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# Water-splitting-based, sustainable and efficient H<sub>2</sub> production in green algae as achieved by substrate limitation of the Calvin–Benson–Bassham cycle

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## Abstract

**Background:** Photobiological H<sub>2</sub> production has the potential of becoming a carbon-free renewable energy source, because upon the combustion of H<sub>2</sub>, only water is produced. The [Fe–Fe]-type hydrogenases of green algae are highly active, although extremely O<sub>2</sub>-sensitive. Sulphur deprivation is a common way to induce H<sub>2</sub> production, which, however, relies substantially on organic substrates and imposes a severe stress effect resulting in the degradation of the photosynthetic apparatus.

**Results:** We report on the establishment of an alternative H<sub>2</sub> production method by green algae that is based on a short anaerobic induction, keeping the Calvin–Benson–Bassham cycle inactive by substrate limitation and preserving hydrogenase activity by applying a simple catalyst to remove the evolved O<sub>2</sub>. Cultures remain photosynthetically active for several days, with the electrons feeding the hydrogenases mostly derived from water. The amount of H<sub>2</sub> produced is higher as compared to the sulphur-deprivation procedure and the process is photoautotrophic.

**Conclusion:** Our protocol demonstrates that it is possible to sustainably use algal cells as whole-cell catalysts for H<sub>2</sub> production, which enables industrial application of algal biohydrogen production.

**Keywords:** Biohydrogen, Calvin–Benson–Bassham cycle, *Chlamydomonas reinhardtii*, Hydrogenase, Oxygen absorbent, Oxygen evolution, Photosynthesis

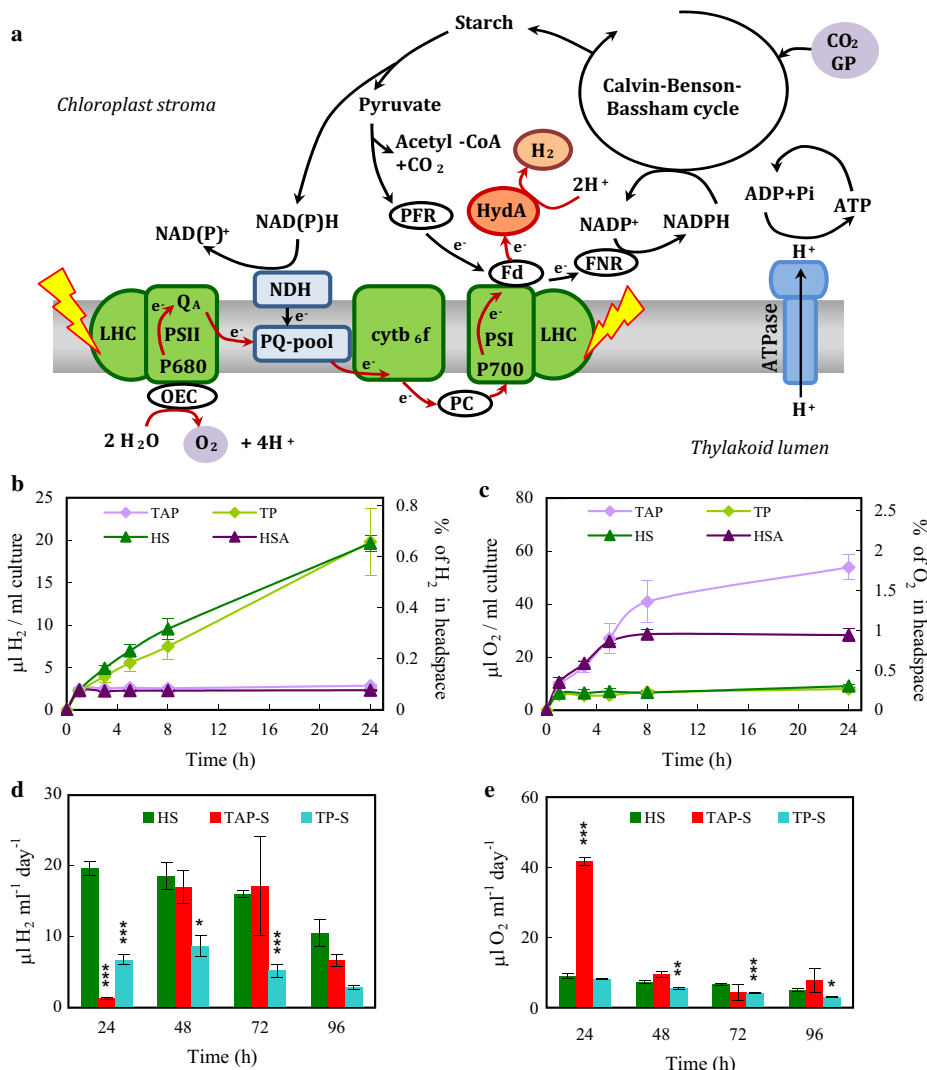
## Background

Among the potential, renewable energy-converting technologies, photobiological H<sub>2</sub> production stands out as an appealing choice, because it is carried out by microorganisms in an aqueous environment, possibly without arable land requirement. Biohydrogen produced by algae may become a genuinely carbon-free energy carrier because, as opposed to bioethanol and biodiesel production, upon the combustion of H<sub>2</sub> only water is produced (reviewed by [1]).

[Fe–Fe]-type hydrogenases found in green algae are one of the most active molecular catalysts known for H<sub>2</sub> production. The green alga *C. reinhardtii* has two [Fe–Fe]-type hydrogenase paralogues, called HydA1 and HydA2; the turnover rate of the major form, HydA1, is several thousands per second, approx. 100-fold higher than that of other type of hydrogenases [2]. Hydrogenases are located in the chloroplasts stroma, at the acceptor side of photosystem I (PSI, Fig. 1a). They may receive electrons from various sources, of which photosynthetic linear electron transport may be the most prominent one. Starch degradation can also feed electrons into the electron transport via the NAD dehydrogenase (NDH) complex, independently of photosystem II (PSII) activity. The third pathway for H<sub>2</sub> production includes pyruvate

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**Fig. 1** H<sub>2</sub> production in *Chlamydomonas reinhardtii*. **a** Schematic presentation of the photosynthetic electron transport chain in green algae. Solar energy is captured by the light-harvesting complexes (LHC) of photosystem II and I (PSII and PSI). Electrons extracted from water by the oxygen-evolving complex (OEC) of PSII are transferred to the photosynthetic electron transport chain via the plastoquinone (PQ)-pool, the cytochrome b<sub>6</sub>f complex (cyt b<sub>6</sub>f), plastocyanin (PC), PSI and ferredoxin (Fd). From Fd, electrons can be transferred by the ferredoxin-NADP<sup>+</sup> oxidoreductase (FNR) to NADP<sup>+</sup> or to the hydrogenase (HydA; for clarity, oxygen-dependent alternative pathways are not shown). H<sup>+</sup> accumulated in the thylakoid lumen are used for ATP production via ATP synthase. The ATP and NADPH generated during primary photosynthetic processes are consumed for CO<sub>2</sub> fixation in the Calvin–Benson–Bassham (CBB) cycle, which produces sugars and ultimately starch. When cultures are grown in the presence of acetate, glycerate 3-phosphate (GP) may also feed the CBB cycle. Hydrogenases are expressed under anoxic conditions; upon illumination, significant H<sub>2</sub> production may occur. The water-oxidation dependent pathway of H<sub>2</sub> production is denoted with red line. Depending on the conditions, starch degradation may also contribute to H<sub>2</sub> production either via the NAD dehydrogenase (NDH) complex, PSII-independent pathway, or via pyruvate-Fd-oxidoreductase (PFR). **b** H<sub>2</sub> production in CC124 *Chlamydomonas* cultures (50 μg chl (a + b)/ml, at 320 μmol photons/m<sup>2</sup>/s) as determined in the headspaces of sealed cultures using gas chromatography during 24 h in acetate-containing (TAP, HSA) and acetate-free media (TP, HS) following dark anaerobic incubation (4 h darkness with 3 × 10 min N<sub>2</sub> flushing). In the right Y axis, the percentage of H<sub>2</sub> in the headspaces of the cultures are shown. Time point 0 indicates the time when the cultures were transferred to the light. **c** Net O<sub>2</sub> production under the same conditions as in **b**. **d** Daily H<sub>2</sub> production in sulphur-containing HS medium of cultures subjected to dark anaerobic incubation and in cultures transferred to sulphur-free acetate-containing (TAP-S) or acetate-free (TP-S) media. All the alga cultures (HS, TAP-S, TP-S) were illuminated continuously and flushed every 24 h with N<sub>2</sub> after determining the gas concentrations in the headspaces of the sealed bottles to avoid excessive H<sub>2</sub> accumulation (cf. [7]) and overpressure in the headspace. **e** Net O<sub>2</sub> production under the same conditions as in **d**. Mean values (± SEM) are each based on 6 biological replicates. Statistical significance levels are presented relative to the *Chlamydomonas* culture subjected to dark anaerobic incubation in HS medium as \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001

oxidation through pyruvate–ferredoxin-oxido-reductase (PFR; reviewed by [1]).

Hydrogenases become highly expressed under hypoxic conditions established for instance during the night when the microbial community consumes the available O<sub>2</sub>; upon illumination, electrons from ferredoxin (FDX) in PSI are transferred to the hydrogenases. Out of the 13 types of FDXs found in *Chlamydomonas* [3], FDX1 and FDX2 contribute to H<sub>2</sub> production [4, 5]. The midpoint redox potential of the major photosynthetic FDX1 is −0.398 V enabling efficient electron donation to HydA [6, 7].

Hydrogenases catalyze the reduction of protons, thereby supporting an alternative electron transport to prevent the over-reduction of the photosynthetic electron transport chain upon dark-to-light transitions. By this reaction, hydrogenases also promote the light-induced increase of stromal pH necessary for the activation of the Calvin–Benson–Bassham (CBB) cycle, ultimately supporting ATP formation (reviewed by, e.g. [8, 9]). Thus, hydrogenases play an essential role upon the induction of photosynthesis in green algae [10]. Once the electron transport is fully functional, the O<sub>2</sub> evolved by PSII reacts with hydrogenases, leaving an inactive [4Fe–4S] subcluster state [11] and with O<sub>2</sub> also inhibiting *HYDA1* gene expression [12, 13].

Owing to the high theoretical efficiency of converting the energy of sunlight into chemical energy [2], photobiological H<sub>2</sub> production by green algae has been studied for decades with O<sub>2</sub> sensitivity of the hydrogenases being a major hurdle in reaching commercial viability. Engineering O<sub>2</sub>-insensitive hydrogenases has been attempted with moderate success (e.g. [14]). Alternatively, hypoxia can be established by downregulating PSII activity, most commonly by sulphur deprivation (e.g. [15], reviewed recently by [16]). However, this method is unspecific and results in the degradation of photosynthetic complexes and cell death on the timescale of days [17]. H<sub>2</sub> production induced by sulphur deprivation is also strongly dependent on acetate or other organic carbon sources, meaning that in a narrow sense it is not a photoautotrophic process [18, 19], and has a low inherent energy conversion efficiency [20]. As it stands, it is very unlikely that sulphur deprivation will represent a viable procedure for industrial H<sub>2</sub> production [20]. Nitrogen [21], phosphorous [22] and magnesium starvations [23] have been also attempted, but pose similar problems to sulphur deprivation.

H<sub>2</sub> production can also be induced in nutrient-replete Tris–acetate–phosphate (TAP) medium, by incubating *Chlamydomonas* cultures in the dark for a few hrs in O<sub>2</sub>-free atmosphere and exposing them to relatively low light. Under these conditions, H<sub>2</sub> production is

temporary, because the evolved O<sub>2</sub> inhibits the hydrogenases within a few hrs (reviewed by [24]). By incubating the cultures under very low light conditions, a balanced O<sub>2</sub> evolution and respiration can be established, resulting in low-yield H<sub>2</sub> production lasting for several days [25]. This type of H<sub>2</sub> production depends largely on photosynthesis and partly on fermentative processes [25]. The yield of H<sub>2</sub> production can be increased by enhancing acetate respiration [26], which, however, represents a severe loss of efficiency, as for each H<sub>2</sub> produced an equivalent amount of substrate is respired [27]. A direct and efficient conversion of solar energy to H<sub>2</sub> in a carbon–neutral way is required for commercial viability [20, 28]. Anaerobiosis-induced H<sub>2</sub> production has been observed in photoautotrophic (i.e. acetate-free, CO<sub>2</sub>-supplemented) cultures as well, in which H<sub>2</sub> production lasted for several days at low light [29, 30]), although with a very low efficiency as compared to the widely used sulphur-deprivation method in TAP medium. Thus, in recent protocols applying anaerobic incubation the CBB cycle was operational, even though the competing nature of CO<sub>2</sub> assimilation and H<sub>2</sub> production was already shown by Gaffron and Rubin [31] upon discovering the photoproduction of H<sub>2</sub> by green algae.

The present approach to improve photobiological H<sub>2</sub> production stem from the ultimate physiological role of the highly efficient hydrogenases found in green algae (Fig. 1a). Their primary function is to serve as a safety valve [32], i.e. alternative electron acceptors upon the induction of photosynthesis in hypoxia [10, 33]; once the photosynthetic apparatus is fully functional, the hydrogenases become inactive by the evolved O<sub>2</sub>. We show here that by preventing the activation of the CBB cycle in the light via substrate limitation (i.e. by omitting CO<sub>2</sub> or acetate), the photosynthetic electron transport chain remains largely reduced, resulting in low O<sub>2</sub> evolution and high H<sub>2</sub> production lasting for several days. The additional application of an iron-based O<sub>2</sub> absorbent resulted in yields that are significantly greater than for the standard sulphur deprivation procedure.

## Results

### Dark anaerobic incubation of *Chlamydomonas* cultures followed by continuous illumination in acetate-free media results in substantial H<sub>2</sub> production lasting for several days

Dark anaerobic incubation treatments to induce hydrogenase expression were carried out with the CC124 strain of *Chlamydomonas reinhardtii*, because it is a relatively efficient H<sub>2</sub> producer and has been successfully used under various conditions (e.g. [34–37]). After growing the cultures for 3 days in TAP medium, the cells were transferred to culture media with (TAP, HSA) or without acetate (TP, HS) with a chlorophyll (chl) (a + b) content

set at 50  $\mu\text{g/ml}$ . Hydrogenase expression was induced by a 4-h dark anaerobic incubation during which  $\text{N}_2$  flushing was applied to remove both  $\text{O}_2$  and  $\text{CO}_2$ . The high chl (a + b) content was set to facilitate the establishment of anaerobiosis, PQ-pool reduction and high hydrogenase expression [38]. For a general scheme of the experimental setup, see Additional file 1: Fig. S1.

As opposed to most earlier studies in nutrient-replete conditions, we subjected the cultures to relatively high light intensities (320  $\mu\text{mol photons/m}^2/\text{s}$  provided by white fluorescent tubes) after the dark anaerobic induction. Upon light exposure,  $\text{H}_2$  production (approx. 2  $\mu\text{l H}_2/\text{ml culture}$ ) was observed during the first hour in all growth media. In acetate-containing media (TAP and HSA), prolonged illumination did not result in further  $\text{H}_2$  production, whereas in acetate-free media (HS, TP)  $\text{H}_2$  production continued (approx. 20  $\mu\text{l H}_2/\text{ml culture}$  in 24 h, Fig. 1b). The amount of  $\text{O}_2$  in the headspace of the vials rapidly accumulated in the presence of acetate (Fig. 1c), whereas in its absence the  $\text{O}_2$  concentration remained at a low level (approx. 9  $\mu\text{l O}_2/\text{ml culture}$  was produced, corresponding to approx. 0.3%  $\text{O}_2$  in the headspace). As cultures kept in HSA or TAP had low  $\text{H}_2$  yields and both TP and HS cultures produced large amounts of  $\text{H}_2$ , the increase in  $\text{H}_2$  production efficiency was attributed to the absence of acetate. Therefore, in the following experiments, we opted for the HS media, as it is commonly used as a minimal media for studying algal physiology (e.g. [39]).

Next, we compared the efficiency of  $\text{H}_2$  production following a dark anaerobic induction in HS medium (performed as described above) with the classical sulphur deprivation method that is largely dependent on acetate [18, 40]. In cultures induced by dark anaerobiosis in HS media approx. 20  $\mu\text{l H}_2/\text{ml}$  (=16.32 nmole  $\text{H}_2/\mu\text{g chl}$  (a + b)) was produced during the first 24 h (Fig. 1d). The produced gases were removed every 24 h by  $\text{N}_2$  flushing after determining the amount of gases produced to promote the establishment of hypoxia, and to avoid a high  $\text{H}_2$  partial pressure [7] and overpressure in the headspace of the cultures. Using this method, the total  $\text{H}_2$  production in HS media was approx. 65  $\mu\text{l H}_2/\text{ml culture}$  in 96 h (Fig. 1d, sum of the green columns). In the case of sulphur deprivation (TAP-S, Fig. 1d),  $\text{H}_2$  production was low during the first 24 h, with the maximum  $\text{H}_2$  output detected after 48 and 72 h; the total production was approx. 42  $\mu\text{l H}_2/\text{ml culture}$  in 96 h (Fig. 1d, sum of the red columns). This productivity is consistent with earlier results obtained with the CC124 strain when using sealed flasks (e.g. [37, 41]), but it is below the yields attained with improved photobioreactor (PBR) systems on the same timescale (approx. 200  $\mu\text{l H}_2/\text{ml culture}$  in 96 h, [42]). When acetate was omitted from the media of

cultures subjected to sulphur deprivation (TP-S, Fig. 1d), the amount of  $\text{H}_2$  produced was strongly diminished, corroborating the notion that the sulphur deprivation procedure is acetate-dependent [18, 40].

After 24 h of illumination, approx. 9  $\mu\text{l O}_2/\text{ml}$  was accumulated in the HS and TP-S cultures, whereas 40  $\mu\text{l O}_2/\text{ml culture}$  accumulated in the headspaces of sulphur-deprived samples (corresponding to approx. 1.3%  $\text{O}_2$ , Fig. 1e).

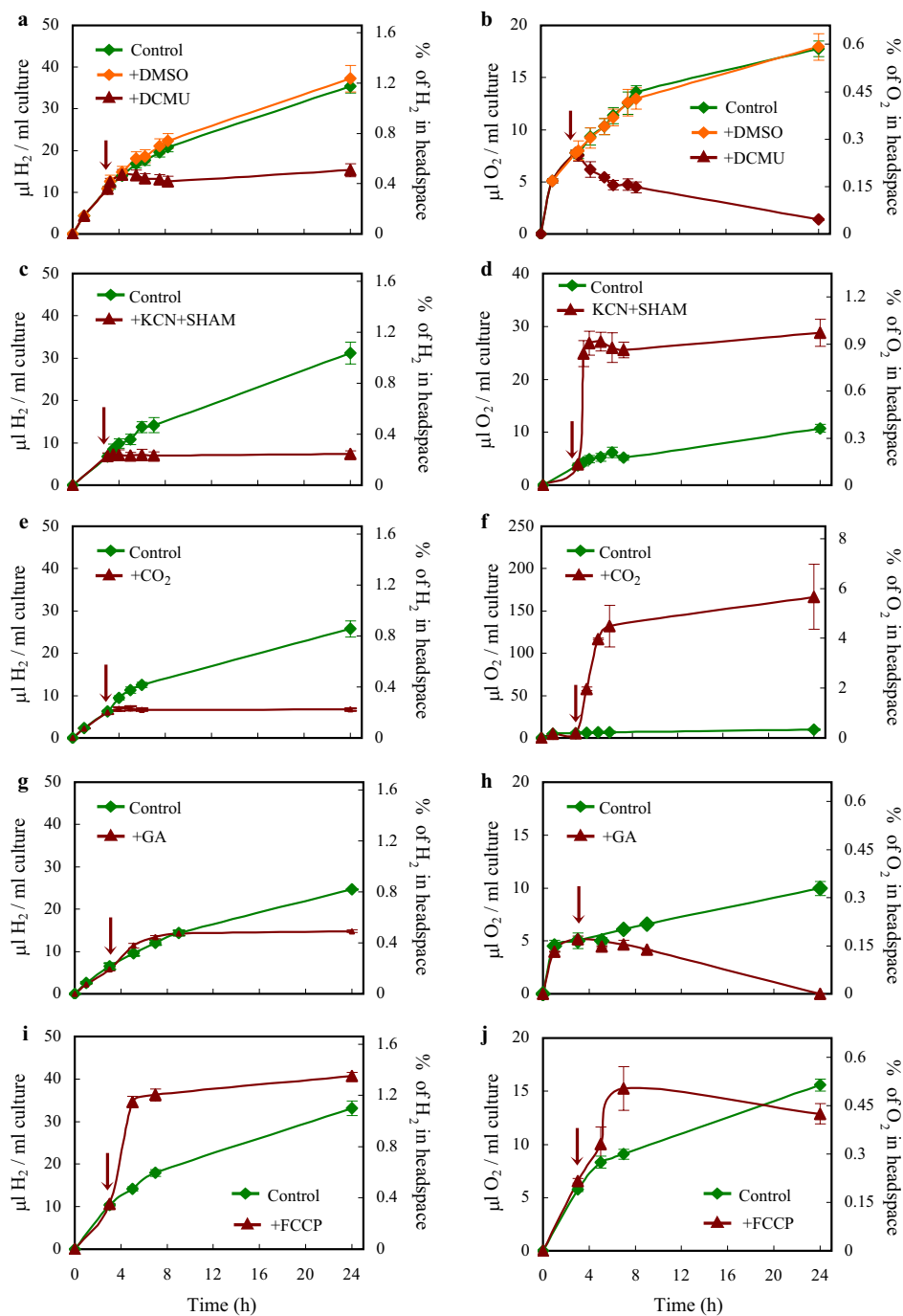
These results demonstrate that anaerobic induction of hydrogenases in minimal (i.e. acetate-free) media can result in sustained  $\text{H}_2$  production of several days, with yields higher than in TAP-S media at equivalent culture conditions.

#### Origin and regulation of $\text{H}_2$ production in acetate-free media

Since the  $\text{H}_2$  production induced by anaerobic incubation is carried out in acetate-free media, and no sulphur deprivation is involved that would induce starch accumulation [17], electrons supporting  $\text{H}_2$  production likely originated directly from water (Fig. 1a). To test this hypothesis, we first measured the starch content throughout the experiment (Additional file 2: Fig. S2). The starch content of control, aerobic cultures was about 0.25 mmol/l, typical of aerobic, non stressed samples [43]. Following the 4-h dark-incubation starch content decreased to about 0.05 mmol/l. Upon transfer to the light (i.e. the start of  $\text{H}_2$  production) the starch content increased but did not reach the control level. Between 24 and 96 h of  $\text{H}_2$  production, there was no major change in the starch content, indicating that starch degradation does not contribute significantly to  $\text{H}_2$  production.

Next, we treated the cells with 3-(3',4'-dichlorophenyl)-1,1-dimethylurea (DCMU), which irreversibly binds to the  $\text{Q}_\text{B}$  site in PSII [44]. Upon the application of DCMU, the amount of  $\text{O}_2$  in the headspace decreased and  $\text{H}_2$  production ceased with a slight delay (Fig. 2a, b), possibly due to degassing of  $\text{H}_2$  from the media. DMSO-treatment used as a control resulted in no discernable effect on  $\text{H}_2$  and  $\text{O}_2$  productions relative to the control (Fig. 2a, b). These results demonstrate the direct dependence of  $\text{H}_2$  production on PSII electron transport and water-splitting, and suggest that the contribution of cellular respiration to  $\text{H}_2$  production is minor.

To test whether respiration contributes to the establishment of hypoxic conditions, we treated the cultures with potassium cyanide (KCN, inhibiting the terminal oxidase) and salicylhydroxamic acid (SHAM, inhibiting the alternative oxidase). Upon their addition, the  $\text{O}_2$  concentration in the headspace of the cultures increased suddenly from approx. 0.3 to 0.9%, and remained at this level thereafter (Fig. 2d). Upon the



**Fig. 2** Photosynthetic electron transport during H<sub>2</sub> production of *Chlamydomonas* in acetate-free HS medium induced by dark anaerobic incubation. **a, b** H<sub>2</sub> and net O<sub>2</sub> productions upon the addition of the PSII inhibitor DCMU, and DMSO, used as a control. **c, d** H<sub>2</sub> and net O<sub>2</sub> productions upon the addition of the respiratory inhibitors potassium cyanide (KCN) and salicylhydroxamic acid (SHAM). **e, f** H<sub>2</sub> and net O<sub>2</sub> productions upon the addition of 2% CO<sub>2</sub>. **g, h** H<sub>2</sub> and net O<sub>2</sub> productions upon the addition of the CBB cycle inhibitor glycolaldehyde (GA). **i, j** H<sub>2</sub> and net O<sub>2</sub> productions upon the addition of and the ionophore carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP). The time points at which the various treatments were applied are indicated by arrow. In the right Y axis, the percentage of the corresponding gases in the headspaces of the cultures are shown. All experiments were carried out at 320 µmol photons/m<sup>2</sup>/s following the 4-h dark anaerobic incubation (0 time point). Mean values (± SEM) are each based on 4 or 5 biological replicates

application of the respiratory inhibitors, H<sub>2</sub> production ceased (Fig. 2c), probably as a result of the increased O<sub>2</sub> concentration.

In *Chlamydomonas*, acetate assimilation may occur via the tricarboxylic acid cycle and the glyoxylate cycle, which are metabolically linked to gluconeogenesis and the oxidative pentose phosphate pathway [45, 46]. The released CO<sub>2</sub> and glycerate 3-phosphate feed the CBB cycle for which the reducing power generated by the photosynthetic electron transport is also utilized (Fig. 1a), as indicated by the extreme light sensitivity of various Rubisco mutants [47, 48]. We hypothesized that in the absence of acetate and CO<sub>2</sub>, the CBB cycle is mostly inactive and the electrons originating from the photosynthetic electron transport chain are transferred to the hydrogenases. To ascertain about this, we added 2% CO<sub>2</sub> (v/v) into the headspace of the cultures. As a result, H<sub>2</sub> production ceased, whereas O<sub>2</sub> production strongly increased (Fig. 2e, f). Concomitantly, the amount of CO<sub>2</sub> in the headspace of the cultures diminished rapidly and after 24 h no CO<sub>2</sub> could be detected (Additional file 3: Table S1).

When the CBB cycle inhibitor glycolaldehyde (GA) was added, H<sub>2</sub> production was unchanged during the initial 9 h, whereas the O<sub>2</sub> concentration in the headspace decreased (Fig. 2g, h). By the 24th hour, H<sub>2</sub> production ceased and no O<sub>2</sub> could be detected, which is most probably due to a side-effect of GA on photosynthesis [49]. These results demonstrate that the CBB cycle activity, which would compete for electrons with the hydrogenases, must be low. There may be some remaining activity only at the beginning of illumination explaining the transitional increase in starch content following the dark-anaerobic incubation (first 24 h in the light, Additional file 2: Fig. S2).

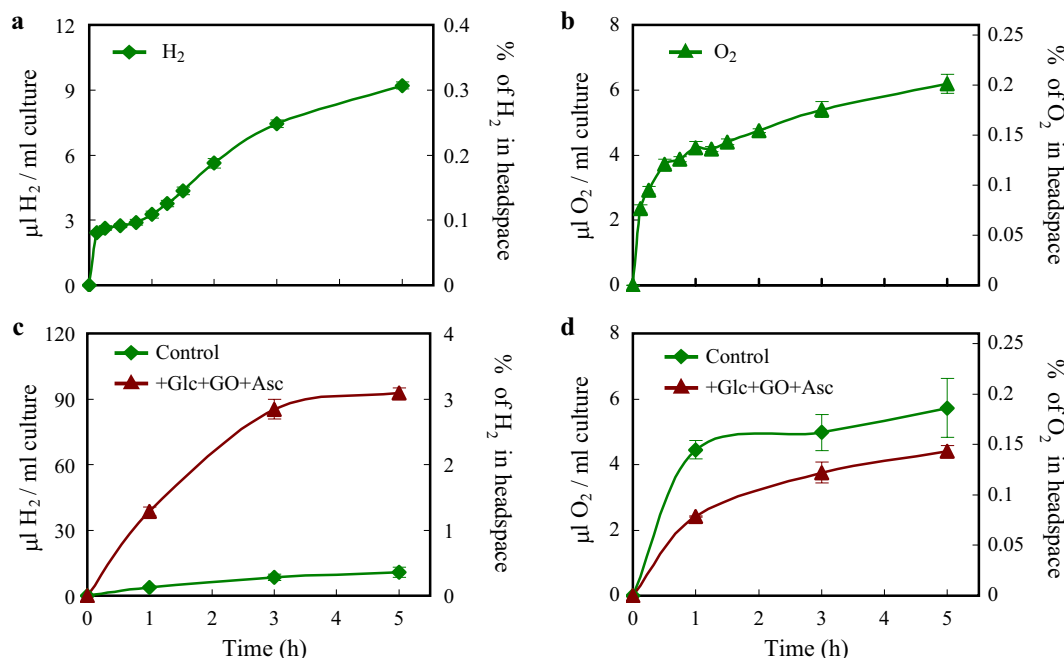
Upon low substrate availability for the CBB cycle, the consumption of ATP and NADPH is decreased, triggering adjustments of the light reactions to prevent damage to the photosynthetic apparatus. Under these conditions, PSI cyclic electron transport is increased, contributing to a strong thylakoid lumen acidification, which triggers photoprotective quenching mechanisms to dissipate excess energy (reviewed by, e.g. [50]). Lumen acidification also slows down the oxidation of plastoquinol by the cytochrome (cyt) b<sub>6</sub>f complex, resulting in a reduced PQ-pool and decreased electron transport from PSII; by this so-called “photosynthetic control” mechanism, the accumulation of electrons on PSI is prevented, which can otherwise lead to photodamage [51–53]. Under these conditions, the rate of charge recombination in PSII increases and the rate of O<sub>2</sub> evolution decreases [54]. The excess reducing power in the photosynthetic electron transport may be alleviated by alternative pathways

in *Chlamydomonas*, including the Mehler reaction, the malate shuttle, the plastid terminal oxidase and the flavodiiron-dependent photoreduction reduction pathways (reviewed by [50, 55]). However, these pathways are O<sub>2</sub>-dependent and under hypoxic conditions electron transport to the hydrogenases may represent a more suitable safety valve. To test the possibility that thylakoid lumen acidification in the light may limit linear electron transport and thereby O<sub>2</sub> and H<sub>2</sub> production, we treated the cultures with the ionophore carbonylcyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) in the light [56, 57]. Upon the addition of FCCP, H<sub>2</sub> evolution increased promptly, followed by a transitory increase in O<sub>2</sub> evolution (Fig. 2i, j), in agreement with the notion that a high ΔpH may limit linear electron flow to the hydrogenases [58].

The fast chl *a* fluorescence (OJIP) transient is a sensitive and widely used indicator of photosynthetic function (e.g. [59]); its F<sub>0</sub> and F<sub>J</sub> values can be used to estimate the redox status of the PQ-pool [60, 61]). The latter was evaluated in cell cultures collected directly from serum bottles during H<sub>2</sub> production experiments, with no dark-adaptation prior to the measurement (Additional file 4: Fig. S3). F<sub>0</sub> and F<sub>J</sub> values were relatively high in the cultures producing H<sub>2</sub>, indicating that the PQ-pool was in a reduced state. This result corroborates that “photosynthetic control” is an important factor in the establishment of the H<sub>2</sub> producing conditions.

When measuring the rate of H<sub>2</sub> production with a higher time resolution in continuous light following dark anaerobic incubation (Fig. 3a), we observed a burst of H<sub>2</sub> production, as typically occurring in such dark–light transitions [62]. Approximately 2.3 μl H<sub>2</sub>/ml culture was produced in 8 min, corresponding to a rate of 15.76 μmol H<sub>2</sub>/mg chl (a + b)/h. This was followed by a lower H<sub>2</sub> production rate (1.31 μmol H<sub>2</sub>/mg chl (a + b)/h between 1 and 5 h) during which approx. 0.2% O<sub>2</sub> accumulated gradually in the gas phase (Fig. 3b). The [Fe–Fe]-type hydrogenases of *C. reinhardtii* have an I<sub>50</sub> of 0.3–0.4% O<sub>2</sub> as determined upon a 2-min incubation in vivo [63], with a very minor fraction remaining active at atmospheric O<sub>2</sub> levels [64]. Thus, a prolonged exposure to 0.2% O<sub>2</sub> may explain the decreasing rate of H<sub>2</sub> production.

A way to keep the hydrogenases active may be eliminating the produced O<sub>2</sub> [65]. As a first approach, we added glucose (Glc), glucose oxidase (GO) and ascorbate (Asc) to the cultures to scavenge O<sub>2</sub> [60]. The separate additions of Glc, GO and Asc had minor effects on the amount of evolved O<sub>2</sub> and H<sub>2</sub> (Additional file 5: Table S2). Upon the combined Glc + GO + Asc treatment, the amount of O<sub>2</sub> in the headspace was reduced (Fig. 3d), and concomitantly, the amount of H<sub>2</sub> produced in 3 h increased tenfold, from approx. 8–85 μl H<sub>2</sub>/ml culture (equal to 1.36



**Fig. 3** The effect of O<sub>2</sub> on the H<sub>2</sub> production of *Chlamydomonas* in acetate-free HS medium induced by dark anaerobic incubation. **a, b** Detailed time courses of H<sub>2</sub> and net O<sub>2</sub> productions during the initial 5 h of illumination at 320 μmol photons/m<sup>2</sup>/s. **c, d** The effects of glucose (Glc, 2 mM), glucose oxidase (GO 0.2 mg/ml) and ascorbate (Asc, 1 mM) on H<sub>2</sub> and net O<sub>2</sub> productions, added after the dark anaerobic incubation. In the right Y axis, the percentage of the corresponding gases in the headspaces of the cultures are shown. Mean values (± SEM) are each based on 4–8 biological replicates

and 23.13 μmol H<sub>2</sub>/mg chl (a + b)/h, respectively; Fig. 3c). These values correspond to light-to-H<sub>2</sub> energy conversion efficiencies of 0.29 and 2.95% in the absence and presence of Glc + GO + Asc, respectively (Table 1, see “Materials and methods” for the calculations). However, this treatment is not a viable option for a long-term H<sub>2</sub> production because of the Glc requirement and the concomitant reactive oxygen species production [66]; thus we searched for another possibility.

**Preserving hydrogenase activity using an iron-salt based O<sub>2</sub>absorbent**

Decreasing the amount of O<sub>2</sub> in the cultures can be achieved by (i) downregulating PSII activity (e.g. [15, 67]), which is precarious, because PSII is the main source of reducing power; (ii) increasing the respiration:photosynthesis ratio [25], which is limited to low light intensities and requires significant amounts of organic substrates overall leading to low energy conversion efficiencies; and (iii) bacterial respiration [35], with the drawback that it also requires acetate or other organic carbon source. Intense flushing with ultra-pure helium has been adopted to preserve hydrogenase activity [65, 68]; hemoglobin, myoglobin and cobalt chelates have

**Table 1 Light-to-H<sub>2</sub> energy conversion efficiency in *Chlamydomonas reinhardtii* in HS medium under various conditions**

Conditions of H <sub>2</sub> production	Time of illumination following dark anaerobic incubation (h)				
	0.25	1	3	5	24
Control (HS)					
H <sub>2</sub> produced (μl/ml)	3.46	3.86	8.41	10.80	27.47
Efficiency (%)	1.43	0.40	0.29	0.22	0.11
+ Glc + GO + Asc					
H <sub>2</sub> produced (μl/ml)	n.d.	38.57	85.33	92.68	117.04
Efficiency (%)	n.d.	4.00	2.95	1.92	0.51
+ O <sub>2</sub> absorbent					
H <sub>2</sub> produced (μl/ml)	3.85	6.73	13.09	19.20	62.10
Efficiency (%)	1.60	0.70	0.45	0.40	0.27

Algal cultures containing 50 μg chl (a + b)/ml and dark-incubated for 4 h were illuminated using cool white fluorescent tubes, at an intensity of 52 W/m<sup>2</sup>, corresponding to approx. 320 μmol photons/m<sup>2</sup>/s. See “Materials and methods” for the calculations on the incident light-to-H<sub>2</sub> conversion efficiency. The experiments were carried out in HS medium (control) and the effects of a combined glucose (Glc, 2 mM), glucose oxidase (GO, 0.2 mg/ml) and ascorbate (Asc, 1 mM) treatment and the effects of an iron-salt O<sub>2</sub> absorbent were also assessed

nd non-determined

also been used in short-term experiments to remove the evolved O<sub>2</sub> [69].

Here, we opted for using a chemical O<sub>2</sub> absorbent, a mixture of iron powder and sodium chloride, widely used in the food industry (for details, see “[Experimental procedures](#)”). It is highly active (1 g absorbent can absorb up to 13 ml O<sub>2</sub> at room temperature; e.g. [70]), biologically safe and very cheap (O20TM; <http://www.o2zero.com>, 20 cc). We placed a small amount (approximately 1.3 g) of O<sub>2</sub> absorbent into a 2-ml vial and introduced it into the headspace of the serum bottle, above the *Chlamydomonas* culture (Fig. 4a). This system resulted in approx. twofold higher H<sub>2</sub> productions (Fig. 4b), reaching approx. 200 μl H<sub>2</sub>/ml culture in 96 h [equal to approx. 163.3 μmol H<sub>2</sub>/mg chl (a + b)]. The concentration of O<sub>2</sub> in the headspace accumulated in 24 h was lowered to approx. 2.1 μl O<sub>2</sub>/ml culture (Fig. 4c), corresponding to 0.07% O<sub>2</sub> in the headspace.

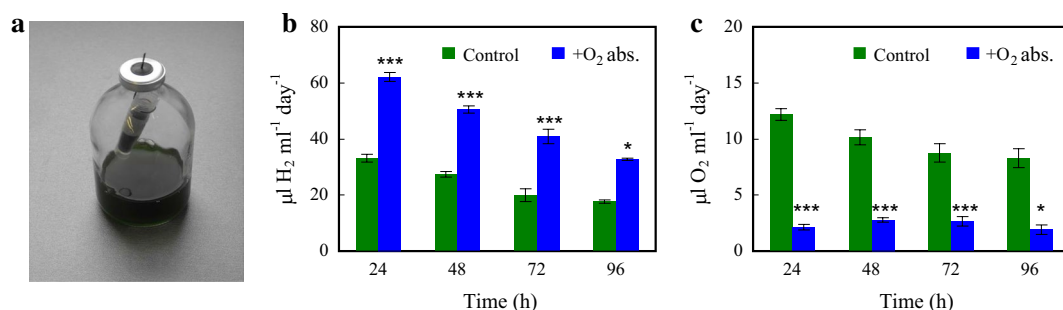
The light-to-H<sub>2</sub> energy conversion efficiency was approx. 1.5% both in the absence and presence of the O<sub>2</sub> absorbent following the anaerobic incubation and 15 min of illumination. However, after 24 h of illumination the light-to-H<sub>2</sub> energy conversion efficiency decreased to approx. 0.11% in the control samples (HS), whereas it was more than two times higher (approx. 0.27%) when the O<sub>2</sub> absorbent was present (Table 1). In earlier sulphur deprivation experiments carried out in PBRs and in the presence of acetate, the maximum light-to-H<sub>2</sub> conversion efficiency was in the range 0.13–3.22%, whereas in nutrient-replete conditions with acetate, it was much lower, approx. 0.1% (see Table 3 in [25], for a comparison on various H<sub>2</sub> producing conditions).

We have also compared the H<sub>2</sub> production yields at three different chl concentrations. In the absence of the O<sub>2</sub> absorbent, the total amounts of H<sub>2</sub> produced in 96 h

were approx. 50, 90 and 105 μl/ml culture at 15, 30 and 50 μg chl (a + b)/ml culture, respectively. In the presence of the O<sub>2</sub> absorbent, productivities increased to approx. 120, 210 and 227 μl H<sub>2</sub>/ml culture, respectively (Additional file 6: Fig. S4). Thus, reducing the chl content from 50 to 15 μg chl (a + b)/ml improved H<sub>2</sub> production on a chl basis even in the absence of the O<sub>2</sub> absorbent (from 2.1 to 3.3 ml H<sub>2</sub>/mg chl (a + b), respectively).

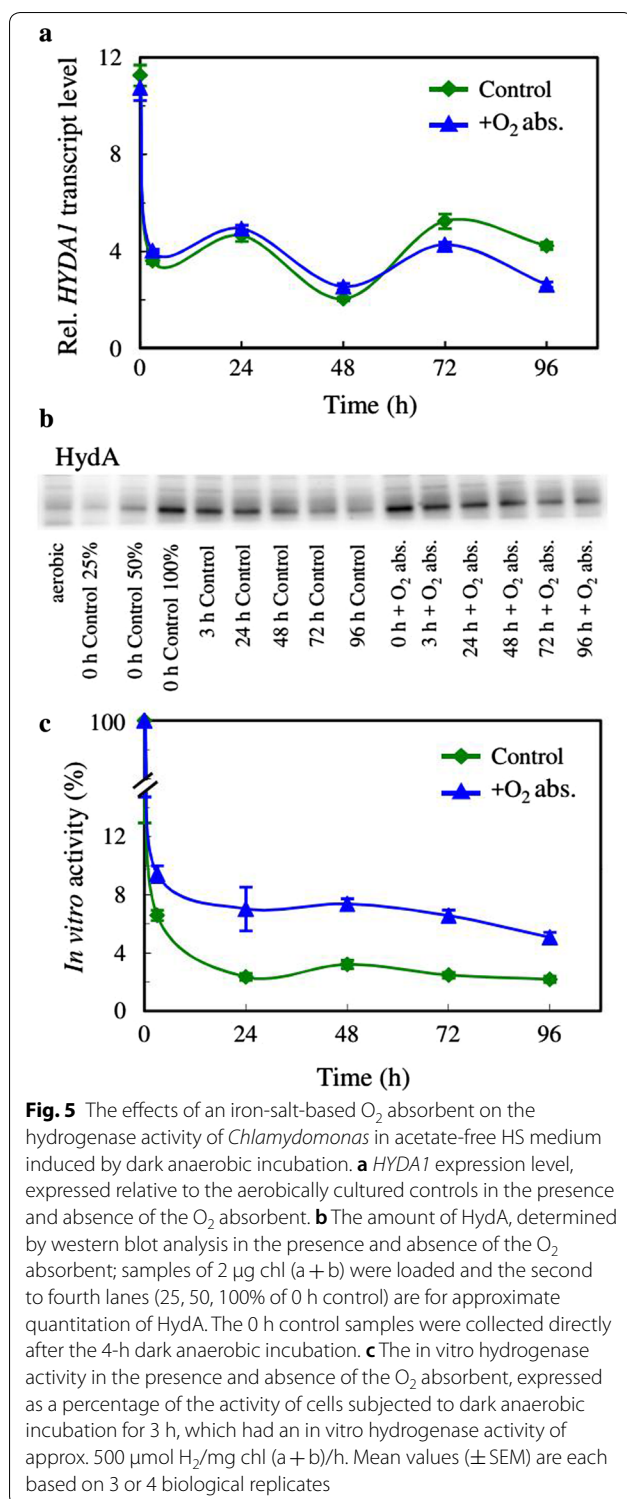
Daily N<sub>2</sub> flushing was applied to periodically remove the produced gases. To test whether this was essential, we carried out an experiment without gas removal. In the absence of the O<sub>2</sub> absorbent and without N<sub>2</sub> flushing, the amount of H<sub>2</sub> produced was reduced by about 50%. However, when O<sub>2</sub> absorbent was present, the amounts of H<sub>2</sub> were in the same range with our without N<sub>2</sub> flushing (Additional file 7: Fig. S5).

Next, we assessed the expression and the activity of hydrogenases in the presence and absence of the O<sub>2</sub> absorbent. During the 4-h dark anaerobic incubation period, the *HYDA1* transcript level increased approx. 11-fold, as determined by quantitative RT-PCR analysis (Fig. 5a), which was followed by a decrease both in the presence and absence of the O<sub>2</sub> absorbent, possibly due to the strong inhibitory effect of O<sub>2</sub> on *HYDA1* expression [12, 13]. The amount of HydA enzyme as determined semi-quantitatively by western blot analysis, also showed a strong upregulation upon the 4-h dark anaerobic incubation period. Later on, the amount of HydA decreased, which was attenuated by the O<sub>2</sub> absorbent (Fig. 5b). Following the dark anaerobic incubation, the in vitro hydrogenase activity, as determined in the presence of reduced methylviologen as an electron donor, was approx. 500 μmol H<sub>2</sub>/mg chl (a + b)/h, in agreement with literature data ([24]; see “[Experimental procedures](#)” for details). Upon 4 h of illumination, the in vitro



**Fig. 4** The effect of an iron-salt-based O<sub>2</sub> absorbent (O20TM) on the H<sub>2</sub> production of *Chlamydomonas* in acetate-free HS medium induced by dark anaerobic incubation. **a** Photograph of the H<sub>2</sub>-producing *Chlamydomonas* cultures in serum bottles with the iron-salt-based O<sub>2</sub> absorbent introduced into the headspace. **b, c** The effects of the iron-salt-based O<sub>2</sub> absorbent on the daily H<sub>2</sub> production and daily O<sub>2</sub> accumulation in *Chlamydomonas* cultures illuminated continuously at 320 μmol photons/m<sup>2</sup>/s. The cultures were flushed with N<sub>2</sub> for 10 min every 24 h after determining the gas concentrations in the headspaces of the sealed bottles. Mean values (± SEM) are each based on 11–15 biological replicates. Statistical significance levels are presented relative to the *Chlamydomonas* culture subjected to dark anaerobic incubation in HS medium as \**p* < 0.05; \*\*\**p* < 0.001





hydrogenase activity decreased to approx. 6 and 9% of the original activity in the absence and presence of the O<sub>2</sub> absorbent, respectively (Fig. 5c), highlighting the extreme O<sub>2</sub> sensitivity of hydrogenases [63]. Following this rapid decline, there was a slower phase of inactivation, and

by the end of the experiment 2% of the original in vitro activity remained in the absence of the O<sub>2</sub> absorbent, while 5% remained in its presence (Fig. 5c).

### Photosynthetic activity during H<sub>2</sub> production

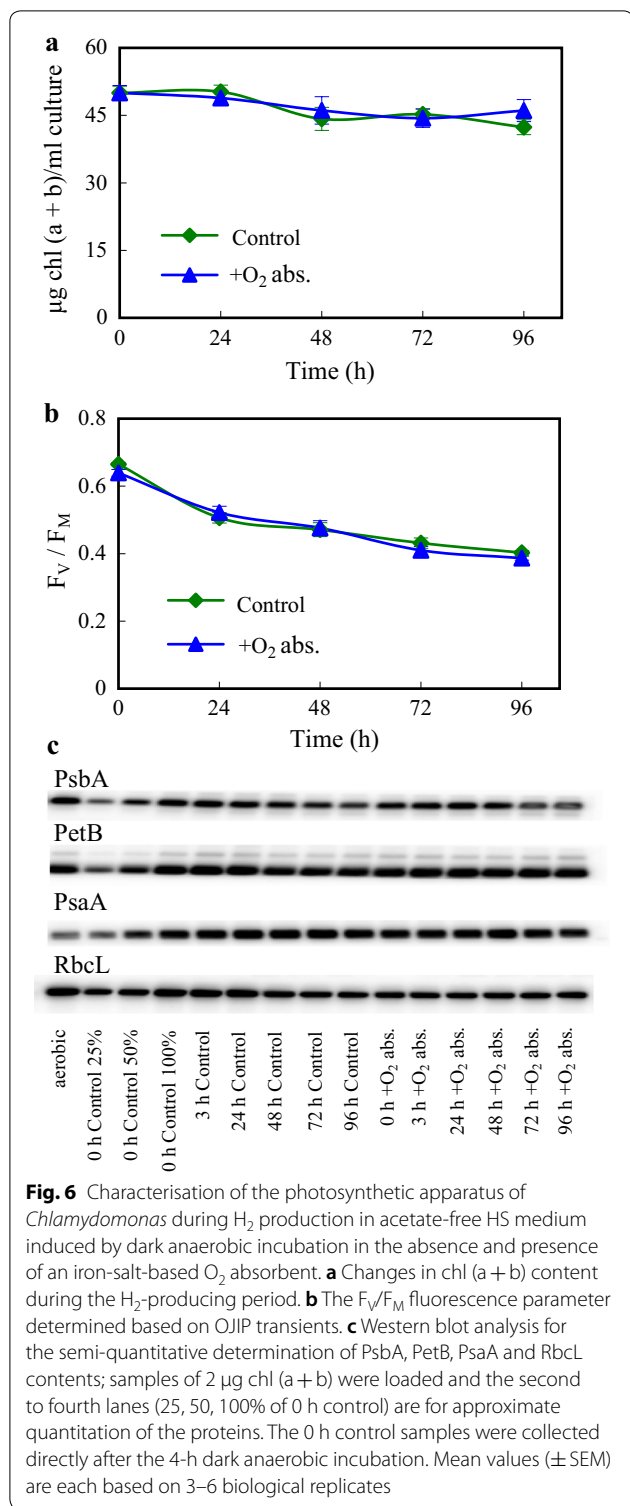
The maintainability of our system was tested by characterization of the photosynthetic apparatus during the 96-h H<sub>2</sub>-producing period. The chl (a + b) content decreased only by approx. 10% during the 96-h experiment (Fig. 6a). The  $F_V/F_M$  value, an indicator of PSII efficiency, slowly decreased during the 96-h H<sub>2</sub> producing period, but remained relatively high (above 0.4) both in the presence and absence of the O<sub>2</sub> absorbent (Fig. 6b).

The amount of PsbA (reaction center protein of PSII), PetB (a subunit of the cytb<sub>6</sub>/f complex) and PsaA (reaction center protein of PSI) remained largely unaltered during the 96-h H<sub>2</sub>-producing period, both in presence and absence of the O<sub>2</sub> absorbent, as determined by western blot analysis (Fig. 6c); the amount of the large Rubisco subunit, RbcL, showed a moderate decrease (Fig. 6c). These findings are in strong contrast to the sulphur deprivation protocol in TAP medium, where the amount of Rubisco and PsbA strongly diminish within 48 h. By the end of a 4–6-day period of sulphur deprivation most photosynthetic complexes are degraded and the cells eventually die [15, 17, 41, 71].

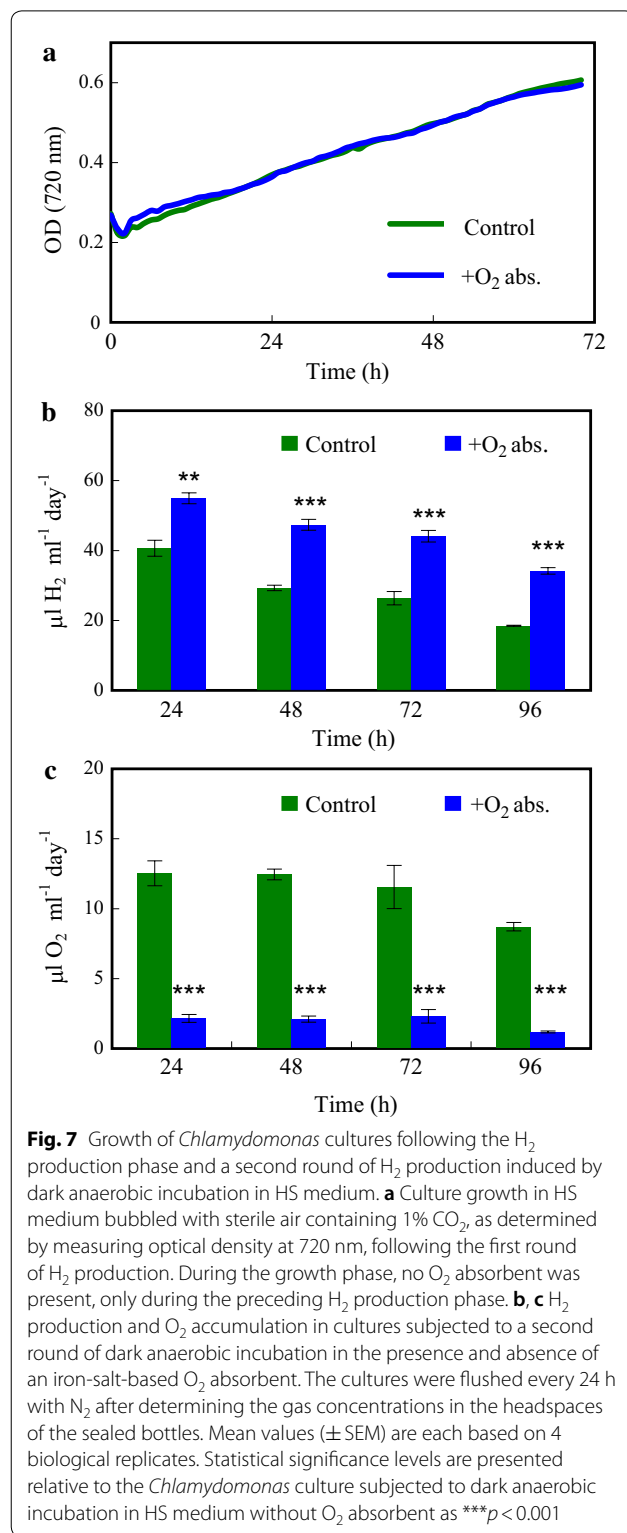
Since our cells remained photosynthetically active, we attempted recovering and reusing the cultures after the H<sub>2</sub>-producing period. Cultures were diluted with HS medium to 6 μg chl (a + b), transferred into a multi-well cultivation instrument and sparged with air containing 1% CO<sub>2</sub> for 72 h. The algal cultures showed discernible growth in the HS medium and the cell density doubled within approx. 24 h, as estimated based on optical density (Fig. 7a). When these cells were subjected to a second dark anaerobic incubation period in HS media (as described above), H<sub>2</sub> production occurred with yields similar to the first cycle (cf. Figs. 4b, c, 7b, c).

### Discussion

The potential energy conversion efficiency from sunlight to H<sub>2</sub> by green algae is in the range of 10–13% [14, 72]. However, in nature H<sub>2</sub> production lasts only for a few minutes due to the inhibition of hydrogenases by the evolved O<sub>2</sub> [20, 27]. Early studies on algal H<sub>2</sub> production based on dark anaerobic incubation were typically unable to sustain the initial high rates of H<sub>2</sub> production for more than a few hrs, if not resorting to continuous flushing with helium [31, 65, 68]. Later, sulphur deprivation became the method of choice to induce long-term H<sub>2</sub> production [15, 17, 42]. However, sulphur deprivation has several drawbacks which impede its industrial application [20, 72]: the procedure requires several washing



steps; H<sub>2</sub> production starts with a delay of about 2 days; it is largely dependent on acetate (H<sub>2</sub> production can be induced under photoautotrophic conditions as well, but with a much lower efficiency [18–20]); it necessitates



the inactivation of PSII; and it results in the degradation of the photosynthetic machinery. Recovery following the terminal phase of H<sub>2</sub> production by re-additions of

sulphur was incomplete and could be performed only a few times [73].

The future of this biotechnology relies on the development of a novel approach at least as efficient as the sulphur deprivation procedure, and which could solve most of the present issues limiting its applicability. Here we report on the establishment of a photoautotrophic and sustainable H<sub>2</sub> production system in *C. reinhardtii*, and demonstrate the applicability of algal cells as whole-cell catalysts for H<sub>2</sub> production. This new protocol shares the early approach by Gaffron and Rubin [31] to induce hydrogenase activity by dark anaerobic treatment and keep the CBB cycle inactive by substrate limitation. As an important addition, the protocol applies a simple O<sub>2</sub> absorbent that preserve hydrogenase activity for several days (Fig. 4). This protocol has fundamental advantages relative to the earlier methods, namely that (i) following a few hours of anaerobic dark incubation, H<sub>2</sub> production starts promptly upon illumination (Fig. 1b); (ii) as opposed to sulphur deprivation, it does not require media exchange (Fig. 7); (iii) it does not depend on starch degradation and does not require acetate, thus it is photoautotrophic; (iv) because no organic carbon source is required, the risk of bacterial contamination is low; (v) the cultures remain photosynthetically active during the H<sub>2</sub> production phase (Fig. 6) and they can be easily recovered afterwards (Fig. 7); (vi) it is based on linear electron transport and the electrons originate mostly from the water-splitting activity of PSII, as demonstrated by a DCMU-treatment (Fig. 2), and has relatively high light-to-H<sub>2</sub> energy conversion efficiencies (Table 1); (vii) during the growth phase, CO<sub>2</sub>, an industrial by-product, can be utilized; and, (viii) it can make use of relatively high light intensities (here, approx. 320 μmol photons/m<sup>2</sup>/s).

The maximum H<sub>2</sub> production yield achieved using this protocol was approx. 200 μl H<sub>2</sub>/ml culture in 96 h, which is almost four times higher than the yield of sulphur-deprived cultures at equal chl content and illumination conditions (compare Figs. 1d, 4b), and it is in the same range as observed earlier for sulphur deprivation experiments using PBRs with even illumination [42], but having the drawbacks listed above. We expect the yield of H<sub>2</sub> production achievable upon anaerobic induction in minimal media to be further improved using advanced PBR designs, including optimized gas-to-liquid ratio, illumination and mixing conditions and efficient removal of the produced gases. There is also a high potential in applying this protocol to various photosynthetic mutants possessing, e.g. truncated light-harvesting antennae [74], or a high PsbA protein content [75]. Cyclic electron transport competes for the electrons with HydA (reviewed by [9]), thus its downregulation may entail a further increase in H<sub>2</sub> production under our conditions as well.

Keeping the CBB cycle inactive was achieved by substrate limitation; it has been shown earlier that the CBB cycle represents a competing pathway for H<sub>2</sub> production (e.g. [31, 47, 49, 68]) and that redirecting the electrons towards HydA from FNR may enhance the rate of H<sub>2</sub> production [76, 77].

The present findings show that by imposing substrate limitation on the CBB cycle, the electrons are largely transferred to HydA, with the lack of carbon sources facilitating the establishment of hypoxia. The effects of CO<sub>2</sub> and FCCP additions (Fig. 2) and the relatively reduced PQ-pool (Additional file 4: Fig. S3) indicate that the mechanism occurs by “photosynthetic control” [51, 54, 78]: since the hydrogenases are less effective at accepting electrons than the CBB cycle, the lumen is acidified and the photosynthetic electron transport is decelerated at the cytb<sub>6</sub>f complex. This results in a reduced PQ-pool, which entails a high charge recombination rate in PSII, resulting in diminished O<sub>2</sub> evolution (Fig. 2i, j).

Another key factor to reach a sustained H<sub>2</sub> production is to protect the hydrogenases from O<sub>2</sub>, which may also shift the balance between O<sub>2</sub> and H<sub>2</sub> production, established by the above-mentioned “photosynthetic control”. We applied an iron-salt-based O<sub>2</sub> absorbent, which decreased the O<sub>2</sub> concentration in the headspace below 0.1%. This very low concentration of O<sub>2</sub> was still inhibitory for hydrogenases (Fig. 5), thus it is desirable to test even more advanced materials in the future, as for instance crystalline salts of cationic multimetallic cobalt complexes [79]. By further decreasing the O<sub>2</sub> level, we expect that hydrogenase activity would be better preserved and act as a more effective electron sink; as a result, lumen acidification and “photosynthetic control” will be attenuated, and the yield of H<sub>2</sub> production further increased. Engineering hydrogenases to tolerate a few percent of O<sub>2</sub> [14] could also be a successful strategy to further increase the efficiency of H<sub>2</sub> production.

## Experimental procedures

### Algal growth conditions and H<sub>2</sub> production

*Chlamydomonas reinhardtii* CC124 strain was grown initially at 22 °C in 250 ml Erlenmeyer flasks containing 50 ml Tris–acetate–phosphate (TAP) medium shaken at 120 rpm in an algal growth chamber under continuous illumination of 80–90 μmol photons/m<sup>2</sup>/s PAR (measured by a LI-250A light meter equipped with a quantum sensor), provided by white fluorescent tubes.

After 72 h of cultivation, the cells were transferred to high-salt (HS) medium, HS supplemented with acetate (HSA), Tris–phosphate (TP) or Tris–acetate–phosphate (TAP) media (<http://www.chlamycollection.org/methods/media-recipes/>) and the chl content was set at 50 μg chl (a + b)/ml [80], corresponding to approx. 15 million

cells/ml as determined by a Millipore Scepter cell counter (described in [81]). In one experiment, 15 and 30  $\mu\text{g chl (a+b)}$ /ml was also set. For  $\text{H}_2$  production, 30 ml culture was placed in a 120-ml serum bottle and sealed off with rubber septa under sterile conditions. For dark anaerobic incubation, the gas phase of the bottle was flushed with  $\text{N}_2$  gas for 10 min and kept in the dark for 4 h, during which the vials were flushed twice more with  $\text{N}_2$ . To produce sulphur-deprived cultures, the cells were washed five times with sulphur-free TAP medium (centrifugation at 1000g, at 24 °C for 5 min, see also [41]) and the chl content was set at 50  $\mu\text{g chl (a+b)}$ /ml. Following these steps, the cultures were placed under T8 cool white fluorescent light tubes (Sylvania luxline plus), providing approximately 52  $\text{W/m}^2$  at the level of the cultures as determined by a Spectra-Physics 404 Power meter; this light intensity corresponded to approx. 320  $\mu\text{mol photons/m}^2/\text{s}$  PAR as measured by a LI-250A light meter equipped with a quantum sensor. The cultures were illuminated continuously and kept at 26 °C for 96 h. For a general scheme of the experiments, see Additional file 1: Fig. S1.

In the regeneration experiment following  $\text{H}_2$  production, a Multi-Cultivator MC 1000-OD instrument (Photon Systems Instruments, Brno, Czech Republic) was used. At the start of cultivation, the chl content was set at 6  $\mu\text{g chl (a+b)}$ /ml in HS medium. The cells were grown in HS medium at 23 °C, 80  $\mu\text{mol photons/m}^2/\text{s}$  in continuous light provided by white LEDs for 72 h and the cultures were bubbled with sterile air containing 1%  $\text{CO}_2$ . Following this, the cultures were subjected to a second round of dark anaerobic incubation in HS medium, as described above.

#### Chemical treatments

In separate experiments, 20  $\mu\text{M}$  3-(3',4'-dichlorophenyl)-1,1-dimethylurea (DCMU) dissolved in dimethyl sulfoxide (DMSO, 100 mM stock solution), 1 mM potassium cyanide (KCN) and 1 mM salicylhydroxamic acid (SHAM), 2% v/v  $\text{CO}_2$ , 10 mM glycolaldehyde (GA) dissolved in water (1.5 M stock solution) and 2  $\mu\text{M}$  carbonylcyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) dissolved in HS medium (20 mM stock solution) were added to the cultures 3 h after the onset of light and start of  $\text{H}_2$  production. To eliminate the produced  $\text{O}_2$ , 2 mM glucose (Glc, 1 M stock solution), 0.2 mg/ml glucose oxidase (GO, 30 mg/ml stock solution) [82] and 1 mM ascorbate (Asc, 1 M stock solution) were added to the cultures before the start of dark anaerobic incubation.

An iron-salt-based, non-cytotoxic  $\text{O}_2$  absorbent ( $\text{O2}_{\text{TM}}$ ; <http://www.o2zero.com>, 20 cc) was used to eliminate  $\text{O}_2$  from the headspace of the serum bottles during the 96-h  $\text{H}_2$  production phase. To this end, 1.3 g of  $\text{O}_2$  absorbing material was placed into a 2 ml-vial, which

was left open and introduced into the headspace of the serum bottles; the algal culture did not get into direct contact with the  $\text{O}_2$  absorbent (Fig. 4a).

#### Determination of net $\text{H}_2$ and $\text{O}_2$ production by gas chromatography

The net amounts of  $\text{H}_2$  and  $\text{O}_2$  produced by the cells were determined by taking 250  $\mu\text{l}$  aliquot from the gas phase of the cultures with a gas tight microsyringe. These samples were injected manually into an Agilent 6890 N gas chromatograph (GC) equipped with a HP-PLOT Molesieve 5 Å column (30 m  $\times$  0.53 mm  $\times$  0.25  $\mu\text{m}$ ) and a TCD detector. The oven temperature was 30 °C. The carrier gas was argon, and a linear velocity of 115 cm/s was used. The bottles were flushed with  $\text{N}_2$  gas every 24 h following the determination of gas production.

#### In vitro hydrogenase activity assay

In vitro hydrogenase activity was measured after the dark anaerobic incubation and during the course of  $\text{H}_2$  production in the light, as described in [24]. The assay was carried out in 13.5-ml serum vials at 37 °C and the reaction mixture consisted of 1 ml of 100 mM potassium phosphate buffer, pH 6.8, 80  $\mu\text{l}$  of deionized water, 200  $\mu\text{l}$  of 10% Triton X-100, 20  $\mu\text{l}$  of 1 M methylviologen, 200  $\mu\text{l}$  of anaerobic 1 M sodium dithionite and 200  $\mu\text{l}$  of algal culture. The  $\text{H}_2$  concentration in the headspace was measured by GC every 15 min and fitted with linear regression. Results are the mean value of tests performed in at least four replicates. Following a 4-h dark anaerobic incubation the hydrogenase activity was approx. 500  $\mu\text{mol H}_2/\text{mg chl (a+b)}/\text{h}$ , in agreement with [24]. The data are presented as percentages of this original (maximum) activity.

#### RNA isolation and qRT-PCR analysis to assess HYDA1 transcript level

For RNA isolation, 1 ml culture, containing approximately 50  $\mu\text{g chl (a+b)}$ , was collected and the Direct-Zol RNA kit was used, following the recommendations of the manufacturer (ZymoResearch). To remove contaminating DNA from the samples, the isolated RNA was treated with DNaseI (ZymoResearch). RNA integrity was checked on a 1% (w/v) MOPS gel. Reverse transcription was primed with oligo dT using 1  $\mu\text{g}$  of total RNA and SuperScript III reverse transcriptase (Life Technologies). To confirm the absence of DNA contaminations, an aliquot of the RNA sample was used without reverse transcriptase.

Real-time qPCR analysis was performed using an Applied Biosystems Prism 7900HT Fast Real Time PCR System using HOT FIREPol® EvaGreen® qPCR Mix Plus (ROX) (Solis BioDyne). Primers were designed using the

NCBI Primer Blast Tool (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/>). The melting temperature was 60 °C and the amplicon length was between 100 and 130 bp. To ensure correct normalization of the *HYDA1* (Cre03.g199800) transcript level, three reference genes showing stable expression during H<sub>2</sub> production were used, namely *bTUB2* (Cre12.g549550), *ACTIN* (Cre13.g603700) and *UBQ* (XP\_001694320). The primers for *HYDA1* were GGCGAGTGGGACAATCCAAT and TGCCCCGTGAACAGCTCATAG; for the reference genes, see [83]. The data are presented as fold-change in mRNA transcript abundance of *HYDA1*, normalized to the average of the three reference genes, and relative to the control sample (cultures grown in TAP medium under normal growth conditions). The analysis was carried out with three technical replicates and two or three biological replicates; the standard error was calculated based on the range of fold-change by calculating the minimum and the maximum of the fold-change using the standard deviations of the  $\Delta\Delta Ct$ .

#### Fast chl a fluorescence (OJIP) measurements

Fluorescence measurements were carried out with a Handy-PEA instrument (Hansatech Instruments Ltd, UK). For  $F_V/F_M$  measurements, *C. reinhardtii* cultures were dark-adapted for 15 min and then 3 ml of cell suspension (50 µg chl (a + b)/ml) was filtered onto a Whatman glass microfibre filter (GF/B) that was placed in a Handy-PEA leaf clip. For the assessment of the PQ-pool redox status, the cultures were measured immediately after taking them from the serum bottles, without any dark adaptation. The algal sample was illuminated with continuous red light (3500 µmol photons/m<sup>2</sup>/s, 650 nm peak wavelength; the spectral half-width was 22 nm; the light emitted by the LEDs is cut off at 700 nm by a NIR short-pass filter). The light was provided by an array of three light-emitting diodes focused on a circle of 5 mm diameter of the sample surface. The first reliably measured point of the fluorescence transient is at 20 µs, which was taken as  $F_0$ .

#### Western blot analysis and determination of starch content

Two ml of culture were collected at each time-point and the analyses were carried out as described in [41] with slight modifications.

#### Calculation of light-to-H<sub>2</sub> energy conversion efficiency

Efficiency of light-to-H<sub>2</sub> energy conversion ( $\eta_c$ ) was calculated as the ratio of the rate of chemical energy production ( $k_H \text{ HHV}_H$ ) to the incident light power ( $E_s A$ ) (similarly to [84]). The rate of chemical energy production (as H<sub>2</sub>) is expressed as the product of the rate of H<sub>2</sub>

evolution ( $k_H$ ) measured in mol/s units and the higher heating value of H<sub>2</sub> ( $\text{HHV}_H = 286,000 \text{ J/mol}$ ).

The incident light power ( $E_s A$ ) was calculated from the incident light intensity ( $E_s$ , in W/m<sup>2</sup>) and the illuminated area ( $A$ ).

$$\eta_c = k_H \text{ HHV}_H / E_s A$$

Light was provided by T8 cool white fluorescent tubes. Its incident intensity was 52 W/m<sup>2</sup> as determined using a Spectra-Physics 404 Power meter. The temperature during H<sub>2</sub> production was 298 K, the diameter of the serum bottles was 0.048 m and the irradiated area was 0.0018 m<sup>2</sup>.

#### Statistics

The presented data are based on at least three independent experiments. When applicable, averages and standard errors (SEM) were calculated. Statistical significance was analysed using Student's *t* test and the significance level are presented as: \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ .

#### Additional files

**Additional file 1: Figure S1.** General scheme of the H<sub>2</sub> production experiment induced by dark anaerobic incubation. Notes: (1) HS media was used in most experiments, except for Fig. 1, where TAP, TP, HSA, HS and TAP-S media were compared. (2) Before the start of dark anaerobic incubation, O<sub>2</sub> absorbent was placed in the headspaces of the cultures (Figs. 4, 5, 6, Additional file 6: Fig. S4, Additional file 7: Fig. S5). (3) Chemicals were added after 3 h of illumination (Fig. 2) or at the beginning of illumination (Fig. 3). (4) Sampling of the cultures at various time intervals (Figs. 5, 6, Additional file 2: Fig. S2, Additional file 4: Fig. S3, Additional file 6: Fig. S4, Additional file 7: Fig. S5). (5) The cultures were regenerated following the 96-h H<sub>2</sub> production using HS medium and CO<sub>2</sub> bubbling; afterwards, a second round of H<sub>2</sub> production was carried out (Fig. 7).

**Additional file 2: Figure S2.** Starch content of *Chlamydomonas* cultures subjected to dark anaerobic incubation followed by continuous illumination at 320 µmol photons/m<sup>2</sup>/s in acetate-free HS medium. Time 0 is the time point when the cultures were transferred to the light.

**Additional file 3: Table S1.** The percentage of CO<sub>2</sub> in the headspaces of sealed cultures of *Chlamydomonas* cultures subjected to dark anaerobic incubation of 4 h in HS medium followed by continuous illumination of 320 µmol photons/m<sup>2</sup>/s, as determined using gas chromatography. Mean values ( $\pm$  SEM in parentheses) are each based on 4–6 biological replicates. bld: below detection limit of 0.01%.

**Additional file 4: Figure S3.** Fast chl a fluorescence (OJIP) transients of *Chlamydomonas* cultures subjected to dark anaerobic incubation followed by continuous illumination at 320 µmol photons/m<sup>2</sup>/s in acetate-free HS medium. Time 0 is the time point when the cultures were transferred to the light. For the fluorescence measurements, the cultures were measured immediately after taking them from the serum bottles, without any dark adaptation.

**Additional file 5: Table S2.** The effects of the separate and combined additions of glucose (Glc, 2 mM), glucose oxidase (GO, 0.2 mg/ml) and ascorbate (Asc, 1 mM) on the net H<sub>2</sub> and O<sub>2</sub> productions of *Chlamydomonas* cultures subjected to dark anaerobic incubation of 4 h in HS medium followed by continuous illumination of 320 µmol photons/m<sup>2</sup>/s, as determined in the headspaces of sealed cultures using gas chromatography. Mean values ( $\pm$  SEM in parentheses) are each based on 4 to 8 biological replicates.

**Additional file 6: Figure S4.** H<sub>2</sub> production yields (a, c) and O<sub>2</sub> concentrations in the headspaces of the serum bottles (b, d) at 15, 30 and 50 µg chl (a + b)/ml culture in the absence (a, b) and the presence (c, d) of an iron-salt-based O<sub>2</sub> absorbent. Apart from changing the chl concentrations, the experimental conditions are identical to Fig. 4. The cultures were flushed with N<sub>2</sub> for 10 min every 24 h after determining the gas concentrations in the headspaces of the sealed bottles. Mean values (± SEM) are each based on 5 to 6 biological replicates.

**Additional file 7: Figure S5.** Continuous H<sub>2</sub> production (a) and O<sub>2</sub> accumulation (b) at 50 µg chl (a + b)/ml culture in the absence and the presence of an iron-salt-based O<sub>2</sub> absorbent. Apart from omitting the daily N<sub>2</sub> flushing, the experimental conditions are identical to Fig. 4. Mean values (± SEM) are each based on 5–6 biological replicates.

#### Authors' contributions

VN, AP, AVM, and RT carried out the experiments. VN, AVM, RT, LK, AS and SZT analysed the data. SZT conceived and planned the experiments with support from VN, LK and AS. SZT wrote the paper with support from AS and GR. All authors read and approved the final manuscript.

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#### Competing interests

The authors declare that they have no competing interests.

#### Ethics approval and consent to participate

Not applicable.

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