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ETHANOL SUPPLEMENTATION AS A NEW APPROACH TO REGULATE GROWTH AND HYDROGEN PRODUCTION OF *ESCHERICHIA COLI* UPON GLYCEROL FERMENTATION

A. A. POLADYAN *

Chair of Biochemistry, Microbiology and Biotechnology, YSU, Armenia

Molecular hydrogen (H_2) and ethanol are the main by-products of glycerol fermentation by *Escherichia coli*. In this study, the growth of *E. coli* BW25113 was investigated with the addition of small amounts (0.05 to 2 %) of ethanol alone and in a combination with glycerol The bacterial growth, the kinetic of the redox potential, and the H_2 production in peptone medium, pH 7.5, were investigated upon various amounts of ethanol supplementation. In the presence of any amount of ethanol, but upon the absence of other sources of carbon, no H_2 production was observed. Whereas ethanol (0.3 to 1 %) with a combination of glycerol stimulated both bacterial growth and H_2 production, pH 7.5. A correlation was observed between the redox potential and stimulated by ethanol bacterial growth. The obtained results can be applied to regulate fermentation processes in biotechnology.

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***Keywords*:** glycerol fermentation, ethanol effects, H_2 production, *E. coli*.

Introduction. Molecular hydrogen (H_2) is an attractive, alternative, eco-friendly and renewable fuel. It can be obtained in various ways, but the most effective is the biological, when bacteria ferment certain organic substances (glucose, glycerol, etc.) [1–3]. Moreover, extensive research efforts have been devoted to strain optimization to improve the cost effectiveness of microbial biofuel production [2, 4]. With the rapid development of biodiesel production, the volume of glycerol as the main by-product has recently increased dramatically: about 1 *kg* of glycerol is generated with every 10 *kg* of biodiesel production by the transesterification process [2]. Fermentative metabolism of glycerol has been reported in species of the genera *Bacillus*, *Citrobacter*, *Enterobacter*, *Klebsiella*, *Clostridium*, *Lactobacillus*, *Propionibacterium*, and *Anaerobiospirillum* [5]. However, the potential for using them in the industry is limited due to many issues that include pathogenicity, the requirement for strict anaerobic conditions, the need for rich nutrients, as well as unavailability of the genetic tools and physiological knowledge necessary to manipulate them effectively. The use of bacteria such as *E. coli*, an organism very amenable to industrial applications, might help overcome the above-mentioned issues [2].

* E-mail: apoladyan@ysu.am

E. coli produces H_2 during mixed fermentation of various carbon sources such as glycerol, glucose, xylose, etc. [6–9]. In *E. coli*, glycerol enters the cells mainly with the help of GlpF protein [10]: phosphorylation by glycerol kinase in the cytoplasm traps glycerol in the cell and initiates its consumption by well-known glycolytic enzymes [6–8]. H_2 is produced upon glycerol fermentation during formate decomposition by formate dehydrogenase H (FDH-H) and hydrogenase (Hyd) enzymes [11–13]. In *E. coli*, four membrane-bound reversible [NiFe] Hyd enzymes participate in H_2 metabolism and have an important role in the cell energetics [14–16]. However, it has been shown that ethanol is the main by-product of glycerol fermentation in *E. coli* [6]. Ethanol is produced from acetaldehyde by alcohol/acetaldehyde dehydrogenase: here NADH is required for the reduction of acetaldehyde [6–8]. Thus, both H_2 and ethanol production contributes to maintain the redox balance of the cell during the glycerol fermentation [6, 7]. On the other hand, ethanol is one of the important microbial end products, whose toxic effects are known to limit their production in both bacteria and yeast [10, 17–19]. It has been shown that ethanol may reduce peptidoglycan cross-linking in *E. coli*, interfere with cell division, affect steady-state growth, lead to disruption of the membrane structure or function and variations in fatty acid composition and protein synthesis in the membrane, inhibit the nutrient transport via membrane-bound ATPases, alter the membrane potential ($\Delta\Psi$) and ΔpH, etc. [4, 10, 17, 19]. Toxic effects have been stated for a wide variety of microbial species using ethanol concentrations ranging from 2.5% to 70% [17].

On the other hand, the ethanol generation by bacteria represents a favorable approach to the biofuel production at an industrial scale. Consequently, a fundamental understanding of the effects of low amounts of ethanol (<2.5%) on bacterial physiology is important.

Therefore, in this study, we set ourselves the purpose to determine the effects of ethanol in amounts below 2.5% alone or together with glycerol on the growth of *E. coli* BW25113 and H_2 production under glycerol fermentation, pH 7.5.

Materials and Methods.

Cultivation Conditions of Bacteria. The wild type parental *E. coli* strain (WT) was kindly provided by Prof. T. Wood (Pennsylvania State University, University Park, PA, USA) [20]. Bacteria were grown under fermentative conditions at pH 7.5, 37°C in peptone medium (PM). Bacterial subcultures were grown on PM under the same anaerobic conditions. PM was composed of 20 $g \cdot L^{-1}$ peptone, 2 $g \cdot L^{-1}$ K_2HPO_4, 5 $g \cdot L^{-1}$ NaCl, pH 7.5 [21, 22]. Various amounts of ethanol (0.05 to 1.5%) and 1% of glycerol were supplemented. The pH was measured by a pH-meter with a selective pH electrode (HJ1131B, Hanna Instruments, Portugal). The pH regulation was done by 0.1 *M* NaOH or 0.1 *M* HCl.

Bacterial Growth Parameters Determination. Bacterial cell formation was estimated using a Spectro UV-Vis Auto spectrophotometer, (Labomed Inc., Los Angeles, CA, USA), by measuring the optical density (OD) values of the bacterial culture at 600 *nm*. The bacterial specific growth rate (μ) was considered as lg2/doubling time, which was calculated during the linear growth of the logarithm of OD over time [22]. The yield of bacterial biomass was expressed in bacterial culture dry weight (CDW), (g CDW)$\cdot L^{-1}$ [22].

H_2 Production Determination. The Platinum (Pt), (EPB-1, GSEEE; or PT42BNC, HANNA Instruments, Portugal) and titanium-silicate (Ti-Si), (EO-02, GSEEE, Gomel, Belarus) redox electrodes were used to control the oxidation-reduction potential (ORP) of bacterial culture [21, 22]. Regardless of the Ti-Si electrode, the data obtained by the Pt electrode revealed the presence of H_2 or O_2: the drop in readings down to negative values (~ –400 *mV*) indicated the H_2 production in the medium under anaerobic conditions. The difference between the readings of the Pt and Ti-Si electrodes permitted to estimate the H_2 production under different conditions [21, 22]. The H_2 production yield was calculated in $(mmol\ H_2)\cdot L^{-1}$ units [21, 22].

The formation of H_2 during the growth of *E. coli* was proved by the presence of gas bubbles in the test Durham tubes. This was also verified by the chemical assay based on bleaching of a $KMnO_4$ solution with H_2 in the presence of H_2SO_4 [21].

Data Processing. Glycerol, peptone, and ethanol (Carl Roths GmbH, Germany) and other reagents used were of analytical grade. For data processing, Microsoft Excel 2016 was used. The values for all data were averaged over 3 replications, and the means were subjected to analysis of variance: the standard errors and Student's criteria were used to confirm the difference in average data between different series of experiments. Thus, the difference was applicable when $p<0.05$.

Results.

Growth and ORP Kinetics of E. coli BW25113 upon the Addition of Ethanol and Glycerol Fermentation. The influence of small amounts of ethanol (0.05 to 2 %) on the biomass formation of *E. coli* BW25113 during the batch growth on PM at pH 7.5 was investigated. Fig. 1 illustrates the growth (OD) of bacteria during 144 *h* when the PM was supplemented with 0.5 and 1 % ethanol. Control experiments were done without carbon source supplementation. Note that PM is a complex mixture of nutrients containing large amounts of amino acids and peptides. Overall, bacterial growth was suppressed upon supplementation with more than 1% ethanol (data not shown). Whereas, in some cases, slightly growth-stimulating effects were observed whit the addition of 0.5 to 1 % ethanol: it was shown that by 144 *h*, at pH 7.5, ethanol in amounts of 0.5% and 1% stimulated bacterial growth ~1.2 fold compared to the control experiment (Fig. 1).

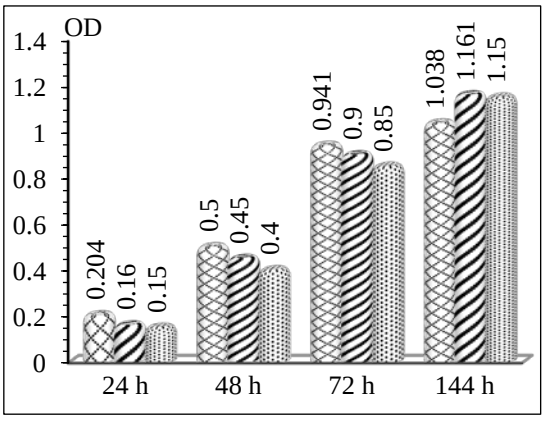

Fig. 1. Batch fermentation characteristics of bacteria *E. coli* BW25113 upon 0.5% and 1% ethanol supplementations. Bacteria were grown anaerobically in PM, pH 7.5. Control was without any carbon source supplementation.

Stimulation of growth under certain conditions by the influence of ethanol indicates that the bacteria have mechanisms to assimilate or counteract ethanol, which needs further study.

The kinetics of ORP was studied using platinum (Pt) and titanium-silicate (Ti-Si) electrodes. Overall, during the batch growth on PM, a drop in the readings of redox (Pt and Ti-Si) electrodes from positive to negative values were observed during 24 *h* of bacterial growth (Fig. 2).

It should be noted, that in all cases H_2 production did not observe. Starting from 24 *h*, ORP tended to return to positive values.

The maximal ORP decline (more reductive conditions) was detected at pH 7.5, after bacterial growth for 72 *h*: the Pt electrode reading reached down to –250±10 *mV* (Fig 2, a). Besides, after bacterial growth for 144 *h*, the ORP values were still negative. Moreover, compared with the control (without ethanol addition), the readings of the Pt and Ti-Si electrodes were ~100 *mV* (Fig. 2, a) and ~50 *mV* (Fig. 2, b) more reductive in ethanol (0.5% and 1%) added samples. Here is seen a correlation with the data on bacterial growth (Fig. 1), when ethanol had a stimulating effect on the bacterial growth by 144 *h* of culturing.

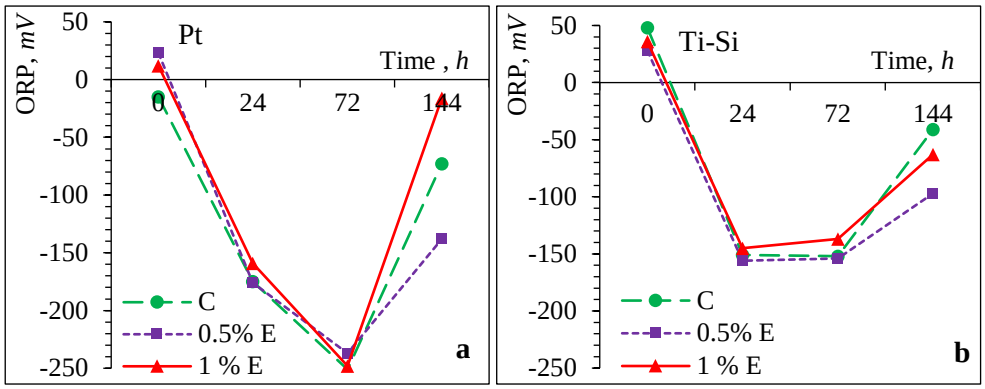

Fig. 2. Kinetics of ORP during the growth of *E. coli* BW25113. Bacteria were grown anaerobically on PM, upon 0.5% and 1% ethanol supplementations, pH 7.5. ORP was determined by a) Pt and b) Ti-Si electrodes. C stands for control, E for ethanol. The error bars were less than 3% and within the designations.

As mentioned above, H_2 is produced during glycerol fermentation [2, 14]. Recently, more attention has been paid to the fermentation of mixed carbon sources. As noted in the Introduction, ethanol is one of the end-products of glycerol fermentation, and from this point of view, it was significant to study its effects on *E. coli*. The effects of mixed carbon sources such as glycerol and ethanol at pH 7.5 on bacterial growth, ORP kinetics, and H_2 production in *E. coli* BW23113 were examined.

The samples were taken both during the first hours and after 24, 48, and 72 hours of growth in the presence of ethanol in various concentrations of 0.1, 0.3, 0.4, 0.5, 1 and 1.5 %, and 1% glycerol. The control experiment was with the supplementation of only 1% glycerol.

Compared to the control, the growth of bacteria was stimulated ~1.2 fold with the introduction of 0.5% and 1% of ethanol, while higher levels of ethanol (1.5%) suppressed the growth of bacteria (data not shown).

After 4 *h* growth, compared to the control, there was a decline in ORP from positive to negative values at all of the above concentrations of ethanol in the medium. After 24 *h*, a decrease in the readings of the Pt and Ti-Si electrodes was observed (Fig. 2). In all samples, the readings of Ti-Si electrode ranged from –70±5 *mV* (control) to –95±5 *mV* (ethanol supplemented). Whereas, in the control experiment (only glycerol), the readings of the Pt electrode reached–400±10 *mV* with a yield of 0.73 $mmol \cdot L^{-1}$ H_2 production. Moreover, upon introduction of 0.3, 0.4, 0.5 % ethanol and 1% glycerol, ORP values were more reductive (–480±10 *mV*) resulting in the 1.4 $mmol \cdot L^{-1}$ H_2 production.

Discussion. To increase the efficiency of the application of glycerol in the production of H_2, it is essential to identify and understand its metabolic pathways. Ethanol is the main end product (~70%) of glycerol fermentation in *E. coli* (Fig. 4) [6, 7]. On the other hand, many researchers are focused on the bactericidal effect of ethanol, however, little is known about the effect of low concentrations on *E. coli* [5, 17].

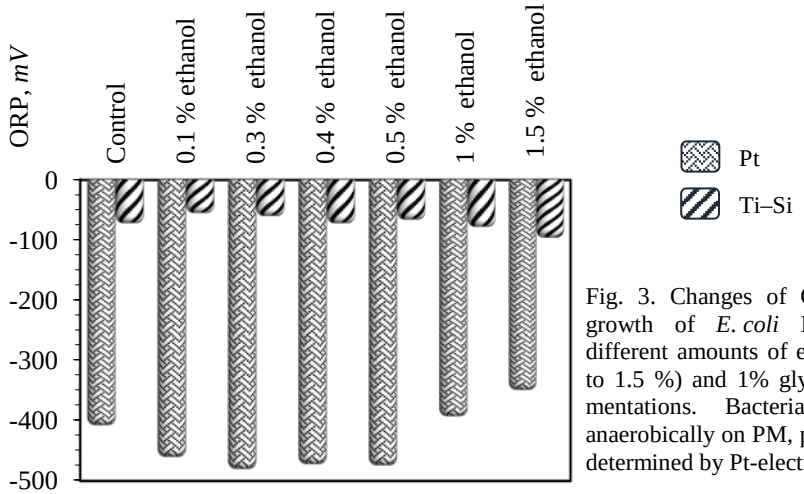

Fig. 3. Changes of ORP at 24 *h* of growth of *E. coli* BW25113 upon different amounts of ethanol (from 0.1 to 1.5 %) and 1% glycerol co-supplementations. Bacteria were grown anaerobically on PM, pH 7.5. ORP was determined by Pt-electrode.

The growth of *E. coli* BW25113 bacteria was followed in nutrient-rich PM. In PM, biomass formation was enhanced by only ethanol addition at pH 7.5 (OD is ~1.2). It should be noted that bacterial growth was prolonged under the conditions without or only with ethanol supplementation. What is important to note is that the ethanol-stimulated bacterial growth was observed at the late stationary growth phase. Thus, the results suggest the possibility of ethanol consumption in *E. coli* under energy-limited conditions.

ORP is one of the significant physicochemical parameters that determine the bacterial growth, as well as a parameter related to the Hyd enzymes and H_2 production [21–23]. Thus, the kinetics of ORP during 144 *h* of bacterial growth on PM was studied with the introduction of ethanol. It should be noted that during the bacterial growth, the ORP values were significantly reductive (Figs. 2, 3). There was

a correlation between ORP drop (Fig. 2) and bacterial growth (Fig. 1), namely, upon ethanol-stimulated bacterial growth, ORP values were more reductive. The results accord with previous findings, pointing to the important role of the reductive conditions for bacterial fermentative growth [21–23].

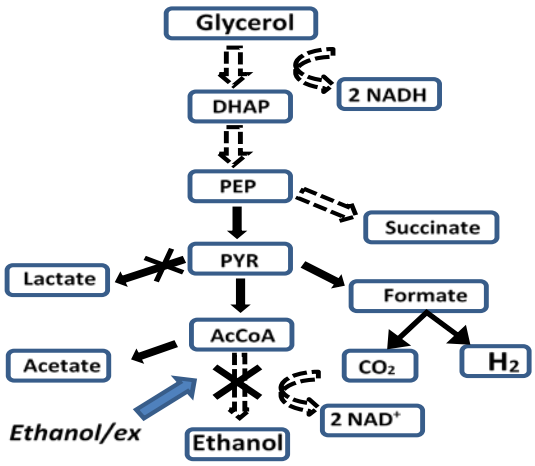

Fig. 4. Simplified representation of glycerol fermentation pathway in E. coli. DHAP stands for dihydroxyacetone phosphate, PEP for phosphoenolpyruvate, PYR for pyruvate, AcCoA for acetyl coenzyme A, ex for external. Dashed arrows indicate multiple reactions, crossed arrows indicate the inhibition of a metabolite production [2, 6].

The situation was quite different when bacteria grew with the simultaneous supplementation of ethanol and glycerol. In this case, low concentrations of ethanol enhanced both bacterial growth and H_2 production at pH 7.5. However, with the combined supplementation of ethanol and glycerol, the stimulatory effect of ethanol on bacterial growth was observed to be weak, suggesting that bacteria resorted to ethanol consumption only under energy-limited conditions. Our results are in line with the study by Chatterjee and co-workers [17], who determined the concentration of supplemented ethanol in the culture medium throughout the growth cycle of *Staphylococcus aureus* populations and showed that in the absence of microorganisms, the concentration of ethanol in the medium remained stable over 24 *h*, while in the presence of *S. aureus*, ethanol was depleted from the culture medium by 24 *h*, suggesting that the bacteria catabolized ethanol. Moreover, increased activity of alcohol-aldehyde dehydrogenase (AdhE) was observed at low amounts of ethanol supplementation in the *S. aureus* culture. Although, as already mentioned, ethanol primarily affects the bacterial membrane structure and functions, therefore, it might affect systems related to H_2 metabolism, such as membrane-bound Hyds and FDH-H or F_oF_1-ATPase, upon glycerol fermentation [2, 17, 22]. However, we have suggested the ethanol supplementation as a possibility of a new approach to affect the metabolic flux towards the H_2 production (Fig. 4): external ethanol supplementation might adversely affect its own (ethanol) production, hence promoting the redox-balanced pathway for H_2 formation (Fig. 4). Future studies are required to reveal the mechanisms of ethanol effect on H_2 production during glycerol fermentation in *E. coli*.

Conclusion. Small amounts of ethanol contribute to the growth of *E. coli* under energy-limited conditions, during the late stationary growth phase. Moreover, small amounts of ethanol co-supplemented with glycerol enhance H_2 production ~2

fold. To our knowledge this is the first report demonstrating the effects of small amounts of ethanol on *E. coli* growth and H_2 production. However, future studies are needed to further explore this phenomenon. Alcohols such as ethanol are important microbial bio-products whose toxic effects are known to limit their production in microorganisms. Although H_2 production was not observed in the presence of only ethanol in PM, our results suggest the possibility of using ethanol with a combination of glycerol to control and enhance H_2 production. These observations are important for understanding H_2 production in *E. coli* mainly under glycerol metabolism, as well as open new perspectives in our understanding of bacterial behavior in the presence of sub concentrations of antiseptic agents.

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Ա. Ա. ՓՈԼԱԴՅԱՆ

ԷԹԱՆՈԼԻ ՆԵՐՄՈՒԾՈՒՄԸ ՈՐՊԵՍ *ESCHERICHIA COLI*–Ի ԱՃԻ ԵՎ ՋՐԱԾՆԻ ԱՐՏԱԴՐՈՒԹՅԱՆ ԿԱՐԳԱՎՈՐՄԱՆ ՆՈՐ ՄՈՏԵՑՈՒՄ ԳԼԻՑԵՐՈԼԻ ԽՄՈՐՄԱՆ ՊԱՅՄԱՆՆԵՐՈՒՄ

Մոլեկուլային ջրածինը (H_2) և էթանոլը *Escherichia coli*-ի կողմից գլիցերոլի խմորման հիմնական արգասիքներն են: Այս հետազոտությունում ուսումնասիրվել է *E. coli* BW25113-ի աճը էթանոլի ցածր քանակների (0.05-ից մինչև 2%) առանձին և գլիցերոլի հետ համատեղ կիրառման պայմաններում: Բակտերիաների աճը, օքսիդա-վերականգողական պոտենցիալի կինետիկան և H_2-ի արտադրությունը հետազոտվել են էթանոլի տարբեր քանակների ներմուծման պայմաններում, պեպտոնային միջավայրում, pH 7,5-ում: Էթանոլի կամայական քանակի առկայության, բայց ածխածնի այլ աղբյուրների բացակայության դեպքում H_2-ի արտադրություն չի դիտվել: Մինչդեռ էթանոլը (0,3–1%) գլիցերոլի հետ համատեղ խթանել է ինչպես բակտերիաների աճը, այնպես էլ H_2-ի արտադրությունը, pH 7,5-ում: Օքսիդա-վերականգողական պոտենցիալի և էթանոլով պայմանավորված բակտերիաների աճման միջև հարաբերակցություն է դիտվել: Ստացված արդյունքները կարող են օգտագործվել կենսատեխնոլոգիայում խմորման գործընթացների կարգավորման նպատակով:

А. А. ПОЛАДЯН

ДОБАВЛЕНИЕ ЭТАНОЛА КАК НОВЫЙ ПОДХОД К РЕГУЛЯЦИИ РОСТА И ВЫДЕЛЕНИЯ ВОДОРОДА ПРИ СБРАЖИВАНИИ ГЛИЦЕРИНА В *ESCHERICHIA COLI*

Молекулярный водород (H_2) и этанол являются основными продуктами сбраживания глицерина в *Escherichia coli*. В данной работе изучено влияние низких количеств этанола (от 0,05 до 2 %) (как отдельно, так и в комбинации с глицерином) на *E. coli* BW25113. Рост бактерий, кинетика окислительно-восстановительного потенциала (ОВП) и производство H_2 в пептонной среде при pH 7,5 были исследованы в присутствии различных объемов этанола. При отсутствии других источников углерода и при наличии всех объемов этанола, выделения H_2 не наблюдалось. При этом этанол (0,3–1%) в комбинации с глицерином стимулировал как рост бактерий, так и производство H_2 при pH 7,5. Обнаружена корреляция между ОВП и ростом бактерий, обусловленным действием этанола. Полученные результаты могут применяться в биотехнологии для регуляции процессов брожения.