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Effect of the photosynthesis inhibitors on hydrogen production by non-heterocyst cyanobacterial strains

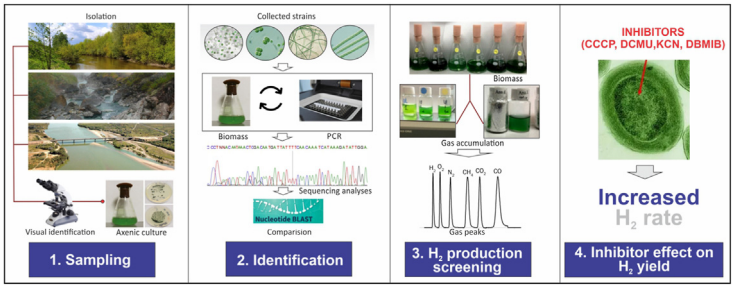
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HIGHLIGHTS

- Six species of cyanobacteria from different ecosystems were isolated and identified.
- The H_2-producing activity of the strains was studied in the dark and in the light.
- The effect of four inhibitors of photosynthesis on H_2 production was studied.
- The effect of light and dark modes was evaluated on H_2 production by cyanobacteria.

GRAPHICAL ABSTRACT



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ABSTRACT

The energy of cyanobacterial hydrogen (H_2) produced via bio-photolysis is being investigated as a potential solution to early-century environmental challenges. The main limiting factors of cyanobacterial H_2 photoproduction are the availability of electrons for [NiFe]-hydrogenase (H_2ase) and the suppression of bidirectional H_2ase activity induced by O_2 acquired from water molecules splitting in photosystem II. The current study investigated how photosynthetic inhibitors (PIs) affected H_2 production in non-N_2-fixing cyanobacteria. Study findings revealed a rather high H_2 yield in *Synechocystis* sp. PSU 1262, as well as a beneficial (14.2-fold) influence of 500 μmol KCN on the H_2 production by the aforesaid strain. A 12/6-h light/dark cycle increased H_2 production by 80.3% in cells supplemented with 500 μmol KCN. Under the optimised conditions, the photobiological H_2 production of *Synechocystis* sp. PSU 1262 increased from 49.6 to 1552 nmol H_2 mg^{-1} *Chl a* h^{-1}. PIs suppressed chlorophyll *a* concentration under illumination, lowering the O_2 levels, which enhanced bidirectional H_2ase activity in *Synechocystis* sp. PSU 1262 cells. Applying varied light modes, preceded by the incorporation of PIs at optimal concentrations in H_2 production by research cyanobacterial strains, improved the H_2 yield and contributed significantly to the research originality.

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Abbreviations

ATP	Adenosine triphosphate
BG_0-11	BG-11 medium without nitrogen source
CCCP	m-chloro-carbonyl-cyanide-phenyl-hydrazone
Chl	Chlorophyll
CO_2	Carbon dioxide
Cyt b_6f	Cytochrome b_6f
DBMIB	2,4-dibromo-3-methyl-6-isopropyl-benzoquinone
DCMU	N-(3,4-dichlorophenyl)-N′,N′-dimethylurea
DMSO	Dimethyl sulfoxide
ETC	Electron transport chain
Fd	Ferredoxin
FNR	Ferredoxin-NADP reductase
GC	Gas chromatograph
H^+	Hydrogen ion
H_2	Hydrogen
H_2ase	Hydrogenase
KCN	Cyanide
N_2	Nitrogen
N_2ase	Nitrogenase
$NADP^+$	Nicotinamide adenine dinucleotide phosphate
NDH-I	NADPH dehydrogenase (complex I)
O_2	Oxygen
OD	Optical density
PC	Plastocyanin
PIs	Photosynthetic inhibitors
PQH_2	Plastoquinone pool
PSI	Photosystem I
PSII	Photosystem II

Introduction

Petroleum products and coal as primary energy sources have greatly damaged the natural landscapes in recent years, raising the global average temperature by 1.5 °C during the past century [1–3]. Scientists have strengthened the efforts to develop physical, biological, and chemically extracted and regenerated energy sources based on inexhaustible substrates in order to find a solution [4,5]. Hydrogen (H_2) is therefore seen as a potential substitute for energy, as it can be produced by capturing sunlight [6–8]. The development of cyanobacterial species that actively absorb a broad spectrum of sunlight utilising the pigments present in their thylakoid membranes has been one of the most important areas of research over the last five decades.

Under stress conditions, cyanobacteria use two primary enzyme complexes, i.e., hydrogenase (H_2ase) in vegetative cells and nitrogenase (N_2ase) in heterocyst cells, to transform excess energy stored as adenosine triphosphate (ATP) into H_2 by utilising two hydrogen ions (H^+) [9]. When oxygen (O_2) molecules are present, the enzyme either has a decreased intensity or does not produce H_2 at all [10–14]. Hydrogen enzymes, on the other hand, can be induced by inhibiting photosystem II (PSII) complex using photosynthetic inhibitors (PIs) [15–18]. Inhibitors of the electron transport chain (ETC) can be utilised efficiently to increase cyanobacterial cell productivity in H_2 production [19–21]. The H_2 production is limited by the presence of O_2 molecules generated by the active operation of the PSII complex. PIs can enhance the H_2 yield by mitigating this limitation [22]. Inhibitors such as m-chloro-carbonyl-cyanide-phenyl-hydrazone (CCCP), N-(3,4-dichlorophenyl)-N′,N′-dimethylurea (DCMU), 2,4-dibromo-3-methyl-6-isopropyl-benzoquinone (DBMIB), and cyanide

(KCN) are regularly utilised in order to impede the ETC in cyanobacterial cells. Furthermore, all of the inhibitors mentioned have the potential to enhance bio-H_2 production. Indeed, Cournac et al. (2004) discovered that under dark anaerobic conditions, 75 μM DCMU led to an increase in H_2 production by *Synechocystis* sp. PCC 6803 [19].

DCMU is strongly linked to the quinone binding site in the PSII reaction centre due to its resemblance in structure to singly reduced plastoquinone (PQ) [23]. KCN and CCCP, on the other hand, inhibit quinol oxidase leading to an increased PQ pool recovery [24]. KCN can obstruct H_2 uptake [25] or electron transport from cytochrome b_6f (Cyt b_6f) to photosystem I (PSI) through plastocyanin [26], as well as DBMIB. Moreover, several studies have shown that DBMIB can induce the activation of genes associated with nitrate assimilation [27,28] and hence increase H_2 production. This occurs because nitrate assimilation serves as an electron sink [29]. On the other hand, DBMIB was discovered to lower photo-H_2 production while not affecting H_2ase activity [19]. In the case of succinate dehydrogenase, it was found to reduce the PQ pool in the dark. Malonate, when tested for its behaviour under light exposure, does not affect the complete recovery of the PQ pool in other mutants, such as those lacking terminal oxidase [30].

Light energy (photons; $\lambda = 680$ nm) is absorbed by chlorophyll *a* (*Chl a*) molecules within the PSII reaction centres, resulting in the movement of an electron within the molecules to a higher energy level [31,32]. Then excited electrons travel through the plastoquinone pool (PQ/PQH_2), the Cyt b_6f complex, plastocyanin, and PSI before reaching the terminal electron acceptor ferredoxin (Fd) [33]. The pigments in PSI absorb light at a wavelength of 700 nm, causing electrons to be emitted and transferred to diminish Fd. PSII obtains electrons from the water-splitting reaction, which leads to the generation of O_2 as a by-product (Fig. 1). PIs may be capable of enhancing H_2 production by diverting the electron flow towards N_2ase and/or bidirectional *Hox*-H_2ase. Under light conditions, *Aphanothece halophytica* can increase both H_2 production and bidirectional *Hox*-H_2ase activity by inhibiting the NADH-dehydrogenase complex I (NDH-I) using a particular inhibitor, CCCP [34,35].

Currently, different studies have highlighted that the improvement of cyanobacterial hydrogen production rates treated with PIs. For example, Pansook et al. (2019) conducted a study on the impact of the PSII inhibitors CCCP and DCMU on H_2 production, photosynthetic activity, and respiratory activity of *A. halophytica* under light and dark conditions. The findings revealed that CCCP- and DCMU-treated cells demonstrated a 3–5 times increase in H_2 production rate when exposed to light compared to untreated cells. *Synechocystis* sp. PCC 6803 was able to produce 30 times more H_2 under light compared to the control by utilising KCN as an inhibitor [36]. The use of DCMU as an inhibitor to hinder PSII function has been extensively studied. Weissman and Benemann (1977) found that treating *A. cylindrica*, a nitrogen (N_2)-fixing cyanobacterium, by DCMU can increase H_2 production by enhancing N_2ase activity and providing protection against O_2 inhibition [37]. A robust H_2 production was observed in the unicellular cyanobacterium *Synechocystis* sp. PCC 6803 and *A. siamensis* TISTR 8012 under dark anaerobic conditions with 75 μmol DCMU [19,35]. A recent study by Bozieva et al. (2023) investigating a novel cyanobacterial strain, *Dolichospermum* sp. IPPAS B-1213 and the effects of DCMU under light and dark conditions found that DCMU-treated cultures produced several times more H_2 than untreated cells [38].

The research on H_2 photoproduction in non-N_2-fixing heterocyst-free cyanobacteria was conducted in three stages. The first stage involved testing for H_2 production in anaerobic conditions without impurities, both in light and dark conditions. In the second stage, the optimal PI and its concentration were identified. Finally, in the third stage, the inhibitors that positively impact H_2 production in various light-dark cycles were investigated. Research findings will contribute to our comprehension of the connection between H_2 metabolism, photosynthesis, and O_2 evolution. Furthermore, the

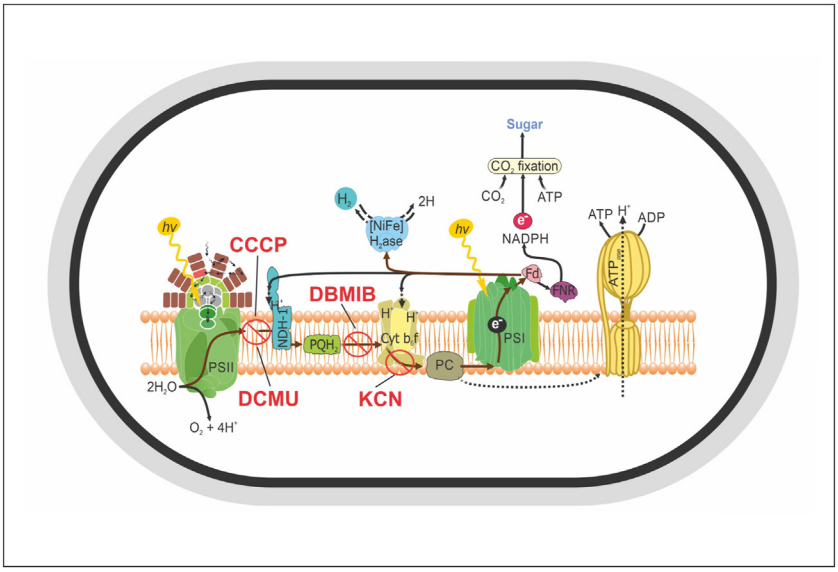


Fig. 1 – Effect of inhibitors on cyanobacterial electron transport chain. The illustration depicts the mechanism of hydrogen distribution within the vegetative cell of cyanobacteria. Modified from Refs. [14,29].

knowledge collected will be valuable in enhancing bio-H_2 production by non-N_2-fixing cyanobacteria, utilising optimal PSII inhibitors in the light-dark mode. The biological production of H_2 by these cyanobacteria holds great promise as a future approach to producing alternative clean energy biofuels.

Materials and methods

Used microorganisms

The investigation utilised cyanobacterial strains that do not develop heterocysts, akinetes, or trichomes. The isolated cyanobacterial strains are classified as filamentous (*Desertifilum* sp. PSU 1129, *Oscillatoria* sp. S-001, *Phormidium* sp. PSU 1120) and unicellular (*Chroococcus* sp. PSU 1008, *Synechococcus* sp. SU 78, and *Synechocystis* sp. PSU 1262). The filamentous, heterocyst-forming *Nostoc* sp. J-1 strain was obtained from a collection [33].

Research materials

The following main research materials were used in the study: m-chloro-carbonyl-cyanide-phenyl-hydrazone (CCCP; Sigma-Aldrich, Darmstadt, Germany); 2,4-dibromo-3-methyl-6-isopropyl-benzoquinone (DBMIB; Fisher Scientific Inc, Dublin, Ireland); N-(3,4-dichlorophenyl)-N′,N′-dimethylurea (DCMU; Sigma-Aldrich, Darmstadt, Germany); cyanide (KCN; Himedia, Maharashtra, India); and dimethyl sulfoxide (DMSO; Sigma-Aldrich, Darmstadt, Germany).

Various species of cyanobacteria were isolated from soil and water samples collected from different natural environments such as Turgen village (GPS: 43.396875 N, 77.586164 E) in the Almaty region, the Syrdarya River (GPS: 43.877411 N, 67.206102 E) in the Kyzylorda region, and the Badam River (GPS: 42.265652 N, 69.687269 E) in the Turkestan region.

Cultivation and hydrogen production

To screen for hydrogen production, the cyanobacterial strains were cultured in a laboratory under specific conditions. The temperature was maintained at 25 °C, and the light intensity was 30 μmol (photons) m^{-2} s^{-1}. A 100 mL flask containing 60 mL of culture medium was used, and 0.5% carbon dioxide (CO_2) gas was added to the air composition to promote cell growth. All six strains used in the study were grown in a BG-11 culture medium with a pH of 7.1. The culture media were aerated using an air blower compressor [21]. The culture medium was centrifuged (5424r, Eppendorf, Germany) at 6000 rpm for 5 min. As the supernatant was drained, 50 mL of BG_0-11 culture medium was added to the biomass and shaken for 5 min. The optical density (OD_{730}) of all cyanobacterial cells was adjusted to 1.5 using a UV-VIS spectrophotometer (UV-2600i, Shimadzu, Tokyo, Japan). Next, the cells were cultured under light with an intensity of 30 μmol (photons) m^{-2} s^{-1} for one day, then cells were separated from the liquid by centrifugation for 5 min at 7000 rpm. Following the supernatant removal, 25 mL of BG_0-11 culture medium was added to the biomass. This procedure was repeated twice, and 25 mL of culture medium was poured over the cell biomass and adjusted to an $OD_{720} = 1.5$ using a spectrophotometer. 15 mL of BG_0-11 culture medium was poured over the thick biomass and modified with 50 mmol HEPES-KOH (pH = 7.4) and 100 mmol $NaHCO_3$. To replace O_2 and thereby create anaerobic conditions, 100% argon gas was injected into the GC vial with a syringe [37].

The impact of inhibitors on H_2 production, cell and chlorophyll *a* concentrations, and the effect of light mode on H_2 production were investigated according to the procedure outlined in Weissman and Benemann (1977) [37]. A light intensity of 30 μmol (photons) m^{-2} s^{-1} was maintained throughout all the experiments.

Different concentrations of various inhibitors were introduced into the cyanobacterial cells before anaerobic conditions were created. To prepare the inhibitors, each PI was dissolved in an appropriate solvent (DMSO for DCMU, ethanol for CCCP and DBMIB, and water for KCN), and stock solutions were made beforehand and kept at −20 °C [21].

The study employed four distinct light/dark conditions to evaluate the influence of varied light cycles on photobiological H_2 production. These conditions included: 1) continuous light; 2) 12/6-h light/dark cycle; 3) 12/12-h light/dark cycle; and 4) 12/24-h light/dark cycle. For versions 2–4, an electromechanical timer (Rev Ritter, D.W.I Service GmbH, Germany) was used to automatically set the light rhythms. During the 12-h light mode, a light intensity of 30 μmol (photons) m^{-2} s^{-1} was utilised to determine the H_2 yield.

The gas chromatograph (GC) vials were covered with aluminium foil and incubated on a shaker under dark conditions for research purposes. A 15 mL gap was created within the GC vials to collect the gases that were separated from the cells [38].

Determination of chlorophyll content

Cyanobacterial cultures were transferred from a sterile agar Petri plate to a 1-L flask and cultivated under 30 μmol (photons) m^{-2} s^{-1} light while being aerated with a SPP-25GA air pump (Takatsuki, Osaka, Japan). When the cell density reached $OD_{730} = 1.5$, the cells were collected by spinning them at 10,000 rpm for 5 min in a centrifuge (5424r, Eppendorf, Germany). The resulting dense biomass was adjusted to an $OD_{730} = 1.5$ using a spectrophotometer (UV-2600i, Shimadzu, Tokyo, Japan) and utilised for further research. To determine the chlorophyll content, 100 μL of the GC vial suspension was mixed in a 1:10 ratio with 100% pure ethanol and incubated for 5 min. After centrifuging at 15,000 rpm, the absorbance of the supernatant was measured using a spectrophotometer to determine the concentration of *Chl a* at 646.6, 663.6, and 730 nm (expressed as *Chl a* mg L^{-1} [39].

Determination of nitrogenase activity

The ability of cyanobacteria to reduce acetylene to ethylene was used to estimate their enzymatic nitrogenase activity [40].

The cyanobacterial strains were cultured in the light until the cells reached the stationary phase. After that, the samples were spun at 6000 rpm for 20 min at 4 °C in a centrifuge (5424R, Eppendorf, Germany). A 15 mL sample of dense biomass was injected into a GC vial, and a gas mixture consisting of 10% acetylene and 90% argon was supplied for 30 min. The cells in the flask were incubated for 24 h at 45 μmol (photons) m^{-2} s^{-1} light intensity. Following incubation, 500 μL gas samples were collected and ethylene (acetylene reductive activity) concentration in the gas mixture was measured using a gas chromatograph (GC-15 A, Shimadzu, Japan) and expressed as μmol ethylene mg^{-1} *Chl a* h^{-1} [41].

Identification

The extraction of DNA was performed using standard protocols. When cells were in the exponential phase (70 mL), 0.5 mL of lysis buffer consisting of 25% sucrose, 50 mmol Tris-HCl, and 100 mmol EDTA was added to the sample. The cells were treated with 5 mg lysozyme for 25 min at 23 °C, followed by incubation with $NaC1_2H_{25}SO_4$ (1%) and proteinase K (100 μg) for 1 h at 50 °C [36]. The DNA material was extracted three times with phenol:chloroform:isoamyl alcohol (25:24:1) and twice with chloroform:isoamyl alcohol (24:1). The DNA material was then precipitated with 70% ethanol and stored at −20 °C in a 100 μL Tris-EDTA buffer. Polymerase chain reactions (PCR) were performed using a PCR Thermal Cycler (T100, Bio-Rad Laboratories, Inc., California, USA) [42].

PCR was used to amplify the 16S rRNA genes using specific forward (AGAGTTTGATCCTGGCTCAG and TGGTTGATCCTG CCAGAGG) and reverse (GGCTACCTTGTTACGACTT and AAA GGAGGTGATCCAGCC) primers. The PCR mixture consisted of 10 μL Taq (10X) commercial buffer, 10 μL purified DNA (50–100 ng), dNTPs (150 μmol of each), and primers (500 ng of each). The reaction was carried out at 95 °C for 3 min and 55 °C for 2 min [43]. The resulting PCR products were loaded onto a 1.5% agarose gel containing 0.5X TAE, and electrophoresis with ethidium bromide (0.5 μg mL^{-1}) dye was performed. The sequences were aligned using the CLUSTAL W multiple sequence alignment tool, and a phylogenetic tree was constructed using the Neighbour-Joining method. Nucleotide sequences of neighbouring species were obtained from the "BLAST: basic alignment search tool" foundation and the topology of the tree was assessed using load analysis with 1000 repeats [44,45].

DNA purification kits were utilised to purify the DNA composition. To identify the isolated strains, DNA fragments of various lengths were amplified with universal primers (27f, M23f, M23′f, 1492r, 1525r) and similar sequences were identified through the online BLAST database [46].

16 sequence records with high similarity were discovered in the collected data, and several alignments were performed using the MUSCLE software. The phylogenetic tree was constructed using the software MEGA 6 based on the evolutionary distances calculated with the Neighbour-Joining method based on the Kimura-2 parameter algorithm. The tree topologies were statistically evaluated using load analysis with 1000 repeated samples [45].

Data processing

The calibration curve of the hydrogen standard was utilised to compute the moles of H_2 produced. The calibration curve was adjusted using Microsoft Excel software. After conducting multiple experiments, a linear correlation between the mV-mL ratio was observed, with an average deviation of 0.9824. The H_2 production data was converted to μmol and then divided by the chlorophyll content (mg). The ultimate H_2 yield was determined in mmol H_2 mg^{-1} *Chl a* h^{-1} by multiplying the result by the space within the vial (15 mL) and then dividing it by the time of H_2 production. Argon was used as the carrier gas during the experiment [47].

Results

Cyanobacterial strains isolation and identification

During the summer months, a study was conducted to isolate new cyanobacterial strains from phototrophic microorganisms collected from various ecosystems across the Republic of Kazakhstan. After analysing samples from three different regions, 29 species of phototrophic microorganisms belonging to 3 sections, 4 classes, and 8 orders were identified as being morphologically distinct from one another. The taxonomic-percentage structure of the phototrophic microorganism diversity was: 12 (41.3%) *Cyanobacteria*, 2 (6.8%) *Bacillariophyta*, 3 (10.3%) *Euglenophyta*, and 9 (31%) *Chlorophyta*. Six heterocyst-free axenic pure cyanobacterial cultures were obtained and cultured under laboratory conditions. *Chroococcus* sp. PSU 1008 and *Synechococcus* sp. SU 78 were isolated from natural water sources (Turgen) in the Almaty region, while *Oscillatoria* sp. S-001 and *Desertifilum* sp. PSU 1129 - from the Syrdarya River in Kyzylorda region, and *Phormidium* sp. PSU 1120 and *Synechocystis* sp. PSU 1262 were obtained from the Badam River in the Turkestan region. Thick green clusters with numerous gaseous vesicles indicating high photosynthetic activity were observed on the water surface in the areas where phototrophic microorganism samples were collected. The water temperature ranged from 19 to 22 °C, and the pH was 6.9–7.2 in the three areas of sample collection.

Predictors along with PCR were used to construct a phylogenetic tree of PSU 1008, PSU 1129, S-001, PSU 1120, PSU 1262 and SU 78 strains. The most closely related species were selected by searching the online database BLAST for similar sequences [48]. The nucleotides of six strains (*Desertifilum* sp. PSU 1129, *Oscillatoria* sp. S-001, *Phormidium* sp. PSU 1120, *Chroococcus* sp. PSU 1008, *Synechococcus* sp. SU 78, and *Synechocystis* sp. PSU 1262) were used to construct a phylogenetic tree, while an external group, *Chlorella vulgaris* SAG 211-11b, was included.

Fig. 2 displays the evolutionary relationships among neighbouring phyla and a diagram that serves as the foundation for comparing taxa sequentially. The phylogenetic tree comprises five main clusters and one group, which mainly consists of distinct representatives of the species *Desertifilum*, *Oscillatoria*, *Phormidium*, *Chroococcus*, and *Synechocystis*. The

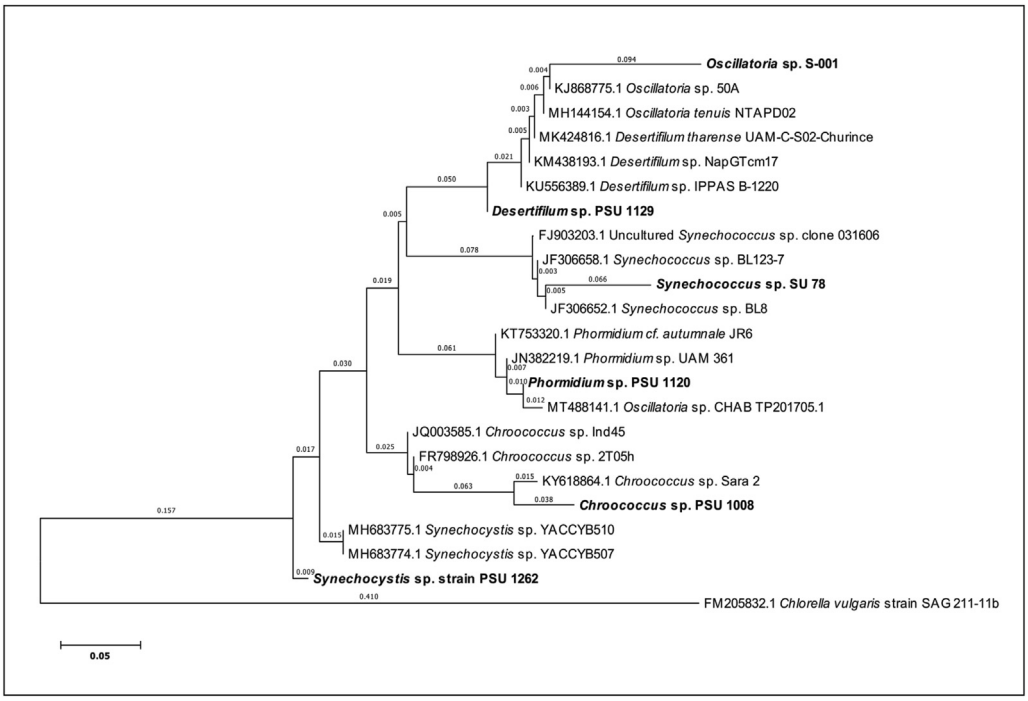


Fig. 2 – Phylogenetic tree of isolated cyanobacterial strains and neighbouring species.

phylogenetic analysis of the PSU 1129 strain was conducted using 890 bp, and the resulting phenogram indicated that the strain is closely related to *Desertifilum* sp. (score = 1631; affinity = 99.78%), *Desertifilum tharense* (score = 1631, affinity = 99.78%), and *Desertifilum* sp. NapGTcm17 (score = 1631, affinity = 99.78%). The strain PSU 1129 is morphologically similar to *Desertifilum* sp. even though it has an affinity for several strains. To sequence the entire S-001 strain, two additional primers were created using 19–20 bp derived from the sequencing results obtained with the universal primers. The Tm values ranged from 56 to 64 °C. According to the analysis, strain S-001 was genetically similar to *Oscillatoria* sp. 50 A by 91.19%, and also showed affinity of 90.98 and 91% with *Oscillatoria tenuis* NTAPD02 (score = 656) and *Geitlerinema calcuttense* NRMC-F 0142 (score = 658) strains, respectively. Morphologically, the strain was similar to *Oscillatoria* sp. Although, PSU 1008 strain showed a comparably high affinity with *Chroococcus* sp. Sara 2 (score = 1188), *Chroococcus* sp. Ind 45, and *Alborzia kermanshahica*, ranging from 89.17 to 95.87%, its morphological traits were closer to *Chroococcus* sp. The PSU 1120 strain showed a genetic similarity of 99.14% to both *Phormidium* sp. UAM 361 (score = 1472) and *Oscillatoria* sp. CHAB TP201705.1, but based on its morphological traits, it was determined that the studied strain was more similar to *Phormidium* sp. The SU 78 strain showed a genetic affinity of 94.44, 94.31, and 94.31 with similar scores (1131) to *Synechococcus* sp. clone 031,606, *Synechococcus* sp. BL8, and *Synechococcus* sp. BL123-7, respectively. Finally, the phylogenetic tree revealed that PSU 1262 strain had a 98.52% genetic affinity with *Synechocystis* sp. CACIAM 05.

Thus, after conducting a genetic analysis of six strains (PSU 1008, PSU 1129, S-001, PSU 1120, PSU 1262 and SU78) and constructing a phylogenetic tree to identify their close groups, it was determined that the heterocyst-free strains of the identified cyanobacteria would be suitable for further research into the production of biological H_2.

Screening for hydrogen production in cyanobacterial strains

Six research cyanobacterial strains incapable of forming heterocysts were utilised to identify strains that actively produce H2. All strains did not show N_2ase during studies on the oxidation of acetylene to ethylene. The ethylene yield of a heterocyst-forming strain *Nostoc* sp. J-1 (control) was found to be 19.3 μmol ethylene mg^{-1} *Chl a* h^{-1}. Phylogenetic studies determined that *Nostoc* sp. J-1 vegetative cells have two types of H2ase enzymes involved in H_2 production, whereas heterocyst cells contain only N_2ase. The fact that the cyanobacterial strains were unable to convert acetylene to ethylene indicated the absence of N_2ase [49].

All newly isolated cyanobacterial strains were found to have the ability to produce H_2 in the dark. Among them, *Synechocystis* sp. PSU 1262 demonstrated the highest rate of H_2 production. When the cells were incubated in a space filled with argon gas, H_2 production continued in the dark for 24–96 h, with a steady increase in H_2 yield. After 24 h, the H_2 yield was 15.3 nmol H_2 mg^{-1} *Chl a* h^{-1}, and after 96 h, it reached a rate of 33.9 nmol H_2 mg^{-1} *Chl a* h^{-1} (Fig. 3). However, there was a slight decline in H_2 production in the following hours, which could be due to a decrease in glycogen reserves in the cell and an increase in the release of O2 into the surrounding environment caused by the operation of photosynthetic apparatus [14].

The strain *Synechocystis* sp. PSU 1262 produced the highest yield of H_2 in the dark compared to the other five strains studied. Among the remaining strains, *Synechococcus* sp. SU 78 and *Desertifilum* sp. PSU 1129 demonstrated relatively high H_2 production, with similar H_2 yields. *Synechococcus* sp. SU 78

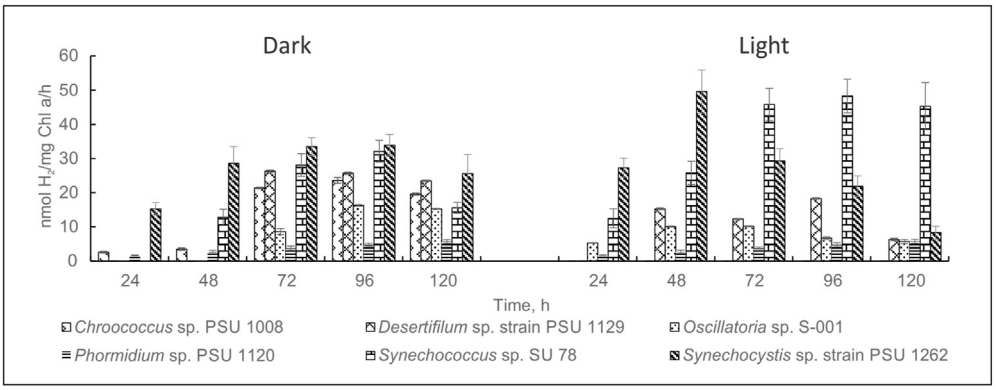


Fig. 3 – Study of H_2 production intensity by cyanobacteria in the light and dark.

accumulated 12.8 nmol H_2 mg^{-1} *Chl a* h^{-1} after 48 h and increased to 32.1 nmol H_2 mg^{-1} *Chl a* h^{-1} after 96 h. *Desertifilum* sp. PSU 1129 produced the maximum amount of H2 (26.3 nmol H_2 mg^{-1} *Chl a* h^{-1}) after 72 h of incubation. Conversely, *Phormidium* sp. PSU 1120 produced the lowest amount of H2 (5.8 nmol H_2 mg^{-1} *Chl a* h^{-1}) in the dark, with H2 production rapidly declining over the next few days after attaining this value after 120 h. Despite being cultured in the dark, the amount of O2 in the medium increased every day for all strains (Fig. 3).

The intensity of H_2 production by isolated cyanobacterial strains in a light-rich environment was then examined. Light is an essential factor for H_2 production since it acts as an electron donor in a process called direct biophotolysis. Direct biophotolysis involves the use of light energy to decompose H_2O molecules into H^+ and O_2 within the cell via the PSII complex in cyanobacteria [50]. The pigments in the thylakoid membranes of cyanobacteria (such as chlorophyll *a* and phycobilisomes) absorb the light energy, which promotes the oxidation of H_2O molecules into PSII. The released H^+ ions are used to activate ATP synthesis, while the electrons are transported to Fd via PSI. Fd protein is the primary electron donor for [FeFe]-H_2ase in cyanobacteria, which helps in the regeneration of H^+ in the cell to the H_2 molecule [51].

The cyanobacterial strains were cultivated under the same conditions as in the dark, with the only difference being light transmission. H_2 production rates were measured for each strain using the same incubation conditions. *Synechocystis* sp. PSU 1262 and *Synechococcus* sp. SU 78 strains showed higher H_2 production rates under light conditions. H_2 production in *Synechocystis* sp. PSU 1262 was observed within 24 h of establishing anaerobic conditions and peaked at 49.6 nmol H_2 mg^{-1} *Chl a* h^{-1} on the second day before gradually declining over the next few days. The maximum H_2 production rate in *Synechococcus* sp. SU 78 was recorded after 96 h, reaching 48.3 nmol H_2 mg^{-1} *Chl a* h^{-1}.

Despite producing considerable amounts of H_2 in the absence of light, the strains *Oscillatoria* sp. S-001 and *Desertifilum* sp. PSU 1129 produced slightly lower yields of H_2 under light conditions. *Synechocystis* sp. PSU 1262, on the other hand, produced 1.4 times more H_2 in the light compared to the dark. Therefore, *Synechocystis* sp. PSU 1262 and *Synechococcus* sp. SU 78 outperformed other strains in terms of H_2 production in light conditions. As a result, *Synechocystis* sp. PSU 1262 was selected for further research as it showed promising results in both light and dark conditions.

Influence of inhibitors on hydrogen production

In addition to selecting active H_2 producers, it is important to conduct scientific research aimed at improving the H_2 production activity of different cyanobacteria. To maximise the efficiency of substrate conversion into H_2, it is necessary to optimize cyanobacteria cultivation. Therefore, it is crucial to identify various cellular factors that can enhance H_2 production [21]. As a means of increasing H_2 production in *Synechocystis* sp. PSU 1262, the impact of varying PI concentrations under light was investigated as a contributing factor to biohydrogen production (Fig. 4).

The study focused on optimising the H_2 production of *Synechocystis* sp. PSU 1262 by examining the effect of different PIs. Among the four PIs tested, the highest H_2 yield was observed at a final concentration of 1 μmol CCCP, a PSII inhibitor, with a production rate of 158.3 nmol H_2 mg^{-1} *Chl a* h^{-1}. Albeit the H_2 production of all CCCP-enriched variants was greater than that of the control, higher concentrations of PIs (5–10 μmol) were found to be toxic, and a low concentration of 1 μmol was the most effective. DBMIB, on the other hand, resulted in the lowest H_2 production compared to other PIs. H_2 production was highest at 20 μmol DBMIB (96.8 nmol H_2 mg^{-1} *Chl a* h^{-1}) but decreased at higher concentrations (1000 μmol). The control group produced the least amount of H_2, with a production rate of 56.9 nmol H_2 mg^{-1} *Chl a* h^{-1} at a concentration of 5 μmol.

Moreover, the DCMU-treated suspensions showed significant photobiological H_2 production, with the highest amount of H_2 produced at 20 μmol (710 nmol H_2 mg^{-1} *Chl a* h^{-1}). However, increasing the concentration of DCMU had a detrimental effect and entirely suppressed electron transport in ETC. H_2 production was nearly four times lower than the control at 100 μmol DCMU, which was the lowest among the four concentrations tested. Even at 10 μmol DCMU, high rates of H_2 production were observed, which were 1.5 times higher than at 40 μmol DCMU. KCN was found to be the most suitable PI for microbiological H_2 production. All concentrations of KCN (100–1000 μmol) enhanced H_2 production, however, the effects varied. Comparable results were obtained at 250–1000 μmol, corresponding to 441.5 and 410.5 nmol H_2

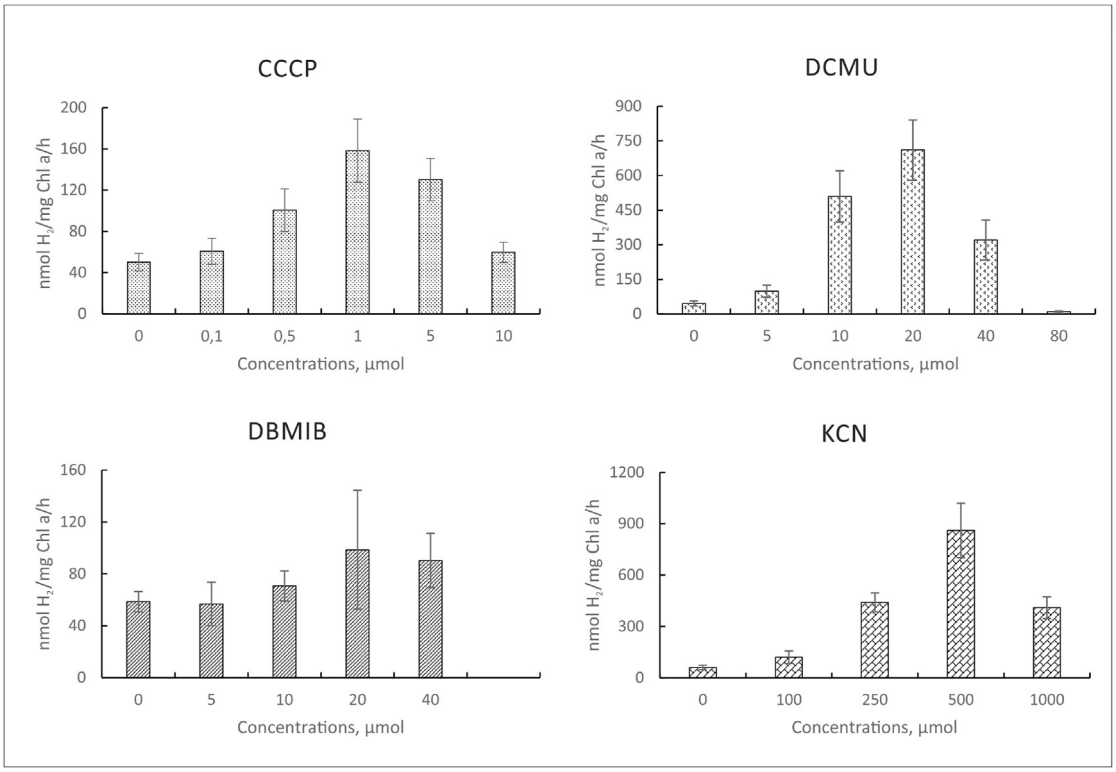


Fig. 4 – Study of four photosynthetic inhibitors influence on hydrogen production.

mg^{-1} *Chl a* h^{-1}, respectively. The highest rate of H_2 production was recorded at 500 µmol KCN (860 nmol H_2 mg^{-1} *Chl a* h^{-1}), which was 5.4, 8.7, and 1.2-fold higher than CCCP, DBMIB, and DCMU, respectively.

Effects of inhibitors on cell and chlorophyll a concentrations

The study examined the impact of different concentrations of inhibitors on the growth of cyanobacterial cells, which have been shown to be effective for H_2 production. The primary cells with an OD_{730} of 0.3 were monitored for 72 h. The growth of cells treated with 500 µmol KCN was only slightly lower than the control (without the inhibitor) over time. After 72 h, the control OD_{730} was 0.52, while the cells treated with KCN had an OD_{730} of 0.45. However, a decrease in optical density was observed throughout the day with all other inhibitors. The OD_{730} of cells treated with 20 µmol DBMIB inhibitor was 0.22 after three days. The lowest results were obtained with 1 µmol CCCP and 20 µmol DCMU (0.12 and 0.15, respectively) in this experiment. Nevertheless, the decrease in optical density was not considered to be a direct factor affecting H_2 production (Fig. 5).

Chl a is essential in the production of biological H_2. *Synechocystis* sp. PSU 1262 cells were cultured in a N_2-free anaerobic environment for 24, 48, and 72 h under light with various concentrations of DBMIB, DCMU, CCCP, and KCN as inhibitors. The concentration of *Chl a* in the thylakoid membranes was then determined (Fig. 6).

The concentration of *Chl a* was observed to decrease in all four variants of *Synechocystis* sp. PSU 1262 cells after being cultured in a BG_0-11 nutrient medium containing a PI. A slight

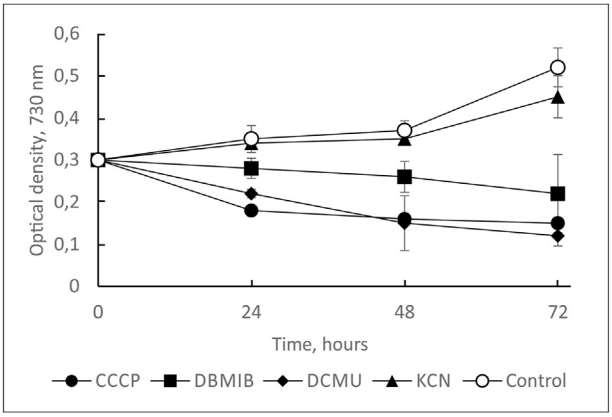


Fig. 5 – Cell growth curves treated with different inhibitors under the light. The inhibitors used were CCCP, DBMIB, DCMU, and KCN, with concentrations of 1, 20, 20, and 500 µmol respectively.

decrease in *Chl a* concentration was observed during the first 24 h of incubation, which continued to decrease linearly until 72 h in all cases. The initial concentration of *Chl a* was 80 µg mL^{-1} in all strains, and higher concentrations of DBMIB, DCMU, CCCP, and KCN as well as longer incubation times led to a significant decrease in both cell and *Chl a* content of the *Synechocystis* sp. PSU 1262. The study found that higher concentrations of CCCP and DCMU inhibitors, at 20–40 and 5–20 µmol, respectively, had a more severe toxic effect compared to the other two PIs. Treatment with CCCP resulted in a *Chl a* concentration of 55 µg mL^{-1}. The DBMIB-treated cells had the

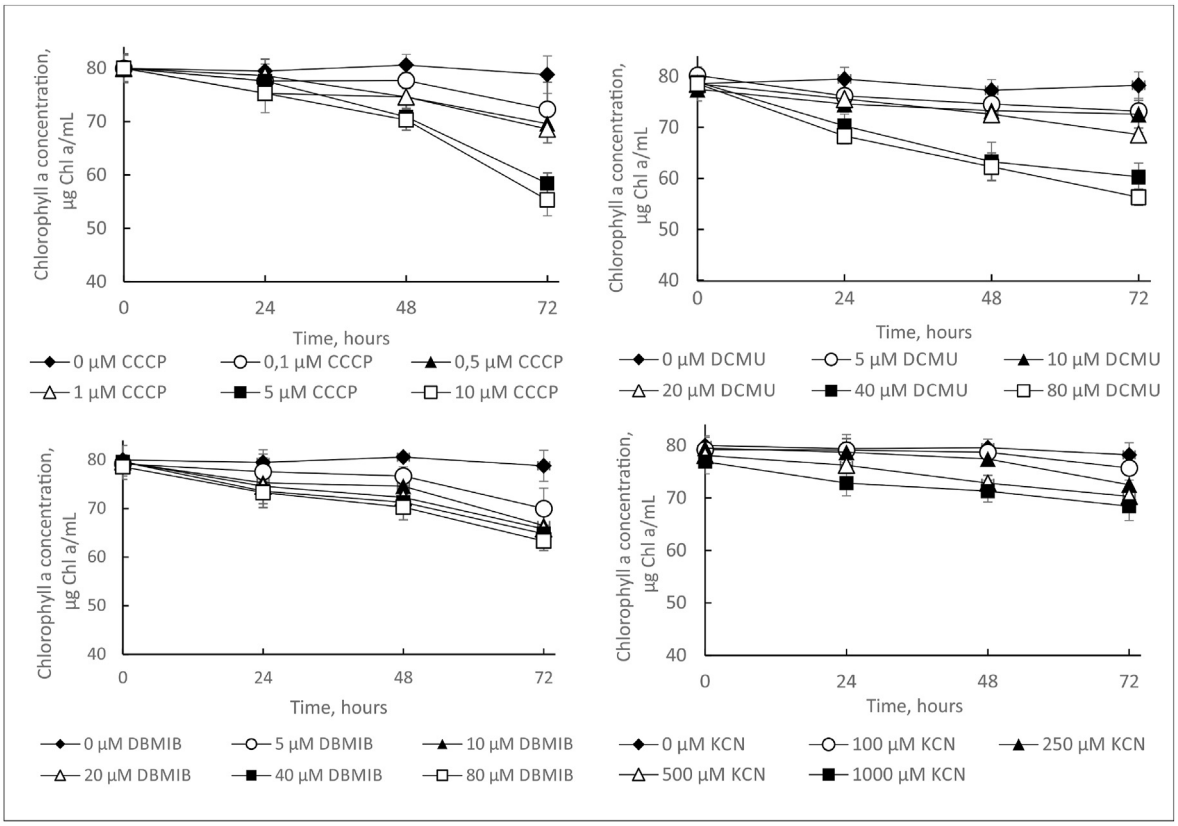


Fig. 6 – Effects of inhibitors on chlorophyll *a* concentrations.

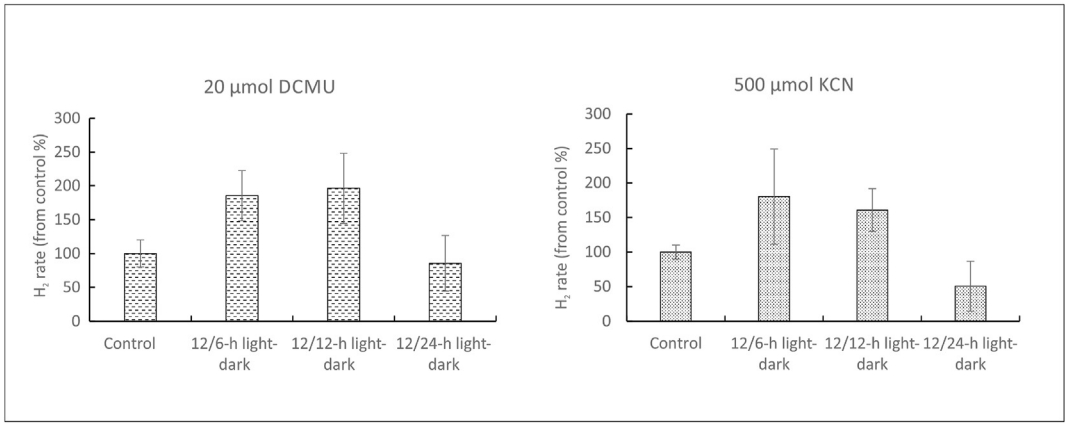


Fig. 7 – Study on the influence of different light modes on hydrogen production.

lowest *Chl a* concentration of 63.3 ± 1.96 µg mL^{-1}, while KCN-treated cells had a *Chl a* concentration of 68.4 ± 2.7 µg mL^{-1}.

Influence of light mode on hydrogen production

The investigation was divided into two stages, with the last stage having three versions. In the first phase, cells were exposed to light for 12 h after being aerated with argon. In the second phase, the variants were subjected to darkness for 6, 12, and 24 h.

Following the discovery of optimal concentrations of DCMU and KCN for H_2 production, the study aimed to investigate how these concentrations affected H_2 production under different light modes. The investigation was divided into two stages, with the last stage having three versions. In the first phase, cells were exposed to light for 12 h after being aerated with argon. In the second phase, the cells were subjected to darkness for 6, 12, and 24 h (Fig. 7).

The results of experiments with two levels indicated that H_2 production is greatly influenced by KCN, DCMU, and the light-dark cycle. In light-dark cycles of 12/6, 12/12, and 12/24 h, cultures treated with 500 µmol KCN and 20 µmol DCMU showed higher H_2 production than control cultures. In a 12/6-h light-dark cycle, *Synechocystis* sp. PSU 1262 cells treated with

500 μmol KCN produced approximately 80.3% more H_2 than control cultures, which was equivalent to 1552 nmol H_2 mg^{-1} *Chl a* h^{-1}. In the 12/12-h light-dark cycle, the cells produced 60.6% more H_2 than control cultures. However, the H_2 yield was 49.4% lower in the 12/24-h light-dark cycle.

Application of 20 μmol DCMU and a 12/12-h light-dark cycle increased H_2 production in the strain by 96.4% compared to the control (1394 nmol H_2 mg^{-1} *Chl a* h^{-1}). In a 12/6-h light-dark cycle, H_2 production was 85.3% higher than the control, but in a 12/24-h light-dark cycle, it decreased by 14.4%. Furthermore, treating *Synechocystis* sp. PSU 1262 with 500 μmol KCN and using a 12/6-h light-dark cycle had a positive effect on H_2 production. Moreover, a 12/12-h light-dark cycle and 20 μmol DCMU resulted in a relatively high H_2 yield.

Discussion

Screening for hydrogen production in cyanobacterial strains

Hydrogen is utilised as a high-quality energy source worldwide. Microbial H_2 interacts with O_2 to produce energy and water. Cyanobacteria, capable of splitting water into H^+ and O_2 using solar energy, might be employed as a potential source of H_2 energy in the future [52,53]. The hydrogen ions from PSII activate H_2-producing enzymes, and some cyanobacterial species can enzymatically produce H_2 in the light. However, the high cost of cyanobacterial H_2 energy has made it commercially impractical. The use of metabolic and genetic engineering techniques can help to improve the efficiency of cyanobacterial bio-H_2 production. One of the primary goals of the industry is to identify and develop active cyanobacterial strains that can produce H_2 [54,55].

Light energy plays a crucial role in the photoproduction of H_2 by cyanobacteria. It facilitates the transfer of electrons from the photosynthetic reaction centres to H_2ase or N_2ase and prevents the release of O_2 in the PSII complex, which affects the activity of H_2ase and the rate of photoproduction. Previous studies have shown that *Oscillatoria thiebauti*, which was isolated from the ocean, actively produces H_2 through direct biophotolysis [56]. Another strain, *Phormidium valderianum* BDU 20041, can produce a significant amount of H_2 within a few hours through both direct and indirect biophotolysis [57]. According to Touloupakis et al. (2016), the non-N_2-fixing strain *Synechocystis* sp. PCC 6803 can produce H_2 through indirect biophotolysis at a maximum rate of 40.6 nmol H_2 mg^{-1} *Chl a* h^{-1} [58].

Study findings revealed that all of the cyanobacterial strains that were tested produced some amount of H_2 under anaerobic dark or solely dark conditions, with the highest levels observed in the unicellular strains *Synechocystis* sp. PSU 1262 and *Synechococcus* sp. SU 78. It has been observed that in cyanobacteria that lack N_2ase, H_2 production occurs exclusively through H_2ase via glycogen fermentation in the photosynthetic reserve [59].

The H_2 yield of cyanobacterial strains was higher after 48 h of incubation in light compared to darkness. The reason for the increase in H_2 production in the light can be attributed to the high amount of electrons required for the activity of the H_2ase enzyme. The *Hox*-H_2ase enzyme utilizes glycogen reserves as a primary substrate for H_2 production, which is accumulated by photosynthesis [60]. Cyanobacteria use cell-directed light energy to build biomass and reserve substances, including glycogen, mostly under N_2-deficient conditions in the culture medium [61]. Therefore, glycogen fermentation in the dark significantly increased the H_2 yield of cyanobacterial cells. The H_2ase enzyme was active until the O_2 content increased in the first 120 h, explaining the increase in H_2 production in the light. Previous studies have also reported similar results [62–64], where non-N_2-fixing *Gloeocapsa alpicola* CALU 743 cultivated under N_2 deficiency showed increased glycogen (40–50% of dry biomass weight) storage under light, leading to higher H_2 production [65].

In this study, a highly active H_2 producer under anaerobic light conditions, *Synechocystis* sp. PSU 1262, was identified among the wild strains. *Synechocystis* sp. PSU 1262 produced 1.46 times higher H_2 production in the light than in the dark, and 1.5-fold higher than *Synechococcus* sp. SU 78. The activity of N_2ase, photosynthesis, and respiration of unicellular cyanobacterial strains, *Synechocystis* sp. RF-1, *Synechococcus* sp. Miami BG43511, and *Gloeothece* sp. ATCC51142 was found to be under circadian regulation when grown in alternating light-dark mode [66].

Influence of inhibitors on hydrogen production

Aside from screening for active H_2 producers, researchers are also exploring ways to increase the activity of H_2 production by diverse cyanobacteria [67]. This involves optimising the conditions in which strains are cultivated to make substrate conversion into H_2 more efficient. It is also critical to identify different cellular stimuli contributing to H_2 production [65]. Thus, as a factor contributing to biohydrogen production, the effect of various concentrations of PIs (DCMU, KCN, CCCP, and DBMIB) under light has been examined. Although many recent studies have focused on minimising the amount of O_2, no particular findings have been reported.

The usage of PIs is intended to limit the activity of the PSII complex, which creates anaerobic conditions that are favourable for producing molecular H_2 [68]. PIs operate by attaching to the PSII complex and preventing the movement of electrons between the first and second acceptors [69]. Consequently, it disrupts the linear chain of electrons in photosynthesis, limiting the breakdown of H_2O molecules and the formation of O_2 by PSII. However, the effectiveness of PIs can vary depending on the particular strain being studied. Thus, determining the optimal concentration of PIs is a crucial factor in investigating H_2 production.

Non-heterocyst cyanobacterial strains produce H_2 through the action of bidirectional H_2ase, particularly when N_2 is absent and under anaerobic light/dark conditions [70–72]. This organism relies solely on H_2ase for catalysing both the production and uptake of H_2 [72]. However, because the bidirectional H_2ase is highly sensitive to O_2 [9], which is a by-product of water cleavage by PSII, H_2 production in the light is reduced [71]. To increase H_2 production in non-heterocyst cyanobacterial strains, it is necessary to remove O_2. Therefore, to prevent O_2 formation during water cleavage through photolysis, PSII inhibitors are used. The study focused on the four most frequently used inhibitors for H_2 production. All these

inhibitors enhance the activity of the enzyme uptake H_2ase by inhibiting the activation of the PSII complex, which lowers the level of O_2 in the medium. Numerous studies on biological H_2 production have also employed the selected inhibitor [12,19,36,73]. However, the preferred inhibitor for H_2 production may vary due to the unique physiological properties of the cyanobacterial strains used in the study.

The study investigated the effects of the four inhibitors mentioned earlier on H_2 production in six different cyanobacterial strains. Among them, *Synechocystis* sp. PSU 1262 was selected for further investigation due to the highest intensity of H_2 production. The application of 500 μmol KCN and 20 μmol DCMU to the strain had a positive impact on H_2 production. The cultures exposed to PIs produced more molecular H_2 under light conditions but less under dark conditions, with H_2 production peaking on the 4th day. However, it is worth to be noted that the concentrations of PIs used showed only a slight increase in O_2 and N_2 content compared to higher concentrations (data not shown), as even small amounts of these gases can deactivate H_2 enzymes. Conversely, when their levels increase, photosynthesis is completely halted, and the electrons required for H_2 production are not released at all [21].

KCN and DCMU were effective in increasing cyanobacterial H_2 production, which is consistent with previous research [74,75]. Another study used the N_2-fixing strain *Anabaena siamensis* TISTR 8012 and an inhibitor to block photosynthetic electron transport, resulting in a slightly higher H_2 yield [35]. The highest H_2 yield was observed after the addition of diuron under the light, but the DCMU concentration used was several times higher (50 μmol) than in this study. The optimal application dose of the inhibitor depends on the specific cyanobacterial strain being targeted. The observed H_2 production rate was 22 μmol H_2 mg^{-1} *Chl a* h^{-1}, approximately 4 times higher than that of the control strains. Numerous studies have also reported increased H_2 production by cyanobacteria in the presence of DCMU [28,41,76]. For instance, Cournac et al. (2004) [19] found that *Synechocystis* sp. PCC 6803 produced more H_2 production in the presence of 75 μmol DCMU under dark, anaerobic conditions. Exposure to 0.1 μmol DCMU has been found to cause a significant decrease in the growth of *Eudorina elegans* and *Nannochloropsis* [77,78]. Moreover, DCMU considerably reduced the chlorophyll content of N_2-fixing *Nostoc* sp. G3 and *A. variabilis* [79]. While in the case of CCCP, the inhibition of the PSII photochemical activity under the light in *Synechocystis* sp. PCC 6803, *Nostoc* sp., and *Lyngbya* sp. have been demonstrated [80,81].

PIs such as DCMU and CCCP are known to inhibit the photochemical activity of PSII and ATP synthesis, which can lead to a decrease in O_2 production and an increase in dark respiration rate in cyanobacterial strains such as *Anabaena variabilis* and *Anacystis* sp [36]. Under the light, CCCP has been shown to suppress the PSII photochemical activity in the strains *Synechocystis* PCC 6803, *Synechococcus* PCC 7942, and *Nostoc* sp [82,83]. Additionally, studies have shown that CCCP can increase H_2 production in *Oscillatoria* sp. and *Synechocystis* PCC 6803. However, high concentrations of DCMU and CCCP, as well as prolonged incubation, have been found to be toxic and cause cell death. KCN is another PI that inhibits quinol oxidases and reduces the PQ pool, which can suppress H_2 production or block electron transfer from Cyt b_6f to PSI by inhibiting plastocyanin activity [24–26]. DBMIB is another PI that regulates the activity of nitrate assimilation genes, leading to an increase in H_2 production [29].

Photosynthesis can boost the supply of electrons available for H_2ase. However, oxygenic photosynthesis can also produce molecular O_2 by breaking down water in PSII, which can hinder enzymatic H_2 production. Studies have shown that inhibiting PSII function with DCMU can address this issue. Generally, the respiratory ETC transfers electrons from an electron donor to an electron acceptor (like O_2) while transporting protons across the membrane. Reducing electron flow through the respiratory ETC can increase the number of electrons accessible for H_2 production [84]. For instance, *Cyanothece* sp. has a single-subunit NADH dehydrogenase complex that can oxidise NADH. Additionally, the heterocysts *A. siamensis* could increase both H_2 production and bidirectional *Hox*-H_2ase activity in light by inhibiting NDH-I with the specific inhibitor rotenone [85].

Research conducted on *A. siamensis* has revealed that inhibiting all terminal respiratory oxidases, including quinol oxidase and cytox, with a specific KCN inhibitor can redirect electron flow to the bidirectional *Hox*-H_2ase during photosynthesis. Inhibition of quinol oxidase was found to be more effective at stimulating H_2 production than inhibiting cytox alone, as no increase in H_2 was observed when only cytotoxic activity was inhibited with an inhibitor. This finding is consistent with earlier studies that demonstrated the ability of KCN to induce H_2 accumulation during a light-dark cycle over 3 days [86]. Furthermore, the effects of DCMU and DBMIB (PSI electron flow inhibitor) on H_2 production in *Synechococcus elongatus* were investigated as well [87].

Effects of inhibitors on cell and chlorophyll a concentrations

Four inhibitors with the optimal effect on H_2 production were selected and their impact on the growth of *Synechocystis* sp. PSU 1262 cells were investigated. The growth of cells treated with CCCP, DBMIB, and DCMU decreased during the first three days. On the other hand, the optical density of cells treated with 500 μmol KCN increased over time, although the increase was lower compared to the control. The findings obtained are consistent with earlier published studies [19,36,41]. Pansook et al. (2019), for example, reported that the addition of 0.5–250 μmol DCMU halted the growth of *Aphanothece halophytica* cells, and suppression also occurred at 0.1–5 μmol CCCP [36].

The impact of the four PIs on the concentration of *Chl a* in an anaerobic environment was also investigated. In the treatments without CCCP, the concentration of *Chl a* remained unchanged. However, in the CCCP-treated *Synechocystis* sp. PSU 1262 cells, the concentration of *Chl a* decreased, particularly in cells treated with high concentrations of CCCP over long-term incubations. No cell growth was observed in any of the treatments, and the concentration of *Chl a* did not increase as cyanobacterial cells were incubated in a BG_0-11 nutrient medium without an N_2 source. CCCP was used to inhibit the electron flow rate in the photosynthetic ETC in the thylakoid membrane of cyanobacterial cells, which led to a decrease in the concentration of *Chl a* in *Synechocystis* sp PSU 1262 [13].

These findings are consistent with previous studies that have shown a decrease in the optical density and concentration of *Chl a* in other phototrophic microorganisms [39,40]. For instance, the treatment of cell cultures with 15 μM CCCP resulted in a reduction in the concentration of *Chl a* in the green algae *C. reinhardtii* [22]. The treatment of *Synechocystis* sp. PSU 1262 with DBMIB resulted in a decrease in chlorophyll concentration within 24 h. DBMIB functions by serving as a potent competitive antagonist of plastohydroquinone in the isolated cellular thylakoid membranes. This disrupts the photosynthetic electron transport at the plastoquinone-reducing site [88]. Otama et al. (1985) showed that electron-transport activities and *Chl a* concentrations were lowered in *Anacystis nidulans* and *Synechocystis* sp. PCC 6714 at 50 μM DBMIB [89]. *Synechocystis* sp. PSU 1262 showed a decrease in chlorophyll concentration when treated with DCMU, similar to the effect of CCCP. This is because DCMU also inhibits photosynthetic activity, leading to inhibited cell growth and decreased chlorophyll concentration. Previous studies have also shown a decrease in *Chl a* concentration in other cyanobacterial strains, such as *Synechocystis* sp. PCC 6803 and *Anabaena variabilis* when treated with DCMU [36]. The concentration of CCCP and DCMU and the duration of incubation are factors that contribute to toxicity and cell death. Although KCN treatment also led to a decrease in *Chl a* concentration, its toxic effects were less than those of the other inhibitors. As the concentration of inhibitor increased, the concentration of *Chl a* continued to decrease over time. KCN serves as a cytochrome oxidase inhibitor, disrupting photosynthetic electron transport at the cytochrome-reducing site. Abdelwahab et al. (2014) studied the effects of low and high KCN concentrations on H_2 production and *Chl a* concentration in *Synechocystis* sp. PCC 6803 under anaerobic conditions. The addition of a low concentration (1 mM) of KCN had no impact on the concentration of *Chl a* but increased the yield of H_2 [90].

The four PIs described above were found to have a negative impact on *Chl a* concentration. Despite the decrease in cell density and *Chl a* concentration, higher concentrations of PIs led to a greater yield of H_2. This can be explained by a reduction in the activity of PSII, the O_2-releasing complex in thylakoid membranes. However, a decrease in H_2 production at high PI concentrations could be due to two reasons: 1) a decrease in *Chl a* concentration, which negatively impacts the thylakoid membrane structure, or 2) a complete halt in proton and electron transport, which is required for H_2 production from the PSII complex. Further investigation is necessary to this concept be explored in detail.

Influence of light mode on hydrogen production

In experiments using *Synechocystis* sp. PSU 1262, the highest yield of H_2 was observed in cells treated with 20 μmol DCMU under a 12/12-h light-dark cycle. Electron transport and glycogen storage play critical roles in maximising the production of molecular H_2 under different light modes. This is important since glycogen content is substantially regulated by natural light-dark cycles, and photobiological H_2 production must be optimised to coincide with daily solar cycles for commercial viability. While the experiments did not show an increase in glycogen storage under a 12/12-h light-dark cycle, compared to 12-h light and 6- and 12-h dark cycles, it is suggested that this is a normal finding.

According to our findings, cells do not deplete their glycogen reserves required for H_2 production when kept in the dark for 6 h. Moreover, cells cultured in a 21/3 light-dark cycle accumulated more glycogen than those in a 21/12 cycle, and 90% of glycogen was consumed after 6 h dark [91]. However, after 12 h of darkness, the glycogen content remained the same as before the dark cycle, as cells only use the glycogen reserves stored for use in the dark.

Another reason for the higher H_2 yield at a 12/6 h light-dark cycle could be the lower O_2 levels in the vial compared to the control (light only). When cyanobacterial cells are cultured under continuous light, photosynthetic activity increases, leading to higher O_2 levels that inhibit the activity of H_2-releasing enzymes. Additionally, Antal and Lindblad (2005) showed that a 12-h light phase is enough to accumulate substantial glycogen reserves in cyanobacterial cells [92]. When the cells are then placed in the dark for 12–24 h after the light phase, O_2 levels decrease, glycogen reserves are depleted, and H_2 production is catalysed, resulting in small amounts of H_2 molecules with the desired electron flow. Research findings indicate that to achieve maximum H_2 yield, electron flow and glycogen storage are crucial. This is significant because the amount of glycogen present is primarily influenced by alternating periods of light and darkness. Utilising natural daily solar cycles is necessary for the economic feasibility of biological H_2 production. It was observed that the O_2 levels in the GC vial were too low to support sufficient electron flow required for H_2ase activity, at both PI concentrations during a 24-h dark. Further investigation is needed to determine the optimal level of glycogen storage and the environmental conditions necessary.

There exist theoretical maximum values for both glycogen storage and electron flow, and the goal is to find the optimal balance between these two factors. H_2ase activity can vary depending on environmental conditions, but it has been observed that *in situ* H_2 production values are considerably lower than H_2ase activity values. For example, Gutthann et al. (2007) reported H_2ase activity of 133 μmol H_2 mg^{-1} *Chl* h^{-1} [29], which was 110 times higher than the brief burst of H_2 production measured by Cournac et al. (2004) at 1.2 μmol H_2 mg^{-1} *Chl a* h^{-1} in light [19].

Moreover, the heterocyst-free unicellular strain *Synechocystis* sp. PSU 1262 has demonstrated rapid growth and a sufficient enzyme system for H_2 production, as well as the capability to maintain favourable intracellular conditions for active H_2 production. The prospect of utilising a light source to prompt cyanobacteria to produce the H_2 molecule in the external environment is an attractive approach.

Conclusion

The utilisation of phototrophic microorganisms for H_2 production is an important field in biotechnology. Research efforts, such as screening for active strains and developing genetic engineering strategies for photobiological H_2 production, are critical for advancing bio-H_2 production. Therefore, the study aimed to identify promising H_2 producers among

newly isolated cyanobacterial strains and enhance bio-H_2 production. Six strains (*Chroococcus* sp. PSU 1008, *Synechococcus* sp. SU 78, *Oscillatoria* sp. S-001, *Desertifilum* sp. PSU 1129, *Phormidium* sp. PSU 1120, and *Synechocystis* sp. PSU 1262) were identified and evaluated for H_2 production capabilities in both dark and light conditions. Among them, *Synechocystis* sp. PSU 1262 exhibited the highest H_2 yield. Furthermore, strain exposure to 500 μmol KCN resulted in increased photobiological H_2 production, with the greatest H_2 production several times higher than the control. Additionally, a 12/6-h light-dark cycle led to an 80.3% increase in H_2 photoproduction, resulting in a yield of 1552 nmol H_2 mg^{-1} *Chl a* h^{-1}. The findings demonstrate the effectiveness of PIs for cyanobacterial H_2 production and indicate that cultures were grown under varied light modes improve cellular glycogen reserves and increase H_2 production. The use of different light modes and appropriate PIs may be the optimal solution for increasing H_2 productivity in cyanobacteria-based H_2 production.

CRediT authorship contribution statement

GKK, literature survey, providing experimental works and writing; BDK, literature, plan survey and analysis; AKS, GAS, main idea and consulting, GAS, MOB, AKS, AAK, experimental works; BKZ, HFA, SIA consulting; BKZ and SIA, main idea, consulting, writing. All the authors contributed to the 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.

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