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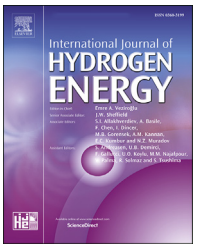

Sustained photobiological hydrogen production by *Chlorella vulgaris* without nutrient starvation

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HIGHLIGHTS

- *Chlorella* produced H_2 by direct photosynthesis without applying sulfur-starvation.
- *Chlorella* G-120 strain can release H_2 both in the light and in the dark.
- *Chlorella* G-120 strain can be grown both autotrophically and heterotrophically.
- Photobiological H_2 production by strain G-120 reached 7.7% efficiency (H_2 to light).
- Photosynthetic rate of *Chlorella* G-120 surpassed that of *C. reinhardtii* CC-124.

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ABSTRACT

This article describes the ability of the *Chlorella vulgaris* BEIJ strain G-120 to produce hydrogen (H_2) via both direct and indirect pathways without the use of nutrient starvation. Photobiological H_2 production reached a maximum rate of 12 mL H_2 L^{-1} h^{-1}, corresponding to a light conversion efficiency (light to H_2) of 7.7% (average 3.2%, over the 8-day period) of PAR, (photosynthetically active irradiance). Cells presented a maximum *in vivo* hydrogenase activity of 25.5 ± 0.2 nmoles H_2 μgChl^{-1} h^{-1} and the calculated *in vitro* hydrogenase activity was 830 ± 61 nmoles H_2 μgChl^{-1} h^{-1}. The strain is able to grow either heterotrophically or photo autotrophically. The total output of 896 mL of H_2 was attained for illuminated culture and 405 mL for dark cultures. The average H_2 production rate was 4.98 mL L^{-1} h^{-1} for the illuminated culture and 2.08 mL L^{-1} h^{-1} for the one maintained in the dark.

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Introduction

Growing concerns about global warming and the limited amount of available fossil energy have increased the need to shift the energy production towards renewable sources. Hydrogen (H_2) is extensively proposed as a future source of alternative energy. Some species of microalgae are currently being investigated as potential sources of bioenergy and biofuels such as H_2 [1–5]. In microalgae, H_2 production is catalyzed by the enzyme hydrogenase in a light-dependent process since hydrogenases are coupled to the photosynthetic electron transport chain via a specific ferredoxin [6,7].

Microalgae and cyanobacteria (blue-green algae) are the only organisms able to combine oxygenic photosynthesis with the production of H_2, an attractive pathway for a direct production of H_2 from solar energy and water [8]. Following the enthusiasm over the sulfur-deprivation process that greatly stimulates algal H_2 production from water in *Chlamydomonas reinhardtii*, many papers, reviews and book, have been recently published on this subject [5,7,9–12]. Recent reports have also shown that certain new isolates of *Chlorella* can produce high amounts of hydrogen when suitable organic substrates and reducing agents are supplied to nutrient replete cultures under low irradiance [13]. It has also been found that some isolates of *Chlorella vulgaris* can produce small amounts of hydrogen even under aerobic conditions, provided that the ratio CO_2:O_2 is much higher than that normally occurring in the atmosphere [14].

The simplest most sustainable and efficient way to produce H_2 with microalgae is the so-called direct biophotolysis, which involves direct transfer of electrons from water to the hydrogenase. However, until to date H_2 production of significant amounts of hydrogen from direct biophotolysis is strongly limited by the O_2 sensitivity of hydrogenase which is the most challenging barrier to overcome [15,16].

Oxygen acts as a transcriptional repressor, an inhibitor of hydrogenase maturation, and an irreversible inhibitor of hydrogenase catalytic activity [17–19].

The sulfur-starvation technique proposed by Melis et al. [8] is still the preferred way to induce H_2 production in *Chlamydomonas reinhardtii* but it imposes a severe stress leading to the progressive degradation of the photosynthetic apparatus, and as a result light conversion efficiency is very low (0.1% of PAR, photosynthetically active irradiance) [5,20]. Since the water splitting function of photosystem II (PSII) is the main source of electrons for H_2 photoproduction, it is important to maintain its activity for as long as possible [1,21–23].

The possibility of improving the biomass and output rates of hydrogen production has been reported in *C. reinhardtii*, by introducing an alternative route to supply H^+ and e^- to the hydrogenase enzyme utilizing glucose an alternative source of electrons [24]. In particular, the authors reported an increased H_2 production by about 50% in a *C. reinhardtii* when glucose was added to the sulfur-deprived medium.

Here we report an evidence of sustained photobiological H_2 production by *Chlorella vulgaris* BEIJ, strain G-120 under continuous illumination without applying any nutrient starvation. Its high respiration rate, coupled to a high light compensation point made it possible to efficiently dispose the O_2 produced by the water splitting process thus maintaining anaerobiosis, even under relatively high irradiance. This strain is able to grow vigorously in the dark using glucose as a source of carbon and energy, while under illumination it can easily convert its metabolism towards photo autotrophy and produce H_2 in a sealed reactor without the need for starvation.

Materials and methods

Microalgal strains and culture medium

The green microalga *Chlorella vulgaris* G-120 (registered as *Chlorella vulgaris* BEIJ., 1996/H 14, CCALA 30001, Culture Collection of Autotrophic Organisms, Institute of Botany, Třeboň, Czech Republic) is a natural, non-GMO strain (cell size between 3 and 5 μm), which grows fast when cultured heterotrophically (Fig. 1).

The *Chlorella* G-120 cultures were grown heterotrophically in a medium (hereafter HM) as already described [25,26]. The composition of nutrients solution related to 10 g of glucose: urea = 0.914 g; KH_2PO_4 = 0.211 g; $MgSO_4 \cdot 7H_2O$ = 0.157 g; $FeSO_4 \cdot 7H_2O$ = 12.51 mg; H_3BO_3 = 2.85 mg; $CuSO_4 \cdot 5H_2O$ = 0.8 mg; $ZnSO_4 \cdot 7H_2O$ = 1.11 mg; $CoSO_4 \cdot 7H_2O$ = 0.97 mg; $MnCl_2 \cdot 4H_2O$ = 1.3 mg; $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ = 0.46 mg; $CaCl_2$ = 6.94 mg pH = 6.5.

Analytical procedures

Determination of dry biomass weight (DW) was performed in triplicate using 10 mL samples. Pre-weighted, 47-mm diameter glass microfiber filter membranes (Whatman GF/F, Maidstone, England) were used; the filters with the cells were washed twice with deionized water and oven-dried at 105 °C for 3 h. Concentrations of chlorophylls and carotenoids were determined spectrophotometrically according to Lichtenthaler [27].

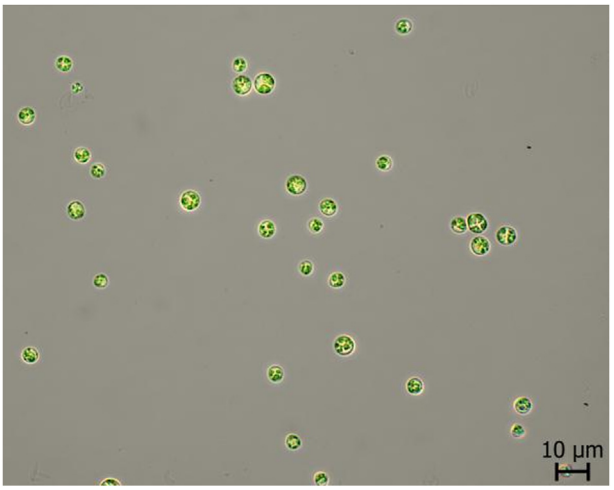

Fig. 1 – Light microscopy image of *Chlorella vulgaris* strain G-120 cells (× 1000) (Photograph by dr. K. Ranglová, Algatech).

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Total carbohydrate content in the cells was assessed using the phenol-sulfuric acid method, using D+ glucose as standard [28]. HPLC analysis of glucose and organic acids in the culture medium were performed with a Varian Prostar 210 HPLC System equipped with Phenomenex Rezex ROA − Organic Acid H+ (300 × 7.8 mm) column using 5 mM H_2SO_4 aqueous solution as an eluent with the flow rate of 0.6 mL min^{-1} [29].

Photosynthesis measurements

Rates of photosynthetic oxygen evolution as a function of irradiance (P vs I curve) and of dark respiration (R_d) were measured with a Clark-type oxygen electrode (DW2/2 Oxygen Electrode, Hansatech Instrument Ltd., Norfolk, UK) connected to a Hansatech Oxylab control box. Algal samples (2 mL) of known chlorophyll concentration (4 μg mL^{-1}) were inserted into the water-jacketed, temperature-controlled chamber (25 °C), and mixed by magnetic stirrer. Oxygen evolution rate was recorded using the Oxygraph Plus software or O_2 ViewXP software (Hansatech Instrument Ltd., Norfolk, UK). The rates (measured in triplicate) are reported in units of μmoles of O_2 evolved per unit of chlorophyll concentration per unit of time. The irradiance was controlled by intensity-controllable white LED light, which was programmed to increase in intensity according to a specific, pre-determined light regime (0–2000 μmol photons m^{-2} s^{-1}) allowing the construction of the production of photosynthetic response curves (P vs I curves). Each curve was generated according to Eilers and Peeters [30] that allowed the determination of several photosynthetic variables, such as the dark respiration (R_d), photosynthetic compensation point (I_c), that is, the light irradiance when the photosynthetic rate is just balanced by respiration, maximum rate of light-saturated photosynthesis (P_{max}), irradiance at the onset of light saturation (I_k), and the light utilization efficiency as determined by the initial slope (α) [30].

Fluorescence measurements

On-line chlorophyll fluorescence measurements were carried out with a pulse-amplitude-modulation fluorometer (PAM-2100, H. Walz, Effeltrich, Germany) operated by means of PC software PamWin (version 2.00f). The fluorescence nomenclature follows van Kooten and Snel [31]. The maximum quantum yield of PSII (F_v/F_m) during the hydrogen production in illuminated cultures was measured at time intervals by temporary switching off the light and covering the reactor with a dark plastic sheet. The effective photochemical quantum yield of PSII, $\Delta F/F_m' = (F_m'-F_s)/F_m'$, F_s and F_m' were measured by putting the fiber-optic probe of the fluorometer directly in contact on the illuminated photobioreactor (PBR) surface. Non-photochemical quenching (NPQ) was calculated by using the Stern-Volmer equation $NPQ=(F_m-F_m')/F_m'$ [32]. Chlorophyll *a* fluorescence transients were recorded using a Handy PEA (Hansatech Instruments, UK) in 2-mL dark adapted samples that were illuminated with continuous light (650 nm peak wavelength, 3500 μmol photons m^{-2} s^{-1}). The relative variable fluorescence, $V_t=(F_t-F_0)/(F_m-F_0)$, was obtained by normalization of the chlorophyll fluorescence data on both F_0 and F_m. Each chlorophyll *a* fluorescence induction curve was analyzed using "Biolyzer HP3" software, and reported according to the JIP-test [33].

- $M_0 = 4(F_{300\mu s}-F_0)/(F_m-F_0)$, which corresponded to the net rate of the reaction center closure, where it increases by means of trapping and decreases by means of electron transport;
- V_J $(F_J-F_0)/(F_m-F_0)$, the variable fluorescence at phase J, which is considered to be a good indicator of the plastoquinone pool redox state;
- $\phi(P_0) = F_v/F_m=(F_m-F_0)/F_m$, the maximum quantum yield of PSII for primary photochemistry;
- $\phi E_0 = \phi(P_0) \times \Psi_0$, the quantum yield of the electron transport, where Ψ_0 is the efficiency with which a trapped exciton can move an electron further than Q_A^- (primary quinone electron acceptor) into the electron transport chain.

Hydrogen production experiments

Chlorella cells were transferred in a 1 L Pyrex Roux-type bottle PBR (5 cm light path) with a flat cross section (12 × 5 cm width), a flat bottom, and five ports (Fig. 2). The PBR was fitted with three probes for the continuous monitoring of pH, redox potential and dissolved O_2 concentration. The main port at the

Fig. 2 – Roux-type bottle photobioreactor (1 L working volume) used for the hydrogen experiments with cultures of *Chlorella vulgaris* strain G-120. The culture behavior was continuously monitored by sensors of pH, DO, and redox potential as well as fluorescence yield by a PAM-2100.

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top (2.5 cm i.d.) was sealed with a stopper equipped with tygon tubes that connected the PBR headspace to the gas-to-liquid conversion bottle. The illumination level of a photon-flux density (PFD) of 150 μmol photons m^{-2} s^{-1} was provided by cool white fluorescent lamps (Dulux L, 55W/840, Osram, Italy). Mixing of the culture was provided by a rotating impeller driven magnetically from the bottom of the reactor [34].

The PFD value at the culture surface was measured with a quantum radio-photometer (LI-250A, Li-Cor Biosciences, Nebraska, USA) equipped with a cosine-corrected sensor. Culture parameters, such as pH, temperature, redox potential, dissolved O_2 concentration and H_2 gas production were monitored as described elsewhere [34]. Culture's temperature was maintained at 26.0 ± 0.2 °C. The quantity of gas produced was monitored continuously according to Kosourov et al. [35] Samples of the gas mixture produced were analyzed with a thermal conductivity detector (TCD) equipped gas chromatograph (model Clarus 500, PerkinElmer, Waltham, Massachusetts), with an at the following operating conditions: isothermal program at 35 °C for 2.25min; nitrogen carrier gas flow 30 mL min^{-1}; injection temperature 150 °C; detector temperature 150 °C. A packed column (model Carbosieve S-II Spherical Carbon, Supelco) was used. Calibration of hydrogen was performed by injecting known amounts of pure gas.

In vitro hydrogenase activity was determined as described in Hemschemeier et al. (2009) [36]. The reaction mixture of this assay contains Triton-X 100 (a mild detergent which lyses the algal cells), methyl viologen as an artificial electron donor and sodium dithionite as an efficient reductant for methyl viologen. The hydrogenase activity of whole cells is defined as nmoles H_2 produced per hour and per μg chlorophyll. All the experiments were carried out in triplicate.

Statistical analysis

Sigma Plot 11.0 was used to determine significant differences between treatments. One-way ANOVA and Holm-Sidak test were conducted for every binary combination of systems. P values lower than 0.05 were considered significantly different.

Results and discussion

Photosynthetic rate

The net photosynthetic rates of *Chlorella* strain G-120 was compared with those of *Chlamydomonas reinhardtii* strain CC-124 which is commonly used for the hydrogen production experiments (Fig. 3). The important photosynthetic variables estimated from the curves of both the strains are reported in Table 1.

Compared to CC-124, the strain G-120 presents significant differences: a 4-fold higher maximum photosynthesis rate (P_{max}), and an 8-fold higher saturation irradiance (I_k) (Table 1). More importantly, for H_2 production, the respiration rate was almost double compared to CC-124. These characteristics make it very attractive for H_2 production. Indeed, a high level of respiration allows to maintain the anaerobic condition for a longer period, thus enhancing the amount of H_2 produced. As can be seen in Fig. 3, compared to CC-124, the compensation

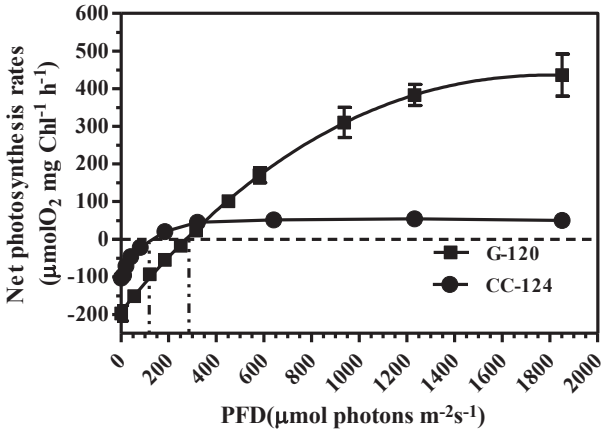

Fig. 3 – Net photosynthesis light-response curve of the *Chlorella vulgaris* strain G-120 cells (square) exposed to a stepwise increase of light intensity to measure photosynthesis vs irradiance curve. For comparison, the net photosynthesis rate of *Chlamydomonas reinhardtii*, strain CC-124 is reported.

Table 1 – Comparison of the main photosynthesis parameters of *Chlamydomonas reinhardtii* strain CC-124 and *Chlorella* sp. Strain G-120. Data are the mean values ± standard deviation (n, 3).

Parameters (units)	CC-124	G-120
P_{max} (μmol O_2 mg chl^{-1} h^{-1})	160.1 ± 18.4	640 ± 77
Initial slope, α (μmol O_2 mg chl h^{-1} μmol^{-1} photons m^2 s)	1.61 ± 0.017	0.796 ± 0.087
I_K (μmol photons m^{-2} s^{-1})	97 ± 9.3	804 ± 9.6
Respiration rate (μmol O_2 mg chl^{-1} h^{-1})	−103 ± 14	−197 ± 23.6
R/P (Respiration/photosynthesis ratio) (–)	0.64 ± 0.07	0.30 ± 0.037

point, i.e. the irradiance at which photosynthesis equals respiration, is almost double in the strain G-120. Moreover, this strain possesses a much higher photosynthetic capacity (P_{max}) which can potentially be addressed towards the H_2 production. On the other hand, the strain CC-124 shows a better light utilization (initial slope α) under low light conditions ($p < 0.005$).

Hydrogen production

H_2 production was tested by *Chlorella* cells, exposed to incident light irradiance of 150 μmol photons m^{-2} s^{-1}, without applying sulfur starvation protocol (Fig. 4A, red curve). A culture in the dark at the same conditions was set-up to evaluate the H_2 production in the dark by fermentation (Fig. 4A, black curve). In the illuminated culture, the H_2 production in the light started immediately (no lag phase) and continued for about 7 and 8 days while culture tested in the dark conditions showed a 12- hour lag phase. The final total outputs of 896 mL of H_2 and 405 mL, for the illuminated and the dark culture respectively, were attained. The average H_2 production rate was 4.98 mL H_2 L^{-1} h^{-1} for the illuminated culture and 2.08 mL H_2

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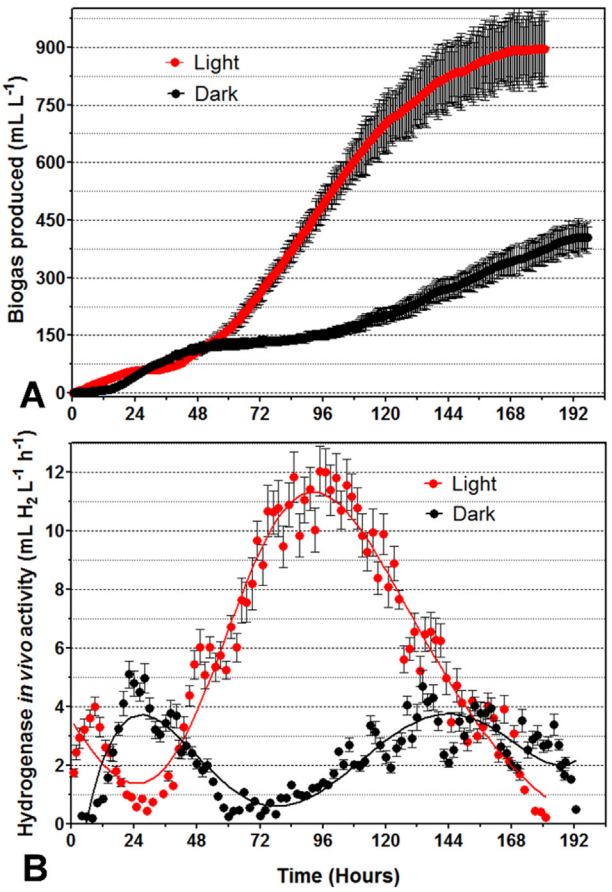

Fig. 4 – A) Time courses in H_2 production of the *Chlorella* cultures; B) Time courses of the *in vivo* hydrogenase activity (red circles: illuminated culture; black circles: dark culture. The fitting line averages the results of three replicates. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

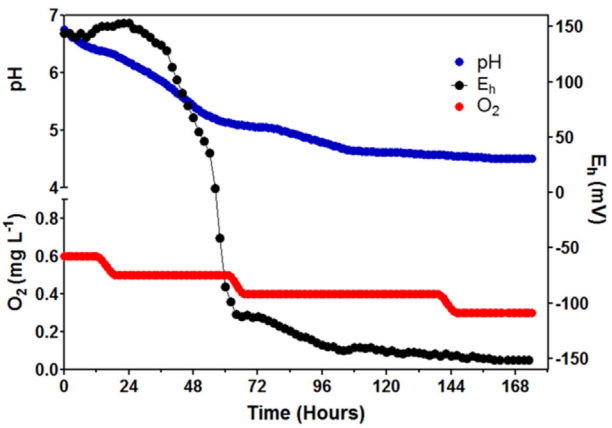

Fig. 5 – Time courses in redox potential (E_h) (black circles), dissolved oxygen (red circles) and pH (blue circles) in the illuminated *Chlorella* cultures during the H_2 production process. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

L^{-1} h^{-1} for the one tested in the dark. The maximum H_2 production rate was 12.0 mL H_2 L^{-1} h^{-1} and 5.1 mL H_2 L^{-1} h^{-1} for the illuminated and non-illuminated cultures respectively. Both H_2 production rate and final produced volume in the illuminated culture are currently among the highest reported in literature for microalgae strains, and were achieved with a reactor of relatively large size compared to those reported in literature. The illuminated culture presented a maximum *in vivo* hydrogenase activity of 25.5 ± 0.2 nmoles H_2μg Chl^{-1} h^{-1}. This value is about 3-fold higher than in the best H_2 producer, the D1-protein mutant strain L159I–N230Y of *C. reinhardtii*, previously tested by us [37].

The maximum hydrogenase activity, calculated *in vitro* using methyl viologen and dithionite, was 830 ± 61 nmol H_2 μg Chl^{-1} h^{-1}. This value is higher than the one reported for *C. reinhardtii* (500 mol H_2 μg Chl^{-1} h^{-1}) [36].

In order to distinguish the contribution of direct and indirect pathways to the hydrogen produced under illumination, we performed an experiment of hydrogen production in the light by adding the herbicide DCMU (3-(3,4-dichlorophenyl)-1,1-dimethylurea), which is known to block the transfer from the electron transporter Q_A to Q_B thus interrupting the water splitting contribution to H_2 production. With DCMU, the amount of hydrogen produced was insignificant (0.333 mL), indicating that indirect pathways played a negligible role (results not shown).

Table 2 shows the fermentation products found at the end of the hydrogen production process, after 192 h of anaerobiosis. The most important compounds were lactic acid, particularly in the light, and acetic acid particularly in the dark hydrogen production process, while formic acid was found only in the dark [38,39].

Time course in the biophysical parameters during the hydrogen production process

During the first 12 h of hydrogen photoproduction measurements, a burst in the hydrogenase activity was recorded (Fig. 4B, red circles) to which corresponded a raise in the effective quantum yield of PSII ($\Delta F/F'_m$) (Fig. 6A), and a reduction in NPQ (Fig. 6B). Within 24 h the hydrogenase activity showed a deep decline, down to almost zero, which was accompanied by a raise of redox potential indicating lesser cellular reductive conditions (Fig. 5). It is conceivable that the reduction of the hydrogenase activity between 12 and 24 h was caused by a reduction of the electron supply due to temporary cellular carbohydrate exhaustion. A similar behavior was observed in the dark hydrogen production (Fig. 4B, black circles), the fermentation of carbohydrate reserve may have supported the first burst of the *in vivo*

Table 2 – Fermentation products (g L^{-1}) found at the end of the H_2 production experiments. Values are mean ± standard deviation. Numbers in columns with different letters are significantly different ($P < 0.05$).

	Acetic Acid	Formic Acid	Lactic Acid	Ethanol
Light	0.16 ± 0.01a	–	0.99 ± 0.05a	0.040 ± 0.002a
Dark	0.19 ± 0.01b	0.14 ± 0.01	0.12 ± 0.01b	0.040 ± 0.002a

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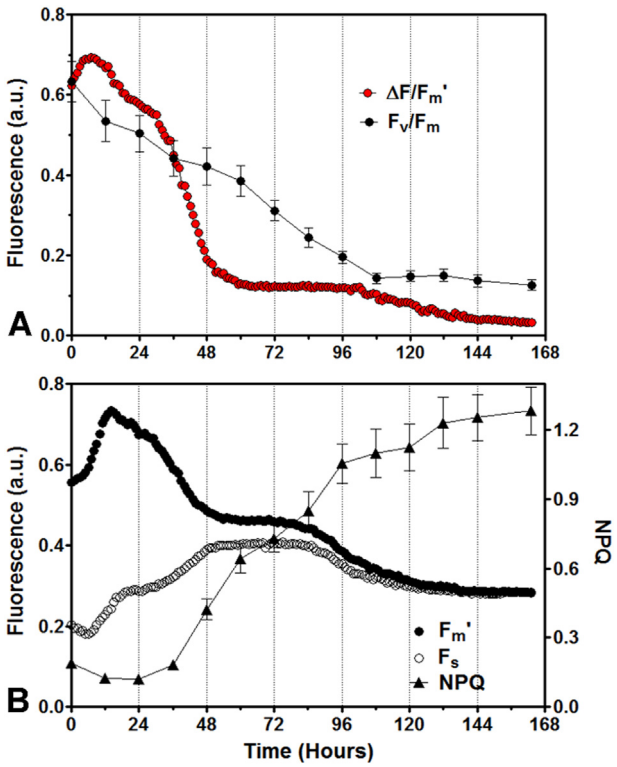

Fig. 6 – Fluorescence measurements carried out in *Chlorella* culture during H_2 production in the light. A) Effective quantum yield of PSII photochemistry $\Delta F/F_m'$ (red circles), Maximum quantum yield of PSII (F_v/F_m) black circles). B) the maximum fluorescence yield in the light exposed culture, F_m' (closed circles), the steady-state fluorescence yield in the light (F_s) (open circles) and non-photochemical quenching NPQ (closed triangles). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

hydrogenase activity during the first 24 h, which was then almost completely depleted within 72 h of the experiment. Then, the glucose fermentation intensely supported the dark hydrogen production.

A fast decline occurred in the redox potential after 48 h of hydrogen production (Fig. 5). The redox potential of the cells (E_h) decreased slowly during the first 38 h, from +144 to +128 mV, then dropped from +128 to −110 mV in the following 26 h, and subsequently plateaued at −150 mV (Fig. 5). This value was considerably less negative than that found with the strain CC124 of *C. reinhardtii* usually considered as a reference strain [34]. Redox potential decreased due to the anoxic conditions while there was an increase in the intracellular reducing power most likely created by cellular respiration.

The on-line measurement of $\Delta F/F_m'$, after the initial increase, it then decreased rapidly for about 60 h, and stabilized at about 18% of the maximum value (Fig. 6A), following a pattern similar to that observed for the redox potential (E_h). The fast decline in the $\Delta F/F_m'$ ratio after 24 h of culture was the result of both a raise of the steady-state of fluorescence in the light (F_s), indicative of an increasing level of reduction of PQ pool, and a decline of the F_m' (maximum fluorescence in light-adapted state) due the progressive closure of the reaction centres (Fig. 6B).

The decline in the effective quantum yield of PSII was already noted by Antal et al.[22], who interpreted it as the consequence of a transition from state 1 to state 2 of the photosynthetic apparatus [40]. The state 1 to state 2 transition represents a photoprotective strategy and consists of a partial migration of the LHCII from PSII to PSI, which is known to occur in *C. reinhardtii* under anaerobic conditions [41–43]. It was reported that the reduction level of the PQ pool is one of the main triggering events involved in the induction of the physiological response to stress conditions affecting the PSII activity [44]. The high reduction state of the PQ pool was confirmed by the increased reduction of plastoquinone pool resulting in a significant increase the J step recorded in the fast fluorescence induction kinetics (Fig. 7).

After 36 h of incubation in anaerobiosis NPQ showed a fast raise which coincided with a strong drop in Eh and $\Delta F/F_m'$. It is conceivable to ascribe this initial increase of NPQ to state transition (qT) due to a reversible migration of antenna protein (LHCII). These results are in agreement with previous findings, reporting the occurrence of state 1 to state 2 transition in *Chlamydomonas* cells during anaerobiosis in the dark [40,45]. Thereafter, the NPQ raise can be associated with the build-up of the *trans*-thylakoid proton gradient and attributable to qE high-energy state quenching associated to the xanthophyll cycle [45,46].

Measurements of rapid fluorescence induction kinetics (OJIP-test) of the illuminated culture were recorded at three time periods of the trials. The transients followed the typical polyphasic OJIP rise (Fig. 7). During the first 24 h of H_2 production, the analysis of the OJIP test parameters showed a 45% increase of M_0 (Table 3), i.e. the initial slope of the transients suggesting an increased rate of closure of the reaction centres. The increase of this variable was associated with changes in the V_J, which raised by 50%, indicating the higher rate of the

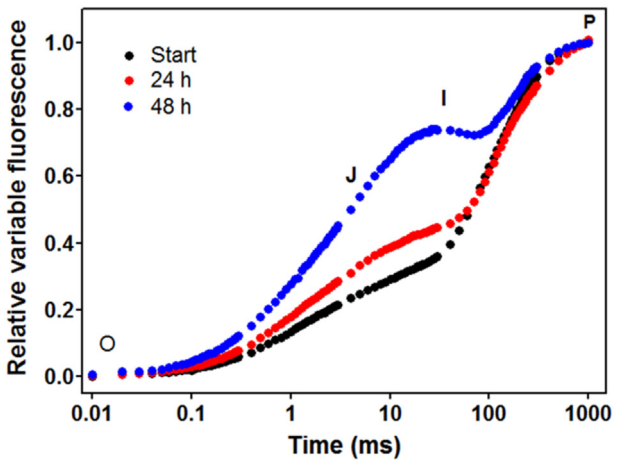

Fig. 7 – Changes of the fast fluorescence induction kinetics during H_2 production in illuminated *Chlorella* culture recorded at the start and after 24 and 48 h of the experiment.

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Table 3 – Changes in variables calculated from OJIP curves in the illuminated *Chlorella* cultures during H_2 production: M_0 (initial slope at the beginning of the variable fluorescence), V_J (the fluorescence yield at the time point J, 2–3 msec from the start of induction), ϕE_0 (the quantum yield of electron transport), $\phi(P_0)$ (the maximum quantum yield of PSII). Values are mean with standard deviation.

	M_0	V_J	ϕE_0	ϕP_0
Start	0.179 ± 0.002	0.158 ± 0.003	0.618 ± 0.004	0.734 ± 0.002
24 h	0.260 ± 0.008	0.237 ± 0.015	0.447 ± 0.007	0.586 ± 0.004
48 h	0.377 ± 0.026	0.334 ± 0.006	0.273 ± 0.056	0.402 ± 0.011

Q_A reduction under anaerobic conditions. These events are well correlated with the redox potential values, which were stable within the first 24 h, but decreased in the following period of H_2 production, due to the increased plastoquinone pool reduction level. Within the same period, changes in other variables related to the electron transport occurred, such as a 28% drop in ϕE_0 reflecting the decrease in the quantum yield of the electron transport, and a 20% decrease in ϕP_0 reflecting a delay in the foreword electron transport beyond Q_A. During the following period of H_2 production, the cells exhibited further increment of M_0 and V_J value, by 45 and 41%, respectively, which reflected the increase of PQ-pool reduction, associated with the decrease of electron transport, ϕE_0 and ϕP_0, by 39 and 31%, respectively (Table 3). Further evidence of the increased level of reduction of the PQ pool was obtained by the measurement of the relative variable fluorescence which showed an increased value at the J step level, which was smaller in the first 24 and increased in the following 24 h, in accordance with the changes of F_s (Fig. 5) [45].

Changes in the dry weight and chlorophyll during hydrogen production

Under illumination, the total chlorophyll concentration was initially 10.53 ± 0.19 mg L^{-1} (0.48% of DW), after 72 h increased to 20.98 ± 0.13 mg L^{-1} (0.42% of DW) and finally decreased to 9.32 ± 0.11 mg L^{-1} (0.20% of DW). In this experiment, DW increased from 2.21 ± 0.09 to 4.97 ± 0.11 g L^{-1} after 72 h and thereafter decreased to 4.55 ± 0.01 g L^{-1}. Initial carbohydrate content of the cells was 24.37 ± 1.75%, increased to 45.98 ± 0.05% after 72 h, and finally decreased to 36.57 ± 0.44% of DW. Carotenoid content in the illuminated culture increased from 1.52 ± 0.07 mg L^{-1} (0.05% of DW) to 7.05 ± 0.06 mg L^{-1} (0.18% of DW) at the beginning and at the end of the H_2 production process respectively.

Under dark conditions total chlorophyll concentration was initially 18.43 ± 0.78 mg L^{-1} (0.65% of DW) and decreased to 10.72 ± 0.35 mg L^{-1} (0.46% of DW) at the end of the experiment. In this experiment DW increased from 2.82 ± 0.03 to 3.05 ± 0.01 g L^{-1} after 48 h and then decreased to 2.32 ± 0.04 g L^{-1}.

During the photobiological experiments carried out in the presence of DCMU a slight decreased of the dry weight was observed. It changed from 3 g L^{-1} at the start to 2.74 after 48 h.

The lack of the photosynthetic activity which releases oxygen for the respiration of the glucose accounts for the lack of growth in cultures grown anaerobically in the dark or treated with DCMU in the light.

Comparison of hydrogen production with other microalgal strains

Comparing the performance of the *Chlorella* strain G-120 with other microalgal strains is made difficult by the differences in culture conditions, particularly light intensity, chlorophyll concentration and size of the PBRs. Hydrogen production performance tested in vials can strongly differ from that attained in scaled up PBRs. Indeed, in a small device the illumination is supposed to be uniform and the mixing of cells optimal for homogeneous exposure of the cells to light. The size of the PBR and the possibility to scale up the process have been chosen by us as a trade-off to compare the performance of different mutant strains of *Chlamydomonas* with G-120 of *Chlorella vulgaris* reported in Table 4.

Recently, an *Chlorella* sp. KLS Sc59 has been reported to produce up to 750 mL H_2 L^{-1} in the presence of reducing agents such as ethanol and dithionite which are good sources of electrons for the hydrogenase thus strongly stimulating the hydrogen output [13]. However, when the said strain was tested with glucose as a carbon substrate, under similar conditions to our experiments, it yielded about 128 μmol H_2 mg Chl^{-1} within 24 h, that is, 0.116H_2 mL H_2 mg Chl^{-1} h^{-1}. This amount is about 50% lower than that recorded with *Chlorella* G-120 (0.236 μmol H_2 mg Chl^{-1}). The different hydrogen yield may be explained by the large difference in the light irradiance to which the cells were exposed, 53 μmol photons m^{-2} s^{-1} (strain KLSc59) and 150 μmol photons m^{-2} s^{-1} (strain G-120). It is worth pointing out that the higher light used in case of *Chlorella* G-120 was possible because this strain had a very high respiration rate which translates in a higher light compensation point (200 μmol photons m^{-2} s^{-1}) and thus the possible to expose the strain to a higher irradiance (Fig. 3). Moreover, contrary to the strain KLSc59, which can endure an irradiance intensity up to 53 μmol m^{-2} s^{-1}, the strain G-120 can perform photosynthesis up to 1850 μmol m^{-2} s^{-1} without any apparent symptoms of photoinhibition.

Both high respiration rates and high saturating irradiance are common features of *Chlorella* strains with reduced antenna size [46,49,50]. Moreover, the strains with reduced antenna size are characterized by a lower non-photochemical quenching capacity, therefore high respiration rate usually observed in low antenna size strains may represent a useful valve to dissipate excess of energy surplus excitation energy [46,49–53].

It is well known that oxygen is toxic to hydrogenase even at low concentration. However, some strains of *Chlorella* have been shown to produce hydrogen under a mixture of CO_2 and O_2 [14]. Although, the amount produced was incomparably

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Table 4 – Comparison of H_2 production rates in various microalgae strains.

Strain	H_2 volume (mL L^{-1})	Mean H_2 production rate (mL L^{-1} h^{-1})	Growth medium	Light irradiance (μmol m^{-2} s^{-1})	Chlorophyll (μg mL^{-1})	PBR size (mL)	Reference
C. reinhardtii CC-124	100	1.4	TAP-S	140	12	1000	[34]
C. reinhardtii stm6	540	4.0	TAP-S	150	26	500	[21]
C. reinhardtii L159I–N230Y	504	5.8	TAP-S	140	12	1000	[1]
C. reinhardtii Pgr 5	850	7.0	TAP-S	60	15	250	[47]
C. reinhardtii ΨH1	nr	3.6	TAP	600	30	1000	[48]
C. vulgaris g120	896	5.3	HM (full medium)	150	21	1000	This work
C. vulgaris g120	405	5.1	HM (full medium)	dark	18	1000	This work

lower than that usually produced under anaerobiosis, these findings indicate that *Chlorella* is a promising strain as a model microalga for further investigation for the photobiological hydrogen production.

We calculated light conversion efficiency (LCE) of the whole process as the ratio between the energy stored in H_2 and the absorbed light energy by the culture, plus the amount of energy consumed by the respiration of carbohydrates to maintain anaerobiosis. We have assumed that: (a) the energy content of H_2 at 25 °C is 12.94 J mL^{-1}; (b) 209 kJ is the average energy content of a mole of photons of visible light; c) 0.9 the transparency of the glass PBR; d) the absorbed light energy was 362009J, which is the product of: 150 μmol photons m^{-2} s^{-1} × 0.209 J μmol photons^{-1} × 180 h × 3.600 s × 0.0198 m^2 × 0.9; (e) the cellular respiration to absorb the O_2 produces by water splitting for the H_2 production (0.544 g carbohydrates equivalent to 8468 J); (f) the total amount of H_2 produced by the culture was 896 mL. Therefore, the LCE for the entire H_2 production process was (896 mL × 12.94 J mL^{-1})/(362,009 J + 8468 J) × 100 = 3.1%. If we calculate the LCE on the basis of the highest H_2 production rate achieved (12 mL L^{-1} h^{-1}), we attain 7.7%. A detailed mass balance of the glucose during the fermentation in the light is shown in Supplementary section. Nevertheless, LCE is at least twice as high asthat attained with *C. reinhardtii* (less than 1%) under sulfur limitation which in turn is overestimated since the consumption of substrate (glucose) to sustain O_2 uptake decreases by half the H_2 the efficiency of the process [54].

Under dark conditions the total amount of produced H_2 was 405 mL, with a mean rate of 2.1 mL L^{-1} h^{-1}; the maximum rate attained was about 4 mL L^{-1} h^{-1} (Fig. 4B dark line). The corresponding energy conversion efficiency (glucose to H_2) reached the value of (405 mL × 12.94 J mL^{-1})/(21,047 J) × 100 = 24.89%. Where 21047J correspond to 1.32 g of glucose consumed to produce 405 mL of H_2. The hydrogen yield was 2.26 mol H_2/mol glucose, which is close to what obtained in fermentative process with bacteria [55,56]. If 10% LCE could be attained (for example using microalgae with an O_2-tolerant hydrogenase) in sunny areas, the cost of biohydrogen could be as low as 2.99 USD per gallon of gasoline equivalent (gge), while with an LCE of 2%, the estimated cost would be 8.15 USD per gge in 2009 [57]. The authors estimated that, if sulfur-deprived microalgae reached 1.5% LCE, the cost of H_2 would be 8.44 USD per gge. Therefore, the 2% LCE (light to H_2) and that obtained with the *Chlorella* G-120 strain might be economical feasible. Of course, the performance of the strain must be tested outdoors where light conditions fluctuates daily and seasonally. For this purpose, it is important to use robust strains, such as *Chlorella*, with high photosynthetic capacity; the strain G-120 is indeed able to cope with higher irradiance increasing its photosynthetic activity (Fig. 3).

Summing up, the features of this *Chlorella* G-120 strain tested in these experiments, compared to data reported in the literature, were: (i) capability to grow vigorously in a fermenter which strongly reduces the time period for biomass production; (ii) high photosynthetic capacity coupled to high respiration rate and high light compensation point which allow to reach rapid anaerobiosis conditions and carry out the process under relatively high irradiance; iii) lower Chl content (<1% of DW) compared to that commonly found in autotrophic *Chlorella vulgaris* cells which strongly enhances the light penetration in the photobioreactor; iv) rapid trophic conversion from heterotrophic to both photoheterotrophic and autotrophic conditions; v) no lag phase at the start of the H_2 process thus improving the overall efficiency, and vi) perspective of producing H_2 outdoors without interruption during the night part of the diurnal cycle.

In light of the results obtained with this strain, we suggest that selection of strains for H_2 production should not only be conducted on the basis of their high respiration rate, as typically done with *Chlamydomonas reinhardtii* strains, but also it should take into account the maximum photosynthetic capacity (P_{max}), which should be as high as possible, and the *Chlorella* strain G-120 is an example. This characteristic translated in a much higher H_2 output, which is expected to further increase if all the factors (light irradiance, temperature, pH, mixing, cell density, hydraulic retention time) are optimized. For example, the increase of light irradiance coupled to a higher cell concentration increased the output of H_2 to 1300 mL (not shown). Further increase in H_2 output may be attained by bioengineering photosynthesis, i.e. bypassing the competition of CO_2 assimilation with H_2 production while preserving the capacity of the photosynthetic machinery, as it was already successfully demonstrated [48,58].

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Conclusions

This study demonstrated that the microalga *Chlorella vulgaris*, strain G-120 is capable to produce a sizeable amount of biohydrogen without nutrient starvation, which represents an important step forward in the scale-up process. Indeed, the use of sulfur starvation strongly reduces the economy of the process since it requires to eliminate sulfur residues by repeatedly centrifugate the cells, enhancing the risk of contamination of the culture. Moreover, the strong down-regulation of the PSII caused by the sulfur deprivation deeply reduces the H_2 output. *Per contra* the heterotrophic growth requires the addition of glucose which is likely to be costly at large scale. Therefore, it will be important to consider potentially much cheaper sources (e.g., wastewater from sugar factories, and paper mills, baker's yeast and brewery). Yet, an important advantage of the O_2 consumption through respiration of organic substrates is represented by the purity of the H_2 produced (close to 98%), which strongly reduced the investment cost for H_2 purification, which can account for up to 50% of total cost.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijhydene.2020.10.257.

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