ՀՀ ԿՐԹՈՒԹՅԱՆ, ԳԻՏՈՒԹՅԱՆ, ՄՇԱԿՈՒՅԹԻ ԵՎ ՍՊՈՐՏԻ
ՆԱԽԱՐԱՐՈՒԹՅՈՒՆ
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ԻՍԿԱՆԴԱՐՅԱՆ ՄԵՐԻ ԿԱՐԵՆԻ

Cupriavidus necator H16 **մանրէի հետերոտրոֆ աճի տարբեր պայմաններում** H_2**-օքսիդացնող հիդրոգենազային ակտիվության և կենսաէներգետիկական չափանիշների ուսումնասիրումը**

**Գ.00.04- կենսաքիմիա մասնագիտությամբ
կենսաբանական գիտությունների թեկնածուի
գիտական աստիճանի հայցման ատենախոսության**

ՍԵՂՄԱԳԻՐ

ԵՐԵՎԱՆ 2025

MINISTRY OF EDUCATION, SCIENCE, CULTURE AND SPORTS OF RA
YEREVAN STATE UNIVERSITY

ISKANDARYAN MERI KAREN

The investigation of H_2-oxidizing Hydrogenase activity and bioenergetic parameters of *Cupriavidus necator* H16 bacterium under different conditions of heterotrophic growth

SYNOPSIS

of dissertation for conferring of science degree of
Candidate of Biological Sciences
In the specialty of 03.00.04-Biochemistry

YEREVAN 2025

Ատենախոսության թեման հաստատվել է Երևանի պետական համալսարանում

Գիտական ղեկավար՝ կ.գ.դ., դոց. Աննա Արշակի Փոլադյան

Պաշտոնական ընդդիմախոսներ՝ կ.գ.դ., պրոֆ.Աստղիկ Զավենի Փեփոյան

կ.գ.թ., դոց. Հռիփսիմե Լյուդվիկի Հայրապետյան

Առաջատար կազմակերպություն՝ ՀՀ ԳԱԱ Հ. Բունիաթյանի անվան կենսաքիմիայի ինստիտուտ

Ատենախոսության պաշտպանությունը տեղի կունենա 2025 թ. փետրվարի 25-ին ժամը 14:00-ին, Երևանի պետական համալսարանում գործող ՀՀ ԲԿԳԿ-ի Կենսաֆիզիկայի 051 մասնագիտական խորհուրդի նիստում (0025, Երևան, Ալեք Մանուկյան փ. 1, ԵՊՀ, կենսաբանության ֆակուլտետ):

Ատենախոսությանը կարելի է ծանոթանալ Երևանի պետական համալսարանի գրադարանում:

Ատենախոսության սեղմագիրը առաքված է 2025թ. հունվարի 24-ին:

051 մասնագիտական խորհրդի գիտական քարտուղար, կ.գ.դ., դոց.՝ Մարինե Աշոտի Փարսադանյան

The theme of dissertation has been approved at Yerevan State University

Academic advisor: D. Sc., Assoc. Prof. Anna Poladyan

Official opponents: D. Sc., Prof. Astghik Pepoyan

Ph. D., Assoc. Prof. Hripsime Hayrapetyan

Leading organization: H. Buniatian Institute of Biochemistry of NAS RA

The defence of the dissertation will be held on 25 February 2025, at 14:00, at the session of 051 Scientific Specialized Council on Biophysics of HESC of RA at Yerevan State University (0025, Yerevan, Alex Manoogian str. 1, YSU, faculty of Biology).

The dissertation is available at the library of Yerevan State University

The synopsis has been sent on 24th January 2025.

Scientific Secretary of 051 Specialized Council, D. Sc., Assoc. Prof. Marine Parsadanyan

INTRODUCTION

Topic's significance: *Cupriavidus necator* H16 is a facultative chemolithoautotrophic β-proteobacterium and a well-known biotechnological model organism. It is an industrially applicable microbe capable of synthesizing bioplastics, biofuels, and various valuable metabolites through both heterotrophic pathways using organic compounds and autotrophic pathways using CO_2, H_2, and O_2 gases.

During lithoautotrophic growth, *C. necator* synthesizes four distinct O_2-tolerant [NiFe]-Hydrogenases (Hyds): a membrane-bound Hyd (MBH), a soluble cytoplasmic Hyd (SH), an actinobacterial-type Hyd (AH), and a regulatory Hyd (RH). The [NiFe]-Hyds catalyze the reversible conversion of H_2 into protons (H^+) and electrons (e^-), depending on the cell's redox state and nutritional conditions. This reaction is important for developing hydrogen-based technologies, for instance microbial or enzymatic fuel cells (MFCs, EFCs) couple H_2 oxidation and O_2 reduction for efficient electricity production. The O_2-tolerant [NiFe]-Hyds of *C. necator* H16 can serve as anodic biocatalysts in FCs. However, the active Hyd enzymes is not always synthesised in *C. necator* cells. Nutrient-rich environments, favorable for high bacterial biomass production, may not promote Hyd synthesis. According to the literature energy-limited and stress conditions enhance Hyd production, as these enzymes enable additional energy supply to the cell by directing H^+ and e^- generated from H_2 oxidation into the respiratory chain. During heterotrophic growth, *C. necator* can utilize various organic carbon and energy sources, including intermediates of the tricarboxylic acid (TCA) cycle, sugars, fatty acids, amino acids, alcohols, and aromatic compounds. It has also been shown that glycerol is assimilated by *C. necator* H16, albeit with slow growth, which nevertheless induces the expression of genes encoding Hyds and enzymes of the Calvin-Benson-Bassham (CBB) cycle. The latter are key components of lithoautotrophic metabolism.

Importantly, Hyd synthesis can be stimulated during heterotrophic growth of *C. necator*. Therefore, it is crucial to elucidate the mechanisms that promote Hyd synthesis and activity under heterotrophic conditions, as well as to investigate the redox potential (ORP), a key physicochemical parameter characterizing microbial growth and metabolism influenced by Hyd activity.

To ensure the efficient and cost-effective synthesis of Hyds, it is advisable to utilize nutrient-rich organic waste, such as whey, which is a major by-product of the dairy industry. Whey contains approximately 50% nutrients, including lactose, proteins,

minerals, and vitamins. Therefore, developing innovative methods and approaches for the sustainable reuse of dairy waste is essential.

As a rich nutrient source, whey can be processed and used for microbial biomass production, as well as for obtaining valuable compounds and enzymes.

Research goals and tasks: The aim of this research is to investigate the activity of H_2-oxidizing Hyds and bioenergetic parameters of *C. necator H16* under various heterotrophic growth conditions, as well as the mechanisms promoting Hyd activity and microbial biomass production, and their potential application in biofuel cells (BFCs).
The research focuses on the following objectives:

1. Study the impact of LB (Luria-Bertani) medium components: amino acids, B-group vitamins, and glycine betaine effect on the growth parameters (optical density, specific growth rate, pH) and H_2-oxidizing Hyd activity of *C. necator* H16.
2. Investigate the bioenergetic parameters of *C. necator* H16 (wild-type) and mutant strains with Hyd defects, including changes in redox potential (ORP), pH, and proton motive force (Δp) (intracellular pH and membrane potential) under various heterotrophic growth conditions.
3. Investigate H^+/K^+ exchange and ATPase activity in membrane vesicles of *C. necator* H16 under heterotrophic growth and oxygen-limited conditions.
4. Process organic waste, analyse the composition of organic compounds before and during microbial growth, and develop optimal conditions for *C. necator* H16 cultivation.
5. Cultivate *C. necator* H16 using organic waste such as glycerol and dairy whey, optimizing conditions for maximum Hyd activity and biomass production.
6. Evaluate *C. necator* H16 whole cells as anodic biocatalysts for electricity (current) production in electrochemical systems.

Scientific novelty and practical value of the study: The growing energy demand, coupled with the scarcity of traditional resources and environmental pollution, necessitates the development of alternative energy sources and sustainable strategies for bioenergy production through eco-friendly systems. The O_2-stable Hyds of *C. necator H16* are promising targets for biofuel cell (BFC) applications. However, there is limited information in the literature about compounds that enhance Hyd synthesis or activity

under heterotrophic conditions. Furthermore, the potential synthesis or activity of Hyds of *C. necator* H16 using organic waste has not been studied.

Identifying mechanisms to promote Hyd synthesis and/or activity of *C. necator* H16 can lead to new approaches for producing highly active enzymes, which could address global energy challenges. This work represents the first comprehensive study of *C. necator* H16 growth under various heterotrophic conditions, emphasizing the mechanisms promoting the activity of O_2-stable H_2-oxidizing Hyds. It demonstrates, for the first time, the role of amino acids, particularly glycine, and glycine betaine in promoting Hyd synthesis and activity. The impact of glycine on the activity and synthesis of four purified and isolated Hyds (MBH, SH, RH, and AH) was also explored, enabling future applications of these enzymes in BFCs.

Platinum-independent hydrogen-based technologies offer an environmentally friendly approach to energy and biosensor production. Utilizing inexpensive organic waste reduces the costs of *C. necator H16* biomass production. This study shows that using dairy whey and glycerol as organic waste enhances the activity of O_2-stable Hyds and biomass production, suggesting novel methods for waste utilization and solving environmental pollution problem.

Bioenergetic parameters during *C. necator* H16 growth, the role of O_2-stable Hyds in proton motive force generation, and their interaction with F_oF_1-ATPase under various heterotrophic conditions were investigated. These experiments involved both the wild-type *C. necator* H16 and mutant strains with Hyd defects. Understanding the bioenergetic parameters of *C. necator* will enable better control of O_2-stable Hyd synthesis with significant biotechnological potential. Moreover, cells containing active Hyds isolated from heterotrophic growth media were tested as anodic biocatalysts in BFCs, leading to efficient bioelectricity production. This research contributes to the development of Hyd-based BFCs and the production of electroactive microbial biomass. It provides fundamental, environmental, and economic advantages, offering a foundation for advancing BFC technology in Armenia.

Main points to present at the defense:

- Precise control of heterotrophic growth conditions promotes O_2-stable Hyd synthesis. Components of LB medium, such as amino acids, and glycine betaine, stimulate not only biomass production but also Hyd synthesis in *C. necator* H16.

- Organic wastes such as dairy whey and glycerol can serve as substrates for *C. necator* H16 biomass production, addressing energy and environmental challenges while reducing pollution.
- Redox potential (ORP) reduction and medium acidification associated with increased H_2-oxidizing Hyd activity in *C. necator* H16.
- Energy homeostasis regulation: Hyds activity and/or their presence are critical for maintaining cellular energy balance in *C. necator* H16.
- Nutrient recovery from whey: Optimized cultivation conditions, aligned with the "3R (reduce, reuse, recycle) concept," led to enhanced biomass growth, high Hyd activity, and the generation of ~0.65 V electrochemical potential.
- Bioelectricity production: Cells with active O_2-stable Hyds are promising candidates as anodic biocatalysts in BFCs.

Work approbation: The results of the dissertation work were presented through oral and poster presentations at several international conferences and congresses, including the FEMS - Microbiology Congress 2022 (Belgrade, Serbia), IUBMB–FEBS–PABMB Congress 2022 (Lisbon, Portugal), the 67th Annual Meeting of the Biophysical Society (San Diego, USA, 2023), the Falling Walls Science Summit 2023 (Berlin, Germany), the 68th Annual Meeting of the Biophysical Society (Pennsylvania, USA, 2024), and the FEBS 2024 Congress (Milan, Italy).

Publications: According to experimental data observed in dissertation, 14 papers, including 4 articles in peer-reviewed journal, 1 article in a journal included in the list of HESC of the RA and 9 abstracts were published.

Volume and structure of dissertation: The dissertation contains the following chapters: introduction, literature review (Chapter 1), experimental part (Chapter 2), results and discussion (Chapter 3), conclusions and cited literature (total 147 papers and books). The dissertation consists of 133 pages, 3 tables and 40 figures.

MATERIALS AND METHODS

Bacterial growth mediums and conditions: *C. necator* H16 (DSM 428), its mutant strains with various Hyd defects (HF371, HF380, HF388), and strains with Strep-tagged Hyds (HF649, HF574 pGE3888, HP23, HP80) were provided by Dr. O. Lenz (Technical

University of Berlin, Berlin, Germany). *C. necator* was cultivated under different heterotrophic conditions in Fructose-Nitrogen minimal salt medium (FN), fructose-glycerol-nitrogen (FGN), glycerol-fructose-nitrogen (GFN) mediums, and in dairy industrial wastes with different dilutions and filtration, both under aerobic (250 ml flasks / 100 ml medium) and microaerobic (1000 ml flasks / 800 ml medium) conditions. The bacteria were cultivated on a shaker at 130 rpm and 30°C, 24 - 172 hours. The inoculant was grown under the same conditions but at 37°C, from glycerol stocks rapidly frozen in liquid nitrogen and stored at -80°C. Depending on the effect of the studied substrate, the medium was supplemented with 7.0 µmol/ml glycine, several L-amino acids (L-AAs), and B vitamins, as well as 7.0–300.0 µmol/ml glycine-betaine [Iskandaryan 2023; Iskandaryan et al., 2023a].

Investigation of the growth parameters of *C. necator:* Bacterial growth was determined by using a spectrophotometer 600nm (Spectro UV-VIS Auto, LaboMed, Los Angeles CA, USA). During bacterial growth the specific growth rate (µ, h^{-1}), colony formic units (CFU) and cell dry weight (CDW, g L^{-1}) were determined. The pH of the medium was measured using a pH electrode of HJ1131B pH-meter (Hanna Instruments, Portugal). The ORP of bacterial culture medium was measured by using a couple of oxidation-reduction platinum (Pt) (EPB-1, Measuring Instruments Enterprise, Gomel, Belarus, or PT42BNC, Hanna Instruments, Portugal) and titanium-silicate (Ti-Si) (EO-02, Measuring Instruments Enterprise, Gomel, Belarus) electrodes [Iskandaryan et al., 2023a; Poladyan et al., 2023].

Hyds purification and biochemical analyses: The purification of the MBH, SH, RH and AH Hyds obtained from *C. necator Strep-tagged* strains (HF649, HP80, HF574 pGE3888, HP23) was conducted through affinity chromatography following the procedures outlined by Lenz et al., 2018. Subsequently, the Hyds H_2-oxidizing activity was measured, and the protein samples were promptly frozen in liquid nitrogen, preserving them at –80°C for subsequent biochemical analyses.

Protein concentrations were assessed using the Pierce BCA Protein Assay Kit (Thermo Scientific) with a Spectra MAX 340 Microplate Reader spectrophotometer (90-240V), bovine serum albumin was used as a standard. Protein purity was assessed by polyacrylamide gel electrophoresis (SDS-PAGE) and immunological analysis (Western blotting).

Investigation of H_2-oxidazing Hyd activity: The H_2-oxidizing activity of whole cells, MBH, RH and AH were assessed through methylene blue (MB) reduction at a

wavelength of 570 nm, 30 °C (Cary 60 UV–vis spectrophotometer, Agilent Technologies, USA). A sample (1-20 μL) was added to an H_2-saturated reaction mixture, containing 1.9 mL of previously H_2-saturated buffer, and 5 μL MB (Lenz et al. 2018). The SH was measured in anaerobic cuvettes using NAD^+ as an acceptor of electrons under 365 nm (Cary 60 UV–vis spectrophotometer, Agilent Technologies, USA). Sample (3-10 μL) and 1 mM 40 μL NAD^+ were added to a 1.9 mL 50 mM H_2-saturated Tris/HCl buffer, pH 8.0. A unit of enzyme activity equivalent to 1 μmol oxidized H_2 (accordingly 1 μmol reduced MB) per minute per 1 mg of protein / 1 mg (g) CDW [Iskandaryan et al., 2023a].

Determination of ATPase activity: Membrane vesicles were isolated from bacteria by the osmotic lysis method. Membrane vesicles were incubated with 0.1 mM DCCD for 10 min. All assays were done at 30 °C. ATPase activity was calculated by determining the amount of inorganic phosphate (P_i) produced during the reaction of membrane vesicles with 5 mM ATP (pH 7.0) in the assay mixture (50 mM Tris–HCl buffer containing 1 mM $MgSO_4$, pH 7.0). The ATPase activity was expressed in nM P_i / (min μg protein). P_i was measured spectrophotometrically at 750 nm (Labomed, Los Angeles, CA, USA).

Identification of dairy whey composition: Sweet and acid whey was collected from the local «Marianna» Dairy products factory (Yerevan, Armenia) and analyzed by HPLC for sugars, amino- and organic- acids composition. The determination of micro- and macro- elements has been carried out with Agilent 5800 ICP-OES system [Poladyan et al., 2023; Iskandaryan et al., 2024a].

Protein nitrogen determination using the Kjeldahl Method based on ammonia containing distillate titration by 0.1 N HCl. Investigation of Volatile solids (VS) (indirect method of organic carbon measurement) in samples performed by thermal treatment of the samples in a muffle furnace (DAIHAN Digital Muffle Furnace, Taiwan) set at 550°C for 4 h [Iskandaryan et al., 2024a].

Investigation of bioenergetics parameters: The intracellular pH (pH_{in}) was measured by the quenching of fluorescence of 9-aminoacridine (9-AA), as described, using a Cary Eclipse spectrofluorimeter (Varian, USA) with excitation at 390 nm and emission at 460 nm. Membrane potential ($\Delta\varphi$) is determined by the potentiometric method using a selective TPP^+ electrode. It was estimated using TPP^+ distribution through the membrane. The proton motive force (Δp) of cells consists of the

transmembrane differences in membrane potential ($\Delta\varphi$) and pH (ΔpH) and it was calculated by the following equation: $\Delta p = \Delta\varphi + (2.3RT/F)\ \Delta pH$

H^+ and K^+ fluxes through the plasma membrane of intact cells were determined potentiometrically using a pH (HI1131, Hanna Instruments) and potassium-selective (HI4114, Hanna Instruments).

Electrochemical measurements: Electrochemical measurements were carried out in a double-electrode electrochemical system, which was equipped with a computerized potentiometer (H_2 fuel cell voltammeter (HFCVA)). Two-electrode electrochemical system with polyvinyl chloride base, carbon conductive thin layers and Ag/AgCl layer (reference electrode) was used as testing micro-strips. Biological samples were immobilized on the micro-strips between two thin layer electrodes using 3 mM KCl solution and 0.8% bacteriological agar support. Samples were in connection with both electrode surfaces to close the electrical circuit by making an electrochemical cell [Seferyan et al., 2024].

Chemicals and data processing: All chemicals of analytical grade were used. Data processing was done using MS Excel 2016 software, which calculated average values (from three and more independent measurements), standard errors, and Student's t-test (P) (used to average the data from the different series of measurements) for data analyses. The difference was valid when $P<0.05$.

Results and Discussion

The impact of amino acids and glycine betaine on growth and H_2-oxidizing hydrogenases activity of *C. necator* H16

The growth parameters, ORP, and pH kinetics of *C. necator* H16 were monitored with supplementation of 0.5% LB medium, YE or tryptone during 72 hours. Bacterial growth on FN standard medium without listed additional components was considered a control experiment [Iskandaryan et al., 2023].

Both LB medium and its components supplementation lead to stimulation of bacterial growth ~1.7 - 2.2 fold compare to control, during 72 hours bacterial growth. YE and tryptone might serve as an additional source of nitrogen (N), carbon (C), and vitamins for bacterial growth.

During bacterial growth, a pH decrease of the mediums ~0.45-0.55 points was recorded after 24 hours of bacterial growth. In contrast to the control, in observed samples, a decrease in ORP values was recorded from positive (+109 ± 10 mV) to

negative (-120 ± 10 mV) values after 24 h. With a pH and ORP decrease in the presence of LB components, the H_2-oxidising total Hyd activity was detected, in contrast to the control. The maximum total (whole cells) H_2-oxidizing Hyd activity ~ 8 ± 0.06 U/g(CDW)/min and 7 ± 0.02 U/g(CDW)/min was shown in the mediums with supplementation of tryptone and LB, respectively after 24 h.

For understanding the role of tryptone and YE components in the synthesis or activation of H_2-oxidizing Hyd enzymes, the *C. necator* H16 growth was examined in an FN medium with 7.0 μmol/ml L-AA (Pro, Glu, Asn, Asp, Tyr, Cys, Ala, Arg, His, Ser and Gly) and vitamins B (Thiamine, Nicotinic acid, Pyridoxine) supplementation for up to 72 hours. After 72 h, compared to control, the supplementation of Pro, Asn, Ala, and Gly significantly stimulated *C. necator* H16 growth by ~ 1.9, 2.3, 2.7, and 2.9 fold, respectively. The specific growth rate (μ) of the samples with AAs was also stimulated ~1.2 to 1.4 ± 0.02 fold, compared to control. Studied B vitamins and other AAs, supplementation also stimulated growth ~1.2-1.7 ± 0.05 fold.

The pH and ORP of bacterial growth mediums with the Gly, Ala, Asn, and Pro L-AAs containing samples was significantly decreased, after 24 h. Moreover, after 48 h the ORP values of the Pt electrode recovered to positive and remained almost unchanged in 72 h of bacterial growth (Fig.1).

It is well known that the ORP (E_h) of the bacterial culture is influenced not only by the ratio of oxidized to reduced fermentation products but also by pH based, whereby as the E_h decreases, the pH increases. However, it is difficult to explain the relationship between the observed decrease in E_h and the decrease in pH during bacterial growth; it may be explained by the complexity of redox processes occurring during fermentation, which cannot be described by the equation above. ORP might be influenced by the activities of both H_2-oxidizing and H_2-evolving Hyd enzymes, as well as other membrane-associated oxidoreductases that affect proton release.

The H_2-oxidizing Hyd activity of whole cells was not observed in control samples, while it was recorded in samples, where Gly, Ala, Asn, Pro, His and Arg were present. The maximal ~ 22 ±0.05 U/g(CDW)/min Hyd activity was determined in Gly containing samples after 24 h of *C. necator* H16 growth (Fig.2).

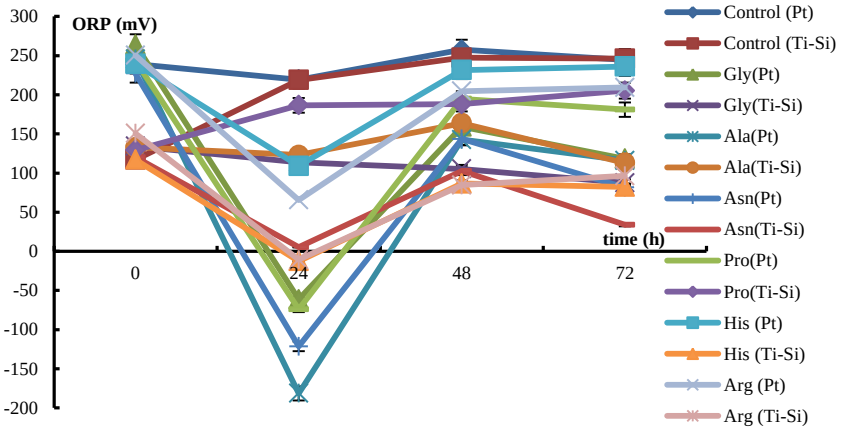

Fig.1. L-AAs influence the kinetics of ORP of *C. necator* H16. Bacteria were grown aerobically, at pH 7.0, for 72 hours (n=5, p <0.05). The ORP was measured by using Pt and Ti-Si electrodes.

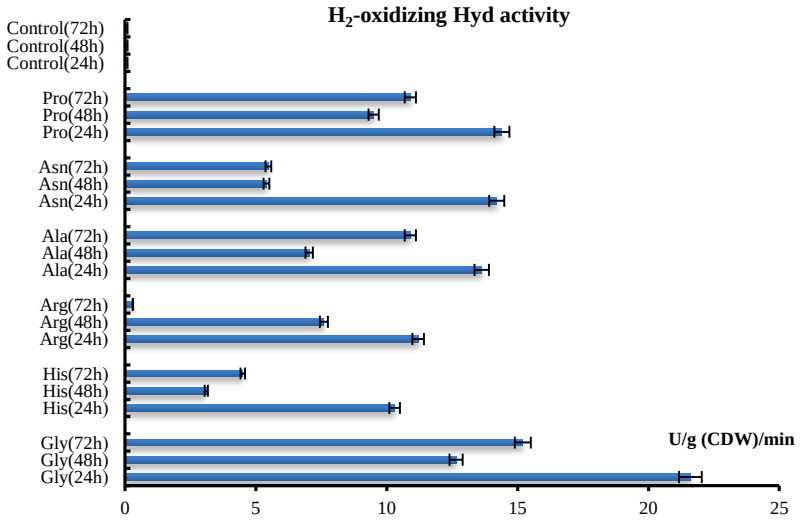

Fig.2. H_2-oxidizing total Hyd activity of whole cells of *C. necator* H16 under influence of L-AAs. L-AAs were added in FN medium at 7.0 μmol/ml concentration (72 hours, n=5, p <0.05).

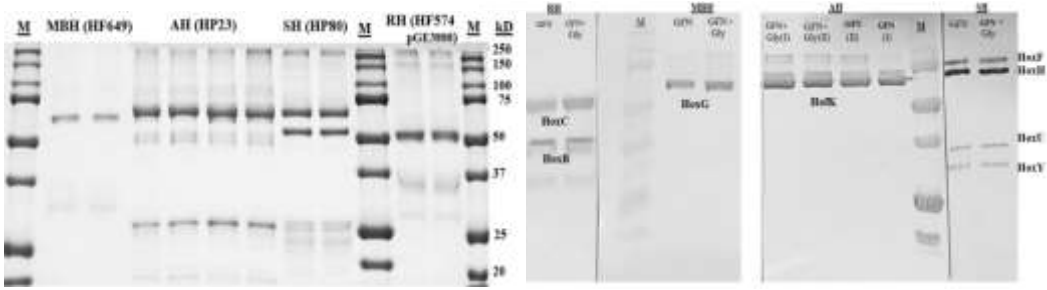

Fig. 3. Coomassie-stained SDS PAGE gel and Western Blotting analysis of Hyd enzyme samples purified from GFN and GFN+Gly media. The gel contains 1 µg of MBH and 5 µg of AH, RH, and SH, **M: Marker (Precision Plus Protein™ Dual** Color Standard 10–250 kDa from Bio-Rad) (n=3, $p<0.05$).

The effect of L-AA on the H_2-oxidizing Hyd activity can be inferred from their metabolic and bioenergetic activities in microbial cells, but how they work in this particular situation is currently unexplained and subject to further investigation [Iskandaryan et al., 2023]. Glycine (Gly) significantly enhanced the activity of individual [NiFe]-Hyds of *C. necator* strains grown in GFN medium under microaerobic conditions. Compared to glycine-free medium, the activity of single H_2-oxidizing Hyds in Gly-containing GFN medium increased by 1.6- to 3-folds. For instance, the SH activity reached ~71 U/mg/min, compared to ~24.5 U/mg/min in GFN medium. AH activity increased 1.6-fold to ~0.8 U/mg/min, while RH and MBH activities were stimulated by ~1.8-fold, reaching ~7.1 U/mg/min and ~31.2 U/mg/min, respectively.

Interestingly, Gly also reduced the lag phase typically observed during AH activity measurements, thereby enhancing the catalytic efficiency of the Hyds. The production of specific enzymes was visualized using SDS-PAGE and Western blotting techniques (Fig.3) [Iskandaryan et al., 2024c].

These findings highlight Gly's potential role as a promoter of Hyds activity, possibly through its effects on enzyme maturation, genetic regulation, or catalytic center stabilization.

Many soil bacteria use GB not only as an osmoprotectant, but also may use it as a sole source of C and N. *C. necator* is one of the soil bacteria and may involve GB in cell metabolism. Different concentrations of GB were supplemented, *µmol/mL*: 7, 15, 25, 50, 75, 100, 150, 200, 250, 300 to the FN medium. Bacterial growth at low concentrations of GB was stimulated ~ 30-40 % after 48h, in contrast to control: the

maximal growth stimulation was detected at 15 *µmol/mL* and 25 *µmol/mL* concentrations (OD_{600} ~4.4 ± 0.02). However, at high concentrations, the growth activity of *C. necator* was partially inhibited (OD_{600} ~2.7 ± 0.02) compared with control by ~10–20%. Bacterial growth led to the decrease of culture medium pH from pH 7.0 up to pH 6.5, after 24 h in all studied samples. In parallel with a decrease in extracellular pH, a slight reduction in ORP was observed. The reduction in the values of both parameters is associated with the adaptation of microorganisms to environmental conditions and with the production of different metabolites. Compared to the control, where was detected a slight H_2-oxidizing Hyd activity, the addition of GB induced Hyd activity. Basically, at concentrations of 250 and 300 *µmol/mL*, the maximum activity of 16.4 ± 0.05 U/g(CDW)/min was observed after 24 h of growth. Interestingly the total H_2-oxidizing Hyd activity of *C. necator* H16 was more significant in the case of high GB concentrations, however, partial inhibition of bacterial growth was observed at the same concentrations. The minimum Hyd activity was respectively observed at low concentrations (7 *µmol/mL*: 4.0 ± 0.05 U/g(CDW)/min, where growth stimulation was recorded, in contrast to the control.

The transport of GB was energy-dependent and occurred against a concentration gradient, GB transport is driven by the electrochemical proton gradient. Therefore, active transport of a large amount of GB may lead to energy depletion of cells, which is the main reason for growth inhibition and stimulation of Hyds activity. GB as osmoprotectant might promote folding of proteins, such as Hyds, beside of the GB may be catabolised into Gly, which is one of the identified L-AAs responsible for the induction of H_2-oxidizing activity during the heterotrophic growth of *C. necator* H16 [Iskandaryan, 2023].

A novel, cost-effective approach for the production of hydrogenases from whey-based by-products

Large-scale production of biomass and biotechnologically valuable products are still an economic challenge. The application of cheap organic materials is an attractive alternative to reduce fermentation costs. Furthermore, it has been demonstrated that specific conditions during heterotrophic growth of *C. necator* favours the catabolic derepression of Hyd gene expression. This feature allows isolating biomass with active Hyds, avoiding an autotrophic fermentation process, which requires the use of dangerous gas mixtures of H_2, O_2, and CO_2.

To cultivate *C. necator*, different approaches of pretreatment of the media were applied. Dilution of whey from 0 to 5 fold using dH_20 and filtration (when needed) was performed. The pH of the mediums was adjusted to pH7 $NaHCO_3$. Favourable conditions for the growth of bacteria and Hyd activity were developed: the aerobic and microaerobic growth of *C. necator* H16 were followed for 72 and 168 h. Results were compared to the control experiment, which was *C. necator* growth on FN (aerobic) and GFN (microaerobic) medium.

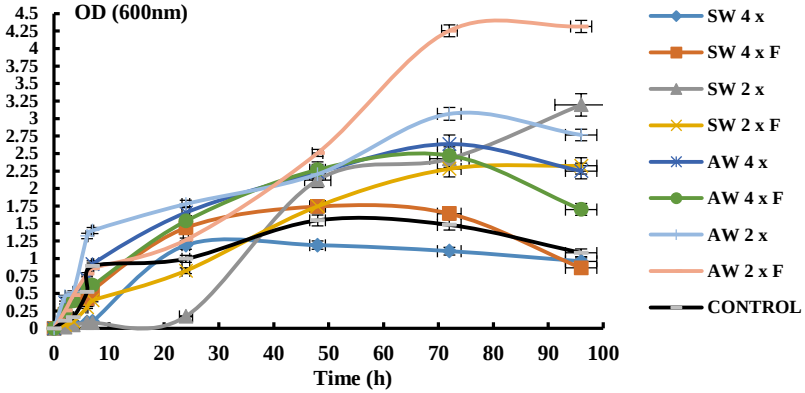

Fig.4. Growth kinetics of *C. necator* H16 on SW and AW. Bacteria were grown aerobically, at 130 rpm, 30ºC. Control is bacterial growth on FN medium; x 2 or x 4 – are 2 or 4-fold dilutions, respectively; F-is filtrated samples (n=5, p<0.05). SW 2 x - 30 g/L, AW 2 x - 25 g/L, SW 4 x - 15 g/L, AW 4 x - 12.5 g/L, SW 2 x F - 18 g/L, AW 2 x F - 16 g/L, SW 4 x - 9 g/L, AW 4 x -8 g/L of dry weight.

Bacteria were grown overnight in the FN medium, then 3% of bacterial pre-cultures were introduced into the whey-containing growth mediums, pH 7. The bacterial biomass formation (OD_{600}) on 2-fold and 4-fold diluted, filtrated, and non-filtrated acid whey (AW) and sweet whey (SW) mediums. Biomass formation was followed by a decrease in medium pH from 7.0 up to 5.9-6.5. Compared to control, bacterial growth was mainly stimulated: maximal growth (OD_{600} ~ 4 ± 0.02 and CFU~ 0.6 10^{15}) was shown for bacteria on 2-fold diluted and filtrated sample (Fig.4). The specific growth rate of bacteria in control reached 0.4 h^{-1} and was slightly stimulated in many samples. When bacteria grow on SW, maximal CFU was 2 (0.7 10^{10}) and 4 (0.5 10^{10}) fold diluted SW.

Compared to control, all samples demonstrated a low ORP profile: maximal decrease from positive +250±10 mV to low negative -150±10 mV was detected in the 2-fold

diluted, filtrated SW. In all samples growing on whey, after 24 h H_2-oxidizing Hyd activity was significant: maximal 13±0.05 U/g(CDW)/min was shown for 4 fold diluted and filtrated SW. Compared to growth on non-filtrated whey, upon bacterial consumption of filtrated whey Hyd-activity is stimulated. Thus, during bacterial growth on whey, the relationship of Hyd enzymes activity with low (more reductive) ORP and pH is suggested [Poladyan et al., 2023; Iskandaryan et al., 2022b].

The growth conditions were modified for *C. necator* by prolonging growth (7 day) under microaerophilic conditions: A mixture of 7.5 g/L SW and 6.5 g/L AW with and without glycerol was explored for growth of *C. necator* H16. Optimal concentrations of nutrients (N, C), pH, and ORP proved critical factors during the lag and exponential phases of bacterial growth, as well as for optimal Hyd activity. Unlike in the GFN medium, after the exponential growth-phase (first day) of *C. necator* H16 an observed increase in pH from 7.0 to 7.2 - 8.0 was noted. However, a pH decrease was observed during the subsequent late stationary phase, reaching ~6.2 in the mixture of AW and SW with glycerol and 5.8 without glycerol. The maximum biomass attained for *C. necator* H16 was observed after the 5th day when utilizing a mixture of SW and AW (OD_{600} ~3.9 ± 0.02). Notably, there was no significant difference recorded between the SW and AW mixture and the mixture containing glycerol. This contrasts with the slow growth of the culture grown in a GFN medium [Iskandaryan et al., 2024a].

A decrease in ORP, with the most significant change recorded on the 2nd day of bacterial growth, dropping from +330 ± 10 mV to +80 ± 10 mV was measured. This decrease in ORP correlated with an increase in Hyd activity. Stimulated H_2-oxidizing Hyd activity was observed from the 2nd day (48 h) of *C. necator* H16 cultivation in the mixtures both with or without glycerol. For comparison, in the GFN medium Hyd activity started on the 1st day (24 hours). The maximum Hyd activity, approximately 5.2 ± 0.05 U/min/mg CDW, was determined for the glycerol-containing sample, which was twice the activity compared to the glycerol-free mixture. This increase in activity persisted until the 6th day of bacterial growth, and this phenomenon can be attributed to the presence of glycerol, which facilitates the expression of the genes encoding Hyd and sustains the notably slow growth of *C. necator* H16. Thus, microaerophilic conditions with the supplementation of glycerol stimulated both bacterial biomass and Hyd activity in whey [Iskandaryan et al., 2024a].

The initial N concentration in all samples was 35 mg/100 ml. The VS in glycerol-containing medium was 40 mg/100 ml higher than in glycerol-free samples (180 mg/100 ml). Cultivation of *C. necator* H16 in glycerol-containing samples doubled the N

concentration compared to glycerol-free samples, suggesting enhanced N incorporation into biomass and protein synthesis. In contrast, C concentration showed an opposite trend. These effects may result from increased Hyd enzyme activity in glycerol-containing samples due to redox stress relief via H_2 release and higher protein levels.

Table. 1. Analysis of waste composition using HPLC and ICP-OES systems. Results are expressed in mg/mL and mg/L units (n=3, p<0.1).

Parameter	Cheese Whey (SW)	Curd Whey (AW)	Mixture of SW+AW
pH	6.5 ± 0.20	5 ± 0.30	5.5 ± 0.30
ORP (Pt, mV)	+180±20	+150±30	+330 ± 10
Protein content (mg/mL)	2.5 ± 0.1	1.5 ± 0.2	
Lactic acid (mg/mL)	1 ± 0.02	0.3 ± 0.05	1.3 ± 0.05
Oxalic acid (mg/mL)	0.5 ± 0.1	0.3 ± 0.05	1.05 ± 0.1
Formic acid (mg/mL)	0.2 ± 0.05	0.1 ± 0.1	0.9 ± 0.05
Citric acid (mg/mL)	1.5 ± 0.05	1.5 ± 0.05	0.86 ± 0.05
Lactose (mg/mL)	28.10 ± 0.80	24.77 ± 1.0	28 ± 1.0
Amino acids (Gly, Asp, Arg, Hs, Ala et al.) (mg/ mL)	0.003-0.09 (each)	0.003-0.09 (each)	0.006-0.18 (each)
Potassium (K) (ppm)	310	240	
Magnesium (Mg) (ppm)	16	210	
Microelements (Al, Fe, Cu, Zn, Ni, etc.) (ppm)	> 3 (each)	> 3 (each)	

HPLC data indicated the presence of several organic acids, significant amount of lactose, trace amounts of fructose, glucose, and galactose in the whey samples (Table 1) [Iskandaryan et al., 2024a].

The impact of hydrogenases on the bioenergetic and electrochemical activity of *C. necator* H16 cells under different heterotrophic growth conditions

Bioenergetic parameters, including transmembrane potential ($\Delta\varphi$), intracellular pH (pH_{in}), proton motive force (Δp) and ATPase activity, were analyzed in relation to the activity of hydrogenases (Hyds) in *C. necator* H16 and Hyd-defective mutant strains (HF388 - SH^-, HF380 - MBH^-, and HF371 - MBH^- and SH^-) under aerobic and microaerobic heterotrophic growth conditions. In wild-type (H16) bacterial cells grown in FN medium, $\Delta\varphi$ was approximately -150 ± 0.05 mV, while in both Gly-supplemented FN and GFN, as well as GFN samples exhibited an increase in $\Delta\varphi$ of approximately 10-40 mV. Nevertheless, Hyd-defective mutants, $\Delta\varphi$ values were elevated by 1.2- to 1.6-fold. Changes in pH_{in} were also noted in the samples with the H_2-oxidizing activity: compared to the control (pH_{in} 6.5±0.01), pH_{in} consistently increased by approximately 0.55 - 1 units in Gly-added FN and GFN, and GFN samples, respectively ΔpH of those samples was equal to 0.55-0.9 [Iskandaryan et al., 2024b]. The ΔpH of the mutants ranged from -0.3 to 0.7 units, compared to the H16 strain, where it was 0.2 units. The Δp of *C. necator* H16 was approximately -161 ± 0.5 mV, while in the mutants, it increased by 97–112 mV. However, when *C. necator* H16 was cultivated under aerobic and microaerobic conditions with Gly supplementation, the Δp increased by 30–84 mV (fig.5).

F_OF_1-ATPase activity was determined by treating membrane vesicles with the F_OF_1-ATPase inhibitor N,N'-Dicyclohexylcarbodiimide (DCC) for 10 minutes. In H16 strain it was markedly varied depend on growth conditions: K^+ stimulated 1.6 fold the DCC-sensitive ATPase activity up to 90 ± 1 nmol P_i /min/mg protein. Addition of glycine and glycerol to the growth medium lead to the 1.1–1.6 fold higher activity of DCC-sensitive ATPase. The HF380 mutant grown in the FN medium exhibited ~1.5 fold lower ATPase activity compared to the wild-type strain, however the supply of Gly, significantly stimulate the enzyme activity to 103 ± 1 nmol P_i /min/mg protein. The results revealed that *C. necator* maintains $\Delta\varphi$, ΔpH, and Δp within specific ranges crucial for cellular processes, such as nutrient uptake and pH homeostasis. Our findings demonstrate the interplay between Hyds, bioenergetic parameters and environmental adaptability of *C. necator*, emphasizing their importance for survival, metabolic diversity, under different growth conditions (fig.6).

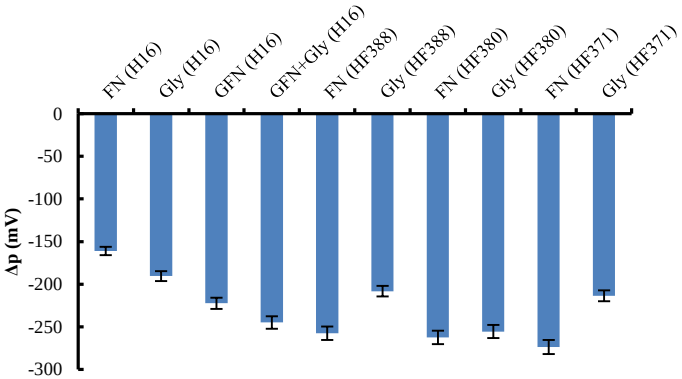

Fig.5. The dependency of electrochemical potential (**Δ**p) on the composition of growth medium and the cultivated strain of *C. necator* (H16 and mutant strains HF388, HF380, HF371) (n=5, p<0.05).

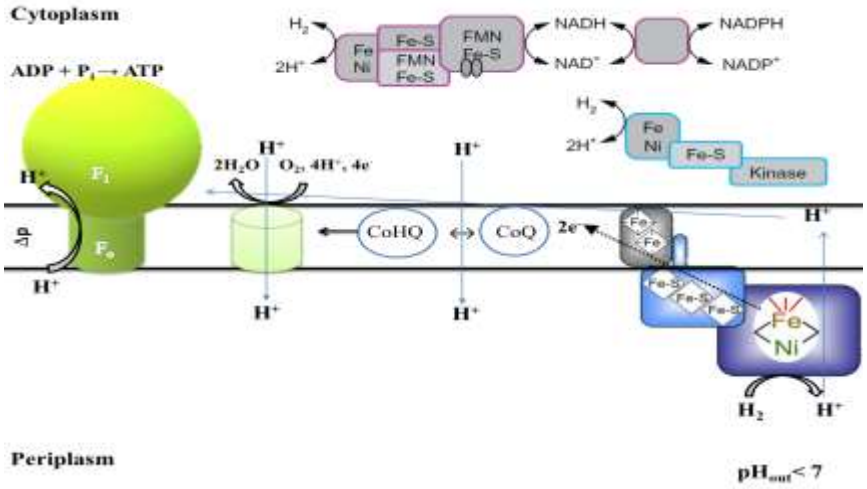

Fig.6. The schematic representation of the bioenergetic metabolism of the *C. necator* H16 bacterium's cell is presented. It includes the activity of the electron transport chain (ETC), F_OF_1-ATP synthase, MBH and SH activity.

Hyd enzymes can exhibit variable functional activity depending on several factors, including the carbon source (e.g., fructose, glycerol) and the pH of the medium, among others [Seferyan et al., 2024]. *C. necator* H16 cells cultivated in GFN medium produced

a current with a potential of 0.46 ± 0.05 V. In contrast, biomass obtained from GFN+Gly medium generated a current with a potential of 0.65 ± 0.05 V (fig.7) [Poladyan et al., 2024]. Cells of *C. necator* H16 grown in dairy waste mixture without glycerol exhibited a potential of approximately 0.35 ± 0.05 V. However, in glycerol-containing samples, the potential was stimulated 1.8-fold, reaching 0.63 ± 0.05 V (fig.7) [Iskandaryan et al., 2024a].

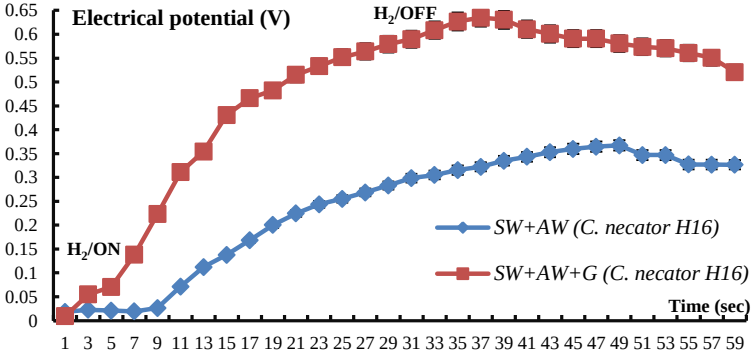

Fig.7. Electrical potential generation based on H_2-oxidizing Hyd activity of *C. necator* H16 cultivated on the mixture of waste streams. *C. necator* H16 was cultivated for 2 days (30°C, 130 rpm). The system operated at a temperature of 30°C (n = 3, p < 0.1).

Current generation was observed in all samples as long as the H_2 supply continued, however, the current began to decrease when the H_2 flow was stopped. Microbial fuel cells hold significant promise for renewable green energy generation and its widespread applications. The Hyd enzymes of *C. necator* H16, with their high sensitivity to H_2 gas and O_2-tolerancy, are ideal candidates as a cathode for use in fuel cells and biosensors. Their advantages include stability in the presence of O_2, low cost, high sensitivity, and the feasibility of developing portable devices.

Conclusions

1. Supplementation (0.5%) of LB medium's components to the FN medium led to a ~2-fold stimulation of growth and H_2-oxidizing total Hyd activity of *C. necator* H16 cells. Hyd activity was observed in the presence of six tested amino acids. The highest Hyd activity,

22 ± 0.05 and 14 ± 0.5 U/g(CDW)/min, were recorded in samples containing Gly and Pro, respectively.

2. Low concentrations of glycine betaine enhanced *C. necator* H16 growth (30–40%) and repressed H_2-oxidizing Hyd activity, while high concentrations slowed the growth (10–20%) but simultaneously stimulated Hyd activity.
3. In all samples, bacterial growth and specific growth rate (μ) were stimulated in the presence of Gly. Depending on Hyd type, the addition of Gly to *C. necator* H16 GFN medium under microaerobic conditions stimulated purified and isolated individual Hyd activities by ~1.6–3-fold. Additionally, Gly reduced the lag phase observed during the expression of AH activity.
4. In curd and cheese whey, the biomass formation of *C. necator* H16 was accompanied with a decrease in the medium's pH and ORP, and the utilization of organic acids (e.g., oxalic acid, lactic acid). Maximum growth (OD_{600} ~4.3) and CFU (~0.6×10^{15}) were observed in 13 g/L curd whey, while the highest total Hyd activity, 13 ± 0.05 mU/mg (CDW)/min, was observed in 7.5 g/L cheese whey under aerobic conditions. A mixture of whey, glycerol supplementation, and microaerobic conditions significantly stimulated the total Hyd activity of the cells.
5. In FN medium, ~ -150 mV Δp and 90 ± 1 nmol P_i (min*mg (protein))$^{-1}$ ATPase activity were recorded. In all samples with Hyd activity, Δp and ATPase activity were stimulated by ~1.2- and 2-fold, respectively. K^+ enhanced FoF_1-ATPase activity. In Hyd-defective mutant cells, Δp increased ~2-fold due to membrane potential, while ATPase activity decreased 1.5-fold, which was restored by Gly supplementation. The results suggest a potential interaction between Hyd and FoF_1-ATPase during heterotrophic growth of *C. necator* and their role in regulation cellular bioenergetic processes.
6. Bacterial whole cells grown heterotrophically with high H_2-oxidizing Hyd activity immobilized on micro-strips exhibited electrochemical activity in MFCs. The highest current generation was observed with the cells isolated from Gly-containing GFN medium, corresponding to a 0.65 ± 0.05 V.

List of Publications as a part of Dissertation Topic

1. Iskandaryan M., Baghdasaryan L., Minasyan E., Trchounian K., Antranikian G., Poladyan A., A novel, cost-effective approach for the production of hydrogenase enzymes and molecular hydrogen from recycled whey-based by-products. Int J Hydrogen Energy (2024(a)). https://doi.org/10.1016/j.ijhydene.2024.10.256

2. Seferyan T, Baghdasaryan L, Iskandaryan M., Trchounian K., Poladyan A, Development of an H_2 fuel cell electrochemical system powered by *Escherichia coli* cells, Electrochemistry Communications (2024), 165, 107746. https://doi.org/10.1016/j.elecom.2024.107746
3. Iskandaryan M., Blbulyan S., Sahakyan M., Vassilian A., Trchounian K., Poladyan A., L-amino acids affect the hydrogenase activity and growth of *Ralstonia eutropha* H16. *AMB Express* (2023(a)), 13(1), 1-12. https://doi.org/10.1186/s13568-023-01535-w
4. Poladyan A., Trchounian K., Paloyan A., Minassyan E., Aghekyan H., Iskandaryan M., Khoyetsyan L., Aghayan S., Tsaturyan A., Antranikian G., Valorization of whey-based side streams for microbial biomass, molecular hydrogen, and hydrogenase production. Appl Microbiol Biotechnol (2023), 107 (14), 4683-4696. https://doi.org/10.1007/s00253-023-12609-x
5. Iskandaryan M., The role of Glycine-Betaine on the growth and Hydrogenases activity of *Ralstonia eutropha* H16. Proceedings of the YSU B: Chemical and Biological Sciences (2023), 57(2(261)), 154-163. https://doi.org/10.46991/PYSU:B/2023.57.2.154
6. Iskandaryan M., Schoknecht J., Lenz O., Poladyan A., Effect of Glycine on the Heterotrophic Growth and [NiFe]-Hydrogenase Activity of *Cupriavidus necator* H16, P-20-020, 48th FEBS Congress, 29 June-3 July, Milan, Italy, 2024(c).
7. Poladyan A., Baghdasaryan L., Iskandaryan M., Seferyan T., Evaluation of an H_2 Fuel Cell Electrochemical System Powered by Microbial Cells, 48th FEBS congress, 29 June-3 July, Milan, Italy, 2024.
8. Iskandaryan M., Poladyan A., The impact of oxygen-tolerant Hydrogenases on cell Bioenergetics of *Cupriavidius necator* H16, Biophysical Journal (2024(b)), Volume 123, Issue 3, 248a - 249a, DOI: 10.1016/j.bpj.2023.11.1572
9. Poladyan A., Iskandaryan M., Karapetyan O., Minasyan E., Vassilian A., Trchounian K., Anatranikian G., A Novel Cost-Effective Approach for Production of Hydrogenase Enzymes and Molecular Hydrogen from Whey-Based By-Products, 14TH International conference on hydrogen production (ICH2P-2023), Hamad Bin Khalifa University Doha, Qatar, December 19-21, 2023
10. Iskandaryan M., Mnatsakanyan L., Poladyan A., The role of Glycine-Betaine in the Hydrogen Metabolism of *Ralstonia eutropha* H16, Federation of European microbiological societies (FEMS) conference, Hamburg, Germany, 9 - 13 July, 2023(b)

11. Iskandaryan M., Seferyan T., Baghdasryan L., Trchounian K., Poladyan A., Constriction of H_2 biofuel cell voltammeter based on *Escherichia coli* microbial cells. Biophysical Journal (2023(c)), 122(3), 156a. https://doi.org/10.1016/j.bpj.2022.11.974
12. Iskandaryan M., Sahakyan M., Poladyan A., Blbulyan S., The effects of some L- amino acids on the growth and hydrogen-oxidizing hydrogenases activity of *Ralstonia eutropha* H16, FEBS Open Bio12 (2022(b)), S1, P-02.4-001 (p. 177), doi:10.1002/2211-5463.13440
13. Iskandaryan M., Blbulyan S., Sahakyan M., Vassilaian A., Poladyan A., Hydrogen-oxidizing Hydrogenases activity of *Ralstonia eutropha* H16 during bacterial growth in organic waste materials, Federation of European microbiological societies (FEMS) conference, Belgrade, Serbia, 30 June-2 July, 2022(a)
14. Poladyan A., Iskandaryan M., Aghekyan H., Minassyan E., Ghevondian D., Trchounian K., Paloyan A., Aghayan S., Antranikian G., The valorization of whey-based side-streams for microbial biomass, hydrogen and hydrogenase enzyme production, Federation of European microbiological societies (FEMS) conference, Belgrade, Serbia, 30 June-2 July, 2022.

ԻՍԿԱՆԴԱՐՅԱՆ ՄԵՐԻ ԿԱՐԵՆԻ

Cupriavidus necator H16 **մանրէի հետերոտրոֆ աճի տարբեր պայմաններում** H_2**-օքսիդացնող հիդրոգենազային ակտիվության և կենսաէներգետիկական չափանիշների ուսումնասիրումը**

Ամփոփագիր

Հանգուցային բառեր՝ *Cupriavidus necator* H16, H_2-օքսիդացնող հիդրոգենազներ, O_2-կայուն, ամինաթթու, գլիցին-բետաին, օրգանական թափոններ, կենսաէներգետիկ չափանիշներ, պրոտոնաշարժ ուժ, ԱԵՖ-ազային ակտիվություն, էլեկտրաքիմիական ակտիվություն

Էներգիայի աճող պահանջարկը, ավանդական աղբյուրների սակավության և շրջակա միջավայրի աղտոտման խնդիրները կարևոր նախապայման են գտնել այլընտրանքային էներգիայի աղբյուրներ և մշակել նոր մեթոդներ ու ռազմավարություն՝ կայուն և էկոլոգիապես մաքուր կենսաէներգիայի ստացման համար: Ցույց է տրվել, որ ոչ ախտածին *Cupriavidus necator* H16-ի O_2-կայուն Հիդ-ները պոտենցիալ թիրախներ են վառելիքային սարքերում՝ որպես անոդային կենսակատալիզատոր կիրառման համար: Ներկայիս հետազոտությունները ցույց

են տալիս, որ ԱԹ-ների հավելումը ստանդարտ Ֆրուկտոզ-Ազոտային միջավայր խթանում է *C. necator* H16-ի աճը և կենսազանգվածի արտադրությունը մոտ 3 անգամ, և նպաստում O_2-կայուն [NiFe]-Հիդ-ների սինթեզին և/կամ ակտիվության խթանմանը բակտերիաների հետրոտրոֆ աճի ընթացքում:

Բակտերիաների աճը ուղեկցվում է միջավայրի թույլ թթվեցմամբ, իսկ ՕՎՊ-ի գրանցված անկումը՝ հանգեցնում Հիդ ֆերմենտների ակտիվության բարձրացմանը: Գլիցինի և Լ-ԱԹ-ների խթանող ազդեցությունը հավանաբար կապված է բջջի ռեդօքս վիճակի հետ, որը կարող է հանգեցնել գեների էքսպրեսիայի ակտիվացմանը: Դրանք կարող են ներգրավված լինել տարբեր նյութափոխանակային ուղիներում՝ Կրեբսի ցիկլում և խթանել ռեդօքս համարժեքների առաջացումը (ՆԱԴH, ՖԱԴH_2): Մասնավորապես Gly-ի խթանիչ ազդեցությունը (~3 անգամ) ակնհայտ է ՆԱԴ$^+$/ՆԱԴH-կախյալ ԼՀ-ի ակտիվության վրա:

Շատ հողային բակտերիաներ, ի դեմս *C. necator*-ի կարող են սինթեզել, ինչպես նաև կլանել ԱԹ-ներ արտաքին միջավայրից: Gly-ի սինթեզման հնարավոր աղբյուր կարող է հանդիսանալ հողում լայն տարածում ունեցող Գլիցին բետաինը (ԳԲ): Ենթադրաբար, ԳԲ-ը նաև կարող է գործել որպես սպիտակուցները կայունացնող միացություն, ենթադրաբար այն կարող է մասնակցել Հիդ-ների ֆոլդինգին: ԳԲ-ի կլանումը էներգատար գործընթաց է. դրա բարձր քանակների կլանումը հանգեցրել է բջջի էներգետիկ սպառմանը՝ նվազեցնելով բակտերիաների կենսազանգվածի արտադրությունը և միևնույն ժամանակ խթանելով Հիդ ակտիվությունը:

Կաթնամթերքի արդյունաբերության ենթամթերք՝ շիճուկի ԲԿՀՔ հետազոտության արդյունքում միջավայրում հայտնաբերվել են շուրջ 15 ԱԹ-ներ, ներառյալ գլիցինը, հիստիդինը, ալանինը և այլն, ինչպես նաև մի շարք օրգանական թթուներ, վիտամիններ և մետաղներ: Ցույց է տրվել, որ կաթնամթերքի արդյունաբերության կողմնակի արտանդրանքները կարող են լինել *C. necator*-ի կուլտիվացման համար նպաստավոր միջավայր շնորհիվ իրենց քիմիական կազմի՝ կենսազանգվածի արտադրության և O_2-կայուն Հիդ-ների ստացման համար: Կաթնային շիճուկի խառնուրդի և այդ խառնուրդի և գլիցերոլի յուրացման արդյունքում կենսազանգվածի վերականգնման արդյունավետությունը համապատասխանաբար կազմել է ~100% և 85.2%: Կենսազանգվածի ձևավորման (ԲՉՔ) վերլուծությունը՝ որպես շիճուկի յուրացման արդյունքում վերականգնված բաղադրիչ,իրականացվել է հիմնվելով սկզբնական C / ՑՊՕՄ-ի և N-ի քանակների վրա:

[Ni-Fe]-Հիդ-ների առկայությունը, որոնք իրականացնում են H_2-ի օքսիդացում և վերականգնում՝ հավանաբար ստեղծում են ներբջջային H_2-ի շրջապտույտ: ԼՀ-ը կարող է ոչ միայն օքսիդացնել, այլ նաև վերականգնել H_2՝ բջջի ռեդօքս համարժեքներից կախված: Այս գործընթացը նպատակաուղղված է բջջի ռեդօքս բալանսի կարգավորմանը և ԹՀ-ների կողմից H_2 օքսիդացմանը՝ բջջի պրոտոնաշարժ ուժը կարգավորելու համար: Հիդ-ների սինթեզը/ակտիվությունը, հավանաբար, կարող է հավասարակշռել Δp-ն՝ H_2-ի ցիկլի միջոցով: Հիդ-ները *C. necator*-ի բջջում էլեկտրոններ են տրամադրում շնչառական շղթայի համալիրներին՝ մասնակցելով էներգիայի առաջացմանը: Բացի այդ, ԹՀ կարող է ունենալ H^+ տեղափոխիչ ակտիվություն, ինչը թույլ է տալիս բջիջներին վերահսկել H^+-ի հոմեոստազը:

Համաձայն էլեկտրաքիմիական վերլուծության, ինչպես Gly պարունակող ԳՖԱ միջավայրում, այնպես էլ թափոնների խառնուրդում կուլտիվացված և մեկուսացված մանրէների միջոցով գեներացվել է 0.65 ± 0.05 Վ պոտենցիալով հոսանք, տվյալ միջավայրերում խթանված Հիդ-ային ակտիվության շնորհիվ:

C. necator-ը մեծ կենսատեխնոլոգիական ներուժ ունեցող մանրէ է: Դրա նյութափոխանակային ուղիների և կենսաէներգետիկ վիճակի ուսումնասիրումը հետերոտրոֆ աճի ընթացքում, թույլ կտա կառավարել բակտերիայի նյութափոխանակությունը և ուծել բազմաթիվ էկոլոգիական (թափոնների յուրացում, կենսահոսանքի ստացում) և էներգետիկ (կենսահոսանքի ստացում) խնդիրներ:

ИСКАНДАРЯН МЕРИ КАРЕНОВНА

Исследование активности H_2-окисляющих гидрогеназ и биоэнергетических показателей бактерии *Cupriavidus necator H16* при различных условиях гетеротрофного роста

РЕЗЮМЕ

Ключевые слова: *Cupriavidus necator* H16, H_2-окисляющие гидрогеназы, O_2-устойчивость, аминокислота, глицин бетаин, органические отходы, биоэнергетические показатели, протон движущая сила, АТФазная активность, электрохимическая активность

Растущий спрос на энергию, нехватка ее традиционных источников и проблемы загрязнения окружающей среды являются важными предпосылками для поиска альтернативных источников энергии и разработки новых методов и стратегий получения устойчивой и экологически чистой биоэнергии. Показано, что O_2-устойчивые гидрогеназы (Гид) непатогенной *Cupriavidus necator* H16 являются потенциальными мишенями для применения в топливных элементах в качестве анодных биокатализаторов.

Исследования показывают, что добавление аминокислот (АК) в стандартную фруктоза азотную среду (ФА) стимулирует рост *C. necator* H16 и производство биомассы примерно в 3 раза, а также способствует синтезу и/или активации O_2-устойчивых [NiFe]-Гид, при гетеротрофном росте бактерий. Рост бактерий сопровождается слабым подкислением среды, а снижение уровня ОВП приводит к повышению активности Гид. Стимулирующее воздействие глицина и L-АК, вероятно, связано с редокс-состоянием клетки, что может активировать экспрессию генов. АК-ы задействованы в различных метаболических путях, таких как цикл Кребса, и соответственно, могут способствовать формированию редокс-эквивалентов (NADH, $ФАДH_2$). В частности, стимулирующее влияние глицина (~3 раза) проявляется в активности NAD^+/NADH-зависимой растворимой Гид.

Многие почвенные бактерии, включая *C. necator*, способны синтезировать аминокислоты, а также поглощать их из внешней среды. Глицин может быть синтезирован из глицин бетаина (ГБ), который является широко распространённым соединением в почве. ГБ также может стабилизировать белки и возможно он участвует в процессе фолдинга Гид. Поглощение ГБ является энергозатратным процессом: поглощение его в больших количествах привело к энергетическому истощению клетки, что снизило производство биомассы бактерий (10-20%), одновременно стимулируя активность Гид.

Побочные продукты молочной промышленности, такие как сыворотка, были исследованы на предмет их химического состава (ВЭЖХ). В результате анализа среды было обнаружено около 15 аминокислот, включая глицин, гистидин, аланин и другие, а также ряд органических кислот, витаминов и металлов. Было показано, что побочные продукты молочной промышленности могут служить благоприятной средой для культивирования *C. necator* благодаря своему химическому составу, что способствует производству биомассы и получению O_2-стабильных Гид.

Анализ формирования биомассы (СВК) как результата переработки сыворотки на основе содержания начального С / летучих твёрдых веществ (ЛТВ) и азота

показал, что эффективность восстановления биомассы составила ~100% и 85.2%, соответственно при переработке молочной сыворотки и её смеси с глицерином.

Наличие [Ni-Fe]-Гид, которые окисляют и восстанавливают H_2, вероятно, создают внутриклеточный цикл H_2. Растворимые Гид способны не только окислять, но и восстанавливать H_2 в зависимости от редокс-эквивалентов клетки, регулируя редокс-баланс клетки и окисление H_2 мембраносвязанной Гид (МСГ) для управления протон движущей силы клетки. Синтез и активность Гид, возможно, балансируют Δp через цикл H_2. Гид в клетке *C. necator* поставляют электроны комплексам дыхательной цепи, участвуя в выработке энергии. Кроме того, МСГ может обладать активностью переносчика H^+, что позволяет клеткам контролировать гомеостаз протонов.

Согласно электрохимическому анализу, в среде ГФА содержащей Gly, а также в смеси отходов, культивированные и выделенные микроорганизмы генерировали ток с потенциалом ~0,65 ± 0,05 В благодаря стимулированной Гид активности в этих средах.

C. necator является микроорганизмом с высоким биотехнологическим потенциалом. Изучение его метаболических путей и биоэнергетического состояния в условиях гетеротрофного роста, а также их корректное управление позволит решить множество экологических (утилизация отходов, получение биоэлектричества) и энергетических (производство биоэлектричества) задач.