IRSTI 62.09.39 https://doi.org/10.26577/ijbch.2021.v14.i1.01

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# Production of biohydrogen from cyanobacteria: current challenges and opportunities

**Abstract.** Sources of renewable energy as a promising alternative to traditional sources operating on oil, natural gas and coal, are currently of great interest. Among the various types of biofuels, biohydrogen is the most clean and valuable fuel, and may be the most promising candidate for the role of environmentally friendly and renewable energy carrier of the future. Photosynthetic microorganisms have attracted special interest as potential cell factories for production of biohydrogen. In combination with photosynthesis, these organisms can use inexpensive inorganic substrates and solar energy to simultaneously biosynthesize and release hydrogen. Most studies focus on using different metabolic strategies to increase the hydrogen yield in different strains of cyanobacteria, including construction of genetic mutants of cyanobacteria with high capacity for biohydrogen production, followed by a well-chosen metabolic approach to increase its yield, and development of the innovative methods for their cultivation. Our laboratory is currently working on the isolation of heterocystic nitrogen-fixing cyanobacteria cultures from natural sources, as well as on the study of their morphological and cultural properties for use in biotechnology. The fundamental achievements made in these areas are summarized in this review.

**Key words:** Biohydrogen, photosystem, cyanobacteria, nitrogenase, hydrogenase.

## Introduction

Today, the scarcity of fossil fuels and the effects of climate change have led humanity to seek new sources of alternative energy. With many advantages, bioenergy is a promising mean to replace traditional energy. However, the production of first- and second-generation biofuels is likely to add even more challenges to water scarcity and food security threats. Meanwhile, third-generation biofuels derived from microalgae and cyanobacteria look promising, due to the rapid growth, ability to fix $CO_2$, grow on non-arable lands and high yields of the latter [1]. Microalgae and cyanobacteria appear to be the only renewable sources capable of producing a wide range of biofuels, including biohydrogen, biomethane, bioethanol, and biodiesel [2]. By expanding transformation of biological raw materials into biofuels, humanity simultaneously reduces the environmental burden on nature, reduces the pollution of territories and water reservoirs as well as $CO_2$ emissions into the atmosphere. In the field of biofuels, biohydrogen is the cleanest and most valuable of the fuels produced and may be the most promising candidate for the role of an environmentally friendly and renewable energy carrier of the future [3]. The current direction in bioenergy is the search for objects capable of producing biohydrogen that do not pollute the environment, as well as the development of high-yield technologies. There are two main groups of phototrophic organisms: 1) anaerobic phototrophic bacteria that do not produce molecular oxygen, and 2) aerobic phototrophic cyanobacteria, microalgae, and green plants that produce oxygen in the light [4]. Photosynthetic microorganisms, including cyanobacteria or blue-green (*Cyanophyta*), and green (*Chlorophyta*) microalgae, which have a high metabolic potential, are of particular interest in this regard [5-7]. Use of cyanobacteria as possible producers of biohydrogen is particularly relevant and very profitable since they form hydrogen as a result of solar energy conversion and do not require complex or expensive nutrient media for *in vitro* cultivation [8].

In this study, we present an overview of cyanobacteria application for production of biofuels, including their biology, characterization of the metabolism of heterocyst forms of cyanobacteria, which provide light-dependent hydrogen release due to the spatial separation of the processes of

hydrogen and oxygen release, systematization of available scientifically based data on the mechanisms of hydrogen formation, enzymes that catalyze this process, and factors that stimulate the release of hydrogen, as well as genetic and metabolic approaches to increase the productivity of hydrogen production by their cells. The review also provides an overall picture of the current state of this renewable energy industry.

**Promising cultures of cyanobacteria for biohydrogen production**

Cyanobacteria are commonly referred to as blue-green algae because of their color and their similarity to microalgae [9]. Unlike microalgae, cyanobacteria are prokaryotic as they lack a nucleus or membrane-bound organelles, such as chloroplasts, endoplasmic reticulum, Golgi apparatus and other. Cyanobacteria are the only bacteria that contain *chlorophyll a*. They have a relatively small genome, and many of them are already fully sequenced, so it is easy to genetically modify their biological characteristics in order to increase the productivity of biofuels [10]. Like microalgae, cyanobacteria are small and grow in aquatic environments. Cyanobacteria survive over a wide range of temperatures, but most tend to have warm optimum temperature for growth (above 20°C to be competitive with eukaryotic phytoplankton taxa, and above 25°C to be competitive with diatoms).

They can grow abundantly under certain conditions (including low ratio between nitrogen and phosphorus concentrations), and this can cause bloom, which in turn leads to high turbidity, anoxia, fish death and alteration of the food web [10].

In the diverse space of ecological niches, microorganisms are able to exist at extreme values of temperature, pH, salinity, atmospheric pressure, relatively high irradiance [11]. Consequently, cyanobacteria have mastered various ecological niches and, along with other habitats, successfully develop in the seas, fresh waters and ice of the Arctic and Antarctic. They are characterized by remarkably high adaptability to extremely unfavorable living conditions [12], and can develop in hypersaline and alkaline lakes, thermal springs, at high concentrations of metals, etc. Together with other microorganisms, cyanobacteria often form microbial mats, which are found in different latitudes – from Antarctic ice to continental hot springs. Also, these prokaryotes can be resistant to xerophilic conditions, forming endolytic communities in desert regions [11].

Thus, a unique feature of cyanobacteria is their ability to survive in extreme conditions, carry out photosynthesis with the participation of unusual pigments. They can fix nitrogen and release various compounds into the culture media, some with antineoplastic, antimicrobial, and antiviral effects [13].

Due to their economic efficiency and environmental sustainability, cyanobacteria can be used to produce biofuels, and can also potentially replace a significant part of traditional fossil fuels [14].

There are several aspects of cyanobacterial biofuel production that are of special interest to researchers around the world:

- Cyanobacteria are able to use water as an electron donor for oxygen photosynthesis;
- They can use a wide variety of water sources (freshwater, brackish water, wastewater and seawater) and have simple growth requirements to light, $CO_2$, inorganic nutrients;
- Cyanobacteria are capable of both producing and subsequently releasing molecular hydrogen. Therefore, mass cultivation of cyanobacteria for commercial hydrogen production can be quite efficient;
- They are non-food raw materials and their cultivation can be used in areas unsuitable for agricultural use.

Creation of a new technology for producing biohydrogen from cyanobacteria is of an urge and great interest in prospects of development of alternative energy on the global range. Biohydrogen production has been studied in a wide variety of cyanobacterial species and strains. The efficiency of biohydrogen production depends on the metabolic potential of microorganisms, which in turn depends on the type of cyanobacteria. To date, it is known that representatives of more than 14 genera of cyanobacteria produce hydrogen under various cultivation conditions. Table 1 shows a group of promising cyanobacteria in production of biohydrogen.

It is necessary to highlight some of them, for example: the unicellular non-nitrogen-fixing cyanobacteria *Gloeocapsa alpicola* demonstrated increased production of hydrogen under conditions of sulfur starvation [16]. In addition, it was found that *Arthrospira* can produce hydrogen (1 μmol/mg/dry cell mass/hour) under dark anaerobic conditions [17]. The hydrogen producing activity in representatives of the genus *Anabaena* has been well studied. For example, *Anabaena cylindrica* produced hydrogen and oxygen simultaneously in an argon atmosphere for 30 days under limited light conditions [18]. *Anabaena spp.* Is capable of

producing a significant amount of hydrogen and *Anabaena cylindrica* produces the highest amount of hydrogen under nitrogen deficiency conditions (30 ml/L culture/hour). Besides, *Cyanothece* 51142 is currently one of the most promising nitrogen-fixing cyanobacteria. Also, there is a complete metabolic model *Cyanothece* 51142 to assess the general theoretical ability of this organism to produce hydrogen [19]. There are some data on the production of hydrogen by halotolerant cyanobacteria, among which *Aphanothece halophytica* demonstrated good potential [20].

**Table 1** – Basic cyanobacteria promising in the production of biohydrogen. Table modified from [15]

| Strain | Characteristics of strain | The maximum $H_2$ yield |
|---|---|---|
| *Anabaena* variabilis 1403/4B | Heterocyst filamentous | 20 μmol $H_2$/mg chl/h |
| *Anabaena* variabilis ATCC 29413 | Heterocyst filamentous | 45.16 mmol $H_2$/mg chl/h |
| *Anabaena* variabilis AVM13 | Heterocyst filamentous | 68 mmol $H_2$/mg chl/h |
| *Anabaena* variabilis PK17R | Heterocyst filamentous | 59.18 mmol $H_2$/mg chl/h |
| *Aphanothece halophytica* | Non-nitrogen-fixing unicellular | 82.79 nmol $H_2$/mg dry wt/h |
| *Calothrix* membrnacea B-379 | Heterocyst filamentous | 0.108 mmol $H_2$/mg dry wt/h |
| *Chroococcidiopsis* thermalis CALU 758 | Non-nitrogen-fixing unicellular | 0.7 μmol $H_2$/mg chl/h |
| *Cyanothece* 51142 | Non-nitrogen-fixing unicellular, diazotrophic | 465 μmol $H_2$/mg chl/h |
| *Desertifilum* sp. IPPAS B-1220 | Nitrogen-fixing, filamentous | 0.348 μmol $H_2$/mg chl/h |
| *Gloebacter* PCC 7421 | Non-nitrogen-fixing unicellular | 1.38 mmol $H_2$/mg chl/h |
| *Gloeocapsa* alpicola CALU 743 | Non-nitrogen-fixing unicellular | 0.58 μmol $H_2$/mg protein |
| *Aphanocapsa* montana | Non-nitrogen-fixing unicellular | 0.40 μmol $H_2$/mg chl/h |
| *Microcoleus* chthonoplasts | Non-nitrogen-fixing filamentous | 1.7 nmol $H_2$/mg prot/h |
| *Mycrocystis* PCC 7806 | Non-nitrogen-fixing unicellular | 11.3 nmol $H_2$/mg prot/h |
| *Nostoc* muscorum IAM M-14 | Heterocyst filamentous | 0.60 mmol $H_2$/mg chl/h |
| *Oscillatoria* limosa strain 23 | Non-nitrogen-fixing filamentous | 19.83 μl $H_2$/mg chl/h |
| *Phormidium valderianum* BDU 20041 | Non-nitrogen-fixing filamentous | 0.2 μl $H_2$/mg dry wt//h |
| *Spirulina* strain | Non-nitrogen-fixing unicellular | 1.22 mol $H_2/m^3$/h |
| *Synechoccus* PCC 6301 | Non-nitrogen-fixing unicellular | 0.09 mmol $H_2$/mg chl/h |
| *Synechocystis* sp. PCC 6803 | Non-nitrogen-fixing unicellular | 300 nmol $H_2$/mg chl/h |

At the University of Helsinki from UHCC collection of cyanobacterial cultures (containing over 1,000 strains), the *Anabaena* PCC 7120 and *Nostoc punctiforme* ATCC 29133 strains were studied for their ability to release biohydrogen under various cultivation conditions: aerobic/light, aerobic/dark, microaerobic/light, anaerobic/dark. The highest rates of $H_2$ production were observed under microaerobic/light conditions [21].

It should be noted that the morphological features of cyanobacteria make them the most promising hydrogen producers. This is especially true for heterocyst forms of cyanobacteria, in which the processes of oxygen and hydrogen evolution are spatially separated [22].

Because of these features, heterocyst cyanobacteria are able to release hydrogen in the presence of molecular oxygen and carry out light-dependent hydrogen release (Figure 1).

The production of biohydrogen, as a by-product of microbial metabolism, is a relatively new area of technological development and a promising source of renewable energy. Since the possibility of such reactions in living organisms was demonstrated more than half a century ago, the process of biophotolysis carried out by cyanobacteria has been actively studied over the past 35 years [23]. During this time, the main molecular mechanisms of hydrogen formation by living systems were studied. Cyanobacteria can release protons and electrons in the process of splitting water using solar energy [24]. Hydrogen is formed by direct absorption of light and the transfer of electrons through two different enzymes, namely hydrogenase and nitrogenase.

Nitrogenase catalyzes the formation of hydrogen and simultaneously reduces nitrogen to ammonia. It is activated in heterocysts of filamentous cyanobacteria under conditions of low nitrogen content. Hydrogen is formed as a by-product. Hydrogenase catalyzes the simplest chemical reaction – reversible reduction of hydrogen from protons and electrons. Both of these enzymes are key cellular enzymes responsible for the biological production of hydrogen in cyanobacteria. The study showed that electrons and protons formed as a result of water splitting reactions are re-mixed with hydrogenase with the release of high-purity $H_2$ (up to 98%) [25].

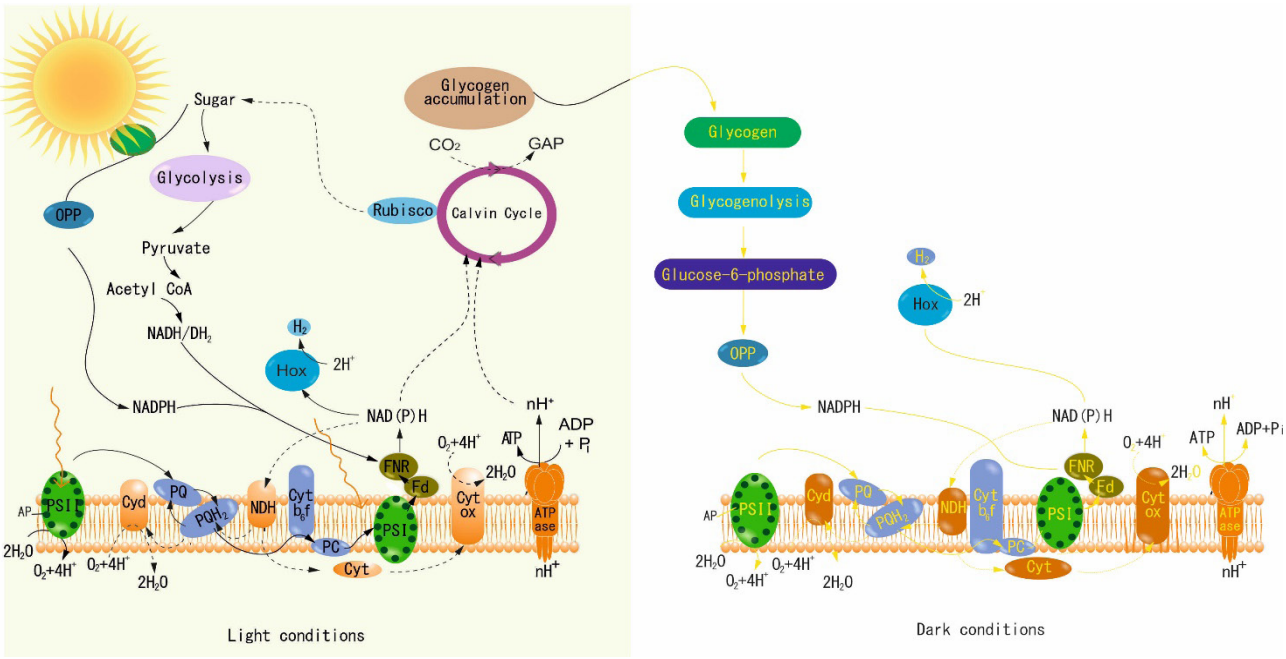


**Figure 1** – The mechanism of hydrogen release in cyanobacteria in the light and dark phases [8].

**Mechanism of hydrogen production. Photosynthesis and photosystem**

Photosynthesis is a sequence of biochemical reactions that convert sunlight into chemical energy. The fixation of $CO_2$ into organic compounds, such as carbohydrates, lipids and proteins through photosynthesis provides food for all living organisms on Earth. It basically consists of two types of reactions: (1) light and (2) dark [26]. In a light reaction, microorganisms absorb photons and produce adenosine triphosphate (ATP) – the main molecule that carries energy in cells, and nicotinamide adenine dinucleotide phosphate (NADPH) – the carrier of electrons.

These products are subsequently used in dark reactions, such as carbon fixation and hydrogen production. Hydrogen sulfide, sulfur in photosynthetic bacteria, and water in plants, algae, and cyanobacteria provide an electron that drives these reactions. When water is used as a source of electrons, oxygen is released as a by-product, and this process is known as oxygenic photosynthesis, whereas when oxygen is not produced as a by-product, it is known as anoxygenic photosynthesis. Photoautotrophic organisms like microalgae and cyanobacteria, as well as photoheterotrophic bacteria, have the capacity to absorb light energy stored as chemical energy through the formation of chemical bonds. The photosystem (PS) is considered as the key unit of the photosynthetic apparatus. It is an antenna complex containing tens or hundreds of carotenoids and chlorophylls that absorb light in the form of photons, and a reaction center composed of a highly specialized molecule called P680 that converts light into chemical energy. When a light particle hits one of the pigment antennas, it is excited and transfers the excited energy to the next antenna of the molecule with a lower excitation energy. The excitation energy will further lift the reaction center into an excited state, where it will transfer one electron from one chemical compound called a donor to another compound called an acceptor. In the reaction center, charge separation occurs, that is, the accumulation

of the excitation energy in an energy-rich chemical bond. When transporting energy from a photon to a reaction center, some energy is always lost, which must be paid for storing light energy [27].

All raw materials for the production of biohydrogen from cyanobacteria are displaced from their oxygenic photosynthesis (Figure 2).

Photosynthesis takes credit for converting solar energy into the chemical energy and ultimately plays an important role in the production of affordable biofuel materials. Cyanobacteria follow the following mechanisms in their photosynthesis, which are divided into two stages: light-dependent reactions, which occur in the thylakoids, and dark reactions (Calvin cycle), which occur in characteristic folds of the outer membrane.

The thylakoid contains a pair of photosystems called Photosystem I (PSI) and Photosystem II (PSII). Their responsibility is to work in tandem to generate the energy needed to produce carbohydrates in the Calvin cycle. The protein light harvesting complexes LCI and LCII embedded in the photosynthetic membrane are responsible for capturing sunlight. These proteins bind to a network of chlorophyll and carotenoid molecules that absorb photons of light. Within these molecules, the absorbed light energy excites the electrons to a higher state. Charged electrons are transferred from the PSII to the electron transport chain. At the same time, a photosynthetic water splitting reaction takes place in PSII, which converts water into protons, oxygen and electrons in order to compensate for the transported charged electrons. Thus, oxygen is a byproduct of photosynthesis. As electrons pass through the transport chain, the energy of the electron is used to pump hydrogen ions into the thylakoid to create a concentration gradient that stimulates ATP production. Hence the electrons entering the PSI are of low energy. In PSI, the process is repeated, when light hits the LCI, the electrons are recharged and passed through the transport chain, where the electron carrier NADPH is generated. In general, the main outputs of light-dependent reactions are ATP and NADPH. Dark reactions or the Calvin cycle are responsible for $CO_2$ fixation. This process uses ATP and NADPH, which have been produced by light reactions. The Calvin cycle consists of a series of reactions that reduce $CO_2$ to glyceraldehyde-3-phosphate (G3P). The cycle consists of three stages: carbon fixation, substrate recovery and regeneration. At the end of each cycle, one molecule of G3P is formed. They are then used to make glucose, fatty acids, or glycerin. Several types of biofuels can be obtained from these three compounds. In some photosynthetic microalgae species, protons and electrons extracted from water (or starch) in two photosystems PSII and PSI can pass through ATP synthase to the hydrogenase enzyme (HydA) to catalyze direct photoproduction of biohydrogen [28, 29].

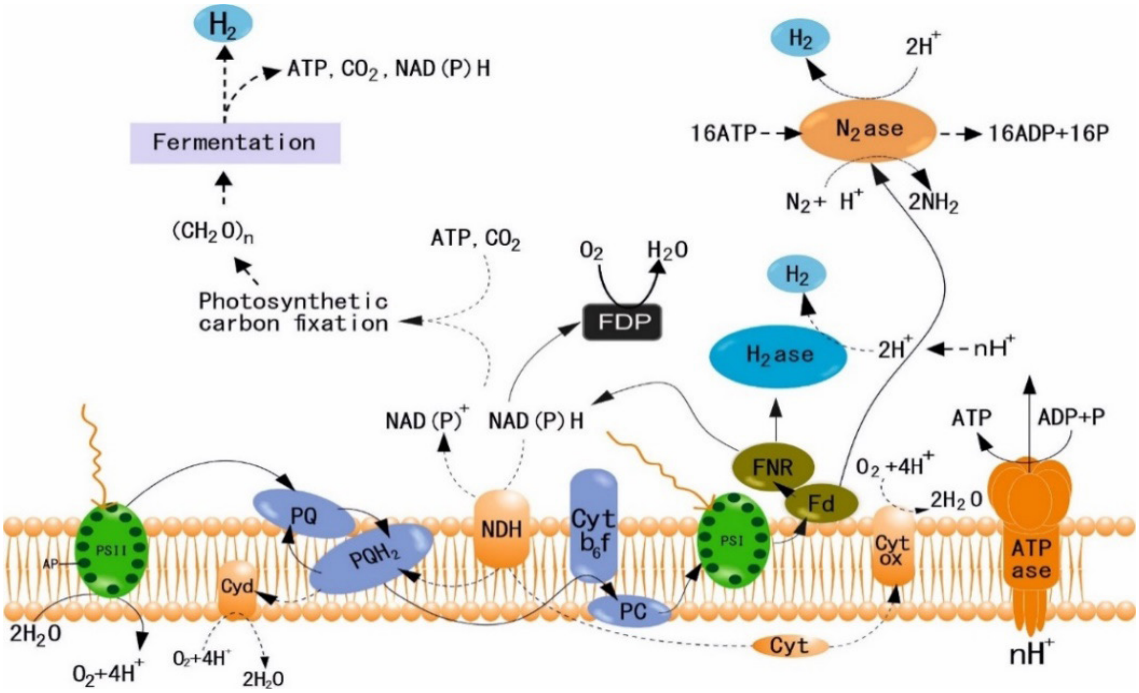


**Figure 2** – Role of Photosystem I and Photosystem II in photo production of biohydrogen [8].

Thus, cyanobacteria are promising biological systems for the conversion of solar energy. Two hydrogen enzymes (hydrogenase and nitrogenase) are key to this progress.

Direct biophotolysis is a process that uses solar energy to break down water into hydrogen and oxygen.

$$2H_2O + \text{solar energy} \rightarrow 2H_2 + O_2 \quad (1)$$

In this process, light energy is absorbed by photosystem II (PSII) or photosystem I (PSI) and forms electrons that are transferred to ferredoxin (FD). The hydrogenase then receives electrons from ferredoxin directly to produce hydrogen [30]. Hydrogenase is one of two general classes of enzymes that catalyze the oxidation of hydrogen to protons and the reduction of protons to hydrogen.

Indirect biophotolysis is known as a nitrogenase-based system. Nitrogenase is a catalyst in the production of hydrogen by reducing nitrogen to ammonia, as seen from reaction (2) [31].

$$N_2 + 8\,H^+ + 8e^- + 16\,ATP \rightarrow 2\,NH_3 + \\ + H_2 + 16\,ADP + 16\,Pi \quad (2)$$

In this process, hydrogen is generated in two successive stages. Photosynthesis for carbohydrate storage and dark fermentation of carbon storage for hydrogen production. The hydrogen formation reaction is illustrated in reactions (3) and (4).

$$12\,H_2O + 6\,CO_2 + \text{light energy} \rightarrow C_6H_{12}O_6 + O_2 \quad (3)$$

$$C_6H_{12}O_6 + 12\,H_2O + \\ + \text{light energy} \rightarrow 12\,H_2 + 6\,CO_2 \quad (4)$$

Thus, the formation of oxygen and hydrogen is spatially separated in time. This separation not only avoids the incompatibility of oxygen and hydrogen evolution, but also facilitates the purification of hydrogen, since $CO_2$ can be conveniently removed from a mixture of hydrogen and $CO_2$. According to the scheme, $CO_2$ is reduced to starch by photosynthesis in the daytime, and the resulting starch is fermented to gaseous hydrogen and organic acids under anaerobic conditions at night.

**Enzymes for biohydrogen production**

The two main hydrogen-producing enzymes are hydrogenases and nitrogenases. Hydrogenase is present in all three areas of life, namely archaea (methanogens and some extremophiles), eubacteria and eukaryotes (green algae). Hydrogenase is a metal enzyme that plays a vital role in energy exchange in various microbial communities [32]. Hydrogenases can be divided into two non-homologous classes based on their metal catalytic subunits – the first is Fe-hydrogenase, which contains only Fe in the active site, and the others are [Ni-Fe] hydrogenase and [Ni-Fe-Se] hydrogenase, which contain Ni, Fe, and sometimes Se [33]. They differ in their metal subunits, subunit composition, molecular weight, specificity of electron carriers, sensitivity to oxygen (Fe-Fe is more sensitive), cellular arrangement, and their physiological roles [34]. Some organisms containing hydrogenases produce hydrogen along with anaerobic energy metabolism. Sources of Fe-Fe hydrogenases are green algae, some anaerobes, protists, and fungi. The hydrogenase gene has been found in a variety of green algal species, including *C. reinhardtii, S. obliquus, C. moewusii,* and *Chlorella fusca*. Only metallic iron is present in Fe-Fe hydrogenases [35]. They contain an iron-sulfur center, which has carbon monoxide and cyanide at the active site of the enzyme. Fe-Fe hydrogenases have a higher turnover [36]. Ni-Fe hydrogenases are not phylogenetically associated with Fe-Fe hydrogenases and are more prevalent than Fe-Fe hydrogenases and are found in archaea, bacteria, and cyanobacteria. The simplest forms of Ni-Fe hydrogenases are heterodimeric in nature, along with complex forms consisting of five subunits of bidirectional hydrogenases that are found in cyanobacteria. These hydrogenases contain a catalytic site that is linked to a large subunit known as HoxH, which has Fe and Ni atoms attached to CO and CN ligands with S atoms in cysteine residues in the surrounding protein. The small subunit known as HoxY contains the [4Fe-4S] cluster, which is required for the transfer of electrons to the large subunit. At the catalytic site, three other subunits known as HoxE, HoxF, and HoxU form the diaphorase portion. Ni-Fe hydrogenases are oxygen tolerant and usually participate in hydrogen uptake reactions, but they can also generate hydrogen [37]. Nitrogenase is found only in prokaryotes and is an irreversible catalyst. Its natural function is to produce nitrogen and some hydrogen, with greater hydrogen production when nitrogen is deficient [38]. Nitrogenases in cyanobacteria are spatially separated by heterocysts, which provides anoxic conditions, since they lack PSII, which is responsible for oxygen production and, therefore, anaerobic hydrogen production occurs. This is a very energy consuming process [36]. Nitrogenase contains two subunits – nitrogenase reductase and dinitrogenase complex. The reductase subunit contains the Fe-S

protein and is encoded by the *nif* gene. It is a homodimer with a molecular weight of 65 kDa and transfers an electron from an external electron donor to the dinitrogenase complex. The Mo-Fe-S protein is present in the dinitrogenase complex, which is a heterotetramer containing α2β2 units and having a molecular weight of 230 kDa. It gradually reduces dinitrogen ($N_2$) to two ammonia molecules [34, 39]. At the same time, proton reduction to molecular hydrogen occurs. Based on metal cofactors in their catalytic centers, nitrogenases are classified as types of iron, molybdenum, and vanadium [40]. Different stoichiometries of ammonia and hydrogen production by different types of nitrogenases are presented as:

Mo-nitrogenase: $N_2$ þ 8Hþ þ 8e– →2$NH_3$ þ $H_2$

Fe-nitrogenase: $N_2$ þ 21Hþ þ 21e⁻ →2$NH_3$ þ 7:5$H_2$

V-nitrogenase: $N_2$ þ 12Hþ þ 12e⁻ →2$NH_3$ þ 3$H_2$

Some stages of electron transfer are endergonic, so ATP is required to produce ammonia and hydrogen. Compressed hydrogen is formed as a result of this irreversible and unidirectional process [37].

**Factors affecting the yield. Metabolic modulation in increasing the hydrogen yield**

Optimization of the cultivation conditions of selected cyanobacteria cultures-potential producers of hydrogen, including such parameters as the intensity of illumination, composition of the nutrient media, the pH value of the medium, etc. are of great importance. Thus, the study of the effect of oxygen stress, nitrogen, sulfide and phosphorus starvation on the physiological and biochemical properties of the cyanobacteria cell – producer of hydrogen, is one of the main points in improving the yield of biohydrogen.

Some environmental conditions, such as light, temperature, salinity, nutrient availability, and a gaseous atmosphere, play a role in the production of hydrogen. The requirements of different types of cyanobacteria are different for optimal hydrogen production.

*Light*. Although most cyanobacteria species absorb deep red light of about 680 nm [41], the need for light to produce hydrogen varies among different species of cyanobacteria. While spirulina (*Arthrospira platensis*) produces hydrogen under anaerobic conditions, both in the dark and in the light [42], some other species produce hydrogen only in the presence of the light [43]. Hydrogen production mediated by native hydrogenases in *Synechococcus* PCC7942 occurs in the dark under anaerobic conditions [44]. *Spirulina platensis* can optimally produce hydrogen at 32°C in a fully anaerobic and dark state [42]. The largest volume production of hydrogen was found in *Anabaena variabilis* ATCC 29413 and its mutant PK84 [42]. The production of hydrogen in *Nostoc muscorum* is catalyzed by nitrogenase; in this strain, more hydrogen is produced in the light than in the dark [45]. *Anabaena cylindrica* produces hydrogen in an argon atmosphere for 30 days under limited illumination (light intensity 6.0 W/$m^2$) and 18 days under increased illumination (light intensity 32 W/$m^2$) [46]. The continuous production of hydrogen by *Anabaena cylindrica* for a long period of time under conditions of limited illumination occurs in the absence of exogenous nitrogen [47]. The effect of light on nitrogenase-mediated hydrogen production in most cyanobacteria has been well studied [44]. The nitrogenase function is saturated only at a much higher light intensity than is required for optimal growth. Thus, the rate of hydrogen production can be doubled if the intensity of light exposure to crops is changed from 20 W/$m^2$ to 60 W/$m^2$ [47].

*Temperature.* The optimal temperature for hydrogen production for most cyanobacteria species is 30-40°C and varies from species to species. For example, *Nostoc* cultured at 22°C showed higher rates of hydrogen production than at 32°C [48], while *Nostoc muscorum* SPU004 showed optimal hydrogen production at 40°C [49]. *Anabaena variabilis* SPU 003, on the other hand, shows optimal hydrogen production at 30°C [50].

*Salinity.* Salinity does affect the production of hydrogen by cyanobacteria [45]. In general, fresh water cyanobacteria exhibit a lower rate of hydrogen formation with increasing salinity. This is probably due to the diversion of energy and reducing agents to extrude $Na^+$ ions from the cells or to prevent the influx of $Na^+$ [51].

*Trace elements.* Trace elements such as cobalt (Co), copper (Cu), molybdenum (Mo), zinc (Zn), and nickel (Ni) affect hydrogen production. Many of these metals have shown a marked increase in hydrogen production and are thought to be related to their involvement in the nitrogenase enzyme. For example, *Anabaena variabilis* SPU003 is highly sensitive to Co, Cu, Mn, Zn, Ni, and Fe ions and does not exhibit hydrogen formation at concentrations of these ions below 10 mm [52]. *Anabaena cylindrica* culture grown with 5.0 mg of iron ions per liter produces hydrogen at a rate about twice as fast as a culture with 0.5 mg of iron ions per liter [46].

*Carbon source.* Carbon sources are also known to have a significant effect on hydrogen production by affecting nitrogenase activity [49]. The presence

of different carbon sources leads to a change in the ability of cofactor compounds to give up electrons to nitrogenase, which affects the formation of hydrogen [49].

*Nitrogen source.* Several inorganic nitrogenous compounds greatly affect the rate of hydrogen production. Nitrites, nitrates, and ammonia have been reported to inhibit nitrogenase in *Anabaena variabilis* SPU003 and *Anabaena cylindrica* [53, 46]. As a rule, all exogenously added nitrogen sources inhibit nitrogenase synthesis [54]. Although in *Anabaena cylindrica* addition of ammonium (0.2 mM $NH_4^+$) at a given time ultimately suppresses the formation of hydrogen, but the periodic addition of smaller amounts (0.1 mm ammonium chloride) does not inhibit the release of hydrogen [46]. However, the influence of the nitrogen source is not always accompanied by pronounced effects, and its interpretation is not unambiguous. While some studies have shown that there are significant differences in hydrogen production depending on the nitrogen content in the medium [42], other studies show the opposite [55]. In the culture of *Anabaena cylindrica*, oxygen production prevails with the gradual addition of $NH_4Cl$ (from 0.1 mm to 0.5 mm) [56]. The ratio of hydrogen production to oxygen (4:1) under conditions of complete nitrogen starvation decreases (1.7:1) with addition of ammonium ions [56].

*Molecular nitrogen.* Molecular nitrogen is a competitive inhibitor of hydrogen production and removal of molecular nitrogen, if it is often very necessary for hydrogen production. The formation of hydrogen can be significantly inhibited in the presence of molecular nitrogen [55].

*Effect of oxygen on hydrogen production*. Due to their extreme sensitivity to oxygen, the photoevolution of hydrogen catalyzed by nitrogenases or hydrogenases can only function under anaerobic conditions. Since oxygen is a by-product of photosynthesis, nitrogenase-containing organisms have developed several spatial and temporal separation/compartmentalization strategies, as described above, to protect the enzyme from inactivation by oxygen [56].

*Effect of sulfur on hydrogen production*. Sulfur starvation increases the rate of hydrogen production in some species of cyanobacteria (e.g., *Gloeocapsa alpicola* and *Synechocystis* PCC 6803). It is possible to inhibit oxygen photosynthesis and enhance hydrogen production by incubating cynobacteria in sulfur-free nutrients [16]. Sulfur is a very important component in PSII recovery cycle as without sulfur, protein biosynthesis is severely disrupted, and the production of cysteine or methionine becomes impossible. This leads to the absence of the D1 protein (32-kDa protein of the reaction center), which is necessary for PSII and needs constant replacement [57]. For these reasons, when deprived of sulfur, photosynthesis and respiration are reduced even in the presence of light. Since photosynthesis decreases much faster than respiration, the equilibrium point is reached after some time (usually 22 hours), and after that the amount of oxygen that is used in respiration is greater than the oxygen released by photosynthesis, thus cell becomes anaerobic, at which point hydrogen production occurs in higher amounts, reaching its peak [16].

*Methane effect.* Increased hydrogen production (up to four times) is observed in *Gloeocapsa alpicola* and *Synechocystis* PCC 6803 during the dark anoxic incubation in the presence of methane and a pH of 5.0 to 5.5. The effect of methane on the release of hydrogen was maximal during the first hour of incubation, followed by a gradual decrease [16, 58].

**Table 2 –** Optimization of methods of $H_2$ production. Modified from [15].

| Methods | Notes | Amount | Strain | $H_2$ rate, μmol $H_2$/mg chl/h |
|---|---|---|---|---|
| Sulfur deprived | – | – | *Nostoc calcicola* | 4.27 |
| Micronutrients | Ferric ions | 5 mg/ | *Anabaena cylindrica* | 110 |
| External factors | pH | 7.5 | *Synechococcus* sp. Miami BG 043511 | 140 |
| | Light | 30 μmol $m^{-2}$ $s^{-1}$ | *Cyanothece* sp. strain ATCC 51142 | 300 |
| | Temperature | 23°C | *Nostoc* sp. PCC 7422 | 100 |
| Anoxia | Ar | 100% | *Anabaena cylindrica* B-629 | 320 |

The preparation of cyanobacteria cultures for fuel requires the metabolic modulation of cyanobacteria for enhanced hydrogen production.

To summarize the above, it should be noted that various metabolic approaches significantly increase the rate and duration of $H_2$ production by cyanobacteria (Table 3).

However, it is very difficult to detect certain concentrations of trace elements, simple organic compounds, or certain doses of other physical, chemical and physiological factors that equally affect the enzymatic activity of cyanobacteria and, accordingly, the production of biohydrogen.

**Table 3** – Basic approaches for increasing hydrogen production by cyanobacteria cells. Modified from [15].

| | | | |
|---|---|---|---|
| **$H_2$** | Metabolic approaches | Optimization of cultivation conditions | Anoxia |
| | | | Creating stressful conditions |
| | | | The use of exogenous electron donors |
| | | | The use of photosystem inhibitors |
| | | | Regulation of external factors |
| | Technological approaches | The construction of genetic mutants | Improving the characteristics of a photobioreactor to increase hydrogen production productivity |
| | | | Optimization of methods for separating hydrogen from impurities of other gases |
| | | | Immobilization of cyanobacterial cells |
| | | | The use of a cheap nutrient substrate (wastewater, waste etc.) |
| | | | Optimization of cultivation conditions of strains of cyanobacteria |
| | Genetic approaches | Improving the technological design of biofermentation of hydrogen producers | Obtaining mutants defective in hydrogen recycling. *HupSL, HydFS* |
| | | | *HoxEFUYH* mutants with oxygen – tolerant hydrogenase |
| | | | Mutants with low sensitivity of Mo-nitrogenase to ammonium ions |
| | | | *HetRFCLN patS* gene mutants responsible for the formation and suppressions of heterocysts |
| | | | Pigment reduced mutants |

**Genetic modulation in increasing the yield of biohydrogen**

One of the ways to optimize the process of biohydrogen uptake is based on the study of the genetic control of hydrogen metabolism and the use of genetic approaches to obtain effective producer strains. That is why the creation of genetic mutants to produce hydrogen is economically feasible. All genetic approaches to the modification of phototrophic microorganisms, including cyanobacteria, are aimed at increasing the yield of hydrogen by suppressing, introducing new or overexpressing existing genes. According to the literature data, it can be said that genetic manipulation of cyanobacteria is necessary to eliminate the following main problems:

1) the presence and activity of absorbing hydrogenase;

2) a decrease in the frequency of heterocyst formation;

3) competition for reducing agents, presence of other methods of assimilation;

4) low photochemical efficiency of light-collecting antennas.

As noted, the release of $H_2$ after genetic modification of several types of cyanobacteria was significantly higher compared to the wild type [59]. On the basis of the literature analysis, all the mutants can be divided into six main groups:

1) mutants defective in hydrogen recycling, *hupSL* and *hypF*;

2) mutants with low sensitivity of Mo-nitrogenase to ammonium ions;

3) mutants defective in the genes *hetRFCLN* and *patS* used for the formation and suppression of heterocysts;

4) mutants with oxygen-tolerant hydrogenase *hoxEFUYH*, etc.;

5) mutants with electron flow, reoriented to hydrogen metabolism;

and 6) mutants with a reduced pigment content.

When observing nitrogen exchange by fixing bacteria, it should be noted that the genetic approaches to increasing their productivity in the release of hydrogen are associated with the removal or inactivation of hydrogen-containing hydrogenases, with the creation of mutants that are insensitive to ammonia, and an artificial increase in the number of heterocysts in the filaments. It is known that during the nitrogen fixation, hydrogenase participates in the recirculation of hydrogen oxidized by nitrogenase. Considering and analyzing the metabolism of cyanobacteria, it can be argued that the removal of hydrogen-absorbing hydrogenase can lead to an increase in the yield of hydrogen. Genetic studies on this manipulation have been conducted for a long time, and they are described in many works. In particular, the genes of the large or small hydrogenase subunit HupSL are knocked out, as well as the regulator (HupR protein), which reduces the level of hydrogenase expression, thereby increasing the hydrogen yield [60].

To increase the production of biohydrogen by filamentous nitrogen-fixing cyanobacteria, mutant strains of *Anabaena variabilis* ATCC 29413, *Nostoc punctiforme* PCC 73102, and *Nosto*c PCC 7422 were obtained by chemical mutagenesis [61]. Interestingly, according to the results presented in these publications, the release of hydrogen by mutant strains under anaerobic conditions almost tripled. Since a high concentration of ammonium ions inhibits the process of hydrogen release by suppressing the synthesis of nitrogenase, which turns off the activity, an increase in the $H_2$ yield is possible due to a decrease in the sensitivity of nitrogenase to ammonium ions [62]. A similar effect can be achieved by point mutations of the *nifA* gene or by switching off one of the glutamine synthase genes, *glnA*, which makes the NifA protein insensitive to ammonium ions, as a result of which nitrogenase synthesis does not depend on the presence of ammonium in the nutrient medium. In addition, by acting on the genes responsible for the transport of ammonium into the cell, it was also possible to obtain mutants whose synthesis and nitrogenase activity did not depend on the concentration of ammonia in the medium.

Resulting mutant strains of cyanobacteria have shown advantages for cultivation in wastewater containing a huge concentration of ammonium; this further helps the environment by using downstream waste to create fuel using hydrogen producers. In addition, the active production of hydrogen by nitrogen-fixing cyanobacteria can also be increased by artificially increasing the number of heterocysts in the filaments, which leads to an increase in the concentration of nitrogenase. This can be achieved either by genetic engineering or by chemical treatment, for example, with 7-azatriptophan. Genetic tools can control the frequency of heterocyst formation if the key genes involved in heterocyst formation and nitrogen fixation are known, which is challenging because 600-1000 genes are specifically activated during heterocyst formation. The primary gene that controls the formation of heterocysts is the *hetR* gene, and the product of the *patS* and *hetN* genes regulates their repression. Genetic manipulations leading to overexpression of the *hetR* gene in *Anabaena* PCC 7120 caused an increase in the frequency of heterocyst formation by 29%, while nitrogen starvation reduced this value to 10% [63]. In addition, the *hetR*, *patS*, and *hetN* genes involved in the formation, maintenance, and regulation of heterocysts may be targets for genetic manipulations.

Thus, by increasing the expression of the *hglK* gene, it is possible to strengthen the glycolipid layer of the heterocyst, thereby minimizing its decay [64]. One of the most interesting genetic tools for increasing the hydrogen productivity of cyanobacteria is the production of mutant strains with a reduced content of pigments, that is, with a lower content of chlorophyll and phycobilin pigments. It is believed that in this case, when the photosynthetic apparatus of the cell absorbs less photons at high light intensity and therefore spends less photons. In addition, the transfer of an electron from water to $CO_2$ or $H_2$ requires only two photons, since two photosystems are involved in this transfer. That is why the creation of mutants with a reduced number of light-collecting pigments in the photosynthetic apparatus allows for a 10% increase in the efficiency of converting solar energy into carbohydrates, from which hydrogen fuel can be obtained [65].

According to Bernat et al., the use of the antenna-deficient mutant strain *Synechocystis* sp. PCC 6803 suggests that up to 200 ml of $H_2$ per hour can be obtained from 1 L of culture with continuous cultivation. They also noted that in a mutant without a phycobilisome, the linear electron flow rate was 5.5 times higher than in the wild-type strain, while the cyclic electron flow rate remained the same for both strains. An increase in the linear velocity of the electron flow is a prerequisite for increasing the release of $H_2$ under photoautotrophic conditions [66]. An important genetic approach for increasing the hydrogen productivity of cyanobacteria is to solve the problem of oxygen sensitivity of hydrogenase.

There is much research on the development of an $O_2$-tolerant hydrogenase enzyme for the sustained

release of $H_2$ in cyanobacteria. In addition, research is underway to obtain new recombinant strains of cyanobacteria that carry acid-resistant hydrogenases. The integration of the necessary structural and auxiliary genes was responsible for the synthesis of the *Thiocaspa roseoperscina* hydrogenase in the *Synechococcus elongatus* PCC 7942 chromosome [67]. The introduction of structural genes encoding clostridial Fe hydrogenase into cyanobacteria *Synechococcus elongatus* PCC 7942 and their subsequent transcription and translation into a functional hydrogen-producing enzyme of a photosynthetic microorganism is of interest and deserves further study.

Thus, an increase in hydrogen production can be achieved by reducing or eliminating competing biochemical pathways. Consequently, each of the described genetic approaches deserves special attention, and in the course of solving many fundamental problems, new opportunities have opened up for improving the technology for producing biohydrogen. Despite the fact that modern technologies do not allow us to successfully overcome all existing problems, the development of genetic engineering methods allows us to manipulate the metabolism of cyanobacteria, which opens up new prospects for improving the efficiency of biological hydrogen production.

**Assessment of the profitability of hydrogen energy and prospects for the future**

The production of hydrogen from cyanobacteria provides high-purity energy, since the conversion of sunlight to hydrogen is much less energy-intensive than methods of converting it to other fuels. The production cycle contains almost no waste, while raw materials can be grown on land use. After the production of biofuels, the spent cyanobacteria biomass can be used as an animal feed. The profitability of this type of business is high, because the demand for biofuels is growing day by day. The economic production of biohydrogen can be improved by improving its production technology. Various complex approaches of genetic and metabolic engineering will increase the productivity of cyanobacteria [69].

Compared to other mechanisms, such as oil refining, coal gasification, fossil fuels, and thermochemical methods, photobiological hydrogen production has many advantages, since the previously mentioned approaches are inherently dangerous and create environmental chaos. For this reason, photobiological hydrogen production can be considered as a competent mechanism for the production of pure hydrogen. Nevertheless, in addition to the advantages, it also has some disadvantages, mentioned below.

The introduction of any new bioenergy technologies requires a technical and economic analysis of the benefits of innovation, an assessment of the cost of the investment project and its impact, and an analysis of the payback period of the project.

Among all energy sources, biohydrogen, as an almost inexhaustible renewable energy source, takes an active position and participates in the process of forming the future model of the world energy system. Also an important driver of bioenergy policy is the growing awareness of the impact of fossil fuels on global climate change. Most countries are aware of the need to take measures to reduce greenhouse gas emissions, and many of them support the use of energy derived from alternative renewable biological sources as a key element of climate change mitigation [42]. An important aspect of the development of bioenergy is also the desire to reduce the level of air pollution in cities. The use of biohydrogen implies a significant reduction in toxic emissions into the atmosphere compared to the use of fossil fuels in electricity production, heating and transport. All of the above determines the impact of various policy aspects on the profitability of biohydrogen production in many sectors, including agriculture, energy, transport, the environment and trade. In the case of the production of biohydrogen by cyanobacteria, despite the prospects for photobiological production of hydrogen by their cells, it should be noted that there are difficulties in its production and use on a large scale [35].

Currently, the level of hydrogen production by cyanobacteria is insufficient for its commercial production as a competitive energy carrier, so its production today in most countries of the world will have a higher cost than traditional fuel. The key problem is the low rate of hydrogen production from cyanobacteria. This factor can become a significant obstacle to the development of photobiological production of hydrogen as a fuel, and therefore requires an in-depth analysis of the processes of increasing the productivity of hydrogen by cyanobacteria and the correct allocation of subsidies and investments. Thus, first of all, incentives for the production of bioenergy should be introduced, and it is also necessary to develop and implement comprehensive programs for the development of biotechnologies, in which bioenergy plays a significant role. There is no doubt that the development of this bioenergy sector will take quite a long time, but many analysts believe that

the development of hydrogen energy can meet the growing needs of the world's population for fuel, along with environmental safety [60]. Therefore, in the case of the production of biohydrogen by cyanobacteria cells, the main subsidies and investments should now be directed primarily to their basic scientific research. For the successful production of hydrogen by cyanobacteria, it is necessary to study additional aspects of improving the producers using the genetic and metabolic approaches described above. At the same time, the development of genetic engineering, high-performance technologies and advances in systems biology using genome-wide tools make it possible to create strains of cyanobacteria with the desired genotype and the required hydrogen characteristics [65].

At-present, an approach to the production of hydrogen from cyanobacteria by direct and indirect biophotolysis is being considered, since it is still a theoretical method, it is quite difficult to assess the cost-effectiveness of this method in comparison with the already developed methods for producing hydrogen in the energy market. The main costs and investments in this method are required for the design and maintenance of photobioreactors for the cultivation of cyanobacteria, as well as for various engineering works for the separation, processing, storage and transportation of gases. Since water and solar energy are used as renewable sources, the cultivation of cyanobacteria does not require expensive nutrient media and even involves the use of wastewater for their cultivation. This is why this technology is significantly cheaper in addition to providing cheap energy while treating waste at the same time. In addition, the absence of greenhouse gas emissions and the reduction of environmental pollution also exclude the capital costs of their restoration. The following conclusions can be drawn (Figure 3).

Thus, the photobiological production of $H_2$ requires an increase in the metabolic capabilities of hydrogen producers based on an integrated approach.

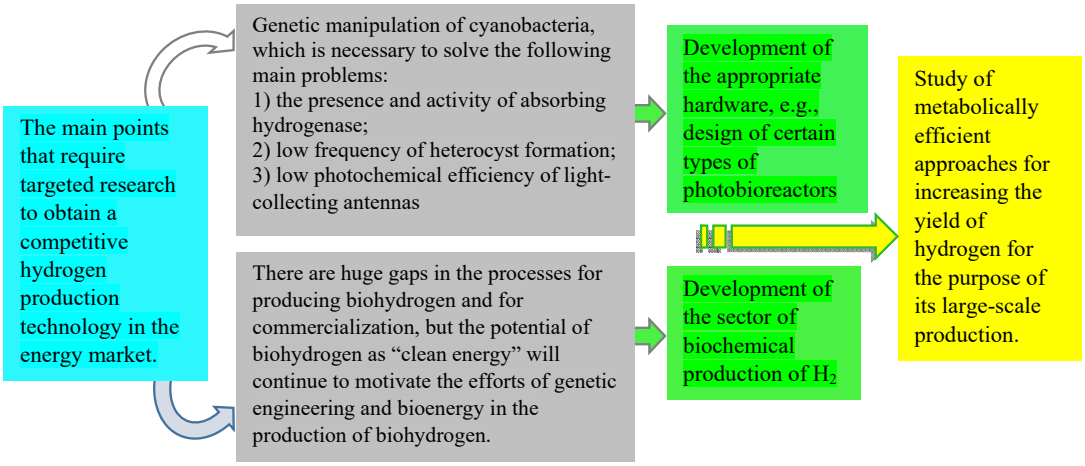


**Figure 3** – The main points that require targeted research to obtain a competitive biohydrogen production technology on the energy market.

**Advantages of photobiological hydrogen production**

This process uses microorganisms to convert solar energy into hydrogen. Basically, photosynthetic microorganisms require clean and transparent technologies along with cheap sources of energy, which means that sunlight is compared to the electrochemical production of hydrogen, which depends on the splitting of water molecules. Therefore, they use sunlight and water only as renewable energy sources. The conversion efficiency for producing biohydrogen from sunlight is quite high, i.e. about 10-16%. Compared to a fossil fuel system, the production of biohydrogen using sunlight is a cheap source. Because they use renewable energy sources, they do not release polluting gases and toxic compounds into the environment. In addition, this process leads to the formation of pure and clean hydrogen [23].

Green algae, cyanobacteria, and photosynthetic bacteria are ubiquitous and have easy growth conditions, when placed under appropriate artificial conditions, and are mostly non-harmful to the environment. Thus, these microorganisms can be easily grown to achieve the appropriate goal [65].

There are several photosynthetic bacteria that use broad-spectrum light energy and organic waste. During the production of hydrogen in an anaerobic environment, the corresponding metabolites are formed, such as lactic, butyric and acetic acids, considered as by-products.

**Disadvantages of photobiological production of hydrogen**

Hydrogenase activity is inactivated when an oxygen molecule is present in microorganisms. The simultaneous production of oxygen and hydrogen in green algae disrupts the hydrogenase activity of oxygen. When considering the uptake of hydrogenase by cyanobacteria and photosynthetic bacteria, a slight decrease in hydrogen production is observed [64].

The release of hydrogen from the photofermentation process slows it down. The exact metabolic pathway of hydrogen production by microorganisms is still unclear. In addition, there is no clear opponent for a reliable, industrially capable microorganism that can be metabolically engineered to increase the rate of hydrogen production.

Growing green algae and cyanobacteria in large quantities is quite a challenge, as it requires a large surface area. In addition, the yield of hydrogen production from these microorganisms is low. Photosynthetic bacteria do not have sufficient capacity to produce more hydrogen and, therefore, will not be able to cope or meet the social requirements.

The scaling strategies and materials needed to create suitable photobioreactors are expensive. According to a number of researchers, the production of hydrogen by direct biophotolysis, provided that the total capital cost of the process is $135/m$^2$.

Storing hydrogen is very expensive, as it is stored in a compressed form. Cryo-compressed hydrogen storage and liquid hydrogen storage are alternative mature approaches where hydrogen is liquidized at −253°C and compressed into vessels that can be pressurized to 250–350 atm. Accordingly, the size of liquid hydrogen requires larger tanks reaching to about 3 times larger than the current used gasoline tank [60]. Moreover, liquid hydrogen requires well-insulated and expensive cryogenic storage vessels to prevent boil-off and maximize dormancy and to maintain the temperature below to 20 K. Unluckily, the gas-liquid transformation process is extremely expensive, consuming approximately ~25%–30% of the energy content of the stored hydrogen [67]. Thus, employing traditional storage approaches in real future applications may be difficult due to their high cost and safety issues.

**Conclusion**

Analysis of the literature data shows that in recent years, various aspects of cyanobacteria application as a source of biohydrogen have become the subject of a number of scientific studies under the influence of the increasing interest in the development of new sources of renewable energy. The prospects for the development of hydrogen energy based on cyanobacteria can be improved by a comprehensive approach to increasing the productivity of hydrogen by cyanobacteria cells. The use of cyanobacteria as possible producers of hydrogen is particularly relevant and profitable, since they form hydrogen due to the conversion of solar energy and do not require complex, expensive nutrient media. In addition, after the production of biofuels, the spent biomass can be used as animal feed. The attention of researchers is focused on the study of the main processes of cyanobacteria photosynthesis; on the processes of biophotolysis of photosynthetic microorganisms; on enzymes involved in the metabolism of hydrogen, which is very promising from the point of view of obtaining fundamental knowledge and their practical application in biotechnology. Most studies focus on using different metabolic strategies to increase the hydrogen yield in different strains of cyanobacteria.

Our laboratory is currently working on the isolation of heterocystic nitrogen-fixing cyanobacteria cultures from natural sources, as well as on the study of their morphological and cultural properties for use in biotechnology. Four non-heterocystic strains of cyanobacteria producing $H_2$ in light and dark processes were studied. The greatest accumulation of $H_2$ was observed in *Synechocystis* sp. PCC 6803 in the dark condition. The photoautotrophic yield of $H_2$ in the strain *Desertifilum* sp. IPPAS B1220 was 0.229 mmol/mg Hcl/h, which was increased by 1.5 times using DCMU inhibitor. This was the first data on the $H_2$-producing strain of *Desertifilum* [28]. The obtained scientific results indicate the promising use, practical significance and the need for further study of the isolated cyanobacteria as biosystems capable of efficiently converting light energy into the chemical energy of hydrogen. A lot of information is given about increasing the hydrogen productivity of cyanobacteria strains by genetic engineering methods. Despite the high theoretical prospects of this technology, there are serious limitations that prevent its introduction into production. The combined efforts of scientists and engineers with the political support of the state and high investment determine the future prospects of this sector of biohydrogen energy based on cyanobacteria.

**Acknowledgements**

This work was written with the support of the grant AP09260785 "Development of technology for producing biohydrogen based on promising strains of cyanobacteria for the production of biofuels" (2021-2023) and grant AP08052481 "Development of technology for producing biodiesel based on active microalgae strains" (2020-2022) funded by the Ministry of Education and Science, Republic of Kazakhstan.

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