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Factors influencing algal photobiohydrogen production in algal-bacterial co-cultures

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ABSTRACT

Algal-bacterial co-cultures represent an alternative way for algal biohydrogen generation. Efficient algal hydrogen production requires anaerobiosis and electrons accessible for the algal FeFe-hydrogenases. A number of factors strongly influence the development of this optimal environment. Various algal strains were tested for hydrogen evolution with a selected bacterial partner, a fully hydrogenase deficient *Escherichia coli*. During the hunt for the most efficient algae strains, gas-to-liquid phase ratio, algal optical density and algal cell size were identified as crucial factors influencing algal hydrogen evolution rate, accumulated algal hydrogen yield, carbon dioxide and oxygen levels as well as acetic acid consumption in illuminated algal-bacterial cultures. The highest accumulated hydrogen yields were observed for the different algal partners under similar experimental setup. The combination of a gas-to-liquid phase ratio of 1/1 with an algae cell density of $3.96 * 10^8$ algae cell ml^{-1} (OD_{750}: 1) resulted in the highest accumulated algal hydrogen yields under continuous illumination of ~50 μmol m^{-2} s^{-1} light at 25 °C irrespective of the applied algae strain. Accumulated hydrogen yield was also strongly influenced by the algal cell size, smaller cell size correlated with higher hydrogen evolution rate. The highest accumulated algal hydrogen yield (88.98 ± 2.19 ml H_2 l^{-1} d^{-1}) was obtained with *Chlorella* sp. MACC 360 -*E. coli* Δ*hypF* co-culture.

1. Introduction

Green algae are able to evolve hydrogen by FeFe-hydrogenases in both fermentative and photochemical ways [1]. Since oxygen is a strong inhibitor of the algal FeFe-hydrogenase activity and expression, anaerobic environment is a prerequisite of algal hydrogen evolution [2]. Plastidic ferredoxin receiving the electrons from the photosystem I (PSI) serves as direct electron donor of the FeFe-hydrogenase. Three different electron pathways were described for algal hydrogen evolution. Two of them are connected to the photosynthetic electron transport, while the third one represents the fermentative degradation of the endogenous stored compounds. However, the photosynthetic electron transport dependent pathways are strikingly different. The electrons are originated from water splitting in the photosystem II (*PSII*) dependent pathway, while electrons are provided by starch degradation in the *PSII* independent pathway and these electrons are transferred to the plastoquinone (PQ) pool in the electron transport chain *via* the NAD(P)H:plastoquinone oxidoreductase [3]. The electrons migrate from the electron transport chain to ferredoxin in both cases and reduced ferredoxin donates electrons to the FeFe-hydrogenases [4,5].

The FeFe-hydrogenases are extremely sensitive to oxygen [2]. Anaerobiosis is fundamental for their efficient operation, but O_2 is generated during photosynthetic activity and inhibits hydrogen evolution [6]. Various approaches have been utilized to overcome this bar-

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rier [7]. During dark adaptation the sealed algal cultures consume the oxygen and establish anaerobic environment by the algal respiration [1,8]. Under illumination the initial rate of hydrogen evolution is high, but the evolving oxygen instantly inhibits the FeFe-hydrogenases. Many attempts have been made to elaborate long-term continuous H_2 photoproduction [7]. It can be achieved during illumination, when the algae cultures are constantly purged with inert gas to maintain a stable anaerobic environment by rapidly removing the photosynthetically evolved oxygen [9]. Establishment of anaerobic environment is possible through nutrient deprivations by depleting either sulfur, phosphorus, nitrogen or magnesium from the medium [7]. The oxygen production rate of *PSII* is diminished in response to nutrient deprivation, however it takes time, anaerobic environment is achieved in 1 to 8 days depending on the applied nutrient deprivation approach [10–13]. The oxygen content of the closed cultures is consumed by the algal respiration, which can be enhanced by the addition of acetic acid [14]. Establishment of anaerobic environment is possible without nutrient deprivation by using low light intensity and adding acetic acid. Under 50 μmol photon $m^{-2} s^{-1}$, the oxygen evolved by the low activity of *PSII* is efficiently respired by the algae which allow hydrogen production [15]. By the addition of bacterial partners to the algal culture in TAP (Tris-Acetate-Phosphate) medium the rate of the net oxygen consumption can be further enhanced in the sealed algal-bacterial cultures. This decreases the time necessary for the establishment of the anaerobic environment in the liquid phase to a few hours (compared to 1 to 8 days). The elevated total respiration rate enables the use of higher illumination resulting in higher hydrogen production. A number of studies focused on the determination of the optimal light intensity for the most efficient hydrogen production [16,17]. Besides changing the incident illumination power, the consumable light yield can be influenced by the density of the cells in the liquid cultures [18]. To obtain the maximal hydrogen evolution rate of an axenic algal or a mixed algal-bacterial culture the optimal cell density values must be determined experimentally.

The hydrogen production capacities of the axenic algal or mixed alga-bacterial cultures are also strongly influenced by the concentration of the H_2. The FeFe-hydrogenases have hydrogen evolving and consuming functions as well [19]. The partial pressure of the ambient hydrogen influences the establishment of the equilibrium between the concentration of H_2 and the level of the reduced ferredoxin (Fd). This phenomenon has been investigated in anaerobic bacteria during dark fermentation [20]. The equilibrium levels show significant differences between the different bacterial (and possibly algal) strains as well. In the case of *Chlamydomonas reinhardtii* the midpoint redox potential of the major photosynthetic electron transport Fd (encoded by *petF*) is around −0.4 V [21]. When the hydrogen redox potential at pH 7 is more negative than this value the hydrogenase starts consuming hydrogen, while hydrogen production is initiated at less negative redox potential [22]. This phenomenon is of high importance when the goal is to achieve the highest possible accumulative hydrogen level. There are two possible solutions to eliminate the inhibitory effect of the accumulated hydrogen; the appropriate setup of the liquid *versus* gas volume ratio, as well as regular purging of the headspace gas to control the concentration of the accumulated hydrogen [15,22]. Both methods have been applied with promising results for pure algae cultures incubated in sulfur-depleted TAP medium.

The application of the bacterial partners allows significantly earlier algal hydrogen production compared to the nutrient deprived axenic algal cultures. Furthermore, carefully selected bacteria enhance algal biomass production leading to even higher hydrogen production rate [23–25]. The aim of this study was the further improvement of the hydrogen production efficiency. To achieve maximal hydrogen accumulation and hydrogen photoproduction rate the most appropriate algae strains were selected, the algal culture density was optimized and the gas-to-liquid ratio was fine-tuned in a strain specific way. The effect of H_2 partial pressure on algal H_2 production was investigated in detail using three selected algae strains (Table 1).

Table 1
List of algal and bacterial strains.

Strain	Relevant genotype or phenotype	Reference or source
Chlamydomonas reinhardtii cc124	Wild type	[26]
Chlorella sp. MACC 360	Wild type	[27]
Chlorella sp. MACC 411	Wild type	This work
Chlamydomonas sp. MACC 530	Wild type	This work
Chlamydomonas sp. MACC 549	Wild type	[28]
Chlamydomonas sp. MACC 772	Wild type	This work
Chlamydomonas sp. MACC 775	Wild type	This work
Chlorella sp. PAG	Wild type	This work
Escherichia coli JW5433	BW25113, Δ*hypF:kan*	[29]

2. Materials and methods

2.1. Cultivation of axenic and mixed algae cultures

Chlamydomonas sp. MACC 549, *Chlorella* sp. MACC 360 and all the MACC algae strains were selected from the Mosonmagyaróvár Algal Culture Collection (MACC) (Table 1). *Chlamydomonas reinhardtii* cc124 and *Chlorella* sp. PAG were received from the Institute of Plant Biology, Biological Research Centre of the Hungarian Academy of Sciences. *E. coli* JW5433 (Δ*hypF*) strain was selected from Institute of Biochemistry, Biological Research Centre of the Hungarian Academy of Sciences. Pure algae strains were grown and maintained on TP (Tris-Phosphate medium) medium supplemented with rifampicin (50 μg ml^{-1}). The TP medium is a modified TAP (Tris-Acetate-Phosphate medium) medium where acetic acid is replaced with HCl to obtain pH 7 [30]. The algae strains were constantly incubated on TP plates under 50 μmol $m^{-2} s^{-1}$ light intensity at 25 °C. Algae used for hydrogen-evolution experiments were directly inoculated from TP plates into liquid TAP medium. Algae cultures in TAP medium were incubated in closed Erlenmeyer flasks, under 50 μmol $m^{-2} s^{-1}$ light intensity at 25 °C shaken at 180 rpm. Algae stock solutions were generated using fresh cultures by centrifugation and re-suspending the cells in fresh TAP medium. *Escherichia coli* JW5433 (Δ*hypF*) was pre-grown for experiments on LB (Luria-Bertani medium) plates at 30 °C in the dark [31]. *Escherichia coli* JW5433 (Δ*hypF*) was harvested from LB plates and suspended into TAP media for the preparation of concentrated bacterial stock solution. Algal-bacterial liquid cultures were established by mixing the axenic algal and pure bacterial stock solutions in 40 ml serum bottles. Six different liquid final volumes were set in the bottles: 10, 15, 20, 25, 30 and 35 ml. First the proper amount of algae was measured into the 40 ml bottles from the algae stock solutions. Second, the proper yield of *E. coli* Δ*hypF* was measured into the 40 ml bottles from the bacterial stock solutions. Finally, TAP medium was added to the dense algal-bacterial co-cultures to obtain the required culture volumes and algal and bacterial optical densities. The final optical densities of the algae cells were set to 0.7, 1, 2, 3, 4 and 5 (OD_{750}) in each volume in the *Chlorella* sp. MACC 360 – *E. coli* Δ*hypF* co-cultures. The final optical densities of the algae cells were set to 0.7, 1, 2, 3 and 4 (OD_{750}) in each volume in the *Chlamydomonas* sp. MACC 549 – *E. coli* Δ*hypF* co-cultures. The final optical densities of the algae cells were set to 0.7, 1 and 2 (OD_{750}) in each volume in the *Chlamydomonas reinhardtii* cc124 – *E. coli* Δ*hypF*. The final optical density of *E. coli* Δ*hypF* was set to 0.5 (OD_{600}). This way the mixed cultures had differential algal cell numbers for the different algae strains (Table 2) while the bacterial cell number was fixed at $8.31 \pm 0.95 * 10^9$ cells ml^{-1}. Mixed cultures were incubated under 50 μmol $m^{-2} s^{-1}$ light intensity at 25 °C shaken at 180 rpm. All

Table 2
The optical densities represent different cell numbers for the various algae strains.

OD_{750}	*Chlamydomonas reinhardtii* cc124 (10^8 cell ml^{-1})	*Chlorella* sp. MACC 360 (10^8 cell ml^{-1})	*Chlamydomonas* sp. MACC 549 (10^8 cell ml^{-1})
0.7	1.03	2.77	0.27
1	1.47	3.96	0.38
1.5	2.21	5.94	0.57
2	2.95	7.93	0.76
3	4.43	11.89	1.15
4	5.91	15.86	1.53
5	7.39	19.83	1.91

experiments were performed with three parallel samples. 40 ml serum bottles were sealed with butyl rubber stoppers and aluminium caps.

2.2. Gas phase analyses

The hydrogen, oxygen and carbon dioxide levels in the headspace of the serum bottles were measured with gas chromatography. An Agilent 7890A gas chromatograph was equipped with a thermal conductivity detector. For hydrogen and oxygen measurements an Agilent HP-Molsieve column (length 30 m, diameter 0.320 mm, film 12.0 μm) was used in splitless mode. For carbon dioxide measurements an Agilent HP-PLOT Q column (length 30 m, diameter 0.530 mm, film 40.0 μm) was used in splitless mode. Linde HQ argon 5.0 gas was used as carrier and reference gas. The temperatures of the injector, the TCD detector and column were kept at 150 °C, 160 °C and 60/55 °C, respectively. Samples of 50 μl volumes were analyzed. Three biological replicates were used for all measurements.

Hydrogen, oxygen and carbon dioxide calibration curves were used to determine accurate gas volumes. Serial dilutions of pure gases were prepared in 25 ml gas-tight vials, identical volumes were injected into the gas chromatograph, data from three replicates were used to draw the calibration curves.

2.3. Microscopy analyses and cell numbers

Microscopic investigations of algal-bacterial cultures were performed using an Olympus Cell^R microscope with 40 × magnification. The cell areas on the pictures were calculated by the following formula: cell area = pixel number of the cell ∗ 0.10075 μm $pixel^{-1}$. Average cell areas of the different algae strains were calculated from 100 cell areas.

For cell counting, axenic algae dilution series were prepared from algae stock solution. Optical densities were set to 0.7, 1, 1.5, 2, 3, 4 and 5 (OD_{750}). Algae cell numbers were counted by hemocytometer under microscope.

2.4. Analytical measurements

The analysis of acetic acid in TAP media was performed by the GC/MS instrument (Perkin Elmer Clarus 600 GC and 600 T MS) equipped with an electron impact (EI) ion source. The analytes were separated on a DB 17 capillary column (60 m × 0.32 mm × 0.15 μm; Agilent, USA). The oven temperature program was as follows: 60 °C initial temperature increased to 140 °C at 20 °C min^{-1}, 140 °C was maintained for 3 min, then increased to 200 °C at 10 °C min^{-1}, 200 °C was maintained for 5 min. Helium (99.999% purity) was the carrier gas set at a constant flow rate of 2.1 ml min^{-1}. The volumes of the injected samples were 1 μl. Solvent delay time was set for 7.5 min for all samples.

2.5. Chlorophyll measurements

The three different co-cultures with maximum hydrogen accumulation were selected for chlorophyll measurements. 1 ml samples were taken from the algal-bacterial co-cultures after 1 h incubation. Co-cultures were incubated in sealed 40 ml bottles under 50 μmol m^{-2} s^{-1} light intensity at 25 °C shaken at 180 rpm. Samples were centrifuged at 13300 rpm for 15 min and resuspended in 1 ml 100% dimethyl formamide (DMF). After 24 h incubation at 4 °C, the optical densities were measured at 664, 647 and 750 nm. Chlorophyll content was then photometrically quantified using the equation of Porra et al. [32].

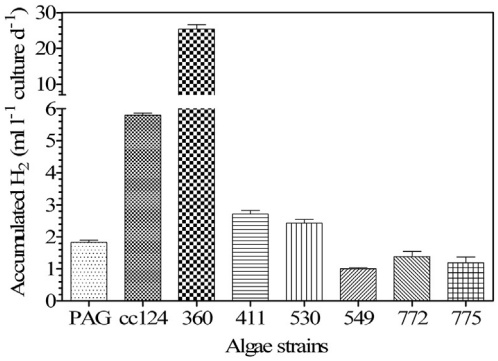

Fig. 1. Daily accumulated hydrogen yields in the headspaces (5 ml/35 ml gas-to-liquid ratio) of the various algal-bacterial co-cultures. Initial optical densities (ODs) were 0.7 (algae, measured at 750 nm) and 0.5 (bacterium, measured at 600 nm). *E. coli* Δ*hypF* was added to the algae strains as bacterial partner. The following algae strains were tested for hydrogen evolution: PAG – *Chlorella* sp. PAG, cc124 – *Chlamydomonas reinhardtii* cc124, 360 – *Chlorella* sp. MACC 360, 411 – *Chlorella* sp. MACC 411, 530 – *Chlamydomonas* sp. MACC 530, 549 – *Chlamydomonas* sp. MACC 549, 772 – *Chlamydomonas* sp. MACC 772, 775 – *Chlamydomonas* sp. MACC 775. Data shown is the mean +/−SD, n = 3.

3. Results

3.1. Selection of algae strains

Our general aim was the optimization of the algal-bacterial co-culture conditions in order to obtain the highest possible algal photobiohydrogen production under conditions permitting algal reproduction and biomass generation (active photosynthesis maintained throughout hydrogen generation). A large number of algae strains were tested for hydrogen evolution in a rapid semi-batch co-culture test system (24 h long monitoring). Three different green algae strains were chosen for detailed analysis based on their clearly different hydrogen production rates under identical conditions (Fig. 1). *Chlamydomonas* sp. MACC 549 was used in our previous study to demonstrate the algal-bacterial co-culture approach for algal hydrogen production [16]. The hydrogen production rate of MACC 549 strain was relatively small in co-culturing approach while negligible amount of hydrogen was generated with this algae when bacterial partners were omitted [28]. *Chlamydomonas reinhardtii* cc124 is a widely investigated green algae model strain in different research fields including biohydrogen production [10]. The third selected green algae was a *Chlorella* species, our screen revealed a *Chlorella* strain (*Chlorella* sp. MACC 360) with remarkable hydrogen production capabilities.

3.2. Initial algal cell number strongly influences the hydrogen yield

The hydrogen production and oxygen consumption rates of the algal-bacterial cultures with varied algal (Table 2) and fixed bacterial ($8.31 \pm 0.95 * 10^9$ cells ml^{-1}, OD_{600}: 0.5) initial cell numbers were investigated for a 24 h long period. The trends of hydrogen production and oxygen consumption were similar in the three co-cultures (Supplementary Figs. 1, 2, 3, 4, 5 and 6). The data of *Chlorella* sp. MACC 360 – *E. coli* Δ*hypF* co-cultures with a 20 ml/20 ml gas-to-liquid volume ratio are shown in Fig. 2. The oxygen consumption rate was remarkably influenced by the initial algae cell numbers of the algal-bacterial co-cultures (Fig. 2, panel A). Oxygen consumption from the headspace

took 8 to 12 h depending on the initial algae cell number. Hydrogen production was initiated during the oxygen consumption phase, it could be measured at 4 h following inoculation in each culture indicating that anaerobic microenvironment was quickly established in the liquid phase. The relative oxygen level was stabilized at about 5% (*v*/v) in the headspace. However, at 16 h after the experiment start, a rise in the relative oxygen level was observed in the co-cultures with $11.89 * 10^8$, $15.86 * 10^8$, $19.83 * 10^8$ algae cells ml^{-1} (OD_{750}: 3, 4, 5). Hydrogen production rate was in clear correlation with the rate of oxygen consumption. More efficient oxygen consumption was accompanied with an earlier initiated hydrogen production. Remarkable differences were observed in the accumulated hydrogen yields at 4 h (Fig. 2, panel B). Dense cultures ($11.89 * 10^8$, $15.86 * 10^8$, $19.83 * 10^8$ algae cells ml^{-1}, OD_{750}: 3, 4, 5) accumulated significantly higher amount of hydrogen than the diluted ones ($2.77 * 10^8$, $3.96 * 10^8$, $7.93 * 10^8$ algae cells ml^{-1}, OD_{750}: 0.7, 1, 2). However, interesting hydrogen evolution kinetics were observed during the 24-hour-long measurements. The maximum hydrogen yield during a given 4-hour period was similar for all cultures, regardless of the initial algal cell concentration, although these maximal amounts were evolved at different 4-hour periods (Table 3). The most concentrated cultures produced the highest amount of hydrogen in the second period (between 4 and 8 h), while the most diluted cultures reached the maximal production in the last period (between 20 and 24 h) (Fig. 2, panel C). Interestingly the more concentrated co-cultures had a lower maximal productivity in 4 h and also the total accumulated hydrogen levels in 24 h were lower for the concentrated cultures. Thus, the total hydrogen yield is rather influenced by the duration of the hydrogen generation period than by the hydrogen production rate. Consequently, the highest accumulated hydrogen yields in 24 h were observed in the most diluted cultures (Fig. 2, panel B) (Table 3). Hydrogen production was limited by two factors. The hydrogen saturation in the headspace of the bottles and the regained algal oxygen production influences the duration of hydrogen evolution. The restart of oxygen production was first observed in the most concentrated co-cultures (Fig. 2, panels A and B). This factor (oxygen production restart) as well as the net oxygen evolution was strongly influenced by the acetic acid concentration in the TAP medium. When the acetic acid concentrations were low (under 200 $\mu l\, l^{-1}$, or 3.49 mM), the respiration of algae cells decreased causing an increase in oxygen evolution and relative level (Fig. 2, panel D). Acetic acid consumption rate was in strong correlation with the initial algae cell concentration, higher algae cell number resulted in higher acetic acid consumption rate.

The highest rate of carbon dioxide accumulation was detected in the first 4 h. Relative level of CO_2 elevated from 0.095% (*v*/v) to 1.35–1.79% (*v*/v) in this period (Fig. 3, panel A). Similar trends were observed for CO_2 accumulation in the 24 h experiments to what was measured for hydrogen. The cultures with higher initial algal concentration accumulated higher amount of carbon dioxide in the headspace in the initial period, while later the carbon dioxide accumulation slowed down and reached a lower maximum relative level in these cultures (Fig. 3, panel B). The highest relative carbon dioxide level was detected in the headspace of the most diluted co-culture (2.65% (*v*/v)). The highest accumulated carbon dioxide yield was 26.54 ± 1.05 ml $CO_2\, l^{-1}$ culture, which was obtained in the most diluted co-culture ($2.77 * 10^8$ algae cells ml^{-1}, OD_{750}: 0.7). The lowest accumulated carbon dioxide yield was 17.38 ± 0.9 ml $CO_2\, l^{-1}$, which was detected in the co-culture started with the highest initial alga concentration ($11.89 * 10^8$ algae cells ml^{-1}, OD_{750}: 3). The highest hydrogen/carbon dioxide ratio (3.79 H_2/CO_2) was obtained by the co-culture starting with an algae cell number of $3.96 * 10^8$ algae cells ml^{-1} (OD_{750}: 1) (Fig. 3, panel C). The trend in the H_2/CO_2 ratio was also highly similar to that of the accumulated hydrogen yields. The lowest H_2/CO_2 ratios were obtained in the co-cultures with a starting algal cell number of $11.89 * 10^8$ algae cells ml^{-1} (OD_{750}: 3).

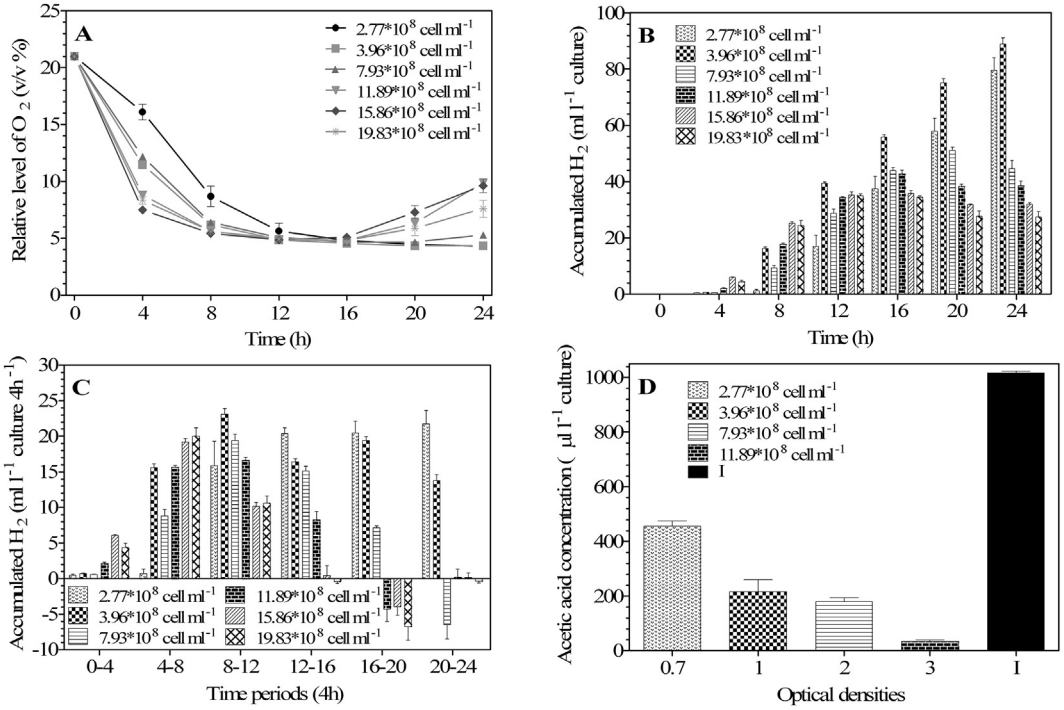

Fig. 2. Daily hydrogen accumulation, oxygen level and acetic acid consumption in *Chlorella* sp. MACC 360 – *E. coli* Δ*hypF* co-cultures. Gas-to-liquid phase ratio was 20 ml/20 ml. Algae cell numbers were $2.77 * 10^8$, $3.96 * 10^8$, $7.93 * 10^8$, $11.89 * 10^8$, $15.86 * 10^8$, $19.83 * 10^8$ algae cells ml^{-1} (OD_{750}: 0.7, 1, 2, 3, 4, 5). Panel A: Relative oxygen levels. Panel B: Daily hydrogen accumulation. Panel C: Accumulated 4 h long period hydrogen yields. Panel D: Acetic acid concentrations after 24 h compared to the initial (I) acetic acid concentration. Data shown is the mean +/−SD, n = 3.

Table 3
Accumulated hydrogen yields in *Chlorella* sp. MACC 360 – *E. coli* Δ*hypF* cultures using a fixed 20 ml/20 ml gas-to-liquid volume ratio and various initial algae cell concentrations.

Initial algae cell numbers (10^8 algae cells ml^{-1}) and algae optical densities (OD_{750})	2.77 (0.7)	3.96 (1)	7.93 (2)	11.89 (3)	15.86 (4)	19.83 (5)
Maximum 4 h long period hydrogen yield (ml H_2 l^{-1} culture 4 h^{-1})	21.74 (20 h–24 h)	23.08 (8 h–12 h)	19.40 (8 h–12 h)	16.64 (8 h–12 h)	19.20 (4 h–8 h)	20.00 (4 h–8 h)
Total accumulated hydrogen yield (ml H_2 l^{-1} culture)	79.60 ± 4.49	88.98 ± 2.18	51.09 ± 1.25	42.69 ± 1.39	35.80 ± 1.07	34.94 ± 0.70

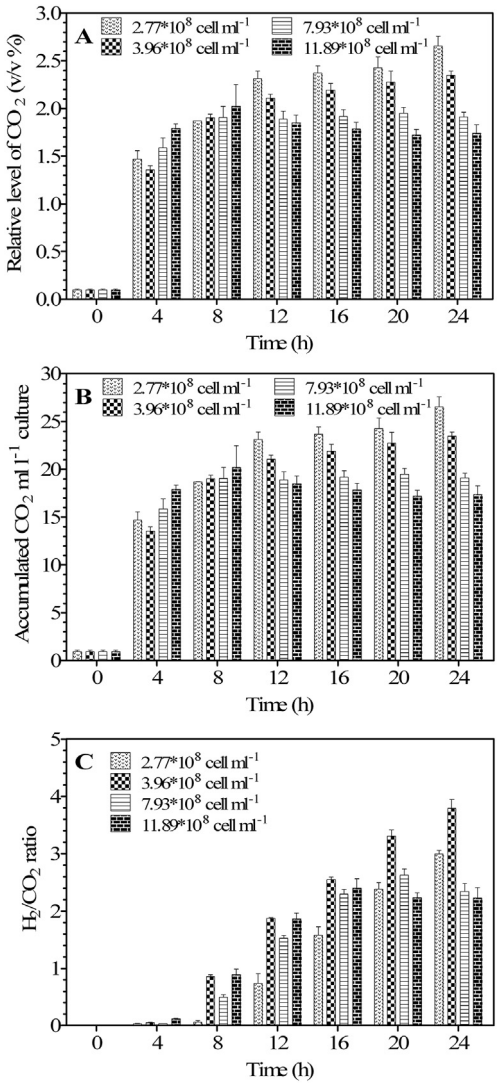

Fig. 3. Carbon dioxide accumulation in the headspace of the *Chlorella* sp. MACC 360 – *E. coli* Δ*hypF* co-cultures with 20 ml/20 ml gas-to-liquid volume ratio in 24 h. Algae cell numbers were set to $2.77 * 10^8$, $3.96 * 10^8$, $7.93 * 10^8$, $11.89 * 10^8$ algae cells ml^{-1} (OD_{750}: 0.7, 1, 2, 3). Panel A: Relative carbon dioxide levels. Panel B: Accumulated carbon dioxide yields. Panel C: Ratio of accumulated hydrogen and carbon dioxide. Data shown is the mean +/−SD, n = 3.

3.3. Gas-to-liquid volume ratio plays important role in algal hydrogen productivity

The hydrogen production and oxygen consumption rates of the different algal-bacterial co-cultures with fixed algal (*Chlorella* sp. MACC 360, $3.96 * 10^8$ algae cells ml^{-1}, OD_{750}: 1) and bacterial (*E. coli* Δ*hypF*, $8.31 \pm 0.95 * 10^9$ bacterial cells ml^{-1}, OD_{600}: 0.5) cell concentrations and various gas-to-liquid volume ratios were investigated in 24 h experiments. The volume of the gas phase was gradually (5 ml in each step) raised from 5 ml to 30 ml while the liquid volume was decreased from 35 ml to 10 ml with the same gradient. The minimum relative oxygen levels in the headspaces showed remarkable differences ranging between 2 and 5% (*v*/v) (Fig. 4, panel A). Co-cultures with 5 ml and 10 ml gas volumes quickly reached the minimum relative oxygen levels in 4 h, while co-cultures with 15 ml and 20 ml gas volumes reached it between 8 and 12 h. Relative levels of oxygen in the co-cultures with 25 ml and 30 ml headspaces did not reach the 5% (*v*/v). Relative levels of oxygen in the cultures with small headspace volumes (5, 10 and 15 ml) were stabilized at the minimum values until the end of the 24 h measurement, while in the bottles with 25 ml and 30 ml headspace volumes the oxygen accumulation restarted after 16 and 12 h, respectively. The initiation of hydrogen production was strongly influenced by the rate of oxygen consumption. Hydrogen accumulation was observed in the first 4 h period in each co-culture with different gas-to-liquid volume ratios. However, the yield of the accumulated hydrogen showed significant differences (Fig. 4, panels B and C). Efficient hydrogen production was detected when the relative level decreased to 10% (*v*/v) (Fig. 4, panel C). Relative oxygen levels in most co-cultures passed this threshold (Fig. 4, panel A). During the 24 h long measurements, each co-culture with different gas-to-liquid volume ratios accumulated similar maximum hydrogen yields in a specific 4 h period (Fig. 4, panel C). However, the maximum accumulated hydrogen yields were achieved in different 4 h periods by the co-cultures with different gas-to-liquid volume ratios. By increasing the gas-to-liquid volume ratios the maximum accumulated hydrogen yields in a 4 h period appeared later. The highest accumulated hydrogen yields in 24 h were observed in the cultures where the gas and liquid volumes were similar (Fig. 4, panel B). The total accumulated hydrogen yields were influenced by two related factors: the volume of the co-culture headspace and the length of the period with minimum relative oxygen level. Maximum accumulated hydrogen yield can be obtained by balancing these two factors. The most efficient hydrogen production was achieved using 1/1 gas-to-liquid volume ratio (20 ml/20 ml) (Fig. 4, panel B). The *Chlamydomonas* sp. MACC 549 – *E. coli* Δ*hypF* and *Chlamydomonas reinhardtii* cc124 – *E. coli* Δ*hypF* co-cultures showed similar results, the most appropriate gas-to-liquid volume ratios in these combinations were 20 ml/20 ml and 25 ml/15 ml, respectively (Fig. 6) (Supplementary Figs. 1, 2, 3, 4, 5 and 6).

The intensity of the carbon dioxide accumulation was in accordance with the gas-to-liquid volume ratio. Carbon dioxide saturation depended on the volume of the gas phase, co-cultures with high gas-to-liquid volume ratios showed higher carbon dioxide accumulation rates compared to co-cultures with lower gas-to-liquid volume ratios (Fig. 5, panels A and B, Supplementary Fig. 7). The highest accumulated carbon dioxide volume was 70.77 ± 0.86 ml CO_2 l^{-1} culture in the co-culture with 30 ml/10 ml gas-to-liquid volume ratio in 24 h, while the lowest total daily carbon dioxide volume (0.86 ± 0.03 ml CO_2 l^{-1}) was measured in co-cultures with 5 ml/35 ml gas-to-liquid volume ratio (Fig. 5, panel B). The highest relative carbon dioxide level was 2.72% (*v*/v) (in the co-cultures with 25 ml/15 ml gas-to-liquid volume ratio), while the lowest relative carbon dioxide level was 0.6% (*v*/v) (measured in co-cultures with 5 ml/35 ml gas-to-liquid volume ratio) in 24 h (Fig. 5, panel A). The highest H_2/CO_2 ratio was obtained in the case of co-cultures with a gas-to-liquid volume ratio of 5 ml/35 ml (Fig. 5, panel C). Hydrogen saturation and carbon dioxide saturation showed

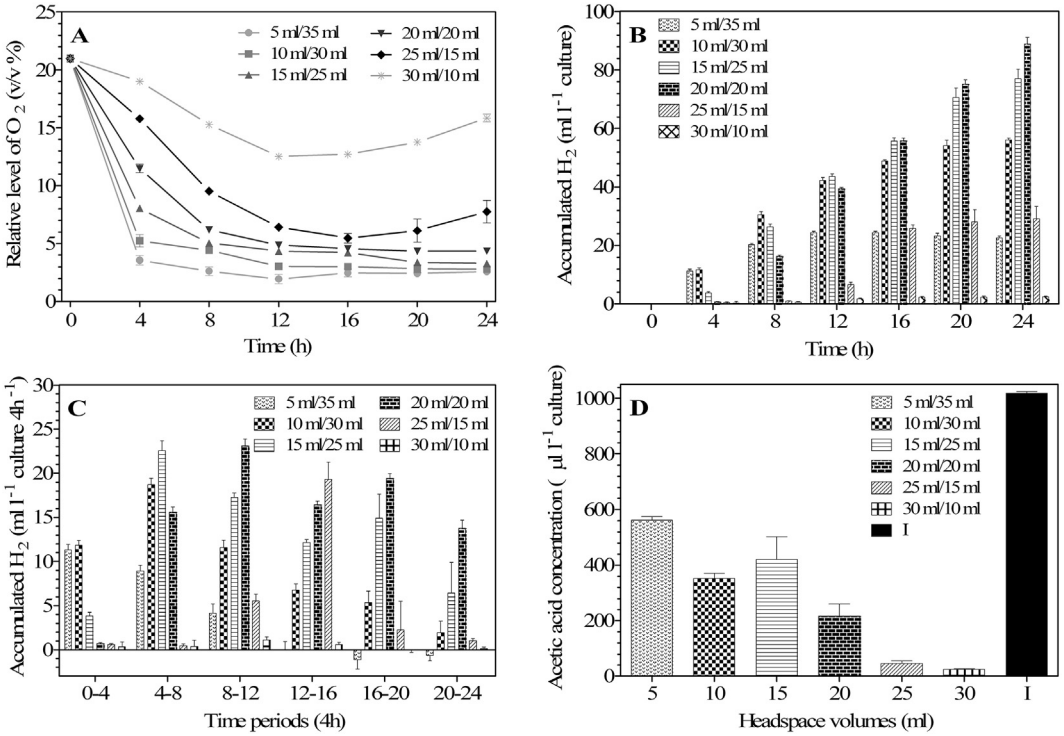

Fig. 4. Hydrogen accumulation, oxygen and acetic acid consumption trends in *Chlorella* sp. MACC 360 – *E. coli* Δ*hypF* co-cultures maintained in bottles with fixed initial algal and bacterial cell numbers (*Chlorella* sp. MACC 360: $3.96 * 10^8$ algae cells ml^{-1}, OD_{750}: 1; *E. coli* Δ*hypF*: $8.31 \pm 0.95 * 10^9$ bacterial cells ml^{-1}, OD_{600}: 0.5) and different gas-to-liquid volume ratios (5 ml/35 ml, 10 ml/30 ml, 15 ml/25 ml, 20 ml/20 ml, 25 ml/15 ml, 30 ml/10 ml) in 24 h experiments. Panel A: Relative oxygen levels. Panel B: Hydrogen accumulation. Panel C: Accumulated hydrogen yields in 4 h periods. Panel D: Acetic acid concentrations measured after 24 h. Data are compared to the initial (I) acetic acid concentration. Data shown is the mean +/−SD, n = 3.

similar trends with more explicit differences in the CO_2 levels.

3.4. The various algae strains require different conditions for the maximum hydrogen yields

Three different algae strains mixed with *E. coli* Δ*hypF* evolved significantly different amounts of hydrogen as measured in the headspace after 24 h (Fig. 6, panel A, Supplementary Fig. 8, panels A and B). *Chlorella* sp. MACC 360 – *E. coli* Δ*hypF* algal-bacterial cultures incubated in 20 ml/20 ml gas-to-liquid phase ratio and containing $3.96 * 10^8$ algae cells ml^{-1} (OD_{750}: 1) accumulated the highest amount of hydrogen: 88.98 ± 2.19 ml H_2 l^{-1} d^{-1} (13.1 ml H_2 mg^{-1} chlorophyll d^{-1}, 20 ml H_2 mg^{-1} chlorophyll *a* d^{-1}). *Chlamydomonas reinhardtii* cc124 – *E. coli* Δ*hypF* algal-bacterial cultures incubated in 25 ml/15 ml gas-to-liquid phase ratio and containing $1.03 * 10^8$ algae cells ml^{-1} (OD_{750}: 0.7) accumulated 18.67 ± 1.01 ml H_2 l^{-1} d^{-1} (6.4 ml H_2 mg^{-1} chlorophyll d^{-1}, 12.35 ml H_2 mg^{-1} chlorophyll *a* d^{-1}), which was 4.8 times less than maximum accumulated hydrogen yield of *Chlorella* sp. MACC 360 – *E. coli* Δ*hypF* algal-bacterial culture. *Chlamydomonas* sp. MACC 549 – *E. coli* Δ*hypF* algal-bacterial cultures incubated in 20 ml/20 ml gas-to-liquid phase ratio and diluted to containing 0.38 algae cells ml^{-1} (OD_{750}: 1) accumulated the smallest amount of maximum hydrogen yield: 6.84 ± 0.26 ml H_2 l^{-1} d^{-1} (0.98 ml H_2 mg^{-1} chlorophyll d^{-1}, 1.46 ml H_2 mg^{-1} chlorophyll *a* d^{-1}), which was 13 times less than the maximum accumulated hydrogen yield of the *Chlorella* sp. MACC 360 – *E. coli* Δ*hypF* algal-bacterial culture.

The highest relative hydrogen levels were obtained in the co-cultures in which the gas-to-liquid phase ratios were 5 ml/35 ml. Remarkable differences were observed between the maximum relative hydrogen levels of the different algal-bacterial cultures (Fig. 6, panel B). Maximum values were the following: *Chlamydomonas reinhardtii* cc124 – *E. coli* Δ*hypF*: 1.4% (*v*/v) ($2.21 * 10^8$ algae cells ml^{-1}, OD_{750}: 1.5, 5 ml/35 ml); *Chlorella* sp. MACC 360 – *E. coli* Δ*hypF*: 24.06% (*v*/v) ($3.96 * 10^8$ algae cells ml^{-1}, OD_{750}: 1, 5 ml/35 ml); *Chlamydomonas* sp. MACC 549 – *E. coli* Δ*hypF* 0.84% (*v*/v) ($0.27 * 10^8$ algae cells ml^{-1}, OD_{750}: 0.7, 5 ml/35 ml). Relative hydrogen levels in the algal-bacterial cultures, which produced the highest yields of hydrogen were lower than the maximum relative hydrogen levels: *Chlamydomonas reinhardtii* cc124 –*l E. coli* Δ*hypF*: 1.12% (*v*/v) ($1.03 * 10^8$ algae cells ml^{-1}, OD_{750}: 0.7, 25 ml/15 ml); *Chlorella* sp. MACC 360 – *E. coli* Δ*hypF*: 9.89% (*v*/v) ($3.96 * 10^8$ algae cells ml^{-1}, OD_{750}: 1, 20 ml/20 ml); *Chlamydomonas* sp. MACC 549 – *E. coli* Δ*hypF*: 0.68% (*v*/v) ($0.38 * 10^8$ algae cells ml^{-1}, OD_{750}: 1, 20 ml/20 ml).

3.5. Correlation between algae cell size and hydrogen production

The three different algae cultured under identical conditions (optical density, bacterial partner, light intensity, temperature and growth media) showed remarkable differences in their maximum accumulated hydrogen amounts in 24 h (Fig. 6, Supplementary Figs. 1, 2 and 3). The oxygen consumption and hydrogen accumulation trends of the three co-cultures were similar (Fig. 2, panels A and B; Fig. 4, panels A and B, Supplementary Figs. 4, 5 and 6).

Algae cell numbers and cell sizes of the mixed cultures were investigated to explain the observed differences in the hydrogen production rates. Clear correlation was detected between the maximum accumulated hydrogen yields and the algae cell numbers (Fig. 6, panel A; Fig. 7, panel A). The highest algae cell number was measured for *Chlorella* sp. MACC 360: $3.96 * 10^8$ algae cells ml^{-1} (OD_{750}: 1) (Fig. 7, panel A). The cell number of *Chlamydomonas reinhardtii* cc124 was $1.03 * 10^8$ algae cells ml^{-1} (OD_{750}: 0.7), which was 3.9 times lower compared to *Chlorella* sp. MACC 360. The lowest cell number was measured for *Chlamydomonas* sp. MACC 549: $0.38 * 10^8$ algae cells ml^{-1} (OD_{750}: 1), this value was 10.4 times lower than that of the *Chlorella* sp. MACC 360 culture.

Significant differences were observed between the average cell sizes of the different algal strains. The algae cell sizes and the maximum

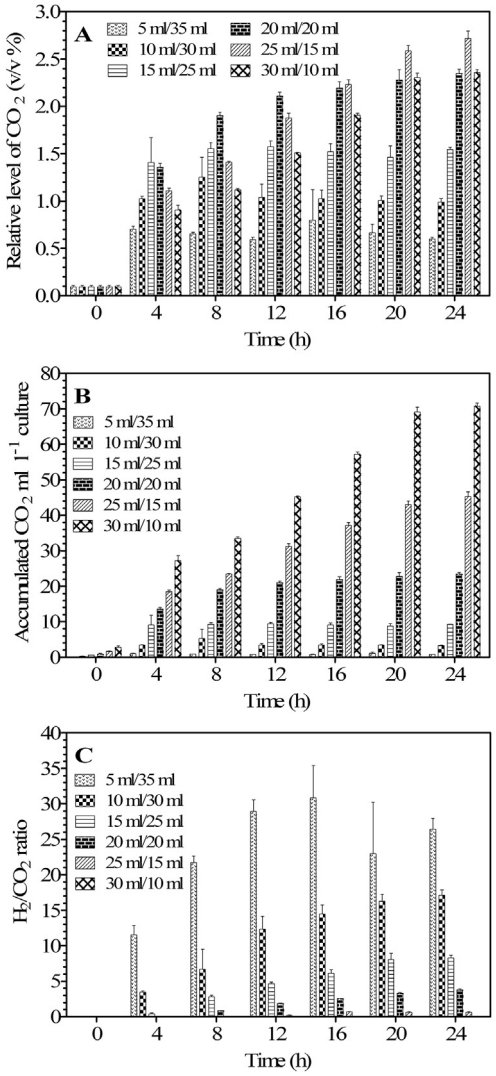

Fig. 5. Carbon dioxide accumulation in *Chlorella* sp. MACC 360 – *E. coli* Δ*hypF* co-cultures with fixed initial algal and bacterial cell numbers (*Chlorella* sp. MACC 360: $3.96 * 10^8$ algae cells ml^{-1}, OD_{750}: 1; *E. coli* Δ*hypF*: $8.31 \pm 0.95 * 10^9$ bacterial cells ml^{-1}, OD_{600}: 0.5) and different gas-to-liquid volume ratios (5 ml/35 ml, 10 ml/30 ml, 15 ml/25 ml, 20 ml/20 ml, 25 ml/15 ml, 30 ml/10 ml) in 24 h. Panel A: Relative carbon dioxide levels. Panel B: Accumulated carbon dioxide yields. Panel C: Accumulated hydrogen and carbon dioxide ratios. Data shown is the mean +/−SD, n = 3.

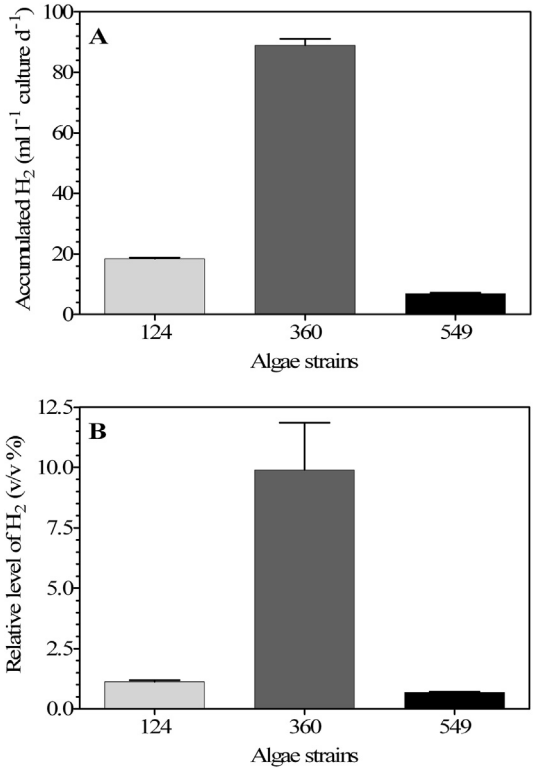

Fig. 6. Maximum hydrogen yields of *Chlamydomonas reinhardtii* cc124 – *E. coli* Δ*hypF* ($1.03 * 10^8$ algae cells ml^{-1}, OD_{750}: 0.7, 25 ml/15 ml) (light grey, 124), *Chlorella* sp. MACC 360 – *E. coli* Δ*hypF* ($3.96 * 10^8$ algae cells ml^{-1}, OD_{750}: 1, 20 ml/20 ml) (grey, 360) and *Chlamydomonas* sp. MACC 549 – *E. coli* Δ*hypF* ($0.38 * 10^8$ algae cells ml^{-1}, OD_{750}: 1, 20 ml/20 ml) (black, 549) co-cultures. Panel A: Maximum accumulated hydrogen yields in 24 h. Panel B: Maximum relative hydrogen levels in 24 h. Data shown is the mean +/−SD, n = 3.

accumulated hydrogen yields showed reciprocal proportionality (Fig. 6, panel A; Fig. 7, panel B). *Chlorella* sp. MACC 360 had the smallest average cell size: 14.464 ± 3.925 μm^2 (Fig. 7, panel B). The average cell size of *Chlamydomonas reinhardtii* cc124 was approximately 3.5 times larger compared to *Chlorella* sp. MACC 360: 51.077 ± 12.86 μm^2. *Chlamydomonas* sp. MACC 549 had the largest average cell size of 105.979 ± 42.727 μm^2, this was approximately 7.3 times larger than the *Chlorella* sp. MACC 360 cell size.

Hydrogen production rates of the algae strains were calculated as the maximum accumulated hydrogen evolved by 10^{11} algae cells in a day (Fig. 7, panel C). Significantly lower differences were observed between the hydrogen production rates of the algae strains compared to what was detected in the maximum accumulated hydrogen yields, cell numbers and average cell sizes (Fig. 6, panel A; Fig. 7, panels A and B). The hydrogen production rate of *Chlorella* sp. MACC 360 was 22.47 ml H_2 by 10^{11} algae cells d^{-1}. *Chlamydomonas reinhardtii* cc124 showed a rate of 18.12 ml H_2 by 10^{11} cells d^{-1}, 1.24 times lower compared to *Chlorella* sp. MACC 360. The *Chlamydomonas* sp. MACC 549 culture displayed a rate of 18 ml H_2 by 10^{11} cells d^{-1} which was 1.25 times lower than that of *Chlorella* sp. MACC 360.

4. Discussion

The co-culture-based algal hydrogen evolution depends on a number of growth parameters. The selected algae partner, the initial algal concentration and gas-to-liquid volume ratio are three major factors determining algal hydrogen productivity in algal-bacterial co-cultures. The concept of co-culture-based algal hydrogen production was described in our previous study, however, the hydrogen productivity was not in the focus of that study [28]. As described, hydrogen production efficiency was low compared to that achieved by other approaches including nutrient deprived methods using axenic algae cultures [28]. In this study we have optimized the experimental setups and achieved significantly higher hydrogen production rates and yields (Table 4). Compared to the original hydrogen production rates of 49.83 ± 0.18 $\mu l\, l^{-1}\, h^{-1}$ and 241.68 ± 2.73 $\mu l\, l^{-1}\, h^{-1}$ achieved by *Chlamydomonas* sp. MACC 549 – *E. coli* Δ*hypF* and *Chlamydomonas reinhardtii* cc124 – *E. coli* Δ*hypF* co-cultures (algae OD_{750}: 0.7, bacterial OD_{600}: 0.5, 5 ml/35 ml gas-to-liquid ratio), respectively, the optimization of the experimental conditions resulted in a clearly higher rate for the investigated *Chlamydomonas* strains as well (Table 5) [28]. *Chlamydomonas* sp. MACC 549 – *E. coli* Δ*hypF* co-culture had a maximum rate of 285.11 $\mu l\, H_2\, l^{-1}\, h^{-1}$ (algae OD_{750}: 1, bacterial OD_{600}: 0.5, 20 ml/20 ml gas-to-liquid ratio) while the *Chlamydomonas reinhardtii* cc124 – *E. coli* Δ*hypF* co-culture showed a maximum of 766.87 $\mu l\, H_2\, l^{-1}\, h^{-1}$ (algae OD_{750}: 0.7, bacterial OD_{600}: 0.5, 25 ml/15 ml gas-to-liquid ratio) (Fig. 6, panel A). The highest hydrogen production rate was achieved by the *Chlorella* sp. MACC 360 – *E. coli* Δ*hypF*

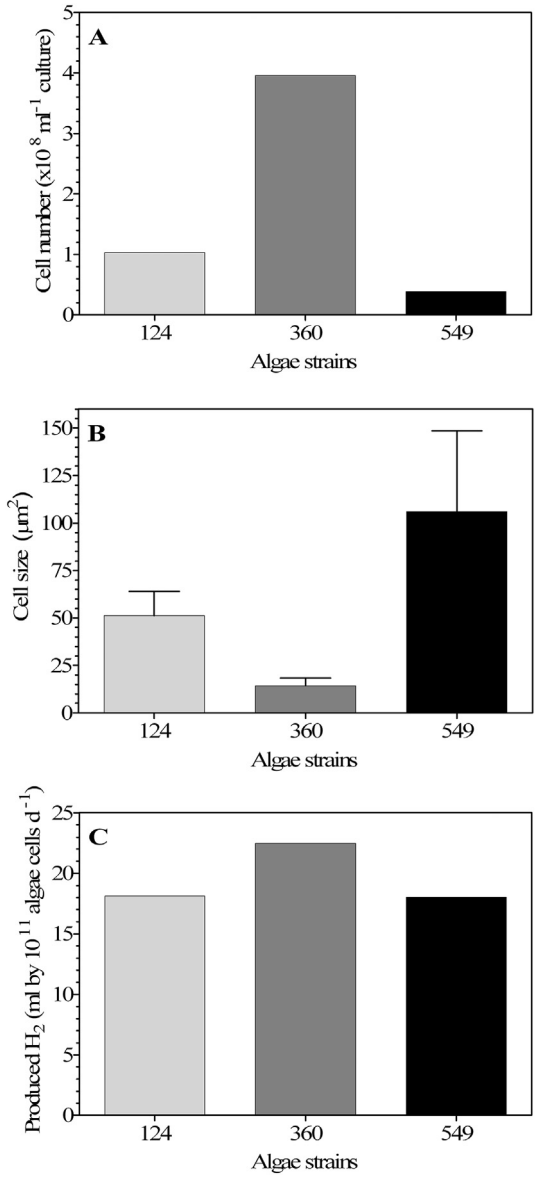

Fig. 7. Algae cell numbers, cell sizes and hydrogen production rates of *Chlamydomonas reinhardtii* cc124 – *E. coli* Δ*hypF* ($1.03 * 10^8$ algae cells ml^{-1}, OD_{750}: 0.7, 25 ml/15 ml) (light grey, 124), *Chlorella* sp. MACC 360 – *E. coli* Δ*hypF* ($3.96 * 10^8$ algae cells ml^{-1}, OD_{750}: 1, 20 ml/20 ml) (grey, 360) and *Chlamydomonas* sp. MACC 549 – *E. coli* Δ*hypF* ($0.38 * 10^8$ algae cells ml^{-1}, OD_{750}: 1, 20 ml/20 ml) (black, 549) co-cultures. Panel A: Algae cell numbers in 1 ml co-cultures containing maximum accumulated hydrogen. Panel B: Average cell sizes of the three different algae strains. Panel C: Accumulated hydrogen yields of 10^{11} algae cells in co-cultures in one day. Data shown is the mean +/−SD, n = 3.

co-culture (algae OD_{750}: 1, bacterial OD_{600}: 0.5, 20 ml/20 ml gas-to-liquid ratio), the rate of 3707.56 μl H_2 l^{-1} h^{-1} was the highest among all tested algal-bacterial co-cultures.

The hydrogen productivity of the *Chlorella* sp. MACC 360 – *E. coli* Δ*hypF* co-culture was superior to all other combinations tested (Supplementary Figs. 1, 2 and 3). Beside the hydrogen production rate, the relative hydrogen level achieved by the *Chlorella* sp. MACC 360 co-culture (24% (*v*/v) of the headspace volume) was also far the highest compared to the *Chlamydomonas reinhardtii* cc124 and *Chlamydomonas* sp. MACC 549 co-cultures (1.4% (*v*/v) and 0.8% (*v*/v)), respectively (Fig. 6, panel B). Further investigations on the correlation of algae cell number, cell size and hydrogen productivity explained this striking difference. The algae cell numbers in the algal-bacterial co-cultures showed strong correlation with the accumulated hydrogen yields and also with the relative hydrogen level. The *Chlorella* sp. MACC 360 – *E. coli* Δ*hypF* co-cultures had a much higher algae cell number at the same optical density – therefore produced more hydrogen – compared to the *Chlamydomonas reinhardtii* cc124 – *E. coli* Δ*hypF* and *Chlamydomonas* sp. MACC 549 – *E. coli* Δ*hypF* co-cultures (Fig. 6, panel A; Fig. 7, panel A). The algae cell numbers showed reciprocal proportionality with the cell size of the specific algae strains, thus, the size of the specific algae cells is one of the most important factor in hydrogen productivity of the algal-bacterial co-cultures. The hydrogen production rates of the different algae strains were defined as the maximum accumulated hydrogen yield achieved by 10^{11} algae cells (Fig. 7, panel C). The correlations between the maximum accumulated hydrogen yields of the mixed cultures and the hydrogen production rates of the specific algae strains were not unambiguous (Fig. 6, panel A; Fig. 7, panel C). The hydrogen production rate of *Chlorella* sp. MACC 360 was higher than that of the two investigated *Chlamydomonas* strains, however, the differences were significantly smaller than in the case of the maximum accumulated hydrogen yields of the mixed cultures. Previous studies concluded that hydrogen production rate can be correlated with the *in vivo* oxygen tolerance of the algae strains [37,38]. The respiration rate and the spatial structure of the algae cells were speculated to be the main reasons for the different *in vivo* oxygen tolerance. However, in our algal-bacterial cultures the algae respiration rate is not a limiting factor at the applied light intensity (50 μmol m^{-2} s^{-1}), since the strong bacterial respiration masks the different algae respiration rates. The spatial structures of the algae strains are more crucial in the algal-bacterial systems. Algae cell size strongly affects the hydrogen production rates by resulting in different shading features. Maximum accumulated hydrogen yields and maximum accumulated hydrogen yields achieved by 10^{11} algae cells (Fig. 6, panel A and Fig. 7, panel C) was also compared to the chlorophyll *a*/b ratios of the specific strains (Supplementary Fig. 8, panel D). Hydrogen yields did not show clear correlation with chlorophyll *a*/b ratios. The chlorophyll *a*/b ratios of *Chlorella* sp. MACC 360 and *Chlamydomonas* sp. MACC 549 were similar (6.79 and 6.99, respectively), while the chlorophyll *a*/b ratio of *Chlamydomonas reinhardtii* cc124 was significantly lower (2.87).

The initial density of the algal-bacterial co-cultures is also important to achieve optimal light utilization efficiency [18]. Only algae growing near the surface of the bottles can efficiently utilize the incident light in more concentrated co-cultures. Algae in the inner part of the cultures absorb less light, therefore the lower *PSII* activity results in lower oxygen as well as algae biomass production rate. At the same time algal respiration is not affected and equally maintained in the entire liquid phase. The lower oxygen production rate and the unaltered respiration (algal and bacterial) establish anaerobic environment earlier in the more concentrated cultures permitting the initiation of algal hydrogen evolution (Fig. 2, panels A and C). On the other hand higher initial algae concentration affects not only oxygen consumption, but results also in faster acetic acid consumption leading to hydrogen saturation at lower level (Fig. 2, panels B and D). Remarkable effects of different bacterial cell concentrations on the hydrogen accumulation were observed in the algal-bacterial co-cultures, however this data is not in the focus of this manuscript. Our studies showed that the decrease of the bacterial cell concentration ($< 8.31 \pm 0.95 * 10^9$ cells ml^{-1}, $< OD_{600}$: 0.5) could prolong the time of the oxygen consumption period caused by the lower bacterial respiration. The initiation of the hydrogen evolution and the hydrogen saturation were also shifted to a later time point. Decreased accumulated hydrogen yield was also observed. Theoretically, the rapid algae oxygen and acetic acid consumption period was elongated which could result in faster acetic acid consumption and declined hydrogen production period. The decrease of the acetic acid concentration strongly correlated with the rate of the algae respiration in our current study as well [28]. The depletion of acetic acid allowed the re-accumulation of photosynthetic oxygen in the headspace of the algal-bacterial cultures (Fig. 2, panel A). Thus, co-cultures containing more algae cells consume acetic acid faster from the TAP medium leading to the elevation of oxygen level and the inhibition of

Table 4
Comparison of various *Chlorella*-based hydrogen production approaches.

Total hydrogen production	Starting time of hydrogen production	Length of hydrogen accumulation	Experimental setup	References
88.98 ml l^{-1}	< 4 h	24 h	Medium: TAP, algae strain: *Chlorella* sp. MACC 360, bacterial partner: *E. coli ΔhypF* (JW5433), liquid volume: 20 ml, light intensity: 50 μmol $m^{-2} s^{-1}$, cellular concentration: $3.97 * 10^8$ algae cells ml^{-1}	This work
233.7 ml l^{-1}	2–4 h	100 h	Medium: TAP-S (0.35 mM NH_4Cl), algae strain: *Chlorella protothecoides*, liquid volume: 650 ml, light intensity: 40–50 μmol $m^{-2} s^{-1}$, cellular concentration: $8 * 10^6$ algae cells ml^{-1}	[33]
25.51 ml l^{-1}	12 h	72 h	Medium: artificial waste water, algae strain: *Chlorella pyrenoidosa* 707S, liquid volume: 20 ml, light intensity: 35–45 μmol $m^{-2} s^{-1}$, cellular concentration: $2.5–3.5 * 10^7$ algae cells ml^{-1}	[34]
~150 ml l^{-1}	45 h	177 h	Medium: TAP-S, algae strain: *Chlorella sorokiniana* strain Ce, liquid volume: 500 ml, light intensity: 120 photons $m^{-2} s^{-1}$, cellular concentration: $5 * 10^6$ algae cells ml^{-1}	[35]
140.4 ml l^{-1}	12 h	84 h	Medium: TAP-S-N, algae strain: *Chlorella protothecoides*, liquid volume: 20 ml, light intensity: 30–40 μmol $m^{-2} s^{-1}$, cellular concentration: $2.5–3.5 * 10^7$ algae cells ml^{-1}	[36]

Table 5
Comparison of our previously published and optimized hydrogen productions achieved by *Chlamydomonas* strains.

Total hydrogen production	Starting time of hydrogen production	Length of hydrogen accumulation	Experimental setup	References
1.2 ml l^{-1}	2–4 h	24 h	Medium: TAP, algae strain: *Chlamydomonas* sp. 549, bacterial partner: *E. coli ΔhypF* (JW5433), gas-to-liquid volume: 5/35 ml, light intensity: 50 μmol $m^{-2} s^{-1}$, algae cellular concentration: $0.27 * 10^8$	[28]
6.8 ± 0.3 ml l^{-1}	< 4 h	24 h	Medium: TAP, algae strain: *Chlamydomonas* sp. 549, bacterial partner: *E. coli ΔhypF* (JW5433), gas-to-liquid volume: 20/20 ml, light intensity: 50 μmol $m^{-2} s^{-1}$, algae cellular concentration: $0.38 * 10^8$	This work
5.8 ml l^{-1}	2–4 h	24 h	Medium: TAP, algae strain: *Chlamydomonas reinhardtii* cc124, bacterial partner: *E. coli ΔhypF* (JW5433), gas-to-liquid volume: 5/35 ml, light intensity: 50 μmol $m^{-2} s^{-1}$, algae cellular concentration: $1.03 * 10^8$	[28]
18.7 ± 1 ml l^{-1}	< 4 h	24 h	Medium: TAP, algae strain: *Chlamydomonas reinhardtii* cc124, bacterial partner: *E. coli ΔhypF* (JW5433), gas-to-liquid volume: 25/15 ml, light intensity: 50 μmol $m^{-2} s^{-1}$, algae cellular concentration: $1.03 * 10^8$	This work

hydrogen evolution (Fig. 2, panel D). Moreover, in these co-cultures starting with higher algae cell number turn into hydrogen uptake; however, this hydrogen uptake also stops due to the inactivation of the FeFe-hydrogenases by the elevated oxygen level. As a conclusion, the increase of the initial algal concentration has a net negative effect on the hydrogen productivity in illuminated batch cultures. Nevertheless, the advantages of the more concentrated algae cultures can be more apparent in photobioreactors, where homogenous light distribution and a generally better light utilization can be achieved [39,40].

The rate of oxygen consumption is influenced by the gas-to-liquid volume ratio as well. The larger the gas phase results in lower oxygen consumption rate, which leads to a delay of hydrogen production initiation (Fig. 4). Acetic acid consumption is faster in the presence of the oxygen compared to that under anoxic conditions [15]. As it was earlier described, algae cell respiration is decreased in the absence of acetic acid. The consequent oxygen accumulation inhibits the FeFe-hydrogenases. Based on these considerations the application of high gas-to-liquid volume ratios leads to shortened hydrogen accumulation periods (Fig. 4, panels B and C). However, increased amount of total accumulated hydrogen can be achieved even beside high gas-to-liquid volume ratios. The hydrogen gas saturation level showed strong correlations with the algae cell size (Fig. 6, panel A; Fig. 7, panel B). Algae strains with a fixed optical density showed highly similar hydrogen saturation rates regardless the actual gas-to-liquid volume ratios (Fig. 6, panel B). As a result of this, the hydrogen accumulation was higher in the co-cultures with larger headspace volume. The maximum hydrogen accumulation rates were not affected by the gas-to-liquid volume ratio. Again, the duration of hydrogen evolution was strongly influenced as discussed previously (Fig. 4, panel C).

One of the most important advantage of the utilization of molecular hydrogen as energy carrier compared to fossil fuels is the lack of carbon dioxide emission. In the algal-bacterial co-cultures the establishment of anaerobic environment essential for hydrogen evolution was achieved by the algal and bacterial cell respiration which process utilizes oxygen and releases carbon dioxide. The rate of the carbon dioxide emission was influenced by the experimental conditions. The highest accumulated carbon dioxide yields were obtained in the cultures having the largest gas-to-liquid volume ratios and the lowest initial algae concentration (Figs. 3 and 5, Supplementary Fig. 7). Carbon dioxide production is continuous until acetic acid is available in the medium and the relative oxygen level decreases to 5% (*v*/v) as measured in the headspace. The accumulated carbon dioxide yields were mainly limited by the available oxygen in the headspace and consequently by the rate of the algal oxygen production. The oxygen production rate of the algae cells were influenced by the initial algae concentration, since the diluted co-cultures were accessible for light. Previous studies showed that the hydrogen production rate was decreased by the elevation of the carbon dioxide concentration [41]. In the current study the inhibition

of carbon dioxide on hydrogen production was not observed (Figs. 2, 4 and 6, panel A). No correlation was detected between the accumulated carbon dioxide and hydrogen yield. For environmental protection considerations it is important to calculate how much carbon dioxide is produced in line with a specific amount of hydrogen. The highest hydrogen-to-carbon dioxide gas ratios were obtained when low gas-to-liquid volume ratios and low initial algae concentrations were applied in batch cultures (Figs. 3 and 5, panel C). The rate of the carbon dioxide evolution was strongly reduced when the oxygen concentration in the gas phase decreased below 5% (*v*/v) (Figs. 2, 3, panel A and Figs. 3 and 5, panel B). In a continuous system where a gas mixture containing oxygen between 3% (*v*/v) and 5% (*v*/v) is used, carbon dioxide emission could be reduced significantly.

Another remarkable advantage of the algal-bacterial co-culture-based hydrogen producing approach is the opportunity for parallel biomass production and hydrogen evolution.

5. Conclusion

Algal hydrogen production is a promising candidate for being a future environmental friendly solution to replace fossil fuel utilization. The application of algal-bacterial consortia has a number of advantages over using axenic algae cultures for hydrogen evolution. The main benefit of this method is the easy implementation, since no pre-growth of algal biomass is necessary for hydrogen production. Also, there is no need to pay attention to the otherwise highly costly culture purity, the appropriately selected algal-bacterial partners form stable consortia. Beside hydrogen evolution, the excess biomass can be harvested to continuously maintain the optimal co-culture density for the maximum hydrogen production rate and the biomass can be used for various further purposes [23–25]. The application of bacterial partners can support the oxygen consumption also in nutrient deprived cultures and enhance the rate of hydrogen production in these combined systems as well [28]. Bacterial respiration accelerates the oxygen consumption rate and reduces the time needed for reaching anaerobiosis to approximately 4 h depending on the experimental setup (from 1 to 8 days observed in nutrient-depleted axenic algae cultures). Acetic acid is a key component in our approach to ensure anaerobic environment. By purging of the culture headspace for a few minutes each day with a designed gas mixture containing < 5% (*v*/v) oxygen the important period of acetic acid consumption can be prolonged, the carbon dioxide production might be reduced and the maximum hydrogen production rate can be kept at constant high level. The required frequency of the gas purging is estimated for between 4 and 24 h depending on the experimental setup. Using a light intensity of 50 μmol m^{-2} s^{-1} for illumination of the batch co-cultures the most promising initial algae concentration density is around OD 1 measured at 750 nm, however, higher algae concentrations might also be effective in case light distribution in the co-culture is solved. Algae cell size proved to be another essential factor for hydrogen productivity as well.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.algal.2017.10.024.

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Statement of informed consent, human/animal rights

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

Author contributions

GL designed and carried out the experiments together with DB and AF. VÖ provided access to the MACC and characterized the selected algae strains. PTN executed analytical measurements, TB added useful recommendations for the co-cultivation of the algae and bacteria. GM designed the experiments and composed the manuscript.

Conflict of interest

There is no conflict of interest.

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