



Evaluation of light energy to H₂ energy conversion efficiency in thin films of cyanobacteria and green alga under photoautotrophic conditions



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ABSTRACT

Cyanobacteria and green algae harness solar energy to split water and to fix CO₂. Under specific conditions, they are capable of photoproduction of molecular hydrogen (H₂). This study compares the light-energy-to-hydrogen-energy conversion efficiency (LHCE) in two heterocystous, N₂-fixing cyanobacteria (wild-type *Calothrix* sp. strain 336/3 and the $\Delta hupL$ mutant of *Anabaena* sp. strain PCC 7120) and in the sulfur-deprived green alga, *Chlamydomonas reinhardtii* strain CC-124, after entrapment of the cells in thin Ca²⁺-alginate films. The experiments, performed under photoautotrophic conditions, showed higher LHCEs in the cyanobacteria as compared to the green alga. The highest efficiency of ca. 2.5% was obtained in films of the entrapped $\Delta hupL$ strain under low light condition (2.9 W m⁻²). *Calothrix* sp. 336/3 films produced H₂ with a maximum efficiency of 0.6% under 2.9 W m⁻², while *C. reinhardtii* films produced H₂ most efficiently under moderate light (0.14% at 12.1 W m⁻²). Exposure of the films to light above 16 W m⁻² led to noticeable oxidative stress in all three strains, which increased with light intensity. The presence of oxidative stress was confirmed by increased (i) degradation of chlorophylls and some structural carotenoids (such as β -carotene), (ii) production of hydroxylated carotenoids (such as zeaxanthin), and (iii) carbonylation of proteins. We conclude that the H₂ photoproduction efficiency in immobilized algae and cyanobacteria can be further improved by entrapping cultures in immobilization matrices with increased permeability for gases, especially oxygen, while matrices with low porosity produced increased amounts of xanthophylls and other antioxidant compounds.

1. Introduction

Photosynthetic production of molecular hydrogen (H₂) by cyanobacteria and green algae via water biophotolysis is a potential source of clean energy. The processes involved have a maximum theoretical light energy to H₂ energy conversion efficiency (LHCE) of 6–7% for heterocystous cyanobacteria and 10–12% for green algae [1–3]. In reality, however, only a fraction of these values has been achieved due to a number of physiological and biochemical barriers limiting H₂ photoproduction yields in phototrophic cultures [3–5].

Hydrogen photoproduction in green algae is catalyzed by the [Fe-Fe]-hydrogenase enzyme, which reduces protons to molecular hydrogen using photosynthetically reduced ferredoxin (Fd) as a donor [6,7]. The process involves both indirect and direct pathways. The former is a photofermentative (photosystem I [PSI]-dependent but photosystem II [PSII]-independent) pathway linked to the photosynthetic electron transport chain at the level of plastoquinone, and the latter is a water-splitting, photosystem II (PSII)-dependent pathway. Both contribute reductants to the [Fe-Fe]-hydrogenase [8]. There is also

a dark fermentative pathway that can donate electrons to the [Fe-Fe]-hydrogenase [9]. Under hypoxic conditions caused by low light in dense algal cultures, the non-O₂-generating photofermentation pathway seems to play a significant role in H₂ evolution [10,11]. The process is not very efficient, but lasts for up to one month [11]. H₂ photoproduction, which relies on PSII and occurs upon exposure of dark-adapted anaerobic cultures to high light, is a temporary phenomenon due to the rapid inactivation of the algal hydrogenase enzymes by O₂ coevolved in PSII [12]. Depending on the light intensity and respiratory activity of the cells, H₂ evolution in dark-adapted algae lasts from few seconds [13] to several hours [10].

Sustained H₂ photoproduction in green algae also occurs after partial inactivation of the water-splitting activity in cells by nutrient deprivation, such as sulfur [14], phosphorus [15], magnesium [16], or in genetically modified cells with a decreased PSII activity [17]. Under nutrient starvation, O₂ produced by the residual PSII is metabolized sufficiently by respiration of acetate in the medium, protecting [Fe-Fe]-hydrogenase from inactivation. However, H₂ evolution in nutrient-deprived algae proceeds at reduced rates, resulting in low LHCEs in

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suspension cultures [18]. On the other hand, H₂ photoproduction under autotrophic conditions is difficult to sustain [19]. Nevertheless, autotrophic algae could generate a substantial amount of H₂ if proper light regime is applied [20,21].

In contrast to green algae, photoautotrophy is not a problem for sustained H₂ photoproduction in N₂-fixing heterocystous cyanobacteria that produce H₂ mainly under diazotrophic conditions as an obligatory by-product of N₂ fixation [22,23]. In these species, oxygenic photosynthesis and N₂ fixation are spatially separated in different cells. While oxygenic photosynthesis occurs in vegetative cells, N₂ fixation is restricted to heterocysts, specialized cells with specific morphology and metabolism that provides a microoxic environment for the O₂-sensitive nitrogenase enzyme [24]. This environment within the heterocysts is maintained by an elevated level of respiration and restricted diffusion of O₂ via a unique cell wall envelope [22,23]. At the same time, the vegetative cells supply heterocysts with the energy needed for N₂ fixation, mainly in the form of sucrose [25,26]. Long-term H₂ photoproduction in N₂-fixing heterocystous cyanobacteria requires elimination of both combined nitrogen from the media and atmospheric N₂, deletion of uptake hydrogenase in heterocysts and the presence of CO₂ in atmosphere [27–29]. However, N-depletion, especially in the excess of CO₂, causes an imbalance in the C/N ratio in cyanobacteria, leads to the inhibition of photosynthetic activity in the vegetative cells, and eventually causes the attenuation of H₂ production in the heterocysts [30,31]. Thus, analogous to green algae, H₂ photoproduction in N₂-fixing heterocystous cyanobacteria is the effect of a stress condition (-N).

Imbalance in photosynthetic activity and cell metabolism under stress conditions, which leads to H₂ photoproduction in green algae and cyanobacteria, may also induce the production of reactive oxygen species (ROS) in photoautotrophic cells. This can cause photoinhibition and oxidative damage, especially under high light conditions [32]. As discussed above, unicellular green algae and filamentous heterocystous cyanobacteria apply different mechanisms for H₂ evolution and, therefore, have different acclimation strategies to oxidative stress under H₂ photoproduction conditions. To our knowledge, a comparative investigation of H₂ photoproduction activities in these two types of organisms under the same conditions has never been reported before.

In the present study, we compare H₂ photoproduction under different light intensities in two heterocystous N₂-fixing cyanobacteria (*Calothrix* sp. 336/3 and the *ΔhupL* mutant of *Anabaena* sp. PCC 7120) and in S-deprived green alga (*Chlamydomonas reinhardtii*) entrapped in thin Ca²⁺-alginate films under autotrophic conditions. The entrapment of phototrophic cells in thin films ensures uniform light distribution to the cells, allows a more precise evaluation of light effects on H₂ photoproduction, and simplifies estimation of LHCEs.

2. Materials and methods

2.1. Strains and growth conditions

Two filamentous heterocystous cyanobacteria (the *ΔhupL* mutant of *Anabaena* sp. strain PCC 7120 and the wild-type *Calothrix* sp. strain 336/3) and the green alga, *Chlamydomonas reinhardtii* strain CC-124 were used in this study. The *ΔhupL* mutant of *Anabaena* sp. PCC 7120 is deficient in the large subunit of the uptake hydrogenase [28] and is thus affected in H₂ uptake activity in heterocysts. This mutant was kindly provided by Prof. H. Sakurai. The wild-type *Calothrix* sp. 336/3 was selected from the University of Helsinki Culture Collection (UHCC, Finland) as one of the best natural H₂ producers [33]. The wild-type *C. reinhardtii* CC-124 (mt–, nit–), which is a common model organism for studying photobiological H₂ production, was obtained from the Chlamydomonas Resource Center at the University of Minnesota, USA.

Stock cultures of *C. reinhardtii* were maintained photoautotrophically in 150-mL Erlenmeyer flasks containing 50 mL of the Tris-Phosphate (TP) medium, which is a modification of the standard

TAP medium [34] that contains acetate. The pH of the TP medium was adjusted to 7.2 with concentrated HCl. Stock cultures of cyanobacteria were kept under diazotrophic conditions in the similar flasks containing 50 mL of Z8x medium [35]. The medium for growing stock cultures of *ΔhupL* mutant was supplemented with 25 μg ml⁻¹ of spectinomycin. The flasks with algae and cyanobacteria were placed on a shaker (~100 RPM) illuminated with fluorescent lamps (Lumilux T8 15W/865; Osram) that provided about 30 μmol photons m⁻² s⁻¹ photosynthetic active radiation (PAR) at the top of the flasks. The cultures were grown at 25 °C and diluted weekly (green alga) or biweekly (cyanobacteria) with fresh medium. Before immobilization, cultures were transferred into 500-mL Erlenmeyer flasks containing 300 mL of either TP medium (*C. reinhardtii*) or Z8x medium (cyanobacteria) and grown under ~60 μmol photons m⁻² s⁻¹ in a growth chamber at 25 °C. During this stage, spectinomycin was not added to the *ΔhupL* cultures. All flasks were sparged continuously with air filtered through 0.2 μm pore-size membrane filters (Acro 37 TF, Gelman Sciences, Inc.) for aeration and mixing.

2.2. Cell immobilization and H₂ photoproduction experiments

H₂ photoproduction in *C. reinhardtii* films was initiated by applying a two-stage sulfur deprivation protocol [14]. The cells at the exponential growth phase (14–16 μg total Chl mL⁻¹ for green alga; 6–8 μg Chl a mL⁻¹ for cyanobacteria) were harvested by centrifugation (3000 g for 5 min at 24 °C) and pelleted biomass was washed once in either T-S-P medium (*C. reinhardtii*) or Z8x medium (cyanobacteria). The T-S-P medium is a modification of the TAP medium, in which acetate and also the phosphate buffer were excluded from the composition and all sulfates were replaced with chloride salts at the same concentrations. Phosphorus exclusion in addition to sulfur deprivation was necessary for stabilization of alginate films as discussed by Kosourov and Seibert [36]. According to previous publications [15,37], simple exclusion of phosphorus from the experimental medium does not lead to the P-deprivation state in cells and, therefore, the immobilized algal cells were considered as S-deprived only. The harvested biomass was entrapped in thin Ca²⁺-alginate films as described previously [30,36]. The films were prepared using a formulation ratio of 1 g of wet cell biomass, 0.5 mL water, and 1 mL 4% sodium alginate (#71238, Sigma-Aldrich). Polymerization of the film was initiated by spraying the surface with a 50 mM CaCl₂ solution. In contrast to previous experiments [36], the thickness of the alginate films was ~180 μm, and was affected by the thickness of the plastic, window insect screen used as a support. Each *C. reinhardtii* strip contained 185–190 μg total Chl, while cyanobacterial strips contained 50–67 μg Chl *a*. Despite the difference in Chl concentrations, the films (as stated above) were formed based on the same fresh biomass content in the formulation.

After fabrication, the alginate films were cut into 6 cm × 1 cm strips and transferred into 75-mL vials containing 10 mL T-S-P medium or Z8x medium for green alga and cyanobacteria, respectively. The initial pH of both media was 7.5. The vials were sparged with argon for 20 min. For H₂ photoproduction, 6% and 3% CO₂ was added into the headspaces of vials with cyanobacterial films and *C. reinhardtii* films, respectively. The addition of CO₂ decreased pH of the media to 6.3 and 6.9, respectively. The vials were placed in a growth chamber and cultivated at 25 °C under continuous illumination from the top. The light intensities at the level of alginate films varied from ~13 to ~209 μmol photons m⁻² s⁻¹ (2.9 to 45.6 W m⁻²).

To ensure active photosynthesis, the alginate films with entrapped green alga and cyanobacteria were re-supplied periodically with CO₂ gas. For *C. reinhardtii* films, the gas phase in the vials was replaced with Ar containing 3% CO₂ every day during the first three days. For cyanobacteria, the headspace was replaced with Ar containing 6% CO₂ on every fourth day throughout the whole experiment (~260 h). In this procedure, the vials were sparged with argon for 20 min, and pure CO₂

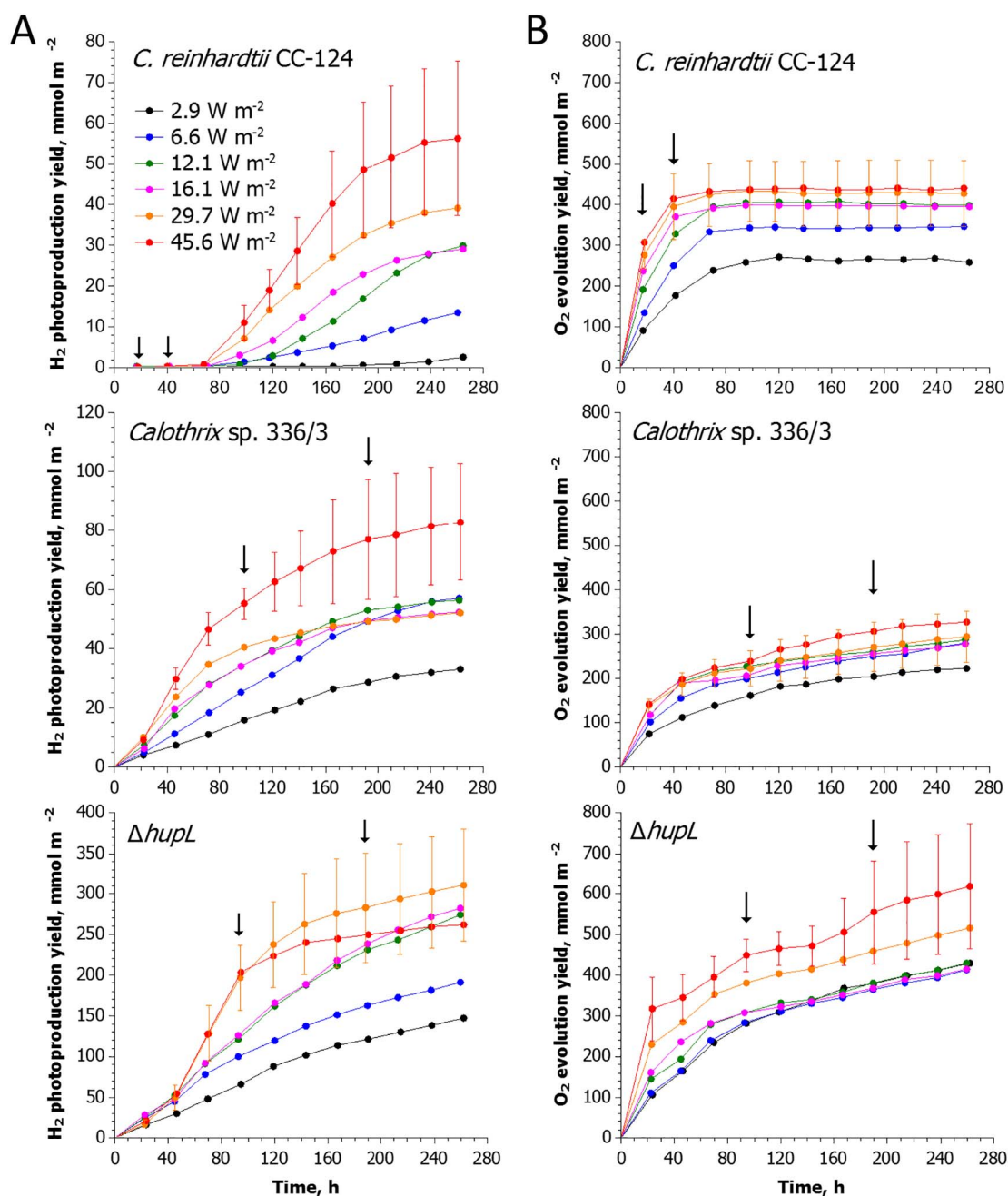


Fig. 1. The effect of light intensity on (A) H₂ photoproduction and (B) net O₂ evolution yields by *C. reinhardtii* and two cyanobacterial strains entrapped in Ca²⁺-alginate films. The arrows indicate the points when the gas phase in the headspace of the vials was renewed with Ar supplemented with 3% (alga) and 6% (cyanobacteria) CO₂. The curves show cumulative H₂ and O₂ yields. Each experimental point represents the average from 3 to 12 vials. Error bars are given for the curve with the highest standard deviations in each set.

was reinjected to the final level (3 and 6%, respectively) in the headspace of the vials with a gas-tight syringe. These two protocols for CO₂ supplementation (for alga and cyanobacteria) were selected based on our previous experimental data [20,30].

The H₂ and O₂ contents in the headspace of the vials were monitored once a day using a gas chromatograph (Clarus 500, PerkinElmer, Inc.) equipped with a thermal conductivity detector and a molecular sieve 5A column (60/80 mesh). The kinetic curves shown in Fig. 1 represent cumulative H₂ and O₂ production yields, from which the maximum rates presented in Fig. 2 were calculated.

2.3. Light energy to hydrogen energy conversion efficiency

Incident light intensities in the photosynthetic active radiation

(PAR) region were measured at the level of alginate surface in the middle of the strip and on both sides with a light meter (LI-250, LI-COR) equipped with the LI-190R quantum sensor. The average light energy on the surface of the films was calculated assuming the conversion coefficient for the cool-daylight fluorescent lamps of 0.218 J per 1 μ mol photons averaged over 400–700 nm.

For precise estimation of LHCEs in the potentially reversible process, the changes in the partial pressure of H₂ gas in the experimental vials were taken into consideration [38], and LHCEs were calculated using Eq. (1) [39]:

$$\eta(\%) = 100 \frac{(\Delta G^\circ - RT \ln \left(\frac{p^0}{p} \right)) R_H}{E_S A}, \quad (1)$$

where ΔG° is the change of the standard Gibb's free energy for the

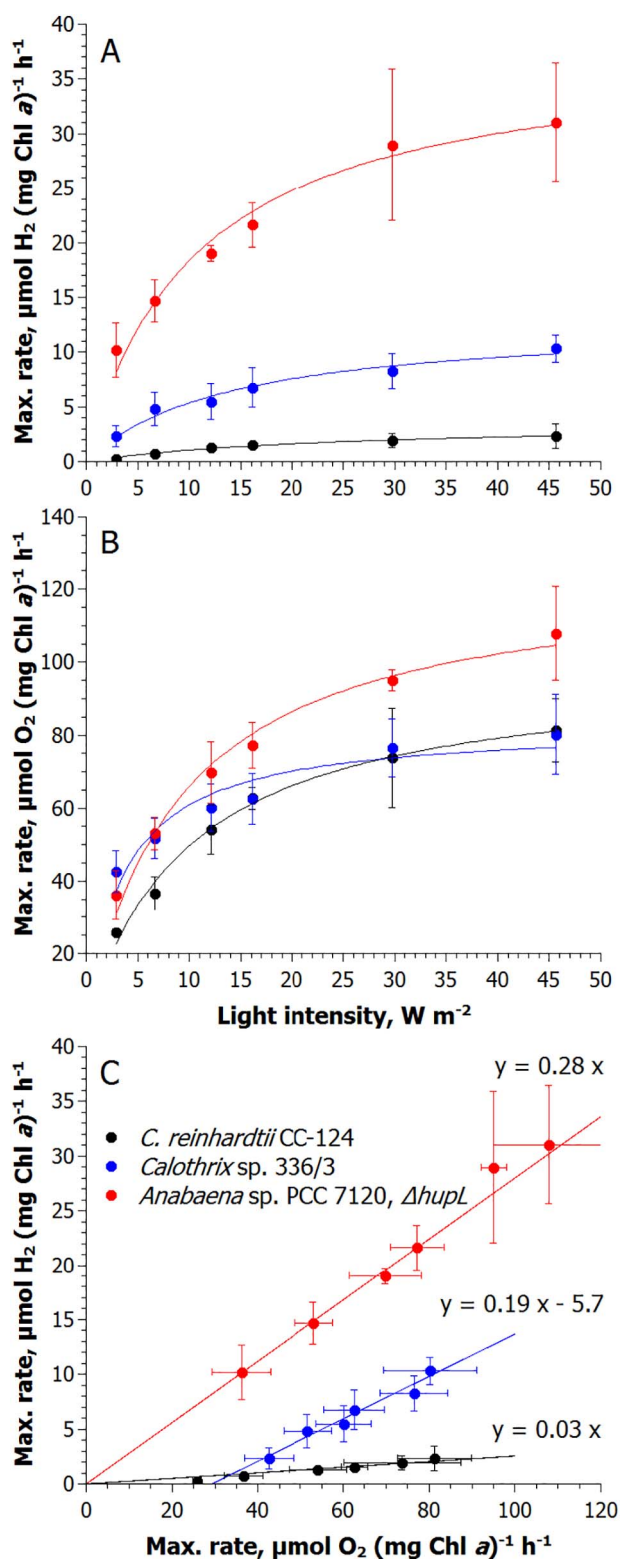


Fig. 2. The effect of light intensity on the maximum rate of (A) H₂ production and (B) net O₂ evolution. The rates were derived by the differentiation of the kinetic curves presented in Fig. 1. The panel (C) shows the correlation between the two rates. Each experimental point represents 3–12 replicates, and the error bars are one standard deviation. Non-linear and linear curve fitting was performed with QtiPlot 0.9.8.9. For non-linear fitting, a scaled Levenberg-Marquardt algorithm with a tolerance = 0.0001 was applied.

water-splitting reaction ($237,200 \text{ J mol}^{-1}$ at 25°C and 1 atm), R is the universal gas constant, T is the absolute temperature, P^0 and P are the standard and observed H₂ pressures (atm), R_H is the rate of H₂

photoproduction (mol s^{-1}), E_S is the energy of the incident light radiation ($\text{J m}^{-2} \text{ s}^{-1}$), and A is the illuminated surface area (0.0006 m^2).

2.4. Chlorophyll determination

The Chl *a* (cyanobacteria) and total Chl (*a* + *b*) (*C. reinhardtii*) contents in Ca²⁺-alginate films were assayed in randomly chosen strips after solubilization of the alginate matrices in 50 mM Na-EDTA solution (pH 7.0). The cells were washed once by centrifugation with either Z8x or T-S-P medium, respectively. The Chl *a*, Chl *b* and the total Chl (*a* + *b*) contents in *C. reinhardtii* samples were assayed at 665 and 649 nm in 95% ethanol extracts [34]. The Chl *a* content in cyanobacterial samples was determined spectrophotometrically at 665 nm minus 720 nm after extraction of the cell pellets with 90% methanol [40].

2.5. Analysis of pigment composition

The Ca²⁺-alginate strips with entrapped cells were collected, and the alginate polymer was destroyed by vortexing in 50 mM Na-EDTA solution (pH 7.0) to release the cells. The recovered cells were washed once by centrifugation with either T-S-P or Z8x medium. The final pellets were frozen in liquid nitrogen and stored at -80°C before processing. Photosynthetic pigments were extracted from the pellets with 100% methanol in the dark at 4°C . The procedure was repeated until the pigments were extracted. The extracts were combined, centrifuged, and filtered through a $0.2\text{-}\mu\text{m}$ polytetrafluoroethylene syringe filter to remove any remaining cell debris. Pigments were separated by the high-performance liquid chromatography (HPLC, Agilent 1100 Series, Agilent Technologies, Palo Alto, CA) equipped with a diode array detector and a reverse-phase C18 column ($4 \times 125 \text{ mm}$, $5 \mu\text{m}$, LiChroCART, Merck KGaA, Darmstadt, Germany). Cyanobacterial pigments were eluted with methanol/water (9:1, v/v) for 20 min and then with 100% methanol at a flow rate of 0.5 mL min^{-1} . For separation of the *C. reinhardtii* pigments, two solvents A and B were applied consecutively at a constant flow rate of 0.75 mL min^{-1} . Solvent A, consisting of acetonitrile/methanol/0.1 M Tris-HCl buffer adjusted to pH 8.0 (72:8:3, v/v), was flowed for 4 min, followed by a linear gradient of solvent B (methanol/Tetrahydrofuran [92:8, v/v]) from 0 to 100% for 15 min. After the gradient, an isocratic run of solvent B was applied for another 26 min. The column was re-equilibrated between samples for about 13 min with solvent A.

Chl *a*, Chl *b*, lutein, violoxanthin, antheraxanthin, neoxanthin, zeaxanthin, myxoxanthophyll, canthaxanthin, echinenone and β -carotene standards were purchased from DHI Lab Products (Hørsholm, Denmark) and used for quantification of identified carotenoids.

2.6. Determination of protein oxidation

In the end of the experiment ($\sim 260 \text{ h}$), the Ca²⁺-alginate strips with entrapped cells were collected and destroyed by vortexing in 50 mM Na-EDTA solution (pH 7.0). The cell pellets were washed with ice-cold STNE buffer (0.4 M sucrose, 10 mM Tris-HCl, pH 8.0, 10 mM NaCl, 20 mM Na-EDTA) supplemented with 50 mM dithiothreitol. The pellets were resuspended in 150 ml of the same buffer, frozen in liquid nitrogen and stored -80°C before processing. The total protein samples were isolated following the procedure of Pollari and co-authors [41]. They were prepared according to the Oxyblot™ Protein Oxidation Detection Kit (Millipore), separated by 12% SDS-PAGE (Mini Gel Pro-tean) and transferred to Immobilon-PVDF membranes (Millipore). The carbonylated proteins were detected with the chemiluminescent HRP substrate (Millipore). The membranes were stained with Coomassie brilliant blue R-250 (Bio-Rad) to verify equal loading and even transfer of the samples.

2.7. Statistical analysis

Statistical analysis of H₂ photoproduction activities in the alginate films was carried out in Microsoft Excel 2016 by processing the data obtained from at least three independent photobioreactor (PBR) vials. Under high light intensities (above 16 W m⁻²), where H₂ photoproduction in alginate films was affected by the oxidative stress, all experiments were repeated in up to 12 independent samples. Due to excessive amount of data, the SDs are plotted for the curves with the highest deviations in each set, but the original data and SDs are provided as supplementary material (Data Profile.xlsx). Non-linear and linear curve fitting was performed in QtiPlot 0.9.8.9. The OLS method was applied in the case of linear regression. For non-linear fitting, a scaled Levenberg-Marquardt algorithm with a tolerance = 0.0001 was used. The adjusted R-squared values were higher than 0.95 in all cases. All pigments were analyzed from 3 to 6 independent alginate films, and the results are presented as a mean ± SD. The Oxyblots were repeated at least two times from independently isolated protein samples collected from different experiments.

3. Results

3.1. Effect of light intensity on H₂ photoproduction activity

Two cyanobacterial strains and S-deprived *C. reinhardtii* were entrapped in thin Ca²⁺-alginate films and transferred to an Ar + CO₂ atmosphere to initiate H₂ production. As shown in Fig. 1 A, increasing the light intensity applied to the surface of the Ca²⁺-alginate films with entrapped cells improved the rates and yields of H₂ photoproduction in *C. reinhardtii* and both cyanobacterial strains. The most significant effect of light on the H₂ photoproduction yield was observed in *C. reinhardtii* films, where an increase from 2.9 to 45.6 W m⁻² PAR increased the H₂ photoproduction yield by > 20 times (from ~2.5 to 56 mmol m⁻² after ~260 h). Nevertheless, under all conditions tested, these films produced less H₂ than the films with entrapped cyanobacterial strains exposed to the similar light intensities. It should be noted that S-deprived *C. reinhardtii* produces H₂ more efficiently in the presence of acetate [42]; however, acetate was not included in the medium in the current research.

Under our conditions, the wild-type *Calothrix* sp. 336/3, which possesses an uptake hydrogenase in the heterocysts [43], produced less H₂ than the $\Delta hupL$ mutant of *Anabaena* sp. PCC 7120 lacking an active uptake hydrogenase (Fig. 1 A). The films with entrapped *Calothrix* sp. 336/3 cells showed the highest H₂ photoproduction yield (83 mmol m⁻² after 260 h) under 45.6 W m⁻² PAR, but even under this illumination, they produced only about two-thirds of the H₂ than films with entrapped $\Delta hupL$ mutant cells placed under the lowest light level. The $\Delta hupL$ films yielded 147 mmol H₂ m⁻² after 260 h under 2.9 W m⁻² PAR (Fig. 1 A). In contrast to *Calothrix* sp. 336/3, the $\Delta hupL$ films produced the highest H₂ level (~310 mmol m⁻²) under moderate light illumination (29.7 W m⁻²). The light intensity at 45.6 W m⁻² caused a noticeable inhibitory effect on the H₂ photoproduction yield in this mutant especially after 96 h. As a result, the $\Delta hupL$ films placed under 12.1 and 16.1 W m⁻² PAR yielded the same amount of H₂ as the films placed under 45.6 W m⁻² light (~260 mmol H₂ m⁻²).

The maximum specific H₂ photoproduction rate, which shows the maximum capacity of the selected strain to produce H₂ under the applied environmental conditions, increased with increasing light irradiation in all three strains (Fig. 2 A). Fitting the light saturation curves with Michaelis-Menten models gave a maximum theoretical rate of H₂ production (P_{max} H₂) of about 38, 13 and 3.6 μ mol H₂ (mg Chl a)⁻¹ h⁻¹ and the half-saturation constant (K_i H₂) of about 10.8, 14 and 24.2 W m⁻² for the *Anabaena* sp. PCC 7120 $\Delta hupL$, *Calothrix* 336/3, and *C. reinhardtii* films, respectively.

3.2. Effect of light intensity on net O₂ evolution

All three alginate-entrapped strains demonstrated higher accumulation of O₂ in the head-space of the vials during the first 24 h. Later, the rates of net O₂ production declined in all strains (Fig. 1 B). S-deprived *C. reinhardtii* stopped producing O₂ within ~72 h, while both cyanobacterial strains continued evolving O₂ throughout the experiment, but at substantially lower rates than during the first 24 h. *C. reinhardtii* films only photoproduced H₂ under the anaerobic environment established after about 72 h of S-deprivation. In contrast, H₂ photoproduction (released in the anaerobic environment of the heterocysts) in the cyanobacterial films occurred simultaneously with the release of O₂ to the vial headspaces.

Similar to H₂ photoproduction, the maximum rate of net O₂ evolution in all three strains depended greatly on the light intensity (Fig. 2 B). The lowest half-saturation constant (K_i O₂) of ~3.6 W m⁻² was obtained for *Calothrix* sp. 336/3. This strain also showed the lowest maximum theoretical rate of net O₂ evolution (P_{max} O₂), which was around 83 μ mol O₂ (mg Chl a)⁻¹ h⁻¹. The K_i O₂ constants were very close in the $\Delta hupL$ mutant (9 W m⁻²) and in *C. reinhardtii* (9.8 W m⁻²), but the P_{max} O₂ was higher in the $\Delta hupL$ strain: 125 compared to 99 μ mol O₂ (mg Chl a)⁻¹ h⁻¹.

3.3. Comparison of H₂ photoproduction and net O₂ evolution activities

As expected for the water biophotolysis process, all three strains showed a positive correlation between the maximum rate of H₂ photoproduction and the maximum rate of net O₂ evolution (Fig. 2 C). Over the range of light intensities studied, the dependence was linear in all three strains with H₂/O₂ ratios of around 0.03, 0.19 and 0.28 for *C. reinhardtii*, *Calothrix* sp. 336/3, and the $\Delta hupL$ films, respectively.

For the whole H₂ photoproduction period, the H₂/O₂ ratio varied depending on the strain and the light intensity applied to the film. In *C. reinhardtii* films, the final H₂/O₂ ratio was close to 0.015 at 2.9 W m⁻² and steadily increased with increasing light intensity to a maximum value of 0.11 at 45.6 W m⁻². The final H₂/O₂ ratio did not vary much on light intensity in *Calothrix* sp. 336/3. The values obtained for different light intensities were very close (0.18–0.24) to the ratio obtained for the maximum rates (0.19). Among three strains the $\Delta hupL$ films placed under moderate light (16.1 W m⁻²) showed the highest H₂/O₂ ratio (0.68). This value is about 34% of the theoretical maximum for water biophotolysis, which is 2 mol H₂ per 1 mol O₂ produced. An increase or a decrease in the light intensity caused a decline in the final H₂/O₂ ratio in the $\Delta hupL$ films to around 0.42 at 45.6 W m⁻² and 0.34 at 2.9 W m⁻².

3.4. Comparison of light-to-H₂ conversion efficiencies

Next, we calculated the LHCEs for the cyanobacterial and *C. reinhardtii* entrapped films (Fig. 3). In Fig. 3 A, the LHCEs (for the maximum rate of H₂ photoproduction) declined with increasing light intensity from 0.57 to 0.12% (*Calothrix* sp. 336/3 films) and from 2.5 to 0.5% ($\Delta hupL$ films). In *C. reinhardtii* films, however, the LHCE declined under light below 12 W m⁻² (Fig. 3 A). As a result, the alga produced H₂ at the same efficiency under both 2.9 W m⁻² and 45.6 W m⁻²: ~0.09 and 0.08%, respectively.

Thus, the highest LHCE (0.14%) in *C. reinhardtii* films was obtained under the moderate light (12.1 W m⁻²). All three strains showed much lower LHCEs calculated for the whole H₂ photoproduction period (~260 h). As shown in Fig. 3 B, the values varied over the range of 0.02 to 0.06% in *C. reinhardtii* films, 0.04 to 0.3% in *Calothrix* 336/3 films and from 0.14 to 1.2% in the $\Delta hupL$ films.

3.5. Composition of carotenoids under different light intensities

Alginate polymer restricts the permeability of gases [44,45]. In

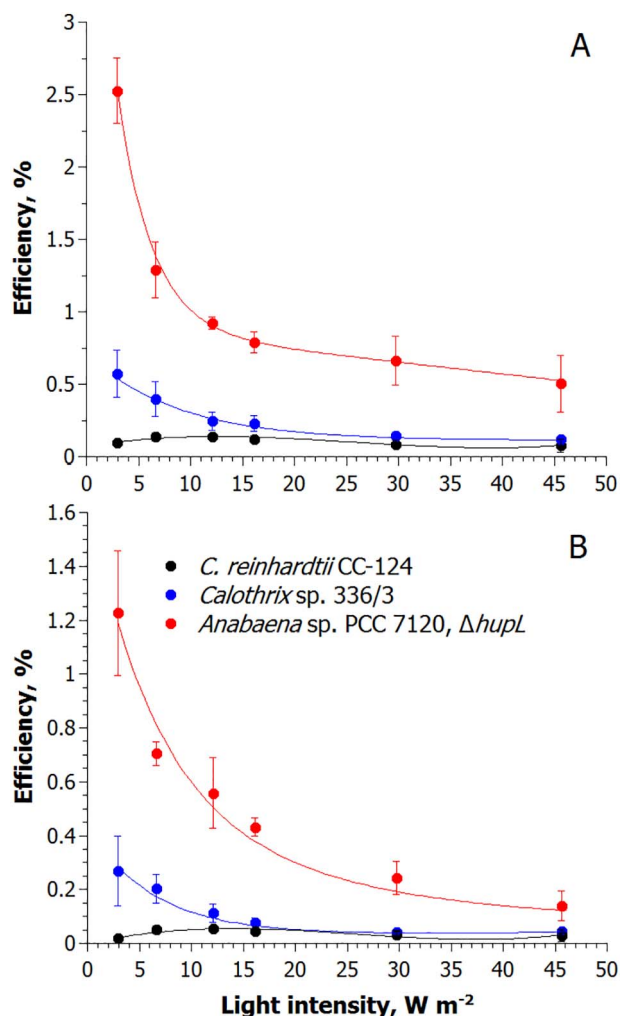


Fig. 3. The light-energy-to-H₂-energy conversion efficiencies exhibited by alginate-entrapped cultures of *C. reinhardtii* CC-124, *Calothrix* sp. 336/3, and the Δ*hupL* mutant. The efficiencies were calculated based on PAR (400–700 nm) during (A) the maximum rates of H₂ photoproduction (presented on Fig. 2 A) and (B) the whole period of H₂ photoproduction. Each experimental point represents 3–12 replicates, and the error bars are one SD.

excess light and CO₂, this limitation may cause enhanced O₂ accumulation inside the immobilization matrix due to the photosynthetic water-splitting activity of entrapped cells [31]. Over-accumulation of O₂ together with carbon/sulfur or carbon/nitrogen imbalance in photosynthetically active cyanobacteria and green algae will inevitably lead to the excess ROS formation, which causes the oxidative damage to the cells. Progressive bleaching of the films throughout the experiment supports the presence of oxidative stress in the entrapped cells (Fig. 4; see also the Oxyblot data below).

The bleaching phenotype was very pronounced in all strains and correlated well with the light intensity at the surface of the films. The Chl *a* content in *C. reinhardtii* and in both cyanobacterial strains decreased almost exponentially with increasing light intensity (Fig. 5). Chl *b* levels in *C. reinhardtii* followed the same pattern, suggesting that both photosystems as well as the light-harvesting complexes are affected by S-deprivation [46]. Bleaching at high light was so strong that even some major carotenoids were affected. By the end of the experiment, the β-carotene content in the *C. reinhardtii* films did not exceed 20% of the original level at 0 h under low light (2.9 W m⁻²) and was only around 2% under high light (45.6 W m⁻²). The β-carotene content was also affected in *Calothrix* sp. 336 films, but only at light intensities above 10 W m⁻². In contrast, Δ*hupL* films showed higher β-carotene levels as

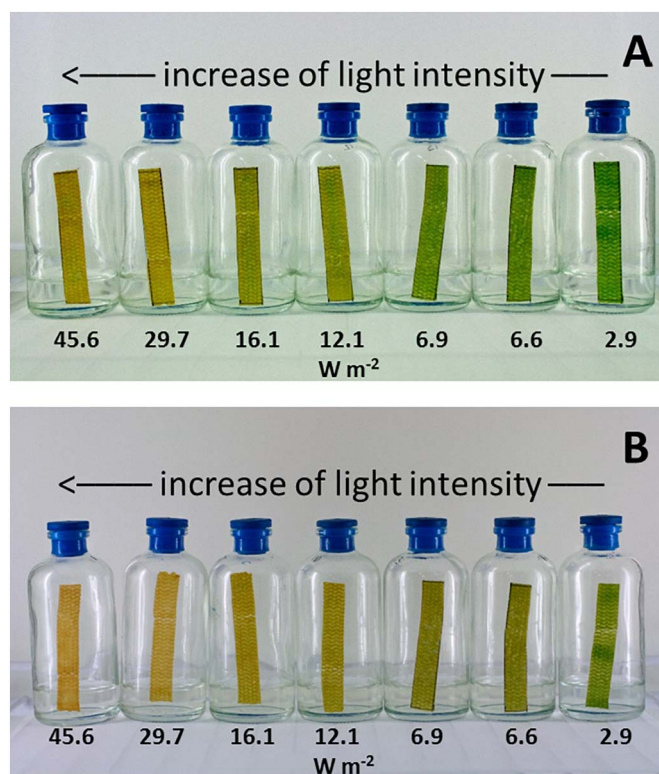


Fig. 4. The visual changes in the pigment composition of the *Anabaena* sp. PCC 7120 Δ*hupL* films placed under different light intensities (45.6–2.9 W m⁻²) during a H₂ photoproduction experiment. The pictures were taken on 4th (A) and 11th (B) days after the start of the experiment. Similar, but less pronounced changes were observed in *Calothrix* sp. 336/3 and *C. reinhardtii* films. The picture is representative of 3 independent experiments.

compared to 0 h samples, but again the levels declined with the increasing light intensity (Fig. 5 C). Similarly to β-carotene, both cyanobacterial strains also demonstrated a noticeable decline of echinenone with increasing the light intensity.

In *C. reinhardtii* films, the lutein level was ~2-times higher than in the initial (0 h) films at light intensities below 12 W m⁻², but decreased with increasing light. The violoxanthin cycle carotenoids behaved as expected for cells under oxidative stress: the levels of zeaxanthin and antheraxanthin were enhanced under all light intensities with significantly higher zeaxanthin content, which in some cases increased > 30-times as compared to 0 h samples (Fig. 5 A). On the other hand, violoxanthin levels were lower in all samples (Fig. 5 A).

In both cyanobacterial strains, we noticed increased levels of myxoxanthophylls and hydroxylated carotenoids as compared to the 0 h samples. Their levels were high under low light and decreased with increasing light intensity (Fig. 5 B and C), except for zeaxanthin and 3'-hydroxyechinenone in the Δ*hupL* cells, which demonstrated the opposite trend (Fig. 5 C). The films with entrapped Δ*hupL* cells showed a significant accumulation of ketomyxol 2'-fucoside, and increased levels of both myxol 2'-fucoside and canthaxanthin. The *Calothrix* sp. 336/3 cells, which could not synthesize ketomyxol [47], accumulated myxol 2'-methylpentoside (most probably, myxol 2'-fucoside).

Calothrix sp. 336/3 cells also showed enhanced levels of hydroxylated carotenoids, such as nostoxanthin and caloxanthin. Under low light (2.9 W m⁻²), the nostoxanthin content increased > 18-times as compared to the 0 h samples, but declined with increasing light intensity. The zeaxanthin level in *Calothrix* 336/3 was also slightly higher, but only under low light (below 10 W m⁻², Fig. 5 B).

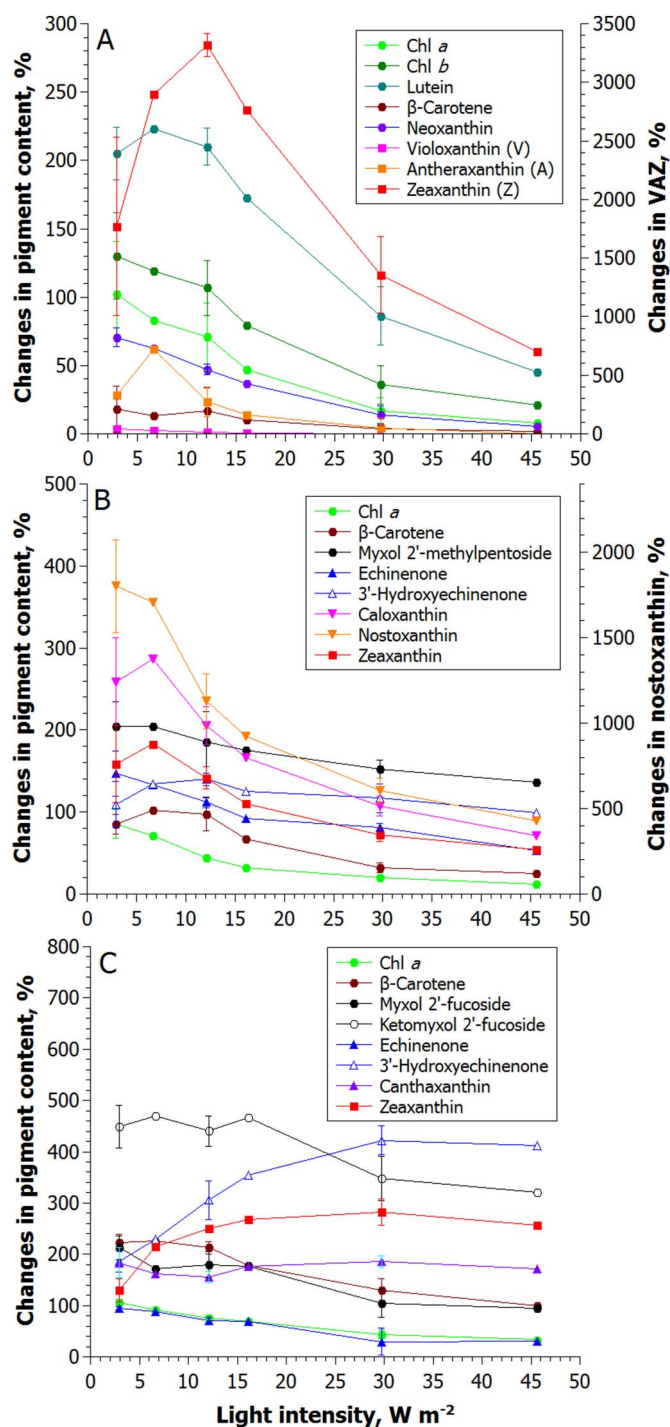


Fig. 5. Changes in pigment composition by the end of H_2 photoproduction. The pigment composition of (A) *C. reinhardtii* CC-124, (B) *Calothrix* sp. 336/3, and (C) $\Delta hupL$ mutant cells entrapped in alginate films were measured after ~ 260 h hours of H_2 photoproduction conditions at different light intensities. The pigment levels in 0 h alginate films of each strain were considered as 100%. The values are the mean of 3 to 6 biological replicates. SDs are presented for 3 light intensities.

3.6. Changes in the protein oxidation

The carbonylation of proteins, which is caused by enhanced production of ROS in cells, is a widespread biomarker of oxidative damage. We found that the degree of protein carbonylation in total protein extracts isolated from the cells in the end of H_2 production experiment enhanced in *C. reinhardtii* samples with increasing light intensity (Fig. 6). The effect was less pronounced in both cyanobacterial films.

4. Discussion

4.1. Increasing light intensity improves the H_2 photoproduction activity in all immobilized strains

Hydrogen photoproduction in green algae and cyanobacteria depends on photosynthetic activity. Therefore, it is not surprising that *C. reinhardtii*, *Calothrix* sp. 336/3 and the $\Delta hupL$ mutant of *Anabaena* sp. PCC 7120 cells entrapped in alginate films demonstrated the improved H_2 photoproduction rates and yields with an increasing light intensity at least up to 45.6 W m^{-2} (Fig. 1). All three strains also showed typical light saturation curves of H_2 photoproduction and O_2 evolution (Fig. 2 A and B). Interestingly, at high irradiances ($29.7\text{--}45.6 \text{ W m}^{-2}$ PAR) we have not noticed any significant inhibition of the maximum H_2 and O_2 production rates despite severe stress conditions. The dependences between the maximum rate of H_2 photoproduction and the maximum rate of net O_2 evolution were linear in the range of light intensities studied (Fig. 2 C), indicating on the indirect involvement of PSII in the H_2 photoproduction yield. Indeed, in all three strains, especially in *C. reinhardtii*, the correlation coefficients between the maximum H_2 and O_2 production rates were much below the theoretical maximum of H_2/O_2 ratio for water biophotolysis.

The maximum rate of net O_2 evolution always precedes the maximum H_2 photoproduction activity in all three strains (Fig. 1). In S-deprived *C. reinhardtii*, this phenomenon is explained by the gradual degradation of PSII centers in cells that leads to the onset of anaerobiosis by respiration, expression of [Fe-Fe]-hydrogenase enzymes followed by a period H_2 photoproduction [14,48]. Thus, the O_2 evolution and H_2 photoproduction stages of S-deprived algal suspensions are separated temporarily [14,49]. The same separation was clearly observed in S-deprived algae immobilized on glass fiber matrices [50], and in agar gel cubes [51]. It is clear that under photoautotrophic conditions H_2 photoproduction in alginate-entrapped *C. reinhardtii* cultures occurs only after inhibition of O_2 -evolving activity in the cells (Fig. 1). Under photoheterotrophic conditions, the same films can produce H_2 during the O_2 -evolution stage (but at reduced rate) or even under an air atmosphere, where anaerobiosis and H_2 production are supported by respiration using acetate as a substrate [36,52].

In N_2 -fixing heterocystous cyanobacteria pre-grown under an air atmosphere, a slow gradual increase in H_2 -photoproduction activity is observed at all light intensities within first 24–48 h after the transfer of films to an $Ar + 6\% \text{ CO}_2$ atmosphere. This is most probably caused by the competition of nitrogenase for the reductants with respiratory pathways in heterocysts due to enhanced production of O_2 in vegetative cells (Fig. 1 B). Nitrogenase activity, however, was not inhibited but even increased at this stage (Fig. S1). The absence of significant inhibition of nitrogenase up to O_2 concentrations of $300 \mu\text{M}$ was also demonstrated in *A. azollae*, *A. variabilis* ATCC 29413 and in the uptake hydrogenase mutant of *A. variabilis* PK84 [53]. Heterocystous cyanobacteria have evolved a wide variety of respiratory mechanisms protecting the nitrogenase from O_2 inactivation, which include heterocyst-specific respiratory terminal oxidases and flavodiiron proteins. In *Anabaena* sp. PCC 7120, the heme-copper terminal respiratory oxidases, Cox2 and Cox3, were shown to be essential for diazotrophic growth [54] and protection of the nitrogenase from O_2 inactivation [55]. The Cox2 was indeed found upregulated in the uptake-hydrogenase-deficient mutant of *Nostoc punctiforme* ATCC 29133 under diazotrophic growth conditions [56]. *Anabaena* sp. PCC 7120 also possesses two heterocyst-specific flavodiiron proteins, Flv1B and Flv3B [57] with Flv3B shown to be involved in light-induced O_2 uptake in heterocysts [58]. Orthologs of these enzymes exist in *Calothrix* sp. 336/3. All these pathways, however, compete with nitrogenase for the reductants, thus decreasing H_2 photoproduction yields.

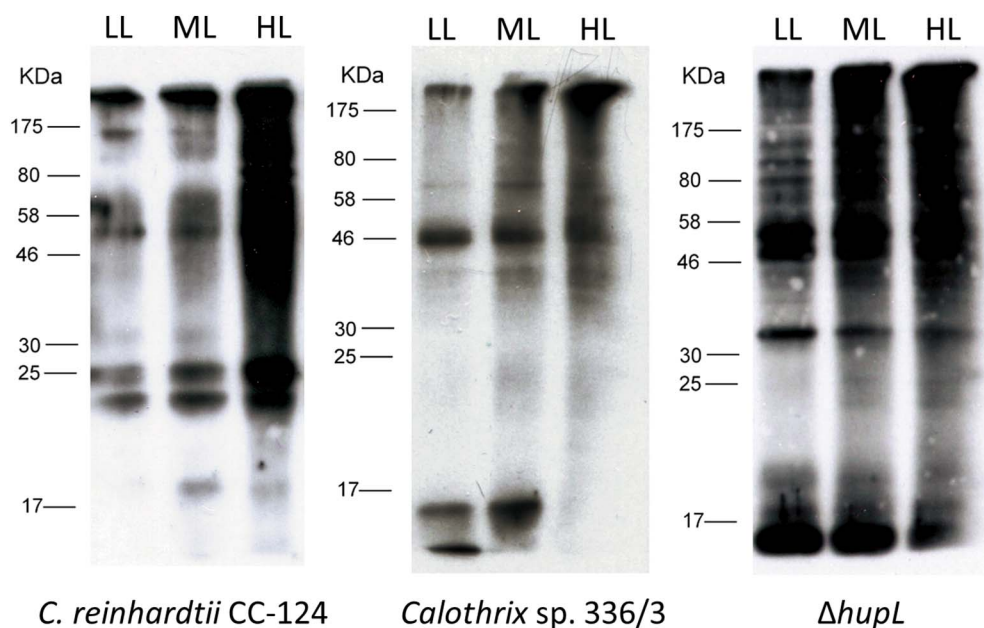


Fig. 6. Oxyblots of total proteins isolated from the algal and cyanobacterial films in the end of H_2 photoproduction experiments. LL - 2.9 W m^{-2} , ML - 16.1 W m^{-2} , and HL - 45.6 W m^{-2} light.

4.2. H_2 photoproduction in nutrient-deprived *C. reinhardtii* under autotrophic conditions is affected by high sensitivity of [Fe-Fe]-hydrogenase to O_2

H_2 photoproduction in nutrient-deprived green algae is driven by two inter-dependent mechanisms [8]. The first is the direct water-biophotolysis pathway involving the splitting of water by PSII with subsequent transport of electrons to the [Fe-Fe]-hydrogenases through PSI. The second is the indirect pathway and depends on the metabolic oxidation of organic substrates coupled to the PSI and the [Fe-Fe]-hydrogenases via the PQ pool [59]. Due to the gradual degradation of PSII centers in nutrient-deprived algae [60,61], the contribution of these two pathways in the overall H_2 photoproduction yield varies depending on the stage of nutrient limitation with less involvement of the direct pathway over time after anaerobiosis [62,63]. Since in the long-term process direct water biophotolysis is potentially more efficient than the indirect, the stabilization of PSII at early stages of nutrient starvation promotes higher H_2 photoproduction yields [64]. On the contrary, the degradation of PSII centers upon nutrient deprivation or inhibition of PSII by DCMU [8,19] would favor the indirect mechanism.

Under autotrophic conditions, H_2 photoproduction in immobilized *C. reinhardtii* cells seems to be limited by high PSII activity and low respiratory rate, conditions that favor accumulation of O_2 inside the films and impair the O_2 -sensitive [Fe-Fe]-hydrogenase enzymes [19,20]. As shown in Fig. 1 A, we indeed observed a noticeable delay ($> 72 \text{ h}$) in the start of H_2 photoproduction by algal films under sulfur-deprived, autotrophic conditions as compared to photoheterotrophic films ($< 24 \text{ h}$ [36,52]). The delay was more pronounced under low light conditions indicating possible limitations on H_2 photoproduction by low level of accumulated carbohydrates [20]. Starch catabolism during H_2 photoproduction is important both for the removal of O_2 produced by PSII, thus, protecting the O_2 -sensitive hydrogenase of inactivation, or for providing electrons to hydrogenase through the photofermentation pathway [8,59]. Thus, the strong dependence of H_2 photoproduction in autotrophic algae on light intensity (Fig. 1 A) could be explained by the dependence of the process on the starch reserves accumulated during the O_2 -evolving stage (first 48–96 h of the experiment, depending on the light intensity), and on PSI-activity during the H_2 -production stage. High light was indeed shown to enhance starch accumulation in nutrient-deprived algae [20].

In beginning of S-deprivation, when the PSII activity is high and cells have limited amounts of carbohydrates, periodic recharging with

argon helps to protect anaerobiosis in the vials and decreases the loss of reductants to respiration, but even under that conditions algae entrapped in alginate films do not produce H_2 as efficiently as photoheterotrophic films placed under the same light conditions. For more H_2 photoproduction, a better immobilization protocol must be introduced with either enhanced porosity in the immobilization matrix or entrapment of the nutrient-deprived cells after the initial period of photosynthetic activity. This would limit accumulation of O_2 inside the matrix prior to H_2 photoproduction and decrease the loss of reductants to respiration.

4.3. Thin films with entrapped cyanobacteria can produce H_2 with PAR efficiency of over 2.5%

Under autotrophic conditions heterocystous cyanobacteria show significantly higher LHCEs than S-deprived green alga *C. reinhardtii* (Fig. 3), but these values were still lower than the maximum theoretical efficiency of 6–7% (considering the total incident solar radiation for nitrogenase-driven H_2 photoproduction in cyanobacteria) [3]. This range is much lower than the theoretical efficiency of the direct water biophotolysis process in green algae, which is estimated at around 10–12% [2]. In the best case, the $\Delta hupL$ mutant of *Anabaena* sp. PCC 7120 produced H_2 with PAR efficiency of 2.52% ($\sim 1.1\%$ if the total solar spectrum at ground level is considered) for at least 24 h and 1.2% ($\sim 0.5\%$ solar) for 260 h. The LHCEs in S-deprived *C. reinhardtii* films under autotrophic conditions did not exceed 0.14% ($\sim 0.06\%$ of sun-light). Nevertheless, the strain is capable of producing H_2 at approximately the same efficiency as the $\Delta hupL$ mutant under photoheterotrophic conditions and the same light intensities.

Although the LHCEs of above 3% in PAR were reported for heterocystous cyanobacteria by several research groups, the direct comparison of the data is difficult due to differences in experimental conditions and calculation approaches applied in their studies. For suspension cultures of the $\Delta hupL$ mutant of *Nostoc* sp. PCC 7422, a 3.7% efficiency was estimated under a light intensity of $70 \mu\text{mol m}^{-2} \text{ s}^{-1}$ PAR [65]. In contrast to our data, the calculations were made based on the absorbed light that would roughly approximate the lowest light condition in our study. Thin alginate films, however, did not absorb all incident light (even under the lowest light intensity), and the absorption properties varied throughout the experiment due to noticeable changes in the pigment composition (Fig. 4). Since the degree of these changes varied in different strains and depended on the light intensity

(Fig. 5), we estimated LHCEs based on the incident light only. In another study, a LHCE of 4% was reported for the $\Delta hupW$ mutant of *Nostoc (Anabaena)* sp. PCC 7120 under an incident light intensity of $44 \mu\text{mol m}^{-2} \text{s}^{-1}$ PAR [66]. The illumination was provided by the combination of white and red LED panels, which resulted in a significant shift to the red area in the overall light spectrum and, therefore, could not be directly compared to the current research. The total energy content of the H_2 gas in the above mentioned studies [65,66] was estimated based on its combustion energy (ΔH_c) of $285.8 \text{ kJ mol}^{-1}$. Using the same computational procedure with the ΔH_c as above, we estimate the highest H_2 -conversion efficiency of the alginate-entrapped $\Delta hupL$ strain (2.52%, Fig. 3 A) at ca. 3.3%, very close to the 3.75% *Nostoc* result.

In most studies, however, LHCE measured with heterocystous filamentous cyanobacteria did not exceed 1.5–2% in laboratory PBRs [67–69] and 0.1–0.2% under outdoor conditions [3,70]. Thus, immobilization into the thin alginate films does not affect the efficiency of H_2 photoproduction by cyanobacteria and green algae, but does simplify their cultivation in PBRs. Indeed, H_2 -producing biofilms do not require expensive gas bubbling and culture mixing, and facilitate the exchange of media in the bioreactors [71].

4.4. Alginate-entrapped cyanobacteria and green algae experience oxidative stress under high light conditions

H_2 photoproduction in green algae and cyanobacteria occurs under the conditions of severe redox imbalance in the cells caused by either sulfur (green algae) or nitrogen (cyanobacteria) deprivation. Imbalance in the redox status together with impaired protein biosynthesis may induce the production of reactive oxygen species in photosynthesizing cells, resulting in photoinhibition and oxidative damage [37,72]. In alginate-entrapped algae, oxidative stress could be even more pronounced than in liquid cultures due to the low diffusion rate of O_2 through the alginate polymer [44], which causes over-accumulation of O_2 inside the matrix during active photosynthesis. On the other hand, when PSII activity is already affected by nutrient limitation, the alginate matrix may provide significant protection for the H_2 -producing hydrogenase enzyme against inactivation by atmospheric O_2 [36]. Thus, immobilization in alginate could result in both positive and negative effects on H_2 photoproduction by green algae and cyanobacteria.

Experimental data obtained in the current study show that algal and cyanobacterial cells entrapped within thin alginate matrices can experience oxidative stress under conditions favorable to H_2 photoproduction. The stress increases with light intensity, which can be seen from the progressive bleaching of the films at higher light (Fig. 4). By the end of the experiment, the films exposed to high light also showed increased levels of protein carbonylation as compared to the films exposed to low light, but the effect was less pronounced in both cyanobacterial strains (Fig. 6). The presence of oxidative stress in S-deprived suspension algae was also confirmed recently by Sáenz and co-authors [72].

As expected, the levels of Chl *a* (Chl *a* and *b* in *C. reinhardtii*) and some major carotenoids, such as β -carotene declined with increasing light intensity, while the amounts of some keto and hydroxylated carotenoids, such as zeaxanthin (all three strains), 3'-hydroxyechinenone (both cyanobacteria), lutein (*C. reinhardtii*), and nostoxanthin (*Calothrix* sp. 336/3) increased at the same time (Fig. 5). It is known, that hydroxylated carotenoids are efficient antioxidants that protect membranes from photooxidation and radical-mediated lipid peroxidation [73]. Some of these carotenoids also play a key role in photoprotection by stimulating energy dissipation within the light-harvesting antenna proteins [74]. In *C. reinhardtii*, carotenoids of the xanthophyll cycle (violaxanthin, antheraxanthin, and zeaxanthin) are major players of the non-photochemical quenching (NPQ) photoprotection mechanism [75]. Similar to other research groups [76–78], we indeed noticed a rise in the levels of antheraxanthin and, especially, zeaxanthin in addition to a

noticeable decline of violoxanthin (Fig. 5 A). In cyanobacteria, NPQ is triggered mostly by the orange carotenoid protein (OCP) [79,80]. Both cyanobacterial strains showed enhanced levels of 3'-hydroxyechinenone, a major carotenoid of OCPs, at the expense of echinenone (Fig. 5 B and C). *Calothrix* sp. 336/3 also showed a significant increase in caloxanthin and nostoxanthin. These two hydroxylated carotenoids may play an important role in photoprotection of the photosynthetic apparatus in this benthic strain under prolonged H_2 photoproduction conditions [47]. Interestingly, the protein carbonylation in *Calothrix* sp. 336/3 was not so pronounced as in the other two strains (Fig. 6).

5. Concluding remarks

Thin layer immobilization brings essential benefits for improving H_2 photoproduction in green algae and cyanobacteria; the advantages include uniform light distribution, ease of media exchange, and high volumetric cell density, which prevent cell division and allow more efficient conversion of light energy to H_2 . However, currently alginate-based biofilms suffer from low porosity of the immobilization matrix, which leads to over-accumulation of O_2 inside the films that causes oxidative damage in the photosynthesizing cells. Although this property can be utilized for the production of hydroxylated carotenoids and other antioxidants, future research efforts relevant to H_2 -photoproduction should be directed toward screening for better cell immobilization materials that will have improved porosity properties and adequate mechanical stability.

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Authors contributions

Conception and design of the study: S.K., Y.A.; collection, analysis and interpretation of the experimental data: S.K.; determination of carotenoids: G.M.; drafting of the manuscript: SK; critical revision of the manuscript: MS, YA; final approval of the manuscript: S.K., Y.A.; obtaining of funding: S.K., Y.A.

Conflicts of interest

The authors declare that they have no conflict of interest.

Statement of informed consent, human/animal rights

No conflicts, informed consent, human or animal rights applicable.

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