Abstract

of the dissertation submitted for the degree of Doctor of Philosophy (PhD)
in the specialty **8D05105 – Biotechnology**

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"Isolation of hydrogen-producing cyanobacteria from different ecosystems and increasing their productivity by metabolic approaches"

General description of the work

The dissertation is devoted to the isolation of axenic cultures of cyanobacteria and a comprehensive study of their hydrogen-producing capacity in order to select promising strains for application in photobioenergetic technologies for hydrogen production. Within the framework of the work, samples were collected from various soil and water resources of Southern Kazakhstan during the spring, summer, and autumn periods of 2020; six cyanobacterial strains were isolated and identified. Key factors determining the efficiency of photobiological hydrogen release were studied, including cultivation regimes, gas-exchange conditions, and lighting. The effect of photosynthetic electron transport inhibitors (CCCP, DBMIB, DCMU, KCN) was investigated, and their optimal concentrations were determined at which increased hydrogen production by cyanobacteria is achieved. It was established that KCN (500 μM) and DCMU (20 μM) show the greatest effectiveness. The new results obtained form a basis for developing technological recommendations for hydrogen production and for further scaling up cultivation processes of phototrophic microorganisms.

Relevance of the research

Hydrogen is considered one of the key components of future energy resources, since its use does not involve direct CO_2 emissions, and it can serve as a universal energy carrier for the electric power industry, transport, and energy storage. Under conditions of global decarbonization and a growing share of renewable energy sources, demand is increasing for "low-carbon" hydrogen production technologies that do not require fossil energy carriers (natural gas, oil, coal) and minimize environmental damage.

Cyanobacteria are potentially capable of producing H_2 via nitrogenase and hydrogenases using light energy, which makes them attractive objects for bioenergy solutions. At the same time, the practical development of biohydrogen technologies is constrained by the fact that natural strains are often characterized by limited productivity, as well as the sensitivity of the process to cultivation conditions and the presence of oxygen. Therefore, the search and selection of cyanobacterial strains with high hydrogen production, optimization of cultivation parameters and initiation regimes for hydrogen production, study of regulatory and enzymatic mechanisms of hydrogen formation and release, and development of approaches to increase hydrogen yield remain relevant.

Aim of the work

To search for and identify promising cyanobacterial strains from aquatic and soil sources, comprehensively study them, and develop approaches to increase their activity.

Research objectives

1. To isolate axenic cultures of cyanobacteria from soil and water resources of the Turkestan, Kyzylorda, and Almaty regions; to perform their molecular-genetic identification and phylogenetic analysis using marker sequences (16S rRNA).

2. To determine the baseline level of hydrogen release and the activity of key enzymes of hydrogen metabolism (hydrogenases and nitrogenases) in the isolated cyanobacterial strains, and to establish the relationship between these indicators and their ability to produce hydrogen.

3. To assess the influence of major physicochemical factors (temperature, illumination, nitrogen content, buffer systems, pH) on hydrogen release by the active cyanobacterial strain *Synechocystis* sp. PSU 1262.

4. To assess the effect of various concentrations of photosynthetic electron transport inhibitors (CCCP, DBMIB, DCMU, KCN) on hydrogen release and determine optimal application regimes.

5. To study the effect of immobilizing *Synechocystis* sp. PSU 1262 cells in a calcium-alginate gel on hydrogen-release indicators.

6. To formulate technological recommendations for increasing H_2 yield in cyanobacteria based on optimization of cultivation conditions, selection of lighting regimes, and application of photosynthesis inhibitors.

Place where the dissertation research was conducted

The dissertation was carried out at Al-Farabi Kazakh National University. Part of the research was conducted at leading specialized research organizations: the K.A. Timiryazev Institute of Plant Physiology, RAS (Russia) and the Tianjin Institute of Industrial Biotechnology (TIB, CAS, China).

Objects of the study

The objects of the study were natural and collection strains of phototrophic microorganisms. The main objects used were: *Desertifilum* sp. PSU 1129, *Oscillatoria* sp. S-001, *Phormidium* sp. PSU 1120, *Chroococcus* sp. PSU 1008, *Synechococcus* sp. SU 78, *Synechocystis* sp. PSU 1262, *Tolypothrix tenuis* J-1 and *Nostoc* sp. J-1, *Anabaena* A-9.

Research methods

A set of microbiological, physiological-biochemical, and molecular-genetic methods was used. Algological samples were collected in spring, summer, and autumn of 2020 from soil and water sources of the Republic of Kazakhstan. Isolation and obtaining of axenic cultures were performed by primary cultivation of samples on liquid media (BG-11/BG_0-11) followed by repeated subculturing onto agar media. Strain identification was carried out by morphological characteristics and by 16S rRNA: DNA extraction (phenol-chloroform method), PCR amplification, electrophoretic verification of products, Sanger sequencing; phylogenetic analysis was performed in MEGA X, selecting the closest sequences via BLAST and using bootstrap evaluation.

H_2 production was determined in sealed vials after argon anaerobization of cell suspensions, followed by measurement of H_2 in the gas phase by gas chromatography. To intensify hydrogen formation pathways, inhibitors of photosynthetic electron transport (CCCP, DBMIB, DCMU, KCN) were used at different concentrations. The activity of the bidirectional [NiFe]-hydrogenase was assessed by the rate of H_2 accumulation (using methyl viologen reduced with dithionite), and nitrogenase activity was assessed by the acetylene reduction test ($C_2H_2 \rightarrow C_2H_4$) followed by GC control.

Scientific novelty

1. The first time, 6 axenic strains of cyanobacteria belonging to three taxonomic orders — *Oscillatoriales, Chroococcales*, and *Synechococcales* — were isolated. The obtained strains expand current understanding of the taxonomic diversity of cyanobacteria in the studied ecosystems and provide a basis for further investigation of their physiological properties and biotechnological potential.

2. For the first time, a comprehensive identification of the isolated strains was carried out based on morphological characteristics and molecular genetic analysis (16S rRNA); phylogenetic analysis confirmed the taxonomic affiliation of the isolates to *Desertifilum* sp. PSU 1129, *Synechocystis* sp. PSU 1262, *Phormidium* sp. PSU 1120, *Chroococcus* sp. PSU 1008, *Synechococcus* sp. SU 78, and *Oscillatoria* sp. S-001, the sequences of which were deposited in the NCBI GenBank database under the following accession numbers: Desertifilum sp. PSU 1129 (No. PZ058579), *Synechocystis* sp. PSU 1262 (No. PZ058575), *Phormidium* sp. PSU 1120 (No. PZ058576), *Chroococcus sp*. PSU 1008 (No. PZ058578), *Synechococcus* sp. SU 78 (No. PZ058580), and *Oscillatoria* sp. S-001 (No. PZ058577).

3. For the first time, the possibility of targeted enhancement of H_2 production through the use of photosynthetic electron transport inhibitors was experimentally demonstrated; the most effective inhibitors and their optimal concentrations were determined: for DCMU — 20 μM (up to 710 nmol H_2/mg chl a/h), and for KCN — 500 μM (up to 860 nmol H_2/mg chl a/h).

4. **Scientific and practical significance**

The scientific significance of the work lies in expanding data on the taxonomic diversity and potential of cyanobacteria in Kazakhstan, as well as clarifying the factors that regulate photobiological hydrogen release. The practical significance consists in obtaining a promising H_2-producing strain and validating conditions that increase hydrogen production through optimization of cultivation regimes and application of photosynthesis inhibitors, as well as preparing technological recommendations that can be used for further scaling and for the design of photobioreactor systems.

Main provisions submitted for defense

1. Six axenic strains of cyanobacteria were isolated and identified. The taxonomic affiliation and phylogenetic position of the isolated cyanobacteria were confirmed by molecular identification based on the 16S rRNA gene and phylogenetic analysis; the nucleotide sequences were deposited in the NCBI GenBank database as follows: *Desertifilum* sp. PSU 1129 (No. PZ058579), *Synechocystis* sp. PSU 1262 (No. PZ058575), *Phormidium* sp. PSU 1120 (No. PZ058576), *Chroococcus* sp. PSU 1008 (No. PZ058578), *Synechococcus* sp. SU 78 (No. PZ058580), and *Oscillatoria* sp. S-001 (No. PZ058577).

2. Based on the results of comparative screening of the isolated cyanobacteria, the strain *Synechocystis* sp. PSU 1262 was selected as the most promising producer, combining a high level of hydrogen evolution with stable growth under cultivation conditions.

3. The hydrogen-producing capacity of cyanobacteria can be purposefully enhanced through optimization of cultivation conditions and inhibition of photosynthetic electron transport. For the strain *Synechocystis* sp. PSU 1262, the baseline level of hydrogen evolution is 80–97 nmol H_2/mg chl a/h. Inhibition of electron transport results in a

multiple increase of this parameter: upon addition of DCMU (20 μM) — up to 710 nmol H_2/mg chl a/h; KCN (500 μM) — up to 860 nmol H_2/mg chl a/h.

4. Immobilization of cells in a Ca-alginate matrix makes it possible to maintain hydrogen production under dark anaerobic conditions for up to 168 h while preserving photosynthetic activity, thereby increasing the duration of the process by 72 h compared with a cell suspension.

Main results and conclusions

The results obtained in the work allow the following conclusions to be drawn:

1. Soil and water samples were collected from the Turkestan, Kyzylorda, and Almaty regions (a total of 56 samples). As a result of multi-stage isolation and purification, six axenic cyanobacterial isolates were obtained. Their molecular-genetic identification and phylogenetic analysis using marker sequences (16S rRNA) were performed, confirming the taxonomic affiliation of the isolates: *Desertifilum* sp. PSU 1129 (99.78%), *Synechocystis* sp. PSU 1262 (98.52%), *Phormidium* sp. PSU 1120 (99.14%), *Chroococcus* sp. PSU 1008 (95.87%), *Synechococcus* sp. SU 78 (94.44%), *Oscillatoria* sp. S-001 (~91% to the closest strains).

2. Baseline indicators of H_2 release and the activity of key enzymes of hydrogen metabolism were determined for the isolated strains. In non-heterocyst isolates, nitrogenase activity was not detected, indicating the predominant role of hydrogenase systems in their hydrogen production. Maximum H_2 production was recorded for *Synechocystis* sp. PSU 1262 (up to 49.6 nmol H_2/mg Chl *a*/h) and *Synechococcus* sp. SU 78 (up to 48.3 nmol H_2/mg Chl *a*/h).

3. For the active strain *Synechocystis* sp. PSU 1262, optimal physicochemical factors for hydrogen production were established: pH 8 (up to 79.2 nmol H_2/mg Chl *a*/h); among buffers at pH 8, HEPES was most effective (up to 81.9 nmol H_2/mg Chl *a*/h, higher than TES and TRIS). The temperature optimum for H_2 production is 27–30 °C (maximum around 27.5 °C – 81.3 nmol H_2/mg Chl *a*/h). Addition of $NaNO_3$ has a pronounced inhibitory effect (decrease from 75 to 3 nmol H_2/mg Chl *a*/h with increasing concentration). Optimal illumination for H_2 production is 120 μmol photons/m²/s (up to 96.6 nmol H_2/mg Chl *a*/h); higher levels cause photoinhibition and cell damage.

4. It was shown that inhibition of photosynthetic electron transport with concentration optimization significantly enhances H_2 production by *Synechocystis* sp. PSU 1262. The most effective inhibitors are DCMU 20 μM (up to 710 nmol H_2/mg Chl *a*/h) and KCN 500 μM (up to 860 nmol H_2/mg Chl *a*/h); for comparison: CCCP 1 μM – 158.3, DBMIB 20 μM – 96.8 nmol H_2/mg Chl *a*/h. Increasing the dose is accompanied by toxicity and suppression of electron transport; at the same time, KCN 500 μM provides maximal stimulation with minimal impact on growth (OD_{730} 0.45 vs 0.52 in the control). The effect depends on the light-dark regime: with KCN 500 μM and 12 h light / 6 h dark, 1552.2 nmol H_2/mg Chl *a*/h is achieved; with DCMU 20 μM and 12/12, up to 1394 nmol H_2/mg Chl *a*/h.

5. The immobilization of *Synechocystis* sp. PSU 1262 in calcium alginate gel was substantiated as a technological approach that ensures the preservation of cellular physiological activity and prolonged hydrogen production. Immobilized cells retain photosynthetic activity (O_2 accumulation rate – 0.012 $\mu mol \cdot ml^{-1} \cdot min^{-1}$) and provide prolonged H_2 accumulation in dark anaerobiosis – up to – 48.1 nmol H_2/mg *Chl* a/h by

168 h, with a maximum in the initial phase (up to – 19.3 nmol H_2/mg *Chl* a/h in the first 9 h) and reaching a plateau after 96 h.

6. Technological recommendations were formulated and an optimized laboratory protocol for increasing H_2 yield in cyanobacteria was described, including: standardized biomass preparation and creation of anaerobiosis; selection of buffer system and pH (pH 8 optimum; HEPES preferred); optimization of temperature (27–30 °C) and illumination (120 μmol photons/m²/s); exclusion/reduction of nitrate nitrogen (an inhibitory factor); use of the most effective inhibitors (KCN 500 μM, DCMU 20 μM) considering light-dark regimes; and, if necessary, immobilization in a calcium-alginate matrix to stabilize and prolong the process.

Personal contribution of the author

The author carried out analysis of scientific literature on the research topic, planning of experiments, collection of natural material, isolation and maintenance of pure cyanobacterial cultures, morphological and molecular studies, experiments on hydrogen release by cyanobacterial cells (including testing photosynthesis inhibitors), processing and interpretation of results, preparation of publications, and writing of the dissertation.

Connection with governmental programs and projects

The dissertation was performed within the projects: AP13067574 “Increasing the activity of hydrogen production by cyanobacterial strains by genetic engineering” and AP14869048 “Constructing cyanobacterial hup-mutants and developing a cultivation technology for increased hydrogen production.”

Approbation of results

The main results and provisions of the dissertation were presented and discussed at the following scientific events:

1. 11th International Conference on Photosynthesis and Hydrogen Energy: Research for Sustainability – June 3-9, 2023, Bahcesehir University Future Campus, Istanbul, Turkey (p. 65).
2. 12th International Conference on Photosynthesis and Hydrogen Energy: Research for Sustainability – July 13-19, 2024, Bahcesehir University South Campus, Istanbul, Turkey (p. 36).
3. International Scientific Forum “Modern Trends in Sustainable Development of Biological Sciences” (IFBioScFU 2024) – March 27-28, 2024, Almaty, Kazakhstan.

Publications

The main results of the dissertation are reflected in 11 published works, including 1 article in a national scientific journal from the list of the authorized body of the Republic of Kazakhstan, 6 articles in first-quartile journals (Q1), and 3 theses in international conference proceedings. Patents were obtained based on the research results: “Photobioreactor for the cultivation of phototrophic microorganisms,” No. 8207 (04.04.2023) and “Cyanobacteria strain *Nostoc* sp. J -1 as a producer of biohydrogen for biofuel production,” No. 7880 (20.12.2022).

Structure and volume of the dissertation

The dissertation is presented in 116 pages of computer text and includes a list of symbols and abbreviations, an introduction, a literature review, a section “Materials and Methods of Research,” a section “Results and Discussion,” a conclusion, and a list of references (205 titles). The work contains 4 tables, 33 figures, and 1 appendix.