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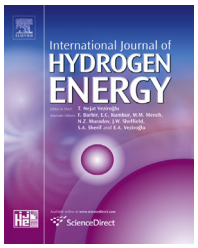

Enhancement of *Escherichia coli* bacterial biomass and hydrogen production by some heavy metal ions and their mixtures during glycerol vs glucose fermentation at a relatively wide range of pH

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ABSTRACT

Escherichia coli growth and H_2 production were followed in the presence of heavy metal ions and their mixtures during glycerol or glucose fermentation at pH 5.5–7.5. Ni^{2+} (50 μM) with Fe^{2+} (50 μM) but not sole metals stimulated bacterial biomass during glycerol fermentation at pH 6.5. Ni^{2+}+Fe^{3+} (50 μM), Ni^{2} +Fe^{3+}+Mo^{6+} (20 μM) and Fe^{3+}+Mo^{6+} (20 μM) but not sole metals enhanced up to 3-fold H_2 yield but Cu^{+} or Cu^{2+} (100 μM) inhibited it. At pH 7.5 stimulating effect on biomass was observed by Ni^{2+}+Fe^{2+}+Mo^{6+}. H_2 production was enhanced 2.7 fold particularly by Ni^{2+}+Fe^{3+}+Mo^{6+} at the late stationary growth phase. Whereas at pH 5.5 increased biomass was when Fe^{2+}+Mo^{6+} or Mo^{6+} were added. H_2 yield was decreased compared with that at pH 6.5, but metal ions again enhanced it. During glucose fermentation at pH 6.5 biomass was increased by the mixtures of metal ions, and 1.2 fold increased H_2 yield was observed. At pH 7.5 Ni^{2+}+Fe^{2+} increased biomass but Cu^{+} or Cu^{2+} had suppressing effect; Fe^{3+}+Mo^{6+} stimulated H_2 production. At pH 5.5 biomass also was raised by Ni^{2+}+Fe^{2+}+Mo^{6+}; H_2 yield was increased upon Mo^{6+} and Mo^{6+}+Fe^{2+} or Mo^{6+}+Fe^{3+} additions. The results point out the importance of Ni^{2+}, Fe^{2+}, Fe^{3+} and Mo^{6+} and some of their combinations for *E. coli* bacterial growth and H_2 production mostly during glycerol but not glucose fermentation and at acidic conditions (pH 5.5 and 6.5). They can be used for optimizing fermentation processes on glycerol, controlling bacterial biomass and developing H_2 production biotechnology.

Abbreviations: DW, dry weight; FHL, formate hydrogen lyase; FDH-H, formate dehydrogenase H; Hyd, hydrogenase; ORP, oxidation–reduction potential; Pt, platinum; Ti-Si, titanium-silicate.

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Introduction

It is an undeniable fact that molecular hydrogen (H_2) is a promising clean energy carrier for the future with high energy content and with only water as major by-product upon combustion. H_2 can be produced using a number of chemical and biological routes [1–5]. H_2 produced biologically, means with the help of microorganisms, has significant advantages over chemical ways, but its technology pathways need a lot of problems to solve. Moreover, the use of cheap and widely available resources particularly organic wastes for H_2 production will provide cost-effective energy generation with immediate waste treatment [5,6]. Consequently, glycerol as a by-product or waste material of biodiesel and other industries can be considered for this proposes. It should be mentioned that recently the price of glycerol was dramatically decreased [2,3,7]. The glycerol became even more attractive carbon source for biotechnological applications, when it was discovered that glycerol as glucose can be fermented by *Escherichia coli* and other bacteria with the formation of H_2, as well as ethanol, acetate and other end products [8–11]. However, pathways of glycerol and glucose fermentation are relatively different: for example, glycerol enters to the cell of *E. coli* by passive transport via GlpF glycerol facilitator protein and, under anoxygenic conditions it converts to the dihydroxyacetone (DHA) phosphate (DHAP), glycolytic intermediate, in a two-step pathway, and the conversion of DHAP to phosphoenolpyruvate and then to pyruvate is coupled to DHA phosphorylation [2,10]. The coupled reaction is essential for glycerol fermentation and generates a loop path in the metabolic pathway. Moreover, upon glycerol fermentation exceed of reducing equivalents in the form of NADH are observed which can affect the end products ratio. As a result, mostly ethanol as a main product of glycerol fermentation was detected [10]. But optimal conditions for glycerol fermentation seem to be pH 6.3 whereas glycerol can be fermented at wide range of pH from pH 5.5 to pH 7.5 [2,9,11]. Redox routes differences are also observed during *E. coli* anaerobic growth upon glucose and glycerol [12].

Thus, H_2 is produced by formate decomposition, the end product of both glycerol or glucose fermentation, due to operation of membrane-associated formate hydrogenase lyase (FHL) [1,13,15,16]. The later consists of [Mo]-formate dehydrogenase H (FDH-H) and [Ni-Fe]-hydrogenase (Hyd) enzymes [15,16]. Four Hyd enzymes (Hyd 1–4) encoded by the *hya*, *hyb*, *hyc* and *hyf* operons, respectively, operate in *E. coli* [1,15–17]. They catalyze both H_2 formation and H_2 oxidation, moreover, their prospective to form a H_2 cycle across the bacterial membrane of *E. coli* was suggested [16,18]. Moreover, Hyd-3 and probably 4 Hyd-4 with FDH-H form two different H_2-evolving FHL pathways during glucose fermentation [1,19,20]. There are many studies on H_2 metabolism in *E. coli* during both glucose and glycerol fermentation, but involvement of four Hyd enzymes in H_2 metabolism and their activity conditions requirements such as pH, carbon source of fermentation, its concentration, oxidation–reduction potential (ORP) and other features lead to a complicated mechanism of H_2 production [1,12,19–21]. For example, Hyd-1 and Hyd-2 are H_2 oxidizing enzymes during glucose fermentation at neutral and low pH. On the other hand they might operate in a reverse, H_2 producing mode during glycerol fermentation at neutral pH. Operation of Hyd-3 in reverse, H_2 oxidizing mode was also shown. Moreover, the study of oxidation–reduction properties of these Hyd-1 and Hyd-2 has reveal that, unlike Hyd-2, Hyd-1 could be suggested to function at higher oxidation–reduction potential (ORP) and/or at higher oxygen concentration [1,3,14–16].

It was stated that metal ions such as iron, nickel, molybdenum and selenium are necessary for maturation and association of a functional FHL complex [17,22–25]. Hyd enzymes are composed of large and small subunits; the active site is located in the large subunit and contains a bimetallic [Ni-Fe] complex, which is inserted through the action of general Hyp accessory proteins. Fe is coordinated by one CO and two CN^- ligands, and two thiolates cysteine residues are bridging the metals, whereas Ni is coordinated by two terminal cysteine thiolates [25,27].

Generally, bacteria have evolved multiple mechanisms to effectively distinguish one metal from another, and keep hemostasis for metal ions by different ways, such as primary and secondary transport systems, membrane channels, functioning together with siderophores [22–24]. For example, Ni^{2+} specific transport system is encoded by the *nikABCDE* operon is synthesized under anaerobic conditions probably to cover the increased demand for nickel needed for hydrogenase synthesis [28]. Besides, FNR (fumarate-nitrate regulator) and the nickel-responsive regulator, NikR regulate the expression of the operon. In addition, it was shown, that Ni and Mo ions might indirectly regulate the expression of *hyc* or *hyp* and *fdhF* and *hyc* genes, respectively, through their transport systems [16,28]. Moreover, recently it was shown that the sensitive to lysis D (SlyD) protein might control Hyd enzymes activity in the late-stationary growth phase cells (when Ni concentration is limited) by regulating Ni delivery for enzyme maturation [26]. Fe ions influence on expression of the *hyc* operon was also proposed: deletion of *fur* gene, which encodes ferric uptake regulator, Fur, reduced FHL activity due to lower transcriptions of *fdhF* and *hyc* genes [23,26]. Selenium is an important component of FDH-H too, which is attached to a cysteine, but, there is no proof that selenium influences transcription of *fdhF* or *sel* genes in *E. coli* [16].

Thus, due to metals evolved in the active centers of Hyd enzymes they proceed redox reactions, consequently provide pathways for H_2, proton and electron transfer during H_2 formation or oxidation. Direct influence of metals on Hyd enzyme activity was also proposed, as the addition of Fe^{2+} affected H_2 production rate by *E. coli in vivo* and increased it *in vitro* at pH 6.5 and pH 7.5 during glycerol fermentation [29]. Similar effects of some metals ions including Ni^{2+}, Fe^{2+} and Mo^{6+} were also shown in other H_2 producing bacteria with different metabolism such as *Clostridium butyricum* [30], *Rhodobacter sphaeroides* [31,32] and other bacteria [33].

So, metalloenzyme expression levels can be strongly regulated in response to changes in environmental conditions. Therefore, taking into account the role of metals in H_2 metabolism of *E. coli*, and for purposes to regulate and enhance H_2 production, the aim of the present research was to study the effects of Fe, Ni, Mo as well as Cu ions of different oxidation states alone and their mixtures on *E. coli* growth and

H_2 production at wide range of pH (pH 5.5–pH 7.5) during both glycerol and glucose fermentation.

Comparison of these substrates fermentation might reveal the conditions when the effects are significant, so the presence of metals should be controlled.

Only recently our research group published data pointing out the effects of Ni^{2+}, Fe^{2+}, Fe^{3+} and Mo^{6+} alone and their mixtures on *E. coli* growth and H_2 metabolism during glycerol fermentation at pH 6.5 [34]. In addition, Cu^{+} and Cu^{2+} effects as well as discrimination between Fe^{2+} and Fe^{3+} were also shown for H_2 production [34].

In the present study was shown that, except Cu^{+} and Cu^{2+}, all mentioned metal ions at low concentrations (of 20 μM–100 μM) had stimulatory effect on *E. coli* bacterial growth during both glucose and glycerol fermentation at pH 5.5–pH 7.5. Nevertheless, the stimulating effects were pH and substrate (glucose or glycerol) dependent: though some stimulation of H_2 production was observed for all cases, but, effects were stronger especially during glycerol fermentation in acidic conditions (pH 5.5 and pH 6.5). Again, Cu^{+} and Cu^{2+} repressed or had no effect on bacterial growth and H_2 production.

Materials and methods

Bacterial strains and growth conditions

The following study performed with the *E. coli* BW25113 wild type under strict anaerobic conditions at 37 °C. 1.5% volume of overnight grown batch culture bacteria was added into the nutrient medium with the composition of 20 g L^{-1} peptone, 2 g L^{-1} K_2HPO_4, 5 g L^{-1} NaCl with the addition of 10 g L^{-1} glycerol or 20 g L^{-1} glucose at pH from 5.5 to 7.5. Batch culture bacteria were grown in 150 mL glass vessels with plastic press-caps as described [12,14,21]. For preparing solid nutrient media it was necessary to add 1.5% agar into the peptone medium without additional carbon source (glucose or glycerol). The pH was measured by a pH-meter with the help of selective pH-electrode (HJ1131B, Hanna Instruments, Portugal), and it was adjusted by 0.1 M NaOH or 0.1 N HCl. Additionally, when needed, metal ions in the form of various salts ($NiCl_2$, $FeSO_4 \cdot H_2O$, $FeCl_3$, $Na_2MoO_4 \cdot 2H_2O$, $CuCl_2$, $CuSO_4$) with the relatively proper concentration added from freshly prepared sterile solutions into the growth medium before bacterial inoculation. Moreover, Cl^{-} or SO_4^{2+} in the concentrations used were determined to have no effects (data not shown).

Bacterial growth was followed every 0.5 h till stationary growth phase, which was specific for each carbon source fermentation, by spectrophotometer (Spectro UV–VIS Auto, Labomed, USA); the bacterial culture absorbance (optical density, OD) at 600 nm was measured. Bacterial yield was estimated by determining of dry weight (DW) of bacterial suspension expressing in g L^{-1}, as before [12]. With analyzing the relation of DW to the plotted absorbance it was possible to obtain the growth curves. The counting number of cells in a unit of volume was done by the number of colonies that were analyzed after re-plating the diluted bacterial suspension on solid nutrient media. All assays and analyses were strictly done at 37 °C.

Determination of oxidation–reduction potential and H_2 production

Using two types of Pt (EPB-1, GSEEE; or PT42BNC, HANNA Instruments, Portugal) and titanium-silicate (Ti-Si, EO-02, GSEEE, Gomel, Belarus) oxidation–reduction electrodes, it became possible to measure ORP of bacterial culture. As opposite to Pt, Ti-Si electrode readings were unaffected by the presence of H_2 (or oxygen) in the medium, allowing discrimination of H_2 during anaerobic growth of bacteria (reference electrode was (Ag/AgCl) one) [12,14,35]. Side note, ORP of the formate:H_2 couple is intensely reduced (–420 mV). The exact measurement of H_2 by the pair of oxidation–reduction electrodes currently delivers relatively more correct results for H_2 determination in liquids [12,35].

The strong appearance of gas bubbles in the test tubes over the bacterial suspension visualized H_2 production by *E. coli* during growth. It was done by Durham tube method. Based on the bleaching of $KMnO_4$ solution in H_2SO_4 with H_2 [11,12,14,21] it was very easy to verify the production of H_2. The calculations of the H_2 yield were done by the decrease of ORP to low negative values, as described [36], and expressed in mol H_2 L^{-1}.

Others and data processing

Glycerol, glucose, peptone (Carl Roths GmbH, Germany) and other reagents of analytical grade were used. Three independent experiments were representing the average data; to validate the difference in average data between different series of experiments the standard errors and Student criteria (p) were calculated, that were successfully employed in order to do that using Microsoft Excel 2010. The differences were valid if $p < 0.05$ (otherwise it was mentioned).

Results

E. coli growth properties in the presence of various heavy metal ions during glycerol and glucose fermentation at different pHs

Some heavy metals ions in low concentrations are necessary for bacterial growth and metabolism: it was shown that heavy metals as nickel (Ni^{2+}), iron (Fe^{3+}, Fe^{2+}) and molybdenum (Mo^{6+}) are essential for proper H_2 formation; particularly they are necessary for biosynthesis and maturation of FDH-H and Hyd enzymes [17,22–26].

In the present part of the study *E. coli* growth was followed during 10 g L^{-1} glycerol or 20 g L^{-1} glucose batch culture fermentation at pH 5.5–pH 7.5 in the presence of various heavy metal ions with different oxidation states, as Ni^{2+}, Fe^{2+} or Fe^{3+}, Mo^{6+} and Cu^{+} or Cu^{2+} and their mixtures at various concentrations. It is worth to mention that the metal ions concentrations were used relaying on the results for *E. coli* and other bacteria, but different substrates of fermentation and conditions were used in the studies before [30–32,37,38].

The growth properties of *E. coli* BW25113 were determined upon each metal alone and their mixtures supplementation to nutrient medium. Earlier it was shown that during glucose

fermentation of *E. coli* exponential growth usually observes for period of ~5–6 h, starting when the culture is ~2 h and ending at ~7–8 h that was non-depending on pH, whereas in glycerol fermented culture exponential growth was prolonged: it was monitored for the period of ~10 h, starting when the culture was of ~2 h and ending at ~12 h [12]. Our research group lately also showed the importance of Ni^{2+} with Fe^{2+} as effective factor for increasing bacterial biomass and H_2 production during *E. coli* glycerol fermentation at pH 6.5 [34]. Indeed, it was shown that bacterial biomass was 0.30 ± 0.01 g L^{-1} (DW) without metal ion addition (control) and was stimulated 1.3–1.5 fold upon Ni^{2+}, Fe^{2+} and Fe^{3+} (in the concentrations of 50 μM) and 1.3–2 fold upon the mixtures of Ni^{2+}+Fe^{2+}+Mo^{6+}; Ni^{2+}+Fe^{3+}; Ni^{2+}+Fe^{2+} or Fe^{3+}+Mo^{6+} (20 μM Mo^{6+}) supplementation (Fig. 1). The maximal biomass was achieved in the presence of Ni^{2+}+Fe^{2+} mixture (see Fig. 1).

Next experiments were done at the same conditions upon *E. coli* glycerol utilization and metal ions concentrations at pH 5.5 and pH 7.5: at acidic conditions (pH 5.5) up to 1.3 fold bacterial growth yield stimulating effect was observed with sole Fe^{2+}, Fe^{3+} or Ni^{2+} and their mixtures of Ni^{2+}+Fe^{3+}, Ni^{2+}+Fe^{2+} or Ni^{2+}+Fe^{2+}+Mo^{6+} and ~1.6 fold with Fe^{2+}+Mo^{6+} or Mo^{6+} supplementations (Fig. 2A). Whereas at pH 7.5 glycerol fermentation the same metal ions and their combinations had less stimulating effect on bacterial biomass, and maximal 1.2 fold stimulation was detected upon Ni^{2+}+Fe^{2+}+Mo^{6+} or Ni^{2+}+Fe^{3+}+Mo^{6+} addition (Fig. 2B).

The correlation of observed effects of the metals with the substrate of fermentation was also studied: bacterial growth was followed during glucose fermentation at pH 5.5–pH 7.5 in the presence of Ni, Fe, Mo and Cu ions. Glucose is accepted as preferable substrate (carbon source) for *E. coli* and other bacteria, its fermentation pathway is characterized well than glycerol one and, as was mentioned, there are differences in glucose and glycerol fermentations pathways, as mentioned above [12,14]. All mentioned metals solely and their mixtures stimulated bacterial growth yield: at pH 5.5 bacterial growth

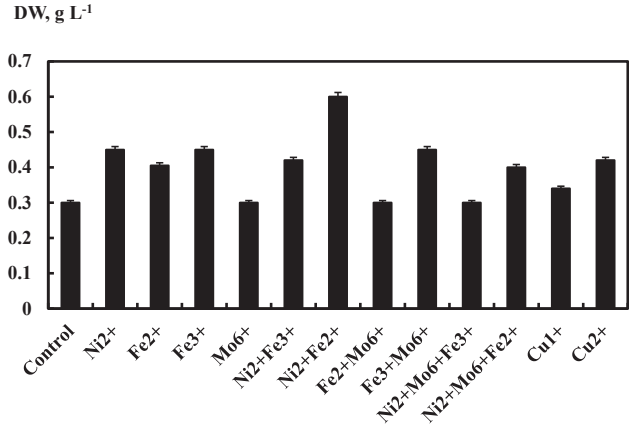

Fig. 1 – The effect of various metal ions and their mixtures on *E. coli* BW25113 growth yield during glycerol fermentation at pH 6.5. 50 μM Ni^{2+}, Fe^{2+}, Fe^{3+}, 20 μM Mo^{6+} and 100 μM Cu^{+} or Cu^{2+} were added into the growth medium when mentioned. Dry cell weight (DW) was determined after 24 h bacterial growth. For the others, see Materials and methods. Data were adapted from Ref. [34].

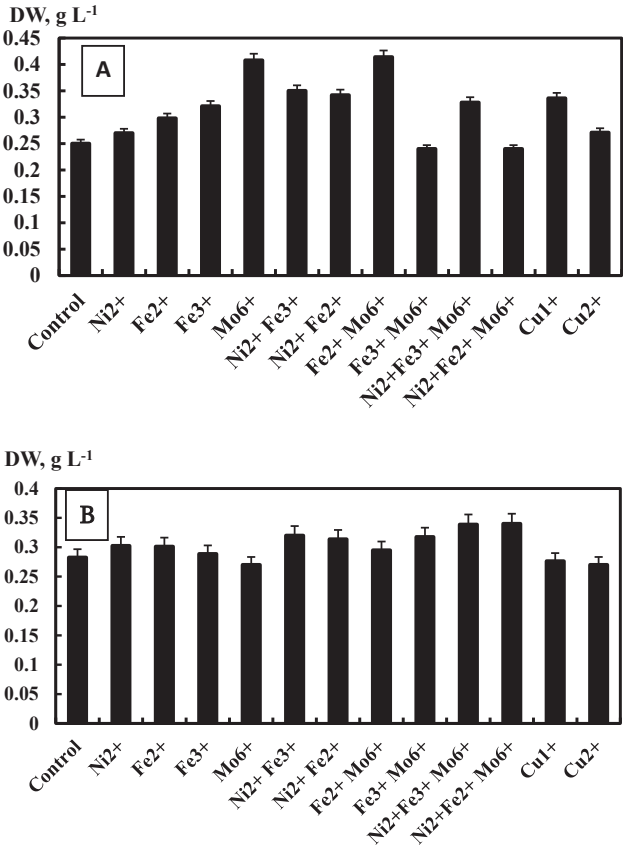

Fig. 2 – The effect of various metal ions and their mixtures on *E. coli* BW25113 growth yield during glycerol fermentation at different pHs. A- pH 5.5; B- pH 7.5. For the others, see legends to Fig. 1 and Materials and methods.

yield was stimulated ~1.4–1.7 fold upon Mo^{6+}+Fe^{2+} and Ni^{2+}+Fe^{2+}+Mo^{6+} mixtures supplementation (Fig. 3A). More increase was observed with metals combinations at pH 6.5: in contrary to pH 7.5, at pH 6.5 upon Fe^{3+}, Ni^{2+}+Fe^{3+}, Ni^{2+}+Fe^{2+} and Mo^{6+}+Fe^{2+} addition ~1.3-fold stimulation was detected (Fig. 3B). At last, at pH 7.5 in the presence of Ni^{2+}+Fe^{2+} growth yield was increased 1.5 fold and Cu^{+} or Cu^{2+} had bacterial growth suppressing effect (Fig. 3C).

The results are interesting to regulate *E. coli* bacterial growth and could be applied in biotechnology. They are in agreement with data obtained with the other bacteria: particularly it was shown that Fe^{3+} stimulated growth and the F_OF_1 ATPase activity of *Enterococcus hirae* [39] and H_2 production of *R. sphaeroides* [32].

H_2 production by E. coli *in the presence of various heavy metal ions during glycerol and glucose fermentation at different pHs*

E. coli wild type ferments glucose and glycerol when H_2 is produced, as an one of by-products. ORP as an important physicochemical parameter can monitor both cell growth and H_2 metabolism of *E. coli*: it's drop to negative values can point out the strengthening of reducing processes and H_2 production during fermentation [12,35,40]. Thus, the drop of ORP with increase of bacterial biomass (dry weight, DW) in *E. coli* BW25113

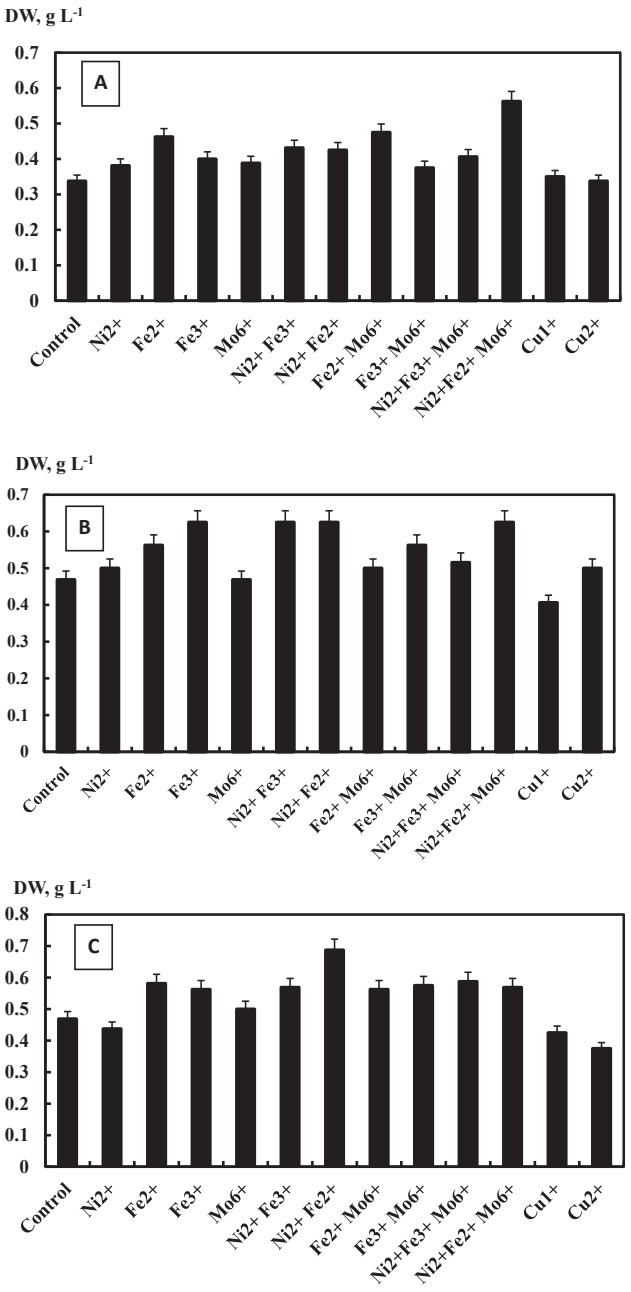

Fig. 3 – The effect of various metal ions and their mixtures on *E. coli* BW25113 growth yield during glucose fermentation at different pHs. A- pH 7.5, B- pH 6.5, C- pH 5.5. For the others, see legends to Fig. 1 and Materials and methods.

batch culture at different pHs was stated (Tables 1–3). It was shown in the case of glycerol fermentation, ORP (measured by platinum (Pt) electrode) dropped down to −400 ± 12 mV at the middle of log growth phase, as before [12]. Consequently, H_2 production was observed and its yield was calculated. In glucose fermenting culture ORP dropped to low negative values down to −550 ± 10 mV earlier, at the beginning of log growth phase, at different pHs [12].

Fe^{2+}, Fe^{3+}, Ni^{2+}, Mo^{6+}, Cu^{+} and Cu^{2+} solely and their mixtures influences on the decrease in ORP and H_2 production of *E. coli* during glucose and glycerol fermentation were investigated at pH 5.5–pH 7.5 (see Tables 1–3). H_2 production yield was 0.75 ± 0.02 mmol H_2 L^{-1} at 12 h growth of *E. coli* at pH 6.5 during glycerol fermentation, and it was noticeably stimulated (1.9–3 fold) in the presence of mixtures of Ni^{2+}+Fe^{3+}, Ni^{2+}+Fe^{3+}+Mo^{6+} or Fe^{3+}+Mo^{6+} Mo^{6+} (see Table 1). While Cu^{+} or Cu^{2+} had H_2 production suppressing effect (see Table 1). These results were expected, since Cu^{2+} can inhibit Hyd enzymes activity in different bacteria [41] and archaea [42]. Similar data were obtained at the certain conditions but at pH 5.5: though the H_2 production yield was ~1.5 fold decreased (0.50 ± 0.02 mmol H_2 L^{-1}) compared with pH 6.5, but metal ions supplementations again stimulated H_2 yield, particularly ~1.5 fold upon addition of Ni^{2+} alone and Ni^{2+}+Fe^{3+}, Ni^{2+}+Fe^{3+}+Mo^{6+} or Ni^{2+}+Fe^{2+}+Mo^{6+} (see Table 1).

Interesting data with *E. coli* was observed during glycerol fermentation at pH 7.5: less stimulation effect on H_2 production yield was determined upon metal ions alone and their mixtures at the end of log growth phase, but effects were ~1.7 fold stimulated ones upon all metals (Ni^{2+}, Fe^{3+}, Fe^{2+} and Mo^{6+}) and 2.7–3 fold upon Ni^{2+}+Fe^{3+}+Mo^{6+} mixture supplementations (see Table 2).

Subsequently, Fe^{2+}, Fe^{3+}, Ni^{2+}, Mo^{6+}, Cu^{+} and Cu^{2+} alone and their mixtures influence on *E. coli* H_2 production was investigated during glucose fermentation at pH 5.5–pH 7.5. As can be seen from Table 3, at pH 5.5 H_2 production yield without metals addition (control) was 0.66 ± 0.02 mmol H_2 L^{-1} and stimulated 1.2 fold upon Mo^{6+} alone and Mo^{6+}+Fe^{2+} or Mo^{6+}+Fe^{3+} mixtures supplementations.

At pH 6.5 H_2 yield without metals supplementation (control) was 0.75 ± 0.02 mmol H_2 L^{-1}; addition of Fe^{2+}+Mo^{6+}or Ni^{2+}+Fe^{2+}+Mo^{6+} increased H_2 ~1.2 fold, whereas at pH 7.5, compared with control (0.70 ± 0.02 mmol H_2 L^{-1}), only Fe^{3+}+Mo^{6+} mixture addition had ~1.5 fold stimulating effect (see Table 3).

Similar data with these metals was stated for photo fermentation performing *R. sphaeroides*, when some metal ions addition increased H_2 production yield [31,32].

Discussion

Some heavy metals have a role as structural components of Hyd enzymes, such as catalytic cofactors of these enzymes in reversible oxidation–reduction reactions, particularly in H_2 oxidation and H_2 production [14,16,17]. Thus, it was suggested that metals involved in H_2 metabolism might affect the bacterial cell growth and H_2 production of *E. coli*.

Low concentrations of Fe^{2+}, Fe^{3+}, Ni^{2+} and their mixtures of Ni^{2+}+Fe^{2+}+Mo^{6+}, Ni^{2+}+Fe^{3+}, Ni^{2+}+Fe^{2+} and Mo^{6+}+Fe^{3+} had stimulating effects on *E. coli* growth during glucose and glycerol fermentation at pH 5.5–pH 7.5. However, upon glycerol utilization metal effects were minor at pH 7.5. In the most cases, cooper ions at low concentrations inhibited or had no effect. As mentioned above, there are four different [Ni-Fe]-Hyd enzymes involved in H_2 metabolism in *E. coli*: with help of each Hyd mutants (lucking of catalytic subunits) the role of Hyd enzymes for bacterial growth was suggested and responsible Hyd enzyme were determined for different pHs and other conditions [1,42]. Probably, Ni, Fe and other metals

Table 1 – The decrease in oxidation–reduction potential (ORP) and *E. coli* BW25113 H_2 production yield during glycerol fermentation in the presence of heavy metal ions.

Growth conditions[a]	pH 5.5		pH 6.5	
	ORP[b], mV	H_2 yield, mmol H_2 L^{-1}	ORP, mV	H_2 yield, mmol H_2 L^{-1}
Control[c]	−300 ± 10	0.50 ± 0.02	−410 ± 10	0.75 ± 0.02
Ni^{2+}	−410 ± 10	0.75 ± 0.03 ($p < 0.01$)[d]	−390 ± 15	0.71 ± 0.03 ($p > 0.05$)
Fe^{2+}	−330 ± 15	0.60 ± 0.02 ($p < 0.05$)	−390 ± 10	0.70 ± 0.02 ($p > 0.05$)
Fe^{3+}	−300 ± 10	0.46 ± 0.04 ($p > 0.05$)	−390 ± 15	0.71 ± 0.04 ($p > 0.05$)
Mo^{6+}	−330 ± 15	0.60 ± 0.03 ($p < 0.05$)	−410 ± 15	0.75 ± 0.03 ($p > 0.05$)
Ni^{2+}+Fe^{3+}	−390 ± 10	0.70 ± 0.01 ($p < 0.01$)	−460 ± 10	1.30 ± 0.01 ($p < 0.001$)
Ni^{2+}+Fe^{2+}	−350 ± 10	0.64 ± 0.02 ($p < 0.025$)	−430 ± 10	0.80 ± 0.02 ($p > 0.05$)
Fe^{3+}+Mo^{6+}	−330 ± 15	0.60 ± 0.01 ($p < 0.05$)	−560 ± 12	2.20 ± 0.01 ($p < 0.001$)
Fe^{2+}+Mo^{6+}	−325 ± 15	0.57 ± 0.03 ($p < 0.05$)	−480 ± 10	1.40 ± 0.03 ($p < 0.01$)
Ni^{2+}+Fe^{3+}+Mo^{6+}	−420 ± 10	0.77 ± 0.01 ($p < 0.002$)	−535 ± 10	1.50 ± 0.01 ($p < 0.01$)
Ni^{2+}+Fe^{2+}+Mo^{6+}	−390 ± 10	0.68 ± 0.01 ($p < 0.01$)	−480 ± 10	0.8 ± 0.01 ($p > 0.05$)
Cu^{1+}	−200 ± 10	–[e]	−240 ± 10	–
Cu^{2+}	−70 ± 15	–	−130 ± 15	–

[a] 10 g L^{-1} glycerol was supplemented into the growth medium; 50 μM Ni^{2+}, Fe^{2+}or Fe^{3+}, 20 μM Mo^{6+}, and 100 μM Cu^{+}or Cu^{2+} were added into the growth medium when mentioned (see Materials and methods).
[b] Oxidation–reduction potential measured by Pt electrode.
[c] Control was without metal supplementation.
[d] p for difference between the experiment and appropriate control.
[e] H_2 production was inhibited.

Table 2 – The decrease in oxidation–reduction potential (ORP) and *E. coli* BW25113 H_2 production yield during glycerol fermentation in the presence of heavy metal ions at pH 7.5.

Growth conditions[a]	Log growth phase		Stationary growth phase	
	ORP, mV	H_2 yield, mmol H_2 L^{-1}	ORP, mV	H_2 yield, mmol H_2 L^{-1}
Control	−415 ± 10	0.75 ± 0.02	−440 ± 10	0.81 ± 0.02
Ni^{2+}	−430 ± 10	0.77 ± 0.03 ($p > 0.05$)	−480 ± 12	1.35 ± 0.01 ($p < 0.001$)
Fe^{2+}	−420 ± 12	0.75 ± 0.02 ($p > 0.05$)	−450 ± 10	0.83 ± 0.02 ($p > 0.05$)
Fe^{3+}	−410 ± 10	0.72 ± 0.04 ($p > 0.05$)	−460 ± 15	1.33 ± 0.04 ($p < 0.001$)
Mo^{6+}	−380 ± 15	0.64 ± 0.03 ($p > 0.05$)	−440 ± 10	0.81 ± 0.03 ($p > 0.05$)
Ni^{2+}+Fe^{3+}	−440 ± 10	0.81 ± 0.01 ($p < 0.05$)	−470 ± 12	1.35 ± 0.01 ($p < 0.001$)
Ni^{2+}+Fe^{2+}	−430 ± 10	0.80 ± 0.02 ($p < 0.05$)	−470 ± 10	1.36 ± 0.02 ($p < 0.001$)
Fe^{2+}+Mo^{6+}	−412 ± 12	0.75 ± 0.01 ($p > 0.05$)	−460 ± 10	1.35 ± 0.01 ($p < 0.001$)
Fe^{3+}+Mo^{6+}	−425 ± 12	0.77 ± 0.03 ($p > 0.05$)	−480 ± 15	1.38 ± 0.03 ($p < 0.001$)
Ni^{2+}+Fe^{3+}+Mo^{6}	−445 ± 11	0.81 ± 0.01 ($p < 0.05$)	−560 ± 10	2.24 ± 0.01 ($p < 0.001$)
Ni^{2+}+Fe^{2+}+Mo^{6}	−455 ± 10	0.83 ± 0.01 ($p < 0.05$)	−520 ± 10	1.50 ± 0.01 ($p < 0.001$)
Cu^{1+}	−210 ± 12	–[b]	−270 ± 15	–
Cu^{2+}	−150 ± 15	–	−200 ± 15	–

[a] Data presented for different growth phases. For other conditions and designations see Table 1.
[b] No H_2 produced.

can affect bacterial growth due to the decrease in ORP when reducing conditions were preferable for bacterial growth under anaerobic conditions [42] and the effects on Hyd enzymes. Thus, metal ions influence on bacterial growth can be regarded with different Hyd enzymes activity.

Fe, Ni and Mo ions alone and some of their mixtures stimulated also H_2 production yield by *E. coli* during batch culture growth on glucose and glycerol at wide range of pHs (pH 5.5–pH 7.5). Discrimination between Fe^{2+} and Fe^{3+} was important for biomass yield and H_2 production. The effects might be regarded with metal ions direct or indirect influence on H_2 metabolism; as was shown, some metals have a role in Hyd enzymes biosynthesis and maturation processes [17,23,25], or they might affect enzymes activity [29]. It seems that H_2 production stimulating effects were substrate of fermentation (glucose or glycerol) and pH dependent: compared with glucose stronger stimulating effects on H_2 production were observed during glycerol fermentation and at pH 5.5 and pH 6.5. H_2 yield stimulation was not significant at pH 7.5 during glycerol fermentation log growth phase, but it was enhanced at stationary growth phase, the stimulation effect might be regarded with external pH drop (from 7.5 to 6.5), as a result the increase in H_2 production yield was observed, as was shown [26] at stationary growth phase the Hyd enzymes maturation and biosynthesis processes are different, which may also affect Hyd activity. In addition, the role of Mo ions solely or with Fe ions seems to be important at acidic pH during glucose fermentation. It seems that Mo ions as structural compounds of FDH-H stimulate the FHL activity, which is essential for bacteria grown on glucose at low acidic pH. The difference in the effects, during glucose and glycerol utilizations, might be due to different pathways stated for glucose and glycerol fermentation as well as different Hyd

Table 3 – The decrease in oxidation–reduction potential (ORP) and *E. coli* BW25113 H_2 production yield during glucose fermentation at different pHs in the presence of heavy metal ions.

Growth conditions[a]	pH 5.5		pH 6.5		pH 7.5	
	ORP, mV	H_2 yield, mmol H_2 L^{-1}	ORP, mV	H_2 yield, mmol H_2 L^{-1}	ORP, mV	H_2 yield, mmol H_2 L^{-1}
Control	−380 ± 10	0.66 ± 0.02	−400 ± 10	0.73 ± 0.02	−390 ± 10	0.70 ± 0.02
Ni^{2+}	−380 ± 15	0.68 ± 0.03 ($p > 0.05$)	−450 ± 12	0.88 ± 0.02 ($p < 0.025$)	−400 ± 15	0.72 ± 0.03 ($p > 0.05$)
Fe^{2+}	−380 ± 15	0.66 ± 0.02 ($p > 0.05$)	−450 ± 15	0.90 ± 0.02 ($p < 0.002$)	−420 ± 10	0.76 ± 0.02 ($p > 0.05$)
Fe^{3+}	−380 ± 10	0.65 ± 0.04 ($p > 0.05$)	−450 ± 12	0.89 ± 0.01 ($p < 0.01$)	−430 ± 15	0.77 ± 0.04 ($p > 0.05$)
Mo^{6+}	−440 ± 10	0.80 ± 0.03 ($p < 0.025$)	−450 ± 10	0.88 ± 0.03 ($p < 0.05$)	−430 ± 15	0.79 ± 0.03 ($p > 0.05$)
Ni^{2+}+Fe^{3+}	−370 ± 10	0.65 ± 0.01 ($p > 0.05$)	−450 ± 10	0.85 ± 0.01 ($p < 0.025$)	−390 ± 10	0.70 ± 0.01 ($p > 0.05$)
Ni^{2+}+Fe^{2+}	−390 ± 10	0.66 ± 0.02 ($p > 0.05$)	−380 ± 10	0.66 ± 0.02 ($p > 0.05$)	−390 ± 10	0.70 ± 0.02 ($p > 0.05$)
Fe^{2+}+Mo^{6+}	−430 ± 15	0.78 ± 0.01 ($p < 0.025$)	−490 ± 15	1.00 ± 0.01 ($p < 0.002$)	−370 ± 10	0.65 ± 0.01 ($p > 0.05$)
Fe^{3+}+Mo^{6+}	−410 ± 15	0.75 ± 0.03 ($p < 0.05$)	−450 ± 15	0.84 ± 0.02 ($p < 0.05$)	−450 ± 10	1.27 ± 0.03 ($p < 0.02$)
Ni^{2+}+Fe^{3+}+Mo^{6+}	−370 ± 10	0.66 ± 0.01 ($p > 0.05$)	−450 ± 15	0.88 ± 0.01 ($p < 0.025$)	−450 ± 15	0.84 ± 0.01 ($p < 0.01$)
Ni^{2+}+Fe^{2+}+Mo^{6+}	−370 ± 15	0.65 ± 0.01 ($p > 0.05$)	−450 ± 15	0.90 ± 0.01 ($p < 0.01$)	−380 ± 15	0.68 ± 0.01 ($p > 0.05$)
Cu^{1+}	−250 ± 15	–[b]	−300 ± 10	–	−60 ± 15	–
Cu^{2+}	−100 ± 10	–	−200 ± 15	–	−90 ± 15	–

[a] For conditions and designations see Table 1.
[b] No H_2 produced.

enzymes involved in H_2 metabolism [1]. The results might also be regarded with stimulated functioning of metal-uptake systems at lower pHs, which can lead to more amount of Hyd enzymes. This might favor the synthesis of the enzymes required for H_2 production from glycerol. By the way, further studies are required to reveal responsive Hyd activity for H_2 production under metal ions alone and their mixtures supplementations upon both glycerol and glucose fermentations at different pHs.

Though, glycerol is inexpensive carbon source for bacterial fermentation, but yet H_2 production yield upon glycerol fermentation compared with other carbon (glucose) is lower. So, it should be significantly increased to be used in H_2 production biotechnology.

Interestingly it has been shown that relatively high concentrations of metals (>0.1 mM) may also affect *E. coli* growth, Hyd and the FOF1-ATPase enzymes activities [37,38].

Thus, the concentrations of heavy metals are important in regulation of glycerol and glucose fermentation, Hyd enzymes and H_2 metabolism by bacteria leading to changed biomass and H_2 production. The results are of significance, as they might be useful for obtaining biomass and increased H_2 production by mixed culture of *E. coli* with different bacteria and two stage technology development [5,6], and effects of heavy metals and their mixtures might be considered as optimization strategy for enhanced H_2 production.

Conclusions and significance

E. coli possesses four [Ni-Fe]-Hyd enzymes for activity of which heavy metals are required; in addition Mo is essential for FHL activity. Ni, Fe and Mo ions alone and their mixtures at low concentrations (Ni^{2+}+Fe^{3+} (50 μM), Ni^{2+}+Fe^{3+}+Mo^{6+} (20 μM) and Fe^{3+}+Mo^{6+} (20 μM)) but not Cu^{+} or Cu^{2+} (100 μM) were shown to stimulate bacterial biomass and H_2 production yields mostly upon glycerol but not glucose fermentation and in acidic conditions (pH 5.5 and pH 6.5).

H_2 is the most promising source of future fuel, so every new finding about H_2 production by bacteria is important. Moreover, glycerol as a cheap and available carbon source is attractive for fermentation processes. The modification of the medium with some heavy metals and their mixtures studied would be a strategy to be applied for the enhancement of biomass and H_2 production. The results are novel and might be used for optimizing fermentation processes on glycerol to develop effective H_2 production biotechnology.

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