ՀՀ ԿՐԹՈՒԹՅԱՆ, ԳԻՏՈՒԹՅԱՆ, ՄՇԱԿՈՒՅԹԻ ԵՎ ՍՊՈՐՏԻ
ՆԱԽԱՐԱՐՈՒԹՅՈՒՆ
ԵՐԵՎԱՆԻ ՊԵՏԱԿԱՆ ՀԱՄԱԼՍԱՐԱՆ

ՎԱՆՅԱՆ ԼԻԱՆԱ ՄԱՆՎԵԼԻ

ESCHERICHIA COLI-ՈՒՄ ՋՐԱԾՆԻ ՆՅՈՒԹԱՓՈԽԱՆԱԿՈՒԹՅՈՒՆԸ և ՊՐՈՏՈՆԱՅԻՆ ՑԻԿԼԸ ԳԼՅՈՒԿՈԶԻ ՏԱՐԲԵՐ ԿՈՆՑԵՆՏՐԱՑԻԱՆԵՐԻ ԽՄՈՐՄԱՆ ՊԱՅՄԱՆՆԵՐՈՒՄ

Գ.00.04 – Կենսաքիմիա մասնագիտությամբ
կենսաբանական գիտությունների թեկնածուի
գիտական աստիճանի հայցման ատենախոսության

ՍԵՂՄԱԳԻՐ

ԵՐԵՎԱՆ 2024

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

VANYAN LIANA MANVEL

HYDROGEN METABOLISM AND PROTON CYCLING IN *ESCHERICHIA COLI* DURING FERMENTATION OF DIFFERENT GLUCOSE CONCENTRATIONS

SYNOPSIS

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

YEREVAN 2024

Ատենախոսության թեման հաստատվել է Երևանի պետական համալսարանում

Գիտական ղեկավար՝ կ.գ.դ., պրոֆ. Կարեն Արմենի Թռչունյան

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 2024թ. հոկտեմբերի 18-ին ժամը 14^{00}-ին, Երևանի պետական համալսարանում գործող ՀՀ ԲԿԳԿ-ի Կենսաֆիզիկայի 051 մասնագիտական խորհրդի նիստում (0025, Երևան, Ալեք Մանուկյան փ. 1, ԵՊՀ, կենսաբանության ֆակուլտետ):

Ատենախոսությանը կարելի է ծանոթանալ Երևանի պետական համալսարանի գրադարանում:

Ատենախոսության սեղմագիրն առաքված է 2024թ սեպտեմբերի 17-ին:

051 մասնագիտական խորհրդի գիտական քարտուղար, կ.գ.դ., դոցենտ՝ Մ.Ա. Փարսադանյան

The theme of dissertation has been approved at Yerevan State University

Academic advisor: D.Sc., Prof. Karen Armen Trchounian

Official opponents: D.Sc., Prof. Astghik Zaven Pepoyan
Ph.D., Assoc. Prof. Hripsime Ludvik Hayrapetyan

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

The defense of the dissertation will be held on 18th of October 2024, at 14:00, at the session of 051 Scientific Specialized Council on Biophysics of SCC 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 17th of September, 2024.

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

INTRODUCTION

Topic's significance. Energy sources in nature are unlimited and non-renewable, which can lead to serious problems. That is why it is necessary to find alternative sources of energy that will be more environmentally friendly and affordable. Under anaerobic conditions, *Escherichia coli* performs a mixed acid fermentation of a carbon source, resulting in the production of molecular hydrogen (H_2) by the cleavage of formic acid over a wide range of environmental pHs (5.5-7.5). H_2 is environmentally friendly and a more efficient energy carrier compared to oil and gas, producing ~142 kJ/g of energy from combustion and only water as a byproduct. One of the ways to increase the yield of hydrogen production is to understand the regulation mechanism of H_2 production and the effect on the overall metabolism of H_2 in bacteria by studying the dependence of the activity of the enzymes responsible for H_2 generation on various environmental factors. The enzymes responsible for the H_2 production process in *E. coli* are 4 hydrogenases (Hyd), which act together to form an H_2-proton cycling system within the membrane.

By discovering the mechanisms of regulation of the activity of these enzymes, it will be possible to fully control the process with high efficiency. The H_2-proton cycle is the basis of energy transformation and energy production of useful work in living organisms and biological systems. At the same time, the processes of energy generation during respiration are more specified and studied, the basis of the main patterns of which is the chemiosmotic theory of proton motive force generation proposed by Mitchell. Meanwhile, the processes of fermentation are less studied and clarified, but nowadays they are more widespread (many pathogenic microbes of the human body survive in oxygen-free conditions, and it is economically beneficial to use fermentation microbes especially for extraterrestrial processes).

Currently, the use of waste as alternative sources of carbon and research in this direction for production purposes is relevant. During waste processing, microbially available carbon sources (e.g., glucose) are generated, the effect of concentration of which is crucial to increase the efficiency of microbial production. In addition, glucose is used in basic production and is the preferred source of carbon and energy for a number of microbes. The pathways of glucose assimilation and the enzymes responsible for its gradual degradation are well studied both in the absence and presence of oxygen. Usually, high concentrations of carbohydrates are used as a basic ingredient in various researches and productions.

By carrying out the functional analysis of the enzymes responsible under different conditions, it is possible to register significant results for the development of hydrogen production technology, as well as for the production of biomass and other organic materials.

Research goals and tasks. The purpose of this work is to study the role of *E. coli* hydrogenase enzymes during the fermentation of glucose concentrations of 2 g L^{-1} (low) and 8 g L^{-1} (high) at different pHs, to distinguish the interaction of specific subunits with other membrane-bound proton motive force generating proteins

depending on glucose concentration, reveal the importance of Hyds in the prpcesses of energy conversion and glucose concentration sensitivity in *E. coli.*

Constituted tasks of the research were:

- Study the role of different subunits of Hyd-3 and Hyd-4 in the H_2 production during the fermentation of different concentrations of glucose at pH values of 5.5, 6.5, and 7.5.
- Investigate the role of specific subunits of the membrane-bound Hyd-4 enzymes of *E. coli* in proton (H^+)/potassium (K^+) transport, the mechanisms of interaction with the F_O/F_1-ATPase during this process, and the dependence of the dithiol-disulfide exchange on the concentration of glucose at pH 7.5.
- Clarify the role of the membrane-bound and additional subunits of Hyd-1 and Hyd-2 in the total and F_O/F_1-dependent H^+/K^+ fluxes during the fermentation of low or high concentrations of glucose at pH 7.5.
- Study the growth of *E. coli* and H_2 production in coffee waste hydrolysate (from spent coffee grounds and waste generated from green coffee roasting) as an alternative carbon source.
- Select the optimal conditions for waste processing, choose the applicable concentration, and evaluate the primary function of the enzymes used.
- Compare the H_2 production yield during the utilization of different coffee wastes by using the *E. coli* wild-type and a septuple mutant with metabolic disruptions.

Scientific novelty and practical value of the study. It has been shown that under growth conditions at pH 5.5 with low glucose concentration, Hyd-4 is partially responsible for H_2 production, while at pH 7.5, it primarily functions in H_2 oxidation or proton transport. The activity of Hyd-4 at pH 6.5 has been mainly observed when cells were grown in the presence of 8 g L^{-1} glucose. In a mutant lacking the genes encoding all subunits of Hyd-4 (*hyfB-R* mutant) except *hyfA*, H_2 production was reduced or was nearly zero regardless of glucose concentration and medium pH, suggesting that Hyd-4 is active and involved in H_2 metabolism. Additionally, some subunits of Hyd-3, such as HycG, HycH, and HycI, apparently exhibit dual functionality depending on external conditions. Specifically, at pH 7.5, when cells were grown with 2 g L^{-1} glucose, HycH did not play any role in H_2 production. HycI is particularly important at pH 5.5 under growth conditions with 8 g L^{-1} glucose, indicating that certain subunits in Hyd-3 and Hyd-4 are sensitive to glucose concentration. This suggests an active working model of the enzymes under these conditions. It has been shown that at pH 7.5, the HyfB, HyfD, HyfF subunits of Hyd-4, which are homologous to the NuoL, NuoM, NuoN subunits of the respiratory complex, contribute differently to the overall and F_O/F_1-dependent H^+/K^+ fluxes depending on the glucose concentration. As a result, a working model has been proposed reflecting the function of these subunits under energy-limited conditions, suggesting that HyfD can transport H^+ across the membrane, possibly in the form of an $H^+/K+$ antiporter. According to the obtained data, H+ can be transferred to HyfD either directly or through F_O/F_1 and possibly through thiol groups to HyfF. A similar idea was also proposed for the individual subunits of Hyd-1 and Hyd-2. Specifically, under conditions of low glucose concentrations, mutants lacking HyaA-HyaC subunits showed an increase in overall

proton flux by ~30% compared to the wild type, whereas in the presence of high glucose, fluxes increased in both the wild type and mutants. With the absence of the HyaB subunit, the proton flux associated with F_OF_1-ATPase increased by ~3 times, highlighting the role of HyaA-C in the mechanisms of proton transfer and energy conservation. It has also been clearly shown that in the *hybC* mutant, the DCCD-sensitive proton flux decreases by ~3.5 times, indicating that HybC is essential for proton transfer and the function of F_OF_1-ATPase. Under these conditions, a working model of possible interaction under low glucose concentration fermentation conditions has also been proposed. It is also interesting that, according to the obtained data, the glucose concentration affects not only membrane-bound subunits but also additional subunits involved in the maturation of hydrogenases. In the *hybF* mutant, an increase in DCCD-sensitive proton flux was observed, which closely resembled the data seen in *hyaB*, clearly demonstrating the cross-regulation between hydrogenases, particularly highlighting the role of HybF in the activity of Hyd-1. The data from *hybE* and *hybF* mutants indicate that they are crucial for the maturation of Hyd-2 when bacteria are grown in low glucose conditions, and high glucose concentration is added during experiments. In *hyaD* and *hyaF* mutants, a 50% increase in DCCD-sensitive proton flux was observed, demonstrating their significant role in the activity of Hyd-1. The role of Hyd enzymes in the assimilation of hydrolysates from various coffee production wastes has also been studied. It is worth noting that the pH of the waste hydrolysates was adjusted to a value of 7-7.5, considering the favorable growth conditions for *E. coli*. It was shown that during utilization of coffee waste hydrolysate the specific growth rate of bacteria decreased in mutants lacking the catalytic subunits of Hyd-3 and Hyd-4, demonstrating the role of these enzymes in growth bioenergetics. Additionally, it was confirmed once again that under these conditions, the primary enzyme responsible for H_2 production is Hyd-3. The possibilities of obtaining maximum yield from coffee wastes have been studied using a mutant with disruptions in 7 metabolic genes. Hydrolysates containing initial concentrations of 2-200 g L^{-1} of waste were applied in various dilutions. It was shown that bacterial growth and H_2 yield were inhibited in waste environments with higher concentrations, which aligns well with the data obtained with glucose alone. This clarifies that high concentrations of carbohydrates can have a negative effect and that the proton-hydrogen cycle operates efficiently under energy-limited conditions.

Main points to present at the defense.

1. At pH 7.5, the major H_2-producing enzyme is Hyd-3, while Hyd-4 is primarily involved in H_2 oxidation and proton transfer. At this pH, Hyd-3 and Hyd-4 form an H_2-producing Hyd complex to maintain proton motive force in cells during the stationary growth phase.

2. At pH 5.5, there is a phenomenon of complementation between Hyd-3 and Hyd-4, and under these conditions, the FHL-1 and FHL-2 complexes unite, forming a H_2-producing supercomplex.

3. At pH 7.5, the physiological role of Hyd-4 under energy-limited conditions is to generate and balance the proton motive force. The HyfF and HyfD subunits are crucial

for regulating F_0F_1-dependent proton/potassium fluxes under varying glucose fermentation conditions.

4. Glucose concentration is a determining factor in the regulation of Hyd-1 and Hyd-2 dependent proton/potassium fluxes in *Escherichia coli* at pH 7.5.

5. During utilization of coffee waste hydrolyzate, Hyd-3 is the main H_2-producing enzyme, and with the combination of waste pretreatment factors and the use of the mutant, the yield of H_2 can be increased manyfold.

Work approbation. Main results of the dissertation were discussed at seminars in Department of Biochemistry, Microbiology and Biotechnology, Faculty of Biology Yerevan State University, and at scientific conferences: FEMS Congress (Hamburg, Germany), FEMS Conference on Microbiology in association with Serbian Society of Microbiology (Belgrade, Serbia), Scientific Conference "Biotechnology: Science and Practice, Innovation and Business" for Young Researchers (Yerevan, Armenia, 2021), "Modern Trends in Biochemistry and Space Biology: The Great Sissakian and the Importance of His Research" International Conference (Yerevan, Armenia, 2019), V International Conference of Biotechnology and Health (Virtual, Yerevan, Armenia, 2020), 7th International Renewable and Clean Energy Conference (Virtual, Yerevan, Armenia, 2020), EFB 2021 Virtual Conference (Virtual, Barcelona, Spain, 2021), World Microbe Forum (Virtual, Online Worldwide, 2021).

Publications. According to experimental data observed in dissertation, 18 papers, including 5 article in peer-reviewed journal, 1 articles in a journal included in the list of HESC of the RA and 13 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 122 papers and books). The dissertation consists of 140 pages, 8 tables and 32 figures.

MATERIALS AND METHODS

All experiments were done using *E. coli* BW25113 or MC4100 wild type (WT) and appropriate mutants

Bacterial cultivation and growth conditions. *E. coli* were grown at 37°C for 18-20 h in anaerobic conditions by direct transfer from nutrient agar surface in Petri dish into high buffered liquid peptone growth medium containing 20 g L^{-1} peptone, 15 g L^{-1} K_2HPO_4, 1.08 g L^{-1} KH_2PO_4, 5 g L^{-1} NaCl **(pH 7.5)**, 20 g L^{-1} peptone, 7.4 g L^{-1} K_2HPO_4, 8.6 g L^{-1} KH_2PO_4,5 g L^{-1} NaCl **(pH 6.5)** or 20 g L^{-1} peptone, 15 g L^{-1} KH_2PO_4, 1.08 g L^{-1} K_2HPO_4, 5 g L^{-1} NaCl **(pH 5.5)**. As a substrate 2 g L^{-1} or 8 g L^{-1} glucose were added.

Determination of specific growth rate. The bacterial growth was monitored measuring the optical density (OD_{600}) spectrophotometrically. Specific growth rate (μ) was determined by regular measurement of optical density (OD) until the stationary phase with doubling time as described before (Trchounian et al., 2013). DCCD with 0.2 mM final concentration was used as indicated.

Determination of ion fluxes. H^+, and K^+ fluxes through the membrane (ΔJ_{H+}, ΔJ_{K+}) were determined potentiometrically upon addition of glucose. Proton flux was determined using a pH electrode, potassium flux-using a potassium-selective electrode

(HI4114, Hanna Instruments, Portugal) with the sensitive module HI4114-51. The results were calculated using the calibration curve. To understand the relationship between the changes in ion fluxes related to bioenergetic processes, cells were treated with a specific inhibitor of H^+-ATPase, *N, N'*-dicyclohexylcarbodiimide (DCCD, 0.5 mM), for 15 minutes. Results were expressed in mmol min^{-1} per 10^9 cells (Vanyan and Trchounian, 2022, Vanyan and Trchounian , 2024).

Measurement of redox potential and determination of hydrogen production. Redox potential (E_h) in bacterial biomass was determined using two different redox, titanium-silicate (Ti-Si) and platinum (Pt) glass electrodes (Trchounian & Trchounian, 2009). The Ti-Si-electrode measures the overall E_h, whereas the Pt-electrode is responsive to H_2 under anaerobic conditions. H_2 production rate (V_{H2}) was calculated as the difference between the initial rates of decrease in Pt- and Ti-Si-electrodes readings and expressed in mV of E_h per min per mg dry weight of bacteria Cumulative H_2 production was determined as described before.

Waste treatment and media preparation. In the studies, coffee wastes from robusta and arabica coffees were used. Two types of waste were used: the residue after coffee brewing, spent coffee grounds (SCG) and the waste produced after coffee roasting, coffee silverskin (CS). Coffee waste was dried at 105 °C to constant mass. 40-100 g L^{-1} of waste in the case of SCG, and 2-200 g L^{-1} in the case of CS, with 7.32 g L^{-1} in each or with a final concentration of 14.64 g L^{-1} H_2SO_4 were used. In order to optimize the waste pretreatment conditions, not only the amounts of waste and sulfuric acid were changed, but also the duration of hydrolysis, which was carried out for 25 or 45 minutes at 121 °C. The hydrolyzate was filtered, the pH was adjusted to pH 7.5 with K_2HPO_4. Final solutions were diluted 2-, 5-, or 10-fold and autoclaved for 15-20 min at 121 °C. For all studies, bacteria were pre-inoculated to peptone medium for overnight culture. Then, after 18-22 hours, the bacterial suspension was transferred from the overnight culture to the main nutrient medium for growth at a final volume ratio of 3% (Petrsoyan et al., 2020, Mirzoyan et al., 2022).

Chemicals and data processing. All chemicals of analytical grade were used. Each data point represented was averaged from independent triplicate cultures: the standard deviation was not more than 3%. Statistical analysis was performed by using two-way ANOVA Tukey's statistical test using GraphPad Prism 8.0.2 software (San Diego, CA, USA) (Shirvanyan A. et al., 2023).

RESULTS AND DISCUSSION

H_2 production during utilization of glucose at different pHs in mutants with defects in Hyd-3 and Hyd-4. Glucose is the preferred carbon and energy source of *E. coli*. The pathways of glucose utilization and the enzymes responsible for its gradual degradation are well studied, in both, the absence and presence of oxygen. It is transported and phosphorylated by PTS system and further glucose oxidation follows via the Embden-Meyerhoff-Parnas pathway. The high concentrations of this carbohydrate ≥100 mM are commonly employed as a fundamental component in various studies. There is still a lack of model working mechanisms of enzymes and membrane proteins corresponding to each condition and concentration of carbon

source. It has been shown that both Hyd-3 and Hyd-4 show different activities and contribution to H_2 production, but their roles vary with pH, carbon sources and its concentration. So as a first point of for this reserch we analyzed the role and contribution of each subunit of Hyd-4 and Hyd-3 in H_2 generation depending on the external pH (range of 5.5-7.5) and glucose concentration (2 g L^{-1} or 8 g L^{-1}). In *hyfA-B* and *hyfB-R* mutants, the H_2 production rate (V_{H2}) was decreased compared to wild type in almost all conditions, which indicates that Hyd-4 has an impact on the H_2 generation process.

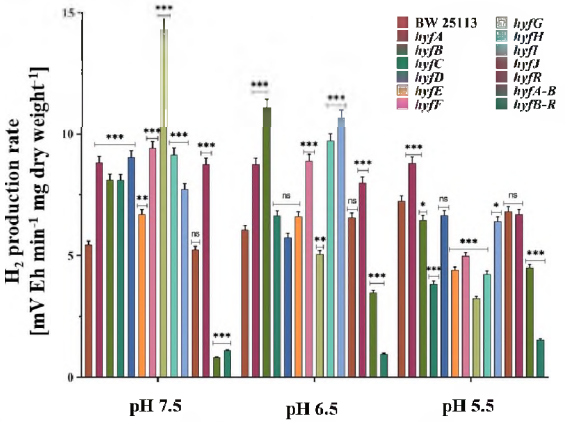

Fig.1 H_2 production rate (V_{H2}) by the *E. coli* BW25113 wild type and mutant strains with various defects in Hyd-4 during glucose fermentation. Cells were grown in a presence of 2 gL^{-1} and supplemented with 2 gL^{-1} glucose at pH 7.5, 6.5 and 5.5.

Notably, when cells were grown at pH 7.5 in the presence of 2 g L^{-1} glucose, and in the enzyme activity assays with 2 g L^{-1} glucose supplemented, it was very intriguing to see that in the single mutants V_{H2} was not disturbed, but rather it was higher than in the wild type. In particular, a *hyfG* single mutant showed V_{H2} to be ~2.6 fold higher than in wild type (see Fig. 1). At pH 6.5 when cells were grown in the presence of 2 g L^{-1} glucose and 2 g L^{-1} glucose was added in the assays, analysis of Hyd-4 single mutants showed that again Hyd-4 functions in H_2-oxidizing mode. Particularly in *hyfA, hyfB, hyfF, hyfH* and *hyfI* single mutants the H_2 production increased by ~1.7 fold compared to wild type (Fig.1). Surprising results were obtained at pH 5.5. In *hyfG* mutants H_2 production was decreased ~2.2 fold compared to wild type which clearly suggests that Hyd-4 has contributed towards H_2 production but not oxidation. Moreover, in *hyfC, hyfE, hyfF* and *hyfH* single subunits V_{H2} was decreased ~1.6 fold compared to wild type. The latter finding was also observed in *hyfA-B* and *hyfB-R* mutants. No differences were detected in other single mutants. When 8 g L^{-1} glucose was added to the assays, V_{H2} of the *hyfG* mutant decreased ~2.35 fold suggesting that under these conditions Hyd-4 is active and responsible for H_2 generation (Petrosyan et al., 2020).

At pH 7.5 when cells were grown in the presence of 2 g L^{-1} glucose and in the assays with 2 g L^{-1} glucose added, a significant decrease in H_2 production rate was detected in *hycB, hycC, hycE, hycF, hycG* single mutants (Fig. 2). In *hycD* and *hycI* single mutants V_{H2} was lowered only by ~1.5 fold but in *hycH* surprisingly H_2 production was

increased ~2.3 fold. Similar results were obtained in the assays supplemented with 8 g L^{-1} glucose.

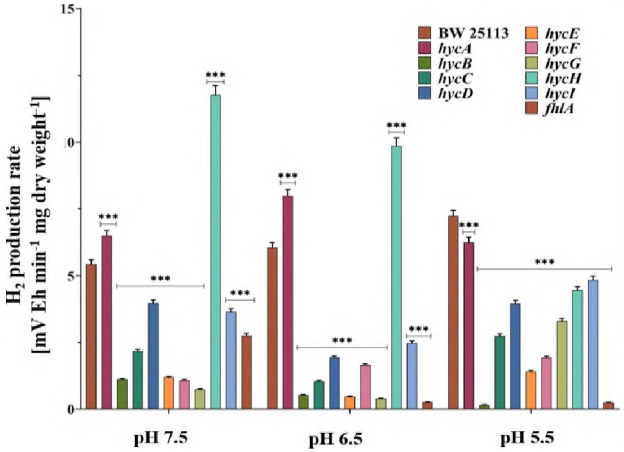

Fig.2 H_2 production rate (V_{H2}) by the *E. coli* BW25113 wild type and mutant strains with various defects in Hyd-3 during glucose fermentation. Cells were grown in a presence of 2 gL^{-1} and supplemented with 2 gL^{-1} glucose at pH 7.5, 6.5 and 5.5.

Based on obtained data schematic model were represented, where the main H_2-producing enzyme at pH 7.5 is Hyd-3 while Hyd-4 is mostly involved in H_2 oxidation and proton translocation to Hyd-3 HycB or HycG subunits. At this pH it is proposed that Hyd-3 with Hyd-4 forms a H_2-producing Hyd complex. This complex is important for maintaining a proton motive force, particularly in stationary phase cells, and is thus mainly involved in energy conservation. At pH 5.5 compensatory H_2-producing function of Hyd-3 and Hyd-4 exists, and again it is suggested that at pH 5.5 FHL-1 and FHL-2 complexes combine to form a H_2-producing supercomplex that works towards H_2 generation. It makes physiological sense to have H_2-producing function at low pH when cells need to recycle excess of protons in the cell and regulate intracellular pH and thus proton motive force generation, because at low pH the FoF_1-ATPase activity is very low compared to at high pH.

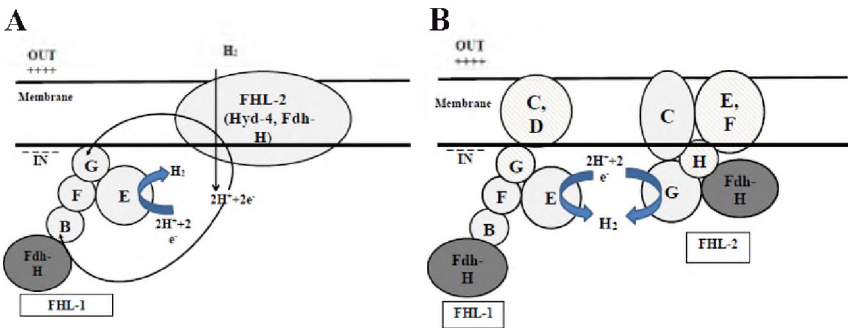

Fig. 3. Schematic representation of *E. coli* FHL-1 and FHL-2 complexes involved in H_2 production at pH 7.5 (A) and pH 5.5 (B). B, C, D, E, F, G represent the subunits of Hyd-3 of FHL-1 complex. The subunits with stripes indicate that they are partially involved in H_2 production, while the subunits with light grey shading are mainly responsible for the H_2 production process. Fdh-H (grey circle) is important for both Hyd-3 and Hyd-4 to produce

H_2. For explanations see section Model of FHL-1 and FHL-2 complexes in E. coli at pH 7.5 and 5.5 during 2 g L^{-1} glucose fermentation in the Results and discussion.

H^+ and K^+ fluxes in *E. coli* wild type and *hyf* mutants grown on only peptone, low concentration (2 g L^{-1}) of glucose and high concentration (8 g L^{-1}) of glucose. When *E. coli* wild type cells were grown on peptone only in the assays supplemented with 2 g L^{-1} glucose total J_{H^+} was similar in wild type and mutants. DCCD-sensitive J_{H^+} was twofold higher in *hyfB* mutant compared to wild type, while in *hyfD* it was decreased twofold compared to wild type. Interestingly, when in the assays 8 g L^{-1} glucose was supplemented total J_{H^+} was similar as in the assays with 2 g L^{-1} glucose but DCCD-sensitive J_{H^+} in wild type was lower ~ twofold compared to the assays with 2 g L^{-1} glucose. The DCCD-sensitive J_{H^+} in *hyfB* mutant was ~2.6 fold higher compared to wild type and stronger effect was detected in 8 g L^{-1} glucose assays compared to 2 g L^{-1} glucose (Fig. 4a). In *hyfD* the DCCD-sensitive J_{H^+} was similar to wild type but in *hyfF* it was increased ~ 60%. The J_{K^+} in wild type cells grown on peptone only was similar when in the assays low and high concentration of glucose was added reaching to 0.2 mmol min^{-1}. But addition of DCCD disturbed J_{K^+} in wild type cells only in the assays when 2 g L^{-1} glucose. The J_{K^+} was higher ~twofold in *hyfB* and *hyfF* mutants compared to wild type while in *hyfD* mutant the J_{K^+} was similar as in wild type. The DCCD-sensitive J_{K^+} was increased in all mutants, especially in *hyfB* and *hyfF* ~1.8 fold compared to wild type (Fig. 4b).

When cells were grown on 2 g L^{-1} glucose and in the assays 2 g L^{-1} glucose was added total J_{H^+} flux in wild type cells and *hyf* mutants was similar but DCCD-sensitive fluxes were absent in *hyfF* mutant and increased ~ twofold in *hyfD* compared to wild type (Fig. 4a). Moreover, DCCD inhibited the fluxes ~ 30% in wild type and *hyfB* mutant while in *hyfD* it was decreased ~ 60%. When in the assays 8 g L^{-1} glucose was added total J_{H^+} fluxes in wild type and *hyfB* mutant was similar to each other and to the assays with 2 g L^{-1} glucose. But in *hyfD* and *hyfF* mutants total J_{H^+} were increased ~ 35–45% compared to wild type. The data suggest that *hyf* subunits might be involved at least to proton translocation to F_0F_1 or to other secondary transporting systems. In 8 g L^{-1} glucose assays J_{K^+} was ~ 1.8 fold higher compared to the assays supplemented with 2 g L^{-1} glucose. When in the assays 2 g L^{-1} glucose supplemented in *hyfD* and *hyfF* mutants but not *hyfB* K^+ uptake was ~ 1.4 fold stimulated compared to wild type (Fig. 3b). DCCD totally disturbed K^+ uptake in all mutants as in wild type. This suggests that the tested subunits in 2 g L^{-1} glucose assays differently contribute to FOF1 dependent K+ uptake. DCCD-sensitive J_{K^+} in *hyfD* and *hyfF* mutants was 40% and 60% higher compared to wild type. Interestingly, in the assays when 8 g L^{-1} glucose is supplemented in *hyfD* mutant only K^+ uptake was stimulated ~ 1.4 fold while in *hyfB* and *hyfF* mutants it was decreased ~ 1.6 and ~ 1.4 fold respectively, compared to wild type (Fig. 3b). DCCD disturbed the K^+ uptake in all tested mutants. DCCD-sensitive J_{K^+} in *hyfB* and *hyfF* mutants was 50% lower compared to wild type, while in *hyfD* mutant it was 45% higher compared to wild type.

Under fermentation of 8 g L^{-1} glucose when in the assays 2 g L^{-1} glucose was added total J_{H^+} in wild type cells reached ~ 2 mmol min^{-1} (Fig. 4a). In *hyfB* and *hyfD*

mutants the J_{H^+} was similar as in wild type but in *hyfF* mutant it was less ~ 30% compared to wild type. DCCD inhibited H^+ fluxes ~ 40–50% in wild type and mutants except *hyfD* where the inhibition reached to ~ 65% (Fig. 4a). In 8 g L^{-1} glucose assays total H^+ flux was similar in wild type and all tested mutants reaching to 1.8 mmol min^{-1}. In wild type and *hyfF* mutant DCCD inhibited the H^+ flux ~ 50% while in *hyfB* and *hyfD* ~ 35% and ~ 70%, respectively. DCCD-sensitive H^+ flux in wild type cells and *hyfB* mutant when in the assays 2 g L^{-1} glucose supplemented was similar but in *hyfD* mutant it was higher ~ 25% and in *hyfF* DCCD-sensitive J_{H^+} flux decreased ~ 45%. The latter might be probably due to different role of *hyfD* and *hyfF* within the metabolic cross talk between F_OF_1 and different Hyd-4 subunits or other secondary membrane bound transporters. It was demonstrated that when wild type cells were grown on 8 g L^{-1} glucose depending on the supplemented glucose concentration in the assays J_{K^+} was similar which exhibites that at higher glucose concentrations K^+ uptake systems are stable which was not the case for low glucose concentrations (Fig. 4b). In 2 g L^{-1} glucose assays J_{K^+} was similar in wild type, *hyfB* and *hyfF* mutants while in *hyfD* it was increased ~ 55%. DCCD disturbed K+ uptake in all strains. Interestingly addition of DCCD stimulated mainly the K^+ efflux in wild type and *hyfB* mutant. DCCDsensitive J_{K^+} was almost identical in wild type, *hyfB* and *hyfF* mutants (Fig. 5B). But in *hyfD* DCCD-sensitive J_{K^+} increased ~ 25%. Same increased data was shown for DCCD-sensitive J_{H^+} flux. Working model for Hyd-4 HyfD and HyfF subunits role during 2 g L^{-1} glucose concentration has been suggested (Fig. 6). Especially, HyfD is possible to translocate H^+ across the membrane and in possibly form H^+/K^+ antiporter. Besides, the H^+ can be transferred via F_OF_1 to HyfD or HyfF via thiol groups (F_OF_1-HyfF-TrkA) as suggested before (Trchounian 2004) for balancing proton gradient and proton motive force (Fig. 6).

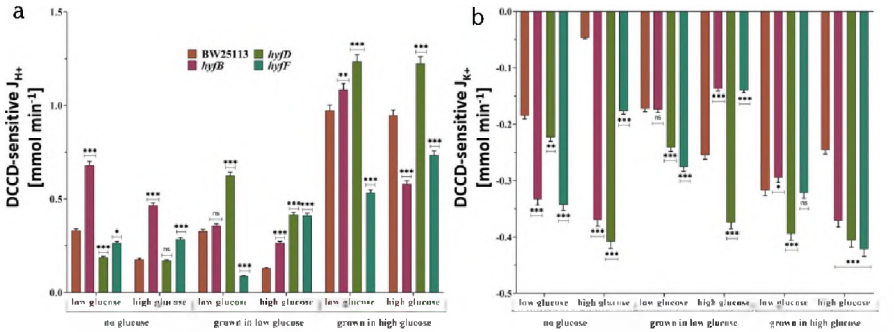

Fig. 4 DCCD-sensitive J_{H+} flux (a) and K + flux (b) by whole cells of *E. coli* wild type and *hyf* mutants. Bacteria were grown under fermentative conditions at pH 7.5 without glucose, in the presence of 2 g L^{-1} and 8 g L^{-1} glucose at 37oC.

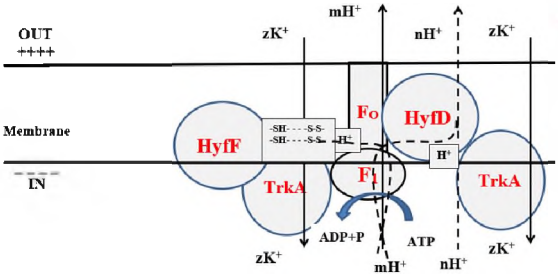

Fig. 5. Schematic working model of HyfD and HyfF subunits of Hyd-4 during glucose fermentation at pH 7.5. Dashed arrows depicts the possibility of H^+ translocation via directly HyfD or through F_OF_1 to HyfD and/or to HyfF via thiol groups to HyfF. Under energy limited conditions HyfD and HyfF work towards conserving energy via interacting with F_OF_1 for efficient proton transfer in membrane or translocation across the membrane for balancing proton gradient and thus proton motive force.

H^+ and K^+ fluxes in *E. coli* wild type, *hya and hyb* mutants grown on low (2 g L^{-1}) or high (8 g L^{-1}) concentration of glucose. The results of our study reveal intriguing findings regarding the impact of specific gene mutations in Hyd-1 and Hyd-2 on J_{H+} in *E. coli* under different conditions.

When bacteria were grown at 2 g L^{-1} glucose medium and during assays low and high glucose was supplemented total J_{H+} at rates of 1.5 mmol min^{-1} per 10^9 cells were observed, respectively. Interestingly, in mutants lacking *hyaA-hyaC* genes J_{H+} increased significantly by 30 % compared to the wild type. Significant changes were determined in DCCD-sensitive J_{H+}. Particularly, in the wild type, DCCD-sensitive J_{H+} constituted 30 % of the total flow, with a rate of 0.45 mmol min^{-1} [Fig. 6a]. In contrast to Hyd-1 single mutants, the DCCD-sensitive J_{H+} increased by 40–50 % in *hyaA* and *hyaC* mutants and more than 60 % in *hyaB* mutant. Ion fluxes were investigated in mutants with defects on membrane-bound subunits of Hyd-2 as well. When low glucose was supplemented DCCD-sensitive fluxes decreased in *hybC* and *hybO* mutants by 70 % and 30 %, respectively, compared to wild type [Fig. 6a]. Thereby, in the absence of HybC, the contribution of F_OF_1-ATPase was strictly declined. It is possible that HybC as a catalytic subunit may supply protons received from H_2 oxidation to F_OF_1. When bacteria were grown at 2 g L^{-1} glucose medium and during assays high glucose was supplemented total proton flux (J_{H+}) at rates of 2.26 mmol min^{-1}.Here, J_{H+} was similar in all *hya* mutants compared to the wild type, but DCCD-sensitive fluxes were equally increased ~60 % and the contribution of F_OF_1-ATPase remained constant at 50 % (Fig. 6a), thus the addition of high glucose in all the three mutants cause similar contribution of proton-ATPase, which highlights the glucose-dependent operational features of the enzyme . In *hybA* and *hybO* DCCD-sensitive fluxes remain similar to wild type. At the same time in *hybB* and *hybC* DCCD-sensitive fluxes increased by 35 % (Fig. 6a).

DCCD-sensitive K^+ uptake in wild type and *hya* mutants was 70 % of the total J_{K+}, which interestingly is lower compared to low glucose, this means that upon addition of high glucose the contribution of F_OF_1-ATPase is declined, which is not depending on subunits of Hyd-1.

When high glucose was supplemented significant changes were not observed in DCCD-sensitive J_{K+} in all *hyb* deletion mutants [Fig. 6b].

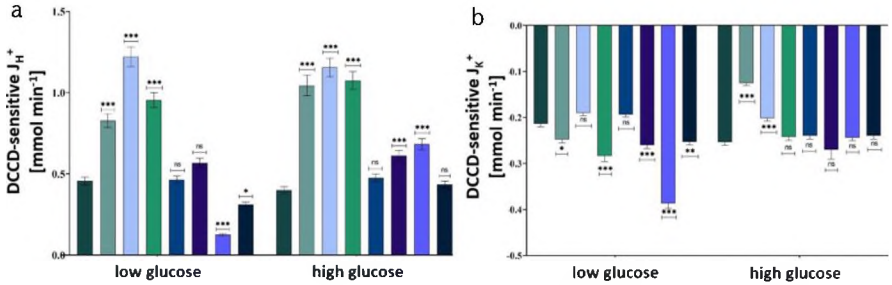

Fig 6. DCCD-sensitive J_{H+} flux (a) and J_{K+} flux (b) by whole cells of *E. coli* wild type and *hyf* mutants. Bacteria were grown under fermentative conditions at pH 7.5 in the presence of 2 g L^{-1} glucose at 37°C. During assays either 2 g L^{-1} (low) or 8 g L^{-1} (high) glucose was supplemented. DCCD (0.2 mM) was added to the assay medium when indicated. DCCD-sensitive fluxes were calculated as a difference between total and DCCD-inhibited fluxes. Positive value indicates efflux and negative value indicates influx.

When cells were grown on high glucose concentration (8 g L^{-1}) and in the assays low glucose was added (2 g L^{-1}) in wild type J_{H+} was ~2 mmol min^{-1} per 10^9 cells. Significant increase only observed in *hyaA* and *hyaC* mutants, and no differences in J_{H+} were observed both in rest *hya* and *hyb* single deletion mutants upon addition of low glucose. Only in *hyaC* mutant DCCD-sensitive J_{H+} decrease was observed constituting 35 % and the contribution of F_OF_1-ATPase reduced as well, meanwhile in mutants lacking other subunits similar increase occurred compared to wild type [Fig. 7a]. Interestingly, in all *hyb* single mutants, no significant differences in total and DCCD-sensitive fluxes were observed. Only in *hyaA* DCCD-sensitive J_{K+} decreased by almost 50 %. In the *hybO* mutant DCCD-sensitive potassium uptake constituted of 0.24 mmol min^{-1}, which decreased by 30–40 % in *hybA*, *hybB*, *hybO* mutants [Fig. 7b]. Generally, it can be also highlighted the fact that in the presence of a high glucose, FoF_1-dependent J_{K+} decreases.

During addition of high glucose in the assays in wild type J_{H+} was ~2 mmol min^{-1} per 10^9 cells [Fig. 7a] similar to low glucose assays. Here, only in *hyaA* total J_{H+} increased by 20 %, and in *hyaB* and *hyaC* no significant differences were observed. In *hybB* and *hybC* mutants total J_{H+} decreased by 30 %. When considering DCCD-sensitive fluxes only in *hybA* and *hybO* significant increased by 30–40 %.

Total J_{K+} was approximately at 0.27 mmol min^{-1} [Fig. 7b]. In all Hyd-1 single deletion mutants similar increase in J_{K+} by 20 % was observed. When high glucose was supplemented DCCD-sensitive potassium uptake was 0.2 mmol min^{-1} with no significant differences between *hyb* single mutants, except *hybC*.

The observation with low glucose concentration (2 g L^{-1}) that in mutants lacking the *hyaA-hyaC* genes, the total J_{H+} increase is attracting attention to this phenomenon. However, this increase was absent when high glucose was supplemented, suggesting a complex interplay between hydrogenases and glucose availability. This indicates that the absence of these genes has a more pronounced impact on J_{H+} when glucose is limited, potentially to enhance energy conservation and metabolic efficiency.

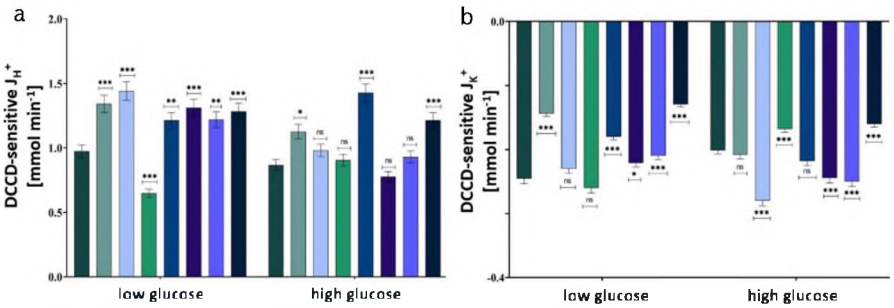

Fig. 7. DCCD-sensitive J_{H+} flux (a) and J_{K+} flux (b) by whole cells of *E. coli* wild type and *hyf* mutants. Bacteria were grown under fermentative conditions at pH 7.5 in the presence of 8 g L^{-1} glucose at 37°C. During assays either 2 g L^{-1} (low) or 8 g L^{-1} (high) glucose was supplemented. DCCD (0.2 mM) was added to the assay medium when indicated. DCCD-sensitive fluxes were calculated as a difference between total and DCCD-inhibited fluxes. Positive value indicates efflux and negative value indicates influx.

Moreover, it was shown that when the whole operon of Hyd-1 was deleted the intracellular NADH concentration increased significantly while in the mutant where Hyd-2 operon was deleted NADH was ~ 5 times lower compared to wild type (Shekhar et al., 2022). These alterations in intracellular NADH concentration showing antagonistic effects in Hyd-1 and Hyd-2 deleted *E. coli* cells might also affect the proton fluxes and generally proton cycling in the cell in the context of *pmf* generation and cellular bioenergetics. This effect might suggest that protons from NADH in *hya* (encoding for Hyd-1 operon) deleted cells might be transported out e.g. via FoF_1, and thus the involvement of F_OF_1 in *hyaA-hyaC* single mutants is higher (Vanyan and Trchounian, 2024). On the contrary, the amount of intracellular NADH and ATP in *hyb* (encoding for Hyd-2 operon) deleted cells is significantly low and our results show that DCCD-sensitive proton flux in *hybC* mutant is almost zero suggesting that F_OF_1 is not involved in this process and which might be due to low amount of intracellular NADH and ATP.

The extensive exploration of ion fluxes in mutants with defects in membrane-bound subunits of Hyd-2 reveals new possible interplay between hydrogenases, F_OF_1-ATPase and TrkA system. The observation that low glucose supplementation during the assay did not significantly alter total H^+ flux in mutants suggests that certain fundamental ion transport mechanisms remain relatively constant under different glucose conditions. However, the notable reduction in DCCD-sensitive fluxes in *hybC* and *hybO* mutants indicates that these specific subunits play a significant role in regulating J_{H+}. Notably, in the absence of HybC, the contribution of F_OF_1-ATPase declined, suggesting that HybC might be involved in catalyzing H_2 oxidation and supplying protons to F_OF_1 as recently suggested for HyfF (Vanyan and Trchounian, 2022). Alternatively, as suggested above, it might be related to intracellular NADH and ATP concentration.

Moreover, cells sense the glucose concentration even when grown in other concentrations. Possibly Hyd-1 can receive protons from Hyd-2 via the quinone cycle and be responsible for proton transport along the membrane, thus when any subunit

of Hyd-1 is absent contribution of F_0F_1-ATPase increases as one direction from Hyd-2 to Hyd-1 is not working (Fig. 8) (Vanyan and Trchounian, 2024).

In this background, we may state that low glucose is an energy-limited condition, under which complete hydrogenases or their several subunits interact with each other or with F_0F_1-ATPase, TrkA system to transport ions and thus contribute to the generation of the proton motive force. Moreover, the function and interaction mechanisms may be changed depending on glucose concentration. It can be predicted that Hyd-1 and Hyd-2 besides complementarity may interact with Hyd-3 or Hyd-4 generating supercomplexes (Trchounian and Trchounian, 2019). Hyd-1 and Hyd-2 may have compensatory H_2 oxidizing and proton transporting functions for maintain overall Δp.

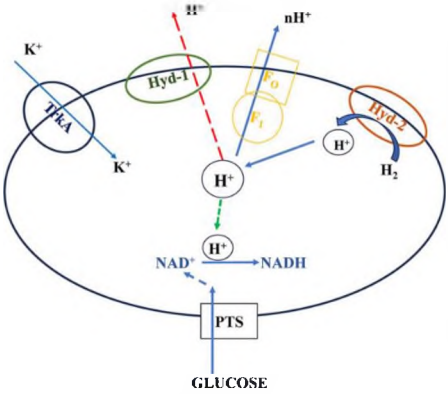

Fig. 8. Schematic working model of Hyd-1 and Hyd-2 in the presence of 2 g L^{-1} glucose at pH 7.5. Hyd-2 is primarily responsible for H_2 oxidation. Generated protons may be directed in one of several ways: for NAD^+ reduction, through F_0F_1 for ATP hydrolysis, or via Hyd-1 for membrane translocation. Therefore, in the absence of Hyd-1, the excess protons are compensated by F_0F_1, which increases proton flux, or by enhancing NAD^+ reduction, which result in intracellular NADH concentration increase. Conversely, when Hyd-2 is absent, protons are not produced for these pathways, resulting in no proton leakage through F_0F_1 and a significant reduction in intracellular NADH levels.

H+/K+ fluxes in *E. coli* mutants with defects in accessory and maturational subunits of Hyd-1 and Hyd-2 when cells were grown low (2 g L^{-1}) or high (8 g L^{-1}) concentration of glucose. In *hyaD* and *hyaF* DCCD-sensitive J_{H+} increased by 50%, in *hyaE* – by 20% (Fig. 9a). It was shown previously that under aerobic conditions HyaE functions as a chaperone protein that is non-essential for the cell's survival or function but plays a role in assisting the β subunit (HyaA) of the Hyd-1. In this study, conducted under fermentative conditions with low glucose levels, it was found that HyaE is not critical for the activity of Hyd-1 or the maturation of HyaA. This conclusion is based on the significant differences observed in the behavior of a *hyaA* deletion mutant, which notably affects proton fluxes, compared to the effects seen with *hyaE*.

Significant increase in DCCD-sensitive J_{H+} was observed in *hyaD* and *hyaF* mutants, suggesting that under implemented conditions these genes are important for Hyd-1 activity and specifically for the functioning of HyaB catalytic subunit. Totally, different DCCD-sensitivity was observed in *hyb* deletion mutants, in *hybD* DCCD-sensitive J_{H+} decreased by ~30%, in *hybG* and *hybE* decreased by ~50%, meanwhile in *hybF* it increased by ~60% (Fig. 9 a).

Data show that HybD can be involved in the maturation of the HybC, but is not as essential as *hybG* and *hybE*, where the contribution of F_0F_1-ATPase was strongly suppressed (Fig. 9a). Data for *hybE* and *hybF* were similar to *hybC* strongly suggesting that in these conditions this two proteins are important for the Hyd-2 activity. Interestingly only in *hybF* DCCD-sensitive flux increased, which states the cross-

regulation between hydrogenases, particularly the role of *hybF* for the activity of Hyd-1 as obtained difference had more similarity with *hyaB* data. However no significant changes were observed in potassium fluxes.

When cells were grown at 8 g L^{-1} glucose medium and during assays low and high glucose was supplemented the mutants demonstrated varied DCCD-sensitivity to the decreased availability of glucose. The *hyaD* mutant showed a substantial decrease in DCCD-sensitive J_{H+}, with a 65% reduction compared to the wild type (Fig. 9a). Similarly, *hyaE* experienced a 30% reduction in DCCD-sensitive J_{H+}.

In contrast, the *hyb* mutants presented a different pattern of response. In *hybD* DCCD-sensitive J_{H+} remained unchanged, indicating its function in the maturation of the HybC subunit, as both in low glucose and high glucose assays data for both *hybD* and *hybC* were similar [Fig 9a and Fig.6a]. *hybE* also showed no significant change, aligning with *hybD*, which may reflect a similar resilience or an alternative pathway ensuring its participation in hydrogenase activity remains unaffected. However, *hybF* exhibited an increase in DCCD-sensitive J_{H+} by 38%, unique among the mutants. HybF is believed to fulfil a role similar to that of HypA, focusing on nickel processing within the hydrogenase enzymes (Menon et al, 1994). Thus, *hybF* in depending on applied glucose concentration is most probably is responsible for Hyd-1, rather than for Hyd-2, as in all discussed conditions data from *hybF* were more similar to *hya* membrane-subunit deletion mutants. The results reveal the crucial roles of specific maturation proteins in influencing hydrogenase activity, showing that mutations in these proteins significantly affect ion fluxes in a glucose presence. Specifically, alterations in *hyaD* and *hyaF* led to a notable increase in proton flux, underlining their essential roles in Hyd-1 activity. The study also suggests a cross-regulation between hydrogenases, with the *hybF* mutant displaying changes in proton flux indicative of its involvement in the maturation or activity of both Hyd-1 and Hyd-2 under certain conditions (Vanyan, 2024).

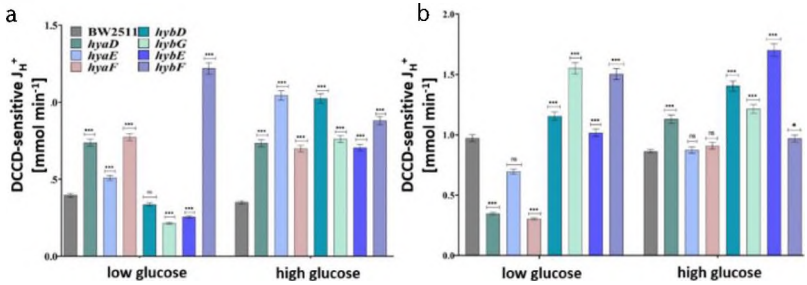

Fig. 9. DCCD-sensitive J_{H+} flux (a) and J_{K+} flux (b by whole cells of *E. coli* wild type, *hya* and *hyb* mutants. Bacteria were grown under fermentative conditions at pH 7.5 in the presence of (a) 2 g L^{-1} and (b) 8 g L^{-1} glucose at 37°C. During assays either 2 g L^{-1} (low) or 8 g L^{-1} (high) glucose was supplemented. DCCD (0.2 mM) was added to the assay medium when indicated. DCCD-sensitive fluxes were calculated as a difference between total and DCCD-inhibited fluxes. Positive value indicates efflux and negative value indicates influx.

H_2 production in *E. coli* during dark fermentation of coffee waste. For the next stage of the research the utilization of coffee by-products, specifically spent coffee

grounds (SCG) and coffee silverskin (CS), as alternative sugar sources for the growth of *Escherichia coli* and the activity of hydrogenase enzymes were investigated. Building upon previous research that examined the effects of glucose concentration on these processes, this study explores the potential of SCG and CS to substitute glucose in microbial cultures.

The experimental approach involves assessing the impact of varying concentrations of SCG and CS on *E. coli* growth and hydrogenase activity. The use of SCG hydrolysates, which are rich in fermentable sugars, has shown potential in supporting *E. coli* growth and enhancing hydrogen production, offering a sustainable alternative to traditional sugar substrates. Similarly, CS, as another by-product of coffee processing, is evaluated for its effectiveness as a carbon source in microbial growth and enzyme activity.

The results obtained have shown that SCGs hydrolyzed with H_2SO_4 for 45min and with 2 times dilution were optimal conditions for *E. coli* to grow and produce H_2 with yield of ~31 mL H_2 (g sugar)$^{-1}$ or 2.75 L (kg SCG)$^{-1}$ (Table 1). Hyd-3 and Hyd-4 had influenced specific growth rate during utilization of SCG, which might be due to change of bioenergetic properties. *Escherichia coli* Hyd-3 was responsible for H_2 production, where deletion of Hyd-1 and Hyd-2 had no significant difference. H_2 production yield and rate were enhanced by ~2-fold in septuple mutant reaching ~72 mL H_2 (g sugar)$^{-1}$ or 5.5 L H_2 (kg SCG)$^{-1}$ (Petrosyan et al., 2020).

Table1. H_2 production yield from coffee waste

Waste type	H_2 production in mL		H_2 production yield mL per gram of waste		H_2 production rate mL per day (small-scale)	
	Wt	mutant	Wt	mutant	Wt	mutant
Initially 65 g L^{-1} containing 2 fold diluted CS	54	80	1.66	2.46	27	26.6
Initially 40 g L^{-1} containing 2 fold diluted SCG	56	180	2.75	5.5		

In case of CS a combination of operating procedures, such as short hydrolyzing duration and higher waste concentration, is optimal for biomass generation. *E. coli* wild-type SGR in a medium of 200 g L^{-1} CS containing medium hydrolyzed for 25 min and twice diluted was 0.64 ± 0.02 h^{-1} yielding 0.495 ± 0.015 g L^{-1} biomass. Longer hydrolysis is efficient for degradation of CS lignocellulosic structures, thus, resulting

in a higher yield for biohydrogen (Mirzoyan et al., 2022). The usage of longer hydrolyzed and twice diluted 65 g L^{-1} CS was the most efficient medium for highest H_2 yield where in septuple mutant grown in this medium, reaching up 2.15 mL (g CS)$^{-1}$ (Table 1).

CONCLUSIONS

The following conclusions were made based on experimentally obtained results:

1. In the presence of 2 g L^{-1} glucose, Hyd-4 is partially responsible for H_2 production at pH 5.5 and works in H_2-oxidizing conditions at pH 7.5, while at pH 6.5 Hyd-4-activity was demonstrated in the presence of 8 g L^{-1} glucose.

2. Hyd-3 is the main enzyme responsible for H_2 production, but HycG, HycH and HycI subunits exhibit dual function depending on external conditions (pH, glucose concentration).

3. HyfB, HyfD, and HyfF subunits of hydrogenase-4 are critical in total and F_OF_1-ATPase-mediated proton/potassium fluxes at pH 7.5. Under energy-limited conditions, in *hyfF*, the DCCD-sensitive proton flux was absent, and in *hyfD*, the DCCD inhibited the flux by 60%.

4. In the presence of low glucose, the total proton flux in *hyaA-hyaC* mutants increased by ~30% compared to the wild type, whereas high glucose resulted in increased flux equally in the wild type and mutants. Absence of the HyaB subunit resulted in a ~3-fold increase in DCCD-sensitive proton flux, whereas in the HybC mutant it was reduced ~3.5-fold, indicating that HybC is essential for F_OF_1-ATPase activity.

5. Glucose concentration affects the activity of the subunits involved in the maturation process of Hyd-1 and Hyd-2, in particular cross-regulation between the maturation proteins of hydrogenases was shown, as evidenced by the effect of the *hybF* mutant on both Hyd-1, and on Hyd-2.

6. Hydrolysis of SCG with H_2SO_4 for 45 min at a 2-fold dilution was optimal for *E. coli* growth and H_2 production: ~31 mL H_2 (g carbohydrate)$^{-1}$ or 2.75 L (kg SCG). Hyd-3 and Hyd-4 affected the specific growth rate in the hydrolyzate. Hyd-3 was responsible for H_2 production, while Hyd-1 and Hyd-2 had no significant role. The yield and rate of H_2 production increased ~2-fold in the septuple mutant to ~72 mL H_2 (g carbohydrate)$^{-1}$ or 5.5 L H_2 (kg SCG)$^{-1}$.

7. The specific growth rate of *E. coli* wild type in 200 g L^{-1} CS hydrolyzed for 25 min and twice diluted medium was 0.64 ± 0.02 h^{-1}, producing 0.495 ± 0.015 g L^{-1} biomass. In 65 g L^{-1} CS hydrolyzed for 45 min and twice diluted, H_2 yield was the highest, reaching 2.15 ml (g CS)$^{-1}$ in the septuple mutant.

LIST OF PUBLICATIONS AS A PART OF DISSERTATION TOPIC

1. **Vanyan L.**, Trchounian K., (2024) Glucose concentration is determinant for the functioning of Hydrogenase 1 and 2 in regulating the proton and potassium fluxes in *Escherichia coli*, *Biochimie*, ePub Jul. 20 ISSN 0300-9084, https://doi.org/10.1016/j.biochi.2024.07.013
2. **Vanyan L.** (2024) Proton and potassium fluxes in mutants with defects in subunits responsible for maturation of Hyd-1 and Hyd-2 during glucose fermentation, *Proceedings of the YSU B: Chemical and Biological Sciences*, 58 (1 (263)):54-67, https://doi.org/10.46991/PYSU:B.2024.58.1.054
3. Mirzoyan S., Aghekyan H., **Vanyan L.**, Vassilian A., Trchounian K. (2022) Coffee silverskin as a substrate for biobased production of biomass and hydrogen by *Escherichia coli*. *International Journal of Energy Research*, 46 (15): 23110–23121, https://doi.org/10.1002/er.8612
4. **Vanyan L.**, Trchounian K. (2022) HyfF subunit of hydrogenase 4 is crucial for regulating F_OF_1 dependent proton/potassium fluxes during fermentation of various concentrations of glucose, *Journal of Bioenergetics and Biomembranes*, 54: 69-79, https://doi.org/10.1007/s10863-022-09930-x
5. Petrosyan H., **Vanyan L.**, Mirzoyan S., Trchounian A., Trchounian K. (2020) Roasted coffee wastes as a substrate for Escherichia coli to grow and produce hydrogen. *FEMS Microbiology Letters*, 367 (11), fnaa088, https://doi.org/10.1093/femsle/fnaa088
6. Petrosyan H., **Vanyan L.**, Trchounian A., Trchounian K. (2020) Defining the roles of the hydrogenase 3 and 4 subunits in hydrogen production during glucose fermentation: A new model of a H_2-producing hydrogenase complex. *International Journal of Hydrogen Energy*, 45, 5192-5201, https://doi.org/10.1016/j.ijhydene.2019.09.204
7. **Vanyan L.**, Trchounian K. (2024). The effect of entire deletion of Hydrogenase-1 and 2 on proton flux during utilization of varied glucose concentration at pH 7.5. *The 48th FEBS Congress*, FEBS Open Bio, V.14 Sup. 2, p. 195, 29 June-03 July, Milano, Italy.
8. **Vanyan L.**, Vassilian A., Poladyan A., Trchounian K. (2024). Biotechnological potential of spent coffee grounds for large-scale hydrogen production. *14th International Conference on Hydrogen Production*, Conference Proceedings, pp. 79-84, Qatar, Doha.
9. **Vanyan L.**, Vassilian A., Poladyan A., Trchounian K. (2023). Understanding the Role of Escherichia coli Hydrogenase-2 subunits in proton flux under different glucose concentrations. *10th Congress of European Microbiologists*, FEMS Abstract Book, Post Num OTS3/6, pp. 1126, Hamburg, Germany.
10. **Vanyan L.**, Trchounian K. (2022). The role of E. coli Hydrogenase-1 in proton flux during glucose utilization at pH 7.5. *FEMS Conference on Microbiology in association with Serbian Society of Microbiology*, Abstract Book, p. 817, Belgrade, Serbia.

11. Mirzoyan S., **Vanyan L.**, Aghekyan H., Trchounian K. (2021). Biohydrogen production from roasted coffee waste: Understanding the role of *E. coli* hydrogenases during fermentation. *World Microbe Forum*, Abstract ID: WMF 21-0438.
12. **Vanyan L.**, Vassilian A., Trchounian K. (2021). Proton/potassium Fluxes Depend on Glucose Concentration in *E. coli* at pH 7.5. *World Microbe Forum*, Abstract ID: WMF 21-2026.
13. **Vanyan L.**, Vassilian A., Trchounian K. (2021). Is FHL complex responsible for sensing glucose concentration? *World Microbe Forum*, Abstract ID: WMF 21-2003.
14. **Vanyan L.**, Gevorgyan H., Petrosyan H., Trchounian A., Trchounian K. (2020). Industrial waste-based hydrogen production technology: the profitability for industrial waste generators. *Proceedings of the 7th International Renewable and Clean Energy Conference*, pp. 56-59, Yerevan, Aremnia.
15. Trchounian K., Petrosyan H., **Vanyan L.**, Trchounian A., Vassilian A. (2020). The Role of *Escherichia coli* F_OF_1-ATPase and Hydrogenases on Specific Growth Rate During Glucose Fermentation. *ASM Microbe 2020*, MBP06, Chicago, USA.
16. **Vanyan L.**, Trchounian A., Trchounian K. (2019). Interaction between *Escherichia coli* Hydrogenase-4 and F_OF_1-ATPase for proton translocation during fermentation of various glucose concentrations at slightly alkaline pH. Modern Trends in Biochemistry and Space Biology: *The Great Sissakian and the Importance of His Research*, Conference Book, pp. 139-141, Yerevan, Armenia.

ՎԱՆՅԱՆ ԼԻԱՆԱ ՄԱՆՎԵԼԻ

ESCHERICHIA COLI-ՈՒՄ ՋՐԱԾՆԻ ՆՅՈՒԹԱՓՈԽԱՆԱԿՈՒԹՅՈՒՆԸ և ՊՐՈՏՈՆԱՅԻՆ ՑԻԿԼԸ ԳԼՅՈՒԿՈԶԻ ՏԱՐԲԵՐ ԿՈՆՑԵՆՏՐԱՑԻԱՆԵՐԻ ԽՄՈՐՄԱՆ ՊԱՅՄԱՆՆԵՐՈՒՄ

Ամփոփագիր

Բանալի բառեր՝ *E.coli*, հիդրոենազներ, գլյուկոզի կոնցենտրացիա, պրոտոն/կալիումական տեղափոխություն, F_OF_1-ԱԵՖազ, ԴՑԿԴ-զգայուն իոնային հոսքեր:

Հետազոտության նպատակն է ուսումնասիրել *E.coli* հիդրոգենազ ֆերմենտների առանձին ենթամիավորների դերը և դրանց ազդեցությունը պրոտոն/կալիումական հոսքերի, թաղանթակապ ֆերմենտների ակտիվության և այլ փոխազդեցությունների վրա գլյուկոզի տարբեր կոնցենտրացիաների խմորման ընթացքում: Ուսումնասիրելով գլյուկոզի տարբեր հասանելիության պայմանները՝ մենք փորձեցինք պարզել, թե ինչպես են այս ենթամիավորներն ազդում *E. coli*-ի նյութափոխանակային հարմարվողականության վրա խմորման պայմաններում՝ փոխելով իոնային հոսքերը, որն էլ թույլ կտա բացահայտել դրանց դերը հիդրոգենազի ակտիվության և բջջին էներգիական

նյութափոխանակության մեջ: Բացահայտվել է , որ pH 7,5-ում հիմնական H_2-արտադրող ֆերմենտը Հիդ-3-ն է, մինչդեռ Հիդ-4-ը հիմնականում ներգրավված է H_2-ի օքսիդացման և պրոտոնի տեղափոխման մեջ դեպի Հիդ-3 HycB կամ HycG ենթամիավորներին: Այս pH-ում առաջարկվում է, որ Հիդ-3-ը Հիդ-4-ը կազմում են H_2-արտադրող Հիդ համալիր: pH 5,5-ում ևս ՄՋԼ-1 և ՄՋԼ-2 համալիրները միավորվում են՝ ձևավորելով H_2-արտադրող գերհամալիր: Դիտակրելով Հիդ-4-ի կարևոր գործառույթը pH 7,5-ում պրոտոնաշարժ ուժի առաջացման գործընթացում, ուսումնասիրվել է մասնավորապես HyfB, HyfD և HyfF ենթամիավորների դերը պրոտոն/ կալիումական տեղափոխությունում: Ցույց է տրվել, որ գլյուկոզի սահմանափակ քանակության պայմաններում HyfD-ն և HyfF-ն աշխատում են էներգիայի պահպանման ուղղությամբ՝ փոխազդելով F_OF_1-ի հետ՝ թաղանթի երկայնքով կամ միջով պրոտոնի արդյունավետ փոխանցման և տեղափոխման համար՝ պրոտոնի գրադիենտը և, հետևաբար, պրոտոնաձշարժ ուժը հավասարակշռելու համար: Դեռ ավելին H^+-ները կարող է փոխանցվել F_OF_1-ի միջոցով HyfD-ին կամ HyfF-ին թիոլ խմբերի միջոցով (F_OF_1-HyfF-TrkA) համալիրի միջոցով պրոտոնային գրադիենտը և պրոտոնաշարժ ուժը հավասարակշռելու համար:

Նման օրինաչափությունները ուսումնասիրվել են նաև Հիդ-1-ի և Հիդ-2-ի առանձին թաղանթակապ, ինչպես նաև լրացուցիչ հասունացմանը նպաստող ենթամիավորների դեպքում: Մասնավորապես ցույց է տրվել, որ կախված գլյուկոզի կոնցենտրացիայից Հիդ ֆերմենտների տարբեր ենթամիավորները պատասխանատու են և ունեն տարբեր ներգրավվածություն տարբեր գործընթացներին: Էներգիայի սահմանափակ պայմաններում Հիդ-1-ը և Հիդ-2-ը աշխատում են էներգիայի պահպանման ուղղությամբ՝ փոխազդելով F_OF_1-ի հետ՝ թաղանթի միջով պրոտոնի արդյունավետ փոխանցման կամ թաղանթի վրայով տեղափոխման համար՝ պրոտոնոնային գրադիենտը և, հետևաբար, պրոտոնաշարժի ուժը հավասարակշռելու համար: Հիդ-2-ը պատասխանատու է F_OF_1-ԱԵՖազի միջոցով պրոտոնների տեղափոխման համար, այն ապահովում է պրոտոններ ջրածնի օքսիդացումից անմիջապես դեպի F_OF_1-ԱԵՖազ կամ թիոլ խմբերի կամ Հիդ-4-ի միջոցով: Ստացված օրինաչափությունները դիտարկվել են նաև սուրճի արտադրության և օգտագործման արդյունքում առաջացած տարբեր թափոնների կիրառությամբ: Ցույց է տրվել, որ սուրճի նստվածքը և թաղանթը խոստումնալից լիգնոցելյուլոզային թափոններ են, որոնց արդյունավետ մշակման, երկարատև ֆիզիկաքիմիական հիդրոլիզի և մուտանտների կիրառության արդյունքում հնարավոր է ստանալ արդյունավետ բարձր կենսազանգվածի և կենսաջրածնի ելք:

ВАНЯН ЛИАНА МАНВЕЛОВНА

ВОДОРОДНЫЙ ОБМЕН И ПРОТОННЫЙ ЦИКЛ У *ESCHERICHIA COLI* В УСЛОВИЯХ БРОЖЕНИЯ ПРИ РАЗЛИЧНОЙ КОНЦЕНТРАЦИИ ГЛЮКОЗЫ

РЕЗЮМЕ

Ключевые слова: *E.coli,* гидрогеназы, концентрация глюкозы, транспорт протонов/калия, F_OF_1-АТФаза, ДЦКД -чувствительные ионные токи.

Цель исследования - изучить роль отдельных субъединиц ферментов гидрогеназы *E.coli* и их влияние на потоки протонов/калия, активность мембраносвязанных ферментов и другие взаимодействия при брожении глюкозы различных концентраций. Исследуя условия различной доступности глюкозы, мы стремились выяснить, как эти субъединицы влияют на метаболическую адаптацию *E. coli* в условиях брожения, путем изменения потоков ионов, что указывает на их роль в активности гидрогеназы и клеточном энергетическом метаболизме. Было обнаружено, что Гид-3 является основным ферментом, продуцирующим H_2, при pH 7,5, тогда как Гид -4 в основном участвует в окислении H_2 и переносе протонов к субъединицам HycB или HycG Гид-3. Предполагается, что при этом pH Гид-3 и Гид-4 образуют комплекс Гид, продуцирующий H_2. При pH 5,5 Гид-3 и Гид-4 выполняют контркомпенсаторную функцию по производству H_2, и снова предполагается, что при pH 5,5 комплексы FHL-1 и FHL-2. Наблюдая за важной функцией Гид-4 в процессе генерации протонной движущей силы при pH 7,5, в частности, была изучена функция субъединиц HyfB, HyfD и HyfF в транспорте протонов/калия. Было показано, что в условиях ограниченного содержания глюкозы HyfD и HyfF способствуют сохранению энергии путем взаимодействия с F_OF_1 для эффективного переноса и транслокации протонов через мембрану или через нее, чтобы сбалансировать градиент протонов и, следовательно, движущую силу протонов. Еще больше H^+ может быть перенесено через F_OF_1 в HyfD или HyfF через комплекс тиоловых групп (F_OF_1-HyfF-TrkA), чтобы сбалансировать протонный градиент и движущую силу протонов. Подобные закономерности были также изучены для отдельно связанных с мембраной Гид-1 и Гид-2, а также для дополнительных субъединиц, способствующих созреванию. В частности, было показано, что в зависимости от концентрации глюкозы разные субъединицы Гид -ферментов несут ответственность и по-разному участвуют в разных процессах. В условиях ограниченной энергии Гид-1 и Гид-2 работают над сохранением энергии, взаимодействуя с F_OF_1 для эффективного переноса протонов через мембрану или транспорта через мембрану, чтобы сбалансировать градиент протонирования и, следовательно, движущую силу протонов. Гид-2 отвечает за транспорт протонов через F_OF_1-АТФаз, он доставляет протоны в результате окисления водорода непосредственно в F_OF_1-АТФаз либо через тиоловые группы, либо через Гид-4. Полученные закономерности также наблюдались при использовании различных отходов, образующихся в результате производства и потребления кофе. Было показано, что кофейная гуща и пленка являются перспективными лигноцеллюлозными отходами, которые можно эффективно перерабатывать с высоким выходом биомассы и биоводорода посредством длительного физико-химического гидролиза и применения мутантов.