Algal Research 28 (2017) 253–263

Contents lists available at ScienceDirect

Algal Research

journal homepage: www.elsevier.com/locate/algal

Evaluation of light energy to H_2 energy conversion efficiency in thin films of cyanobacteria and green alga under photoautotrophic conditions

Sergey Kosourov[a], Gayathri Murukesan[a], Michael Seibert[b], Yagut Allahverdiyeva[a,*]

[a] Molecular Plant Biology, Department of Biochemistry, University of Turku, Turku, FI 20014, Finland
[b] National Renewable Energy Laboratory, 15013 Denver West Parkway, Golden, CO 80401, USA

ARTICLE INFO

Keywords:
Hydrogen photoproduction
Thin-layer immobilization
Microalgae
Cyanobacteria
Oxidative stress
Carotenoids

ABSTRACT

Cyanobacteria and green algae harness solar energy to split water and to fix CO_2. Under specific conditions, they are capable of photoproduction of molecular hydrogen (H_2). This study compares the light-energy-to-hydrogen-energy conversion efficiency (LHCE) in two heterocystous, N_2-fixing cyanobacteria (wild-type *Calothrix* sp. strain 336/3 and the Δ*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 Ca^{2+}-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 Δ*hupL* strain under low light condition (2.9 W m^{-2}). *Calothrix* sp. 336/3 films produced H_2 with a maximum efficiency of 0.6% under 2.9 W m^{-2}, while *C. reinhardtii* films produced H_2 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 H_2 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 (H_2) 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 H_2 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 H_2 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-O_2-generating photofermentation pathway seems to play a significant role in H_2 evolution [10,11]. The process is not very efficient, but lasts for up to one month [11]. H_2 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 O_2 coevolved in PSII [12]. Depending on the light intensity and respiratory activity of the cells, H_2 evolution in dark-adapted algae lasts from few seconds [13] to several hours [10].

Sustained H_2 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, O_2 produced by the residual PSII is metabolized sufficiently by respiration of acetate in the medium, protecting [Fe-Fe]-hydrogenase from inactivation. However, H_2 evolution in nutrient-deprived algae proceeds at reduced rates, resulting in low LHCEs in

* Corresponding author.
E-mail address: allahve@utu.fi (Y. Allahverdiyeva).

http://dx.doi.org/10.1016/j.algal.2017.09.027
Received 24 April 2017; Received in revised form 28 September 2017; Accepted 28 September 2017
Available online 14 October 2017

suspension cultures [18]. On the other hand, H_2 photoproduction under autotrophic conditions is difficult to sustain [19]. Nevertheless, autotrophic algae could generate a substantial amount of H_2 if proper light regime is applied [20,21].

In contrast to green algae, photoautotrophy is not a problem for sustained H_2 photoproduction in N_2-fixing heterocystous cyanobacteria that produce H_2 mainly under diazotrophic conditions as an obligatory by-product of N_2 fixation [22,23]. In these species, oxygenic photosynthesis and N_2 fixation are spatially separated in different cells. While oxygenic photosynthesis occurs in vegetative cells, N_2 fixation is restricted to heterocysts, specialized cells with specific morphology and metabolism that provides a microoxic environment for the O_2-sensitive nitrogenase enzyme [24]. This environment within the heterocysts is maintained by an elevated level of respiration and restricted diffusion of O_2 via an unique cell wall envelope [22,23]. At the same time, the vegetative cells supply heterocysts with the energy needed for N_2 fixation, mainly in the form of sucrose [25,26]. Long-term H_2 photoproduction in N_2-fixing heterocystous cyanobacteria requires elimination of both combined nitrogen from the media and atmospheric N_2, deletion of uptake hydrogenase in heterocysts and the presence of CO_2 in atmosphere [27–29]. However, N-depletion, especially in the excess of CO_2, 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 H_2 production in the heterocysts [30,31]. Thus, analogous to green algae, H_2 photoproduction in N_2-fixing heterocystous cyanobacteria is the effect of a stress condition (-N).

Imbalance in photosynthetic activity and cell metabolism under stress conditions, which leads to H_2 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 H_2 evolution and, therefore, have different acclimation strategies to oxidative stress under H_2 photoproduction conditions. To our knowledge, a comparative investigation of H_2 photoproduction activities in these two types of organisms under the same conditions has never been reported before.

In the present study, we compare H_2 photoproduction under different light intensities in two heterocystous N_2-fixing cyanobacteria (*Calothrix* sp. 336/3 and the Δ*hupL* mutant of *Anabaena* sp. PCC 7120) and in S-deprived green alga (*Chlamydomonas reinhardtii*) entrapped in thin Ca^{2+}-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 H_2 photoproduction, and simplifies estimation of LHCEs.

2. Materials and methods

2.1. Strains and growth conditions

Two filamentous heterocystous cyanobacteria (the Δ*hupL* 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 Δ*hupL* mutant of *Anabaena* sp. PCC 7120 is deficient in the large subunit of the uptake hydrogenase [28] and is thus affected in H_2 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 H_2 producers [33]. The wild-type *C. reinhardtii* CC-124 (mt−, nit−), which is a common model organism for studying photobiological H_2 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 diazotropic conditions in the similar flasks containing 50 mL of Z8x medium [35]. The medium for growing stock cultures of Δ*hupL* 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 Δ*hupL* 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 H_2 photoproduction experiments

H_2 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 Ca^{2+}-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 $CaCl_2$ 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 H_2 photoproduction, 6% and 3% CO_2 was added into the headspaces of vials with cyanobacterial films and *C. reinhardtii* films, respectively. The addition of CO_2 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 CO_2 gas. For *C. reinhardtii* films, the gas phase in the vials was replaced with Ar containing 3% CO_2 every day during the first three days. For cyanobacteria, the headspace was replaced with Ar containing 6% CO_2 on every fourth day throughout the whole experiment (~260 h). In this procedure, the vials were sparged with argon for 20 min, and pure CO_2

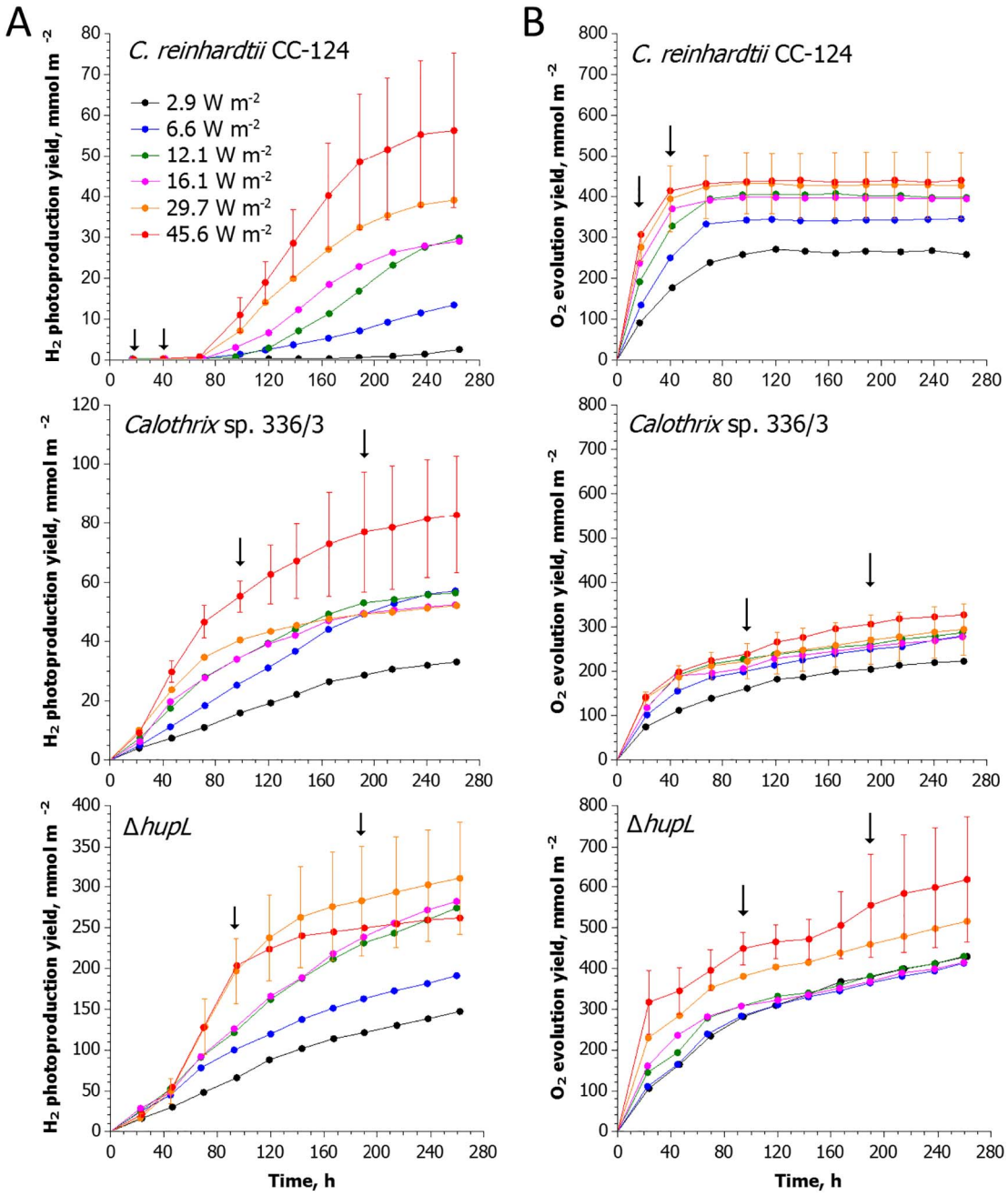

Fig. 1. The effect of light intensity on (A) H_2 photoproduction and (B) net O_2 evolution yields by *C. reinhardtii* and two cyanobacterial strains entrapped in Ca^{2+}-alginate films. The arrows indicate the points when the gas phase in the headspace of the vials was renewed with Ar supplemented with 3% (alga) and 6% (cyanobacteria) CO_2. The curves show cumulative H_2 and O_2 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 CO_2 supplementation (for alga and cyanobacteria) were selected based on our previous experimental data [20,30].

The H_2 and O_2 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 H_2 and O_2 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 μmol photons averaged over 400–700 nm.

For precise estimation of LHCEs in the potentially reversible process, the changes in the partial pressure of H_2 gas in the experimental vials were taken into consideration [38], and LHCEs were calculated using Eq. (1) [39]:

$$\eta(\%) = 100\,\frac{\left(\Delta G^{o} - RT\ln\left({}^{P^{o}}/_{P}\right)\right)R_H}{E_S A}, \tag{1}$$

where ΔG^{o} is the change of the standard Gibb's free energy for the

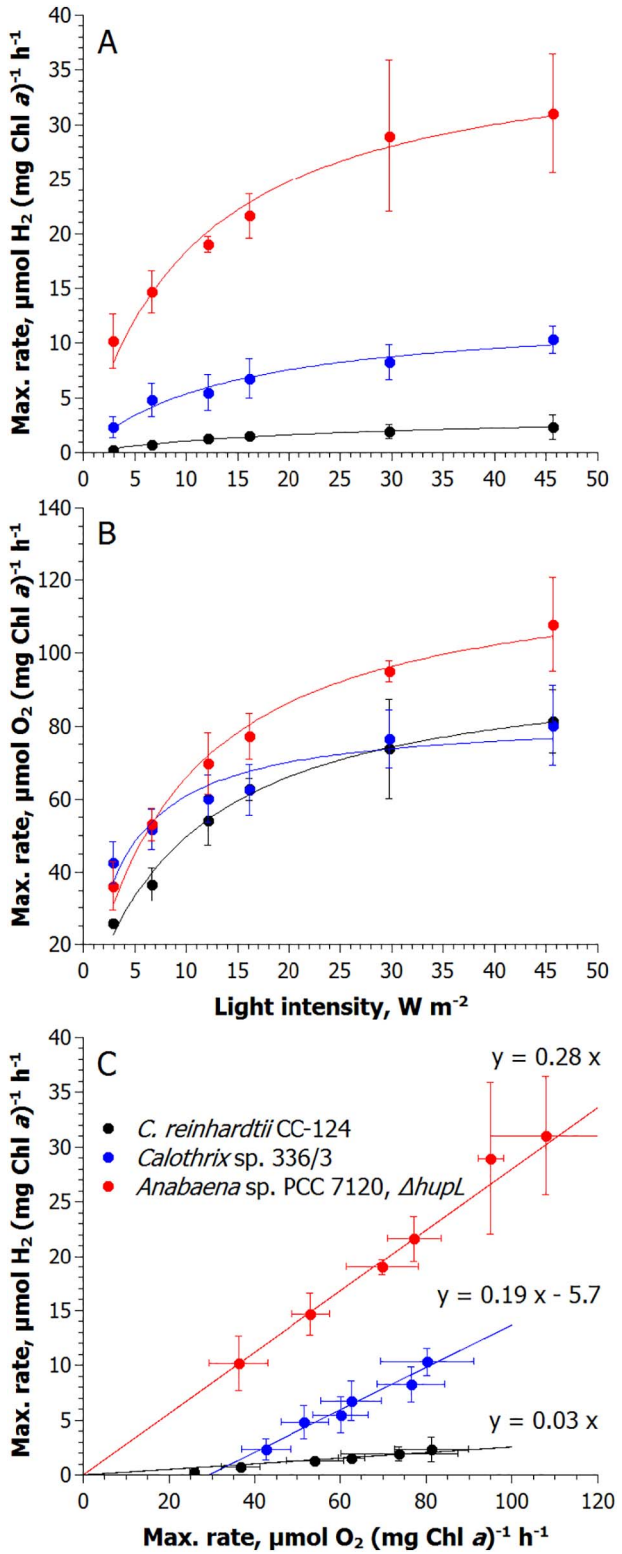

Fig. 2. The effect of light intensity on the maximum rate of (A) H_2 production and (B) net O_2 evolution. The rates were derived by the differentiation of the kinetic curves presented in Fig. 1. The panel (C) shows the correlation between the two rates. Each experimental point represents 3–12 replicates, 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 J mol^{-1} at 25 °C and 1 atm), R is the universal gas constant, T is the absolute temperature, P^o and P are the standard and observed H_2 pressures (atm), R_H is the rate of H_2 photoproduction (mol s^{-1}), E_S is the energy of the incident light radiation (J m^{-2} 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 Ca^{2+}-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 Ca^{2+}-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 of the pigments were extracted. The extracts were combined, centrifuged, and filtered through a 0.2-µm 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 × 125 mm, 5 µm, 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 (~260 h), the Ca^{2+}-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 Protean) 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 H_2 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 H_2 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 H_2 photoproduction activity

Two cyanobacterial strains and S-deprived *C. reinhardtii* were entrapped in thin Ca^{2+}-alginate films and transferred to an Ar + CO_2 atmosphere to initiate H_2 production. As shown in Fig. 1 A, increasing the light intensity applied to the surface of the Ca^{2+}-alginate films with entrapped cells improved the rates and yields of H_2 photoproduction in *C. reinhardtii* and both cyanobacterial strains. The most significant effect of light on the H_2 photoproduction yield was observed in *C. reinhardtii* films, where an increase from 2.9 to 45.6 W m^{-2} PAR increased the H_2 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 H_2 than the films with entrapped cyanobacterial strains exposed to the similar light intensities. It should be noted that S-deprived *C. reinhardtii* produces H_2 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 H_2 than the Δ*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 H_2 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 H_2 than films with entrapped Δ*hupL* mutant cells placed under the lowest light level. The Δ*hupL* films yielded 147 mmol H_2 m^{-2} after 260 h under 2.9 W m^{-2} PAR (Fig. 1 A). In contrast to *Calothrix* sp. 336/3, the Δ*hupL* films produced the highest H_2 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 H_2 photoproduction yield in this mutant especially after 96 h. As a result, the Δ*hupL* films placed under 12.1 and 16.1 W m^{-2} PAR yielded the same amount of H_2 as the films placed under 45.6 W m^{-2} light (~260 mmol H_2 m^{-2}).

The maximum specific H_2 photoproduction rate, which shows the maximum capacity of the selected strain to produce H_2 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 H_2 production (*Pmax* H_2) of about 38, 13 and 3.6 μmol H_2 (mg Chl *a*)$^{-1}$ h^{-1} and the half-saturation constant (*Ki* H_2) of about 10.8, 14 and 24.2 W m^{-2} for the *Anabaena* sp. PCC 7120 Δ*hupL*, *Calothrix* 336/3, and *C. reinhardtii* films, respectively.

3.2. Effect of light intensity on net O_2 evolution

All three alginate-entrapped strains demonstrated higher accumulation of O_2 in the head-space of the vials during the first 24 h. Later, the rates of net O_2 production declined in all strains (Fig. 1 B). S-deprived *C. reinhardtii* stopped producing O_2 within ~72 h, while both cyanobacterial strains continued evolving O_2 throughout the experiment, but at substantially lower rates than during the first 24 h. *C. reinhardtii* films only photoproduced H_2 under the anaerobic environment established after about 72 h of S-deprivation. In contrast, H_2 photoproduction (released in the anaerobic environment of the heterocysts) in the cyanobacterial films occurred simultaneously with the release of O_2 to the vial headspaces.

Similar to H_2 photoproduction, the maximum rate of net O_2 evolution in all three strains depended greatly on the light intensity (Fig. 2 B). The lowest half-saturation constant (*Ki* O_2) of ~3.6 W m^{-2} was obtained for *Calothrix* sp. 336/3. This strain also showed the lowest maximum theoretical rate of net O_2 evolution (*Pmax* O_2), which was around 83 μmol O_2 (mg Chl *a*)$^{-1}$ h^{-1}. The *Ki* O_2 constants were very close in the Δ*hupL* mutant (9 W m^{-2}) and in *C. reinhardtii* (9.8 W m^{-2}), but the *Pmax* O_2 was higher in the Δ*hupL* strain: 125 compared to 99 μmol O_2 (mg Chl *a*)$^{-1}$ h^{-1}.

3.3. Comparison of H_2 photoproduction and net O_2 evolution activities

As expected for the water biophotolysis process, all three strains showed a positive correlation between the maximum rate of H_2 photoproduction and the maximum rate of net O_2 evolution (Fig. 2 C). Over the range of light intensities studied, the dependence was linear in all three strains with H_2/O_2 ratios of around 0.03, 0.19 and 0.28 for *C. reinhardtii*, *Calothrix* sp. 336/3, and the Δ*hupL* films, respectively.

For the whole H_2 photoproduction period, the H_2/O_2 ratio varied depending on the strain and the light intensity applied to the film. In *C. reinhardtii* films, the final H_2/O_2 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 H_2/O_2 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 Δ*hupL* films placed under moderate light (16.1 W m^{-2}) showed the highest H_2/O_2 ratio (0.68). This value is about 34% of the theoretical maximum for water biophotolysis, which is 2 mol H_2 per 1 mol O_2 produced. An increase or a decrease in the light intensity caused a decline in the final H_2/O_2 ratio in the Δ*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-H_2 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 H_2 photoproduction) declined with increasing light intensity from 0.57 to 0.12% (*Calothrix* sp. 336/3 films) and from 2.5 to 0.5% (Δ*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 H_2 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 H_2 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 Δ*hupL* films.

3.5. Composition of carotenoids under different light intensities

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

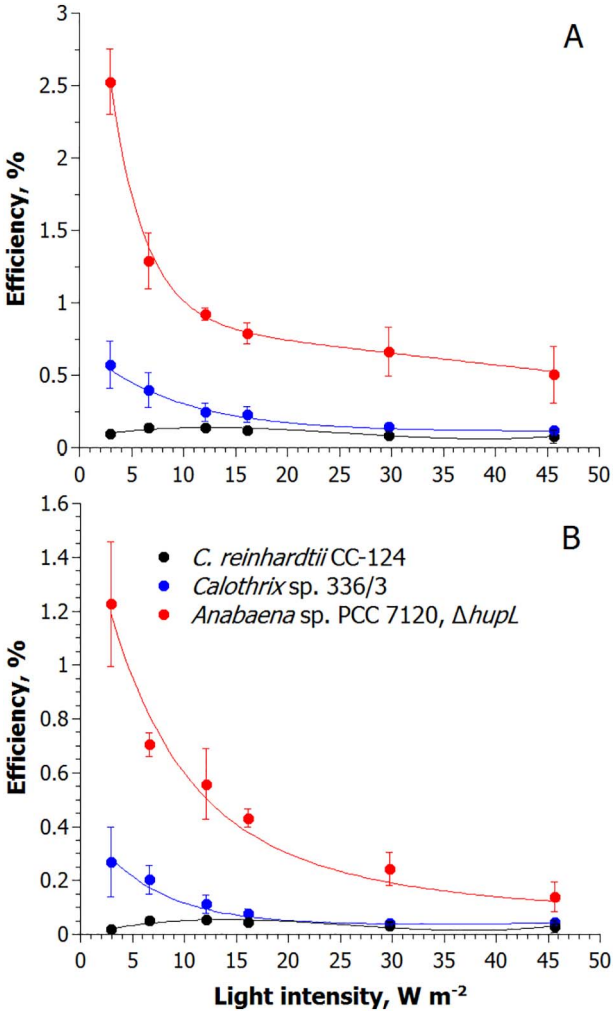

Fig. 3. The light-energy-to-H_2-energy conversion efficiencies exhibited by alginate-entrapped cultures of *C. reinhardtii* CC-124, *Calothrix* sp. 336/3, and the Δ*hupL* mutant. The efficiencies were calculated based on PAR (400–700 nm) during (A) the maximum rates of H_2 photoproduction (presented on Fig. 2 A) and (B) the whole period of H_2 photoproduction. Each experimental point represents 3–12 replicates, and the error bars are one SD.

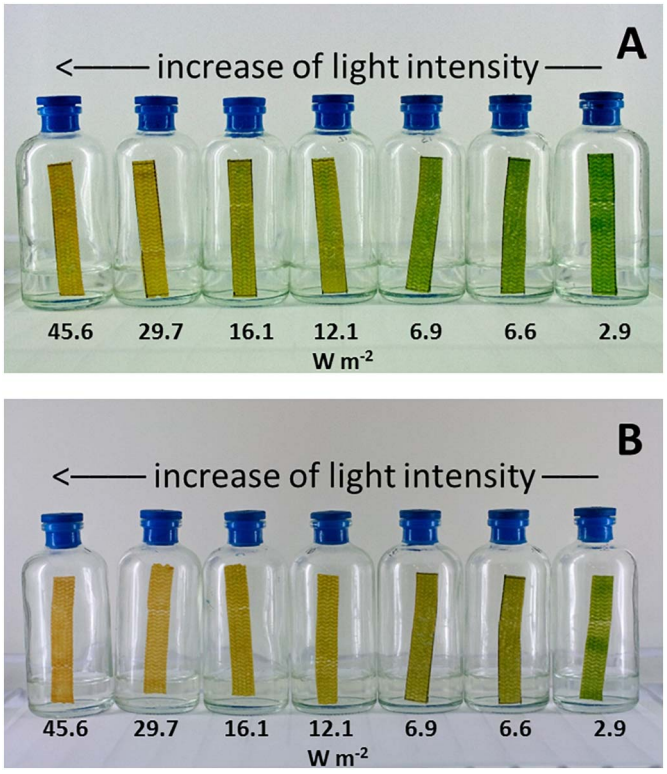

Fig. 4. The visual changes in the pigment composition of the *Anabaena* sp. PCC 7120 Δ*hupL* films placed under different light intensities (45.6–2.9 W m^{-2}) during a H_2 photoproduction experiment. The pictures were taken on 4th (A) and 11th (B) days after the start of the experiment. Similar, but less pronounced changes were observed in *Calothrix* sp. 336/3 and *C. reinhardtii* films. The picture is representative of 3 independent experiments.

excess light and CO_2, this limitation may cause enhanced O_2 accumulation inside the immobilization matrix due to the photosynthetic water-splitting activity of entrapped cells [31]. Over-accumulation of O_2 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 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).

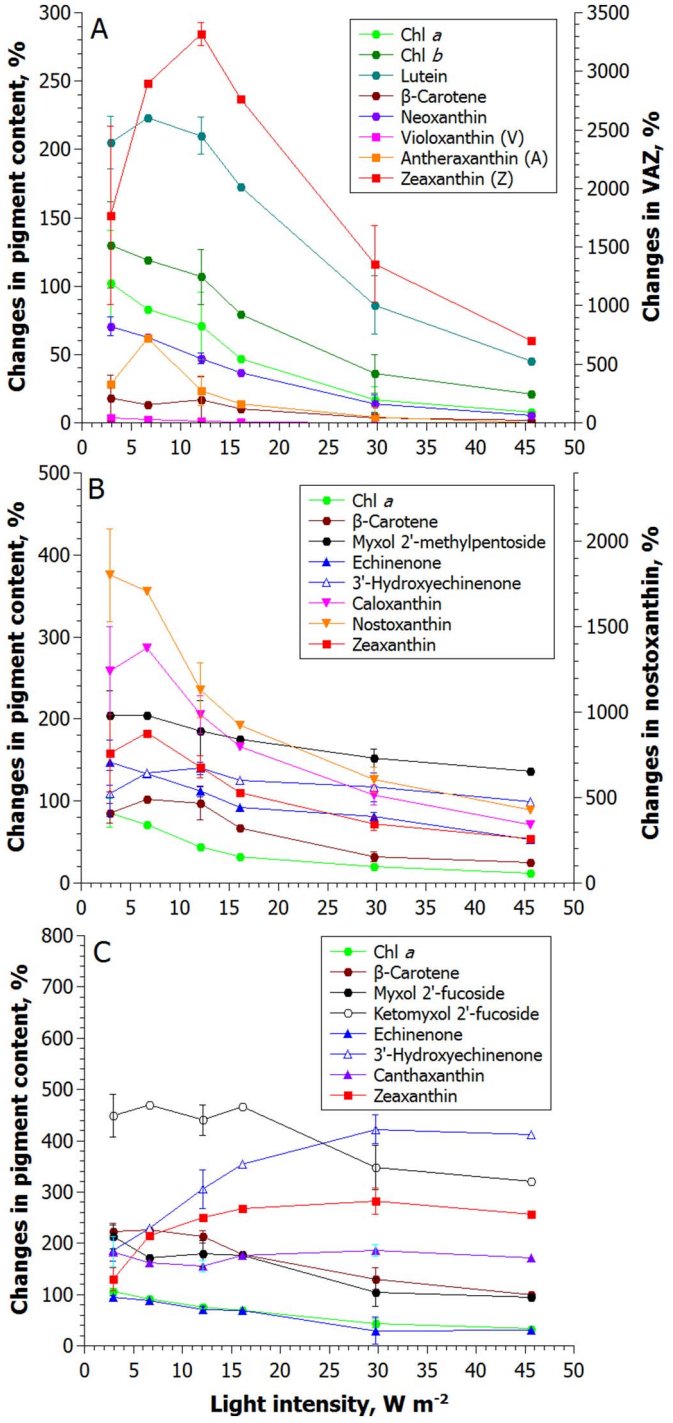

Fig. 5. Changes in pigment composition by the end of H_2 photoproduction. The pigment composition of (A) *C. reinhardtii* CC-124, (B) *Calothrix* sp. 336/3, and (C) Δ*hupL* mutant cells entrapped in alginate films were measured after ~260 h hours of H_2 photoproduction conditions at different light intensities. 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 Δ*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 (29.7–45.6 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. reinhadrtii*, 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. reinhadrtii* 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% 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 μM 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.

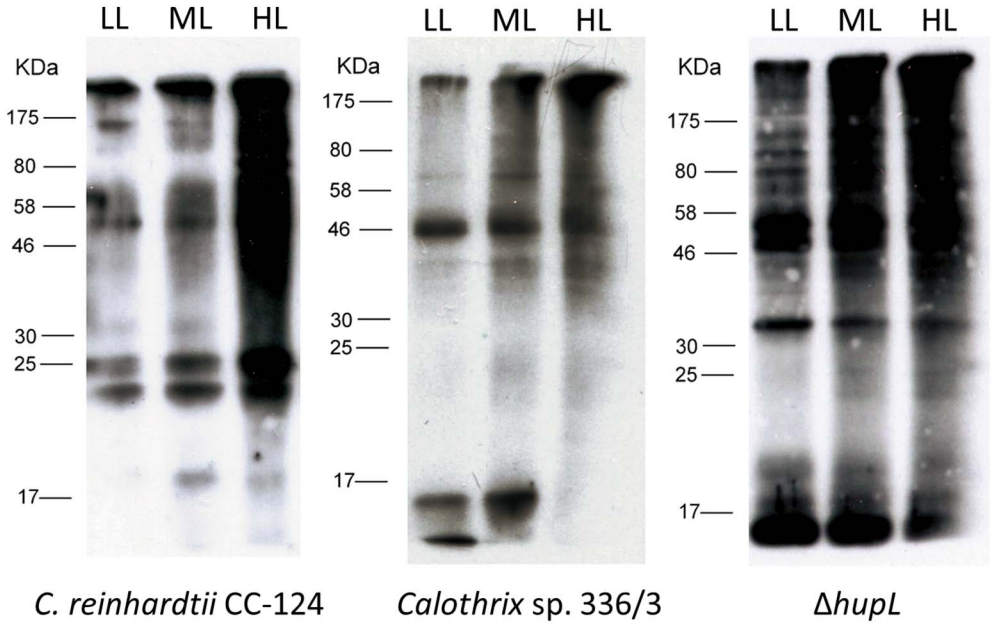

Fig. 6. Oxyblots of total proteins isolated from the algal and cyanobacterial films in the end of H_2 photoproduction experiments. LL – 2.9 W m^{-2}, ML – 16.1 W m^{-2}, and HL – 45.6 W m^{-2} light.

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 anaerobisis [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 h) in the start of H_2 photoproduction by algal films under sulfur-deprived, autotrophic conditions as compared to photoheterotrophic films (< 24 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 Δ*hupL* mutant of *Anabaena* sp. PCC 7120 produced H_2 with PAR efficiency of 2.52% (~1.1% if the total solar spectrum at ground level is considered) for at least 24 h and 1.2% (~0.5% solar) for 260 h. The LHCEs in S-deprived *C. reinhardtii* films under autotrophic conditions did not exceed 0.14% (~0.06% of sunlight). Nevertheless, the strain is capable of producing H_2 at approximately the same efficiency as the Δ*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 Δ*hupL* mutant of *Nostoc* sp. PCC 7422, a 3.7% efficiency was estimated under a light intensity of 70 μmol m^{-2} 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 Δ*hupW* mutant of *Nostoc* (*Anabaena*) sp. PCC 7120 under an incident light intensity of 44 μmol m^{-2} 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 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 Δ*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 alga 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.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.algal.2017.09.027.

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.

Acknowledgements

The authors acknowledge financial support from the NordForsk NCoE program “NordAqua” (project # 82845), the Kone Foundation, the Maj and Tor Nessling Foundation (# 201600294), and the Academy of Finland FCoE program (# 307335). MS acknowledges the NREL Emeritus Program.

References

[1] J.R. Bolton, D.O. Hall, The maximum efficiency of photosynthesis, Photochem. Photobiol. 53 (1991) 545–548, http://dx.doi.org/10.1111/j.1751-1097.1991.tb03668.x.
[2] M.L. Ghirardi, A. Dubini, J. Yu, P.-C. Maness, Photobiological hydrogen-producing systems, Chem. Soc. Rev. 38 (2009) 52–61, http://dx.doi.org/10.1039/b718939g.
[3] H. Sakurai, H. Masukawa, M. Kitashima, K. Inoue, How close we are to achieving commercially viable large-scale photobiological hydrogen production by cyanobacteria: a review of the biological aspects, Life 5 (2015) 997–1018, http://dx.doi.org/10.3390/life5010997.
[4] M.L. Ghirardi, S. Kosourov, P. Maness, S. Smolinski, M. Seibert, M.C. Flickinger, Algal hydrogen production, in: M.C. Flickinger (Ed.), Encycl. Ind. Biotechnol, John Wiley & Sons, Inc., 2010, pp. 184–198, , http://dx.doi.org/10.1002/

9780470054581.eib362.
[5] T.K. Antal, T.E. Krendeleva, E. Tyystjärvi, Multiple regulatory mechanisms in the chloroplast of green algae: relation to hydrogen production, Photosynth. Res. 125 (2015) 357–381, http://dx.doi.org/10.1007/s11120-015-0157-2.
[6] M. Seibert, P.W. King, M.C. Posewitz, A. Melis, M.L. Ghirardi, Photosynthetic water-splitting for hydrogen production, in: J. Wall, C. Harwood, A. Demain (Eds.), Bioenergy, American Society of Microbiology, Washington, DC, 2008, pp. 273–291, , http://dx.doi.org/10.1128/9781555815547.ch22.
[7] M. Winkler, A. Hemschemeier, J. Jacobs, S. Stripp, T. Happe, Multiple ferredoxin isoforms in *Chlamydomonas reinhardtii* - Their role under stress conditions and biotechnological implications, Eur. J. Cell Biol. 89 (2010) 998–1004, http://dx.doi.org/10.1016/j.ejcb.2010.06.018.
[8] S. Kosourov, M. Seibert, M.L. Ghirardi, Effects of extracellular pH on the metabolic pathways in sulfur-deprived, H_2-producing Chlamydomonas reinhardtii cultures, Plant Cell Physiol. 44 (2003) 146–155.
[9] F. Mus, A. Dubini, M. Seibert, M.C. Posewitz, A.R. Grossman, Anaerobic acclimation in *Chlamydomonas reinhardtii*, J. Biol. Chem. 282 (2007) 25475–25486, http://dx.doi.org/10.1074/jbc.M701415200.
[10] P.J. Aparicio, M.P. Azuara, A. Ballesteros, V.M. Fernández, Effects of light intensity and oxidized nitrogen sources on hydrogen production by *Chlamydomonas reinhardii*, Plant Physiol. 78 (1985) 803–806, http://dx.doi.org/10.1104/pp.78.4.803.
[11] A. Scoma, L. Durante, L. Bertin, F. Fava, Acclimation to hypoxia in *Chlamydomonas reinhardtii*: can biophotolysis be the major trigger for long-term H2 production? New Phytol. 204 (2014) 890–900, http://dx.doi.org/10.1111/nph.12964.
[12] M.L. Ghirardi, R.K. Togasaki, M. Seibert, Oxygen sensitivity of algal H_2- production, Appl. Biochem. Biotechnol. 63–65 (1997) 141–151, http://dx.doi.org/10.1007/BF02920420.
[13] T.S. Stuart, H. Gaffron, The gas exchange of hydrogen-adapted algae as followed by mass spectrometry, Plant Physiol. 50 (1972) 136–140, http://dx.doi.org/10.1104/pp.50.1.136.
[14] A. Melis, L. Zhang, M. Forestier, M.L. Ghirardi, M. Seibert, Sustained photobiological hydrogen gas production upon reversible inactivation of oxygen evolution in the green alga *Chlamydomonas reinhardtii*, Plant Physiol. 122 (2000) 127–136, http://dx.doi.org/10.1104/pp.122.1.127.
[15] K.A. Batyrova, A.A. Tsygankov, S.N. Kosourov, Sustained hydrogen photoproduction by phosphorus-deprived *Chlamydomonas reinhardtii* cultures, Int. J. Hydrog. Energy 37 (2012) 8834–8839, http://dx.doi.org/10.1016/j.ijhydene.2012.01.068.
[16] A. Volgusheva, G. Kukarskikh, T. Krendeleva, A. Rubin, F. Mamedov, Hydrogen photoproduction in green algae *Chlamydomonas reinhardtii* under magnesium deprivation, RSC Adv. 5 (2015) 5633–5637, http://dx.doi.org/10.1039/C4RA12710B.
[17] R. Surzycki, L. Cournac, G. Peltier, J.-D. Rochaix, Potential for hydrogen production with inducible chloroplast gene expression in *Chlamydomonas*, Proc. Natl. Acad. Sci. U. S. A. 104 (2007) 17548–17553, http://dx.doi.org/10.1073/pnas.0704205104.
[18] M.L. Ghirardi, Hydrogen production by photosynthetic green algae, Indian J. Biochem. Biophys. 43 (2006) 201–210.
[19] S. Fouchard, A. Hemschemeier, A. Caruana, J. Pruvost, J. Legrand, T. Happe, G. Peltier, L. Cournac, Autotrophic and mixotrophic hydrogen photoproduction in sulfur-deprived *Chlamydomonas* cells, Appl. Environ. Microbiol. 71 (2005) 6199–6205, http://dx.doi.org/10.1128/AEM.71.10.6199-6205.2005.
[20] A.A. Tsygankov, S.N. Kosourov, I.V. Tolstygina, M.L. Ghirardi, M. Seibert, Hydrogen production by sulfur-deprived *Chlamydomonas reinhardtii* under photoautotrophic conditions, Int. J. Hydrog. Energy 31 (2006) 1574–1584, http://dx.doi.org/10.1016/j.ijhydene.2006.06.024.
[21] I.V. Tolstygina, T.K. Antal, S.N. Kosourov, T.E. Krendeleva, A.B. Rubin, A.A. Tsygankov, Hydrogen production by photoautotrophic sulfur-deprived *Chlamydomonas reinhardtii* pre-grown and incubated under high light, Biotechnol. Bioeng. 102 (2009) 1055–1061, http://dx.doi.org/10.1002/bit.22148.
[22] A.A. Tsygankov, Nitrogen-fixing cyanobacteria: a review, Appl. Biochem. Microbiol. 43 (2007) 250–259, http://dx.doi.org/10.1134/S0003683807030040.
[23] H. Bothe, O. Schmitz, M.G. Yates, W.E. Newton, Nitrogen fixation and hydrogen metabolism in cyanobacteria, Microbiol. Mol. Biol. Rev. 74 (2010) 529–551, http://dx.doi.org/10.1128/MMBR.00033-10.
[24] P. Tamagnini, E. Leitão, P. Oliveira, D. Ferreira, F. Pinto, D.J. Harris, T. Heidorn, P. Lindblad, Cyanobacterial hydrogenases: diversity, regulation and applications, FEMS Microbiol. Rev. 31 (2007) 692–720, http://dx.doi.org/10.1111/j.1574-6976.2007.00085.x.
[25] L. Curatti, E. Flores, G. Salerno, Sucrose is involved in the diazotrophic metabolism of the heterocyst-forming cyanobacterium *Anabaena* sp. FEBS Lett. 513 (2002) 175–178.
[26] R. Lopez-Igual, E. Flores, A. Herrero, Inactivation of a heterocyst-specific invertase indicates a principal role of sucrose catabolism in heterocysts of *Anabaena* sp. J. Bacteriol. 192 (2010) 5526–5533, http://dx.doi.org/10.1128/JB.00776-10.
[27] A.A. Tsygankov, V.B. Borodin, K.K. Rao, D.O. Hall, H(2) photoproduction by batch culture of *Anabaena variabilis* ATCC 29413 and its mutant PK84 in a photobioreactor, Biotechnol. Bioeng. 64 (1999) 709–715.
[28] H. Masukawa, M. Mochimaru, H. Sakurai, Disruption of the uptake hydrogenase gene, but not of the bidirectional hydrogenase gene, leads to enhanced photobiological hydrogen production by the nitrogen-fixing cyanobacterium *Anabaena* sp. PCC 7120, Appl. Microbiol. Biotechnol. 58 (2002) 618–624, http://dx.doi.org/10.1007/s00253-002-0934-7.
[29] W. Khetkorn, P. Lindblad, A. Incharoensakdi, Inactivation of uptake hydrogenase leads to enhanced and sustained hydrogen production with high nitrogenase activity under high light exposure in the cyanobacterium *Anabaena siamensis* TISTR 8012, J. Biol. Eng. 6 (2012) 19, http://dx.doi.org/10.1186/1754-1611-6-19.
[30] H. Leino, S.N. Kosourov, L. Saari, K. Sivonen, A.A. Tsygankov, E.-M. Aro, Y. Allahverdiyeva, Extended H_2 photoproduction by N_2-fixing cyanobacteria immobilized in thin alginate films, Int. J. Hydrog. Energy 37 (2012) 151–161, http://dx.doi.org/10.1016/j.ijhydene.2011.09.088.
[31] S. Kosourov, H. Leino, G. Murukesan, F. Lynch, K. Sivonen, A.A. Tsygankov, E.-M. Aro, Y. Allahverdiyeva, Hydrogen photoproduction by immobilized N_2-fixing cyanobacteria: understanding the role of the uptake hydrogenase in the long-term process, Appl. Environ. Microbiol. 80 (2014) 5807–5817, http://dx.doi.org/10.1128/AEM.01776-14.
[32] K. Hakkila, T. Antal, A.U. Rehman, J. Kurkela, H. Wada, I. Vass, E. Tyystjärvi, T. Tyystjärvi, Oxidative stress and photoinhibition can be separated in the cyanobacterium *Synechocystis* sp. PCC 6803, Biochim. Biophys. Acta Bioenerg. 1837 (2014) 217–225, http://dx.doi.org/10.1016/j.bbabio.2013.11.011.
[33] Y. Allahverdiyeva, H. Leino, L. Saari, D.P. Fewer, S. Shunmugam, K. Sivonen, E.-M. Aro, Screening for biohydrogen production by cyanobacteria isolated from the Baltic Sea and Finnish lakes, Int. J. Hydrog. Energy 35 (2010) 1117–1127, http://dx.doi.org/10.1016/j.ijhydene.2009.12.030.
[34] E.H. Harris, The Chlamydomonas sourcebook: a comprehensive guide to biology and laboratory use, Academic Press, San Diego, 1989.
[35] J. Kotai, Instructions for Preparation of Modified, Nutrient Solution Z8 for Algae. Publication B-11/69, Norwegian Institute for Water Research, Oslo, 1972.
[36] S.N. Kosourov, M. Seibert, Hydrogen photoproduction by nutrient-deprived *Chlamydomonas reinhardtii* cells immobilized within thin alginate films under aerobic and anaerobic conditions, Biotechnol. Bioeng. 102 (2009) 50–58, http://dx.doi.org/10.1002/bit.22050.
[37] D.D. Wykoff, J.P. Davies, A. Melis, A.R. Grossman, The regulation of photosynthetic electron transport during nutrient deprivation in *Chlamydomonas reinhardtii*, Plant Physiol. 117 (1998) 129–139, http://dx.doi.org/10.1104/pp.117.1.129.
[38] S.N. Kosourov, K.A. Batyrova, E.P. Petushkova, A.A. Tsygankov, M.L. Ghirardi, M. Seibert, Maximizing the hydrogen photoproduction yields in *Chlamydomonas reinhardtii* cultures: the effect of the H_2 partial pressure, Int. J. Hydrog. Energy 37 (2012) 8850–8858, http://dx.doi.org/10.1016/j.ijhydene.2012.01.082.
[39] J.R. Bolton, Solar photoproduction of hydrogen, IEA technical report of the IEA agreement of the production and utilization of hydrogen, Oak Ridge, TN, 1996, http://dx.doi.org/10.2172/776257.
[40] H.K. Lichtenthaler, Chlorophylls and carotenoids: pigments of photosynthetic biomembranes, Methods Enzymol. 148 (1987) 350–382.
[41] M. Pollari, S. Rantamäki, T. Huokko, A. Kårlund-Marttila, V. Virjamo, E. Tyystjärvi, T. Tyystjärvi, Effects of deficiency and overdose of group 2 sigma factors in triple inactivation strains of *Synechocystis* sp. strain PCC 6803, J. Bacteriol. 193 (2011) 265–273, http://dx.doi.org/10.1128/JB.01045-10.
[42] S. Kosourov, E. Patrusheva, M.L. Ghirardi, M. Seibert, A. Tsygankov, A comparison of hydrogen photoproduction by sulfur-deprived *Chlamydomonas reinhardtii* under different growth conditions, J. Biotechnol. 128 (2007) 776–787, http://dx.doi.org/10.1016/j.jbiotec.2006.12.025.
[43] H. Leino, S. Shunmugam, J. Isojärvi, P. Oliveira, P. Mulo, L. Saari, N. Battchikova, K. Sivonen, P. Lindblad, E.-M. Aro, Y. Allahverdiyeva, Characterization of ten H_2 producing cyanobacteria isolated from the Baltic Sea and Finnish lakes, Int. J. Hydrog. Energy 39 (2014) 8983–8991, http://dx.doi.org/10.1016/j.ijhydene.2014.03.171.
[44] D.J. Hassett, Anaerobic production of alginate by *Pseudomonas aeruginosa*: alginate restricts diffusion of oxygen, J. Bacteriol. 178 (1996) 7322–7325, http://dx.doi.org/10.1128/JB.178.24.7322-7325.1996.
[45] W. Sabra, A.P. Zeng, H. Lünsdorf, W.D. Deckwer, Effect of oxygen on formation and structure of *Azotobacter vinelandii* alginate and its role in protecting nitrogenase, Appl. Environ. Microbiol. 66 (2000) 4037–4044, http://dx.doi.org/10.1128/AEM.66.9.4037-4044.2000.
[46] L. Zhang, T. Happe, A. Melis, Biochemical and morphological characterization of sulfur-deprived and H_2-producing *Chlamydomonas reinhardtii* (green alga), Planta 214 (2002) 552–561, http://dx.doi.org/10.1007/s004250100660.
[47] S. Kosourov, G. Murukesan, J. Jokela, Y. Allahverdiyeva, Carotenoid biosynthesis in *Calothrix* sp. 336/3: composition of carotenoids on full medium, during diazotrophic growth and after long-term H_2 photoproduction, Plant Cell Physiol. 57 (2016) 2269–2282, http://dx.doi.org/10.1093/pcp/pcw143.
[48] M.L. Ghirardi, L. Zhang, J.W. Lee, T. Flynn, M. Seibert, E. Greenbaum, A. Melis, Microalgae: a green source of renewable H(2), Trends Biotechnol. 18 (2000) 506–511.
[49] S. Kosourov, A. Tsygankov, M. Seibert, M.L. Ghirardi, Sustained hydrogen photoproduction by *Chlamydomonas reinhardtii*: effects of culture parameters, Biotechnol. Bioeng. 78 (2002) 731–740, http://dx.doi.org/10.1002/bit.10254.
[50] T. Laurinavichene, A. Fedorov, M. Ghirardi, M. Seibert, A. Tsygankov, Demonstration of sustained hydrogen photoproduction by immobilized, sulfur-deprived *Chlamydomonas reinhardtii* cells, Int. J. Hydrog. Energy 31 (2006) 659–667, http://dx.doi.org/10.1016/j.ijhydene.2005.05.002.
[51] W. Song, N. Rashid, W. Choi, K. Lee, Biohydrogen production by immobilized *Chlorella* sp. using cycles of oxygenic photosynthesis and anaerobiosis, Bioresour. Technol. 102 (2011) 8676–8681, http://dx.doi.org/10.1016/j.biortech.2011.02.082.
[52] T.K. Antal, D.N. Matorin, G.P. Kukarskikh, M.D. Lambreva, E. Tyystjärvi, T.E. Krendeleva, A.A. Tsygankov, A.B. Rubin, Pathways of hydrogen photoproduction by immobilized *Chlamydomonas reinhardtii* cells deprived of sulfur, Int. J. Hydrog. Energy 39 (2014) 18194–18203, http://dx.doi.org/10.1016/j.ijhydene.2014.08.135.
[53] A. Tsygankov, L. Serebryakova, K. Rao, D. Hall, Acetylene reduction and hydrogen photoproduction by wild-type and mutant strains of *Anabaena* at different CO_2 and O_2 concentrations, FEMS Microbiol. Lett. 167 (1998) 13–17, http://dx.doi.org/10.

1111/j.1574-6968.1998.tb13201.x.
[54] A. Valladares, A. Herrero, D. Pils, G. Schmetterer, E. Flores, Cytochrome c oxidase genes required for nitrogenase activity and diazotrophic growth in *Anabaena* sp. PCC 7120, Mol. Microbiol. 47 (2003) 1239–1249.
[55] A. Valladares, I. Maldener, A.M. Muro-Pastor, E. Flores, A. Herrero, Heterocyst development and diazotrophic metabolism in terminal respiratory oxidase mutants of the cyanobacterium *Anabaena* sp. strain PCC 7120, J. Bacteriol. 189 (2007) 4425–4430, http://dx.doi.org/10.1128/JB.00220-07.
[56] M. Ekman, S.Y. Ow, M. Holmqvist, X. Zhang, J. van Wagenen, P.C. Wright, K. Stensjö, Metabolic adaptations in a H_2 producing heterocyst-forming cyanobacterium: potentials and implications for biological engineering, J. Proteome Res. 10 (2011) 1772–1784, http://dx.doi.org/10.1021/pr101055v.
[57] M. Ermakova, N. Battchikova, Y. Allahverdiyeva, E.-M. Aro, Novel heterocyst-specific flavodiiron proteins in *Anabaena* sp. PCC 7120, FEBS Lett. 587 (2013) 82–87, http://dx.doi.org/10.1016/j.febslet.2012.11.006.
[58] M. Ermakova, N. Battchikova, P. Richaud, H. Leino, S. Kosourov, J. Isojärvi, G. Peltier, E. Flores, L. Cournac, Y. Allahverdiyeva, E.-M. Aro, Heterocyst-specific flavodiiron protein Flv3B enables oxic diazotrophic growth of the filamentous cyanobacterium *Anabaena* sp. PCC 7120, Proc. Natl. Acad. Sci. U. S. A. 111 (2014) 11205–11210, http://dx.doi.org/10.1073/pnas.1407327111.
[59] A. Hemschemeier, S. Fouchard, L. Cournac, G. Peltier, T. Happe, Hydrogen production by *Chlamydomonas reinhardtii*: an elaborate interplay of electron sources and sinks, Planta 227 (2008) 397–407, http://dx.doi.org/10.1007/s00425-007-0626-8.
[60] T.K. Antal, T.E. Krendeleva, T.V. Laurinavichene, V.V. Makarova, A.A. Tsygankov, M. Seibert, A.B. Rubin, The relationship between the photosystem 2 activity and hydrogen production in sulfur deprived *Chlamydomonas reinhardtii* cells, Dokl. Biochem. Biophys. 381 (2001) 371–375.
[61] L. Zhang, A. Melis, Probing green algal hydrogen production, Philos. Trans. R. Soc. Lond. B. Biol. Sci. 357 (2002) 1499–1509, http://dx.doi.org/10.1098/rstb.1152.
[62] T. Laurinavichene, I. Tolstygina, A. Tsygankov, The effect of light intensity on hydrogen production by sulfur-deprived *Chlamydomonas reinhardtii*, J. Biotechnol. 114 (2004) 143–151, http://dx.doi.org/10.1016/j.jbiotec.2004.05.012.
[63] V. Chochois, D. Dauvillée, A. Beyly, D. Tolleter, S. Cuiné, H. Timpano, S. Ball, L. Cournac, G. Peltier, Hydrogen production in *Chlamydomonas*: photosystem II-dependent and -independent pathways differ in their requirement for starch metabolism, Plant Physiol. 151 (2009) 631–640, http://dx.doi.org/10.1104/pp.109.144576.
[64] A. Volgusheva, S. Styring, F. Mamedov, Increased photosystem II stability promotes H_2 production in sulfur-deprived *Chlamydomonas reinhardtii*, Proc. Natl. Acad. Sci. U. S. A. 110 (2013) 7223–7228, http://dx.doi.org/10.1073/pnas.1220645110.
[65] F. Yoshino, H. Ikeda, H. Masukawa, H. Sakurai, High photobiological hydrogen production activity of a *Nostoc* sp. PCC 7422 uptake hydrogenase-deficient mutant with high nitrogenase activity, Mar. Biotechnol. 9 (2007) 101–112, http://dx.doi.org/10.1007/s10126-006-6035-3.
[66] M. Nyberg, T. Heidorn, P. Lindblad, Hydrogen production by the engineered cyanobacterial strain Nostoc PCC 7120 Δ*hupW* examined in a flat panel photobioreactor system, J. Biotechnol. 215 (2015) 35–43, http://dx.doi.org/10.1016/j.jbiotec.2015.08.028.
[67] S.A. Markov, P.F. Weaver, M. Seibert, Spiral tubular bioreactors for hydrogen production by photosynthetic microorganisms: design and operation, Appl. Biochem. Biotechnol. 63–65 (1997) 577–584, http://dx.doi.org/10.1007/BF02920455.
[68] J. Liu, V. Bukatin, A. Tsygankov, Light energy conversion into H_2 by *Anabaena variabilis* mutant PK84 dense cultures exposed to nitrogen limitations, Int. J. Hydrog. Energy 31 (2006) 1591–1596, http://dx.doi.org/10.1016/j.ijhydene.2006.06.025.
[69] H. Berberoğlu, J. Jay, L. Pilon, Effect of nutrient media on photobiological hydrogen production by *Anabaena variabilis* ATCC 29413, Int. J. Hydrog. Energy 33 (2008) 1172–1184, http://dx.doi.org/10.1016/j.ijhydene.2007.12.036.
[70] A.A. Tsygankov, A.S. Fedorov, S.N. Kosourov, K.K. Rao, Hydrogen production by cyanobacteria in an automated outdoor photobioreactor under aerobic conditions, Biotechnol. Bioeng. 80 (2002) 777–783, http://dx.doi.org/10.1002/bit.10431.
[71] A. Tsygankov, S. Kosourov, Immobilization of photosynthetic microorganisms for efficient hydrogen production, in: D. Zannoni, R. De Philippis (Eds.), Microb. BioEnergy Hydrog. Prod, Springer Netherlands, Dordrecht, 2014, pp. 321–347, , http://dx.doi.org/10.1007/978-94-017-8554-9_14.
[72] M.E. Sáenz, K. Bišová, E. Touloupakis, C. Faraloni, W.D. Di Marzio, G. Torzillo, Evidences of oxidative stress during hydrogen photoproduction in sulfur-deprived cultures of *Chlamydomonas reinhardtii*, Int. J. Hydrog. Energy 40 (2015) 10410–10417, http://dx.doi.org/10.1016/j.ijhydene.2015.06.124.
[73] H.P. McNulty, J. Byun, S.F. Lockwood, R.F. Jacob, R.P. Mason, Differential effects of carotenoids on lipid peroxidation due to membrane interactions: X-ray diffraction analysis, Biochim. Biophys. Acta 1768 (2007) 167–174, http://dx.doi.org/10.1016/j.bbamem.2006.09.010.
[74] Y. Kusama, S. Inoue, H. Jimbo, S. Takaichi, K. Sonoike, Y. Hihara, Y. Nishiyama, Zeaxanthin and echinenone protect the repair of photosystem II from inhibition by singlet oxygen in *Synechocystis* sp. PCC 6803, Plant Cell Physiol. 56 (2015) 906–916, http://dx.doi.org/10.1093/pcp/pcv018.
[75] B. Demmig-Adams, Linking the xanthophyll cycle with thermal energy dissipation, Discov. Photosynth, Springer-Verlag, Berlin/Heidelberg, 2003, pp. 923–930, , http://dx.doi.org/10.1007/1-4020-3324-9_83.
[76] T.K. Antal, A.A. Volgusheva, G.P. Kukarskih, A.A. Bulychev, T.E. Krendeleva, A.B. Rubin, Effects of sulfur limitation on photosystem II functioning in *Chlamydomonas reinhardtii* as probed by chlorophyll *a* fluorescence, Physiol. Plant. 128 (2006) 360–367, http://dx.doi.org/10.1111/j.1399-3054.2006.00734.x.
[77] A. Scoma, L. Giannelli, C. Faraloni, G. Torzillo, Outdoor H_2 production in a 50-L tubular photobioreactor by means of a sulfur-deprived culture of the microalga *Chlamydomonas reinhardtii*, J. Biotechnol. 157 (2012) 620–627, http://dx.doi.org/10.1016/j.jbiotec.2011.06.040.
[78] C. Faraloni, G. Torzillo, Xanthophyll cycle induction by anaerobic conditions under low light in *Chlamydomonas reinhardtii*, J. Appl. Phycol. 25 (2013) 1457–1471, http://dx.doi.org/10.1007/s10811-013-9986-6.
[79] A. Wilson, C. Punginelli, A. Gall, C. Bonetti, M. Alexandre, J.-M. Routaboul, C.A. Kerfeld, R. van Grondelle, B. Robert, J.T.M. Kennis, D. Kirilovsky, A photoactive carotenoid protein acting as light intensity sensor, Proc. Natl. Acad. Sci. U. S. A. 105 (2008) 12075–12080, http://dx.doi.org/10.1073/pnas.0804636105.
[80] R. López-Igual, A. Wilson, R.L. Leverenz, M.R. Melnicki, C. Bourcier de Carbon, M. Sutter, A. Turmo, F. Perreau, C.A. Kerfeld, D. Kirilovsky, Different functions of the paralogs to the N-terminal domain of the orange carotenoid protein in the cyanobacterium *Anabaena* sp. PCC 7120, Plant Physiol. 171 (2016) 1852–1866, http://dx.doi.org/10.1104/pp.16.00502.