Photosynthetic hydrogen production: Novel protocols, promising engineering approaches and application of semi-synthetic hydrogenases

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Abstract
Photosynthetic production of molecular hydrogen (H₂) by cyanobacteria and green algae is a potential source of renewable energy. These organisms are capable of water biophotolysis by taking advantage of photosynthetic apparatus that links water oxidation at Photosystem II and reduction of protons to H₂ downstream of Photosystem I. Although the process has a theoretical potential to displace fossil fuels, photosynthetic H₂ production in its current state is not yet efficient enough for industrial applications due to a number of physiological, biochemical, and engineering barriers. This article presents a short overview of the metabolic pathways and enzymes involved in H₂ photoproduction in cyanobacteria and green algae and our present understanding of the mechanisms of this process. We also summarize recent advances in engineering photosynthetic cell factories capable of overcoming the major barriers to efficient and sustainable H₂ production.

1 INTRODUCTION

Molecular hydrogen (H₂) represents the highest energy density per mass unit among currently employed fuels and is a zero-carbon emission solution for the sustainable economy. Besides excellent properties as a fuel, H₂ is an important feedstock for many industrial processes, including methanation of CO and CO₂, methanol synthesis from CO₂ hydrogenation, and hydrogenation of nitrogen (N₂) for the production of ammonia. H₂ is also used as a reducing agent in a number of different applications. Compared to conventional methods of H₂ generation, photobiological H₂ production represents an attractive renewable alternative since it utilizes water and solar light, the world’s most abundant resources.

Many species of cyanobacteria and eukaryotic green algae are capable of photosynthetic water splitting to H₂ and oxygen (O₂) under specific conditions using dedicated enzymes. Although a
theoretical maximum of light energy to H2 energy conversion efficiency (LHCE) is as high as 10–13% for green algae and 6% for N2-fixing cyanobacteria, only a fraction of these values has been achieved (1.5–2% in algae and up to 4% in cyanobacteria) under controlled laboratory conditions typically employing low light illumination (Bolton & Hall, 1991; Kosourova et al., 2017; Sakurai et al., 2015). A recent study showed that green alga *Chlorella vulgaris* is capable of producing H2 with the maximum LHCE up to 8% (3% on the average basis), though in the presence of glucose (Touloupakis et al., 2021). Importantly, volumetric H2 yields in algal and cyanobacterial cultures are still quite low even in the controlled environment due to a number of physiological and biochemical barriers limiting H2 production rates.

A deep understanding of the photosynthetic H2 metabolism and its effect on cell bioenergetics opens new possibilities for the development of H2-producing cell factories with improved performance and enhanced H2 production yields. This mini-review will summarize recent advances in understanding the mechanisms of light-driven H2 production in cyanobacteria and green algae and give a short overview of strategies and approaches for engineering efficient H2-producing cell factories, including whole-cell applications of semi-synthetic hydrogenases.

### 2. A SHORT OVERVIEW OF H2 METABOLISM IN CYANOBACTERIA

Cyanobacteria are a large and diverse group of oxygenic photoautotrophic prokaryotes. Many of them have an innate capacity to produce H2. Cyanobacteria have two sets of enzymes involved in H2 metabolism: nitrogenases and hydrogenases (Figure 1A,C). Nitrogenases (encoded by *nifHDK*) are found in N2-fixing unicellular or filamentous cyanobacteria, which produce H2 as a byproduct of N2 fixation and its reduction into ammonia. Cyanobacteria also possess two types of [NiFe]-hydrogenases: bidirectional hydrogenase (encoded by *hoxEFUYH*) and uptake hydrogenase (encoded by *hupSL*) enzymes (Barz et al., 2010; Lindblad, 2018; Puggioni et al., 2016). Unlike uptake hydrogenase, which recycles H2 produced by nitrogenase, the bidirectional hydrogenase both oxidizes and produces H2 and can be present in diazotrophic and non-diazotrophic strains. Since O2 is a byproduct of photosynthesis and all enzymes involved in H2 metabolism are O2 sensitive, cyanobacteria have developed spatial and temporal strategies for their protection: (1) heterocystous filamentous cyanobacteria spatially separate oxygenic photosynthesis (occurring in vegetative cells) and N2 fixation (occurring in heterocysts, specialized cells that provide a low-oxygen environment) and...
(2) non-heterocystous cyanobacteria (e.g., filamentous Trichodesmium, unicellular Cyanothece) perform N₂ fixation, and thus H₂ production, typically under anoxic conditions but also under oxic conditions (e.g., due to the control of their circadian rhythm or intracellular compartmentation). The processes that support H₂ production in these organisms under oxic conditions have yet to be fully resolved. In contrast, the bidirectional hydrogenase particularly operates under microoxic or anaerobic environment and could utilise reductants produced directly by the photosynthetic electron transport chain (PETC). Cyanobacteria bearing this enzyme typically photoproduce H₂ on exposure to light, but only for a very short period followed by H₂ uptake. The O₂ sensitivity of the bidirectional hydrogenase thus represents a major biotechnological hurdle for efficient H₂ photoproduction via this enzyme.

3 | A SYSTEM BIOLOGY APPROACH TO UNDERSTANDING THE MECHANISM OF H₂ PRODUCTION IN CYANOBACTERIA

To achieve a greater understanding of how cell metabolism and bioenergetics navigate during H₂ photoproduction and to identify potential targets for further metabolic engineering, a system-level omics approach, which includes genomics, transcriptomics, proteomics, and metabolomics analysis, is necessary. Most of the omics approaches have been focused on understanding the metabolism of nitrogenase-based H₂ production (see, e.g., Aryal et al., 2013; Bernstein et al., 2015; Ekman et al., 2011; Kourpa et al., 2019; Sadler et al., 2016; Stensjö et al., 2007). The differential proteomes of N₂-fixing and non-N₂-fixing cultures of both Nostoc sp. and Cyanothece sp. have been resolved, and the heterocyst-specific Cys-proteome was detailed (Sandh et al., 2014). The reprogramming of metabolism in the H₂-producing mutant (ΔHupL) of Nostoc (also known as Anabaena) sp. PCC 7120, which lacks the uptake hydrogenase in heterocysts (Figure 1C), involved >100 differentially expressed proteins (Ekman et al., 2011). Also, in the unicellular diazotrophic cyanobacterium Cyanothece sp., which produces H₂ at very high rates (Bandyopadhyay et al., 2010), proteomics was used to identify targets for improving H₂ production yields (Aryal et al., 2013). General important factors to drive nitrogenase-dependent H₂ production in both Cyanothece sp. and Nostoc sp. were (1) to provide sugars, that is, glycogen oxidation in the dark for Cyanothece and import of sucrose from the vegetative cell to heterocysts in Nostoc; (2) to provide enough reducing equivalents by an efficient oxidative pentose phosphate pathway; and (3) to provide ATP by cyclic electron flow (CEF) around Photosystem I (PSI).

For Cyanothece, this view was later challenged when it was shown that the defined key components of H₂ metabolism in Cyanothece differed substantially from that in Nostoc. For example, a positive quantitative correlation between the H₂ production profile and the abundance of proteins of Photosystem II (PSII) was demonstrated (Bernstein et al., 2015). This suggests that in contrast to heterocystous cyanobacteria, the PSII-driven linear electron flow in unicellular Cyanothece directly provides reducing equivalents and ATP for nitrogenase-dependent H₂ photoproduction. These findings bring new strategies for future metabolic engineering and optimization of growth conditions for efficient and sustainable photobiological H₂ production.

Although the usefulness of multi-omics and in silico strategies are well accepted, the attempts to use them for designing H₂-producing cyanobacteria are still infrequent. One of the delays in the effort to use systems biology as a fundament for bioengineering is the lack of genome-scale metabolic models. Recently, two such models have been constructed to predict pathways and protein targets for improvement of H₂ photoproduction in the diazotrophic heterocyst-forming model organisms, Anabaena sp. PCC 7120 (Malatinszky et al., 2017) and Anabaena variabilis sp. ATCC 29413 (Malek Shakhkouhi & Motamedian, 2020). The in silico studies, for the most part, confirmed the targets of importance for improving heterocyst-based H₂ production emphasized in the quantitative proteomics investigations. Thus, the initial simulations and modeling attempts display the value for further development of these methods.

4 | ENGINEERING CYANOBACTERIA FOR SUSTAINABLE AND EFFICIENT H₂ PRODUCTION

Since cyanobacteria are capable of catalyzing H₂ photoproduction by the direct (e.g., in unicellular cyanobacteria via bidirectional hydrogenase) and indirect water biophotolysis (e.g., in N₂-fixing heterocystous cyanobacteria via nitrogenase localized in heterocysts) mechanisms, challenges and engineering strategies for improving H₂ photoproduction yields also differ to some extent. In general, the major metabolic bottlenecks of cyanobacterial H₂ production include (1) high O₂ sensitivity of the enzymes involved in H₂ metabolism (nitrogenases and/or hydrogenases), (2) low photosynthetic yield, (3) alternative electron transport pathways competing for photosynthetic electrons, and (4) H₂ uptake by the cells (via bidirectional or uptake hydrogenases). Therefore, the following challenges are to be addressed: (1) modulation of competing pathways for boosting the H₂ production yield without compromising cell fitness; (2) improvement of photosynthesis, including optimization of NADPH/ATP ratio, for efficient bioproduction; (3) screening available cyanobacterial culture collections; (4) mining and introducing novel, more efficient, and O₂-tolerant enzymes; and (5) development of novel cultivation protocols for efficient H₂ production. Throughout the years, there have been numerous attempts to overcome these barriers and challenges by employing a diversity of genetic engineering approaches. Here we focus on the most recent and novel advances in engineering cyanobacteria for sustainable H₂ photoproduction.

4.1 | Deletion of the capacity to take up hydrogen

There are many examples in the literature of either disrupting or deleting a structural gene, or a gene encoding a protein of the
maturation machinery, of the uptake HupSL hydrogenase in cyanobacteria (see, e.g., Lindblad, 2018). The result will be H₂-producing cells, where H₂ evolution is catalyzed by a native nitrogenase. Using a ΔHupW strain of Anabaena sp. PCC 7120 (a strain lacking the last step in the maturation machinery) analyzed in a 5.0 L flat-panel photo-bioreactor system with a 3.0 L culture volume and illuminated with a mixture of red and white LED lights, Nyberg et al. (2015) showed a maximal LHCE of ca. 4.0% (in the PAR region) and a maximal hydrogen concentration of 6.89%. However, it is also important to note that inactivation of uptake hydrogenase reprograms cell metabolism (Ekman et al., 2011; Kourpa et al., 2019) and may significantly decrease the duration of the H₂ production process and cell fitness, particularly under a high C/N environment (Kosourov et al., 2014).

Another approach is to modify the flow of electrons from H₂ uptake toward H₂ evolution in the HupSL hydrogenase. The NiFe-containing active site is present in the large subunit, whereas the small subunit (HupS) harbors three FeS-clusters. HupS of the filamentous cyanobacterium Nostoc punctiforme ATCC 29133 was heterologously expressed as a fusion protein in Escherichia coli (f-HupS), purified, and characterized (Raleiras et al., 2013). Thereafter, the proximal Fe-S cluster of HupS was modified by changing the cysteine in position 12 to proline (C12P). C12P f-HupS investigated by EPR spectroscopy demonstrated a conversion from a 4Fe4S to a 3Fe4S proximal cluster of HupS was modified by changing the cysteine in position 12 to proline (C12P). C12P F-HupS investigated by EPR spectroscopy demonstrated a conversion from a 4Fe4S to a 3Fe4S proximal cluster. C12P HupSL was then introduced and expressed in N. punctiforme, which resulted in cells with increased H₂ production. The modified proximal Fe-S cluster in HupS changed the flow of electrons toward hydrogen production (Raleiras et al., 2016).

### 4.2 Modulation of alternative electron transport routes

Regardless of hydrogenase, a steady flow of electrons to the hydrogenase enzyme, provided by primary electron donors such as reduced ferredoxin, is needed for efficient and sustainable photobiological H₂ production (Guttekunst et al., 2014). However, there is no “bag-of-electrons”; the available photosynthetic electrons are utilized in other assimilatory pathways, such as the respiratory electron transport system, nitrate assimilation, and carbon fixation via the Calvin-Benson-Bassham (CBB) cycle. From a biotechnological aspect, these are competing routes. Moreover, alternative electron transport pathways such as the flavodiiron proteins (FDPs)-driven water–water cycle and the cyclic electron transport around PSI can be considered as “waste” points for photosynthetic production. Therefore, a genetic engineering strategy should be applied for re-directing the electron flow away from the existing native assimilatory and auxiliary pathways toward H₂ production. This has been experimentally demonstrated in Synechocystis sp. PCC 6803 by eliminating the electron flow to (1) the respiratory terminal oxidases (Gutthann et al., 2007), (2) the nitrate-assimilation pathway (Baebprasert et al., 2011), and (3) the NDH-1 complex involved in CEF, respiration, and carbon concentrating mechanism (Cournac et al., 2004). These examples all resulted in increased H₂ production yields. Specifically, removing nitrate from the growth medium, or creating strains lacking a functional pathway to assimilate nitrate, significantly increases photobiological H₂ production.

Despite the fact that filamentous heterocystous N₂-fixing cyanobacteria are the most fascinating organisms for biotechnological applications, the electron-transport pathways inside heterocysts are largely unknown. A recent study demonstrated that the heterocyst-specific Flv3B enzyme actively eliminates O₂ thus enabling microoxic interior inside of the heterocyst, which supports diazotrophic growth and nitrogenase enzyme activity under illumination (Ermarkova et al., 2014). Accordingly, overexpression of the Flv3B protein resulted in high H₂ photoproduction, even though this phenotype was not linked to enhanced nitrogenase activity (Roumezi et al., 2020). The latter requires further investigations.

### 4.3 PSI–hydrogenase fusion in vivo

Semiartificial in vitro techniques can be created in which hydrogenases are attached to isolated photosystems for H₂ production. However, generally such systems are short lived. Recently, Appel et al. (2020) reported photosynthetic H₂ production using a PSI-hydrogenase fusion in vivo. HoxYH of the NiFe-hydrogenase of Synechocystis sp. PCC 6803 was fused to its PSI subunit PsaD. The recombinant Synechocystis strain showed long-lived H₂ production in a light-dependent manner with otherwise undisturbed metabolism.

### 4.4 Introduction of [FeFe]-hydrogenases in cyanobacteria

Cyanobacteria contain [NiFe]-hydrogenases. However, [FeFe]-hydrogenases, found in green algae and diverse bacteria, are metabolically much more active toward H₂ production and with higher turnover numbers. Already in 2011, Ducat and co-authors introduced and expressed a [FeFe]-hydrogenase (HydA) together with the HydEFG maturation machinery from Clostridium acetobutylicum in the non-N₂-fixing cyanobacterium Synechococcus elongatus PCC 7942. The heterologously expressed [FeFe]-hydrogenase showed the most significant in vivo hydrogenase activity connected to the light-dependent reactions of PETC under anoxic conditions (Ducat et al., 2011). Moreover, the introduced hydrogenase supported limited growth in the light using solely H₂ as a source of reducing equivalents. A challenge with introducing a very oxygen-sensitive [FeFe]-hydrogenase was addressed by Avilan et al. (2018) who expressed the clostridial [FeFe]-hydrogenase in the heterocysts of Anabaena sp. PCC 7120. Unlike wild type, the mutant strain showed negligible H₂ photoproduction under argon atmosphere, however substantially more H₂ photoproduction was observed in the presence of DCMU (3-[3,4-dichlorophenyl]-1,1-dimethylurea), an inhibitor of PSII. To further decrease the oxygen levels in the heterocysts, an oxygen scavenger, a GlbN cyanoglobin from Nostoc commune, was co-expressed with HydA from Clostridium acetobutylicum. The obtained mutant strain showed significantly higher H₂ production under an argon
atmosphere, but weak H₂ photoproduction was also recorded in the presence of air (Avián et al., 2018).

As further discussed below, another strategy is now available that circumvents the biological maturation of [FeFe]-hydrogenase by an artificial synthetic activation of the heterologously expressed HydA protein in living cells. Whereas the structural HydA apoprotein (from e.g. the green alga *Chlamydomonas reinhardtii*) is expressed using a designed gene fragment, a functional HydA is created by the addition of a synthetic analogue of the [2Fe]₃ subsite outside the cells (Wegelius et al., 2018). The experiments showed that the non-native, semi-synthetic enzyme retains its H₂ production capacity for several days after synthetic activation with the regulation of activity based on the availability of electrons.

### 4.5 Whole-cell immobilization approach

The immobilization of enzymes and microbes within polymer matrices is an attractive approach to extending the lifetime of catalysts and enabling an easy and continuous production platform. Improved cell fitness and H₂ photoproduction activity have been demonstrated in cyanobacteria and green algae immobilized in thin-films (Jämsä et al., 2018; Kosourov et al., 2017; Wutthithien et al., 2019). Recently, a proof-of-concept for the sustained nitrogenase-based H₂ photoproduction was demonstrated by engineered thin-layer alginate films with entrapped heterocysts cells (Volgusheva et al., 2019). Immobilized heterocysts were able to produce H₂ in the presence of externally added sucrose for about 20 days, whereas heterocysts in suspensions showed similar activity for only 24 h.

### 5 RECENT ADVANCES IN UNDERSTANDING H₂ METABOLISM IN GREEN ALGAE

Hydrogen metabolism in eukaryotic green algae is mediated by [FeFe]-hydrogenase enzymes that catalyze the reversible reduction of protons to molecular hydrogen using a biologically unique diiron cofactor (the [2Fe]₃ subsite). Depending on the redox state of the cells and the partial pressure of H₂ in the environment, algae could either produce or oxidize H₂. Concerning H₂ metabolism, *C. reinhardtii* is the most studied alga and thereafter it would be a primary focus of this review.

Similar to many other green algae, *C. reinhardtii* possesses two distinct hydrogenase genes in the nuclear genome that encode for two proteins, CrHYDA1 and CrHYDA2 (Forestier et al., 2003; Happe & Kaminski, 2002). Some algal species carry three hydrogenases (Skjånes et al., 2010). Until recently, the physiological basis for the presence of multiple hydrogenases in cells has not been yet determined. The electrochemical study performed by Engelbrecht et al. (2021) showed the higher catalytic preference of CrHYDA2 to H₂ oxidation, thus revealing its potential role in H₂ uptake in *C. reinhardtii* cells. The biosynthesis and assembly of the complete active site of [FeFe]-hydrogenases is a comparatively simple process that involves three specific maturation enzymes: HydE, HydF, and HydG (Posewitz et al., 2004). In *C. reinhardtii*, genes encoding HydE and HydF proteins are fused, but in the majority of other organisms hydE, hydF, and hydG exist as separately transcribed genes.

It has been widely accepted that *C. reinhardtii* induces [FeFe]-hydrogenases and activates H₂ metabolic pathways only under strictly anoxic conditions (see, e.g., Dubini & Ghirardi, 2015). The extreme sensitivity of isolated [FeFe]-hydrogenases to atmospheric O₂ with oxidation and irreversible inactivation of their catalytic center supported the anaerobic nature of these enzymes (Stripp et al., 2009). The first evidence on the presence of hydrogenase transcripts in aerobic samples was reported by Forestier et al. (2003), who observed a prominent level of hydA2 transcripts in aerobic samples. Later, the presence of hydA1, hydA2 transcripts at the beginning of sulfur deprivation, when cultures are still aerobic, was shown by microarray analysis (Nguyen et al., 2008) and RNA-Seq (González-Ballester et al., 2010). The authors suggested that O₂ may not be the only trigger for the induction of hydA transcription. The presence of a very small amount of HYDA1 in aerobic samples was later confirmed at the protein level (Nikolova et al., 2018).

The availability of transcripts or even proteins in cells does not always mean the presence of active enzymes. Employing a high-sensitive membrane-inlet mass spectrometry (MIMS) approach, Liran et al. (2016) showed H₂ photoproduction in *C. reinhardtii* cultures under aerobic conditions, and in particular, on a shift from low to high light conditions. The authors proposed the existence of microoxic niches in the thylakoid stroma, where the enzyme resides and remains active (Liran et al., 2016). In these microoxic niches, O₂ is eliminated either by chlororespiration catalyzed by the plastid terminal oxidase (PTOX) or O₂ photoreduction driven by FDPs. Though, the role of FDPs in the creation of microoxic niches was put under question soon after Liran’s et al. publication (Burlacot et al., 2018). Similarly, the presence of the [FeFe]-hydrogenase in aerobic samples was proposed by Kosourov et al. (2018), but in contrast to Liran’s et al.’s (2016) study, authors could not detect the production of H₂ in aerobic cultures. Instead, they observed the appearance of the active [FeFe]-hydrogenase in cells within less than 3 min of transition from fully aerobic (21% of O₂) to microoxic (0.01–0.04% of O₂) environment (Kosourov et al., 2018, 2020). Since [FeFe]-hydrogenases are nuclear-encoded and their transportation to the chloroplast with following maturation requires time, the authors suggested the expression of hydrogenases during aerobic cultivation and activation of these enzymes under a microoxic environment. It should be noted, however, that Kosourov and co-authors worked with synchronized cultures, which were sampled during the period of active photosynthesis. Therefore, it is possible that Liran et al. study (employing unsynchronized cultures) monitored H₂ production from a fraction of algae in a different metabolic state, either cells with reduced photosynthetic activity or cells with enhanced respiration. Further experiments are necessary to prove this suggestion. Anyway, the presence of the [FeFe]-hydrogenase in aerobically-grown *C. reinhardtii* algae is now proved by two independent physiological studies. The most obvious role of
hydrogenases in aerobically grown algal cells is the protection of the photosynthetic machinery of overreduction during (or immediately after) a shift to the microoxic environment. In such a scenario, algae should constitutively express [FeFe]-hydrogenases and activate the enzymes as soon as microoxic conditions are established. On the contrary, the transition from microoxic to aerobic conditions results in degradation of the major pool of the [FeFe]-hydrogenase enzyme as demonstrated in the pulse-illuminated algae on the switch to continuous light (Kosourov et al., 2018).

Interestingly, another alga *Chlorella vulgaris* that possesses the [FeFe]-hydrogenase has been shown to sustain H₂ photoproduction under air atmosphere, though at relatively low production rates (Hwang et al., 2014). The mechanism of such O₂ tolerance in photosynthetically grown algae has not been revealed yet. This work demonstrates that the inhibitory threshold of [FeFe]-hydrogenases to extracellular levels of O₂ could be different in different strains and species and may vary depending on photosynthetic and respiratory activities of algae. Thus, the adverse effect of O₂ on [FeFe]-hydrogenase activity, or yet unknown protection mechanisms.

The most efficient H₂ photoproduction in *C. reinhardtii* cultures occurs on their exposure to light after the period of the dark anaerobic adaptation. According to Boichenko et al. (2004), some active algal producers placed under optimal conditions could reach production rates up to 300 μmol H₂ h⁻¹ (mg Chl)⁻¹, which is close to the maximal steady-state rate of CO₂-dependent O₂ evolution (Boichenko et al., 2004). Thus, H₂ photoproduction in green algae has a great potential for industrial applications, but the process proceeds for only a short time (from a few seconds to a few minutes).

Hydrogen photoproduction in algae (Figure 1B) depends on two metabolic pathways: (1) direct water biophotolysis, which involves water oxidation at PSII, and (2) indirect process, which supplies reductants originating from the oxidation of organic substrates to the PQ-pool via the Type II NADPH dehydrogenase (NDA2) (see, e.g., Dubini & Ghirardi, 2015). The contribution of each pathway varies to a different degree depending on the metabolic state of the cell and stage of hydrogen production. While the availability of the indirect pathway in algae was shown by multiple studies with photosynthetic inhibitors, the role of the PSII-dependent process in H₂ photoproduction has been mostly probed indirectly. The reason for that was the absence of O₂ evolution during the period of efficient H₂ photoproduction and inhibition of the latter as soon as O₂ starts to accumulate in the culture. Even if the absence of net O₂ production could be explained by the removal of O₂ in respiration (especially for the sulfur-deprived algae with their residual PSII activity; Melis et al., 2000; Tsygankov et al., 2006), the other research data indicate the domination of PSII-independent pathway in algal cells. Many short-term experiments performed with dark-adapted algae showed the quantum yield of H₂ evolution significantly exceeding 0.25 (Boichenko et al., 2004), the maximum theoretical value for the process depending on two photosystems. The latter means that H₂ photoproduction in green algae occurs at least partially through the mechanism independent of water oxidation. Moreover, PSII-deficient strains do not lose the capacity to photoproduce H₂ at least in the short-term process (Kosourov et al., 2020).

The direct photosynthetic pathway involving both photosystems is the most promising for the development of sustainable H₂ production. It utilizes water oxidation by PSII as an electron source for the hydrogenase-driven H⁺ reduction but also co-evolves O₂, a by-product of water oxidation. Since O₂ inhibits the [FeFe]-hydrogenase enzyme, it is clear that the PSII activity must be contained to a level where respiration overtakes O₂ evolution thus creating an anoxic state in the cells (or at least in the pockets where the [FeFe]-hydrogenase operates) and allowing prolonged H₂ evolution. Early strategies for sustaining H₂ production in algal cultures pursued this goal. The nutrient limitation approach and, in particular, the most-studied sulfur (S)-deprivation protocol is one of them. This approach allows effective termination of the de novo PSII synthesis leading to a gradual inhibition of the PSII-dependent water oxidation activity about 20% of the original level and enabling spontaneous anoxia in algal cultures with following induction of hydrogenase enzymes in cells (Melis et al., 2000; Volgusheva et al., 2013). Another important consequence of S-deprivation is the reduced state of the PQ pool, which in some cases enables H₂ photoproduction for more than a week (Volgusheva et al., 2013). Since S-deprivation gradually degrades PSII centers in algal cells, their contribution to H₂ photoproduction declines over time and is replaced by the degradation of organic substrates through the PSII-independent pathway, primarily by the degradation of starch. The involvement of the residual PSII activity in the H₂ photoproduction yield in S-deprived algae has been proven by spectroscopic studies, fluorescence data, and inhibitory analysis (Melis et al., 2000; Volgusheva et al., 2013, 2016).

Alternatively, the PSII activity could be restricted by specific mutations leading to similar results. However, we have to emphasize that strategies aiming at the restriction of PSII activity in cells are a temporary solution for sustaining H₂ production in algal cultures. They significantly decrease the efficiency of the process and should be replaced by the methods aiming at the natural regulation of photosynthetic electron flow from PSII to the [FeFe]-hydrogenase.

### Multi-omics approaches

Multi-omics approaches, which include data from genomics, transcriptomics, proteomics, and metabolomic analyses, have been used for identification of genes and proteins involved in H₂ production in *C. reinhardtii* and some other algal species (see, e.g., Xu et al., 2019). These genes and proteins are potential new targets for metabolic engineering and improving H₂ production yields. Although genetic analysis has been plentifully employed, the analytical proteome studies of H₂-producing algae are quite rare. As a result, the experimental data at the protein level are still limited, especially in combination with
other omics approaches. Comparative proteomics has been applied to sulfur- and nitrogen-deprived algae at different stages of nutrient starvation (Chen et al., 2010; Li et al., 2021) and to anaerobically adapted C. reinhardtii cultures under continuous illumination (Terashima et al., 2010). In dark-adapted anaerobic C. reinhardtii, the proteome analysis has been combined with transcriptomics and metabolomics data (Subramanian et al., 2014). The authors of these studies identified new candidates for further investigations and targeted engineering, which could clarify peculiarities of H2 metabolism in algae. However, no complex metabolic engineering strategies, which are based on findings from multi-omics data, have been implemented yet for improving the algal H2 production yield.

7 | NOVEL APPROACHES FOR SUSTAINING H2 PHOTOPRODUCTION IN GREEN ALGAE

7.1 | Balancing PSII to PSI ratio

One of the important conclusions derived from S-deprivation studies of C. reinhardtii is that the complete reduction of the PQ-pool is the initial condition for induction of H2 photoproduction in algal cells (Volgusheva et al., 2015, 2016). The reduction of the PQ-pool reflects the overall reduced state in the chloroplast and also results in the decreased PSI activity. Both conditions (the reduced PQ-pool and the decrease PSII activity) facilitate H2 evolution as a way to relieve the electron pressure in the cell.

Although the redox state of the PQ-pool in the thylakoid membrane of C. reinhardtii depends on many factors, a proper condition for the induction of H2 photoproduction can be achieved by simply altering the balance between two photosystems, PSII and PSI. A decreased amount of PSI can potentially modulate the electron transfer from the PSII by generating the reduced PQ-pool, thus also the decreased PSII activity. Indeed, the C3 mutant strain of C. reinhardtii with an abnormally low PSI content exhibits a reduced state of the PQ-pool already at standard growth conditions (Krishna et al., 2019). Incubation of C3 mutant in closed photobioreactor resulted in the establishment of anaerobiosis within 17 h (allowing respiration to overtake O2 evolution) and stimulated H2 photoproduction for an unprecedented period of at least 6 weeks under the standard continuous light and growth conditions (Krishna et al., 2019). Although a low amount of PSI was pre-request for quick initiation H2 photoproduction, after 6 days it has significantly exceeded the amount of PSII, demonstrating the importance of PSI at later stages. It is possible that by optimizing PSII/PSI ratio in the cells even longer H2 photoproduction can be achieved.

7.2 | Avoiding competition with CO2 fixation and other metabolic pathways

As mentioned above, H2 photoproduction in the nutrient-replete green algae is a transient phenomenon, which is typically observed in dark-adapted anaerobic cultures after their exposure to light. Under saturating illumination, algae evolve H2 for only a few seconds and start accumulating O2. Therefore, the inactivation of H2 photoproduction in cells was primarily attributed to the irreversible inhibition of hydrogenase enzymes by O2 accumulated in algal cultures. For a while, the O2-sensitivity hypothesis became dominating despite (1) the proposed role of hydrogenases in the activation of the CBB cycle (see, e.g., Godaux et al., 2015) and (2) the available data on the direct competition of H2 photoproduction for reducing equivalents with CO2 fixation. The O2-sensitivity concept has been reconsidered after three independent studies (Kosourov et al., 2018; Milrad et al., 2018; Nagy et al., 2018), which were published almost simultaneously.

By employing a high-sensitive MIMS approach, Milrad et al. (2018) showed that inactivation of H2 photoproduction in C. reinhardtii during the shift from dark to light conditions is caused by an increasing flow of electrons toward photosynthetic CO2 fixation. The competition for photosynthetic reductants between these two pathways occurs long before the irreversible inactivation of [FeFe]-hydrogenases by O2 accumulated in the culture. Thus, the hydrogenase remains active during the period of induction of oxygenic photosynthesis and could even participate in H2 uptake later on. Not only CO2 fixation but also O2 photoreduction driven by FDPs could compete with H2 evolution for photosynthetic reductants. Similar to hydrogenases, FDPs are crucial for the rapid activation of the CBB cycle but negatively affect H2 production yields in C. reinhardtii cultures under increasing O2 levels (Burlacot et al., 2018). Elimination of FDPs as a competing electron sink resulted in the substantial increase in the long-term H2 photoproduction yield (Jokel et al., 2019). These studies indicate that the adverse effect of O2 on H2 photoproduction also occurs long before the direct inhibition of [FeFe]-hydrogenases by constantly increasing levels of O2 co-produced in photosynthesis during the shift from dark anaerobic to light aerobic conditions.

Since activation of the CBB cycle in dark-adapted algae requires time, Kosourov et al. (2018) proposed that a sequence of short light pulses applied to cultures instead of continuous light will prevent induction of CO2 fixation, eliminate the competition, and thus, make a substantial number of photosynthetic electrons available to hydrogenase. The performed research corroborated the prediction. A train of 1-s light pulses interrupted by 9-s dark phases indeed induced efficient H2 photoproduction in C. reinhardtii cultures and sustained the process for up to 3 days. The maximum rates were observed in the first 8 h. In contrast to the classical dark adaptation protocol, only a very short period (3–5 min) of purging with argon was needed for activation of the hydrogenase enzyme in cells. The cell-wall deficient strain (CC-4533) of C. reinhardtii showed H2 evolution already on the first light pulse (Kosourov et al., 2020), indicating that hydrogenase proteins were already available in aerobic cultures. As expected, the pulse-illuminated algae could not fix CO2 and produce biomass (Jokel et al., 2019; Kosourov et al., 2018). Instead, the PSII-dependent CO2 release was detected by MIMS in pulse-illuminated algal cultures (Kosourov et al., 2020). The CO2 consumption and biomass accumulation were detected when longer light pulses (5–8 s) activated the CBB cycle (Jokel et al., 2019). The latter was accompanied by the boost in the production of O2 in the cultures and pronounced H2 uptake.
The application of $^{18}$O$_2$-labeled water, DCMU, and PSII- and NDA2-deficient mutants showed that up to 92% of H$_2$ in the pulse-illuminated algae originate from water oxidation activity, while the remaining H$_2$ is evolved by fermentation during the prolonged dark periods (Kosourov et al., 2020).

Another method for preventing competition with the CBB cycle was devised by Nagy et al. (2018). The method relies on the substrate limitation of the CBB cycle in very dense CO$_2$- and acetate-limited algal cultures. In this approach, anaerobic C. reinhardtii suspensions are dark-adapted for several hours with periodic re-flushing by N$_2$ (or Ar) for the CO$_2$ removal. The dark adaptation step is followed by exposure of algae to continuous illumination, which enables H$_2$ photoproduction. Periodic purging of H$_2$-producing algae with N$_2$ prevents the accumulation of CO$_2$ in cultures and sustains H$_2$ photoproduction for several days. It should be noted, however, that N$_2$ re-flushing removes not only CO$_2$ but also O$_2$ and H$_2$ from the cultures. The latter prevents inhibition of [FeFe]-hydrogenases and, in principle, should limit H$_2$ oxidation through the reversible process. The authors suggested that H$_2$ photoproduction in CO$_2$-limited algae is driven by direct water biophotolysis since DCMU completely blocks the process.

Besides competition with CO$_2$ assimilation and O$_2$ photoreduction, H$_2$ photoproduction in C. reinhardtii cells is negatively affected by the induction of CEF around PSI. Elimination of state transition and disruption of PGR5 and PGR1 proteins, essential components of CEF, resulted in significantly enhanced H$_2$ photoproduction yields in S-deprived algae (Kruse et al., 2005; Steinbeck et al., 2015; Tolleter et al., 2011). The performance of these mutants may be further improved by eliminating the competition of H$_2$ photoproduction with the CBB cycle, thus redirecting most of photosynthetic electrons to [FeFe]-hydrogenase.

### 7.4 | Eliminating the H$_2$ uptake component

Under an increased H$_2$ partial pressure, algal hydrogenases could also function in H$_2$ uptake. In 2012, Kosourov and co-authors evaluated the effect of the H$_2$ partial pressure in S-deprived cultures and found an exponential decay in the H$_2$ photoproduction yield with the increased H$_2$ level (Kosourov et al., 2012). The authors also observed a transient H$_2$ uptake after injecting extra H$_2$ in the photobioreactor headspace. The presence of active H$_2$ uptake in sulfur-deprived algae was later confirmed by Scoma and Hemschemeier (2017). Yet, the mechanism and metabolic relevance of this reaction have not been revealed. The possibility of CO$_2$ photoreduction in sulfur-deprived algae is questionable because of the noticeable degradation of the RuBisCo enzyme by the time H$_2$ photoproduction begins. Nevertheless, as Scoma and Hemschemeier (2017) suggested, H$_2$ oxidation could be linked to fermentative CO$_2$ reduction via PFR1, which also interacts with [FeFe]-hydrogenases. Meanwhile, the oxyhydrogen reaction should be limited by the low levels of O$_2$ in algae due to the degradation of PSII centers, the only source of O$_2$ in the sealed sulfur-deprived cultures. Recently, the presence of strong H$_2$ uptake was demonstrated in the pulse-illuminated algae on the shift to darkness (Kosourov et al., 2018). The reaction occurred even in the DCMU-treated cells (thus under strong anaerobiosis) and does not show a strong correlation with CO$_2$ consumption (Kosourov et al., 2020). These data thus indicate another unknown terminal acceptor for H$_2$ oxidation. Recently, Milrad et al. (2021) presented evidence that H$_2$ uptake in the darkness is accompanied by restoration of the NADPH pool. The latter suggests that H$_2$ may serve as an electron donor to NADP$^+$ via [FeFe]-hydrogenase. Anyway, the elimination of H$_2$ uptake component should significantly improve the H$_2$ production yield in algal cultures. This could be achieved by physical removal of H$_2$ from H$_2$-producing algae or by disruption of H$_2$-uptake pathway(s) in cells. The latter, though, requires understanding the molecular mechanisms of H$_2$ oxidation and regulation of this process.

The findings presented in this section bring some optimism in the development of H$_2$-producing algal-based photosynthetic cell factories. Altogether they show the way how to (1) control the photosynthetic electron flow between photosystems for keeping the reduced state of the PQ-pool, (2) balance the PSII activity for preventing inactivation of hydrogenases by O$_2$ co-produced during water oxidation, and (3) eliminate H$_2$ uptake and competition of H$_2$ photoproduction with other metabolic pathways, primarily with CO$_2$ fixation (biomass production) and FDPs.

### 8 | SEMI-SYNTHETIC HYDROGENASES

Hydrogenases possess a remarkable capacity for the interconversion of H$^+$ to H$_2$ on par with noble metal artificial catalysts; however, the enzymes achieve this activity with first row transition metals and can thus be readily produced in abundant quantities (Kleinhaus et al., 2021; Land, Senger, et al., 2020). Therefore, they have become increasingly employed in H$_2$ production applications, especially
[FeFe]-hydrogenases. They possess the highest catalytic activity among different hydrogenases and a comparatively simple maturation, which involves three specific enzymes denoted as HydE, -G, and -F (Posewitz et al., 2004). Through the combined activities of two radical 5-adenosyl-1-methionine enzymes (HydG, HydE), a pre-catalyst of the [2Fe]H subsite is assembled on HydF. The pre-catalyst is subsequently transferred to the apo [FeFe]-hydrogenase, containing a pre-assembled [4Fe4S]H cluster, to yield the active enzyme (Britt et al., 2020). Despite its relative simplicity, the requirement for co-expression of the [2Fe]H maturation enzymes to obtain active [FeFe]-hydrogenases initially complicated both large-scale screening as well as detailed studies of these biotechnologically relevant enzymes, as isolation was dependent on anaerobic isolation of the enzyme following homologous expression or the use of specifically engineered E. coli strains.

In 2013, synthetic chemists reported that the complex biological H-cluster maturation machinery of [FeFe]-hydrogenases could be avoided by insertion of chemically synthesized diiron sites into the apo protein (Berggren et al., 2013; Esselborn et al., 2013). More specifically, the synthetic [2Fe]H subsite analogue [μ-adt]Fe2(CN)2(CO)4(C2H4)Cl2 (adt = azadiithiolate) generates a fully active enzyme, but modified cofactors can also be introduced to generate “organometallic mutants” with new properties. Utilizing this “artificial maturation” technique, target [FeFe]-hydrogenases can now be expressed in a wide range of host organisms and activated without the need for co-expression of the HydEFG machinery. Critically, this can be achieved both in vitro as well as in whole cells, as will be further outlined below.

In short, key applications of this technique in the context of biotechnology include (1) large-scale isolation of the active enzyme utilizing standard expression hosts, enabling detailed mechanistic investigations, (2) manipulation of the enzyme through synthetic chemistry, (3) screening of new [FeFe]-hydrogenases, and (4) screening of host organisms.

The large degree of freedom in creating new semi-synthetic enzymes obtained by artificial maturation was demonstrated by Siebel et al. (2015). They created enzymes with novel properties by introducing a range of synthetic analogues of the diiron site into apo [FeFe]-hydrogenase from C. reinhardtii (CrHydA1). These non-native diiron sites with alternated dithiolate ligands and/or variations of the diatomic (CO/cyanide) ligands led to [FeFe]-hydrogenase enzymes with drastically different characteristics regarding hydrogen oxidation and proton reduction. For example, ~50% H2 evolution activity was observed when the native diiron site was replaced by its mono cyanide variant. The importance of the azadiithiolate amine moiety was demonstrated by the nearly complete loss of activity upon replacement of nitrogen by other elements. In a more mechanistic study, Duan et al. investigated the reactivity of CrHydA1 diiron site variants with H2 and CO by in situ and transient IR spectroscopy (Duan et al., 2019). The substrate affinity was monitored by tracing the redox state composition and the kinetics of the redox state (de)population. The differences observed suggest diverse interactions of ligands (e.g., CO or a terminal hydride) with the dithiolate head group, amino acid residues, and water molecules close to the distal Fe atom. This indicates a function beyond only proton transfer for the dithiolate head group.

9 | WHOLE-CELL APPLICATIONS OF THE SEMI-SYNTHETIC SYSTEMS

Inspired by the in vitro applications, the concept of artificial maturation of [FeFe]-hydrogenase was extended to in vivo conditions. In 2017, Khanna et al. reported the successful incorporation of the diiron site into heterologously expressed apo-hydrogenase inside living E. coli cells for the first time (Khanna et al., 2017). Importantly, the maturation occurs spontaneously via the simple addition of the synthetic cofactor to the growth medium. Perhaps, the most immediate advantage in the context of biotechnology is the more rapid screening for new [FeFe]-hydrogenases enabled by omitting the time-consuming process of enzyme isolation. Moreover, it has proven to be a highly useful tool for screening also non-native host organisms for [FeFe]-hydrogenases with higher throughput and lower experimental effort. Thus, both the catalyst ([FeFe]-hydrogenase) and the host organism can be screened for the optimal design of H2 producing cells.

From an enzyme re-design perspective, it should be noted that artificial maturation in vivo also preserved the possibility to optimize [FeFe]-hydrogenases itself by creating organometallic variants by modified diiron sites. In addition, it opened the door for detailed mechanistic investigations of [FeFe]-hydrogenases in a more native cellular environment compared to studies in vitro.

E. coli cells containing heterologously expressed [FeFe]-hydrogenases have been successfully matured with a monoyanide derivative of the diiron site, as well as synthetic cofactors in which the bridgehead amine of the adt-ligand has been replaced with a non-protonatable methylene group (pdt) (Khanna et al., 2017; Mészáros et al., 2018). Utilizing EPR spectroscopy, the latter study provided the first spectroscopic verification of the successful assembly of a semi-synthetic enzyme in the cytoplasm of a living cell. Both synthetic cofactor variants resulted in lower whole-cell H2 producing activities as compared to cells containing the H-cluster generated by the addition of [μ-adt]Fe2(CN)2(CO)4 to the growth medium. However, both activity measurements and spectroscopy studies indicate that activity observed in vitro translates into whole-cell activity and that alternative diiron sites can be utilized in vivo as well.

To gain more molecular level insights into the hydrogenase mechanism inside living cells, Meszaros et al. expanded the analytic toolbox by FTIR spectroscopy, electrochemistry, and scattering scanning near field optical microscopy on whole cells (Mészáros et al., 2020). In combination with gas treatments, pH titrations and isotope editing a number of proposed catalytic intermediates of [FeFe]-hydrogenase CrHydA1 were observed in living E. coli cells. In addition to the first observation of a reactive metal hydride species in living cells, only partially in vitro characterized hydride species could be characterized in greater detail under whole-cell conditions. This finding highlights how
investigations in vivo can contribute to the understanding of H₂ catalysis in general. We have just started to gain insight into the enzyme chemistry on a molecular level in cellulo and in vivo, and these findings can now be used to optimize enzymes via rational design and directed evolution.

A proof-of-concept study by Land et al. showed how artificial maturation of heterologously expressed apo-hydrogenase in living cells can be applied for new screening procedures in the search for novel [FeFe]-hydrogenases (Land et al., 2019). A bioinformatic approach motivated the selection of the putative [FeFe]-hydrogenases encoding genes. Representative examples of uncharacterized [FeFe]-hydrogenases were expressed in different strains of E. coli, artificially matured, and screened in vivo and in vitro for H₂ evolution. As a result, two newly found active [FeFe]-hydrogenases were subsequently subjected to whole-cell (in vivo) and in vitro biophysical and electrochemical characterization. In the wider context of understanding H₂ metabolism, it is noteworthy that the screening study subsequently enabled the first detailed characterization of a putative sensory group D [FeFe]-hydrogenase, phylogenetically distinct from previously studied groups A and C [FeFe]-hydrogenases (Land, Sekretareva, et al., 2020).

In parallel, Wegelius et al. pushed artificial maturation beyond the E. coli system and introduced apo [FeFe]-hydrogenase into the model cyanobacterium Synechocystis sp. PCC 6803 (Wegelius et al., 2018), and showed how that could be activated via simple cofactor addition to the growth medium. The resulting semi-synthetic in vivo functional [FeFe]-hydrogenase links to the native metabolism of the cyanobacterial cells and is active both in light conditions and in darkness. Impressively, the heterologously introduced [FeFe]-hydrogenase outperformed the native [NiFe]-hydrogenase and deletion of [NiFe]-hydrogenase amplified the H₂ evolution activity of these cells even more. Importantly, once activated, the enzyme remained stable on the time-scale of days in this non-native host, underscoring the high stability of these artificially matured [FeFe]-hydrogenases.

The increased H₂ evolution activity of [FeFe]-hydrogenase introduced into cyanobacteria serves as an example of how an artificial metalloenzyme can tune the performance of host cell systems and outperform the native catalysis pathways. Additionally, the possibility to re-design the H-cluster with synthesized metal cofactors opens the perspective of creating enzymes that catalyze abiotic reactions not present in natural systems. In this respect, the concept of artificial maturation/semi-synthetic hydrogenases also provides a promising perspective for the design of non-natural metabolic pathways for the production of high-value chemicals.

10 | CONCLUSIONS

The release of H₂ by algae and cyanobacteria in the environment is bioenergetically expensive and results in a significant loss of metabolic energy. Therefore, photosynthetic microbes typically photoproduce H₂ only “on-demand” for a very short period or recycle produced H₂ via dedicated metabolic pathways. H₂ recycling has already been known for years in N₂-fixing cyanobacteria, where H₂ is intimately co-produced during N₂ fixation and then oxidized by the uptake hydrogenase. Elimination of HupSL and then its modification for H₂ evolution instead of H₂ oxidation, which resulted in the boost production of H₂ in N₂-fixing heterocystous cyanobacteria, were the first and the second most noticeable achievements in this field. Recently, the presence of the active H₂ uptake component has also been confirmed in green algae, but molecular mechanisms of this process are still not clear. We expect that elimination of this pathway, if achievable, may enhance H₂ photoproduction yields in green algae as well.

Besides H₂ recycling, photosynthetic H₂ production competes for the reducing equivalents with a number of assimilatory and auxiliary pathways, from which CO₂ fixation in the CBB cycle and O₂ photoreduction by FDPs represent the strongest sinks both in cyanobacteria and green algae. For years, the decline in H₂ photoproduction via the direct water biophotolysis pathway in green algae (but not in cyanobacteria where H₂ photoproduction via the bidirectional hydrogenase is followed by noticeable H₂ uptake) has been primarily attributed to O₂ inactivation of [FeFe]-hydrogenase. Recent studies mentioned in this mini-review have demonstrated that the competition for photosynthetic electrons occurs before inactivation of [FeFe]-hydrogenase by O₂ co-produced in photosynthesis. Suggested novel protocols (pulse-illumination and CO₂ limitation) specifically designed for prevention of the CBB cycle activation resulted in sustainable H₂ photoproduction in algal cultures without nutrient deprivation. Additional elimination of FDPs in algal cells enhanced H₂ photoproduction yields in these protocols. Similar strategies for preventing competition for photosynthetic reductants have been shown to improve H₂ photoproduction in cyanobacteria.

Without a doubt, the O₂ sensitivity of H₂ producing enzymes and pathways still remains one of the major issues of photosynthetic H₂ production. Although some progress with the heterologous expression of more O₂-tolerant hydrogenases has been achieved, direct modification of the enzymes for enhanced O₂ tolerance has not been successful yet. In this context, the development of strategies and approaches for the assembly of semi-synthetic hydrogenases in vivo with novel catalytic properties brings some optimism in this field.

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