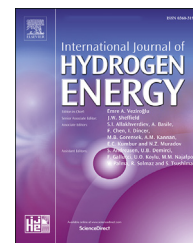


Available online at www.sciencedirect.com

ScienceDirect

journal homepage: www.elsevier.com/locate/he

Screening and optimisation of hydrogen production by newly isolated nitrogen-fixing cyanobacterial strains

Gulzhanay K. Kamshybayeva^{a,b}, Bekzhan D. Kossalbayev^{a,b,**},
Asemgul K. Sadvakasova^a, Meruyert O. Bauenova^a,
Bolatkhon K. Zayadan^a, Ayshat M. Bozieva^c, Hesham F. Alharby^d,
Tatsuya Tomo^e, Suleyman I. Allakhverdiev^{c,f,*}

^a Faculty of Biology and Biotechnology, Al-Farabi Kazakh National University, Al-Farabi 71, Almaty 050038, Kazakhstan

^b Department of Chemical and Biochemical Engineering, Institute of Geology and Oil-Gas Business Institute Named after K. Turyssov, Satbayev University, Almaty 050043, Kazakhstan

^c K.A. Timiryazev Institute of Plant Physiology, Russian Academy of Sciences, Botanicheskaya Str. 35, Moscow 127276, Russia

^d Department of Biological Sciences, Faculty of Science, King Abdulaziz University, Jeddah 21589, Saudi Arabia

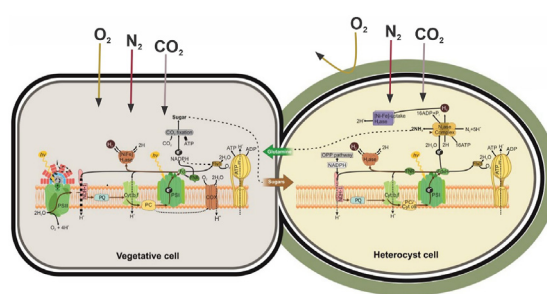
^e Department of Physics, Graduate School of Science, Tokyo University of Science, 1-3 Kagurazaka, Shinjuku-ku, Tokyo 162-8601, Japan

^f Faculty of Engineering and Natural Sciences, Bahcesehir University, Istanbul 34353, Turkey

HIGHLIGHTS

- Eleven nitrogen fixing cyanobacteria species were isolated and identified from different ecosystems.
- Four cyanobacterial strains were distinguished by nitrogenase activity and hydrogen production.
- 2% CO₂ and O₂ gases influence was favorably for most strains to produce molecular hydrogen.
- Supply with 1% O₂:2% CO₂ gas ratio increased H₂ production activity in *Anabaena variabilis* BTA-1047 by 2-fold.

GRAPHICAL ABSTRACT



Abbreviations: ATP, adenosine triphosphate; Chl, chlorophyll; CO₂, carbon dioxide; H₂, hydrogen; H₂ase, hydrogenase; H₂O, water; N₂, nitrogen; Nase, nitrogenase; O₂, oxygen; OD, optical density; PSII, photosystem II.

* Corresponding author. K.A. Timiryazev Institute of Plant Physiology, Russian Academy of Sciences, Botanicheskaya Str. 35, Moscow 127276, Russia.

** Corresponding author. Faculty of Biology and Biotechnology, Al-Farabi Kazakh National University, Al-Farabi 71, Almaty 050038, Kazakhstan.

E-mail addresses: kossalbayev.bekzhan@gmail.com (B.D. Kossalbayev), suleyman.allakhverdiev@gmail.com (S.I. Allakhverdiev).
<https://doi.org/10.1016/j.ijhydene.2023.01.163>

0360-3199/© 2023 Hydrogen Energy Publications LLC. Published by Elsevier Ltd. All rights reserved.

ARTICLE INFO

Article history:

Received 3 December 2022

Received in revised form

7 January 2023

Accepted 14 January 2023

Available online 4 February 2023

Keywords:

Biohydrogen

Isolation

Filamentous cyanobacteria

Nitrogenase

Hydrogenase

ABSTRACT

Recently, there has been a propensity to postpone dealing with the world's climate concerns until later, resulting in a 1.5 °C rise in temperature over the last century. Therefore, interest in biologically derived, inexhaustible energy sources based on solar energy is growing. Cyanobacteria have the potential to produce clean, renewable fuels in the form of hydrogen (H₂) gas, derived from solar energy and water. The current study reports the screening 11 cyanobacterial strains isolated from rice paddies and hot springs for efficient H₂ producers. According to our findings, H₂ concentrations in the species ranged from 3.6 to 48.9 μmol mg⁻¹ Chl a h⁻¹. H₂ production by isolated species was shown to have a 2% positive influence on oxygen (O₂) and carbon dioxide (CO₂) concentrations and a 2% negative effect on all nitrogen gas (N₂) concentrations. It was discovered that at high CO₂ concentrations, photosynthesis is enhanced but H₂ production is suppressed. *Anabaena variabilis* BTA-1047 was found to be the most active H₂-producing species, with an H₂ production activity of 21.3 μmol mg⁻¹ Chl a h⁻¹. Moreover, a 1% O₂: 2% CO₂ gas mixture doubled the strain activity of H₂ production. The findings of the study called into the question the notion that only an anaerobic environment is required for H₂ production by N₂-fixing cyanobacterial species and explored whether H₂ productivity can be increased by stimulating the micro-anaerobic environment with a carbon source.

© 2023 Hydrogen Energy Publications LLC. Published by Elsevier Ltd. All rights reserved.

Introduction

Renewable hydrogen (H₂) energy based on phototrophic microorganisms has a promising future, as solar energy, water (H₂O), air, and simple salts are sufficient for its production [1]. No hazardous compounds are utilised or produced in cyanobacterial H₂ production. All carbonates used are neutral, and the societal repercussions are minimal [2]. One of the current priorities in H₂ production is the development of simple and inexpensive photobioreactors. Obtaining phototrophic bacteria that actively produce H₂ through genetic manipulation is a priority as well. Since no species with substantial H₂ production activity has been discovered among species with fully sequenced genomes, identifying the H₂ production capacity of wild species is critical. Despite efforts to establish genetically modified species under laboratory conditions [3–5], H₂ has not piqued the interest of producers since production costs outweigh returns.

Numerous phototrophic microorganisms and bacteria carrying nitrogenase (N₂ase) [6,7] and hydrogenase (H₂ase) [8,9] enzymes have been studied as model organisms for photobiological H₂ production to date. Among them are the bacterium *Rhodospseudomonas palustris* conducting violet photosynthesis [10], the green microalga *Chlamydomonas reinhardtii* [11], and the cyanobacteria *Anabaena* sp. [12], and *Synechocystis* sp. [13], whose strains are distinguished by unique H₂ production. In heterocysts, filamentous, diazotrophic *Anabaena* sp. species produce H₂ molecules [14], which protect them from O₂ and N₂ (Fig. 1). The low abundance of heterocysts in the filament (roughly one in every twenty cells is generally split into heterocysts) lowers the yield of pure H₂ produced. It should be emphasised that H₂ production in *Chlamydomonas reinhardtii* and *Synechocystis* sp. is aided by H₂ase enzymes, and that these two organisms have long-term

H₂ production under strictly anaerobic conditions [15]. Although these strains have long been used as model organisms for research into bio-H₂ production, the lack of N₂ase enzymes makes it impossible to produce H₂ at high rates. Recent studies have revealed that several species of N₂-fixing heterocyst/heterocyst-free cyanobacteria so far have greater H₂ production rates than certain previously studied mutant strains. This paper describes the utilisation of N₂-fixing cyanobacterial strains for effective photobiological H₂ production under microaerobic conditions. Because the enzymes involved in biological H₂ production are O₂ sensitive, using oxygenic photosynthetic organisms as a platform for H₂ production in general is extremely challenging. Thus, photobiological studies of H₂ production have largely relied on artificial interventions to aid in the creation of an anaerobic environment [16].

Over the last 30 years, a considerable number of review articles have compared the H₂ production of all active producer strains [17–21]. Hundreds of freshly isolated cyanobacterial strains from natural environments were examined, with H₂ production rates ranging from 1 nmol to 300 μmol mg⁻¹ Chl a h⁻¹ [22]. The H₂ production rate of many genetically modified species [23–25] might be several times lower than that of wild species [26], which belong to a different genus. However, to optimise all isolated species for H₂ production, the impact of physiological and chemical factors (light, nutrient salts, biomass density, temperature, pH, and O₂, N₂, and CO₂ content) is evaluated first, followed by genetic analysis. Due to the various research methods, it is difficult to conduct comparative research on H₂ yield even within species belonging to the same genus. Leino et al. (2014) [27] compared the N₂ase and H₂ase activity of ten different N₂-fixing cyanobacteria isolated from the Baltic Sea and Finnish lakes, particularly their ethylene and H₂ yields. When the active species were studied using the Southern hybridisation

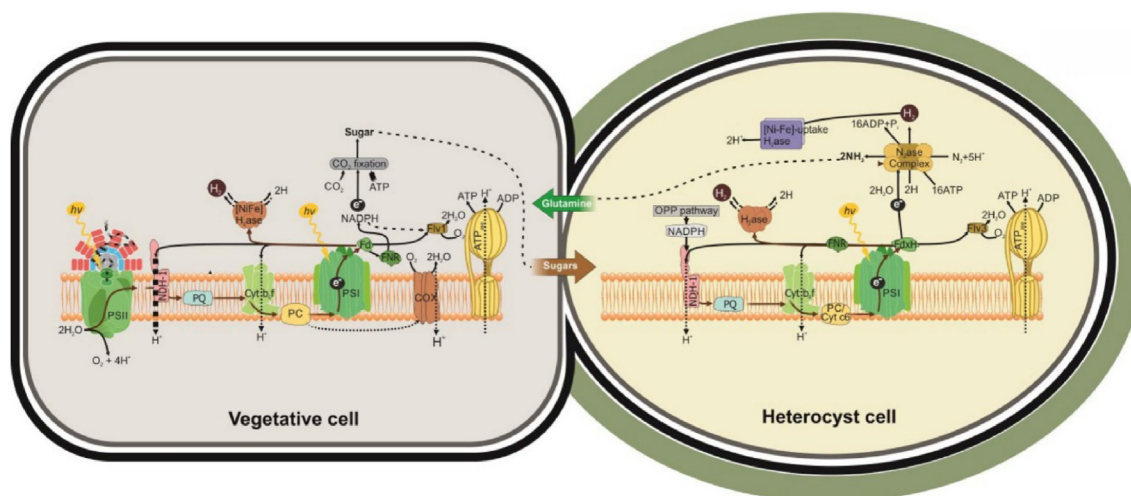


Fig. 1 – Scheme of H_2 production by heterocyst cyanobacterial strains. Vegetative cells contain only [NiFe]-hydrogenase, while heterocyst cells – activated [NiFe]-hydrogenase and nitrogenase enzymes. Electrons were transferred from photosystem II (PSII) to the heterocysts by photosynthetic proteins in the form of polysaccharides. Modified from reference: [6].

method, it was discovered that both primary (*nifHDK1*) and secondary (*nif* etc.) N_2 ase enzymes exist. Allahverdiyeva et al. (2010) [22] studied H_2 production in 400 species from the above locations and discovered it in half of them. Schutz et al. (2004) [28] studied the intensity of molecular H_2 production in various cyanobacterial strains. The presence or absence of *Hup* and *Hox* genes in isolated and collectable species was analysed, and several gene complexes were found to be involved in H_2 production. While the receptive H_2 ase was found in all species investigated, the bidirectional H_2 ase was found in only a few of them. In most non- N_2 -fixing species, only bidirectional H_2 ase was detected. Weissman and Benemann (1977) [29] assessed the H_2 production rate by N_2 -starved cyanobacteria and found that *Anabaena* strains had the highest H_2 production capacity in a short time.

Numerous studies [14,26,30,31] have shown that H_2 ase activity lasts for a long time, and although N_2 ase activity is extremely high, intense H_2 production does not last long. In addition, the N_2 ase enzyme is more sensitive to O_2 than H_2 ase, so, its activity reduces in a vacuum environment when PSII produces more O_2 . However, N_2 ase activity was found to increase when CO_2 and glucose were added to the medium.

Although numerous cyanobacterial species have been examined for H_2 production, few studies have attempted to identify strains with significant H_2 production in an anaerobic environment [30,32]. Testing the natural diversity of H_2 -producing cyanobacteria is a globally recognised challenge to uncover species and strains with significantly higher H_2 production capacities than model organisms [33].

The study aimed to identify naturally efficient H_2 producers among N_2 -fixing cyanobacterial strains. The screening conditions for cyanobacteria isolated from rice fields and hot springs are discussed, and the influence of the gases O_2 , N_2 , and CO_2 and their ratios on the rate of H_2 production in the newly isolated cyanobacterial strains are shown.

Materials and methods

Research objects

To isolate all cyanobacteria species, samples were collected from natural ecosystems in the Republic of Kazakhstan throughout the summer and fall. New species were isolated and investigated from the samples in the laboratory (Table 1).

Identification procedure

DNA extraction was performed according to standard procedures [34]. The exponentially growing cells (70 mL) were centrifuged, resulting in their conversion to granules, and then 0.5 mL of lysis buffer (25% sucrose, 50 mmol Tris-HCl, and 100 mmol EDTA) was added. Cells were treated with 5 mg lysozyme for 25 min at room temperature. After adding $NaC_{12}H_{25}SO_4$ and proteinase K at final concentrations of 1% and 100 $\mu g\ mL^{-1}$, respectively, samples were incubated at 50 °C for 1 h. DNA was extracted three times using phenol: chloroform: isoamyl alcohol (25:24:1) and twice using chloroform: isoamyl alcohol (24:1). DNA was precipitated in 70% ethanol, then transferred to a 100 μL Tris-EDTA buffer and stored at –20 °C. Polymerase chain reactions (PCR) were performed with the Mastercycler® Nexus device (Eppendorf, Hamburg, Germany). Universal 16S rRNA primers were used for PCR and sequencing.

Cultivation procedure

The isolated cyanobacteria were cultured in liquid media. All species were grown at 25 °C under a light intensity of 30 $\mu mol\ (photons)\ m^{-2}\ s^{-1}$ while the biomass was intensively harvested. All 11 strains studied were cultured on BG-11/BG₀-11 medium with a pH of 7.1. BG-11 culture medium consisted of (g

Table 1 – Isolated cyanobacteria species.

Strain	Type	Cell forming	Growth/H ₂ production medium	Source of isolation	Sampling place (Kazakhstan)
<i>Anabaena cylindrica</i> PPC-701	Filamentous, N ₂ -fixing	Heterocyst	BG-11, Z8/BG ₀ -11	Rice paddies	Zhanakorgan region (GeoCoordinate: 43°55'42.8"N 67°11'01.2"E)
<i>Nostoc commune</i> PCC 793	Filamentous, N ₂ -fixing	Heterocysts	BG-11/BG ₀ -11	Rice paddies	
<i>Tolypothrix tenuis</i> PCP-489	Filamentous, N ₂ -fixing	Akinets, trichome, heterocyst	BG-11/BG ₀ -11	Rice paddies	
<i>Anabaena variabilis</i> BTA-1047	Filamentous, N ₂ -fixing	Heterocyst	BG-11/BG ₀ -11	Alfalfa paddies	Sayram region (GeoCoordinate: 42°15'56.4"N 69°41'14.2"E)
<i>Nostoc</i> sp. BTA-1201	Filamentous, N ₂ -fixing	Heterocysts	BG-11/BG ₀ -11	Alfalfa paddies	
<i>Anabaena</i> sp. ZTB-1	Filamentous, N ₂ -fixing	Heterocyst	BG-11/BG ₀ -11	Rice paddies	Balkhash region (GeoCoordinate: 45°50'24.9"N 74°47'53.1"E)
<i>Stigonema ocellatum</i> ZTB-63	Filamentous, N ₂ -fixing	Akinets	BG-11/BG ₀ -11	Rice paddies	
<i>Calothrix</i> sp. BUY-14.2	Filamentous, N ₂ -fixing	Heterocyst, akinet, sheath-encased trichome	BG-11/BG ₀ -11	Rice soil	Shieli region (GeoCoordinate: 44°09'22.3"N 66°40'44.7"E)
<i>Tolypothrix</i> sp. BUY-03.1	Filamentous, N ₂ -fixing	Akinets, trichome	BG-11/BG ₀ -11	Rice soil	
<i>Scytonema</i> sp. SSP-9697	Filamentous, N ₂ -fixing	Akinets	BG-11, Z8/BG ₀ -11	Plankton	Uigur region (GeoCoordinate: 43°23'48.8"N 77°35'10.2"E)
<i>Nostoc punctiforme</i> SSP-01	Filamentous, N ₂ -fixing	Heterocysts	BG-11, Z8/BG ₀ -11	Plankton	

L⁻¹): 0.001 EDTA; 0.006 ferric ammonium citrate; 0.006 citric acid, 0.036 CaCl₂ × 2H₂O; 0.075 MgSO₄ × 7H₂O; 0.031 K₂HPO₄; 0.02 Na₂CO₃; 2.9 × 10⁻³ H₃BO₃; 1.8 × 10⁻³ MnCl₂ × 4H₂O; 0.22 × 10⁻³ ZnSO₄ × 7H₂O; 0.08 × 10⁻³ SiSO₄ × 5H₂O; 0.05 × 10⁻³ CoCl₂ × 6H₂O; and 0.39 × 10⁻³ NaMoO₄ × 2H₂O. During active growth, air purging was carried out using a special compressor (BOYO, China).

Hydrogen production evaluation research

Cyanobacterial cultures were grown in 40 mL tubes and aerated with sterile gas. 1% CO₂ was computed using Rotamer (RMA-0.063 G, Promcomplekt, Rostov-na-Donu, Russia) and then added to the air composition. It was then centrifuged for 5 min at 10,000 rpm. After draining the supernatant, the cell cultures were given 100 mL of BG₀-11 culture medium and stirred for 3 min. The pre-cultured cells were adjusted to an optical density of 0.4 (OD₇₃₀) using a spectrophotometer (UV-VIS Spectrophotometer UV-2600i, Shimadzu, Tokyo, Japan) at a wavelength of ~730 nm. Cultures were then cultivated for 24 h under light (intensity: 45 mmol (photons) m⁻² s⁻¹) and collected after 5 min in a centrifuge (5424R, Eppendorf, Hamburg, Germany) at 10,000 rpm. After draining the supernatant, 30 mL of BG₀-11 culture medium was added. The cells were then centrifuged for 5 min at 10,000 rpm. A spectrophotometer was used to adjust the cells isolated from the supernatant to OD₇₃₀-1.5 after two washes. 15 mL of BG₀-11 culture medium, adjusted with 50 mmol of HEPES-KOH (pH of 7.4) and 100 mmol of NaHCO₃ as chemical additives, was poured over the condensed cells. A 15 mL space was provided within the GC vial for the accumulation of gases evolved by the cells [28]. A gas chromatograph (GC-15A, Shimadzu, Tokyo, Japan) was used to determine H₂ production activity,

and the result was stated in the indication μmol H₂ mg⁻¹ Chl *a* h⁻¹. Argon was employed as the carrier gas.

Determination of nitrogen, oxygen, and carbon dioxide influence

Using a 15-gauge medical syringe, 99.9% pure argon gas was introduced into hermetically sealed GC vials for 40 min to create an anaerobic environment. A rotameter (Rotameter-25 AR, Dohmet, Russia) was used to monitor the argon atmosphere, and a second 15-gauge needle served as an outlet during purge.

To achieve the concentrations of the gases N₂, O₂, and CO₂ in the free area of the vial, argon gas was extracted with the help of a syringe and replaced by pure 99.9% gases in varying volumes. In this manner, an atmosphere of 5, 10, 20, and 40% gases was created in the 15 mL space of the vial. The experimental vials were incubated in a special spinner (IKA KS 4000, Osaka, Japan) under continuous light (100 μmol (photons) m⁻² s⁻¹) for up to 14 days after the composition of the gas mixture in the headspace was adjusted. During this time, 100 μL samples were taken daily from the headspace until the H₂ production rate of the cyanobacterial species decreased [31].

Investigation of carbon dioxide and oxygen ratio effects

30 mL gas chromatograph vials (containing a 10 mL suspension) were used to investigate the effect of CO₂ and O₂ ratios. The argon stimulation lasted 40 min. Different O₂ and CO₂ ratios were made to detect their effects on H₂ production. O₂ and CO₂ residues of about 1% were detected in all experiments, and the amount may be estimated by injecting a sample into a gas chromatograph using a syringe. The vials with tightly sealed caps were continually incubated in dark and light conditions. The H₂ production of cells was examined for 14 days in all experimental variants. Each day, an aliquot of

160 μL was taken from the headspace with a syringe and injected into a gas chromatograph. A column was used to determine the amount of H_2 produced by measuring the amounts of H_2 , N_2 , and O_2 . The obtained molecular H_2 was measured as directed by the manufacturer (GC-15A, Shimadzu, Tokyo, Japan). The injector and column were set to 80°C , while the detector was set at 120°C . The amount of O_2 and H_2 was measured every 24 h and divided by the total number of hours. A calibration curve was established via 100% pure universal gases to acquire a proportion of the gases [26].

Investigation of light intensity influence

30 mL GC gas vials were used to explore the influence of light (the maximum volume was 30 mL). 10 mL of the suspension was put into the vials, 50 mM HEPES (pH = 7.5) concentration was placed on top, and the lids of the vials were closed with a specific clamp for 40 min while being stimulated with 100% pure argon. The 2% argon was then replaced with CO_2 in the vial's free space. The light intensity was measured using the Quantum Q 40555 Li-250A light metre (LI-COR Biosciences, Lincoln, NE, USA) at 5 sides in the vial, and a common reading in μmol (photons) $\text{m}^{-2} \text{s}^{-1}$ was shown in current research.

Determination of chlorophyll content

The 100 μL cell suspension was filled with 100% pure methanol, shaken once, and placed on ice for 5 min. After centrifugation at 15,000 rpm, the supernatant absorbance was measured. A spectrophotometer was used to measure chlorophyll a concentration at 646, 663.4, and 730 nm (Chl a mg L^{-1}).

Determination of nitrogenase activity

N_2 ase activity was determined by passing a gas mixture of 10% acetylene and 90% argon through the vial for 30 min, as described in David et al. (1980) [35]. Cells were grown for 24 h in 30 mL vials at a light intensity of $250 \mu\text{mol}$ (photons) $\text{m}^{-2} \text{s}^{-1}$. After incubation, 500 μL gas samples were collected, and the ethylene concentration in the gas mixture was measured. Acetylene reduction activity was measured in μmol ethylene $\text{mg}^{-1} \text{Chl a h}^{-1}$ using a gas chromatograph.

Statistical analysis

A calibrated curve of the H_2 standard was utilised to compute the produced H_2 moles. The Microsoft Excel software (ANOVA) was used to alter the H_2 concentrations. The linear line of the mV: mL ratio was calculated after several experiments, and the average deviation was 0.9. The transformed H_2 was converted to mol and divided into chlorophyll concentrations (mg). Then it was divided by the hour of H_2 production and multiplied by the free space within the vial (10 mL).

Results

Screening of isolated species concerning hydrogen production rate

Cyanobacteria are phototrophic microorganisms that can produce gaseous, ecologically favourable H_2 through the

action of cellular proteins (H_2 ase and N_2 ase). Only H_2O , simple salts, and sunshine are required under certain conditions to produce molecular H_2 . All currently explored species produce small amounts of H_2 , and the benefit from the product does not surpass the loss. In this context, isolating novel active cyanobacterial species from natural habitats to examine the H_2 ase and N_2 ase enzymes activities are critical and a research topic of global interest.

The isolated strains were sorted into taxonomic groups using 16S rRNA primers. All isolated cyanobacterial species were amplified using universal primers, and sequencing samples were obtained. The number of nucleotide sequences obtained from cyanobacterial species 16S rRNA regions ranged between 400 and 1400 bp. Based on the findings, a phylogenetic tree was constructed, and the closest strains were chosen for the species name. Bioinformatic screening discovered neighbouring species with 99–100% affinity for each species.

Eleven species capable of N_2 fixation were examined to identify the cyanobacterial species active in H_2 production. The screening was then carried out under strictly anaerobic conditions along with the species' H_2 production. *Anabaena* sp. strain PCC 7120 was used as a model organism actively producing H_2 . For 7 days, the H_2 production rate of all 11 species was investigated, and the maximum value is shown in Fig. 2. However, since the O_2 content in the culture medium increased from day to day, the maximum value of H_2 production was found in the first four days and decreased in the subsequent days. Four strains obtained from the control (*Anabaena* sp. PCC 7120) performed better or equally well in the research. The strain *Anabaena variabilis* BTA-1047 produced more H_2 ($5.3 \mu\text{mol mg}^{-1} \text{Chl a h}^{-1}$) than the other species. *Nostoc punctiforme* SSP-01 actively produced H_2 for the first two days, reaching a maximum of $4.1 \mu\text{mol mg}^{-1} \text{Chl a h}^{-1}$ on the day 2. On day three, the *Calothrix* sp. BUY-14.2 strain produced H_2 at a rate of $3.1 \mu\text{mol mg}^{-1} \text{Chl a h}^{-1}$. *Anabaena* sp. ZTB-1 is another active H_2 producer, with a massive yield on 3rd day ($3.65 \mu\text{mol mg}^{-1} \text{Chl a h}^{-1}$). The following species had the lowest yields under light: *Tolypothrix tenuis* PCP-489, *Nostoc commune* PCC 793, and *Nostoc* sp. BTA-1201.

N_2 -fixing cyanobacteria have several major groups of genes responsible for H_2 production and their alternative genes. According to our results, the principle of H_2 production of isolated species corresponds to that of *Anabaena cylindrica* B629 and *Tolypothrix* sp. B379. Yeager et al. (2011) [36] studied the H_2 production of the 2 strains for 8 h and found that the highest value was recorded on the 1st day and that H_2 production was suppressed in the following days by an increase in O_2 concentration. Furthermore, Lindberg et al. (2004) [37] discovered that the enzyme N_2 ase consumes 4 times more electrons than H_2 ase in each scenario. In turn, the latter rapidly destroys the stored glycogen reserves under anaerobic conditions, providing the energy required for the H_2 . Consequently, the intense H_2 production of examined strains in the first four days and the lower one in the following days fits with the previous investigations. According to Tamagnini et al. (2007) [38], the lack of the enzyme bi- H_2 ase explains the high H_2 yield of *Nostoc punctiforme*. Since this enzyme reversely transforms the H_2 molecule produced by N_2 ase into H^+ , the high H_2 production of *Nostoc commune* PCC 793 in our study

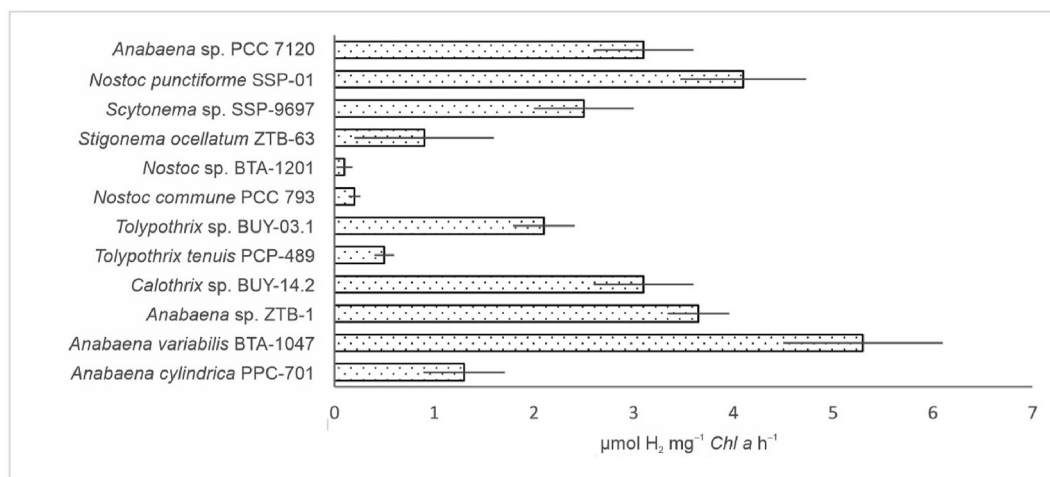


Fig. 2 – The rate of cyanobacterial H₂ production under the light. A culture cultivated under 30 μmol (photons) m⁻² s⁻¹ light and constant air supply yielded biomass. Fresh cells were centrifuged at 6000 rpm for 10 min before being suspended in BG₁₁ media. The suspended cells were placed in 30 mL GC vials and bubbled with argon gas for 40 min at room temperature. The GC vials were then cultured under the illumination of 30 μmol (photons) m⁻² s⁻¹. The H₂ content of the sample recovered from the vials was determined using GC.

corresponds with prior investigations. Allahverdiyeva et al. (2010) [22] reported that both forms of H₂ase enzyme exist in *Anabaena variabilis*, moreover, the existence of N₂ase (nifHDK1), N₂ase alternative genes (nifHDK2, vnfDGK), H₂ase host genes (hupLS, hoxY), and genes involved in maturation (HydA1, HydA2, HydB1, HydB2) can yield greater H₂ production rates. As shown in Fig. 3, no matter how much H₂ is catalysed into protons by the hoxY-controlled bi-H₂ase, the yield of H₂ rises due to the high N₂ase activity of *Nostoc commune* PCC 793. Related research has been utilised to screen heterocyst-forming cyanobacterial strains for the presence of different forms of N₂ase and H₂ase [39].

Nitrogen fixation is an energy-intensive process that requires 16 (adenosine triphosphate) ATP molecules for each N₂ molecule fixed and each H₂ produced (Fig. 1). However, this process is especially important for diazotrophic species that inhabit ecological niches with very low levels of nitrogenous nutrients (e.g., deep oceans) [40]. Biological N₂ fixation is known as the enzymatic reduction of atmospheric N₂ to ammonium. The nifHDK genes, contiguous in the genome, encode the common enzyme N₂ase. Alternative N₂ases (alternative and second alternative) contain nifH as well as a third protein encoded by nifG (nifDGK) in the Mo-protein analogue. N₂ase genes can be detected and characterised by amplification in biological samples using PCR. Amplification of N₂ase genes indicates the presence of N₂-fixing microorganisms, but not whether they actively fix N₂ [41].

Genetic investigation revealed that the extra genes nifd1, nifG1, and nifk1 for N₂ase were identified in *A. variabilis* ATCC 29413, though the strains *Tolypothrix tenuis* PCP-489 and *Nostoc commune* PCC 793 were not amplified. Furthermore, the alternative N₂ase genes nifHDK2 and vnfDGK were amplified in *Anabaena variabilis* BTA-1047, *Nostoc punctiforme* SSP-01, and *Anabaena* sp. ZTB-1, indicating that alternative genes play a role in H₂ production.

The gas chromatography data revealed that all strains of N₂-fixing cyanobacteria exhibit N₂ase activity in an acetylene

medium. Among the examined species, strain PCC 793 (*Anabaena*) had a comparatively low ethylene concentration (3.6 μmol ethylene mg⁻¹ Chl a h⁻¹) whereas strain ZTB-1 had a high ethylene content (21.3 μmol ethylene mg⁻¹ Chl a h⁻¹). Furthermore, strains *Anabaena variabilis* BTA-1047 (19.2 μmol ethylene mg⁻¹ Chl a h⁻¹) and *Nostoc punctiforme* SSP-01 (20.5 μmol ethylene mg⁻¹ Chl a h⁻¹) also performed well. *Tolypothrix tenuis* PCP-489 produced 16.3 μmol ethylene mg⁻¹ Chl a h⁻¹ (Fig. 3).

Thus, a high N₂-fixing capacity was found in the strains *Anabaena* sp. ZTB-1, *Nostoc punctiforme* SSP-01, and *Anabaena variabilis* BTA-1047. Based on the experiments conducted, it was found that all isolated cyanobacterial strains able to fix N₂ to varying degrees. No gaseous ethylene oxidation was observed in strain *Synechocystis* sp. PCC 6803 without heterocyst serving as a control. The research findings correlate with Lindberg et al. (2004) [34]. Accordingly, a rise in the frequency of heterocysts in *Nostoc punctiforme* ATCC 29133 leads to increased activity of the enzyme N₂ase. It was cautioned, however, that the rise in heterocysts is not proportionate to the H₂ yield. Although the activity of the N₂ase enzyme was low under high light and dark conditions, medium light had a favourable influence on ethylene oxidation. Lindberg et al. (2004) [37] and Ernst et al. (1990) [42] reported a reduction in ethylene yield after 6 days, however, our findings showed N₂ase activity in all strains in the first 2 days and a decline in subsequent days. It can be explained by the individual characteristics of the species. *Stigonema ocellatum* ZTB-63 and *Tolypothrix tenuis* PCP-489 strains produced a high amount of ethylene: 18.7 and 16.3 μmol ethylene mg⁻¹ Chl a h⁻¹, respectively. Our results coincide with Lindberg et al. (2014) [37] study, which reported that strains *Nostoc Becid* 16, *Calotrix Becid* 19, *Nodularia* AV33, and *Anabaena* sp. PCC 7120 with a scarce H₂ yield showed strong N₂ase activity. The lack of uptake H₂ase genes in the studied strains was considered to explain this phenomenon.

Thus, the strains *Anabaena variabilis* BTA-1047, *Anabaena* sp. ZTB-1, *Calotrix* sp. BUY-14.2, and *Nostoc punctiforme* SSP-01

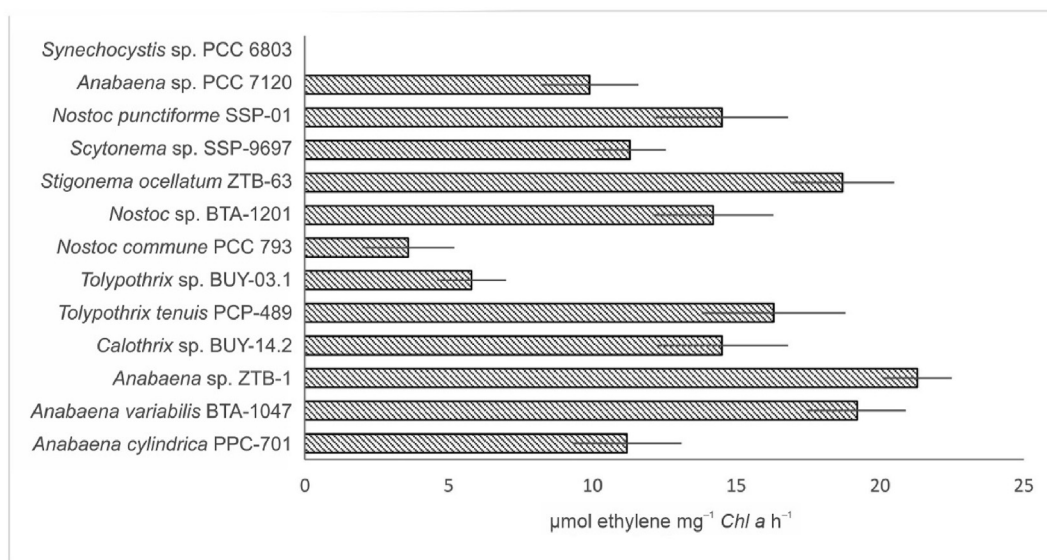


Fig. 3 – Nitrogenase activity of isolated cyanobacterial strains. Cyanobacterial strains were cultivated under continuous air supply at 30 μmol (photons) $\text{m}^{-2} \text{s}^{-1}$ light. The cells were then centrifuged and transferred to a 20 mL GC vial. All samples were adjusted to a concentration of 20 mg Chl a mL^{-1} using a spectrophotometer. Following bubbling for 30 min in a 10% acetylene:90% argon mixture, the vials were placed under 30 μmol (photons) $\text{m}^{-2} \text{s}^{-1}$ light. The ethylene concentrations were monitored by GC during the next two days.

were chosen as active H_2 producers for the next stage of research.

Investigation of nitrogen, oxygen, and carbon dioxide influence on hydrogen production

N_2 ase and H_2 ase are sensitive to inactivation by O_2 and therefore require an anoxic medium [43]. Many filamentous cyanobacteria, such as *Anabaena* sp., form differentiated cells known as heterocysts. Heterocysts account for 5–10% of the filament cells and provide a microaerobic environment in the cell that feeds on the photoreduction of neighbouring vegetative cells [44]. Thus, when aerobic conditions prevail, the heterocysts become a site of N_2 fixation and H_2 production.

According to the findings, cell density and growth duration have a direct influence on the ultimate yield of H_2 . Under anaerobic conditions, cells release O_2 molecules via PSII [43]. However, not all of the 8-h strains investigated released O_2 via PSII. The strains investigated were shown to be affected differently by free space O_2 concentrations. The O_2 resistance of four active N_2 -fixing cyanobacterial strains was studied. The investigations discovered two distinct manifestations: 1) *Anabaena variabilis* BTA-1047 and *Nostoc punctiforme* SSP-01 strains were discovered to promote H_2 production at concentrations lower than 6% O_2 , whereas concentrations higher than 6% O_2 were found to inhibit it; 2) *Anabaena* sp. ZTB-1 and *Calothrix* sp. BUY-14.2 strains displayed limited H_2 production activity in oxygenic environments. A concentration of 8% O_2 had a deleterious effect on *Calothrix* sp. BUY-14.2, resulting in a 90% drop in H_2 yield.

It was discovered that even 1% O_2 negatively affected the H_2 production of the heterocyst-free, non- N_2 -fixing *Synechocystis* sp. PCC 6803 used as a control (data not shown). It should be emphasised, however, that H_2 production for 48 h was not

observed at high O_2 concentrations (4–8%). In the initial hours, N_2 ase is activated in heterocyst cells and rapidly depletes the stored glycogen reserves, while H_2 catalysis by the H_2 ase enzyme situated in the vegetative cell is hampered by high O_2 concentrations [45].

As shown in Fig. 1, the enzyme N_2 ase, which is found in heterocyst cells with a thick protective O_2 layer generated by photosynthetic activity, produces H_2 at a high rate [46]. Inactivation of N_2 ase did not occur in the *Anabaena variabilis* BTA-1047 and *Nostoc punctiforme* SSP-01 strains due to a thick heterocyst layer, even though the heterocyst cells cannot tolerate high (8%) O_2 concentrations (3.74 $\mu\text{mol H}_2 \text{mg}^{-1} \text{Chl a h}^{-1}$) (Fig. 4). However, low O_2 concentrations are required for proper heterocyst operation, ATP molecules that activate N_2 ase are generated in strictly anaerobic conditions in small quantities, and the essential carbohydrates from vegetative cells are not transported to heterocyst cells [47]. This explains why there were strong peaks of H_2 activity at concentrations of more than 6% O_2 in free space. Furthermore, changes in the H_2 : O_2 ratio have an immediate impact on the activity of H_2 enzymes (N_2 ase and uptake H_2 ase) in the heterocyst. However, at high O_2 concentrations, H_2 ase is completely inactivated in a vegetative cell [48]. The essential energy from the vegetative cells enables the enzyme N_2 ase to actively catalyse H_2 in the first view of the investigation (*Anabaena variabilis* BTA-1047 and *Nostoc punctiforme* SSP-01). As a result, at 2% O_2 , the H_2 yield in strain BTA-1047 increased by 89% (10 $\mu\text{mol H}_2 \text{mg}^{-1} \text{Chl a h}^{-1}$) compared to the control (0% O_2). The resulting results are consistent with Yeager et al. (2011) [36] investigation on favourable H_2 -producing O_2 concentrations for *Nostoc commune* MFG-1 and *Fischerella muscicola* 7414 strains, which were less than 5%.

Leino et al. (2014) [27] investigated the effects of long-term CO_2 exposure on the immobilized strain *Calothrix* 336/3 in

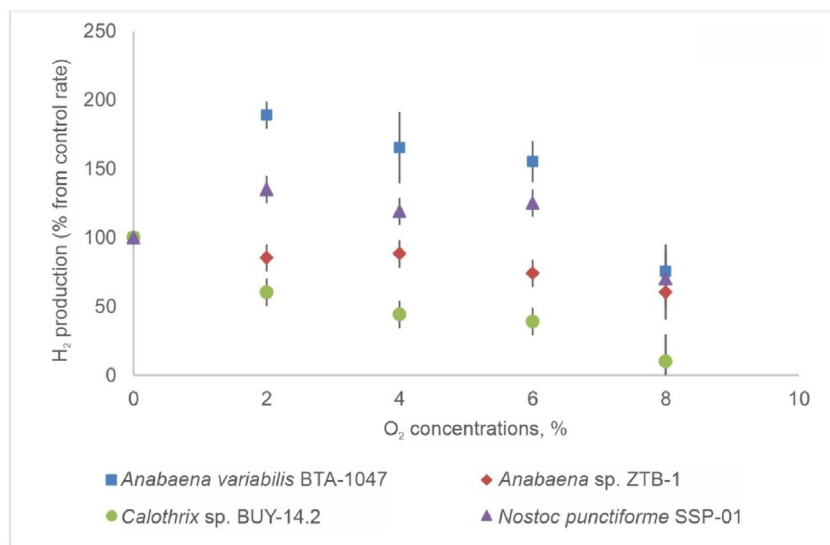


Fig. 4 – Influence of different O₂ concentrations on cyanobacterial H₂ production. Cultures were grown under continuous air supply at 30 μmol (photons) $\text{m}^{-2} \text{s}^{-1}$ light. The cells were then collected by centrifugation and transferred to a 20 mL GC vial. The biomass in the GC vials was adjusted to 1.5 OD at 730 nm, then the cells were incubated under an argon atmosphere with an initial O₂ headspace concentration of 0.

alginate gel. 1 and 6% O₂ concentrations increased H₂ production for up to 40 h, whereas 6% O₂ concentrations increased production for 84–120 h. Tsygankov et al. (1998) [49] discovered that the *A. variabilis* strain had a low H₂ production rate at 50 $\mu\text{mol L}^{-1}$ of O₂ dissolved in H₂O. Furthermore, it was revealed that the uptake H₂ase enzyme catalysed the formation of H₂ molecules at this O₂ concentration. Furthermore, *A. variabilis* possesses bi-H₂ases in vegetative cells [50]. Unlike N₂ase, however, these enzymes are only weakly shielded from O₂, and their activity is impaired at low O₂ concentrations [51].

It has been discovered that in heterocyst cyanobacteria, there is an intricate link between H₂ yield and O₂ generation, and that understanding their interaction is required to achieve consistent H₂ production. Numerous studies have found that producing H₂ simply creates an anaerobic environment. While this method is suitable for studying species with vegetative cells, low O₂ levels in N₂-fixing species may promote normal H₂ production. However, research indicates that O₂ resistance varies between species, so screening for O₂ resistance is the first step.

N₂ase, the primary enzyme in filamentous cyanobacteria responsible for H₂ production, catalyses the reduction of N₂ to ammonia (NH₃) as part of the H⁺ to H₂ reduction pathway [38]. It should be noted that decreasing the N₂ content in the medium has no effect N₂ase activity, and the H₂ production mechanism of N₂ase enzyme continues in anaerobic and N₂-free conditions [52]. An uptake [NiFe]-H₂ase enzyme functions in the heterocyst of some cyanobacterial strains to recapture the H₂ produced by the N₂ase enzyme [38]. In addition, the enzyme bi-H₂ase has been discovered in certain heterocyst cells. However, whereas the function of this enzyme in vegetative cells has been thoroughly studied, its role in heterocyst cells remains unclear [53].

The influence of varying N₂ gas concentrations on the H₂ production activity of N₂-fixing cyanobacterial species was

then examined. N₂ concentrations of 2% were found to have a deleterious effect on H₂ production. However, when H₂ production was tracked over time, the yield of time-dependent H₂ rose somewhat in all strains. This behaviour could be explained by N₂ase not having enough H⁺ to cleave H₂ [54]. Because of the enhanced activity of N₂ fixation in an environment with high N₂ content, the N₂ase enzyme's indirect function is to catalyse H₂, reducing the flow of ATP molecules and H⁺ [55].

The study used concentrations of 0, 5, 10, and 20% N₂ gas, as shown in Fig. 5, to determine the time-dependent H₂ yield. In each of the cyanobacterial strains studied, the effect of N₂ gas caused a rapid and concentration-dependent decrease in H₂ production rate (see Fig. 3). The H₂ production rate was high in all species (*Anabaena variabilis* BTA-1047, *Anabaena* sp. ZTB-1, *Calothrix* sp. BUY-14.2, and *Nostoc punctiforme* SSP-01), with *Anabaena* sp. ZTB-1 producing the most at the hour 6 (2.56 $\mu\text{mol H}_2 \text{mg}^{-1} \text{Chl a h}^{-1}$). While *Anabaena variabilis* BTA-1047 and *Calothrix* sp. BUY-14.2 strains were found to have low rates of H₂ production at 8% N₂, *Anabaena* sp. ZTB-1 and *Nostoc punctiforme* SSP-01 were not assigned any H₂ production at all.

The strong N₂ase activity of *Anabaena* sp. ZTB-1 and *Nostoc punctiforme* SSP-01 strains might explain the results, as shown in Fig. 3. N₂ase competes for ATP molecules from vegetative cells by oxidising gaseous N₂ to NH₃, resulting in a reduction of H₂ yield in free space [56]. It should be noted that while *Anabaena variabilis* BTA-1047 demonstrated relative resistance to a 5% N₂ gas concentration, the H₂ yield at this concentration is 10% higher in *Anabaena* sp. ZTB-1, 250% higher in *Nostoc commune* MFG-1, and 70% higher in *Fischerella muscicola* 7414. Meanwhile, the impact of a 10% N₂ gas concentration had the same negative effect on H₂ production in all strains, and no H₂ production was observed in *Nostoc punctiforme* SSP-01 since the first hours. At this concentration, the maximal H₂ production of three strains was 0.32 $\mu\text{mol H}_2 \text{mg}^{-1} \text{Chl a h}^{-1}$.

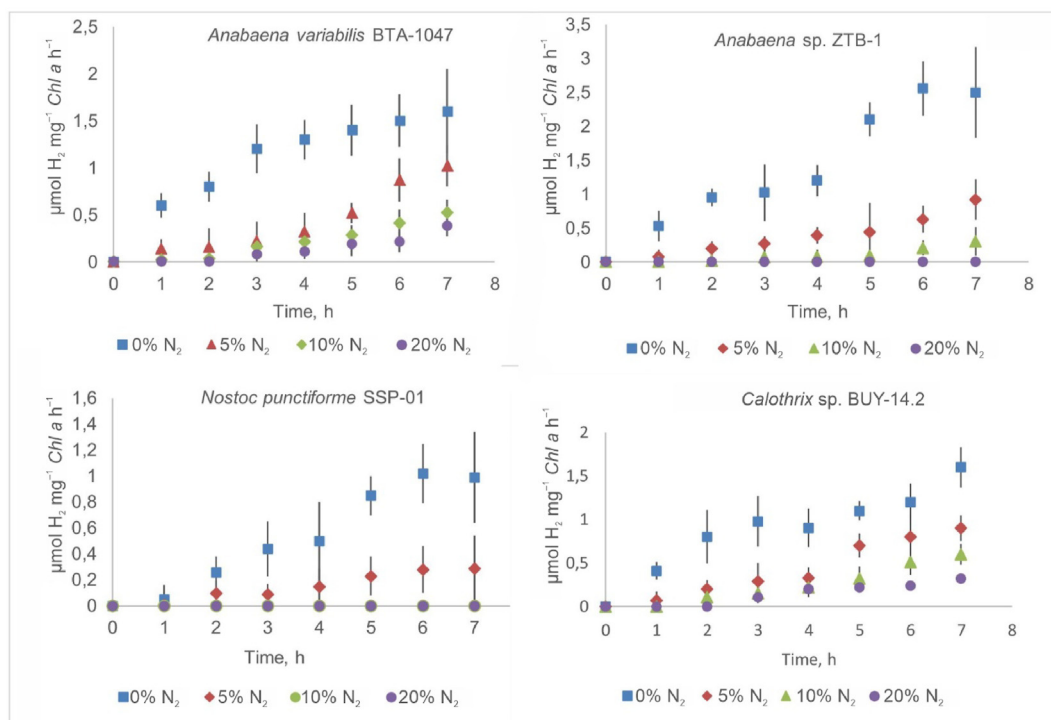


Fig. 5 – H₂ production profiles of four cyanobacterial species, immediately (0–7 h) after N₂ injection. The vials headspace was composed of argon with N₂ at concentrations of 1, 10, 20, and 40%. The experiment lasted for 7 h.

The following concepts were developed based on the research findings.

- 1) all N₂ concentrations have a detrimental influence on H₂ production;
- 2) the yield of H₂ produced by N₂-fixing cyanobacteria rises over time.

This time-dependent increase in H₂ production rate might be attributed to the depletion of cellular energy reserves required for N₂ase operation (16 ATP molecules must be hydrolysed for N₂ fixation; Fig. 1) [57].

The influence of CO₂ on H₂ production by isolated cyanobacterial species was then studied. Through air-water interaction, increased atmospheric CO₂ levels can raise dissolved CO₂ levels in freshwater systems, promoting primary production in water bodies and watersheds. Although elevated CO₂ promotes cyanobacterial growth and biomass, the consequences on their internal physiological processes may be more complicated. Phosphorylation and N₂ fixation in cyanobacteria are intimately tied to CO₂ levels in the air. A 20-fold increase in biomass yield was reported when the strain *Microcystis* HUB5-2-4 was cultivated in air containing 2.8% CO₂ [58], but highly intensive development of *Trichodesmium erythraeum* was detected at a concentration of 0.9% CO₂ [59]. However, some cyanobacteria can thrive at low CO₂ levels: *Crocospira watsonii* – 0.38% [60]; *Nostoc punctiforme* – 0.43% [61]; *Trichodesmium* – 0.8% [62]; *Dolichospermum circinale* – 0.33% [63]. Despite numerous studies on the effects of CO₂ on cyanobacterial biomass accumulation [64], phosphorylation metabolism [65,66], and light absorption mechanisms [67,68],

there has been relatively little study of the influences on H₂ production. The response of cyanobacterial cultures to different CO₂ concentrations varied. At a CO₂ concentration of 2%, all species produced a higher yield of H₂ when compared to the control and other experimental options. 2% CO₂ enhanced the H₂ production of strains *Anabaena variabilis* BTA-1047, *Anabaena* sp. ZTB-1, *Calothrix* sp. BUY-14.2, and *Nostoc punctiforme* SSP-01 by 25, 24, 36, and 46%, respectively, as compared to the control. However, in the other experimental options, the production of H₂ decreased linearly due to an increase in CO₂ concentrations (4%). The 8% CO₂ concentration, as predicted, had a detrimental influence on H₂ yield, with the lowest values obtained in this option.

According to the results, a 2% CO₂ concentration stimulates H₂ production. It should be mentioned that at CO₂ concentrations of 2 and 4%, cell growth was vigorous, and the cells did not enter the lethal phase for 4 weeks. Meanwhile, at 6% CO₂, the number of cells decreased and foams began to develop on the surface of the culture medium on the day 13, indicating that high CO₂ concentrations inhibit cyanobacterial cell proliferation [69]. The results demonstrated that 2% CO₂ had a favourable influence on the H₂ production activity of isolated strains (Fig. 6). The obtained results are consistent with the findings of Yeager et al. (2011) [36], who found that at 4% CO₂, six strains had high H₂ yields, with *Anabaena cylindrica* B629 having the highest value (55 $\mu\text{mol H}_2 \text{ mg}^{-1} \text{ Chl a h}^{-1}$). In the case of *Calothrix* 336/3 immobilisation, Leino et al. (2014) [27] observed that CO₂ concentrations favourable for H₂ production range in the 2–10% diapason, with a beneficial effect of 6% CO₂ on long-term (130 days) H₂ production. Furthermore, Touloupakis et al. (2016) [69] discovered that low CO₂

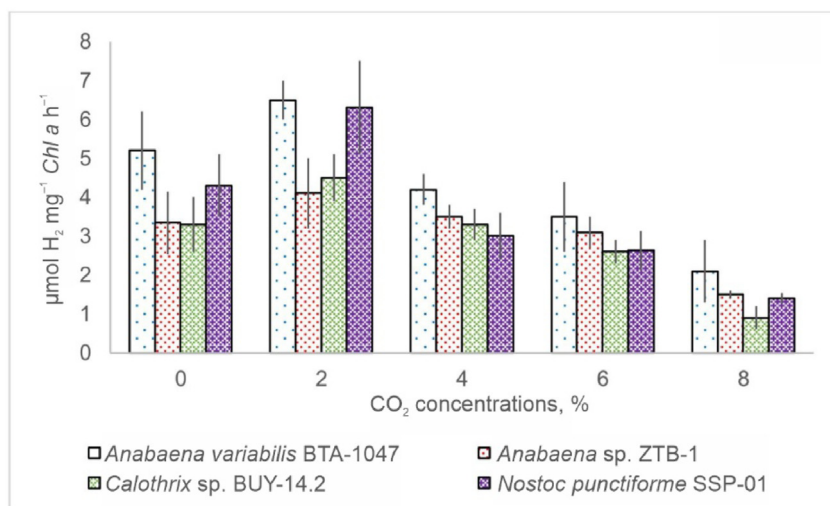


Fig. 6 – H₂ production profiles of four cyanobacterial species, immediately (0–7 h) after CO₂ injection. The vials headspace was composed of argon with CO₂ at 2, 4, 6, and 8%. The maximum yield of H₂ produced by strains at different time points is shown.

concentrations increase the H₂ production of immobilized cyanobacterial strains.

The rate of photosynthesis and N₂ fixation in cyanobacterial cells increases as carbon levels rise. Enhanced N₂ fixation increases the yield of H₂ molecules produced indirectly [50,70]. It should be highlighted, however, that the link between N₂ fixation and H₂ production, which should occur via N₂ase, is extremely intricate and warrants additional exploration. It is critical to note that once a strain has reached its maximum H₂ yield, it gradually decreases. This reduction is most likely related to a shortage of nutrition and N₂. After this alleged H₂ production experiment and the addition of a new growth medium to keep the cells growing, H₂ production will resume.

It can be concluded that a relatively low CO₂ concentration (2%) supplies or recovers the energy source required for H₂ production. PSII pigments serve as the primary energy donor in heterocyst cyanobacteria. The strain *Anabaena variabilis* BTA-1047 has high H₂ production and can effectively dispose of electrons gained during photosynthesis. The active strain *Anabaena variabilis* BTA-1047 was chosen for further research as an active H₂-producer with a high yield.

Influence of O₂:CO₂ ratio on hydrogen production

The primary goal of this study was to conduct optimisation to enhance H₂ production in N₂-fixing cyanobacteria. The effects of O₂, N₂, and CO₂ on H₂ production by cyanobacterial strains were studied in this context. The N₂-fixing cyanobacteria examined were extremely efficient at producing H₂ in the presence of O₂, and a 2% O₂ concentration encouraged normal H₂ production. In N₂-fixing cyanobacteria, there is a dynamic link between O₂ concentration and H₂ production rate, hence, the influence of the O₂:H₂ ratio on H₂ production may alter between strains [70].

Another study looked at the effect of O₂ and CO₂ on H₂ productivity in the strain *Anabaena variabilis* BTA-1047, which produced a high H₂ yield when stimulated with the gases O₂ and CO₂. N₂ gas was not employed in subsequent studies since

its low concentration reduced H₂ productivity. The influence of varying concentrations of O₂ and CO₂ gases on H₂ production was investigated, as shown in Fig. 7.

It was found that the ratio of O₂ to CO₂ has a different influence on H₂ production. At an O₂:CO₂ ratio of 1%:2%, high H₂ production was obtained (110% of the control). However, remarkable results were obtained at O₂:CO₂ ratios of 1%:1% and 2%:1%. High CO₂ (1%:8%) and O₂ (8%:1%) concentrations resulted in very low H₂ production (15 and 58% of the control, respectively). It should be emphasised that at high CO₂ concentrations (1%:8%), the O₂ volume was 16.2% higher than at baseline. At the same time, at low CO₂ concentrations (8%:1%), O₂ volume increased by 4.3% compared to baseline. This phenomenon occurs when the cells grow vigorously at high CO₂ levels and the activity of the PSII complex increases, resulting in the intense release of O₂ molecules into free space. The data we obtained were pooled with those of other researchers. Yeager et al. (2011) [36] found that low concentrations of O₂ and CO₂ (1%:3%) positively affect H₂ production. At high CO₂ concentrations (8%), Bandyopadhyay et al. (2010) [16] discovered that strain *Cyanospora* 51142 produced extremely high H₂ yields (230 μmol H₂ mg⁻¹ Chl a h⁻¹). The authors attributed the strain tolerance to high-dose released O₂ concentrations to the use of the circadian rhythm. It has been found that adding glycerol to the culture medium as a supplementary carbon source increased H₂ yield in the 12/12-h light/dark cycle. Furthermore, Touloupakis et al. (2016) [69] reported that an O₂:CO₂ ratio of 1%:3% positively influenced H₂ production by the strain *Synechocystis* sp. PCC 6803. The H₂ production activity of the strain *Anabaena variabilis* BTA-1047 has been reported to be enhanced at low concentrations of O₂ and CO₂. The high concentration of CO₂ enhances the photosynthetic activity and releases a large number of O₂ molecules into the environment, inhibiting N₂ase and H₂ase activity. The optimal H₂ production environment for the N₂-fixing strain *Anabaena variabilis* BTA-1047 was discovered to have an O₂:CO₂ ratio of 1%:2%.

Decades of research have brought numerous principles of bio-H₂ production to light. However, increasing bio-H₂

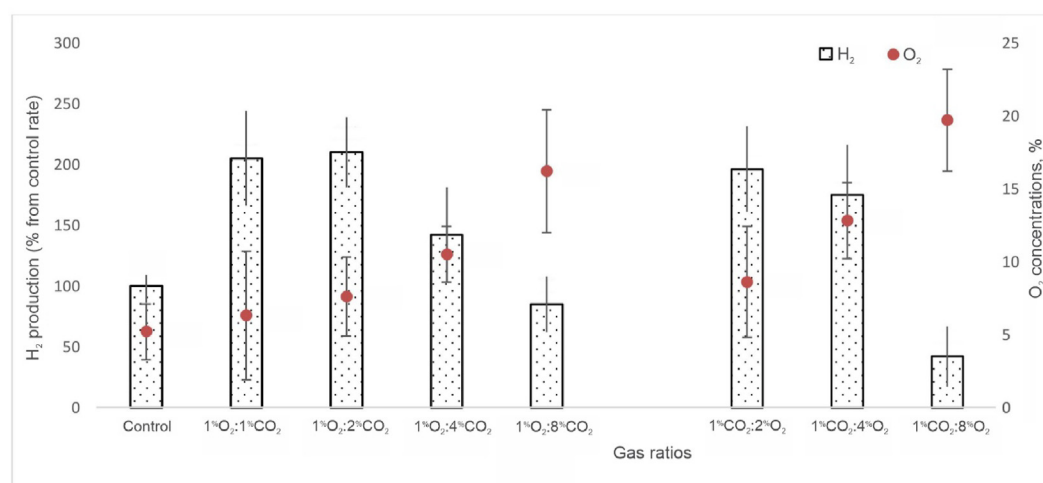


Fig. 7 – Influence of O₂:CO₂ gas ratios on hydrogen production. The vial headspace consisted of argon with different O₂:CO₂ gas ratios. A vacuum excited with 100% argon was used as a control, and the H₂ yield in control was 5.3 μmol H₂ mg^{−1} Chl *a* h^{−1} (100%). The share (%) of O₂ was estimated at the peak of H₂ production.

production significantly has proven to be a considerable obstacle. Compared to the respective wild strains, genetic modifications of the H₂ production pathways led to higher production rates. However, since the yield of H₂ produced by these wild strains is low, even a 20-fold increase in the yield of the mutant strains is not enough to attain industrial-scale production. Our discovery of cyanobacterial strains that exhibit high H₂ production rates under aerobic conditions offers new avenues for photobiological studies on H₂ production. The 11 cyanobacterial strains discovered, four of which are active H₂ producers, can potentially be considered model organisms for future genetic engineering studies. The thorough investigation of this phenomenon in *Anabaena variabilis* BTA-1047 will disclose previously undiscovered cellular components and regulatory systems that control this process, allowing them to be improved to produce even more H₂ as an energy carrier.

Conclusions

Governments, industries, and many countries throughout the world continue to prioritise the use of fossil fuels over human health. Environmental issues caused by the uncontrolled use of conventional energy sources have enhanced the relevance of alternative energy development initiatives. 11 strains of N₂-fixing cyanobacteria were isolated from natural sources for the current study, and four strains were identified as active H₂ producers. During the H₂ yield optimisation, it was discovered that the strains *Anabaena variabilis* BTA-1047 and *Nostoc punctiforme* SSP-01 have a beneficial influence on H₂ production at a 2% O₂ concentration. All investigated concentrations of N₂ gas in free space (2–8%) were shown to have a detrimental influence on the H₂ yield of all four strains. Meanwhile, 2% CO₂ favorably influenced the long-term production of H₂ for all strains examined, although high CO₂ concentrations reduced H₂ yield due to an increase in O₂ content in free space. The strain *Anabaena variabilis* BTA-1047, selected as the most active H₂ producer based on optimisation studies, was

positively affected by the presence of an O₂:CO₂ gas ratio of 1%:2%, i.e., H₂ yield increased twofold. According to the results, H₂ production by N₂-fixing cyanobacterial strains requires a micro-anaerobic environment, and low CO₂ concentrations (2%) allow for long-term H₂ production and cell viability.

CRediT authorship contribution statement

GKK, literature, experimental works and writing; BKD, literature, plan survey and analysis; AKS, main idea and consulting, MOB and AMB, experimental works and writing; BKZ, TT consulting; HFA, TT and SIA, main idea, consulting, writing. All the authors contributed to writing of the final version and approved the manuscript.

Declaration of competing interest

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

Acknowledgements

This research was supported by the Ministry of Higher Education and Science of the Republic of Kazakhstan in the framework of the following projects: AP14869048, AP13067574, and AP09260077. HFA gratefully acknowledges support from the Distinguished Scientists Fellowship Programme, King Abdulaziz University, Saudi Arabia. AMB and SIA were supported by the grant from Russian Science Foundation (22–44–08001). Fig. 1 was supported under the state contract of the Ministry of Science and Higher Education of the Russian Federation (theme No. 122050400128-1). The present work was also financially supported in part by Grants-in-Aid for Scientific Research (No. 21K06101 (TT)).

REFERENCES

- [1] Lau N-S, Matsui M, Abdullah AA-A. Cyanobacteria: photoautotrophic microbial factories for the sustainable synthesis of industrial products. *BioMed Res Int* 2015;754934. <https://doi.org/10.1155/2015/754934>.
- [2] Sadvakasova AK, Kossalbayev BD, Zayadan BK, Kirbayeva DK, Alwasel S, Allakhverdiev SI. Potential of cyanobacteria in the conversion of wastewater to biofuels. *World J Microbiol Biotechnol* 2021;37:140. <https://doi.org/10.1007/s11274-021-03107-1>.
- [3] Sadvakasova AK, Kossalbayev BD, Zayadan BK, Bolatkhan K, Alwasel S, Najafpour MM, et al. Bioprocesses of hydrogen production by cyanobacteria cells and possible ways to increase their productivity. *Renew Sustain Energy Rev* 2020;133:110054. <https://doi.org/10.1016/j.rser.2020.110054>.
- [4] Masukawa H, Kitashima M, Inoue K, Sakurai H, Hausinger R. Genetic engineering of cyanobacteria to enhance biohydrogen production from sunlight and water. *Ambio* 2012;41:169–73. <https://doi.org/10.1007/s13280-012-0275-4>.
- [5] Akimbekov N, Digel I, Abdieva G, Ualieva P, Tastambek K. Lignite biosolubilization and bioconversion by *Bacillus* sp.: the collation of analytical data. *Biofuels* 2021;12:247–58. <https://doi.org/10.1080/17597269.2020.1753936>.
- [6] Sadvakasova AK, Kossalbayev BD, Token AI, Bauanova MO, Wang J, Zayadan BK, et al. Influence of Mo and Fe on photosynthetic and nitrogenase activities of nitrogen-fixing cyanobacteria under nitrogen starvation. *Cells* 2022;11:904. <https://doi.org/10.3390/cells11050904>.
- [7] Weyman PD, Pratte B, Thiel T. Transcription of *hupSL* in *Anabaena variabilis* ATCC 29413 is regulated by NtcA and not by hydrogen. *Appl Environ Microbiol* 2008;74:2103–10. <https://doi.org/10.1128/AEM.02855-07>.
- [8] Zayadan B, Ussebayeva A, Bolatkhan K, Akmukhanova A, Kossalbayev B, Baizhigitova A, et al. Screening of isolated and collection strains of cyanobacteria on productivity for determining their biotechnological potential. *Eur J Entomol* 2018;2:121–30. <https://doi.org/10.26577/EJE-2018-2-823>.
- [9] Min H, Sherman LA. Hydrogen production by the unicellular, diazotrophic cyanobacterium *Cyanothece* sp. strain ATCC 51142 under conditions of continuous light. *Appl Environ Microbiol* 2010;76:4293–301. <https://doi.org/10.1128/AEM.00146-10>.
- [10] Wang Z, Gao D, Geng H, Xing C. Enhancing hydrogen production by photobiocatalysis through *Rhodospseudomonas palustris* coupled with conjugated polymers. *J Mater Chem* 2021;9:19788. <https://doi.org/10.1039/D1TA01019K>. –95.
- [11] Hemschemeier A, Fouchard S, Cournac L, Peltier G, Happe T. Hydrogen production by *Chlamydomonas reinhardtii*: an elaborate interplay of electron sources and sinks. *Planta* 2008;227:397–407. <https://doi.org/10.1007/s00425-007-0626-8>.
- [12] Ferreira AF, Marques AC, Batista AP, Marques PASS, Gouveia L, Silva CM. Biological hydrogen production by *Anabaena* sp. – yield, energy and CO₂ analysis including fermentative biomass recovery. *Int J Hydrogen Energy* 2012;37:179–90. <https://doi.org/10.1016/j.ijhydene.2011.09.056>.
- [13] Ortega-Ramos M, Jittawuttipoka T, Saenkham P, Czarnecka-Kwasiborski A, Bottin H, Cassier-Chauvat C, et al. Engineering *Synechocystis* PCC6803 for hydrogen production: influence on the tolerance to oxidative and sugar stresses. *PLoS One* 2014;9:e89372. <https://doi.org/10.1371/journal.pone.0089372>.
- [14] Dutta D, De D, Chaudhuri S, Bhattacharya SK. Hydrogen production by cyanobacteria. *Microb Cell Factories* 2005;4:36. <https://doi.org/10.1186/1475-2859-4-36>.
- [15] McIntosh CL, Germer F, Schulz R, Appel J, Jones AK. The [NiFe]-Hydrogenase of the cyanobacterium *Synechocystis* sp. PCC 6803 works bidirectionally with a bias to H₂ production. *J Am Chem Soc* 2011;133:11308–19. <https://doi.org/10.1021/ja203376y>.
- [16] Bandyopadhyay A, Stöckel J, Min H, Sherman LA, Pakrasi HB. High rates of photobiological H₂ production by a cyanobacterium under aerobic conditions. *Nat Commun* 2010;1:139. <https://doi.org/10.1038/ncomms1139>.
- [17] Bolatkhan K, Sadvakasova AK, Zayadan BK, Kakimova AB, Sarsekeyeva FK, Kossalbayev BD, Bozieva AM, Alwasel S, Allakhverdiev SI. Prospects for the creation of a waste-free technology for wastewater treatment and utilization of carbon dioxide based on cyanobacteria for biodiesel production. *J Biotechnol* 2020;324:162–70. <https://doi.org/10.1016/j.jbiotec.2020.10.010>.
- [18] Singh RP, Yadav P, Gupta RK. Chapter 11 – modern strategy of cyanobacterial biohydrogen production and current approaches toward its enhancement. In: Singh PK, Fillat MF, Sittler V, Kumar A, editors. *Expanding horizon of cyanobacterial biology*. Academic Press; 2022. p. 219–38. <https://doi.org/10.1016/B978-0-323-91202-0.00002-6>.
- [19] Sivaramakrishnan R, Incharoensakdi A. Cyanobacteria as renewable sources of bioenergy (biohydrogen, bioethanol, and bio-oil production). In: Rastogi RP, editor. *Ecophysiology and biochemistry of cyanobacteria*. Singapore: Springer Nature; 2021. p. 431–54. https://doi.org/10.1007/978-981-16-4873-1_19.
- [20] Kamshybayeva GK, Kossalbayev BD, Sadvakasova AK, Zayadan BK, Bozieva AM, Dunikov D, et al. Strategies and economic feasibilities in cyanobacterial hydrogen production. *Int J Hydrogen Energy* 2022;47:29661–84. <https://doi.org/10.1016/j.ijhydene.2022.06.277>.
- [21] Bolatkhan K, Kossalbayev BD, Zayadan BK, Tomo T, Veziroglu TN, Allakhverdiev SI. Hydrogen production from phototrophic microorganisms: reality and perspectives. *Int J Hydrogen Energy* 2019;44:5799–811. <https://doi.org/10.1016/j.ijhydene.2019.01.092>.
- [22] Allahverdiyeva Y, Leino H, Saari L, Fewer DP, Shunmugam S, Sivonen K, et al. Screening for biohydrogen production by cyanobacteria isolated from the Baltic Sea and Finnish lakes. *Int J Hydrogen Energy* 2010;35:1117–27. <https://doi.org/10.1016/j.ijhydene.2009.12.030>.
- [23] Lindberg P, Schütz K, Happe T, Lindblad P. A hydrogen-producing, hydrogenase-free mutant strain of *Nostoc punctiforme* ATCC 29133. *Int J Hydrogen Energy* 2002;27:1291–6. [https://doi.org/10.1016/S0360-3199\(02\)00121-0](https://doi.org/10.1016/S0360-3199(02)00121-0).
- [24] Yoshino F, Ikeda H, Masukawa H, Sakurai H. High photobiological hydrogen production activity of a *Nostoc* sp. PCC 7422 uptake hydrogenase-deficient mutant with high nitrogenase activity. *Mar Biotechnol* 2007;9:101–12. <https://doi.org/10.1007/s10126-006-6035-3>.
- [25] Lindberg P, Hansel A, Lindblad P. *hupS* and *hupL* constitute a transcription unit in the cyanobacterium *Nostoc* sp. PCC 73102. *Arch Microbiol* 2000;174:129–33. <https://doi.org/10.1007/s002030000186>.
- [26] Kossalbayev BD, Kakimova AB, Bolatkhan K, Zayadan BK, Sandybayeva SK, Bozieva AM, et al. Biohydrogen production by novel cyanobacterial strains isolated from rice paddies in Kazakhstan. *Int J Hydrogen Energy* 2022;47:16440–53. <https://doi.org/10.1016/j.ijhydene.2022.03.126>.
- [27] Leino H, Shunmugam S, Isojärvi J, Oliveira P, Mulo P, Saari L, et al. Characterization of ten H₂ producing cyanobacteria isolated from the Baltic Sea and Finnish lakes. *Int J Hydrogen Energy* 2014;39:8983–91. <https://doi.org/10.1016/j.ijhydene.2014.03.171>.
- [28] Schütz K, Happe T, Troshina O, Lindblad P, Leitão E, Oliveira P, et al. Cyanobacterial H₂ production – a comparative analysis. *Planta* 2004;218:350–9. <https://doi.org/10.1007/s00425-003-1113-5>.
- [29] Weissman JC, Benemann JR. Hydrogen production by nitrogen-starved cultures of *Anabaena cylindrica*. *Appl*

- Environ Microbiol 1977;33:123–31. <https://doi.org/10.1128/aem.33.1.123-131.1977>.
- [30] Kumar D, Kumar HD. Hydrogen production by several cyanobacteria. Int J Hydrogen Energy 1992;17:847–52. [https://doi.org/10.1016/0360-3199\(92\)90034-T](https://doi.org/10.1016/0360-3199(92)90034-T).
- [31] Kossalbayev BD, Tomo T, Zayadan BK, Sadvakasova AK, Bolatkhan K, Alwasel S, et al. Determination of the potential of cyanobacterial strains for hydrogen production. Int J Hydrogen Energy 2020;45:2627–39. <https://doi.org/10.1016/j.ijhydene.2019.11.164>.
- [32] Vyas D, Kumar HD. Nitrogen fixation and hydrogen uptake in four cyanobacteria. Int J Hydrogen Energy 1995;20:163–8. [https://doi.org/10.1016/0360-3199\(94\)E0008-M](https://doi.org/10.1016/0360-3199(94)E0008-M).
- [33] Ghirardi ML, Posewitz MC, Maness P-C, Dubini A, Yu J, Seibert M. Hydrogenases and hydrogen photoproduction in oxygenic photosynthetic organisms. Annu Rev Plant Biol 2007;58:71–91. <https://doi.org/10.1146/annurev.arplant.58.032806.103848>.
- [34] Barbier FF, Chabikwa TG, Ahsan MU, Cook SE, Powell R, Tanurdzic M, et al. A phenol/chloroform-free method to extract nucleic acids from recalcitrant, woody tropical species for gene expression and sequencing. Plant Methods 2019;15:62. <https://doi.org/10.1186/s13007-019-0447-3>.
- [35] David KAV, Apte SK, Banerji A, Thomas J. Acetylene reduction assay for nitrogenase activity: gas chromatographic determination of ethylene per sample in less than one minute. Appl Environ Microbiol 1980;39:1078–80. <https://doi.org/10.1128/aem.39.5.1078-1080.1980>.
- [36] Yeager CM, Milliken CE, Bagwell CE, Staples L, Berseth PA, Sessions HT. Evaluation of experimental conditions that influence hydrogen production among heterocystous Cyanobacteria. Int J Hydrogen Energy 2011;36:7487. <https://doi.org/10.1016/j.ijhydene.2011.03.078>.
- [37] Lindberg P, Lindblad P, Cournac L. Gas exchange in the filamentous cyanobacterium *Nostoc punctiforme* strain ATCC 29133 and its hydrogenase-deficient mutant strain NHM5. Appl Environ Microbiol 2004;70:2137–45. <https://doi.org/10.1128/AEM.70.4.2137-2145.2004>.
- [38] Tamagnini P, Leitão E, Oliveira P, Ferreira D, Pinto F, Harris DJ, et al. Cyanobacterial hydrogenases: diversity, regulation and applications. FEMS Microbiol Rev 2007;31:692–720. <https://doi.org/10.1111/j.1574-6976.2007.00085.x>.
- [39] Bothe H, Schmitz O, Yates MG, Newton WE. Nitrogen fixation and hydrogen metabolism in cyanobacteria. Microbiol Mol Biol Rev 2010;74:529–51. <https://doi.org/10.1128/MMBR.00033-10>.
- [40] Soumare A, Diedhiou AG, Thuita M, Hafidi M, Ouhdouch Y, Gopalakrishnan S, et al. Exploiting biological nitrogen fixation: a route towards a sustainable agriculture. Plants 2020;9:1011. <https://doi.org/10.3390/plants9081011>.
- [41] Imran A, Hakim S, Tariq M, Nawaz MS, Laraib I, Gulzar U, et al. Diazotrophs for lowering nitrogen pollution crises: looking deep into the roots. Front Microbiol 2021;12:637815. <https://doi.org/10.3389/fmicb.2021.637815>.
- [42] Ernst A, Liu Y-D, Reich S, Böger P. Diurnal nitrogenase modification in the cyanobacterium *Anabaena variabilis*. Bot Acta 1990;103:183–9. <https://doi.org/10.1111/j.1438-8677.1990.tb00146.x>.
- [43] Vignais PM, Billoud B. Occurrence, classification, and biological function of hydrogenases: an overview. Chem Rev 2007;107:4206–72. <https://doi.org/10.1021/cr050196r>.
- [44] Golden JW, Yoon H-S. Heterocyst development in *Anabaena*. Curr Opin Microbiol 2003;6:557–63. <https://doi.org/10.1016/j.mib.2003.10.004>.
- [45] Ekman M, Ow SY, Holmqvist M, Zhang X, van Wagenen J, Wright PC, et al. Metabolic adaptations in a H₂ producing heterocyst-forming cyanobacterium: potentials and implications for biological engineering. J Proteome Res 2011;10:1772–84. <https://doi.org/10.1021/pr101055v>.
- [46] Kumar K, Mella-Herrera RA, Golden JW. Cyanobacterial heterocysts. Cold Spring Harbor Perspect Biol 2010;2:a000315. <https://doi.org/10.1101/cshperspect.a000315>.
- [47] Yoon K-S, Nguyen NT, Tran KT, Tsuji K, Ogo S. Nitrogen fixation genes and nitrogenase activity of the non-heterocystous cyanobacterium *thermoleptolyngbya* sp. O-77. Microb Environ 2017;32:324–9. <https://doi.org/10.1264/jisme.2017015>.
- [48] Mishra AK, Kaushik MS, Tiwari DN. Chapter 8 – nitrogenase and hydrogenase: enzymes for nitrogen fixation and hydrogen production in cyanobacteria. In: Mishra AK, Tiwari DN, Rai AN, editors. Cyanobacteria. Academic Press; 2019. p. 173–91. <https://doi.org/10.1016/B978-0-12-814667-5.00008-8>.
- [49] Tsygankov AA, Borodin VB, Rao KK, Hall DOH. (2) photoproduction by batch culture of *Anabaena variabilis* ATCC 29413 and its mutant PK84 in a photobioreactor. Biotechnol Bioeng 1999;64:709–15. [https://doi.org/10.1002/\(sici\)1097-0290\(19990920\)64:6<709::aid-bit10>3.0.co;2-c](https://doi.org/10.1002/(sici)1097-0290(19990920)64:6<709::aid-bit10>3.0.co;2-c).
- [50] Madamwar D, Garg N, Shah V. Cyanobacterial hydrogen production. World J Microbiol Biotechnol 2000;16:757–67. <https://doi.org/10.1023/A:1008919200103>.
- [51] Berberoğlu H, Barra N, Pilon L, Jay J. Growth, CO₂ consumption and H₂ production of *Anabaena variabilis* ATCC 29413-U under different irradiances and CO₂ concentrations. J Appl Microbiol 2008;104:105–21. <https://doi.org/10.1111/j.1365-2672.2007.03559.x>.
- [52] Benemann JR, Weare NM. Hydrogen evolution by nitrogen-fixing *Anabaena cylindrica* cultures. Science 1974;184:174–5. <https://doi.org/10.1126/science.184.4133.174>.
- [53] Tamagnini P, Axelsson R, Lindberg P, Oxelfelt F, Wünschiers R, Lindblad P. Hydrogenases and hydrogen metabolism of cyanobacteria. Microbiol Mol Biol Rev 2002;66:1–20. <https://doi.org/10.1128/MMBR.66.1.1-20.2002>.
- [54] Harris DF, Lukoyanov DA, Shaw S, Compton P, Tokmina-Lukaszewska M, Bothner B, et al. Mechanism of N₂ reduction catalyzed by Fe-nitrogenase involves reductive elimination of H₂. Biochemistry 2018;57:701–10. <https://doi.org/10.1021/acs.biochem.7b01142>.
- [55] Allakhverdiev ES, Khabatova VV, Kossalbayev BD, Zadneprovskaya EV, Rodnenkov OV, Martynyuk TV, et al. Raman spectroscopy and its modifications applied to biological and medical research. Cells 2022;11:386. <https://doi.org/10.3390/cells11030386>.
- [56] de Vries W, Stam H, Stouthamer AH. Hydrogen oxidation and nitrogen fixation in rhizobia, with special attention focused on strain ORS 571. Antonie Leeuwenhoek 1984;50:505–24. <https://doi.org/10.1007/BF02386223>.
- [57] Heiniger EK, Oda Y, Samanta SK, Harwood CS. How posttranslational modification of nitrogenase is circumvented in *Rhodospseudomonas palustris* strains that produce hydrogen gas constitutively. Appl Environ Microbiol 2012;78:1023–32. <https://doi.org/10.1128/AEM.07254-11>.
- [58] Verspagen JMH, Waal DBV de, Finke JF, Visser PM, Donk EV, Huisman J. Rising CO₂ levels will intensify phytoplankton blooms in eutrophic and hypertrophic lakes. PLoS One 2014;9:e104325. <https://doi.org/10.1371/journal.pone.0104325>.
- [59] Eichner M, Basu S, Gledhill M, de Beer D, Shaked Y. Hydrogen dynamics in *Trichodesmium* colonies and their potential role in mineral iron acquisition. Front Microbiol 2019;10. <https://doi.org/10.3389/fmicb.2019.01565>.
- [60] Fu F-X, Mulholland MR, Garcia NS, Beck A, Bernhardt PW, Warner ME, et al. Interactions between changing pCO₂, N₂ fixation, and Fe limitation in the marine unicellular cyanobacterium *Crocospaera*. L&O 2008;53:2472–84. <https://doi.org/10.4319/lo.2008.53.6.2472>.

- [61] Lindo Z, Griffith DA. Elevated atmospheric CO₂ and warming stimulates growth and nitrogen fixation in a common forest floor cyanobacterium under axenic conditions. *Forests* 2017;8:73. <https://doi.org/10.3390/f8030073>.
- [62] Li X, Fonseca-Batista D, Roevros N, Dehairs F, Chou L. Environmental and nutrient controls of marine nitrogen fixation. *Prog Oceanogr* 2018;167:125–37. <https://doi.org/10.1016/j.pocean.2018.08.001>.
- [63] Symes E, van Ogtrop FF. The effect of pre-industrial and predicted atmospheric CO₂ concentrations on the development of diazotrophic and non-diazotrophic cyanobacterium: *Dolichospermum circinale* and *Microcystis aeruginosa*. *Harmful Algae* 2019;88:101536. <https://doi.org/10.1016/j.hal.2018.10.005>.
- [64] Sandybayeva SK, Kossalbayev BD, Zayadan BK, Sadvakasova AK, Bolatkhan K, Zadneprovskaya EV, et al. Prospects of cyanobacterial pigment production: biotechnological potential and optimization strategies. *Biochem Eng J* 2022;187:108640. <https://doi.org/10.1016/j.bej.2022.108640>.
- [65] Abdrahimov R, Amirgaliyeva A, Tastambek K, Zhumalipov A, Polyakova S. Annual river runoff of the ile-balkash basin and prospects of its assessment due to climatic changes and water economy activities. *Int J GEOMATE* 2020;18:230–9. <https://doi.org/10.21660/2020.69.32068>.
- [66] Price GD, Badger MR, Woodger FJ, Long BM. Advances in understanding the cyanobacterial CO₂-concentrating-mechanism (CCM): functional components, Ci transporters, diversity, genetic regulation and prospects for engineering into plants. *J Exp Bot* 2008;59:1441–61. <https://doi.org/10.1093/jxb/erm112>.
- [67] Tastambek K, Akimbekov N, Yernazarova A, Kayyrmanova GK, Abdiyeva G, Uliyeva PS. The evaluation of microbial diversity in water and soil samples from Atyrau and Mangystau regions. *Ecology series* 2016;3:77–83. <https://doi.org/10.26577/EJE-2016-3-889>.
- [68] Zhubanova AA, Xiaohui Q, Ualieva PS, Abdieva G, Tastambek KT, Kayrmanova GK, et al. Metagenomic analysis reveals correlation between microbiome structure and leonardite characteristics from Kazakhstan coal deposits. *Eur Ch-Techn J* 2019;21:135–41. <https://doi.org/10.18321/ectj823>.
- [69] Touloupakis E, Rontogiannis G, Silva Benavides AM, Cicchi B, Ghanotakis DF, Torzillo G. Hydrogen production by immobilized *Synechocystis* sp. PCC 6803. *Int J Hydrogen Energy* 2016;41:15181–6. <https://doi.org/10.1016/j.ijhydene.2016.07.075>.
- [70] Fay P. Oxygen relations of nitrogen fixation in cyanobacteria. *Microbiol Rev* 1992;56:340–73. <https://doi.org/10.1128/mr.56.2.340-373.1992>.