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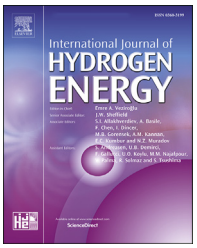

Genetic engineering contribution to developing cyanobacteria-based hydrogen energy to reduce carbon emissions and establish a hydrogen economy

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HIGHLIGHTS

- Genetic engineering findings of cyanobacterial nitrogenase enzyme are described.
- Recent genetic engineering methods to obtain hydrogenase mutants are discussed.
- Engineering photosynthetic H_2 production is shown as an efficient method.
- Development of a non-waste H_2 production technology is explained.

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ABSTRACT

Growing concerns over greenhouse gas emissions and energy insecurity caused by the depletion of conventional fuels have led to a search for sustainable fuel alternatives. As an alternative energy carrier, hydrogen (H_2) is particularly attractive as only water is released

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during combustion. The process of H_2 production from genetically engineered phototrophic microorganisms through biophotolysis leads the way to solve energy shortages. Genetically engineered cyanobacteria species are potential candidates due to their superior properties for reducing greenhouse gases and using solar energy as an energy source. The review discusses the mechanisms and enzymes involved in H_2 production by cyanobacteria and applications of genetic engineering. A critical analysis of the fundamental issues attributed to the technical advancement of photobiological cyanobacteria-based H_2 production is provided, as well as the perspectives for future research to reduce carbon dioxide emissions through the creation of waste-free technology.

Introduction

The major environmental challenge of the new millennium is global warming and air pollution caused by fossil fuel burning. This problem has necessitated the search for new renewable energy sources that are non-polluting and could replace non-renewable energy sources when they become scarce and more expensive [1]. Therefore, scientists focus on producing biofuels from secure, inexhaustible feedstocks, such as hydrogen (H_2), by photocatalytic decomposition of water (H_2O). Among the various gaseous and liquid biofuels, H_2 is one of the most valuable and cleanest fuels, and it may serve as an environmentally safe and forthcoming renewable energy carrier [2]. As of today, H_2 is primarily produced by thermochemical processes [3]. However, biological H_2 production is of great scientific and practical relevance since it avoids the use of fossil fuels, whose reserves are diminishing each year, and the environmental pollution resulting from using conventional energy sources [4–6]. In conjunction with the issue related to energy production, the intermittency of renewable energy sources is another point to be considered, from which the necessity to accumulate and store part of the H_2 energy produced emerges. Although there are several excellences in the field of photovoltaic PV and H_2 storage around the world, both at the academic and industrial levels, only a portion of the scientific community has prioritized the appropriate and innovative integration of energy generation and storage devices [7–10].

Currently, in numerous research studies, cyanobacteria are being investigated as a solution to such environmental problems [11,12]. Moreover, only cyanobacteria are capable of performing oxygenic photosynthesis among prokaryotes. Solar energy, H_2O, carbon dioxide (CO_2), and mineral nutrients are used to create a considerable amount of oxygen (O_2) and organic assimilation for an aerobic food chain [9]. In addition, cyanobacteria are considered prospective "low-cost"" cell factories to capture and store carbon, as well as to produce sustainable biofuel, due to the basic nutritional requirements, physiological stability (as they can colonize many biotopes, transportation costs can be potentially reduced by enabling industrial production nearby), and strong genome-based properties of some model strains [13,14]. It should be noted that cyanobacteria are up-and-coming H_2 producers considering their morphological and metabolic characteristics [15,16]. Indeed, heterocystic cyanobacteria having spatially separated O_2 and H_2 evolution processes and producing H_2 in an O_2-contained environment can carry out light-dependent H_2 production. This process is appealing because both solar energy and the substrate (water) are inexhaustible and renewable, and the by-product (O_2) is non-toxic [17]. The genetic ability to synthesize the essential enzymes, as well as the internal and external metabolic and environmental conditions that supply the necessary energy, control the rate of cyanobacteria-based H_2 production.

A growing variety of molecular tools has recently unleashed the potential of cyanobacteria [18]. These advancements have coincided with an increase in the ability to modify endogenous genetic sequences and transfer exogenous DNA into a wide range of cyanobacterial strains [19,20]. Furthermore, breakthroughs in sequencing technology have enabled the genomes of over 200 cyanobacteria to be sequenced [21], considerably simplifying the use of system-level approaches such as transcriptomics and proteomics. With the help of new sequencing tools, modification of the genes of the nitrogenase enzyme, obtaining hydrogenase mutants, increasing electron transport by changing the photosynthesis complex, and obtaining mutants resistant to external environmental factors can be the most effective methods of biological H_2 production. Three main aspects must be considered simultaneously to produce H_2 from modified cyanobacterial cells: technological limitations, metabolic characteristics, and genetic engineering [22–24] (Fig. 1).

Although many cyanobacterial species worldwide have been studied in the laboratory for H_2 production, high H_2 amounts have not yet been obtained under industrial conditions. In this context, this review analyzes the main ways of obtaining promising H_2 production mutants that can be used for industrial purposes and evaluates their economic and social effects. Next, an examination was made to reduce the amount of CO_2 in production fields by creating a zero-waste technology for H_2 energy production based on cyanobacterial biomass.

Cyanobacteria-based H_2 production enzymes

Two groups of cyanobacterial enzymes are involved in H_2 metabolism: nitrogenases (N_2ase) and hydrogenases (H_2ase) [25]. Nitrogenases are typically present in unicellular or filamentous cyanobacteria that fix nitrogen (N_2) and reduce it to ammonium ion (NH_4^+), thereby producing H_2 as a by-product

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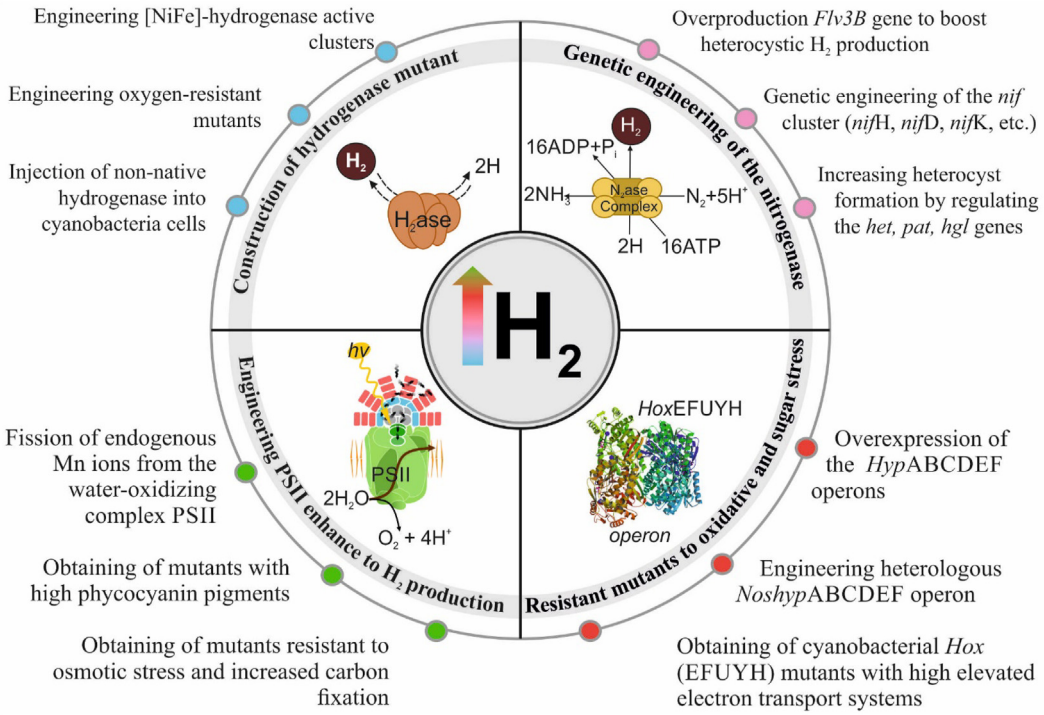

Fig. 1 – Pathways to increase H_2 cleavage in cyanobacterial strains.

[26]. Furthermore, two types of cyanobacterial [NiFe]-N_2ases, namely bidirectional and uptake N_2ases, are found. The difference between bidirectional N_2ase and bidirectional H_2ase (reabsorbs H_2 produced by N_2ase) is that the latter is found primarily in diazotrophic microorganisms [27].

(1) heterocyclic filamentous cyanobacteria spatially engage in O_2 evolving photosynthesis (in vegetative cells) and N_2 fixation (in heterocysts with an O_2 deficiency) [28,29];
(2) heterocystic cyanobacteria accomplish N_2 fixation and are capable of H_2 production, usually under O_2 deficiency, rarely under O_2-rich conditions (e.g., by managing the daily circadian rhythm or intracellular sections) [30].

Nitrogenases

Nitrogenases function differently, depending on the structure of the clusters they contain, and genes directly control the function of the clusters within them. The overall structure of N_2ase consists of two parts [31]: while the di-N_2ase protein occupies the first part, the second part consists of the di-N_2ase reductase protein. The di-N_2ase is an $\alpha_2\beta_2$-heterotetramer (M_r = 220–240 kDa) that splits N_2 atoms [4], whereas the di-N_2ase reductase is a homodimer (M_r = 60–70 kDa). The protein's primary function is transporting electrons from the external environment (ferredoxin) to di-N_2ase. Three distinct di-N_2ase proteins have been identified, each with a unique metal cofactor. The first type contains molybdenum (Mo), the second is vanadium (V), and the third type is iron (Fe) [32].

In cyanobacteria, Mo nitrogenase consists of two proteins (Fe protein and MoFe protein), which are connected by their ends. The nifH gene encodes the small homo-dimeric ($\gamma 2$) protein. The significant component, which is a tetrameric ($\alpha_2\beta_2$) protein, has a molecular weight of 240 kDa and binds to each $\alpha\beta$ dimer. Two types of molybdenum metal-dependent nitrogenase enzymes have been found in cyanobacteria. The first enzyme (nitrogenase, encoded by the *nif1* gene cluster) is activated in heterocysts; the second enzyme (encoded by the *nif2* gene cluster) functions only under anaerobic/anaerobic conditions in vegetative cells.

In general, all H_2ase types follow the following basic formula (eq. (1)):

$$16ATP + 16H_2O + N_2 + 10H^+ + 8e^- \rightarrow (N_2ase)\ 16ADP + 16Pi + 2NH_4 + H_2 \quad (1)$$

The primary function of N_2ase is to convert atmospheric N_2 into NH_4^+; H_2 production is negligible, considering it a negative and undesirable side reaction [33]. Nitrogenases exclusively catalyze H_2 production on N_2-free substrates [34]. In native, intact cyanobacterial cultures, H_2 production by N_2ases is functionally tied with H_2-scavenging *Hup*-H_2ase, leading to zero net H_2 production. However, by changing certain environmental conditions, such as switching from an O_2-rich to an O_2-free phase, a considerable H_2 yield might be achieved [35]. As shown in Fig. 2, cyanobacteria can be divided into three categories depending on the H_2 enzymes they contain.

(1) multicellular cyanobacteria (*Synechocysts* sp. PCC6308) with [NiFe]-hydrogenase (bidirectional hydrogenase) located only in vegetative cells;
(2) types of filamentous cyanobacteria (*Anabaena* sp. PCC7120) with [NiFe]-hydrogenase (bidirectional and uptake hydrogenases) in vegetative cells and

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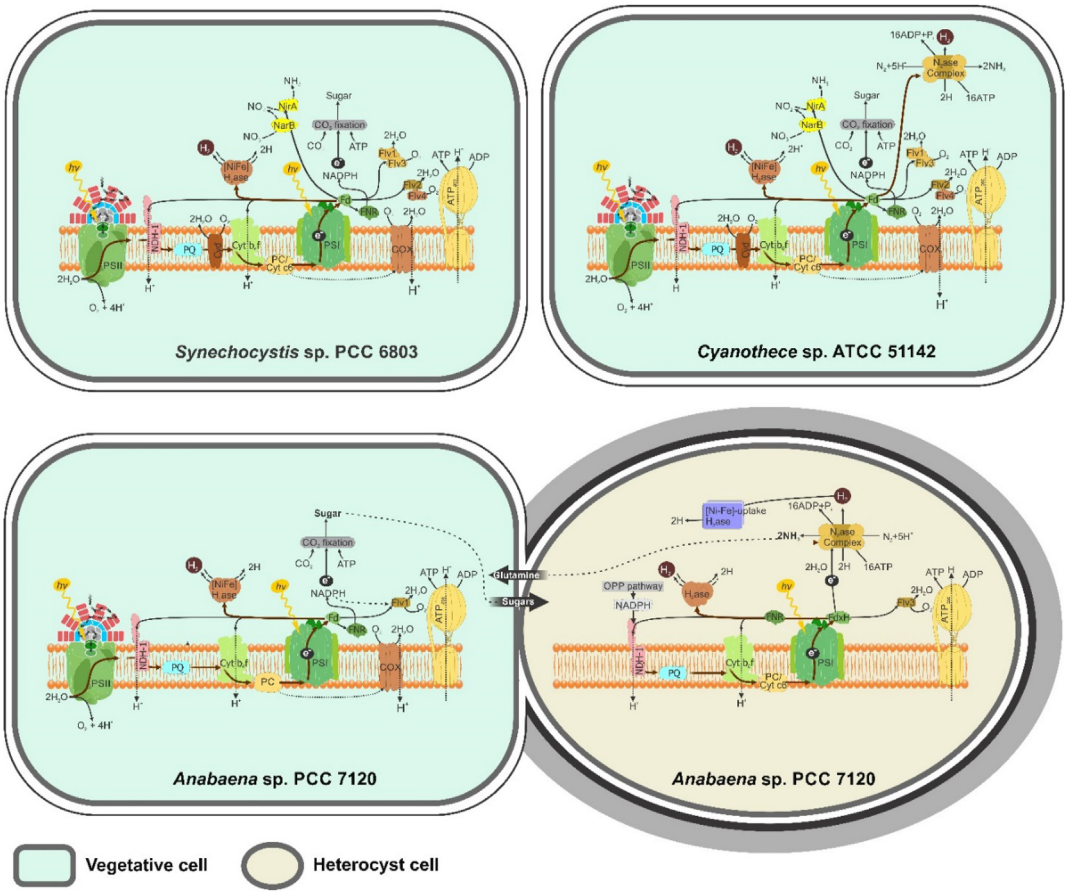

Fig. 2 – Mechanisms of H_2 production in different cyanobacterial species. Modified from Refs. [34,35]. *Abbreviations:* PSI – photosystem I; PSII – photosystem II; NDH-1 – NAD(P)H dehydrogenase complex; PQ pool – plastoquinone pool; Cyt b_6f – cytochrome b_6f complex; PC – plastocyanin; Fdx – ferredoxin; FNR – ferredoxin-NADP$^{(+)}$ reductase; NADPH – nicotinamide adenine dinucleotide phosphate; CO_2 – carbon dioxide; ATP – adenosine triphosphate; ATPase – adenosine triphosphatase; Flv – flavodiiron proteins; H^+ – protons; OPP pathway – oxidative pentose phosphate pathway; N_2ase – nitrogenase; H_2ase – hydrogenase.

nitrogenase and bidirectional [NiFe)-hydrogenase in heterocyst cells;

(3) multicellular cyanobacterial species with active bidirectional [NiFe)-hydrogenase and weak nitrogenase (*Cyanothece* sp. ATCC 51142).

Hydrogenase

Hydrogenase is the second most abundant enzyme involved in H_2 production in cyanobacteria. Many cyanobacteria contain various types of H_2ase enzymes. Uptake H_2ases (encoded *hup*SL) can oxidize H_2, while the bidirectional H_2ase (encoded *hox*FUYH) enzyme can absorb or produce H_2 [36]. Uptake H_2ase is mainly observed in the thylakoids of filamentous bacterial heterocysts. Oxy-hydrogenation, or the *Knallgas* reaction, implies a transfer of electrons from H_2 via the respiratory chain to perform O_2 reactions [37]. This enzyme is composed of two subunits. The *hup*L-encoded protein functions as an uptake H_2ase, while the *hup*S-encoded component carries out the reduction process (Fig. 2). As the resulting H_2 is usually re-oxidized by H_2ase (according to the *Knallgas* reaction), strains containing uptake H_2ase do not produce pure H_2 under environmental conditions [38]. Thus, if H_2 production needs to be scaled up to an industrial scale, a considerable amount of net H_2 production is required between the uptake and H_2-producing H_2ases. The uptake H_2ase catalyzes the reaction, which follows the formula (eq. (2)) [39]:

$$H_2 \rightarrow (H_2\text{ase})\ 2H^+ + 2e^- \quad (2)$$

The biological function of bidirectional and uptake H_2ases is not considerably clear, and maintaining ionic concentration in cells could be a possible purpose. Uptake H_2ase is bound to the cytoplasmic membrane and serves as an electron acceptor for NADH and H_2 [40]. Reversible H_2ase is a multimeric enzyme consisting of four or five (depending on the species) different subunits. It is encoded by the *hox*YH gene, which controls [NiFe]-H_2ase. The activity of auxiliary proteins known as *hyp* is required to develop bidirectional H_2ase [41]. Contrary to the uptake H_2ases, the bidirectional ones are directly involved in H_2 production [42].

[FeFe]-hydrogenases are another type of hydrogenase found in microorganisms. This type of hydrogenase has two different cofactors: iron and sulfur. In addition, this enzyme is

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attached to the protein skeleton by three cysteine residues. The iron cluster is connected to the di-iron part by a fourth cysteine residue. Hydrogenase in this cluster is not found in cyanobacteria. However, as a group of foreign genes, it can activate in cyanobacterial cells.

An anoxic environment is created in cyanobacterial cells by separating the processes of O_2-evolving photosynthesis and O_2-sensitive enzymes spatially or temporally, in combination with efficient cellular respiration [43]. The phosphorylation process that generates the necessary ATP energy requires the bidirectional H_2ase found in about half of all cyanobacteria. This evolutionary process demonstrates that bidirectional (*hox*) H_2ase has functions other than cell-specific H_2 separation [44]. Many cyanobacterial species, however, can live freely in nature without bidirectional (*hox*) H_2ase and are competitive with other organisms. The biological functions of bidirectional (*hox*) H_2ase in cyanobacteria include cellular redox processes, such as the release of energy stored as H_2 to the external environment under stress conditions, ensuring cell safety [35,45].

Several known cyanobacterial H_2 production reactions can be utilized in large-scale production [46]. While growing in an alternating dark light mode (i.e., photosynthesis and respiration occurring under circadian regulation in response to light similar to a sleep-wake cycle), some unicellular and filamentous cyanobacteria (e.g., *Spirulina* sp. [47], *Synechocystis* sp. RF-1 [48], *Cyanothece* sp. Atcc51142 [49], *Desertifilum* sp [31]. and *Oscillatoria* sp [31]) contain an active N_2ase. The circadian rhythm regulates N_2 fixation, photosynthesis, and respiration and prevents O_2 from inhibiting N_2ase. N_2 fixation via N_2ase catalysis is primarily observed in a wide range of heterocyst-forming cyanobacteria and azotobacter [50]. However, the strain *Cyanothece* devoid of heterocysts can fix N_2 using light by N_2ase. The circadian rhythm is most often observed in a dark environment. The cell is considered to revert to a reduced state when photosynthesis resumes in the light. When O_2 release occurs, however, this activity is rapidly suppressed [51].

Genetic engineering of cyanobacterial enzymes for efficient H_2 production

The use of modern methods to increase or eliminate the desired characteristics of wild species is called genetic engineering [52]. Genetic engineering methods are mainly implemented via the transfer of genes [53]. Transcription and translation processes are implemented using advanced synthetic biology techniques [54]. Despite scientific advances, only about 2000 species of cyanobacteria have been fully sequenced, including *Cyanothece* sp. ATCC 51142, *Synechocystis* sp. PCC6803, *Anabaena* sp. PCC 7120, and *Synechococcus* sp. PCC 7942 [55–57]. As whole gene groups have not yet been identified and only partial sequencing has been conducted, the remaining cyanobacterial species are unsuitable for genetic studies and modifications (Fig. 2). Natural, artificial, causative, and inducible promoters are required to regulate the transcription of heterologous genes [58]. Non-natural (orthogonal) promoters do not affect cellular regulation or metabolism [59]. The capacity of cyanobacteria to produce H_2 directly from solar energy and H_2O is of great interest to the scientific community [60]. However, photobiological H_2 production by natural cyanobacteria faces several challenges, including inefficient use of solar energy, enzyme O_2 sensitivity, and inefficient metabolism [61]. In general, the highest production of a particular molecule or product from biological systems is typically uncommon, and competition and survival exert evolutionary constraints on natural cyanobacteria [62]. Natural cyanobacterial cells that produce and release H_2 lose energy and electrons, negatively influencing their competitive environment *in situ* [63].

Exploring the genetic control of H_2 production and using genetic approaches to obtain efficient, productive strains is one way to optimize the process of bio-H_2 production [64]. Therefore, creating genetic mutants to obtain photobiological H_2 is economically viable and the only way to achieve a higher yield in the future [65]. Genetic approaches to increasing H_2 yield by phototrophic microorganisms, including cyanobacteria, include gene knockout, insertion, and overexpression [66]. Thus, H_2 evolution in some cyanobacterial species is significantly higher after genetic modification than in wild species. N_2ase and H_2ase enzyme engineering are primary genetic engineering approaches to increase H_2 yield. Studies on H_2ase enzymes are primarily concerned with eradicating or inactivating the uptake H_2ase and generating NH_4-insensitive mutants. In contrast, studies on N_2ase enzymes are concerned with increasing the number of heterocysts in filamentous cyanobacteria (Table 1) [67].

Genetic engineering of the nitrogenase

Nitrogenase converts molecular nitrogen into NH_4^+ while releasing H_2. For the following reasons, the improvement of an N_2ase-based H_2 production system may be of particular interest.

- N_2ase biochemistry and proteins crystal structures are well-studied and available [78];
- N_2ase in heterocysts is naturally protected from O_2 released during photosynthesis in PSII (photosystem II) [79];
- some filamentous cyanobacteria of the order *Nostocales* (*Nostoc*, *Anabaena*, etc.) can fix carbon via photosynthesis and simultaneously release H_2 using N_2ase [80];
- at high partial pressure, the enzyme can produce H_2 [81].

H_2ase-mediated H_2 production lacks the above features. However, H_2ase engineering is also promising to boost cyanobacteria-based H_2 yield [82]. N_2ase converts atmospheric N_2 to NH_4^+ and protons to H_2 [83]. The N_2ase system is the principal source of H_2 during photosynthesis, and N_2 limitation occurs in many photosynthetic bacteria. Moreover, bio-H_2 production efficiency is heavily reliant on N_2ase, as well as the availability of ATP and electrons in the cell [62,84]. Skizim et al. [85] evaluated the ratio of H_2 produced by N_2ase compared to H_2ase in *Cyanothece* Miami BG 043511 (light-vs. dark-induced). This species is capable of actively releasing hydrogen during illumination. Therefore, the transition between light and dark and the intensity of light have a direct effect on the amount of hydrogen produced. Moreover, the generation of the N_2ase mutant library by random

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Table 1 – Hydrogen production in engineered native enzymes of cyanobacterial strains using different strategies.

Cyanobacterial strains	Engineered genes	H_2 production rate	H_2 production assay condition	References
Synechocystis PCC6803	*HoxW*	4.5 nmol H_2 10^{-9} cells s^{-1}	2500 lux; 31.25 μE m^{-2} s^{-1}, on BG-11 medium enriched with 3.78 mM Na_2CO_3	[24]
Anabaena variabilis AVM13	*hupSL*	135 μmol H_2 mg *Chl* a^{-1} h^{-1}	Ar; 100 μEm^{-2} s^{-1}; N_2-fixing	[68]
Nostoc punctiforme NHM5	*hupL*	14 μmol H_2 mg *Chl* a^{-1} h^{-1}	Light and N_2-fixing	[69]
Anabaena sp. PCC 7120	*hupL/hoxH*	53 μmol H_2 mg *Chl* a^{-1} h^{-1}	Ar; 10 Wm^{-2}; N_2-fixing	[70]
Nostoc sp. PCC 7422	*hupL*	100 μmol H_2 mg *Chl* a^{-1} h^{-1}	Ar + 5% CO_2, 70 μEm^{-2} s^{-1}; N_2-fixing	[71]
Anabaena siamensis TISTR 8012	*hupS*	29.7 μmol H_2 mg *Chl* a^{-1} h^{-1}	Ar; 200 μEm^{-2} s^{-1}; N_2-fixing	[72]
Synechocystis M55	*ndhB*	200 nmol H_2 mg *Chl* a^{-1} h^{-1}	Anaerobic and N_2 deprivation	[73]
Synechocystis sp. PCC 6803	*ctaI/cyd*	190 nmol H_2 mg *Chl* a^{-1} h^{-1}	Anaerobic and N_2 deprivation	[74]
Synechococcus sp. PCC 7002	*ldhA*	14.1 mol H_2 day^{-1} 10^{17} $cell^{-1}$	Dark anaerobic fermentation	[75]
Anabaena sp. PCC 712	*hupW*	3.3 μmol H_2 mg *Chl* a^{-1} h^{-1}	Dark anaerobic fermentation	[76]
Cyanothec 51,142	*hupS*	373 μmol H_2 mg *Chl* a^{-1} h^{-1}	Photoautotrophic growth under ambient CO_2 concentrations, argon incubation	[77]
Anabaena 29,413 (PK84 mutant)	*hup*$^-$	167.6 μmol H_2 mg *Chl* a^{-1} h^{-1}	2% CO_2, 24 argon incubation	
Synechocystis 6803 (M55 mutant)	*ndhB*$^-$	56 μmol H_2 mg *Chl* a^{-1} h^{-1}	Glucose, glucose oxidase, sulfur deprivation, argon incubation	

mutagenesis and gene shift could significantly contribute, although developing high-throughput screening methods is required. Furthermore, understanding the biochemical criteria for efficient H_2 synthesis by [Mo]-N_2ase is essential as a foundation for its re-engineering [85].

The N_2ase enzyme requires an O_2-free environment to function properly [86]. This process can occur in cyanobacteria under two conditions: transiently in unicellular cells and permanently in filamentous cells, where O_2 accumulates as a result of photosynthesis in vegetative cells. As a result, filamentous cyanobacteria can fix N_2 in free space without restriction and form an efficient respiratory system [87]. Filamentous cyanobacteria (*Anabaena*, *Nostoc*, etc.) are dominant in N_2- and O_2-free environments. Heterocysts contribute 5–10% of vegetative cells. This process occurs in response to N_2 deficiency to maintain the vital activity of cells [88]. Moreover, H_2ase is sensitive to O_2 and N_2. Studies show that many filamentous cyanobacterial heterocysts form in N_2-free environments, although heterocysts also form in nitrogen-rich environments [89,90]. N_2ase occurs in heterocysts, and some studies have found it in multicellular species of *Oscillatoria*, *Desertifilum*, and *Synechococcus* [91].

The following genetic engineering studies are conducted in conjunction with obtaining NH_4-insensitive mutants. The degree of N_2 fixation by filamentous cyanobacteria is based on the highly effective regulatory genes responsible for NH_4 concentration. A high NH_4^+ concentration hinders the process of H_2 cleavage due to the suppression of N_2ase catalysis and the discontinuation of its activity. A reduction in nitrogenase activity could boost H_2 production by lowering the sensitivity of N_2ase to NH_4^+ ions [92]. Point mutations in the *nif*A gene or knockout of the *gln*A (glutamine synthetase) gene make the *Nif*A protein NH_4-insensitive and lead to average H_2 production and N_2 fixation even in an NH_4-containing environment, which can yield such outstanding outcomes [92]. With 2- and 4-mM ammonium concentrations in the medium, the wild-type cells produced 1558 ± 12 mL L^{-1} of H_2. Mutant cells produced 1678 ± 57 mL L^{-1} of H_2, much higher than the wild type [93].

Furthermore, N_2ase activity can be increased or normalized by modulating the genes that transport NH_4^+ into the cell. This is because mutants obtained by these methods are not NH_4-sensitive, which increases H_2 yield [94]. In the case of specific residual mutant strains that are not NH_4-sensitive, the cyanobacterial mutant strains obtained in this manner may have advantages when using wastewater with very high NH_4^+ concentrations [95].

Another study on genetic engineering of the enzyme N_2ase was conducted by transferring 35 genes of the *nif* cluster (*nif*H, *nif*D, *nif*K, etc.) from the strain *Cyanothece* sp. ATCC 51142 to *Synechocystis* sp. PCC 6803. The O_2 resistance of H_2ase in *Synechocystis* 6803 was improved by the introduction of N_2ase reception genes, which showed that this was a functional way to enhance the activity of H_2ase under microoxic conditions. As a result, the N_2 fixation capacity of the *Synechocystis* sp. 6803 strain was 30% higher than in wild species, which is the highest activity found in a non-diazotrophic photosynthetic organism [96]. Furthermore, Li et al. [97] showed that the *suf* operon electron transport genes of *Paenibacillus* sp. WLY78 can increase H_2ase activity. Therefore, about 28 genes were selected from WLY78 and expressed in recombinant *E. coli* 78–7. The derived mutant cells produced 50.1% more H_2 than the wild-type cells.

Genetic engineering of heterocyst formation

Certain N_2-fixing filamentous cyanobacteria develop heterocysts under N_2 deficiency. Heterocysts have stronger cell walls than the neighboring vegetative cells, lack PSII, and show enhanced respiratory activity, resulting in microoxic conditions that preserve O_2-sensitive N_2ases [45,98]. Fdx reduced by PSI yields electrons for N_2 fixation. Clostridial [FeFe]-H_2ases produced in heterocyst cells can scavenge photosynthetic electrons before they reach the N_2ase and produce a higher H_2 yield (0.22 μmol H_2 dry^{-1} wt h^{-1}) at a rate exceeding that of the original strain [46]. However, [FeFe]-H_2ase proved to be more O_2-sensitive than N_2ase and was even inactivated in heterocysts. The *Flv3B* gene (encoding a flavo-diiron protein) overproduction boosted heterocystic H_2 production, most likely via increased O_2 consumption, and may promote the expression of [FeFe]-H_2ases as well. However, H_2 production in heterocysts is laborious and needs electrons and carbon

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skeletons from functioning vegetative cells, which is less efficient than direct photolysis [98].

The process of active H_2 evolution in N_2-fixing cyanobacteria is accompanied by an increase in heterocyst numbers in the filaments. The number of heterocysts can be artificially increased, which raises the concentration of N_2ase [99]. This method can now be implemented by treatment with 7-azatriptophan or other genetic methods. Therefore, key genes involved in or controlling the formation of heterocysts need to be deciphered [100]. By over-expressing or introducing these genes, the number of heterocysts in the filaments can be regulated. It is currently known that more than 1000 genes directly or indirectly influence the formation of heterocysts. All genes mainly belong to the group of *het* genes, of which the *hetR* gene is the most actively studied. While the *hetR* gene is responsible for heterocyst formation, the gene products of *patS* and *hetN* [101] are mainly responsible for heterocyst elimination. So far, numerous researchers have investigated the turnover of heterocysts under various conditions. For example, Buikema et al. [102] increased heterocyst formation in *Anabaena* PCC 7120 by 30% by regulating the *hetR* gene, although this number decreased to 30% under N_2 starvation.

During heterocyst formation, the *HetR* (serine protease) and *PatA* (responsible regulator) genes impart signals that are crucial for the differentiation process [103]. However, there are no comprehensive studies on the production of bio-H_2 by genetically modified free-living cells to increase heterocyst content. Although such elements can theoretically produce a higher yield of H_2, they still need to be investigated. However, despite continued research efforts, N_2ase-based H_2 production requires increased energy in the form of ATP: for each mole of H_2 produced, at least 4 mol of ATP are needed [104].

The *patS* and *hetN* genes are often used to increase the activity of N_2ase. Moreover, they have been shown to influence the structure of heterocysts indirectly. For example, black et al. [105] revealed that the *hglK* gene's main function was to form the outer, thick layer of the heterocysts. The thick layer stimulates the N_2ase enzyme by blocking O_2 passage from the external environment to the heterocysts. It was observed that, in cells deprived of the *hglK* gene, H_2 yield decreased sharply.

As mentioned earlier, heterocyst formation in filamentous cyanobacteria in an O_2-free environment affects the N_2 cycle [106]. However, the process of heterocyst formation is not fully understood, although some studies suggest that heterocyst cells arise from older vegetative ones. Interestingly, free-living cyanobacterial strains show a heterocyst abundance of 5–10%, while modified strains gradually increase to 30–35% with a concomitant increase in N_2 fixation. Moreover, the mutant strains increase the transport of cellular products by performing high levels of N_2 fixation [107]. The H_2 content of Δ*nblA* mutant filamentous cyanobacterium *Anabaena* sp. 33,047 grown in bioreactors is 2–10-fold higher (100 μm H_2 mg^{-1} *Chl a* h^{-1}) than that of wild species, and the number of heterocysts increases. Genetically modified cyanobacteria can be ideal model systems for effective cell development that can be used in future photobioreactors. This is due to the availability of various theories and genetic methods that control the differentiation of heterocysts.

Furthermore, enhanced H_2 production requires the separation of metabolic H_2 from microbial growth by increasing heterocyst abundance. Jeffries et al. [108] physiologically established constant H_2 production in *Anabaena cylindrica* under N_2-deficient conditions. In heterocyst cells, H_2 was produced using stored carbohydrates and PSI. In addition, ferredoxin and RNR play a crucial role in cyanobacterial H_2 yields [109]. In addition, some studies have shown that the Fe content of the culture medium positively influences heterocyst formation. The abundance of heterocysts is significant as they serve as a chassis for H_2 production [106].

Only [NiFe]-H_2ases that can be active during ethylene and H_2 production by the N_2ase enzymes are found in heterocyst cells. Introducing non-native [FeFe]-H_2ase to cyanobacteria cells resulted in a higher H_2 yield than its wild type. One of the most significant tasks in bio-H_2 research is introducing H_2-producing [FeFe]-H_2ase into heterocysts of filamentous diazotrophic cyanobacteria to boost H_2 production [46]. Despite the incorporation of clostridial [FeFe]-H_2ase into *Nostoc* sp. PCC 7120, sufficient levels of H_2 production have not yet been attained. When Fe-limited filaments are effectively converted into heterocysts, [FeFe]-H_2ase requires Fe during Fe-limited culture. [FeFe]-H_2ase requires the same amount of Fe as other H_2ases: more than 10 Fe atoms for every functional H_2ase protein complex [46]. Therefore, all heterologously expressed H_2ases can influence the photosynthetic Fe requirement and remaining Fe-containing enzymes such as N_2ase [110]. Longstanding Fe deficiency (~90 days) in wild-type *Nostoc* PCC 7120, on the other hand, revealed that the Fe homeostasis mechanism is tightly controlled and maintains photosynthesis and N_2 fixation at a reduced but stable level after a few days of culture. Given H_2ases fast turnover rate [111], the primary goal should be a well-regulated expression level that fits the metabolic capability of H_2 production while minimizing the metabolic burden of H_2ase on available intracellular Fe.

Moreover, a robust process of H_2ase activation can be implemented at the transcriptional level to synchronize expression with the onset of Fe-limiting effects. Since N_2ase activity naturally decreases under Fe deficiency [112], the remaining Fe used for N_2 fixation can be redistributed to [FeFe]-H_2ase. On the other hand, a basic level of N_2 fixation is required to stabilize the culture and maintain the proteome, i.e., the PSII repair mechanism. Therefore, Fe-limited cultures can be used to support heterocyst-based H_2 production while the activity of N_2ase remains negligible. Since Fe-limitation inhibits the growth of cyanobacterial cells and increases intracellular carbohydrate storage, this is a promising culture strategy for utilizing energy stored photosynthetically for H_2 biosynthesis. Rather than enabling cyanobacteria to grow continuously in photobioreactors, as has been advocated in the last 30 years of biofuel research [113], solar energy should be converted directly into H_2 or other fuels.

Genetic engineering of heterocysts can be performed to increase their abundance, the thickness of the cell membrane, the amount of sugar coming from the vegetative cell, and the speed of arrival. In addition, the modification of genes contributing to the formation of heterocysts in a nitrogen or oxygen environment is also of interest.

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Construction of the O_2-resistant hydrogenase

Biocatalysts will play an increasing role in processes aimed at providing alternative and clean fuel sources [114]. However, producing enzyme cells in the presence of hydrogenases is currently difficult because O_2 inhibits their activity. The enzyme sensitivity to O_2 is the main impediment that must be addressed to develop effective photobiological H_2 production. [NiFe]-H_2ases are cyanobacterial hydrogenases that include both uptake *Hup*-H_2ase ($2H^+ \rightarrow H_2$) and bidirectional *Hox*-H_2ase ($2H^+ \Leftrightarrow H_2$). Common [NiFe]-H_2ases are resistant to microaerobic conditions and are only transiently inhibited by O_2. Several microorganisms with O_2-resistant [NiFe]-H_2ases, such as *Ralstonia eutropha*, have O_2-resistant H_2ases that can oxidize H_2 in the presence of atmospheric pO_2 [115]. Although not all species can accommodate these plasmids, the research on transporting genes encoding natural proteins in this organism to the necessary cyanobacteria appears very promising [116,117]. The genes responsible for the growth of the bacterium *R. eutropha* in a microanaerobic medium were fully studied. Nevertheless, *R. eutropha* H_2ase is considered a model system to inspire the development of effective O_2-resistant hydrogenases [118].

Genetic engineering techniques to increase H_2 synthesis by shifting electron flow towards H_2 metabolism have recently received much interest. An engineering approach to removing competing electron routes, such as the respiratory ETC, the nitrate absorption system, and C fixation via the Calvin-Benson cycle, might be a potential technique to improve H_2 production. Thus, decreasing nitrate uptake reduces electron flow for H_2 metabolism, while increasing its yield. *Synechocystis* PCC 6803 Δ*Har* and Δ*Hir* mutants (deletions of the Δ*Har* and Δ*Hir* genes) with impaired nitrate uptake produced a higher H_2 yield. Furthermore, inactivating hiloxidase in *Synechococcus* PCC 7002 increased H_2 production *in vivo* [97]. According to McNeely et al. [75], increasing the NADPH/$NADP^+$ ratio also increases H_2 production by NADPH-dependent, bidirectional [NiFe]-H_2ase. H_2 synthesis can thus be enhanced by lowering or removing competing metabolic processes.

Fritsch et al. [119] presented a novel crystalline biological active substance (membrane-bound H_2ase from *R. eutropha* H16-MBH). This crystal enters the thylakoid membrane and retains O_2 without allowing it to enter the domain of enzymes that cleave H_2 molecules. Since there is no O_2-resistant component in the structures of [NiFe]-H_2ases, the crystal structure of O_2-resistant H_2ase is a mechanism used by some of these enzymes to protect them from O_2, and this control may pave the way for H_2ases in many biotechnological developments. Goris et al. [120] also engineered a membrane of *R. eutropha* H16 that allows the O_2-resistant [NiFe]-H_2ase to produce H_2 in an O_2-rich environment. The authors demonstrated that O_2 resistance is critically related to changes in the internal ETC. Six instead of four conservative coordination cysteines surrounded the [Fe–S] cluster near the active site. O_2 tolerance is not only based on the limited O_2 access to the active site but also on reducing O_2 species guided by the electronic relay's unique structure.

Genetic engineering of O_2-resistant hydrogenase enzymes is carried out by modification of their responsible genes. In this context, studies aimed at reducing the amount of O_2 released by PSII in an anoxic environment will undoubtedly bear fruit. The hydrogenase enzyme can be made O_2-resistant by replacing the cluster.

Engineering of [NiFe]-hydrogenase to enhance hydrogen production

The indicators of H_2 production by cyanobacteria species obtained from many natural fields have been studied, and optimization work has been carried out in laboratory conditions. However, despite the efforts made for half a century, industrially useable H_2 has not yet been isolated from cyanobacteria. In this context, the importance of obtaining mutant strains through genetic engineering before implementing metabolic and technical strategies is increasing. [NiFe]-hydrogenase is the main enzyme activated in cyanobacterial strains under anoxic conditions [18].

Hydrogenase reabsorbing H_2 produced by N_2ase is one of the major constraints to the efficient conversion of solar energy into H_2 [2,121]. *Hup*-H_2ase catalyzes the almost unidirectional uptake of H_2, whereas *Hox*-H_2ase catalyzes both H_2 uptake and production. Generally, heterocystic cyanobacteria have *Hup* and *Hox*, though some only have *Hup* [122]. To improve N_2-based H_2 production, *Anabaena* sp. PCC 7120 was selected as a model strain [123] as its entire genome sequence is available. Since *Anabaena* sp. contains two types of H_2ases, targeted gene disruption was used to inactivate each type and both H_2ases, resulting in single (Δ*Hup* and Δ*Hox*) and double (Δ*Hup*Δ*Hox*) mutants [124]. In an argon environment, inactivating *Hup* activity increased the H_2 production rate by 4–7-fold relative to wild-type cells, whereas inactivating *Hox* activity did not affect H_2 production. *Hup*-damaged mutants have also been shown to increase H_2 production in another *Anabaena* and *Nostoc* sp [125].

Since O_2-resistant [NiFe]-H_2ases in *R. eutropha* are always inactive, studies suggest that the *NiB* genes are responsible for tolerance. Instead, structural changes in proximal [FeS] clusters facilitate O_2 transport. The crystal structure of [NiFe]-H_2ase bound to an O_2-resistant membrane from *R. eutropha* H16 has a high resolution [119]. A new [3Fe4S] cluster coordinated to six S atoms of cysteine residues, two of which are identical to O_2-resistant hydrogenases, is responsible for O_2 tolerance [126]. When Lenz et al. [127] substituted glycine for conventional cysteines, the enzyme became more sensitive to O_2. As a result, the previously unique proximal [$3Fe_4S$] cluster was converted to the normal [$4Fe_4S$] cluster. In addition, all future research should concentrate on the proximal [FeS] cluster and its probable significance in determining O_2 sensitivity.

A chemical mutagenesis study was conducted using mutant strains of the filamentous N_2-fixing cyanobacteria *Nostoc punctiforme* PCC 73102, *Anabaena variabilis* ATCC 29413, and *Nostoc* PCC 7422 to increase H_2 production by several folds [69,76]. Some authors [22,128] claim that changes in H_2ase activity or functional relationships with the N_2ase complex in the mutant cells are linked to a general regulatory system that regulates cell differentiation. Furthermore, H_2ase is influenced by a disruption of ETC linked to active N_2 and H_2 metabolism. Masukawa et al. [129] compiled the amino acids dc-Q193S and dc-R284H, which are in proximity to the [FeMo] cofactor of N_2ase, and described mutants of *Anabaena* sp. PCC 7120 that lacks *Hup*-H_2ase. The wild Δ*Hup* strain had a lower

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optimal temperature for H_2 production, and unlike the parent strain, H_2 splitting in the mutant strain was not completely inhibited in the N_2 gas phase. From a practical standpoint, this is very promising, as it authorizes utilizing a less expensive gas phase instead of argon in the process. A defective *Synechocystis* sp. PCC 6803 M55 mutant in the *ndh*B gene with limited *Hup*-H_2ase activity also produced a higher H_2 yield [73].

In the case of N_2 fixation, H_2ase is involved in processing the H_2 produced by N_2ase. Therefore, H_2ase elimination can potentially yield a higher H_2 volume when considering and analyzing cyanobacterial metabolism. For a long time, genetic research on this alteration has been conducted and described in numerous papers. In particular, the gene knockout of the regulator *HupR* protein reduces *Hup*SL-H_2ase expression and thus increases H_2 yield [130].

A possible technique for improving photobiological H_2 production under oxygenated conditions is starting with parent strains with high H_2ase activity and further inactivating *Hup* activity. The acetylene reduction assay revealed that *Nostoc* sp. PCC 7422 had the highest H_2ase activity among the 13 heterocystic strains tested [94]. Following the identification of the nucleotide sequences of *Nostoc* sp. PCC *Hup*-encoding genes, a *Hup*-minus (Δ*Hup*) mutant was engineered by insertion and *hup*L disruption. When grown in the original headspace over Ar + 5% CO_2 gas under constant light, Δ*Hup* mutant cultures collected H_2 at rates of 20–30% (w/w) with O_2 evolution. H_2 accumulation was reduced by <20% when 20% O_2 was added to the initial airspace of the Δ*Hup* cultures, indicating that the mutant H_2ase is not sensitive to O_2. At incoming light energy of 70 μmol photons m^{-2} s^{-1}, the Δ*Hup* mutant converted light energy to H_2 with a high efficiency of 1.8% relative to total solar radiation. Under lab (optimal) conditions, more than 1% of efficiencies relative to total solar irradiance have been reported [129]. However, this was achieved only at a low light intensity, while with increasing light intensity, efficiency decreases significantly, as demonstrated for the Δ*Hup* mutant PCC 7120 [129,131,132]. The maximum efficiency under bright sunlight was around 0.1% [133]. Targeted mutagenesis to lower the quantity of light-harvesting complexes and/or the concentration of reaction centers, as well as a selection of wild-type strains with greater tolerance to high light intensity, might be used to solve the problem of low light saturation in cyanobacteria.

Engineering O_2-resistant mutants

Manipulating O_2-resistant H_2ase genes is another approach for cyanobacteria-based H_2 production [134]. Many studies [135–137] aim to increase H_2 production by replacing or deleting hydrogenase structural genes. The primary goal of recent H_2ase studies has been to solve problems associated with increasing H_2 production by identifying, copying, and cutting genes not sensitive to O_2 to maintain stable H_2 production in cyanobacteria [138]. Many studies have been conducted, even though the results are minimal [139,140]. In addition, several recombinant cyanobacterial species with O_2 resistance have been obtained. Thus, some of the structural and auxiliary plasmid genes required for the synthesis of H_2ase in *Thiocaspa roseoperscina* were introduced into *Synechococcus* PCC 7942 [141]. One of the most important fundamental studies is incorporating [Fe]-encoding structural genes of H_2ase into *S. elongatus* PCC 7942 and synthesizing a functional H_2-producing enzyme. In addition, two recombinant structural H_2ase genes from *Synechocystis* PCC 6803 were obtained using *Rubrivivax gelatinosus* [142]. When such mutant cyanobacterial strains with high production of O_2-resistant H_2ase are developed and employed, it is unnecessary to aerate the culture medium with an inert gas to induce anaerobic conditions.

The main properties of oxygen-resistant enzymes are directly related to the metals they contain. The unique cluster [3Fe4S] not only ensures O_2 transport through the H_2ase bound to the *R. eutropha* membrane but also suggests that auxiliary *HoxR* and *HoxT* proteins may be involved in O_2 transport [119]. The disruption of *Hox*R or *HoxT* genes leads to an increase in O_2 sensitivity and a change in the unique [3Fe4S] cluster composition, as demonstrated experimentally. The predictive corresponding *HoxR* functions in forming a unique [$3Fe_4S$] cluster are inconsequential for cofactor stability development.

Obtaining O_2-insensitive mutants in cyanobacteria is currently an essential issue, so research in this direction will undoubtedly be of interest. In this context, *Hox*R and *Hox*T proteins from *R. eutropha* are important factors that need further investigation.

Hydrogenase behavior in an anoxic environment

Accidental gene insertion (*hox*, *hyp*, *hup*) can increase the activity of the natural enzyme H_2ase. Neighboring relatives of cyanobacteria or other prokaryotic microorganisms act as donors. Research has been conducted on plasmid introduction into the cytoplasm by cross-cutting cells. Related studies [143,144] reported that the rate of H_2 production in the [FeFe]-H_2ase encoding strains *C. acetobutylicum* and *C. saccharobutylicum* increased by 67% on average compared to the natural strain. In addition, the *Synechococcus elongatus* strain UTEX 2973 showed a relatively higher H_2 yield. It co-existed with *C. saccharobutylicum* in nature, and the sequencing showed that this cyanobacteria strain contains a unique [FeFe]-H_2ase gene responsible for H_2 production. H_2ase activity was found to be 1.3-fold higher in this study. The utility of such strategies in producing O_2-sensitive or non-O_2-sensitive mutant species is enormous, and all genetic studies aimed at increasing H_2 production use cyanobacterial [NiFe]-H_2ase and N_2ase enzymes.

Active species containing new and unique hydrogenases with the functions and properties required for H_2 production can be isolated from the environment, particularly in cells living in specific environmental conditions (extreme regions). For example, numerous changes in [FeFe]-hydrogenase adapted to living in an O_2-free environment have been observed in studies of the H_2ase enzyme diversity. It was discovered that strictly conservative amino acids undergo distinct changes in redox properties and active site O_2 sensitivity. Similar methods can be used to create effective and non-O_2-sensitive H_2ases, such as [NiFe]-H_2ase [145]. Ideas for genetic engineering research can be obtained by studying microorganisms that have altered their properties to survive and adapt to such natural conditions.

Moreover, research in this realm involves expressing and introducing O_2-resistant [NiFe]-H_2ases into the cell. For

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example, *Hyd*SL H_2ase expressed by the sulfur bacterium purple *Thiocapsa roseopersicina* was incorporated into unicellular *Synechococcus* sp. PCC 7942 [141]. However, the introduced foreign [NiFe]-H_2ase requires an extra maturation system and a consistent electron supply for H_2 production. Therefore, the O_2- and heat-resistant NiFe-HynSL H_2ase (*Hyn*SL) was incorporated into the *S. elongatus* chromosome along with 11 indirect *Atheromonas macleodii* genes [141]. As a result, a 3-fold higher H_2 production was obtained in the recombinant strain.

Genetic engineering accomplishments also include enhanced expression of the inserted genes, O_2 tolerance, and effective integration of the H_2ase into the photosynthetic ETC [16].

Incorporation of heterologous hydrogenase into cyanobacteria cells

The N_2ase or natural bidirectional [NiFe]-H_2ase activity is tightly linked to cyanobacteria-based bio-H_2 production. However, H_2 production with natural enzymes remains very low and far from economic productivity. Therefore, genetic engineering studies need to be conducted, albeit at high intensity, for which the host cell and primary enzymes are necessary. All N_2ases have a low cycle number and require 2 ATP molecules to reduce one electron. In addition, they secrete at least 1 mol of H_2 for each mole of N_2 absorbed [146]. In cyanobacterial cells, uptake, or bidirectional [NiFe]-H_2ase, degrades H_2 molecules in relatively small amounts [147]. In contrast, H_2 production based on [NiFe]-H_2ase is not dependent on the ATP molecule and has a high rate. [FeFe]-H_2ase is a relatively well-studied enzyme for genetic engineering. Therefore, [FeFe]-H_2ase can be used as a model enzyme when implementing recombinant expressions in synthetic systems [148]. However, no naturally occurring [FeFe]-H_2ase has been discovered in cyanobacteria [66]. Thus, one of the most important strategies is to isolate [FeFe]-H_2ase and introduce it into cyanobacterial cells. This enzyme is most commonly found in enzymatically aerobic *Clostridium* [149]. Another model system is [FeFe]-H_2ase (*Hyd*A1) from the microalga *Chlamydomonas reinhardtii*, which consists only of the F-domain and lacks the FeS cluster [150]. The ability of metabolic [FeFe]-H_2ases isolated from microalgae to connect with water oxidation by PSII and photosynthetic ETCs sets them apart. However, the expression of [FeFe]-H_2ase in natural cyanobacteria is fraught with complications. This is because cyanobacteria can only produce gene signals that target the maturation of their [NiFe]-H_2ase under natural conditions. With the extraction of [FeFe]-H_2ase from *Clostridium* sp. and its successful introduction into the unicellular cyanobacterium *Synechococcus* sp. [151], there has been a small breakthrough in this direction in the last decade. *In vitro* and *in vivo*, the heterologous, foreign [FeFe]-H_2ase was also active. *In vivo* [FeFe]-H_2ase activity was light-dependent and associated with ETC reactions. This study showed that AMC414 lacks Hup activity, and increased H_2 (3.75 nmol H_2 Chl^{-1} *a* h^{-1}) was observed compared to the wild type (1.5 nmol H_2 *Chl a* h^{-1}). Again, [FeFe]-H_2ase was degraded in filamentous heterocystic *Nostoc* PCC 7120 blue-green algae. The *hetR* gene, which is only expressed and functions in heterocystic cyanobacteria, was used as a promoter for this purpose [152]. The [FeFe]-H_2ase protein is found not only in cyanobacteria but also in *Escherichia coli*. The H_2 yield increased when [FeFe]-H_2ase was delivered into the cell along with *HydE, HydF*, and *HydG* genes [153]. However, no biochemical or genetic evidence supports the maturation of [FeFe]-H_2ases in cyanobacteria. This research is not limited to bacteria but is also carried out in eukaryotes. It has been shown that the [FeFe]-H_2ase of *Clostridium* sp., which lacks the genes responsible for the maturation system, is expressed in the cyanobacterium *Synechococcus* PCC 7942 under *in vivo* and *in vitro* conditions [154]. Similar findings were attained in a study on transferring the *HydA* gene from *Chlamydomonas* to *Synechocystis* PCC 6803. Activation of an enzyme transferred into a foreign cell may occur due to the taxonomy of genes copied along with the maturation system. As shown in the preceding study [155], when [FeFe]-H_2ase was transferred to *Synechocystis* sp. PCC 6803, without additional maturation genes, was activated differently in each organism. Why [FeFe]-H_2ase is activated in some organisms without maturation operons and why some organisms do not need to express it is an area of research that still requires clarification (Table 2).

Table 2 – Hydrogen production in engineered non-native enzymes of cyanobacterial strains using different strategies.

Cyanobacterial strains	Engineered genes	H_2 production rate	H_2 production assay condition	References
Synechosystis sp. PCC 6803	*hydA* from *Chlamydomonas reinhardtii*	130 nmol H_2 μg *Chl* a^{-1} h^{-1}	Anaerobic; 50 μEm^{-2} s^{-1}; 5 μm DCMU	[155]
Anabaena sp. PCC 7120 (*Hup*$^-$)	H_2ase operon (*hydA*, *hydB*, *hydE*, *hydF*, *hydG* along with two additional genes, S03922 and S03924, from *Shewanella oneidensis* MR-1)	3.4 nmol H_2 μg *Chl* a^{-1} h^{-1}	Light and nitrate deprivation	[152]
Synechococcus elongatus	*hydA* and maturation operon (*hyd*EFG) from *Clostridium acetobutylicum*	2.8 μmol H_2 μg *Chl* a^{-1} h^{-1}	Light; 5 μm DCMU; bubbling with 2.5% CO_2 and 97.5% N	[151]
Synechococcus elongatus	[NiFe]-H_2ase from *Thiocapsa roseopersicina*	~0.07 nmol H_2 mg protein h^{-1}	Anaerobic; 40 μEm^{-2} s^{-1}	[141]
Synechococcus elongatus	[NiFe]-H_2ase (*hyn*SL along with 11 adjacent proteins) from *Alteromonas macleodii*	~4.2 nmol H_2 mg protein h^{-1}	Anaerobic; 40 μEm^{-2} s^{-1}	[141]
Synechococcus PCC7942	*hydA* from *C. acetobutylicum*	~6.8 μmol H_2 mg *Chl* a^{-1} h^{-1}	Ar, dark adaptation	[154]

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Studies on [FeS] cluster ligation in [NiFe]-H_2ases small subunits have recently changed the enzyme proclivity to accept H_2ases. Yonemoto et al. [156] developed an O_2-tolerant enhanced H_2 production scheme in cyanobacterial strains by introducing site-directed mutations into *A. macleodii* O_2-tolerant [NiFe] HynSL hydrogenase, a membrane-associated H_2ase. Due to its 2% O_2 tolerance, this H_2ase is appealing for biotechnological applications. The ligand modification was based on the mutagenesis of uptake H_2ase from *D. fructosovorans* [157]. Yonemotto et al. [158] also continued the investigation of *A. macleodii* H_2ase to obtain an enzyme with considerably higher H_2 production activity than previously reported. The substitutions in *A. macleodii HynSL* were thoroughly investigated, with aspartic acid, histidine, asparagine, or glutamine substituted for each of the 12 amino acid sites linking three [FeS] clusters in the small subunit, as well as alternate coordinating ligands.

In general, all H_2ase use direct or auxiliary mechanisms to transport electrons to the donor, i.e., Fdx. An unanswered question is whether the functional foreign [FeFe]-H_2ase can effectively interfere with the main metabolism, specifically whether it can transfer or accept electrons to the cell redox zone. Artificial [FeFe]-H_2ase introduction into the whole redox system and complete contact with the host cell is a very questionable process. This statement is supported by research on *Synechococcus elongatus* [151]. The electron transfer to the introduced [FeFe]-H_2ase and the energy stored in reserve are handled differently by distinct Fdx. As a result, Fdx that can modulate the introduced foreign H_2ase into the cell must be created. Consequently, continuous electrons can reach the introduced artificial [FeFe]-H_2ase.

Cyanobacteria-based H_2 production can achieve higher yields with O_2- and heat-resistant enzymes. This might offer benefits over O_2-labile [FeFe]-H_2ases.

Engineering a photosynthetic apparatus to enhance H_2 production

The mechanism of photosynthesis evolved over millions of years to operate under unfavorable environmental conditions and competition, but not as efficiently as possible. The ratio of PSI and PSII, linear-cyclic electron transport, the volume of the LHC, and the number of cells all play essential roles in the efficient operation of the photosynthetic process [110]. The traits mentioned above can be genetically engineered to improve the photosynthetic structure operation. Cyanobacteria have a lower PSII/PSI ratio than microalgae and plants, resulting in better cyclic electron transport performance than linear electron transport [159]. Many mutant strains were obtained to increase the H_2 yield by enlarging the antennae. For example, the mutant Olive (*apcE* and *apcAB* operons deleted) cyanobacterium *Synechocystis* sp. PCC 6803 without phycocyanin antennae produced more H_2 than the wild species [160]. As a result, the cyanobacterial species do not consume redundant energy sources, leading to an increment in PSI/PSII ratio. According to the findings, the Olive mutant is an encouraging H_2 production contender. An increase in the pool of photosynthetically derived NADPH regenerators and linear electron transport between photosystems of the Olive mutant were weakened by the pigment phycocyanin [160]. Moreover, to compensate for the excessive reduction in cellular metabolism, the mutant strain enhanced the amounts of oxidative and osmotic stress-related enzymes and carbon fixation. It can be concluded that the mutant Olive, whose phycocyanin pigment was removed, is the most promising candidate for further genetic modification as a constructive cyanobacterial cell [161].

Indeed, genetic engineering methods are being used to reduce phycobilin pigments and chlorophyll antennae to increase cyanobacteria-based H_2 yield. As only two photons are required for the transfer of electrons acquired from the water decomposition, theoretically, at high light intensities, a cell PSA absorbs and wastes fewer photons. Therefore, engineering mutants by reducing the quantity of light-absorbing pigments in the PSA allows for a 10% efficiency in turning solar energy into carbohydrates. Moreover, H_2 can be obtained from these cells [162]. Bernat et al. [160] demonstrated that continuous growth of a mutant *Synechocystis* PCC 6803 with shortened or reduced antennae could produce 200 mL H_2 h^{-1} L^{-1} culture. Linear electron transport was 6-fold faster in a *Synechocystis* PCC 6803 mutant missing a phycobilisome than in a wild strain, whereas cyclic electron transport was the same in both strains. The main prerequisite for increasing H_2 production under conditions of increasing sunlight absorption is an enhancement of the linear electron flow rate.

Recently, it was discovered that protein synthesis technology could significantly improve photosynthetic electron transport efficiency to the H_2ase. To prevent competing with Fdx-$NADP^+$ oxidoreductase (FNR), Fdx can be combined with H_2ase [163]. Eilenberg et al. [117] found that combining [FeFe]-H_2ase with Fdx *in vivo* increased photosynthesis 4-5-fold and improved O_2 resistance over wild-type [FeFe]-H_2ase. Alternatively, PSI can also directly combine [FeFe]-H_2ase. Kanygin et al. [52] used this approach to develop the PSI H_2ase chimaera, which is integrated into the *Hyd*A chain unit. Under anoxic conditions, the engineered *C. reinhardtii* strain produced stable H_2 for 5 days at a rate of 14 μmol H_2 mg Chl^{-1} h^{-1}. Moreover, H_2 photoproduction occurs independently of the Calvin cycle in nutrient-rich algae.

Cyanobacteria *Acaryochloris marina* having a high *Chl d* content could be another impressive H_2 producer. Its plasmid (pREB4) contains the *hyp*ABCDEF gene, which encodes a complete set of bidirectional H_2ase subunits [164]. *A. marina* can utilize light until the near-infrared region, as the absorption maximum of *Chl d* is near red. *A. marina* is a natural mutant because its unique redox potential of electron-carrier components in photosynthesis is directly tied to it. One of the fundamental studies is the study of H_2 splitting under the red color spectrum of a strain with unique photosynthetic pigments [165].

Bernát et al. [160] used *Synechocystis* PCC 6803 strains with genetically modified antennae to produce H_2. The authors made a potential hydrogen production strain using antenna-deleted *Synechocystis* sp. PCC 6803, and their results showed that decreasing or missing PBS antenna gave the mutant strains (Olive and PAL mutants) the capability to grow under limited light. Also, the group showed that the linear electron transport rate increased by 1.5-fold in PC- and PBS-depleted Olive and PAL mutants. Furthermore, the effects of the strain on the ETC and the environment were determined.

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When photoautotrophic mutants with shortened phycobilisome antennas were grown under the light, they produced the most H_2 (200 mL H_2 h^{-1} by a 1 L culture).

Numerous literature data cover H_2 production pathways in anoxygenic organisms and their relevance to photosynthesis [166–169]. However, further studies are needed to increase the efficiency, productivity, and reliability of photosynthetic H_2 production in direct solar energy conversion.

Effects of resistance to oxidative and sugar stress

Cyanobacteria are of global interest as biological instruments for carbon capture and storage. They are considered producers of sustainable biofuels due to their excellent photosynthetic endowment. Their genetic simplicity and physical stability may one day make them the most important industrial species. A well-developed complex group of enzymes is expected to contribute to the current energy deficit, making cyanobacteria-based H_2 production a promising industry [170].

With each study, the structure of the cyanobacterial enzyme types involved in H_2 production becomes more complex [39]. Therefore, the strain *Synechocystis* PCC6803, whose genome has been fully sequenced, was used. A bidirectional pentameric H_2ase that is reversibly O_2-inactivated can produce H_2. For photosynthesis and/or sugar catabolism, the H_2 reaction ($2H^+ + 2e^- \leftrightarrow H_2$) relies on electrons from NAD(P)H, as well as redox cofactors from the [NiFe] center and multiple [FeS] clusters. The *HoxEFUYH* operon is controlled by a feeble promoter [171] expressed by 168 bp in the upper stream of the proximal *hox*E gene codon. Meanwhile, the *Hox*EFU subunits work together to form a diaphorase component, which transports electrons to the *Hox*HY H_2ase unit, which uses NAD(P)H to produce H_2 from protons. Physiological studies have revealed that H_2ase operates as an emergency electrical valve, releasing extra photosynthetic electrons, such as switching from (anaerobic) dark to light conditions, resulting in debilitated and temporary H_2 production.

According to Ortega-Ramos et al. [171], overexpression of the *Hox*EFUYH operon protects cells from stress caused by H_2O_2, glycerine, and glucose. Simultaneous overexpression of the *Hox*EFUYH and *Hyp*ABCDEF proteins resulted in a 20-fold increase in H_2ase activity. The authors concluded that the increased cellular production of H_2 proteins positively affects H_2 yield and that future increases in H_2 yield can only be achieved through genetic engineering.

Germer et al. [103] used the *psb*AII promoter to increase H_2ase activity in *Synechocystis* sp. via the expression of the endogenous *hox*EFUYH operon of strain *Nostoc* PCC7120 and the heterologous *Nosthyp*ABCDEF operon. As a result, the mutant strain H_2 yield increased from 2.9 (wild type) to 9.4 (mutant) nmol H_2 mg *Chl* a^{-1} min^{-1} [103].

Despite the preceding investigations, H_2 metabolism remains a mystery. More profound knowledge is required to identify favorable environmental conditions and to carry out powerful genetic alterations to speed up the rate of H_2 production [172]. Obtaining mutants of cyanobacteria that are resistant to sugar and various salts, in turn, opens the way to rapid biomass production and increases the H_2 yield. This is because the amount of biomass is vital in obtaining any biological component.

Developing a zero-waste H_2 production technology using genetically engineered cyanobacteria

A promising future research direction is the development of a zero-waste H_2 technology based on cyanobacterial strains. Many technologies are currently proposed to produce biodiesel and bioethanol from cyanobacteria [170], but there are no plans yet to develop a zero-waste energy technology based on cyanobacterial H_2. According to the current environmental policies, one of the most important activities is to develop energy production technologies that do not pollute nature.

Nutrients (macro- and microelements) are extracted from municipal wastewater (1) to cultivate mutant strains, as shown in Fig. 3. This technology is implemented by constructing special photobioreactors in manufacturing areas. Household or industrial wastewater is treated in two stages: (2) before being purified in natural ponds and (3) until entering the photobioreactor. The conditions for H_2 production are then created, and the nutrients are transported into the photobioreactor, where the cyanobacteria grow. Since the photobioreactor is located in an external environment (4), solar energy is sufficient. The CO_2 required for cell growth is transported via special hoses from nearby manufacturing facilities and fed directly or indirectly to the photobioreactors (5). The H_2 energy produced is stored in special H_2 cylinders (6). The resulting pure H_2 energy is stored in bacteria (7) as electrical energy or used for lighting and motor vehicles (8). Hydrogen energy can also be used directly as a high-power energy source in the heating system (9) and the space industry (10).

As shown in Fig. 3, in the future, by creating smart cities, it will be possible to reduce the current main problem - the temperature of the Earth's surface. At the same time, the technologies of waste remediation, reuse of wastewater, and creation of new, inexhaustible energy sources can be considered the main plan to prevent the energy crisis. Furthermore, by constructing the photobioreactors for growing cyanobacteria in production facilities, industrial CO_2 can be directly used for cell growth. In this context, one of the future genetic research's main goals is to create new strains capable of actively growing and releasing H_2 energy in an environment with high CO_2 (1–10% of air).

The main advantage of the cyanobacteria-based H_2 production technology is higher biomass productivity. However, since cell division occupies an important place in the production of any biological product, it is also critical that the mutant strain's growth intensity is high for H_2 energy. Table 3 summarises the most important characteristics of the cyanobacterial strains for H_2 production technology.

However, culturing cyanobacterial strains in wastewater has its challenges. This is because wastewater contains very high amounts of lignocellulosic materials (LMs) [173]. LM is a composite based on cross-linked biopolymers on a dry basis,

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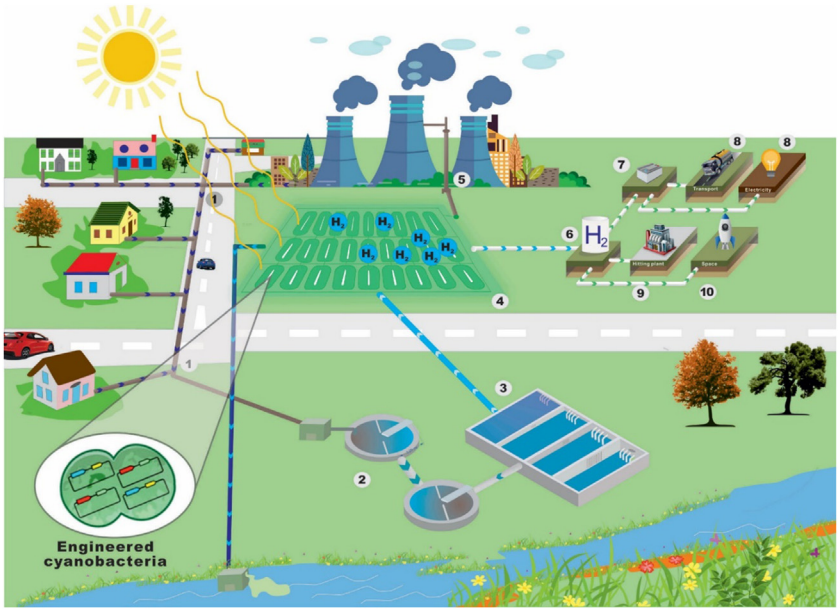

Fig. 3 – Zero-waste technology for H_2 production based on genetically modified cyanobacteria. *Note:* 1 – municipal wastewater; 2 – treatment facilities; 3 – aero tank; 4 – open bioreactor for cyanobacterial biomass; 5 – transfer of CO_2 from flue gas of thermal power plants to the bioreactor; 6 – H_2 storage; 7 – storage of H_2 generated electricity; 8 – the electricity generated can be used in the transport industry and as a light source; 9 – H_2 can be used as an energy source for heating generators; 10 – H_2 can be used as the main energy source in the space industry.

with 35–45% cellulose, 25–30% hemicellulose, and 25–30% lignin [174]. These are classified into four main proportions based on their source, namely woody biomass, agricultural waste, energy crops, and cellulosic waste groups [175]. It is likely that LMs negatively affect the growth of cyanobacterial cells and lead to a decrease in H_2 content. However, using LMs as biofilms, their toxicity can be eliminated, and cell growth is stimulated. For example, Zhang et al. [173] made the first attempt to develop a new biofilm technology using LMs as biofilm carriers. The authors selected LMs pine sawdust, rice husks, sugar cakes, and oak sawdust, which are produced in large quantities around the world every year. Finally, the toxicity of LMs to cells was determined by measuring the growth parameters and chemical composition of *C. vulgaris* grown in biocarrier leachate. Next, Zhang et al. [176] obtained five different LMs and used microalgae strains for their utilization. The effect of the physicochemical properties and toxicity of biocarriers on the growth and attachment of cells was determined. *C. vulgaris* cell growth was promoted by pine sawdust filtrate (15.5%), whereas shoot bark filtrate (15.5%) and sugarcane (13.2%) inhibited cell growth during light cultivation.

Phototrophic cells attach to LMs and use them as natural materials for biofilm formation. After cultivation, the cyanobacterial biomass can be harvested together with LMs or separately for disposal [177]. Interestingly, the collected mixture (LMs and cyanobacterial biomass) can be used directly in the laboratory or industrial conditions as a raw material for H_2 production. However, although the use of LMs as carriers in industrial photobioreactors is economically beneficial, the following conditions should be taken into account first.

(1) Choosing the optimal materials for fixing the cells that increase the efficiency of the new technology;
(2) Assessment of the chemical composition of the collected cyanobacterial biomass;
(3) Determining the effect of material surface properties on algae productivity;
(4) Identification of factors that positively affect the growth of cyanobacterial cells.

The abundance of fossil fuels, which is still running out, is the primary reason for the lack of development of biological H_2 energy. Since the cost of implementing investment projects is so high, many countries purchase cheap fossil fuels to compensate for household needs. Despite intensive study, bio-H_2 production is lagging, and most H_2 produced is derived via cracking.

Challenges and future perspectives

Although the mechanics behind the primary steps of H_2 production by phototrophic microbial cells are fully established, bio-H_2 production is still a long way from being implemented. The main constraints for sustainable H_2 production are the enzyme O_2 sensitivity and electron competition across distinct pathways that use reduced electrons. Therefore, cyanobacteria-based H_2 production has only been observed in lab conditions thus far. To reach an industrial scale, the pace and duration of H_2 production must be increased. Many alternative ways are being investigated to increase H_2 production. These techniques have three major components, namely, metabolic, genetic, and technological (Fig. 4) [33,34].

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Table 3 – SWOT analysis of zero-waste H_2 production technology [2,19].

Strengths	Uses only regenerating substrates: sun and water High growth rate Flexible to genetic modifications Can grow on cheap salts Independent of geographical location and weather conditions Easy to handle
Weaknesses	Expensive photobioreactor materials Reliant on electricity Difficulty in separating H_2 and O_2 The complexity of H_2 gas storage technology High transport costs Lack of supporting infrastructure in the fuel industry
Opportunities	Large-scale development of mutants with higher H_2 yields The necessity of high-level governmental and international programs Launch of high-level H_2 capture and storage projects Granting of rebates for the transport of bio-H_2 energy
Threats	High cost of bio-H_2 energy Lack of international support Low demand for non-renewable energy sources Inability to withstand competition

The emerging demand for the development of sustainable solutions to utilize solar energy with the subsequent production of energy sources for the social community arouses scientific interest [15]. However, along with solar cells to produce electricity, there are other ways to produce solar fuel. One of the most challenging fields of biotechnology to tackle environmental concerns is the development of photosynthetic cyanobacterial cells to generate sustainable solar fuels. Native and photosynthetic cyanobacteria have long been utilized as model systems to investigate, demonstrate, and develop photobiological H_2 production. However, the level of H_2 production attained is negligible from the perspective of industrial production of competitive energy carriers. Therefore, solutions to boost bio-H_2 production in cyanobacteria must be discovered through research. As shown in Fig. 4, recent breakthroughs in genetic engineering, which employs synthetic biology to engineer a cyanobacterial cell to produce the desired product, such as H_2, open up new possibilities and alternatives.

High-throughput assays and screening technologies, along with computational and systems biology breakthroughs, have enabled the examination of vast volumes of data to predict cell metabolism and physiology at the systems level. In addition, synthetic biology will enable the production of specifically tailored cyanobacterial cells capable of producing considerable volumes of renewable H_2 [97].

To investigate an organism at the systems level, systems biology can utilize a number of genome-wide technologies,

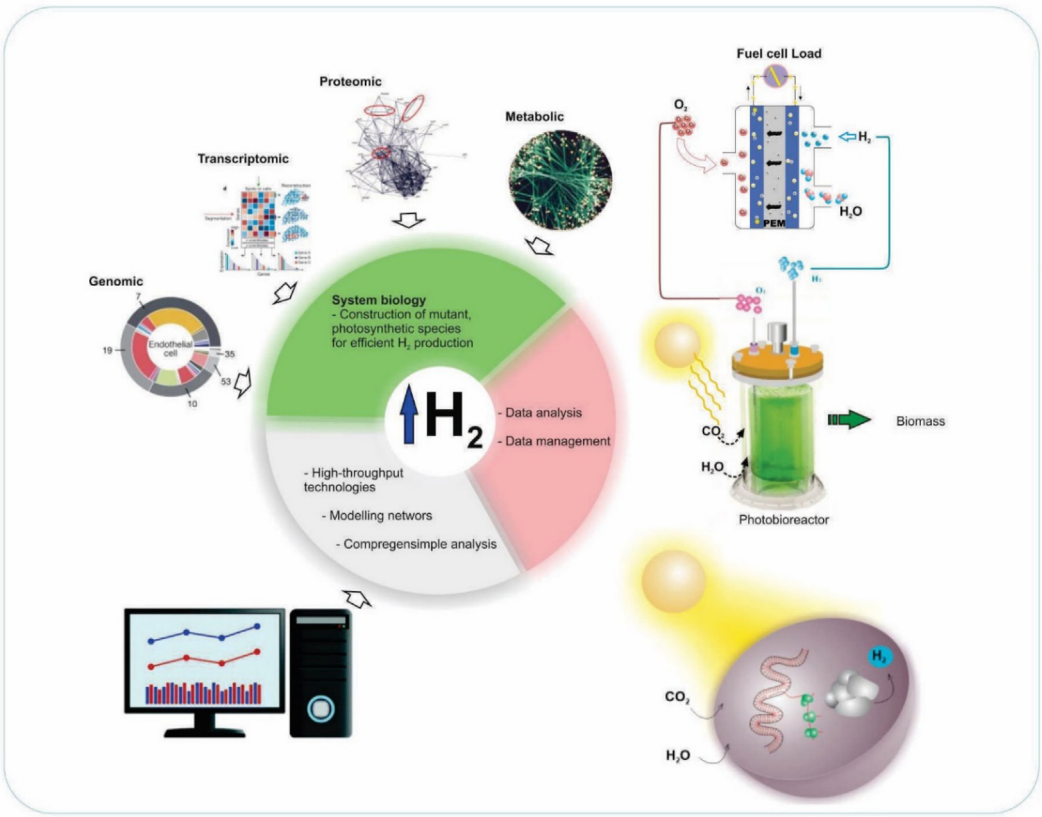

Fig. 4 – Prospects for cyanobacteria-based H_2 production using metabolic engineering systems to design and create cyanobacterial strains that convert solar energy and water directly into the preferred solar fuel, H_2.

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such as high-throughput analytical methods and computational and ohmic studies encompassing the genome, transcriptome, proteome, and metabolome levels. The findings of such studies can be used extensively in strain construction using metabolic engineering. Generally, the entire procedure is repeated until the desired phenotype and performance are achieved. Based on *in silico* modeling, knockouts and inserts will be possible in the future. The development of synthetic biology will allow the creation of modified cyanobacterial cells with predicted responses and/or the development of bioreactors. Still, it will also have implications for economic costs, public acceptance, and the development and use of H_2 energy systems in our society. Due to their rapid growth and simplicity, Cyanobacteria offer an ideal foundation for ambitious metabolic engineering projects.

Conclusion

Hydrogen production has only been determined on a laboratory scale thus far, as the yield remains insufficient for the industrial scale. Consequently, to regulate the H_2 production rate, the design and operational parameters must be optimized for maximal H_2 yield. Currently, efforts to increase the H_2 production capabilities of cyanobacteria species are carried out mainly in four directions: modification of nitrogenase enzyme genes, obtaining hydrogenase mutants, increasing electron transport by changing the photosynthesis complex and obtaining mutants resistant to external environmental factors. However, due to the low H_2 yields of the obtained mutants and the high costs involved, their introduction into production is ineffective. In this context, developing a waste-free technology using a genetically modified active H_2 producer is of great importance. The zero-waste technological system realizes hydrogen production and contributes to the reduction of the price of bio-H_2 in the future. Furthermore, the construction of zero-waste technology with mutant species in the production of bio-H_2 will not only alleviate future energy concerns but also lower CO_2 levels in the environment.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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