2007). This pathway of H_2 generation may be significantly less efficient than light-driven processes. However, a complete understanding of the anaerobic sulfur deprived metabolism of this microalga should not neglect any of the known pathways, as it appears evident that H_2 production in *Chlamydomonas* is an elaborate interplay of electron sources and sinks (Hemschemeier et al., 2008b).

Several molecular approaches were proposed to enhance H_2 productivity and overcome the main barriers that were found limiting the process. One of the most important is the developtment of an O_2 tolerant hydrogenase. This solution would permit the fully exploitation of the photosynthetic apparatus for H_2 production purposes. Depending on the physiological conditions adopted for the H_2 production, competition for photosynthetically-generated reductant among the pathways that depend on reduced FDX may also become an important issue. In fact, reduced FDX provides reductants to nitrite reductase, sulfite reductase, glutamate synthase, thioredoxin, and others (Ghirardi et al., 2009a). Another problem is represented by the fact that anaerobic cultures use very little ATP. As a consequence, the proton gradient generated by photosynthetic electron transport is not dissipated properly, and this causes a decrease in the rate of electron transport (Antal et al., 2001; 2003; Lee and Greenbaum, 2003; Johnson and Melis, 2004), which ends up reducing also electron feeding to the FDX for H_2 evolution.

A severe reduction of the photosynthesis yields under sulfur starvation is due to state transition of the photosynthetic apparatus (Antal et al., 2001; 2003), triggered by PQ pool reduction (Horton and Black, 1980; Allen et al., 1981) and by Rubisco inactivation (Ghirardi et al., 2009a). The relevance of this limiting mechanism has been recently demonstrated by mutants blocked in state 1 which had

13 times higher productivities respect to wild types (Kruse et al., 2005). Finally, strains with a reduced antenna complex would allow greater transmittance of irradiance through ultrahigh cell density cultures without significant dissipation of light energy and, as a result, would exhibit higher photosynthetic productivity. Higher biomass concentrations would be reached without incurring in the so-called low light acclimation effect that leads to an increased amount of pigment antenna per cell. This represents a strong limitation in the microalgae mass cultures where high cell density is desirable.

Aside from engineered algae, a positive support may also be provided by optimized photobioreactors (PBRs). It is widely known that stirring represents the most practical mean by which efficient light utilization in photosynthetic cultures can be achieved (Richmond, 1990). Light dilution imposed on properly stirred cultures may prevent saturating (or inhibiting) intensities experienced by the cells. As a result, cells would be exposed to regular light/dark (L/D) cycles, which for high light intensities are translated into short flashes separated by long dark periods. If the frequency and intensity of the flashes, their relative duration and their subsequent dark periods have appropriate values, the organisms can then carry on photosynthesis at nearly the same rate in flashing light as they do in continuous light of the same average intensity (for a Review, see Fredrickson and Tsuchiya, 1970; Rabinowitch, 1956). This problem is not new, as one of the major biotechnological challenge in outdoor massive cultivation in PBRs has historically always been solar light dilution to lower light intensities, in order to reproduce productivity as found in the laboratory controlled conditions.

The role played by the D1 protein respect to photosynthesis and hydrogen production

It is known that the sulfur starvation protocol proposed by Melis and co-workers (2000) acts on a number of metabolic pathways of *Chlamydomonas*. In particular, the lack of inorganic sulfur in the medium affects the activity of a key protein of the PSII (the D1 protein), thus down-regulating photosynthesis to the level of respiration and leading to the anaerobiosis in the light in sealed cultures. This protein of the PSII reaction center turns over in a light-dependent manner more rapidly than any other chloroplast protein and is the primary target for photoinhibitory damage. The D-E loop of this protein, the amino acid sequence of which is greatly conserved among cyanobacteria, algae and higher plants, is involved in binding both Q_b, the second stable quinone acceptor in PSII, and several classes of herbicides that inhibit photosynthetic electron transport at the Q_b docking site (Bowyer et al., 1991; Gokhale and Sayre, 2009). Only a few of the many amino acid substitutions made in this region result in the loss of D1 function photosynthetic capability (Nir

and Hirschberg, 1992; Lardans et al., 1998) suggesting that most positions can tolerate considerable variations in residue group conformation or charge, and still permit D1 function. In support of this hypothesis, recent progresses both in chloroplast engineering and in crystal structure analysis increased manipulative possibilities as well as the knowledge of structure-function relationships in PSII so much, that in PSII-based biosensors, by applying a molecular "Lego" approach (Perham, 1994; Gilardi and Fantuzzi, 2001), small protein modules with the desired properties can be fused with the D1 subunit of PSII without compromising its function (Johanningmeier et al., 2005). Nonetheless, particular residues may be necessary for optimal PSII activity or may provide functional advantages under certain environmental conditions (Etienne and Kirilovsky, 1993). For example, the extended D-E loop is involved in the rapid turnover of the D1 protein: particularly, it has been shown that a primary cleavage site exists between amino acid residues 234 and 242 (Kettunen et al. 1996) and that mutations in this region can affect the functional properties of PSII (Kless et al., 1994; Mäenpää et al. 1995).

Other than the D-E loop, the D1 protein is known to bind the majority of the cofactors involved in the PSII mediated electron transport (like chlorophylls, phaeophytins, carotenoids and the metal ions manganese and iron [Johanningmeier et al., 2005]). For instance, most of the amino acid residues between S155 and D170 (Xiong et al., 1998) may be crucial in mediating the electron transfer from the D1-Y161 (or donor Z) to $P680^{+}$ (Xiong et al., 1996) at the level of the oxygen evolving complex and some of them (e.g., D170) have been already discovered to be crucial for the binding of the manganese cluster (Nixon and Diner, 1992; Whitelegge et al., 1995; Chu et al., 1995; Xiong et al, 1998).

Mutations which deal with the possibility to improve photosynthetic capacity of a biotechnologically significant microorganism are of great interest for any process aimed to an economical sustainability. As concern the H_2 production in *Chlamydomonas*, it was already stated that mutations involving PSII may positively influence the process by (i) leading to anaerobic conditions more rapidly upon sulfur deprivation (Makarova et al., 2007), (ii) improving starch storage capacity (Posewitz et al., 2004) and sustaining for a long period the direct remaining PSII contribution (Torzillo et al., 2009), often referred to as "biophotolysis".

AIM OF THE WORK

Several *Chlamydomonas reinhardtii* D1 protein mutant strains are considered for H_2 production purposes. Mutations regard regions which involve Q_b interaction, oxygen evolving complex (OEC) interaction and D1 degradation, and were induced by means of random and site-directed mutagenesis, as well as by peptide insertion. Colonies were selected when showing significant deviations from the WT fluorescence characteristics. The rationale was to individuate strains which had different photosynthetic activities, in order to test their effects on H_2 production. Recent investigations on *C. reinhardtii* D1 protein mutants for H_2 evolution purposes (Makarova et al., 2007) support the interest for this research. In particular, much attention has to be addressed towards both high and non-producing strains, which could differently support our aim to deepen the understanding of the physiology of the process.

On the other hand, the potential H_2 production capacity of microalgae is considered with respect to the optimal photobioreactor, able to maximize photosynthetic activity and give indications on the other relevant pathways which concomitantly contribute to the sustainability of the process. Results from the physiological studies will be gathered with those concerning the reactor to address H_2 productivity to optimal levels. High light intensities under these conditions will be adopted also to test the feasibility of H_2 evolution under more stressing conditions. Indeed, this step is intended to give further suggestions in order to successfully carry out in a horizontal outdoor tubular 50-liter photobioreactor a H_2 production under direct sunlight radiation, which until now has never been reported in literature yet.

MATERIALS AND METHODS

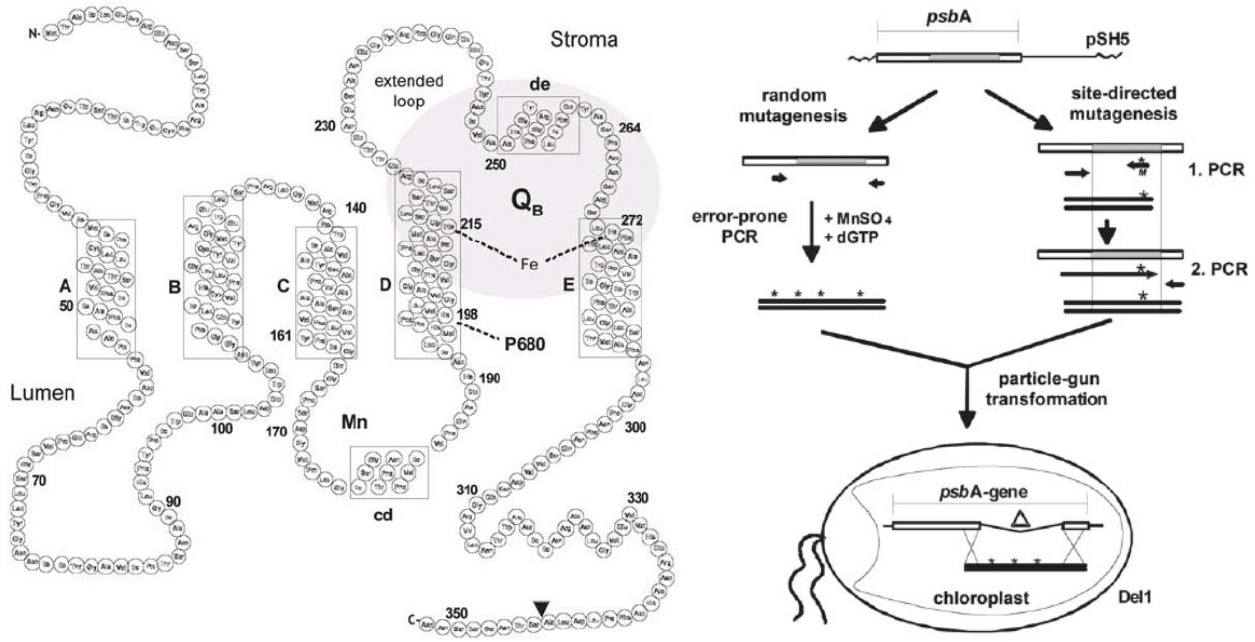
Section 1. Strains and growth conditions

1.1 D1 protein mutant mutagenesis procedures

C. reinhardtii D1 protein mutant strains adopted in this work were kindly provided by Prof. Udo Johanningmeier (Institut für Pflanzenphysiologie, Martin-Luther Universität, Halle, Germany). Concerning the other wild types, we are grateful to Dr. Micheal Seibert (NREL, Golden, Colorado) for providing us with the strain CC124 and with Prof. Roberto Bassi (University of Verona, Verona, Italy) for the WT (cw15).

All D1 protein mutants were obtained after WT (11-32b) genetic manipulation. First, an intron-less mutant (IL) was built by removing 4 large introns in *psbA* gene encoding for D1 protein (Johanningmeier and Heiss, 1993). Such a removal became necessary since *psbA* gene manipulation is a tedious task including the work with large DNA constructs and subtle selection procedures involving herbicide or antibiotic resistance markers (Erickson et al., 1984; Przibilla et al., 1991; Roffey et al., 1991; Schrader and Johanningmeier, 1992; Heiss and Johanningmeier, 1992). A Del1 mutant strain was then obtained by deleting a region of the *psbA* intron-less gene encoding from Ala 153 to Ala 294 (Preiss et al., 2001). This mutant was unable to grow photoautotrophically, but grew normally on media containing acetate as a carbon source. Its gene product was a truncated D1 protein which did not accumulate due to its rapid, ATP-dependent proteolysis (Preiss et al., 2001). Upon transformation of the deletion mutant with a plasmid carrying an intact *psbA* gene, photosynthetic growth was restored. The Del1 mutant strain was used as a recipient for all mutated fragments obtained by: (1) random mutagenesis, (2) site-direct mutagenesis and (3) peptide insertion.

Concerning the first group, the Del1 mutant strain was bombarded with mutated PCR fragments that were randomly generated. These mutated fragments were obtained, by means of amplification, from the plasmid pSH5, which contains the complete intron less *psbA* gene and 3'-flanking regions (Preiss et al., 2001), using an error-prone PCR in the presence of $MnSO_4$ and dGTP. The transformation method restored the full *psbA* intron-less gene by means of a homologous recombination of the PCR fragments with the *psbA* gene deleted region (Figure 1).



Figures 01. Secondary structure profile of the *C. reinhardtii* D1 protein (left) and PCR-based mutagenesis procedures for the introduction of random and site-directed mutations into the recipient strain Del1 (right). ***(Left)*** Transmembrane helices A-D and parallel helices cd and de are shown together with locations for the primary donor P680, the nonheme iron, Fe, the manganese, Mn, and the secondary plastoquinone Q_B. The shaded area roughly outlines the binding niche for Q_B and some herbicides. ***(Right)*** For random mutagenesis an error-prone PCR in the presence of $MnSO_4$ and dGTP was used. For site-directed mutagenesis a mutagenic primer M was used. Template for PCR was the intron less *psbA* gene in vector pSHc5 (Johanningmeier et al., 2000). PCR fragments were precipitated directly onto tungsten particles and introduced by particle gun transformation without further cloning or purification steps (Dauvillee et al., 2004). Homologous recombination in the recipient cell Del1 is indicated by crosses. Stars indicate point mutations. The shaded area and Δ indicate the deleted sequence in the Del1 mutant (found in Johanningmeier et al., 2005).

To generate pools of PCR fragments under error-prone reaction conditions (but controlled mutation frequencies) commercially available kits were used. In order to largely avoid transformants with WT *psbA* genes, algal colonies growing under photoautotrophic conditions were initially screened directly on plates for their fluorescence characteristics with the help of an imaging fluorometer (Johanningmeier et al., 2005). Colonies with significant deviations from the WT fluorescence characteristics were further analyzed by sequencing that part of the *psbA* gene which could have been modified by the incoming PCR fragment. Unlike other procedures, the selection for photosynthetic growth represents a very robust method that can be easily extended to also screen for herbicide-, temperature or radiation-tolerance (Johanningmeier et al., 2005).

In the second group of mutants (obtained from site-directed mutagenesis), a mutagenic primer M was used. Amino acid residues were selected according to their sensitivity to the most commercial herbicides, known to inhibit photosynthesis by displacing Q_B from its binding site in D1 and thus blocking electron transport from Q_A to Q_B (Velthuys, 1981). All algal colonies were able to grow

photoautotrophically. Substitutions were confirmed by DNA sequencing.

In the third group (peptide insertion), a small metal-binding domain was inserted into the extended D-de loop structure. The only strain created using this technique was HIS10, in which 10 histidines were inserted between Gly236 and Tyr237. The rationale behind this was that such domains would undergo conformational changes upon metal binding and that binding-induced changes at one (allosteric) site can be propagated over considerable distances (Yu and Koshland, 2001). Although it is not yet possible to reliably predict the transmission of conformational changes from an allosteric site through the protein structure (Mizoue and Chazin, 2002), it appeared possible that the structural effect upon metal binding would extend to the nearby Q_B binding niche and change PSII fluorescence (Johanningmeier et al., 2005). However, it was also demonstrated that a primary cleavage site exist between amino acid residues 234 and 242 (Kettunen et al. 1996), thus such an insertion (between Gly236 and Tyr237) could have an effect also on D1 degradation process. Insertion was made using appropriate oligonucleotides cloned into a single BstEII site in the intron-less *psbA* gene and was verified by DNA sequencing. The strain HIS10 was able to grow photoautotrophically. Table 1 shows a list of the all the tested D1 protein mutants.

Table 1. List of all the *Chlamydomonas reinhardtii* strains adopted in this work. The table shows the mutagenesis procedure and the region of interest of the D1 protein for each of the tested strain. Keys: *, in the His10 strain, 10 histidines were inserted between the amino acid residues Gly236 and Tyr237.

Strain	Mutagenesis procedure		D1 region
L159I-N230Y	Random	Substitution	Oxygen evolving complex interaction
V160A	Random	Substitution	Oxygen evolving complex interaction
I163N	Random	Substitution	Oxygen evolving complex interaction
D170E-G178S	Random	Substitution	Oxygen evolving complex interaction
D240	Random	Amino acid deletion	D1 degradation
D240-41	Random	Amino acid deletion	D1 degradation
D239-40	Random	Amino acid deletion	D1 degradation
HIS10*	Random	Insertion	Q_b interaction-D1 degradation
V185I-I283M-L288V	Random	Substitution	Q_b interaction
A251C	Site-directed	Substitution	Q_b interaction
V219M	Site-directed	Substitution	Q_b interaction
A250L	Site-directed	Substitution	Q_b interaction
S264K	Site-directed	Substitution	Q_b interaction
A251G	Site-directed	Substitution	Q_b interaction
S264C	Site-directed	Substitution	Q_b interaction
N230S-G289D	Random	Substitution	Q_b interaction
A250S	Site-directed	Substitution	Q_b interaction
A250I	Site-directed	Substitution	Q_b interaction
WT (cw15)	Wild type	-	-
CC124	Wild type	-	-
WT (11-32b)	Wild type	-	-
IL	-	Introns deletion	-

Mutations were indicated as follows: for example, strain A251C reported a substitution of the amino acid residue in position 251, whereas an alanine (A) was substituted with a cysteine (C). In Table 2 a list of the three and single letter code for each amino acid is reported.

Amino acid	Three letter code	Single letter code
glycine	Gly	G
alanine	Ala	A
valine	Val	V
leucine	Leu	L
isoleucine	Ile	I
methionine	Met	M
phenylalanine	Phe	F
tryptophan	Trp	W
proline	Pro	P
serine	Ser	S
threonine	Thr	T
cysteine	Cys	C
tyrosine	Tyr	Y
asparagine	Asn	N
glutamine	Gln	Q
aspartic acid	Asp	D
glutamic acid	Glu	E
lysine	Lys	K
arginine	Arg	R
histidine	His	H

Table 2. List of the 20 amino acids and their abbreviations. Amino acids from Gly to Pro are considered non polar amino acid (hydrophobic); from Ser to Gln, amino acid are within the hydrophilic group. Asp and Glu are electrically charged (negative) while from Lys to His, amino acid are positively charged (Source: http://www.bio.davidson.edu/Biology/aatable.html, as found 2010-01-20).

1.2 Growth conditions

D1 protein mutant strain collection was maintained photoheterotrophically on agar plates and tubes, provided with low light intensity (about 20 µmol photons m^{-2} s^{-1}) by means of cool white lamps (Dulux L, 55W/840, Osram, Italy), on Tris-Acetate-Phosphate (TAP) medium (Harris, 1989), pH 7.2 ± 0.1, temperature 20 ± 1 °C (Figure 2). Incident light was measured with a flat quantum radio-photometer (LI-250A, LI-COR).

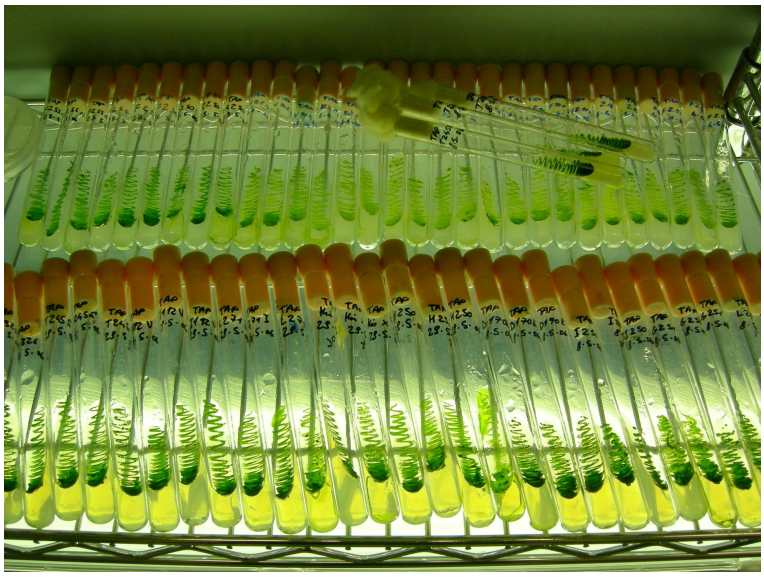


Figure 2. *Chlamydomonas reinhardtii* strains maintained on TAP agar medium.

Afterwards, cultures were moved to liquid suspension in TAP medium (Harris, 1989), pH 7.2 ± 0.1, in 50 mL flasks, and incubated at 28 ± 0.5 °C under constant shaking. Light intensity ranged from 30 to 50 µmol photons m^{-2} s^{-1} (Figure 3).

Figure 3. *Chlamydomonas reinhardtii* strains incubated under low light intensity in liquid suspensions. (Picture courtesy of Dr. Luca Giannelli).

1.2.1 Photomixotrophic growth

Cultures were then transferred in 400 mL cylindrical air-lift reactors (5-cm i.d.) to experience a photomixotrophic growth. Reactors were irradiated on both sides with 70 µmol photons m^{-2} s^{-1} and mixed bubbling a mixture of air and CO_2 (v/v, 98.5/1.5). Temperature was set to 28 ± 0.5 °C by immersing column reactors in a water bath filled with thermo-stated deionized water. To increase biomass volume, cultures were finally transferred to 1-liter Roux-type bottles (800 mL working volume, 5 cm light path) at the same conditions here described (Figure 4).

Figure 4. *Chlamydomonas reinhardtii* cultures growing in air-lift reactors and flat Roux-bottle type reactors during growth. Picture on the left courtesy of Dr. Luca Giannelli.

1.2.2 Photoheterotrophic growth

Photoheterotrophic growth was adopted during specific set of experiments (Prof. Thomas Happe is gratefully acknowledged for his technical support during these experiments): (1) anaerobic induction experiments (Section 3a.3, Results and Discussion); (2) H_2 production in sulfur depleted cultures with Suba sealed PBRs (Section 3a.4, Results and Discussion); (3) experiment devoted to fermentative pathways analysis (Section 3a.5, Results and Discussion); H_2 oxidation (Appendix).

In these cases, cultures were initially maintained on agar plates with TAP medium (Harris, 1989), initial pH 7.2, at 20 °C. Afterwards, they were moved to liquid suspensions (TAP medium) and irradiated with a light intensity that varied between 50 and 100 μmol photons m^{-2} s^{-1}, at the same conditions temperature and pH value, and maintained under constant shaking. Cultures were then collected and treated as described in the text for each experiment.

1.3 Hydrogen production experiments in standard conditions

H_2 production experiments were initially carried out in culture conditions that were similar to what generally adopted in literature by other authors (Melis et al., 2000; Ghirardi et al., 2000a; Cournac et al., 2002; Zhang et al., 2002; Kosourov et al., 2002; 2003; 2005; 2007; Tsygankov et al., 2002; 2006) and are indicated in this work as "standard conditions". The latter concerned H_2 production experiments performed in 1.1-liter photobioreactors (PBRs), Roux-bottle type (5 cm light path), with an illuminated area of 0.02235 m^2 (on each side of the PBR). Cultures grown photomixotrophically as described in Section 1.2.1 (Materials and Methods) were collected in the exponential phase of growth and washed in TAP-S up to 5 times (Melis et al., 2000). Afterwards, they were resuspended in TAP-S, pH 7.2 ± 0.1, to an initial chlorophyll (chl) concentration of 12 mg L^{-1}. Light was provided by both sides and was equal to 70 μmol photons m^{-2} s^{-1}; temperature was maintained at 28 ± 0.5 °C by immersing the PBRs in a water bath filled with thermo-stated deionized water (Figure 5). Mixing was allowed by a magnetic stir bar placed at the bottom of the PBR.

Figure 5. Hydrogen production experiment with the screening system set up (left) and particular of the Roux-bottle type PBR during H_2 gas evolution (right). After saturating the liquid suspension, the H_2 gas is collected as foam and bubbles in the upper part of the PBR.

1.4 Anaerobic induction experiments

Cultures were grown photoheterotrophically as reported in Section 1.2.2 (Materials and Methods). After reaching about 20-25 mg chl L^{-1} (Hemschemeier et al., 2009), cultures were collected and concentrated up to 100-120 mg L^{-1} in complete TAP medium (initial pH 7.2), and placed in the dark in 50 mL Sarstedt (Sarstedt, Numbrecht, Germany) or Falcon tubes wrapped with aluminum foil. Dark incubated cultures were continuously flushed with inert gas (like N_2 or Ar) and maintained at room temperature (20-25 °C). At specific time points, aliquots of algal cells were taken and properly incubated to evaluate *in vitro* and *in vivo* H_2 productions.

1.5 Hydrogen production experiments in sealed photobioreactors

Cultures were grown photoheterotrophically as reported in Section 1.2.2 (Materials and Methods). At the mid-exponential phase of growth, they were collected and sulfur deprived by means of 3 centrifugations (2200 rpm for 3 min) in TAP-S. Aftwards, cultures were placed in 325 mL PBRs (gas phase equal to 210 mL, the remaining part [125 mL] being liquid phase), at room temperature (20-25 °C) and irradiated with about 60-70 µmol photons m^{-2} s^{-1} per side. Initial chl concentration was 12 mg L^{-1}, the light path being roughly 6 cm (o.d.). Contrary to what previously shown, in these experiments the produced biogas remained trapped inside the PBR headspace. This implies a different treatment before sealing cultures for H_2 production purposes. In fact, to get rid of the O_2 in the gas and liquid phase due to the initial air presence, sealed cultures were flushed with Ar for at least three minutes at the onset of the experiment. Contrary to Roux-bottle type PBR, the evaluation of the illuminated area of such PBRs would be meaningless. As a matter of fact, PBR head space

volume was so relevant that it can be assumed a three sided illumination, meaning that the upper liquid surface of the culture was significantly irradiated (Figure 6).

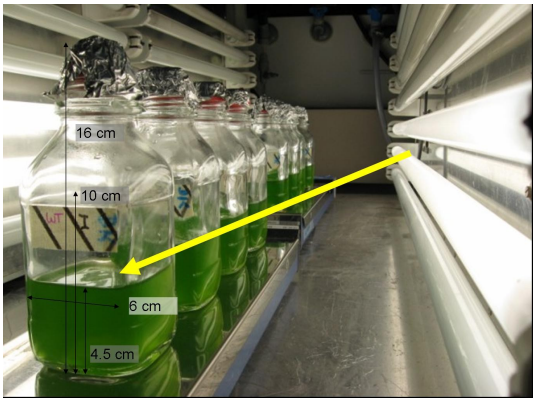


Figure 6. H_2 production experiments in sealed cultures.

1.6 Laboratory growth conditions for hydrogen production outdoor

Outdoor H_2 production experiments were initially carried out with laboratory grown cultures. First, cultures were grown as reported in Section 1.2.1 (Materials and Methods). Then, culture volume was increased by means of a massive growth conducted in 5 and 8 liters Pyrex bottles. Light was increased up to 1000 µmol photons m^{-2} s^{-1} supplied on both sides with cool white lamps (Dulux L, 55W/840, Osram, Italy). A mixture of air and CO_2 (v:v, 97:3) was supplied to the cultures by means of bubbling, thus allowing also culture mixing. Note that this concentration of CO_2 was increased respect to the previous part of the growth (1.5%). Temperature was set to 26 ± 2 °C. This set up is presented in Figure 7.

Figure 7. Massive cultivation of *Chlamydomonas reinhardtii* strains carried out in the laboratory for outdoor H_2 production experiment.

1.7 Outdoor growth conditions

In order to reach a sufficient amount of biomass, culture were initially grown as reported in Section

1.6 (Materials and Methods). Afterwards, they were moved outdoor in a horizontal tubular 50-liter PBR placed in the outside area of the Istituto per lo Studio degli Ecosistemi (ISE) of the National Council of Research (CNR) located in Sesto Fiorentino (Florence, Italy).

The initial concentration of about 5 mg L^{-1} was chosen to let the culture experience a full acclimation outdoor. A detailed description of this reactor is given in Section 2.4 (Materials and Methods).

As concerns the outdoor growth of the strain CC124, it was tested during the August of 2008. Direct total solar radiation during this period of the year and at this site (latitude 48.818° North, longitude 11.202° East) is particularly intense and may reach up to 2000 μmol photons m^{-2} s^{-1}. On the other hand, while testing outdoor growth of the D1 protein mutant L159I-N230Y (September-October of 2008) solar radiation was never higher than 1500 μmol photons m^{-2} s^{-1}. Cultures were subjected to a natural day/night cycles and were not irradiated during nights with artificial illumination.

Both strains were cultivated photomixotrophically in complete TAP medium (Harris, 1989). CO_2 gas was supplied in order to maintain pH between 7.2 and 7.5 during the growth, temperature was maintained at 28 ± 1 °C. Culture velocity was adjusted at about 0.20 m s^{-1}, which corresponded to a Reynolds number of 10,000.

1.8 Hydrogen production conditions outdoor with direct solar light

When testing H_2 production outdoor with laboratory grown cultures, the rationale proposed by Laurinavichene et al. (2002) was adopted. As the outdoor PBR volume was 50 liters, an inoculum of 20-25 liters grown as described in Section 1.6 (Materials and Methods) was diluted by means of addition of 25-30 liters of TAP-S. On the other hand, when acclimated cultures were tested for the H_2 production outdoor, 40 liters grown as described in Section 1.7 (Materials and Methods) were collected and repeatedly washed with TAP-S by means of centrifugation (up to 4 times).

In both cases, the initial pH was adjusted to pH 7.2 ± 0.1, and temperature was set to 28 ± 1 °C. Culture velocity was maintained to 0.20 m s^{-1}, which corresponded to a Reynolds number of 10,000. Solar radiation and chl concentration are reported specifically for each experiment.

During H_2 production experiments with outdoor acclimated cultures, artificial light was provided during nights (17:30 - 9:00) with a 1000 W lamp, which intensity was about 100 μmol photons m^{-2} s^{-1} at the reactor surface (incident light was measured with a flat quantum radio-photometer [LI-250A, LI-COR]). Artificial light was controlled automatically by a timer.

1.9 Hydrogen production experiment outdoor with continuous artificial light supply

The reliability of the outdoor PBR for H_2 production purposes with *Chlamydomonas* was evaluated by setting up specific experiments during which continuous artificial light was always provided to the culture throughout the entire experiment. Cultures grown as reported in Section 1.7 (Materials and Methods) were placed in the outdoor PBR to a final concentration of 12 ± 1 mg L^{-1}. Artificial light was continuously provided by a parabolic system (M2M Engineering, Naples, Italy) equipped with a set of 20 neons (Osram L 58 W/940, Luminux de Lux, cool white) 1.60 m long. The illumination was homogenous upon the all PBR surface. To prevent solar light during days, the PBR was entirely covered with a green pass filter. The initial pH was adjusted to pH 7.2 ± 0.1, and temperature was set to 28 ± 1 °C. Culture velocity was maintained to 0.20 m s^{-1}, which corresponded to a Reynolds number of 10,000.

Section 2. Photobioreactors and monitoring systems for hydrogen production purposes

2.1 Screening system

The experiments carried out during the screening system adopted a 1.1-liter PBRs, Roux-bottle type (5 cm light path), with an illuminated area of 0.02235 m^2 (on each side of the PBR). The headspace of the PBR (the volume above the culture level) was 40 mL (3.6% of the PBR volume), corresponding to a surface area of 0.012 m^2, (5.4% of the total illuminated area of the PBR). Culture stirring was provided by a stir bar placed at the bottom of the PBR (see also Section 2, Results and Discussion). H_2 production was evaluated by water displacement, using calibrated cylinders (Figure 8), as already done by other authors (Melis et al., 2000; Ghirardi et al., 2000b). Cylinders were maintained at room temperature 25 ± 1 °C. The PBRs were operated with a negative pressure, which facilitated both the O_2 and H_2 degassing of the cultures. The negative pressure in the collecting system was obtained using a vacuum pump connected to the calibrated cylinders. Unlike Melis et al. (2000), and similarly to Laurinavichene et al., 2004, PBRs were sealed from the beginning of the experiment, and connected to the calibrated cylinders filled up with water (Fig. 8).

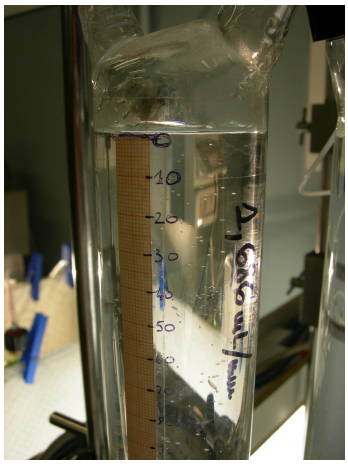


Figure 8. Particular of calibrated cylinders for H_2 gas collection with the screening system set up at the onset of the experiment

However, as long as biogas production was carried out, the negative pressure varied from -4.04 kPa to virtually zero, in the case biogas production could reach cylinders overall volume (700 mL).

2.2 Continuous monitoring system

The continuous monitoring system was developed according to Kosourov et al. (2002), and consisted of five elements: (1) a PBR equipped with four probes for the continuous monitoring of culture parameters, for example, culture temperature, pH, redox potential (estimated with a platinum/gold electrode), dissolved O_2 concentration; (2) a gas-to-liquid conversion bottle; (3) a liquid accumulating bottle; (4) a digital balance; (5) a lap-top computer for continuous data recording. An overview of the system is presented on Figure 9.

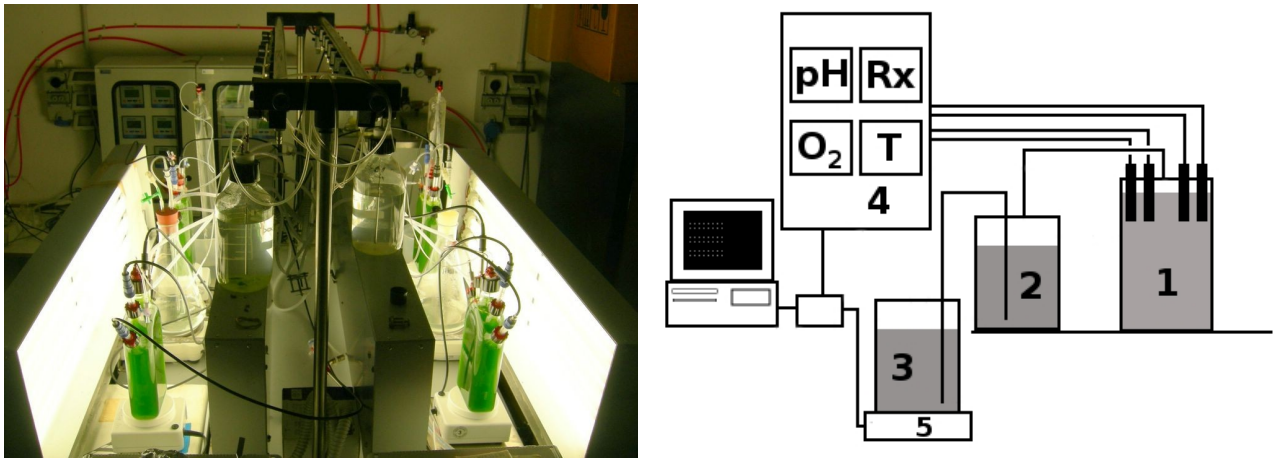


Figure 9. Continuous monitoring system: (*on the left*) Picture taken during 4 parallel hydrogen production experiments; (*on the right*) General scheme of the system (Kosourov et al., 2002, mod.). Keys: 1, photobioreactor; 2, gas-to-liquid conversion bottle; 3, liquid collecting bottle; 4, instruments interface; 5, digital balance.

The digital balance, monitoring the changes in the weight of the liquid accumulating bottle

(Acculab, ALC models, Sartorius Group, Goettingen, Germany), was connected to the lap-top computer via a RS232 to RS485/422 converter (Intelligent DA&C module ND 6520 UDAM, Chemitec, Florence, Italy). A software for automatic culture control and data acquisition was developed (Chemitec). Raw data recorded during the experiments were then processed using a Linux-based software written by us. As reported above, unlike Melis et al. (2000), PBRs were sealed from the beginning of the experiment in such a way that both the O_2 and H_2 production/consumption were continuously recorded by the system (Laurinavichene et al., 2004). The PBRs were operated at a gauge pressure of -4.04 kPa, which was constant throughout the entire experiment, unlike the screening system (Section 2.1, Materials and Methods). The negative pressure in the PBR was obtained as previously reported (Section 2.1, Materials and Methods).

The main differences respect to Kosourov and coworkers (2002), were (1) the establishment and maintainance of a slight negative pressure (-4.04 kPa) throughout the experiment, (2) the Linux-based software for data processing (which was written by us) and (3) the multiple impeller mixing device design and developed by us. The latter consisted of five turbines of the same diameter (25 mm) fitted on a vertical shaft (Figure 10).

Figure 10. Frontal view of the multiple impeller device inside the PBR.

At the bottom, a magnet holder was designed in order to contain the highest number of magnets achievable (represented by small cylinders of 2x2 mm). It must be noted that the multiple impeller device could be taken out from the PBR, to permit cleaning after each experiment. However this limited the diameter of turbines and holder itself to the Roux-bottle neck one (25 mm) (Figure 11, on the left). Magnets were disposed in five parallel lines, which faced perpendicularly formed a

couple of triangles (Fig. 11, in the middle). This conformation allowed us to maximize the effect of the magnetic fields spread by each pile of small magnets.

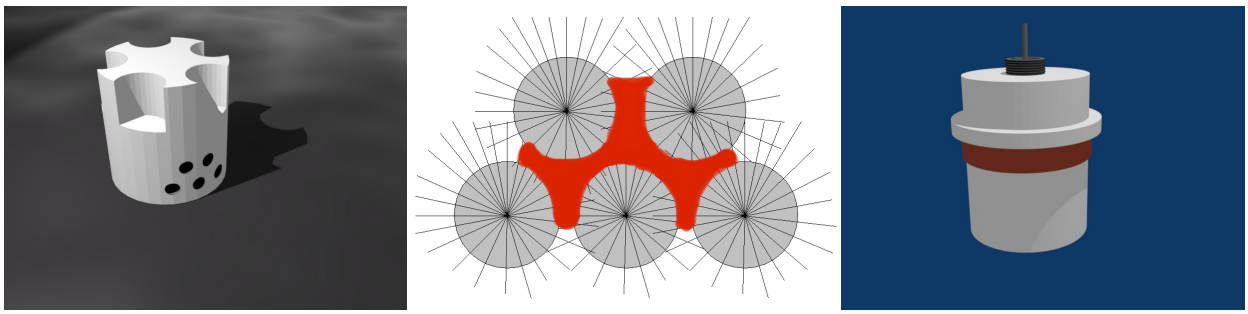


Figure 11. Magnet holder (*on the left*), final magnetic field determined by piled magnets (*in the middle*) and photobioreactor stopper (*on the right*). Images courtesy of Dr. Luca Giannelli.

Both the magnet holder and the stopper at the top of the PBR (Fig. 11, on the right) were made of polyethylene terephthalate (commercially known as PET). The stopper was studied to permit the insertion of the upper part of the vertical shaft. The final hydrodynamic flow imposed by this stirring system is presented in Figure 12.

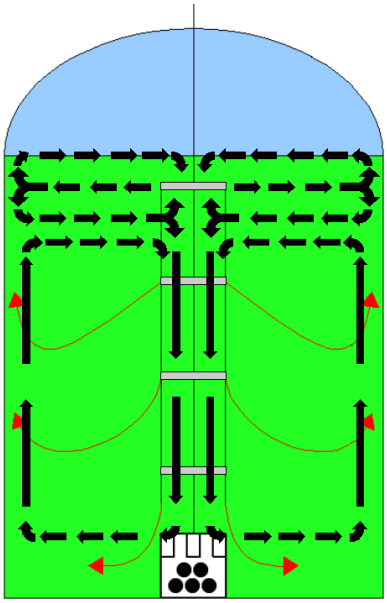


Figure 12. Fluid dynamics imposed by the multiple impeller stirring device in a flat Roux-bottle type photobioreactor. Image courtesy of Dr. Luca Giannelli.

A detailed discussion of such a mixing device in comparison with a stir-bar mixing system is described in Section 2 of Results and Discussion.

2.3 Outdoor PBR

The reactor was made of 10 parallel Pyrex glass tubes (length 2 m, i.d. 4.85 cm, 1 m^2 illuminated

area) connected by PVC U-bends with watertight flanges. The tubes were placed horizontally in a stainless-steel basin containing thermo-stated deionized water (Figure 13). The culture was recycled by means of a PVC pump that had three stainless steel flat blades at an angle of 120° to each other on the propeller shaft. The distance between the blades and the casing was 0.5 cm. The velocity of the culture can be adjusted to any required value from 0.20 to 0.80 m s^{-1}. At the end of the circuit (length 23 m), the culture flowed into a 2 liters transparent PVC cylindrical degasser. The degasser contained several hose-fittings for connecting the reactor to medium supply, and air or N_2 supply. The head space of the PBR, that is, the volume above the culture level, was about 0.2 liters (0.4% of the total volume).

Figure 13. General overview of the outdoor PBR (*clockwise*): Panoramic view of the outside area, with six parallel 50-liter horizontal tubular outdoor PBRs; Particular of the tubular outdoor PBR during hydrogen production experiments; Picture showing the overall PBR system; Particular of the PVC pump for culture mixing and of the degasser.

During outdoor experiments, the continuous monitoring system (Kosourov et al., 2002, mod.) was successfully adopted. In addition, remote control and monitoring of the culture (from the laboratory or from home) was made possible by the freely available software Tight VNC Viewer, which was

associated with an internet site created on purpose (http://algotrone.altervista.org/).

Section 3. Physiological analysis of algal cells

3.1 Chlorophyll fluorescence measurements

Chlorophyll fluorescence measurements of the cultures were carried out using pulse-amplitude-modulation fluorometer PAM-101-103 coupled with the emitter-detector unit ED-101US, (H. Walz, Effeltrich, Germany). An estimate of the overall linear photosynthetic electron transport rate (ETR) by PSII, i.e. the product of the effective photochemical yield of PSII and absorbed light (PFD x a*), ETR= PFD x $\Delta F/F'_m$ x a* x 0.5 (µmol e^- mg^{-1} chl *a* s^{-1}) (Krompamp and Forster, 2003), was measured using 1.5 mL cell samples (5 mg chl L^{-1}) by increasing the PFDs stepwise from 0 to 2000 µmol photons m^{-2} s^{-1}. The effective photochemical quantum yield of PSII [$\Delta F/F'_m = (F'_m - F_s) / F'_m$] of the cultures was measured using a portable pulse-amplitude-modulation fluorometer PAM-2100 (H. Walz, Effeltrich, Germany); a*, is the optical cross-section of the cells normalized to chl *a*. This equation assumes that the quantum yield of the electron transfer of trapped photons within a reaction center is 1 (Kolber and Falkowski, 1993) which means 50% of the absorbed light goes to PSII, and 50% to PSI in order to achieve a balanced pressure on both photosystems, and that no cyclic electron transport by PSI is occurring. Actinic light was provided by a halogen lamp (Shott, KL 1500 electronic, H. Walz) and adjusted by a set of neutral filters. Finally, in order to evaluate photosynthetic performances of the cultures, during growth and H_2 production experiments, $\Delta F/F'_m$ was evaluated by means of the optical fiber of the fluorometer, which was pointed directly onto the illuminated PBR surface.

3.2 Photosynthetic oxygen evolution and respiration measurements

Oxygen measurements were carried out at 28 °C in triplicate 2-mL culture samples, which concentration varied according to the experiment (see within the text), using a PC-controlled Oxylab electrode control unit (Hansatech Instruments, Norfolk, UK) connected to a closed thermo-stated chamber (model DW2/2, Hansatech Instruments) provided with a magnetic stirrer. The O_2 concentration dissolved in the culture was continuously monitored at an acquisition rate of 0.2 r s^{-1}. Data were analyzed with Oxylab-32 software. The light source consisted of 11 red LEDs centered on 650 nm (model LH11/2R, Hansatech Instruments). Light irradiance was measured with a spherical micro-quantum sensor US-SQS/B (H. Walz, Effeltrich, Germany) connected to a LI-250A

light meter (LI-COR Biosciences). Dark respiration rates were always measured after the measurement of photosynthesis rates. The total O_2 evolved by the cultures (gross photosynthetic rate) was calculated as the sum of the O_2 evolution in the light (net photosynthesis) and uptake in the dark (respiration). Light response curves (P/I) of photosynthetic O_2 evolution were generated by increasing incident irradiance stepwise from 0 to 640 µmol photons m^{-2} s^{-1}. Except when differently reported, maximum rates were generated at 800 µmol photons m^{-2} s^{-1}. The relative apparent quantum yield ($Ø_{rel}$) was estimated by means of linear regression from the initial slopes of the O_2 evolution curve plotted against the absorbed irradiance. The intercept of the initial slope of the photosynthesis-irradiance curve (α) and the maximum photosynthesis rate (P_{max}) can be defined as $I_k = P_{max}/\alpha$, and gives the saturation irradiance. Accordingly, I_h is defined as the intensity at which a photoinhibition is observed, I_s is the intensity at which P_{max} is reached, and I_c is defined as the compensation point (i.e., when net photosynthesis is zero). In vivo chlorophyll-specific optical absorption cross-sections (a*) of the cells were measured according to reference (Falkowsky and Raven, 1997).

3.3 Light attenuation measurements

Light attenuation at different culture depths was measured using a homemade flat black plastic chamber. Cool-white florescent light was supplied by one side. The transmitted light intensity was measured at different distances (2.5; 3.5; 5.0; 7.0; 10.5; 14.0 cm). Light attenuation measurements were performed using a 4π sensor connected to a quantum radio-photometer (LI-250A, LI-COR) and were carried out with *C. reinhardtii* cultures that were grown at three different chl concentrations (5; 12; 24 mg L^{-1}).

3.4 Dry weight and cell number determination

Dry weight was evaluated with triplicate samples of at least 5 mL (according to culture concentration), with nitrate cellulose filters (Sartorius, 0.45 µm) and incubated at 105 °C for at least 2 hours, until they reached a constant value. Cell counting was performed at least on 6 independent cultures by means of a Burker chamber.

3.5 Hydrogenase in vivo and in vitro activity

The reaction mixture of the in vitro hydrogenase activity assay for anaerobic induction experiments contained 1 mL 100 mM potassium phosphate buffer pH 6.8, 380 µL deionized water, 200 µL 10% Triton X-100, 20 µL 1 M methyl viologen, 200 µL anaerobic 1 M sodium dithionite and 200 µL

algal sample. The 8 mL vessels were incubated in a shaker filled with thermo-stated water at 37° C for a 20 minutes. When the same analysis was conducted during sulfur deprivation experiments, the mixture was composed as follows: 1 mL of 100 mM potassium buffer pH 6.8, 80 µL deionized water, 200 µL 10% Triton X-100, 20 µL of 1 M methylviologen, 200 µL anaerobic 1 M sodium dithionite and 500 µL algal culture. In this case, incubation at 37 °C lasted for 30 minutes.

For *in vivo* measurements, 1-mL or 2-mL culture samples (in anaerobic induction and sulfur deprivation experiments) were incubated in an anaerobic 8 mL vessel, and placed under constant shaking with an irradiation of 100 µmol photons m^{-2} s^{-1} for 1 hour, at 20°C.

3.6 Fermentative products measurements

Ethanol and formate were assayed with alcohol-dehydrogenase and formate-dehydrogenase, respectively (Boehringer, Mannheim / R-biopharm, Darmstadt, Germany), using a UV-method that take advantage of the absorbance peak of the NADH (334, 340 or 365 nm). The test kits were utilized following the instructions for the supplier.

3.7 Calculation of apparent light-conversion efficiency

Light-conversion efficiency was evaluated as the ratio between the energy content of the H_2 produced by the cultures and the amount of incident light supplied on both the sides of the PBR as follows:

Light conversion efficiency (%) = (H_2 production rate * H_2 energy content) / Incident light

It must be pointed out, however, that this ratio does not take into account the amount of acetate consumed by the culture, which may differently affect H_2 productivities.

Section 4. Biochemical and analytical procedures

4.1 Hydrogen gas measurements

Biogas volume was estimated as reported in Section 2.1 and 2.2 of Materials and Methods. As concerns biogas composition, it was analyzed with a gas chromatograph (model Clarus 500, Perkin Elmer), using a packed column (model Carbosieve S-II Spherical Carbon, Supelco). H_2 was determined by using nitrogen as a carrier gas, while O_2, N_2 and CO_2 were determined by using helium as a carrier gas. Calibrations of H_2, N_2, O_2 and CO_2 were performed by injecting known amounts of pure gas. Alternatively, when experiments were carried out at the Ruhr Universität

(Bochum) in the laboratory of Prof. Thomas Happe, gas chromatography was performed with a GC-2010, Shimadzu, (Kyoto, Japan) equipped with a PLOT fused silica coating molsieve column (5Å, 10 m by 0.32 mm) from Varian (Palo Alto, CA).

4.2 Chlorophylls and carotenoids determination

Chlorophyll (*a* and *b*) and total carotenoids concentration was determined spectrophotometrically according to Lichtenthaler (1987). Cells were centrifuged (3500 rpm, 5 minutes) and the pellet resuspended in 90% acetone solution. After a second centrifugation, the supernatant was analyzed at the following wavelengths: 450, 630, 645, 663, 750. Alternatively, when experiments were carried out at the Ruhr Universität (Bochum) in the laboratory of Prof. Thomas Happe, 1-mL samples were collected by means of centrifugation and resuspended in 100% acetone solution, then incubated for 5 minutes at 80°C. The supernatant was analyzed at a wavelength of 652 nm.

The concentrations of individual carotenoids were assessed with a reversed-phase Beckman System Gold HPLC (module 125 solvent) equipped with a diode array detector, model 168 Nouveau, according to Gilmore and Yamamoto (1991). This involved two columns placed in sequence, an ODS-1 (5 µm particle size, 250 mm x 4.6 mm i.d.) from Higgins and an ODS-1 (5 µm particle size, 250 mm x 4.6 mm i.d.) from Alltech.

4.3 Endogenous substrates determination

The carbohydrates content of the biomass was measured using the phenol-sulfuric acid method (Dubois et al., 1956) (three replicates), with D+ glucose as a standard. Protein was measured according to the Lowry method (Lowry et al., 1951) (three replicates), using Folin-Ciocalteu's reagent 2N and bovine serum albumin as a standard. For starch measurements, samples were treated with Lugol solution (3% KI and 3% I_2; Southgate, 1976) as described by Monma et al. (1991).

4.4 Western blotting analyses

From the physiologically active culture, a sample of 1 mL was spun down, the pellet was resuspended in 200 µL of sample buffer (5x buffer: 250 mM Tris/HCl, pH 8.0; 7.5% (w/v) SDS; 25% (v/v) glycerol; 0.25 mg mL bromphenol blue; 12.5% (v/v) ß-mercaptoethanole) and the lysate was heated for 5 min at 95°C. After centrifugation at top speed for 1 min, protein extracts were loaded onto denaturing gels. SDS-polyacrylamide-gelelectrophoresis (SDS-PAGE) was conducted as described before (Laemmli and Favre, 1973) using 10% separating and 5% collecting gels. After gel electrophoresis, proteins were transferred onto polyvinylidene difluoride membranes (PVDF) by

electroblotting with a fastblot apparatus, semi-dry, Biometra. Blocking and wash steps were performed in 1x PBS (phosphate buffered saline; 4 mM KH_2PO_4; 16 mM Na_2HPO_4; 115 mM NaCl) with 0.1% Tween 20 and, in case of blocking, 3% non fat skimmed milk powder (Biomol Feinchemikalien GmbH, Hamburg, Germany). The first antibodies, according to the different protein, were: polyclonal rabbit-anti-*C. reinhardtii* HydA1 1:5000; rabbit-anti *E. coli* AdhE 1:5000 (Kessler et al., 1991); rabbit-anti Rubisco (large subunit, 1:5000); rabbit-anti *A. thaliana* D1 1:20.000 (Park and Rodermel, 2004). Super Signal West chemiluminescent substrate from Pierce (Rockford, IL, USA) was used. Chemiluminescence was detected by the FluorChem 8800 apparatus from Alpha Innotech, San Leandro, CA, USA.

RESULTS AND DISCUSSION

Results and discussion are divided into 4 main sections, as follows:

- Section 1 concerns the initial screening of several mutant strains of the D1 protein according to their H_2 production and photosynthetic capabilities;
- Section 2 deals with the biotechnological optimization of a photobioreactor for photosynthetic process, with a particular attention for H_2 production;
- Section 3 is a wide study of the physiological features of selected D1 protein mutant strains, after which an optimization of H_2 production conditions is presented;
- Section 4 is dedicated to outdoor experiments carried out with a 50 liters horizontal tubular photobioreactor.

In each section, conclusions and future perspectives are also discussed.

SECTION 1.

Screening of D1 protein mutant strains for hydrogen production purposes

1.1 D1 protein mutant strains of *C. reinhardtii*

The photosynthetic capacity of several D1 mutants of *Chlamydomonas*, involving a DNA sequence encoding from the middle of helix C to the complete helix E (namely, from Ala 153 to Ala 294), was studied. Mutations regarded specific regions of the D1 protein which involve Q_b interaction, oxygen evolving complex (OEC) interaction and D1 degradation. The mutants here investigated were kindly provided by Prof. Johanningmeier (Institut für Pflanzenphysiologie, Martin-Luther Universität, Halle, Germany) and represent just a small part out of a wider collection of D1 protein mutants. Colonies were selected when showing significant deviations from the WT fluorescence characteristics. All the mutants were able to grow under photoautotrophic conditions.

The rationale of this study was that as H_2 production is connected directly or indirectly to photosynthesis (thus to the PSII), relevant insights would have been provided by studying the effects of such mutations on a protein like D1, which plays a key role in the PSII activity. The D1 protein is known to bind the majority of the cofactors involved in the PSII mediated electron transport, like chlorophylls (chl), phaeophytins, carotenoids (car), plastoquinones and the metal ions manganese and iron (Johanningmeier et al., 2005). Moreover, concerning the H_2 production in *Chlamydomonas*,

it was already stated that mutations involving PSII may positively influence the process by (i) leading to anaerobic conditions more rapidly upon sulfur deprivation (Makarova et al., 2007), (ii) improving starch storage capacity (Posewitz et al., 2004) and sustaining for a long period the direct remaining PSII contribution (Torzillo et al., 2009), often referred to as "biophotolysis".

1.2 Hydrogen production and photosynthetic activity screening

In a first wide screening, up to 22 *C. reinhardtii* strains were tested with respect to (1) H_2 production upon sulfur starvation, (2) quantum yield of PSII (in both growing and H_2-producing conditions) and (3) photosynthetic and respiratory capability. All strains were subjected to the same growing conditions (see Materials and Methods), which let them reach the late exponential phase of growth, when they were collected for sulfur depletion. For the H_2 production screening, culture conditions were chosen according to what already reported in literature (Melis et al., 2000; Ghirardi et al., 2000a; 2000b; Cournac et al., 2002; Zhang et al., 2002; Kosourov et al., 2002; 2003; 2005; 2007; Tsygankov et al., 2002; 2006) in order to have reliable and comparable data.

Figure 1. A panoramic view of the screening system adopted for the H_2 production under sulfur deprivation. Initial chl content was set at 12 mg L^{-1}, initial pH was 7.2. Cultures were irradiated with 70 µmol photons m^{-2} s^{-1} per side. Temperature was adjusted at 28°C by placing PBRs in a bath filled with deionized thermo-stated water. Mixing was allowed by a magnetic stir bar placed at the bottom of the PBR. Biogas production was evaluated by water displacement.

Thus, algal cells were subjected to an optimal dilution of light within the culture layers by providing a low light intensity to a low concentrated culture (i.e., with a low chl content), using a simple 1.1-

liter-Roux-bottle-type photobioreactor (PBR) (5 cm light path). The main difference respect to what usually adopted in literature was a double sided illumination to obtain a more homogeneous light distribution between the surface and the core of the reactor (Figure 1).

Results showed a wide range of productivity between zero and more than 500 mL H_2 L of culture^{-1}. This simple observation gives a hint of the relevance of the D1 protein role within the PSII, particularly when considering that most of these strains do not share just two or three amino acid on the *psbA* gene. Furthermore, contrary to what previously reported (Makarova et al., 2007), it points out that H_2 production can effectively be increased by means of mutations on this protein. A summary of all the 22 strains tested for H_2 production is presented in Table 1.

Table 1. Hydrogen production screening of D1 protein mutant strains upon sulfur deprivation. Strains are listed according to their productivity (H_2 total volume). Note that up to three controls (CC124, WT[11-32b] and WT[cw15]) were tested to compare productivities of the mutants. Results are the mean value of experiments made at least in triple on independent cultures. Keys: $^+$, H_2 maximal rates were sustained for at least 10 hours. The value of this column represents the time- after H_2 production start- at which rates strongly declined; *, strain S264C did not evolve H_2 for at least 10 hours at a stable value.

N.	Strain	Lag Phase (aerobic)	H_2 production phase (anaerobic)	H_2 total volume	H_2 production rates		Max H_2 production rates	
		Hours	Hours	ml H_2 L of culture^{-1}	ml H_2 L of culture^{-1} Hour^{-1}	ml H_2 g of chlorophyll^{-1} Hour^{-1}	ml H_2 L of culture^{-1} Hour^{-1}	Rates expressed until hour$^+$
1	L159I-N230Y	35	263	**510**	2.14	196	7.75	26
2	V219M	34	280	**456**	1.64	150	6.28	28
3	D240	40	221	**448**	2.69	247	5.18	70
4	D240-41	34	234	**386**	1.59	145	4.36	55
5	V185I-I283M-L288V	31	211	**364**	1.73	158	5.71	39
6	A251C	44	162	**284**	1.76	162	5.08	30
7	D239-40	30	307	**274**	0.91	83	2.58	64
8	A250L	38	49	**248**	5.15	472	6.27	29
9	S264K	42	99	**219**	2.20	201	3.73	37
10	A251G	45	88	**213**	2.42	222	4.26	18
11	HIS10	35	134	**207**	1.55	142	3.61	41
12	V160A	39	163	**167**	0.98	89	5.16	28
13	A250S	51	90	**156**	1.41	129	2.72	21
14	I163N	38	70	**152**	2.42	222	4.40	26
15	A250I	39	87	**150**	1.45	133	4.59	22
16	D170E-G178S	38	102	**126**	1.23	113	3.08	32
17	WT (cw15)	19	120	**82**	0.68	57	2.20	9
18	CC124	16	53	**80**	1.51	139	2.09	31
19	WT (11-32b)	50	98	**29**	0.30	27	1.36	16
20	S264C	16	24	**17**	1.06	98	-*	-*
21	N230S-G289D	-	-	**-**	-	-	-	-
22	IL	-	-	**-**	-	-	-	-

Strains could be divided into four different groups according to their H_2 productivity: (1) high productivity (more than 350 mL L^{-1}), (2) medium (between 350 and 100 mL L^{-1}), (3) low (less than 100 mL L^{-1}) and (4) no productivity (unable to evolve H_2 in the supplied conditions). This group

distinction will be adopted further in this section. It must be pointed out that strain CC124 performance (about 80 mL L^{-1}) which was considered as a control for our system reliability, was equal to what reported in literature under similar conditions (Ghirardi et al., 2000b; Kosourov et al., 2002; Tsygankov et al., 2002). It is interesting to note that all producing strains generally needed the same amount of time before starting to evolve H_2, about 35-40 hours. However, this general rule was not followed by the low productivity group (less than 100 mL L^{-1}). This observation was confirmed by reduced antenna and state transition mutants whose results are not shown within this work. Most likely, these strains suffered of an unbalanced photosynthesis-respiration ratio under sulfur deprivation, which led to a reduced accumulation of starch when H_2 production started. Indeed, if the initial aerobic phase is particularly reduced, endogenous substrates (e.g., starch) are not stored in a high amount as a consequence of a mere lack of time. On the other hand, an overly too long aerobic phase can cause an excessive down-regulation of the PSII (leading to a reduced efficiency of starch synthesis) and, moreover, it may cause a sustained starch respiration to remove the O_2 photosynthetically generated (Ghirardi et al., 2000b; Fouchard et al., 2005; Kosourov et al., 2003; 2007; Melis, 2002; 2007; Melis and Happe; 2001; Makarova et al., 2007). Consistent with this hypothesis, previous results with D1 mutant strains suggest that a low amount of starch can result in a lower transcription of the hydrogenase gene (Posewitz et al., 2004).

Contrary to what observed concerning the lag phase, the H_2 production phase vary strongly between strains, thus suggesting that, aside from starch metabolism, all the pathways underlaying the H_2 production process were very differently involved. Even if within the high productivity group a constant production time was observed (generally about 240 hours), final productions were influenced by both production times and/or maximum rates. For instance, in several of the tested strains (e.g., L159I-N230Y, V219M, A250L, V160A, A251C and V185I-I283M-L288V) final productivity was particularly influenced by maximum rates, which were higher than 5 mL L^{-1} h^{-1} (and were sustained for at least 10 hours). In some other cases (e.g., strains reporting a deletion of some amino acid residue, i.e. D240, D240-41 and D239-40), as soon as maximum rates declined significantly, production could stop for a short period (ranging between 5 to 20 hours), starting then once again at a very low rate for several hours.

The best performance, considering both final H_2 volume (510 mL L^{-1}) and maximum rate of production (7.75 mL L^{-1} h^{-1}), was obtained with the double mutated strain L159I-N230Y. When expressed in terms of chl, the former is 2 times higher than what reported in literature with the highest producing mutant known to date (*Stm6*, Kruse et al., 2005) and still higher than *Stm6Glu4* (Doebbe et al., 2007) which production was enhanced by means of glucose addition thanks to a

HUP1 (hexose up-take protein) hexose symporter.

The main aim of this screening was to discover both high and very low productive strains, which could differently support our interest for the physiology of the process. However, it was rather surprising to find out that one of the strains to which all mutants belong (the intron-less mutant, IL) was not able to produce H_2 at all, at the supplied conditions. The H_2 production capability showed by its wild-type (WT [11-32b]), which was actually low, was completely lost by removing the four large introns in the *psbA* gene. However, when considering both their maximum and effective quantum yields of PSII, no significant changes were observed (Table 2). This observation turned out to be true generally for all the tested strains. Eventually, though a slight decrease was noted in F_v/F_m values of D1 mutants respect to their WT (11-32b), when exposed to the culture conditions supplied for the H_2 production experiments, $\Delta F/F'_m$ values at the start of the experiment (Tab. 2) were generally comparable between all the tested strains, and no clear correlation could be found with respect to their H_2 productivity.

N.	Strain	F_v/F_m	$\Delta F/F'm$	
		Start of the experiment	Start of the experiment	Start of H_2 production
		aerobic	aerobic	anaerobic
1	L159I-N230Y	0.700	0.562	0.140
2	V219M	0.680	0.536	0.110
3	D240	0.680	0.621	0.093
4	D240-41	0.680	0.639	0.057
5	V185I-I283M-L288V	0.740	0.560	0.061
6	A251C	0.720	0.656	0.211
7	D239-40	0.700	0.618	0.135
8	A250L	0.660	0.643	0.170
9	S264K	0.650	0.433	0.113
10	A251G	0.660	0.576	0.097
11	HIS10	0.650	0.631	0.058
12	V160A	0.620	0.569	0.122
13	A250S	0.690	0.619	0.088
14	I163N	0.670	0.543	0.095
15	A250I	0.680	0.635	0.075
16	D170E-G178S	0.590	0.427	0.054
17	WT (cw15)	0.680	0.640	0.180
18	CC124	0.750	0.708	0.207
19	WT (11-32b)	0.790	0.536	0.110
20	S264C	0.550	0.531	0.336
21	IL	0.770	0.630	n.d.$^+$
22	N230S-G289D	0.740	0.535	n.d.$^+$

Table 2. Maximum (F_v/F_m) and effective ($\Delta F/F'_m$) quantum yields of PSII of *C. reinhardtii* D1 protein mutants and three controls (namely, WT [11-32b], WT [cw15] and CC124). Strains are listed according to their H_2 productivity (see Tab. 1). To estimate F_v/F_m values, samples were taken before sealing PBRs. The effective quantum yield of PSII ($\Delta F/F_m'$) was evaluated *in situ* by pointing the optical fiber of the fluorometer directly onto the illuminated PBR surface. Results are the mean value of experiments made at least in triple on independent cultures. Keys: $^+$, these strains did not start to evolve H_2 gas.

As soon as cultures underwent anaerobic conditions, $\Delta F/F'_m$ values typically dropped due to the migration of the LHC-II proteins from the PSII to the PSI, within the transition from state 1 to state

2 (Antal et al., 2001; 2003). Low productive strains showed a higher value than other groups at the onset of H_2 production, most likely due to their reduced lag phase, which may have prevented an excessive degradation of the photosynthetic apparatus- though not leading to high productivities. Nonetheless, in the other producing groups, a high maximum rate (i.e., more than 5 mL L^{-1} h^{-1}) was generally correlated to a relatively high $\Delta F/F'_m$ value (about 0.100) at the onset of H_2 production. In order to evaluate photosynthetic and respiratory capabilities of the strains, photosynthesis-light response (P/I) curves were done in optimal growing conditions. Results are presented in Table 3.

Table 3. Photosynthesis and dark respiration rates in optimal growing conditions of *C. reinhardtii* D1 protein mutants and three controls (namely, WT [11-32b], WT [cw15] and CC124). Strains are reported according to their H_2 productivity (see Tab. 1). Light was increased stepwise from 10 to 640 µmol photons m^{-2} s^{-1}. Dark respiration rates were evaluated at the end of photosynthesis measurements. Cultures were grown to the initial exponential phase (10-15 mg chl L^{-1} of culture), diluted to 3 mg L^{-1} and maintained at 28°C throughout the entire analysis. Results are the mean value of analysis made at least in triple on independent cultures.

Strain	Gross Photosynthesis and respiration rate (µmoles O_2 mg chl^{-1} h^{-1})							
	Light (µmol photons m^{-2} s^{-1})							Dark Respiration
	10	20	40	80	160	320	640	
L159I-N230Y	38.0	56.2	80.6	143.0	243.6	383.1	487.8	211.6
V219M	1.0	29.6	47.6	93.1	134.5	166.6	188.2	83.2
D240	9.5	36.9	49.1	66.3	78.7	81.0	83.2	68.7
D240-41	27.4	58.5	85.0	139.4	223.3	282.1	301.9	174.8
V185I-I283M-L288V	33.9	63.2	93.0	157.5	231.9	285.0	308.0	207.4
High productivity group average	**22.0**	**48.9**	**71.1**	**119.9**	**182.4**	**239.6**	**273.8**	**149.1**
A251C	28.1	42.8	74.9	134.1	207.3	248.5	266.5	126.6
D239-40	8.2	13.5	30.7	48.5	72.3	101.8	113.1	68.5
A250L	21.3	47.7	76.4	138.0	204.1	233.3	213.4	114.6
S264K	15.2	36.8	64.1	104.3	161.1	221.1	268.3	160.6
A251G	10.8	25.7	64.6	120.8	183.6	198.2	194.1	120.8
HIS10	39.3	52.0	90.2	152.1	234.9	307.1	359.6	150.7
V160A	27.6	54.7	88.6	148.9	248.3	315.6	318.8	175.2
A250S	10.8	34.5	73.3	128.4	212.4	284.6	332.3	84.2
I163N	21.5	46.1	78.5	119.8	211.6	262.3	264.1	144.8
A250I	23.3	47.2	88.2	158.4	260.5	374.0	402.8	97.3
D170E-G178S	19.4	32.8	55.1	102.7	124.0	125.0	111.5	55.1
Medium productivity group average	**20.5**	**39.4**	**71.3**	**123.3**	**192.7**	**242.9**	**258.6**	**118.0**
WT (cw15)	10.8	34.5	73.3	128.4	212.4	284.6	332.3	134.5
CC124	25.7	50.4	75.4	100.3	141.7	167.1	173.6	121.9
WT (11-32b)	14.6	32.3	60.8	101.5	169.7	204.8	223.1	120.7
S264C	9.9	34.0	67.3	99.7	142.4	171.2	153.0	78.4
Low productivity group average	**15.3**	**37.8**	**69.2**	**107.5**	**166.6**	**206.9**	**220.5**	**113.9**
IL	21.6	49.3	80.6	133.4	191.8	220.0	245.0	125.5
N230S-G289D	6.6	17.6	40.6	66.6	98.1	101.4	92.6	59.8
No productivity group average	**14.1**	**33.5**	**60.6**	**100.0**	**145.0**	**160.7**	**168.8**	**92.7**

In a previous study, it was reported that *Chlamydomonas* D1-R323 mutants showed a reduction in photochemical activity that resulted in a reduction in the H_2 output (Makarova et al., 2007). However, amino acid substitutions made in the region involving the loop between the D and E helix resulted in the loss of D1 function and photosynthetic capability in only a few cases (Lardans et al., 1998). Mutations described in this work concerned several different amino acid positions, which implies that no detailed description could be done concerning this aspect, but rather general observations. In fact, respect to the WT (11-32b), the ratio between strains that reported an increase in the photosynthesis rate versus those who suffered of a reduction is about 50:50. Nevertheless, as a matter of fact, when comparing the final average of different groups of H_2 productivity in Tab. 3, it turns out that the higher the photosynthesis rate, the higher the respiration capacity, the higher the H_2 productivity. Maximizing photosynthetic efficiency is one of the key factors for improving H_2 production performances (Happe et al., 2002) as a high photosynthesis rate may lead to a high synthesis of endogenous substrates like starch, which is essential for several reasons, namely: (1) for maintaining anaerobic conditions (Ghirardi et al., 2000b; Fouchard et al., 2005; Kosourov et al., 2003; 2007; Melis, 2002; 2007; Melis and Happe; 2001; Makarova et al., 2007); (2) as an additional source of electrons for the plastoquinone pool (Bamberger et al., 1982; Gfeller and Gibbs, 1985; Godde and Trebst, 1980; Mus et al., 2005) and (3) for maintaining [FeFe]-hydrogenase gene expression (Posewitz et al., 2004). On the other hand, a high respiration rate is a fundamental prerequisite to induce and maintain anaerobic conditions, which permit hydrogenase expression and function (Ghirardi et al., 1997; Melis, 2002; Flynn et al., 2002; Happe et al., 2002; Happe and Kaminski, 2002; Forestier et al., 2003; Posewitz et al., 2004).

Considering P/I curve analyses, when exposed up to 640 µmol photons m^{-2} s^{-1} most of the tested strains did not reach a photoinhibition irradiance (I_h) (data not shown), except strains N230S-G289D, S264C and D170E-G178S, which were also found to produce low amounts of H_2 (Tab. 1). Most interestingly, when plotting the relative apparent quantum yields ($Ø_{rel}$) of P/I curves versus the maximum rates of H_2 production, a linear correlation was observed (Figure 2): the higher the relative apparent quantum yield, the higher the maximum rate.

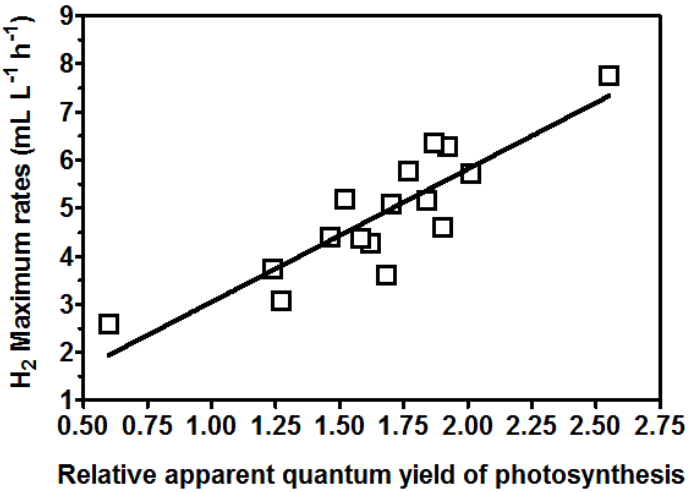


Figure 2. Relative apparent quantum yield of photosynthesis plotted versus maximum rates of H_2 production. Values refer to *C. reinhardtii* strains, either wild types or D1 protein mutants, which are partially presented in Tab. 1.

The coefficient of determination (R^2) of such a linear regression is relatively high (0.764) if considered that H_2 production in *Chlamydomonas* is an elaborate interplay of electron sources and sinks (Hemschemeier et al., 2008b). Mostly, Figure 2 shows how strongly maximum rates sustained during the first hours of H_2 production depend on the remaining activity of the PSII. In support of this hypothesis, previous results (Antal et al., 2001; 2003; Lee and Greenbaum, 2003; Fouchard et al., 2005; Kosourov et al., 2003; Kruse et al., 2005; Antal et al., 2009) showed by means of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) addition (an urea class inhibitor of PS II [Bishop, 1958; Draber et al., 1991]) that up to 80-85% of reductants are initially supplied directly from the remaining water splitting activity of the PSII.

As previously mentioned, H_2 production screening was carried out with a 1.1 liters PBR, using a water displacement system to estimate the overall biogas production (Ghirardi et al., 2000b; Melis et al., 2000). Even if such a system is particularly convenient for screening purposes, it can suffer of some drawbacks when accurately measuring biogas composition. The main one is the presence of other gases due to a number of head spaces (i.e., PBR head space, connecting tubes, and calibrated cylinder head space). In order to overcome these problems, the biogas composition of *C. reinhardtii* cultures was followed during the entire experiment. In Figure 3, results obtained with the highest producing strain (L159I-N230Y) are reported.

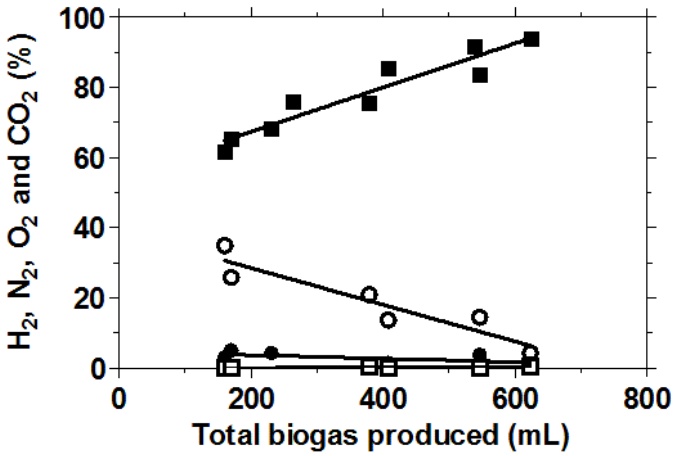


Figure 3. Changes in biogas composition during the H_2 production process with the *C. reinhardtii* mutant strain L159I-N230Y. Keys: H_2 (■); N_2 (○); O_2 (●); CO_2, (□).

As long as the biogas volume increased, relative H_2 amount raised linearly. On the contrary, N_2 and O_2 content was dramatically reduced. It is interesting to note that the amount of CO_2 measured in the gas phase was always negligible, and started to slightly accumulate only after 400 mL of produced biogas. There are a number of factors contributing to this: (1) CO_2 Henry's law constant is one order of magnitude lower than H_2 one (Perry and Green, 1999), which implies that (upon the same conditions) the former has a higher solubility in a liquid solution, (2) starch catabolism, other than supplying electrons to the [FeFe]-hydrogenase through the PQ pool, is known to feed other fermentative pathways, which generally led to acetate, ethanol and formate production (Winkler et al., 2002; Happe et al., 2002; Kosourov et al., 2003; Hemschemeier and Happe, 2005), and (3) as aforementioned, during the first hours of biogas production, most of the electrons derived from the remaining water splitting activity (Antal et al., 2001; 2003; Lee and Greenbaum, 2003; Fouchard et al., 2005; Kosourov et al., 2003; Kruse et al., 2005; Antal et al., 2009). The final composition was 99.5% of H_2 and 0.5% of CO_2. Similar results, respect to the H_2 content, were previously reported with high producing strains (98%, Kruse et al., 2005), and only slightly lower values were reported with strains which produced up to 4 times lower final volumes (between 120-160 mL L^{-1}, 87% of which was H_2 [Ghirardi et al., 2000b; Melis et al., 2000]).

Section 1 Conclusions and future perspectives

The experiments carried out so far pointed out mutations upon the D1 protein may lead to enhanced H_2 productions, most likely by means of a higher photosynthetic activity counteracted by a high respiration rate. In order to obtain new information, the most productive mutant L159I-N230Y was further tested and characterized along with the mutant IL, which was not able to produce at all.

Their WT (11-32b), together with the commonly used strain CC124, was adopted as a control. However, in consideration of these results, it would be of great interest for future applications to investigate, by means of more sophisticated molecular biology approaches, the role of the D1 protein mutants on the H_2 production, particularly with respect to mutations concerning the Oxygen Evolving Complex (OEC).

SECTION 2.

Design, construction and testing of a photobioreactor equipped with an optimized stirring system for photosynthetic microbiological processes

2.1 The rationale of light/dark cycles in photosynthetic microorganisms

In principle, in order to reach the maximum efficiency in algal photosynthesis it is necessary to irradiate a single cell so that the incident light seen by it is close to the point at which the P/I curve starts to flatten out. Unfortunately, this condition is difficult to realize in dense cultures, because they are exposed to a light gradient across the reactor that depends on the culture depth, chl concentration, and mixing rate. As a result of the light gradient, cells are exposed to a certain light/dark (L/D) cycle, the frequency of which is regulated by the mixing rate. Thus, cells growing in dense cultures are exposed intermittently to light, which is the most practical way to "dilute" strong light intensity among cells and to attain a more efficient way to use light. Algal cells can be influenced by three ranges of intermittent illumination (L/D cycles) (Grobbelaar, 1989; Jansen et al., 2000a): (1) high frequency fluctuations of 100 ms (10 Hz) or less; (2) medium frequency fluctuations of seconds to minutes and (3) low frequency cycles of hours to days and years. L/D fluctuations in algal reactors usually fall into the category of the medium frequency fluctuations, the beneficial effect on light transformation efficiency of which is still controversial (Grobbelaar, 1989; Janssen, 1999; Barbosa et al., 2003). On the other hand, studies on the effect on algal photosynthesis have demonstrated that high light intensity may be used with high efficiency if supplied in short flashes separated by long dark periods (flashing effect). In fact, it can be assumed that the catalytic reactions limiting the rate of photosynthesis in high light can continue for about 100 ms after the cessation of illumination, which for dense cultures would correspond to the dark part of the reactor. This condition will correspond to a full integration of the light intensity experienced by the cells. As long as an optimal L/D cycle is not achieved, only a part of the possible benefit in yield can be attained. In an optically-dense algal culture, the light irradiance seen by individual algal cells will be

strongly dependent on the position of that cell across the light gradient. For a cell near the surface, the photosynthesis will be over-saturated, and will dissipate excess of light as heat and fluorescence, while the one located in the deep layers will be exposed to a weak light or to a complete darkness and will consume energy by means of respiration. This non-homogeneity of the irradiation intensity in optically-dense cultures, coupled with the existence of a saturation level for photosynthesis, leads to an engineering dilemma (Myers and Graham, 1958). Nevertheless, if the irradiation is interrupted by replacing a batch of algae in the light path while the first one is "digesting" the flash products in darkness, an increase in the H_2 productivity could be expected. Empirical studies on algal photosynthesis as a function of "intermittent light" have been carried out on optically-thin layers of *Chlorella* suspension in relation to a mass culture of this organism (Kok, 1953; Phillips and Myers, 1954). Kok showed that, with a ratio $(t_f+t_d)/t_f$= 5.5 and a frequency period of about 60 Hz (flash length t_f= 3 ms and dark length t_d= 13.5 ms), a yield practically equal to the one attainable in constant light of the same integrated intensity could be reached. However, the achievement of an intermittent effect pattern that would permit complete light integration, whereas the catalyst is fully occupied for the whole duration of the dark fraction is, at present, beyond the capability of any sort of engineering. However, if we were satisfied with somewhat less than the maximal rate, with only a partial light integration, then much longer periods, of the order of 200 ms and more, could still yield some improvement in productivity (Kok, 1953). Moreover, it has been demonstrated that relatively long intervals, of the order of 1 s, may also give rise to an improvement in the energy conversion yield (Laws et al., 1987; Terry, 1986). As long as the frequency of the L/D cycle increases over 1 s, the benefit become insignificant, as demonstrated by Janssen et al. (2000b) with *C. reinhardtii*.

Thus, in order to find out to which extent mixing could positively influence the H_2 production process in *Chlamydomonas*, a PBR equipped with an optimized stirring system was designed and built. The fluid dynamic and hydrodynamic characterization of such a PBR was studied and compared to the one achieved with a conventional stir bar. Then, H_2 production experiments carried out at different light regime (by means of different light intensities and/or chl concentrations) were done with both PBRs using the strain CC124, taking advantage of a continuous monitoring system (Kosourov et al., 2002, mod, see Materials and Methods). Results reported in this section were published on Biotechnology and Bioengineering (Giannelli et al., 2009).

2.2 Fluid dynamics in the photobioreactors

Stirring of the cultures was achieved by using either a stir bar or a rotating impeller, both driven

magnetically at the bottom. The fluid dynamics of the PBR stirred with a conventional magnetic stir bar (3.5 cm length, 0.7 cm diameter) is discussed first. The rotation of the magnetic stir bar (rotational speed, 31 rps) generates a flow pattern similar to that of any other low off-bottom clearance impeller (Halàsz et al., 2007). A single circulating loop was produced that consisted of one outgoing radial flow at the PBR bottom (Figure 4a), with the liquid moving from the center towards the walls, an ascending flow, and an axial descending flow back to the bar stirrer. A tangential flow was also induced by the stirrer rotation superimposed to this bottom-to-top/top-to-bottom circulation, due to the variable cross section of the vertical planes that depended on the angular position (Figure 4b).

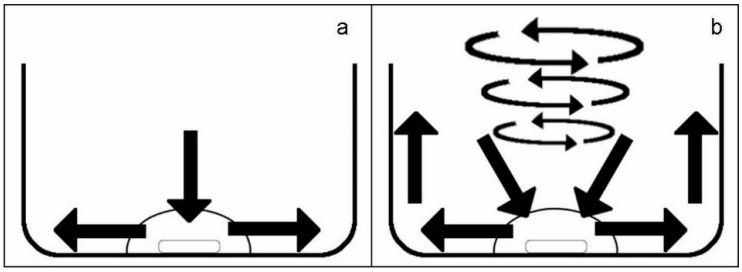


Figure 4. General scheme of fluid dynamics in a PBR with a flat cross section and a flat bottom stirred by a magnetic stir bar.

The solid-body rotation of the liquid was impeded, and a negligible vortex appeared at the surface with this PBR configuration- contrary to what happens in a system of cylindrical symmetry (Halàsz et al., 2007). Liquid circulation in the loop propagated among fluid layers because of a shear mechanism, while the layers closer to the bar and the bottle bottom were characterized by an intensive movement, the ones involving the uppermost zones resulted almost static. Consistent with this, during H_2 production experiments in Roux bottle type PBR, H_2 bubbles are clearly visible in the upper part of a stir bar-mixed culture. Consequently, both the aforementioned top-to-bottom fluid exchange and the liquid transfer from the external zones to the central PBR core were rather limited, meaning that parts of the culture were subjected for a long time to light, while the internal part remained in the dark. This light exposure pattern led to a poor L/D cycle that entailed a reduction in light utilization efficiency by the culture.

Because of these limits and due to inadequacies on the part of the stir bar, a more effective stirrer system was devised, i.e. an impeller that determined intensive flow movement from the upper part of the PBR towards the bottom and from the core region to the walls. To achieve this goal, a multiple impeller was designed that consisted of five turbines of the same diameter (25 mm) fitted on a vertical shaft, as depicted in Figure 5.

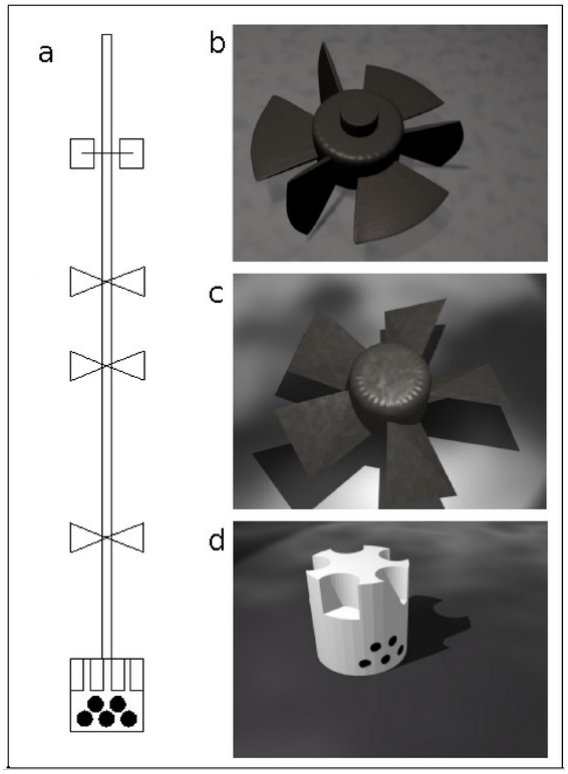


Figure 5: Schematic representation of the multiple-impeller stirrer. (a) A front view of the impeller; (b) the three-bladed radial turbine placed at the top; (c) one of the three six-bladed pitched-blade turbines; (d) the plastic magnet holder at the bottom of the impeller, with a radial-turbine design in its upper part.

The uppermost element (Fig. 5b) was a radial, three-bladed turbine intended to create radial flow just a few centimeter below the surface of the liquid. This avoided foam accumulation during H_2 production, and generated sufficient liquid movement in the upper part of the culture. Three axial-flow pitched-blade turbines pumping downwards (Fig. 5c) were placed beneath the radial one, with the function of pumping the fluid from the top to the bottom of the PBR. Here, another radial turbine pumped the liquid towards the wall from which an ascending flow originated. The bottom element, which was also the holder for the magnets that provided the rotation of the whole impeller, was obtained from a cylindrical PET rod and its upper part was carved so as to obtain five vertical blades that determined a radial flow (Fig 5d). The flow pattern produced by the uppermost and lowest radial turbines consisted of a single loop: indeed, their small clearance from the liquid surface and PBR bottom, respectively, was such that the second loop typical of radial turbines was suppressed (Montante et al., 1999). All the pitched-blade turbines had six identical, slightly curved blades and produced an axial flow that gave rise to a single, overall top-to-bottom vertical loop, as well as limited radial flows (Zhou and Kresta, 1996). The former guaranteed vertical liquid exchange between the loops of the radial turbines, while the secondary radial flows at each pitched-blade turbine determined culture movement from the core to the illuminated wall at the various elevations. To enhance this effect, the blade pitch was different for each axial impeller: namely, 60°,

40° and 20° from the horizontal plane from the first to the lowest one, and were set following the speed triangle rule. Moreover, with this stirrer configuration the superficial vortex due to tangential flow was barely noticed. The liquid and cell exchange from the center to the walls was, therefore, improved and so were the L/D cycles. Complete characterization of the fluid dynamics of each turbine in terms of flow numbers (Hemrajani and Tatterson, 2004) is beyond the scope of this study. The rotational speed of this impeller system was N=30 rps, which allowed a fully turbulent regime. A complete overview of the final fluid dynamic achieved is also shown in Fig. 12 of Materials and Methods.

2.3 Hydrodynamic characterization of the photobioreactors

To compare the fluid dynamic performance of the new impeller with that of the standard stir bar in creating convective mixing, two different techniques were used: flow visualization with flow followers, and mixing time evaluation. According to the first technique, neutrally-buoyant spherical particles (3.0 ± 0.3 mm diameter) were added to the liquid and their motion was filmed (at 30 fps). Frame-by-frame analysis of the video made it possible to determine particle position at different times and to reconstruct their trajectories, as shown in Figure 6 for the multiple impeller system.

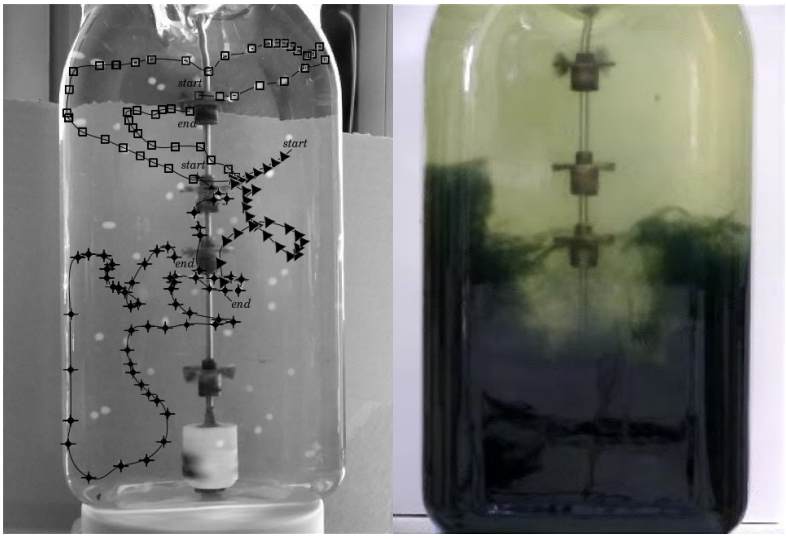


Figure 6. (Left) Photograph showing some real fluid trajectories of selected tracer particles produced by the multiple-impeller stirring device, and (right) spatial distribution of black dye drops added at the bottom of the impeller-mixed PBR. Note on the right picture the loop created at the third turbine (from the top), which clearly demonstrates the liquid exchange between the outer and the inner zone of the PBR.

The graph confirmed the main behavior discussed in the previous section and, in particular, the effective liquid exchange from the core to the outer zones (and vice versa) also in the upper part of the PBR. Instead, significant segregation between the core and the outer zones was apparent with the stir bar, except at the PBR bottom. From the trajectories it was easy to calculate the center-to-wall walk time in each part of the PBR (the results are shown in Table 4), and confirmed that, at

least for the top and the bottom turbines, the time required for a particle to run along the light gradient was within the time required in order to promote an intermittent light effect in the culture.

Table 4. Times and speeds required by cells to cover the path from center-to-wall and from the top layer towards the bottom of the photobioreactor measured at different turbine positions on the impeller.

Turbine positions	Center-to-Wall		Top-Down	
	Time (s)	Speed (m s^{-1})	Time (s)	Speed (m s^{-1})
Upper three-bladed radial turbine	0.053	0.47	~0.00	< 0.10
Pitched Blades Turbine n°1	0.100	0.25	0.20	0.13
Pitched Blades Turbine n°2	> 0.500	<0.10	0.33	0.16
Pitched Blades Turbine n°3	> 0.500	<0.10	0.12	0.21
Bottom six-bladed radial turbine	0.058	0.43	n.d.	~0.00

The second technique adopted for comparing the two stirring devices involved following the evolution of the homogeneity degree after a concentration pulse of a liquid tracer. For this purpose, conductivity was measured with a small probe (1 x 1 cm square electrodes, 1 cm apart) placed on the surface of the liquid after the injection of a 33% w/w NaCl solution at the bottom. In order to assess the effective response time of the electrode itself, measurements were also carried out in a well-agitated system, by injecting an identical saline solution (33% NaCl, w/w). Conductivity was recorded at 0.5-s intervals and plotted against time, as depicted in Figure 7.

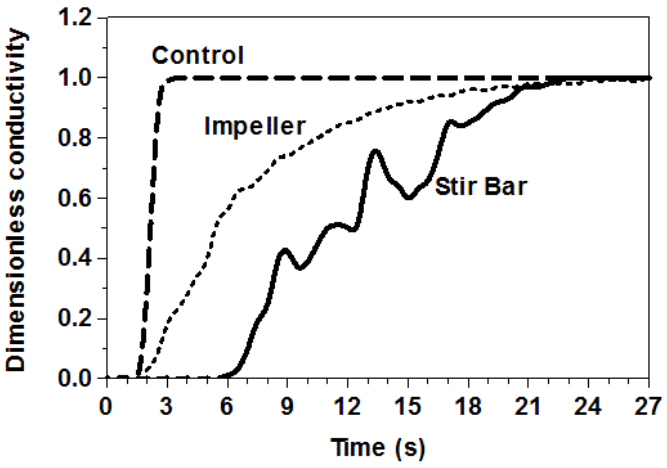


Figure 7. Time course of conductivity time response to the injection of a NaCl solution.
Keys: *continuous line*, liquid stirred with the stir bar; *dotted line*, liquid stirred with the multiple impeller; *dashed line*, response time of the sensor.

It was found that the response time of the electrode was 1.5 seconds (Fig. 7 dashed line, control). The initial response time (i.e., the moment in which the conductivity response curve started) was practically coincident in both the well-mixed system used as a control, and the multiple impeller

system, while in the stir bar this time was longer. Other differences were noted in the shape and smoothness of the curves: the mixing curve obtained with the stir bar system exhibited significant spikes that could be attributed to blobs of traced liquid that propagated irregularly through the culture like a cloud. These spikes were never observed with the multiple impeller, thus indicating much reduced local concentration gradients and better axial and, especially, radial homogenization processes. This behavior is typical of multiple-impeller systems (Jahoda et al., 1994). Additional evidence of such behavior was obtained by visually following the spatial distribution of a few drops of a black dye rapidly added to the liquid in the same position as that of the electrolytic tracer pulse. While the homogenization progressed smoothly and rapidly along the PBR with the multiple impeller, layers of colored liquid were clearly visible at any position when the stir bar was used- even at advanced stages in the mixing process- thus confirming poor radial mixing (Figure 8). The latter type of behavior has been defined as the Taylor regime (Ndonga and Tatterson, 2006). The so-called mixing time, i.e. the time necessary for obtaining a given degree of homogeneity, gives a quantitative index of homogenization. It was found in this case for the 95% homogenization level: in other words, a ±5% deviation from the value of the end point of mixing was accepted.

The effective initial response times, and the 95% mixing times, t_{mix} (average of six experiments), was 4 seconds for the system mixed with the stir bar, and only 0.8 second for the one mixed with the multiple impeller (Fig. 7). Accordingly, the mixing time was 15.5 seconds, with a time of 12.9 seconds for the stir bar and impeller system, respectively. These values demonstrate the superiority of the multiple impeller in the homogenization process. It is interesting to note that the dimensionless mixing time, $N\text{-}t_{mix}$, for the two mixing devices (i.e., 420 and 510, respectively) are of the same order of magnitude as those reported in the literature for a flat-bottomed cylindrical bottle (Ndonga and Tatterson, 2006) and multiple-impeller stirrers (Montante and Magelli, 2004).

Figure 8. Boundary level relative to a fully turbulent mixing in a stir bar-mixed PBR with a flat cross section. Fluid was colored by means of black dye addition at the bottom of the PBR, near to the rotating stir bar. Note that black dye stops at about 3/4 of the PBR height. Blobs of traced liquid propagating irregularly like a cloud, responsible for the spikes noted in Fig. 7 with NaCl solution, can also be appreciated.

Although these differences do not appear to be particularly remarkable, the segregation of liquid zones and poor radial exchange- as revealed by the longer initial response time and the spikes in the mixing curve- suggest that the L/D cycles are supported much less effectively when stirring with the single magnetic bar than with the multiple impeller.

2.4 Photosynthetic characterization of the strain CC124

P/I curve analyses of the strain CC124 were done with diluted culture suspensions (5 mg chl L^{-1}), and photosynthesis rates were plotted vs. the absorbed light irradiance (Figure 9).

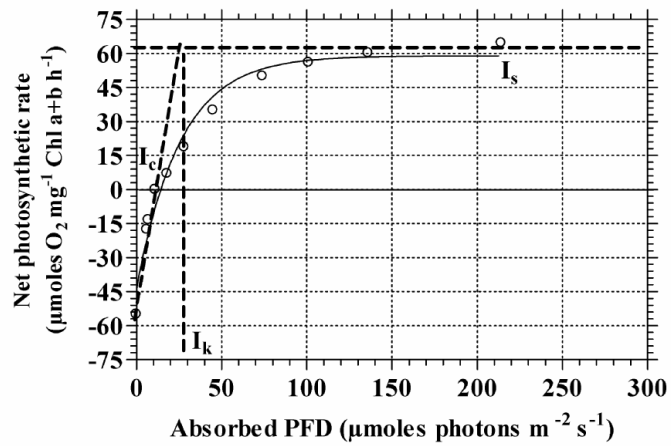


Figure 9: Photosynthesis-light response curve in CC124 strain. Oxygen measurements were carried out with a culture having a 5 mg L^{-1}. Light absorbed by the cultures was obtained as the difference between the incident light measured in the cuvette containing TAP medium and that transmitted by the culture. Dark respiration rates were measured after the measurement of photosynthesis rates. Results are the mean value of 3 independent experiments.

The saturation irradiance (I_k) was found to be close to 30 µmol photons m^{-2} s^{-1}; the light-saturated rate (I_s) was achieved at about 200 µmol photons m^{-2} s^{-1}; the compensation point (I_c, i.e., when net photosynthesis is zero) was found at a PFD of 11 µmol photons m^{-2} s^{-1}. Respiration rate accounted for about 46% of the gross photosynthetic capacity (net photosynthesis plus respiration). Results of light attenuation by *C. reinhardtii* cultures having different chl concentrations are shown in Figure 10.

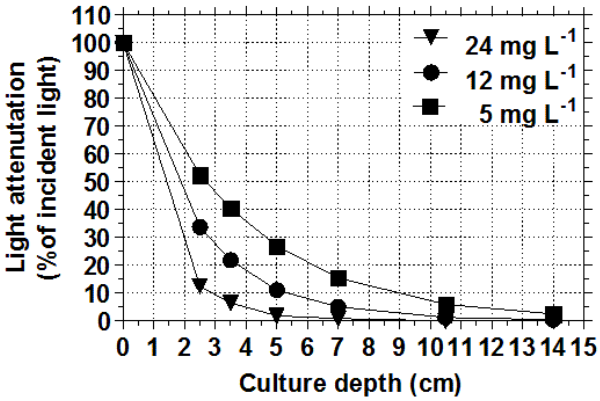


Figure 10. Light attenuation measurements in *Chlamydomonas reinhardtii* cultures (strain CC124) having different chl concentrations. Measurements were carried out with a spherical micro-quantum sensor US-SQS/B (H. Walz, Effeltrich, Germany) connected to a LI-250A light meter (LI-COR Biosciences).

The attenuation of about 50% of the incident light was achieved within a culture depth of roughly 1.5 cm at the highest chl concentration tested (24 mg L^{-1}). The same light attenuation was achieved within 1.9 cm and 2.7 cm culture depth as the concentration was decreased from 12 and 5 mg L^{-1}, respectively. Light irradiance was almost totally extinguished within a depth of 5 cm when the highest concentration (24 mg L^{-1}) was used. As can be observed, with a 5 mg L^{-1} concentration the light extinction followed the Lambert-Beer law and departed from it as the cultures became more dense as a consequence of the increase in light scattering due to an increase in the number of cells. Information gathered with light extinction curves was combined with that gathered from the P/I one and was used to make a careful choice of the range of light intensities and chl concentrations to be tested for experiments involving H_2 production. Given that the optimal light utilization efficiency is achieved under photolimited conditions (i.e., within the I_k value), a series of experiments at two light irradiances were planned, and for each of them chl concentration was adjusted in order to test H_2 productivity under different light dilutions.

2.5 Interplay of chlorophyll concentration and light intensity on hydrogen production by testing different mixing system with the strain CC124

The experiments involving H_2 production at different light irradiances and chl concentrations were carried out in PBRs equipped with two different stirring systems. In one reactor the mixing was provided by a conventional magnetic stir bar and, in the other, by an impeller designed by us.

The first experiment was carried out using the photon flux density (PFD) of 70 µmol photons m^{-2} s^{-1} supplied on both sides of the PBR (total amount of light: 140 µmol photons m^{-2} s^{-1}), and with a chl concentration of 12 mg L^{-1}. These culture conditions were already adopted during the H_2 production screening and are the ones most commonly used by laboratories for H_2 production with

Chlamydomonas (Melis et al., 2000; Ghirardi et al., 2000a, 2000b; Cournac et al., 2002; Zhang et al., 2002; Kosourov et al., 2002; 2003; 2005; 2007; Tsygankov et al., 2002; 2006). Under these conditions, cells move within a light gradient that is very close to the I_k value, which ranges from 70 µmol photons m^{-2} s^{-1} on the walls of the PBR to 40 µmol photons m^{-2} s^{-1} in the center of it. Thus, cells were exposed to light irradiances above the onset of saturation.

Contrary to the simple H_2 production screening system aforementioned, by taking advantage of the continuous monitoring system (Kosourov et al., 2002, mod., see Materials and Methods), it was possible to divide the entire H_2 production process into 5 consecutive steps (as already noted by Kosourov et al., 2002): (1) an O_2 production phase, characterized by accumulation of O_2 in the culture; (2) an O_2 consumption phase, during which photosynthesis cannot keep up with respiration; (3) an anaerobic lag phase, during which neither O_2 nor H_2 is evolved by the culture; (4) a H_2 production phase, during which H_2 is released and (5) a termination phase during which the accumulation of H_2 ceases and an eventual consumption of the H_2 itself can occur.

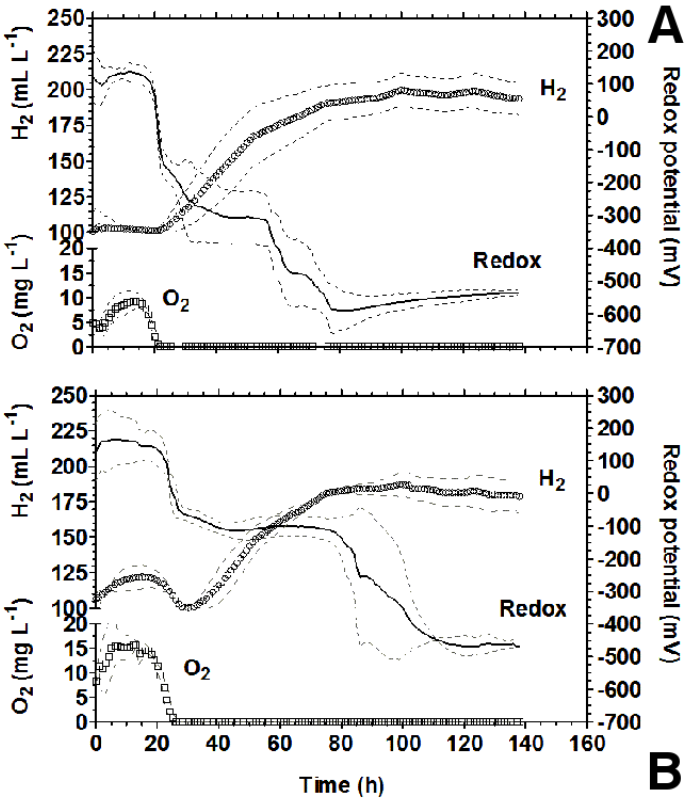


Figure 11: Time courses in the H_2 outputs recorded in the strain CC124 mixed with a stir bar (a) and with an impeller (b). The experiments were carried out with cultures exposed to a PFD of 70 µmol photons m^{-2} s^{-1} supplied on both sides, and a 12 mg chl L of $culture^{-1}$. Dashed lines indicate the standard deviation of four independent experiments. Note in (b) the initial rise of oxygen produced by the culture during the aerobic phase within the first 24 h of the experiment and detected by the electronic balance.

In the culture mixed using the stir bar, the concentration of O_2 remained within 10 mg L^{-1} (Figure 11a), while it increased up to 16 mg L^{-1} in the one mixed using the impeller (Figure 11b). In this case, the O_2 concentration increased over the saturation level and was released in the head space,

causing a water displacement that was detected by the digital balance. That was possible as in our experiments, unlike Melis et al. (2000) and similarly to Laurinavichene et al. (2004), PBRs were sealed from the beginning of the experiment, permitting the evaluation of O_2 production. In this respect, the higher photosynthetic rate of O_2 evolution sustained by the impeller mixed-culture was a first confirm of the better mixing system. Nevertheless, the higher O_2 concentration in this culture caused a delay of about 7 hours in the start of the H_2 production (23 vs. 30 hours in stir bar and impeller, respectively), as more O_2 had to be consumed. Similar observations were done previously (Laurinavichene et al., 2004). As a consequence, respiration phase was about 3-times longer respect to the stir bar-mixed culture and a similar delay was observed also in the lag phase (5.3 vs. 1.3 hours, respectively). This delay must probably account for the reduced production of H_2 (100 vs. 87 mL L^{-1} with stir bar and impeller, respectively) as more endogenous substrates were likely used to get rid of the PSII-generated O_2 (Ghirardi et al., 2000b; Fouchard et al., 2005; Kosourov et al., 2003; 2007; Melis, 2002; 2007; Melis and Happe; 2001; Makarova et al., 2007). However, the mean rate of the H_2 production was found to be higher with the impeller (1.39 mL L^{-1} h^{-1}) compared to the stir bar (0.99 mL L^{-1} h^{-1}) and, similarly, maximum rates (typically occurring within the first hours of H_2 production) were found to be slightly higher in the impeller-mixed culture, 2.44 vs. 2.23 mL L^{-1} h^{-1}.

Redox potential followed a typical pattern in both cultures, showing a sudden drop as soon as they entered anaerobiosis. It must be stressed that in the culture mixed with the impeller, the redox potential remained more stable, and at a less negative value (-100 mV), for a longer period of time compared to the other culture (-300 mV). This clearly reflected a less reductive condition experienced by the former as a result of a greater capability to dissipate an excess of energy, due to the better mixing achieved. Concerning this, it is interesting to note that as soon as H_2 accumulation with the impeller-mixed culture flattened out (T=80 hours, Fig. 11b), redox potential started to decrease sharply, reaching about -500 mV at the end of the experiment. Such a low value was reached at the end of the process also by the stir bar-mixed culture. No relevant differences occurred when measuring the pH value (data not shown), which typically went up to 8.0 in both cultures during the aerobic phase, as a result of a sustained acetate consumption (Kosourov et al., 2003; 2007), and thereafter decreased almost to the initial value (pH= 7.4) as a consequence of the fermentative pathways involved in the H_2 production process.

The second set of experiments was carried out using the same incident PFD of 70 μmol photons m^{-2} s^{-1} per side, while the chl concentration was increased up to 24 mg L^{-1}. The increased concentration reduced the amount of light available for single cells, compared to the previous set of experiments. According to the light extinction curves (Fig. 10), cells moved across a light gradient ranging from

70 (near the walls) to 15 µmol photons m^{-2} s^{-1} in the center of the PBR, which means that under these conditions, cells were subjected to a strong photolimitation.

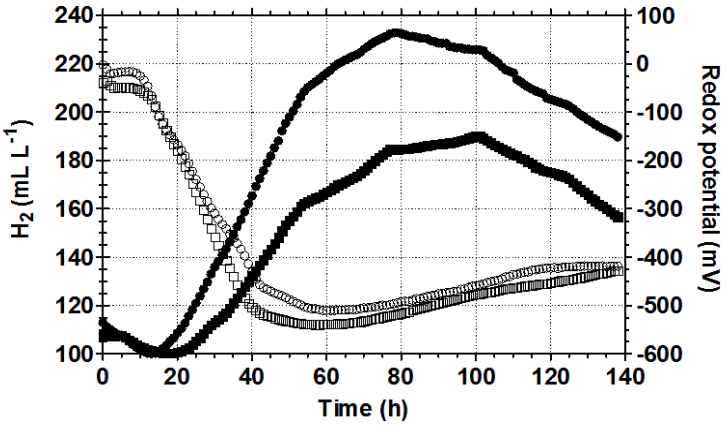


Figure 12. Time courses in the H_2 outputs recorded in the strain CC124 mixed with a stir bar (■) and with an impeller (●). The experiments were carried out with cultures under a PFD of 70 µmol photons m^{-2} s^{-1} per side and a 24 mg L^{-1} chl concentration. Keys: impeller redox potential (○); stir bar redox potential (□).

The culture mixed with the impeller performed much better that the one mixed with the stir bar (Figure 12). In the former, H_2 output reached a final 133 mL L^{-1}, while in the other one the output was about 82 mL L^{-1}. This result was a consequence of very different maximum rates of H_2 production. Moreover, when compared to the previous set of experiments (PFD 70 µmol photons m^{-2} s^{-1}, 12 mg L^{-1}), the time during which maximum rates were sustained did not significantly change in the culture mixed with the stir bar, while it was almost doubled (from 19 to 36 hours) in the one mixed with the impeller. Such a better performance in dense impeller-mixed cultures was not surprising if considered that time cycles (t_c) within 50 and 100 ms were found attainable in this PBR (Tab. 4). This short L/D cycle was made possible because the PBR was illuminated from both sides, thus reducing the light path by 50%. It must be pointed out that impeller-mixed cultures were circulated in accordance with two main patterns. One of these involved radial flow along the light gradient and cell movement determined by the turbines, with a culture velocity ranging from a minimum of 0.25 m s^{-1} to a maximum of 0.47 m s^{-1}, which determined L/D cycles of 100 ms to 53 ms, respectively (Tab. 4). The other involved a vertical flow (downwards) of cells of the core region of the PBR to its bottom, with a characteristic time ranging from 120 ms to 300 ms, followed by an ascending flow along the PBR walls. On the other hand, due to photolimited conditions, cells in the stir bar-mixed culture were likely trapped for a long period within the dark core of the reactor, while impeller-mixed ones could effectively exploit the low light intensity at the surface of the PBR, processing the accumulated reducing power when moved in the dark core of the reactor. This situation does not entirely apply to the previous set (70 µmol photons m^{-2} s^{-1} per side and 12 mg L^{-1}), as low chl concentrations let light penetrate much deeper within the culture layers.

As both were very dense cultures (24 mg L^{-1}), respiration rates per unit of volume were very high, and this induced a condition of anaerobiosis from the start of the experiments on, which led to a strong reduction in the lag time before the cultures started to produce H_2 (13.5 and 19 hours in impeller and stir bar, respectively). In fact, no trace of O_2 could be recorded in either culture during the experiment, and the redox potential remained in the negative field from the moment that the cultures were inoculated. Consistent with these observations, it was previously noted that transition time is a function of cell density (Kosourov et al., 2002; Laurinavichene et al., 2004). The pH value was only slightly higher in the impeller-mixed culture (8.27 vs. 8.16, end of the aerobic phase), and declined thereafter to 7.60 in both systems (data not shown). Finally, it was very interesting to note that at the end of the H_2 production, in both cultures a strong consumption occurred. A detailed description of the H_2 oxidation process is discussed in the Appendix.

In a third set of experiments (Figure 13), the incident PFD was increased to 140 µmol photons m^{-2} s^{-1} per side, in combination with a chl concentration of 12 mg L^{-1}. Under these conditions, cells were expected to move within a light field ranging from 140 (walls of the PBR) and 85 µmol photons m^{-2} s^{-1} in the middle of the reactor and were thus subjected to irradiances well above the I_k saturation level.

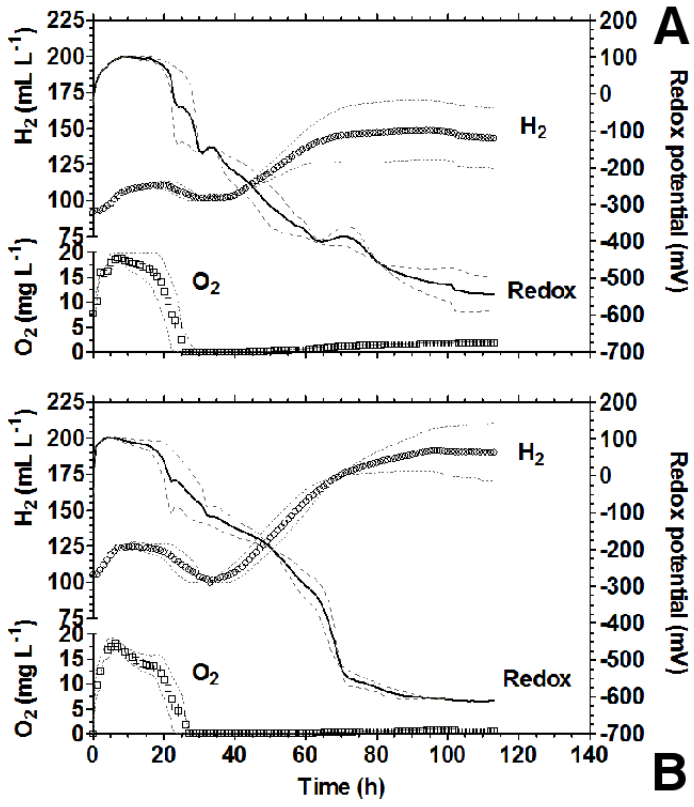


Figure 13. Time courses in the H_2 outputs recorded in the strain CC124 under a PFD of 140 µmol photons m^{-2} s^{-1} (supplied on both sides) and 12 mg L^{-1} chl concentration, in stir bar (a) and impeller (b) mixing cultures. Dashed lines indicate the standard deviation of four independent experiments. Note in (a) the onset of oxygen trace at time 60 h during the course of the experiment observed with cultures mixed using the stir bar system. Keys shown in the graph.

The exposure to higher light intensities did not produce any increase in the H_2 output. Eventually, it led to about a 50% reduction in the stir bar-mixed culture (Fig. 13a). The higher photosynthetic activity of the cultures, due to the higher PFD to which they were exposed, caused an increase in the dissolved O_2 concentration which led to an appreciable water displacement in both PBRs (Fig. 13). Nonetheless, the impeller-mixed culture showed a higher respiratory capability as, before the start of H_2 production, such a water displacement was fully "recovered" (Fig. 13b), contrary to what observed with the stir bar one. As a consequence, H_2 production started later than in the impeller mixed-culture and led to a reduced ability to maintain anaerobic conditions, as demonstrated by the trace of O_2 (0.9 mg L^{-1}) observed after about 60 hours (Fig. 13a). This situation may hold also for the first part of the H_2 production, as demonstrated by the significant lower rates of maximum production found in the culture mixed with stir bar, 1.61 vs. 2.38 mL L^{-1} h^{-1} with the impeller. Indeed, the incapacity to measure O_2 does not preclude the possibility that micromolar quantities of O_2 are present on a transient basis in the cell or in the PBR itself (Kosourov et al., 2002). In addition, it was shown that after the migration of LHC-II proteins from the PSII to PSI within the state 2 induction (Antal et al., 2001; 2003), PSII recovers some activity (Antal et al., 2001). This happens in combination with H_2 release by the culture, as a consequence of hydrogenase activity which partly oxidizes photosynthetic carriers like the plastoquinone (PQ) pool, thus increasing the rate of the electron transfer from PSII to plastoquinones (Antal et al., 2001). In this connection, it is likely that such a PSII recover had pushed O_2 release over respiratory consumption, acting like a negative feedback on the hydrogenase activity. A high dissolved O_2 concentration in combination with high light can be toxic to cells, because O_2 radicals can be generated, which promote photoinhibition (Krause and Kornic, 1994; Torzillo et al., 1998). Moreover, an overly long exposure of cells to excessive light as result of inadequate mixing (like that achieved with stir bar), may have also caused a reduction in the accumulation of starch during the aerobic phase to consume the O_2 present in the medium (Ghirardi et al., 2000b; Fouchard et al., 2005; Kosourov et al., 2003; 2007; Melis, 2002; 2007; Melis and Happe, 2001; Makarova et al., 2007). Finally, the inability of maintaining the anaerobiosis naturally led to the inhibition of the hydrogenase itself, which expression is strongly inactivated by O_2 both at transcriptional and post-translational level (Ghirardi et al., 1997; Melis, 2002; Flynn et al., 2002; Happe et al., 2002; Happe and Kaminski, 2002; Forestier et al., 2003; Posewitz et al., 2004).

The renewed increase in the O_2 concentration in the stir bar-mixed culture was clearly mirrored by a rise in the redox potential recorded at about 70 hours (Fig. 13a). Subsequently, the O_2 concentration increased up to 2 mg L^{-1} at the end of the process. Notwithstanding this very limited presence of O_2,

the redox potential continued to decrease, most likely as a result of the increasing degree of reduction in the photosynthetic chain. In fact, at this point the H_2 release was terminated: thus, the capability of the cells to rid themselves of the excess of reducing power generated by photosynthesis was reduced as well. It is worth mentioning that such problems were completely absent in the impeller-mixed culture throughout the entire experiment, even if an equal O_2 productivity was observed (Fig. 13) soon after sealing PBRs (i.e., when PSII activity was not affected by sulfur starvation yet). In this respect, a more rapid and homogeneous spatial distribution (see also Fig. 7) of respiration substrate (i.e., O_2)- like that achieved with the impeller- is consistent with an effective increased respiration capacity of cultures.

As observed in the previous set, impeller-mixed culture pH value was slightly higher than stir bar one (8.11 vs. 8.03, respectively) at the end of aerobic phase. However, as a consequence of H_2 production interruption after only 60 hours in the stir bar-mixed culture, a higher value was found in the latter at the end of the experiment (7.80 vs. 7.70, in stir bar and impeller, respectively) (data not shown).

The study of the interrelationship between light intensity and chl concentration continued by comparing the H_2 output of cultures in which the chl concentration was increased to 24 mg L^{-1}, while the light intensity was unchanged as compared with previous experiment (140 μmol photons m^{-2} s^{-1} per side). The rationale of this experiment was to reduce the effect of excessive light at which cultures were exposed in the previous set, by increasing the chl concentration so as to "dilute" the light on a larger number of cells. If that were the case, it should have produced an increased amount of H_2 per unit of reactor volume, provided that cells were subjected to an adequate mixing in order to expose them to a more homogeneous light field. The results of this experiment are shown in Figure 14.

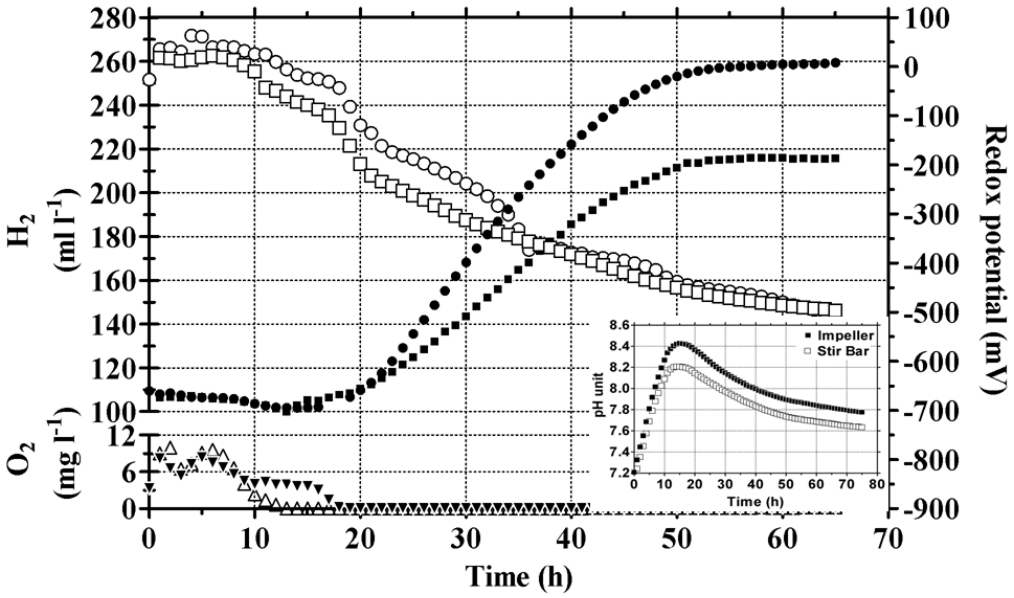


Figure 14. Time courses in the H_2 outputs recorded in the strain CC124 under a PFD of 140 µmol photons m^{-2} s^{-1} supplied on both sides and a 24 mg L^{-1} chl concentration, mixed using a stir bar (■) and an impeller (□). Keys: Redox potentials, impeller mixed culture (○), stir bar (□); O_2, impeller mixed culture (▼), stir bar (△). Insert: pH behavior during the experiment observed with the use of an impeller (■) and a stir bar (□).

Both cultures performed much better than in the previous experiments. The total amount of H_2 produced reached almost 120 mL L^{-1} in the culture mixed with the stir bar, and rose to almost 170 mL L^{-1} in the impeller-mixed one. Moreover, maximum rates reached 3.72 and 5.66 L L^{-1} h^{-1}, respectively. These productivities, translated on an PBR areal basis, corresponded to about 90 and 140 mL m^{-2} h^{-1}, respectively. No important differences were observed in the cultures as regards the redox potential value during the experiment, while certain differences were noted for the pH (Fig. 14, insert). Indeed, it increased up to 8.45 and 8.20 in the impeller- and stir bar-mixed cultures, respectively, and decreased to about 7.70 at the end of the experiment in both cultures. A summary of all the information gathered during the different set of experiments carried out in this section is provided in Table 5.

Table 5. Summary of the H_2 production experiments carried out with *Chlamydomonas reinhardtii* cultures (strain CC124) tested in PBRs equipped with two mixing systems and exposed to different combinations of light intensities and chl concentrations. The data represent an average of 4-10 independent experiments. Max rate duration was estimated as the time during which H_2 accumulation was rather constant. Keys: SD, standard deviation.

Culture conditions				Aerobic phase				Anaerobic phase				H_2 total volume		H_2 final rate		H_2 max rate		H_2 final rate		H_2 max rate		Max rate duration	
Incident PFD	Chl content	Mixing system		O_2 prod phase	S.D.	O_2 resp phase	S.D.	Lag phase	S.D.	H_2 prod phase	S.D.												
µmol photons $m^{-2}s^{-1}$	mg L^{-1}			hours				hours				mL H_2 L^{-1}		mL H_2 L^{-1} h^{-1}				mL H_2 g Chl^{-1} h^{-1}				hours	
70+70	12	Stir bar		13.67	1.53	7.66	0.41	1.34	0.03	101.00	0.47	99.7	11.5	0.99	0.11	2.23	0.32	82.5	9.2	185.8	26.7	29	4
		Impeller		4.50	0.71	20.17	1.25	5.33	0.25	62.50	6.21	87.2	8.1	1.39	0.04	2.44	0.23	115.8	3.3	203.3	19.2	19	2
70+70	24	Stir bar		-	-	-	-	-	-	83.00	2.00	82.1	26.3	0.98	0.34	1.90	0.07	40.8	14.2	79.2	2.9	31	1
		Impeller		-	-	-	-	-	-	64.75	0.83	132.9	17.8	2.05	0.28	2.87	0.51	85.4	11.7	119.6	21.3	36	6
140+140	12	Stir bar		8.00	1.41	18.00	2.94	6.00	1.06	62.50	0.47	48.8	29.1	0.78	0.39	1.61	0.12	65.0	32.5	134.2	10.0	22	2
		Impeller		5.50	0.71	22.00	5.09	6.00	1.14	70.50	15.34	95.0	25.7	1.35	0.38	2.38	0.18	112.5	31.7	198.3	15.0	25	2
140+140	24	Stir bar		5.67	0.58	6.33	0.74	1.50	0.39	47.83	7.37	118.9	1.7	2.49	0.04	3.72	0.38	103.8	1.7	155.0	15.8	23	2
		Impeller		4.60	1.67	13.65	1.12	0.55	0.07	41.70	1.29	167.6	17.1	4.02	0.21	5.66	0.05	167.5	8.5	235.8	2.1	20	0

While in the third set a benefit was achieved mainly by means of respiration, in the second and forth set during which high chl concentrations were adopted (24 mg L^{-1}), an effective benefit was

achieved by means of L/D cycles. It is well known that the benefit deriving from the intermittent light pattern is better realized in dense cultures exposed to intense light, much above the saturation level. Although even the highest PFD used to illuminate cultures (140 μmol photons m^{-2} s^{-1} per side) was much lower than the one normally tested in the flashing light effect experiments by Kok (1953) (which was usually about 5 times higher than that necessary to saturate photosynthesis), it must be pointed out that CC124 strain P/I curve starts to flatten out (I_k) at about 30 μmoles m^{-2} s^{-1} (Fig. 9). This means that above such a value the control of photosynthesis passes from light absorption and photochemical energy conversion to reductant utilization. Moreover, during the H_2 production phase, it has been found that the photosynthesis rate declines considerably, as a result of an increased photoinhibition of PSII. The latter is exacerbated by sulfur starvation which leads to a strong reduction in the initial slope of the P/I curve, as well as to a more than 3-time reduction in the saturation level of photosynthesis (Zhang and Melis, 2002).

Fast cycles to which cells were subjected in the impeller-mixed culture likely led to a partial light integration: photosynthesis rates were shifted back from saturation to the linear part of the P/I curve, thus improving the efficiency of light utilization. This hypothesis seems to be supported by the observation that cultures exposed to the same light intensity could not benefit from the L/D cycles when their chl concentration was 50% lower. In these cultures, an excess of light led to an increased photoinhibition rate, and this was particularly evident in the culture with stirring provided by the stir bar, as shown by its lower maximum rate compared with the impeller-mixed-culture one.

In order to obtain further insight into the physiological changes caused to the cultures by the two different mixing systems, biochemical analysis of the main cell components were performed with respect to the last experiment (140 μmol photons m^{-2} s^{-1} per side and 24 mg L^{-1}). These analyses were accompanied by parallel measurements of the chl fluorescence (Table 6).

Table 6. Changes in the carbohydrate and protein contents measured in a sulfur-deprived *Chlamydomonas reinhardtii* culture mixed with the use of different devices. Changes in effective quantum yield of PSII are also shown. The experiments were carried out with a PFD of 140 µmol photons m^{-2} s^{-1} supplied on both sides and 24 mg chl L^{-1}.

Culture mixed with a stir bar				
Time	Carbohydrates	Proteins	P/C ratio	Fluorescence
Hours	%			$\Delta F/F'_m$
Start of the experiment	100	100	3.20	0.460
Start of the H_2 production	195	112	1.84	0.090
End of the experiment	110	104	3.03	0.030
Culture mixed with an impeller				
Time	Carbohydrates	Proteins	P/C ratio	Fluorescence
Hours	%			$\Delta F/F'_m$
Start of the experiment	100	100	3.39	0.590
Start of the H_2 production	322	112	1.18	0.120
End of the experiment	128	93	2.45	0.050

In accordance with what reported in literature (Melis et al., 2000; Zhang et al., 2002; Tsygankov et al., 2002), a strong accumulation of carbohydrates occurred before the H_2 production took place. However, the culture mixed with the impeller performed much better than the stir bar one (322 vs. 195%, Tab. 6). Such a difference was also reflected by the higher pH value reached by the impeller-mixed culture (Fig. 14, insert), as a consequence of a higher acetate consumption, which is strongly utilized during the aerobic phase to store endogenous substrates such as carbohydrates (Kosourov et al., 2003; 2007). Proteins showed just a small increase during the aerobic phase in both cultures (12%), probably due to a slight increase in the number of cells, which is then inhibited by sulfur starvation (Zhang et al., 2002).

The better performance showed by the culture mixed with the impeller was also evidenced by the chl fluorescence measurement taken during the H_2 production process. The effective quantum yield of PSII ($\Delta F/F'_m$) was significantly higher in the culture mixed with the impeller, particularly during the first hours of the aerobic phase, when an accumulation of carbohydrates takes place (Tab. 6). Moreover, a slightly higher residual PSII activity was measured at the onset of H_2 production, as well as at the end of the process, indicating that the L/D cycles somehow protected the PSII from rapid photoinhibition, since it could recover in the dark portion of the cycle.

In summary, better L/D cycles realized with the multiple impeller system enabled a better culture performance and influenced the H_2 production process in different ways: (1) directly, by providing the PSII with a higher production of electrons for the hydrogenase; (2) indirectly, through a higher

synthesis of carbohydrates, which were subsequently used to feed electrons to the PQ pool and to the hydrogenase and (3) by means of a quick spatial distribution of the photosynthetically produced biogas, which increased the respiration capacity thus maintaining anaerobiosis in the culture even at high light intensities.

2.6 Apparent light conversion efficiency

A direct comparison of culture performances based on the amount of H_2 produced per unit of volume is difficult, since it is affected by several parameters, such as: (1) the light intensity and its spectra, (2) the chl concentration, (3) the geometry of the PBR and its surface-to-volume ratio, (4) the stirring system and the rate of mixing. For these reasons, in order to compare the culture performances with what is available in literature, the light transformation efficiency of the process was evaluated as the ratio between the energy stored in H_2 and the incident irradiance on the PBR. This parameter includes all factors affecting the above-mentioned performance of the culture, except acetate consumption which was not considered. This choice was facilitated by the flat geometry of the PBR (Roux bottle type), which made it possible to easily obtain an accurate measurement of the incident irradiance. Apparent light conversion efficiency was calculated for final H_2 outputs and maximum production rates, which were sustained for at least 20 hours (see Tab. 5). In general, cultures mixed using the impeller performed better than those mixed using the stir bar during all set of experiments (Table 7).

Table 7. Apparent light conversion efficiency of the *Chlamydomonas reinhardtii* CC124 strain achieved in different culture conditions.

Culture conditions		Total H_2 output		Max rate H_2 output	
PFD	Chl content	Stir Bar	Impeller	Stir Bar	Impeller
µmol photons m^{-2} s^{-1}	mg L^{-1}	Energy Conversion Efficiency (%)			
70+70	12	0.468	0.547	1.260	1.420
70+70	24	0.477	0.965	1.100	1.670
140+140	12	0.150	0.265	0.460	0.690
140+140	24	0.563	0.805	1.080	1.640

As expected, the highest value of light conversion efficiency was attained when the cultures were very photolimited, i.e. when they were exposed to combination of low light and high chl concentration (70 µmol photons m^{-2} s^{-1} and 24 mg L^{-1}) and mixed with impeller (0.97%). To calculate this apparent light conversion efficiency, it was assumed that: (a) the energy content of H_2

at 25 °C was 12.94 J mL^{-1} (for energy calculations, the upper combustion value- or gross caloric value- of H_2 was used); (b) an amount of 4.6 μmol of photons m^{-2} s^{-1} was equivalent to 1 W m^{-2}; (c) the incident energy was 195903 J, which is the product of 70 μmol photons m^{-2} s^{-1} x 80 h x 3600 s x 0.0447 m^2 (both sides of the PBR) / 4.6 μmol photons m^{-2} s^{-1}); (d) the total amount of H_2 produced by the culture was 146.2 mL PBR^{-1}. The light conversion efficiency thus obtained was:

Light conversion efficiency (%) = 146.2 mL PBR^{-1} x 12.94 J mL^{-1} / 195903 J x100 = 0.97%.

Notwithstanding the fact that the highest conversion efficiency was found in photolimited conditions, it was interesting to note that the light conversion efficiency remained quite high even when a high light condition was combined with a high chl concentration (PFD of 140 μmol photons m^{-2} s^{-1}, 24 mg L^{-1}), particularly when using the impeller system. This indicates that a light dilution was effectively achieved in well-mixed cultures. In this set, the light transformation efficiency during the entire H_2 production experiment reached the values of 0.81% and 0.56%, in the impeller- and stir bar-mixed cultures, respectively. It is worthwhile noting that such a relatively high light conversion efficiency (0.81%) was reduced by only 17% respect to what previously obtained with the impeller at same chl concentration (24 mg L^{-1}, 0.97%) even though the light intensity was doubled (140 μmol of photons m^{-2} s^{-1}). Indeed, the conversion efficiency increased up to 1.64% if the maximum rates were considered, equal to what obtained when photolimited conditions were provided (1.67%, Tab. 7). These values are considerably higher than those reported by Fouchard et al. (2008) with the same strain under sulfur-deprivation, and are comparable with those attained by Kosourov and Seibert (2009) and Ghirardi (2006) with immobilized *Chlamydomonas* cultures in which a considerably higher amount of chl was used. Unfortunately, these better yields are still very far from the theoretical maximum achievable with direct biophotolysis, which would predict a 10-13% light conversion of the total solar radiation (Tredici et al., 1997; Ghirardi et al., 2009b), provided that organisms with hydrogenase resistant to O_2 were available and that neither light saturation nor photoinhibition would affect H_2 production outdoors.

Section 2 Conclusions and future perspectives

In conclusion, results demonstrate that a proper stirring can contribute to an improvement of H_2 productivity, and this may be particularly relevant if mutants with higher H_2 production capacity are considered (see also Section 3b). Moreover, the impeller mixing system demonstrated to be a valuable tool for studying H_2 production with photosynthetic microorganisms and for obtaining a

better insight into the process physiology. To confirm the goodness of such a reactor and its flexibility towards other photosynthetic biological processes, H_2 production experiments with purple non sulfur bacteria are now in progress (Figure 15).

Figure 15. H_2 production experiments by means of photo-fermentative processes carried out with purple non sulfur bacteria, using the impeller mixing device developed by us.

SECTION 3.

Physiological features of selected D1 protein mutant strains and optimization of the H_2 production in batch conditions

a) Physiological characterization of the D1 protein mutant L159I-N230Y in growing and hydrogen producing conditions

3a.1 Reduction of the antenna size and increased photosynthetic capability

The H_2 production screening (Section 1) showed that the D1 mutant L159I-N230Y was the most productive strain out of 22 tested strains of *Chlamydomonas*. Previous observations pointed out that photosynthetic and respiratory capacity of the mutant L159I-N230Y were particularly higher than both CC124 and WT (11-32b) (namely, P_{max} was 2.8 and 2.2 times higher, respectively; respiration rate was about 1.7 times higher respect to both controls; see Tab. 3). Part of the results shown in this section were published on the International Journal of Hydrogen Energy (Torzillo et al., 2009). The phenotypic characterization of the mutant showed relevant changes with respect to the WT. Figure

16 reports the chl accumulation measured during photomixotrophic growth. As can be seen, the rate of chl accumulation in the mutant proceeded at a much slower rate than in the WT and CC124. However, the total amount of chl accumulated by the mutant was comparable to CC124 strain and about 25% higher than the WT.

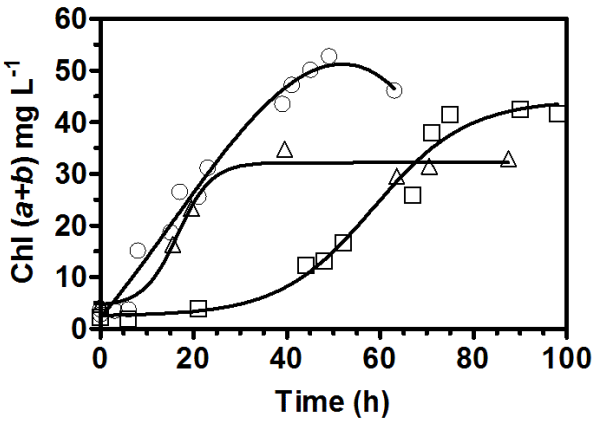


Figure 16. Time course of *C. reinhardtii* strains growth followed as chl accumulation. The cultures were grown photomixotrophically on TAP medium, irradiated with 70 µmol photons m^{-2} s^{-1} per side, at 28°C, light path 5 cm (diameter of cylindrical PBRs). Results are the mean value of three experiments made on independent cultures. Keys: WT, (△); mutant L159I-N230Y, (□); CC124, (○).

In Table 8, quantification of some fundamental parameters evaluated during the growth are reported.

Table 8. Characterization of *C. reinhardtii* mutant L159I-N230Y during the logarithmic phase of growth on TAP, and comparison with its wild type (WT [11-32b]) and CC124.

Strains	Chl	Chl a/b ratio	a*	Maximum dry weight +
	% of dry weight		m^2 mg^{-1} Chl *a*	g L^{-1}
WT (11-32b)	3.20	2.90	0.0263	1.42
L159I-N230Y	1.80	3.00	0.0336	2.05
CC124	3.40	2.80	0.0250	1.52

The main feature of the mutant was a lower chl content per dry weight (1.8%) compared to the WT (3.2%) and the CC124 (3.4%). Such a lower chl content resulted in an increased optical absorption cross-section, which positively affected biomass accumulation (dry weight) during the photomixotropical growth: the latter was indeed found to be between 35% and 44% higher in the mutant respect to the CC124 and the WT, respectively. A lower chl content confers a number of advantages on the strain. It can be grown at higher light intensity and reach higher biomass concentrations without incurring in the so-called "low light acclimation effect", which leads to an increased amount of pigment antenna per cell. This represents a strong limitation in the microalgae

mass culture where high cell density is desirable. A number of studies have been carried out to reduce the antenna size of technologically important microalgae (Nakajima and Ueda, 1997, 1999; Neidhardt et al., 1998; Melis et al., 1999; Nakajima et al., 2001; Masuda et al., 2003), including *Chlamydomonas* (Polle et al., 2000; 2001; 2002; Melis, 2005; Tetali et al., 2007). It was shown that chl b-less *C. reinhardtii* strains report a chl antenna size that is truncated (Michel et al., 1983; Picaud and Dubertret 1986; Allen and Staehelin 1994). However, that was not the case as the reduction in the amount of chl in the mutant L159I-N230Y involved both chl *a* and *b*, since the chl a/b ratio remained substantially similar to that found in its WT. Figure 17 shows the morphological aspect of the mutant L159I-N230Y and WT streaked on the TAP agar medium grown photoeterotrophically.

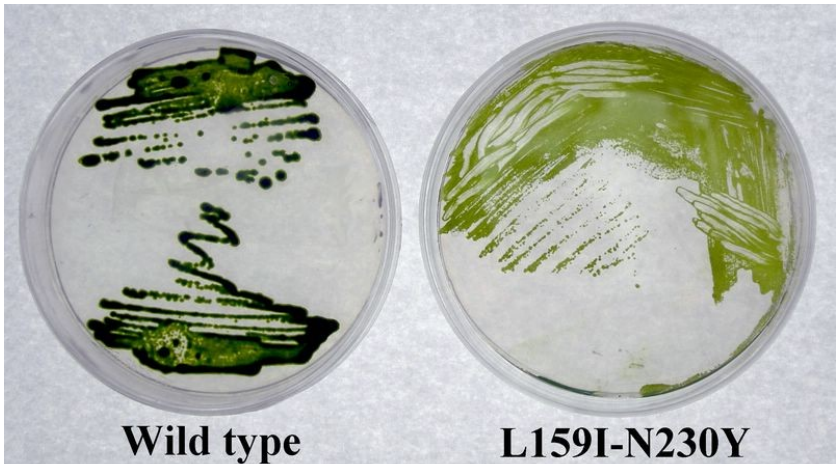


Figure 17. Picture of *C. reinhardtii* WT (11-32b) and D1 mutant strain L159I-N230Y streaked on TAP agar medium.
Cultures were grown photoeterotrophically for 10 days at 50 μmol photons m^{-2} s^{-1} and 25 °C.

Visual observations of the mutant compared to the WT supported the hypothesis of a lower chl content also per cell amount: upon the same amount of chl (12 mg L^{-1}), D1 mutant L159I-N230Y showed to have 2.6 more cells than the WT. However, it must be noted that a first slight increase in cell number was already achieved by removing the four large introns at the level of the *psbA* gene, that is with strain IL, which had 1.2 times more cells than the WT. While in WT cultures single algal cells- most of which with a high motility- were typically observed, mutant strain L159I-N230Y showed always numerous cells grouped together, with reduced or no motility at all (and with no flagella), typical of cultures which are undergoing a very high number of divisions (Cavalier-Smith, 1974) (Figure 18).

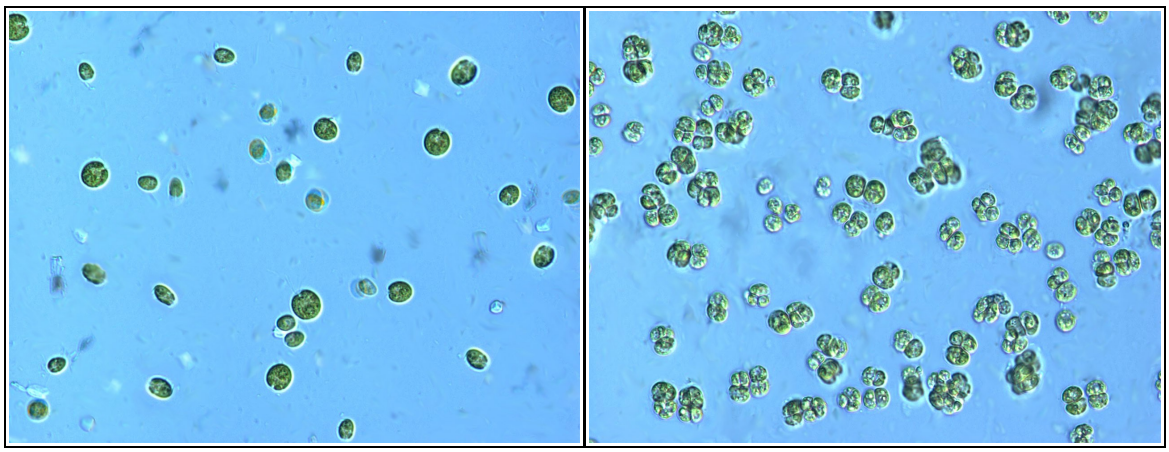


Figure 18. *C. reinhardtii* algal cells in WT (11-32b) (left) and D1 protein mutant strain L159I-N230Y (right), in photomixotrophical growing conditions on TAP medium.

On the whole, the phenotypic adjustment of the photosynthetic apparatus of the mutant corresponded to that of a high-light acclimated photosynthetic organism, with a similar number of traps but increased antenna size (Ramus, 1981). Examples of the theoretical aspects of such a phenotype are reported in Figure 19a,b.

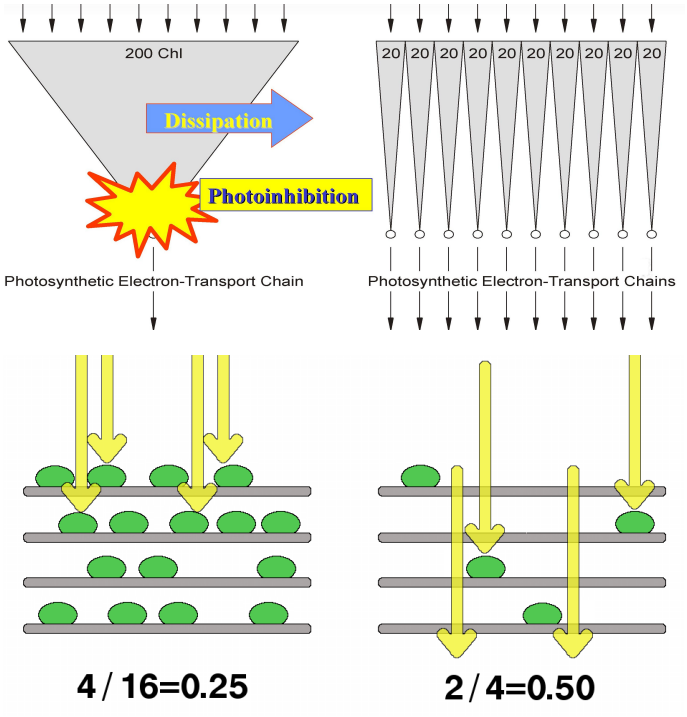


Figure 19a. Theoretical benefits of a reduced light harvesting antenna (right) compared to a control (left), on a chl basis. Note that, upon the same amount of chl, the number of traps is increased (right), thus leading to a higher number of electrons driven into the electron transport chain (Courtesy of Dr. John Benemann, modified).

Figure 19b. Theoretical benefits of a larger chl cross-section of the light harvesting antenna (right) respect to a control (left), on a cell basis. Note that, upon the same amount of cells, reduced amounts of light harvesting antenna complexes may lead to a higher efficiency per absorbed photon (Courtesy of Dr. Luca Giannelli, modified).

Loss of chl in D1 mutants is not surprising, as this fundamental PSII core protein, in association with its sister protein D2, forms a heterodimer that is involved in binding cofactors such as pheophytin, plastoquinone, metals like iron and manganese, and chl (Johanningmeier et al., 2005). Results of the quenching analysis of chl fluorescence performed by means of a stepwise increase in

the actinic light intensity for the WT and the mutant are shown in Figure 20. Interestingly enough, the mutation (L159I-N230Y) did not affect the effective quantum yield of PSII. Indeed, both mutant and WT showed the same changes in the $\Delta F/F'_m$ value. The decline in the $\Delta F/F'_m$ in both strains was the result of an increased level of steady-state fluorescence (F'_s), while the maximum fluorescence yield F'_m was little affected in the case of the WT or completely unaffected in the mutant (Fig. 20). As a result, when cells were exposed to the maximum light irradiance of 2000 μmol photons m^{-2} s^{-1}, in the WT only a small rise in the non-photechemical quencing (NPQ, up to 0.21) was found, while it remained close to zero in the mutant (data not shown). An estimation of the linear electron transfer rate (ETR) by means of PSII (Figure 21) confirmed a higher performance in the mutant due to the increase in the optical absorption cross-section (Tab. 8).

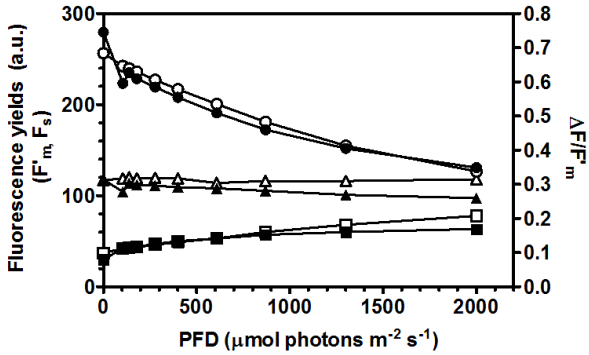


Figure 20. Chl *a* fluorescence parameters recorded during quenching analysis of *C. reinhardtii* WT, and D1 mutant L159I-N230Y. Keys: F'_s, steady-state level of fluorescence in the light, WT (■), L159I-N230Y (□); F'_m, maximum yield of PSII fluorescence in the light, WT (▲), L159I-N230Y (△); $\Delta F/F'_m$, effective quantum yield of PSII, WT (○), L159I-N230Y (●).

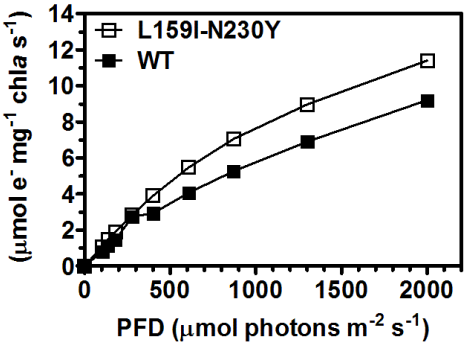


Figure 21. Light response curve of electron transport rate (ETR) of *C. reinhardtii* WT, and D1 mutant L159I-N230Y. For ETR calculation details, see Materials and Methods. Keys: reported in the graph.

3a.2 Hydrogen production in sulfur starvation: redox potential, pH value, O_2 production/consumption and PSII photoprotection in standard conditions

A further characterization of the mutant L159I-N230Y was carried out by taking advantage of the continuous monitoring system (Kosourov et al., 2002, mod.) in order to find out redox potential, O_2 production/consumption and pH ongoing during the H_2 production process when supplying standard conditions (i.e., the ones adopted during the H_2 production screening, Section 1). Mixing was allowed by a conventional stir bar placed at the bottom of the PBR. Strain CC124 (which was already fully characterized under these conditions, Section 2) and WT (11-32b) are also reported for

comparison purposes (Figure 22).

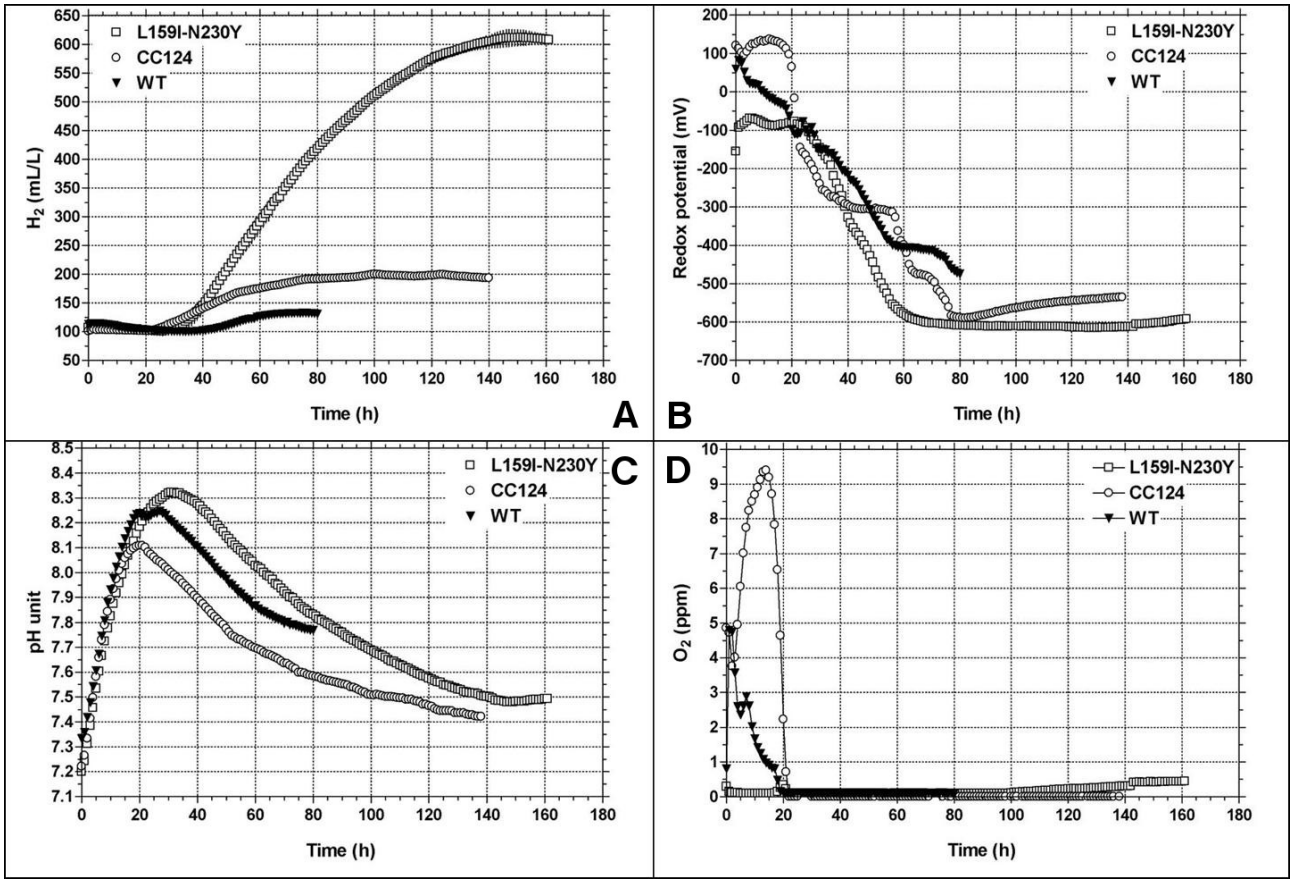


Figure 22: Time course of: (A) Cumulative H_2 gas production, (B) redox potential, (C) pH value and (D) dissolved O_2 concentration, in the D1 mutant strain L159I-N230Y, strain CC124 and WT (11-32b). Cultures were resuspended in TAP-S to an initial chl content of 12 mg L^{-1}, initial pH 7.2 and irradiated with 70 µmol photons m^{-2} s^{-1} per side. Culture volume was equal to 1.1 liters, temperature was adjusted at 28°C by placing PBRs in a bath filled with deionized thermo-stated water. Mixing was allowed by a magnetic stir bar placed at the bottom of the PBR. Results were obtained taking advantage of the continuous monitoring system (Kosourov et al., 2002, mod; see Materials and Methods for details). Note that for graphical reasons the start of H_2 accumulation (A) is equal to 100 mL/L. Tzero represents the time at which cultures were sealed. Vertical bars represents the standard deviation. Results are the mean value of experiments repeated at least three times on independent cultures. Keys are reported in the graph.

The performance of the mutant L159I-N230Y in the H_2 gas final production confirmed to be more than 5-times higher than what is usually found with the strain CC124 and more than 16 times higher than its WT (Tab. 1). However, it must be noted that the H_2 production phase was strongly reduced in the mutant compared to what observed previously (Tab. 1), most likely as a consequence of the biogas collecting system. Indeed, aside from the possibility to evaluate the end of the process in any moment thanks to the continuous recording of the data, in this last set of experiments the collecting system was set up in order to maintain a constant pressure (-4.04 kPa) throughout the entire experiment. On the contrary, in the former set up (Fig. 1), the degassing pressure naturally changed

as long as H_2 production occurred, ranging from -4.04 kPa (at the start of the experiment) to virtually zero, when reaching the maximum supported volume of the calibrated cylinder (about 700 mL). Thus, also considering that the PBR volume was 1.1 liters, when testing high H_2 productive strains (about 500 mL L^{-1}), this situation may have held for a strong delay in the effective evaluation of production times because of the time required to the biogas to reach a stable value within the calibrated cylinder.

Interestingly, in the mutant L159I-N230Y dissolved O_2 was absent since the onset of the experiment, while a typical O_2 production followed by a sharp consumption occurred in both controls (Fig. 22d). This was reflected in the redox potential pattern of the mutant (Fig. 22b), which was always in the negative field (about -100 mV at the onset of the experiment). Concerning the pH value, it increased up to 8.32, 8.25 and 8.10 in L159I-N230Y, WT and CC124 respectively. As aforementioned, a higher increase in the pH value during the aerobic phase is an indirect measure of the consumption of acetate contained in the medium, which may reflect on a proportional increase in the amount of starch that is stored (Tab. 6 and Fig. 14; Kosourov et al., 2007). At the end of the experiment, pH value in the WT was equal to 7.80, while it decreased to about 7.45 in CC124 and L159I-N230Y. It is not clear what could be responsible for WT reduced H_2 production, but it appeared evident that some physiological limitation occurred. Some other observations can be done by considering the summary of all the specific phases and production rates obtained by adopting the continuous monitoring system (Table 9).

Table 9. Summary of the H_2 production experiments carried out in standard conditions with *Chlamydomonas reinhardtii* strains WT (11-32b), D1 protein mutant L159I-N230Y and CC124.

Results are the mean value of experiments repeated at least three times on independent cultures.

Strain	Aerobic phase		Anaerobic phase		H_2 total volume	H_2 Final rate	H_2 Maximum rate	H_2 Final rate	H_2 Maximum rate
	O_2 production phase	O_2 respiration phase	Lag phase	H_2 production phase	hours			mL H_2 L^{-1}	mL H_2 L^{-1} h^{-1}	µmoles H_2 mg^{-1} chl h^{-1}	
WT (11-32b)	1.5 ± 0.7	18.0 ± 1.4	15.5 ± 2.1	42.0 ± 0.0	32.2 ± 0.8	0.77 ± 0.02	1.36 ± 0.18	249 ± 0.06	4.40 ± 0.58
L159I-N230Y	-	-	26.0 ± 3.52	139.5 ± 24.7	513.6 ± 11.5	3.75 ± 0.75	6.86 ± 0.22	1214 ± 2.43	21.63 ± 0.71
CC124	13.7 ± 1.5	7.7 ± 0.4	1.3 ± 0.0	101.0 ± 0.5	99.7 ± 11.5	0.99 ± 0.11	223 ± 0.19	3.20 ± 0.36	7.22 ± 0.62

The O_2 production phase of the WT (11-32b) was particularly short when compared to the CC124, while O_2 respiration phase and the following anaerobic lag phase were much longer. It is likely that the supplied conditions may have led to an excessive consumption of energy reserves in the WT

(e.g., starch) that negatively affected H_2 production (Ghirardi et al., 2000b; Fouchard et al., 2005; Kosourov et al., 2003; 2007; Melis, 2002, 2007; Melis and Happe, 2001; Makarova et al., 2007). Similar observations were done when optimizing the mixing system of the PBR using another strain (CC124, Tab. 5): indeed the reduction of both these phases (O_2 respiration and anaerobic lag phase) during batch experiments is crucial to reach the highest H_2 productivity.

Another very peculiar feature of the mutant was its ability to accumulate high amounts of the carotenoid (car) zeaxanthin during the H_2 production process. It is well known that zeaxanthin, together with violaxanthin and antheraxanthin, is involved in the xanthophylls cycle, which is fundamental to the energy dissipation within the antenna (Demmig-Adams and Adams, 1996; Niyogi et al., 1997; Masojiedeck et al., 1999). In the mutant, the zeaxanthin level at the end of the H_2 production process reached 114 mmol mol^{-1} chl *a*, while in the WT and CC124 it was less than 70 mmol mol^{-1} chl *a*. The ability to convert very large amounts of violaxanthin to zeaxanthin during the H_2 production process is a prerequisite for protecting PSII from rapid degradation and thus for prolonging the PSII-based H_2 production period.

3a.3 Hydrogen production by means of anaerobic induction

Other than sulfur deprivation, the anaerobic induction (AI) can represent a very fast and smart way to evaluate H_2 production capability in different strains (Prof. Thomas Happe is gratefully acknowledged for his technical support during this phase of the experimental work). In fact, given that in such conditions production occurs only for few hours (up to about 4-6 hours, for a Review, see Hemschemeier et al., 2009), hydrogenase expression can be quickly induced by means of anaerobiosis, which is reached thanks to three different conditions: (1) inert gas bubbling (e.g., Argon or Nitrogen); (2) use of very dense cultures (up to 100-120 mg chl L^{-1}), and (3) dark conditions provided throughout the entire experiment. In this way, O_2 inhibition is quickly avoided and hydrogenase activity can be evaluated either by *in vitro* or *in vivo*. *In vitro* analysis represents the evaluation of the quantity of active hydrogenase present in the culture: the H_2 production obtained this way is the highest possible, since there is no limitation due to the electron source, as usually happens *in vivo*. The latter is obtained by providing intense light to anaerobically incubated samples (using complete TAP medium). It is of interest to note that the H_2 production occurring in these conditions is achieved basically because of direct photosynthesis from PSII: it represents a boost in H_2 evolution due to a high accumulation of reducing power that, once the culture is illuminated and hydrogenase enzyme is expressed, can be utilized to evolve H_2 gas. By applying the AI protocol, the D1 protein mutant L159I-N230Y was characterized with respect to two control

strains, CC124 and WT (11-32b), and strain IL (to which all D1 protein mutants belong, being the first step of WT genetic manipulation). The latter was tested as it showed to be unable to evolve H_2 gas during the initial screening described in Section 1, under standard conditions (Tab. 1).
The kinetic of induction of the hydrogenase expression was different in each strain (Figure 23).

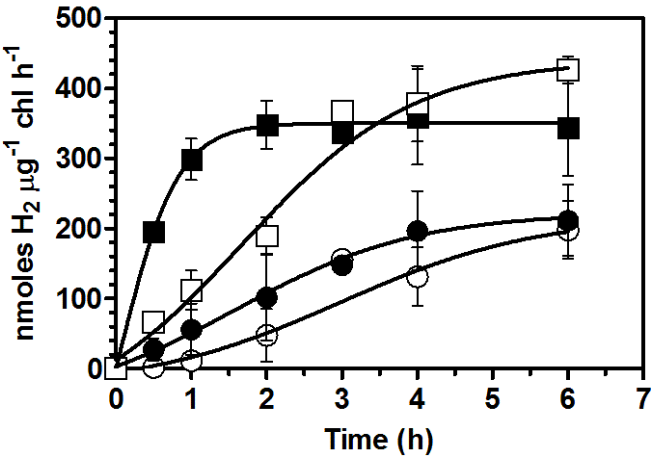


Figure 23: *In vitro* hydrogenase activity during anaerobic induction in two D1 protein mutant strains (L159I-N230Y and IL) and two controls (WT [11-32b] and CC124) of *Chlamydomonas reinhardtii*.
Results are the mean value of 3-5 independent experiments. Vertical bars indicate standard deviation. Keys: WT, (●); CC124, (■); L159I-N230Y, (□); IL, (○).

The CC124 promptly induced the enzyme expression, reaching the level of saturation within the first-second hour of experiment. On the other hand, the WT and its D1 protein mutant strains (L159I-N230Y and IL) had a similar pattern although showing different levels of expression: hydrogenase activity was still comparable among them within the first-second hour of experiment after which strain L159I-N230Y quickly reached the level of CC124 (third hour), slightly increasing thereafter. No significant difference was observed between WT and IL, even though the latter was always lower. Notwithstanding a high *in vitro* expression of the hydrogenase enzyme, the CC124 strain had a very low *in vivo* H_2 productivity, comparable to the other strain used as a control (WT) and roughly 3 times lower than both D1 mutant strains (Figure 24).

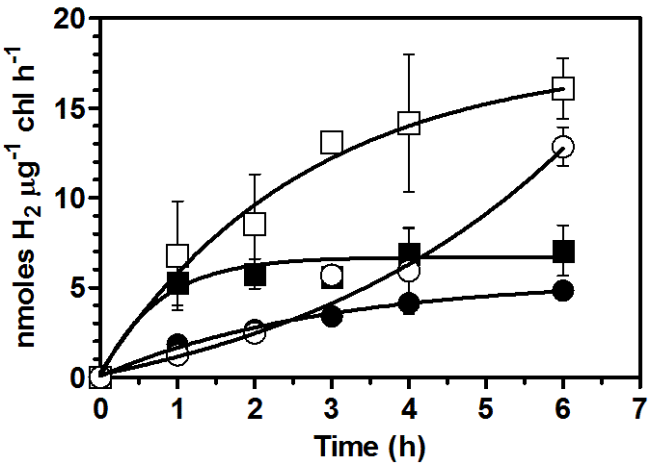


Figure 24: *In vivo* hydrogenase activity during anaerobic induction in two D1 protein mutant strains (L159I-N230Y and IL) and two controls (WT [11-32b] and CC124) of *C. reinhardtii*. Results are the mean value of 3-5 independent experiments. Vertical bars indicate standard deviation. Keys: WT, (●); CC124, (■); L159I-N230Y, (□); IL, (○).

It was not surprising to ascertain in the mutant L159I-N230Y the highest *in vivo* H_2 production, as it was already seen that a higher PSII-mediated ETR (Fig. 21), together with a lower chl per cell, contributed to such a result. On the other hand, IL strain showed an incredibly high capacity of photoproducing H_2 gas, almost reaching L159I-N230Y strain at the sixth hour (13 vs. 16 nmoles of H_2 μg^{-1} chl h^{-1}, in IL and L159I-N230Y respectively). This result clearly demonstrates that IL strain capability to drive electrons to the hydrogenase directly from the PSII is not simply allowed, but even much more effective than in its WT. Thus, its inability to evolve H_2 gas must be searched within the complex interrelationship of different pathways (Wykoff et al., 1998) and genes expression (Davies et al., 1994; Takahashi et al., 2001; Ravina et al., 2002; Zhang et al., 2004; Eberhard et al., 2006) triggered or inhibited by sulfur deprivation. It must be noted once again that *in vitro* hydrogenase expression of IL strain remained always very low throughout the AI (Fig. 23). This observation is relevant when considering the efficiency of utilization of the hydrogenase enzyme. To evaluate it, *in vivo* measurements were plotted versus *in vitro* ones (and expressed as a percentage; Figure 25). Both the D1 protein mutant strains (L159I-N230Y and IL) had a higher efficiency compared to the controls (WT and CC124). This was evident particularly in the first hour of the experiment, when H_2 production was induced.

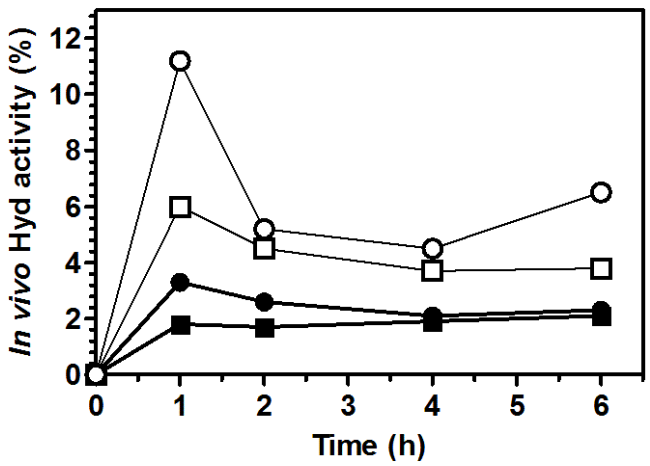


Figure 25: Efficiency of utilization of the hydrogenase enzyme during anaerobic induction in two D1 protein mutant strains (L159I-N230Y and IL) and two controls (WT[11-32b] and CC124) of *Chlamydomonas reinhardtii.* Keys: WT, (●); CC124, (■); L159I-N230Y, (□); IL, (○).

Nonetheless, both mutants reported higher efficiencies than the controls (roughly 2 times higher) even when higher *in vivo* productivity was observed (i.e., at the end of the experiment, Fig. 24).

Why mutations on the *psbA* gene interfere with the capability to drive electrons from the PSII directly to the hydrogenase and can affect hydrogenase expression itself? The only possible explanation is that by dealing with their photosynthetic capabilities, under certain conditions mutations may end up changing the level of reduction of the PQ pool. The latter is known to account for several relevant metabolic changes in *Chlamydomonas*, such as state transitions (Horton and Black, 1980; Allen et al., 1981) and target gene expression (Escoubas et al., 1995; Bellafiore et al., 2005). Moreover, it was already claimed PQ pool reduction state played a key role in the hydrogenase expression (Posewitz et al., 2004). Nevertheless, recent studies (Chochois et al., 2009) proposed the proton gradient (or ATP) generated by cyclic electron flow around PSI to be the main mechanism involved in the hydrogenase induction process. In addition, such an induction seems to be PSII-independent (Chochois et al., 2009). However, the same authors suggested that the fact that suppression of a pathway does not lead to significant changes in H_2 production rates does not necessarily mean that this pathway is not operating. Controversial results were lately reported also by Antal et al. (2009). This topic will need further investigations to be elucidated.

3a.4 Hydrogenase enzyme expression, cell number, starch metabolism and D1 protein amount in sulfur starvation conditions

In a new set of experiments, H_2 production under sulfur starvation was carried out usingd different culture conditions respect to what above reported, which permitted a fast evaluation of a number of physiological parameters (Prof. Thomas Happe is gratefully acknowledged for his technical support

during this phase of the experimental work). In accordance to previous experiments (Fig. 1, Tab. 1), the amount of light impinging PBR's surface was set at about 60-70 µmol photons m^{-2} s^{-1} per side and initial chl concentration was 12 mg L^{-1}, the light path being roughly 6 cm (o.d.). The most important changes concerned the PBR: other than a reduced volume (325 mL), it was sealed by means of Suba stoppers, meaning that the biogas produced by the culture was trapped into the gas phase. Thus, as a high H_2 production was desired, the ratio between liquid and gas phase was studied in order to avoid any problem of inhibition due to an excess of H_2 produced. The working volume of the reactor was set at 115 mL, the remaining part being gas phase (210 mL). Similarly, to circumvent also a possible delay in the induction of the H_2 production due to the high amount of O_2 present in the gas phase (filled with air), sealed cultures were flushed with argon just before the start of the experiment (roughly 3 minutes). Another relevant change was that PBRs were operated at room temperature (20-25 °C, that is 3-8 °C lower than what adopted previously, Tab. 1, Tab. 5 and Tab. 9). An overview of this set up is presented in Figure 26.

Figure 26: Overview of the final set up for the H_2 production in sulfur deprivation with sealed PBRs. Light intensity was provided by both sides and adjusted at about 60-70 µmol photons m^{-2} s^{-1}. Initial chl concentration was 12 mg L^{-1} and PBR volume was 325 mL. The working volume of the reactor was 115 mL, with the remaining part (210 mL) being gas phase. PBRs were operated at room temperature (20-25 °C). Cultures adopted in this set of experiments were cultivated in photoeterotrophic conditions at 20°C.

During this experimental set, other than D1 protein mutant L159I-N230Y, strain CC124 and WT (11-32b) were tested as a control; in addition, strain IL was also adopted to evaluate which conditions were responsible for its inability to evolve H_2. The light dilution to which all cultures were exposed determined a clear output of O_2 in the gas phase, even in the L159I-N230Y mutant strain. However, as the gas phase had a high volume respect to the liquid one and cultures were previously flushed with argon, although they were sealed O_2 concentration did not reach sufficient levels to inhibit the induction of the H_2 production (note that all cultures start to produce H_2 when in

the gas phase O_2 presence is below 1 µmol mL^{-1} gas phase) (Figure 27).

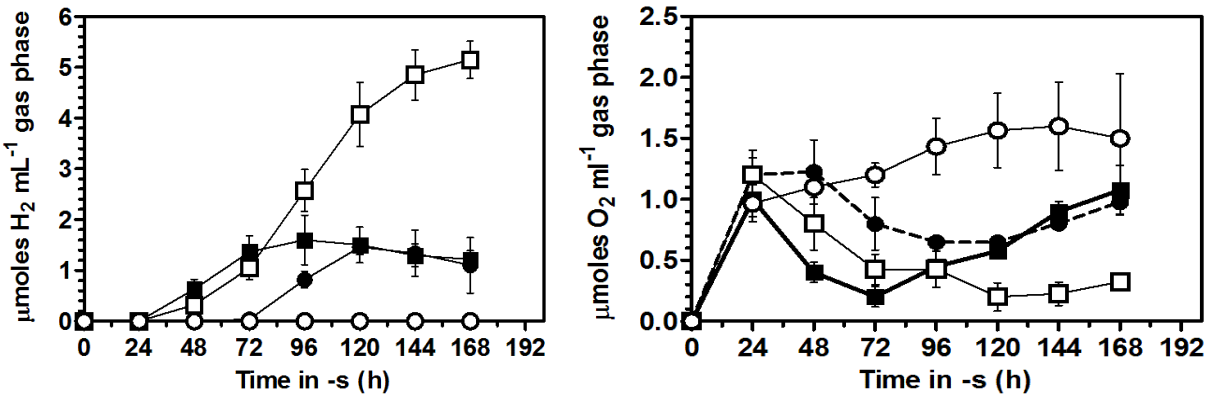
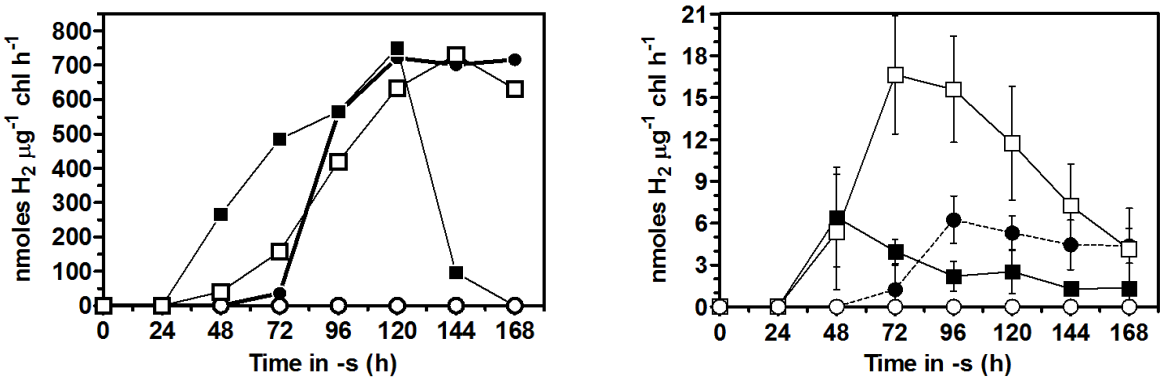


Figure 27: H_2 (left) and O_2 (right) gas content in the gas phase of sealed *C. reinhardtii* strains under sulfur starvation. Cultures were subjected to a light intensity of about 60-70 µmol photons m^{-2} s^{-1} on both sides, with an initial chl concentration of 12 mg L^{-1}. Results are the mean value of 6 experiments made on independent cultures. Vertical bars indicate standard deviation. Keys: WT, (●); CC124, (■); L159I-N230Y, (□); IL, (○).

In these conditions, strain L159I-N230Y produced about 3.2 times more than both controls (CC124 and WT) while IL did not produce H_2 at all. In comparison to what previously shown (Tab. 1 and Tab. 9) these values are lower, as they would correspond to 196, 60 and 53 mL L^{-1} in L159I-N230Y, CC124 and WT, respectively. This was not surprising as cultures were subjected to very different growing and operational conditions. In particular, cultures were grown under photoeterotrophic conditions instead of photomixotrophic ones (Tab. 1 and Tab. 9). Consistent with these results, Kosourov et al. (2007) showed that the latter condition respect to the former may lead to: (1) a higher acetate consumption during the initial O_2 production phase (+32%); (2) a higher starch storage during the overall aerobic phase (+42%) and (3) a higher H_2 photoproduction (+500%). Nevertheless, it is noteworthy WT productivity was increased (+70% respect to Tab. 1 and Tab. 9). This is not controversial, as it confirms that in the previous conditions a problematic induction of the H_2 production process (Tab. 9), with respect to the very long time needed to go over both the O_2 respiration and anaerobic lag phase, was effectively occuring (Tab. 9), and was due to culture conditions rather than WT capacities. It is unclear, however, which specific pathway could account for this. At the present moment, we can only speculate on a partial temperature effect, which was substantially different in growing (-8°C) and experimental (between -3 and -8°C) conditions, respect to the previous set (Tab. 1 and Tab. 9). Temperature may have affected all strains according to their own characteristics: in fact, it appears evident that optimal culture conditions may vary strain by strain. Unfortunately, no study has ever been reported in literature with respect to temperature effect

on the H_2 production with *Chlamydomonas*. Recently, Singh and coworkers (2005) found in *Synechocystis* loss of photochemical efficiency of PSII and sustained degradation of D1 are temperature-dependent. Although they dealt with light intensities up to 600 µmol photons m^{-2} s^{-1} , it is known that sulfur depletion ends up exacerbating PSII photoinhibition even at low light intensities (Zhang et al., 2002). The present topic will be object of future researches.

Contrary to what found with the AI (Fig. 23), *in vitro* activity of the hydrogenase enzime was equal in all producing strains (Figure 28), probably as an effect of the sulfur starvation, which was seen to strongly increase hydrogenase expression (Winkler et al., 2002). The only difference was represented by the onset of such an expression, due to the different range of time cultures needed to undergo anaerobic conditions.



Figures 28: *In vitro* (left) and *in vivo* (right) hydrogenase activity during sulfur deprivation in *C. reinhardtii* strains. Samples were taken from a PBR irradiated on both sides with a light intensity of about 60-70 µmol photons m^{-2} s^{-1} and incubated as reported in Materials and Methods. Initial chl concentration was 12 mg L^{-1}. Results are the mean value of 6 experiments made on independent cultures. Vertical bars indicate standard deviation. Keys: WT, (●); CC124, (■); L159I-N230Y, (□); IL, (○).

In the IL culture, *in vitro* activity was never detected even 168 hours after sulfur starvation. That was not surprising considering O_2 ongoing in the gas phase (Fig. 27). Western blot analysis with antibodies against HydA1 confirmed the complete absence of protein until 144 hours (data not shown). *In vivo* H_2 production rates were clearly higher in the mutant L159I-N230Y compared to both controls CC124 and WT (11-32b) (roughly 3 times higher). These measurements are usually carried out with a combination of light intensity, exposure time and light path that boost H_2 production to the highest extent and confirmed in the mutant L159I-N230Y the possibility to sustain rates of H_2 production that are 2.5 times higher than both controls, as shown in different culture conditions (Tab. 9). Interestingly enough, between 72 and 96 hours, H_2 production rates remained almost unchanged at very high levels (about 16 nmoles H_2 mg^{-1} chl h^{-1}), while concomitantly an

exponential accumulation of H_2 in the gas phase was observed (Fig. 27). A partial explanation for such a high productivity was found when cells amount was estimated as a function of sulfur depletion (Figure 29).

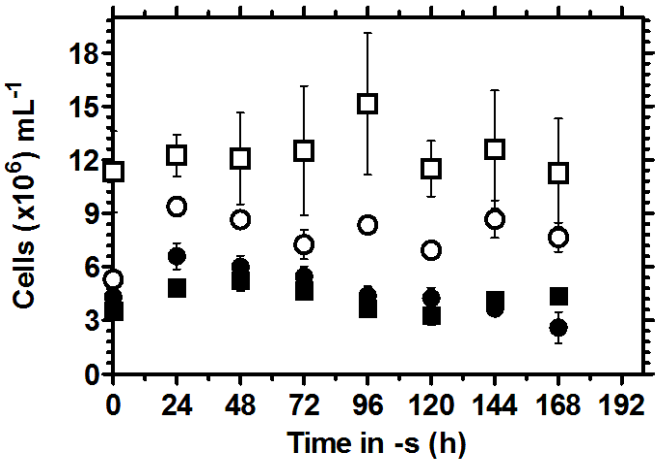


Figure 29: Cell number during sulfur starvation in *C. reinhardtii* strains (see Fig. 27). Results are the mean value of 6 experiments made on independent cultures. Vertical bars indicate standard deviation. Keys: WT, (●); CC124, (■); L159I-N230Y, (□); IL, (○).

Throughout the entire experiment, L159I-N230Y maintained a higher number of cells when compared to both controls (WT [11-32b] and CC124) and strain IL. As previously noted (Fig. 18, *right*) groups of sticked cells (up to 10-15) were observed during the growth phase in L159I-N230Y. However, during sulfur starvation, cells gradually lost the capability of remaining sticked each other. This could be due to the block of cell division (Zhang et al., 2002), to a secondary effect of the increased cell volume (because of starch synthesis) or to the degradative processes that take place due to the sulfur deprivation. After 168 hours, only groups of 2-3 cells were observed.

It can be assumed that a high H_2 production in the mutant L159I-N230Y was achieved by means of a high number of cells. However, as a high cell density is known to reduce photosynthetic yields because of the so-called self-shading (Myers et al., 1951; Agustì et al., 1986), the high amount of cells throughout the entire experiment in the mutant L159I-N230Y (about 2.8 times more respect to both controls CC124 and WT [11-32b]) did suffer no lack of productivity. This is one of the positive consequences of reduced antenna mutants (Tab. 8). However, as H_2 production in the mutant L159I-N230Y respect to both controls was 3.2 times higher and the number of cells was only 2.8 times higher, even considering no reduction of photosynthetic efficiency at all, other physiological effects may have contributed rather than a mere high cell number. Consistent with this hypothesis, a high amount of cells was not a sufficient condition in the strain IL to sustain a high productivity of H_2 gas (neither to sustain high respiration rates, Fig. 27). Interestingly enough, if *in vitro* hydrogenase activity is plotted *per cell* content (Figure 30) instead of chl content (Fig. 28), results show that the

highest H_2 production was achieved in the mutant L159I-N230Y with the lowest amount of enzyme, respect to both controls (CC124 and WT [11-32b]).

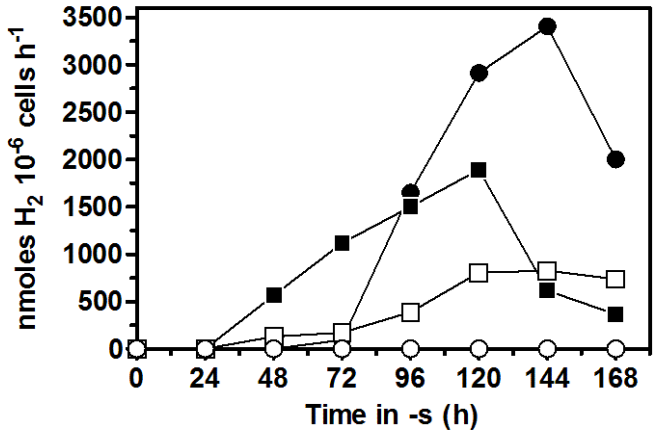


Figure 30: *In vitro* hydrogenase activity normalized per cell content during sulfur deprivation in *C. reinhardtii* strains. Samples were taken from a PBR irradiated on both sides with a light intensity of about 60-70 µmol photons m^{-2} s^{-1} and incubated as reported in Materials and Methods. Initial chl concentration was 12 mg L^{-1}. Results are the mean value of 6 experiments made on independent cultures. For a direct comparison, see Fig. 27. Keys: WT, (●); CC124, (■); L159I-N230Y, (□); IL, (○).

It is widely known that the amount of hydrogenase enzyme does not represent a limiting factor for the H_2 production, since the rates *in vivo* are greatly lower than those achieved *in vitro* (Figure 23-24). In addition, as previously mentioned, under sulfur starvation (Fig. 28) hydrogenase expression is greatly enhanced (Winkler et al., 2002). However, question arises about what was responsible for such a low amount of enzyme *per cell* in the mutant L159I-N230Y.

As above aforementioned, Posewitz et al. (2004) noted that hydrogenase expression was triggered by the redox state of the PQ pool. Recently, Chochois et al. (2009) stated that hydrogenase induction is mainly triggered by the light. As a consequence of DCMU, DBMIB and FCCP addition, they concluded that the proton gradient (or ATP) generated by cyclic electron flow around PSI is involved as the main responsible for the hydrogenase induction. Thus, both PSII electron feeding and starch breakdown may not play a primary role in hydrogenase induction (Chochois et al., 2009). At the present moment we can only speculate on what was responsible for such differences. However, assuming that culture conditions investigated in the present work are comparable to those discussed by Chochois and co-workers, some interesting observations can be done. When reporting *in vitro* and *in vivo* hydrogenase activities during the AI (Fig. 23 and 24) *per cell* content, it turns out that the final efficiency of hydrogenase utilization (sixth hour of AI) is still roughly 35% higher in the mutant L159I-N230Y than in its WT (data not shown). The latter findings would imply that a higher photosynthetic electron transfer (Fig. 25) through the PSI could reduce the level of reduction of the PQ pool, and may act as a negative feedback on hydrogenase expression.

However, the possibility that a high O_2 content poisoning the enzyme within cells, because of the

strain improved photosynthetic capacity (Tab. 3), could not be completely ruled out. This aspect will need further investigations to be elucidated.

Starch amount was followed in all cultures during sulfur deprivation in the supplied conditions described above (Fig. 27). Starch storage was perfectly in accordance with visual observations of cells: the higher the amount of starch, the bigger the cell volume (Zhang et al., 2002). All producing strains reported a high number of spherical cells after 72 hours, while IL strain showed a higher number of ellipsoidal ones, typical of the growth phase (Zhang et al., 2002). The strong accumulation of starch within the first days was incredibly higher in the mutant L159I-N230Y respect to both controls of about 3 times (T=24 hours, Fig. 31). Interestingly enough, in all the H_2 producing strains (and especially in the highest producer, L159I-N230Y), the final amount of starch is higher than what present at time zero.

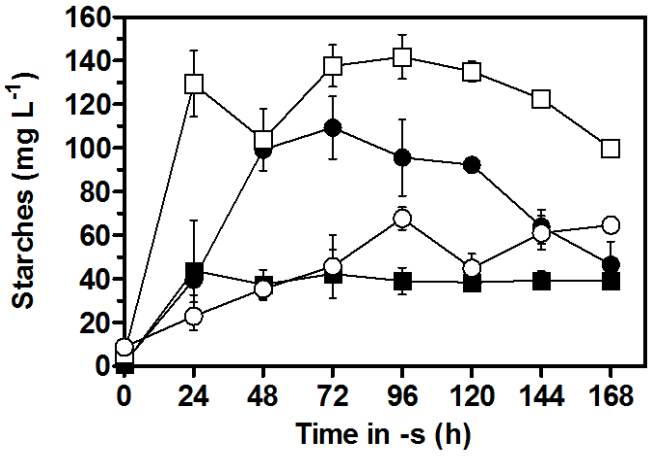


Figure 31. Starch accumulation and consumption during sulfur deprivation in *C. reinhardtii* strains. Samples were taken from a PBR irradiated on both sides with a light intensity of about 60-70 µmol photons m^{-2} s^{-1}, with an initial chl concentration of 12 mg L^{-1}. Results are the mean value of 2-4 experiments made on independent cultures. Vertical bars indicate standard deviation. Keys: WT, (●); CC124, (■); L159I-N230Y, (□); IL, (○).

When an exponential phase of H_2 gas accumulation was observed in the L159I-N230Y culture (i.e. until 120 hours, Fig. 27) no substantial decrease in the starch amount was reported. This result was rather surprising, particularly when considered that in the WT H_2 gas accumulation and starch degradation occurred concomitantly (Fig. 27 and Fig. 31, respectively). Two explanations were suggested for this phenomenon in the mutant: either starch production/consumption rates were equal during this period or PSII direct contribution in this strain was higher. It is worth noting the latter implies starch could be partially saved from catabolism when PSII direct contribution is more effective, a possibility which would have a wide interest although never reported in literature yet. This would fit with the idea that *C. reinhardtii* produces H_2 for survival purposes (Melis et al., 2000) as it permits generating sufficient amounts of ATP (Schlegel and Schneider, 1978; Melis and Happe, 2001) required for the survival of the organism under sulfur-depleted anaerobic conditions.

The main processes for ATP formation, mitochondrial respiration and oxygenic photosynthesis, are not available to sealed and sulfur-deprived *C. reinhardtii* cells due to the lack of O_2 and inactivation of PSII function, respectively (Melis et al., 2000). However, as long as the remaining PSII activity is maintained relatively high after state 1-2 transition (Antal et al., 2001; 2003), there would be the possibility for saving the amount of energy already stored into biochemical compounds. In both cases, the role of the D1 protein (and of photosynthesis) turns out to be fundamental. Consistent with this, western blotting with antibodies against D1 protein at time zero (i.e., growing conditions) showed a higher amount in the mutant L159I-N230Y respect to all the other strains (Figure 32).

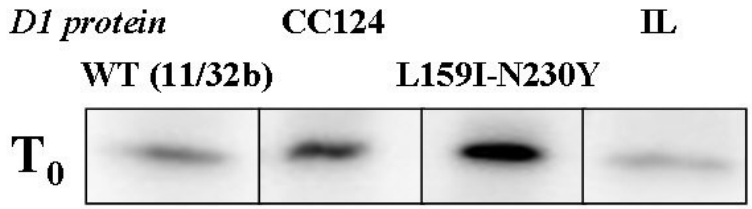


Figure 32. Western blot analysis with antibodies against D1 protein in selected *C. reinhardtii* strains. The analysis was normalized per cell content. Cultures were grown photoeterotrophically at 20 °C and irradiated with 50-70 µmol photons m^{-2} s^{-1}.

This result is much more impressive when considered that the analysis was performed *per cell* content and that, as previously shown (Fig. 29), a higher number of cells per chl content is a typical feature of the mutant L159I-N230Y. Western blot analysis performed during H_2 production confirmed also that L159I-N230Y mutant had generally a higher D1 protein amount respect to the WT (11-32b) (data not shown). However, that was due also to the fact that H_2 production in the mutant L159I-N230Y started about 48 hours earlier, meaning that such a delay in the WT during sulfur starvation caused much more severe damage to the overall PSII complexes (Zhang et al., 2002). Nonetheless, a high amount of D1 protein is crucial for sustaining a high photosynthesis rate. Kosourov et al. (2005) showed the reactivation of PSII activity upon sulfur re-addition causes *de novo* D1 biosynthesis. In their experiments, renewed photosynthetic activity was not counteracted by a sufficient respiration, blocking the H_2 release. Contrarily, in the mutant L159I-N230Y, the physiological high amount of D1 protein can be exploited since the onset of the experiment for both the initial starch accumulation and, later on, the direct PSII electron supply.

To point out to which extent starch synthesis was still in competition with the hydrogenase as the main sink for the PSII-generated reducing power in the mutant L159I-N230Y, the remaining activity of the Calvin-Benson cycle at the onset of H_2 production was evaluated (Figure 33).

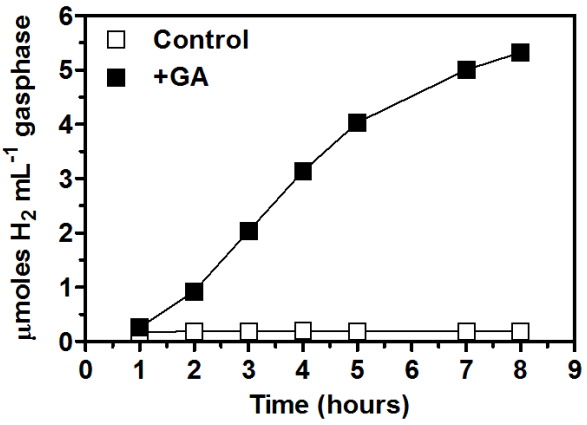


Figure 33. Long term effects of glycolaldehyde addition on sulfur deprived L159I-N230Y cultures. Cultures were sealed in a 325 ml PBR (300 ml working volume), flushed with argon for 3 minutes and exposed to 60-70 µmol photons m^{-2} s^{-1} irradiated on both sides, with an initial chl content of 12 mg L^{-1}. glycolaldehyde was added (T= 0 hours in the graph) after 24 hours of sulfur deprivation, at the onset of H_2 accumulation. Results are the mean value of 2 experiments on independent cultures. Keys are shown in the graph.

Cultures were sealed in a 325 mL PBR (300 mL working volume), flushed with argon (3 minutes) and exposed to 60-70 µmol photons m^{-2} s^{-1} irradiated on both sides, with an initial chl content of 12 mg L^{-1}. Glycolaldehyde (GA), a specific inhibitor of the Calvin cycle, was added up to 10 mM (final concentration, as described by Ruehle et al. [2008]), after 24-28 hours of sulfur deprivation (T=0 hours in Fig. 33). Upon GA addition, the treated culture was able to perform 5 times better than the control (i.e., a L159I-N230Y culture without the inhibitor). This experiment confirmed that PSII activity was still high when anaerobiosis was set in the culture and that it could be exploited for starch synthesis. Moreover, it proved that in the mutant L159I-N230Y there would be still room for enhancement in H_2 productivity, as most of this reducing power is exploited to store starch, a part of which is not even fermented at the end of the process (Fig. 31). Finally, it points out photosynthetically generated O_2 may be concentrated to a relatively high level within the cell, which support previous observations related to a lower amount of hydrogenase in this mutant respect to both WT and CC124 (Fig. 30).

Aside from L159I-N230Y characterization, some basic observations were done with respect to the strain IL. As previously said, the IL strain did not produce H_2 because unable to express the hydrogenase enzyme, as a consequence of an excess of O_2 (Fig. 27). Nonetheless, other effects may have contributed. For example, a slower increase in the starch amount per chl content was seen to occur within the first hours of sulfur starvation respect to the WT (Fig. 31). That was rather surprising if considered that the IL strain had generally an almost doubled amount of cells throughout the entire experiment (8.1 vs. 4.7 x10^6 cells mL^{-1}, Fig. 29). The latter was a consequence of a strong increase within the first 24 hours after sulfur starvation, contrary to what observed in all the other tested strains (Fig. 29). That was completely unexpected considering that sulfur deprivation causes a severe inhibition on cell division (Zhang et al., 2002), and that even re-addition

of sulfate (12.5 to 50 μM $MgSO_4$ final concentration) affects culture density mainly through an acceleration of cell growth (Kosourov et al., 2002). Finally, a western blot analysis pointed out that the amount of Rubisco enzyme *per cell* was generally higher in the IL strain rather than in the WT (Figure 34).

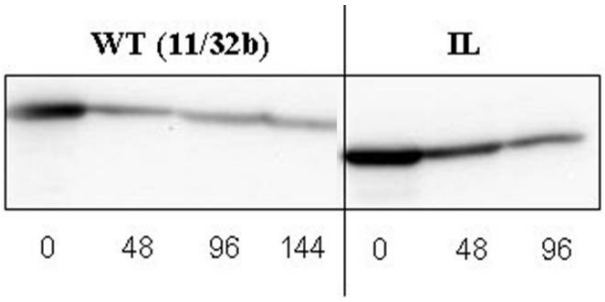


Figure 34. Western blot analysis with antibodies against Rubisco protein in selected *C. reinhardtii* strains. The analysis was normalized per cell content and represent the amount of protein expressed during sulfur starvation at specif time points (see Fig. 26). Keys are shown in the graph.

As a matter of fact, the strain IL demonstrated a physiological limitation to store starch reserves and this could probably contribute to its inability to induce the process (Posewitz et al., 2004) even when proper conditions are supplied.

3a.5 Fermentative pathways

During sulfur deprivation experiments, fermentative products such as ethanol and formate were followed (Figure 35) (Prof. Thomas Happe is gratefully acknowledged for his technical support during this phase of the experimental work). It is known that the anaerobic metabolism of this microalga is coupled with a fermentative metabolism (Winkler et al., 2002; Happe et al., 2002; Kosourov et al., 2003; Hemschemeier and Happe, 2005) in which pyruvate production (due to starch breakdown) is utilized by a pyruvate formate lyase (Pfl) for the coincident production of ethanol, formate and acetate (Hemschemeier et al., 2008a). In some cases, traces of D-lactate and glycerol (Gfeller and Gibbs, 1984) or malic acid (Mus et al., 2007) were also found. However, fermentative metabolism may be harmful as high concentrations of ethanol or formate are known to be toxic, and organic acids (e.g. lactic or acetic acid) may excessively acidify the cell (Kennedy et al., 1992).

In *C. reinhardtii*, a Pfl system was proposed when fermentative analyses of dark-adapted algae revealed a ratio of formate, ethanol, and acetate of 2:1:1 (Gfeller and Gibbs, 1984; Kreuzberg, 1984). As concerns formate and ethanol analyzed during the reported set of experiments, a 2:1 ratio was effectively obtained with all the tested strains but IL (Fig. 35).

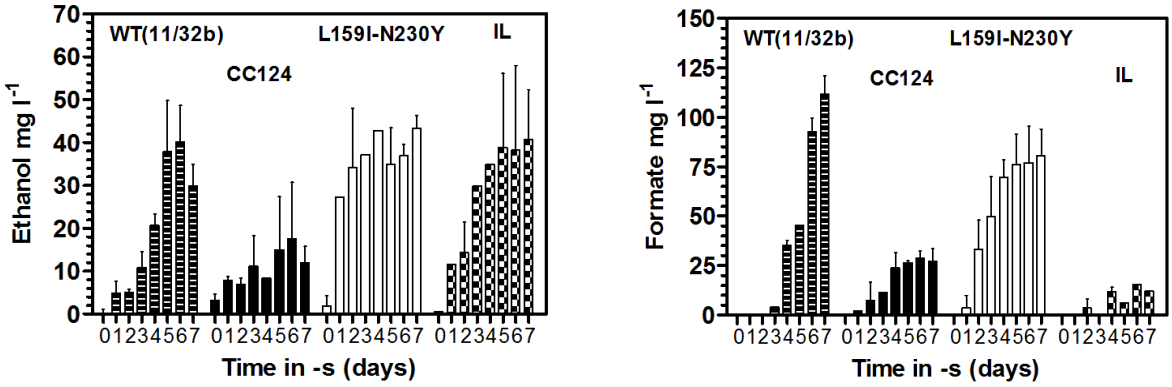


Figure 35. Ethanol (left) and formate (right) production in sulfur deprived *C. reinhardtii* strains. Samples were taken from a PBR irradiated on both sides with a light intensity of about 60-70 µmol photons m^{-2} s^{-1}, with an initial chl concentration of 12 mg L^{-1}. Keys are shown in the graph.

Strain IL produced negligible amounts of formate. However, the most surprising observation concerned the presence of relevant amounts of ethanol, considering that the IL culture was always aerobic throughout the experiment (Fig. 27). Western blot *per cell* analysis with antibodies against AdhE revealed that the induction of its expression occurred already after 48 hours, while in the WT this happened after 96 hours (Figure 36), that is when in this culture anaerobiosis was completely set and H_2 production started.

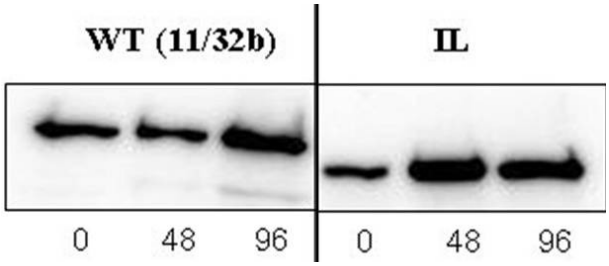


Figure 36. Western blot analysis with antibodies against AdhE protein in selected *C. reinhardtii* strains. The analysis was normalized per cell content and represent the amount of protein expressed during sulfur starvation at specif time points (see Fig. 26).

The typical enzyme responsible for ethanol production in plants is a pyruvate decarboxylase (Pdc), but in many cyanobacteria and other bacterial species, pyruvate can be oxidized by a pyruvate ferredox/flavodoxin-oxidoreductase (PFO) (Hemschemeier et al., 2008a). However, as IL culture was always aerobic, only Pdc enzyme could be responsible for ethanol production, as PFO strictly needs anaerobiosis (nowithstanding this, when anaerobiosis was induced in the culture, like during AI experiments, a valuable production of formate can be appreciated even in IL cultures [data not shown]). When sulfur deprived, strain IL started to produce ethanol from the onset of the experiment (Day 1, Fig. 35), independently by an eventual H_2 production that could occur later on in the experiment. In the WT, a real accumulation of ethanol in the media was seen to occur only at the third/forth day of sulfur deprivation (Fig. 35), concomitantly with starch breakdown (Fig. 31) and H_2

production (Fig. 27). This observation represents another hint for the physiological inability of the strain IL to easily induce the H_2 production in sulfur starvation.

Other interesting observations may be done if fermentation processes are considered on a *per cell* basis, rather than on a *per chl* one. Indeed, if fermentation products are expressed per cell amount, it turns out that the WT had the highest production of ethanol and formate, while CC124, L159I-N230Y and IL were comparable. In this point of view, the fermentative nature of the strain IL was evidenced by the fact that such a comparable ethanol production was reached with a 10 times lower starch amount *per cell* respect to all the other H_2-producing strains (data not shown). The latter experienced an increase in the amount of starch per cell which peaked at the onset of H_2 production (data not shown). In this respect, WT showed at this timepoint a doubled amount of starch per cell respect to both L159I-N230Y and CC124 (data not shown), although it did not lead to a high H_2 production, but rather to a high accumulation of ethanol and formate.
Taken together, these observations suggest in the WT an easier capability to fermentate starch content towards other pathways aside from the photosynthetic H_2 production, a consideration that fits well with the reduced H_2 production previously described (Section 3a.2) and with some of the IL strain behaviors.

3a.6 Conclusions and future perspectives

The wide characterization of several physiological features of the mutant L159I-N230Y pointed out its improved H_2 production capacity respect to its WT (11-32b) depended on a number of factors, some of which are strictly correlated, such as:

1. a higher photosynthetic capacity, as pointed out in particular by PSII-mediated ETR analyses;
2. a larger chl optical cross-section, which permitted a better light penetration within culture layers and a reduced photoinhibition;
3. a higher amount of D1 protein (also during sulfur starvation conditions), which led to both a higher direct and indirect PSII contribution;
4. a higher respiration rate per chl content;
5. a higher efficiency of utilization of the hydrogenase enzyme by means of direct electron feeding from the PSII;
6. a higher number of *producing* cells per chl content;
7. a higher conversion of violaxanthin to zeaxanthin, which allowed higher photoprotection,

hence a prolonged period of production of H_2 via PSII.

All these informations are relevant to the H_2 production and further increases in productivity are expected when proper PBRs and culture conditions are provided according to the strain features.
On the other hand, it is also interesting to point out that in the strain IL H_2 production under sulfur starvation was limited mainly by four factors: (1) inability to establish anaerobic conditions; (2) low efficiency of starch storage; (3) use of starch in favor of other fermentation pathways since the onset of sulfur deprivation; (4) utilization of energy resources to increase the cell amount during the first hours of sulfur starvation. However, the ability to produce sustained ethanol amounts even under aerobic conditions shown by IL cultures could represent a new topic for future biotechnological applications with this strain.

b) Improved hydrogen productivity by means of optimized culture conditions, PBR stirring system and high performance mutant strains

3b.1 Optimizing productivity with the mutant L159I-N230Y

In the last set of experiments carried out in the laboratory, previously gained experience on the optimal PBR system was gathered with the knowledge of the main physiological parameters of the best producing strain, the mutant L159I-N230Y. It is commonly accepted the optimization of any biotechnological process must deal with the typical features of the strain adopted. Thus, culture conditions were specifically studied in order to promote the best H_2 production with the mutant L159I-N230Y. After a specific set of experiments (data not shown), culture conditions were finally adjusted to 140 μmol photons m^{-2} s^{-1} per side of light irradiance, with an initial chl content of 18 mg L^{-1}, mixing performed by the multiple impeller system developed by us (described and tested in Section 2) and a doubled amount of acetate in the TAP-S medium respect to Harris (1989) (2 mL L^{-1} final concentration). The chl content and light intensity were chosen as a result of the best light dilution concerning L159I-N230Y features (also with respect to the stirring system), in order to attain a prolonged PSII direct contribution. On the other hand, the amount of acetate was doubled to avoid it becoming a limiting factor. Indeed, when optimizing culture conditions of reduced antenna mutants, it must always be considered that cells amount is higher than wild types upon the same amount of chl, a fact that leads to a number of physiological consequences (e.g., a higher respiration rates, Tab. 3).

Improved H_2 productivity would have given further insights on the reliability of the process on an economical and practical point of view when using strains with enhanced performances. In the meantime, the study of the physiology of the process when forcing culture conditions would have given relevant information for the following up-scale in a 50-liter PBR placed outdoor. During this last set, together with the D1 mutant L159I-N230Y, the well-known CC124 and the D1 mutant IL were also tested. The WT (11-32b) was not adopted as its production was previously proved to be particularly affected by photomixotrophic growing conditions (Tab. 9 and Fig. 27). In fact, the latter were chosen as the best growing conditions for H_2 production purposes, according to previous results (Tab. 9 and Fig. 27; Kosourov et al., 2007).

The H_2 production of the mutant L159I-N230Y was much better than CC124 (Figure 37): the higher total H_2 output was the result of both higher rates, typically occurring within the first hours of production, and longer production times. Interestingly enough, L159I-N230Y culture had a rapid change in the slope of H_2 gas accumulation (T=80 hours) which is absent in strain CC124 pattern. On the other hand, IL strain was unable to evolve H_2 gas even in these conditions (as already noted in other culture conditions, Tab. 1 and Fig. 27).

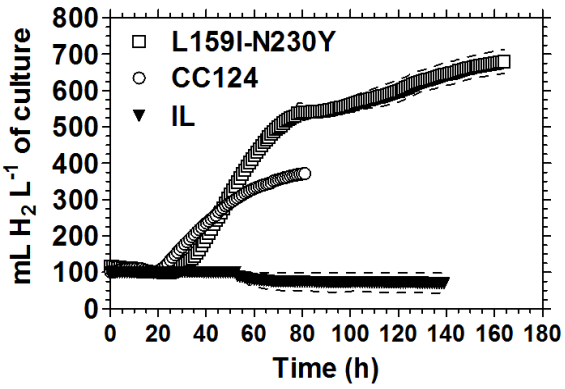


Figure 37: H_2 gas accumulation in *C. reinhardtii* D1 mutant strains L159I-N230Y and IL, and in the control strain CC124.

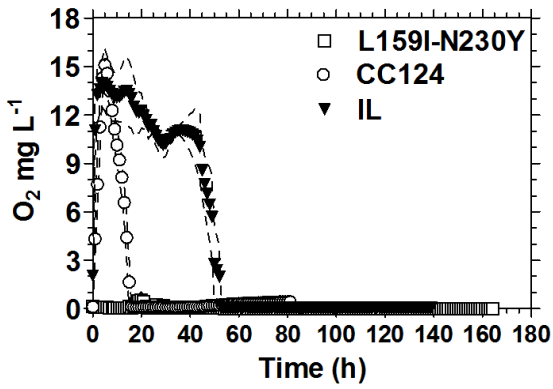


Figure 38: O_2 gas accumulation and consumption in *C. reinhardtii* D1 mutant strains L159I-N230Y and IL, and in the control strain CC124.

Cultures were grown photomixotrophically, sulfur deprived an re-suspended in TAP-S with an initial chl content of 18 mg L^{-1}; light irradiance was set to 140 µmol photons m^{-2} s^{-1} on both sides and mixing provided by means of an impeller designed, built and tested by us (see Section 2). Results are the mean value of experiments made at least in triple on independent cultures. Dashed lines represent standard deviations. Keys reported in the graphs.

Curiously, IL culture started to consume small amounts of O_2 gas (T=55 hours), as shown by a decrease in the collection system (Fig. 37). As above noted, gas consumption was made possible as communication between cultures and produced biogas was always allowed. As concern dissolved O_2 concentration (Figure 38), strains CC124 and IL reached rather high values during the initial aerobic phase (up to 15 mg L^{-1}). Nevertheless, strain IL needed a much longer time to get anaerobic (T=55 hours), which confirmed its reduced ability to consume O_2 under sulfur starvation conditions. As a matter of fact, when respiratory capacity was evaluated in standard growing conditions (on complete TAP medium), no significant differences were evaluated with respect to the WT (11-32b) and CC124 (Tab. 3). On the other hand, strain L159I-N230Y photosynthetic activity did not lead to an O_2 accumulation and remained under electrode sensitivity for almost the entire experiment. In this strain, a net O_2 output under sulfur starvation was only seen when stirred with the impeller system designed by us and irradiated with rather high light intensities, that is 210 µmol photons m^{-2} s^{-1} per side [420 µmol photons m^{-2} s^{-1} in total], with an initial chl content of 18 mg L^{-1} (data not shown).

In accordance with the dissolved O_2 concentration pattern, mutant L159I-N230Y redox potential (Figure 39) ranged around zero mV during all the aerobic phase, while it reached about 100 mV in CC124 and IL in the first 10-20 hours of experiment.

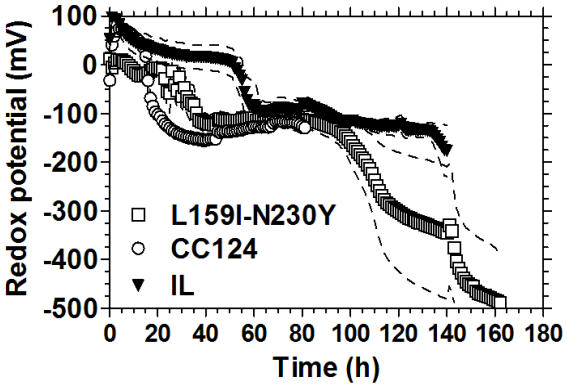


Figure 39: Changes in the redox potential value in *C. reinhardtii* D1 mutant strains L159I-N230Y and IL, and in the control strain CC124.

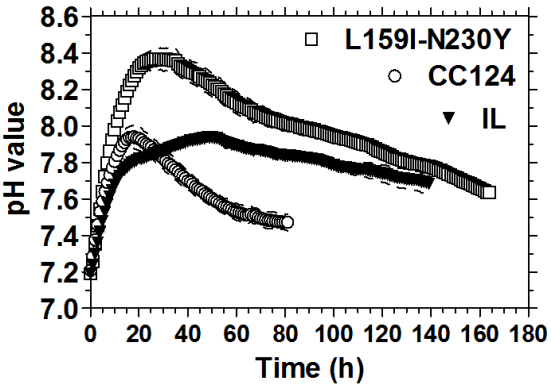


Figure 40 Changes in the pH value in *C. reinhardtii* D1 mutant strains L159I-N230Y and IL, and in the control strain CC124.

Cultures were grown photomixotrophically, sulfur deprived an re-suspended in TAP-S with an initial chl content of 18 mg L^{-1}; light irradiance was set to 140 µmol photons m^{-2} s^{-1} on both sides and mixing provided by means of an impeller system designed, built and tested by us (see Section 2). Results are the mean value of experiments made at least in triple on independent cultures. Dashed lines represent standard deviations. Keys reported in the graphs.

As soon as cultures underwent anaerobic conditions, a clear drop occurred up to -100/150 mV. This value remained stable for about 2-3 days thereafter. During this period, H_2 production in both CC124 and L159I-N230Y was performed at the maximum rates. It must be stressed that this parameter represents a clear indication of the prolonged capability of cultures to get rid of the reducing power. In fact, L/D cycles induced by the multiple-impeller device enhanced photosynthetic electron transport to the hydrogenase and led to a more oxidized PQ pool. As regards pH (Figure 40), strains CC124 and IL went up to 7.95, while strain L159I-N230Y was able to reach about 8.40. Such a high increase in the latter (also considering that a doubled amount of Tris-Base was used to neutralize acetate) was a confirm that the doubled amount of acetate was effectively exploited by the mutant.

Interestingly, pH increase in the strain IL showed a dramatic change in the slope concomitant with the start of the respiration phase, which suggest that the incapability to carry on a quick consumption of O_2, like that achieved by strain CC124, may have led to a competition between production and consumption of starch. Finally, in both producing strains (i.e., L159I-N230Y and CC124) pH strongly decreased to 7.60 and 7.50, respectively, while IL incapacity to evolve H_2 was reflected in a lower pH decrease (7.70 at the end of the experiment). A summary of all the productivities and phases of production is reported in Table 10.

Table 10. H_2 production phases and productivities of selected *C. reinhardtii* D1 protein mutant strains (L159I-N230Y and IL) and of the strain CC124 used as a control. Keys: $^+$, IL strain did not produce H_2. The culture remained anaerobic until 164 hours after sulfur starvation, when the experiment was stopped.

Strain	Aerobic phase		Anaerobic phase		H_2 total volume	H_2 Final rate	H_2 Maximum rate	H_2 Final rate	H_2 Maximum rate
	O_2 production phase	O_2 respiration phase	Lag phase	H_2 production phase					
	hours				mL L^{-1}	mL L^{-1} h^{-1}		µmol H_2 mg^{-1} chl h^{-1}	
L159I-N230Y	-	-	24.8 ± 6.5	145.0 ± 3.4	570.5 ± 50.6	3.94 ± 0.39	11.08 ± 1.02	8.52 ± 0.85	23.96 ± 2.21
CC124	6.5 ± 1.7	10.3 ± 1.5	25 ± 1.3	63.5 ± 4.7	2620 ± 4.5	4.02 ± 0.15	7.47 ± 1.68	8.70 ± 0.32	16.15 ± 3.64
IL	3.3 ± 2.3	525 ± 15.0	-	-$^+$	0.0 ± 0.0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

3b.2 Photosynthetic apparatus physiological changes

Measurements of photosynthesis and respiration rates were carried out in aliquots of algal cells removed from the PBR to evaluate the influence of sulfur starvation (Figure 41 and 42, respectively). The increase of these values within 3 hours from the start of the experiment was a consequence of the adaptation to culture conditions. Both D1 protein mutants (L159I-N230Y and IL) sustained a higher rate of net photosynthesis respect to CC124, reaching a maximum value of 180, 150 and 100 μmoles O_2 mg chl^{-1} h^{-1}, respectively.

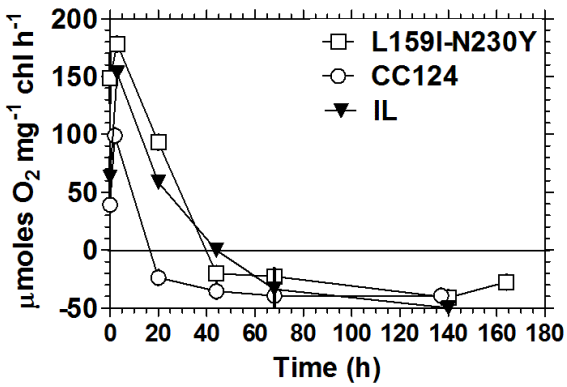


Figure 41. Net photosynthesis rates of selected *C. reinhardtii* D1 protein mutant strains (L159I-N230Y and IL) and of the strain CC124 used as a control.

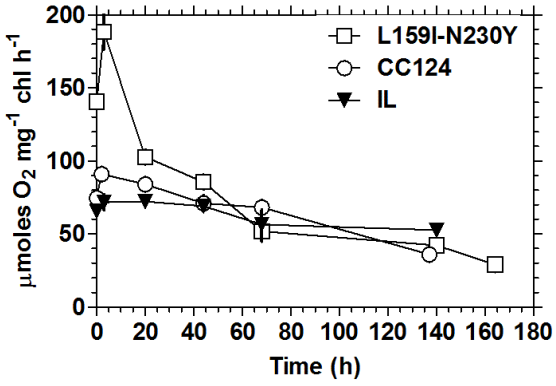


Figure 42. Respiration rates of selected *C. reinhardtii* D1 protein mutant strains (L159I-N230Y and IL) and of the strain CC124 used as a control.

Aliquots of algal cells were taken from the PBRs and tested at 28°C. Photosynthesis was measured at 800 μmol photons m^{-2} s^{-1} and respiration rates were evaluated thereafter. Values were estimated after reaching a linear pattern. Results are the mean value of experiments made at least in triple on independent cultures. Vertical bars represent standard deviations. Keys are reported in the graphs

Assuming the H_2 production in the main PBR was carried out at the highest rates achievable, maximum rates of production (not corrected for cellular respiration) would have a H_2/O_2 ratio (mol/mol) of 0.15:1 and 0.18:1, in L19I-N230Y and CC124, respectively. These values are equal to the first findings of Melis and coworkers (2000), who reported a 0.17:1 ratio with *Chlamydomonas* strain C137. If photosynthesis was entirely devoted to the H_2 production, in theory a maximum 2:1 ratio would be attained (Benemann et al., 1973; Bishop et al., 1977; McBride et al., 1977; Greenbaum, 1982, 1988; Miura, 1995), provided that the photosynthetic apparatus remained in state 1 (Antal et al., 2001; 2003) and that an O_2-tolerant hydrogenase could be available. If such a ratio was expressed by the mutant L159I-N230Y, it would turn into a 150 mL L^{-1} h^{-1} H_2 production.

Sulfur starvation severely inactivated photosynthesis in all cultures. Nevertheless, in both D1 protein mutants net photosynthesis was found in the negative field only since 40 hours after sulfur depletion and a comparable value between all cultures was observed only after 70 hours (roughly 3 days). One of the most surprising data was found measuring respiration rates (Figure 42). It is commonly believed that sulfur starvation has little or no effect on respiration rates (Melis et al., 2000; Zhang et al., 2002; Zhang and Melis, 2002; Kosourov et al., 2007), even after long periods (up to 5 days, Kosourov et al., 2005). This observation is not true when applied on reduced antenna mutants like L159I-N230Y, which have a very high number of cells per chl content. As soon as O_2 evolution dropped in the mutant L159I-N230Y (first 20 hours of the experiment, Fig. 41) a concomitant strong reduction occurred in the respiration rates (namely, from 190 to 100 μmoles O_2 mg chl^{-1} h^{-1}). For purpose of comparison, it must be noted that after 70 hours of experiment both CC124 and IL cultures were still slightly influenced (-20%), while L159I-N230Y experienced a 4-fold decrease.

It was clear that the high photosynthetic performance of the IL strain was not properly counteracted by respiration, leading to a net output of O_2 in the medium (Fig. 38). This was controversial, as in complete TAP medium no significant difference was noted between IL and CC124 photosynthesis rates (Tab. 3). As a whole, gross photosynthesis rates (net photosynthesis plus respiration) were found to be equal in all cultures only 70 hours after sulfur depletion (data not shown).

The effective photochemical efficiency of the PSII ($\Delta F/F'_m$) was followed in all cultures by pointing the optical fiber directly onto the illuminated surface of the PBR (Figure 43).

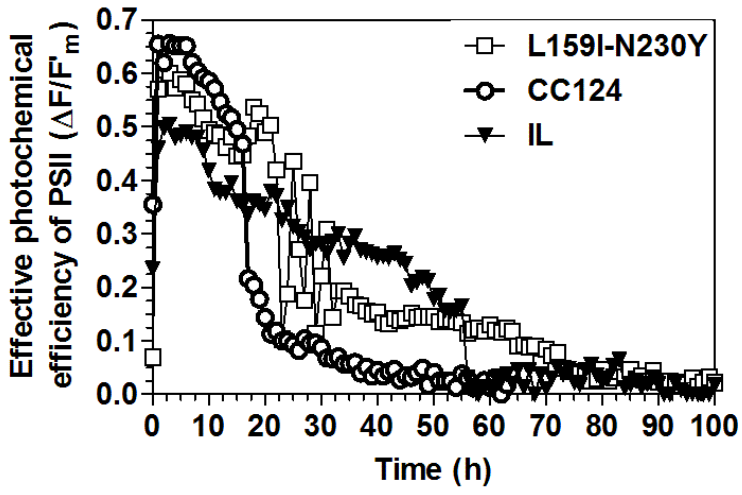


Figure 43. Effective photochemical efficiencies under sulfur deprivation in *C. reinhardtii* D1 protein mutant strains (L159I-N230Y and IL) and a control (CC124). Tzero represents the time

when cultures were sulfur deprived. Cultures had an initial chl content of 18 mg L^{-1}; light irradiance was set to 140 µmol photons m^{-2} s^{-1} on both sides and mixing provided by means of an impeller designed, built and tested by us (see Section 2). Note that $\Delta F/F'_m$ values are reported only for the first 100 hours of experiment, as after that time fluorescence was substantially equal to zero in all cultures. Keys reported in the graph.

Each strain went under a state 1-2 transition of the photosynthetic apparatus of the PSII (Antal et al., 2001; 2003) though following a very different pattern. Strain CC124, used as a control, showed a typical behavior: first a slow decrease in $\Delta F/F'_m$ value was observed (from 0.660 to about 0.500), then a sudden drop occurred (to about 0.220) in a 15 minutes range (Antal et al., 2001; 2003). After the transition to state 2 the efficiency of PSII constantly decreased, to reach the complete inactivation after 50 hours of sulfur starvation. The low $\Delta F/F'_m$ value showed by strain IL in the first hours of experiment (0.510) suggests an excess of light irradiation. Most interestingly, state 1-2 transition in this culture occurred very late (only 55 hours after sulfur starvation), when PSII activity in state 1 was about 0.165: such a value was even lower than what observed in the mutant L159I-N230Y and CC124 *after* the transition to state 2 (0.200 and 0.220, respectively), which gives a hint of the level of the extremely stressing conditions to which strain IL was exposed. Such a delay, entirely due to the time needed to consume all the O_2 in the liquid suspension (Fig. 38), led to a dramatic consequence on the PSII activity, which dropped directly to zero after transition to state 2, poorly recovering over 0.050 in the following 100 hours, when the experiment was stopped (Fig. 43).

As regards L159I-N230Y strain behavior, as soon as it was adapted to the culture conditions, $\Delta F/F'_m$ value reached up to 0.600 decreasing slowly thereafter for 10-15 hours. Then, a little recover in the $\Delta F/F'_m$ value was observed concomitantly with a small O_2 output in the liquid phase (that was recorded for the first time since the onset of the experiment), which anticipated a surprising number of transitions from state 1 to state 2 and back, finally reaching an equilibrium after about 10 hours around 0.200 in state 2 (Figure 44).

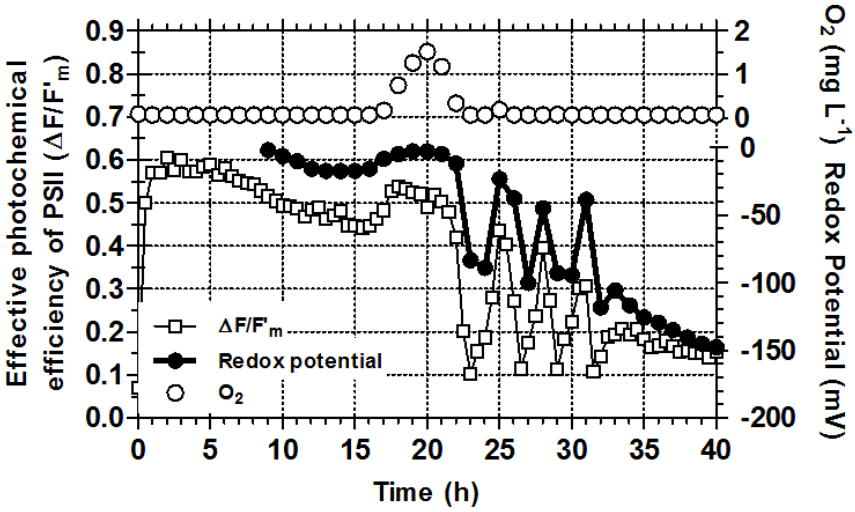


Figure 44. Fast inactivation of the respiration rate in the D1 protein mutant L159I-N230Y and its effects on the effective photochemical efficiency of PSII, the redox potential and the dissolved O_2 concentration. Each time respiration drops because of the lack of intracellular O_2, photosynthesis leads to the re-establishment of aerobiosis. This happens until sulfur deprivation definitely down-regulates PSII activity. Tzero represents the time when culture was sulfur deprived. Note the parallel pattern followed by the redox potential and effective quantum yield of PSII, which both express the level of reduction of the PQ pool.

In Figure 44, strain L159I-N230Y undergoes up to 4 transitions to state 2 before remaining stable, but it must be pointed out this number of transitions could vary in each repetition. The first positive output of O_2 in the culture (up to 2 mg L^{-1}, T=20 hours, in Fig 44) was preceded by a small recover in the redox potential and, even sooner, by a new increase in the $\Delta F/F'_m$ value, which suggests that intracellular accumulation of O_2 started well in advance (please note that in Fig. 44 time is expressed in hours). These events may be due to the strong inactivation of the high respiratory rates showed by this mutant (Fig. 42), which seem to be much faster than photosynthesis ones (Fig. 41). As previously mentioned, it was shown that after the migration of LHC-II proteins from the PSII to PSI within the state 2 induction, PSII recovers some activity (Antal et al., 2001, 2003). This happens in combination with H_2 release by the culture (as effectively occurred in our case, data not shown), as a consequence of hydrogenase activity which partly oxidizes photosynthetic carriers like the PQ pool, thus increasing the rate of the electron transfer from PSII to plastoquinones (Antal et al., 2001; 2003). However, the explanation suggested by Antal and co-workers only partially applies to this case, as the first recover of $\Delta F/F'_m$ value occurred well before the first state 1-2 transition (Fig. 44 T=15 hours). In agreement with this observation, when the strain CC124 was subjected to high rates of photosynthesis (Fig. 13a), anaerobiosis was maintained only for few hours. This was due to the

fact that the respiration rate of this strain (CC124) is little or no affected by sulfur deprivation (Fig. 42), as already stated by others (Melis et al., 2000; Zhang et al., 2002; Zhang and Melis, 2002; Kosourov et al., 2005; 2007). These findings are also consistent with observations made above with this strain, that is respiration may be rapidly inactivated by the lack of substrate (i.e., O_2). In fact, such renewed O_2 output may have acted as a positive feedback on respiration, which increased once more, thus completely up-taking all the O_2 and leading to the first state 1-2 transition, as confirmed by the parallel redox potential decrease (T=23 hours, Fig. 44). This event repeatedly occurred until sulfur starvation did not prevent photosynthesis recover over respiration (T=32 hours, Fig. 44). A similar pattern was observed in very few cases with this mutant even when applying standard conditions (Tab. 9), in cultures mixed by a magnetic stir bar (data not shown). When finally a stable H_2 production was achieved, $\Delta F/F'_m$ value remained relatively high for a longer period respect to the strain CC124. This part will be discussed further in the text with respect to H_2 production rates.

Concerning the total amount of chl, carotenoids (car) and dry weight some basic observations can be done. Although starting from the same initial chl content (18 ± 1 mg L^{-1}, Fig. 45a) D1 protein mutants (L159I-N230Y and IL) spent less energy on chl synthesis respect to the strain CC124.

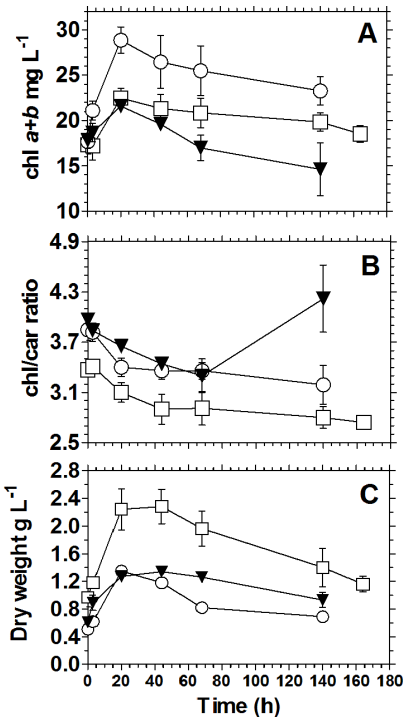


Figure 45. The effects of sulfur deprivation on chl synthesis (A), chlorophylls/carotenoids (chl/car) ratio (B) and dry weight (C) in *C. reinhardtii* D1 protein mutant strains (L159I-N230Y and IL) and a control (CC124). Tzero represents the time when cultures were sulfur deprived. Light irradiance was set to 140 µmol photons m^{-2} s^{-1} on both sides and mixing provided by means of an impeller designed, built and tested by us (see Section 2). Vertical bars represent standard deviations. Keys: L159I-N230Y, (□); CC124, (○); IL, (▼).

Nevertheless, all strains reached their higher chl concentration after 20 hours (and decreased thereafter), despite they needed very different periods of time to undergo anaerobic conditions or

start to evolve H_2. This observation implies that sulfur deprivation down-regulates a number of metabolic pathways independently by other major changes experienced by the culture, such as anaerobiosis establishment. Strain IL was the most affected and eventually reached a value below its initial amount (15 mg L^{-1}), which reflected its severe stressing conditions already described above. On the other hand, the chl/car ratio showed by the mutant L159I-N230Y (Fig. 45b) was always lower respect to the other strains, which may have favored PSII photoprotection (and prolonged PSII contribution), as confirmed by fluorescence measurements (Fig. 44). Finally, the dry weight ongoing is reported (Fig 45c). The graph showed in both producing strains (L159I-N230Y and CC124) a strong imbalance due to carbohydrates synthesis, which is finally recovered when H_2 production ceases.

3b.3 PSII direct and indirect contribution

As cultures were deprived of sulfur, a typical carbohydrates accumulation was observed (Table 11).

Table 11. Carbohydrate and protein analyses in *C. reinhardtii* D1 protein mutant strains (L159I-N230Y and IL) and a control (CC124). Cultures had an initial chl content of 18 mg L^{-1}; light irradiance was set to 140 µmol photons m^{-2} s^{-1} on both sides and mixing provided by means of an impeller designed, built and tested by us (see Section 2).

Strain	Time	Carbohydrates	S.D.	Proteins	S.D.	Proteins / Carbohydrates Ratio	S.D.
	hours	mg L^{-1}					
L159I-N230Y	0	309.7	65.4	310.6	71.6	1.00	0.02
	3	456.3	12.8	338.5	79.1	0.74	0.00
	20	1387.8	206.2	416.3	111.3	0.30	0.04
	44	1341.1	131.1	473.0	93.0	0.35	0.04
	68	1219.3	80.9	412.4	10.0	0.34	0.03
	140	676.3	273.8	428.7	22.4	0.63	0.24
	164	474.9	167.7	393.4	2.2	0.83	0.32
CC124	0	136.0	25.4	244.2	46.0	1.81	0.26
	3	189.2	29.4	260.1	40.6	1.38	0.17
	20	609.0	44.6	334.4	77.4	0.55	0.09
	44	385.5	44.6	330.9	67.6	0.86	0.09
	68	261.7	18.2	273.1	14.3	1.04	0.05
	140	187.2	10.7	269.3	14.4	1.44	0.12
	164	n.d.	-	n.d.	-	n.d.	-
IL	0	175.7	7.5	293.6	1.1	1.67	0.08
	3	260.4	4.1	308.6	1.9	1.19	0.01
	20	514.7	12.9	406.8	5.9	0.79	0.01
	44	636.2	67.5	398.3	19.0	0.63	0.10
	68	609.4	6.5	381.0	13.8	0.63	0.02
	140	518.0	30.4	378.4	29.6	0.73	0.01
	164	n.d.	-	n.d.	-	n.d.	-

Despite the fact that chl synthesis, aerobiosis and state 1-2 transition were affected only after a period of 10-20 hours, a proteins-carbohydrates (p/c) imbalanced ratio was recorded only 3 hours after sulfur depletion. The highest amount was reached in all cultures at the end of the their own aerobic phase, and was equal to 1.39, 0.61 and 0.64 g L^{-1} in L159I-N230Y, CC124 and IL respectively. These amounts corresponded, respectively, to the 62%, 45% and 48% of the overall dry weight. The ability to produce the highest net amount of carbohydrates showed by the mutant L159I-N230Y (more than 1 g L^{-1} over 20 hours) is consistent with its increased photosynthetic capability and with a strong acetate consumption (Kosourov et al., 2007), reflected by the highest increase in the pH value (8.4, Fig. 40). However, it must be noted that in this strain (L159I-N230Y) an imbalanced ratio towards carbohydrates was observed even at the onset of the experiment (i.e., growing conditions), as shown by the p/c ratio compared to the other strains (1.00, 1.81 and 1.67 in L159I-N230Y, CC124 and IL, respectively). Carbohydrates are of extreme relevance to the H_2 production for several factors: (1) for maintaining anaerobic conditions (Ghirardi et al., 2000b; Fouchard et al., 2005; Kosourov et al., 2003; 2007; Melis, 2002; 2007; Melis and Happe; 2001; Makarova et al., 2007); (2) as an additional source of electrons for the plastoquinone pool (Bamberger et al., 1982; Gfeller and Gibbs, 1985; Godde and Trebst, 1980; Mus et al., 2005) and (3) for maintaining [FeFe]-hydrogenase gene expression (Posewitz et al., 2004). However, it is likely that another physiological limit may exist to carbohydrates accumulation, that is the cell volume. Visual observation of the mutant L159I-N230Y confirmed a very high increase of the cell volume at the end of the aerobic phase, which is unclear to which extent may have played a role.

In contrast with what observed previously (Fig. 31), at the end of the H_2 production process the synthetized carbohydrates were completely fermented. Most likely, this was due to culture conditions (i.e., mixing and light irradiation), which were more stressing and forced cultures to produce more H_2. This may support the hypothesis that most of the H_2 produced was due to an indirect PSII contribution. However, in the strain CC124 about 40% (i.e., 225 mg L^{-1}) of the stored carbohydrates were already consumed within the first day of H_2 production (between 20 and 44 hours after sulfur deprivation), while in the mutant L159I-N230Y only 9% of the carbohydrates content (about 125 mg L^{-1}) were consumed within the same period of time (first day of H_2 production, between 44 and 68 hours after sulfur starvation). Moreover, after that period strain L159I-N230Y had already produced about 70% of its total H_2 final volume (400 mL L^{-1}, Fig. 37); the remaining 750 mg L^{-1} of carbohydrates were consumed while the culture produced only about 170 mL L^{-1}.

It was already shown that in the mutant L159I-N230Y Rubisco activity at the onset of H_2 production may divert most of the reducing power from the hydrogenase (Fig. 33) to ensure starch synthesis (Fig. 31). Nevertheless, when H_2 production rates were plotted together with $\Delta F/F'_m$ values in both L159I-N230Y and CC124 (Figure 46 a and b, respectively) a parallel decay was observed. In Figure 46a (where T_{zero} is the onset of H_2 production), the highest H_2 production rate in L159I-N230Y (about 11.08 ± 1.02 mL L^{-1} h^{-1}) was maintained as long as PSII activity was sustained to a relatively high value (21.8 ± 7.7 hours at about 0.150).

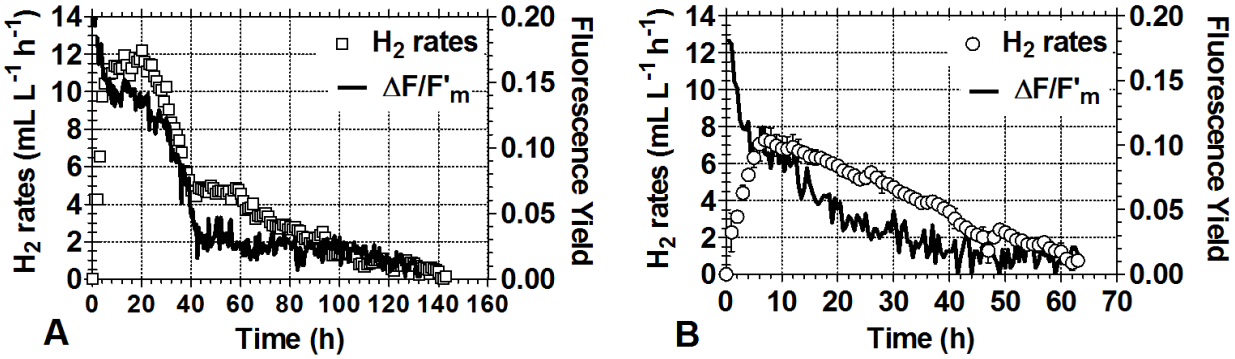


Figure 46. Parallel pattern in H_2 production rates an $\Delta F/F'_m$ decay in the D1 protein mutant strain L159I-N230Y (A) and a control (CC124 [B]). Vertical bars represent the standard deviations. Tzero represents the onset of H_2 production. Rates were obtained with an initial chl content of 18 mg L^{-1}, a light irradiance of 140 µmol photons m^{-2} s^{-1} per side and a mixing provided by an impeller designed, built and tested by us (see Section 2). Results are the mean value of 4 independent experiments. Keys reported in the graphs.

The occurrence of rates of H_2 production that were both high and sustained was surprising. Concerning the latter aspect, it was suggested by Ghirardi and co-authors (2001) that a steady-state H_2 evolution is possibly limited by factors that lower the effective capacity of photosynthetic electron transport. In addition, it was also stated that the rate of PSII-catalyzed O_2 evolution limited H_2 production (Ghirardi et al., 2001). In agreement, it was noted that the mutant L159I-N230Y had incredibly high rates of respiration (Fig. 42) and a higher PSII-mediated ETR compared to its WT (Fig. 21), which was reflected in both a higher efficiency of hydrogenase utilization (Fig. 25) and a higher *in vivo* PSII-based H_2 production (Fig. 24). Indeed, as soon as PSII activity dropped below 0.150-0.100, a linear decrease was observed in the rates of H_2 production (Fig. 46a). On the contrary, strain CC124 (Figure 46b) showed no steady-state in the H_2 production rates or in the effective photochemical efficiency of PSII, rather a linear decrease in both these parameters. As a whole, $\Delta F/F'_m$ value remained above 0.100 for 35 vs. 10 hours in L159I-N230Y and CC124, respectively.

If expressed per chl content, the maximum rates performed by L159I-N230Y corresponded to 23.96 ± 2.21 μmoles H_2 mg^{-1} chl h^{-1} (at 0.96 atm and 28 °C), that is about 4 times more than what obtained with the highest producing strains known to date (*Stm6*, Kruse et al., 2005, and *Stm6Glu4*, Doebbe et al., 2007). It must be stressed that such a relevant increase was due to both the photosynthetic capacity of the strain L159I-N230Y and the optimized PBR provided with the impeller mixing system described in Section 2. In fact, not all the strains may be suitable to fully exploit L/D cycle effects, as observed with CC124 which was not able to sustain a constant rate of H_2 production. In these cases, however, a partial benefit can be achieved: indeed, if expressed in terms of chl, CC124 maximum rates (16.15 ± 3.64 μmoles H_2 mg^{-1} chl h^{-1}) are considerably higher than those reported with the same strain by other authors (Tsygankov et al., 2002; Kosourov et al., 2002; 2003; 2005; 2007; Ghirardi, 2006; Kosourov and Seibert, 2009; Oncel and Vardar-Sukan, 2009). The better results obtained with this strain (CC124), particularly with respect to the previous experiments (Section 2), was due to mainly two factors: (1) a doubled acetate amount in the medium; (2) a deeper light penetration within the culture layers. We believe that a better light supply was reached in this case, respect to Section 2 experiments, as proved by the redox potential value, which remained steady at less negative values for a prolonged period. Concerning the doubled acetate amount in the medium, it was seen that in photomixotrophically grown cultures (Kosourov et al., 2007), significant acetate consumption can take place even during H_2 production. Most likely, the higher amount of acetate supplied electrons for PSII generated O_2 respiration (Chochois et al., 2009), which probably saved part of the endogenous reserves for the H_2 evolution.

To estimate the effective PSII direct contribution in L159I-N230Y maximum rates, as soon as they were set in the culture, DCMU was added to a final concentration of 10^{-5} M (Figure 47).

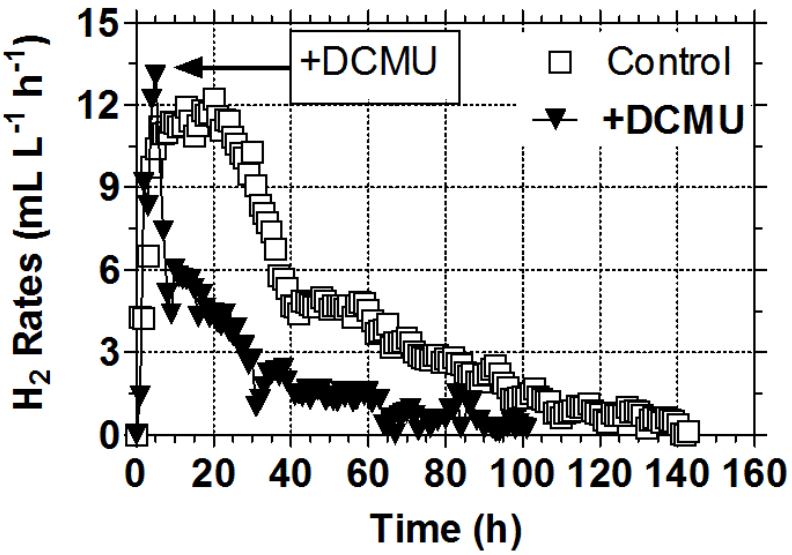


Figure 47. The effective PSII direct contribution to the H_2 production in the mutant strain L159I-N230Y as pointed out by DCMU addition. DCMU was added as soon as the highest rates of H_2 production were set in the culture, to evaluate the effective contribution of the PSII remaining water splitting activity. Results are the mean value of three independent experiments. Tzero represents the onset of H_2 production. Keys reported in the graph.

When DCMU was added, $\Delta F/F'_m$ value dropped to zero within seconds, H_2 production rates were reduced by 54% (from 13 to 6 mL L^{-1} h^{-1}) and the overall H_2 output was finally down-regulated of about 60% (244 vs 622 mL L^{-1}). The inhibitory effect of DCMU may vary from 0 to 85% (Ghirardi et al., 2000a, Antal et al., 2001; 2003; Kosourov et al., 2003, Lee and Greenbaum, 2003; Fouchard et al., 2005; Kruse et al., 2005; Hemschemeier et al., 2008b; Antal et al., 2009) according to a number of factors, such as cell density, light availability and time after sulfur starvation. Such a decrease (54%) is actually lower than what shown by others. However, it must be stressed in these conditions, such a value corresponded to an impressive 7 mL L^{-1} h^{-1} H_2 production rate evolved only by means of biophotolysis. Moreover, it was observed that a 3 hours period was needed to estimate such a decrease (Fig. 47), most likely because of the collection system. This suggests the possibility that the effective contribution of PSII might be higher. As noted by others (Chochois et al., 2009), it seems highly probable that direct and indirect PSII contribution, which are somehow competing for electron carriers, may complement each other to some extent and there could also be some subtle synergetic modes of interactions between pathways.

Notwithstanding a 62% increase in maximum rates respect to the standard conditions (from 6.86 to 11.08 mL L^{-1} $hour^{-1}$, Tab. 9 and Tab. 10 respectively), the final H_2 productivity in the mutant L159I-

N230Y was increased of only 11% (from 513.6 to 570.5 mL L^{-1}). This situation may reflect the fact that sulfur depletion ends up inhibiting photosynthesis so much (Zhang et al., 2002) that an improvement in H_2 productivity could not overcome a certain level. However, as a matter of fact, the remaining micromolar quantities of sulfur could be exploited as a reserve to sustain PSII activity (as, for instance, for the D1 turnover), in the same manner carbohydrates act as a reserve of reducing power for H_2 production itself. This would explain why maximum rates were so much increased but did not lead to a proportional increase in the final volume produced. Similarly, final apparent light conversion efficiencies were found to be 0.97% and 0.92% in L159I-N230Y and CC124, respectively (which are similar to the highest values previously found out of a range of different conditions with CC124, see Section 2.6), while efficiencies of maximum rates corresponded to much greater values, namely 3.22% and 2.17% in L159I-N230Y and CC124, respectively.

Possibly, further increases in H_2 productivity could be achieved only by addition of micromolar quantities of sulfur as soon as maximum rates are about to cease. Such re-additions should not stop the process to turn back towards the aerobic phase (Ghirardi et al., 2000a, Kosourov et al., 2005; Fouchard et al., 2008), but rather maintain PSII survival enough to prolong the maximum productivity as much as possible. From a certain point of view, this rationale was already proposed by continuous H_2 production experiments (Fedorov et al., 2005; Mullner, 2008), in which very long periods of production (weeks to months) were attained, although to the detriment of production rates (that were not higher than 2 mL L^{-1} $hour^{-1}$). Of course, the substitution of old batches of algae with fresh ones contributed to the prolonged production phase in a way that would not be attainable by any fed-batch experiment. Nevertheless, sulfur re-addition to maintain PSII direct contribution to the H_2 production may explain the equilibrium between electrons coming from water splitting activity and from fermentation. In fact, interestingly enough, when DCMU was added and biophotolysis was blocked (Fig. 47), the experiment lasted about 40 hours less. This observation is in agreement with previous assumptions (Section 3a.4) which deal with the possibility that endogenous substrates may be saved upon a higher PSII direct contribution. Contrarily, Doebbe et al. (2007) showed that in the mutant strain *Stm6Glc4* (into which a hexose symporter was introduced), a 45-50% increase in the total yield (respect to the control *Stm6*) was observed when externally supplying 1mM of glucose, without affecting production times. Furthermore, authors affirmed that maximum production rates were maintained for a 215% longer period but remained comparable to the control (*Stm6*, Kruse et al., 2005). More research must be addressed to elucidate these topics.

3b.4 First hydrogen production by sulfur deprivation with the strain IL: the role played by PSII

Although previous indications revealed in strain IL a number of physiological limitations (Section 3a), it is unclear why H_2 production did not start in the conditions supplied in Section 3b.1, considering that: (a) anaerobiosis was established during the experiment (T=55 hours, Fig. 38); (2) a state 1-2 transition occurred (T=55 hours, Fig. 43); (3) the redox state of the culture was maintained for 1-2 days at levels comparable to those achieved by both producing strains (-100/150 mV, Fig. 39); (4) a sufficient amount of carbohydrates was stored (0.64 g L^{-1}, Tab. 11), comparable to what produced by the strain CC124 and (5) p/c ratio pointed out that the biomass was imbalanced towards carbohydrates as much as in the producing strain CC124 (about 0.6). The aforementioned conditions are generally considered sufficient to induce the H_2 production in most of the *Chlamydomonas* strains reported in literature. As concerns hydrogenase enzyme, it can be observed even when DCMU is added immediately after sulfur starvation, which means that no starch is stored and no PSII can lead to H_2 evolution. Moreover, it was shown that under anaerobic conditions, strain IL was able to express and efficiently utilize the hydrogenase enzyme (Figs. 23-25). Thus, once O_2 is consumed (and hydrogenase is expressed), and endogenous substrates like carbohydrates are stored in sufficient amounts, H_2 production should eventually occur.

A last relevant experiment on this topic was set up. The aim of the experiment was to point out if a functional PSII was responsible for the induction of the H_2 production process. To overcome the excess of dissolved O_2 in the IL culture, due to a photosynthesis/respiration ratio imbalanced in favor of the former, per liter respiration was improved by increasing the initial chl content up to 24 mg L^{-1}. Concomitantly, light irradiance was reduced to 70 µmol photons m^{-2} s^{-1} (irradiated on both sides). All the other conditions were left unchanged respect to the last experiment above discussed (Section 3b.1). The results of this experiment are shown in Figure 48.

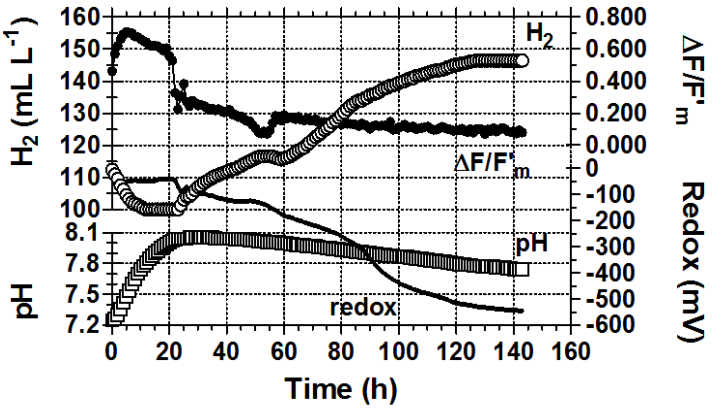


Figure 48. H_2 production under sulfur starvation in the D1 protein mutant IL. H_2 production was reached inducing a timely transition of the PSII apparatus to the state 2. Initial chl concentration was 24 mg L^{-1}, light intensity was set to 70 µmol photons m^{-2} s^{-1} per side. Mixing was provided by the impeller system described in Section 2. The culture was always anaerobic. Tzero represents the time of sulfur starvation. Keys reported in the graph.

As expected, O_2 was never recorded since the onset of the experiment and redox potential remained always in the negative field. In these conditions, H_2 production was finally reached in this strain, even if low amounts were evolved (47 mL L^{-1}). This phenomenon was clearly the result of a timely transition to the state 2, occurring just 20 hours after sulfur starvation. The strong PSII-based H_2 production of this strain (hence, its reduced ability to mobilize endogenous substrates for H_2 production purposes) was evidenced by the effects caused by a sudden drop in the $\Delta F/F'_m$ value after about 50 hours of experiment (Fig. 48). Although H_2 production was already going on for 20 hours, H_2 gas accumulation concomitantly stopped and promptly re-started as soon as $\Delta F/F'_m$ recovered its previous level. This phenomenon did not occurred in each repetition.

This experiment demonstrates for the first time that to successfully reach the induction of the H_2 production under sulfur starvation a *timely* transition to the state 2 is also needed. The exact mechanisms are not clear yet and at the present moment we can only speculate that the excessive photosaturating conditions may deviate energy reserves towards processes other than H_2 production. Similarly, Tolstygina et al. (2009) found that cultures grown under high light experienced irreversible inhibition of PSII, leading to irrelevant H_2 productions. The relevance of this indication was crucial when moving outdoor under sunlight irradiance in an up-scaled PBR of 50 liters.

3b.5 Conclusions and future perspectives

The optimization of culture conditions in batch experiments with the mutant L159I-N230Y revealed the possibility to carry out H_2 production at incredibly high rates (11 mL L^{-1} h^{-1}, which corresponded to an apparent light conversion efficiency of 3.2%), particularly sustained by means of a direct PSII contribution. Notwithstanding the fact that these rates are still far from the theoretical 10-13%

previously suggested for an economical H_2 production (Tredici et al., 1997; Ghirardi et al., 2009b), they represent a relevant step forward to this goal.

The fact that the final H_2 production was not increased as expected (+11%) suggests that other solutions may be adopted. The aim of future studies with strain L159I-N230Y must be the maintenance of the highest rates for the longest time. A first step, before a continuous H_2 production system, could be done by adding small amounts of sulfur to sustain PSII contribution without reverting the process to aerobic conditions, as preserving PSII activity represents a basic goal to improve the productivity for *in vivo* H_2 production with algae, until an O_2-tolerant hydrogenase will be available.

Section 4

Hydrogen production experiment in outdoor horizontal tubular photobioreactors

a) Experiments with strain CC124

4a.1 Sunlight hydrogen production attempts with laboratory-grown cultures

The final part of the PhD experimental activity was conducted with a tubular outdoor PBR (50 liters volume), placed in the outside area of the ISE-CNR, Sesto Fiorentino, Florence. This PBR was extensively adopted in the past for the photosynthetic growth of bacteria, cyanobacteria and algae, as well as for the biological H_2 production (Bocci et al., 1987; Torzillo et al., 1991a; 1991b; 1996; 1998; Vonshak et al., 1994; 1996; Pushparaj et al., 1994; 1995; Prakash et al., 1995; Masojideck et al., 1999; 2000; Carlozzi and Sacchi, 2001; Carlozzi et al., 2006; 2008; Carlozzi, 2008). A detailed description of the PBR is given in Materials and Methods.

The experiments were carried out during a 5-month period between the summer and autumn of 2008 and concerned initially the well-known strain CC124 and later on the D1 protein mutant L159I-N230Y. It is worth mentioning that no report of H_2 production outdoor (that is, by means of direct sunlight) with *Chlamydomonas reinhardtii* strains is present in literature to date.

The first attempt to produce H_2 outdoor was conducted with cultures grown in the laboratory. This solution guaranteed a prolonged axenicity of the culture but suffered of a number of practical concernes. For instance, to have enough culture to be collected in its late exponential phase of growth, a very high volume had to be treated. Thus, when sulfur depriving cultures by means of

centrifugation and re-suspension in TAP-S (up to 5 times, Melis et al., 2000) the risk of contamination was rather high. To partially overcome these problems, the rationale proposed by Laurinavichene et al. (2002) was adopted. As the outdoor PBR volume was 50 liters, an inoculum of 20-25 liters was diluted by means of addition of 25-30 liters of TAP-S. The final concentration of sulfur was not evaluated, but it must be considered that the massive growth imposed to the cultures had appreciably reduced the amount of sulfur in the medium when collected for H_2 production experiments. This solution permitted a very fast set up of H_2 production experiments, while ensuring the axenicity of algal cultures for a prolonged period of time. These growing conditions were applied in a set of experiments during which cultures were subjected to increasing solar light irradiances. The solar radiation was reduced covering the PBR by means of white filters, which did not alterate sunlight spectra. Light supply in each experiment was, namely: low (up to 300 µmol photons m^{-2} s^{-1}), medium (up to 650 µmol photons m^{-2} s^{-1}) and high solar light intensity (up to 2000 µmol photons m^{-2} s^{-1}). The irradiances impinging the PBR surface during the above mentioned experiments are presented in Figure 49.

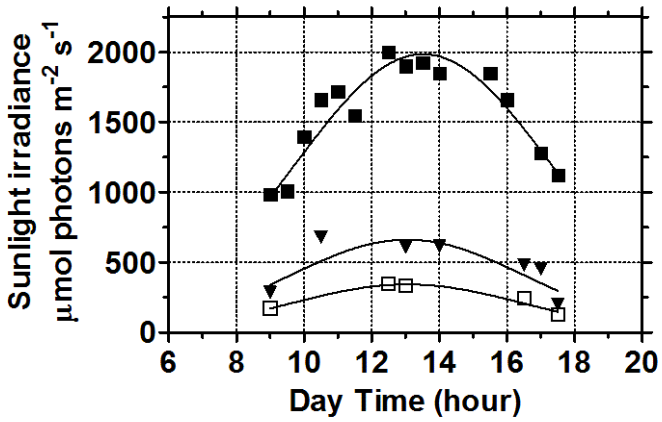


Figure 49. Direct solar radiation impinging PBR surface. Note that the latitude and longitude of the outside area where the PBR was operated were 48.818° North and 11.202° East, respectively. Light intensities were measured at the PBR surface with a flat quantum radio-photometer (LI-250A, LI-COR).

Solar light exploitation encounters several drawbacks respect to artificial laboratory one, such as: (1) solar radiation impinging PBR's surface has a very different spectra compared to cool-white fluorescent light usually adopted in the laboratories; (2) light dilution in outdoor cultures is not easily achievable, as by means of a double sided illumination; (3) the apparent sun rotation implies a constant change in the supplied light radiation to the culture; (4) the natural day/night cycles lead to L/D cycles, which implies culture will remain in the dark for several hours if not artificially

illuminated. All these parameters are relevant to the H_2 production, as they may exacerbate photoinhibition of PSII, which is already inhibited by high light (Kandler and Sironval, 1959; Kyle et al., 1984), particularly in a sulfur-deprived culture where the recovery of the D1 is suppressed.

When low solar light was provided (Figure 50), culture did not show any significant change in the tested parameters (chl content, dry weight, maximum and effective quantum yield of PSII, redox potential, pH value and dissolved O_2 concentration) over a 3-days period.

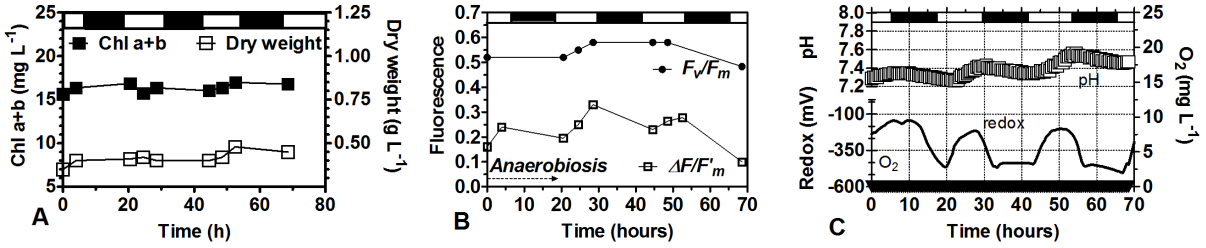


Figure 50. H_2 production experiments under solar light radiation with laboratory-grown cultures of the strain CC124 exposed to low light intensities (up to 300 µmol photons m^{-2} s^{-1}).
The culture was monitored according to (A) chlorophyll and dry weight, (B) F_v/F_m and $\Delta F/F'_m$ values and (C) pH value, redox potential and dissolved O_2 concentration. White and black bars reported in the upper part of the graphs represent the day/night cycles, respectively, to which cultures were exposed due to the apparent sun rotation. Keys reported in the graph

The aim of this experiment was to illuminate cultures with a highest intensity (up 300 µmol photons m^{-2} s^{-1} at midday) comparable to what already adopted in the laboratory. However, the chl content and the dry weight on the one hand, and both fluorescence parameters (F_v/F_m and $\Delta F/F'_m$) on the other showed that the culture suffered a relevant photolimitation. No trace of O_2 was ever recorded and, in accordance, the highest redox potential value remained below -100 mV.

When exposed to a medium solar light intensity (up to 650 µmol photons m^{-2} s^{-1} at midday) (Figure 51), cultures showed a different behavior with respect to the previous set, which however was not enough to induce the H_2 production process.

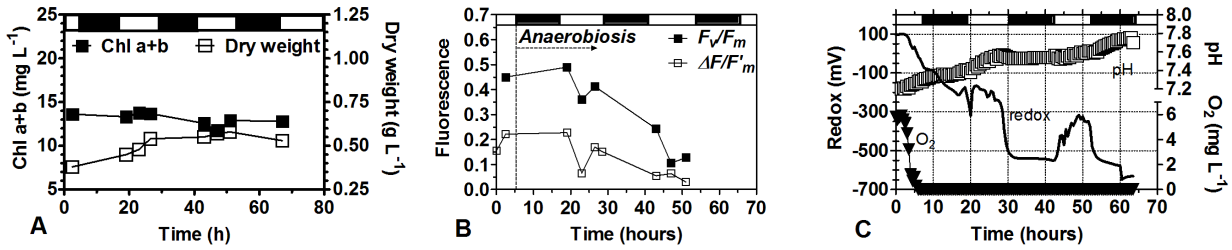


Figure 51. H_2 production experiments under solar light radiation with laboratory-grown cultures of the strain CC124 exposed to medium light intensities (up to 650 µmol photons m^{-2}

s^{-1}). The culture was monitored according to (A) chlorophyll and dry weight, (B) F_v/F_m and $\Delta F/F'_m$ values and (C) pH value, redox potential and dissolved O_2 concentration. White and black bars reported in the upper part of the graphs represent the day/night cycles, respectively, to which cultures were exposed due to the apparent sun rotation. Keys reported in the graph.

Interestingly, the culture had an initial aerobic phase of about 5 hours, after which a lasting anaerobiosis was established. Both fluorescence parameters showed a strong decrease within the first 45 hours. The reduction in the F_v/F_m value proved the occurrence of a strong photoinhibition (Vonshak et al., 1994; Torzillo et al., 1996; 1998). As no H_2 production was recorded, solar light intensity was increased up to 2000 µmol photons m^{-2} s^{-1} (at midday) in a last set of experiments (Figure 52).

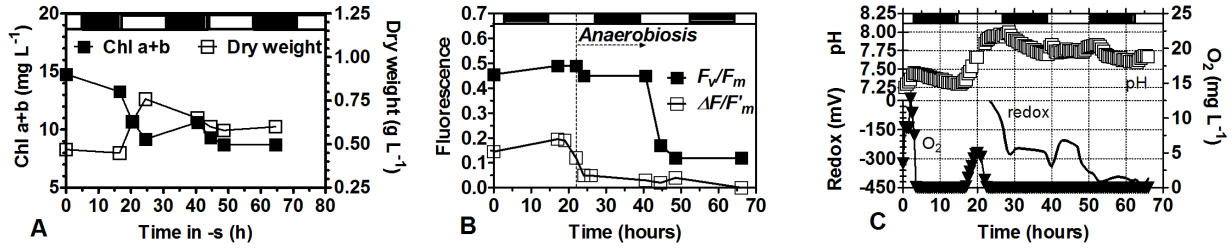


Figure 52. H_2 production experiments under solar light radiation in laboratory-grown cultures of the strain CC124 exposed to high light intensities (up to 2000 µmol photons m^{-2} s^{-1}). The culture was monitored according to (A) chlorophyll and dry weight, (B) F_v/F_m and $\Delta F/F'_m$ values and (C) pH value, redox potential and dissolved O_2 concentration. White and black bars reported in the upper part of the graphs represent the day/night cycles, respectively, to which cultures were exposed due to the apparent sun rotation. Keys reported in the graph.

Between 17 and 23 hours after sulfur starvation (that is, at the 2nd day of cultivation), a strong consumption of acetate (reflected in the sharp increase of the pH value) occurred concomitantly with an increase of the dry weight. In the mean time, the culture, which underwent anaerobic conditions due to the night period, produced a net amount of O_2 (up to 5 mg L^{-1}), which was up-taken by respiration and led to the anaerobiosis in the light. $\Delta F/F'_m$ value suffered a strong reduction (from 0.200 to 0.050), while F_v/F_m remained stable. Although this typical changes should have led to the induction of H_2 production, no gas evolution was noticed. Both fluorescence parameters declined thereafter, and redox potential did not recover over -150 mV during the following day.

The increase in solar light intensity appreciably decreased the lifetime of PSII (Fig. 50b, 51b and 52b). Similarly, chl degradation was enhanced at higher solar light intensities (Fig. 50a, 51a and 52a). As noted above, the coupling of sulfur depletion with high solar light may exacerbate PSII photoinhibition and, like observed with the strain IL (Section 3b.2), a sufficiently active PSII is needed for the induction of the H_2 production process before energy reserves are driven towards

other metabolic pathways. Thus, in order to enable a prolonged PSII activity, cultures were adapted to the outdoor conditions by growning them directly into the outdoor PBR.

4a.2 Sunlight hydrogen production with outdoor grown cultures

To adapt cultures to the outdoor conditions, the inoculum was first grown in the laboratory as previously described. Afterwards, cultures were diluted into the outdoor PBR (50 liters final volume) to an initial concentration of about 5 mg L^{-1} to experience a complete growth outdoor under full sunlight radiation. The pH was maintained between 7.2 and 7.5 by means of sterile CO_2 bubbling. Temperature and mixing speed were left unchanged respect to previous H_2 production experiments. A typical growth followed as chl and dry weight is shown in Figure 53.

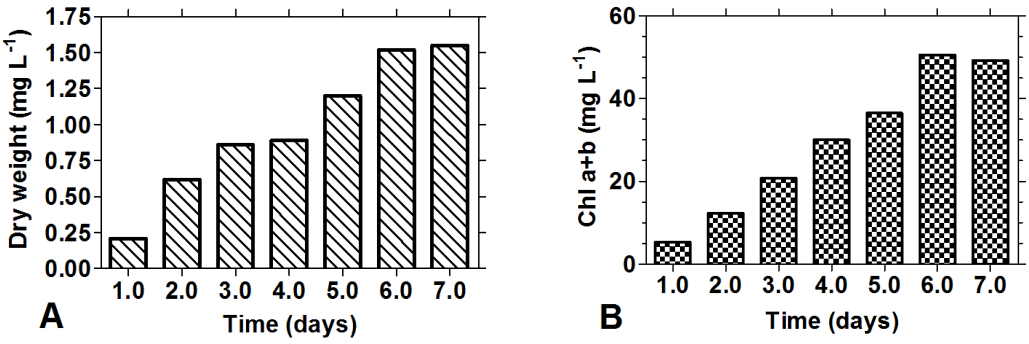


Figure 53. Time course of *C. reinhardtii* CC124 strain growth according to the dry weight (A) and the chlorophyll content (B). The growth was performed during the Summer of 2008 (see Fig. 49 for a typical solar radiation pattern). Results are the mean value of 3 independent cultures.

The culture reachead the maximum values of chl and dry weight (50 mg L^{-1} and 1.5 g L^{-1}, respectively) after 6 days outdoor (the growth was performed during the August 2008). No reduction in the chl content per dry weight was observed (3.33%), although light supply was particularly different from laboratory standard one. After two days of growth outdoors, the culture was able to fully acclimate to high light, as demonstrated by the recovery of the photosynthetic activity (Figure 54, circles), which surpassed that attained with laboratory cultures (Fig. 54, triangles). However, such a result could be also partially due to the fact that day/night cycles have led to a synchronization of cells division, which was shown to have a partial effect on photosynthesis and respiration rates (Kosourov et al., 2002).

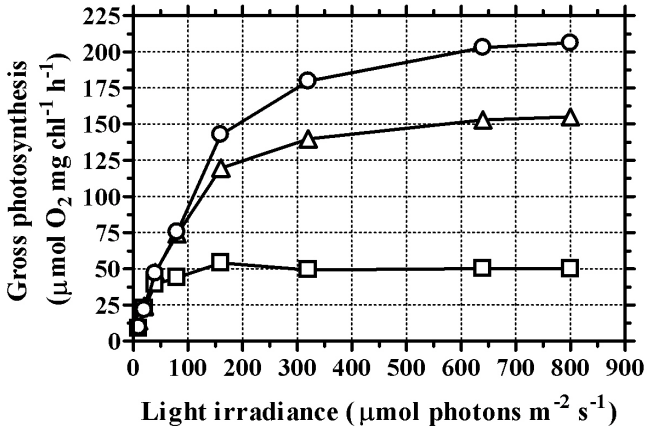


Figure 54. Gross photosynthetic activity (net photosynthesis plus respiration) in a *C. reinhardtii* CC124 culture grown outdoor. Analyses were carried out with a chl concentration of 10 mg L^{-1}. Keys: Day 1, (△); Day 2, (□); Day 3, (○).

Acclimated cultures were set up for H_2 production after sulfur depletion as proposed by Melis et al. (2000). Cultures were collected in the mid-exponential phase of growth outdoor (after at least 3 days, Fig. 53), washed with TAP-S and finally resuspended at the desired final concentration. In order to evaluate the effect of outdoor acclimation on cultures, part of the same inoculum tested outdoor was used to test H_2 production in laboratory standard conditions (70 μmol photons m^{-2} s^{-1} on both sides and 12 mg L^{-1} initial chl content, mixing allowed by a stir bar; Tab. 9). In order to partially reduce the stressing conditions due to day/night cycles, well described by the oscillatory redox potential pattern which followed sunlight illumination, artificial light was provided during nights (17:30 - 9:00) with a 1000 W lamp, which intensity was about 100 μmol photons m^{-2} s^{-1} at the reactor surface.

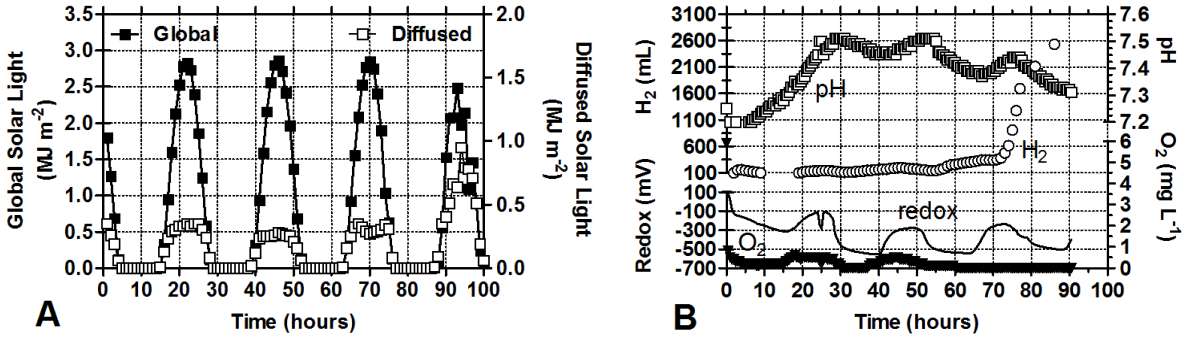


Figure 55. H_2 production experiments under solar light radiation in outdoor acclimated cultures of the strain CC124 with an initial chlorophyll content of 13.5 mg L^{-1}. (A) Solar light radiation during the outdoor experiment. Note that the latitude and longitude of the outside area where the PBR was operated were 48.818° North and 11.202° East, respectively. Data were kindly provided by La.M.M.A. - Regione Toscana, Laboratorio per la Meteorologia e la Modellistica Ambientale, which has a weather station next to the outside area of the ISE-CNR where the outdoor PBR was operated. Keys reported in the graph.

The first H_2 production outdoor (Figure 55) occurred with a very low chl concentration (13.5 mg L^{-1}). Acclimated cultures were able to evolve O_2 in the outdoor PBR until the 3rd day of cultivation, that is until about 50 hours. Accordingly, redox potential values followed a similar pattern. H_2 production started after 55 hours (as confirmed by gas cromatographic analyses). Nonetheless, rates of production remained rather low until the start of the 4th day (T=70 hours): since then, rates increased up to 360 mL PBR^{-1} h^{-1}. H_2 production ended as soon as the sun declined, the final production being 2.4 L PBR^{-1} (96% H_2 and 4% CO_2). On the other hand, H_2 production in laboratory standard conditions of the outdoor acclimated culture showed a very typical behavior (Figure 56).

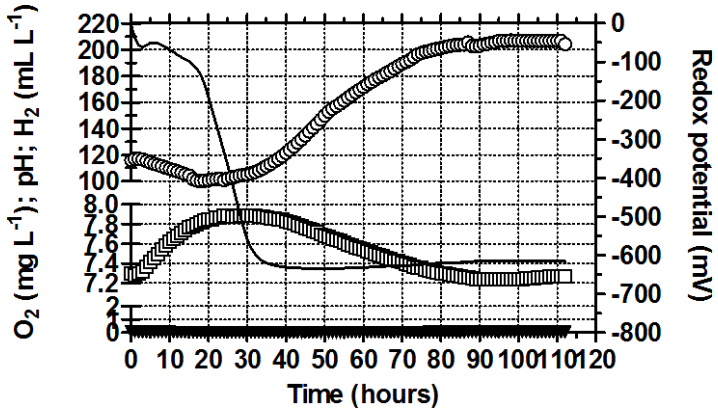


Figure 56. H_2 production by an outdoor acclimated CC124 culture under laboratory standard conditions. (see also Materials and Methods for details). Results refer to the same culture tested outdoor (Fig. 55b). Keys: pH value, (□); redox potential, (continuous line); dissolved O_2 concentration, (▼); H_2 gas accumulation, (○).

The continuous and stable illumination of the culture led to a very linear pattern of the followed parameters, respect to what observed with the same culture tested outdoor (Fig. 55). Contrary to previous observations (Section 3a), O_2 was never detected, as a result of an increased respiratory capacity of the strain. Finally, the culture evolved about 100 mL L^{-1} (Fig. 56), similar to previous findings (Tab. 9).

This very high production outdoor was actually rare (it corresponded to only 50% reduction respect to the laboratory, on a per liter basis) and was rather surprising considering the low chl content (13.5 mg L^{-1}). Generally, a concentration of about 20 mg L^{-1} was found to be the most successful for outdoor H_2 production purposes (Figure 57). This chl concentration was considered the best compromise between the mixing regime and light irradiance, in order to promote a sufficient light penetration and lower the PSII photoinhibition. Nonetheless, in several experiments, PSII activity was particularly low and, as above reported, H_2 process did not start notwithstanding a high amount of carbohydrates and the complete anaerobiosis reached very soon by the culture.

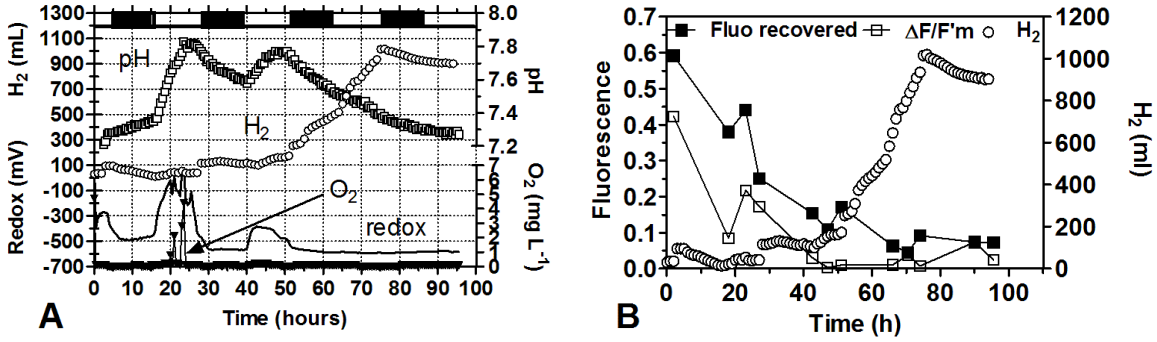


Figure 57. (A) H_2 production experiment under solar light radiation in outdoor acclimated cultures of the strain CC124 with an initial chlorophyll content of 20 mg L^{-1}, and (B) fluorescence parameters during the H_2 production. (A) White and black bars reported in the upper part of the graphs represent the day/night cycles, respectively, to which cultures were exposed due to the apparent sun rotation. Keys reported in the graph.

H_2 production started at the end of the 2nd day of cultivation (T=27 hours), stopped due to the night period and started again the day after. The artificial light supply during nights was not enough to sustain redox potential towards less negative value and it was unclear to which extent it may have contributed. $\Delta F/F'_m$ measurements (Fig. 57b) confirmed PSII activity was sufficient to induce the H_2 production process, but its contribution ceased immediately after. Thus, the production obtained during these experiments was only due to fermentation processes.

Interestingly, measurements of fluorescence taken directly from the PBR (thus, in anaerobic conditions), after a very short period in the dark, showed a partial recover of the PSII activity (Fig. 57b, Fluo recevered). This measure gives a hint of the level of photoinhibition suffered by the culture in that specific moment. The sharp increase in the pH value during the first 30 hours is consistent with carbohydrate and protein analyses which showed in both a strong increase (Figure 58). However, even if H_2 production was already started, between 40 and 50 hours after the onset of the experiment (3rd day of cultivation), another sharp increase in the pH was observed, which was reflected in an increase in the protein content. This may be due to a remaining Rubisco activity, which can compete for electrons with the hydrogenase (as demonstrated by Ruehle et al., 2008; see also Fig. 33). Biogas composition was found to be 98% H_2 and 2% CO_2.

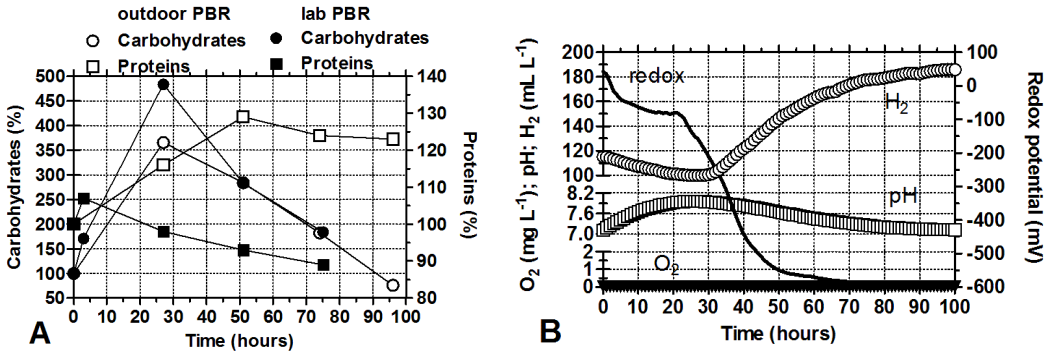


Figura 58. (A) Carbohydrate and protein measurements in outdoor acclimated cultures of CC124 tested in an outdoor PBR under sunlight and in laboratory standard conditions. (B) H_2 production by an acclimated culture of CC124 tested in laboratory standard conditions. Keys reported in the graph.

As a whole, H_2 final volumes were found to be 18.4 vs. 86.0 mL L^{-1} in the outdoor and laboratory tested cultures, respectively. It is of interest to note that the latter were always found competent for the H_2 production, even when in the outdoor experiment no production was obtained. However, no increase or reduction in productivity was ever observed with respect to previous findings (Tab. 9). This observation must also include the fact that day/night cycles led to synchronized cultures, which was already shown had little or no effect respect to unsynchronized ones for H_2 production purposes under sulfur depletion (Kosourov et al., 2002; Tsygankov et al., 2002). Nevertheless, when comparing H_2 productivity between the outdoor and the laboratory PBR, a relevant reduction was observed (-78% H_2 production per liter of culture). A number of factors must account for this lowered efficiency: (1) in the cultures tested in the outdoor PBR, a relevant part of the reducing power is used for protein synthesis, most likely to sustain other metabolic pathways devoted to the survival of the cells; a part of them is synthesized even during the H_2 production process; on the other hand, in laboratory PBRs proteins degradation may have actively contributed to the H_2 production; (2) the decreasing pattern followed by the pH during nights suggests that in the culture tested outdoor a consumption of endogenous substrates may have taken place: most likely they were used as a source of reducing power during prolonged periods of darkness (Torzillo et al., 1991a; 1991b). This evidence represent also another hint for the insufficient support given by the artificial light radiation supplied during nights; (3) although cultures tested in the laboratory had a lower biomass concentration and were subjected to much lower light intensities respect to the one cultivated outdoor, carbohydrates accumulation reached a higher value in the former (500 vs. 350% of the initial value, respectively); (4) fluorescence measurements pointed out that in the outdoor PBR a severe photoinhibition of the PSII took place since the onset of the experiment, as $\Delta F/F'_m$

value was rarely detected over 0.200.

Aside from demonstrating that laboratory grown cultures may very hardly produce H_2 when transferred outdoor, as an acclimation is crucial to overcome PSII severe photoinhibition, the previous set of experiments pointed out that light availability in the outdoor reactor suffered some critical concern. Visual observations ruled out a possible *shear stress* imposed to the cells due to the pump. Obviously, the main concern is the lack of light irradiance during nights, which leads to an excessive consumption of energy reserves and causes a strong change in the redox state of the culture. It was shown previously in this work (Section 3b) that an optimal redox potential for H_2 production purposes should be maintained around -100/150 mV as long as possible. Unfortunately, this is very hardly achievable in cultures irradiated with direct solar light, and artificial light illumination will never solve this problem. As a matter of fact, after being illuminated with direct solar light (up to 2000 μmol photons m^{-2} s^{-1}), any other irradiation is perceived by the sulfur-deprived culture as a low intensity, a new adaptation requiring a very long time. This was particularly evident when observing sudden drops in the redox potential towards extremely negative values (e.g., -500/600 mV) as soon as clouds reduced sunlight radiation for few minutes by the half (that is, still 1000 μmol photons m^{-2} s^{-1}). The only possible solution to overcome both problems (high intensities at midday and darkness during night) would be a sunlight collection system able to store part of the light energy during daytime and irradiate the remaining part during the night period. In this case, according to the on average impinging solar radiation, one could irradiate cultures with a more constant (and selected) intensity throughout the entire experiment. However, such a solution could be too expensive for any biological H_2 production application.

4a.3. Hydrogen production comparison, under artificial light illumination, in laboratory and outdoor photobioreactors

H_2 production was tested with a continuous artificial illumination provided by a parabolic system (M2M Engineering, Naples, Italy) equipped with a set of 20 neons (Osram L 58 W/940, Luminux de Lux, cool white) 1.60 m long (Figure 59).

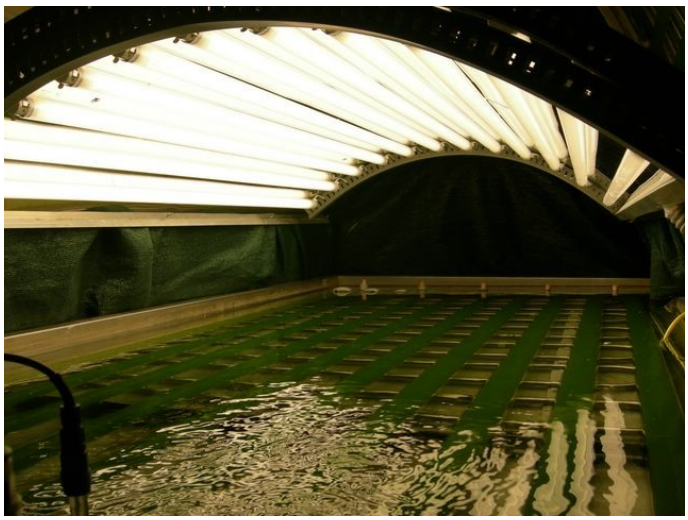


Figure 59. Overview of the parabolic system supplying continuous artificial light for H_2 production experiments with the outdoor PBR. Note that illumination was homogenous upon the all PBR surface. The PBR was entirely covered with a green pass filter (clearly visible in the picture) to avoid sunlight radiation to illuminate the cultures during days.

The aim of such experiments was to test the PBR reliability for H_2 production purposes with green algae, aside from solar light supply. Cultures were grown outdoor as described above; the initial chl content was set to 12 ± 1 mg L^{-1} in both outdoor and laboratory tested culture used as a control. Light intensity was set to about 250 µmol photons m^{-2} s^{-1} in the outdoor PBR and to 70 µmol photons m^{-2} s^{-1} on both sides in the control. H_2 outputs are shown in Figure 60.

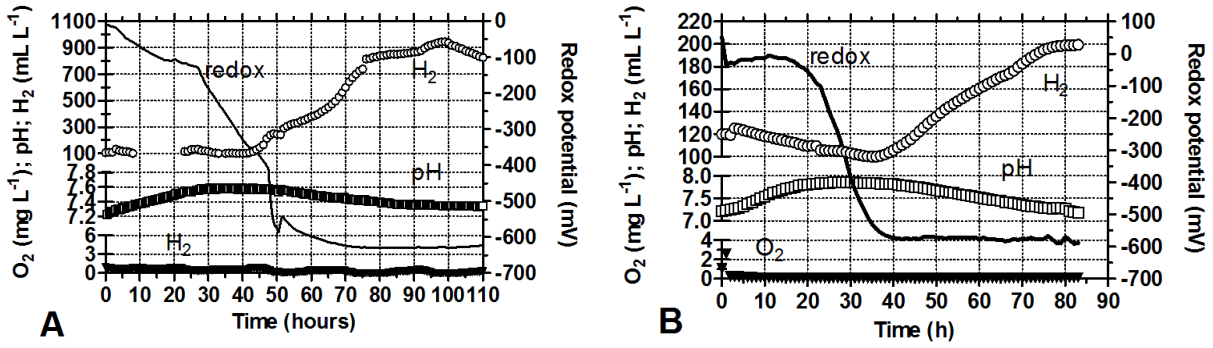


Figure 60. H_2 production experiments under continuous artificial light radiation (strain CC124) in (A) a 50-liter tubular PBR, and in (B) laboratory standard conditions. Keys reported in the graph.

Although all the parameters usually followed showed to be much more linear in the outdoor PBR (as a result of constant illumination), the H_2 productivity achieved was still much lower compared to the control (17 vs. 99 mL L^{-1}).

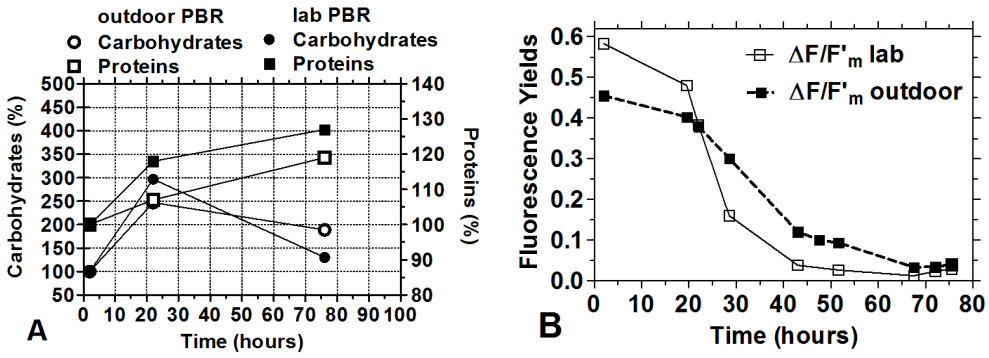


Figure 61. Physiological changes in CC124 cultures tested under continuous artificial light in an outdoor PBR and in laboratory standard conditions: (A) carbohydrate and protein measurements; (B) effective quantum yields of PSII. Keys reported in the graph.

Fluorescence measurements carried out directly onto the illuminated surface of the PBR showed no severe photoinhibition in the culture tested outdoor, contrary to previous findings while supplying solar light. However, comparing $\Delta F/F'_m$ values between the outdoor and laboratory PBR (Fig. 61b), the same culture performed much better in the latter. Moreover, $\Delta F/F'_m$ values in the outdoor PBR were rather low also considered that: (1) F_v/F_m value at the onset of the experiment was as much as 0.700 (versus 0.450 in the $\Delta F/F'_m$ value outdoor); (2) light dilution (due to chl content and light intensity at the surface of the PBR) could not justify such a reduced value. Accordingly, the culture tested outdoor showed a reduced capability to both store and utilize carbohydrates compared to the culture tested in the laboratory (Fig. 61a); this was also reflected in a different increase in the pH value within the first hours of experiment.

Thus, other than the unconstant sunlight supply, light itself was not properly exploited in the outdoor PBR probably as a consequence of inadequate mixing. It was estimated that with a culture velocity of about 0.20 m s^{-1}, the corresponding Reynolds number was about 10,000, which for smooth tubes like the one used for the PBR construction did not enable a wholly turbulent flow to be reached (Munson et al, 1990). Nevertheless, it must be noted that a lowered and constant irradiation led to an effective enhancement in H_2 productivity when comparing outdoor PBR performances in solar (Fig. 57a) and artificial light (Fig. 60a). In fact, the final volume produced in these experiments, in terms of chl, increased from 46 to 77 mL H_2 g^{-1} chl, respectively.

b) Experiments with the mutant strain L159I-N230Y

4b.1 Up-scaling hydrogen production from 1 to 50 liters under artificial light supply

Outdoor experiments for the H_2 production were carried out also with the D1 mutant strain L159I-

N230Y, during the autumn of 2008. In this period of the year, at our latitude (43° North) the solar irradiance constancy is compromised and it was possible to properly execute with direct sunlight only some growth experiments (Figure 62).

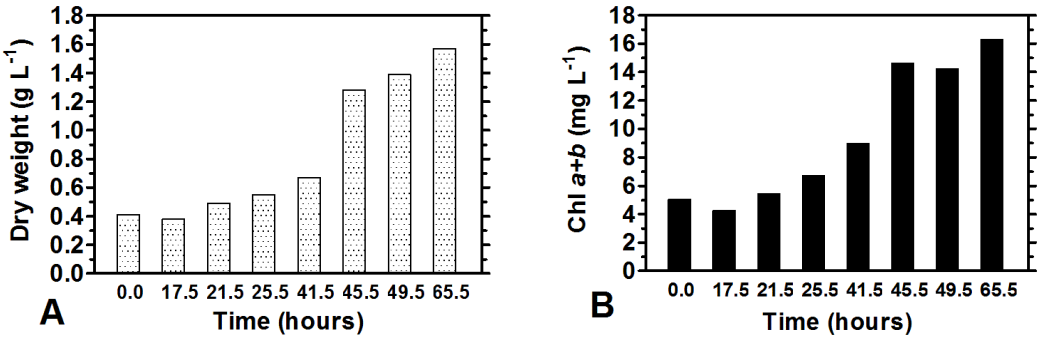


Figure 62. Time course of *C. reinhardtii* D1 protein mutant strain L159I-N230Y growth according to the dry weight (A) and the chlorophyll content (B). The growth was performed during the Autumn of 2008. Results are the mean value of 2 independent cultures.

As observed in the laboratory, this strain synthesized reduced amounts of chl per dry weight unit: after 65 hours, only about 16 mg L^{-1} where measured along with roughly 1.6 g L^{-1} of dry weight, with a corresponding ratio of 1.0-1.3% chl/dry weight. This ratio was appreciably lower then the one noted in laboratory standard conditions (1.8%, Tab. 8), notwithstanding the relatively low solar light irradiances. This behavior was not observed in the CC124 strain grown outdoor, which maintained its typical chl/dry weight ratio (Fig. 53 and Tab. 8). As a consequence of the enhanced reduction of the antenna, this strain could theoretically perform even better than what shown in the laboratory. Unfortunately, H_2 production experiments outdoor with this strain were only carried out under artificial light, and further investigations on its H_2 productivity under sunlight were postponed to future projects.

As H_2 production in the outdoor PBR most likely suffered of poor mixing regime, a last attempt to circumvent this problem (and reach laboratory H_2 production) was done improving light penetration within the culture layers by means of the interplay between chl content and light intensity. The utilization of a reduced antenna mutant such as L159I-N230Y would have fitted with such a purpose. Thus, in a first set H_2 productivity was evaluated by providing 200 µmol photons m^{-2} s^{-1} to 12 and 6 mg L^{-1} concentrated cultures, respectively. Then, in a second set light intensity was increased up to 350 µmol photons m^{-2} s^{-1} while chl concentrations were maintained unchanged. Cultures were grown in the laboratory as above described for the 50 liters PBR, and continuous

artificial light was irradiated with the parabolic system already utilized with the strain CC124 (Fig. 59). The results obtained are summarized in Table 12.

Table 12. Summary of all the H_2 production phases and yields achieved with the D1 protein mutant strain L159I-N230Y tested in the laboratory and in the outdoor PBR under continuous artificial light. Results are the mean value of two experiments made on independent cultures.

Culture Conditions		Aerobic phase		Anaerobic phase		H_2 total volume		H_2 Final rate	H_2 Maximum rate	H_2 Final rate	H_2 Maximum rate	Max rate duration
Light	Chl Content	O_2 production phase	O_2 respiration phase	Lag phase	H_2 production phase							
µmol photons m^{-2} s^{-1}	mg L^{-1}	hours	Hours	Hours	Hours	mL H_2 L^{-1}	mL H_2 PBR^{-1}	mL H_2 L^{-1} h^{-1}		mL H_2 g chl^{-1} h^{-1}		hours
200	12	-	-	43	56	9.8	491.5	0.18	0.37	15.5	32.7	11
200	6	-	-	27	72	27.4	1368.4	0.38	0.59	60.7	94.2	31
350	12	-	-	16	60	19.0	947.7	0.32	0.41	28.5	37.1	26
350	6	-	-	18	76	15.7	786.8	0.21	0.42	39.5	80.7	32

The H_2 productivity obtained with laboratory standard conditions (70 µmol photons m^{-2} s^{-1} on both sides and 12 mg L^{-1} initial chl content) was never reached in any of the tested conditions. It was clear that photosynthesis was carried out at very low rates, as $\Delta F/F'_m$ was hardly found over 0.350 even during the initial aerobic phase of the process (data not shown). Accordingly, carbohydrates accumulation was never higher than 210% of the initial content, which for this strain is rather low (Tab. 11). Moreover, it was found that the protein accumulation was particularly sustained and interested the entire H_2 production phase, as also found during the experiments with strain CC124. The best performance was obtained with the lowest chl concentration and light supply (6 mg L^{-1} and 200 µmol photons m^{-2} s^{-1}, respectively) and was about 3 times lower (on a chl basis) than usually obtained in the laboratory.

Section 4 Conclusions and future perspectives

H_2 production was evaluated using a tubular outdoor PBR by means of two different strains (CC124 and the D1 protein mutant L159I-N230Y), two different light supply (solar and artificial) and by testing two different kind of cultures (grown in standard laboratory conditions or acclimated outdoor). First of all, it must be stressed that the present work represents, to the best of our knowledge, the first H_2 production with *Chlamydomonas reinhardtii* carried out by means of sunlight radiation.

An appreciable reduction in H_2 productivity was generally observed in all the tested conditions. Indeed, results showed that, while productivities were left substantially unchanged with respect to biomass growth in both strains under solar light, much more attention must be taken when producing H_2 even under artificial light supply.

The reduction of productivity was due to three main factors: (1) culture volume was increased up to 50 times (from 1.1 to 50 liters), which implies that optimal laboratory conditions can hardly be reproduced without a consistent loss of efficiency; (2) up to 22% of the culture volume was in the dark because of the PVC U-bends connecting PBR tubes and because of the pump; this means that on average a relevant part of the culture was maintained in the darkness. By blacking out the illumination at intervals no practical benefit could be achieved with respect to an intermittent light effect (L/D cycle). On the contrary, cells in the darkness will consume energy by respiration; (3) the mixing regime to which cultures were exposed was not sufficiently turbulent and led to a reduced photosynthetic activity. A clear evidence for that was given by the H_2 bubbles, which usually run in the upper part of the tubes due to buoyancy and were never cought by the flow turbulence.

Aside from a constant and homogeneus illumination, in order to optimize H_2 productivity outdoor, light experienced by the cells must be improved by means of appropriate L/D cycles. Of course, light dilution achieved by changing light intensities and/or chl concentration can only partially prevent photoinhibition or photolimitation of the cultures. On the other hand, an increase in the velocity must be obtained by minimizing the hydrodynamic stress of the cells and avoiding the formation of foam, which can favor culture contamination. Very likely, one possibility for circumventing the problem could be the use of static mixers, which may promote a regular L/D cycle in the culture depth, thus an effective light dilution within the culture, even if a higher shear stress would be very likely to be induced by the head losses localized on the boundary layer surrounding the mixers.

CONCLUSIONS

The role of the D1 protein within the H_2 production in the green microalga *Chlamydomonas reinhardtii* was estimated by studying several mutants reporting deletions or substitutions in a specific region of this protein. Mutants were selected according to their capacity to grow photoautotrophically and because showing fluorescence characteristics different from the WT. The H_2 production was found to range between 0 and more than 500 mL L^{-1}. One of the most interesting mutants was L159I-N230Y, which showed a 5 times higher productivity respect to the widely used CC124, when subjected to standard conditions (low light and low chlorophyll content). Its improved productivity was due to a number of key features, the most important of which, with respect to its WT, are: (1) a larger chlorophyll optical cross-section; (2) a higher electron transfer rate by PSII; (3) a higher respiration rate; (4) a higher efficiency of utilization of the hydrogenase; (5) a higher starch synthesis capability; (6) a higher *per cell* D1 protein amount; (7) a higher zeaxanthin synthesis capability

On the other hand, the mutant L159I-N230Y was able to fully exploit the positive effects of well-agitated cultures, like those provided by the multiple-impeller device developed by us (Giannelli et al., 2009). Indeed, it was found that an effective light/dark cycle, particularly in dense cultures, was induced with the impeller mixing device, leading to improved photosynthetic yields. In addition, the better spatial distribution of the photosynthetically generated O_2 enhanced respiration rates in comparison with stir-bar-mixed cultures. When the best mutant (L159I-N230Y) was operated in optimal culture conditions with the impeller mixing device, it produced steadily for more than 20 hours at rates equal to 11 mL L^{-1} h^{-1}, corresponding to a 3.2% apparent light conversion efficiency (Scoma et al., 2010a, *submitted*). Up to 54% of these rates (and 61% of the final H_2 productivity) were due to the direct contribution of the PSII, meaning that they were produced by means of biophotolysis. Such a result in the mutant was made possible also by the fact that a high PSII photoprotection delay PSII complete down-regulation (Torzillo et al., 2009). As a whole, both the mutant and the multiple-impeller device were found to be useful tools to deepen the physiological changes occurring in H_2 production conditions.

Other than the mutant L159I-N230Y, an accurate characterization of the mutant strain IL was also carried out. This strain was the first mutant created after genetic manipulation of the WT. It was found that with most of the supplied culture conditions this strain was unable to produce H_2. That was due to the fact that physiological limitations reduced its capacity of degrading endogenous

substrates in favor of the H_2 production. Moreover, in a specific set of experiments it was also clearly evidenced that in the absence of a sufficiently active PSII, the H_2 production process could not be induced, meaning that aside from (1) a state 1-2 transition, (2) the establishment of anaerobic conditions and (3) the storage of high amounts of carbohydrates, a *timely* transition to the H_2 production is fundamental to induce the process in sulfur starvation, before energy reserves are deviated towards other processes needed for the survival of the cell.

This last information was crucial when moving outdoors. In fact, the H_2 production process was tested in a horizontal tubular 50-liter photobioreactor placed outside. Results demonstrated that cultures needed to be acclimated to sunlight during growth, in order to prevent extreme PSII photoinhibition because of the occurrence of both high light and sulfur deprivation. Finally, the H_2 production with *Chlamydomonas reinhardtii* under sunlight radiation was successfully achieved (Scoma et al., 2010b, *submitted*). To the very best of our knowledge, the experiments here reported represent the first H_2 production ever obtained with a green microalga by means of direct solar light. Outdoor productivity respect to laboratory standard condition was reduced of about 4-5 times due to several factors, the most relevant of which are: (1) a low photosynthetic yield, (2) an appreciable consumption of endogenous substrates during night periods and (3) a scarce turbulence of the culture. However, preliminary studies showed that enhancements can be achieved by optimizing culture conditions (e.g., chlorophyll concentration and mixing rate) in order to photoprotect PSII from a rapid inactivation.

APPENDIX

Hydrogen oxidation by the microalga *Chlamydomonas reinhardtii*

The first claim of a hydrogenase activity in green algae (e.g. *Scenedesmus*) was reported in 1939 by Gaffron, who discovered that H_2 could be used to photoreduce CO_2 under strictly anaerobic conditions (Gaffron, 1939). Later on, H_2 production in *Scenedesmus* was also observed by the same author (Gaffron and Rubin, 1942). During the last 70 years, dozens of papers have been published on the H_2 metabolism in green algae, particularly after the discover made by Melis and co-workers (2000) that a sustained production could be obtained by depriving a *Chlamydomonas* culture of inorganic sulfur. Nonetheless, only few of these papers concerned the first discover made by Gaffron: the oxidation of H_2, its metabolism and biological meaning (Horwitz, 1957; Russell and Gibbs, 1968; McBride et al., 1997; Maione and Gibbs, 1986; Chen and Gibbs, 1991, 1992; Singh et al., 1992). Moreover, none of them concerned the *in vivo* H_2 consumption under sulfur starvation.

The interest for this topic arose when observing a relevant biogas consumption at the end of the H_2 production process, in sulfur starvation conditions, in CC124 cultures supplied with 70 μmol photons m^{-2} s^{-1} on both sides, and an initial chl content of 24 mg chl L^{-1} (Figure 1).

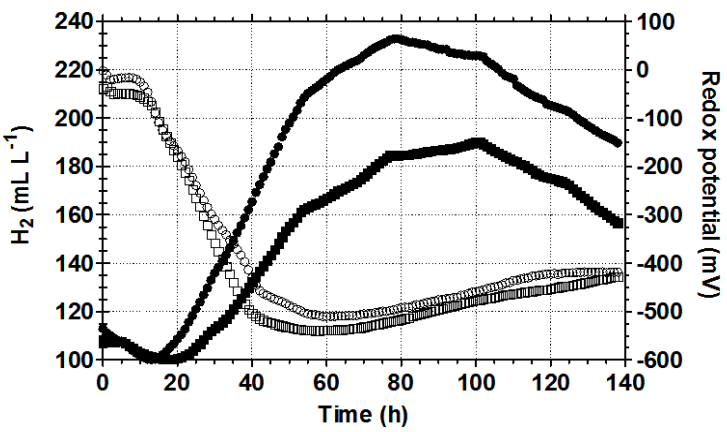


Figure 1. Biogas consumption in sulfur-deprived *C. reinhardtii* cultures (strain CC124) at the end of the H_2 production phase. Cultures were stirred by means of different devices. Keys: H_2 gas in impeller-stirred cultures, (●); H_2 gas in stir bar-mixed cultures, (■); redox potential in impeller-stirred cultures, (○); redox potential in stir bar-mixed cultures, (□).

Cultures were provided with two different mixing devices, a multiple impeller system and a conventional stir bar (see Section 2). As H_2 gas production ended, a biogas consumption took place in both photobioreactors (PBRs), soon after reaching a constant value equal in both mixing devices (about 0.73 mL^{-1} biogas L^{-1} of culture h^{-1}, between 100 and 140 hours in Fig. 1). It must be noted that

cultures remained always in contact with the previously produced biogas (either H_2 or O_2). At the same time, redox potentials moved towards less negative values (from -500 to -400 mV). These few results led to a number of observations: (1) as the rate of consumption was equal in both stirring systems, mixing did not represent a factor; (2) as mixing did not represent a factor, most likely the process was not light dependent; in accordance, the H_2 production process that was just ended (between 20 and 80-100 hours, Fig. 1) showed that light-driven processes can appreciably benefit of proper stirring; (3) if the consumed biogas was all O_2, this would have meant that both H_2 productions were very strongly underestimated, which is unlikely considering rates of production. However, that was not the case, as experiments already showed that the impeller mixing device was able to enhance also respiration rates (see Section 2). Thus, even though a concomitant O_2 consumption could not be completely ruled out, most likely the main biogas consumed was H_2.

To point out if light was really unnecessary, in a similar experiment light was switched off at the end of the exponential phase of H_2 production. This time consumption took place immediately and reached comparable rates respect to the previous set (Figure 2).

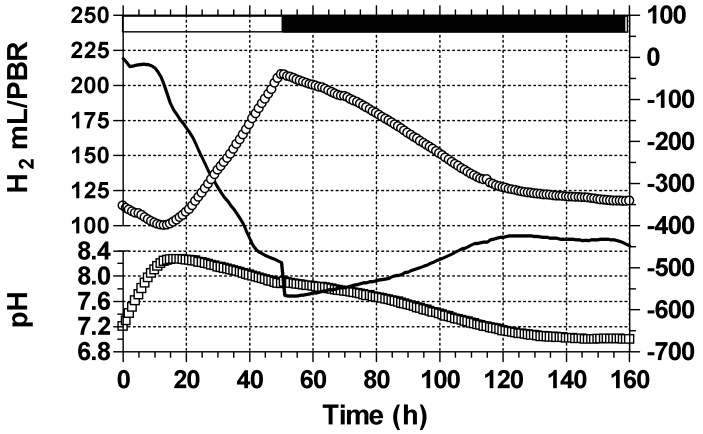


Figure 2. H_2 production-consumption pattern in a CC124 culture depending on light availability. The pH value is also reported. White and black bar in the upper part of the graph indicate the presence of absence of light, respectively. Keys: H_2, (○); redox potential, (continuous line); pH, (□).

Interestingly, all the measured parameters flattened out at the same time (T=120 hours, after more than 48 hours from the start of consumption, Fig. 2), when roughly 80% of the previously produced biogas was consumed. This experiment led to the conclusion that most likely the enzyme responsible for the biogas production under light irradiation (i.e., the hydrogenase) was the same responsible for its consumption. Indeed, O_2 content in the gas phase was not finished when consumption rates declined.

In literature, two pathways are proposed for the H_2 oxidation in *Chlamydomonas* (for Review, see Posewitz et al., 2009): (1) an oxy-hydrogen reaction and (2) a dark CO_2 fixation. Both of them have in common the first step in which H_2 is oxidized by the hydrogenase enzyme to reduce ferredoxin.

Then, in the former, ferredoxin is utilized to reduce $NADP^+$ to NADPH. Consequently, PQ pool reduction is performed by a NAD(P)H/PQ oxidoreductase and in a final step PQ pool is oxidized with the use of O_2 through chlororespiration. On the other hand, dark CO_2 fixation can be performed directly from the reduced ferredoxin with the use of ATP and probably mitochondrial respiration: thus, in both cases, the concomitant presence of small amounts of O_2 turns out to be necessary. On the one hand, CO_2 fixation is taking advantage of a source of electrons to store energy reserves when few other processes could be sustained; on the other, the oxy-hydrogen reaction has been proposed as a mechanism to counteract an excess of reduction of the PQ pool, though the precise reactions associated with this pathway are not completely understood (Posewitz et al., 2009). Most interestingly, both pathways may act together and lead to the concomitant consumption of O_2 and H_2 (Gaffron, 1942; 1944; Kessler, 1974). In a new set of experiments, the H_2 oxidation under sulfur starvation was studied with respect to both these pathways. The main aim was to evaluate which pathway accounted the most of the observed H_2 oxidation, and to understand the physiological circumstances that lead to this consumption in sulfur-deprived cultures (Prof. Thomas Happe is gratefully acknowledged for his technical support during these experiments).

Materials and Methods

A CC124 culture was collected in the exponential phase of growth and deprived of inorganic sulfur as reported by Melis et al. (2000). Cultures were then sealed in a PBR irradiated with 70 µmol photons m^{-2} s^{-1} (on both sides), initial chl a+b content 20 mg L^{-1}. PBR volume was 325 mL, working volume was 115 mL and gas phase was 210 mL. PBRs were operated at room temperature (20-25 °C) and sealed sulfur-deprived cultures were flushed with argon (3 minutes) before the onset of the experiment. A picture of this set up is presented in Figure 3.

Figure 3. Overview of sealed cultures of *Chlamydomonas reinhardtii* under sulfur deprivation. Samples were taken at specific time points and incubated in the dark to estimate the H_2 consumption capabilities.

At specific time points (each 24 hours), 2-mL samples were taken from the PBR and incubated in the dark (20 °C) in 8 mL vessels, under constant shaking. To evaluate H_2 *in vivo* consumption activity, samples were flushed with argon to avoid the presence of any other gas in the liquid and gas phase; then, known amounts of H_2 and air (that is, O_2) were injected in the gas phase of the vessels. O_2 presence is fundamental because the oxy-hydrogen reaction can take place only when low amounts of O_2 are provided (up to 1-2% of the gas phase, Maione and Gibbs, 1986). In the 8 mL vessels adopted for the incubation of the samples, pure H_2 gas was supplied up to a final amount of 2 mL (25% of the vessel volume), while 0.4 mL of air were added to have a final 0.08 mL O_2 content (that is, 1% of the vessel volume). H_2 consumption was estimated as the difference of H_2 content in the gas phase of incubated vessels, with respect to a vessel used as a blank, in which the culture sample was substituted by 2 mL of TAP-S medium. Rates of consumption were the average of 3 analyses per sample (after 1, 2 and 4 hours in the dark). Results are the mean value of 4 experiments on independent cultures (thus, each point represent the mean value of 12 measurements). A measurement of consumption after 20-22 hours was also evaluated, but it turned out that a strong reduction of H_2 consumption was always going on. Most likely, that was due to the fact that the supplied O_2 amount (1%) was already consumed after about 4 hours of incubation, negatively affecting both pathways. Finally, the contribution of the two pathways previously described, was distinguished by injecting in some samples the inhibitor of the Calvin cycle glycolaldehyde (GA) (10 mM final concentration): in this way, only the oxy-hydrogen reaction could have taken place. In the others, used as a control, both pathways would have accounted for the H_2 oxidation.

Results and Discussion

The H_2 production in the main PBR started immediately after 24 hours, and lasted until 120 hours after sulfur starvation (Figure 4).

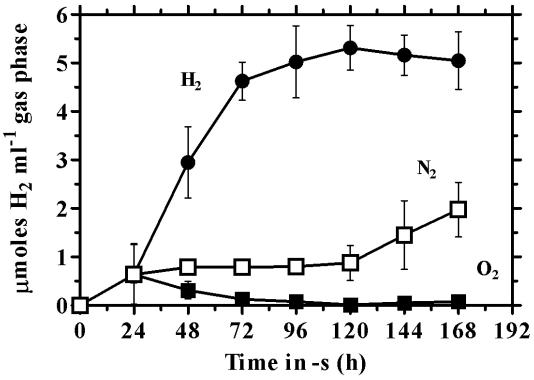


Figure 4. H_2 gas accumulation upon sulfur deprivation in the main PBR. At specific time points, aliquots of algal cells were collected and incubated as described to evaluate the H_2 oxidation capability under sulfur deprivation.

As a whole, the pattern followed by both the control and the samples with GA (Figure 5 and 6, respectively) was similar.

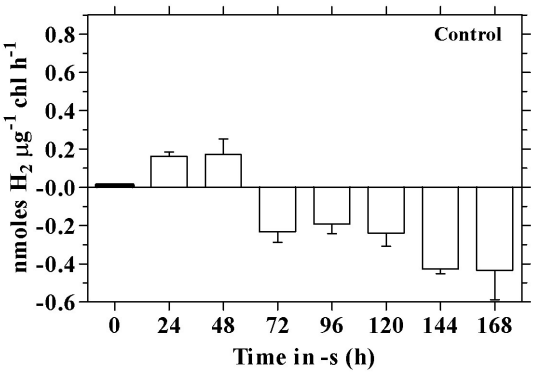


Figure 5. H_2 oxidation in dark incubated algal cells of *C. reinhardtii*, strain CC124. Rates are the sum of the oxy-hydrogen reaction plus CO_2 fixation.

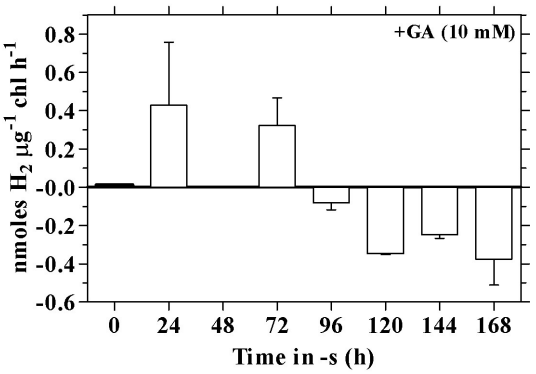


Figure 6. H_2 oxidation after glycolaldehyde addition in dark incubated algal cells of *C. reinhardtii*, strain CC124. Rates represent the H_2 oxidation only due to the oxy-hydrogen reaction.

During the first 48-72 hours of experiment a low but clear H_2 production occurred in both samples even if incubated in the dark. During this time, H_2 production in the main PBR was in the exponential phase (Fig. 4). When finally H_2 consumption in incubated samples prevailed, H_2 production in the main PBR was substantially over (Fig. 4). However, it can be noted that when GA was added (Fig. 6) a lower consumption (or a higher production), with respect to the other sample (Fig. 5), was recorded until about 120 hours after sulfur deprivation. Interestingly, this time was coincident with the end of the H_2 production in the main PBR. Since then, rates of H_2 consumption are equivalent in both control and GA treated cultures, and equal to 0.4 nmoles of H_2 μg^{-1} chl h^{-1}. This value is of the same order of that found in this strain (CC124) under different culture conditions, as described previously (1.7 nmoles of H_2 μg^{-1} chl h^{-1}, Fig. 2). Moreover, as previously

noted, the latter rate must be reduced of a certain value because of a concomitant O_2 consumption that could not be estimated with the previous set up.

According to these results, some considerations can be done: a) if Rubisco inhibition upon GA addition ends up increasing the rates of productivity compared to the control (Fig. 5 and 6), it means that *in vivo* H_2 consumption can take place as soon as hydrogenase enzyme is expressed, even if a number of concomitant effects may hide this phenomenon; b) after 120 hours in the above mentioned conditions, Rubisco activity becomes irrelevant; c) the final consumption rate (equal to 0.4 nmoles of H_2 μg^{-1} chl h^{-1}) was due only to the oxy-hydrogen reaction. It is noteworthy that this timepoint (120 hours) was perfectly coincident with the end of H_2 production in the main PBR. As noted by other authors (White and Melis, 2006), there is a cause-and-effect relationship between a light-dependent and specific catabolism of Rubisco, starch accumulation and the ability of the cell to perform a light-dependent H_2-evolution. Thus, in our experiments it is likely that as long as Rubisco could act as a sink for electrons in the light (Fig. 4), its activity was effectively maintained and revealed also in the dark.

It was surprising to find out that a H_2 production occurred in both dark incubated samples, in the first days of experiment. The physiological pathways responsible of a H_2 production in the dark in *Chlamydomonas* are already known (Beer et al., 2009), even though considered hypothetical yet (for Review, Posewitz et al., 2009). Starch catabolism is known to provide high amounts of pyruvate, a key molecule in the fermentative metabolism of *Chlamydomonas*. The PFO enzyme could decarboxylate pyruvate to acetyl-CoA and reduce ferredoxin at the same time (Mus et al., 2007; Hemschemeier et al., 2008a). Then, the reduced ferredoxin can be oxidized by the hydrogenase to evolve H_2, with no need for light irradiance. In perfect agreement with this hypothesis, in Figure 7 *in vivo* H_2 production rates and starch catabolism show the fermentative nature of the H_2 production of a CC124 culture cultivated as reported above.

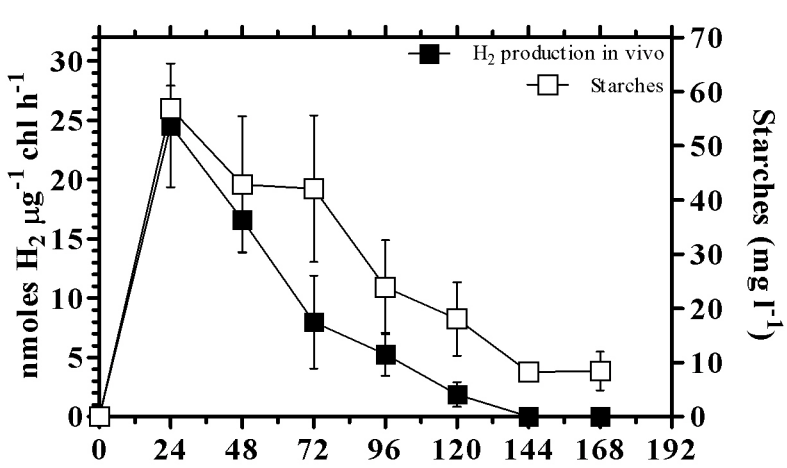


Figure 7. *In vivo* H_2 production rates and starches pattern in a sulfur deprived culture of CC124, cultivated at a light intensity of about 60-70 µmol photons m^{-2} s^{-1} irradiated on both sides, with an initial chl concentration of 20 mg L^{-1}. Keys reported in the graph.

It is of interest to note that when a net H_2 up-take finally took place in both treated cultures (T=96 hours in Fig. 5 and 6), starch amount was more than 2.5 times lower respect to the onset of the H_2 production. Thus, it seems that only when a big part of the reducing power previously stored was finally consumed, a H_2 oxidation can be definitely appreciated.

Conclusions and future perspectives

The *in vivo* H_2 oxidation under sulfur deprivation in *Chlamydomonas reinhardtii* was studied with respect to the oxy-hydrogen reaction and the dark CO_2 fixation. Results showed that when proper conditions are provided, dark CO_2 fixation can occur since the very early phase of H_2 production, but it is reduced as a function of time under sulfur starvation. Indeed, a similar reduction was also observed in the H_2 production rates in the dark, most likely because the latter was due to a dark fermentative metabolism. These results show once again that the H_2 metabolism in *Chlamydomonas* is a complex interplay between several processes, some of which can even occur in the dark. Concerning them, the evaluation of any specific contribution was made difficult by the fact that rates of production/consumption were extremely low. This means also that no appreciable phenomenon can affect H_2 production in the light, and that even when a very dense and poorly illuminated or mixed culture is considered, no substantial H_2 consumption could reduce H_2 productivity.

The absence of Rubisco activity since 120 hours after sulfur deprivation made it possible to evaluate the contribution of the oxy-hydrogen reaction only (equal to 0.4 nmoles of H_2 μg^{-1} chl h^{-1}). The occurrence of the oxy-hydrogen reaction deals with the PQ pool degree of reduction, but it is not clear whether it could take place in the light during H_2 production, when electrons move along the transport chain by means of photosynthetic processes.

However, further experiments are needed to individuate any specific pathway, as it appears evident that the final measured output of H_2 gas is always the result of a number of processes that operate concomitantly.

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