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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**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 \( \text{H}_2 \) during mixed fermentation of various carbon sources such as glycerol, glucose, xylose, etc. [6–9]. In *E. coli*, glycerol enters the cell 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]. \( \text{H}_2 \) 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 \( \text{H}_2 \) 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 \( \text{H}_2 \) 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 \( \Delta \text{pH} \), 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 \( \text{H}_2 \) 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·L\(^{-1}\) peptone, 2 g·L\(^{-1}\) K\(_2\)HPO\(_4\), 5 g·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 (\( \mu \)) 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)·L\(^{-1}\) [22].
**H₂ 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 H₂ or O₂: the drop in readings down to negative values (~ −400 mV) indicated the H₂ production in the medium under anaerobic conditions. The difference between the readings of the Pt and Ti-Si electrodes permitted to estimate the H₂ production under different conditions [21, 22]. The H₂ production yield was calculated in (mmol H₂)·L⁻¹ units [21, 22].

The formation of H₂ 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 KMnO₄ solution with H₂ in the presence of H₂SO₄ [21].

**Data Processing.** Glycerol, peptone, and ethanol (Carl Roth 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 with 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).

![Graph showing OD of bacteria growth over time with different ethanol supplements](attachment:fig1.png)

**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 H2 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.

As mentioned above, H2 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 H2 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 L⁻¹ H₂ 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 L⁻¹ H₂ production.

**Discussion.** To increase the efficiency of the application of glycerol in the production of H₂, 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].

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 H₂ 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).
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].

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 H₂ 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 H₂ metabolism, such as membrane-bound Hyds and FDH-H or *F₂F₁*-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 H₂ production (Fig. 4): external ethanol supplementation might adversely affect its own (ethanol) production, hence promoting the redox-balanced pathway for H₂ formation (Fig. 4). Future studies are required to reveal the mechanisms of ethanol effect on H₂ 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 H₂ 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\textsubscript{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\textsubscript{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\textsubscript{2} production. These observations are important for understanding H\textsubscript{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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А. А. Поладян

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

Молекулярный водород (H₂) 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 H₂ в пептонной среде при рН 7,5 были исследованы в присутствии различных объемов этанола. При отсутствии других источников углерода и при наличии всех объемов этанола, выделения H₂ не наблюдалось. При этом этанол (0,3–1%) в комбинации с глицерином стимулировал как рост бактерий, так и производство H₂ при рН 7,5. Обнаружена корреляция между ОВП и ростом бактерий, обусловленной действием этанола. Полученные результаты могут применяться в биотехнологии для регуляции процессов брожения.