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գիտական աստիճանի հայցման ատենախոսության

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MIRZOYAN SATENIK NORIK

OXIDATION OF MIXTURE OF CARBON SOURCES DURING FERMENTATION IN *ESCHERICHIA COLI*

SYNOPSIS

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

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Ատենախոսության թեման հաստատվել է Երևանի պետական համալսարանում

Գիտական ղեկավար՝ Կենս. գիտ. դոկտոր, դոցենտ Կ. Ա. Թռչունյան

Պաշտոնական ընդդիմախոսներ՝ Կենս. գիտ. դոկտոր, պրոֆեսոր
Պ. Ա. Ղազարյան
Կենս. գիտ. թեկնածու, դոցենտ
Հ. Լ. Հայրապետյան

Առաջատար կազմակերպություն՝ Հայաստանի ազգային ագրարային համալսարան

Ատենախոսության պաշտպանությունը տեղի կունենա 2020թ. մայիսի 5-ին ժամը 14^{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 2020թ. մարտի 25-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: Dr. of Biological Sciences,
Associate Professor K. A. Trchounian

Official opponents: Dr. of Biological Sciences,
Professor P. A. Ghazaryan
PhD., Associate Professor H. L. Hayrapetyan

Leading organization: Armenian national agrarian university

The defense of the dissertation will be held on 5th May, 2020, at 14^{00}, at the session of 051 Scientific Specialized Council on Biophisics 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 25th March 2020.

Scientific Secretary of 051 Specialized Council,

PhD., Associate Professor M. A. Parsadanyan

INTRODUCTION

Topic's significance. The current situation of shrinking fossil fuel resources and continually increasing energy demand requires the identification of efficient, renewable and "eco-friendly" new sources of energy (Hosseini & Wahid, 2016). As an alternative source of energy, H_2 can be obtained by various methods such as electrolysis of water, conversion of natural gas, and hydrogen production from coal. Many of these ways are not economically feasible; the price and the sources of its production are quite expensive and inaccessible for many countries. For this reason, it is recommended to obtain H_2 biologically, which is the oxidation of carbon-containing compounds (glucose, glycerol, lactose, formate, acetate, etc.) by microorganisms during dark- and photo-fermentation (Trchounian, 2015; Trchounian et al., 2017). For this purpose, the use of H_2 producing bacteria is of great interest. Among known bacteria *Escherichia coli* is the best genetically and biochemically characterized bacterium which produces H_2 during mixed-acid fermentation under different environmental conditions (Sawers, 2005; Trchounian, 2015). *E. coli* produces H_2 by multiple and reversible hydrogenase (Hyd) enzymes. This bacterium has the ability to encode four membrane bound [Ni-Fe] Hyd enzymes which are active towards H_2 generation or uptake depending on carbon source/s, pH and other factors (Trchounian & Trchounian, 2009; Trchounian et al., 2012). Important factors for H_2 production are anaerobic conditions, pH, temperature, carbon sources (Pinske et al., 2015; Trchounian et al., 2013).

In recent years biodiesel production has reached large volumes generating glycerol as a by-product: with every 10 kg biodiesel produced 1 kg glycerol is being generated (Valerio et al., 2015). This determines the availability of glycerol as carbon source for *E. coli*.

Power stations, engines, cars, and other vehicles are created based on the usage or application of H_2. Some car makers have already developed engines using H_2, not petrol or diesel (Alazemi et al., 2015; Eckert & Trinh, 2016).

Nowadays, there is a great interest in the H_2 production from organic wastes, which contain mixtures of different carbon sources. It is necessary to understand and clarify the oxidation and biochemical properties of mixtures of carbon sources by regulation of metabolic pathways.

Research goals and tasks. The main purpose of the work was to study oxidation of mixtures of different carbon sources (glucose, glycerol, lactose, acetate, etc.) in *E. coli* during fermentation at different pHs (7.5, 6.5, 5.5).

Constituted tasks of the research are the followings:

1. To study the effect of various external factors (pH, carbon sources and their concentrations) on H_2 production during fermentation.
2. To reveal the enzymes responsible for H_2 production during fermentation of different carbon sources.
3. To investigate the metabolic cross-talk and interaction of Hyd enzymes under fermentation of different carbon sources mixtures using mutants with defects in Hyd enzymes.
4. To determine and optimize H_2 production conditions by applying different mutants with defects in Hyds, which will promote the oxidation of carbon sources to H_2.

Scientific novelty and practical value of the study. Research has found that carbon sources have an effect on the physiological working direction of Hyd enzymes. Especially the formate dependent H_2 production of Hyd-4 function has been demonstrated during fermentation of glucose, glycerol and formate mixtures at pH 5.5 and 7.5. This observation about Hyd-4 is novel, and gives new insights into understanding the H_2 metabolism, the Hyd activity and the H_2 cycling regulation. In *E. coli hybC* (lacking large subunit of Hyd-2) mutant prolonged and enhanced yield of H_2 production up to 240 h was determined, which is a novelty that was not previously shown. This may have an important impact for the large scale H_2 production by different biochemical pathways. In addition, in *E. coli* wild type ~3 fold higher H_2 was observed during glycerol and lactose mixture fermentation, which means that crude glycerol and dairy waste containing large amounts of lactose can be mixed and significantly improve H_2 production yield. It has been shown that pH 6.5 and 5 g L^{-1} of lactose concentration are the most optimal conditions for cells growth. It has been found that low pH has inhibitory effect on the cell growth during utilization of a mixture of the acetate and/or glycerol. The obtained results indicate the ways of regulating the activity of various Hyd enzymes, their interactions and the biochemical properties of fermentation of different carbon sources in *E. coli*. From the results obtained it can be concluded that different carbon sources mixtures and their oxidation to H_2 can contribute to increase its yield by applying different Hyd mutants and different organic wastes materials. This approach has practical impact for the development of advanced biological H_2 production technology.

Main points to present at the defense.

1. *E. coli* performs mixed fermentation in the presence of mixtures of different carbon sources (glucose, glycerol, formate) and produces H_2; Hyd-4 has a formate-dependent

function during fermentation of this mixture under acidic and slightly alkaline conditions.

2. In *E. coli hybC* mutant H_2 production is prolonged and stimulated for up to 240 h during fermentation of mixed carbon sources (glucose, glycerol, formate) at pH 7.5.

3. Hyd-3 is mainly and Hyd-4 partially responsible for H_2 production during fermentation of the mixture of acetate and glycerol.

4. *E. coli* utilizes lactose and/or glycerol at different pHs; The most optimal conditions for cells growth are pH 6.5 and 5 g L^{-1} of lactose concentration.

Work approbation. Main results of the dissertation were discussed at seminars in Department of Biochemistry, Microbiology and Biotechnology, Faculty of Biology, Yerevan State University (Armenia), and at scientific conferences and meetings, namely US Biophysical Society 60th Annual Meeting (Los Angeles, USA, 2016), VAAM Annual Conference 2016 of the Association for General and Applied Microbiology (Jena, Germany, 2016), ASM MICROBE 2017 General Meeting (New Orleans, USA, 2017), FEMS 2017 - 7th Congress of European Microbiologists (Valencia, Spain, 2017), 22nd World Hydrogen Energy Conference (Rio de Janeiro, Brazil, 2018), The 20th European Bioenergetics Conference (Budapest, Hungary, 2018), ASM MICROBE 2019 (San Francisco, USA, 2019), 44th FEBS Congress (Krakow, Poland, 2019).

Publications. According to experimental data observed in dissertation, 15 publications, including 6 articles and 9 abstracts in peer-reviewed journals were published.

Volume and structure of dissertation. The dissertation contains following chapters: introduction, literature review (Chapter 1), experimental part (Chapter 2), results and discussion (Chapter 3), concluding remarks, conclusions and cited literature (total 121 papers and books). The document consists of 116 pages, contains one table and 32 graphs and figures.

MATERIALS AND METHODS

Bacteria. The *E. coli* BW 25113 or MC 4100 parental wild type strains and single mutants with defects in subunits of different Hyds from the Keio collection used are listed in Table 1. The strains were provided by Prof. Sawers (Martin-Luther University of Halle-Wittenberg, Germany), Prof. Thomas Wood (Penn State University, University Park, PA, USA) and Prof. Simon Andrews (University of Reading, UK).

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

Strain	Genotype	Appropiate absent or defective proteins	Reference
BW 25113	*rrnB DlacZ4787 HsdR514 D(araBAD)567 D(rhaBAD)568 rph-1*	Wild type	Baba et al., 2006
JW 2472*	BW 25113 *ΔhyfG*	Large subunit of Hyd-4	Andrews et al., 1997
JW 0955*	BW 25113 *ΔhyaB*	Large subunit of Hyd-1	Maeda et al., 2007
JW 2962*	BW 25113 *ΔhybC*	Large subunit of Hyd-2	Maeda et al., 2007
JW 2691*	BW 25113 *ΔhycE*	Large subunit of Hyd-3	Baba et al., 2006
MW 1000	BW 25113 *ΔhyaB ΔhybC*	Large subunits of Hyd-1 and Hyd-2	Trchounian et al., 2015
MC 4100	*araD139 ΔlacU169 rpsL thi fla*	Wild type	Bagramyan et al., 2002
JRG 3633	MC4100 *ΔhycE ΔhyfB-R;*	Subunits of Hyd-3 and Hyd-4	Mnatsakanyan et al., 2002
FTD 147	MC 4100 *ΔhyaB ΔhybC ΔhycE*	Large subunits of Hyd-1-Hyd-3	Skibinski et al., 2002
FTD 150	MC4100 *ΔhyaB ΔhybC ΔhycE ΔhyfG*	Large subunits of Hyd-1-Hyd-4	Skibinski et al., 2002
FM 460*	MC 4100 *ΔselC*	$tRNA^{sec}$	Trchounian et al., 2012

*Resistant to kanamycin

Bacterial growth and cultivation conditions. Bacterial cells were grown under anaerobic fermentative conditions at 37 ^{0}C for 18-20 h in buffered growth medium containing peptone (20 g L^{-1}) at pH of 7.5, 6.5 and 5.5, with salt compositions as follow: 15 g L^{-1} K_2HPO_4, 1.08 g L^{-1} KH_2PO_4 and 5 g L^{-1} NaCl (pH 7.5); 7.4 g L^{-1} K_2HPO_4, 8.6 g L^{-1} KH_2PO_4 and 5 g L^{-1} 1 NaCl (pH 6.5), and 1.08 g L^{-1} K_2HPO_4, 15 g L^{-1} KH_2PO_4 and 5 g L^{-1} NaCl (pH 5.5). The medium was simultaneously supplemented when indicated with the following carbon sources: glucose (2 g L^{-1}), glycerol (10 g L^{-1}), formate (0.68 g L^{-1}), acetate (1-5 g L^{-1}) and lactose (1-5 g L^{-1}). Kanamycin (25 μg ml^{-1}) was also added when appropriate (see Table 1). In some experiments bacteria were incubated for 5-7 minutes with *N,N'*- dicyclohexylmethanediimine (DCCD).

Determination of optical density and specific growth rate of bacteria. The bacterial biomass growth was monitored with spectrophotometer following the optical density (OD) readings of bacterial culture absorbance under 600 nm wavelength. The bacterial specific growth rate (μ) stated, as lg2/doubling time, was calculated where the logarithm of OD was grown linearly with time (Trchounian et al., 2012).

Determination of external or medium pH during growth. The medium or external pH was determined using a pH-meter with selective pH-electrode and appropriately adjusted using 0.1 M NaOH or HCl.

Determination of redox potential and H_2 production assays. Oxidation-reduction potential (E_h) measurements and H_2 production determination were done by using two different redox titanium-silicate (Ti-Si) and platinum (Pt) glass electrodes. The Ti-Si-electrode measures the overall E_h, whereas the Pt electrode is sensitive to H_2 under anaerobic conditions (Poladyan et al., 2017). The H_2 production rate (V_{H2}) of bacteria calculated as the difference between the initial rates of decrease in Pt and Ti-Si electrodes readings and expressed in mV of E_h per min per mg dry weight of bacteria (Trchounian, 2012). The H_2 production yield was calculated by ORP values and expressed in mmol H_2 per L of growth medium (mmol L^{-1} H_2). H_2 production was confirmed by the chemical assay (Maeda and Wood, 2008) and Durham tube method (Bagramyan et al., 2002). Dry weight of bacteria was determined as described (Mirzoyan et al., 2017).

Data processing. Three independent experiments were done and the average data were calculated with the standard errors, was not more than 3% if they are not represented. The validity of differences between experimental and control data was evaluated by Student's criteria (p); the difference was valid if $p < 0.05$.

RESULTS AND DISCUSSION

Effect of mixture of carbon sources on *E. coli* wild type and hydrogenase mutants growth at different pHs

To investigate the growth properties of *E. coli* and to study role of Hyd enzymes during utilization of the mixture of glucose, glycerol and formate wild type and mutants with defects in various Hyd enzymes (see Table 1) were grown at different pH values (pH 7.5-5.5). *E. coli* wild type cell's μ was highest (1.05 ± 0.05 h^{-1}) at pH 6.5 compared to other tested pHs (Fig. 1). The cells were grown at pH 7.5 μ was 0.945 ± 0.05 h^{-1} and it was less at low pH resulting in μ of 0.72 ± 0.03 h^{-1}. The data can be explained if external formate added in the growth medium might affect cell growth rate and thus at low pH it is the least. It is known that formate, like acetate, as a weak acid might have negative effect on cells via being imported into the cells and thus being toxic have uncoupler effect on the proton motive force generation (Russel & Diez-Gonzalez, 1998; Trchounian & Trchounian, 2019). Earlier it was shown that when cells were grown upon fermentation of mixture of glycerol and formate had the similar μ as in the presence of mixture of glucose, glycerol and formate (Fig. 1) (Trchounian & Trchounian, 2015). Moreover, when cells were grown in the presence of mixture of glycerol and acetate μ was less than in the mixture of glycerol and formate (Trchounian & Trchounian, 2015) or in mixture of glucose, glycerol and formate.

Interestingly, in the case when wild type cells were grown in the presence of mixture of glucose, glycerol and formate at pH 7.5 μ was ~1.3 fold stimulated compared to the cells grown on mixture of glucose and glycerol (Fig. 1). This data might indicate that formate has some effects on cell growth via effecting enzyme activities or having up-regulation of genes which products might be important for cell growth and further energy generation during anaerobic conditions (Kirkpatrick et al., 2001; Sawers & Bock, 1998). At low pH when formate is imported into the cells one of the ways for neutralization of formate is to oxidize it via FHL-1 complex, which consists of Hyd-3 and FDH-H, to H_2 and CO_2. This idea is confirmed by analyzing the growth of Hyd mutants with defects in large subunits of Hyd-1 to 4.

In *hycE* single mutant (lacking large subunit of Hyd-3, see Table 1), μ was 0.17 ± 0.02 h^{-1} which is ~4.14 fold less than in wild type. These data directly suggest that when Hyd-3 is absent cell growth is disturbed due to being not able to neutralize formate. It has been suggested that Hyd enzymes might compensate each other, the

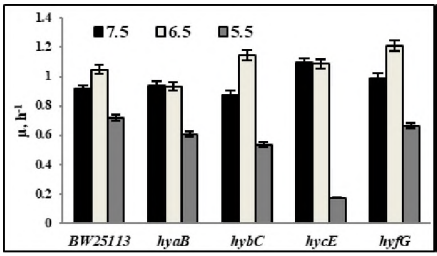

Fig. 1. Specific growth rate (μ) of *E. coli* BW 25113 wild type and mutants with defects in Hyd-1 to Hyd-4 in batch culture in buffered peptone medium at 37 ^{0}C in the presence of the mixture of 2 g L^{-1} glucose, 10 g L^{-1} glycerol and 0.68 g L^{-1} formate at different pHs. For strains see Table 1, for others see Materials and methods.

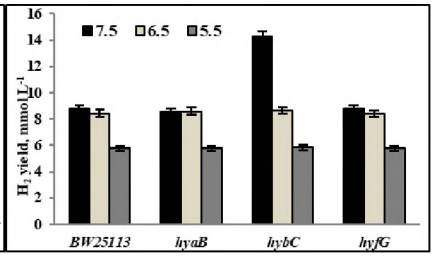

Fig. 2. H_2 production yield by *E. coli* wild type and Hyd mutants in the presence of the mixture of glucose, glycerol and formate at pH range of 5.5-7.5. For others see Materials and methods and legends to Fig. 1.

idea has been also suggested by Lukey et el. (2010), but in this case no any other Hyd enzyme has big impact on growth. Especially, in *hyaB* or *hybC* single mutants μ was 0.61 ± 0.03 h^{-1} and 0.54 ± 0.02 h^{-1}, respectively. This was ~1.17 and ~1.33 fold less than in wild type, respectively (see Fig. 1). In general, it is known that Hyd-1 and Hyd-2 are H_2 uptake enzymes and has no any impact on cell growth (Vignais & Billoud, 2007) but the data obtained might spread new idea that different Hyd enzymes depending on external pH and maybe other factors are playing important role during growth. In *hyfG* single mutant cells have similar μ as in wild type suggesting that the only Hyd enzyme which can neutralize formate at pH 5.5 is Hyd-3. When cells were grown at pH 7.5 either wild type or Hyd mutants had similar μ suggesting that in this case both Hyd-3 and Hyd-4 are active and neutralize formate via producing H_2. The same results were obtained for pH 6.5. When compared to the cells grown on the mixture of glycerol and formate, it was shown that Hyd-2 had significant impact on cell growth (Trchounian & Trchounian, 2015). These data again confirm the idea that depending on various factors Hyd enzymes might play important role in cell growth.

Prolonged and enhanced H_2 production during utilization of mixture of glucose, glycerol and formate at different pHs in *E. coli*

As was mentioned above the main aim of the work was to establish if *E. coli* can grow and evolve H_2 during fermentation of glucose, glycerol and formate and what are the common growth and other properties of external parameters. Next step of the study will be to clarify carbon balance and H_2 yield per substrate utilized. More biochemical study is required to understand clearly the metabolic shift and cross talk between various membrane bound enzymes during *E. coli* growth. It was shown that at all tested pHs (5.5-7.5) *E. coli* wild type was producing H_2. Moreover, H_2 generation was detected

during relatively long period (Fig. 3). Especially, at pH 7.5 wild type cells produced H_2 during ~216 h and yielded 8.8 mmol L^{-1} H_2 (Fig. 3).

There are numerous data on H_2 production from different carbon sources, but they vary depending on external factors such as pH, carbon sources and their concentration, bacteria etc. (Trchounian et al., 2017). The obtained data clearly showed the enhancing effect of using triple carbon source on H_2 yield in *E. coli* BW 25113 wild type and *hybC* mutant strains. This is important from economic point of view for largescale H_2 production. The prolongation of the H_2 production is significant in the presence of three different carbon sources. Moreover, in *hybC* mutant H_2 production was monitored up to ~240 h, besides, H_2 yield was ~1.62 fold higher than in wild type reaching 14.24 mmol H_2 L^{-1} (see Fig. 3A). So this mutant could be further applied for enhanced H_2 production. In *hyfG* single mutant H_2 production was detected during ~192 h (see Fig. 3B). H_2 yield in *hyaB* and *hyfG* mutants at this pH is same as in wild type. In *hycE* mutant H_2 production was absent clearly demonstrating that Hyd-3 is responsible for H_2 evolution.

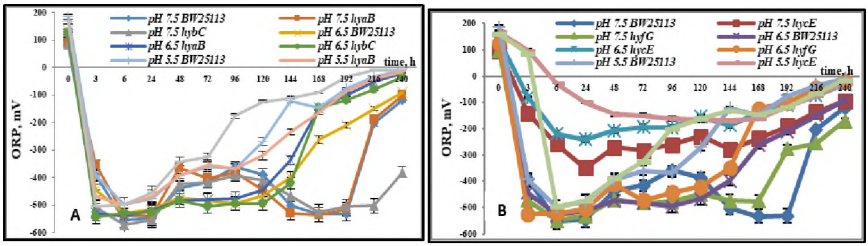

Fig. 3. The ORP kinetics and H_2 production of *E. coli* BW 25113 wild type and mutants with defects in Hyd-1 and Hyd-2 (A) and Hyd-3 and Hyd-4 (B) grown in buffered peptone medium at 37 °C in the presence of the mixture of glucose, glycerol and formate. For others see Materials and methods and legends to Fig. 1.

When wild type cells were grown at pH 6.5 H_2 generation time was shortened and monitored during ~144 h which was less that at pH 7.5. This might be due to changed metabolism and low external pH. At pH 6.5 in all mutants H_2 production and H_2 yield was the same as in wild type (see Fig. 2). At pH 5.5 wild type cells produced H_2 during ~120 h which was least compared to pH 7.5 and pH 6.5 (see Fig. 3). At pH 6.5 and pH 5.5 again Hyd-3 was responsible for H_2 production. Interestingly, in *hybC* and *hyfG* single mutants H_2 evolution was abolished ~48 h which was not the case for wild type (see Fig. 3). This might be due to metabolic changes affecting Hyd enzymes, and all enzymes started to produce H_2 for neutralizing the external formate and maintaining stable the cytoplasmatic pH and thus proton motive force generation (Trchounian & Sawers, 2014). Moreover, disturbance of H_2 cycling between H_2 uptake and producing enzymes might change the operation direction of Hyd enzymes towards H_2 production.

At this pH in wild type and mutants H_2 yield (5.76 mmol H_2 L^{-1}) (see Fig. 2) was similar to each other and was ~1.5 fold less than at pH 7.5.

H_2 production during utilization of glucose, glycerol and formate mixture at various pHs

To determine the role of different Hyd enzymes in H_2 producing metabolic pathways during utilization of various carbon sources (glucose, glycerol and formate), the H_2 production rate by different *E. coli* Hyd mutants was investigated and compared with the wild type strain. In BW 25113 wild type (Fig. 4, A) or MC 4100 in glycerol assays at pH 7.5, V_{H2} was ~4 mmol min^{-1} (g dry weight)$^{-1}$. Similar V_{H2} (Fig. 4, A) was determined for single and double mutant strains devoid of large subunits of Hyd-1 and/or Hyd-2 (see Table 1). As observed in Fig. 4, A, the assay of the *hyfG* mutant revealed a ~2 fold increase in V_{H2} suggesting that under these conditions (glycerol assay, pH 7.5) Hyd 4 functions in the H_2 uptake direction. To further understand the role of the Hyd-4 enzyme in H_2 metabolism under these conditions, the FTD147 triple and FTD150 quadruple mutants (see Table 1) were assayed for H_2 production at pH 7.5. In FTD147 the assay with glycerol revealed that V_{H2} was decreased ~4 fold compared with wild type and negligible in FTD150 (Fig. 4, A), indicating that H_2 production was hardly affected. Moreover, the results for FTD147 demonstrate that Hyd-4 must be responsible for the residual H_2 production.

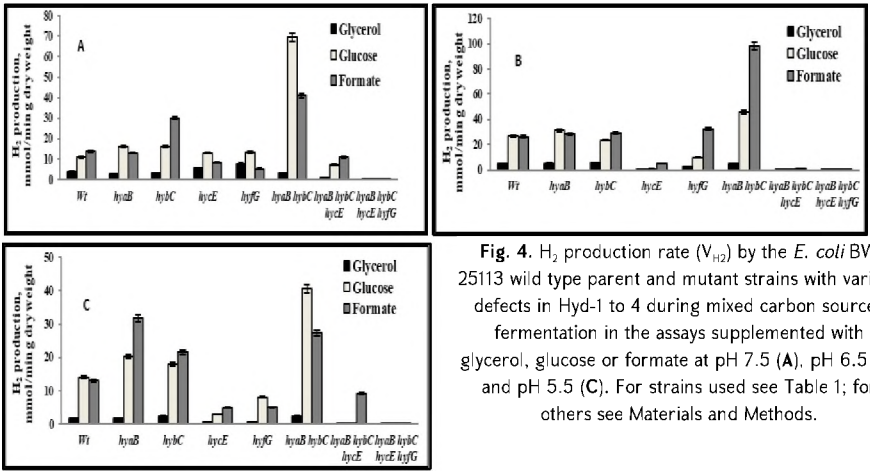

Fig. 4. H_2 production rate (V_{H2}) by the *E. coli* BW 25113 wild type parent and mutant strains with various defects in Hyd-1 to 4 during mixed carbon sources fermentation in the assays supplemented with glycerol, glucose or formate at pH 7.5 (**A**), pH 6.5 (**B**) and pH 5.5 (**C**). For strains used see Table 1; for others see Materials and Methods.

Biohydrogen production rate by wild type cells was doubled at pH 7.5 in the glucose assay in comparison to supplementation of glycerol (see Fig. 4, A). The increase

in V_{H2} was especially significant for the *hyaB hybC* double mutant, being of ~6.3 fold higher (see Fig. 4, A). V_{H2} in the FTD147 triple mutant decreased only slightly (~1.5 fold), indicating that Hyd-4 was responsible for 62% of the H_2 production under these conditions. This was confirmed by analysis of the FTD150 mutant (see Table 1) where H_2 production was essentially absent. Based on the observation of the large decrease (~10 fold) in V_{H2} when comparing the FTD147 triple mutant and the *hyaB hybC* double mutant, it can be confirmed that Hyd-3 also makes a major contribution towards H_2 production. Addition of glucose, glycerol or formate in the assays at pH 7.5 revealed the ability for a dual role of the same Hyd enzyme, confirming the reversibility and the activation of all enzymes.

It was shown that at pH 6.5 for continuous H_2 production, the activity of two Hyd enzymes might be important, and Hyd-3 and Hyd-4 seem to such a pair of enzymes establishing a H_2 cycle in both directions: producing and oxidizing H_2, respectively. When the *hyfG* mutant was grown on glycerol and formate mixture, or on glucose only, and employed consequently for formate assays, no activity or contribution of Hyd-4 in H_2 metabolism and evolution was found (Trchounian & Trchounian, 2015). Therefore, the carbon source added to the assays has clear effect on total Hyd enzyme activity whose suggested function is to maintain the H_2 cycle and cytoplasmic pH.

H_2 production by *E. coli* during mixed carbon sources fermentation in wild type cells at pH 5.5 upon glycerol supplementation was the same as at pH 6.5 and pH 7.5 (see Figs. 4 A, B, C). However, in glucose-supplemented assays it was comparable only to pH 7.5 (see Figs. 4 A, B, C). Glycerol assay of *hycE* or *hyfG* single mutants resulted in a similar V_{H2} decrease of ~3 fold compared to wild type (see Fig. 4C). No role for Hyd-1 or Hyd-2 was detected, which is in contrast with the fact that the same Hyd enzymes were reported as responsible for H_2 oxidation when grown on glycerol only (Trchounian et al., 2011).

Hydrogen production in glucose assays was strongly Hyd-3 but not Hyd-4 dependent. That is inferred from the following observations: decrease of V_{H2} in *hycE* compared to wild type (~4.8 fold) and negligible H_2 production in *hyaB hybC hycE* mutant (see Fig. 4C). The main H_2 uptake activity is contributed by Hyd-1 in glucose assay. H_2 production in formate assays was similar in wild type cells at pH 5.5 and pH 7.5, but not at pH 6.5 (see Fig. 4, A). Moreover, in *hycE* or *hyfG* single mutants H_2 production was decreased ~2.6 fold, and in *hyaB hybC* double mutant V_{H2} was increased ~2.1 fold, compared to wild type. Taken together it can be concluded that Hyd-3 and Hyd-4 were responsible for H_2 production upon formate supplementation, probably for neutralization of formate via FHL complex.

As reported for other pHs, during mixed carbon sources fermentation at pH 5.5 the activities of Hyd enzymes differ depending on the substrate added. The clear difference might be explained via physiological conditions and requirements of the cell to act in one or another direction. So, for example when a weak acid such as formate is

externally added, the cells act to neutralize it and switch two using Hyd enzymes (Hyd-3 and 4) for neutralization via H_2 generation. A relevant consequence is that Hyd-4 is also formate-dependent at pH 5.5.

E. coli growth and H_2 production during mixed carbon fermentation in assays supplemented with acetate and glycerol at different pHs

The role of acetate and glycerol on ORP kinetics and H_2 production and the relactionship to F_0F_1-ATPase was investigated in single *hyaB, hybC, hycE, hyfG*, multiple *hycE hyfB-R* and *hyaB hybC hycE* mutants (see Table 1) during growth at both alkaline and acidic pHs up to ~200 h. In *E. coli hyaB* and *hycE* single mutants the μ was same compared with wild type at pH 7.5 (Fig. 5, A). In *hycE hyfB-R* the μ was decreased ~1.2 fold, however in *hybC* and *hyfG* mutant strains the μ was increased ~1.3 and ~1.2 fold, respectively, compared with wild type (Fig. 5, A). It was determined the μ in the presence of DCCD inhibitor, during which only in wild type was stimulated a little, but in all mutant strains the DCCD had an inhibitory effect. This can suggest that F_0F_1-ATPase has a role in bacterial growth at pH 7.5.

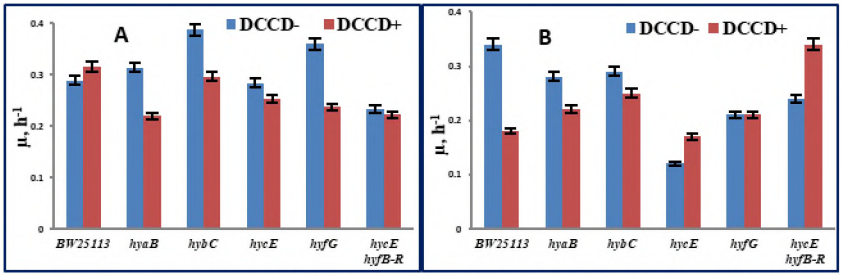

Fig. 5. Specific growth rate (μ) of *E. coli* BW 25113 wild type and mutants with defects in Hyd-1 to Hyd-4 during mixed carbon fermentation in the assays supplemented with acetate and glycerol at pH 7.5 (**A**) and pH 6.5 (**B**). For mutant strains see Table 1.

It was determined that H_2 production was produced in *hyaB* and *hybC* single mutant strains from 6 h, however in wild type from 24 h (Fig. 6, A). In *hyfG* mutant strain the H_2 production started from 48 h and lasted up to 200 h. It suggests that *hyfG* is the best mutant strain in this condition for H_2 production. However, it was not detected in *hycE* and multiple mutant strains suggesting that Hyd-3, but not Hyd-4, is responsible for H_2 production during fermentation of acetate and glycerol at pH 7.5 (Fig. 6, A).

The μ in *hycE* mutant strain was decreased ~2.8 fold, which suggests, that Hyd-3 has an important role during acetate and glycerol fermentation at pH 6.5. DCCD had inhibitory effect on μ in wild type, *hyaB* and *hybC* single mutants ~1.9, ~1.2 and ~1.2

fold, respectively. But μ was stimulated ~1.4 fold in *hycE* and *hycE hyfB-R* mutants compared to wild type (Fig. 5, B).

The H_2 production at pH 6.5 was determined in wild type from 24 h, but in *hyaB* and *hybC* mutant strains it was detected from 6 h, which suggests that the absence of Hyd-1 and Hyd-2 enzymes had negative effect on H_2 production generation time (Fig. 6B). It was not observed for *hycE* and *hyfG* mutant strains, which suggests that Hyd-3 and Hyd-4 are responsible for H_2 production at pH 6.5. The double and triple mutants clarified that Hyd-3 and Hyd-4 together are responsible for H_2 production (Fig. 6, B).

At pH 5.5 no H_2 production was determined in all assays during acetate and glycerol utilization. It suggests that pH is important for H_2 production and pH 5.5 is not optimal condition during fermentation of mixture of acetate and glycerol.

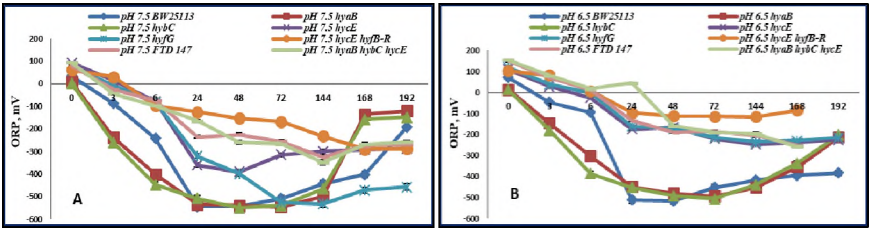

Fig. 6. The ORP kinetics and H_2 production of *E. coli* BW 25113 wild type and mutants with defects in Hyd-1 to Hyd-4 during mixed carbon fermentation in the assays supplemented with acetate and glycerol at pH 7.5 (**A**) and pH 6.5 (**B**). For mutant strains see Table 1.

Hyd-3 and Hyd-4 contribution to H_2 production in *E. coli* during lactose fermentation at different pH

During fermentation of lactose in *E. coli* batch cultures ORP decreased to low negative values (≥-450-500 mV) in all tested concentrations of lactose showing that *E. coli* produced H_2 starting from 6 h of growth at pH 7.5 and 6.5 (Fig. 7 A, B). Note, that without any carbon source ORP decreased till -150 mV which clearly shows that no H_2 production can be detected. Compared to glucose fermentative conditions, the H_2 generation was delayed by 3 h which might be possible as lactose must be further metabolized into glucose and galactose and after the cells will generate H_2 which needs time (Rosales-Colunga et al., 2012). Moreover, at high pH the H^+ symport might occur with different mechanism which also affects the cell growth and thus the end product generation. But at low pH (pH 5.5) H_2 was evolved at 3 h of growth (Fig. 7, C). It might be due to the higher flow of protons into the cells for lactose utilization and thus cells start to produce H_2 earlier to equilibrate the proton gradient across the membrane and thus proton motive force generation.

Further to understand which Hyd enzyme is responsible for H_2 production, Hyd-3 *(hycE)* or Hyd-4 *(hyfG)* single and Hyd-3 and Hyd-4 *(hycE hyfB-R)* mutants had been

studied. At all pHs in *hycE* mutant no any H_2 was detected during 48 h. The data suggested that Hyd-3 is active and responsible for H_2 production disregarding pH value and lactose concentration (Fig. 7).

But interestingly, the same situation occurred with *hyfG* single mutant, which means that besides Hyd-3, also Hyd-4 is responsible for H_2 generation (see Fig. 7). In addition, at all tested pHs no H_2 production was detected in *hycE hyfB-R* mutant. The absence of H_2 generation in Hyd-3 or Hyd-4 mutants might be explained also if these enzymes are involved in H_2 and proton cycling, as suggested (Pinske & Sargent, 2016) and the absence of one Hyd enzyme results in the alternation of the other one.

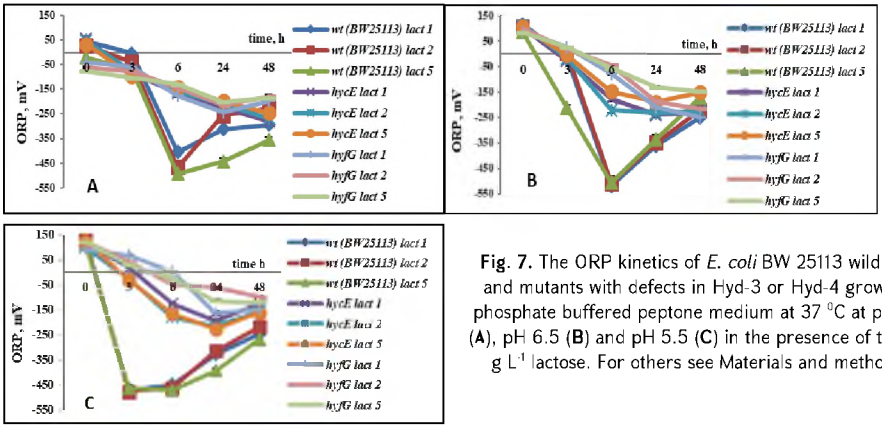

Fig. 7. The ORP kinetics of *E. coli* BW 25113 wild type and mutants with defects in Hyd-3 or Hyd-4 grown in phosphate buffered peptone medium at 37 °C at pH 7.5 (**A**), pH 6.5 (**B**) and pH 5.5 (**C**) in the presence of the 1-5 g L^{-1} lactose. For others see Materials and methods.

CONCLUDING REMARKS

Co-utilization of the mixture of carbon sources (sugar, alcohol and organic acid) might significantly increase the H_2 yield by applying Hyd mutants. It was determined in *E. coli* batch cultures at pH 7.5 the prolonged H_2 production during ~240 h which is a new phenomenon not considered or revealed before. This is important for applying in large scale H_2 production. Moreover, it has been clearly demonstrated pH dependent effect of Hyd enzymes on *E. coli* growth, especially Hyd-3 at pH 5.5 had big impact on growth of *E. coli*. This would lead to the double benefit in effective energy production and management of organic wastes.

The physiological direction of Hyd enzymes is affected by the fermentation of carbon sources. In particular, formate dependent Hyd-4 activity was demonstrated. At low pH mainly Hyd-4 was responsible for H_2 production in formate assays. Enhanced H_2 production was detected in formate supplemented assays when Hyd-1 and Hyd-2 activity were absent. Thus, all Hyd enzymes can either work in H_2 uptake or production directions at a wide range of external pH depending on the carbon source added. They

are likely to regulate the pH gradient across the membrane via producing or taking up H_2. It is suggested that Hyd enzymes are proton sensors and act in H_2 production or uptake direction when proton concentration inside or outside the cell is changed.

E. coli is able grow well during anaerobic utilization of lactose at different pHs: pH 6.5 and 5 g L^{-1} lactose concentration were optimal conditions for cell growth. This is important in optimization of technological conditions for biomass and H_2 production if lactose is applied. New previously unidentified role of Hyd-4 has been shown: disregarding the pH and lactose concentration Hyd-4 with Hyd-3 are responsible for H_2 evolution.

It has been shown that *E. coli* can be applied to utilize lactose or mixture of lactose and glycerol for H_2 production during prolonged duration of 240 h. Moreover, the optimal external conditions (concentrations of lactose or mixture with glycerol; external pH) with effective strains have been developed to significantly (up to 7-fold) enhance and to markedly prolong (up to 8-fold) H_2 evolution.

The results obtained might be significant in development of effective bacterial strains and optimization of conditions for H_2 production biotechnology using lactose or lactose containing wastes as feedstock.

The results indicate that Hyd-3 is responsible for H_2 production during acetate and glycerol fermentation at pH 7.5. Moreover, the data suggest that both Hyd-3 and Hyd-4 are responsible for H_2 production at pH 6.5.

The data are significant in biofuel production technology, especially for H_2 production using bacteria when applying mixture of carbon sources by regulation of metabolic pathways and biochemical properties.

CONCLUSIONS

The following conclusions were made based on experimentally obtained results:

1. It has been revealed that carbon sources and their various mixtures depending on their concentration and environmental acidity have an influence on the activity and interaction of Hyd enzymes in *E. coli*.

2. It has been shown that *E. coli* can perform mixed-acid fermentation of various carbon sources mixtures (glucose, glycerol, formate). It has been established the role of Hyd-3 on cell's growth at pH 5.5.

3. Formate dependent Hyd-4 activity has been demonstrated during mixed carbon sources fermentation in acidic and slightly alkaline conditions.

4. Prolonged and stimulated H_2 production in *E. coli* at pH 7.5 during fermentation of mixed carbon sources has been found using mutants with defects in different Hyd enzymes. The highest yield and H_2 production up to 240 h was demonstrated in *hybC* mutant.

5. It has been shown that *E. coli* can grow well during anaerobic utilization of lactose at different pHs: pH 6.5 and 5 g L^{-1} lactose concentration were optimal conditions for cell growth; and Hyd-3 and Hyd-4 are responsible for H_2 evolution.

6. The highest yield and improved conditions for H_2 production were determined in *hyaB* mutant during 1 g L^{-1} lactose and 10 g L^{-1} glycerol fermentation.

7. The results indicate that in *E. coli* Hyd-3 is responsible for H_2 production during acetate and glycerol fermentation at pH 7.5, but at pH 6.5 both Hyd-3 and Hyd-4 are responsible for H_2 production. Growth of bacterial cells was inhibited at low pH and no H_2 production was determined.

LIST OF PUBLICATIONS AS A PART OF DISSERTATION TOPIC

1. **Mirzoyan S.,** Trchounian A., Trchounian K. (**2019**) Hydrogen production by *Escherichia coli* during anaerobic utilization of mixture of lactose and glycerol: Enhanced rate and yield, prolonged production. *Int. J. Hydrogen Energy,* 44, 18, 9272-9281.
2. **Mirzoyan S.** (**2019**) H_2 production and role of hydrogenases in *Escherichia coli* batch cultures during fermentation of mixture of glycerol and acetate at different pHs. *Biolog. J. Arm.*, 71, 2, 66-73.
3. **Mirzoyan S.,** Trchounian A., Trchounian K. (**2019**) Role of acetate in hydrogen producing hydrogenas 3 activity during glycerol fermentation in *E. coli* at pH 7.5. *ASM MICROBE 2019*, MBP, p.130, San Francisco, USA.
4. **Mirzoyan S.,** Trchounian A., Trchounian K. (**2019**) The effect of the mixture acetate and glycerol on *E. coli* growth and H_2 production during fermentation. *44th FEBS Congress, FEBS OPEN BIO,* p. 314, Krakow, Poland.
5. **Mirzoyan S.,** Trchounian A., Trchounian K. (**2018**) Role of hydrogenases 3 and 4 in *Escherichia coli* growth and H_2 producing hydrogenase activity during anaerobic utilization of lactose. *Int. J. Hydrogen Energy* 43, 18151-18159.
6. **Mirzoyan S.,** Trchounian A., Trchounian K. (**2018**) Role of F_0F_1-ATPase in H^+ flux by *Escherichia coli* during lactose fermentation at different pHs. *The 20th European Bioenergetics Conference,* P10 /12, Budapest, Hungary.
7. **Mirzoyan S.,** Vassilian A., Trchounian A., Trchounian K. (**2018**) H_2 production by *Escherichia coli* during utilization of lactose or mixture of lactose and glycerol: prolongation of production and role of hydrogenases 1 and 2 at different pH. *22nd World Hydrogen Energy Conference* P1: 11, Rio de Janeiro, Brazil.
8. **Mirzoyan S.,** Vassilian A., Trchounian A., Trchounian K. (**2018**) Prolongation of H_2 production during mixed carbon sources fermentation in *E. coli* batch cultures: New findings and role of different hydrogenases. *Int. J. Hydrogen Energy 43*, 18, 8739-8746.
9. Trchounian K., **Mirzoyan S.,** Vassilian A., Trchounian A. (**2017**) Compensatory H_2 producing activity of *Escherichia coli* hydrogenases during mixed carbon sources fermentation. *FEMS 2017, 7th Congress of European Microbiologists,* 095, p. 095, Valencia, Spain.
10. **Mirzoyan S.,** Poladyan A.,Trchounian K., Trchounian A. (**2017**) H_2 production by *Escherichia coli* batch cultures during fermentation of glycerol, lactose at different pHs. *FEMS 2017, 7th Congress of European Microbiologists,* 107, p. 107, Valencia, Spain.
11. **Mirzoyan S.,** Romero-Pareja Pablo M., Dolores Coello M., Trchounian K., Trchounian A. (**2017**) Growth and hydrogen production properties of *Escherichia coli* during fermentation of the mixture of glucose, glycerol and formate at Di. *ASM*

MICROBE 2017 General Meeting, Saturday-612, p. 612, New Orleans, USA.

12. **Mirzoyan S.,** Romero-Pareja Pablo M., Dolores Coello M., Trchounian K., Trchounian A. (**2017**) Evidence for hydrogenase-4 catalyzed biohydrogen production in *Escherichia coli. Int. J. Hydrogen Energy 42,* 21697-21703.
13. Poladyan A., **Mirzoyan S.**, Trchounian K., Trchounian A. (**2017**) Hydrogen production by *Escherichia coli* growing in different nutrient media with glycerol: Effects of formate, pH, production kinetics and hydrogenases involved. *Int. J. Hydrogen Energy 42*, 24026-24034.
14. 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. VAAM Annual Conference 2016 of the Association for General and Applied Microbiology, *Biospektrum* 22, 156, Jena, Germany.
15. Poladyan A., **Mirzoyan S.**, Trchounian A. (**2016**) Effect of different substrates on growth and redox potential kinetics of *Escherichia coli* wild type and hydrogenases lacking mutant. US Biophysical Society 60th Annual Meeting, *Biophys. J.* 110, 315A, Los Angeles, USA.

ՄԻՐԶՈՅԱՆ ՍԱԹԵՆԻԿ ՆՈՐԻԿԻ

ԽՄՈՐՄԱՆ ԸՆԹԱՑՔՈՒՄ ԱԾԽԱԾՆԻ ԱՂԲՅՈՒՐՆԵՐԻ ԽԱՌՆՈՒՐԴՆԵՐԻ ՕՔՍԻԴԱՑՈՒՄԸ *ESCHERICHIA COLI*-ՈՒՄ

Ամփոփագիր

Հանգուցային բառեր՝ Escherichia coli, խառը խմորում, տարբեր ածխածնի աղբյուրների խառնուրդներ, հիդրոգենազներ (Հիդ), H_2-ի արտադրություն, pH:

Այս աշխատանքը նվիրված է *E. coli* բակտերիայում խառը խմորման ընթացքում տարբեր ածխածնի աղբյուրների (գլյուկոզ, գլիցերոլ, լակտոզ, քացախաթթու, մրջնաթթու և այլն) խառնուրդների օքսիդացմանը: Ուսումնասիրվել է խմորման ընթացքում H_2-ի արտադրության գործընթացում տարբեր արտաքին գործոնների՝ pH-ի, ածխածնի աղբյուրների և դրանց խտության ազդեցությունը: Ինչպես նաև պարզվել են տարբեր ածխածնի աղբյուրների խառնուրդների խմորման պայմաններում H_2-ի արտադրության համար պատասխանատու Հիդ ֆերմենտները: Այս խնդրի լուծման համար օգտագործվել են տարբեր Հիդ-ային խանգարումներով մուտանտներ, որոնց միջոցով հնարավոր կլինի նաև խթանել ածխածնի աղբյուրների օքսիդացումը մինչև H_2: Բացահայտվել է տարբեր ածխածնի աղբյուրների խառնուրդների խմորման պայմաններում Հիդ ֆերմենտների նյութափոխանակային կապը և փոխազդեցությունը միմյանց հետ՝ օգտագործելով տարբեր Հիդ-ային խանգարումներով մուտանտներ:

Հետազոտությունների արդյունքում ցույց է տրվել, որ ածխածնի աղբյուրներն ունեն ազդեցություն Հիդ ֆերմենտների աշխատանքի վրա: Ապացուցվել է գլյուկոզի, գլիցերոլի և մրջնաթթվի խառնուրդի խմորման ժամանակ մրջնաթթու-կախյալ Հիդ-4-ի H_2 արտադրող ֆունկցիան pH 7.5-ի և pH 5.5-ի պայմաններում: Հիդ-4-ի մասին այս դիտարկումը նոր է և հնարավորություն է տալիս ավելի լավ պատկերացնել H_2-ի նյութափոխանակությունը, Հիդ-ային ակտիվության և ջրածնային ցիկլի կարգավորումը: Ցույց է տրվել *E. coli hybC* մուտանտում հիմնային պայմաններում երկարածգված և բարձր ելքով H_2-ի արտադրություն մինչև 240 ժամ, ինչը նորույթ է, որը նախկինում հայտնի չէր: Սա կարող է կարևոր նշանակություն ունենալ կենսաքիմիական ուղիների կարգավորման մեթոդներով H_2-ի լայնածավալ արտադրության համար:

E. coli վայրի տիպում գլիցերոլի և լակտոզի խառնուրդի յուրացման ժամանակ դիտվել է ~3 անգամ ավելի շատ H_2, ինչը նշանակում է, որ չմշակված գլիցերոլի և կաթնամթերքի թափոնները, որտեղ առկա է մեծ քանակությամբ լակտոզ, կարող են խառնվել և զգալիորեն բարելավել H_2-ի արտադրության ելքը: Որոշվել են *hyaB* մուտանտում H_2-ի արտադրության բարձր ելքը և բարելավված պայմանները 1 գ l^{-1} լակտոզի և 10 գ l^{-1} գլիցերոլի խառնուրդի խմորման ժամանակ:

Ցույց է տրվել, որ pH 6.5–ը և լակտոզի 5 գ l^{-1}-ը խտությունը բջիջների աճման ամենանպաստավոր պայմաններն են:

Պարզվել է, որ ցածր pH-ն ունի արգելակիչ ազդեցություն բակտերիաների բջիջների աճի վրա քացախաթթվի և/կամ գլիցերոլի խառնուրդի յուրացման ժամանակ: Այսպիսով, ստացված արդյունքները բացահայտում են *E. coli*-ում ածխածնի տարբեր աղբյուրների խառնուրդների խմորման կենսաքիմիական առանձնահատկությունները, տարբեր Հիդ ֆերմենտների ակտիվության կարգավորման ուղիները և դրանց միջև փոխազդեցությունը:

Ստացված արդյունքներից կարելի է փաստել, որ տարբեր ածխածնի աղբյուրների խառնուրդների համատեղ օգտագործումը և դրանց օքսիդացումը մինչև H_2 կարող է նպաստել վերջինիս ելքի բարձրացմանը՝ կիրառելով տարբեր Հիդ-ային խանգարումներով մուտանտներ և տարբեր արտադրական թափոններ: Այս մոտեցումը ունի գործնական նշանակություն կենսաբանական եղանակով H_2-ի արտադրության տեխնոլոգիայի կատարելագործման համար:

МИРЗОЯН САТЕНИК НОРИКОВНА

ОКИСЛЕНИЕ СМЕШАННЫХ ИСТОЧНИКОВ УГЛЕРОДА В ПРОЦЕССЕ БРОЖЕНИЯ В *ESCHERICHIA COLI*

РЕЗЮМЕ

Ключевые слова: *Escherichia coli*, смешанное брожение, смеси различных источников углерода, гидрогеназы (Гид), производство H_2, pH.

Данная работа посвящена изучению окисления различных источников углерода (глюкозы, глицерина, лактозы, уксусной и муравьиной кислот и др.) при смешанном брожении в *E. coli*. Изучено действие различных внешних факторов, таких как pH, источники углерода и их концентрации, на производство H_2 в процессе брожения. Выявлены Гид ферменты, ответственные за выработку H_2 в условиях сбраживания комбинированных источников углерода. Для решения данной задачи использовались Гид мутанты, с помощью которых также возможно ускорение окисления источников углеродов до H_2. Обнаружена взаимосвязь между Гид ферментами при сбраживании комбинированных источников углерода в мутантах с различными нарушениями в Гид.

Полученные данные свидетельствуют о том, что источники углерода оказывают существенное влияние на активность Гид ферментов. Доказана H_2-продуцирующая функция Гид-4 при pH 7.5 и pH 5.5 при совместном сбраживании глюкозы, глицерина и формиата. Это наблюдение о Гид-4 является новым и дает

новое представление о метаболизме H_2, активности Гид и регуляции водородного цикла. Впервые при щелочном pH показано пролонгированное и высокоэффективное выделение H_2 мутантами *E. coli hybC* до 240 ч , что может иметь важное значение для биохимического регулировании широкомасштабного производства H_2.

Выделение H_2 *E. coli* дикого типа при совместном использовании глицерина и лактозы в 3 раза превышало выход H_2 при сбраживании отдельных источников углерода, что свидетельствует о том, что необработанные отходы глицерина и кисломолочного производства, содержащие большое количество лактозы, способны значительно повысить выход H_2. Оптимизированы условия продуцирования H_2 мутантом *hyaB* при сбраживании 1 г $л^{-1}$ лактозы и 10 г $л^{-1}$ глицерина. Показано, что концентрация лактозы 5 г $л^{-1}$ и pH 6.5 являются наиболее благоприятными условиями для роста клеток.

Обнаружено, что низкий pH ингибирует рост бактериальных клеток при сбраживании смеси ацетата и/или глицерина. Таким образом, полученные данные раскрывают биохимические особенности сбраживания различных источников углерода в *E. coli*, пути регуляции активности Гид ферментов и взаимосвязь между ними.

Полученные результаты свидетельствуют о том, что использование смешанных источников углерода и их окисление до H_2 может способствовать увеличению его выхода при использовании мутантов с нарушениями в Гид и различных промышленных отходов. Этот подход может иметь практическое значение для усовершенствования технологии производства H_2 биологическим путем.