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REVIEW

Implementation of photobiological H_2 production: the O_2 sensitivity of hydrogenases

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Abstract The search for the ultimate carbon-free fuel has intensified in recent years, with a major focus on photoproduction of H_2. Biological sources of H_2 include oxygenic photosynthetic green algae and cyanobacteria, both of which contain hydrogenase enzymes. Although algal and cyanobacterial hydrogenases perform the same enzymatic reaction through metallo-clusters, their hydrogenases have evolved separately, are expressed differently (transcription of algal hydrogenases is anaerobically induced, while bacterial hydrogenases are constitutively expressed), and display different sensitivity to O_2 inactivation. Among various physiological factors, the sensitivity of hydrogenases to O_2 has been one of the major factors preventing implementation of biological systems for commercial production of renewable H_2. This review addresses recent strategies aimed at engineering increased O_2 tolerance into hydrogenases (as of now mainly unsuccessful), as well as towards the development of methods to bypass the O_2 sensitivity of hydrogenases (successful but still yielding low solar conversion efficiencies). The author concludes with a description of current approaches from various laboratories to incorporate multiple genetic traits into either algae or cyanobacteria to jointly address limiting factors other than the hydrogenase O_2 sensitivity and achieve more sustained H_2 photoproduction activity.

Keywords Hydrogenases · Photosynthetic microbes · O_2 sensitivity

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Introduction

Fuels derived from biological renewable energy sources (e.g., bioethanol or biodiesel) are carbon neutral, since fuel production through photosynthesis captures CO_2 from the atmosphere and fuel utilization re-emits CO_2 back into the atmosphere. The ultimate carbon-free fuel is hydrogen, when produced directly from inorganic sources such as water. A number of photosynthetic microbes are known to uncouple H_2 photoproduction from photosynthetic carbon fixation, using reductant generated directly from photosynthetic water oxidation to reduce protons (Weaver et al. 1980; Brand et al. 1989; Boichenko et al. 2004; Skjanes et al. 2008). If these organisms were to be utilized as photocatalysts, they would produce H_2 fuel in a non-polluting, carbon-independent manner, and thus bypass the issue of CO_2 emissions that plagues most of the biofuel industry (Fargione et al. 2008; Searchinger et al. 2008). Theoretically, production of H_2 linked to photosynthetic water oxidation has a maximum solar to hydrogen (STH) conversion efficiency of about 10–13 % (Ghirardi et al. 2009; Walker 2009; Blankenship et al. 2011), which is lower than the 24 % that more expensive photovoltaic/electrolysis approaches can be expected to achieve (Blankenship et al. 2011; Hanna and Nozik 2006). However, in practice, photosynthetic microbes are only capable of photoproducing H_2 from water for short periods of time, following prolonged (hours) anaerobic incubation. In order to achieve sustained H_2 photoproduction, numerous challenges related to the organism's metabolism need to be addressed (Dubini and Ghirardi 2014; Posewitz et al. 2008; Rupprecht 2009), the major being the O_2 sensitivity of the hydrogenase catalysts. Extensive research has been done in the last 50 years to further understand, solve, or bypass this challenge. We will describe the past research and current

state of the art in this review, and discuss perspectives for the future.

Hydrogenases

The photoproduction of H_2 gas by microalgae was first discovered in the 1940s (Gaffron and Rubin 1942) and was the focus of extensive research starting in the 1970–1980s (Healey 1970; Kessler 1974; Godde and Trebst 1980; Maione and Gibbs 1986a, b; Ben-Amotz and Gibbs 1975; Roessler and Lien 1984). At that time, it was recognized that the various capabilities required for H_2 production were distributed among photoautotrophic and non-photoautotrophic microbes in communities (Adams and Mortenson 1984; Adams et al. 1980; Vignais et al. 1985). These include: (a) hydrogenase-containing photoautotrophic microbes (green algae and cyanobacteria) and anaerobic hetero- or lithotrophic bacteria, in which the H_2 production reaction is easily reversible, due to the isopotential or slightly negative ΔG^0 between hydrogenases and their electron donors; and (b) nitrogenase-containing cyanobacteria and other N_2-fixing microbes, using a reaction that requires ATP as a co-factor and is essentially irreversible. Although promising, due to its irreversible nature, the ATP requirement lowers the potential solar to hydrogen (STH) conversion efficiency and renders nitrogenase-containing organisms inadequate as commercial H_2 producers (Ghirardi et al. 2009). Indeed, STH values are directly correlated with the overall cost of the process, and technoeconomic analyses have been used by the U.S. Department of Energy to limit funding only to the most promising technologies (James et al. 2009).

Algal and cyanobacterial hydrogenases belong to different classes of enzymes. The first contain a [2Fe2S] catalytic cluster linked by a cysteine residue to a [4Fe4S] center (H-cluster), while the latter operate through a catalytic center comprised a [NiFe]-cluster attached to a conventional [4Fe4S] unit through 3 cysteine residues (Adams and Mortenson 1984; Adams et al. 1980; Vignais et al. 2001). [FeFe]-hydrogenases, in general, favor the H_2 production reaction, while [NiFe]-hydrogenases are mostly involved in H_2 uptake, the reversible reaction. This advantage of the green algal enzyme is counteracted by the fact that [FeFe]-hydrogenases are irreversibly inhibited by O_2, while the O_2-inhibition of [NiFe]-hydrogenases has a reversible character (English et al. 2009). The prompt inactivation of hydrogenases by O_2 is considered to be a major limiting factor preventing sustained and efficient H_2 production by photosynthetic microbes (Ghirardi et al. 2007; Goldet et al. 2009; Lambertz et al. 2011).

Algal hydrogenases were purified in the late 1980s (Roessler and Lien 1984; Happe and Naber 1993). It was found that Chlamydomonas harbors two separate hydrogenase genes that encode for two proteins, HYDA1 and HYDA2 (Forestier et al. 2003). The two proteins have molecular weights of about 47 kDa and interact physiologically with Ferredoxin (Winkler et al. 2009), which shuttles electrons to hydrogenases from either the photosynthetic electron transport chain or from the fermentative pyruvate-ferredoxin oxidoreductase (PFOR) enzyme (Hemschemeier and Happe 2011). To date, the differences in the physiological roles of the two hydrogenases are not known, since both are involved in fermentative and photoproduction of H_2, as shown by Meuser et al. (2012) in their seminal paper describing the functions of single and double knock-out mutants of *Chlamydomonas reinhardtii* hydrogenases. Although the algal enzymes have not been crystallized in their mature form [the apoprotein's structure was solved by Mulder et al. (2010)], homology models were constructed (Cohen et al. 2005a, b), based on the available crystal structures of the *Clostridium pasteurianum* (Peters et al. 1998) and *Desulfovibrio desulfuricans* (Nicolet et al. 1999) hydrogenases. These models show that the major differences between these two classes of [FeFe]-hydrogenases are (a) the lack of additional FeS centers in the algal enzymes; and (b) the presence of additional amino acid loops of unknown function in the algal hydrogenases. Interestingly, non-algal hydrogenases are about two orders of magnitude more tolerant to O_2 inactivation (Cohen et al. 2005a); the hypothesis that their higher O_2 tolerance was due to the lack of the additional FeS clusters was proved not to be true (King et al. 2006).

In contrast with algal [FeFe]-hydrogenases, cyanobacterial [NiFe]-hydrogenases are pentameric complexes, consisting of a large and small hydrogenase subunits (HoxH and HoxY) and three diaphorase subunits (HoxE, HoxF, and HoxU), the first of which is a membrane-bound protein. All three diaphorase subunits harbor additional FeS clusters, and they are responsible for creating an interface for interaction of the hydrogenase with NAD(P)H (Boison et al. 1998; Aubert-Jousset et al. 2011). Cyanobacterial hydrogenases, thus, are linked to both photosynthetic and respiratory electron transport chains (Cournac et al. 2004; Schmitz and Bothe 1996; Appel and Schulz 1996). Moreover, recent evident suggests that both ferredoxins and flavodoxins could act as electron donors to the cyanobacterial hydrogenase (Gutekunst et al. 2014). Besides the major enzymatic differences between [FeFe] and [NiFe]-enzymes described above, it must be noted that [NiFe]-hydrogenases are constitutively expressed, while the expression of [FeFe]-hydrogenases requires anaerobic induction. Both enzymes have similar turnover numbers when measured on an electrode surface (Jones et al. 2002; Armstrong 2004), although the redox potential of NAD(P)H is more positive than that of ferredoxin (−0.32 vs. −0.42 eV),

which favors H_2 production by [FeFe]-hydrogenases and H_2-uptake by [NiFe]-hydrogenases at pH7 and standard conditions. Moreover, recent work (Maness et al. 2002) has shown that it is possible to purify truncated [NiFe]-hydrogenases containing only two subunits that are active in H_2 production and are able to interact with ferredoxin. However, the expression of heterologous O_2-tolerant hydrogenases in cyanobacteria has been extremely challenging, due to the fact that the maturation of [NiFe]-hydrogenases requires a large number of species-specific maturation enzymes (Ghirardi et al. 2014).

Research effort to understand the factors responsible for the high O_2 sensitivity of hydrogenases is taking place in many parts of the world. The use of cyclic voltammetry to study hydrogenases made a major impact in the field (Armstrong 2004; Armstrong and Albracht 2005; Lamle et al. 2005; Leger et al. 2002; Vincent and Armstrong 2005; Vincent et al. 2005a, b), and provided a tool to measure the performance of hydrogenases under a variety of redox conditions, following exposure to H_2, O_2, or CO. These measurements confirmed previously observed major differences between [FeFe]- and [NiFe]-hydrogenases, which included the reversibility of inactivation by the latter (Vincent et al. 2007), and the existence of [NiFe] enzymes with lower sensitivity to O_2 (Cracknell et al. 2008). Additionally, these studies, combined with more refined EPR and FTIR techniques, are still unveiling a series of redox states of [FeFe]-hydrogenases that have been shown to affect its catalytic activity and O_2 sensitivity (Mulder et al. 2013; Stripp and Happe 2009), such as the fact that reduced hydrogenases and CO-treated [FeFe]-hydrogenases are less sensitive to O_2 inactivation (Vincent et al. 2005b; Stripp et al. 2009), probably because of competition between H_2, CO, and O_2 for binding at the catalytic site.

Engineering O_2-tolerant [FeFe]-hydrogenases

The O_2 inactivation of hydrogenases has been the major factor precluding their commercial application (Dubini and Ghirardi 2014; Rupprecht 2009; Lee et al. 2010). Earlier research efforts demonstrated that the *Chlamydomonas reinhardtii* hydrogenases had an I_{50} of 0.3–0.4 % O_2 following a 2-min incubation in vivo, and that using chemical mutagenesis one could select for strains with O_2 I_{50}s up to 9 times higher than the wild-type strain (Ghirardi et al. 1997; Seibert et al. 1998, 2001a). At the time, the lack of inexpensive genome sequencing technologies and of high-throughput screening approaches precluded the investigation of the genotype responsible for these results. However, these observations provided the evidence that it was possible to genetically alter the overall sensitivity of algal H_2 production to O_2.

The subsequent search for O_2-tolerant [FeFe]-hydrogenases focused on two approaches: (a) the use of site-directed mutagenesis to address the origin of O_2 sensitivity; and (b) the generation of random hydrogenase mutants followed by high-throughput screening for H_2-producers in the presence of or following exposure to O_2. These studies were only possible due to the discovery of the maturation proteins required for assembly of the [FeFe]-hydrogenases catalytic site (Posewitz et al. 2004a) and the demonstration that they were the only requirement for expression of the structural genes in *E. coli* (King et al. 2006) or in *Clostridium acetobutylicum* (Girbal et al. 2005; von Abendroth et al. 2008). This capability opened up the door for studies requiring large amounts of these enzymes. Indeed, by co-expressing the maturation genes with the [FeFe] structural hydrogenase genes, it has been possible to detect heterologous expression of the *Clostridium acetobutylicum* hydrogenases in *Synechococcus elongatus* (Ducat et al. 2011) and of the *Shewanella* MR-1 hydrogenase in the heterocystis of *Anabaena* PCC 7120 (Gartner et al. 2012). Similarly, the development of high-throughput screening methods to detect H_2 production by individual colonies proved essential for progress in this area. Among the various techniques devised, the following have been successfully used by various research groups to screen large populations of recombinant hydrogenases of H_2-producers:

(a) Chemochromic films consisting of layers tungsten oxide (WO_3) and platinum, which turn blue as the WO_3 becomes reduced (Seibert et al. 2001b); the response time of the films is fast and they are able to sense down to 1 nmol H_2 emitted from a 3-mm-diameter colony. However, they are not available commercially and their signal is transient. The films have been successfully used to screen chemical mutagenesis-based libraries of Chlamydomonas for mutants unable to produce H_2 (Seibert et al. 1998, 2001b), and they identified novel factors required for expression of an active hydrogenase from random insertional mutagenesis libraries (Posewitz et al. 2004a, b).

(b) Sulfonated Wilkinson's catalyst coupled with a tetrazolium indicator. This method is less sensitive than the WO_3 films, detecting down to 20 nmol H_2 in microwell plates. It has been used to detect H_2 production by cyanobacterial libraries (Katsuda et al. 2006; Schrader et al. 2008) and for identifying algal strains with lowered Photosynthesis/Respiration capacity ratios (Ruhle et al. 2008; Hemschemeier et al. 2008)—see "By-passing the O_2 sensitivity of [FeFe]-hydrogenases" section for context.

(c) Reduction of resazurin to resorufin by H_2 (Stapleton and Swartz 2010a) or production of H_2 by reduced

ferredoxin or methyl viologen (Bingham et al. 2012a; Stapleton and Swartz 2010b). Since resazurin is not specific for H_2 and can be reduced by other metabolic intermediates, the assay's specificity was optimized by absorption of the dyes to beads displaying recombinant hydrogenases. This method was used for detecting O_2-tolerant, H_2 uptake activity by libraries of recombinant hydrogenases.

(d) Biosensor based on the H_2-sensing promoter of Rhodobacter uptake hydrogenases coupled to the GFP marker. This method relies on an overlay of GFP-expressing Rhodobacter that fluorescences when in contact with H_2-producing single colonies of various organisms (Wecker et al. 2011). The assay is extremely sensitive to H_2, and can detect fermentatively produced H_2 gas as well. It has been successfully used to identify Chlamydomonas strains that photoproduce H_2 upon exposure to high light, conditions that normally inhibit the wild-type activity under the assay conditions (Wecker and Ghirardi 2014).

A major hypothesis regarding the factors responsible for the high O_2 sensitivity of [FeFe]-hydrogenases was derived from comparative studies of hydrogenases from different organisms, which revealed the presence of putative hydrophobic gas channels connecting the surface of the protein to the catalytic site. Such channels have been proposed to be pathways by which O_2 and/or H_2 diffuse in and out of a protein (Cohen et al. 2005a; Montet et al. 1997). These features suggested that O_2 sensitivity could be altered by interfering with the gas diffusion pathway, as long as the rate of diffusion is limiting the overall H_2 production reaction, compared to the rate of catalysis (Fontecilla-Camps et al. 2007). In order to further investigate this hypothesis and apply it to [FeFe]-hydrogenases, molecular dynamic simulations (MD), volumetric solvent accessibility maps (VOAM), and potential energy (PMF) plots of the diffusion of O_2- and H_2-sized particles from the surface to the catalytic site of clostridial and algal [FeFe]-hydrogenases were performed (Cohen et al. 2005a, b).

The studies indicated that there were two major H_2 diffusion pathways converging at a large central cavity located near the catalytic site of [FeFe]-hydrogenases, while a small percentage of the H_2 molecules diffused through other less-defined pathways, suggesting that the *Clostridium pasteurianum* CpI hydrogenase is fairly porous to H_2 gas. In contrast, O_2 molecules diffuse at much slower rates through the same pathways and often stay within the channels, without equilibrating with the solvent. The O_2 diffusion motion suggested that it depends on the opening/closing of transient channels. Indeed, when the enzyme was probed for transient cavities using the VOAM method, it was found that the two major pathways seen by the MD simulations correspond to major trajectories predicted by VOAM. These structures seem to correspond to transient protein conformations that are formed spontaneously, as a result of the equilibrium dynamics of the protein structure. Moreover, VOAMs were used to identify specific residues that might be responsible for transiently blocking gas diffusion along each pathway. The relative relevance of each residue was assessed through PMF maps. It was found that the major barrier to O_2 diffusion along either pathway was at the intersection of both pathways, while minor barriers (or wells) were observed along both pathways. Specific residues in CpI were selected for mutagenesis, aimed at replacing them with bulkier residues. However, only minor improvements in O_2 tolerance were detected with the L283 W mutant, accompanied by decreased activity and increased O_2 sensitivity in all other single or double mutants (unpublished). A few years later, an explanation for the lack of success in generating high H_2-producing, O_2-tolerant mutants at those specific positions appeared, when Mulder et al. (Mulder et al. 2010) showed that the residues lining the hydrophobic cavity in the algal hydrogenases were required for the proper folding of the apoprotein; mutation of any of the residues would be expected to prevent maturation of the protein and result in loss of activity, improper protein folding, and higher permeability to O_2. Interestingly, the narrowing of gas channels to slow down O_2 inactivation has been successful when implemented with [NiFe]-hydrogenases (Dementin et al. 2009), as described in more detail in "Engineering O_2-tolerant [NiFe]-hydrogenases" section.

Swartz' group at Stanford took advantage of in vitro cell-free translation systems and used directed evolution to generate hydrogenase mutants, followed by the methyl viologen assay [see (c) above] to screen for H_2 uptake activity after exposure of the enzyme to O_2 (Stapleton and Swartz 2010a, b). After a series of rounds of mutagenesis using error-prone PCR to amplify the CpI gene, the authors detected 20 mutants with H_2 uptake activity levels above the CpI control but only one mutant with decreased O_2 sensitivity. Among the 13 mutations present in this strain, 3 were found to be responsible for the higher O_2 tolerance phenotype. Site-directed saturation mutagenesis was then used to optimize the phenotype. However, neither of the mutants showed increased O_2 tolerance during active H_2 production (Bingham et al. 2012b).

All these discouraging results, however, have been accompanied by progress in the understanding of the factors that confer O_2 tolerance to [NiFe]-hydrogenases (see "Engineering O_2-tolerant [NiFe]-hydrogenases" section), suggesting that cyanobacteria may be a more appropriate organism for expressing heterologous O_2-tolerant hydrogenases.

Engineering O_2-tolerant [NiFe]-hydrogenases

In contrast with [FeFe]-hydrogenases, the O_2 sensitivity of [NiFe]-hydrogenases has been found to be amenable to molecular engineering, and O_2 tolerance was conferred to the *Desulfovibrio fructosovorans* hydrogenase through two major strategies: a reduction in O_2 access to the active site (as described in "Engineering O_2-tolerant [FeFe]-hydrogenases" section with respect to [FeFe]-hydrogenases), and scavenging O_2 from the active site by methionine residues located close to it (Dementin et al. 2009; Leroux et al. 2008). These modifications were achieved by exchanging a valine residue within the enzyme's hydrophobic channel by a methionine, and by substituting another residue near the active site by a second methionine. Both modifications were shown to additively contribute to spontaneous enzyme reactivation after O_2 exposure. These genetic modifications were shown to be further associated with changes in the gas diffusion characteristics and they did affect the directionality of the enzyme (Abou Hamdan et al. 2012). Recent reports have shown that a similar strategy could be developed on the hydrogenase of *Synechocystis*, resulting in the expression of an engineered hydrogenase exhibiting a sustained activity in the presence of O_2 with a bias toward production (Cano et al. 2014). Other engineering strategies involving different targets can be considered and might even be required as part of a larger scale combination of unique redesigning in order to obtain an efficient O_2-tolerant hydrogenase. Indeed, O_2 tolerance has also been tightly linked to electron transfer reactions involving the proximal Fe-S cluster (Fargione et al. 2008; Wu et al. 2011; Lukey et al. 2011) which can be altered through its coordinating cysteine residues (Fargione et al. 2008; Pandelia et al. 2011). Mechanisms involving water molecules and protons transport pathways have also been suggested to play a role in [NiFe]-hydrogenase O_2 tolerance (Cornish et al. 2011; Sumner and Both 2012).

Other approaches toward efficient biohydrogen photoproduction include the heterologous expression of [NiFe]-hydrogenases more tolerant toward O_2 in cyanobacteria, which has proved to be particularly difficult, since their maturation proteins seem to be extremely species specific (English et al. 2009) and, to date, very few successful efforts have been reported (Weyman et al. 2011). Strategies aiming at sequestrating the intracellular O_2 have also been contemplated (Shestakov and Mikheeva 2006), but no reports of such approaches are currently available.

Bypassing the O_2 sensitivity of [FeFe]-hydrogenases

A physiological method to bypass the O_2 inactivation of algal hydrogenases was first demonstrated in 2000 by Melis et al. (2000). These studies relied on the effect of sulfur deprivation on algal physiology. When sulfur is depleted from the growth medium, Chlamydomonas cultures gradually lose their O_2-evolution capacity, over-accumulate starch, and eventually become anaerobic. Anaerobiosis induces both the expression of the hydrogenase enzyme and starch degradation. As a result, the culture produces H_2 in the light using reductant from the residual water oxidation activity and from the indirect, starch degradation-linked photosynthetic pathway. Recent papers showed a similar effect by depriving the cultures of other nutrients, such as nitrogen (Philipps et al. 2012) which was shown to also induce H_2 production but not always as promptly or as specifically as the sulfur-deprivation method.

The sulfur-deprivation process consists of two phases: a photosynthetic phase, during which O_2 evolution is gradually inhibited and starch overaccumulates; and a H_2 production anaerobic phase that is characterized by three major metabolic activities—residual O_2 evolution and H_2 photoproduction from water oxidation, respiration of organic substrates, and starch fermentation, yielding additional H_2 gas and other fermentative products such as formate, acetate, and ethanol (Kosourov et al. 2003). The effects of sulfur deprivation on algal physiology have been extensively studied; the loss of O_2 evolution was attributed to the inhibition of D1 (a major protein forming the Photosystem II reaction center) turnover, which requires sulfurylated amino acid residues, followed by loss of Rubisco, the most abundant protein in Chlamydomonas. It was hypothesized that the latter was a result of an attempt by the organism to recycle its sulfurylated residues from abundant proteins in order to preserve its minimum metabolic functions and survive the deprivation period ((Zhang et al. 2002). Various studies aimed at deconvoluting the complexities associated with the process and optimizing the H_2 production yield of sulfur-deprived cultures, either by engineering additional properties into Chlamydomonas or by further manipulating the process followed. It was observed that, although the original method was implemented under photoheterotrophic conditions (acetate was present in the growth medium), the process could be reproduced under photoautotrophic conditions (using CO_2 as the only carbon source), as long as optimum conditions were maintained for starch accumulation and degradation (Tolstygina et al. 2009).

Indeed, one of the major questions regarding the sulfur-deprivation method is the role of starch. Many reports showed that DCMU, an inhibitor of O_2 evolution, partially inhibited H_2 production when added at the beginning of the anaerobic phase, and the relative level of DCMU inhibition decreased during the 3–4-day duration period (Laurinavichene et al. 2004). This suggested that the contribution from the direct (reductant originated from PSII-catalyzed water oxidation) and indirect (reductant originated from the initial steps of starch degradation and transferred to the photosynthetic plastoquinone pool by the NAD(P)H/plastoquinone oxidoreductase) pathways varies during the process. The H_2 photoproduction capability of starch-less mutants *sta6* (Zabawinski et al. 2001) and *sta7* (Posewitz et al. 2004) following dark anaerobic induction showed transient and low levels of H_2 photoproduction; however, wild-type rates of H_2 photoproduction by both starch-less mutants were detected under sulfur deprivation (Chochois et al. 2009). The latter was explained by the observation that significant acetate consumption occurs in starch-deficient mutants during sulfur deprivation, and acetate consumption is responsible for maintaining anaerobiosis and providing reductant through the indirect H_2 photoproduction pathway during sulfur deprivation. Interestingly, though, the low H_2 production of starch-less mutants under dark anaerobic conditions correlates with low hydrogenase genes transcription (Posewitz et al. 2004). It was hypothesized that, since electrons generated from starch degradation reduce the PQ, this triggers a regulatory process that affects the expression of various photosynthetic components (Escoubas et al. 1995) (Jans et al. 2008), including hydrogenases (Hemschemeier et al. 2008; White and Melis 2006). Alternatively, based on inhibitor studies under sulfur-deprivation conditions, Chochois et al. (2009) proposed that the regulation of hydrogenase activity occurred instead by the proton gradient generated by cyclic electron flow during the process, thus confirming the ATP requirement for hydrogenase induction reported previously by others (Lien and Pietro 1981).

Curiously, two Rubisco mutants that are unable to fix CO_2 have been reported to exhibit either low (White and Melis 2006) or high H_2 photoproduction activity under sulfur deprivation (Hemschemeier et al. 2008). Since neither mutant accumulates starch, it was finally agreed that the two different phenotypes were due to the production of an unstable truncated Rubisco, while the other completely lacks starch (Hemschemeier et al. 2008).

Although not directly related to the O_2 sensitivity limitation, the non-dissipation of the proton gradient during H_2 photoproduction and the induction of state transitions (which direct light energy preferentially toward PSI and CEF) during anaerobiosis are major issues responsible for the low rates of the process (Dubini and Ghirardi 2014; Hankamer et al. 2007). A recent manuscript (Tolleter et al. 2011) describes the effects of genetically uncoupling the proton gradient from photosynthetic electron transport, which is accompanied by an increase in the rates of H_2 photoproduction, either under anaerobically induced or sulfur-deprived conditions. Similarly, efforts to eliminate state transitions while increasing the rates of respiration have successfully led to higher rates of H_2 photoproduction ((Kruse et al. 2005).

The success of partial inactivation of Photosystem II activity to achieve culture anaerobiosis led to the design of alternative processes to inhibit D1 turnover. Initial experiments targeted the sulfate permease enzyme, which translocates sulfate from the cytosol into the chloroplast. Sulfate permease mutants had a phenotype similar to that of sulfur-deprived wild-type algae and produced H_2 at similar levels (Chen et al. 2005). A different approach focused on the feasibility of using D1 mutants with reported lower O_2 evolution capability as H_2-producers in closed photobioreactors (Makarova et al. 2007). Unfortunately, the specific mutants used in this study were unable to over-accumulate starch, a *sine-qua-non* for efficient H_2 photoproduction during anaerobiosis. Markov et al. (Markov et al. 2006) demonstrated sustained H_2 photoproduction for a total of about 90 min by algal cells previously photoinhibited to decrease their O_2 evolution capability. More recently, Surzycki et al. (2007) devised a cyclic process based on the properties of *cytochrome c* promoter sequence, which is responsive to anaerobiosis and low Cu levels, as a regulator expression of the nucleus-encoded Nac2 chloroplast protein. The latter is required for stable accumulation of the psaD RNA (encoding the reaction center D2 protein) and, if it is not expressed, Photosystem II activity is inhibited. The cycle operates by manipulating the copper concentration in the growth medium; in the presence of copper, PSII is inhibited, the culture gradually becomes anaerobic, the hydrogenase is induced, and H_2 production is observed. Hydrogen accumulates for a total of about 1.5 h, at which point anaerobiosis induces the synthesis of the Nac2 protein and PSII activity is restored. The cycle can be repeated upon re-addition of Cu^{2+} to the medium, and the authors demonstrated the occurrence of two successive cycles during a total period of 50 h. These results compare well with the cyclic production of H_2 using sulfur deprivation (Ghirardi et al. 2000), which showed three cycles over 350 h of operation.

Recent reports show that the engineered D1 double mutant in which L159 was replaced by isoleucine and N230 by tyrosine exhibits higher rates of photosynthesis and respiration, lower Chl and higher D1 content (but only slightly higher Chl a/b ratio, resembling high light-grown cells), and higher rates of H_2 photoproduction under sulfur

deprivation than the common cc124 wild-type strain (Scoma et al. 2012; Torzillo et al. 2009). The two mutated amino acid residues are located, respectively, near Tyrosine Y_Z (the primary electron donor to Photosystem II), and in the loop between helixes IV–V. The double mutant accumulates more biomass during photomixotrophic growth and has higher levels of zeaxanthin during the H_2 production phase, suggesting higher photoprotection by the violoxanthin/zeaxanthin cycle, resulting in a longer H_2 production phase.

Finally, a novel method to bypass the O_2-sensitivity property of hydrogenases was reported by Wu et al. (2011). The authors expressed two codon-optimized O_2-binding proteins, leghemoglobin (from *Glycine max*), and ferrochelatase (from *Bradyrhizobium japonicum*) in the chloroplast of Chlamydomonas. Both genes were shown to be transcribed and translated, although biochemical activity measurements were unsuccessful. Nevertheless, faster O_2 consumption under sulfur deprivation and higher H_2 yields were reported, although the strain chosen for transformation is a known low H_2-producer and the levels of the transgenes were still too low.

It is difficult to compare literature data on the application of sulfur deprivation to H_2 photoproduction, since different researchers use different cell densities, report either maximum or average rates, and are able to sustain the process for different amounts of time. Table 1 summarizes the highest rates that have been currently achieved using a combination of sulfur deprivation with other genetic or cultivation approaches to increase productivity. It is important to point out that neither of the reported processes has achieved economic parity with the current cost of gasoline. More details about the genotypes of each mutant can be found in the original reference (4th column).

Future directions

Although most of the research to date has focused on *Chlamydomonas reinhardtii* and *Synechocystis* sp. PCC 6803 as model organisms, H_2 photoproduction has been observed in other algal species (Brand et al. 1989; Boichenko et al. 2004; Skjanes et al. 2008; Timmins et al. 2009; Wang et al. 2011). It is interesting to notice that, instead of using sulfur deprivation, a large number of researchers cultivate algae under low light intensity in a sealed reactor, as an alternative method to induce anaerobiosis (Wang et al. 2011; Maneeruttanarungroj et al. 2010). Although very useful from a research point of view, such systems are incompatible with commercial applications, where decreases in light intensity require larger areas of cultivation which would increase capital and operational costs (James et al. 2009; Hankamer et al. 2007).

The limited success in generating organisms that photoproduce H_2 from water at high STH conversion efficiency by genetically engineering hydrogenases or through physiological approaches to prevent O_2 accumulation is forcing the community to re-evaluate the relevance of O_2 sensitivity in limiting H_2 photoproduction *vis a vis* other known barriers. Those have been mentioned above and were discussed in detail by Dubini and Ghirardi (2014). Suffice it to say that success will involve engineering multiple traits into a single organism, and that various research groups are currently involved in approaches to achieve this goal, as shown in Table 1. A recent report on the transformation of the algal [FeFe]-hydrogenase in the Chlamydomonas chloroplast (Reifschneider-Wegner et al. 2014) of a double hydrogenase knock-out strain (Meuser et al. 2012) may represent a potential useful method to increase the availability of the enzyme, which may be a rate-limiting step in H_2 production. However, current

Table 1 Rates, yield, and long-term duration of H_2 photoproduction by different mutants of *Chlamydomonas reinhardtii* under sulfur-deprivation conditions

Strain name	Maximum rates under S deprivation	Final Yield and duration under S deprivation	Reference
Wild-type (strain cc124)	9.4 μmol mg Chl^{-1} h^{-1} (measured by water–displacement)	276 ml L^{-1} in 4 days (measured by water–displacement)	Kosourov et al. (2003)
stm6	7 μmol mg Chl^{-1} h^{-1} (measured by gas chromatography)	271 mL L^{-1} in 10–14 days (measured by gas chromatography)	Kruse et al. (2005)
pgrl1	Not reported	540 mL L^{-1} in 4 days	Tolleter et al. (2011)
D1 *L195I-N230Y*	Not reported	700 mL L^{-1} in 7 days	Scoma et al. (2012)
stm6GLC4lo1	14.22 μmol mg Chl^{-1} h^{-1} (measured by gas chromatography)	361 mL L^{-1} in 8 days (measured by gas chromatography)	Oey et al. (2013)

See references for more detail about each genotype

expression levels and rates are still too low and need to be substantially increased to demonstrate its benefit over the wild-type strain.

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Conflict of interest The author declares that she has no conflict of interest.

References

Abou Hamdan A, Dementin S, Liebgott PP, Gutierrez-Sanz O, Richaud P, De Lacey AL, Rousset M, Bertrand P, Cournac L, Leger C (2012) Understanding and tuning the catalytic bias of hydrogenase (vol 134, p 8368, 2012). J Am Chem Soc 134:9828

Adams MW, Mortenson LE (1984) The physical and catalytic properties of hydrogenase II of *Clostridium pasteurianum*. A comparison with hydrogenase I. J Biol Chem 259:7045–7055

Adams MW, Mortenson LE, Chen JS (1980) Hydrogenase. Biochim Biophys Acta 594:105–176

Appel J, Schulz R (1996) Sequence analysis of an operon of a NAD(P)-reducing nickel hydrogenase from the cyanobacterium *Synechocystis* sp. PCC 6803 gives additional evidence for direct coupling of the enzyme to NAD(P)H-dehydrogenase (complex I). Biochim Biophys Acta 1298:141–147

Armstrong FA (2004) Hydrogenases: active site puzzles and progress. Curr Opin Chem Biol 8:133–140

Armstrong FA, Albracht SP (2005) [NiFe]-hydrogenases: spectroscopic and electrochemical definition of reactions and intermediates. Philos Trans 363:937–954 **discussion 1035–1040**

Aubert-Jousset E, Cano M, Guedeney G, Richaud P, Cournac L (2011) Role of HoxE subunit in *Synechocystis* PCC6803 hydrogenase. FEBS J 278:4035–4043

Ben-Amotz A, Gibbs M (1975) H2 metabolism in photosynthetic organisms. II: light-dependent H_2 evolution by preparations from *Chlamydomonas*, *Scenedesmus* and spinach. Biochem Biophys Res Commun 64:355–359

Bingham AS, Smith PR, Swartz JR (2012a) Evolution of an [FeFe] hydrogenase with decreased oxygen sensitivity. Int J Hydrog Energy 37:2965–2976

Bingham AS, Smith PR, Swartz JR (2012b) Evolution of an [FeFe] hydrogenase with decreased oxygen sensitivity. Int J Hydrog Energy 37:2965–2976

Blankenship RE, Tiede DM, Barber J, Brudvig GW, Fleming G, Ghirardi M, Gunner MR, Junge W, Kramer DM, Melis A, Moore TA, Moser CC, Nocera DG, Nozik AJ, Ort DR, Parson WW, Prince RC, Sayre RT (2011) Comparing photosynthetic and photovoltaic efficiencies and recognizing the potential for improvement. Science 332:805–809

Boichenko VA, Greenbaum E, Seibert M (2004) Hydrogen production by photosynthetic microorganisms. In: Barber J, Archer MD (eds) Photoconversion of solar energy, molecular to global photosynthesis. Imperial College Press, London, pp 397–452

Boison G, Schmitz O, Schmitz B, Bothe H (1998) Unusual gene arrangement of the bidirectional hydrogenase and functional analysis of its diaphorase subunit HoxU in respiration of the unicellular cyanobacterium anacystis nidulans. Curr Microbiol 36:253–258

Brand JJ, Wright JN, Lien S (1989) Hydrogen production by eukaryotic algae. Biotechnol Bioeng 33:1482–1488

Cano M, Volbeda A, Guedeney G, Aubert-Jousset E, Richaud P, Peltier G, Cournac L (2014) Improved oxygen tolerance of the *Synechocystis* sp PCC 6803 bidirectional hydrogenase by site-directed mutagenesis of putative residues of the gas diffusion channel. Int J Hydrog Energy 39:16872–16884

Chen HC, Newton AJ, Melis A (2005) Role of SulP, a nuclear-encoded chloroplast sulfate permease, in sulfate transport and H_2 evolution in *Chlamydomonas reinhardtii*. Photosynth Res 84:289–296

Chochois V, Dauvillee D, Beyly A, Tolleter D, Cuine S, Timpano H, Ball S, Cournac L, Peltier G (2009) Hydrogen production in Chlamydomonas: photosystem II-dependent and -independent pathways differ in their requirement for starch metabolism. Plant Physiol 151:631–640

Cohen J, Kim K, King P, Seibert M, Schulten K (2005a) Finding gas diffusion pathways in proteins: application to O_2 and H_2 transport in CpI [FeFe]-hydrogenase and the role of packing defects. Structure 13:1321–1329

Cohen J, Kim K, Posewitz M, Ghirardi ML, Schulten K, Seibert M, King P (2005b) Molecular dynamics and experimental investigation of H(2) and O(2) diffusion in [Fe]-hydrogenase. Biochem Soc Trans 33:80–82

Cornish AJ, Gartner K, Yang H, Peters JW, Hegg EL (2011) Mechanism of proton transfer in [FeFe]-hydrogenase from *Clostridium pasteurianum*. J Biol Chem 286:38341–38347

Cournac L, Guedeney G, Peltier G, Vignais PM (2004) Sustained photoevolution of molecular hydrogen in a mutant of *Synechocystis* sp. strain PCC 6803 deficient in the type I NADPH-dehydrogenase complex. J Bacteriol 186:1737–1746

Cracknell JA, Vincent KA, Ludwig M, Lenz O, Friedrich B, Armstrong FA (2008) Enzymatic oxidation of H_2 in atmospheric O_2: the electrochemistry of energy generation from trace H_2 by aerobic microorganisms. J Am Chem Soc 130:424–425

Dementin S, Leroux F, Cournac L, de Lacey AL, Volbeda A, Leger C, Burlat B, Martinez N, Champ S, Martin L, Sanganas O, Haumann M, Fernandez VM, Guigliarelli B, Fontecilla-Camps JC, Rousset M (2009) Introduction of methionines in the gas channel makes [NiFe] hydrogenase aero-tolerant. J Am Chem Soc 131:10156–10164

Dubini A, Ghirardi ML (2014) Engineering photosynthetic organisms for the production of biohydrogen. PhotosynthRes

Ducat DC, Sachdeva G, Silver PA (2011) Rewiring hydrogenase-dependent redox circuits in cyanobacteria. Proc Natl Acad Sci USA 108:3941–3946

English CM, Eckert C, Brown K, Seibert M, King PW (2009) Recombinant and in vitro expression systems for hydrogenases: new frontiers in basic and applied studies for biological and synthetic H_2 production. Dalton Trans 45:9970–9978

Escoubas JM, Lomas M, Laroche J, Falkowski PG (1995) Light intensity regulation of cab gene transcription is signaled by the redox state of the plastoquinone pool. Proc Natl Acad Sci USA 92:10237–10241

Fargione J, Hill J, Tilman D, Polasky S, Hawthorne P (2008) Land clearing and the biofuel carbon debt. Science 319:1235–1238

Fontecilla-Camps JC, Volbeda A, Cavazza C, Nicolet Y (2007) Structure/function relationships of [NiFe]- and [FeFe]-hydrogenases. Chem Rev 107:4273–4303

Forestier M, King P, Zhang L, Posewitz M, Schwarzer S, Happe T, Ghirardi ML, Seibert M (2003) Expression of two [Fe]-hydrogenases in *Chlamydomonas reinhardtii* under anaerobic conditions. Eur J Biochem FEBS 270:2750–2758

Gaffron H, Rubin J (1942) Fermentative and photochemical production of hydrogen in algae. J Gen Physiol 26:219–240

Gartner K, Lechno-Yossef S, Cornish AJ, Wolk CP, Hegg EL (2012) Expression of Shewanella oneidensis MR-a [FeFe]-hydrogenase genes in *Anabaena* sp. strain PCC 7120. Appl Environ Microbiol 78:8579–8586

Ghirardi ML, Togasaki RK, Seibert M (1997) Oxygen sensitivity of algal H_2-production. Appl Biochem Biotechnol 63–65:141–151

Ghirardi ML, Zhang JP, Lee JW, Flynn T, Seibert M, Greenbaum E, Melis A (2000) Microalgae: a green source of renewable H-2. Trends Biotechnol 18:506–511

Ghirardi ML, Posewitz MC, Maness P-C, Dubini A, Yu J, Seibert M (2007) Hydrogenases and hydrogen photoproduction in oxygenic photosynthetic organisms. Annu Rev Plant Biol 58:71–91

Ghirardi ML, Dubini A, Yu JP, Maness PC (2009) Photobiological hydrogen-producing systems (vol 38, p. 52, 2009). Chem Soc Rev 38:3505

Ghirardi ML, King PW, Mulder DW, Eckert C, Dubini A, Maness PC, Yu J (2014) Hydrogen production by water biophotolysis. In: Zannoni D, De Philippis R (eds) Microbial bioenergy: hydrogen. Springer, Dordrecht

Girbal L, von Abendroth G, Winkler M, Benton PM, Meynial-Salles I, Croux C, Peters JW, Happe T, Soucaille P (2005) Homologous and heterologous overexpression in *Clostridium acetobutylicum* and characterization of purified clostridial and algal Fe-only hydrogenases with high specific activities. Appl Environ Microbiol 71:2777–2781

Godde D, Trebst A (1980) NADH as electron donor for the photosynthetic membrane of *Chlamydomonas reinhardtii*. Arch Microbiol 127:245–252

Goldet G, Brandmayr C, Stripp ST, Happe T, Cavazza C, Fontecilla-Camps JC, Armstrong FA (2009) Electrochemical kinetic investigations of the reactions of [FeFe]-hydrogenases with carbon monoxide and oxygen: comparing the importance of gas tunnels and active-site electronic/redox effects. J Am Chem Soc 131:14979–14989

Gutekunst K, Chen X, Schreiber K, Kaspar U, Makam S, Appel J (2014) The bidirectional NiFe-hydrogenase in *Synechocystis* sp. PCC 6803 is reduced by flavodoxin and ferredoxin and is essential under mixotrophic, nitrate-limiting conditions. J Biol Chem 289:1930–1937

Hankamer B, Lehr F, Rupprecht J, Mussgnug JH, Posten C, Kruse O (2007) Photosynthetic biomass and H_2 production by green algae: from bioengineering to bioreactor scale-up. Physiol Plant 131:10–21

Hanna MC, Nozik AJ (2006) Solar conversion efficiency of photovoltaic and photoelectrolysis cells with carrier multiplication absorbers. J Appl Phys 100:74510(1–8)

Happe T, Naber JD (1993) Isolation, characterization and N-terminal amino acid sequence of hydrogenase from the green alga *Chlamydomonas reinhardtii*. Eur J Biochem FEBS 214:475–481

Healey F (1970) Hydrogen evolution by several algae. Planta 91:220–226

Hemschemeier A, Happe T (2011) Alternative photosynthetic electron transport pathways during anaerobiosis in the green alga *Chlamydomonas reinhardtii*. Biochim Biophys Acta 1807:919–926

Hemschemeier A, Fouchard S, Cournac L, Peltier G, Happe T (2008) Hydrogen production by *Chlamydomonas reinhardtii*: an elaborate interplay of electron sources and sinks. Planta 227:397–407

James BD, Baum GN, Perez J, Baum KN (2009) Technoeconomic boundary analysis of biological pathways to hydrogen production

Jans F, Mignolet E, Houyoux PA, Cardol P, Ghysels B, Cuine S, Cournac L, Peltier G, Remacle C, Franck R (2008) A type II NAD(P)H dehydrogenase mediates light-independent plastoquinone reduction in the chloroplast of Chlamydomonas. Proc Natl Acad Sci USA 105:20546–20551

Jones AK, Sillery E, Albracht SP, Armstrong FA (2002) Direct comparison of the electrocatalytic oxidation of hydrogen by an enzyme and a platinum catalyst. Chem Commun (Camb) 8:866–867

Katsuda T, Oshima H, Azuma M, Kato J (2006) New detection method for hydrogen gas for screening hydrogen-producing microorganisms using water-soluble Wilkinson's catalyst derivative. J Biosci Bioeng 102:220–226

Kessler E (1974) Hydrogenase, photoreduction and anaerobic growth. In: Steward WDP (ed) Algal physiology and biochemistry. Blackwell, Oxford, pp 456–473

King PW, Posewitz MC, Ghirardi ML, Seibert M (2006) Functional studies of [FeFe] hydrogenase maturation in an *Escherichia coli* biosynthetic system. J Bacteriol 188:2163–2172

Kosourov S, Seibert M, Ghirardi ML (2003a) Effects of extracellular pH on the metabolic pathways in sulfur-deprived, H-2-producing *Chlamydomonas reinhardtii cultures*. Plant Cell Physiol 44:146–155

Kosourov S, Seibert M, Ghirardi ML (2003b) Effects of extracellular pH on the metabolic pathways in sulfur-deprived, H_2-producing *Chlamydomonas reinhardtii* cultures. Plant Cell Physiol 44:146–155

Kruse O, Rupprecht J, Bader KP, Thomas-Hall S, Schenk PM, Finazzi G, Hankamer B (2005) Improved photobiological H_2 production in engineered green algal cells. J Biol Chem 280:34170–34177

Lambertz C, Leidel N, Havelius KGV, Noth J, Chernev P, Winklers M, Happe T, Haumann M (2011) O_2 reactions at the six-iron active site (H-cluster) in [FeFe]-hydrogenase. J Biol Chem 286:40614–40623

Lamle SE, Albracht SP, Armstrong FA (2005) The mechanism of activation of a [NiFe]-hydrogenase by electrons, hydrogen, and carbon monoxide. J Am Chem Soc 127:6595–6604

Laurinavichene T, Tolstygina I, Tsygankov A (2004) The effect of light intensity on hydrogen production by sulfur-deprived *Chlamydomonas reinhardtii*. J Biotechnol 114:143–151

Lee HS, Vermaas WFJ, Rittmann BE (2010) Biological hydrogen production: prospects and challenges. Trends Biotechnol 28:262–271

Leger C, Jones AK, Roseboom W, Albracht SP, Armstrong FA (2002) Enzyme electrokinetics: hydrogen evolution and oxidation by *Allochromatium vinosum* [NiFe]-hydrogenase. Biochemistry 41:15736–15746

Leroux F, Dementin S, Burlatt B, Cournac L, Volbeda A, Champ S, Martin L, Guigliarelli B, Bertrand P, Fontecilla-Camps J, Rousset M, Leger C (2008) Experimental approaches to kinetics of gas diffusion in hydrogenase. Proc Natl Acad Sci USA 105:11188–11193

Lien S, Pietro AS (1981) Effect of uncouplers on anaerobic adaptation of hydrogenase activity in *C. reinhardtii*. Biochem Biophys Res Commun 103:139–147

Lukey MJ, Roessler MM, Parkin A, Evans RM, Davies RA, Lenz O, Friedrich B, Sargent F, Armstrong FA (2011) Oxygen-tolerant [NiFe]-hydrogenases: the individual and collective importance of supernumerary cysteines at the proximal Fe-S cluster. J Am Chem Soc 133:16881–16892

Maione TE, Gibbs M (1986a) Association of the chloroplastic respiratory and photosynthetic electron transport chains of *Chlamydomonas reinhardii* with photoreduction and the oxyhydrogen reaction. Plant Physiol 80:364–368

Maione TE, Gibbs M (1986b) Hydrogenase-mediated activities in isolated chloroplasts of *Chlamydomonas reinhardii*. Plant Physiol 80:360–363

Makarova VV, Kosourov S, Krendeleva TE, Semin BK, Kukarskikh GP, Rubin AB, Sayre RT, Ghirardi ML, Seibert M (2007) Photoproduction of hydrogen by sulfur-deprived *C. reinhardtii* mutants with impaired photosystem II photochemical activity. Photosynth Res 94:79–89

Maneeruttanarungroj C, Lindblad P, Incharoensakdi A (2010) A newly isolated green alga, *Tetraspora* sp. CU2551, from Thailand with efficient hydrogen production. Int J Hydrog Energy 35:13193–13199

Maness PC, Smolinski S, Dillon AC, Heben MJ, Weaver PF (2002) Characterization of the oxygen tolerance of a hydrogenase linked to a carbon monoxide oxidation pathway in *Rubrivivax gelatinosus*. Appl Environ Microbiol 68:2633–2636

Markov SA, Eivazova ER, Greenwood J (2006) Photostimulation of H-2 production in the green alga *Chlamydomonas reinhardtii* upon photoinhibition of its O-2-evolving system. Int J Hydrog Energy 31:1314–1317

Melis A, Zhang L, Forestier M, Ghirardi ML, Seibert M (2000) Sustained photobiological hydrogen gas production upon reversible inactivation of oxygen evolution in the green alga *Chlamydomonas reinhardtii*. Plant Physiol 122:127–136

Meuser JE, D'Adamo S, Jinkerson RE, Mus F, Yang W, Ghirardi ML, Seibert M, Grossman AR, Posewitz MC (2012a) Genetic disruption of both *Chlamydomonas reinhardtii* [FeFe]-hydrogenases: insight into the role of HYDA2 in H(2) production. Biochem Biophys Res Commun 417:704–709

Meuser JE, D'Adamo S, Jinkerson RE, Mus F, Yang WQ, Ghirardi ML, Seibert M, Grossman AR, Posewitz MC (2012b) Genetic disruption of both *Chlamydomonas reinhardtii* [FeFe]-hydrogenases: insight into the role of HYDA2 in H-2 production. Biochem Biophys Res Commun 417:704–709

Montet Y, Amara P, Volbeda A, Vernede X, Hatchikian EC, Field MJ, Frey M, Fontecilla-Camps JC (1997) Gas access to the active site of Ni–Fe hydrogenases probed by X-ray crystallography and molecular dynamics. Nat Struct Biol 4:523–526

Mulder DW, Boyd ES, Sarma R, Lange RK, Endrizzi JA, Broderick JB, Peters JW (2010) Stepwise [FeFe]-hydrogenase H-cluster assembly revealed in the structure of HydA(Delta EFG). Nature 465:248-U143

Mulder DW, Ratzloff MW, Shepard EM, Byer AS, Noone SM, Peters JW, Broderick JB, King PW (2013) EPR and FTIR analysis of the mechanism of H_2 activation by [FeFe]-hydrogenase HydA1 from *Chlamydomonas reinhardtii*. J Am Chem Soc 135:6921–6929

Nicolet Y, Piras C, Legrand P, Hatchikian CE, Fontecilla-Camps JC (1999) Desulfovibrio desulfuricans iron hydrogenase: the structure shows unusual coordination to an active site Fe binuclear center. Structure 7:13–23

Oey M, Ross I, Stephens E, Steinbeck J, Wolf J, Radzun K, Kugler J, Ringsmuth A, Kruse O, Hankamer B (2013) RNAi knock-down of LHCBM1, 2 and 3 increases photosynthetic H_2 production efficiency of the green alga *Chlamydomonas reinhardtii*. PLoS ONE 8:e61375

Pandelia ME, Nitschke W, Infossi P, Giudici-Orticoni MT, Bill E, Lubitz W (2011) Characterization of a unique [FeS] cluster in the electron transfer chain of the oxygen tolerant [NiFe] hydrogenase from *Aquifex aeolicus*. Proc Natl Acad Sci USA 108:6097–6102

Peters JW, Lanzilotta WN, Lemon BJ, Seefeldt LC (1998) X-ray crystal structure of the Fe-only hydrogenase (CpI) from *Clostridium pasteurianum* to 1.8 angstrom resolution. Science 282:1853–1858

Philipps G, Happe T, Hemschemeier A (2012) Nitron deprivation results in photosynthetic hydrogen production in *Chlamydomonas reinhardtii*. Planta 235:729–745

Posewitz MC, King PW, Smolinski SL, Zhang L, Seibert M, Ghirardi ML (2004a) Discovery of two novel radical S-adenosylmethionine proteins required for the assembly of an active [Fe] hydrogenase. J Biol Chem 279:25711–25720

Posewitz MC, Smolinski SL, Kanakagiri S, Melis A, Seibert M, Ghirardi ML (2004b) Hydrogen photoproduction is attenuated by disruption of an isoamylase gene in *Chlamydomonas reinhardtii*. Plant Cell 16:2151–2163

Posewitz M, Dubini A, Meuser JE, Seibert M, Ghirardi ML (2008) Hydrogenases, hydrogen production, and anoxia. Chlamydomonas Sourceb 2:215–255

Reifschneider-Wegner K, Kanygin A, Redding KE (2014) Expression of the [FeFe hydrogenase in the chloroplast of *Chlamydomonas reinhardtii*. Int J Hydrog Energy 39:3657–3665

Roessler P, Lien S (1984) Purification of hydrogenase from *Chlamydomonas reinhardtii*. Plant Physiol 75:705–709

Ruhle T, Hemschemeier A, Melis A, Happe T (2008) A novel screening protocol for the isolation of hydrogen producing *Chlamydomonas reinhardtii* strains. BMC Plant Biol 8:107

Rupprecht J (2009) From systems biology to fuel–Chlamydomonas reinhardtii as a model for a systems biology approach to improve biohydrogen production. J Biotechnol 142:10–20

Schmitz O, Bothe H (1996) The diaphorase subunit HoxU of the bidirectional hydrogenase as electron transferring protein in cyanobacterial respiration? Die Naturwissenschaften 83:525–527

Schrader PS, Burrows EH, Ely RL (2008) High-throughput screening assay for biological hydrogen production. Anal Chem 80:4014–4019

Scoma A, Krawietz D, Faraloni C, Giannelli L, Happe T, Torzillo G (2012) Sustained H_2 production in *Chlamydomonas reinhardtii* D1 protein mutant. J Biotechnol 157:613–619

Searchinger T, Heimlich R, Houghton RA, Dong F, Elobeid A, Fabiosa J, Tokgoz S, Hayes D, Yu TH (2008) Use of US croplands for biofuels increases greenhouse gases through emissions from land-use change. Science 319:1238–1240

Seibert M, Flynn T, Benson D, Tracy E, Ghirardi ML (1998) Development of selection/screening procedures for rapid identification of H_2-producing algal mutants with increased O_2-tolerance. In: Zaborsky OR (ed) Biohydrogen. Plenum Publishing Corporation, New York, pp 227–234

Seibert M, Flynn T, Ghirardi ML (2001a) Strategies for improving oxygen tolerance of algal hydrogen production. In: Miyake J, Matsunaga T, Pietro AS (eds) BioHydrogen II. Pergamon Press, Amsterdam, pp 65–76

Seibert M, Benson DK, Flynn TM (2001) Method and apparatus for rapid biohydrogen phenotypic screening of microorganisms using a chemochromic sensor. US Patent

Shestakov SV, Mikheeva LE (2006) Genetic control of hydrogen metabolism in cyanobacteria. Russ J Genet 42:1272–1284

Skjanes K, Knutsen G, Kallqvist T, Lindblad P (2008) H-2 production from marine and freshwater species of green algae during sulfur deprivation and considerations for bioreactor design. Int J Hydrog Energy 33:511–521

Stapleton JA, Swartz JR (2010a) Development of an in vitro compartmentalization screen for high-throughput directed evolution of [FeFe] hydrogenases. PLoS ONE 5:e15275

Stapleton JA, Swartz JR (2010b) A cell-free microtiter plate screen for improved [FeFe] hydrogenases. PLoS ONE 5:e10554

Stripp ST, Happe T (2009) How algae produce hydrogen—news from the photosynthetic hydrogenase. Dalton Trans 45:9960–9969

Stripp ST, Goldet G, Brandmayr C, Sanganas O, Vincent KA, Haumann M, Armstrong FA, Happe T (2009) How oxygen attacks [FeFe] hydrogenases from photosynthetic organisms. Proc Natl Acad Sci USA 106:17331–17336

Sumner I, Both GA (2012) Proton transport pathways in [NiFe]-hydrogenase. J Phys Chem B 116:2917–2926

Surzycki R, Cournac L, Peltier G, Rochaix JD (2007) Potential for hydrogen production with inducible chloroplast gene expression in Chlamydomonas. Proc Natl Acad Sci USA 104:17548–17553

Timmins M, Thomas-Hall SR, Darling A, Zhang E, Hankamer B, Marx UC, Schenk PM (2009) Phylogenetic and molecular

analysis of hydrogen-producing green algae. J Exp Bot 60:1691–1702

Tolleter D, Ghysels B, Alric J, Petroutsos D, Tolstygina I, Krawietz D, Happe T, Auroy P, Adriano JM, Beyly A, Cuine S, Plet J, Reiter IM, Genty B, Cournac L, Hippler M, Peltier G (2011) Control of hydrogen photoproduction by the proton gradient generated by cyclic electron flow in *Chlamydomonas reinhardtii*. Plant Cell 23:2619–2630

Tolstygina IV, Antal TK, Kosourov SN, Krendeleva TE, Rubin AB, Tsygankov AA (2009) Hydrogen production by photoautotrophic sulfur-deprived *Chlamydomonas reinhardtii* pre-grown and incubated under high light. Biotechnol Bioeng 102:1055–1061

Torzillo G, Scoma A, Faraloni C, Ena E, Johanningmeier U (2009) Increased hydrogen photoproduction by means of a sulfur-deprived *Chlamydomonas reinhardtii* D1 protein mutant. Int J Hydrog Energy 34:4529–4536

Vignais PM, Colbeau A, Willison JC, Jouanneau Y (1985) Hydrogenase, nitrogenase, and hydrogen metabolism in the photosynthetic bacteria. Adv Microb Physiol 26:155–234

Vignais PM, Billoud B, Meyer J (2001) Classification and phylogeny of hydrogenases. FEMS Microbiol Rev 25:455–501

Vincent KA, Armstrong FA (2005) Investigating metalloenzyme reactions using electrochemical sweeps and steps: fine control and measurements with reactants ranging from ions to gases. Inorg Chem 44:798–809

Vincent KA, Cracknell JA, Parkin A, Armstrong FA (2005a) Hydrogen cycling by enzymes: electrocatalysis and implications for future energy technology. Dalton Trans 21:3397–3403

Vincent KA, Parkin A, Lenz O, Albracht SP, Fontecilla-Camps JC, Cammack R, Friedrich B, Armstrong FA (2005b) Electrochemical definitions of O_2 sensitivity and oxidative inactivation in hydrogenases. J Am Chem Soc 127:18179–18189

Vincent KA, Parkin A, Armstrong FA (2007) Investigating and exploiting the electrocatalytic properties of hydrogenases. Chem Rev 107:4366–4413

von Abendroth G, Stripp ST, Silakov A, Croux C, Soucaille P, Girbal L, Happe T (2008) Optimized over-expression of [FeFe] hydrogenases with high specific activity in *Clostridium acetobutylicum*. Int J Hydrog Energy 33:6076–6081

Walker DA (2009) Biofuels, facts, fantasy and feasibility. J Appl Phycol 21:509–517

Wang H, Fan X, Zhang Y, Yang D, Guo R (2011) Sustained photohydrogen production by *Chlorella pyrenoidosa* without sulfur depletion. Biotechnol Lett 33:1345–1350

Weaver PF, Lien S, Seibert M (1980) Photobiological production of hydrogen. Sol Energy 24:3–45

Wecker MS, Ghirardi ML (2014) High-throughput biosensor discriminates between different algal H_2-photoproducing strains. Biotechnol Bioeng 111:1332–1340

Wecker MSA, Meuser JE, Posewitz MC, Ghirardi ML (2011) Design of a new biosensor for algal H_2 production based on the H_2-sensing system of *Rhodobacter capsulatus*. Int J Hydrog Energy 36:11229–11237

Weyman PD, Vargas WA, Tong Y, Yu J, Maness PC, Smith HO, Xu Q (2011) Heterologous expression of *Alteromonas macleodii* and *Thiocapsa roseopersicina* [NiFe] hydrogenases in *Synechococcus elongatus*. PLoS ONE 6:e20126

White A, Melis A (2006) Biochemistry of hydrogen metabolism in *Chlamydomonas reinhardtii* wild type and a Rubisco-less mutant. Int J Hydrog Energy 31:455–464

Winkler M, Kuhlgert S, Hippler M, Happe T (2009) Characterization of the key step for light-driven hydrogen evolution in green algae. J Biol Chem 284:36620–36627

Wu S, Xu L, Huang R, Xang Q (2011) Improved biohydrogen production with an expression of codon-optrimized hemH and lba genes in the chloroplast of *Chlamydomonas reinhardtii*. Bioresour Technol 102:2610–2616

Zabawinski C, Van den Koornhuyse N, D'Hulst C, Schlichting R, Giersch C, Delrue B, Lacroix JM, Preiss J, Ball S (2001) Starchless mutants of *Chlamydomonas reinhardtii* lack the small subunit of a heterotetrameric ADP-glucose pyrophosphorylase. J Bacteriol 183:1069–1077

Zhang L, Happe T, Melis A (2002) Biochemical and morphological characterization of sulfur-deprived and H_2-producing *Chlamydomonas reinhardtii* (green alga). Planta 214:552–561