ՀՀ ԿՐԹՈՒԹՅԱՆ ԵՎ ԳԻՏՈՒԹՅԱՆ ՆԱԽԱՐԱՐՈՒԹՅՈՒՆ
ԵՐԵՎԱՆԻ ՊԵՏԱԿԱՆ ՀԱՄԱԼՍԱՐԱՆ

ԹՌՉՈՒՆՅԱՆ ԿԱՐԵՆ ԱՐՄԵՆԻ

ՄԹՆԱՅԻՆ ԽՄՈՐՈՒՄ ԻՐԱԿԱՆԱՑՆՈՂ ԲԱԿՏԵՐԻԱՆԵՐՈՒՄ ՄՈԼԵԿՈՒԼԱՅԻՆ ՋՐԱԾՆԻ ԱՐՏԱԴՐՈՒԹՅԱՆ ԵՎ ՀԻԴՐՈԳԵՆԱԶԱՅԻՆ ԱԿՏԻՎՈՒԹՅԱՆ ԽԹԱՆՄԱՆ ՈՒՂԻՆԵՐԸ

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

ՍԵՂՄԱԳԻՐ

Երևան 2016

MINISTRY OF EDUCATION AND SCIENCE OF RA
YEREVAN STATE UNIVERSITY

TRCHOUNIAN KAREN ARMEN

IMPROVING ROUTES OF MOLECULAR HYDROGEN PRODUCTION AND HYDROGENASE ACTIVITY IN BACTERIA DURING DARK FERMENTATION

S Y N O P S I S

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

Yerevan 2016

Ատենախոսության թեման հաստատվել է Երևանի պետական համալսարանում

Գիտական խորհրդատու՝	ՀՀ ԳԱԱ թղթակից անդամ, կենս. գիտ. դոկտոր, պրոֆեսոր Ա. Հ. Թռչունյան
Պաշտոնական ընդդիմախոսներ՝	ՀՀ ԳԱԱ թղթակից անդամ, կենս. գիտ. դոկտոր, պրոֆեսոր Ժ. Ի. Հակոբյան
	ՀՀ ԳԱԱ թղթակից անդամ, գյուղ. գիտ. դոկտոր, պրոֆեսոր Ս. Խ. Մայրապետյան
	կենս. գիտ. դոկտոր, պրոֆեսոր Հ.Ռ. Վարդապետյան
Առաջատար կազմակերպություն՝	ՀՀ ԳԱԱ Հ. Բունիաթյանի անվան կենսաքիմիայի ինստիտուտ

Ատենախոսության պաշտպանությունը տեղի կունենա 2017թ. փետրվարի 10-ին, ժամը 14:00-ին, Երևանի պետական համալսարանում գործող ՀՀ ԲՈՀ-ի Կենսաֆիզիկայի 051 մասնագիտական խորհրդի նիստում (0025, ք. Երևան, Ալեք Մանուկյան փ. 1, ԵՊՀ, Կենսաբանության ֆակուլտետ):
Ատենախոսությանը կարելի է ծանոթանալ Երևանի պետական համալսարանի գրադարանում:
Ատենախոսության սեղմագիրն առաքված է 2016թ. դեկտեմբերի 22-ին:

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

The theme of the dissertation has been approved at Yerevan State University

Academic consultant:	Corresponding Member of NAS RA, Doctor of Biological Sciences, Professor A. Trchounian
Official opponents:	Corresponding Member of NAS RA, Doctor of Biological Sciences, Professor J. Akopian
	Corresponding Member of NAS RA, Doctor of Agricultural Sciences, Professor S. Mayrapetyan
	Doctor of Biological Sciences, Professor H. Vardapetyan
Leading organization:	H. Buniatian Institute of Biochemistry of NAS of RA

The defense of the dissertation will be held on 10th February, 2017, at 14:00, at the session of 051 Scientific Specialized Council on Biophysics of HAC of RA at Yerevan State University (0025 Yerevan, 1 Alex Manoogian str., YSU, Faculty of Biology).
The dissertation is available at the library of Yerevan State University.
The synopsis has been sent on 22nd December, 2016.

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

INTRODUCTION

Topic relevance. It is known that the fossil fuels, especially oil and gas, stocks are decreasing, and it is very important to find alternative and renewable energy sources. Molecular hydrogen or dihydrogen (H_2) as efficient, alternative and renewable energy source has high impact of interest because from its burning high amount of energy (~142 kJ/g) is released; it is ecologically clean; when burning H_2 only water is formed as a side product [Momirlan & Veziroglu, 2005; Trchounian & Trchounian, 2015; Hosseini & Wahid, 2016]. Much investigation is on-going for developing and enhancing H_2 production and to introduce and use this type of fuel in different branches of economy, especially in H_2 fuel cells, in cars (Toyota Mirai first commercial car using H_2), portable generators etc. It is necessary to understand which substrates are needed for developing and processing cheap and efficient H_2 production technology.

H_2 can be produced by different ways: chemical – from water or carbohydrates electrolysis or by their heating. But the chemical way of producing H_2 is complex, inefficient and not profitable. Therefore, H_2 production from bacteria utilizing various substrates (glucose, glycerol, formate) during dark or photo-fermentation is widely spread. Already the possible use of various agricultural or industrial wastes is developed.

The H_2 formation process in bacteria is catalyzed by reversible enzymes - hydrogenases (Hyd) [Sawers, 1994; Trchounian et al., 2012; Peters et al., 2015; Flanagan & Parkin, 2016; Ogata et al., 2016]. Hyd enzymes have been identified and described in 1930s and determined in many prokaryotes and some eukaryotes [Thauer, 1998; Trchounian & Trchounian, 2015; Sargent, 2016]. Importantly, *Escherichia coli* encodes four membrane-bound [Ni-Fe] Hyd enzymes [Kim & Kim, 2011; Trchounian et al., 2012; Peters et al., 2015; Trchounian & Trchounian, 2015; Sargent, 2016]. It is supposed that in different environmental conditions at least one of them or two are working towards H_2 production direction and at least one or two rest Hyd enzymes in H_2 uptake direction. Such operating directions of Hyd enzymes in the cell make new **H_2 cycle**. Some Hyd enzymes can form formate hydrogen lyase complexes (FHL) [Trchounian et al., 2012]. But the structure, biosynthesis, activity, genetic regulation and working mechanisms of various enzymes are not clear yet. By defining and describing the regulation of the activity and working mechanisms of Hyd enzymes, they might be efficiently applied in H_2 production biotechnology. The basis of these is the high H_2 producing strains constructed by the genetic and metabolic engineering methods [Maeda et al., 2008; Hu & Wood, 2010; Tran et al., 2014].

In 2006, it has been discovered that glycerol can be anaerobically utilized or fermented by *E. coli* [Dharmadi et al., 2006; Gonzalez et al., 2008]. This finding has interest not only for basic studies but also from economical point of view, as glycerol, compared to sugars, is very cheap and readily available in huge amounts [Trchounian & Trchounian, 2015; Anitha et al., 2016; Garlapi et al., 2016]. It has been shown that during dark fermentation at pH 6.3 among various fermentation end products H_2 gas is also generated. But it has negative impact on the cell growth and glycerol fermentation.

The use of glycerol for fermentation might have enormous application in H_2 production biotechnology by reaching to industrial scales [Khanna et al., 2012; Clomburg & Gonzalez, 2013; Mattam et al., 2013; Trchounian & Trchounian, 2015]. The differentiation of Hyd enzymes, determination of their activity and revelation of working mechanisms at various conditions during glycerol fermentation would have big impact. In H_2 and other various compounds production biotechnology new approach is applied by investigating different substrates and their mixtures (glycerol+glucose, glycerol+formate, glycerol+acetate etc.).

Research goals and tasks. The main goal was dedicated to the investigation of H_2 production and hydrogenase activity and their improved routes by *E. coli* and other bacteria during dark fermentation of various carbon sources and their mixtures.

Constituted tasks of the research were to:

- reveal the growth of *E. coli* and H_2 production in the presence of various carbon sources, especially glycerol and their mixtures;
- construct *E. coli* mutants with defects in Hyd enzymes, transcriptional factors and determine in the whole cells - Hyd enzymes responsible for H_2 production, in cell extracts – Hyd activity;
- clarify interaction between *E. coli* Hyd enzymes during dark fermentation of various carbon sources;
- investigate the effects of different external factors, among them – pH, osmotic stress, concentrations of carbon sources, various inhibitors, on *E. coli* H_2 production;
- determine proton motive force generated by *E. coli* during glycerol fermentation and ascertain the relationship between H_2 production, hydrogenases and proton F_oF_1-ATPase activities;
- reveal the growth of *C. beijerinckii* and H_2 production in the presence of various carbon sources, especially glycerol, and determine Hyd activity and formate dehydrogenase activity in cell extracts;

- investigate the effects of different factors, among them – pH, concentrations of carbon sources, various inhibitors and some heavy metal ions on *C. beijerinckii* H_2 production;
- develop improved routes of H_2 production in bacteria by selection of appropriate mutants, various carbon sources and their mixtures, optimization of technological conditions.

Topic scientific novelty and applied value. During the conducted research it has been revealed which Hyd is (are) responsible for H_2 production during dark fermentation of glycerol, as a cheap substrate, and various mixtures of carbon sources. This was determined with mutants lacking different Hyd enzymes in *E. coli* during glycerol fermentation. In contrast to glucose fermentation, It was established that at slightly alkaline pH mainly Hyd-2 and to some extent Hyd-1 are responsible for H_2 production. It was suggested that Hyd-1 is related to proton F_0F_1-ATPase. At slightly acidic and acidic pHs FHL-1 complex, where Hyd-3 is one of the components of FHL-1, is responsible for H_2 production. H_2 cycling via multiple Hyd enzymes is proposed. It was shown significant changes in Hyd activities during glycerol or glucose fermentation depending on pH, osmotic pressure and inhibitors. The bioenergetics of glycerol fermentation was described.

H_2 production by *C. beijerinckii* was revealed during dark fermentation of glycerol, various carbon sources and their mixtures. It had been shown the peculiarities of Hyd activity during fermentation of glycerol, various carbon sources and their mixtures depending on pH, various inhibitors and some heavy metals ions.

It has been suggested new improved routes for H_2 production during dark fermentation of glycerol, various carbon sources and their mixtures in bacteria.

The results obtained has significant impact on improvement of H_2 production biotechnology and widening the use of glycerol.

Main points to present at defense.

1. *E. coli* and *C. beijerinckii* produce H_2 during glycerol fermentation at slightly alkaline pH; all *E. coli* Hyd enzymes are reversible and mainly Hyd-2 is responsible for H_2 production.
2. In bacteria Hyd activity depends on type of carbon source and its concentration, mixture of carbon sources, pH, osmotic stress and various inhibitors, different metal ions and their concentrations.
3. The proton motive force during glycerol fermentation, in contrast to glucose fermentation, is low. Hyd enzymes have impact on proton motive force generation.

4. The improved H_2 production in bacteria occurs with the choice of appropriate mutants, carbon sources and their mixtures, pH, osmotic stress and some metal ions and their concentrations conditions.

Work approbation. Main results of the dissertation were discussed at seminars in Department of Biochemistry, Microbiology and Biotechnology, Biology Faculty of Yerevan State University (Armenia), Institute of Biology/Microbiology Martin-Luther University of Halle-Wittenberg (Germany), Department of Microbial Ecology, Limnology and General Microbiology University of Konstanz (Germany), Department of Biochemistry and Molecular Cell Biology University of Georgia, Athens, (USA), Institute of Biosciences and Bioengineering, Rice University in Houston, Texas (USA) and presented at different scientific congresses and meetings, namely 11th International Hydrogenase Conference, Marseille, France (2016), 21st World Hydrogen Energy Congress, Zaragoza, Spain (2016), 116th General Meeting of American Society for Microbiology (ASM) in Boston, USA (2016); 115th General Meeting of ASM in New Orleans, USA (2015); 6th World Congress on Biotechnology, New Delhi, India (2015); 6th FEMS Congress in Maastricht, Netherlands (2015); BIT's 5th Annual World Congress of Molecular and Cell Biology, Nanjing, China (2015); 114th General Meeting of ASM in Boston, USA (2014); 39th FEBS Congress in Paris, France (2014); 18th EBEC Congress in Lisbon, Portugal (2014); 18th International Biophysics Congress in Brisbane, Australia (2014); 38th FEBS Congress in St. Petersburg, Russia (2013); etc. in total more than 20 seminars and 25 scientific congresses and meetings.

Publications. Based on experimentally obtained data 57 articles, papers and thesis, including 29 full papers in international peer-reviewed journals and books were published.

Volume and structure of dissertation. Dissertation contains introduction, literature review (Chapter 1), experimental part (Chapter 2), results and discussion (Chapter 3), conclusions and cited references (total 300 papers and books). The dissertation consists of 230 pages, contains 11 tables and 84 graphs and figures.

MATERIALS AND METHODS

Bacteria: In experiments *E. coli* BW25113 or MC4100 wild type (wt) strains and appropriate mutants were used (Table 1). Strains with multiple deletions were generally constructed by introduction of mutations from *E. coli* donor strains into recipient strains of BW25113 by P1*kc* phage transduction according to Miller [Miller, 1972].

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

Strain	Genotype	Absent or defective gene product	Reference
BW 25113	*lacI^q rrnB$_{T14}$ ΔlacZ$_{W116}$ hsdR514 ΔaraBAD$_{AH33}$ Δrha BAD$_{LD78}$*	Wild type	[Maeda et al., 2007]
JW 0955*	BW 25113 *ΔhyaB*	Large subunit of Hyd-1	[Baba et al., 2006]
JW 2472*	BW 25113 *ΔhyfG*	Large subunit of Hyd-4	[Baba et al., 2006]
JW 2701*	BW 25113 *ΔfhlA*	FHL activator	[Maeda et al., 2007]
JW 2962*	BW 25113 *ΔhybC*	Large subunit of Hyd-2	[Maeda et al., 2007]
MW 1000	BW 25113 *ΔhyaB ΔhybC*	Large subunits of Hyd-1 and Hyd-2	[Maeda et al., 2007]
SW1001*	BW 25113 *ΔhyfG ΔfhlA*	FHL activator and large subunit of Hyd-4	[Trchounian et al., 2011]
SW1002*	BW 25113 *ΔhycG ΔfhlA*	FHL activator and large subunit of Hyd-3	[Trchounian et al., 2011]
KT 2110	BW 25113 *ΔhyaB ΔhybC ΔselC*	Large subunits of Hyd-1, Hyd-2 and tRNA^{sec}	[Trchounian et al., 2012]
JW 0887	BW 25113 *ΔfocA*	Formate channel	[Baba et al., 2006]
JW 2477	BW 25113 *ΔfocB*	Formate channel	[Baba et al., 2006]
MC 4100	*araD139 ΔlacU169 rpsL thi fla*	Wild type	[Bagramyan et al., 2002]
JRG 3615**	MC 4100 *Δ(hyfA-B)*	Subunits of Hyd-4	[Bagramyan et al., 2002]
JRG 3621**	MC 4100 *Δ(hyfB-R)*	Subunits of Hyd-4	[Bagramyan et al.,

			2002]
HDK 103	MC 4100 *Δhya Δhyc*	Hyd-1 and Hyd-3	[Trchounian et al., 2013]
HDK 203	MC 4100 *Δhyb Δhyc*	Hyd-2 and Hyd-3	[Trchounian et al., 2013]
FM 460*	MC 4100 *ΔselC*	$tRNA^{sec}$	[Soboh et al., 2011]
FTD 147	MC 4100 *ΔhyaB ΔhybC ΔhycE*	Large subunits of Hyd-1, Hyd-2 and Hyd-3	[Trchounian et al., 2012]
FTD 150	MC 4100 *ΔhyaB ΔhybC ΔhycE ΔhyfG*	Large subunits of Hyd-1, Hyd-2, Hyd-3 and Hyd-4	[Trchounian et al., 2012]
DHP-F2	MC4100 *ΔhypF*	Maturation of all hydrogenases	[Trchounian et al., 2012]
DK8	*bglR thill rell Δ(uncB-uncC) ilv::Tn 10*	F_0F_1-ATPase	[Trchounian et al., 2011]

* Resistant to Kanamycin

** Resistant to Spectinomycin

Clostridium beijerinckii DSM791 type strain (identical with ATCC 25752) was obtained from DSMZ, Germany.

Bacterial growth and preparation for assays. *E. coli* was cultivated at 37°C for 18-22 h in anaerobic conditions by direct transfer from nutrient agar surface in Petri dish into high buffered liquid peptone growth medium containing peptone 20 g/l, K_2HPO_4 15 g/l, KH_2PO_4 1 g/l, NaCl 5 g/l, pH 7.5; peptone 20 g/l, K_2HPO_4 7.5 g/l, KH_2PO_4 8.6 g/l, NaCl 5 g/l, pH 6.5; peptone 20 g/l, K_2HPO_4 1 g/l, KH_2PO_4 15 g/l, NaCl 5 g/l, pH 5.5. 2 g/l (11 mM) glucose and/or 10 g/l (110 mM) glycerol and/or 0.68 g/l (10 mM) formate was added. Kanamycin (25 μg/ml final concentration) was added where appropriate (see Table 1). In some experiments bacteria were incubated for 5-7 minutes with N,N′-dicyclohexylcarbodiimide (DCCD) or washed with 0.8 M sucrose for the following hypo-osmotic stress. For *C. beijerinckii* the following medium was used: yeast extract 0.5 g/l, K_2HPO_4 1.5 g/l, KH_2PO_4 1.5 g/l, $MgSO_4$ x 7 H_2O 492 mg/l, L-cystein 500 mg/l, $MnSO_4$ x H_2O 15 mg/l, $FeSO_4$ x 7 H_2O 20 mg/l, resazurin 1 mg/l, p-aminobenzoic acid 2 mg/l, thiamine-HCl 2 mg/l, biotin 0.4 mg/l, pH was adjusted to pH 7.5. In the medium 10 g/l glycerol (110 mM) and/or 7.2 g/l (40 mM) glucose and/or 0.68 g/l (10 mM) formate was added.

Preparation of cell extracts. Harvested *E. coli* cells were washed in MOPS-buffer (50 mM MOPS at either, pH 5.5, 6.5 or 7.5) by centrifugation and the cell pellet was re-suspended typically in three volumes of 50 mM MOPS buffer, pH 7.5, including 5 µg DNase/ml and 0.2 mM phenylmethylsulfonyl fluoride. 1-2 g wet weight of cells were disrupted by sonication (30 W power for 5 min with 0.5 s pulses). Unbroken cells and cell debris were removed by centrifugation for 30 min at 50,000 x g and at 4^0C. *Clostridium* cultures were harvested in an anoxic chamber by centrifugation in anoxic polypropylene centrifuge bottles at 16,270 x g for 10 min at 4°C. Cells were washed and re-suspended in anoxic phosphate buffer (50 mM, pH 7.5). The pellet was re-suspended in 5 ml of washing buffer, and the cells were broken by repeated passage through a cooled French-pressure cell at 137 MPa under anoxic conditions. Protein concentration was determined by Lowry or Bradford methods [Lowry et al., 1951; Bradford, 1976] with bovine serum albumin, as standard.

Determination of total hydrogenase enzyme activity and formate dehydrogenase activity. Hyd enzyme activity (H_2-dependent reduction of benzyl viologen (BV)) was determined according to [Ballantine & Boxer, 1985] except that the buffer used was 50 mM MOPS, pH 7.0. The wavelength used was 578 nm and an E_M value of 8,600 M^{-1} cm^{-1} was assumed for reduced BV. One unit of activity corresponded to the reduction of 1 µmol of hydrogen per min. Formate dehydrogenase enzyme activity was measured with BV according to [Müller et al., 2008]. The reaction was initiated by adding 25 mM sodium formate

Non-denaturing polyacrylamide gel electrophoresis (PAGE) and in-gel hydrogenase activity staining. Non-denaturing PAGE was performed using 7.5% (w/v) polyacrylamide gels, pH 8.5 and included 0.1% (v/v) Triton X-100 in the gels and Hyd activity-staining was done as described [Ballantine & Boxer, 1985] except the buffer used was 50 mM MOPS pH 7.0.

Determination of proton motive force. Δp was calculated as a sum of $\Delta\varphi$ and ΔpH according to $\Delta\mu_{H^+}/F=\Delta\varphi-Z\Delta pH$ (negative value in mV) [Skulachev et al., 2010], where Z is RT/F equal 61.1 mV at 37^0C. $\Delta\varphi$ was measured determining tetraphenylphosphonium cation (TPP^+) distribution between the bacterial cytoplasm and the external medium at a steady-state level as described [Zakharyan & Trchounian, 2001]. The assay was done in a thermo-stated vessel of 2 ml with 150 mM Tris-HCl buffer containing 1 µM TPP^+. The changes in the TPP^+ concentration were determined by using a TPP^+-selective electrode. The pH_{in} was determined by the distribution of weak base - 9-aminoacridine (9-AA) across the membrane according to [Puchkov et al., 1983]. The 9-AA fluorescence was

measured with a spectrofluorimeter (SPEX Fluoro Max, France) with excitation at 324 nm and emission at 451 nm.

Determination of redox potential and H_2 production. Redox potential (E_h) in bacterial suspension was measured using the redox, a titanium-silicate (Ti-Si) and platinum (Pt) electrodes [Trchounian et al., 2011]. In contrast to Ti-Si-electrode measuring the overall E_h, a Pt electrode is sensitive to H_2 under anaerobic conditions (in the absence of O_2) allowing detection of H_2. H_2 production rate (V_{H2}) was calculated through the difference between the initial rates of decrease in Pt- and Ti-Si-electrode readings per time and expressed as mV of E_h or mmol H_2 per min per mg dry weight of bacteria [Trchounian et al., 2011; Trchounian, 2012]. H_2 production was also determined by the chemical assay [Maeda & Wood, 2008] and Durham tube method [Bagramyan et al., 2002]. Dry weight of bacteria was determined, as described [Trchounian & Vassilian, 1994].

Analytical methods. Gas samples were taken from the headspace of sealed serum bottles and H_2 gas production was analyzed by gas chromatography (GC) using a thermal conductivity detector; the carrier gas was nitrogen. H_2 production rate was calculated as produced H_2 in mmol/per hour/per g cell mass (mmol/h/g cell mass). Organic compounds such as glucose, glycerol, ethanol, butanol, acetate etc. were measured by high-performance liquid chromatography (HPLC). Samples and standards were prepared by acidification with H_2SO_4 to a final concentration of 91 mM followed by centrifugation for 5 min at 16,100 x g to remove cells. The supernatant was used for analysis and applied to the HPLC system with a 234 auto-injector. Samples were separated at 60°C with an Aminex HPX-87H ion-exchange resin using an isocratic mobile phase of 5 mM H_2SO_4 at a flow rate of 0.6 ml/min provided by a LC-10AT vp pump. Organic compounds were detected with a refractive index detector RID-10A, and the detector signal was recorded with the Shimadzu LC solution software.

Data processing. The average data are represented from at least three independent experiments; the standard errors were calculated using Microsoft Excel 2010 and Student criteria (P) were employed to validate the difference in average data between different series of experiments, as described [Trchounian et al., 2011; Gabrielyan et al., 2014]; the difference was valid if $P < 0.01$, if not valid will be mentioned in the text.

RESULTS AND DISCUSSION

H_2 production by *E. coli* during dark fermentation of glycerol and various mixtures of carbon sources with glycerol at different pHs.

Molecular H_2 is one of the end products of mixed-acid fermentation in *E. coli* and other bacteria which can be used and applied in various branches of industry and human life [Dharmadi et al., 2006; Trchounian, 2015]. Gonzalez group [Dharmadi et al., 2006] has discovered that, glycerol like sugars (glucose), can be anaerobically fermented by *E. coli* and generate H_2 at acidic pH (pH 6.3). But glycerol metabolism pathways are not clear. Due to the fact that glycerol is cheap, it could be also used as a source for H_2 production.

E. coli has the ability to encode four Hyd enzymes catalyzing the reaction of H_2 to $2H^+$. Depending on the carbon source and pH Hyd enzymes can operate either in H_2 uptake or production direction [Trchounian & Trchounian, 2009].

Hyd-1 encoded by the *hya* operon is induced under anaerobic conditions at acidic pH [King & Przybyla, 1999]. Hyd-2 encoded by the *hyb* operon [Laurinavichene et al., 2002] reaches its maximal expression at alkaline medium [King & Przybyla, 1999]. Hyd-3 and Hyd-4 encoded by the *hyc* and *hyf* operons, respectively [Trchounian et al., 2012], with formate dehydrogenase H (Fdh-H), form FHL-1 and FHL-2 complexes. FHL-1 produces H_2 mainly at acidic pH. For slightly alkaline pH, FHL-2 becomes responsible for H_2 production [Trchounian et al., 2012].

Note that the activity of Hyd-3 and Hyd-4 is related with the proton F_0F_1-ATPase [Trchounian et al., 2012]. At pH 7.5 it might result from Hyd-4 interaction with F_0F_1 to supply reducing equivalents ($H^+ + e^-$) for energy transfer to the secondary transport system [Trchounian, 2004]. To establish responsible Hyd for H_2 production under the above mentioned conditions, different mutants were constructed. In addition, to understand mechanisms of Hyd activity and regulation as well as relationship with H^+ transport, the inhibitor's effects were determined.

V_{H2} at pH 7.5 was ~2.2 mV E_h/min/mg dry weight (Fig. 1). It was ~2.5 fold lower compared with cells grown on glucose. When *E. coli* cells grew on glycerol at pH 7.5 medium pH decreased with 0.25 unit which was little compared with glucose fermentation where pH dropped pH 7.5 to pH 6.8. This huge distinction may denote lower acidification of the medium due to less formation of different acids or their changed proportion during dark fermentation of glycerol, as supposed [Murarka et al., 2008]. Moreover, V_{H2} was increased ~2-fold (Fig. 1) in *fhlA* mutant. The results suggested that Hyd-3 at least is operating in a H_2 uptake or oxidation direction during glycerol fermentation at pH 7.5. In *hyaB* mutant V_{H2} was lowered ~3-fold and less or only residual in the *hybC* mutant (Fig. 1). In contrast, during glucose fermentation V_{H2} was almost the same for wild type and mutants studied.

These data revealed that during glycerol fermentation Hyd-2 mostly and Hyd-1 is less responsible for H_2 production.

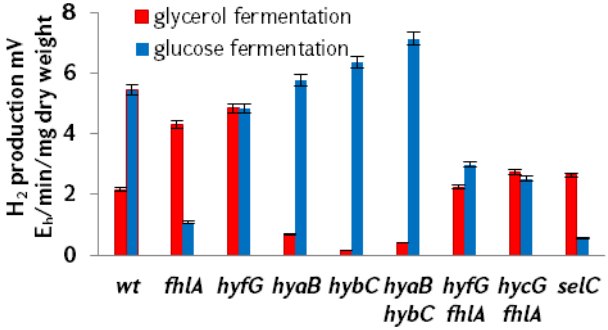


Fig. 1. H_2 production by *E. coli* wt and different mutants defective in Hyd enzymes during glucose or glycerol fermentation at pH 7.5. For others see Materials and Methods.

In addition, the results implied that Hyd-2 can be a reversible enzyme operating in H_2 production direction during glycerol fermentation whereas this enzyme has been shown [Trchounian et al., 2012] to be functioning as H_2 oxidizing one under glucose fermentation.

When *E. coli* cells were grown in the presence of mixture of glycerol and formate in FM460 (*selC*) single mutant H_2 production by *E. coli* decreased ~2 fold suggesting that FDH-H is involved in H_2 production probably supplying reducing equivalents to Hyd-3 or to the other enzymes which are contributing to H_2 evolution (Fig. 2). However, the deletions of genes for three Hyd enzymes in FTD147 (*hyaB hybC hycE*) or KT2110 (*hyaB hybC selC*) mutants resulted in the marked decrease of V_{H2} ~2 fold and ~3.5 fold, respectively, compared to the wild type (Fig. 2). The remaining H_2 seems to be produced by Hyd-4. These data about H_2 producing activity at pH 7.5 are in conformity with the findings obtained previously by Redwood et al. [2008].

The results propose that there are compensatory H_2 oxidizing or producing functions of Hyd enzymes but one Hyd enzyme cannot compensate the other three ones. This causes disturbance of H_2 cycling between different Hyd enzymes where H_2 production already is not favored for the cell. It may be also suggested that each Hyd enzyme cannot function by its own: all Hyd enzymes in *E. coli* are associated each with other to form some assemble and operate together for maintaining H_2 cycling. This idea is novel but it should be ruled out for further study.

At pH 6.5 during mixed carbon fermentation by *E. coli* in glycerol assays the overall situation regarding the study of single or double mutants with defects in Hyd-3 and Hyd-4 was similar to that at pH 7.5. But surprisingly in *selC* mutant no decrease of V_{H2} was detected (Fig. 2). As following, no FDH-H was needed.

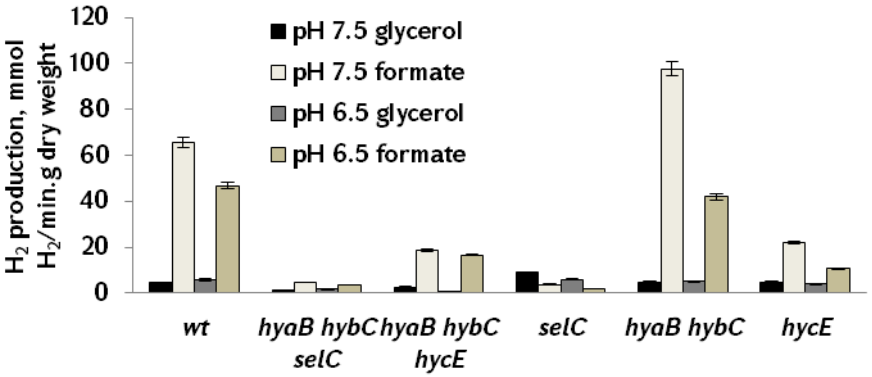


Fig. 2. H_2 production rate (V_{H2}) by *E. coli* wt and mutant strains with defects in FDH-H, Hyd-1, Hyd-2 and Hyd-3 during mixed carbon (glycerol and formate) fermentation in the assays supplemented with glycerol or formate at pH 7.5 and 6.5.

In *hyaB hybC selC* triple mutant V_{H2} was decreased ~3.6 fold (see Fig. 2). But in contrast to the findings at pH 7.5, at pH 6.5 H_2 production was completely absent in *hyaB hybC hycE* triple mutant (see Fig. 2). These data clearly point out that H_2 production is lowered but not totally abolished due to partial destabilization of FHL-1 complex in *hyaB hybC selC* mutant, where FDH-H was absent, and Hyd-3 can work in H_2 producing mode.

In formate supplemented assays, in *hycE* single and *hyaB hybC selC* triple mutants V_{H2} was lowered ~4.5 fold and ~13 fold, respectively, compared to wt (see Fig. 2). Thus, at pH 6.5 during mixed carbon fermentation, in the assays with added glycerol, in wt cells V_{H2} was similar to that with the cells grown on glycerol only. Interestingly, no role of FDH-H has been observed for pH 6.5. The deletion of single or double genes or even the whole operons for one or more Hyd enzymes don't reveal Hyd enzymes mainly responsible for H_2 production. Obviously, as at pH 7.5, in formate assays, Hyd-3 is major H_2 producing Hyd enzyme at pH 6.5.

Taken together, the main results have been obtained with *hyaB hybC selC* and *hyaB hybC hycE* triple mutants. Only deletions of genes for three Hyd enzymes affect H_2 production at both pHs in glycerol assays. These data suggest that during mixed carbon fermentation the disturbance of H_2 cycling reduced the production of H_2 and one of Hyd enzymes cannot work simultaneously either in H_2 uptake or production direction.

To investigate the growth of *E. coli* on acetate in the buffered peptone medium wt cells were grown on glycerol and/or acetate. At first, wt cells of *E. coli* BW25113 were grown on acetate with 1, 2 and 5 g L^{-1} concentrations as a sole carbon source at slightly

alkaline (pH 7.5) and slightly acidic (pH 6.5) pHs (Fig. 3, A, B). According to available reports, acetate exceeding 5 g L^{-1} concentration might be an inhibitor of growth and act as uncoupler [Jensen & Carlsen, 1990]. When wt cells were grown at pH 7.5 under anaerobic conditions on 1 g L^{-1} acetate at early log phase the specific growth rate (μ) was 0.35±.0.03 h^{-1} (Fig. 3, A). The same result was obtained at pH 6.5 (Fig. 3, A). In comparison, cells grown on formate alone either at pH 7.5 or pH 6.5 had ~3 fold higher μ, compared to the cells grown on acetate only. Moreover, in wt cells grown in the same conditions but in the presence of 2 g L^{-1} acetate μ did not change. In addition, cells grown at acidic (pH 5.5) pH had μ of 0.15±0.01 h^{-1} which was lower ~2.3 fold (Fig. 3, A). This might be due to acidic environment and acetic acid, as formate, can suppress the cell growth due to uncoupling effects by dissipation of the transmembrane pH gradient (ΔpH).

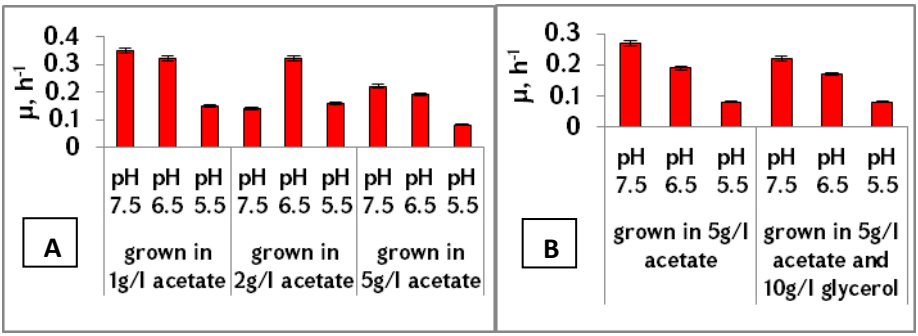


Fig. 3. Specific growth rate (μ) of *E. coli* BW25113 wt grown in buffered peptone medium at 37^{0}C during concentration-dependent acetate (A) and mixture of acetate and glycerol (B) utilization at different pHs.

As in the nature, higher concentrations of acetic acid are formed wt cells were grown on 5 g L^{-1} acetate. At pH 7.5 μ was 0.22±0.01 h^{-1} (see Fig. 3, A, B). The same growth rate was achieved with *E. coli* wt cells under aerobic conditions in the presence of 10 g L^{-1} acetate [Lakshmanaswamy et al., 2011]. After ~10 h of growth cells were able to consume all acetate at μ of 0.23±0.01 h^{-1} [Lakshmanaswamy et al., 2011]. The decrease of pH resulted in less μ, and at pH 6.5 the μ was 0.19±0.01 h^{-1} ~1.7 fold less, compared to the cells grown on 1 or 2 g L^{-1} acetate. Even worse cells grew at pH 5.5 with μ of 0.08±0.01 h^{-1} (see Fig. 3, A). It is important to address that different groups were investigating acetate consumption in the presence of various sugars e.g. glucose and xylose [Lakshmanaswamy et al., 2011]. The critical growth inhibition at low pH might be due to combined effect of high concentration of acetate and acidic pH.

As in different industrial and agricultural wastes not only acetic acid but also glycerol was present it was of great interest to explore the effects of mixture of glycerol and acetate on growth of *E. coli*, and thus, use mixture for producing valuable chemicals, biofuels and other products. As it was shown before, optimal concentration of glycerol for growth was 10 g L^{-1} [Poladyan et al., 2013], and the highest H_2 yield was determined at 5 g L^{-1} acetate, the mixture of 10 g L^{-1} glycerol and 5g L^{-1} acetate was taken to study (see Fig. 3, B). Either cells were grown on acetate or in the mixture with glycerol, μ for all pHs tested above was the same (see Fig. 3, B). This indicate that acetate inhibits cell growth even in the presence of other carbon source. These data are in good conformity with the results obtained previously showing that acetate inhibits growth at early log growth phase in the presence of glucose [Luli & Strohl, 1990]. It is noteworthy that different groups have employed mainly pH 7.0 for acetate fermentation and optimized acetate removal or utilization by *E. coli* for neutral pH [Luli & Strohl, 1990; Russel & Diez-Gonzalez, 1998]. As we were interested in converting acetate to H_2, wide range of pH 5.5 to 7.5 was applied (see Fig. 3). Moreover, this pH range was optimal for H_2 production by *E. coli* as Hyd enzyme was active at this pH range, which was described previously [Trchounian et al., 2012].

To investigate if acetate is reused and transmuted to H_2, H_2 evolution kinetics in *E. coli* batch cultures at different pHs in the presence of varying concentrations of acetate (see Materials and methods) were studied (Fig. 4). H_2 production by *E. coli* was tracked for 48 h. The highest H_2 yield of 5.07±0.15 mmol H_2 L^{-1} was detected at early log growth phase with acetate concentration of 5 g L^{-1} at pH 7.5 and late growth phase at pH 6.5.

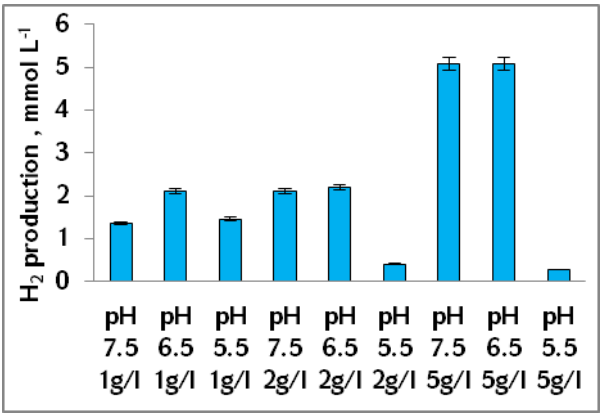


Fig. 4. H_2 production by *E. coli* wt strain in the presence of different concentrations of acetate at different pHs.

At pH 5.5 H_2 yield was residual 0.27± 0.01mmol H_2 L^{-1}. When wt cells were grown on 2 g L^{-1} acetate either at pH 6.5 or pH 7.5, the same H_2 yield (2.2±0.07 mmol H_2 L^{-1}) was reached early at log growth phase. This was less ~2.3 fold than at the same pH but with 5 g L^{-1} acetate (see Fig. 4).

In addition, at pH 5.5, H_2 evolution was detected only at the late stationary phase, and H_2 yield was 0.70±0.02 mmol H_2 L^{-1}. *E. coli* wt cells grown at pH 7.5 and pH 5.5 in the presence of 1 g L^{-1} acetate resulted in production of 1.36±0.04 and 1.45±0.04 mmol H_2 L^{-1} at the late log growth phase, respectively. This was quite intriguing finding because at acidic pH, when 1g L^{-1} acetate was employed, at log growth phase H_2 production was ~3.9-fold more than at the same conditions but in the presence of 2 g L^{-1} acetate (see Fig. 4). The fact clearly demonstrated that substrate limitation resulted in a higher product formation. These data are similar to the outcome shown before that high concentration of glucose inhibits H_2 production [Trchounian & Trchounian, 2014].

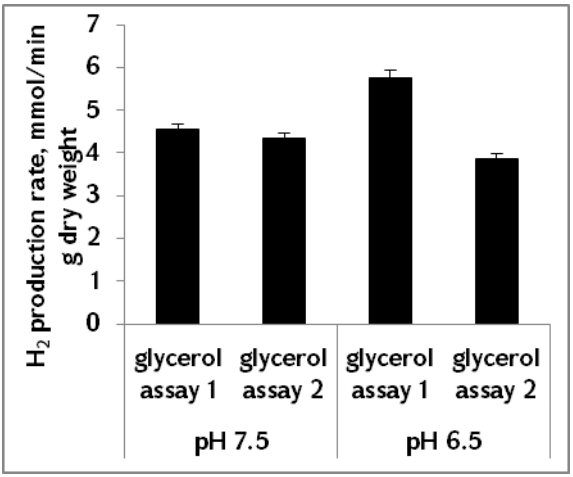


Fig. 5. V_{H2} in *E. coli* wt cells upon supplementation of glycerol. Cells were grown in 10 g L^{-1} glycerol alone or in the mixture of 10 g L^{-1} glycerol and 5 g L^{-1} acetate under anaerobic conditions at 37^0C at pH 7.5 and pH 5.5. Glycerol assay 1 corresponds to the cells grown on acetate and glycerol; glycerol assay 2 corresponds to the cells grown on glycerol only.

Moreover, the uncoupling effect of acetic acid at pH 5.5 might result in low and delayed H_2 production. On the contrary, at pH 6.5, H_2 production was disclosed at early log growth phase and had similar level, as for the data obtained for 2 g L^{-1}. In overall, optimal pH for H_2 generation was pH 6.5, and optimal concentration of acetate is 5 g L^{-1}.

In addition, wt cells grown in the presence of the mixture of acetate and glycerol were harvested and assayed for H_2 production (see Fig. 5). Interestingly, at pH 7.5 V_{H2} in glycerol supplemented assays was the same compared to the cells grown on glycerol

only but at pH 6.5 V_{H2} was ~1.5-fold higher compared to the cells grown on glycerol (see Fig. 5). This might be used further for pre-cultivation of the cells and enhanced H_2 production from glycerol.

Glucose concentration as an important factor for Hyd-4 activity.

During fermentative growth of *E. coli* in the presence of 0.2% glucose and in the assays supplemented with 0.2% glucose at pH 7.5 JRG3615 (*hyfA-B*) and JRG3621 (*hyfB-R*) mutant strains (see Table 1) had H_2 production rate (V_{H2}) ~6.7 fold and ~5 fold less, respectively, than wt cells (Fig. 6, A). In the same conditions addition of 0.8% glucose in the assays had the same effect on H_2 generation. At pH 6.5 in JRG3615 and JRG3621 strains V_{H2} was decreased ~2.2 fold and ~7.8 fold, respectively, compared to wt (Fig. 6, A). But at pH 5.5 in JRG3621 strain it was decreased ~3.8 fold, compared to wt (Fig. 6, A).

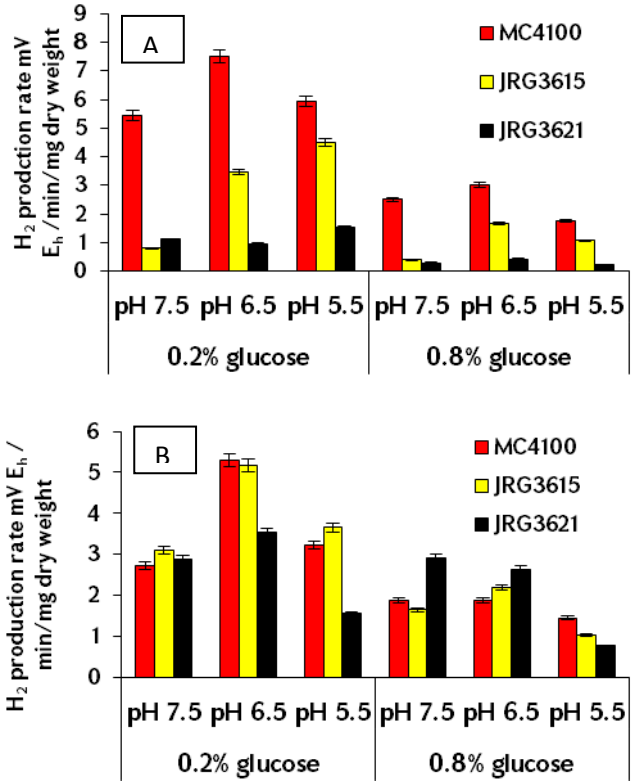


Fig. 6. H_2 production rate by *E. coli* MC4100 wt, JRG3615 (*hyfA-B*) and JRG3621 (*hyfB-R*) mutant strains grown on peptone medium supplemented with 0.2% (A) or 0.8% (B) glucose at different pHs. In the assays 0.2% or 0.8% glucose were added.

These findings point out that at pH < 7.0 only the deletion of the most of Hyd-4 operon genes disturbs H_2 production, unless V_{H2} of JRG3615 is ~3.5 fold higher than that of JRG3621 strain (see Fig. 6, A). The decreased H_2 production might be due to interaction between Hyd-4 and Hyd-3 and the lacking major part of Hyd-4 which affects Hyd-3 activity. The data with Hyd-4 mutants suggest that the deletion of single gene has no clear effect on H_2 production activity of Hyd-3 or Hyd-4 because of compensatory effects between these Hyd enzymes [Trchounian et al., 2012], but deleting most of the *hyf* operon genes, which might be involved in electron transfer chain from FDH-H to Hyd-3 or Hyd-4 or protons from the F_0F_1-ATPase to secondary transport systems, might disturb the pathways and has strong impact on H_2 production overall. During fermentative growth of *E. coli* in the presence of 0.2% glucose but in the assays supplemented with 0.8% glucose the same effects of Hyd-4 impact on H_2 generation was observed (see Fig. 6, A). In this respect it is suggested that supplementation of glucose concentration is important for H_2 formation and addition during the assays of different concentrations of the carbon source has no effect.

During growth of *E. coli* in the presence of 0.8% glucose but in the assays supplemented with 0.2% glucose at pH 7.5 wt and mutants showed similar H_2 producing activity (see Fig. 6, B). In JRG3621 strain V_{H2} was lowered, compared to wt, but this decrease at pH 5.5 was not significant and had no any effect. In the assays with 0.8% glucose in wt and mutants no any difference in V_{H2} was observed (see Fig. 6, B). The data obtained suggest that at 0.8% glucose concentrations no any Hyd-4 activity in H_2 production or other processes can be detected (see Fig. 6, B). Moreover, excess of glucose in the cells can change the fermentative metabolism, and it might be that Hyd-4 is switching on when there are limited conditions for the cell. In addition, an association of the F_0F_1-ATPase with secondary transport systems or key enzymes of fermentation has been proposed under energy limited processes when transfer of energy from ATPase to the other membrane protein might lead to the work increasing efficiency of energy using [Trchounian, 2004; Trchounian & Sawers, 2014].

It can be concluded that Hyd-4 is active mainly at low concentrations of glucose at pH 7.5. Probably Hyd-4 switches on when there are glucose limited, not reach conditions for cells. These results are of significance for regulation of Hyd enzyme activity to enhance H_2 production during fermentative conditions.

Role of formate channels in H_2 production during fermentation of glucose or glycerol.

During growth on glycerol at pH 7.5 the H_2 production rate in the *focA* mutant was ~2.4 fold higher compared to the wt (Fig. 7, A). These results render that deletion of *focA* disturbs the export of formate; so it could be suggested that FocA can export formate and, moreover, enhanced H_2 production is observed. H_2 production in the *focB* was similar to the wt (Fig. 7, A). When the cells were grown on glycerol and 10 mM formate was added to the assays, the V_{H2} increased ~2.8 fold in the *focA* mutant, respectively, relative to the level in the wt. Surprisingly, in the absence of FocB, formate addition failed to affect H_2 production (Fig. 7, A). These data show for the first time that the predicted formate channel FocB has an important role in H_2 production during glycerol fermentation at high pH. At pH 6.5, *E. coli* wt and all mutants demonstrated similar V_{H2} in glycerol assays but in the formate assay the *focA* mutant resulted in ~1.8 fold increase, compared to the wt (Fig. 7, A).

In contrast, the *focB* mutant showed similar V_{H2} to that of the wt (see Fig. 7, A). However, after growth with glycerol at pH 5.5 a different effect was observed. The V_{H2} of *focA* and *focB* mutants was decreased ~1.8 fold compared to the wt. These data denote that *focA* and *focB* genes have a role in H_2 production by *E. coli* during glycerol fermentation and formate supplementation. Taken together the results obtained suggest that at pH 7.5, when glycerol is present, formate enters the cell through FocB.

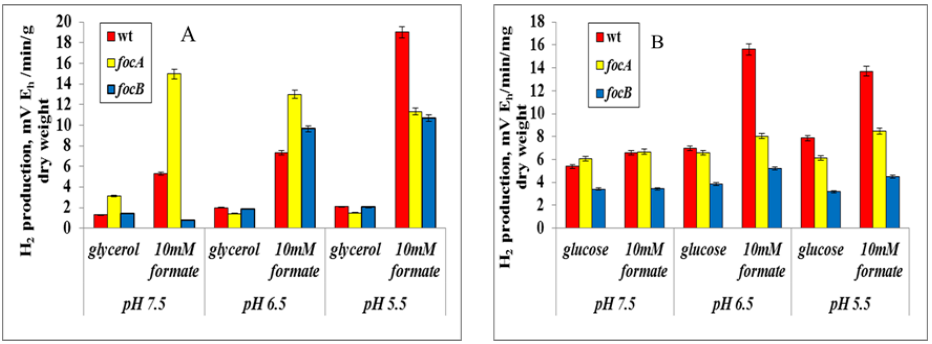


Fig. 7. H_2 production rates of *E. coli* wild type and mutants lacking the genes encoding formate channels FocA or FocB. For strains, see Table 1. Strains were grown at different pHs in peptone medium supplemented with glycerol (A) glucose (B). In the assays glycerol, glucose or formate were added.

This is confirmed by analyzing the *focB* mutant where H_2 production was residual and, in contrast, at more acidic pHs in glycerol-grown cells both channels are operating

in formate import direction and formate is neutralized by converting it to H_2 and CO_2 to maintain intracellular pH at neutrality.

During glucose fermentation at pH 7.5 in the glucose supplemented assays, the V_{H2} by *E. coli* wt and *focA* mutant was similar but it was decreased ~1.6 fold in the *focB* mutant, when compared to the wt (see Fig. 7, B). The same effect was observed in the assays when 10 mM exogenous formate was added. In the *focB* strain V_{H2} was reduced ~1.9 fold (see Fig. 7, B). This might indicate that during glucose fermentation either glucose or exogenously supplied formate has the same impact on V_{H2} in both wt and mutant strains. At pH 6.5 in the assays with supplemented glucose the same results were attained for wt and mutants as at pH 7.5. Moreover, in the presence of exogenously supplied formate H_2 production was decreased ~2 fold and ~3 fold in *focA* and *focB* mutants, respectively, compared to the wt. At pH 5.5 in the presence of externally supplemented formate H_2 production had similar behavior and was lowered ~1.6 fold and ~3 fold in *focA* and *focB* strains, respectively, compared to the wild type.

But at this pH in glucose assays in *focB* single mutant H_2 production was decreased ~2.5 fold, compared to the wt (see Fig. 7, B). These data indicate that *focA* and *focB* genes have different role in H_2 production during glucose fermentation. Moreover, the effects depend on pH. The results point out that external formate has different effects on H_2 production, which might reflect an interaction between FocA and PflB [Beyer et al., 2013]. In addition, during glucose fermentation FocB might also interplay with PflB. To conclude, the results suggest that during glucose fermentation FocB has a role in H_2 production. As FocA is exporting formate, FocB might import back the formate to produce H_2 and, therefore, in the absence of FocB the import of formate is impaired and, thus H_2 production decreased. These might also indicate relationship between intracellular formate generating, formate neutralizing and H_2 producing mechanisms.

Sensitivity of *E. coli* hydrogenases to osmotic stress during dark fermentation of glycerol. Role of DCCD as an inhibitor in H_2 production.

Osmotic stress has been shown to affect H_2-evolving activity by *E. coli* Hyd enzymes during glucose fermentation at pH 7.5 [Bagramyan et al., 2002]. However, during glycerol fermentation at pH 7.5 V_{H2} by wt cells was the same under hyper- or hypo-stress (Fig. 8). But at pH 6.5, V_{H2} decreased approx. 5-fold upon hypo-stress compared to hyper-stress conditions (Fig. 8). Thus, osmotic stress effects on H_2 evolving activity during glycerol fermentation was pH dependent. This finding was of interest as mainly Hyd-2 and to a lesser extent Hyd-1 are responsible for H_2 evolving activity during glycerol fermentation at pH 7.5 [Trchounian et al., 2011; 2013; Trchounian &

Trchounian, 2009], whereas Hyd-3 or Hyd-4 probably are main H_2 producing enzymes [Trchounian, 2015; Trchounian et al., 2011; 2012]. During different stress conditions, also for hypo-stress, sizes or volume of the periplasmic space of the bacterial cell might be changed: the role of periplasmic proteins could be suggested to regulate membrane-associated proteins conformational structure and activity including F_0F_1 [Trchounian et al., 1994]. Due to this change it is possible that these enzymes which are situated on the periplasmic side of the membrane and include Hyd-1 and Hyd-2 [Dubini et al., 2002; Trchounian, 2015; Trchounian et al., 2012] would become osmosensitive. As mentioned above, mainly Hyd-2 and partially Hyd-1 are working in H_2 producing direction at pH 7.5 during glycerol fermentation [Trchounian et al., 2011; 2013; Trchounian & Trchounian, 2009] but no effect of osmotic stress was determined before.

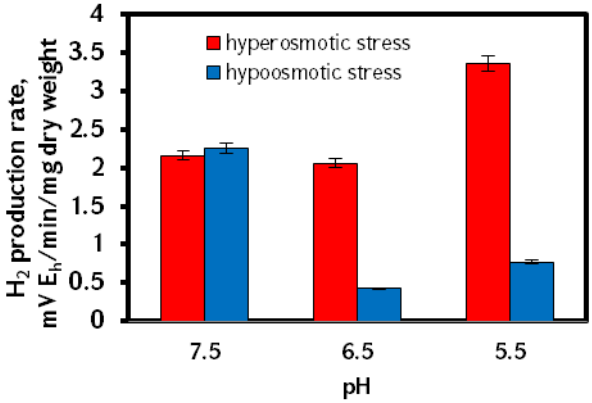


Fig. 8. H_2 production rates (V_{H2}) by *E. coli* BW25113 wt under hyper- and hypo-osmotic stress during glycerol fermentation at different pHs. For others see materials and methods.

This might be explained by Hyd-2 and Hyd-1 changing their conformation or localization within the membrane and, thus, were not directly affected by osmotic stress. This seems likely to be due to a major contribution of Hyd-2 to H_2 production during glycerol fermentation resulted from changed metabolism and surprisingly influenced H^+ reduction [Trchounian et al., 2013]. The other possibility is that under osmotic stress metabolic cross-talk and interaction between some membrane-associated enzymes including Hyd and F_0F_1 [Trchounian et al., 2012; Trchounian, 2015] can be disrupted. This would be with Hyd-3 and Hyd-4 since H_2 production was markedly decreased under hypo-stress at low pH. The effect of pH, which is very complex, should not be ruled out in this context. Importantly, the same pH dependent effect was shown for inhibition of H_2 production with DCCD during glycerol fermentation [Trchounian & Trchounian, 2009; 2013; Trchounian et al., 2011]; therefore comparison with DCCD effects was

done with mutant, which will be described below. Actually, during glycerol fermentation at pH 7.5 *E. coli hyaB* and *hybC* single or *hyaB hybC* double mutants did not show any H_2 production nor did they show an effect of osmotic stress or DCCD (Fig. 9).

Upon hyper-stress at pH 7.5 in *hyfG* single mutant V_{H2} was inhibited by DCCD ~ 6.8 fold but in *fhlA* single and *fhlA hyfG* double mutants it was decreased markedly ~ 10 fold (Fig. 9). V_{H2} under hypo-stress in *fhlA* and *hyfG* mutants was decreased by only ~ 1.7 fold and threefold, respectively; and in *fhlA hyfG* mutant it was decreased ~ 2.5 fold (Fig. 9). These data indicated that Hyd-3 and Hyd-4 were osmosensitive; alternatively, FhlA could be involved in osmotic stress effects on H_2 production. There was no contradiction with the fact that Hyd-4 might work in H_2 uptake mode or supply H^+ to Hyd-2: H^+ could be transferred from F_0F_1, as suggested [Trchounian & Trchounian, 2009].

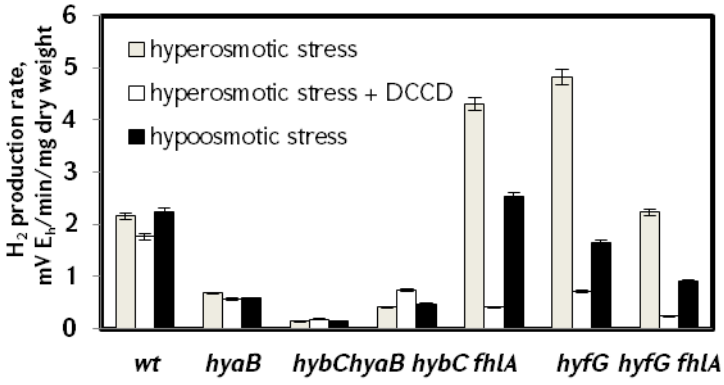


Fig. 9. H_2 production rates by *E. coli* wt and different mutants with defects in Hyd enzymes under hyper- and hypo-osmotic stress at pH 7.5.

Interestingly, osmoregulation of transcription was suggested by change in intracellular solute concentration but under other conditions [Trchounian & Trchounian, 2013].

***E. coli* hydrogenase activity during dark fermentation of glucose or glycerol at different pHs.**

Wild type cells grown on glucose at pH 7.5 yielded a total Hyd specific activity of 3.34 U (mg of protein)$^{-1}$ (Fig. 10), while the activities at pH 6.5 and 5.5 were 2.05 U and 0.82 U (mg of protein)$^{-1}$, respectively. Analysis of *hyaB* mutant showed that Hyd-1 contribution was little to the total Hyd specific activity at pH 7.5. Interestingly, in *fhlA* single or *fhlA hyfG* double mutants ~ 60% reduction in total Hyd specific activity (1.38 U (mg of protein)$^{-1}$ (Fig. 10) was detected, denoting that Hyd-3 made a significant

contribution to total Hyd specific activity under these conditions. The rest of the activity comes from Hyd-2 because a mutant (*hyaB hybC selC*) phenotypically devoid of Hyd-1, Hyd-2 and the FDH-H component of the FHL complex essentially lacked Hyd activity at pH 7.5 (Fig. 10). When cells were grown at pH 7.5 on glucose the total Hyd specific activity in *hybC* mutant was ~ 30% of the wt, yet in *hyaB hybC* double mutant retained 60% of the wt activity (Fig. 10). Hyd specific activity of wt cells grown on glycerol was highest (2.7 U (mg of protein)$^{-1}$) at pH 6.5. The Hyd specific activity was same at pH 7.5 or pH 5.5. Note, that Hyd activity of wt cells grown in peptone medium without addition of carbon source was 0.2 U (mg of protein)$^{-1}$, 0.3 U (mg of protein)$^{-1}$ and 0.44 U (mg of protein)$^{-1}$ after growth at pH 7.5, 6.5 and 5.5, respectively.

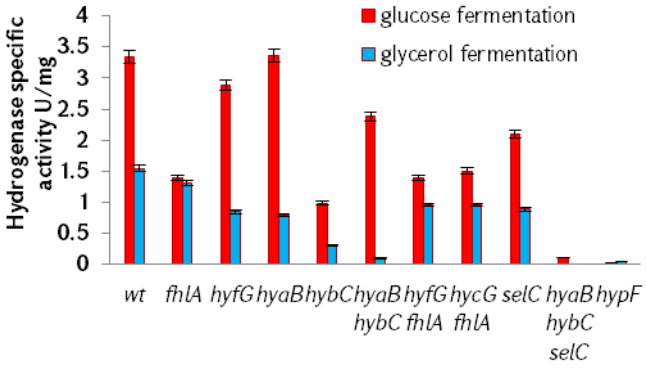


Fig. 10. Hyd specific activity of *E.coli* wt and different Hyd mutants grown during glucose or glycerol fermentation at pH 7.5. For others see Materials and Methods.

This clearly demonstrated that supplementation of glycerol as a carbon source resulted in a 6- to 10-fold increase in Hyd specific activity, depending on pH. Compared to glucose-grown cells, cells grown in the presence of glycerol at pH 7.5 Hyd-1 and Hyd-2 were the main contributors to total Hyd enzyme activity (see Fig. 10) because the specific activity measured after growth of a *hyaB hybC* double mutant was approximately 5% of the activity observed in wt. In *hybC* mutant 80% decrease in enzyme activity was observed (see Fig. 10), while in a single *hyaB* mutant the Hyd specific activity decreased by 50%.

Role of proton F_0F_1-ATPase in Hyd-1 and Hyd-2 specific activities during glucose or glycerol fermentation at various pHs.

First of all, the Hyd specific activity in DK8 mutant (see Table 1), was determined during glucose fermentation at different pH values. At pH 7.5 under glucose fermentation no Hyd activity was detected in the mutant while a high Hyd activity was determined in the wt (Fig. 11, A). Analysis of Hyd-1 and Hyd-2 activity by in-gel staining uncovered that no activity bands corresponding to Hyd-1 or Hyd-2 (Fig. 11, A) could be

detected. In in-gel Hyd activity assay, in DK8 mutant very weak activity bands corresponding to Hyd-1 and Hyd-2 were detected at pH 6.5 (Fig. 11, B). At pH 5.5 activity of Hyd-2 could not be observed and that of Hyd-1 was barely detectable, indicating that the activity of both H_2-oxidizing enzymes was severely affected in *atp* mutant, particularly at extreme pHs (Fig. 11, C). As a control, in *hyaB hybC* double mutant no any activity band was observed (Fig. 11, C). Notably, the in-gel staining data indicated that the weak Hyd-independent enzyme activity due to FDH, and designated by an asterisk, was observed under all growth conditions with equal intensity in wt and DK8 mutant. These data show that the effects observed in DK8 mutant grown on glucose at pH 5.5 did not affect all oxidoreductases and the residual Hyd activity in the mutant was contributed by Hyd-3. This effect could be also due to either the lack of active F_0F_1 directly or may be mediated by a deficient $\Delta\mu_{H^+}$ [Trchounian, 2004].

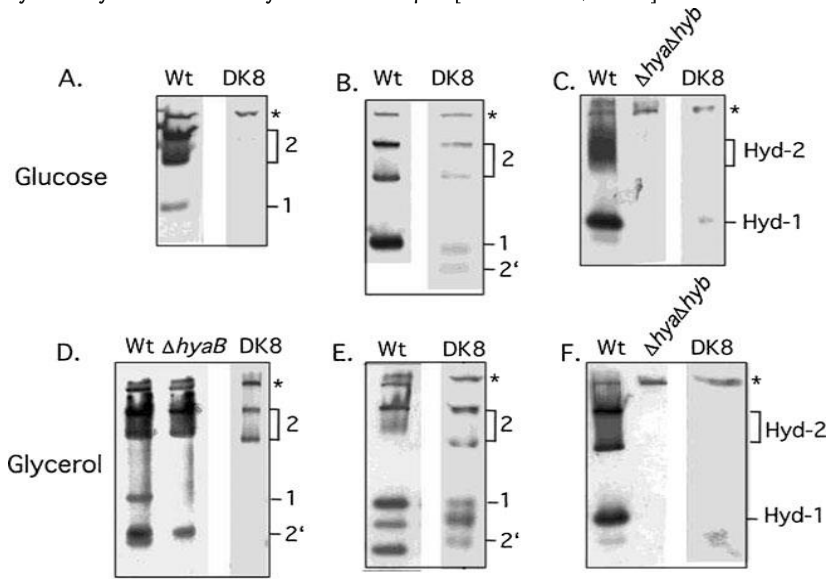


Fig. 11. Analysis of active Hyd-1 and Hyd-2 in *E. coli* by activity staining after non-denaturing-PAGE. Strains were grown either with glucose (A, B, C) or glycerol (D, E, F) as indicated and at pH 7.5 (A, D), pH 6.5 (B, E) or pH 5.5 (C, F).

During growth on glycerol, a double mutant lacking both Hyd-1 and Hyd-2 had elevated F_0F_1-ATPase activity at pH 7.5 but not at pH 5.5 [Blbulyan et al., 2011]. These results indicate that the activity of Hyd-1 and Hyd-2 has a strong influence on the activity of F_0F_1. At pH 7.5, DK8 had a lower Hyd-activity (by 50%) than wt (see Fig. 11, B). In-gel

activity staining revealed that this activity was mainly due to Hyd-2 but not Hyd-1 (see Fig. 11, D).

Wild type cells grown at pH 6.5 had similar Hyd specific activity as during glucose fermentation, and the mutant had a value of 10% of the activity in wt (see Fig. 11, B).The in-gel assay demonstrated that Hyd-2 and Hyd-1 were active under these conditions (see Fig. 11, E). Wild type cells grown at pH 5.5 on glycerol resulted in a Hyd specific activity of 1.5 U (mg protein)$^{-1}$ (see Fig. 11, B). In contrast, the Hyd specific activity of DK8 mutant was barely detectable (see Fig. 11, B). In-gel activity-staining revealed that neither Hyd-1 nor Hyd-2 activity could be detected (see Fig. 11, F). The results indicated that F_0F_1 is needed for the activity of Hyd-1 and Hyd-2 during glucose or glycerol fermentation. These data demonstrated a metabolic link between *E. coli* F_0F_1 and H_2-oxidizing activity and underscore the key role of Hyd-1 and Hyd-2 in energy conservation.

Role of Hyd enzymes in Δp generation.

Δp generation by *E. coli* during glycerol fermentation was first studied. At pH 7.5, the increase of Δφ was established to be only partially compensated by a reversed ΔpH, resulting in a low Δp (Fig. 12). It should be noted that pH_{in} and Δφ were lower and consequently Δp was also not higher when compared with glucose fermentation. This difference between glycerol and glucose fermentation might be due to distinguished mechanisms for H^+ transport, especially Hyd, besides of F_0F_1. Therefore, a role of Hyd in Δp generation during glycerol fermentation can be suggested. That could be different depending on pH.

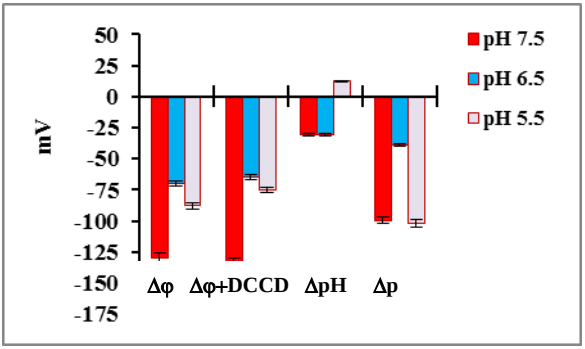


Fig. 12. The values of Δφ, ΔpH and Δp by *E. coli* wt whole cells grown during glycerol fermentation at different pHs. The DCCD (0.3 mM) was added into the assays mixture when indicated. ΔpH was calculated in mV (ZΔpH). For the others, see Materials and methods.

H_2 production in *C. beijerinckii* during dark fermentation of various carbon sources and their mixtures.

To investigate H_2 production during glycerol fermentation, *Clostridium beijerinckii* DSM 791 was assayed for hydrogen formation with glycerol at pH 7.5 and pH 5.5. Besides glycerol, for comparison glucose and various mixtures of carbon sources like glucose plus formate, glycerol plus formate and glucose plus glycerol were taken to test if it is possible to enhance the H_2 production and increase Hyd enzyme activity by applying different carbon sources. The latters were of importance as in nature no single carbon sources are present. The optimal pH for *C. beijerinckii* growing on glycerol was pH 7.5.

The H_2 production rate during fermentation of glycerol (110 mM) at pH 7.5 was ~3.5 mmol/h/g cell mass which was ~1.2 fold lower compared to glucose (40 mM) (Fig. 13). But interestingly, a mixture of 110 mM glycerol and 10 mM formate increased the rate by ~1.4 fold compared to glycerol only. With a mixture of 40mM glucose plus 10 mM formate, the H_2 production rate was decreased ~1.8 fold (Fig. 13). This might point out the differences between glucose and glycerol fermentations and thus differences in Hyd enzyme activity. At pH 5.5, the H_2 production rate was lower in all variations of single or mixed carbon sources. In general, during glycerol fermentation the H_2 production rate at pH 5.5 was 1 mmol/h/g cell mass and ~ 3.4 fold lower than at pH 7.5 (Fig. 13).

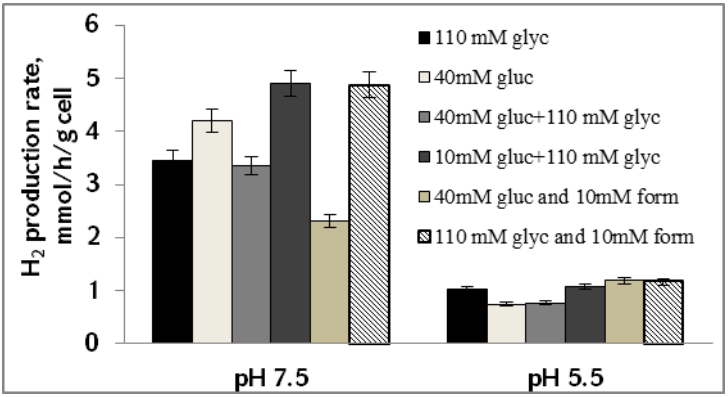


Fig. 13. H_2 production rate of *C. beijerinckii* grown at pH 7.5 or pH 5.5 during utilization of single or mixtures of carbon sources. For details see Materials and methods.

During glucose fermentation at this pH, the H_2 production rate was lower compared to glycerol which was not the same for pH 7.5 (see Fig. 13).

To understand the role of Hyd enzymes in H_2 production during fermentation of different substrates and their mixtures, Hyd specific activity was determined. In all tested conditions the Hyd activity was higher at pH 7.5 than at pH 5.5. When cells were grown at pH 7.5 on glucose, the Hyd activity was ~3.3 fold higher than at pH 5.5 (Fig.

14). Moreover, at pH 7.5, Hyd activity of the cells grown on glycerol was ~1.4 fold higher than cells grown at the same pH but on glucose only. Interestingly, cells grown in the presence of glycerol plus formate at pH 7.5 showed increased Hyd activity compared to cells grown at pH 5.5 (Fig. 14). When the cells were grown at pH 7.5 in the presence of glycerol plus formate Fe^{2+} (0.01 mM) had a stimulatory effect on Hyd activity; it was increased ~1.3 fold. The same effect was obtained when Fe^{3+} (0.5 mM) or Ni^{2+} (1 μM) was added in the assays (Fig. 15).

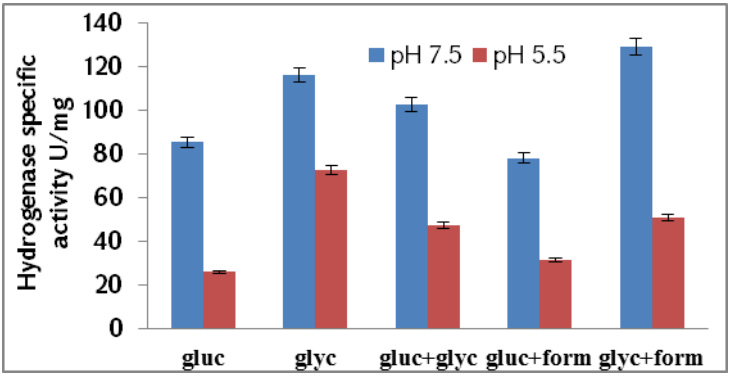
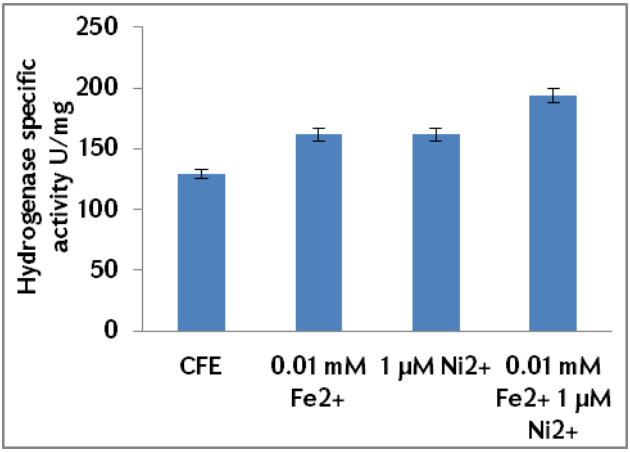


Fig. 14. Hyd activity of *C. beijerinckii* grown at pH 7.5 and pH 5.5 in the presence of various carbon sources.

Fig. 15. Hyd activity of *C. beijerinckii* grown at pH 7.5 in the presence of glycerol and formate. In the assays single or mixtures of different metals (Fe^{2+} and Ni^{2+}) were added. For others see Materials and methods.



As the results above showed that single Fe^{2+} (0.01 mM) and Ni^{2+} (1 μM) increased Hyd activity the mixture of these metals in the same concentrations were studied, and Hyd activity was increased by ~50% compared to the cell free extracts only or by ~25% compared to the assays with single metals (see Fig. 15).

CONCLUSIONS

1. Growth and H_2 production of *E. coli* and *C. beijerinckii* were revealed during dark fermentation of glycerol and various carbon sources and their mixtures. It has been determined that 10 g/l (110 mM) glycerol is optimal concentration for H_2 production rate and yield.
2. It has been established that in *E. coli* mutants with defects in various Hyd enzymes Hyd-2 mainly and Hyd-1 partially are responsible for H_2 production at slightly alkaline pH (pH 7.5), and Hyd-3 and Hyd-4 can operate in H_2 uptake or oxidation direction. At slightly acidic (pH 6.5) and acidic (pH 5.5) pHs Hyd-3, component of FHL-1 complex, is responsible for H_2 production.
3. It has been shown that total Hyd activity during dark fermentation of glucose at acidic (pH 5.5) conditions was formed only by Hyd-1. The role of Hyd-4 was revealed during dark fermentation of mixture of carbon sources (glucose+glycerol) at slightly acidic (pH 6.5) and acidic (pH 5.5) pHs. The dependence of Hyd-4 from glucose concentration was shown.
4. It has been revealed the sensitivity of *E. coli* various Hyd enzymes to osmotic stress during dark fermentation of glycerol at different pHs.
5. It has been shown that DCCD inhibits the H_2 production during dark fermentation of glycerol at acidic (pH 5.5) but not at slightly alkaline (pH 7.5) pH and during fermentation of glucose the inhibition effect of DCCD was inversed.
6. In the mutant lacking proton F_0F_1-ATPase in non-denaturing polyacrylamide gel it was found that in *E. coli* during glucose or glycerol fermentation Hyd activity depends on the F_0F_1-ATPase.
7. The proton motive force in *E. coli* during glycerol fermentation at different pH values was determined. It has been shown the low value of proton motive force and cytoplasmatic pH during glycerol fermentation at pH 7.5 compared to glucose fermentative conditions. The investment of Hyd enzymes in proton motive force generation was revealed.
8. It has been revealed the high activities of Hyd and formate dehydrogenase enzymes in *C. beijericnkii* compared to *E. coli* during glycerol and various carbon sources and their mixture dark fermentation at pH 7.5.
9. It has been shown that in *C. beijericnkii* at pH 7.5 during glucose fermentation 0.1 mM Fe^{3+} or 10 μM Ni^{2+}, during glycerol and formate fermentation mixture of 0,01 mM Fe^{2+} and 1 μM Ni^{2+} enhances Hyd enzyme activity. The inhibitory effect of Cu^{2+} depends on its concentration, medium pH and carbon source.

10. It is suggested to apply glycerol, as cheap carbon source, and different mixtures of carbon sources with glycerol, for H_2 production improvement. The choice of optimal values for technological conditions (pH, concentration of carbon source, inhibitors, some metal ions) substantially increases H_2 production.

LIST OF PUBLICATIONS AS A PART OF DISSERTATION TOPIC

1. ***Trchounian K.,*** *Poladyan A., Trchounian A.* (2016) Optimizing strategy for *Escherichia coli* growth and hydrogen production during glycerol fermentation in batch culture: Effects of some heavy metal ions and their mixtures. ***Appl. Energy 177***, 335-340.
2. ***Trchounian K.,*** *Trchounian A.* (2016) H_2 production by *Escherichia coli* during utilization of acetate and mixture of glycerol and acetate. ***21st World Hydrogen Energy Conf. Proc.*** *Zaragoza, Spain,* 277-278.
3. *Poladyan A., Mirzoyan S.,* ***Trchounian K.,*** *Trchounian A.* (2016) Hydrogen production by *Escherichia coli* wild type and hydrogenase mutants upon formate and glycerol fermentation under different growth conditions. ***Biospektrum* 22**, 156.
4. ***Trchounian K.****, Trchounian A.* (2016) New role of *Escherichia coli* hydrogenase 4 during glucose fermentation. ***11th Int. Hydrogenase Conf. Abstract Book****, Marseille, France,* p. 53.
5. *Poladyan A.,* ***Trchounian K.,*** *Trchounian A.* (2016) Glycerol Fermentation and Hydrogen Metabolism by *Escherichia coli*: New Approaches to Enhance Hydrogen Production. ***ASM Microbe 2016 Abstracts****, Boston (USA)* p.72.
6. ***Trchounian K.*** (2015) Effects of carbon sources mixtures on hydrogen production by *Escherichia coli* during mixed-acid fermentation. ***Rep. Natl. Acad. Sci. Armenia*, 115**, 148-155.
7. ***Trchounian K.,*** *Abrahamyan V., Poladyan A., Trchounian A.* (2015) *Escherichia coli* growth and hydrogen production in batch culture upon formate alone and with glycerol co-fermentation at different pHs. ***Int. J. Hydrogen Energy* 40,** 9935-9941.
8. ***Trchounian K.,*** *Sargsyan H, Trchounian A.* (2015) H_2 production by *Escherichia coli* batch cultures during utilization of acetate and mixture of glycerol and acetate. ***Int. J. Hydrogen Energy* 40,** 12187-12192.
9. ***Trchounian K.,*** *Trchounian A.* (2015) Hydrogen production from glycerol by *Escherichia coli* and other bacteria: An overview and perspectives. ***Appl. Energy 156***, 174-184.
10. ***Trchounian K.,*** *Trchounian A.* (2015) Clean energy technology development: hydrogen production by *Escherichia coli* during glycerol fermentation. ***Progress in Clean Energy*** v. 2, Novel Systems and Applications, (eds. **Dincer,** I. et al.), Springer, pp. 539-549, ISBN 978-3-319-17030-5.
11. ***Trchounian K.,*** *Trchounian A* (2015) *Escherichia coli* hydrogen gas production from glycerol: effects of external formate. ***Renew. Energy*, 83**, 345-351.

12. ***Trchounian K.,*** *Vardanyan A., Poladyan A., Trchounian A* (2015) *Escherichia coli* growth and hydrogen production upon glycerol fermentation at slightly acidic pH:effects of formate and some heavy metal ions. **6th Int. Conf. on Hydrogen Production,** Oshawa (Canada), Proc. pp. 303-313, ISBN 978-0-9781236-3-5.
13. ***Trchounian K.*** *and Trchounian A.* (2015) Different carbon sources for H_2 production by *Escherichia coli*. ***3rd Int. Conf. "Dialogues on Sciences". Book of Abstracts. Yerevan (Armenia),*** P. 40-41. ISBN 978-9939-1-0196-5.
14. ***Trchounian K.*** (2015) Application of mixture of carbon sources to enhance H_2 production by *Escherichia coli*. ***J. Biotechnol. Biomater.*** **5**: 6.
15. ***Trchounian K.*** *and Trchounian A.* (2015) *Escherichia coli* [Ni-Fe]-hydrogenases activity during glycerol fermentation upon formate supplementation. ***FEMS 2015. 6th Cong. Eur. Microbiologists, Abstracts****. Maastricht (The Netherlands)*, 0646.
16. ***Trchounian K.*** *and Trchounian A* (2015) Effect of mixture of different carbon sources on H_2 production by *Escherichia coli* during mixed-acid fermentation at slightly alkaline pH. ***ASM 115th General Meeting. Abstracts****, New Orleans (USA)* N 239.
17. ***Trchounian K.*** *and Trchounian A.* (2015) Structural and functional peculiarities of bacterial hydrogenases during mixed-acid fermentation. ***BIT 5th World Congress on Molecular and Cell Biology****, Nanjing, China, Proceedings*, p. 334.
18. ***Trchounian K.,*** *Trchounian A* (2014) Different role of focA and focB encoding formate channels for hydrogen production by *Escherichia coli* during glucose or glycerol fermentation. ***Int. J. Hydrogen Energy*** **39**, 20987-20991.
19. ***Trchounian K.,*** *Sargsyan H., Trchounian A.* (2014) Hydrogen production by *Escherichia coli* depends on glucose concentration and its combination with glycerol at different pHs. ***Int. J. Hydrogen Energy*** **39,** 6419-6423.
20. ***Trchounian K.,*** *Trchounian A.* (2014) Hydrogen producing activity by *Escherichia coli* hydrogenase 4 (*hyf*) depends on glucose concentration. ***Int. J. Hydrogen Energy*** **39**, 16914-16918.
21. ***Trchounian K.,*** Trchounian A. (2014) Clean energy technology development: hydrogen production by *Escherichia coli* during glycerol fermentation. ***Proc. 13th Int. Conf. Clean Energy*** (eds. I. Dincer, C.O. Colpan, O.Kizilkan et al.), Istanbul (Turkey), pp. 1322-1328. ISBN: 978-605-64806-0-7 www.icce-2014.net)
22. ***Trchounian K.,*** *Abrahamyan V., Vassilian A., Trchounian A.* (2014) Role of formate channel coding *focA* and *focB* genes in H_2 production by *Escherichia coli* upon glucose fermentation at slightly alkaline pH. ***Biochim. Biophys. Acta - Bioenergetics.*** **1837:** S10. p e115.
23. ***Trchounian K.,*** *Trchounian A.* (2014) Glucose concentration is distinctive for *Escherichia coli* hydrogenase 4 (*hyf*) activity. ***The FEBS J.*** **281**, 566-567.
24. ***Trchounian K.*** *and Trchounian A.* (2014) Hydrogen producing *Escherichia coli* [Ni-Fe]-hydrogenase activity during glycerol or glucose fermentation. ***Abstract***

book of Int. workshop "Trends in Microbiology and Microbial Biotechnology" *ISBN 978-5-8084-1895-0, Yerevan, Armenia.* p. 24

25. ***Trchounian K.*** *and Trchounian A.* (2014) Interrelationship between H_2 cycling and proton motive force generation in *Escherichia coli* during glycerol fermentation. ***18th Int. Biophysics Congress Abstracts***, #26, p. 5-6.
26. ***Trchounian K.*** *and Trchounian A.* (2014) Role of formate transport coding *focA* and *focB* genes in H_2 production activity by *Escherichia coli* during glycerol fermentation at slightly alkaline pH. ***ASM 114th General Meeting. Abstracts****, Boston (USA)* K-748.
27. *Poladyan A.,* ***Trchounian K.****, Sawers R. G., Trchounian A.* (2013) Hydrogen-oxidizing hydrogenases 1 and 2 of *Escherichia coli* regulate the onset of hydrogen evolution and ATPase activity, respectively, during glucose fermentation at alkaline pH. ***FEMS Microbiol. Lett.*** **348**, 143-148.
28. *Poladyan A.,* ***Trchounian K.,*** *Minasyants M., Trchounian A.* (2013) Glycerol fermentation and molecular hydrogen production by *Escherichia coli* batch cultures affected by some reducing reagents and heavy metal ions // **Black Sea Energy Resource Development and Hydrogen Energy Problems.** NATO Science for Peace and Security Series C: Environmental Security, (eds. A. Veziroglu, M. Tsitskishvili), Springer, pp 153-163, ISSN 1874-6519; ISBN 978-94-007-6151-6153.
29. ***Trchounian K.****, Blbulyan S., Trchounian A.* (2013) Hydrogenase activity and proton-motive force generation by *Escherichia coli* during glycerol fermentation. ***J. Bioenerg. Biomembr.*** **45**, 253-260.
30. ***Trchounian K.****, Soboh B., C., Sawers G., Trchounian A.* (2013) Contribution of hydrogenase 2 to stationary phase H_2 production by *Escherichia coli* during fermentation of glycerol. ***Cell Biochem. Biophys.*** **66**, 103-108.
31. ***Trchounian K.,*** *Trchounian A.* (2013) *Escherichia coli* multiple [Ni-Fe]-hydrogenases are sensitive to osmotic stress during glycerol fermentation but at different pHs. ***FEBS Lett.*** **587**, 3562-3566.
32. ***Trchounian K.,*** *Trchounian A.* (2013) H_2 producing activity by *Escherichia coli* during mixed carbon fermentation at slightly alkaline and acidic phs: novel functions of hydrogenase 4 (*hyf*) and hydrogenase 2 (*hyb*). **Black Sea Energy Resource Development and Hydrogen Energy Problems.** NATO Science for Peace and Security Series C: Environmental Security, (eds. A. Veziroglu, M. Tsitskishvili), Springer, pp 137-151, ISSN 1874-6519; ISBN 978-94-007-6151-3.
33. ***Trchounian K.,*** *Trchounian A.* (2013) *Escherichia coli* hydrogenase 4 (*hyf*) and hydrogenase 2 (*hyb*) contribution in H_2 production during mixed carbon (glucose and glycerol) fermentation at pH 7.5 and pH 5.5. ***Int. J. Hydrogen Energy*** **38**, 3919-3927.

34. ***Trchounian K.,*** *Trchounian A.* (2013) *Escherichia coli* hydrogenases and the F_0F_1-ATPase are coupled via H_2 forming and H^+ transporting pathways. ***The FEBS J.*** **280**, 186.
35. ***Trchounian K.,*** *Trchounian A.* (2013) Hydrogenase 4 activity at pH 6.5 and Fhla protein Interaction with F_0F_1-ATPase during mixed carbon fermentation by *Escherichia coli*. ***Biophys. J.*** **104**, 486a.
36. ***Trchounian K.*** *and Trchounian A.* (2013) Glucose or Glycerol: Which Fermentation Substrate is better for H_2 Production by Bacteria? ***Book of Articles and Abstracts 2nd Int. conf. of young researchers "Contribution of the young generation in the development of biotechnology"*** *Dedicated to the 70th Anniversary of the National Academy of Sciences. ISBN 978-99941-2-892-1. Yerevan, Armenia.* p. 9
37. ***Trchounian K.,*** *Sawers R.G., Trchounian A.* (2013) Proton motive force generation during glycerol fermentation: an important factor for reversibility of *Escherichia coli* hydrogenases. ***FEMS 2013, 5th Congr. Eur. Microbiologists,*** *Leipzig (Germany)*, 1677.
38. ***Trchounian K.,*** *Trchounian A.* (2013) Osmosensitivy of hydrogenases in *Escherichia coli* during glycerol fermentation at different pH. ***ASM 113th General Meeting. Abstracts,*** *Denver (USA)* K-1486.
39. ***Trchounian K.*** (2012) Transcriptional control of hydrogen production during mixed carbon fermentation by hydrogenases 4 (*hyf*) and 3 (*hyc*) in *Escherichia coli*. ***Gene*** **506**, 156-160.
40. ***Trchounian K.***, *Pinske C., Sawers G., Trchounian A.* (2012) Characterization of *Escherichia coli* [NiFe]-hydrogenase distribution during fermentative growth at different pHs. ***Cell Biochem. Biophys.*** **62**, 433-440.
41. ***Trchounian K.***, *Poladyan A., Vassilian A., Trchounian A.* (2012) Multiple and reversible hydrogenases for hydrogen production by *Escherichia coli*: Dependence on fermentation substrate, pH and the F_0F_1-ATPase. ***Crit. Rev. Biochem. Mol. Biol.*** **47**, 236-249.
42. ***Trchounian K.,*** *Marutyan S., Trchounian* A. (2012) Role of *E. coli* hydrogenases in proton motive force generation during glycerol fermentation at pH 7.5. ***Biochim. Biophys. Acta - Bioenergetics*** **1817**, Suppl 1. S153.
43. ***Trchounian K.,*** *Trchounian A.* (2012) Which hydrogenases are responsible for H_2 production by *Escherichia coli* during mixed carbon fermentation? ***The FEBS J.*** **279**, Suppl., 330.
44. ***Trchounian K.,*** *Trchounian A.* (2012) Role of different hydrogenases in H_2 production activity by *Escherichia coli* during mixed carbon fermentation at low pH. ***ASM 112th General Meeting. Abstracts,*** *San Francisco (USA)* K-2423.
45. ***Trchounian K.***, *Pinske C., Sawers G., Trchounian A.* (2011) Dependence on the F_0F_1-ATP synthase for the activities of the hydrogen-oxidizing hydrogenases 1 and 2

during glucose and glycerol fermentation at high and low pH in *Escherichia coli*. ***J. Bioenerg. Biomembr.*** **43**, 645-650.

46. *Soboh B., Pinske C., Kuhns M., Waclawek M., Ihling C.,* ***Trchounian K.,*** *Trchounian A., Sinz A., Sawers G.* (2011) The respiratory molybdo-selenoprotein formate dehydrogenases of *Escherichia coli* have hydrogen: benzyl viologen oxidoreductase activity. ***BMC Microbiology***, **11**, 173.
47. ***Trchounian K.***, *Sanchez-Torres V., Wood T.K., Trchounian A.* (2011) *Escherichia coli* hydrogenase activity and H_2 production under glycerol fermentation at a low pH. ***Int. J. Hydrogen Energy*** **36,** 4323-4331.
48. *Poladyan A., Poghosyan A.,* ***Trchounian K.,*** *Trchounian A.* (2011) Molecular hydrogen formation by *Escherichia coli* hydrogenase 3 during fermentation of glucose at slightly acidic pH. ***Biophys. J.*** **100**, Suppl. 1, 488a.
49. ***Trchounian K.***, *Sawers G., Trchounian A.* (2011) Influence of *Escherichia coli* F_0F_1-ATPase on hydrogenase activity during glycerol fermentation. ***Eur Biophys J.*** **40,** Suppl. 1, S186.
50. ***Trchounian K.,*** *Vassilian A., Sawers G., Trchounian A.* (2011) Hydrogenase activity in *Escherichia coli*: the H_2-oxidizing activity of hydrogenases 1 and 2 is modulated by the carbon source. ***17th Int. Biophys. Congress & 12th Chinese Biophys. Congress. Abstract***. *Bejing (China).* 479.
51. ***Trchounian K.***, *Sawers G., Trchounian A.* (2011) Metabolic cross-talk between *Escherichia coli* formate dehydrogenase-H and hydrogenases in hydrogen production. ***FEMS 2011. 4th Cong. Eur. Microbiologists "Advancing Knowledge of Microbes"***, Geneva (Switzerland), 2149.
52. ***Trchounian K.***, *Wood T., Trchounian A.* (2010) Hydrogen producing hydrogenases in *Escherichia coli* under glycerol fermentation at different pH. ***The FEBS J.*** **277**, Suppl. 1, 171-172.
53. ***Trchounian K.***, *Trchounian A.* (2010) Role of pH in bio-hydrogen production by *E. coli* under glycerol fermentation. ***ASM 110th General Meeting. Abstracts***, *San Diego CA (USA),* I-2174.
54. ***Trchounian K.,*** *Poladyan A., Trchounian A.* (2009) Relation of potassium uptake to proton transport and activity of hydrogenases in *Escherichia coli*, grown at a low pH. ***Biochemistry (Moscow): A. Membr. Cell Biology*** **3,** 144-150.
55. ***Trchounian K***, *Trchounian A.* (2009) Hydrogenase 2 is most and hydrogenase 1 is less responsible for H_2 production by *Escherichia coli* under glycerol fermentation at neutral and slightly alkaline pH. ***Int. J. Hydrogen Energy*** **34,** 8839-8845.
56. ***Trchounian K.,*** *Trchounian A.* (2009) Redox activity and H_2 production upon glycerol fermentation in *Escherichia coli:* are hydrogenases revesible? ***Biophys. J.*** **96**, Suppl.1, 442a.

57. ***Trchounian K.***, *Trchounian A.* (2008) The *Escherichia coli* hydrogenase activity under glycerol fermentation. ***Biochim. Biophys. Acta - Bioenergetics.*** **1777,** Suppl. 1, S95-96.

ԹՌՉՈՒՆՅԱՆ ԿԱՐԵՆ ԱՐՄԵՆԻ

Մթնային խմորում իրականացնող բակտերիաներում մոլեկուլային ջրածնի արտադրության և հիդրոգենազային ակտիվության խթանման ուղիները

Ամփոփագիր

Հանգուցային բառեր՝ *Escherichia coli*, *Clostridium beijerinckii*, մթնային խմորում, մոլեկուլային ջրածնի (H_2) կամ կենսաջրածնի արտադրություն, հիդրոգենազներ (Հիդ), հիդրոգենազային ակտիվություն, մրջնաթթու դեհիդրոգենազային ակտիվություն, pH, պրոտոնային F_0F_1-ԱԵՖազ, գլիցերոլի խմորում, ածխածնի տարբեր աղբյուրների և դրանց խառնուրդների մթնային խմորում:

Տվյալ աշխատանքը նվիրված է *E. coli*-ում և *C. beijerinckii*-ում միջավայրի pH-ի տարբեր արժեքներում ածխածնի տարբեր աղբյուրների և դրանց խառնուրդների մթնային խմորման ընթացքում Հիդ-ային ակտիվության և կենսաջրածնի արտադրության ուսումնասիրմանը:

Որպես ածխածնի աղբյուրներ օգտագործվել են գլիցերոլը, գլյուկոզը, մրջնաթթուն, քացախաթթուն: *E. coli*-ում Հիդ-ային ակտիվության և H_2-ի արտադրության ուսումնասիրման համար ստացվել և օգտագործվել են տարբեր Հիդ-ները, պրոտոնային F_0F_1-ԱԵՖազը որոշող գեներում խանգարումներով 21 մուտանտներ: Բացի այդ ուսումնասիրվել է նաև տարբեր արգելակիչների և օսմոսային սթրեսի ազդեցությունը H_2-ի արտադրության կենսագործընթացի վրա, որոշվել է *E. coli*-ում պրոտոնաշարժ ուժը և ուսումնասիրվել H_2-ի արտադրության կապը պրոտոնային F_0F_1-ԱԵՖազի հետ: Ավելին, ուսումնասիրվել է կենսաջրածնի արտադրության կախումը ածխածնի աղբյուրների կոնցենտրացիաներից: Այս խնդիրների լուծման նպատակով օգտագործվել են Հիդ-ային ակտիվության որոշման կենսաքիմիական, H_2-ի արտադրության և բջջի թաղանթում պրոտոնաշարժ ուժի որոշման մանրէաբանական, կենսաէլեկտրաքիմիական և այլ մեթոդներ: Բացի այդ *C. beijerinckii*-ում հետազոտվել է գլիցերոլի յուրացումը և H_2-ի արտադրության կախումը գլիցերոլի և տարբեր ածխածնի աղբյուրների խառնուրդներից և դրանց կոնցենտրացիաներից: Որոշվել են Հիդ-ային և մրջնաթթու դեհիդրոգենազային ակտիվությունները, խմորման արդյունքում

առաջացած վերջնանյութերը, տարբեր ծանր մետաղների ազդեցությունը Հիդ-ային ակտիվության վրա:

Կատարված հետազոտությունների արդյունքում բացահայտվել են *E. coli*-ի և *C. beijerinckii*-ի բակտերիաների աճը և H_2-ի արտադրությունը գլիցերոլի և ածխածնի տարբեր աղբյուրների ու դրանց խառնուրդների մթնային խմորման ընթացքում: Ցույց է տրվել, որ գլիցերոլի 10 գ/լ (110 մՄ) կոնցենտրացիան նպաստավոր է H_2-ի արտադրության (արագության և ելքի) համար: Հիդ-ային խանգարումներով մուտանտների օգնությամբ ցույց է տրվել, որ *E. coli*-ում Հիդ-2-ը մեծամասամբ և Հիդ-1-ը մասնակիորեն պատասխանատու են H_2-ի արտադրության համար թույլ հիմնային (pH 7,5) միջավայրում, իսկ Հիդ-3-ը և Հիդ-4-ը կարող են աշխատել H_2-ի կլանման և օքսիդացման ուղղությամբ: Թույլ թթվային (pH 6,5) և թթվային (pH 5,5) պայմաններում H_2-ի արտադրության համար պատասխանատու է Հիդ-3-ը, որը ՄՋԼ-1 համակարգի բաղադրիչներից է:

Ցույց է տրվել Հիդ-4-ի ակտիվության կախումը գլյուկոզի կոնցենտրացիայից pH 7.5-ում: Բացահայտվել է օսմոսային սթրեսի նկատմամբ *E. coli*-ի տարբեր Հիդ-ների զգայունությունն գլիցերոլի մթնային խմորման ընթացքում միջավայրի pH-ի տարբեր արժեքներում:

Որոշվել է *E. coli*-ում բջջի թաղանթով պրոտոնաշարժ ուժը գլիցերոլի խմորման ընթացքում միջավայրի pH-ի տարբեր արժեքներում: Ցույց է տրվել նրա, ինչպես նաև ներբջջային pH-ի ցածր արժեքը միջավայրի pH 7,5-ի պայմաններում գլյուկոզի խմորման համեմատ: Հայտնաբերվել է Հիդ-ների ներդրումը պրոտոնաշարժ ուժի ստեղծման մեջ:

Բացահայտվել են *C. beijerinckii*-ում Հիդ-ային և մրջնաթթու դեհիդրոգենազային բարձր ակտիվությունները գլիցերոլի և ածխածնի տարբեր աղբյուրների ու դրանց խառնուրդների մթնային խմորման ընթացքում pH 7.5-ում՝ *E. coli*-ի համեմատ: Ցույց է տրվել, որ *C. beijerinckii*-ում գլյուկոզի խմորման պայմաններում pH 7.5-ում 0.1 մՄ Fe^{3+} կամ 10 մկՄ Ni^{2+} խթանում է Հիդ-ային ակտիվությունը, իսկ գլիցերոլի և մրջնաթթվի խառնուրդի մթնային խմորման պայմաններում՝ 0.01 մՄ Fe^{2+}-ի և 1 մկՄ Ni^{2+}-ի խառնուրդը:

Առաջարկվում է H_2-ի արտադրության բարելավման նպատակով կիրառել գլիցերոլը, որպես ածխածնի էժան աղբյուր, և ածխածնի տարբեր աղբյուրների հետ նրա խառնուրդները: Տեխնոլոգիական պայմանների (pH, ածխածնի աղբյուրի կոնցենտրացիա, արգելակիչներ, որոշ մետաղների իոններ) նպաստավոր արժեքների ընտրությունը էապես մեծացնում է H_2-ի արտադրությունը և Հիդ-ային ակտիվությունը:

ТРЧУНЯН КАРЕН АРМЕНОВИЧ

Пути стимулирования производства молекулярного водорода и гидрогеназной активности у бактерий, осуществляющих темновое брожение

РЕЗЮМЕ

Ключевые слова: *Escherichia coli, Clostridium beijerinckii*, темное брожение, производство молекулярного водорода (H_2) или биоводорода, гидрогеназы (Гид), гидрогеназная активность, формиат дегидрогензная активность, pH, протонная F_0F_1-АТФаза, брожение глицерина, темновое брожение разных источников углерода и их смесей.

Настоящая работа посвящена исследованию Гид-ной активности и производства биоводорода у *E. coli* и *C. beijerinckii* при сбраживании разных источников углерода и их смесей при различных pH.

Из источников углерода были использованы глюкоза, глицерин, формиат, ацетат. Для исследования Гид-ной активности и производства биоводорода у *E. coli* получены и использованы 21 мутант с дефектами в генах, кодирующих разные Гид-ы и трансляционные факторы, а также протонную F_0F_1-АТФазу. Также были изучены эффекты разных ингибиторов и осмотического стресса на производство H_2 у *E. coli*, определена протоно-движущая сила и связь производства H_2 с протонной F_0F_1-АТФазой. Были исследованы также зависимость производства биоводорода от концентраций источников углерода. Для решения поставленных задач использовали биохимические методы определения Гид-ной активности, микробиологические, биоэлектрохимические и другие методы определения производства H_2 и протон- движущей силы. Кроме того, были исследованы утилизация глицерина и зависимость производства H_2 от глицерина и разных смесей источников углерода и их концетраций у *C. beijerinckii*. Были определены Гид-ная и формиат дегидрогензная активность, конечные продукты брожения, влияние разных тяжелых металлов на Гид-ную активность.

В результате исследований были показаны рост *E. coli* и *C. beijerinckii* и производство H_2 при сбраживании глицерина и разных источников углерода и их смесей. Было установлено, что оптимальной концетрацией глицерина для производства H_2 является 10 г/л (110 мМ). Более того с помощью разных Гид-ных мутантов выяснено, что за производство H_2 у *E. coli* в слабощелочной среде (pH 7,5)

ответственны Гид-2 и частично Гид-1, а Гид-3 и Гид-4 работают в направлении окисления H_2. В слабокислой (pH 6,5) и кислой (pH 5,5) среде за производство H_2 ответственна Гид-3, которая является компонентом комплекса формиат водород лиазы-1 (ФГЛ-1). Была показана зависимость активности Гид-4 от концентрации глюкозы при pH 7,5. Обнаружена чувствительность Гид к осмотическому стрессу при разных pH при брожении глицерина. Определена протон-движушая сила на мембране клеток в условиях сбраживания глицерина при разных pH. Показано низкое значение протон-движушей силы и внутриклеточного pH по сравнению с брожением глюкозы. Выявлена роль Гид при генерировании протон-движушей силы.

Была показана высокая специфическая активность Гид и формиат дегидрогеназ у *C. beijerinckii* по сравнению с *E. coli* при брожении глицерина и разных источников углерода и их смесей при pH 7,5. Также установлено, что у *C. beijerinckii* при брожении глюкозы при pH 7,5 0.1 мМ Fe^{3+} или 10 мкМ Ni^{2+} стимулирует Гид-ную активность, а при брожении глицерина и формиата стимулирование было показано только при использовании 0.01 мМ Fe^{2+} и 1 мкМ Ni^{2+}.

Было предложено для улучшения производства H_2 использовать глицерин, как дешевый источник углерода, и разные смеси источников углерода со смесью с глицерином. Выбор оптимальных технологических условий (pH, концентрация усточников углерода, ингибиторы, некоторые ионы металлов) может привести к существенному росту Гид-ной активности и производства H_2.