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Effects of Carbon Sources Mixtures on Hydrogen Production by *Escherichia coli* during Mixed-Acid Fermentation

(Submitted by corresponding member of NAS RA A. H. Trchounian 10/IX 2015)

Keywords: *Escherichia coli; H_2 production; carbon sources mixtures; pH; mixed-acid fermentation.*

1. Introduction. Nowadays expeditious wane of fuel and gas has strong requirement to find and process alternative energy sources. One of these energy sources which might replace existing fuel and gas is molecular hydrogen (H_2) which is ecologically clean – when burning H_2 gas water is produced; effective - when burning ~3 times more energy is released compared to fuel and gas and renewable one; H_2 can be produced from glycerol during microbe-mediated biological conversion [1]. Glycerol is a very cheap carbon source - crude glycerol costs ~30 cents/kg [2]. Moreover, the main side product of biodiesel production is glycerol.

Dharmadi et al. [2] have established that glycerol can be anaerobically fermented by *Escherichia coli* at slightly acidic pH (pH 6.3). Among the fermentation end products H_2 gas is detected not only at acidic but also at slightly alkaline pH (pH 7.5) [3]. However, no precise data exists about metabolic pathways of glycerol fermentation by *E. coli* and its dependence on external factors and co-fermentation with other carbon substrates. Currently, several studies are on-going using various mixtures of carbon sources like sugars with glycerol (glucose, xylose, etc.) to enhance H_2 production [4-6]/

It is known that H_2 is evolved by *E. coli* via hydrogenases (Hyd), which catalyze the reaction of $H_2 <-> 2H^+ + 2e^-$ [1]. *E. coli* has the capacity to encode four membrane-associated [Ni-Fe]-hydrogenases [1]. Hyd-1 and Hyd-2 are reversible Hyd enzymes: during glycerol or glucose fermentation they operate in H_2 evolving or uptake mode, respectively [7]. Hyd-3 and Hyd-4 are H_2 producing Hyd enzymes under glucose fermentation but are able to work in reverse mode during glycerol fermentation [8]. The mode or direction of Hyd enzymes operation depends not only on fermentation substrate but also on external pH and other factors [1].

In the present paper H_2 production activity by *E. coli* during mixed-acid fermentation of single and mixture of carbon sources has been studied at alkaline and acidic pHs. The possibility of using various carbon sources mixtures might lead to enhanced bio-hydrogen production which can be employed in different biotechnological applications.

Material and methods. ***2.1. Bacterial strains, their growth and preparation for assays.*** *E. coli* BW25113 or MC4100 wild type strains were used in the study (Table 1).

Table 1

Characteristics of *E. coli* wild type strains used

Strains	Genotype	Absent hydrogenase subunit or related protein	References
BW25113	$lacI^q$ $rrnB_{T14}$ Δ $lacZ_{W116}$ *hsdR514* Δ $araBAD_{AH33}$ Δ *rha* BAD_{LD78}	wild type	[6]
MC4100	F^- *araD139* Δ (*argF-lac*)*U169* λ-*rpsL150 relA1 deoC1* *flhD5301* Δ (*fruK-yeiR*)*725*(*fruA25*) *rbsR22* Δ (*fimB-fimE*) *632* (::IS*1*)	wild type	[9]

Bacteria from an overnight (O/N) growth culture were transferred into the buffered peptone medium containing 20 g/l peptone, 15 g/1 K_2HPO_4, 1.08 g/l KH_2PO_4, 5 g/l NaCl (pH 7.5), 20 g/l peptone, 7.4 g/1 K_2HPO_4, 8.6 g/l KH_2PO_4, 5 g/l NaCl (pH 6.5), 20 g/l peptone, 1.08 g/1 K_2HPO_4, 15 g/l KH_2PO_4, 5 g/l NaCl (pH 5.5) and supplemented with glucose (2g/l) and/or glycerol (10 g/l) and/or sodium formate (0.68 g/l). Bacteria were grown in batch culture for 18-22 h at 37 ^{0}C; anaerobic conditions were described previously [4, 6, 7]. Bacterial growth was monitored by measuring bacterial culture absorbance at 600 nm with a spectrophotometer (Spectro UV-Vis Auto, Labomed, USA).

2.2. ***Redox potential determination and hydrogen production assays***. Redox potential (E_h) in bacterial suspension was measured using the oxidation-reduction, titanium-silicate (Ti-Si) (EO-02, Gomel State Enterprise of Electrometric Equipment (GSEEE), Gomel, Belarus) and platinum (Pt) (EPB-1, GSEEE, or PT42BNC, Hanna Instruments, Portugal) glass electrodes [4, 7-10]. Ti-Si-electrode is measuring the overall E_h, whereas Pt-electrode is sensitive to H_2 under anaerobic conditions (in the absence of O_2). This difference between Ti-Si and Pt electrodes properties is allowing detection of H_2 evolution in bacterial suspension. Therefore, H_2 production rate (V_{H2}) by bacteria was calculated as the difference between the initial rates of decrease in Pt- and Ti-Si-electrodes readings and expressed as mV of E_h per min per mg dry weight of bacteria as represented in different papers [4, 7-10].

The E_h measurements were performed upon glucose (glucose assay), glycerol (glycerol assay) or formate (formate assay) supplementation to bacterial suspension. All substrates were supplemented in the concentrations used for the bacterial growth in the culture.

H_2 production by bacteria was confirmed also by the chemical method as described [11].

2.3. Others, reagents and data processing. Preparation of whole cells for H_2 production assays was done as described elsewhere [4, 6, 7]. Dry weight of bacteria was measured as before [4, 6, 7].

Agar, glycerol, peptone, sodium formate, Tris (Carl Roths GmbH, Germany) were used, the other reagents were of analytical grade.

Each data point represented was averaged from independent triplicate cultures at least. The standard errors of average data were calculated as described [7-10]: they were not more than 3 % if not represented. The validity of data differences between experimental and control assays was evaluated by Student's criteria (p) [7-10], the difference was valid if $p<0.01$ or less; otherwise, the difference was not valid if $p>0.5$ (not represented).

3. **Results and discussion**. It is well-established that *E. coli* is capable to ferment different sugars (e.g. glucose) and formate at different pHs producing H_2 [4, 7]. Besides sugars, this bacterium can grow on glycerol in the presence of pepton under anaerobic conditions at different pHs and produce H_2 [2, 9].

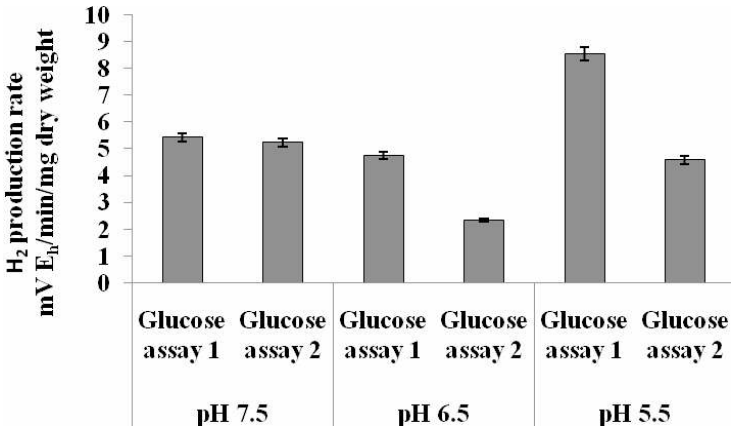
As it was mentioned *E. coli* performs mixed-acid fermentation and among end products formate, succinate, ethanol, CO_2 and H_2 etc. can be detected.

E. coli wild type cells were grown at various pHs in different mixtures of carbon sources and assayed for H_2 production. First wild type cells were grown on mixture of glucose and glycerol and compared to the cells grown either on glucose or glycerol (see Fig. 1 A, B). H_2 production rate (V_{H2}) of wild type cells grown on mixture of 0.2% glucose and 1% glycerol at pH 7.5 in glucose supplemented assays was ~5.25 mV E_h /min mg dry weight (see Fig. 1, A) which was similar to the cells grown on glucose only. At pH 7.5 wild type cells V_{H2} in glycerol assays was ~4 times lower compared to the cells grown on glycerol only. At pH 6.5 and pH 5.5 cells grown on mixed carbon in glucose assays evolved ~2 fold less V_{H2}, compared to the cells grown on glucose only. V_{H2} in glycerol supplemented assays in the cells grown on glycerol and glucose at pH 6.5 was ~0.43 mV E_h /min mg dry weight. Interestingly, at acidic pH (pH 5.5) no any H_2 production was detected when glycerol was supplemented.

Interestingly, at acidic pH (pH 5.5) no any H_2 production was detected when glycerol was supplemented. This suggests that during mixed carbon fermentation glucose inhibits enzymes responsible for glycerol uptake and its further metabolism which is in accordance with previously obtained data for *Klebsiella Pneumoniae* [12]. Further investigation was carried out using mixture of glycerol and formate as glycerol is very cheap carbon source and formate can be found in different industrial and agricultural wastes and by formate hydrogen lyase (FHL) complex is converted to H_2 and CO_2 [1]. The studies were carried out at pH 7.5 and pH 6.5. pH 5.5 was not taken as the wild type cells growth was inhibited. Actually, at pH 7.5 wild type cells V_{H2} grown on glycerol and formate in glycerol supplemented assays was ~2.2 mV E_h /min mg

dry weight (see Fig. 2). No difference was determined when the cells were grown on glycerol only. But when 10mM formate was added in the assays V_{H2} was ~35.34 mV E_h/min mg dry weight. The same result for cells grown on glycerol was obtained for pH 6.5 in glycerol supplemented assays but when formate was added in the assays V_{H2} was ~1.5 fold lower (see Fig. 2)

A



B

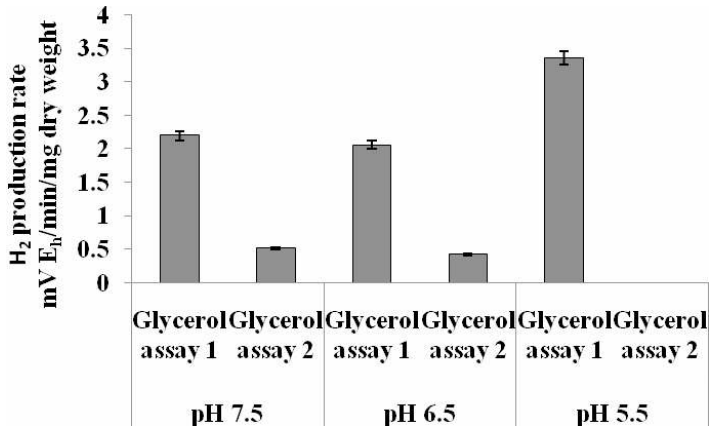


Fig. 1. H_2 production rate (V_{H2}) by *E. coli* BW25113 or MC4100 wild type during single or mixed carbon (glucose and glycerol) fermentation in assays supplemented with glucose or glycerol at different pHs. Glucose assay 1 – cells grown on glucose only; Glucose assay 2 – cells grown on glucose and glycerol; Glycerol assay 1 – cells grown on glycerol only; Glycerol assay 2 – cells grown on glucose and glycerol. For others, see Materials and methods.

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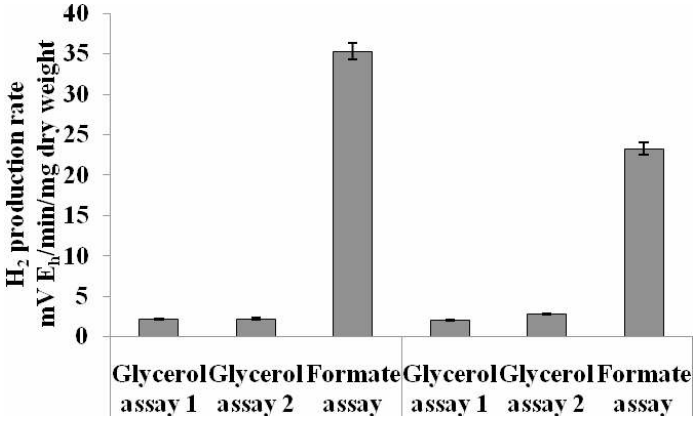


Fig. 2. H_2 production rate (V_{H2}) by *E. coli* BW25113 or MC4100 wild type during glycerol or mixed carbon (glycerol and formate) fermentation in assays supplemented with glycerol or formate at pH 7.5 and pH 6.5. Glycerol assay 1 – cells grown on glycerol only; Glycerol assay 2 – cells grown on glycerol and formate. For others, see Materials and methods.

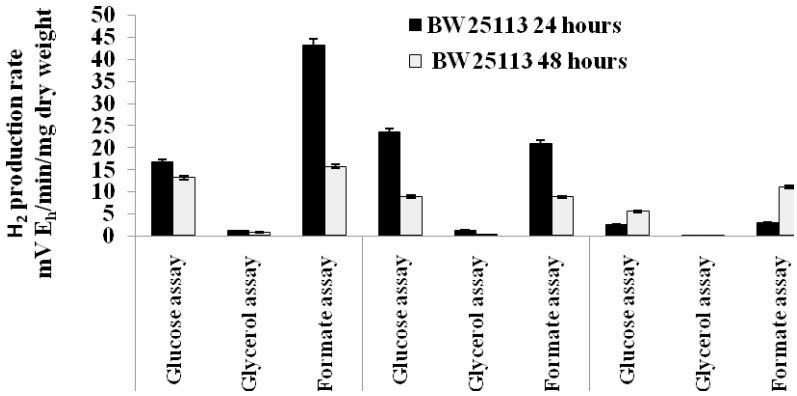
From these results it might be suggested that for enhanced H_2 production cells must be grown at pH 7.5 in the presence of glycerol and formate. As from the results it was obvious that from formate H_2 production rate was higher compared to glycerol. The studies might be used in further developing biohydrogen production technology by growing initially the cells in low concentration of glycerol and formate and producing H_2 from formate.

To further control and optimize conditions for detecting enhanced H_2 production mixture of glucose, glycerol and formate fermentation was investigated. At pH 7.5 wild type cells V_{H2} grown on mixture of carbon sources and harvested after 24 hours in glucose supplemented assays was ~16.8 mV E_h /min mg dry weight (see Fig. 3A). The V_{H2} was ~3 fold higher compared to the cells grown on glucose only (see Fig. 3A).

Surprisingly, at the same conditions but in the assays added with glycerol V_{H2} was ~1.14 mV E_h /min mg dry weight which was ~2 fold higher compared to the cells grown on glucose and glycerol. When formate was added in the assays V_{H2} was similar as the cells grown on glycerol and formate. Moreover, at pH 6.5 wild type cells grown on mixed carbon in glucose assays had ~23.53 mV E_h /min mg dry weight V_{H2} (~4.9 fold more) compared to the cells grown on glucose only. At pH 5.5 in glucose assays V_{H2} was ~ 3.3 fold lower compared to the cells grown on glucose only. Further interest for biotechnology is that cells must be grown and assayed at pH 6.5 and pH 7.5. Cells were also tested for glycerol and formate assays at pH 6.5 and pH 5.5. In glycerol supplemented assays at pH 6.5 V_{H2} was the same as for pH 7.5 but at acidic pH (pH 5.5) residual H_2 gas was detected. In formate assays cells produced ~2 fold less H_2

compared to pH 7.5 but at pH 5.5 V_{H2} was the same as in glucose assays. From these data it can be suggested that for enhanced H_2 production the optimal pH for cell growth is pH 7.5 and glucose or formate assays.V_{H2} was also tested with the cells grown after 48 hours and assayed for H_2 production and compared to the cells grown 24 hours (see Fig. 3, A). Interestingly, only at pH 7.5 in glucose or glycerol assays V_{H2} was similar to the cells grown 24 hours. But when formate was added to the assays H_2 production was decreased ~2.5 fold either at pH 7.5 or pH 6.5.

A



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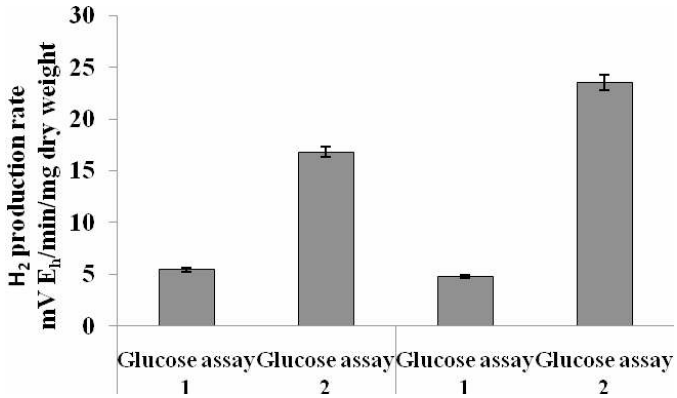

Fig. 3. H_2 production rate (V_{H2}) by *E. coli* BW25113 or MC4100 wild type cells grown either 24 or 48 hours (A) at different pHs. Glucose assay 1 – cells grown on glucose only; Glucose assay 2 – cells grown on glucose, glycerol and formate (B). For others, see Materials and methods.

Note, that the cell count was the same in assays harvested for 24 or 48 h. It might be suggested that after 48 h H_2 production is decreased due to the change of metabolism which might affect the enzymes responsible for H_2 evolution. Further study is required to reveal the role of these enzymes when mixed carbon sources fermentation is occurring.

4. Concluding remarks. The obtained results identified the optimal conditions for enhanced H_2 production when cells are grown on mixture of different carbon sources. Especially, when the cells are grown on triple (glucose, glycerol, formate) carbon source H_2 production is ~3 fold higher compared to the cells grown on glucose only.

The results are good basis for employing various carbon sources, especially mixed carbons, in biohydrogen production technology by *E. coli*.

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Effects of Carbon Sources Mixtures on Hydrogen Production by *Escherichia coli* during Mixed-Acid Fermentation

H_2 production by *Escherichia coli* during various single and/or mixtures of carbon sources at different pHs was investigated. The obtained results showed that in the presence of mixture of glucose, glycerol and formate H_2 production rate at pH 7.5 is ~3 fold higher compared to the cells grown in glucose only as a carbon source. Moreover, in the presence of triple carbon source in glycerol supplemented assays H_2 production rate was ~2 fold higher compared to the cells grown on glucose and glycerol.

Կ. Ա. Թռչունյան

Escherichia coli-ի կողմից մոլեկուլային ջրածնի արտադրության վրա ածխածնի խառն աղբյուրների ազդեցությունը խառը խմորման ընթացքում

Ուսումնասիրվել է միակի և/կամ ածխածնի խառն աղբյուրների խմորման ընթացքում մոլեկուլային ջրածնի (H_2) արտադրությունը *Escherichia coli*-ի կողմից pH-ի տարբեր արժեքների դեպքում: Արդյունքները ցույց են տալիս, որ եռակի ածխածնի՝ գլյուկոզի, գլիցերոլի և մրջնաթթվի առկայության դեպքում H_2-ի արտադրման արագությունը ~3 անգամ ավելին է, քան միայն գլյուկոզում աճեցված բջիջներում: Ավելին, երբ փորձին ավելացվել է գլիցերոլ H_2-ի արտադրման արագությունը ~2 անգամ աճել է համեմատած գլիցերոլի և գլյուկոզի առկայությամբ աճեցված բջիջներում:

К. А. Трчунян

Влияние смеси источников углерода на производство водорода бактериями *Escherichia coli* при смешанном брожении

Изучено производство водорода (H_2) у *Escherichia coli* при брожении одного и/или смеси источников углеродов при разных pH. Результаты показали, что при смеси глюкозы, глицерина и формиата при pH 7.5 скорость производства H_2 в ~3 раза больше по сравнению с клетками, выращенными только при глюкозе. Более того, при смеси трех источников углерода, когда в эксперимент добавляли глицерин, скорость производства H_2 была в ~2 раза больше, чем при смеси глюкозы и глицерина.

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