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ԽՄՈՐՄԱՆ ՎԵՐՋՆԱՓՈՒԼԵՐԻ ՕՔՍԻԴԱՎԵՐԱԿԱՆԳՆՈՂԱԿԱՆ ԿԱՐԳԱՎՈՐՈՒՄԸ ԵՎ ԴՐԱ ԿԻՐԱՌՄԱՆ ՀՆԱՐԱՎՈՐՈՒԹՅՈՒՆԸ ՕՐԳԱՆԱԿԱՆ ԹԱՓՈՆՆԵՐԻ ՄՇԱԿՄԱՆ ԳՈՐԾՈՒՄ

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

ՍԵՂՄԱԳԻՐ

ԵՐԵՎԱՆ 2021

MINISTRY OF SCIENCE AND EDUCATION OF RA
YEREVAN STATE UNIVERSITY

POLADYAN ANNA ARSHAK

Redox regulation of the final steps of fermentation and the possibility of its application for organic waste treatment

SYNOPSIS

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

YEREVAN 2021

Ատենախոսության թեման հաստատվել է Երևանի պետական համալսարանում

Գիտական խորհրդատու՝ ՀՀ ԳԱԱ թղթակից անդամ, կենս. գիտ. դոկտոր, պրոֆեսոր Ա. Հ. Թռչունյան

Պաշտոնական ընդդիմախոսներ՝ կենս. գիտ. դոկտոր, պրոֆեսոր Ա. Զ. Փեփոյան
կենս. գիտ. դոկտոր, պրոֆեսոր Կ. Բ. Ենկոյան
կենս. գիտ. դոկտոր, պրոֆեսոր Մ. Ա. Սիմոնյան

Առաջատար կազմակերպություն՝ ՀՀ ԳԱԱ Հր. Բունիաթյանի անվան կենսաքիմիայի ինստիտուտ

Ատենախոսության պաշտպանությունը տեղի կունենա 2021թ. հուլիսի 14-ին ժամը 12^{00}-ին, Երևանի պետական համալսարանում գործող ՀՀ ԲՈԿ-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 2021թ. հունիսի 3-ին:

051 մասնագիտական խորհրդի գիտական քարտուղար, կենս. գիտ. թեկնածու, դոցենտ՝ Մ.Ա. Փարսադանյան

The theme of dissertation has been approved at Yerevan State University

Academic advisor: Corresponding Member of NAS RA, Dr. of Biological Sciences, Professor A. Trchounian

Official opponents: Dr. of Biological Sciences, Professor A.Pepoyan
Dr. of Biological Sciences, Professor K. Yenkoyan
Dr. of Biological Sciences, Professor M. Simonyan

Leading organization: H. Buniatian Institute of Biochemistry NAS RA

The defense of the dissertation will be held on 14th July, 2021, at 12:00, 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 3th June, 2021.

Scientific Secretary of 051 Specialized Council,
PhD, Associate Professor M. A. Parsadanyan

INTRODUCTION

Topic's significance. Nowadays, the issues of climate change, waste pollution, and energy consumption attract the greatest attention (Scamman et al., 2019; Ripple et al., 2020). Much attention is paid to molecular hydrogen (H_2), as it is an ecologically clean, highly efficient energy source, especially in the field of transportation. In recent years, large-scale projects have focused on the production of H_2 by dark fermentation using various types of biomass, the most common being lignocellulose biomass (Kumar and Sharma, 2017; Poladyan et al., 2018; 2020; Petrossyan et al., 2020; Kim et al., 2021).

Gram-negative, γ-proteobacteria *Escherichia coli* perform mixed fermentation of using glucose or other sugars and glycerol as external sources of carbon and producing formic acid, succinic acid, acetic acid, etc. as the final products (Trchounian, 2019; Gonzalez et. Al., 2008; Trchounian 2012a,b; 2014; 2015; McDowall et al., 2014; Pinske and Sawers, 2016; Valle and Bolívar, 2021). Formate the hydrogenlyase (FHL) complex of *E. coli* membranes breaks down formic acid to produce CO_2 and H_2. Special enzymes, hydrogenases (Hyd), are involved in this process, which carry out the reversible oxidation of H_2 ($H_2 \rightleftarrows 2H^+ + 2e^-$) (Pinske and Sawers, 2016; Sargent, 2016; Trchounian et al., 2017). *E. coli* is known to have four Hyds, which are encoded by *hya, hyb, hyc, and hyf* operons, respectively. It has been shown that the reversibility of Hyds depends on environmental conditions, particularly on carbon source, redox potential, pH, etc (Maeda et al., 2007; Trchounian et al., 2017). Numerous data have been obtained to support the important role of the proton F_oF_1-ATP synthase/ase (primary transporter) and potassium ion transporter TrkA system (secondary transporter) in H_2 metabolism (Baghramyan et al., 2002; Trchounian, 2004; Trchounian et al., 2012; 2019; Blbulyan et al., 2011; Blbulyan and Trchounian, 2015). The cooperation of the two transport systems is explained by the possibility of energy transfer between them inside the membrane to save energy during fermentation. The formation of H_2 and H^+ cycles in *E. coli* was suggested to be involved in the stability of energy exchange and its efficient use under fermentation conditions, with the participation of Hyds and F_oF_1-ATP synthase/ase, respectively (Trchounian and Sawers, 2014; Trchounian and Trchounian, 2019). Moreover, a similar interaction of membrane transport systems (F_oF_1-ATP synthase/ase and potassium ion-transport system) has been shown for intestinal gram-positive, facultative anaerobic, lactic acid bacteria *Entereccocus hirae* (Poladyan and Trchounian; 2011; Vardanyan et al., 2012; Ramsey et al., 2014; Hanchi et al., 2018; Pinke et al. 2019), which lack H_2 metabolism. The interconnection of the F_oF_1-ATP synthase/ase and TrkA system (*E. coli*) (or *KtrI* in *E. hirae*) system presumably occurs through dithiol-disulfide conversions involving redox equivalents ($H^+ + e^-$) (Baghramyan et al., 2002; Poladyan and Trchounian, 2006). It is undeniable that K^+ ions play an important role in bacteria, the absorption of which is stimulated by an increase in the osmotic concentration of the environment. Moreover, they act as stimulus molecules, inducing or activating enzymes, participating in the regulation of intracellular pH, formation of membrane potential, etc. Large amounts of K^+ are required during the logarithmic phase of bacterial growth, moreover, K^+ stimulates the activity of key fermentation enzymes: phosphofructokinase, aldolase, pyruvate kinase (Trchounian, 2009). At present, one of the tasks in the field of H_2 production is the determination and management of the activity of different enzymes. Hyds are used in the production of molecular hydrogen in bacteria; moreover, they can be used as catalysts in biofuel systems (BFS) to participate in the generation of electricity. The development of BFSs, in which glycerol oxidase, Hyds, etc., are used as biocatalysts, is a precondition for the creation of the next generation of small electronic devices used in the fields of production, medicine, and ecology.

Facultative chemolithoautotrophic β-proteobacteria *Ralstonia eutropha* H16 is one of the best-studied model organisms reproducing by the consumption of H_2 and CO_2 (Cramm, 2009). *R. eutropha* have a great biotechnological potential that can synthesize oxygen-resistant Hyds and bioplastic polyhydroxyalkanoate, both heterotrophically from organic substrates and autotrophically from H_2 and CO_2 (Lenz et al., 2015; 2018). However, the cost of producing biomass and biotechnologically valuable products is still an economic challenge. The possibility of cultivating bacteria, using cheap sources for the production of biomass and H_2, in particular glycerol and other industrial wastes, will not only contribute to the production of cheap energy (electricity) but also solve the ecological problem of waste utilization.

The purpose and objectives of the research. The work aims to study the redox regulation of the final stages of fermentation in bacteria, the biochemical transformation of organic waste, the development of new approaches to the production of H_2 and biomass, as well as the use of Hyds.

The following tasks have been set for the work:

1. Study the changes in thiol groups of membrane enzymes and H^+/K^+ exchange in *E. hirae* and *E. coli* under anaerobic conditions;
2. Identify the role of medium pH and different carbon sources in regulating the activity of Hyd enzymes;
3. Study the regulation of H_2 production and H^+/K^+ exchange in *E. coli* using redox and fermentation end products;
4. Determine the effect of some metals on the biomass and H_2 production during fermentation of different carbon sources by *E. coli*;
5. Investigate the activity of Hyds under energy-limited conditions;
6. Identify the favorable conditions for the biochemical transformation of organic waste by *E. coli* and *R. eutropha*;
7. Develop new ways to apply Hyds as biochemical catalysts.

Scientific novelty and practical value of the study. The results show that the interaction of membrane proteins/protein complexes (F_oF_1-ATPase and potassium transporters) and their mechanisms of action can be universal in several bacteria, which is responsible for the occurrence and regulation of the most important energy processes in the cell. These complexes can be targeted to limit the growth of several pathogenic bacteria, but at the same time, they can be used in biotechnological bacteria as a way to manage life processes and promote the production of desired products. Regulation of the activity of Hyd enzymes using redox and fermentation byproducts and the role of redox conditions in the production of H_2 have been demonstrated in the work. The use of energy-restricted low-salt environments and mutants with various disturbances of H_2 metabolism suggested its key role in bacterial metabolism and implicated the possibility of its control by changing external environmental conditions. Moreover, the stimulating effect of some metals involved in H_2 metabolism (Ni, Fe, Mo) on the growth and H_2 production by *E. coli* during glycerol and glucose fermentation has been demonstrated. Combining the results, a new technological approach to enhanced H_2 production was proposed. Hyd enzymes responsible for the production of H_2 during the fermentation of xylose have been identified; the possibility of application of xylose in combination with glycerol has been suggested, the role of the buffering capacity of the medium for the enhanced and prolonged hydrogen production has been revealed. Physico-chemical pretreatment of paper production and brewery wastes, optimization of their hydrolysates to ensure maximum *E. coli* biomass, and H_2 production has been performed. Furthermore, the activity of Hyds during the growth of *R. eutropha* on brewery spent grain hydrolysate (BSGH) has been shown. Although *R. eutropha* H16 exhibits maximum heterotrophic

growth in the presence of several organic substrates, they do not guarantee favorable conditions for Hyd synthesis. Studies of several bioenergetic parameters and the kinetics of ORP of *R. eutropha* have been carried out under different conditions. Moreover, the stimulation of H_2-oxidizing activity of Hyds due to negative ORP has been shown. Bacterial whole cells and Hyds have been considered as anode catalysts. It is noteworthy that high H_2- oxidizing Hyd activity was observed during glycerol fermentation. *R. eutropha* membrane Hyds and *E. coli* whole cells were immobilized on sensors and their effectiveness was tested using various redox mediators as anode catalysts. The stimulating effect of ferrocene carboxylic acid on electron anode transfer and the current generation has been demonstrated.

Thus, low-cost and affordable carbon sources, can be used by bacteria to produce biomass and Hyds, which will later be applied to generate electricity. At the same time, environmental ORP can be a useful tool for regulating the metabolism of bacterial suspension, as well as improving the release of anaerobic and aerobic fermentation end products, identifying and regulating biochemical pathways during waste utilization.

Main points to present at the defense.

1. K^+ ions regulate the activity of enzymes involved in several fermentation biochemical reactions in bacteria,
2. Primary transporter F_oF_1-ATPase plays a crucial role in the formation of fermentation end products,
3. Redox regulation of the final stages of fermentation (particularly, the conversion of formic acid to hydrogen) is mediated by the regulation of thiol groups of key enzymes, which determine the different states of enzymes.
4. Biomass and Hyd enzymes from the biochemical conversion of waste are used in biofuel cells as anode catalysts.

Work approbation. The main results of the dissertation were discussed at seminars in the Department of Biochemistry, Microbiology, and Biotechnology, Biology Faculty of YSU, and at scientific conferences: 55th Annual Meeting of Biophysical Society, Baltimore, USA, 2011; NATO advanced research Workshop "The black Sea: Strategy for Addressing its Energy Resource Development and Hydrogen Energy Problems", Batumi, Georgia, 2012; 17th and 18th European Bioenergetics Conference (EBEC), Freiburg, Germany, 2012, and Lisbon, Portugal, 2014, 38th FEBS Congress, St. Petersburg, Russia, 2013, Int. Scientific Workshop 'Trends in Microb. and Microbial Biotech.', Yerevan, Armenia, 2014; 13th Intern. Conference on Clean Energy, Istanbul, Turkey, 2014; Intern. Conference on Hydrogen Production, UOIT–Oshawa, Canada, 2015; Annual conference of VAAM, Jena, Germany, 2016; ASM microbe, 2016, Boston, USA; 7th FEMS congress,Valencia, Spain, 2017, 19th IUPAB and 11th EBSA Congress, Edinburgh, UK, 2017; 12th Intern. Hydrogenase Conference, Lisbon, Portugal, etc.

Publications. According to experimental data observed in the dissertation, 62 papers, including 28 articles in peer-reviewed journals and 32 abstracts were published.

Volume and structure of the dissertation. The dissertation contains the following chapters: introduction, literature review (Chapter 1), experimental part (Chapter 2), results and discussion (Chapter 3), concluding remarks, conclusions, and cited literature (total 246 papers and books). The document consists of 247 pages, 13 tables, and 62 figures.

MATERIALS AND METHODS

Objects. *E. coli* BW25113 or MC4100 wild type (WT) strains and corresponding mutants (Table 1) as well as *Enterococcus hirae* ATCC7090 and MS116, *Ralstonia eutropha* H16 used in experiments:

Table 1. Characteristics of *E.coli* strains used in this study

Strain	Genotype	Absent hydrogenase subunit or related protein	Reference
BW 25113	*rrnB DlacZ4787 HsdR514 D(araBAD)567 D(rhaBAD)568 rph-1 (հին գենոտիպը՝ lacI*^{q} *rrnB*$_{T14}$ Δ*lacZ*$_{W116}$ *hsdR514* Δ*araBAD*$_{AH33}$ Δ*rha BAD*$_{LD78}$*)*	Parental strain (wild type)	Maeda et al, 2007; Pinske and Sawers, 2016
MC 4100	*araD139* Δ*(argF-lac)U169 ptsF relA1 fib5301 rpsL150*	Parental strain (wild type)	Maeda et al, 2007; Pinske and Sawers, 2016
JW 0955*	BW 25113 Δ*hyaB*	Large subunit of Hyd-1	Maeda et al, 2007; Pinske and Sawers, 2016
JW 2962*	BW 25113 Δ*hybC*	Large subunit of Hyd-2	Maeda et al, 2007; Pinske and Sawers, 2016
JW 2691*	BW 25113 Δ*hycE*	Large subunit of Hyd-3	Maeda et al, 2007; Pinske and Sawers, 2016
JW 2472*	BW 25113 Δ*hyfG*	Large subunit of Hyd-4	Maeda et al, 2007; Pinske and Sawers, 2016
JRG 3633	BW25113 Δ*hycE* Δ*hyfB-R*	B-R subunit of large subunits of Hyd-3 and Hyd-4	Maeda et al, 2007; Pinske and Sawers, 2016
FTD 147	MC 4100 Δ*hyaB* Δ*hybC* Δ*hycE*	Large subunits of Hyd-1, Hyd-2, Hyd-3	Maeda et al, 2007; Pinske and Sawers, 2016
FTD 150	MC 4100 Δ*hyaB* Δ*hybC* Δ*hycE* Δ*hyfG*	Large subunits of Hyd-1, Hyd-2, Hyd-3, Hyd-4	Maeda et al, 2007; Pinske and Sawers, 2016
JW 2691*	BW 25113 Δ*hycE*	Large subunits of Hyd-3	Maeda et al, 2007; Pinske and Sawers, 2016
FM 460*	MC 4100 Δ*selC*	$tRNA^{SEC}$	Maeda et al, 2007; Pinske and Sawers, 2016
MW 1000	BW 25113 Δ*hyaB* Δ*hybC*	Large subunits of Hyd-1 and Hyd-2	Maeda et al, 2007; Pinske and Sawers, 2016
DK8/pACWU 1.2	*bglR thi1rel1 frp01ilv::Tn10*	DK8 strain with pACWU1.2 plasmid, carrying *atp* operon՝ –ի 8 structural genes of F_0F_1 (*atpB-C*)	Mnatsakanyan et al., 2002
DK8/pACWU 1.2/Δ$CysF_0$	DK8/pACWU 1.2/βFlag	Cysteine's of F_0 subunit is replaced by alanine	Mnatsakanyan et al., 2002
DK8/pACWU 1.2/Δ$CysF_1$	DK8/pACWU 1.2/βFlag	Cysteine's of F_1 subunit is replaced by alanine	Mnatsakanyan et al., 2002
FRAG115	FRAG90 Δ(atpB-D)	Absence of ATPase	Mnatsakanyan et al., 2002

* Resistant to Kanamycin

Bacteria and growth conditions. The *E. coli* wild type parental strain (PS) and different Hyd mutants (described in Table 1) have been kindly provided by Prof. G. Sawers, Institute of the Biology/Microbiology Martin Luther University of Halle-Wittenberg, and *R. eutropha* H16 by Dr. O. Lenz, Technical University Berlin (TUB), Berlin, Germany. The wild-type strain *E. hirae* ATCC9790 and the *atpD* mutant MS116 strain (defective in the ß subunit of F_1) were supplied by Prof. H. Kobayashi (Chiba University, Chiba, Japan). The *E. coli* and *E. hirae* were grown under anaerobic conditions at 37 °C in batch culture, peptone growth medium (20 g/l peptone, 2 g/l K_2HPO_4, 5g/l NaCl and 10 g/l glycerol or 2 g/l glucose), different pHs and buffering capacity salt soultions were used; and tryptone growth medium (10 g/l tryptone, 10 g/l K_2HPO_4, 5g/l yeast extract, 10 g/l glucose), pH 8.0, respectively. *R. eutropha* H16 bacteria were grown in FN (Fructose-Nitrogen) minimal medium containing 0.4% fructose, FGN (Fructose-Glycerol-Nitrogen) medium with 0.2% fructose and 0.2% glycerol, or GFN (Glycerol-Fructose-Nitrogen) containing 0.4 % glycerol and 0.05% fructose, or GN (Glycerol-Nitrogen) 0.4% glycerol, under aerobic or micro-aerobic conditions, 30 °C. Brewery spent grains (BSG) were kindly supplied by "Kilikia" beer factory (Yerevan, Armenia) and Prof. Frank-Jürgen Methner, (Department of Brewing Science, TUB, Germany), and the BSG and paper waste (PW) was pre-treated by dilute acid methods in a steam sterilizer for 1 h, 121°C (Kumar et al., 2017) to obtain waste hydrolysates. The bacterial specific growth rate, μ, was determined as lg2/doubling time; CDW (cell dry wight) of bacteria was applied to estimate bacterial biomass yield and expressed in g L^{-1} (Trchounian et al., 2012).

Preparation of membrane vesicles, determination of ATPase activity, and intracellular pH. Membrane vesicles were isolated from bacteria by the osmotic lysis method (Blbulyan et al. 2015). All assays were done at 30 or 37 °C. *E. coli* K12 cell extracts were prepared using Branson Ultrasonics Sonifier™ S-450 Digital Ultrasonic Cell Disruptor/Homogenizer. 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). *N,N'*-dicyclohexcylcarbodiimide (DCCD) or sodium azide were used as an inhibitor of F_OF_1. For DCCD inhibition studies, whole cells or vesicles incubated with 0.2 and/or 0.5 mM DCCD for 10 min.P_i was measured spectrophotometrically (Labomed, Los Angeles, CA, USA). The intracellular pH ($[pH]_{in}$) was measured by the quenching of fluorescence of 9-aminoacridine (9-AA) (Hakobyan et al., 2012), using a Cary Eclipse spectrofluorimeter (Varian, USA) with excitation at 390 nm and emission at 460 nm. The accumulation of 9-AA by cells of bacteria was determined from the disappearance of 9-AA from the assay media. To study the effects of carbonyl cyanide m-chlorophenylhydrazone (CCCP) on cells, the reagent was added at the final concentration of 2 μM and incubation was carried out for 10 min.

Proton and potassium ions transport study. H^+/K^+ transport through the bacterial membrane was determined with a fine pH-potentiometer with H^+/K^+ selective electrodes (Hanna Instruments, Portugal) and analyzed using a computer program (LabVIEW, USA); Ion fluxes were expressed in mMol/min per number of cells in a unit of volume (ml) (Trchounian et al., 2012).

ORP and H_2 production determinations. ORP and H_2 production of bacterial culture cells was investigated with the help of the platinum and titanium-silicate (Ti-Si) redox electrodes (Vassilian and Trchounian, 2009), H_2 yield measured using gas chromatograph Agilent 7820A GC, with FIP и TCP electrodes (USA); The cumulative H_2 yield was measured in 500 mL glass vessels with stirring

conditions; the gases bubbled (H_2 and CO_2) were treated by 1 M NaOH solution (to eliminate CO_2 from the gas mixture), and H_2 gas was collected and estimated by the volume of water displacement.

Electrochemical measurements. Electrochemical measurements were carried out in a two-electrode electrochemical system equipped with a computerized potentiostat (GSEEE, Gomel, Belarus). Biological samples were further immobilized on the sensors using polyvinyl acetate support. The first conductive layer essential electrode design was made of graphite ink using a screen printing technique. A second profiled conductive layer of the contact pad was formed from gold. This test system was distinguished by a low background signal and high sensitivity (Semashko et al., 2013).

Activity measurement of Hyds. The H_2-oxidizing activity of Hyds was quantified in anaerobic cuvettes by monitoring H_2-dependent methylene blue reduction at 570 nm or with NAD^+ as the electron acceptor at 365 nm, 30 °C with a Cary 50 UV–vis spectrophotometer (Lenz et al., 2018). One unit of Hyd activity (U) was defined as the amount of enzyme catalyzing the conversion of 1 μmol of H_2 per min and mg of protein.

Accessible SH-groups. Accessible SH-groups were determined by the reaction with Ellmann's reagent as described (Mnaztsakanyan et al., 2004) using glutathione as a standard. Membrane vesicles were treated with the reagent until the latter was fully reacted and the optical density became constant. The level of SH-groups was expressed in nmol per mg protein.

Protein concentration was estimated by the BCA (bicinchoninic acid) method using the Pierce® BCA Protein Assay Kit (Thermo Fisher Scientific, USA) and bovine serum albumin, as the standard (Schwartz et al., 2009; Lenz et al., 2018). Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) of proteins based on the Laemmli method (Laemmli et al., 1970) was performed for the evaluation of enzyme purity.

Data processing. The average data obtained from 3 independent assays were represented, and the standard deviation of values did not exceed 3 %. For the differences between different series of experiments, Student validity criteria (p) were determined using Microsoft Excel 2016; the difference was valid if $p<0.05$.

RESULTS AND DISCUSSION

Conversions of dithiol-disulfide in membrane transporters of *E. coli* grown under fermentative conditions: effect of thiol reagents on the ion transport, ATPase activity, and H_2 production

Different thiol reagents that able to penetrate the bacterial membrane and have different mechanisms of action may provide insight into the specific role of the thiol groups in the function of the FoF1-ATPase and TrkA and to identify the mechanism of dithiol-disulfide conversions (Fig. 1). N-ethylmalemide (NEM) has been shown to completely suppress both acidifications of the medium and H+/K+ exchange in E. coli (Fig.1,A), whereas modification of thiol (SH-) groups of membrane proteins by Ellman's reagent (5,5′-dithiol-bis-(2-nitrobenzoic) acid) (ER) reduces the H+ efflux and K^+ influx in anaerobically grown (pH 7.5) wild-type strains of *E.coli*. The effect on H^+ efflux depends only on the reagent density up to 0.5 mM, and K^+ uptake is almost completely suppressed even at a density of 0.05 mM (Fig. 1,A). ATPase activity of DK8/pACWU1.2 strain membrane vesicles was significantly enhanced by 100 mM K^+ and K^+ - dependent ATPase activity was suppressed by DCCD and sodium azide (NaN_3) (Fig. 1,A). K^+ -dependent ATPase activity in this strain was significantly reduced by ~ 2.6-3.4 times when the vesicles were treated with ER and 1.7 times by another specific SH-reagent, succinimidyl-6(β-maleimidopropionamido) hexanoate (SMPH) (Fig. 1,B).

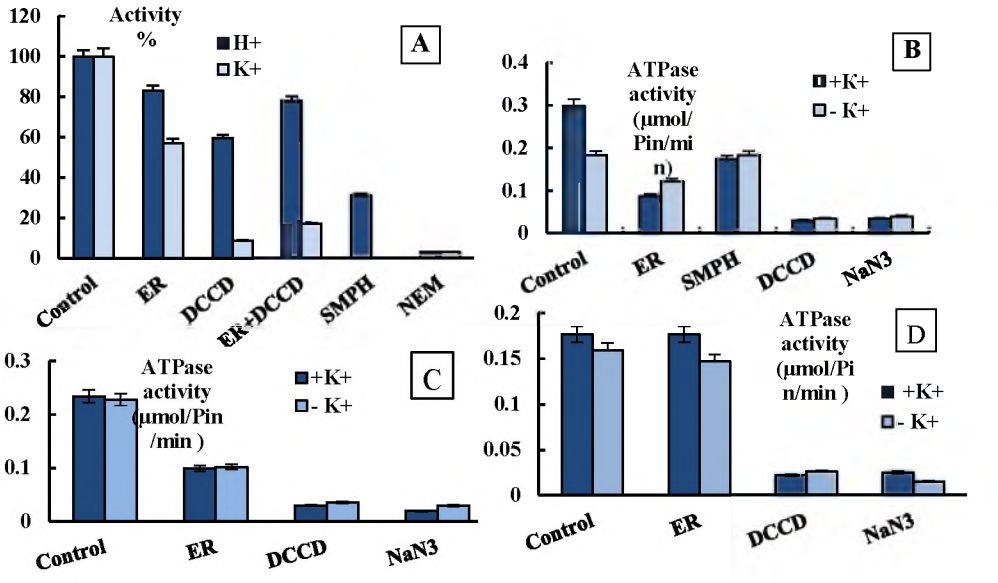

Fig. 1. Activities of K^+ and H^+ fluxes and K^+ - dependent ATPase activity of membrane vesicles of *E.coli*. A) Ions activities of the parental strain of *E. coli* (DK8/pACWU1.2), expressed as a percentage, B) Parental strain DK8/pACWU1.2, C) DK8/pACWU/ΔCysFo, D) DK8/pACWU/ΔCysF$_1$. Ion fluxes were studied in an experimental Tris -phosphate buffer solution. 1 mM SMPH, 10 µM Elman reagent (ER), 1 mM NaN_3, 0.5 mM DCCD and 5 mM ATP was introduced into the reaction mixture, pH 7.5.

However, *E. coli* DK8/pACWU1.2/ΔCysFo strain (with substitution of the cysteine residue in the b-subunit of F_o of proton FoF$_1$-ATPase for alanine) exhibited low ATPase activity that was not K^+-dependent (Fig. 1,C).

Table 2. H_2 production rate (mV/min/mg DW)[a] of *E. coli* parental and mutant strains.

Experimental conditions	DK8/pACWU 1.2		DK8/pACWU1.2/ ΔCysFo	
	Whole cells	Membrane vesicles	Whole cells	Membrane vesicles
Formate	4,2 ± 0.2	5,2 ± 0.2	0.9 ± 0.05	0.8 ± 0.04
Formate+ ER	1.2 + 0.06	0.9 + 0.04	0.8 + 0.04	-

[a] Bacteria and membrane vesicles were washed with distilled water and were introduced into the experimental medium. 5 µM formate and 10 µM Elman reagent (ER) was added. Bacteria were grown under fermentation of glucose, pH 7.5, DW-cell dry wight.

The production of H_2 in *E. coli* whole cells and membrane vesicles was also studied. *E. coli* produces H_2 during the fermentation of sugar (glucose) with the participation of FHL. This may be the result of a dithoil-disulfide conversion of membrane proteins involving the SH-groups of FoF$_1$-ATPase cysteine residues, particularly the FoF$_1$-ATPase b subunit (Mnatsakanyan et al., 2002). In this case, the change of the mentioned groups will lead to the cessation of H_2 production. Indeed, the production of *E. coli* DK8/pACWU1.2 whole-cell H_2 in the presence of glucose or formate was significantly suppressed by the ER (Table 2).

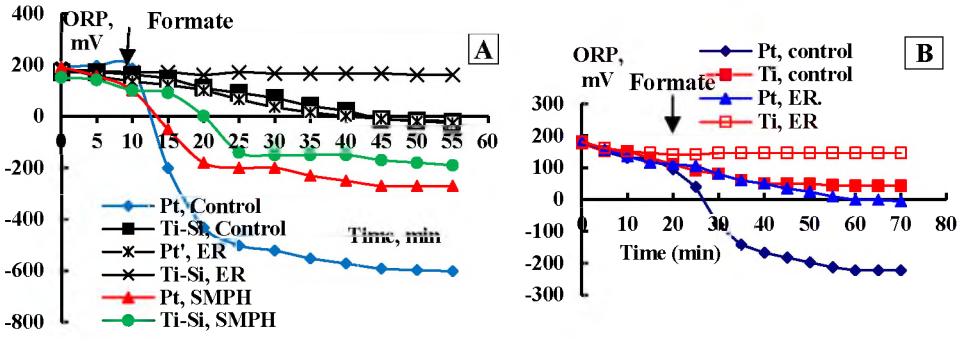

Fig. 2. ORP kinetics of the parental strain of *E. coli*: DK8/pACWU1.2 A) and mutant: DK8 /pACWU/ΔCysFo B) in the presence of thiol reagents. Bacteria were grown upon glucose fermentation, pH 7.5. ORP was studied in an experimental solution in tris-phosphate buffer with Pt-platinum ы Ti-Si-titanium-silicate electrodes and expressed in mV Vs K/Cl saturated Ag/AgCl. 1 mM SMPH, 10 μM Elman reagent (ER) were introduced. The bacteria were treated with 1 μl/ml toluene. The number of bacteria was 0.8 x 10^{10} cells/cm^3. The error rate was up to 3%.

In contrast to the Ti-Si electrode, the Pt electrode reading declined to -570 mV over time (Fig. 2), and the production rate of H_2 was 4.2 mV ORP/min DW (Table 2). Moreover, the redox potential (ORP) decreased as a result of H_2 release during glucose fermentation and formate utilization was partly restored in the presence of ER (Fig. 2, Table 2). Similar changes were established when SMPH, was used. Another thiol reagent, NEM, did not exert such effects despite its inhibitory action on ion transport and ATPase activity.

The data obtained provide conclusive evidence in favor of an essential role of thiol groups and the cysteine residue in the b-subunit of F_o of F_oF_1-ATPase in proton-potassium exchange and H_2 production in *E. coli* cells. The results also point to a possible involvement of SH-groups in the TrkA system of K^+ uptake and involvement of hydrogenases 3 or 4 in the interactions of these integral proteins with each other.

Enterococcus hirae ATCC9790 growth and H^+/ K^+ exchange. The effect of the uncoupler and role of ORP

The acidification and the change in proton motive force (Δp) (Vardanyan et al., 2012) of the medium were observed during *E. hirae* anaerobic growth, which is due to the pH of the medium, proton permeability, or the activity of enzyme cell transport systems. The ability of growth of this bacteria in the presence of protonophores (reducing the Δp) (Kobayashi et al., 1994) is still unexplained. This may indicate the potential role of another environmental parameter, ORP, which can replace this force and determine the processes necessary for growth. The decline in ORP indicates an intensification of the reductive processes associated with the production of fermented sugars, the synthesis of amino acids, proteins, etc., which have been shown in various bacteria (Vassilyan and Trchounian, 2009). In the late stationary phase, the value of ORP returns to some extent to the initial positive values (15 ± 4 mV), which indicates an increase in oxidative processes in the bacterial suspension. In general, the dynamics of ORP during growth show the unique role of redox processes in the bioactivity of bacteria. As bacteria grow, ORP decreases in the presence of protonophore (CCCP), although bacterial growth slows slightly and environmental acidification decreases (Fig. 3).

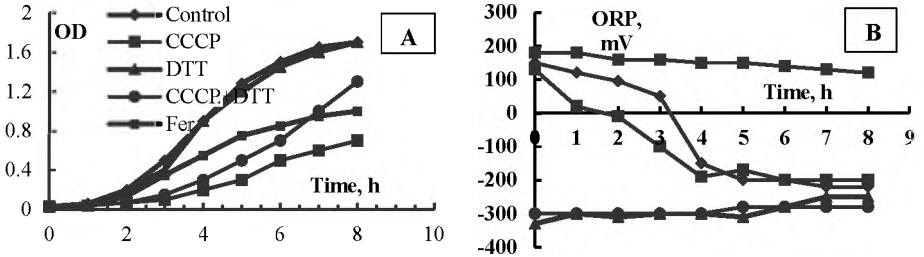

Fig. 3. *E. hirae* ATCC9790 growth (A) and ORP (B) kinetics in the presence of protonophore, oxidizer, and reductant. 0.1 mM CCCP, 3 mM ferricyanide (Fer), and 3 mM DTT were introduced into the medium. ORP was recorded using a Ti-Si electrode.

Meanwhile, the reductant 3 mM DTT, creating the negative values of ORP in the range of -300 ± 14 mV, stimulates the growth of bacteria in the presence of protonophore (Fig. 3), proving the importance of the restored state of the environment. The effect of oxidant and reductant on ion transport in *E. hirae* has been studied (Fig. 4, A; Table 3). *E. hirae* releases protons and uptakes potassium ions when glucose is introduced (Trchounian and Kobayashi, 1998).

Table 3. Proton-potassium exchange in *E. hirae* ATCC9790 bacteria under the influence of oxidant/reducing agents. 10^{10} cell/ml was added.

Experimental conditions	Ion fluxes (mM/min)[a] (overall)		Ion fluxes (mM/min)[a] DCCD-sensitive		The ratio of DCCD-sensitive fluxes
	H^+	K^+	H^+	K^+	
Control	1.11±0.04	0.20±0.02	0.38±0.03	0.17±0.01	2.3
3 mM Fer	0.69±0.02	0.09±0.01	0.14±0.02	0.07±0.01	2
3 mM DTT	1.00±0.03	0.16±0.01	0.27±0.02	0.13±0.01	2.07

Moreover, the absorption of K^+ is suppressed by protonophores. CCCP suppresses K^+ uptake, but the rate of environmental acidification remains high. DTT restores the uptake of CCCP-sensitive K^+ (Fig. 4,A). Moreover, the H^+ efflux and K^+ uptake ratio are 2:1 (Table 3), which is typical of bacteria in the absence of protonophores. In this case, it can be assumed that DTT restores the effect of protonophore by replacing Δp.

Effect of ATP and NAD^+/NADH on available SH-groups of *E. hirae* bacterial vesicles, H^+/K^+ exchange, and ATPase activity

As was mentioned, *E. hiare* ferment sugar (glucose), releasing protons and uptake K^+ (Fig. 4). The mechanisms of that exchange have been studied in the work. Experiments have shown that *E. hiare* ATCC9790 absorbs K^+ when NAD^+/NADH is introduced into the experimental solution, while no H^+ transfer is observed (Fig. 4,B). However, it is not suppressed by the DCCD with an average content of K^+ (2.5 mM), which probably occurs through KtrI or, less likely, through a K transport system operating independently of F_oF_1-ATPase (Fig. 5).

The number of accessible SH groups was determined in membrane vesicles prepared from *E. hirae* grown under anaerobic conditions at alkaline pH (pH 8.0). The addition of ATP or nicotinamide adenine dinucleotides (NAD^++NADH) to the vesicles caused a ~1.4-fold increase in the number of SH-groups (Fig. 5,A). This was inhibited by treatment with *N*-ethylmaleimide (NEM). The increase was significant when ATP and NAD^+/NADH both were added.

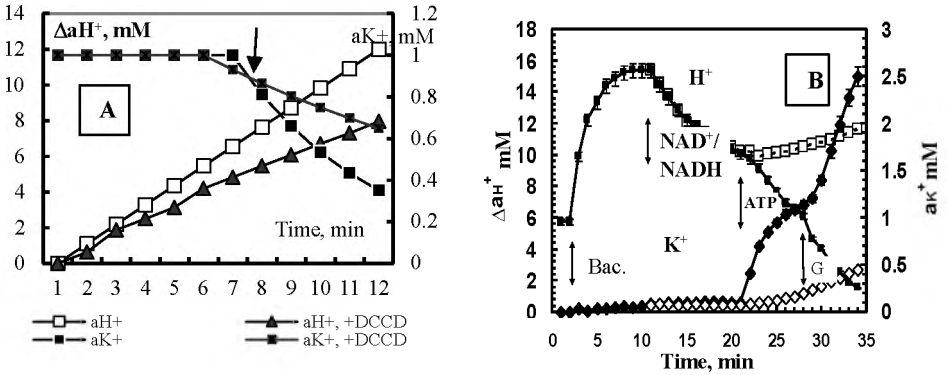

Fig. 4. Effect of DTT (A) and NAH$^+$/NADH (B) on proton-potassium metabolism of *E. hirae* ATCC9790. 0.01 mM CCCP and 20 mM glucose were supplemented. Ion fluxes are studied by the potentiometric method.

The change has lacked in the presence of the F_OF_1-ATPase inhibitors DCCD or sodium azide. This was also absent in *atp* mutant with a defect in the F_OF_1-ATPase and, in addition, it was less in potassium ions-free medium (Fig. 5,B). If a dithiol-disulfide interchange is a pathway for energy transfer from ATP used by the F_0F_1 -ATPase to the solute secondary transport system, namely low-affinity K^+ uptake Trk-like one, then the level of accessible SH-groups may be affected by ATP utilization in the presence of K^+, which can be transported via Trk-like system.

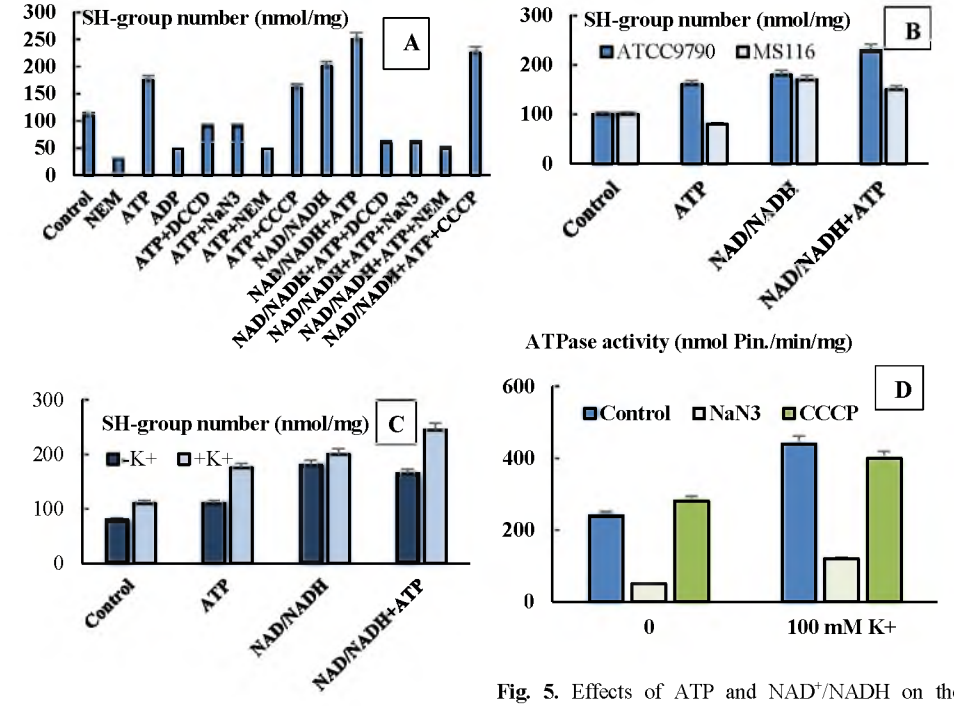

Fig. 5. Effects of ATP and NAD$^+$/NADH on the accessible SH-groups number of membrane vesicles (A) of *E.hirae* ATCC9790 grown in anaerobic conditions on glucose. Increase in SH-groups number *atpD* mutant (MS116) strains (B); K^+-dependence of effects of ATP and NAD$^+$/NADH (C); ATPase activity of membrane vesicles of *E. hirae* ATCC9790 in the K^+-free and 100 mM K^+-containing medium (D). Sodium azide (1 mM) was used to inhibit the F_0F_1-ATPase. 3 mM ATP, 3 mM ADP, 0.05 mM DCCD, 0.5 mM NEM, 1 mM NAD or 3 mM NADH was present in the assay medium as indicated.

Indeed, the level of SH-groups on membrane vesicles was similar despite the presence and absence of K^+ in the medium, but the increase stimulated by ATP was observed only in the medium containing K^+ (Fig. 5, C). These results indicate that the increase in the level of reactive SH-groups is associated with the F_OF_1-ATPase. These results are correlated with data about K^+-dependent F_OF_1-ATPase activity in *E. hirae* (Trchounain and Kobayashi, 1998) suggesting a relationship between the F_OF_1-ATPase and K^+ uptake Trk-like system.

The latter may be regulated by NAD or NADH mediating conformational changes. To confirm this, ATPase activity was determined in membrane vesicles prepared and the most significant (~7-fold) increase in ATPase activity by 100 mM K^+ was demonstrated (Fig. 5,D).

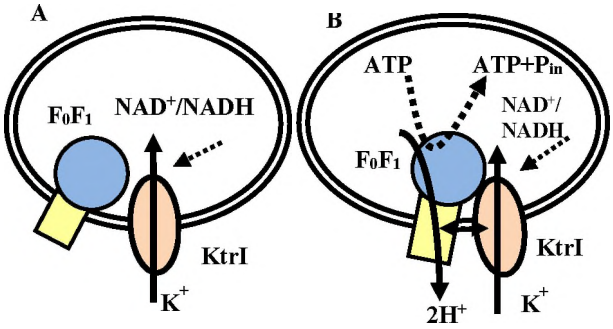

Fig. 6. The interaction of F_OF_1-ATPase and KtrI in *E. hirae*. A) KtrI operates separately, B) KtrI operates in conjunction with F_OF_1-ATPase.

The increase was inhibited by sodium azide. Thus, an increase in thiol groups available in *E. hirae* is observed depending on ATP and NAD^+/NADH (pH 8.0), no increase in thiol groups is observed in *atp* mutation in ATPase and upon the presence of inhibitors such as sodium azide. DTT restores the effect of the uncoupler. The results indicate the interaction of F_OF_1-ATPase and K^+ Trk-like uptake system, which is regulated by NAD^+/NADH-mediated conformational changes (Fig. 6).

Fermentation of glycerol in *E. coli* under different pH and redox conditions

Glycerol is recognized as an important waste, which can be used for ecologically clean and cheap fuel - molecular hydrogen bio-production by bacteria and bacterial culture oxidation-reduction potential (ORP) can be useful for optimizing the yield of glycerol and glucose aerobic or anaerobic fermentation end-products. In the study, concentration-dependent (0-15 g/l) glycerol and glucose anaerobic fermentation by *E. coli* BW25113 at pH of 5.5 to 7.5 was investigated: with the bacterial growth the decrease of ORP, measured by platinum (Pt) and titanium-silicate (Ti-Si) electrodes both (Fig. 7), and of medium pH was observed. Maximal growth yield was determined with 10 g/l glycerol at pH 6.5 and 7.5 although growth was maximal for a glucose concentration range of 5 to 15 g/l at pH 7.5. During glucose fermentation after 8 h growth Ti-Si was positive (0-80 mV), but through glycerol utilization, it dropped to negative values (up to -150 mV).

ΔpH, which is the difference between initial and end - after 24 h growth medium pH values, was 3.8-fold lower during glycerol utilization at pH 7.5 compared with glucose. In glucose-fermenting bacteria, H_2 production, measured by Pt electrode, was observed during an early log growth phase, while in the case of glycerol fermentation H_2 was evolved at the middle of the log phase.

Moreover, oxidant potassium ferricyanide (1 mM) inhibited both bacterial growth and H_2 formation (Table 4). Reducing agents DTT and sodium dithionite (1 and 3 mM) slightly inhibited bacterial growth but stimulated 2-3 fold H_2 production during log phase upon glycerol fermentation at pH 7.5 (Table 4).

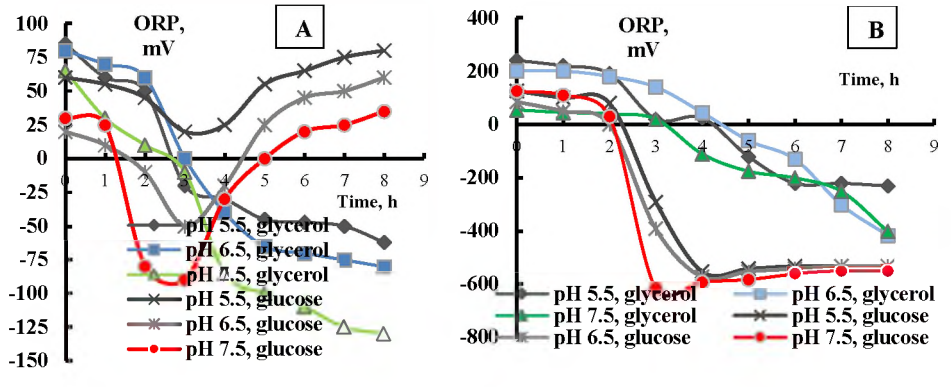

Fig. 7. Kinetics of ORP by *E. coli* BW25113 during glycerol and glucose anaerobic fermentation. Readings of Ti-Si (A) and Pt (B) electrodes are presented, 5.5, 6.5, 7.5 pHs. 10 g/l glycerol or 2 g/l glucose were added to the growth medium.

DTT might increase the formate concentration, which then can be oxidized to H_2 and CO_2. These findings indicate the more strengthening of reduction processes during growth upon glycerol fermentation and point out that appropriate concentrations of carbon sources and reductive conditions (low ORP) are essential for H_2 production by bacteria.

Table 4. Influence of ferricyanide and reducers DTT and dithionite on H_2 yields expressed in mol L^{-1} during log phase of glycerol anaerobic fermentation at different pH in *E. coli* BW25133.

pH of growth medium	Control	DTT 3 mM	Dithionite 1 mM	Ferricyanide 1 mM
5.5	-[a]	0.8	not determined	-
6.5	0.73[b]	1.3[c]	not determined	-
7.5	0.7[b]	1.4[c]	2[b]	-

a nonappearance of H_2
b H_2 yields when the bacterial culture was about 8 hour growth
c H_2 yields when the bacterial culture was about 3-4 hour growth

The role of the Hyd-1 and Hyd-2 in the H_2 metabolism of *E. coli* upon glucose fermentation: redox regulation

Oxidizer $K_3[FeCN_6]$ and reducers DTT and dithionite were used for the application of positive and negative ORP values, respectively. Specific growth rate (μ) determination showed some differences between *E. coli* wild type (BW25113) and Δ*hyaB* or Δ*hyaB*Δ*hybC* (not shown) mutants despite different values at pH 5.5 and 7.5: this characteristic was higher with Δ*hyaB* in the presence of 1 mM oxidizer at pH 5.5 and lower at pH 7.5 if compared with wild type and in the absence of this reagent. For wild type, the μ was almost the same with reducer (3 mM DTT) at pH 5.5 and less at pH 7.5 whereas in Δ*hyaB* it was less at both pHs compared with the absence of this redox reagent added. Whereas in contrast to wild type, in Δ*hyaB* it was less at pH 5.5 and higher at pH 7.5 in the presence of DTT. For Δ*hybC* μ was similar to the wild type in the presence of $K_3[FeCN_6]$ at pH 5.5 and to Δ*hyaB*at pH 7.5 (not shown). ORP simultaneous decrease was observed with *E. coli* wild type (Fig. 8) and Δ*hybC* (not shown) mutant during growth upon fermentation of glucose. Pt-electrode readings dropped down to low negative values (-493±8 mV at pH 5.5; -545±16mV at pH 7.5) also showing the formation of H_2, whereas Ti-Si-electrode readings lowered to +36±7 mV (pH 5.5) or -100±6 mV (pH

7.5) indicating redox processes level. Such kinetics in ORP was observed at pH 5.5 and 7.5 both despite some differences. In the presence of $K_3[FeCN_6]$, ORP kinetics was changed (Fig. 8): both electrode readings were kept on a positive level (+196±6 mV at pH 5.5; +123±7 at pH 7.5) during logarithmic growth phase - ~5 h (pH 5.5) and ~3 h (pH 7.5); then they were decreasing to the appropriate levels as in the absence of oxidizer (not shown). The presence of DTT ORP kinetics was similar to that in the absence of a reducer, despite the difference between pH 5.5 and 7.5 for initial time (~2 h).

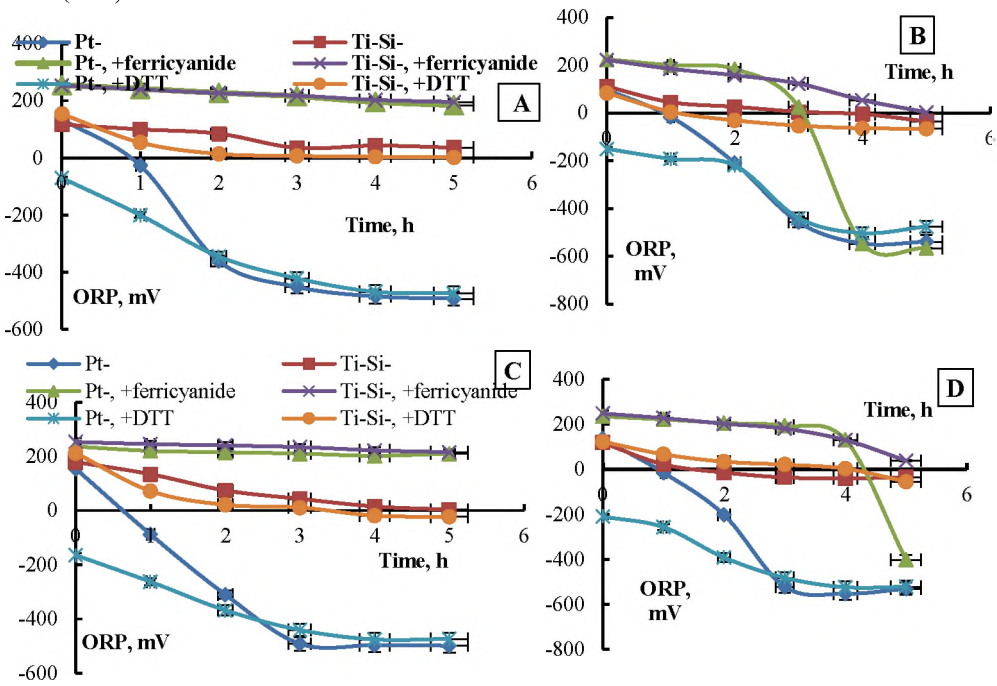

Fig. 8. ORP kinetics by *E. coli* (BW25113, wt) and Δ*hyaB* mutant during growth upon fermentation of glucose and effects of redox reagents at different pH. A) wt, pH 5.5; B) wt, pH 7.5; C) Δ*hyaB*, pH 5.5; D) Δ*hyaB*, pH 7.5. Bacteria were grown under fermentation of glucose (0.2 %) at different pH; 1 mM $K_3[FeCN_6]$ or 3 mM DTT was added into the growth medium.

The distinguishing mode in ORP kinetics was obtained with Δ*hyaB* (Fig. 8) and Δ*hyaB*Δ*hybC* (not shown) mutants in the presence of $K_3[FeCN_6]$ at pH 7.5: a drop in Pt-electrode readings was delayed. Together these results point out the different role of Hyd-1 and Hyd-2 in *E. coli* during fermentation of glucose: Hyd-1 has an activity in the presence of oxidizer (at ORP positive level) at pH 5.5 and 7.5 and can be regulated by a reducer (at ORP negative level), whereas Hyd-2 seems to be non-significant. However, Hyd-1 activity regulation by DTT should be clarified: it is due to low ORP or redox state of protein's thiols which could be changed by the reducer. The results obtained are likely to the data at different conditions (e.g. different growth and assay pH) that Hyd-1 maximal activity is observed at a positive level of ORP and Hyd-2 activity is not controlled by ORP. Interesting findings on the role of Hyd-1 and Hyd-2 were obtained by investigating *E. coli* membrane vesicles ATPase activity at pH 7.5. The overall (not shown) and DCCD-inhibited ATPase activity for Δ*hyaB* and Δ*hybC* mutants both was of lower value compared with wild type and almost similar value with each other (not shown). But in Δ*hybC* mutant DCCD-inhibited ATPase activity was increased ~1.3 fold by DTT ($p<0.01$) (Fig. 9). This was the F_0F_1-ATPase activity since DCCD is an inhibitor for this ATPase in *E. coli*. An increase in ATPase activity by DTT could be explained by the effect of low ORP (see above)

and by reduced dithiol form of disulfide groups on membrane proteins which are important for the F_0F_1-ATPase activity.

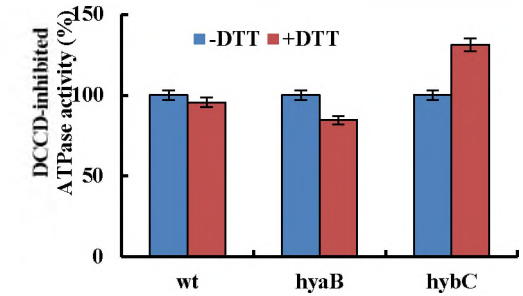

Fig. 9. Changes of membrane vesicles ATPase activity from *E. coli* wild type (BW25113, wt), Δ*hyaB* and Δ*hyabC* mutants by a reducer (DTT). DCCD-inhibited ATPase activity for wt and mutants was 100 %. It was ~70-80 % from overall ATPase activity depending on mutants. 3 mM DTT was added when mentioned.

The latter could be considered a redox-active enzyme. The results might indicate a role of Hyd-2 in the F_0F_1-ATPase activity and its regulation by DTT. It could be possible due to some cross-talk between Hyd enzymes when Hyd-4 is suggested to interact with the F_0F_1-ATPase supplying reducing equivalents. It is not ruled out that Hyd-2 can supply electrons itself for a protein-protein interaction suggested: a further study is required. This is in favor with our previous findings that Hyd-1 and Hyd-2 require the F_0F_1-ATPase.

H_2 production was measured by the difference of pair of Pt and Ti-Si electrodes readings: in the mutant, this difference was higher compared with wild type upon DTT supplementation at both pH 5.5 and pH 7.5.

Table 5. Oxidizer and reducers effects on H_2 yield by *E. coli* Δ*hyaB* Δ*hybC* double mutant during log-phase growth upon glucose fermentation at different pH.

Growth medium pH	H_2 yield, mol L^{-1}		
	[d]control	DTT[c]	Ferrycyanide[c]
5.5	1.21±0.01	[c]1.45±0.04	[a]0,00
7.5	[b]1.70±0.03	[c]2.20±0.05	0,00

[a] H_2 was not produced;
[b] H_2 yield when the bacterial culture was in ~3-4 h growth growth;
[c] 3mM DTT, 1 mM ferrycyanide were added into the growth medium;
[d] control is without reagents supplementation.

Thus, H_2 production was stimulated ~1.2 fold and ~1.3 fold during the bacterial log growth phase at acidic and alkaline pHs, respectively, in the mutant (Table 5). Effects of DTT on bacterial H_2 production can be regarded by its direct or indirect influences on accessible thiol groups of H_2 metabolizing key enzymes, and thus regulate their activity. On the other hand, it was demonstrated that external DTT supplementation may lead to the greater formation of formic acid, which is one of the end products during glucose fermentation (Riodent et al., 2000) resulting in the higher H_2 yield.

The growth and H_2 production of *E. coli* upon formate and glycerol co-fermentation at different conditions

To understand the effect of formate on growth in batch culture, H_2 production at different pHs (5.5-7.5) during glycerol fermentation, *E. coli* BW25113 wild type, and Δ*hyaB,* Δ*hybC,* Δ*hycE,* Δ*hyfG* Hyd mutants (Table 1) with deletions of different key subunits of Hyd-1 to 4, respectively, were studied. 10 mM formate ~2 fold and 30 or 50 mM formate, completely inhibited wild-type growth during glycerol fermentation at pH 5.5, whereas at pH 7.5 and 6.5 formate in the same concentrations stimulated or had no effect on bacterial growth. Note, 10 mM formate was preferred as optimal concentration, so in other experiments when needed 10 mM formate was supplemented to the growth medium. The impact of deleting the large subunits of each Hyd (1-4) enzymes for bacterial growth during the log-phase was evaluated: compared with wild type, the specific growth rate was inhibited

in all Hyd 1-4 inactivated mutants: this inhibited the effect of μ pointed out the significant role of each Hyd enzyme during the log growth phase, and it is partly following the statement that the Hyd-2 and Hyd-3 activity, but not Hyd-1 or Hyd-4 is required for bacterial optimum growth and the maintenance of redox balance using glycerol as a sole carbon source and seem to contradict data as a result of different growth conditions and assays.

Moreover, it was shown that Hyd-2 is important for the growth of the bacterium when it grows with H_2 as an electron donor and fumarate as an electron acceptor. As was mentioned above, Hyd 1-4 enzymes of *E. coli* form an H_2 cycle across the membrane (Trchounian and Sawers, 2014), which together with an H^+ cycle is suggested to have an important role in modulating the cell's energetics. Disturbance of the cycle due to lack of each Hyd enzyme might affect bacterial growth. The formate inhibitory effect during bacterial log growth might be explained by that weak acid as an uncoupler may destroy Δp leading to the bacterial growth inhibition.

From the beginning of the lag growth phase, the drop of two redox Pt and Ti-Si electrodes from positive to negative values were detected in wild type upon glycerol or formate alone or their combined conditions at pH 6.5 (Fig. 10). H_2 formation was observed in wild type with the yield of 0.75 ± 0.03 mM L^{-1} at the end of the log growth phase, formate supplementation leads to 0.83 ± 0.05 mM L^{-1} H_2 generation at early log phase, which was stimulated ~1.1 fold upon formate and glycerol co-supplementation at pH 6.5 (Fig. 10). The same, situation of H_2 evolution stimulating effect by formate or in combination with glycerol was observed at pH 7.5 (data not shown).

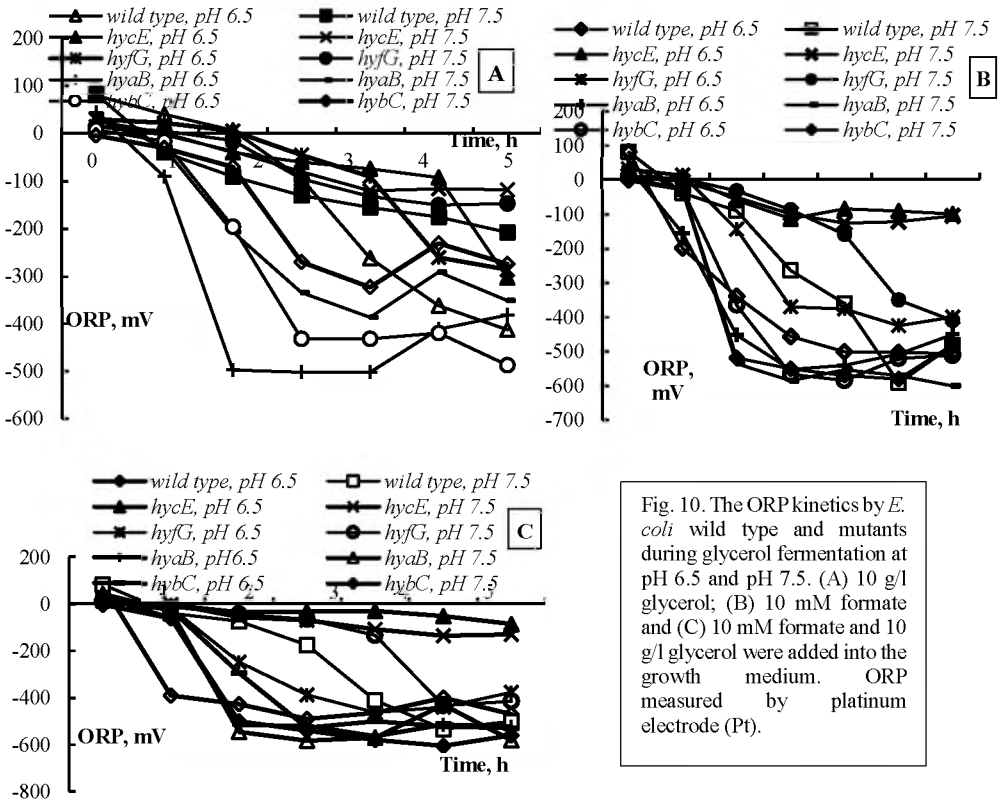

Fig. 10. The ORP kinetics by *E. coli* wild type and mutants during glycerol fermentation at pH 6.5 and pH 7.5. (A) 10 g/l glycerol; (B) 10 mM formate and (C) 10 mM formate and 10 g/l glycerol were added into the growth medium. ORP measured by platinum electrode (Pt).

Increased, 1.45 to 2.2 ±0.05 mM L^{-1} H_2 production was detected during growth of *hyaB* mutant upon all substrates combinations utilization at pH 6.5 and formate alone at pH 7.5 (Fig. 10,B), except upon glycerol only fermentation at pH 7.5 at the beginning of log growth phase (Fig.10,A). H_2 was also

produced in *hybC* mutant during glycerol fermentation at pH 6.5 with the yield of 0.8 ± 0.05 mM L^{-1} and again stimulated ~1.2 fold to ~1.3 fold during formate alone or with glycerol combination (Fig. 10, C). H_2 production was absent at pH 7.5 upon glycerol only fermentation in *hybC* mutant and recovered upon formate supplementation. This is following the recent observation that in contrast to glucose fermentation Hyd-2 has H_2 producing activity upon glycerol fermentation at pH 7.5 and formate increases the activity of Hyd 3. In contrast, H_2 production was absent in *hycE* mutant during log growth phase upon glycerol or formate alone or with glycerol combination (Fig.10).

The results point out the key role of Hyd 3 in H_2 production upon glycerol fermentation. Low and delayed H_2 production was observed at 7.5 pH upon glycerol only fermentation in Δ*hyfG* mutant, whereas at pH 6.5 H_2 production was detected at the middle of log growth phase with the yield of 0.75 ± 0.05 mM L^{-1} (Fig. 10), which was stimulated ~1.5 fold upon formate alone or with glycerol combination at the beginning of log growth phase at 6.5 and the end of log growth phase at 7.5 pHs.

Thus, in the cells grown both with external formate and glycerol H_2 production was stimulated. This stimulation was not pH-dependent. The effect can be contributed to Hyd 3 enzyme increased activity upon bacterial glycerol fermentation.

As medium composition are significant both for bacterial growth and H_2 metabolism the aim of the present part of the work was to continue the study of the physiology of *E. coli* in different nutrient media with various substrates utilization and at different pHs. The role of formate alone or with glycerol on ORP kinetics and H_2 production was investigated in double *hyaB hybC* (lacking large subunits of Hyd 1 and 2; triple *hyaB hybC hycE* (lacking large subunits of Hyds 1-3), and *selC* (lacking FDH-H) mutants during growth in bacterial batch culture up to 72 h in minimal salt medium (MSM).

Table 6. ORP drops and H_2 production yields of the *E. coli* BW25113 strain upon growth on different growth mediums upon 10 g L^{-1} glycerol fermentation.

Growth medium pH	MSM		PM	
	ORP[a], mV	H_2 production yield[b], mmol H_2 L^{-1}	ORP, mV	H_2 production yield[b], mmol H_2 L^{-1}
7.5	-580 ± 10	3.62 ± 0.02	-400 ± 10	0.73 ± 0.02
6.5	-550 ± 15	2.20 ± 0.02	-415 ± 15	0.75 ± 0.02
5.5	-490 ± 10	1.40 ± 0.02	-380 ± 15	0.64 ± 0.03

[a]ORP measured by Pt electrode,

[b]H_2 production yields after 24 h of bacterial growth in MSM and 6 h growth in PM.

The salts of the medium provide basic ionic buffering for the cells, and also provide an environment with comfortable osmotic properties. Moreover, it has the advantage of being cheap. It should be mentioned, that different inoculum (MSM with glucose or glycerol, or, PM with glucose or glycerol) were tasted to initiate the growth on MSM. PM with glucose was the preferred medium to cultivate bacteria under such energy-limited conditions as growth on MSM and glucose was used to have all Hyds expressed and to get well-adapted culture for further growth in different media. Along with BW25133 wild-type parental strain (PS), the MC4100 and K12 were also used in the study. Compared with the data of bacterial growth on PM, the physiological adaptation of *E. coli* cells to MSM was prolonged; a longer lag-phase was observed during anaerobic growth on MSM. The BW25113 growth was ~10 fold inhibited in MSM at all pHs at the 6th h of growth (Fig. 11). Whereas, after 24 h growth bacterial biomass formation was achieved with 0.143 g L^{-1}, 0.204 g L^{-1}, and 0.2 g L^{-1} (CDW), at pH 5.5, pH 6.5, and pH 7.5, respectively. Moreover, at the 72nd h of bacterial growth, surprisingly, biomass reached up to ~0.26 g L^{-1}, 0.423 g L^{-1}, and 0.474 g L^{-1} at different pHs, respectively. The medium pH drop was noticeable when bacterial growth reached 72 h: it was 4.7, 6.0, and 6.7 upon growth in MSM with initial pH 5.5, pH 6.5, and pH 7.5, respectively.

There were no important differences among the data of BW25113 and K12 strains, except MC4100, which had significant difficulties growing at pH 5.5. It was stated, that starting from the end of the log growth phase, the drop of two redox Pt and Ti-Si electrodes readings from positive to low negative values (-450±5 mV for (Pt)) was detected in *E. coli* wild type upon glycerol fermentation

in PM, pointing out the H_2 yield of ~0.75 mmol H_2 L^{-1}, as shown before. *E. coli* wild type parental strain BW25133 ORP drops and H_2 production were also observed in MSM at pH 5.5, pH 6.5, and pH 7.5 (Fig. 11, Table 6): but the drop of Pt electrode reading was delayed and reached up to -490±10 mV, -550±15 mV and -580±10 mV with the ~1.4 mmol H_2 L^{-1}, ~2.2 mmol H_2 L^{-1} and ~3.62 mmol H_2 L^{-1} yields of H_2 at different pHs (as above), respectively.

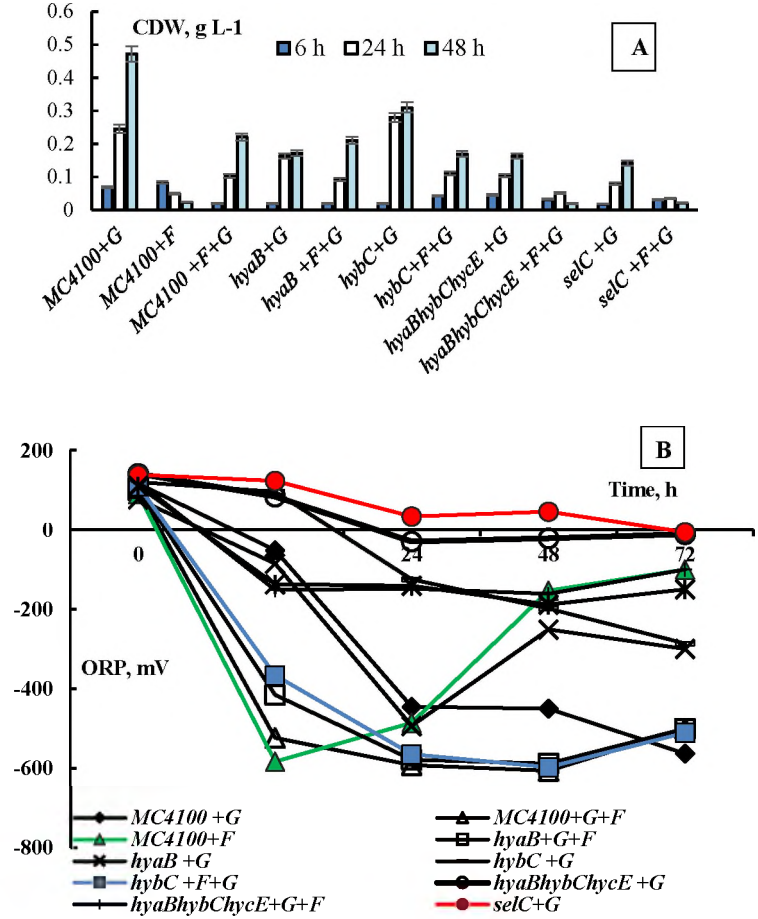

Fig. 11. The growth (A) and kinetics of ORP (B) of the *E. coli* MC 4110 wild-type parental and mutant strains during glycerol fermentation. Bacteria were grown at pH 7.5. 10 g L^{-1} glycerol (G) was supplemented and 0.68 g L^{-1} formate (F) was supplemented into MSM when mentioned Pt electrode readings are presented.

The growth and ORP kinetics, as well as H_2 producing activities, were also investigated in *E. coli* MC4100 and *hyaB, hybC*, triple *hyaB hybC hycE* and sole *selC* mutants (see Table 1) for 48 h of bacterial batch culture growth on MSM upon 10 g L^{-1} glycerol alone or with 0.68 g L^{-1} formate supplementation at pH 6.5 and pH 7.5. In contrast to bacterial growth on PM, formate alone or formate with glycerol supplementation leads to ~ 2-3 fold less MC4100 biomass formation compared with only glycerol fermentation (Fig. 11,A). It should be noted that the biomass formation of all mutants (defective in H_2 metabolism) was suppressed upon growth on MSM at all pH 5.5 to 7.5 compared with wild type parental strains data, moreover, again, formate had an inhibitory effect on mutant strains

grown under these conditions (Fig. 11,A). The results point out the critical role of hydrogen metabolism for bacterial growth.

Though in the experiment with formate only supplementation formate lead to less biomass (0.084 CDW, g L^{-1}) formation (11,A), but enhanced up to ~3.62 mmol H_2 L^{-1} yield of H_2 was already observed at 6th h of growth (Fig. 12). The addition of formate stimulated H_2 production in *hyaB* mutant ~ 2.5 fold (11,B). As formate cannot be neutralized by FHL defective mutant strain, formate excess both in and out of the cells might have a toxic effect on cell metabolism. H_2 production was abolished in *selC* mutant during bacterial growth upon glycerol or/and formate fermentation at pH 7.5; Pt electrode reading drops up to only -130±2 mV or -150±3 mV were observed. Moreover, combined supplementation of formate and glycerol did not affect at all (Fig. 11,B). H_2 production was not observed too upon mutation of Hyd 2 (*hybC*), and with functional only Hyd 4 (*hyaB hybC hycE*), but upon formate addition in *hybC* mutant H_2 producing activity was observed with the yield of 2.2 mmol L^{-1} (Fig. 11,B). The addition of formate in the culture of *hybC* might lead to Hyd 3 enzyme activation and thus recover H_2 production. Thus, bacterial growth on MSM formate leads to enhanced H_2 production, but on the other hand, weak acid formate may negatively affect bacterial growth.

The obtained results at pH 7.5 were not significantly different from those at pH 6.5, except *hybC* mutation: in contract to pH 7.5, at these conditions, H_2 was produced with the yield of 0.82 mmol H_2 L^{-1} (data not shown). Hence, the results confirm, that under certain conditions, pH 7.5 Hyd 2, Hyd 3, and Hyd 4 are responsible for H_2 production. The results are of interest to obtain bacterial biomass, using different media with glycerol and/or formate supplemented.

Growth and ORP kinetics of *E. coli* BW25113 upon addition of ethanol and glycerol fermentation

It was shown that ethanol is the main by-product of glycerol fermentation of *E. coli* (Cintolesi et al., 2012). Ethanol is produced from acetaldehyde by alcohol/acetaldehyde dehydrogenase: two molecules of NADH are utilized for ethanol generation. Thus, both H_2 and ethanol production is directed to possess the redox balance of the cell during glycerol fermentation (Murarka et al., 2008; Cintolesi et al., 2012). Therefore, the purpose of this study was to determine the effects of ethanol amounts below 2.5 % alone or with glycerol on *E. coli* BW25113 growth and H_2 production under glycerol fermentation, pH 7.5.

Control experiments were done without carbon source supplementation. Note, PM is a complex mixture of nutrients, containing large amounts of amino acids and peptides. Overall, bacterial growth was suppressed upon more than 1% ethanol supplementation (data not shown). Whereas, in some cases, slightly growth-stimulating effects were observed when 0.5 to 1 % ethanol was added: it was shown, that compared to the control experiment, at pH 7.5, 0.5% and 1% ethanol after 144 h stimulated bacterial growth ~1.2 fold. Stimulation of growth under certain conditions by the influence of ethanol indicates that the bacteria have mechanisms for assimilating or counteracting ethanol, which needs further study. The kinetics of ORP was studied with the help of Pt and Ti-Si electrodes. Overall, during the batch growth on PM the redox (Pt and Ti-Si), electrode readings drop from positive to negative values are observed during 24 h of bacterial growth.

It should be noted, that in all cases H_2 production did not observe. The maximal ORP decline (more reductive conditions) is detected at pH 7.5, after bacterial 72 h of growth: Pt electrode reading reaches up to -250±10 mV. Yet, after bacterial 144 h growth, ORP values are still negative. Moreover, compared with control (without ethanol addition), the readings of Pt and Ti-Si electrodes are ~ 100 mV more reductive and ~50 mV in ethanol (0.5% and 1%) added samples. There is a correlation with bacterial growth data when ethanol had bacterial growth-stimulating effects at 144 h of growth. The effects of mixed sources of carbon, such as glycerol and ethanol at a pH value of 7.5 were examined on bacterial growth, ORP kinetics, and H_2 production of *E. coli* BW23113. Compared to the control, the growth of bacteria was ~1.2 fold stimulated in the case of the introduction of 0.5% and 1% of ethanol, and higher (1.5 %) levels of ethanol suppress the growth of bacteria (data not shown). 24 hours later, a decrease in Pt and Ti-Si electrode readings was observed (Fig. 12). In all samples, Ti-Si electrode readings were between -70±5 mV (control) to -95±5 mV (ethanol supplemented) values.

Whereas, in the control experiment (only glycerol) Pt electrode value reached up to -400±10 mV with the yield of 0.73 mmol/L H_2 productions. Moreover, upon introduction of 0.3, 0.4, 0.5% ethanol, and 1 % glycerol ORP values were more reductive (-480±10 mV) resulting in the 1.4 mmol/L H_2 productions.

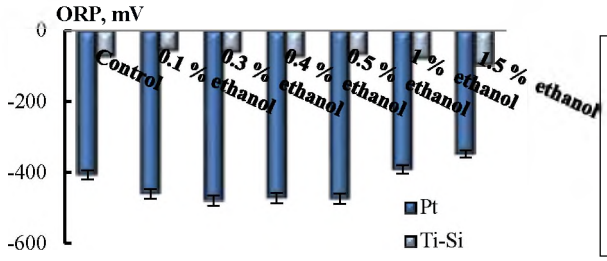

Fig.12. Changes of ORP at 24 h of growth of *E. coli* BW25113 upon different amounts of ethanol (0.1% to 1.5 %) and 1% glycerol co-supplementations. Bacteria were grown anaerobically on PM, pH 7.5. ORP was determined by platinum (Pt)-electrode.

Thus, low amounts of ethanol contribute to the growth of *E. coli* under energy-limited conditions, during the late stationary growth phase. Moreover, low amounts of ethanol co-supplemented with glycerol enhance H_2 production ~2 fold. This is the first report demonstrating the effects of low amounts of ethanol on *E. coli* growth and H_2 production. However, future studies are required to explain this phenomenon. Alcohols such as ethanol are important microbial bio-products whose toxic effects are known to limit their production in microorganisms. Although H_2 production did not observe in the presence of only ethanol in PM, our results suggest the possibility of using the ethanol with a combination of glycerol to control and enhance H_2 production. These observations are important for understanding H_2 metabolism in *E. coli* mainly upon glycerol metabolism, as well as open new perspectives in our understanding of bacterial behavior in the presence of sub concentrations of antiseptic agents.

Enhanced hydrogen production by heavy metal ions and their mixtures during *E. coli* glycerol and glucose fermentation at the wide range of pH

Metal ions play critical roles in the cell that cannot be achieved by any other entity and are therefore essential for all of life. Some heavy metals ions in low concentrations are necessary for bacterial growth and metabolism: it was shown that heavy metals as nickel (Ni^{2+}), iron (Fe^{3+}, Fe^{2+}), and molybdenum (Mo^{6+}) are essential for proper H_2 formation; particularly they are necessary for biosynthesis and maturation of Hyd enzymes.

The mixture of Ni^{2+}+Fe^{2+} (50 μM concentration for each) but not sole metals stimulated up to 1.5-fold bacterial biomass yield during glycerol fermentation at pH 6.5. Moreover, Ni^{2+}+Fe^{3+} (50 μM), Ni^{2}+Fe^{3+}+Mo^{6+} (20 μM) and Fe^{3+}+Mo^{6+} (20 μM) but not sole metals enhanced up to 3-fold H_2 yield but Cu^{+} or Cu^{2+} (100 μM) inhibited it. At pH 7.5 metal ions had minor effects on bacterial biomass and a maximal 1.2 fold stimulating effect was observed by Ni^{2+}+Fe^{2+}+Mo^{6+} only. Whereas at pH 5.5 metal ions raised 1.6 fold the biomass yield when Fe^{2+}+Mo^{6+} or Mo^{6+} were added. H_2 yield was decreased 1.5 fold compared with that at pH 6.5, but metal ions supplementations again enhanced it, particularly up to 1.5 fold by Ni^{2+}+Fe^{3+}+ Mo^{6+}. Moreover, at pH 7.5 H_2 production was enhanced 2.7 fold particularly by Ni^{2+}+Fe^{3+}+Mo^{6+} at the late stationary growth phase. During glucose fermentation bacterial growth was stimulated by metal ions combinations at pH 6.5: maximal 1.3 fold increase was detected by Ni^{2+}+Fe^{3+}, Ni^{2+}+Fe^{2+}, and Mo^{6+}+Fe^{2+} whereas of 1.2 fold increase was with Fe^{2+}+Mo^{6+} and Ni^{2+}+Fe^{2+}+Mo^{6+}; 1.2 fold increased H_2 yield was observed. At pH 7.5 up to 1.5 fold upon Ni^{2+}+Fe^{2+} supplementation increased bacterial biomass but Cu^{+} or Cu^{2+} had to suppress effect. Only Fe^{3+}+Mo^{6+} stimulated 1.5 fold H_2 production. At pH 5.5 bacterial biomass also was raised (particularly 1.7 fold) by Ni^{2+}+Fe^{2+}+Mo^{6+}. H_2 yield was raised 1.2 fold upon Mo^{6+} and Mo^{6+}+Fe^{2+} or Mo^{6+}+Fe^{3+} addition.

Consequently, the results point out the importance of Ni^{2+}, Fe^{2+}, Fe^{3+} and Mo^{6+} alone and some of their combinations for *E. coli* bacterial growth and H_2 metabolism mostly during glycerol

fermentation at acidic pH (pH 5.5 and pH 6.5). They might be used for optimizing fermentation processes on glycerol and developing H_2 production biotechnology.

Batch fermentation characteristics and hydrogen production of *E. coli* wild type and Hyd mutants using xylose and glycerol as feedstock

Xylose is one of the most abundant sugars derived from the breakdown of lignocellulosic biomass. *E. coli* can uptake and utilize many natural sugars to form biomass and to produce H_2. The latter can be evolved from formate decomposition via formate hydrogen lyase (FHL) during *E. coli* xylose or glucose fermentation. FHL consists of formate dehydrogenase H (FDH) and membrane-associated [Ni-Fe]-hydrogenase (Hyd) enzymes.

In the present study xylose different concentrations (0.05% to 1%) utilization was investigated by *E. coli* BW25113 wild type parental strain (PS) and Δ*hyaB,* Δ*hybC,* Δ*hycE,* Δ*hyfG* mutants with deletions of genes for different key subunits of Hyd-1 to Hyd-4, respectively, in minimal salts (MSM) and peptone (PM) medium, pH 5.5 and 7.5. Some data were compared with that of glucose fermentation. Compared with 0.1%, the biomass yield was enhanced ~2 fold upon 1% xylose fermentation (0.60±0.04 cell dry weight, g L^{-1}). Though upon 1% xylose utilization specific growth rate (μ) value of bacteria was ~1.6-fold less compared with 0.2% glucose fermentation, the biomass was almost the same at the stationary growth phase.

Table 7. Comparison of some growth characteristics of *E. coli* BW25113 during xylose and glycerol fermentation at different conditions after 96th h of growth.

Growth conditions[a]	HPM			LPM		
	H_2 yield (mmol/L)	ΔpH[b]	CDW (g/L)	H_2 yield (mmol/L)	ΔpH	CDW (g/L)
pH 5.5						
Glycerol	**0.80±0.03**	**0.0**	**0.30±0.05**	**0.80±0.02**	**0.05**	**0.46±0.05**
Xylose	**-[c]**	**0.7**	**0.40±0.04**	**-**	**0.90**	**0.37±0.05**
Xylose+Glycerol	**0.50±0.03**	**0.7**	**0.40±0.04**	**-**	**0.80**	**0.32±0.05**
pH 7.5						
Glycerol	**1.40±0.02**	**0.6**	**0.70±0.02**	**1.20±0.02**	**1.5**	**0.43±0.05**
Xylose	**0.70±0.02**	**0.6**	**0.60±0.02**	**-**	**2.2**	**0.52±0.03**
Xylose+Glycerol	**2.20±0.01**	**1.1**	**0.90±0.02**	**-**	**2.4**	**0.57±0.02**

[a] 0.4% xylose and 1% glycerol were added when mentioned;
[b] the difference of initial and after 72nd h bacterial growth pHs values;
[c]- H_2 was not produced.

Upon 0.05% xylose fermentation H_2 production, measured with the drop of Pt redox electrode readings, was observed at 4th and 5th h log growth phase with the yield of ~0.8 mmol L^{-1} upon glucose and xylose fermentation, respectively, at pH 7.5. Compared to PS data the biomass formation was ~1.2-fold less, and H_2 production was absent in Δ*hycE* and Δ*hyfG* mutants, with defects in Hyd-3 and Hyd- 4 at all conditions used. An interesting situation was observed with the Δ*hyaB* and Δ*hybC* mutant lucking Hyd-1 and Hyd-2, respectively: H_2 production was observed a one h early (at 4th h growth) and stimulated during growth of Δ*hybC* upon 0.05% xylose fermentation, but upon 1% xylose the same stimulating effect was observed both in Δ*hyaB* and Δ*hybC* mutants. At pH 5.5, compared to pH 7.5, biomass formation is reduced ~2-fold upon both 0.05 and 1 % xylose utilization, particularly with the mutations in Hyd-3 and Hyd-4. In contrast to pH 7.5, during *E. coli* PS growth at pH 5.5, H_2 was produced at 2 h early, at the 3rd h of bacterial growth at both 0.05% and 1 % xylose fermentation with the yield of 0.75±0.05 mmol H_2 L^{-1}; there was no marked stimulation upon mutations in Hyd-1 and Hyd-2. Physiological adaptation of *E. coli* cells to MSM was prolonged; a longer lag-phase was

detected during batch growth on MSM. Moreover, the *E. coli* BW25113 and Hyd-mutants growth were reduced ~3 fold in MSM at all pHs. In contrast to rich PM medium, H_2 formation was delayed, but, again, was observed at ~3 h early at pH 5.5 than at pH 7.5.

Obtained data indicate that during *E. coli* xylose fermentation Hyd-3 and Hyd-4 are important for both bacterial growth and H_2 production at all conditions used. Moreover, high concentrations of xylose might stimulate Hyd-1 enzyme activity during bacterial growth at pH 7.5. At pH 5.5 upon bacterial growth on both PM and MSM H_2 production was observed at the early beginning of the log phase, whereas at pH 7.5 it was delayed ~2 and 4 h. At acidic pH, H_2 production is observed at the early beginning of the bacterial log growth phase. These results are of significance to develop H_2 production biotechnology using xylose as a feedstock.

Growth characteristics and H_2 production were investigated upon consumption of 0.4 % xylose and 1% glycerol alone (which were optimal) or their mixture by *Escherichia coli* BW25113 wild type parental strain (PS) and Δ*hyaB,* Δ*hybC,* Δ*hycE,* Δ*hyfG* mutants with genes deletions for key subunits of hydrogenase (Hyd)-1 to Hyd-4, respectively, in high and low buffer capacity peptone (HPM, LPM) mediums, pH 5.5 and 7.5. Overall, pH 5.5 negatively affected bacterial growth and H_2 production. At pH 7.5, apart from Hyd-3 and Hyd-4 mutants, upon the growth of PS, Hyd-1 and Hyd-2 mutants drop of Pt redox electrode from positive (~ +150 mV) to negative (of -400 to -550 mV) values was detected during log growth phase mentioning H_2 formation. Xylose and glycerol co-utilization did not affect PS and Hyd-1 and Hyd-2 mutant's biomass and H_2 formation during the log growth phase in LPM, but ~1.5 fold stimulated these parameters, especially in HPM, pH 7.5, during prolonged 96 h bacterial growth. Roles of Hyd-3 and Hyd-4 in H_2 production; and Hyd-1 and Hyd-2 in H_2 oxidation during bacterial log growth phase were stated under xylose and glycerol co-fermenting conditions. The results obtained might be valuable for industrial long-term H_2 production by bacteria using a mixture of carbon sources and combining various organic waste materials.

Therefore, in this study, a new approach for long-term H_2 production using organic waste materials is presented: it is the first time that glycerol and xylose co-utilization by *E. coli* PS and Hyd (1 to 4) mutant cells was investigated during prolonged growth in media with different buffer capacities, LPM and HPM. Glycerol with xylose co-supplementation in optimal concentrations (1% glycerol and 0.4% xylose) into the growth medium with high buffering capacity (pH 7.5) impacted on biomass and H_2 formation by *E. coli*: marked stimulation has been shown vs glycerol or xylose alone. The results obtained indicate that during xylose and glycerol co-fermentation Hyd-3 and Hyd-4 are significant for both bacterial growth and H_2 production at all conditions used. Moreover, Hyd-1 and Hyd-2 probably operate in reversed, H_2 oxidation mode in *E. coli* upon xylose and glycerol co-utilization. These results are novel, and they demonstrate the effectiveness of the use of a mixture of xylose, the main component of various organic waste, as lignocellulose, and glycerol, as a by-product of biodiesel production, for H_2 production, in this manner contributing to both low-cost energy generation and the problem of waste recycling and disposal.

Hydrogen production by *Escherichia coli* using brewery and paper wastes: optimal pretreatment and role of different hydrogenases

Brewery spent grains (BSG), one of the by-products of brewery production and paper waste (PW) (office paper and cardboard) were applied for *E.coli* growth and hydrogen (H_2) production. The dilute acid pretreatment method was used to hydrolyze the rough lignocellulose structure; and the BSG and PW hydrolysates (BSGH, PWH) optimal conditions for bacterial growth and H_2 production were designed to be effective (Fig. 13). *E. coli* BW25113 parental strain (PS) and Hyd mutants with deletions of genes for key subunits of Hyd 1-4 (Δ*hyaB,* Δ*hybC,* Δ*hycE,* Δ*hyfG*), respectively, as well as Δ*hyaB*Δ*hybC* double mutant growth, ORP kinetics, and H_2 production, were investigated upon BSGH and PW utilization. All mentioned mutants were able to grow on BSGH and PWH: after 24 h growth on BSGH biomass yields were ~0.3 (g dry weight) L^{-1}, closed to parental strain value, and ~1.8 fold less compared with the data of PS bacteria grown on peptone medium (PM) with glycerol. Whereas specific growth rates were almost similar, even ~1.1 fold stimulated upon Δ*hyaB*Δ*hybC* double mutation.

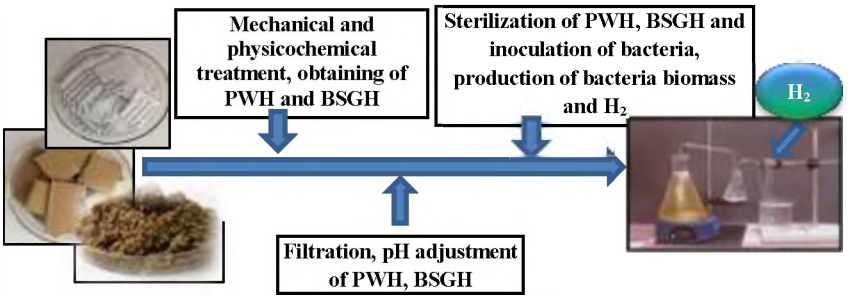

Fig. 13. Pretreatment steps of waste materials. PWH - paper waste hydrolysate; BSGH- brewery spent grains hydrolysate.

Medium pH dropped from 7.5 to 5.5 upon bacterial growth (at 6 h) on BSGH for all strains used, whereas it was to 6.9 during parental strain glycerol fermentation in PM. Readings of redox Pt electrode dropped up to -400±10 mV, with H_2 yield of 0.75 mmol L^{-1} at the 3rd h of parental strain growth and persisted until 48 h. Whereas, redox Ti-Si electrode readings drop was negligible and kept positive in contrast to glycerol fermentation in PM. H_2 production was not observed with defective Hyd 3 and Hyd 4, therefore, Hyd 3 and Hyd 4 are responsible for H_2 production using BSGH, whereas defective Hyd 1 and Hyd 2 led to up to 2 fold stimulation of H_2 yield.

Table 8. Cumulative H_2 yield of *E. coli* wild-type parental strain (PS) BW25113 and Δ*hyaB*Δ*hybC* mutant MW1000 upon growth in different conditions.

Growth Peculiarities[a]	H_2, mL(g CDW)$^{-1}$	Estimated price (USD)[b]
Wild-type parental strain was grown on BSGH	**100±3**	**1.28**
Mutant strain grown on BSGH	**175±5**	**0.7**
PS was grown on PM with glucose	**290±5**	**2.20**
PS was grown on MSM with glucose	**160±5**	**4**

[a]Data on the 11th h of bacterial growth are presented; PM is peptone medium; MSM is minimal salt medium (see Materials and methods)
b Prices are calculated for 1000 L of H_2yieldconsidering only the prices for glucose and BSG. CDW-cell dry wight

Data of cumulative H_2 production per bacterial CDW are presented in Table 8: so, PS growth on rich PM with glucose led to ~3 and ~1.7 fold higher amount of H_2 yield compared to growth of PS and mutant on BSGH, respectively. However, glucose and peptone are much more expensive substrates for H_2 production than BSG. Significant amounts of BSG are annually derived from breweries. Thus, providing wet BSG with the cheapest (non-valuable) price to local consumers, like biogas stations or farmers (for use as cattle feed, etc.) will be the good solution of the breweries for the BSG elimination, since this is a cheap alternative that avoids the energy spend needed for drying of BSG. However, the costs of transporting BSG are significant and should be thought over. Generally, an average of 16 USD per ton of wet BSG transported a distance of 5 miles (~8 km) is considered. So, comparing the prices for glucose and BSG and yields of H_2 produced by PS and Δ*hyaB*Δ*hybC* mutant under different conditions suggests the possibility of BSG application for low-priced H_2 generation (see Table 8).

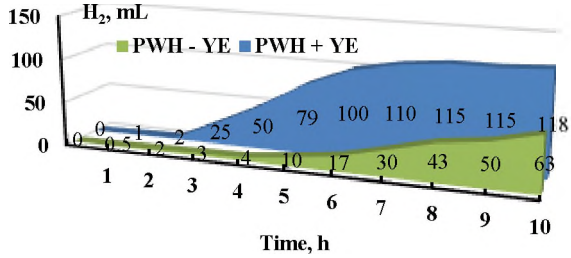

Fig. 14. The cumulative H_2 yield by *E. coli* BW25113 wild type PS PWH (paper waste hydrolysate) utilization. Bacteria were grown anaerobically, pH 7.5.

Readings of redox Pt electrode dropped up to -500±10 mV, mentioning the H_2 yield of ~1.45 mmol H_2 L^{-1} at the 4th h of PS growth using PWH with the formation of 0.20±0.02 g bacterial cell dry weight L^{-1}. Bacterial biomass formation was stimulated ~3-fold upon addition of 0.5% yeast extract and H_2 production was observed at the early beginning of the growth log phase. Moreover, mutations of Hyd-1 and Hyd-2 enhanced H_2 production (Fig. 14).

The main role of Hyd-3 in H_2 formation and Hyd-1 in H_2 oxidation upon fermentation of PWH was proposed. These findings would be useful for the development of energy especially H_2 production biotechnology using different organic wastes.

Growth of the facultative chemolithoautotroph *Ralstonia eutropha* on organic waste materials: growth characteristics, redox regulation, and Hyd activity

Growth and bioenergetics properties, ORP kinetics were investigated during cultivation of *Ralstonia eutropha* H16 on fructose and glycerol or lignocellulose-containing brewery spent grain hydrolysate (BSGH). BSGH was used as a carbon and energy source for *R. eutropha* H16, and the activities of the membrane-bound hydrogenase (MBH) and cytoplasmic, soluble hydrogenase (SH) were measured in different growth phases. Growth of *R. eutropha* H16 on optimized BSGH medium yielded ~0.7 g cell dry weight L^{-1} with 3.50 ± 0.02 (SH) and 2.3 ± 0.03 (MBH) U·(mg protein)$^{-1}$ activities (Fig.15).

High SH activity was observed after bacterial 72 h growth (Fig. 15, A); and buffering components or metal ions (Ni, Fe) or addition of fructose had no effects on Hyd activities (Fig.15, AB). Upon growth on fructose and glycerol, a pH dropped from 7.0 to 6.7 and a concomitant decrease of ORP was observed. During growth on BSGH, by contrast, the pH and ORP stayed constant. The growth rate was slightly stimulated through the addition of 1 mM $K_3[Fe(CN)_6]$, whereas temporarily traded growth was observed upon the addition of 3 mM DTT. Bioenergetics properties of bacteria such as F_OF_1-ATPase activity and $[pH]_{in}$ were investigated upon bacterial growth on glycerol (FGN) and fructose (FN). The overall ATPase activity of right-side-out membrane vesicles of *R. eutropha* H16 grown heterotrophically on FN or FGN was investigated. To determine the F_OF_1-ATPase activity, membrane vesicles were treated with the inhibitor (DCCD) for 10 min. The ATPase activity upon utilization of only fructose (FN medium) was 92±5 nMol Pi (min μg protein)$^{-1}$and 0.2 mM DCCD inhibited ATPase activity ~2.3-fold ($P<0.01$)). It is worth mentioning that compared to growth in FN, membrane vesicles demonstrated ~4.2- ($P<0.002$) and ~2.5-fold ($P<0.05$) lower F_OF_1 -ATPase activity, respectively, upon fructose and glycerol co-utilization (FGN medium). The distribution of 9-AA between external and intracellular spaces in the bacterial cells reflects the pH gradient across the cytoplasmic membrane. The intensity of fluorescence remained constant and decreased insignificantly at pH below 7.50. The fluorescence quenching by addition of *R. eutropha* H16 occurred when $[pH]_{out}$ was higher than $[pH]_{in}$ The $[pH]_{in}$ measured by the 9-AA quenching was 8.00±0.05. As a control experiment, 9AA fluorescence quenching was investigated upon protonophore supplementation: The fluorescence quenching was eliminated by supplementation of 2μM CCCP.

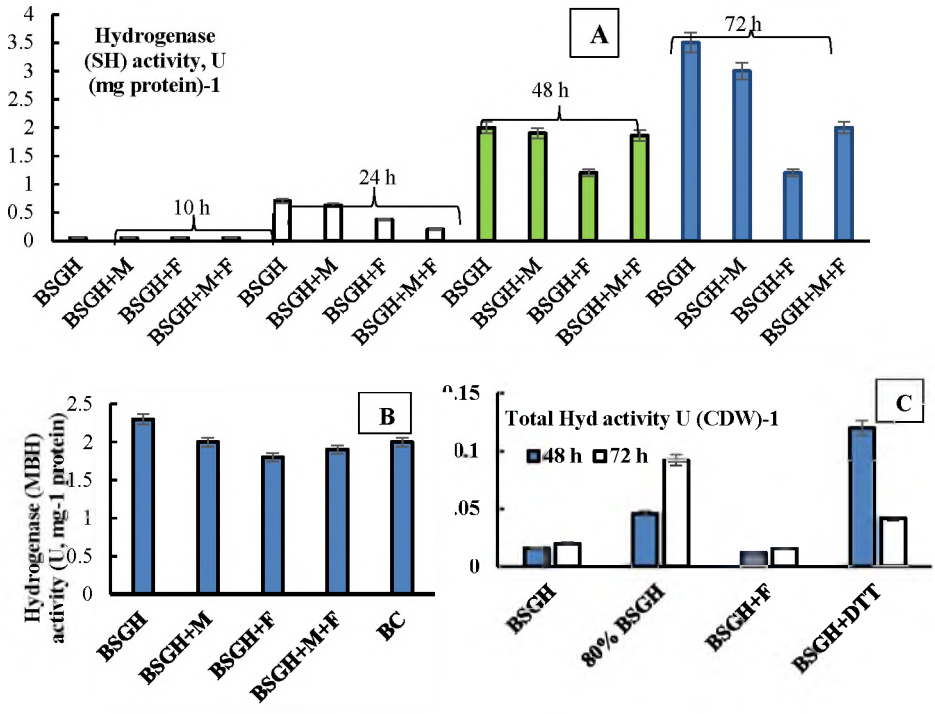

Fig. 15. Hyd activity of *R. eutropha* H16 bacterial cell extracts during bacterial growth under different conditions. F- 0.05 % fructose, M- Ni and Fe ions, BC- buffering components. a) SH activity on BSGH; b) MBH activity on BSGH, C) Total Hyd activity of *R. eutropha* H16 whole cells under different conditions. 3 mM DTT and 1 mM ferricyanide (F) were supplemented where indicated. One unit of Hyd activity was defined as the amount of enzyme which catalyzes the conversion of 1 μmol of H_2 per min.

R. eutropha H16 can grow upon cheap carbon sources: bacterial growth and SH and MBH activity were observed during the degradation of lignocellulosic BSGH. Redox regulation of bacterial growth was investigated and oxidizing conditions stimulated bacterial specific growth rate. In contrast, reducing conditions suppressed bacterial growth, but total Hyd activity of the cells was markedly stimulated (Fig.15,C). It is interesting that a decrease in ORP during the growth of the bacteria was observed, which was more significant upon growth on BSGH and FGN. The relationship of ORP with Hyd enzymes activity might be suggested by these data. Further studies are required to reveal the mechanisms of redox stress adaptation by *R. eutropha* H16 and Hyd activity, where the main role might be contributed by FoF_1 -ATPase.

Application of organic waste glycerol to produce crude extracts of bacterial cells and microbial hydrogenases – the anode enzymes of bio-electrochemical system

[NiFe]–Hyds of *E. coli* and *R. eutropha* can be applied as potential anode biocatalysts in MFC. Bacterial growth parameters and Hyd activities were followed upon utilization of different carbon sources (glucose, fructose, or glycerol) and consequently, the comparative analysis is presented in Table 9. At a pH of 5.5 bacterial (*R. eutropha* and *E. coli*) biomass and Hyd activity were not high, they were almost negligible in growth experiments with *R. eutropha* (data not shown). *E. coli* BW25113 has grown anaerobically and fermented 0.2% glucose or 1% glycerol in peptone medium. Compared to glycerol, upon glucose fermentation, after 24 h growth, bacterial biomass and specific growth rate were enhanced ~2.2 ($P<0.002$) and 1.5-fold ($P<0.001$), respectively. H_2 production was controlled during *E. coli* BW25113 growth with the help of ORP Pt electrode: H_2 formation was stated

at the beginning and late log growth phase upon glucose and glycerol fermentation, with the yields of 0.70±0.02 mmol H_2 L^{-1} and 0.80±0.05 mmol H_2 L^{-1}, correspondingly. Upon glucose fermentation, H_2-oxidizing the Hyd activity of *E. coli* whole cells was 0.40 ± 0.01 U (mg CDW)$^{-1}$ L^{-1} (Table 9).

Table 9. Growth properties and H_2 oxidizing Hyd activities of *E. coli* BW25113 and *R. eutropha* H16 under different growth conditions (Trchounian et al., 2012, 2017; Poladyan et al., 2018, 2019).

Parameters	Bacteria			
	E. coli BW 25113		*R. eutropha* H16	
Growth conditions	Glucose	Glycerol	Fructose	Glycerol
μ, h^{-1}	0.95±0.02	0.60±0.02	0.30 ±0.01	0.16 ±0.02
Cell dry weight, g L^{-1}	1.10 ±0.02	0.50±0.05	2.80[a] ±0.02	3.30[b] ±0.01
Hyd activity, U (mg CDW)$^{-1}$	0.40[c] ±0.01	1.73[d] ±0.02	0.48[d] ±0.02	5.90[d] ±0.02

[a] Bacteria were grown for 48 h;
[b] Bacteria were grown for 168 h, under oxygen limitation conditions;
[c] Total Hyd activity of whole cells (U (g CDW)$^{-1}$ L^{-1});
[d] MBH activity (U (mg protein)$^{-1}$) in crude cell extracts.

Elevated H_2-oxidizing Hyd activity was also observed upon bacterial glycerol fermentation: it reached up to 1.73±0.02 U (mg CDW)$^{-1}$ L^{-1} ($P<0.001$). *R. eutropha* H16 cultures were grown upon 0.4% fructose (FN) and 0.4% glycerol and 0.05% fructose (GFN) utilization under aerobic and micro-aerobic conditions, respectively. Increased biomass formation (2.80±0.02 (g CDW) L^{-1}) was observed in FN after 48 h of growth. Moreover, it reached 3.30±0.02 (g CDW) L^{-1} in GFN after 168 h of growth (Table 1). Compared to GFN, the specific growth rate (μ) was ~ 2-fold higher in FN; the H_2-oxidizing Hyd activity of MBH was rose ~12 fold ($P<0.002$) in bacteria grown in GFN (Poladyan et al, 2019).

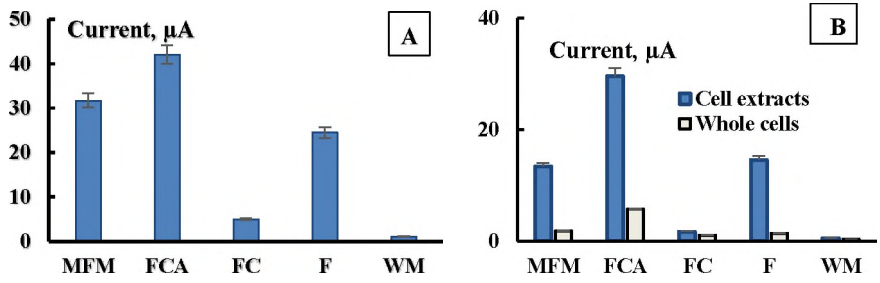

Fig.16. MBH (A) and *E. coli* K12 whole cells and cells extracts (B) as anode catalyst with different mediators using hydrogen as the substrate (b). MFM- methylferrocene methanol; F-ferrocene; FCA- ferrocene-carboxylic acid; WM- without mediators.

R. eutropha HF649 synthesizing *Strep*-tagged MBH was cultivated under oxygen limiting conditions in GFN medium, 168 h. The activity of the purified MBH was ~140 U·mg^{-1}. The purified enzyme used in the experiments was immobilized on the sensors with the aid of polyvinyl acetate (this is powder) support in 50 % concentrations (1% = 1g/100ml, 50 % =50 g/100 ml or 500 mg/1 ml). Ferrocene (F) and its derivatives (ferrocene-carboxy-aldehyde (FC), ferrocene-carboxylic acid (FCA), methyl-ferrocene-methanol (MFM)) were used, as mediators: stock solutions were in initial concentrations of 50 mM, and 3 – 5 μl of mediators were added in the experiments. H_2 (the substrate for Hyd activity) was constantly supplemented during experiments. At 500 mV voltage enzyme demonstrated a maximal level of bioelectrocatalytic activity. Depending on the specific mediator, the current intensity was different: without mediator current generation was negligible, whereas upon addition of FC it reached ~ 5 ± 0.6 μA (Fig. 16,A). Moreover, maximum current flow up to ~ 42 ± 0.5 μA was detected upon FCA supplementation (Fig 16,A). *E. coli* K12 wild-type strain was cultivated under anaerobic conditions. Bacterial cell extracts were prepared by sonication; and 3 μl (10^{10}/ml)

bacterial whole cells or cell extracts were immobilized on sensors using 50 % polyvinyl acetate. H_2 was constantly supplemented during experiments. Upon using different mediators, the current strength varied from 1 to 30 μA. Compared to whole cells of *E. coli* upon using bacterial cell extracts current strength was enhanced ~ 2-5 fold (Fig.16,B). Without a mediator current generation was negligible. Again, in both cases with cell extracts and whole cells, the current strength was enhanced up to 29.58 ±0.7 μA and 5.73 ±0.3 μA upon FCA supplementation, respectively.

Moreover, the maximal up to 150 μA value of current strength was achieved in cell extracts of bacteria with two-fold H_2 supplementation (data not shown). Control experiments were performed using reagent-less electrodes with supplementation of H_2.

Consequently, organic waste materials such as glycerol used in the study for the cultivation of microorganisms and obtaining valuable enzymes (Hyds) will lead to economical electricity generation.

CONCLUDING REMARKS

In an experimental approach, using thiol reagents (succinimidyl-6 [(β-maleimide-propionic-amide) hexanoate] (SMPH), Ellman's reagent), formic acid, and potassium ions with the help of F_oF_1-ATPase mutants the essential role of SH- groups in the transfer of energy from. The obtained results suggest that the low values of ORP compensate for the negative effect of protonophore in *E. hirae*. The redox regulation of proton/potassium exchange in *E. hirae* and the role of thiol groups has been studied: ferricyanide slows down the acidification of the medium and the K^+-uptake, while DTT stimulates the proton-potassium exchange. Moreover, K^+-uptake is suppressed by protonophores, however, DTT restores the DCCD-sensitive K^+-uptake. Moreover, the number of accessible SH-groups in *E. hirae* membrane vesicles is stimulated by ATP or NAD^+/NADH. Also, proton-potassium exchange is observed depending on ATP and NAD^+/NADH. No increase in thiol groups is observed in ATPase mutants (no changes were recorded in *atp* MS116 mutants with non-functional F_oF_1-ATPase (with inactive β-subunit of F_1)) and in the presence of inhibitors DCCD and sodium azide, as well as in the absence of potassium ions. DTT restores the effect of the protonophore. This phenomenon is linked to F_oF_1-ATPase. Moreover, it is observed in the presence of K^+.

It has been shown that modified kinetics of ORP is observed depending on the carbon source: the decrease in pH during glycerol fermentation is insignificant due to the fermentation products, however, more reduction processes are observed during glycerol fermentation, which indicates the essential importance of reducing conditions (more negative values of ORP) for *E. coli* growth and H_2 production. Moreover, reducers significantly stimulate the production of H_2 by creating negative values of ORP. As mentioned, the 4 Hyd enzymes together can form a hydrogen cycle in the membrane of *E. coli* (Sawers and Trchounian, 2014). The disturbance of this cycle due to the absence of two and more Hyd enzymes can be informative. With the help of single, double, or triple Hyd mutants of *E. coli*, it was found that, unlike Hyd-2, Hyd-1 exhibits activity in the presence of an oxidizer (positive values of ORP) at pH 5.5 and 7.5, and can be regulated with a reducer (negative ORP values). However, the regulation of Hyd-1 activity by the DTT still needs to be clarified. The results also showed the role of Hyd-2 in the function of F_oF_1-ATPase and the possibility of its regulation by DTT. During glucose fermentation in the Hyd-1 and Hyd-2 double mutant, an increase in hydrogen yield was observed at basic pH, which was significantly enhanced in the presence of DTT. Thus, studies have shown that the H_2 production in *E. coli* during glucose and glycerol fermentation is regulated by the reducing ORP conditions, which are necessary for the production of biohydrogen. The role of different Hyds has been demonstrated in the presence of external formic acid alone, as a fermentation end product, or in combination with glycerol in *E. coli* wild type and Hyd mutants with inactive catalytic subunits under different pHs. The conditions were found when formic acid alone or its combination with glycerol or ethanol with glycerol or metal ions (Ni, Fe) had a stimulating effect on bacterial growth and H_2 production. The possibility of combined use of xylose and glycerol has been suggested, and the role of the buffering capacity of the medium for the long-term, improved hydrogen production has been identified. Physicochemical pretreatment of paper and brewery wastes and optimization of their hydrolysates were performed. Hyd enzymes responsible for the hydrogen production during waste hydrolysate utilization by *E. coli* were identified. The enhanced H_2 yield in

E. coli ΔhyaBΔhybC double mutant strains has been shown. Moreover, the synthesis of Hyd enzymes during the growth of *E. coli* and *R. eutropha* on BSGH has been shown. Hyd synthesis and activity mainly depend on external factors. Although *R. eutropha* H16 exhibits maximum heterotrophic growth in the presence of several organic substrates, they do not guarantee favorable conditions for Hyd synthesis. Studies of several bioenergetic characteristics and the kinetics of ORP of *R. eutropha* in the presence or absence of various wastes were carried out in this work. Bacterial growth and Hyd enzyme synthesis during BSGH utilization were shown for the first time. Moreover, DTT-induced ORP significantly stimulated the H_2-oxidizing activity of Hyds in cells. It is noteworthy that high H_2-oxidizing Hyd activity was observed during glycerol fermentation. *R. eutropha* MBH and *E. coli* whole cells were immobilized on sensors and their effectiveness as anode catalysts was tested using various redox mediators. The stimulating effect of carboxylic acid ferrocene on electron anode transfer and the current generation has been demonstrated. Moreover, based on the obtained results, with the cooperation of the researchers of the Institute of Biochemistry RA NAS, it was possible to create hydrogen (H_2) fuel cell voltmeter (HFCV). It can work with the sample transmitters of the test specimens and it has a separate external signal input for the H_2 FC system.

Therefore, low-cost and affordable carbon sources, such as various organic wastes, can be used by bacteria to produce biomass and Hyds, which will later be used to generate electricity.

CONCLUSIONS

1. The role of SH-groups in H^+/K^+ exchange in *E. coli* and *E. hirae* and their regulation by NAD^+/NADH-mediated conformational changes in *E. hirae* have been demonstrated. Moreover, the important role of ORP in bacterial growth, and especially in the replacement of Δp in *E. hirae* has been clarified.
2. The key role of the reducing processes of medium ORP in the glycerol fermentation and the H_2 production under anaerobic glycerol fermentation conditions in *E. coli* BW25113 was revealed.
3. In *E. coli* BW25113, Hyd-1, in contrast to Hyd-2, was found to exhibit activity in the presence of an oxidizer (positive values of ORP) during anaerobic glucose fermentation at pH 5.5; and at pH 7.5 it is involved in the H_2 generation. Meanwhile, the role of Hid-2 in the functions of F_oF_1-ATPase and its regulation with DTT has been demonstrated.
4. Conditions have been identified where the utilization formic acid and glycerol combination by *E. coli* BW25113 enhances the production of biomass and H_2; and the significant role of Hyd-3 at pH 6.5 and 7.5, as well as the role of Hid-2 and Hyd-4 at pH 7.5, were noted. The potential role of H_2 metabolism in bacteria in the conditions of energy (nutrient) restriction in MSM has also been demonstrated.
5. Metals of different valency (0.02-0.1 mM Fe^{2+}, Fe^{3+}, Ni^{2+}) and their mixtures (Ni^{2+}+Fe^{2+}+Mo^{6+}, Ni^{2+}+Fe^{3+}, Ni^{2+}+Fe^{2+}, and Mo^{6+}+Fe^{3+}) have been shown to have a stimulating effect on the growth and H_2 production by *E. coli* BW25113 during glucose and glycerol fermentation at pH values of 5.5-7.5.
6. The results showed that the main product of glycerol fermentation in *E. coli*, ethanol, was involved in the regulation of H_2 production and bacterial growth.
7. The primary role of Hyd-3 and Hyd-4 in the H_2 production by *E. coli* from the fermentation of xylose alone and its combination with glycerol has been identified. The role of the buffer capacity and the medium pH during the long-term co-fermentation of xylose and glycerol by *E. coli* has been demonstrated.
8. Favorable conditions for the *E. coli* biomass and H_2 production from organic waste hydrolyzates, BSGH and PWH, have been developed. Moreover, the Hyds responsible for the H_2 production under these conditions (Hyd-3 and Hyd-4) were identified and the defects in Hyd-1 and Hyd-2 contributed to a significant increase in H_2 production by *E. coli*.
9. The growth of *R. eutropha* H16, as well as MBH and SH high activity, have been shown in the conditions of BSGH. The results show that low ATPase activity and negative values of

medium ORP (including because of DTT presence) contribute to the synthesis of Hyds in *R. eutropha*.

10. High H_2-oxidizing activity is observed during fermentation of glycerol by *R. eutropha* and *E. coli*. Moreover, *R. eutropha* MBH and *E. coli* K12 whole cells and broken cells serve as effective anode catalysts in the electrochemical system, and intermediates (carboxylic acid ferrocene) stimulate the generation of electricity.

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ԽՄՈՐՄԱՆ ՎԵՐՋՆԱՓՈՒԼԵՐԻ ՕՔՍԻԴԱՎԵՐԱԿԱՆԳՆՈՂԱԿԱՆ ԿԱՐԳԱՎՈՐՈՒՄԸ և ԴՐԱ ԿԻՐԱՌՄԱՆ ՀՆԱՐԱՎՈՐՈՒԹՅՈՒՆԸ ՕՐԳԱՆԱԿԱՆ ԹԱՓՈՆՆԵՐԻ ՄՇԱԿՄԱՆ ԳՈՐԾՈՒՄ

Ամփոփում

Հանգույցային բառեր՝ *Escherichia coli*, *Enterococcus hirae, Ralstonia eutropha*, սպիտակուց-սպիտակուցային փոխազդեցություն, խմորման վերջնափուլեր, կենսաջրածին, ռեդօքս կարգավորում, հիդրոգենազներ (Հիդ), օրգանական թափոնների մշակում

Տվյալ աշխատանքում թիոլային ռեագենտների (սուկցիմիդիլ-6[(β-մալեիմիդ-պրոպիոն-ամիդ) հեքսոնատ]-ը (ՍՄՊՀ), էլլմանի ռեագենտ), մրջնաթթվի և K^+-ի ներգործությամբ և F_0F_1-ԱԵՖազային մուտանտների օգնությամբ առավել հիմնովին ցույց է տրվել SH-խմբերի էական դերը ԱԵՖազից K^+-ական տեղափոխիչին էներգիայի փոխանցման և H_2-ի արտադրության մեջ: *E. hirae*-ում ևս գործում է H^+-ային F_0F_1-ԱԵՖազ, որն անթթվածին պայմաններում փոխազդում է երկրորդային տեղափոխիչների հետ, ինչպիսին է K^+ կլանող Trk կամ Ktrl համակարգը՝ ձևավորելով H^+/K^+-ական պոմպ: Պոմպի աշխատանքի ընթացքում դիտվել է միջավայրի թթվեցում և ինչպես նաև Δp-ի գեներացում: Աշխատանքում ցույց է տրվել մանրէների աճի և թթվայնացման խթանում վերականգնիչի՝ ԴԹԹ-ի և պրոտոնաֆորի առկայության պայմաններում: Ստացված արդյունքները թույլ են տալիս ենթադրել, որ ՕՎՊ-ի ցածր արժեքները կոմպենսացնում են պրոտոնաֆորի բացասական ազդեցությունը. *E. hirae*-ում մանրէներում ՕՎՊ-ը կարող է փոխարինել Δp-ին և որոշիչ լինել աճի համար անհրաժեշտ գործընթացների համար: *E. hirae*-ում ուսումնասիրվել է նաև H^+/K^+-ական փոխանակության ռեդօքս կարգավորումը և թիոլային խմբերի դերը. ֆերրիցիանիդը դանդաղեցնում է միջավայրի թթվայնացումը և K^+-ի կլանումը, մինչդեռ ԴԹԹ-ն՝ խթանում H^+/K^+-ական փոխանակումը: Ավելին, K^+-ի կլանումը ճնշվում է պրոտոնաֆորների ազդեցությամբ, սակայն, ԴԹԹ-ն վերականգնում է ԴՑԿՂ-զգայուն K^+-ի կլանումը: Ավելին, *E. hirae* –ի թաղանթային բշտիկներում հասանելի SH-խմբերի քանակն և H^+/K^+-ական փոխանակությունը խթանվում է ԱԵՖ-ով կամ ՆԱԴ/ՆԱԴH-ով: Թիոլային խմբերի աճ չի դիտվում ԱԵՖազային *atp* MS116 (F_1-ի β ենթամիավորի խախտումով) մուտանտում և արգելակիչների՝ ԴՑԿՂ և NaN_3-ի ներմուծման դեպքում, ինչպես նաև K^+-ի բացակայության դեպքում: Կրկին, ԴԹԹ-ն վերականգնում է փեղեքչի ազդեցությունը: Արդյունքները վկայում են, որ F_0F_1-ԱԵՖազը գործում է K^+ կլանման համակարգի հետ սերտ կապի ներքո, և այդ 2 սպիտակուցային համալիրների միջև առկա է ՆԱԴ/ՆԱԴH-ով կարգավորվող երկթիոլ-երկսուլֆիդային անցումներ: Սա կարող է լինել ԱԵՖ-ի էներգիան F_0F_1-ԱԵՖազից K^+ կլանման համակարգ տեղափոխման միջոց:

Ցույց է տրվել, որ ածխածնի աղբյուրից կախված դիտվում է ՕՎՊ-ի փոփոխված կինետիկա. Չնայած գլիցերոլի խմորման ընթացքում pH-ի անկումը չնչին է, այնուամենայնիվ գլիցերոլի խմորման ընթացքում առավել վերականգնողական գործընթացներ են դիտվում, որը մատնանշում է ՕՎՊ-ի առավել բացասական արժեքների էական դերը *E. coli* աճի և H_2 արտադրության համար: Ավելին, վերականգնիչները, ստեղծելով ՕՎՊ-ի բացասական արժեքներ, էապես խթանում են H_2–ի արտադրությունը:

Ինչպես նշվեց, *E. coli*- ի 4 Հիդ ֆերմենտները միասին կարող են թաղանթով ջրածնային շրջապտույտ ստեղծել (Sawers and Trchounian, 2014): Շրջապտույտի խանգարումը երկու և առավել Հիդ ֆերմենտների բացակայության պատճառով կարող է տեղեկատվական լինել: *E. coli* –ի տարբեր Հիդ-ային եզակի, կրկնակի կամ եռակի մուտանտների օգնությամբ պարզվել, որ ի տարբերություն Հիդ-2-ի, Հիդ-1-ը ակտիվություն է դրսևորում օքսիդիչի առկայությամբ pH 5.5 և 7.5-ում և կարող է կարգավորվել վերականգնիչով: Այնուամենայնիվ, ԴԹԹ-ի կողմից Հիդ-1 գործունեության կարգավորումը դեռևս պարզաբանման կարիք ունի: Արդյունքները ցույց տվել նաև Հիդ-2- ի դերը F_0F_1-ԱԵՖազի գործունեության մեջ և դրա ԴԹԹ-ով կարգավորման հնարավորությունը: Գլյուկոզի խմորման ընթացքում Հիդ-1 և Հիդ-2 կրկնակի մուտանտում, հիմնային pH-ում դիտվել է ջրածնի ելքի ավելացում, որը զգալիորեն խթանվել է ԴԹԹ-ի առկայությամբ: Այսպիսով, ուսումնասիրությունների

ընթացքում ցույց է տրվել, որ *E. coli*- ում H_2-ի արտադրությունը գլյուկոզի և գլիցերոլի խմորման ընթացքում կարգավորվում է ՕՎՊ-ի վերականգնողական պայմանների միջոցով, որոնք անհրաժեշտ են կենսաջրածնի արտադրության համար: Ցույց է տրվել տարբեր Հիդ-ների դերը արտաքին մրջնաթթվի, որպես խմորման վերջնանյութի, առանձին և գլիցերոլի հետ համատեղ ազդեցության պայմաններում *E. coli* վայրի տիպի և Հիդ-ների կատալիզային ենթամիավորների խախատումներով մուտանտների օգնությամբ, տարբեր pH- ներում (5.5-7.5) և պարզվել են այն պայմանները, երբ մրջնաթթուն միայնակ կամ գլիցերոլի հետ մեկտեղ խթանող ազդեցություն է ունեցել մանրէների աճի և H_2 արտադրության վրա: Ցույց է տրվել նաև գլիցերոլի խմորման ևս մեկ այլ՝ հիմնական արգասիքի՝ էթանոլի ցածր խտությունների ազդեցությամբ *E. coli*-ի աճի և H_2-ի արտադրության կարգավորումը:

Աշխատանքում որոշվել են *E. coli* բակտերիաների աճի և H_2-ի արտադրության խթանման համար որոշ մետաղների (Ni, Fe, Mo) համադրությունները գլիցերոլի և գլյուկոզի խմորման պայմաններում: Ցույց է տրվել *E. coli* –ի *ΔhyaBΔhybC* կրկնակի մուտացիա կրող շտամներում խթանված H_2-ի ելքը: Համադրելով ստացված արդյունքները՝ առաջարկվում է H_2-ի խթանված արտադրություն համար նոր տեխնոլոգիական մոտեցում: Ուսումնասիրվել է լիգնոցելյուլոզային շաքարի՝ քսիլոզի յուրացումը *E. coli*-ում, որոշվել H_2-ի արտադրության համար պատասխանատու Հիդ-ները, ինչպես նաև քսիլոզի և գլիցերոլի բարձր բուֆերայնությամբ միջավայրում երկարատև H_2-ի արտադրության հնարավորությունը: Իրականցվել է գարեջրի արտադադրության և թղթի թափոնների ֆիզիկաքիմիական մշակում, մանրէների կենսազանգվածի և H_2-ի արտադրության օպտիմալացում: Մեր հաշվարկները ենթադրում են գարջրի աճիկի հիդրոլիզատի (ԳԱՀ-ի) կիրառմամբ ցածրարժեք H_2-ի արտադրության հնարավորությունը: Ավելին, ցույց է տրվել Հիդ ֆերմենտների սինթեզը ԳԱՀ-ի վրա *E. coli*-ի և *R. eutropha* –ի աճման ընթացքում: Ինչպես նշվեց, ֆերմենտների սինթեզը և ակտիվությունը հիմնականում կախված են միջավայրի տարբեր գործոններից: Աշխատանքում առաջին անգամ իրականցվել է *R. eutropha*-ի մի շարք կենսաէնեգատիկական պարամետրերի, ՕՎՊ-ի կինետիկայի ուսումնասիրություններ տարբեր թափոնների առկայության և բացակայության պայմաններում:

Առաջին անգամ ցույց է տրվել բակտերիաների աճ և Հիդ ֆերմենտների սինթեզը ԳԱՀ-ի յուրացման պայմաններում: Ավելին, ՕՎՊ-ի ցածր՝ բացասական արժեքները զգալի խթանել են բջիջներում Հիդ-ների H_2-օքսիդացնող ակտիվությունը: Հատկանշանական է, որ բարձր H_2 - օքսիդացնող Հիդ-ային ակտիվություն է դիտվել գլիցերոլի խմորման դեպքում. *R. eutropha*-ի ԹՀ-ը և *E. coli*-ի ամբողջական բջիջները անշարժացվել են սենսորների վրա և դրանց արդյունավետությունը փորձարկվել է որպես անոդային կատալիզատորներ՝ տարբեր ռեդօքս միջնորդանյութերի կիրառմամբ: Ցույց է տրվել ֆերրոցեն կարբօքսիլաթթվի խթանիչ ազդեցությունը էլեկտրոնի անոդային փոխանցման և հոսանքի գեներացման մեջ: Ավելին, հիմնվելով ատացված արդյունքերի վրա, ՀՀ ԳԱԱ Կենսաքիմիայի ինստիտուտի գիտաշխատողների համագործակցության միջոցով, հնարավոր եղավ ստեղծել ջրաածնային (H_2) վառելիքային սարք վոլտաչափ (ՋՎՍՎ)՝ Վառելիքային սարքի պրոտոտիպ: Այն կարող է աշխատել փորձարկվող նմուշների միկրոհաղորդիչներով, ինչպես նաև այն ունի առանձնացված արտաքին ազդանշանային մուտք՝ H_2-ային ՎՍ համակարգի համար:

Այսպիսով, ցածրարժեք և մատչելի ածխածնի աղբյուրները, օրինակ, տարբեր օրգանական թափոնները կարող են օգտագործվել մանրէների կողմից կենսազանգվածի և Հիդ-ների արտադրության համար, որոնք հետագայում ուղղվելու են էլեկտրաէներգիայի արտադրության համար:

ПОЛАДЯН АННА АРШАКОВНА

ОКИСЛИТЕЛЬНО ВОССТАНОВИТЕЛЬНАЯ РЕГУЛЯЦИЯ КОНЕЧНЫХ ЭТАПОВ БРОЖЕНИЯ И ВОЗМОЖНОСТЬ ЕГО ПРИМЕНЕНИЯ ПРИ ПЕРЕРАБОТКЕ ОРГАНИЧЕСКИХ ОТХОДОВ

РЕЗЮМЕ

Ключевые слова: *Escherichia coli*, *Enterococcus hirae, Ralstonia eutropha,* белок-белковое взаимодействие, конечные этапы брожения, биоводород, окислительно-восстановительная регуляция, гидрогеназы (Гид), обработка органических отходов

В экспериментальном подходе с использованием тиоловых реагентов (сукцинимидил-6 [(β-малеимид-пропионовый амид) гексаноат] (SMPH), реагент Эллмана), муравьиной кислоты и ионов калия с помощью мутантов F_oF_1-АТРазы выявлена существенная роль SH-групп в переносе энергии. Полученные результаты позволяют предположить, что низкие значения ОВП компенсируют негативное действие протонофора на *E. hirae*. Было исследовано окислительно-восстановительное регулирование протонно-калиевого обмена в *E. hirae* и роль тиоловых групп: феррицианид замедляет подкисление среды и поглощение K^+, тогда как ДТТ стимулирует протон-калиевый обмен. Более того, поглощение K^+ подавляется протонофорами, однако ДТТ восстанавливает ДЦКД-чувствительное поглощение K^+. Кроме того, количество доступных SH-групп в мембранных везикулах *E. hirae* стимулируется АТФ или $НАД^+$/НАДН. Также наблюдается протон-калиевый обмен в зависимости от АТФ и $НАД^+$/НАДН. У мутантов АТФазы не наблюдается увеличения тиоловых групп (не было зарегистрировано изменений у мутантов *atp* MS116 с нефункциональной F_oF_1-АТФазой (с неактивной β-субъединицей F1)) в присутствии ингибиторов ДЦКД и азида натрия, а также в отсутствие ионов калия. ДТТ восстанавливает действие протонофора. Очевидно, это явление связано с работой F_oF_1-АТФазы.

Было показано, что изменение кинетики ОВП зависит от источника углерода: снижение pH при сбраживании глицерола незначительно из-за продуктов брожения, так же наблюдаются больше восстановительных процессов (в сравнении с глюкозой), что указывает на важность восстановительных условий (отрицательные значения ОВП среды) для роста *E. coli* и выделения H_2. Более того, восстановители значительно увеличивают выделение H_2, создавая отрицательные значения ОВП. Как уже упоминалось, 4 гидрогеназных ферментов (Гид) вместе могут образовывать водородный цикл в мембране *E. coli* (Sawers and Trchounian, 2014). Информативным может быть нарушение этого цикла из-за отсутствия двух и более Гид ферментов. С помощью гидрогеназных мутантов *E. coli* (одиночные, двойные или тройные мутанты) было обнаружено, что в отличие от Гид-2, Гид-1 (отсутствуют большие субъединицы гидрогеназы-1 и гидрогеназы-2, соответственно) проявляет активность в присутствии окислителя (положительные значения ОВП среды) при pH 5.5 и 7.5, и может регулироваться восстановителем (отрицательные значения ОВП). Однако регуляция активности Гид-1 с помощью ДТТ нуждается в уточнении. Результаты также показали роль Гид-2 в функционировании F_oF_1-АТФазы и возможности ее регуляции с помощью ДТТ. При сбраживании глюкозы в двойных мутантах Гид-1 и Гид-2 наблюдалось увеличение выделение водорода при щелочном pH, которое значительно увеличивалось в присутствии ДТТ.

Таким образом, исследования показали, что производство H_2 в *E. coli* при сбраживании глюкозы и глицерола регулируется условиями восстановления ОВП, что и необходимо для производства биоводорода. Роль различных Гид ферментов дикого вида *E. coli* и Гид-ых мутантов была продемонстрирована в присутствии только муравьиной кислоты в качестве конечного продукта брожения или в сочетании с глицерином, при различных pH.

Были обнаружены условия, когда только формиат или его комбинация с глицерином, или этанол с глицерином, или ионы металлов (Ni, Fe) оказывали стимулирующее действие на рост бактерий и выделение H_2. Была предложена возможность использования смешанных источников углерода – ксилозы и глицерола, и была определена роль буферной способности среды для долгосрочного улучшения производства водорода.

Проведена физико-химическая обработка бумажных и пивоваренных отходов и оптимизация их гидролизатов. Идентифицированы Гид-ферменты *E. coli*, ответственные за образование водорода при утилизации отработанных гидролизатов отходов. Показан повышенный выход H_2 у двойных мутантных штаммов *E. coli ΔhyaBΔhybC*. Кроме того, был продемонстрирован синтез ферментов Гид во время роста *E. coli* и *R. eutropha* при утилизации пивоваренных отходов. Синтез и активность Гид в основном зависят от внешних факторов. *R. eutropha* H16 демонстрирует максимальный гетеротрофный рост в присутствии нескольких органических субстратов, но эти субстраты не гарантируют благоприятных условий для синтеза Гид.

В данной работе были проведены исследования ряда биоэнергетических характеристик и кинетики ОВП бактерии *R. eutropha* при утилизации различных отходов. Впервые показан рост бактерий и синтез ферментов Гид при утилизации пивоваренных отходов. Более того, ДТТ-индуцированный ОВП значительно стимулировал H_2-окислительную активность Гид ферментов. Примечательно, что при сбраживании глицерина наблюдалась высокая H_2-окислительная активность Гид-ов. Мембранносвязанная гидрогеназа *R. eutropha* и целые клетки *E. coli* были иммобилизованы на сенсорах, и их эффективность в качестве анодных катализаторов была протестирована с использованием различных окислительно-восстановительных медиаторов. Было продемонстрировано стимулирующее действие ферроцена карбоновой кислоты на перенос электронов на анод и генерацию тока. Более того, на основе полученных результатов при сотрудничестве с сотрудниками Института биохимии НАН Армении удалось создать водородный (H_2) вольтметр топливных элементов (ВВТЭ). ВВТЭ может работать с передатчиками образцов для испытаний и имеет отдельный вход для внешнего сигнала для системы H_2 ТЭ.

Таким образом, недорогие и доступные источники углерода, такие как различные органические отходы, могут использоваться бактериями для получения биомассы и Гид ферментов, которые позже будут использоваться для производтвая электроэнергии.