

Photobiohydrogen Production and Strategies for H₂ Yield Improvements in Cyanobacteria



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The original version of the chapter was revised. The Abstract of this chapter has been corrected. The correction to this chapter can be found at https://doi.org/10.1007/978-3-031-33274-6_219

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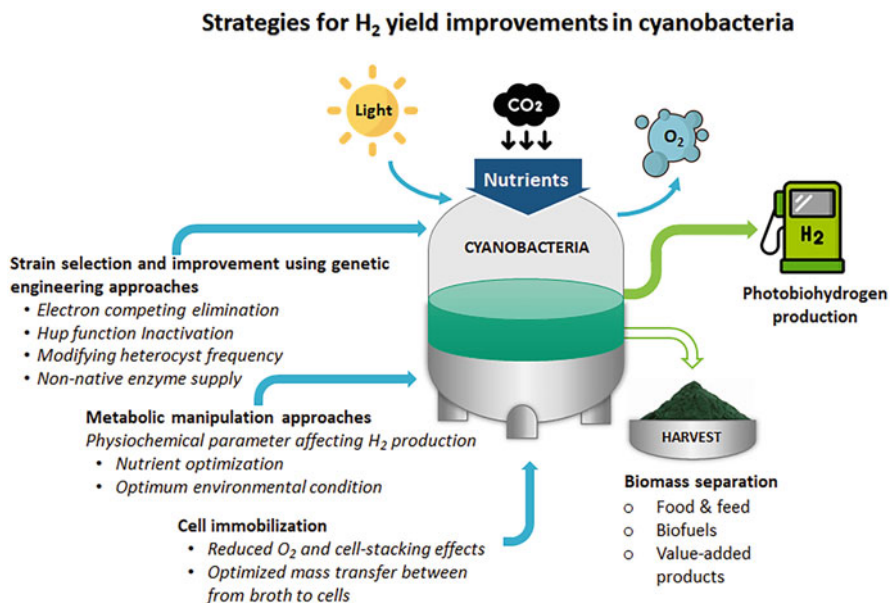
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Abstract H₂, an environmentally friendly energy source, can be generated using a fermentative biological method. Cyanobacteria, with their photosynthetic ability, utilize water as an electron source for H₂ production catalyzed by a bidirectional hydrogenase and/or a nitrogenase. Unfortunately, these enzymes are irreversibly inactivated when exposed to atmospheric molecular oxygen, so optimization of production is needed. Various physicochemical parameters, such as carbon (C), nitrogen (N), phosphorus (P), and sulfur (S) sources, impact H₂ yield, ranging from 0.12 ± 0.01 to 31.79 ± 0.54 $\mu\text{molH}_2/\text{mg chl a/h}$. Genetic modification in many cyanobacterial strains resulted in an increased H₂ yield, ranging from 2.8–101.33 $\mu\text{mol H}_2/\text{mg chl a/h}$. Cell immobilization, primarily in agar and alginate, is another approach to increase H₂ yield during biological production over several production cycles by reducing gas diffusion and cell stacking effects. Although commercialized biological hydrogen has undergone many challenges, numerous scientific methods are still required to be developed to turn these efforts into reality.

Graphical Abstract



Keywords Photohydrogen, Cyanobacteria, Metabolic manipulation, Genetic engineering, Cell immobilization

1 Introduction

Hydrogen gas (H₂) is one of the potential future sustainable and clean energy carriers that may substitute the use of fossil resources including fuels since it has a high energy content (heating value of 141.65 MJ/kg) when compared to traditional hydrocarbon fuels [1]. Water is a byproduct of combustion being a most significant advantage of H₂ being environmentally friendly with the capacity to reduce global greenhouse gas emissions. H₂ is used in various applications. It generates electricity in fuel cells, including applications in transportation, and can be applied as fuel in rocket engines [2]. Moreover, H₂ is an important gas and raw material in many industrial applications. However, the high cost of the H₂ production processes requiring the use of other energy sources is a significant disadvantage. At present, H₂ can be prepared in many conventional ways, such as steam reforming, electrolysis, and biohydrogen production processes. Steam reforming uses high-temperature steam to produce hydrogen gas from fossil resources including natural gas. Electrolysis is an electrolytic process to decompose water molecules into O₂ and H₂. However, both these two methods are energy-intensive and producing hydrogen from natural gas, which is mostly methane (CH₄) and in steam reforming generates CO₂ and pollutants as by-products. On the other hand, biological hydrogen production is more environmentally sustainable and less energy intensive than thermochemical and electrochemical processes [3], but most concepts are not yet developed to production scale.

The production of H₂ using microorganisms has attracted public interest due to its potential as a renewable energy carrier that can be produced using nature's plentiful resources. There are various approaches for biological H₂ production using microorganisms such as green algae, cyanobacteria, photosynthetic anoxygenic bacteria, and dark fermentative bacteria. These microorganisms are physiologically very diverse, occupy different ecological niches, and use distinct metabolic pathways generating H₂. Cyanobacteria, a group of microorganisms performing an oxygenic photosynthesis, can be utilized for H₂ production via biophotolysis [4–8]. They are autotrophic organisms and thereby fix CO₂ from the atmosphere as carbon source. In addition, many strains have the capacity to reduce atmospheric N₂. This chapter addresses and discusses H₂ metabolic pathways involved in cyanobacterial H₂ production and summarizes available and future potential strategies for H₂ yield improvements. The focus is on metabolic manipulation and genetic engineering approaches and on immobilization technologies for enhancing H₂ productivity in cyanobacteria.

2 Biophotolysis and H₂ Metabolism in Cyanobacteria

Cyanobacteria are photoautotrophic organisms that use sunlight as energy source together with atmospheric CO₂ and water for growth. The thylakoid membranes in the cytoplasm of cyanobacteria contain pigment molecules such as chlorophyll *a*,

phycocyanin, phycoerythrin, and allophycocyanin used to absorb light energy (i.e., photons) for oxygenic photosynthesis. The photosynthetic electron transfer reaction is divided into two parts, the light and dark reaction, respectively. The light reaction is involved in transferring electrons through an electron transport chain from PSII to plastoquinone (PQ) pool, cytochrome b_6f complex (Cyt b_6f), photosystem I (PSI), and ferredoxin (Fd), respectively, for generating ATP and reductants, NAD(P)H. For the dark reaction, CO_2 is fixed and reduced into organic compounds using chemical energy obtained from the light reaction [2].

Cyanobacteria constitute a highly diverse group of prokaryotes that have different morphologies, unicellular to heterocystous and non-heterocystous filamentous forms. They are potential microbial chassis for H_2 production by biophotolysis [9]. Biophotolysis is a process that involves the use of water as an electron donor, leading to the generation of O_2 and H_2 in the biological systems in a photosynthetic process. It can be divided into two pathways: direct and indirect biophotolysis pathways (Fig. 1). During direct biophotolysis, H_2 is derived from the electrons generated by water splitting at PSII, whereas for indirect biophotolysis, protons and electrons are mainly supplied for hydrogen generation by degradation of intracellular carbon compound(s), the so-called fermentation [3].

3 H_2 -Catalyzing Enzymes in Cyanobacteria

In cyanobacteria, there may be three enzymes directly involved in H_2 metabolism [5, 6]. (1) Nitrogenase catalyzes the fixation of atmospheric N_2 to produce ammonia (NH_3) under limiting nitrogen condition and concomitantly produces H_2 as a by-product. (2) Uptake (Hup) hydrogenase catalyzes the consumption of the H_2 evolved during N_2 -fixation, which reduces the energy loss during nitrogenase catalysis. (3) Bidirectional (Hox) hydrogenase catalyzes both consumption and production of H_2 . Both nitrogenase and hydrogenase are highly O_2 sensitive and have been a popular target for enzyme improvement. Figure 2 shows an overview of the structural organization of the different hydrogen catalyzing enzymes in cyanobacteria.

Nitrogenase in N_2 -fixing, diazotrophic, cyanobacteria is a multiprotein enzyme complex consisting of the dinitrogenase (MoFe protein) and dinitrogenase reductase (Fe protein). The MoFe protein is a heterotetramer $\alpha_2\beta_2$ with a molecular weight of about 220–240 kDa encoded by *nifD* and *nifK* for α and β subunits, respectively. However, it has also been found that several strains of *Anabaena*, including *Anabaena variabilis*, are able to synthesize an alternative nitrogenase, encoded by the *vnf* gene cluster, where molybdenum is replaced by vanadium in the active center of the enzyme [13]. The function of dinitrogenase is a reduction of N_2 bonds leading to the formation of ammonia (NH_3). The Fe protein is a homodimer with a molecular weight of about 60–70 kDa and encoded by *nifH*. It transfers electrons from the external electron donor to the dinitrogenase protein [13]. This enzyme catalyzes the reduction of atmospheric N_2 to NH_3 and is also responsible for reducing protons

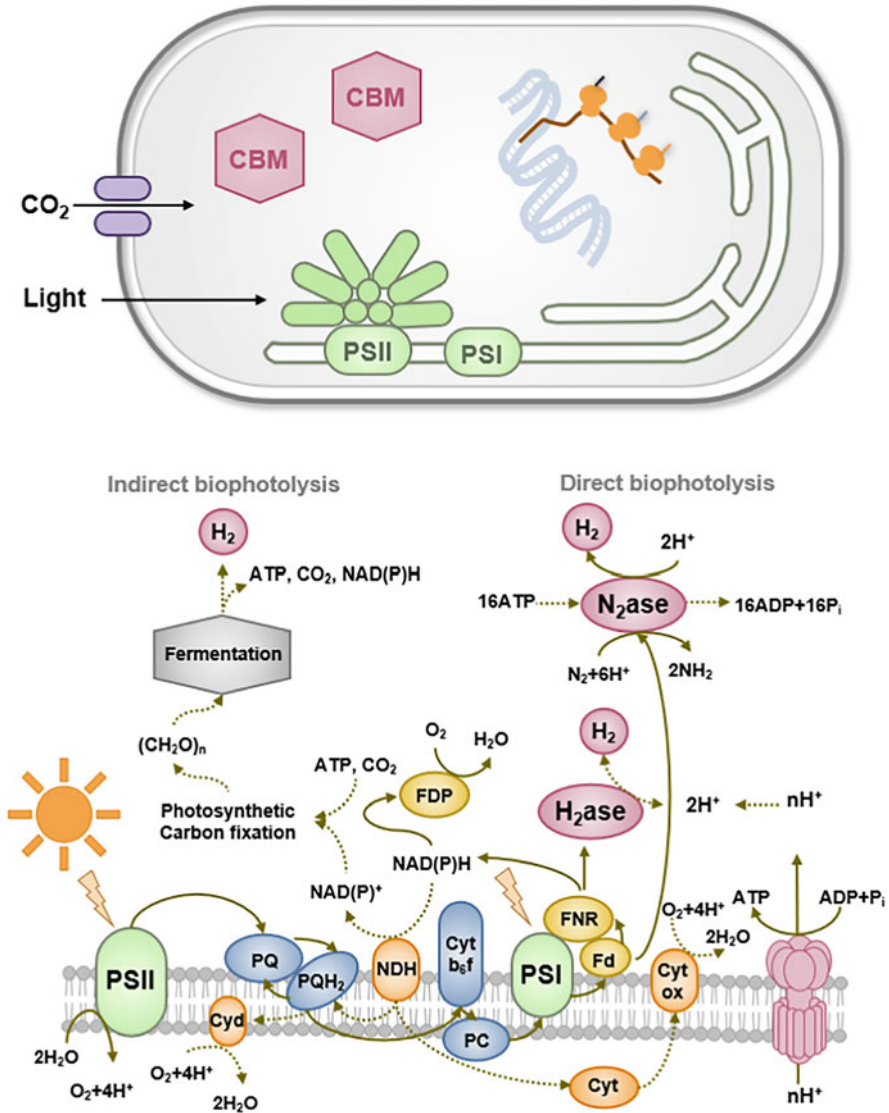


Fig. 1 Simplified view of direct and indirect biophotolysis for hydrogen metabolism involving photosynthetic system in thylakoid membrane of cyanobacterial cell. *PSII* photosystem II, *PSI* photosystem I, *Cyd* cytochrome *bd* quinol oxidase, *PQH₂/PQ* plastoquinol/plastoquinone, *Cyt b₆f* cytochrome *b₆f* complex, *PC* plastocyanin, *Fd* ferredoxin, *FNR* ferredoxin NAD(P) reductase, *NDH* NAD(P)H dehydrogenase, *N₂ase* nitrogenase, *H₂ase* hydrogenase, *H₂* hydrogen (This view was modified from previous articles [10, 11])

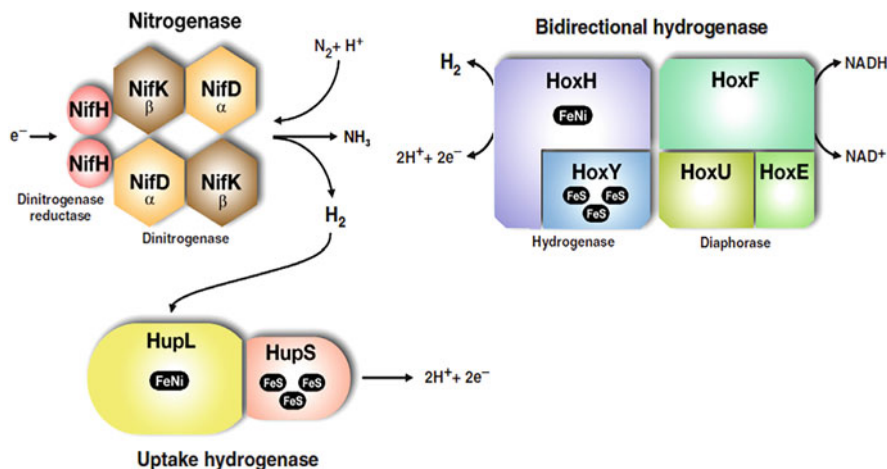


Fig. 2 The enzymes involved in H₂ metabolism in cyanobacteria. Nitrogenase catalyzes N₂-fixing from the atmosphere to produce ammonia and H₂ as a by-product. The produced H₂ is consumed by the uptake Hup-hydrogenase. The bidirectional Hox-hydrogenase can either consume or produce a molecule of H₂ depending on the redox potential (Modified from Tamagnini et al. [12])

(H⁺) into H₂. However, nitrogenases have a rather low turnover rate [14] and H₂ production by nitrogenase requires a considerable number of electrons, reductants, and ATP molecules provided from photosynthesis or by carbohydrate degradation in the cell.

Moreover, nitrogenases are extremely O₂ sensitive, and diazotrophic cyanobacteria have evolved several strategies to separate the photosynthetic evolution of O₂ from the process of N₂ fixation. In filamentous cyanobacteria (e.g., *Anabaena variabilis* ATCC 29413 and *Anabaena* sp. PCC 7120), the vegetative cells can differentiate into heterocyst cells (Fig. 3). Mature heterocysts are individual cells providing a microaerobic environment suitable for the enzymes involved in N₂ fixation and H₂ metabolism. Heterocysts contain a thick cell wall and lack active photosystem II (PSII) complexes resulting in the absence of photosynthetic O₂ evolution [16]. The vegetative cells perform photosynthetic and CO₂ fixing processes, whereas CO₂ fixation is absent in heterocysts due to the lack of the primary CO₂ fixing enzyme ribulose biphosphate carboxylase (Rubisco). Heterocysts import carbohydrates, most likely as sugars, from vegetative cells and use the oxidative pentose phosphate (OPP) pathway for carbohydrate degradation to generate energy and reduce power for nitrogen fixation. In return, the heterocysts export nitrogen in the form of glutamine to the vegetative cells through the GS-GOGAT pathway (Fig. 3) [17, 18]. In some unicellular cyanobacteria, such as *Cyanothece* sp. and *Trichodesmium* sp., N₂ fixation may be controlled by the circadian clock. They separate the production of O₂ and H₂ by performing oxygenic photosynthesis during the daytime and nitrogen fixation at night [19–21].

Uptake (Hup) hydrogenase has been reported for all known N₂-fixing cyanobacteria [6, 22]. It is a heterodimeric enzyme consisting of at least two

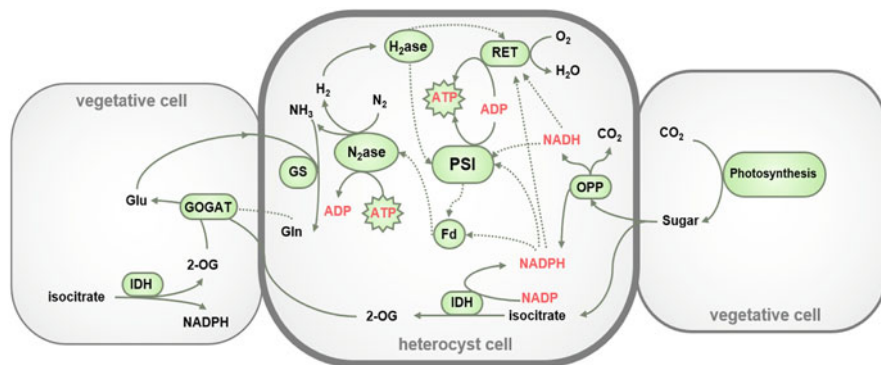


Fig. 3 Simplified view of heterocyst metabolism and exchange with vegetative cells of filamentous heterocystous cyanobacteria under nitrogen starvation. Carbohydrates are imported from vegetative cells into the heterocyst, where they supply reducing power for N₂-fixation. In turn, N₂ is bound in glutamine and exported into vegetative cells through the GOGAT pathway. Dotted lines represent a flow of reducing equivalents. *PSI* photosystem I, *OPP* oxidative pentose phosphate pathway, *RET* respiratory electron transport chain, *IDH* isocitrate dehydrogenase, *GS* glutamine synthetase, *GOGAT* glutamate synthase, *Fd* ferredoxin, *Gln* glutamine, *Glu* glutamate, *2-OG* 2-oxoglutarate, *N₂ase* nitrogenase, *H₂ase* uptake-Hup-hydrogenase (Modified from Lindberg [15])

subunits: HupS (encoded by *hupS*) and HupL (encoded by *hupL*). The HupS subunit has a molecular weight of about 35 kDa containing three FeS clusters. The HupL subunit containing the active site is about twice as large with about 60 kDa. It consists of four conserved cysteine residues involved in coordinating the metallic NiFe at the center of the active site [23, 24]. The uptake (Hup) hydrogenase is involved in the efficient recycling or consumption of the H₂ produced by the nitrogenase. Utilization of H₂ in N₂-fixing cyanobacteria is associated with (1) providing additional reducing equivalents to PSI and various cell functions, (2) generating ATP from oxyhydrogen reaction, and (3) preventing inactivation of nitrogenase by removing O₂ [25]. The structural *hupS* and *hupL* genes have been characterized in many cyanobacteria such as *Nostoc* sp. PCC 73102, *Anabaena variabilis* ATCC 29413, and *Gloeothece* sp. ATCC 27152 [26–28]. *hupS* is usually located upstream of *hupL*. The analysis of gene expression using RT-PCR technique revealed that *hupS* and *hupL* are co-transcription and an enhanced transcription level was found when cells were grown under N₂-fixing condition or the addition of external Ni²⁺ in the culture medium [12]. In some N₂-fixing cyanobacteria, e.g., *Anabaena* sp. PCC 7120, *hupL* in the vegetative cells is interrupted by a DNA element which is excised during heterocyst differentiation by a site-specific recombinase (XisC) resulting in a contiguous *hupL* (Fig. 4) [22, 29, 30].

Bidirectional (Hox) hydrogenase is commonly, though not universally, found in cyanobacteria, catalyzing both consumption and production of molecular H₂ [31, 32]. It is a heteropentamer encoded by *hoxEFUYH* and consists of two protein complexes: a hydrogenase complex (HoxY and HoxH) and a diaphorase complex (HoxE, HoxF, and HoxU). The large subunit, HoxH contains the active metal NiFe

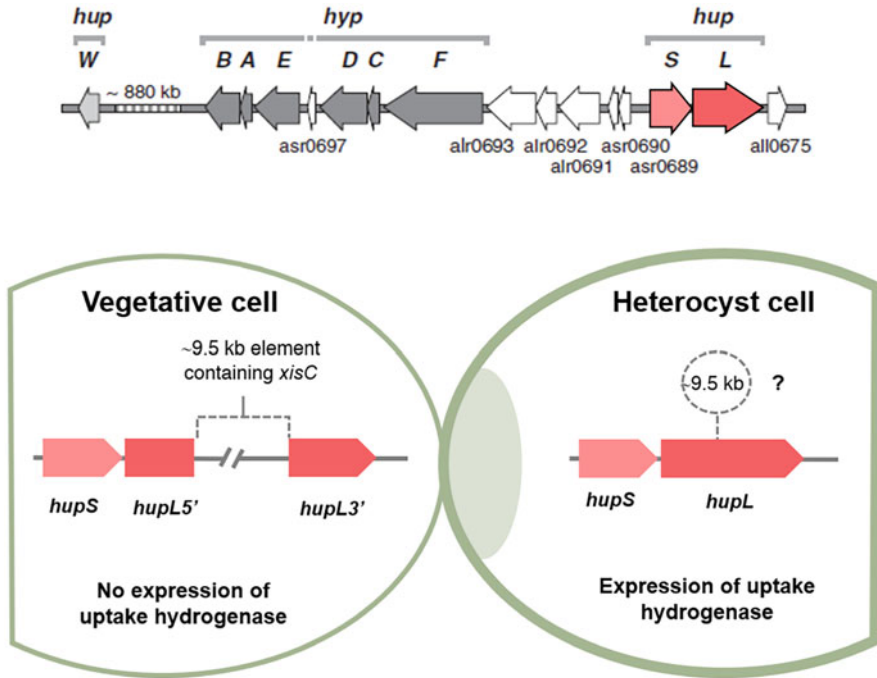


Fig. 4 Schematic representation of the *hupL* rearrangement occurring in *Anabaena* sp. PCC 7120. In the vegetative cell, *hupL* is interrupted by a 9.5-kb DNA element containing site-specific recombination (*xisC*). In contrast, the structure of the *hupL* gene is restored, allowing its expression only in the heterocyst cell. The question marks indicate unclear data explanation (Modified from Tamagnini et al. [12])

center like the uptake hydrogenase (Fig. 2). The physiological role of this enzyme is still under debate. It was found that the bidirectional hydrogenase of *Synechocystis* sp. PCC 6803 acts as an electron sink, storing excess electrons from PSI in the form of hydrogen [33]. Gutekunst et al. [34] reported that Hox-hydrogenase probably functions as an electron sink for reduced ferredoxin/flavodoxin under mixotrophic and nitrate-limiting condition. In addition, this enzyme has been proposed to be a mediator in the release of excess reducing power under anaerobic conditions [35]. Studies in *Synechocystis* PCC 6803 found that the enzyme was insensitive to light, reversibly inactivated by O_2 , and quickly reactivated by NADH or NADPH [36].

4 Strategies for H₂ Yield Improvements in Cyanobacteria

Cyanobacteria are potential H₂-producers, as they can produce H₂ from water as a result of solar energy conversion. However, the main obstacle for the biotechnological process is the low yield of cyanobacteria strains producing H₂ (in the range of 0.06–31.8 μmol H₂/mg Chl *a*/h). Increasing the H₂-productivity by cell improvement has been widely studied using diverse technologies. This section summarizes recent improvements of H₂-metabolism in cyanobacteria by focusing on metabolic manipulation and genetic engineering approaches to understand the metabolic pathways further and increase their respective H₂ yields. An overview of selected cyanobacterial strains and their corresponding rates of H₂ production are summarized in Tables 1 and 2.

4.1 Metabolic Manipulation Approaches

4.1.1 Physiochemical Parameters Affecting H₂ Production

Several parameters may enhance H₂ production, such as nutrient and culture compositions, inorganic mineral supplements, the pH and temperature of culture media, and light intensity. Carbon (C), nitrogen (N), phosphorus (P), and sulfur (S) are all required nutrients for cyanobacterial growth and have been examined for optimizing cellular H₂ production by various microalgae, see Table 1. Changes in the composition of nutrients affect the H₂ production rates. Addition of a carbon source supports by providing energy for cell metabolism. Some cyanobacteria can consume organic carbon sources such as glucose, fructose, galactose, lactose, mannitol, sorbitol, sucrose [39, 44, 49], acetate, succinate, and malate [55] having an effect on hydrogenase or nitrogenase activity and thus on H₂ production. In *Synechocystis* PCC 6803 it was shown that addition of glucose increases the level of reduced NAD (P) which is beneficial for bidirectional Hox-hydrogenase activity, resulting in enhanced H₂ production [49]. Besides, in *Anabaena* sp. PCC 7120, fructose mediated an increase of H₂-production with increased nitrogenase activity and *nifD* expression, in conjunction with elevated electron flow from utilization of fructose through the oxidative pentose phosphate pathway [39].

Although nitrogen and sulfur are essential nutrients for microbial growth, an enhanced H₂ production rate was detected when cells were grown in the nitrogen- or sulfur-deprived condition. This phenomenon was observed in several cyanobacteria such as *Aphanothece halophytica* [40], *Anabaena siamensis* [38], *Arthrospira* sp. PCC8005 [43], *Gloeocapsa alpicola*, and *Synechocystis* sp. PCC 6803 [46]. Furthermore, exogenously added nitrogen sources inhibit nitrogenase activity [13]. Phosphorus (P) is an essential heteroelement in compounds such as ATP, NAD(P)H, nucleic acids, and sugar phosphates, all of which play important roles

Table 1 H₂ production in different cyanobacteria and their optimum environmental condition for enhanced H₂ production

Strains	Maximum H ₂ production	Growth condition	H ₂ production condition	References
<i>Anabaena siamensis</i> TISTR 8012	31.79 ± 0.54 μmol H ₂ /mg Chla/h	Air, BG11 _o , 30°C, 40 μE/m ² /s	Ar, BG11 _o , 30°C, 40 μE/m ² /s, 0.5% fructose, 200 μE/m ² /s	[37]
<i>Anabaena siamensis</i> TISTR 8012	0.057 μmol H ₂ /mg Chla/h	Air, BG11 _o , 30°C, 30 μE/m ² /s	Ar, BG11 _o , 30°C, 30 μE/m ² /s, 4 μM Ni ²⁺	[38]
<i>Anabaena</i> sp. PCC 7120	21.69 μmol H ₂ /mg Chla/h	Air, BG11 _o , 30°C, 40 μE/m ² /s	Ar, BG11 _o , 30°C, 40 μE/m ² /s, 60 mM fructose	[39]
<i>Aphanothece halophytica</i>	13.804 ± 0.373 μmol H ₂ /mg Chla/h	Air, BG11 with Turk Island salt solution, 30°C, 30 μmol photons/m ² /s	Ar, BG11 _o , 30°C, 30 μE/m ² /s, 0.5 M NaCl, 0.4 μM Fe ³⁺	[40]
<i>Arthrospira maxima</i> CS-328	4.5–5.2 ml H ₂ /dry wt/day	Air, Zarrouk medium, 1 μM Ni ²⁺ , 30°C, 12 h light/dark	Ar, Zarrouk medium, 1 μM Ni ²⁺ , darkness	[41]
<i>Arthrospira</i> sp. PCC 8005	5.91 ± 0.14 μmol H ₂ /mg Chla/h	Air, Zarrouk medium, 30°C, 40 μE/m ² /s	Air, ZN _o -S-deprived, 0.15 mM Fe ²⁺ , β-mercaptoethanol, 30°C	[42]
<i>Arthrospira</i> sp. PCC 8005	7.24 ± 0.25 μmol H ₂ /mg Chla/h	Air, Zarrouk medium, 32°C, 40 μE/m ² /s	Air, ZN _o , 0.17 μM Ni ²⁺ , 30°C, darkness	[43]
<i>Calothrix elenkinii</i>	3.21 ± 0.19 μmol H ₂ /mg Chla/h	Air, BG11 _o , 30°C, 50 μmol photons/m ² /s	Ar, BG11 _o , 30°C, 0.3% glucose, 50 μmol photons/m ² /s	[44]
<i>Fischerella muscicola</i>	8.73 ± 0.43 μmol H ₂ /mg Chla/h	Air, BG11 _o , 30°C, 50 μmol photons/m ² /s	Ar, BG11 _o , 30°C, 0.3% glucose, 50 μmol photons/m ² /s	[44]
<i>Fischerella muscicola</i> TISTR 8215	0.32 ± 0.01 mmol H ₂ /L	Air, BG11 _o , 30°C, 40 μmol photons/m ² /s	Ar, BG11 _o , 30°C, 40 μmol photons/m ² /s	[45]
<i>Gloeocapsa alpicola</i>	140 nmol H ₂ /mg protein/h	Air, BG11 _o , 24°C, 25 μE/m ² /s	CH ₄ , BG11 _o with S-deprived, darkness	[46]
<i>Lyngbya perelegans</i>	18.9 ± 0.28 mmol H ₂ /kg dry wt/h	Air, BG11, 3,000 lx, pH 8.0, 27°C	Ar, BG11, (2,000 lx), light: Dark (21:3 h), pH 8.0, 25°C	[47]
<i>Nostoc calcicola</i>	4.27 ± 0.17 μmol H ₂ /mg Chla/h	Air, BG11 _o , 30°C, 50 μmol photons/m ² /s	Ar, BG11 _o , 30°C, 50 μmol photons/m ² /s, 0.3% glucose	[44]
<i>Nostoc muscorum</i>	9.3 nmol H ₂ /mg dry mass/h	Arnon's medium, 3,000 lx, 16 h light/8 h dark, 25°C	Arnon's medium combined N-free, 3,000 lx, 16 h light/8 h dark, 40°C	[48]

(continued)

Table 1 (continued)

Strains	Maximum H ₂ production	Growth condition	H ₂ production condition	References
<i>Nostoc punctiforme</i> ATCC 29133	20.7 ± 0.72 μmol H ₂ /mg Chla/h	Air, BG11 _o , 30°C, 40 μE/m ² /s	Ar, BG11 _o , 30°C, 40 μE/m ² /s	[37]
<i>Scytonema bohneri</i>	7.63 ± 0.26 μmol H ₂ /mg Chla/h	Air, BG11 _o , 30°C, 50 μmol photons/m ² /s	Ar, BG11 _o , 30°C, 0.3% glucose 50 μmol photons/m ² /s	[44]
<i>Synechocystis</i> sp. PCC 6803	0.12 ± 0.01 μmol H ₂ /mg Chla/h	Air, BG11, 30°C, 30 μE/m ² /s	Ar, BG11, 30°C, 0.1% glucose, darkness	[49]
<i>Tolypothrix distorta</i>	10.95 ± 0.22 μmol H ₂ /mg Chla/h	Air, BG11 _o , 30°C, 50 μmol photons/m ² /s	Ar, BG11 _o , 30°C, 0.3% glucose, 50 μmol photons/m ² /s	[44]

in photosynthesis. NAD(P)H is the electron donor to the bidirectional Hox-hydrogenase in cyanobacteria [56].

Generally, trace elements act as essential cofactors, which play an important role in activities of both hydrogenase and nitrogenase enzymes involved in H₂ evolution. For example, a culture of *Fischerella muscicola* TISTR 8215 grown with higher levels of Mo⁶⁺ showed increased nitrogenase activity leading to increased H₂ production [45]. Additionally, the relevance of concentrations of Fe³⁺, Ni²⁺, and Mo²⁺ ions for H₂ production has been investigated and optimized for several strains of cyanobacteria [38, 42, 43, 57], with results suggesting that availability of these elements is a critical factor in controlling H₂ production and N₂ fixation, including effects on expression of hydrogenase and nitrogenase genes.

Furthermore, pH and temperature are crucial parameters influencing the H₂ production process. The pH ranges from 6 to 9 were examined for enhanced H₂ production in several cyanobacteria. In tests using *Lyngbya perelegans* the highest H₂ production was obtained at pH 8.0 [47]. Regarding the temperature, the optimum temperature for H₂ production for most cyanobacteria varies between 23 and 40°C but with differences between strains. *Nostoc muscorum* and *Lyngbya perelegans* showed optimum hydrogen production at 40°C [47, 48] whereas in *Arthrospira* sp. PCC 8005 the maximum rate of H₂ production was observed at 30°C [42]. Moreover, *Calothrix* sp., *Nodularis* sp., and *Microcystis* sp. showed optimum H₂ production at 23°C [58].

Light intensity is a most critical factor affecting the efficiency of cyanobacterial H₂ production. Under artificial illumination, microalgal cultivation under different light intensities alters the metabolic capacity of the cells. Photobiological H₂ production in microalgae and cyanobacteria results from the contribution of a direct and an indirect electron transfer pathway [59–61]. The direct biophotolysis involves a PSII-dependent pathway, which links water-splitting activity to H₂ production. In indirect biophotolysis, electrons, which are derived from the degradation of stored carbohydrates entering the electron chain at the plastoquinone pool are hereafter

Table 2 H₂ production comparison among cyanobacteria using immobilization techniques vs. cell suspension (free cell)

Strains	Matrix	Media	No. cycle	Maximum H ₂ yield/rate				References
				μmol/g DW	mmol/mg DW/h	mL H ₂	mmol/L	
<i>Synechocystis</i> sp. PCC 6803	Alginate bead	BG110	2			5.8 (144 h)		[50]
<i>Lyngbya perelegans</i>	Alginate bead	BG11	1		21.5			[51]
<i>Anabaena</i> sp. PCC 7120	Agar cube	BG11	1		19.8			
	Alginate film	Z8x	3				9	[52]
	Free cell	Z8x	1				13	
<i>Anabaena</i> sp. PCC 7120 mutant strain <i>ΔhupL</i>	Alginate film	Z8x	3				30	[52]
	Free cell	Z8x	2				30	
	Alginate film	Z8x	6				25	[52]
<i>Calothrix</i> 336/3	Free cell	Z8x	3				35	
	Agar cube	BG11	3	3,700				[53]
<i>Fischerella muscicola</i> TISTR 8215	Free cell	BG11	3	1,200				
	Agar cube	BG110	3				7.5	[45]
	Free cell	BG110	-				0.3	
<i>Microcystis aeruginosa</i>	Agar cube	MA	3					[54]

transferred to the hydrogenase to produce H₂ [62]. Previous studies reported that the impact of light intensity varies among different species and strains. The heterocystous cyanobacteria *Nostoc muscorum* and *Anabaena* PCC 7120 produce H₂ from nitrogenase in heterocysts under light conditions [48, 63]. Enhanced light intensity resulted in increased H₂ production in *A. siamensis* TISTR 8012 with a saturation at 200 $\mu\text{E}/\text{m}^2/\text{s}$ of light intensity after 12 h. The cells generated less H₂ above 200 $\text{E}/\text{m}^2/\text{s}$, along with decreased chlorophyll *a* and cell lysis [37].

4.1.2 Cell Immobilization for Reduced O₂ and Cell-Stacking Effects

Hydrogenase catalyzes the incorporation of two protons and two electrons to form H₂, which is the smallest molecule in the universe. H₂ is produced inside the cytoplasm of the cell, diffuses toward liquid broth through the lipid bilayer cell membrane, and finally to the headspace, driven by the partial pressure of the gas. The cytoplasm has the highest H₂ partial pressure, followed by liquid broth and headspace, accordingly. Consequently, the H₂ yield can be determined by quantifying the gas in the headspace using, e.g., gas chromatography. O₂, the strong competitive hydrogenase inhibitor generated in PSII by the water-splitting reaction, has highly similar physicochemical properties, which makes it challenging to separate both gases. Apart from being a strong inhibitor for most H₂ producing enzymes, it also forms an explosive mixture with H₂ (Knallgas reaction) and thus poses a significant safety issue if it comes to scale-up. The amount of molecular oxygen released by the photosynthetic activity depends on the cellular respiration process consuming O₂ as the final electron acceptor. Therefore, one strategy to keep O₂ levels low in H₂ producing cultures is the balancing of PSII activity and cellular respiration [41].

Apart from the parameters discussed above also the cell concentration in the culture was reported to affect the H₂ yield, which is decreasing with increasing cell densities [45, 51]. Too dense cells culture led to the so-called cell-stacking effect, in which cells shade each other and thus run into a light limitation (Fig. 5), which is also difficult to solve by vigorous shaking or mixing [64]. Especially for filamentous cyanobacteria strong mixing is not an option, as the filaments prevent a homogenous mixing, and will also be negatively impacted by high shear forces. Cell immobilization may be a promising solution to relieve the problem of cell-stacking.

Cell immobilization is an essential technique for reducing the cell-stacking effect. For immobilization, the cells are embedded into a supporter material, which is polymerizing during the process. Depending on the physicochemical properties and the concentration of the supporter material, in situ gas removal strategies (like O₂ or H₂) can be implemented into the system. To obtain the highest H₂ yield many studies have been performed with production cycles. Several common biomaterials have been used as cell support. These include carrageenan [53], agar [45, 51, 53, 54], agarose [53], and alginate [52, 65–68]. After immobilizing the cells in a selected support, the mixtures can be molded into different shapes. Thin films [52, 67], cubes [45, 53, 54], and beads [51, 65, 66, 68] are commonly used. Reported yields and corresponding production system parameters are summarized in Table 2.

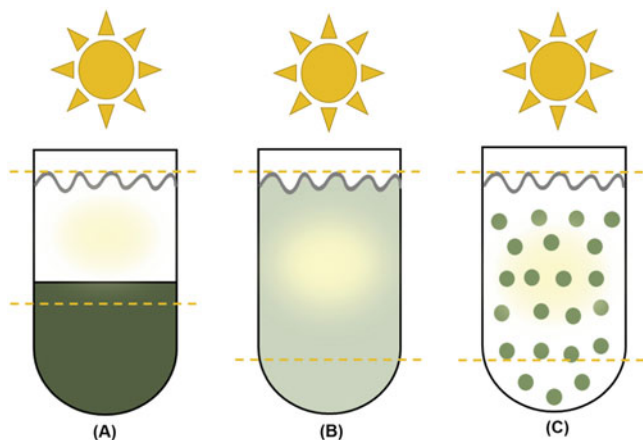


Fig. 5 Schematic shows light penetration power to stacked cells/precipitated cells (a), shaking cells (b), and immobilized cells (c). Dotted lines represent the light penetration path from the surface toward the center of a container

Rashid et al. [54] demonstrated that the unicellular cyanobacterium *Microcystis aeruginosa* immobilized in 1.5% agar in cubes can sustain a hydrogen production phase for up to 95 h with a yield of about 65–70 mL H₂/L culture. This can be increased by the addition of glucose to the culture, which may be degraded through glycolytic pathways, generating the reducing equivalent NADH, which support the flow of electrons to the plastoquinone pool between PSII and PSI and thereby increase the yield of H₂. Wuthithien et al. [45] also reported that immobilizing cells of the N₂-fixing filamentous cyanobacterium *Fischerella muscicola* TISTR 8215 in 1.5% agar improved the H₂ yield significantly, and increasing the Mo⁶⁺ ion concentration also resulted in an increase in H₂ production rate. It seems that stimulation of nitrogenase activity occurs through an addition of molybdenum into their active site [38].

The beneficial effect of immobilization on H₂ production may be explained as follows: (1) The immobilization matrix reduced the O₂ concentration in the direct environment of the cells [69]. (2) The cell-stacking effect was reduced by the immobilization resulting in improved light supply. (3) Optimized mass-transfer between nutrients from broth to cells [70]. (4) The initial cell numbers to agar concentration was appropriate for increasing agar mechanical stability [70].

Pansook et al. [53] compared different materials for immobilization and reported that immobilized unicellular cyanobacterium *Aphanothece halophytica* in 3% (w/v) agar showed the highest H₂ production compared to carrageenan, agarose, and free cells. Carrageenan might encounter the problem of low stability during gel formation due to the presence of NaCl, as previously reported [71], whereas agarose showed lower stability than agar in agreement with Semenчук et al. [72]. The high production rate in cells immobilized in agar was related to: (1) improved cell survival rate and mechanical stability, (2) better nutrient diffusion rate from broth to cells, and

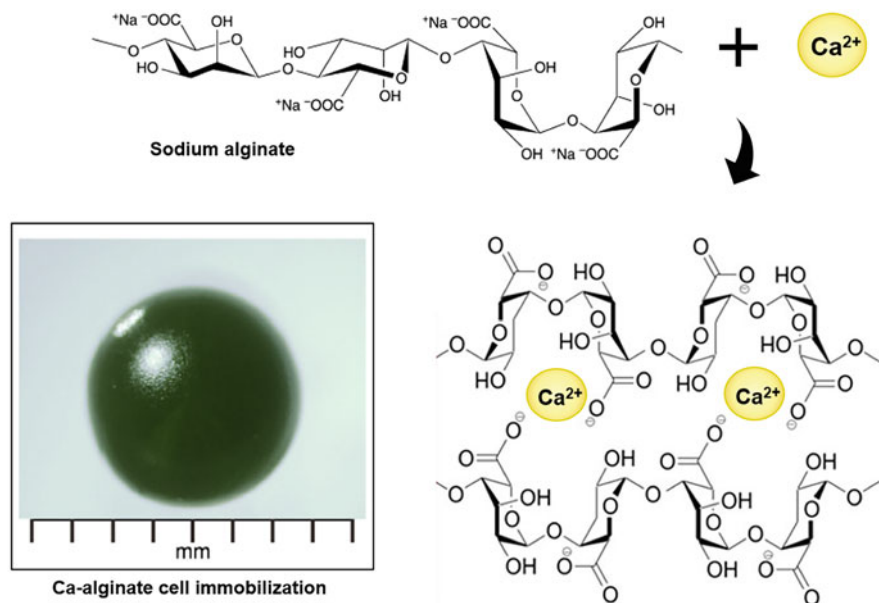


Fig. 6 Cells immobilized in calcium alginate beads prepared using sodium alginate dissolved in algal medium, and Ca-alginate formation from Na-alginate. Negatively charges of Na-alginate chains repulse each other, leading to a uniform structure. Once Ca²⁺ ions are present, positively charges attempt to combine each negative strand close to each other, forming a gel structure (Modified from Touloupakis et al. [50])

(3) small size of the immobilization particles (0.125 cm³) facilitating H₂ and O₂ diffusion from cells toward the bulk.

Another commonly used immobilization matrix is alginate. It is a water-soluble carbohydrate polymer, which will polymerize when interacting with CaCl₂ in solution. Ca²⁺ will replace Na⁺ ions and cross-interact with carboxylate groups (-COO⁻) and negatively polar groups (-OH), leading to carbohydrate strand incorporations and gel formation over time (Fig. 6). Immobilized filamentous cyanobacterium *Lyngbya perelegans* in 4% agar cubes was studied and compared to alginate beads [51]. There were only slight differences between the two materials tested, but both showed about 1.8 times higher H₂ productivity than free cells. Interestingly, this immobilized culture was used to investigate the impact of various gas mixtures, and it was found that a CH₄:Ar (11:2) mix resulted in the highest productivities [51]. Furthermore, Leino et al. [52] screened for H₂ producing cyanobacteria from the University of Helsinki Culture Collection and identified the N₂-fixing heterocystous filamentous cyanobacterium *Calothrix* 336/3 as the strain with the highest H₂ production. Immobilized in a Na-Alginate thin film, it showed a maximum H₂ production rate higher than the rate from free cells. In a study using the model strain *Anabaena* PCC 7120 wildtype and a $\Delta hupL$ mutant of the same strain, there were only small differences between free cells and cells immobilized in alginate films. The

study also found that periodically purging the system with CO₂ balanced Ar led to increased H₂ yield as CO₂ was used as a signal to enhance N₂ fixation [52], consequently prolonging H₂ production.

One of the essential parameters for H₂ production is the photosynthetic activity since PSII generates O₂ with an electron flow to the bidirectional hydrogenase and nitrogenase. Restoring photosynthetic activity between the production cycles thus plays a vital role in prolonging H₂ production as observed in *Calothrix* 336/3, *Anabaena* PCC 7120 wildtype, and its $\Delta hupL$ strain [67].

Finally, cell immobilization enables cell retention and the recycling of the cellular biocatalyst for multiple batches. This facilitates process optimization, as various reaction conditions can be tested with one batch of biocatalysts, like purging with inert gas or gas mixtures, applying different media, etc. Furthermore, it allows to operate the reactors in a continuous or semi-continuous mode, positively influencing process economics.

In summary, cyanobacterial immobilization is an interesting option to enhance H₂ production and process stability by facilitating gas (H₂ and O₂) removal from the cultures. However, optimal conditions in terms of immobilization material and reaction environment will differ from strain to strain and we are still far from defining general process parameters for optimized H₂ production, as up to now only case (strain) specific examples are reported and general operation protocols are missing.

4.2 Genetic Engineering Approaches

4.2.1 Eliminating of Electron Competing Pathways for Promoting H₂ Metabolism

The principal reason for H₂ metabolism through bidirectional Hox-hydrogenase in cyanobacteria may be a disposal of excess reducing equivalents during fermentative metabolism associated with photosynthesis or/and dark anaerobic conditions. Therefore, the bidirectional Hox-hydrogenase requires numerous electrons and reductants as substrates supporting its activities. However, electrons generated through oxygenic photosynthesis are under most conditions not primarily shuttled to H₂ metabolism. Instead, these electrons can be transferred to other competing pathways, such as the respiratory electron transport chain, nitrogen assimilation, and carbohydrate metabolism, shown in Fig. 7. Therefore, diverse genetic engineering strategies for enhanced H₂ production by re-direction of electrons flow toward H₂ metabolism have been extensively examined (Table 3). In *Synechocystis* sp. PCC 6803, interruption of all respiratory terminal oxidases ($\Delta ctaI$, $\Delta ctaII$, and Δcyd) induce the bidirectional Hox-hydrogenase activity leading to a higher H₂-production rate than in wildtype cells under light condition [73]. Moreover, inactivation of type I NADPH-dehydrogenase complex (NDH I) by deleting the large subunit NdhB in a mutant *Synechocystis* strain M55 resulted in prolonged H₂-production and a lower level of O₂ being produced under light condition [36]. Engineering strains with

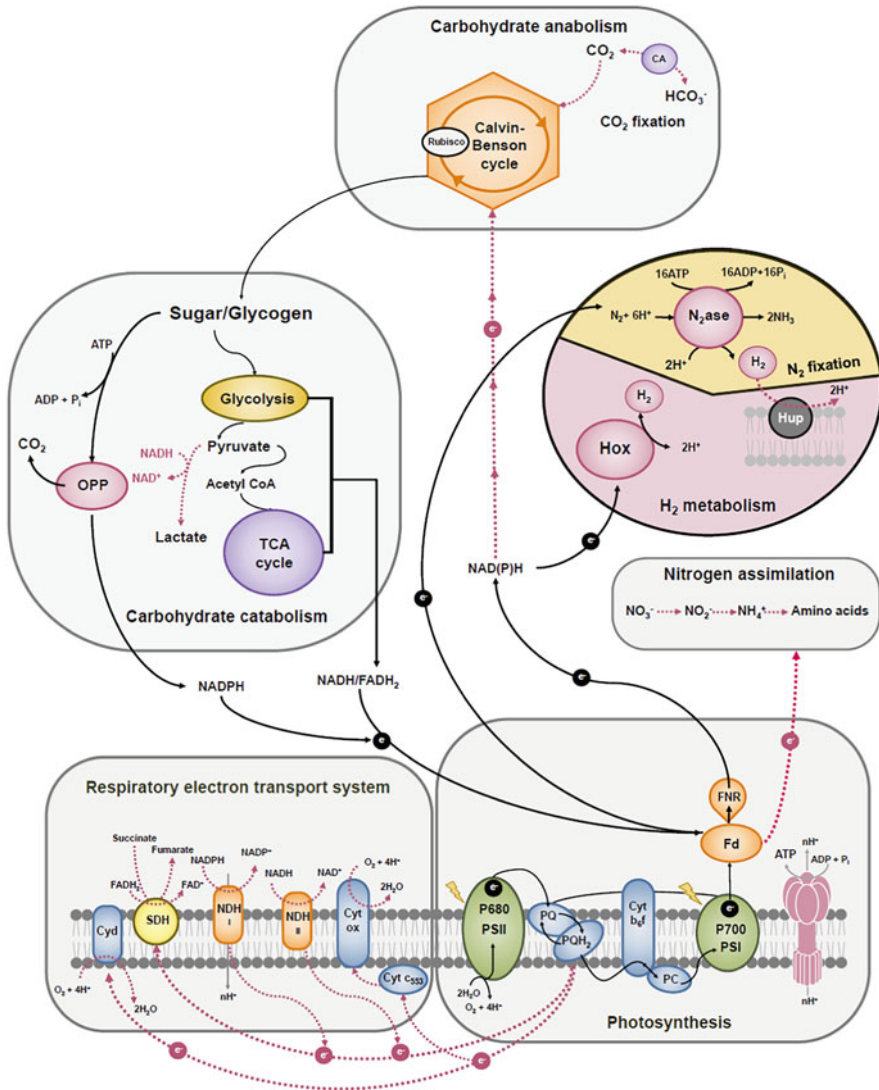


Fig. 7 Different pathways of electron flow involved in H₂ metabolism of cyanobacteria. Dotted lines represent electrons that can be transferred to other assimilatory or competing pathways. *Cyd* cytochrome *b₆*, *Cyt b₆f* cytochrome *b₆f*, *Cyt c₅₅₃* cytochrome *c₅₅₃*, *Cyt ox* cytochrome *c* oxidase, *Fd* ferredoxin, *FNR* ferredoxin-NADP reductase, *Hox* bidirectional Hox-hydrogenase, *Hup* uptake Hup-hydrogenase, *N₂ase* nitrogenase, *NDH* NADPH dehydrogenase, *OPP* oxidative pentose phosphate pathway, *PC* plastocyanin, *PSI* photosystem I, *PSII* photosystem II, *PQ* plastoquinone pool, *Rubisco* ribulose-1,5-bisphosphate carboxylase oxygenase, *SDH* succinate dehydrogenase (Modified from Khetkom et al. [10])

Table 3 H₂ production in engineered cyanobacterial strains using different strategies (Modified from Khetkom et al. [10])

Strains	Engineered genes	H ₂ production rate	H ₂ production condition	References
<i>Synechocystis</i> strain M55	<i>ndhB</i>	200 nmol H ₂ /mg chl <i>a</i> /min	Anaerobic and nitrogen deprivation	[36]
<i>Synechocystis</i> sp. PCC 6803	<i>ctaI/cyd</i>	190 nmol H ₂ /mg chl <i>a</i> /min	Anaerobic and nitrogen deprivation	[73]
<i>Synechocystis</i> sp. PCC 6803	<i>ctaII/cyd</i>	115 nmol H ₂ /mg chl <i>a</i> /min	Anaerobic and nitrogen deprivation	[73]
<i>Synechocystis</i> sp. PCC 6803	<i>ctaI/ctaII/cyd</i>	100 nmol H ₂ /mg chl <i>a</i> /min	Anaerobic and nitrogen deprivation	[73]
<i>Synechocystis</i> sp. PCC 6803	<i>narB</i>	86 nmol H ₂ /mg chl <i>a</i> /min	Ar, darkness, nitrogen deprivation	[73]
<i>Synechocystis</i> sp. PCC 6803	<i>nirA</i>	174 nmol H ₂ /mg chl <i>a</i> /min	Ar, darkness, nitrogen deprivation	[74]
<i>Synechocystis</i> sp. PCC 6803	<i>narB/nirA</i>	300 nmol H ₂ /mg chl <i>a</i> /min	Ar, darkness, nitrogen deprivation	[74]
<i>Synechococcus</i> sp. PCC 7002	<i>ldhA</i>	14.1 mol H ₂ day/10 ¹⁷ cell	Dark anaerobic fermentation	[75]
<i>Anabaena variabilis</i> strain AVM13	<i>hupSL</i>	135 μmol H ₂ /mg chl <i>a</i> /h	Ar, 100 μE/m ² /s, N ₂ -fixing	[26]
<i>Nostoc punctiforme</i> strain NHM5	<i>hupL</i>	14 μmol H ₂ /mg chl <i>a</i> /h	Light and N ₂ -fixing	[76]
<i>Anabaena</i> sp. PCC 7120	<i>hupL/hoxH</i>	53 μmol H ₂ /mg chl <i>a</i> /h	Ar, 10 W/m ² , N ₂ -fixing	[77]
<i>Anabaena</i> sp. PCC 7120	<i>hupW</i>	3.3 μmol H ₂ /mg chl <i>a</i> /h	Ar, 10 W/m ² , N ₂ -fixing	[78]
<i>Nostoc</i> sp. PCC 7422	<i>hupL</i>	100 μmol H ₂ /mg chl <i>a</i> /h	Ar + 5% CO ₂ , 70 μE/m ² /s, N ₂ -fixing	[79]
<i>Anabaena siamensis</i> TISTR 8012	<i>hupS</i>	29.7 μmol H ₂ /mg chl <i>a</i> /h	Ar, 200 μE/m ² /s, N ₂ -fixing	[37]
<i>Anabaena</i> sp. PCC 7120	<i>hupL</i>	101.33 μmol H ₂ /mg Chl <i>a</i> /h	Ar, nitrogen deprivation, 30°C, 40 μE/m ² /s, 60 mM fructose	[39]
<i>Synechococcus elongatus</i>	<i>hydA</i> and maturation operon (<i>hydEFG</i>) from <i>Clostridium acetobutylicum</i>	2.8 μmol H ₂ /mg Chl <i>a</i> /h	Light, 5 μM DCMU, bubbling with 2.5% CO ₂ and 97.5% N ₂	[80]

(continued)

Table 3 (continued)

Strains	Engineered genes	H ₂ production rate	H ₂ production condition	References
<i>Anabaena</i> sp. PCC 7120	Hydrogenase operon, <i>hydA</i> , <i>hydB</i> , <i>hydE</i> , <i>hydF</i> , <i>hydG</i> along with two additional genes, S03922 and S03924, from <i>Shewanella oneidensis</i> MR-1	3.4 nmol H ₂ /μg chl <i>a</i> /h	Light and nitrate deprivation	[81]
<i>Synechococcus elongatus</i>	[NiFe] hydrogenase from <i>Thiocapsa roseopersicina</i>	~0.07 nmol H ₂ /mg protein/h	Anaerobic, 40 μE/m ² /s	[82]
<i>Synechococcus elongatus</i>	[NiFe] hydrogenase (<i>hynSL</i> along with 11 adjacent proteins) from <i>Alteromonas macleodii</i>	~4.2 nmol H ₂ /mg protein/h	Anaerobic, 40 μE/m ² /s	[82]
<i>Synechocystis</i> sp. PCC 6803	O ₂ -tolerant, and NAD (H)-dependent hydrogenase from <i>Ralstonia eutropha</i> (ReSH)	177.6 μmol H ₂ /gCDW	Anaerobic and fermentative condition, 30°C, 50 μE/m ² /s, 10 mM glucose	[83]

disrupted nitrate assimilation, either nitrate reductase ($\Delta narB$) or nitrite reductase ($\Delta nirA$) or both genes ($\Delta narB/\Delta nirA$), in *Synechocystis* sp. PCC 6803 were found to induce significantly higher H₂ production than in wildtype cells [74]. In addition, a mutant *Synechococcus* sp. PCC 7002 ($\Delta ldhA$), lacking the enzyme for the NADH-dependent reduction of pyruvate to D-lactate, showed an increased ratio of NADPH to NADP⁺ and a five-times higher H₂-production when compared with wildtype cells [75]. This work supported that by eliminating competing fermentative carbon metabolism such as the pathway to produce lactate it may be possible to redirect the electron flux to H₂ metabolism in cyanobacteria. Accordingly, an engineering approach by eliminating competitive electron pathways is an effective and promising method to improve cyanobacteria potential for H₂ production, which should be further explored.

4.2.2 Modifying Heterocyst Frequency for Increased H₂ Production

In heterocystous filamentous cyanobacteria, nitrogenase is a key player for H₂ production. The heterocyst provides a partially microoxic environment suitable for oxygen-sensitive enzymes such as nitrogenase since it lacks the PSII activity and has an increased respiration rate [84]. Furthermore, it is surrounded by a thick envelope limiting O₂ diffusion through the cell wall (Fig. 3). Therefore, increasing the heterocyst frequency may enhance H₂ production by promoting nitrogenase activity. The heterocyst differentiation process has been primarily studied in *Anabaena*

sp. PCC 7120 in which it takes approximately 24 h to develop a mature heterocyst from a vegetative cell under nitrogen limited condition [17]. One of the key genes in the regulation of heterocyst pattern formation, *hetR*, encodes a serine-type protease, which is expressed early during heterocyst differentiation. Inactivation of *hetR* inhibits early steps in the differentiation process, while overexpression of the gene increases heterocyst frequency [85]. Recently, it was demonstrated that the addition of fructose rapidly induced the development of mature heterocysts and led to upregulation of *hetR* transcription, resulting in enhanced N₂-fixation and H₂-production in *Anabaena* sp. PCC 7120 Δ *hupL* strain [39]. HetF (a protease) influences heterocyst development by inhibiting *hetR* expression during cell differentiation [86]. PatA, a response regulator, is also known to effect post-translational modification of HetR [87]. However, a practical study with strains exhibiting a genetically engineered high heterocyst frequency with enhanced H₂ production is yet to be reported.

4.2.3 Inactivation of Uptake (Hup) Hydrogenase Function for Enhanced H₂ Production

Uptake hydrogenase activity is a major obstacle for enhanced H₂-production in N₂-fixing cyanobacteria since it catalyzes the consumption of H₂ produced by nitrogenase. Therefore, the disruption of uptake hydrogenase function has been widely studied in many N₂-fixing cyanobacteria. Generally, the structural genes encoding uptake Hup-hydrogenases are clustered in a similar physical organization forming a transcript unit, *hupS* being located upstream of *hupL* (Fig. 4). Inactivation of *xisC* in *Anabaena* sp. PCC 7120 resulted in a strain incapable of forming a functional uptake hydrogenase [29]. A mutant strain AMC 414 (Δ *xisC*) showed high potential for H₂-production compared to wildtype strain under higher light intensity [63]. Moreover, target genes (*hupS*, *hupL*, and *hupW*) that affect H₂-uptake deficiency in N₂-fixing cyanobacteria have been extensively investigated, see Table 3. All generated strains produce H₂ at significantly higher rates than their respective wildtype cells. These experiments indicate that the genetic inactivation of *hup* is an effective strategy for improving cyanobacterial H₂ production.

4.2.4 Introduction of Non-native Hydrogenase for Enhanced H₂ Productivity

Cyanobacteria produce H₂ through bidirectional Hox-hydrogenase ([NiFe]-hydrogenase) with a low rate of H₂-evolution. Therefore, the expression of non-native hydrogenase has been a focus for improving H₂ productivity in cyanobacteria. These include high turnover [FeFe] hydrogenase and some O₂-tolerant [NiFe] hydrogenases from other organisms using advanced synthetic biology techniques. However, successful heterologous expression of [FeFe]-hydrogenase in cyanobacteria remains a challenge, and to date, only very few reports are available, Table 3. The first report by Ducat et al. [80] demonstrated the expression of a [FeFe] hydrogenase (HydA)

and the accessory HydEFG from the anaerobic fermentative bacterium *Clostridium acetobutylicum* into *Synechococcus elongatus* PCC 7942. Interestingly, the results showed both in vitro and in vivo activity of non-endogenous hydrogenase connected to the light-dependent reactions of the electron transport chain. Gärtner et al. [81] have been successfully expressed the FeFe-hydrogenase operon (*hydA*, *hydB*, *hydE*, *hydF*, *hydG*) and two additional genes, S03922 and S03924, from *Shewanella oneidensis* MR-1 into the filamentous cyanobacterium *Anabaena* sp. PCC 7120. Avilan et al. [88] expressed a clostridial [FeFe]-hydrogenase specifically in the heterocysts together with a GlnN cyanoglobin to decrease the O₂ levels in the cell. The obtained strain showed H₂ production concomitantly with oxygenic photosynthesis in the vegetative cells of the filaments. Furthermore, Weyman et al. [82] reported expressing [NiFe] hydrogenases from *Thiocapsa roseopersicina*, as well as *hynSL* along with 11 adjacent proteins from *Alteromonas macleodii* in *Synechococcus elongatus*. The advantage of using [NiFe] homolog over the [FeFe] hydrogenases was their increased half-life and enhanced tolerance toward oxygen stress [89]. The results showed in vitro activity of the expressed protein. Expression of such oxygen-tolerant hydrogenases in photosynthetic systems may open new avenues in cyanobacterial H₂ production. Recently, another strategy that circumvents the biological maturation of [FeFe]-hydrogenase by an artificial synthetic activation of a heterologously expressed HydA protein in living cells of, e.g., *Synechocystis* PCC 6803 was developed. A functional HydA was created by the addition of a synthetic analogue of the [2Fe] subcluster mimicking the active site outside the cells [7]. The experiments showed that the non-native, semisynthetic FeFe-hydrogenase retain its H₂ production capacity for several days after synthetic activation with a regulation of activity based on availability of electrons. The artificial activation technology was expanded to a newly discovered [FeFe]-hydrogenase which when expressed in *Synechocystis* showed stable expression and significant H₂ production under different environmental conditions [8]. The developed technology opens up unique possibilities to investigate not only [FeFe]-hydrogenases but also other metalloenzymes in a photosynthetic microbial cell environment, completely bypassing the many challenges of, e.g., biological maturation and regulations.

In another recent development, Lupacchini et al. [83] introduced an O₂-tolerant hydrogenase from *Ralstonia eutropha* (ReSH) into *Synechocystis* genome. The resulting engineered strain was able to produce H₂ in the dark under fermentative conditions, as well as in the light, under conditions promoting intracellular NADH excess. This opens new possibilities for efficient cyanobacterial H₂ production also under O₂ replete conditions.

5 Conclusions and Perspectives

Due to the growing emphasis on developing renewable energy sources, cyanobacteria have been intensively studied as green cell factories for sustainable H₂ production. Researchers are concentrating their efforts on the main native

processes of cyanobacterial photosynthesis, fermentative metabolism, and on the enzymes involved in H₂-metabolism, which holds great promise in terms of gaining fundamental knowledge and practical applications in biotechnology. The majority of research focuses on applying various metabolic manipulation strategies to enhance H₂ yield in cyanobacteria. Additionally, genetic engineering is used to increase the H₂ yield as well as the technology of cell immobilization for H₂ scale-up challenges. Despite the enormous theoretical potential of cyanobacterial based H₂ production, there are still significant barriers to its commercialization. The prospects of the biohydrogen energy sector will be determined by the combined efforts of scientists and engineers, state political support, and substantial R&D efforts.

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