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Article *in* International Journal of Hydrogen Energy · December 2019

DOI: 10.1016/j.ijhydene.2019.11.164

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Determination of the potential of cyanobacterial strains for hydrogen production

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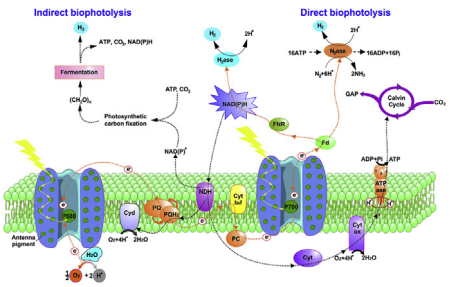
HIGHLIGHTS

- Four non-heterocystous cyanobacterial strains were investigated for H_2 production.
- Best H_2 accumulation was observed with *Synechocystis* sp. PCC 6803 in the dark.
- Photoautotrophic H_2 production yield of *Desertifilum* sp. IPPAS B-1220 was 0.229 μmol/mg Chl/h.
- H_2 yield was increased with the *Desertifilum* sp. IPPAS B-1220 by 1.5-fold in the presence of DCMU.

GRAPHICAL ABSTRACT



Abbreviations: Ar, argon gas; BG_0-11, nitrogen-deprived medium; Chl, chlorophyll *a*+*b*; DCMU, 3-(3,4-dichlorophenyl)-1, 1-dimethylurea; Fd, ferredoxin; FNR, Fd:NADP(H) oxidoreductase; NADP, nicotinamide adenine dinucleotide phosphate; NADPH, nicotinamide adenine dinucleotide phosphate hydrogen; GC, gas chromatography; PET, photosynthetic electron transport; PFR, pyruvate ferredoxin oxidoreductase; PSI, photosystem I; PSII, photosystem II; H_2, hydrogen; UV, ultraviolet; CET, cyclic electron transport.

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https://doi.org/10.1016/j.ijhydene.2019.11.164

Please cite this article as: Kossalbayev BD et al., Determination of the potential of cyanobacterial strains for hydrogen production, International Journal of Hydrogen Energy, https://doi.org/10.1016/j.ijhydene.2019.11.164

ARTICLE INFO

Article history:
Received 24 September 2019
Received in revised form
19 November 2019
Accepted 21 November 2019
Available online xxx

Keywords:
Biohydrogen
Cyanobacteria
Direct biophotolysis
Indirect biophotolysis
DCMU

ABSTRACT

Hydrogen (H_2) is a renewable, abundant, and nonpolluting source of energy. Photosynthetic organisms capture sunlight very efficiently and convert it into organic molecules. Cyanobacteria produce H_2 by breaking down organic compounds and water. In this study, biological H_2 was produced from various strains of cyanobacteria. Moreover, H_2 accumulation by *Synechocystis* sp. PCC 6803 was as high as 0.037 μmol/mg Chl/h within 120 h in the dark. The wild-type, filamentous, non-heterocystous cyanobacterium *Desertifilum sp.* IPPAS B-1220 was found to produce a maximum of 0.229 μmol/mg Chl/h in the gas phase within 166 h in the light, which was on par with the maximum yield reported in the literature. DCMU at 10 μM increased H_2 production by *Desertifilum* sp. IPPAS B-1220 by 1.5-fold to 0.348 μmol H_2/mg Chl/h. This is the first report on the capability of *Desertifilum* cyanobacterium to produce H_2.

Introduction

The growth of energy consumption and environmental protection are two main trends that have driven the search for new sources of energy [1]. Millions of years ago, cellular organic material formed by biochemical reactions played key roles in the formation of coal, oil, and natural gas [2]. Currently, due to advances in biotechnology, many living organisms can also be considered as raw materials for new, cheaper, and eco-friendly energy sources, especially various types of biofuels [3].

Among the microorganisms capable of releasing hydrogen, photosynthetic cyanobacteria and microalgae have received the most attention [4–7]. As it is well known, that these microorganisms can produce H_2 in the light. Cyanobacteria and microalgae have two photosystems, PS II participates in water cleavage and further oxygen release [8,9]. Under normal conditions, photosynthetic microorganisms do not produce H_2, as the energy created by photosynthesis is used for metabolism [10]. Enzymatic system of hydrogen metabolism and its regulation in cyanobacteria include two groups of enzymes: nitrogenases and hydrogenases. Nitrogenases catalyze hydrogen production simultaneously with the reduction of nitrogen to ammonia. Hydrogenases catalyze the simplest chemical reaction – reversible hydrogen reduction from protons and electrons. They are necessary for the release of excess reducing agent during the transition from anaerobic darkness to light conditions. It should be noted that enzyme characterized by high sensitivity to oxygen and it requires aerobic or anaerobic conditions for functioning. In this case hydrogen is formed until concentration of released oxygen have reached the value at which the total inactivation of hydrogenase occurs. Moreover, despite the fact that the quantum yield of H_2 released by phototrophic microorganisms under optimal conditions is close to the theoretical maximum, the toxic effect of oxygen significantly limits the duration of H_2 production. Exclusion of O_2, which forms as a byproduct of PSII and H_2O oxidation, is necessary for gene expression coding hydrogenase synthesis, and it provides sustainable light dependent hydrogen release [11].

Cyanobacteria, like green algae, have attracted much attention of researchers in the production of hydrogen by biophotolysis [12]. Biophotolysis is a process at which water dissociates to oxygen and molecular hydrogen in biological systems under the light condition. Photosynthetic microorganisms, such as cyanobacteria and microalgae, are capable of oxygenic photosynthesis [13–16]. During photosynthesis, chlorophyll pigments in thylakoid membrane of photosynthetic organisms absorb light and produce molecular oxygen. Then, carbon dioxide is converted to triose phosphate in enzymatic reactions with the help of ATP and NADPH [17]. Cyanobacteria are physiologically and morphologically diverse and have the capacity to produce H_2 *via* direct and indirect biophotolysis (Fig. 1) [18]. As it is known, the process of direct biophotolysis involves the use of light energy absorbed by photosynthetic apparatus for the water cleavage with oxygen formation and for generation of low-potential reducing agents followed by protons reduction and hydrogen formation. During direct biophotolysis reducing agent produced by photosynthesis, ferredoxin or NADPH reduce hydrogenase directly. A significant disadvantage of the process is the high sensitivity of hydrogenases to oxygen. The efficiency of conversion of light energy to hydrogen is low, and this indicator can be increased with the continuous removal of oxygen from the medium [19].

Indirect biophotolysis is a type of direct process at which water cleavage and further ferredoxin reduction are used for carbon dioxide fixation and resulted reduced carbohydrate compound could be used for stimulation of hydrogen release in separate reaction. This process, proceeding in two stages separates the stages of oxygen and hydrogen evolution, both in space and in time in order to avoid oxygen inhibition.

According to published articles, *Oscillatoria* sp. 23 [24] and *Oscillatoria chalybea* [25] evolved H_2 photoproduction *via* direct biophotolysis, and *Anabaena* sp. UTEX 1448 synthesizes a large amount of H_2 by direct and indirect biophotolysis for 12 h [26]. According to Touloupakis et al. [27], immobilization of *Synechocystis* sp. PCC 6803 in the process of indirect biophotolysis actively produced H_2 at a maximum rate of 40.6 ± 4.9 mmol/mg Chl/h.

However, it should be noted that despite significant advances in the study of direct and indirect biophotolysis processes in recent years, there are still difficulties limiting the

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use of cyanobacteria for the conversion of solar energy in such ways [28]. In addition, to solving the problems associated with the simultaneous release of oxygen and H_2, thus ensuring a high sensitivity of the process to oxygen, it is necessary to conduct fundamental research related to increasing the efficiency of hydrogen evolution by photosynthetic cultures and increasing the reaction rate of this process.

In addition to the simultaneous release of oxygen and H_2, the sensitivity of this process to oxygen is primarily due to its low efficiency and speed. In this regard, research aimed at increasing the rate of H_2 release by photosynthetic microorganisms is currently very relevant [29,30]. Generally, studies in this field should be aimed at finding new and more productive strains of photosynthetic microorganisms that actively produce H_2 and optimizing the cultivation process in order to increase the efficiency of substrate transformation into H_2 [31–33]. Most importantly, an approach is needed to improve the efficiency and stability of H_2 production by cyanobacteria [34–36].

In this regard, the aim of this study was to identify new strains of cyanobacteria that actively produce H_2 and to optimize the cultivation conditions to improve the efficiency of light-dependent H_2 production. This article presents the results of the selection, identification, and capabilities of four strains of cyanobacteria H_2 production (in the dark and light) and the effects of the photosynthesis inhibitor 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) on H_2 photoproduction by cyanobacteria in the light.

Methods and materials

Strain isolation and characterization

The experiments were performed with four cyanobacteria strains – *Synechocystis* sp. PCC 6803, *Desertifilum* sp. IPPAS B-1220, *Synechococcus* sp. I12, and *Phormidium corium* B-26. *Synechocystis* sp. PCC 6803 was taken from Tomo Laboratory (Tokyo University of Science, Japan). *Synechocystis* sp. PCC 6803 was the first unicellular cyanobacteria to be completely sequenced [37,38] and has been used as a model organism for H_2 production. *Desertifilum* sp. IPPAS B-1220 is a filamentous cyanobacterium that was isolated from Lake Shar-Nuur, a freshwater lake in Bayan-Olgiy Aymag (Mongolia) [39]. *Synechococcus* sp. I12 was isolated from Hot spring Turgen (Almaty, Kazakhstan), and morphological and cultural properties of this strains were described in Userbayeva et al. [40]. The filamentous cyanobacterium *Phormidium corium* B-26 was isolated from Sorbulak lake (Almaty, Kazakhstan).

Culture condition

The cyanobacteria strains were cultivated under artificial light (intensity: 45 μmol photons/m^2/s) supplied from three sides of glass tubes containing 70 mL of liquid BG-11 growth medium and aerated using an SPP-25GA air pump (Techno Takatsuki, Osaka, Japan).

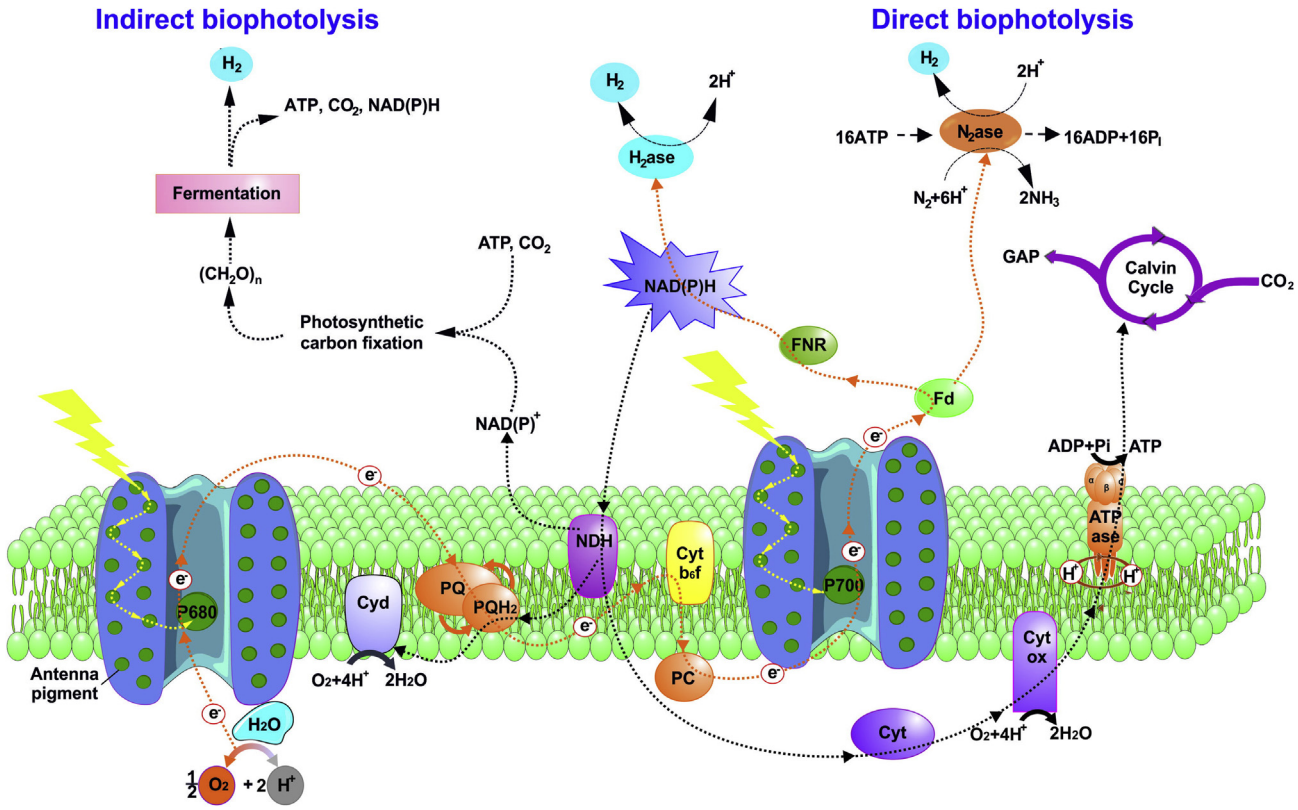

Fig. 1 – Direct and indirect biophotolysis processes of photosynthetic microorganisms Abbreviation: Cyd, cytochrome *bd* quinol oxidase; PQH_2/PQ, plastoquinol/plastoquinone; ATPase, ATP synthase; Cyt b_6f, cytochrome b_6f complex; Fd, ferredoxin; FNR, ferredoxin NAD(P) reductase; H_2ase, hydrogenase; NDH, NAD(P)H dehydrogenase; PC, plastocyanin; PQ, plastoquinones; P680, Photosystem II; P700, Photosystem I; N_2ase, nitrogenase; H_2, hydrogen. This view was modified from previous articles [19–23].

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Cell preparation for H_2 production

The cell cultures were transferred to 40-mL tubes, which were centrifuged for 5 min at 10,000×*g*. After discarding the supernatant, 100 mL of BG_0-11 medium was added to the cell cultures and mixed for 3 min. Pre-cultured cells were adjusted to an optical density at 730 nm (OD_{730}) of 0.4, as determined with a spectrophotometer (V-630; JASCO International Co., Ltd., Tokyo, Japan). The cultures were incubated for 24 h under artificial light (intensity: 30 μmol photons/m^2/s) supplied from one side and collected by centrifugation (Himac CR 22G high-speed refrigerated centrifuge; Hitachi Co., Ltd., Tokyo, Japan) at 10,000×*g* for 5 min. After discarding the supernatant, 30 mL of BG_0-11 medium was added to the cells, and the tube was centrifuged for 5 min at 10,000×*g*. After washing twice, the cells were collected and concentrated to OD_{730} of 1.5 in 7.5 mL of BG_0-11 medium modified using 50 mM HEPES-KOH (pH 7.4) and 100 mM $NaHCO_3$ in a gas chromatography (GC) vial (leaving 10-mL headspace for gases). This procedure was modified from a method reported by Schutz et al. [41].

Argon gas was injected to the GC vial using a GC syringe for 1 h to replace the oxygen and placed under light or dark conditions at room temperature. For the light procedure, the GC vial was placed under continuous light (intensity: 30 μmol photons/m^2/s) supplied from one side and shaken using microstirring bars and a HS-10VA vial stirrer (AS ONE International, Inc., Santa Clara, CA, USA) at 150 rpm. For the dark procedure, the GC vial was covered with foil and cultivated in a BioShaker BR-22FP (Taitec Corporation, Saitama, Japan) at 25 °C.

H_2 measurement

The accumulated molecular H_2 gas was measured by GC (3210; 2-m stainless column packed with a 5A molecular sieve; 60/80 mesh; GL Sciences, Inc., Tokyo, Japan), in accordance with the manufacturer's instructions. Injector and column were at 80 °C and the detector at 120 °C. A gas-tight syringe (Hamilton Company, Reno, NV, USA) was used to directly withdraw 0.15 mL of the gaseous headspace, which was injected, into the gas chromatograph. A hydrogen standard curve was used to calculate moles of hydrogen from the reported peak areas. During this hydrogen production phase of the experiment, temperature was maintained at 25.0 ± 0.5 °C, with an initial pH of 7.4. H_2 production is presented as μmol H_2/mg Chl/h. Ar was used as a carrier gas. Statistical analysis data on growth, chlorophyll *a*, and H_2 was examined by one-way analysis of variance (ANOVA) with confidence level of 96%.

Light intensity measurement

At various times during the culture period, the light intensity (μmol photons/m^2/s) was measured at five locations (i.e., at the four corners and in the middle) in front of and behind each Quantum Q 40555 bottle using a LI-250A Light Meter (LI-COR Biosciences, Lincoln, NE, USA).

Chl measurement

A 1-mL aliquot of each sample was collected in a 1.5-mL tube and centrifuged using a SS-1500X high-speed, refrigerated microcentrifuge (Sakuma Seisakusho, Tokyo, Japan) at 10,000×*g* for 5 min. After discarding the supernatant, 1 mL of 100% methanol was added to the cell culture, and the tube was centrifuged at 10,000×*g* for 5 min. The supernatant was used for absorbance measurements at 665.2 and 750 nm, with 100% methanol as a reference.

Addition of DCMU

Before exposure to the anaerobic condition, 0.046 g DCMU was dissolved in 5 mL of dimethyl sulfoxide and concentrated to 10–45 μM, which was added to the GC vials using a syringe. After adding the DCMU, the cell cultures were shaken for 30 min in order to mix the DCMU into the suspension [42].

Results

Determination of H_2 release by the cyanobacteria cultures

Since representatives of cyanobacteria possess different nitrogenases and hydrogenases, the ways of metabolism leading to hydrogen production in various species are different [43,44]. Certain types of cyanobacteria can synthesize both nitrogenase and hydrogenase [45]. It is known that the release of hydrogen by heterocystic cyanobacteria due to the action of nitrogenase is considered the most promising, since oxygen-sensitive proton reduction reactions are separated in space from oxygen-producing photosynthesis [46]. However, an energy-efficient process for producing biohydrogen is the production of hydrogen based on cyanobacteria that do not form heterocysts, since it is known that the formation of heterocysts is accompanied by the consumption of a huge amount of energy [47]. However, the most studies focus on hydrogen production involving nitrogenase of cyanobacteria containing heterocysts. Information about the production of hydrogen with the non-heterocystic strains of cyanobacteria using the nitrogenase and hydrogenase enzymes is not enough [48]. In order to find more productive strains of phototrophic microorganisms actively producing hydrogen, three strains of cyanobacteria that do not form heterocysts were studied: *Desertifilum* sp. IPPAS B-1220, *Synechococcus* sp. I12, *Phormidium corium* B-26. The strain of cyanobacteria *Synechocystis* sp. PCC 6803 was chosen as a positive control, strain that actively produces hydrogen due to the hydrogenase enzyme.

During preliminary study of nitrogenase activity, it was revealed that the strains *Synechococcus* sp. I12 and *Phormidium corium* B-26 did not provide nitrogen-fixing activity. The strain *Desertifilum* sp. IPPAS B-1220 contains nitrogenase and hydrogenase [39]. All four strains present an interest during the search of prospective producer of hydrogen without a huge energy consumption, as in the case of nitrogen fixing heterocystic cultures.

The results showed that all of the studied strains released H_2 in the dark. The highest productivity was noted with the control strain *Synechocystis* sp. PCC 6803. Cells of this strain started to release H_2 in the dark during 24 h and 48 h after replacement of free space with argon (Fig. 2).

The yield of H_2 to this time made up 0.007 μmol H_2/mg Chl/h. The maximum accumulation of hydrogen by this culture

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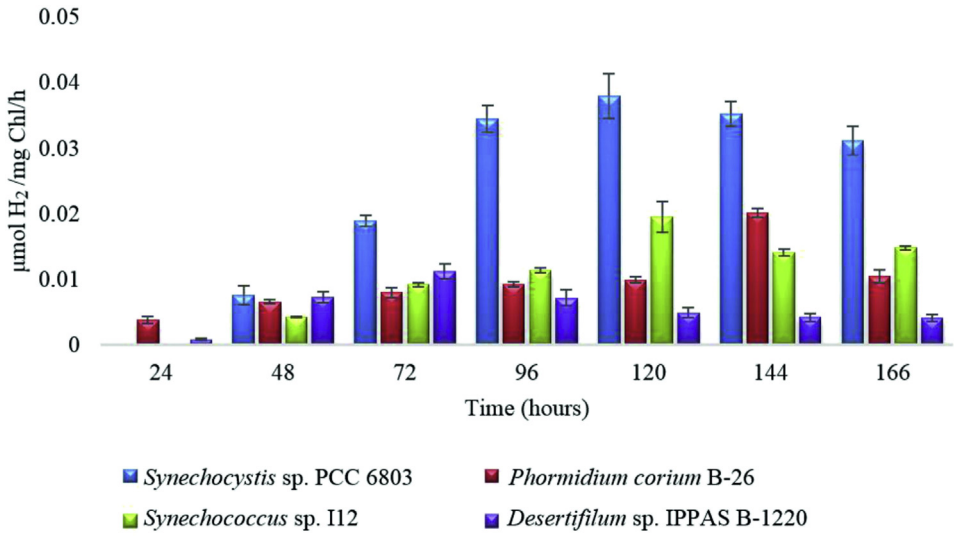

Fig. 2 – Hydrogen release by investigated cyanobacteria strain in anaerobic dark conditions.

was noted after 120 h of incubation and amounted to 0.037 μmol H_2/mg Chl/h, in the next hours of the experiment, a slow decrease in hydrogen evolution is observed, which is naturally associated with the depletion of intracellular substrates, in particular glycogen which limits the rate of fermentation.

The studied experimental strains had lower H_2 productivity in the dark, compared with the control strain. Among the experimental strains, *Phormidium corium* B-26 and *Synechococcus* sp. I12 produced the greatest amounts of H_2. These two strains were characterized by similar amounts of H_2 release, but differed in the maximum amount of accumulated H_2. The accumulation of H_2 by *Phormidium corium* B-26 cells after 24 h was 0.003 μmol H_2/mg Chl/h, which increased to a maximum of 0.02 μmol H_2/mg Chl/h after 144 h. The maximum H_2 release of *Synechococcus* sp. I12 was detected after 120 h of incubation (0.019 μmol H_2/mg Chl/h). Among the studied cyanobacteria strains, *Desertifilum* sp. IPPAS B-1220 released the smallest amount of H_2 in the dark (maximum H_2 productivity = 0.011 μmol H_2/mg Chl/h). However, it should be noted that this value was observed after 72 h of incubation. Afterward, H_2 release had gradually decreased.

Next, H_2 accumulation by cyanobacteria strains under light conditions was investigated. Light energy is important for the release of H_2 and acts as an electron donor for direct biophotolysis [25]. Direct biophotolysis is widely used by cyanobacteria to cleave water into H^+ and O_2 with the use of light energy. When the energy of light is absorbed by the cells of cyanobacteria, it enhances the oxidation of H_2O molecules in PSII, the released protons are used to generate ATP, and the electrons are transported to chloroplast-ferredoxin via PSI. Fd plays the role of an electron donor for [FeFe]-hydrogenases, which helps to restore H^+ to H_2 molecule [49]. In this experiment, the studied species of cyanobacteria were cultivated similarly to the previous experiment, the conditions of cell incubation in the study of hydrogen productivity were the

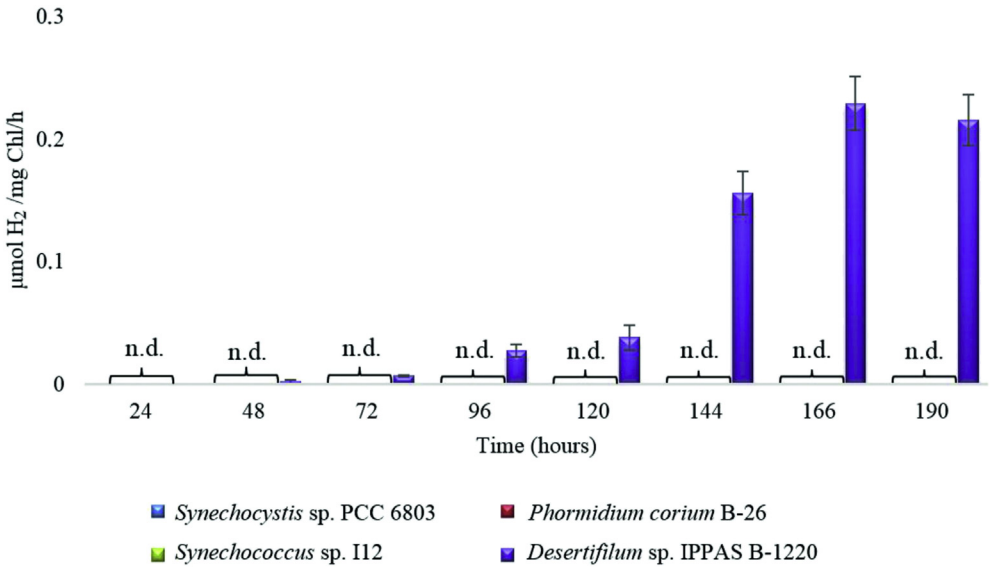

Fig. 3 – Hydrogen release by investigated cyanobacteria strains in anaerobic light conditions (n.d. – not detected).

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same and differed only in the presence of lighting. In the result of conducted experiment, it was established that the only one producer of hydrogen on the light was strain *Desertifilum* sp. IPPAS B-1220.

Hydrogen production by *Desertifilum* sp. IPPAS B-1220 cells was observed on the second day after establishment of anaerobic light conditions (Fig. 3). In the first day cells did not produce hydrogen. The active hydrogen release was reserved during 6 days, and it was decreased. The highest values of hydrogen accumulation noted after 166 h and made up 0.229 μmol H_2/mg Chl/h.

It should be noted that during screening under light conditions, the other three strains, including the control strain, produced no H_2, even though H_2 accumulation was relatively high in the dark. H_2 release by *Desertifilum* sp. IPPAS B-1220 was 20-fold greater in the light than in the dark and was sixfold greater than the control strain *Synechocystis* sp. PCC 6803. Thus, among the studied strains, *Desertifilum* sp. IPPAS B-1220 was prioritized due to its ability to produce H_2 in the light.

Influence of DCMU on H_2 release by the investigated cyanobacteria strains

Next, the effect of the DCMU inhibitor of photosynthesis in the light was investigated as a factor influencing the photosynthetic processes in cyanobacteria cells that contributes to H_2 release. The structure of DCMU, as an inhibitor of electron transport, is most similar to the structure of reduced plastoquinone, which explains the strong binding of the molecules in the quinone-binding pocket in the PSII reaction center.

DCMU was employed to inhibit the activity of the photosystem and create anaerobic conditions favorable for the production of molecular H_2. DCMU was added to the suspension of cyanobacterial cultures once to create an anaerobic state, after which the effect of its influence on the accumulation of hydrogen was analyzed every 24 h for 3 days.

According to obtained results in all investigated strains the maximum yield of H_2 at DCMU 10 μM and 30 μM concentrations was observed after 24–48 h after experiment when oxygen in the medium were depleted, then in the following days the decrease of hydrogen concentration was observed (Fig. 4).

The investigated cyanobacteria strains produced H_2 at 10 μM DCMU. Under these conditions, the H_2 yield of the control strain *Synechocystis* sp. PCC 6803 after 24 h was 0.0013 μmol H_2/mg Chl/h, and after 48–72 h, the yield was 0.001–0.0009 μmol H_2/mg Chl/h. The H_2 yield of *Phormidium corium* B-26 cells was 0.003 μmol H_2/mg Chl/h after 24 h, which decreased to 0.0016–0.0007 μmol H_2/mg Chl/h after 48 h. Similar data were obtained for the experimental strain *Synechococcus* sp. I12 (H_2 accumulation of 0.001 μmol H_2/mg Chl/h after 24 h). Afterward, the H_2 concentration also decreased. At this inhibitor concentration, *Desertifilum* sp. IPPAS B-1220 had the highest H_2 accumulation.

Thus, after 48 h, photoproduction of H_2 was 0.348 μmol H_2/mg Chl/h, which decreased to 0.308 μmol H_2/mg Chl/h after 72 h. It should be noted that higher values were expected than the H_2 productivity of the same strain under light conditions without a photosynthesis inhibitor.

In addition, despite the short-term (2 days) stimulatory effect of this photosynthesis inhibitor on H_2 accumulation, longer

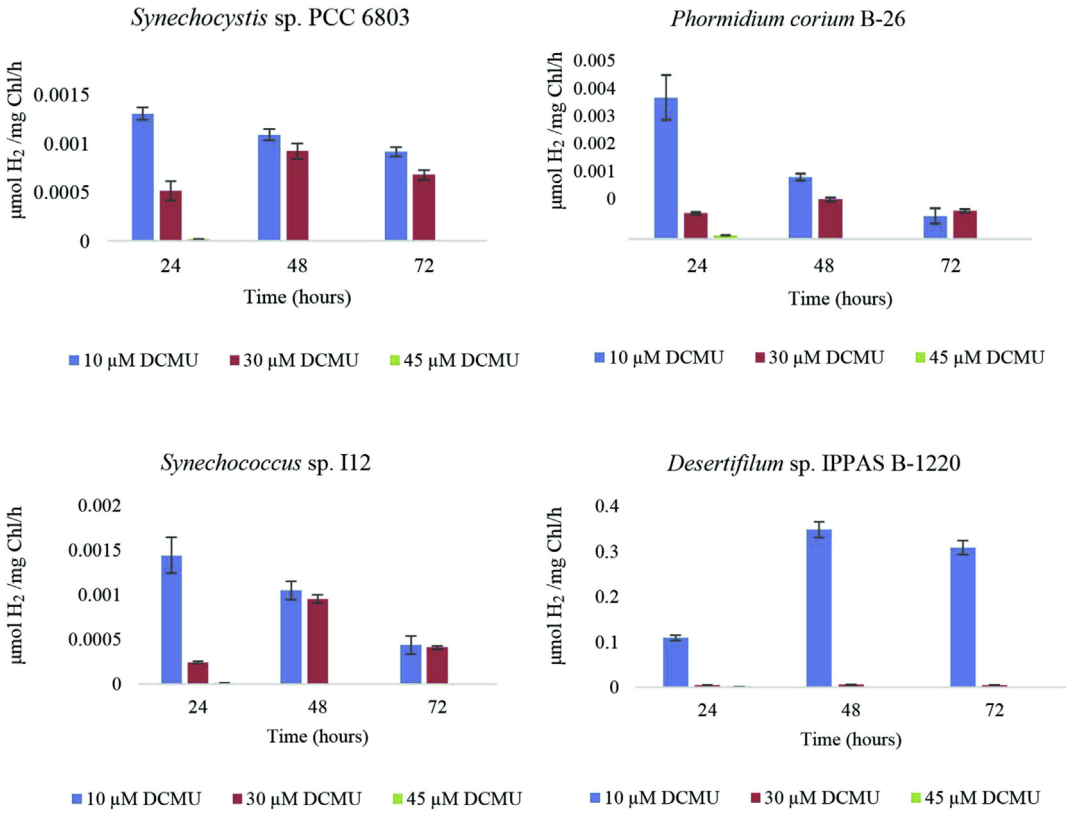

Fig. 4 – Influence of different DCMU concentrations (10, 30, 45 μM) on molecular hydrogen accumulation by cells of various cyanobacteria.

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exposure resulted in a decrease in the photoproduction of molecular H_2. The inhibitory action of DCMU on H_2 photoproduction is probably due to the suppression of the PSII-dependent pathway of H_2 formation due to its toxic effect. As a result, oxygen is released into the medium, which additionally inhibits the photoproduction of H_2. This is evidenced by the accumulation of three gases (i.e., oxygen, hydrogen, and nitrogen) in the medium of *Desertifilum* sp. IPPAS B-1220 under anaerobic conditions with 10 μM of DCMU. During the first 2 days after the addition of 10 μM DCMU under anaerobic conditions, *Desertifilum* sp. IPPAS B-1220 began to actively produce H_2, whereas on day 3, the release of H_2 had decreased, and there was a noticeable increase in the production of oxygen and nitrogen.

The increase of photosynthesis DCMU inhibitor concentration up to 30 μM and 45 μM in the medium did not influenced positively on photoproduction of hydrogen by cyanobacteria cells. So, at a concentration of DCMU 30 μM, significantly lower values of the maximum hydrogen output by cyanobacteria cells were obtained (Fig. 4).

An increase in the concentration of DCMU of up to 30 and 45 μM had not a positive effect on H_2 production by cyanobacteria. Thus, DCMU at a concentration of 30 μM had significantly lowered the maximum yield of H_2 produced by cyanobacteria.

Under these conditions, the maximum H_2 yield by *Desertifilum* sp. IPPAS B-1220 was 0.005 μmol H_2/mg Chl/h, which was 61-fold lower than that of the same strain under the influence of 10 μM DCMU. The remaining cyanobacteria strains showed varying amounts of molecular H_2 released at a given concentration of the photosynthesis inhibitor. However, as a pattern common to all of the studied strains, the maximum H_2 yield was significantly lower at a given concentration of DCMU. Thus, the maximum H_2 accumulation for the control strain *Synechocystis* sp. PCC 6803 was 0.0009 μmol H_2/mg Chl/h after 2 days. For *Phormidium corium* B-26 and *Synechococcus* sp. I12, these values were 0.001–0.0009 μmol H_2/mg Chl/h, respectively.

At 45 μM DCMU, hydrogen releasing was not observed in all studied strains. Thus, the optimum DCMU concentration for H_2 production was 10 μM. It was established that DCMU stimulates the "photoyield" of H_2 by cyanobacteria cells during the first 2 days, and then reverse effects are observed.

Discussion

H_2 can be used as a clean energy source for the high-efficiency production of electricity. Cyanobacteria can be used as a potential source of H_2 energy due to the ability of these organisms to split water into H_2 and oxygen using solar energy [50]. In addition, a number of microalgae are capable of enzymatically releasing hydrogen in the dark, catalyzed by either nitrogenase or hydrogenase. Such production is considered more efficient and less energy intensive. However, at present, H_2 energy from cyanobacteria is less economically viable due to high costs. In this regard, the efficient production of biohydrogen from cyanobacteria can be accelerated with the latest technological advances in metabolic and genetic engineering [11,51]. Also, strains characterized by high H_2 productivity could be used for this purpose.

This article presents the results of the study of H_2 release by various cyanobacteria strains, which are unable to fix molecular nitrogen. To identify prospective producers of H_2, three strains of cyanobacteria (i.e., *Desertifilum* sp. IPPAS B-1220, *Synechococcus* sp. I12, and *Phormidium corium* B-26) were investigated. Strain *Synechocystis* sp. PCC 6803 was chosen for the study as a positive control that actively produces H_2. The H_2 accumulation by cyanobacteria strains were studied under dark and light conditions.

For the photoproduction of H_2 by cyanobacteria, light is fundamentally important for two processes: 1) electron transfer from the photosynthetic electron transport centers to hydrogenase and 2) oxygen release to PSII, which influences hydrogenase activities and the rate of photoinhibition. Previous studies reported that *Oscillatoria limosa* strain 23 isolated from the ocean produced H_2 by direct biophotolysis [52]. Moreover, *Phormidium valderianum* strain BDU 20041 synthesized a large amount of H_2 by direct and indirect biophotolysis over the period of several hours [53]. According to Burrows et al. [61], during the process of indirect biophotolysis, non-nitrogen-fixing *Synechocystis* sp. PCC 6803 had actively produced H_2 at a maximum rate of 18.4 μmol H_2/mg Chl/h.

According to obtained data, all investigated cyanobacteria strains to some extent released hydrogen in anaerobic dark conditions while the highest hydrogen productivity was observed in the wild-type strain *Synechocystis* sp. PCC 6803 used as a control. It is known that along with light-dependent hydrogen release due to the action of nitrogenase, cyanobacteria are capable of hydrogen release under anaerobic conditions in the dark during fermentation of both exogenous and sugars stored during photosynthesis [41]. Thus, interpreting our findings, we can assume that the level of glycogen reserve and hydrogenase activity in the cells after 24 h of incubation were sufficient for H_2 production under dark anaerobic conditions.

It is assumed that the hydrogen release in this case is ensured by the action of Hox-hydrogenase in the dark after photosynthetic accumulation of glycogen. So, since in the process of balanced growth in the light, cyanobacteria use light energy to synthesize biomass and accumulate a small amount of reserve substances, the studied cultures were grown under conditions of nitrogen deficiency in order to direct photosynthesis mainly to the accumulation of endogenous glycogen.

Subsequent fermentation of glycogen in the dark significantly increases the yield of hydrogen by cyanobacteria cells. Similar data were obtained by a number of researchers [4], where the unicellular non-fixing nitrogen *Gloeocapsa alpicola* during growing under conditions of nitrate deficiency accumulated glycogen during the light period up to 40–50% of the weight of dry biomass, whereas at a normal nitrate concentration the glycogen content did not exceed 10% [54]. During subsequent fermentation in the dark, about 4 mol of H_2, 2 mol of carbon dioxide and 2 mol of acetate were released per mole of glucose.

Despite the fact that the separation of the photosynthesis process with the formation of oxygen and the synthesis of reserve substances in time from the process of hydrogen release which is free from oxygen may seem like a promising way to convert solar energy, it is nevertheless necessary to

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note its low efficiency which requires additional further research for increase the speed of these processes.

Active hydrogen production in anaerobic light conditions was noticed only in wild type strain *Desertifilum* sp. IPPAS B-1220. Moreover, it should be noted that the strain *Desertifilum* sp. IPPAS B-1220 under the light released 20 times more hydrogen than in the dark, and 6 times more hydrogen comparing to control strain *Synechocystis* sp. PCC 6803. Hydrogen production by the cells of *Desertifilum* sp. IPPAS B-1220 was observed on the second day after establishment of anaerobic light conditions. In view of the fact that *Desertifilum* sp. IPPAS B-1220 contains nitrogenase and hydrogenase, the most probable explanation for hydrogen production under anaerobic conditions in the light is the induction of nitrogenase in cells during the transition from normal cultivation conditions to a nitrogen-free medium. According to literature data, it is known that several unicellular cyanobacteria such *Synechocystis* sp. strain RF-1, *Synechococcus* sp strain Miami BG43511, *Gloethece* sp. strain ATCC51142 nitrogenase is active during cultivation in alternating light-dark mode, where the processes of photosynthesis and respiration are under circadian control [20]. The circadian rhythm is manifested in the fact that the maximum activity of nitrogen fixation, respiration and photosynthesis are repeated at a cyclic interval, thus preventing oxygen inhibition of nitrogenase. A number of cyanobacteria have the ability to release hydrogen, catalyzed in the light by nitrogenase and hydrogenase in the dark. It is reported that in unicellular cyanobacteria *Cyanothece* sp. Miami BG 043511 [55] the dark, oxygen-free formation of H_2 occurs through hydrogenase using a reducing agent from glycolytic carbohydrate catabolism as a result of autofermentation, and H_2 photoproduction occurs through nitrogenase and requires PSI lighting. According to obtained results, the strain *Cyanothece* sp. Miami BG 043511 is able to maintain a lower level of intracellular oxygen in the dark, possibly by the rhythm of the circadian cycle. Thus, they are supposed that it could be connected with higher level of respiratory metabolism of this strain, or the special properties of its enzyme complex, in particular the resistance of nitrogenase to oxygen, which allows it to produce H_2 in response to visible light [55]. Thus, among investigated strains *Desertifilum* sp. IPPAS B-1220 by ability to produce hydrogen considered as priority. The strain has the ability to produce hydrogen in an argon atmosphere in the dark, presumably due to hydrogenase, and it is capable of producing light-induced hydrogen production through nitrogenase. It should be noted that strains of *Desertifilum* species have not been previously studied by the productivity of molecular hydrogen. These species are close to the species *Oscillatoria*. Undoubtedly, the obtained results require additional research.

Table 1 shows the results of the analysis of the data of previous studies regarding H_2 productivity by various cyanobacteria.

In addition to the selection of active producers of molecular H_2, an important factor in this direction is scientific research aimed at increasing the activity of H_2 production by various cyanobacteria. Therefore, optimizing the cultivation of cyanobacteria is necessary to increase the conversion efficiency of the substrate into H_2. Hence, the identification of various cellular factors that stimulate the release of H_2 is relevant and necessary. In this regard, we studied the effect of different concentrations of the photosynthesis inhibitor DCMU (10, 30, 45 μM) under illumination as a factor contributing to the release of H_2 in order to stimulate H_2 release.

DCMU is one of the widely used inhibitors of electron transport, the molecular structure of which is most similar to the structure of once reduced plastoquinone, which explains the strong binding of these molecules to the quinone-binding site in the PSII reaction center [68]. The use of DCMU is aimed at inhibiting the activity of this photosystem and creating anaerobic conditions favorable for the production of molecular hydrogen. Acting as quinone analogue DCMU binds to QB PSII and blocks electron transfer between primary and secondary electron acceptors, which interrupts the photosynthetic transport chain of electrons during photosynthesis and, thus, reduces the formation of oxygen from the splitting of water molecules through PSII.

However, taking into account the fact that the action of electron transport inhibitors is specific enough for each type of cyanobacteria and can cause growth inhibition of the cyanobacterial culture, it is necessary to choose the inhibitor concentration that is optimal for increasing hydrogen release with minimal effect on the growth parameters of the culture.

According to our results, concentration of DCMU 10 μM concentration of DCMU at 10 μM has a stimulating effect on the accumulation of hydrogen. As expected, the yield of molecular hydrogen in the presence of an inhibitor is higher than the hydrogen productivity of the same strain, achieved under lighting conditions in the absence of its effect.

Our results are consistent with data from other researchers showing an increase in hydrogen production by cyanobacterial cells under the influence of DCMU [47,68,69]. The same result of DCMU inhibition of photosynthetic electron transport system, with a subsequent increase in hydrogen productivity, was obtained in cyanobacteria *Anabaena* siamensis TISTR 8012 [44]. According to their results, the highest H_2 production was found in the nitrogen-fixing culture *Anabaena* siamensis TISTR 8012, with addition of DCMU under the light condition. However, it should be noted that the concentration of DCMU used by them was much higher and amounted to 50 μM, what may be due to the properties of particular species of cyanobacteria. The observed H_2 production rate was 22 mmol H_2/mg Chl/h, which is almost four times higher than in control cells.

Cournac et al. [54] reported about higher H_2 production by cells of unicellular cyanobacteria *Synechocystis* sp. PCC 6803 at presence of 75 μM of DCMU in dark, anaerobic conditions.

In our investigations, photoproduction of molecular hydrogen increased by cell inhibitor treatment was observed at 10 μM concentration of DCMU on hydrogen production. Thus, it should be noted that all of the studied strains released higher amounts of H_2, compared with untreated cyanobacteria cells. However, increased concentrations of DCMU and the subsequent prolonged effect decreased the photoproduction of molecular H_2, which was possibly related to the toxic effects of the inhibitor. At a DCMU concentration of 30 μM, the maximum yield of H_2 decreased. The addition of 45 μM DCMU to the cell suspension resulted in almost full suppression of H_2 photoformation of all studied strains. This effect indicates the strict need for photosynthetic electron transport in the region

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Table 1 – Comparison of H_2 production by cyanobacteria strains.

Organism name	Organism description	Maximum H_2 evolution	Growth condition	H_2 evolution assay condition	H_2 enzymes	References
Cyanothece sp. ATCC 51142	nitrogen fixing, unicellular	465 μmol/mg Chl/h	air; ASP2 medium, 30 °C (12 h light, 12 h dark), light intensity of 30 μE m^2/s	Ar (100%); 30 μE m^2/s	N_2ase, H_2ase	[55]
Cyanothece sp. Miami BG 043511	nitrogen fixing, unicellular	15.8 mL/L	air; ASP2 medium, 30 °C (12 h light, 12 h dark), light intensity of 30 μE m^2/s	Ar (100%); 30 μE m^2/s	N_2ase, H_2ase	
Cyanothece sp. strain ATCC 51142	nitrogen fixing, unicellular	300 μmol/mg Chl/h	ASP2 medium with shaking at 125 rpm at 30 °C, under cool-white fluorescent illumination of 30 μE m^2/s, 50 mM glycerol	Ca^{+2} reduced medium with enhanced iron and at pH-7.4	N_2ase, H_2ase	[56]
Synechocystis strain M55	insertional mutagenesis, engineered genes is ndhB	200 nmol/mg Chl/min	air; photon fluence rate of 50 μE m^2/s, 30 °C	Ar (100%); 2.5 mM NH_4Cl and 5 mM Hepes;	H_2ase	[57]
Synechocystis sp. PCC 6803	ctaI/cyd insertion mutagenesis	190 nmol/mg Chl/min	air; modified Allen's medium at 34 °C, 70 μmol of photons m^2/s^1	Ar (100%); 300 μmol of photons m^2/s	H_2ase	[54]
Synechocystis sp. PCC 6803	ctaI/cyd insertional mutagenesis	115 nmol/mg Chl/min	air; cultured at 28 °C; 50 μE m^2/s photon fluence rate	Ar (100%); 800 μE m^2/s photon fluence rate	H_2ase	[58]
Synechocystis sp. PCC 6803	Insertional mutagenesis is narB/nirA	300 nmol/mg Chl/h	air; 25–40 mmol of photons/m^2/s	Ar; darkness; nitrogen deprivation	H_2ase	[59]
Synechocystis PCC 6308	non-nitrogen fixing	3.1 μl/mg Chl/h	air; 20 μE/m^2/s photon fluence rate	Ar with CO (13.4 μmol); 20 −30 μE/m^2/s photon fluence rate	H_2ase	[60]
Synechocystis PCC 6714	non-nitrogen fixing	1.67 μl/mg Chl/h	air; 20 μE/m^2/s photon fluence rate	Ar with CO (13,4 μmol); 20 −30 μE/m^2/s photon fluence rate	H_2ase	[60]
Synechocystis sp. PCC 6803	non-nitrogen fixing	18.4 μl/mg Chl/h	air; 90 μE/m^2/s photon fluence rate	Ar; BG-11 medium, pH 7.8; 0.36 mM N_2, 30 °C, 90 μE/m^2/s	H_2ase	[61]
Oscillatoria sp. Miami BG7	filamentous marine	5.9 μl/mg dry wt/h	air; NH_4Cl used as combined nitrogen source; 100 μE/m^2/s photon fluence rate	Ar (100%); 90 μE/m^2; 11 day old cells cultivated at 37 °C	H_2ase	[62]
Oscillatoria limosa strain 23	filamentous marine	19.83 μl/mg Chl/h	air; the nitrate-free medium; 1.2 klux photon fluence rate	Ar (100%); dark anaerobic incubation	H_2ase	[52]
Oscillatoria sp. Miami BG7	filamentous non-heterocystous	260 μmol/mg Chl/h	air; photon fluence rate 30 −50 μE/m^2/s	Ar; 2 × 30 W fluorescent lamp	H_2ase	[63]
Synechococcus sp. Miami BG 043511	nitrogen fixing, unicellular	220 μmol/mg Chl/h	air; 150 μE/m^2/s light intensity, 30 °C	Ar (100%); medium without Na_2CO_3 (pH-7.6), 150 μE/m^2/s, 30 °C	N_2ase, H_2ase	[64]
Synechococcus sp. Miami BG 043511	nitrogen fixing, unicellular	0.5 mL/per ml cell/12 h	air; 150 μE/m^2/s light intensity, 30 °C	Ar (100%); 150 μE/m^2/s, 30 °C	N_2ase, H_2ase	[65]

(*continued on next page*)

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Table 1 – (continued)

Organism name	Organism description	Maximum H_2 evolution	Growth condition	H_2 evolution assay condition	H_2 enzymes	References
Synechococcus PCC 6830	non-nitrogen fixing	6.2 μmol/mg Chl/h	air; 20 μE/m^2/s photon fluence rate	Ar with CO (13.4 μmol), C_2H_2 (1.34 μmol); darkness	H_2ase	[60]
Synechococcus sp. Miami BG 043511	non-nitrogen-fixing, unicellular	140 μmol/mg Chl/h	air; combined nitrogen limited medium; photon fluence rate 200 μE/m^2/s.	Ar; 25 W/m^2 (PAR)	H_2ase	[65]
Synechococcus PCC 602	non-nitrogen fixing, unicellular	15.77 μl/mg Chl/h	air; 20 μE/m^2/s photon fluence rate	Ar with CO (13.4 μmol); photon fluence rate 20 −30 μE/m^2/s	H_2ase	[60]
Synechococcus PCC7942	Gene overexpression, hydA from *Clostridium acetobutylicum*	162.52 μmol/mg Chl/h	air; photon fluence rate 20 −50 μmol of photons m^2/s	Ar; dark adaptation	H_2ase	[66]
Synechococcus PCC 6307	Non-nitrogen-fixing, unicellular	0.47 μl/mg Chl/h	air; 20 μE/m^2/s photon fluence rate	Ar (100%); 20−30 μE/m^2/s photon fluence rate	H_2ase	[60]
Phormidium valderianum BDU 20041	filamentous, non-heterocystous, marine cyanobacterium	0.2 μl/mg dry wt/h	air; cultured at 27 °C; 20 μmol of photons m^2/s	Ar; under 5.5 pmol photon m/s; 18 h dark-6 h light cycle	H_2ase	[53]
Phormidium valderianum BDU 20041	filamentous, non-heterocystous marine cyanobacterium	0.22 μl/mg dry wt/h	air; 30 W/m^2 under fluorescent light	Ar; light/dark cycle	H_2ase	[67]

between the plastoquinone (PQ) pool and ferredoxins (Fd) for the photoproduction of H_2.

In general, a slight residual H_2 release in cells treated with a higher concentration and prolonged exposure to the inhibitor may be associated with the reverse recovery of Fd from NAD(P)H with a high content of the latter. Also, insignificant H_2 generation is possible *via* enzymatic fermentation reactions.

Thus, during the study, it was found that the optimal concentration of the DCMU photosynthesis inhibitor to stimulate the process of hydrogen evolution was 10 μM.

It was found that DCMU stimulates the photo-output of hydrogen by cyanobacterial cells during the first 2 days, after which the opposite effect of its influence is observed. Following after stimulating effect inhibitory action of DCMU on hydrogen photoproduction of hydrogen is possibly due to the suppression of the PSII-independent pathway of hydrogen production, due to its toxic effect, which results in the release of oxygen into the medium, which in turn additionally inhibits the photoproduction of hydrogen. In conclusion, the strain *Desertifilum* sp. IPPAS B-1220 due to its ability to grow on a minimal medium, possessing a sufficient enzymatic system of hydrogen metabolism and the ability to maintain low acid intracellular conditions, which allows for the active production of H_2 in response to lighting, is of great interest as a promising object in bioenergy field.

Conclusion

Obtaining biohydrogen by phototrophic microorganisms is both a promising and complex biotechnology that can play an important role in the global quest to reduce greenhouse gas emissions. Perspective of biohydrogen depends on such scientific achievements as the search for active strains with the required characteristics and the selection of appropriate strategies for strain improvements for photobiological production of hydrogen. The aim of our research was to find promising hydrogen-producers among cyanobacteria and understanding the mechanisms of this process and the conditions for increasing bio-hydrogen production. According to the obtained results, a higher H_2 accumulation was observed with *Synechocystis* sp. PCC 6803 of 0.037 μmol H_2/mg Chl/h within 120 h in the dark. Moreover, *Desertifilum* sp. IPPAS B-1220 produced 0.229 μmol H_2/mg Chl/h after 166 h of incubation in the light. A DCMU concentration of 10 μM increased H_2 release (0.348 μmol H_2/mg Chl/h) by *Desertifilum* sp. IPPAS B-1220 by 1.5-fold. The obtained results indicate the prospects, practical importance and the need for further study of cyanobacteria as biosystems capable of efficiently converting light energy to molecular hydrogen – an eco-friendly fuel.

Acknowledgements

This study was supported by the Ministry of Education and Science, the Republic of Kazakhstan, under the framework of the project: «Development of waste-free technology of wastewater treatment and carbon dioxide utilization based on cyanobacteria for potential biodiesel production», 2018–2020

(grant AP05131218). Also, by Grants-in-Aid for Scientific Research (Nos. 17726220801, 17K07453, 18H05177, T.T.) from the Ministry of Education, Culture, Sports, Science, and Technology, Japan. SA would like to thank the Researchers Supporting Project (RSP-2019/59) for their support. SIA was supported by the grant from Russian Science Foundation (no: 19-14-00118).

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