Applied Energy 177 (2016) 335–340

Contents lists available at ScienceDirect

Applied Energy

journal homepage: www.elsevier.com/locate/apenergy

Optimizing strategy for *Escherichia coli* growth and hydrogen production during glycerol fermentation in batch culture: Effects of some heavy metal ions and their mixtures

Karen Trchounian, Anna Poladyan, Armen Trchounian *

Department of Microbiology & Plants and Microbes Biotechnology, Yerevan State University,1 A. Manoukian Str., 0025 Yerevan, Armenia
Research Institute of Biology, Biology Faculty, Yerevan State University, 1 A. Manoukian Str., 0025 Yerevan, Armenia

HIGHLIGHTS

- Effects of heavy metals on *Escherichia coli* were revealed during glycerol fermentation at pH 6.5.
- $Ni^{2+} + Fe^{2+}$ (0.05 mM) mixture stimulated up to 1.5-fold bacterial biomass yield.
- $Ni^{2+} + Fe^{3+}$ (0.05 mM), $Ni^{2+} + Fe^{3+} + Mo^{6+}$ (0.02 mM) and $Fe^{3+} + Mo^{6+}$ enhanced (up to 3-fold) H_2 production.
- Discrimination between Fe^{2+} and Fe^{3+} was important for H_2 production.
- Cu^{+} and Cu^{2+} (0.1 mM) inhibited H_2 production.

ARTICLE INFO

Article history:
Received 21 January 2016
Received in revised form 8 May 2016
Accepted 21 May 2016

Keywords:
Optimizing strategy for applied energy
Glycerol fermentation
Bacterial growth and biomass
H_2 production
Heavy metal ions
Escherichia coli

ABSTRACT

Hydrogen (H_2) is well-known effective, ecologically clean and renewable fuel. Bacterial H_2 production is a promising one and its use in industrial level is expected to increase in the nearest future to establish sustainable and renewable energy source. *Escherichia coli* wild type BW25113 growth yield was shown to be stimulated 1.3–1.5-fold by nickel (Ni^{2+}), iron (Fe^{2+}, Fe^{3+}) ions and by some metal ion mixtures: $Ni^{2+} + Fe^{2+}$ + molybdenum (Mo^{6+}), $Ni^{2+} + Fe^{3+}$, $Ni^{2+} + Fe^{2+}$ and $Mo^{6+} + Fe^{3+}$ in low concentrations (<0.05 mM) stimulated the growth during glycerol (10 g L^{-1}) fermentation up to stationary phase at pH 6.5; $Ni^{2+} + Fe^{2+}$ mixture showed the maximal effect. However, the same concentrations of these metals and their mixtures had no effects or slightly inhibited bacterial specific growth rate: it was suppressed ~1.2-fold upon Ni^{2+}, Fe^{3+}, Mo^{6+} and $Ni^{2+} + Mo^{6+}$ mixture supplementation. H_2 production by *E. coli* from glycerol was observed with the yield of 0.75 ± 0.02 mmol L^{-1}. Moreover, H_2 yield was markedly stimulated 1.7–3-fold in the presence of $Ni^{2+} + Fe^{3+}$, $Ni^{2+} + Fe^{3+} + Mo^{6+}$ and $Fe^{3+} + Mo^{6+}$ mixtures, but not sole metals: maximal stimulation was established by $Fe^{3+} + Mo^{6+}$ mixture with the concentrations of 0.05 mM and 0.02 mM, respectively. While copper (Cu^{+}, Cu^{2+}) ions in low concentration (0.1 mM) had H_2 production suppressing effect. The results point out that some heavy metal ions and their mixtures can stimulate *E. coli* growth, as well as enhance bio-hydrogen production. Discrimination between Fe^{2+} and Fe^{3+} was important for H_2 production. Some interaction of Ni^{2+} with Fe^{2+} was suggested to be effective factor increasing bacterial biomass and determining activity of H_2 producing hydrogenases together with Fe^{3+}. Mo^{6+} was significant for H_2 production. The results obtained were important to develop H_2 production biotechnology using glycerol as cheap substrate and optimizing the technological conditions by some heavy metals and their mixtures at low concentrations.

1. Introduction

Hydrogen (H_2) is well-known effective, ecologically clean and renewable fuel. It can be produced by different methods among which bacterial H_2 production (bio-hydrogen) is the promising one and expected to be increased in the nearest future [1]. Bio-hydrogen has advantages since it could be performed at

* Corresponding author at: Department of Microbiology & Plants and Microbes Biotechnology, Yerevan State University, 1 A. Manoukian Str., 0025 Yerevan, Armenia.
E-mail address: Trchounian@ysu.am (A. Trchounian).

http://dx.doi.org/10.1016/j.apenergy.2016.05.129
0306-2619/

Nomenclature

Hyd	hydrogenase
FHL	formate hydrogen lyase
F_OF_1-ATPase	proton-translocating ATPase
DW	dry weight
OD	optical density
ORP	oxidation reduction potential
Pt	platinum
Ti–Si	titanium–silicate
μ	specific growth rate

relatively low temperatures, atmospheric pressure and with relatively high rates; all these can reduce the costs for H_2 production [2]. In addition, developing decentralized energy systems is of importance for bio-hydrogen when energy production plants can be located not so far from carbon containing resources [2]. To develop biotechnology of H_2 production the by-products use and utilization of carbon containing organic wastes, construction of effective strains and optimization of bioprocessing conditions should be studied. The economic value is prioritized.

Glycerol, by-product of biodiesel industry, is a very cheap substrate [2]; it is fermented by *Escherichia coli*, resulting in the formation of H_2, ethanol, acetate and other end products [3]. Optimal conditions for glycerol fermentation are still under the study: it is suggested to be pH 6.3 [4,5]. Moreover, glycerol has highly reduced carbon and results in a higher production of reducing equivalents, such as reduced nicotinamide adenine dinucleotide (NADH), during its fermentation by *E. coli* than upon sugar (glucose) fermentation [4–6]. Consequently, reducing equivalents excess has a significant effect on the whole metabolic network. The ratio of the end products formed during glycerol and glucose fermentations in *E. coli* varies and depends on the concentration of glycerol or glucose, pH, oxidation–reduction potential (ORP) and other factors [7,8]. It is also important for H_2 economy that pure and crude glycerol gave similar H_2 yield by *E. coli* [2].

In *E. coli*, H_2 has been determined to be produced from formate, which is one of the end products of glucose or glycerol fermentation, by formate hydrogen lyase pathways (FHL) [1,9,10]. Four membrane-associated [Ni-Fe]-hydrogenase (Hyd) enzymes, Hyd 1 (*hya*), Hyd 2 (*hyb*), Hyd 3 (*hyc*) and Hyd 4 (*hyf*), are responsible for H_2 production and oxidation by *E. coli*; they can be reversible and operate in opposite directions during glucose or glycerol fermentation [9,11–15]. Interestingly, four Hyd enzymes together have the potential to form a H_2 cycle across the bacterial membrane of *E. coli* [16]. However, responsible enzymes in *E. coli* under different technological conditions including glycerol as a cheap substrate and at different pHs are not clear yet [1,2,11,12,14].

Hyd enzymes require low concentrations of some heavy metals, as nickel (Ni^{2+}) and iron (Fe^{3+}, Fe^{2+}), molybdenum (Mo^{6+}); these metals are necessary for bacterial growth, biosynthesis and maturation of Hyd enzymes [9,17–19]. The metals mentioned are proposed to participate in redox reactions transferring electrons via [Fe-S] clusters to active centers of Hyd enzymes [9,19–21]. Therefore, heavy metal ions can be suggested to regulate the activity of Hyd enzymes improving H_2 production. Interestingly, various metal ions including Ni^{2+}, Fe^{2+} and Mo^{6+} have been shown to increase H_2 production by different bacteria, specifically *Clostridium butyricum* [22] or *Rhodobacter sphaeroides* [23,24]; but the concentrations of metals, as well as substrates for fermentation and technological conditions used were different. On the other side, copper ions (Cu^{2+}) have been shown to inhibit some membrane enzymes in *E. coli*, including the proton F_OF_1-ATPase and Hyd enzymes [25], and Hyd enzymes in archaea [26]. However, their effects on bacterial growth are not clear, and the effects of heavy metals and especially their mixtures on the H_2 production by *E. coli* during glycerol fermentation have not been reported yet.

The aim of the present study was to determine the effects of some heavy metal ions in different oxidation states, as Ni^{2+}, Fe^{3+}, Fe^{2+} and Mo^{6+}, and their mixtures on *E. coli* growth and H_2 production upon glycerol fermentation at pH 6.5. In addition, Cu^{+} and Cu^{2+} effects on H_2 production were shown.

2. Materials and methods

2.1. Bacteria and growth conditions; growth rate and yield

The *E. coli* wild type BW25113 was used in this study. Bacteria from overnight culture were grown under anaerobic conditions at 37 °C in batch culture (150 mL glass vessels with plastic press-caps) in the peptone growth medium with the composition of 20 g L^{-1} peptone, 2 g L^{-1} K_2HPO_4, 5 g L^{-1} NaCl; 10 g L^{-1} pure glycerol added [7,8,12,14], at pH 6.5. The pH was measured by a pH-meter with selective pH-electrode (HJ1131B, Hanna Instruments, Portugal) and adjusted by 0.1 M NaOH or 0.1 N HCl. Metal ions in the form of different salts ($NiCl_2$, $FeSO_4{\cdot}H_2O$, $FeCl_3$, $Na_2MoO_4{\cdot}2H_2O$, $CuCl_2$, $CuSO_4$) and their mixtures were supplemented with the appropriate concentrations from freshly prepared sterile solutions into the growth medium before bacterial inoculation. Cl^- or SO_4^{2+} in the concentrations used had no effects (data not shown).

Bacterial growth was monitored by measuring bacterial culture absorbance (optical density, OD) at 600 nm using a spectrophotometer (Spectro UV–vis Auto, Labomed, USA). OD was measured in samples taken every 0.5 h till stationary growth phase (12 h). The specific growth rate (μ) was calculated over the interval, where the logarithm of OD was increased linearly with time, and expressed as lg2/doubling time; μ was expressed in h^{-1}. Bacterial yield was estimated by determination dry weight of bacterial suspension (DW) expressing in g L^{-1}, as before [8].

2.2. Determination of H_2 production

ORP of bacterial culture was measured with the help of the coupled oxidation–reduction platinum (Pt; EPB-1, GSEEE; or PT42BNC, HANNA Instruments, Portugal) and titanium–silicate (Ti–Si); EO-02, GSEEE, Gomel, Belarus) electrodes [27,28]. In contrast to Pt, Ti–Si electrode readings were not affected by the presence of H_2 (or oxygen) in the medium; this difference allows the determination of H_2 under anaerobic conditions (in bacterial suspension upon fermentation of glucose or glycerol), as reported previously [7,12,14,29]. This electrochemical method for H_2 determination has given reproducible and correct results for cumulative H_2 yield in liquids [2,9]. The H_2 yield by bacteria was calculated by the decrease of ORP to low negative values, as described [30], and expressed in mmol L^{-1}.

H_2 production during the growth of *E. coli* was visualized by the appearance of gas bubbles in the test tubes over the bacterial suspension using Durham tubes and verified by the chemical assay based on the bleaching of $KMnO_4$ solution in H_2SO_4 with H_2 [27,28].

2.3. Reagents and data processing

Glycerol, peptone (Carl Roths GmbH, Germany) and other reagents of analytical grade were used.

The average data are represented from three independent experiments; the standard errors were calculated using Microsoft Excel 2010 and Student criteria (P) were employed to validate the difference in average data between different series of experiments, as described previously [7,12,14,28]; the difference was valid if $P < 0.1$.

3. Results and discussion

3.1. E. coli growth properties in the presence of various heavy metal ions

E. coli growth was investigated during glycerol fermentation till stationary growth phase at pH 6.5 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. The metal ions concentrations were applied based on the results for *E. coli* and other bacteria, however not all of these metal ions effects were studied and, moreover, the substrates of fermentation and conditions were different [22–25,31].

The growth properties of *E. coli* BW25113 were determined upon each metal alone and their mixtures addition. It was shown that bacterial biomass or growth yield was 0.30 ± 0.01 g L^{-1} (DW) without metal ion supplementation and was increased 1.3–1.5-fold upon Ni^{2+}, Fe^{2+}, Fe^{3+} (in the concentrations of 0.05 mM) and 1.3 to 2-fold upon the mixtures of $Ni^{2+} + Fe^{2+} + Mo^{6+}$; $Ni^{2+} + Fe^{3+}$; $Ni^{2+} + Fe^{2+}$ or $Fe^{3+} + Mo^{6+}$ (the concentration of Mo^{6+} was 0.02 mM) supplementation (Fig. 1). The highest growth yield was obtained in the presence of $Ni^{2+} + Fe^{2+}$ mixture (see Fig. 1). The similar increase was obtained by absorbance measurements (data not shown). Without metal ion supplementation, μ was 0.63 ± 0.02 h^{-1}. While, metal ions of the same concentrations as above had no or slightly inhibitory effects on μ: it was ~1.2-fold inhibited upon Ni^{2+}, Fe^{3+}, Mo^{6+} and $Ni^{2+} + Mo^{6+}$ supplementations (Fig. 2). Importantly, μ with $Ni^{2+} + Fe^{2+}$ was the same as with $Ni^{2+} + Fe^{3+}$ (see Fig. 2). In addition, Cu^{+} or Cu^{2+} had also some stimulatory effects on *E. coli* growth (see Figs. 1 and 2). The effects had concentration dependent manner and were not observed at high concentrations (>0.1 mM) (data not shown).

These data indicate that some heavy metals and their mixtures in low concentrations (<0.1 mM) are required for *E. coli* growth during glycerol fermentation and can increase the growth yield obtaining a higher bacterial biomass. Some interaction of Ni^{2+} with Fe^{2+} could be suggested. These effects of heavy metal ions are obvious due to their requirement for redox processes and enzymes activity [17–21]; a further study is required. The effects of heavy metals at low concentrations studied could be different for various bacteria: Ni^{2+} increased *E. coli* (see Fig. 2) and *R. sphaeroides* [23] growth but had no effect on *Enterococcus hirae* growth [32]. This difference might be due to requirement of Ni^{2+} for Hyd activity by *E. coli* and *R. sphaeroides*. But the inhibitory effects of Cu^{2+} (>0.1 mM) were similar to those for *E. coli* [25,31] or *E. hirae* [33] reported with sugar (glucose) fermentation. Moreover, $Cu^{2+} + Fe^{3+}$ combination decreased *E. hirae* growth [33].

3.2. H_2 production by E. coli in the presence of various heavy metal ions

ORP is known as one of the important physicochemical parameters determining bacterial growth [34]. On the other hand, ORP is a parameter connected to the activity of Hyd enzymes and H_2 production [29]. Thus, ORP kinetics and H_2 production were investigated in *E. coli* during glycerol fermentation at pH 6.5. Drop of Pt electrode readings from positive to low negative values (~−450 mV) (comp. with decrease of Ti–Si–electrode readings) from the beginning of the lag growth phase to stationary growth phase of *E. coli* (Fig. 3) indicated the formation of H_2 with the yield of 0.75 ± 0.02 mmol L^{-1} at 12 h of growth (Table 1). The similar value for H_2 yield by *E. coli* during glycerol fermentation has been reported previously [35]. In spite of glycerol being very cheap substrate for fermentation, the value of H_2 yield by *E. coli* with glycerol fermentation reported by different groups [3,4,35] is not high enough to be employed in H_2 production biotechnology [2]; that is why the significant increase in H_2 yield by optimization of the technology conditions should be required.

H_2 yield was markedly stimulated (1.9–3-fold) in the presence of some heavy metal ion mixtures of 0.02–0.1 mM $Ni^{2+} + Fe^{3+}$, $Ni^{2+} + Fe^{3+} + Mo^{6+}$ and $Fe^{3+} + Mo^{6+}$ Mo^{6} (see Table 1). Indeed, the modification of the medium with some heavy metals and their mixtures studied would be a strategy to be applied for the increased H_2 production. While 0.1 mM Cu^{+} or Cu^{2+} had H_2 production suppressing effect (see Table 1), it seems to be likely

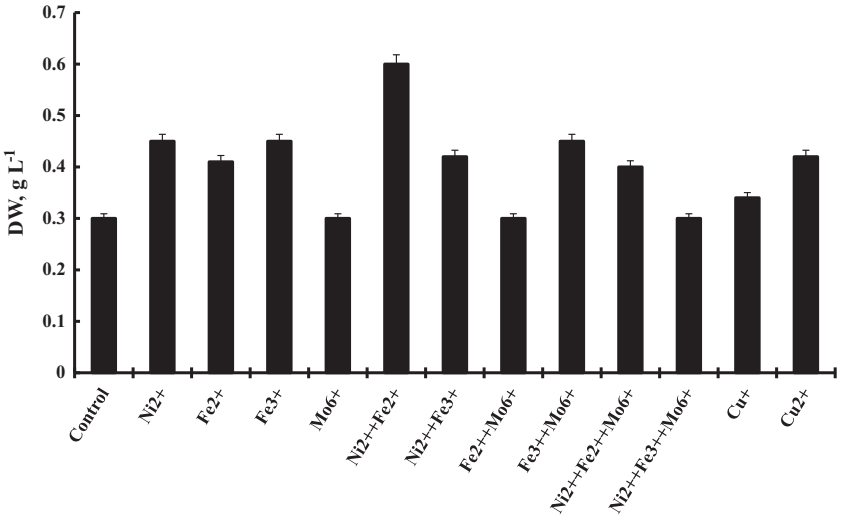

Fig. 1. The effect of various metal ions and their mixtures on *E. coli* BW25113 growth yield during glycerol fermentation at pH 6.5. 0.05 mM Ni^{2+}, Fe^{2+}, Fe^{3+}, 0.02 mM Mo^{6+} and 0.1 mM Cu^{+}, Cu^{2+} were added into the growth medium when mentioned. Dry cell weight (DW) was determined after 24 h bacterial growth. For the others, see Section 2.

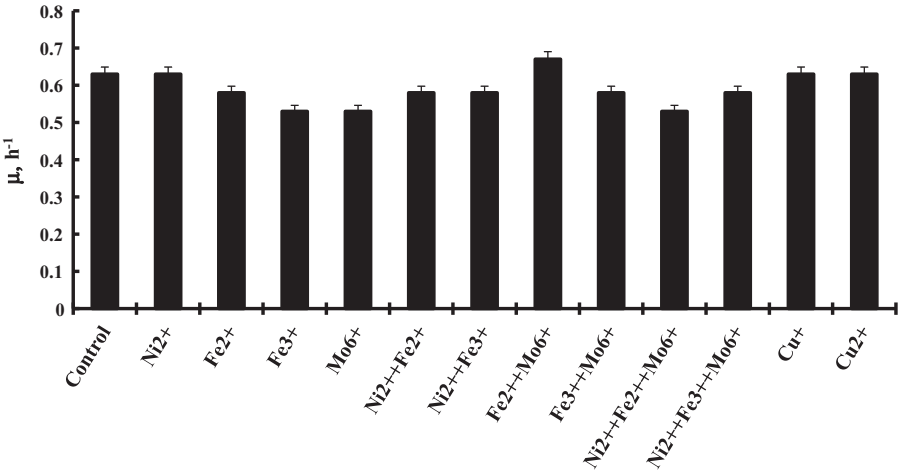

Fig. 2. The effect of various metal ions and their mixtures on *E. coli* BW25113 specific growth rates (μ) during glycerol fermentation at pH 6.5. For details, see the legends to Fig. 1.

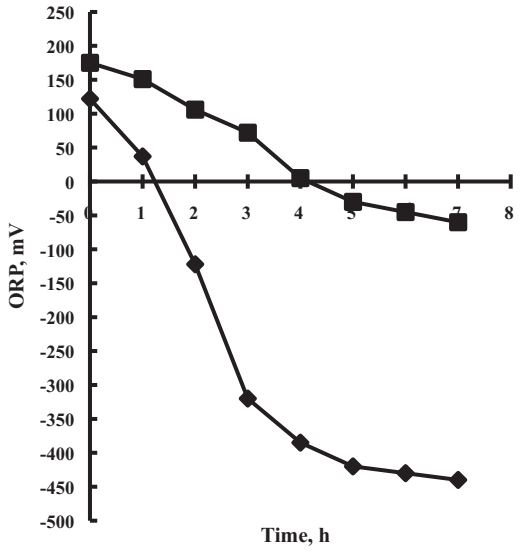

Fig. 3. ORP changes of *E. coli* BW25113 during glycerol fermentation at pH 6.5 without metals ions added into the growth medium. ORP was measured by platinum (Pt, ◆) and titanium–silicate (Ti–Si, ■) electrodes and expressed in mV (vs Ag/AgCl (saturated by KCl)). The error bars were <2% and within the designations. For the others, see Section 2.

Table 1
H_2 production yield of *E. coli* BW25113 during glycerol fermentation at pH 6.5 in the presence some heavy metal ions.

Additions of metal ions and their mixtures[a]	H_2 yield, mmol L^{-1}	Increase of H_2 yield
Control[b]	0.75 ± 0.02	1.00[d]
Ni^{2+}	0.71 ± 0.03 $P > 0.5$[c]	0.95
Fe^{2+}	0.70 ± 0.02 $P > 0.5$	0.93
Fe^{3+}	0.71 ± 0.04 $P > 0.5$	0.95
Mo^{6+}	0.75 ± 0.03 $P > 0.5$	1.00
Ni^{2+} + Fe^{3+}	1.30 ± 0.01 $P < 0.01$	1.73
Ni^{2+} + Fe^{2+}	0.80 ± 0.02 $P > 0.5$	1.07
Fe^{2+} + Mo^{6+}	1.40 ± 0.03 $P < 0.01$	1.87
Fe^{3+} + Mo^{6+}	2.20 ± 0.01 $P < 0.001$	2.93
Ni^{2+} + Fe^{3+} + Mo^{6+}	1.50 ± 0.01 $P < 0.01$	2.00
Ni^{2+} + Fe^{2+} + Mo^{6+}	0.80 ± 0.01 $P > 0.5$	1.07
Cu^{+}	–[e]	
Cu^{2+}	–	

[a] 0.05 mM Ni^{2+}, Fe^{2+}, Fe^{3+}, 0.02 mM Mo^{6+}, and 0.1 mM Cu^{+}, Cu^{2+}were added into the growth medium when mentioned.
[b] control was without metals supplementation.
[c] for the difference between the series and control.
[d] 1.00 was for control.
[e] H_2 production was absent.

that Cu^{2+} is an inhibitor for Hyd in different bacteria [25] and archaea [26].

Thus, the obtained results indicated the significance of some heavy metal ions and their mixed combinations in H_2 metabolism of *E. coli* during glycerol fermentation at pH 6.5. The stimulatory effects of metal ions at low concentrations (<0.1 mM) on H_2 production might be regarded as a result of their involvement in maturation and assembly or functioning of Hyd enzymes responsible for H_2 production: Ni-Fe ions as cofactors are necessary for the formation of active sites in Hyd [15,19,21] as well as Mo ions – for formate dehydrogenase, component of FHL [10]. Some interaction of Ni^{2+} with Fe^{3+} but not Fe^{2+} (comp. with *E. coli* growth yield, see Fig. 1) could be suggested to determine the activity of Hyd enzymes and enhanced H_2 production by *E. coli* (see Table 1). Fe^{3+} might stimulate electron transfer to H^{+} forming bridge of Ni with Fe and reducing H^{+} into H_2 within active site of Hyd enzymes, as proposed [9,15,19]. This complex mechanism of [Ni–Fe]-active site is in a good correlation with crystallography results of Hyd [21]. Therefore, some direct effect of Ni^{2+} and Fe^{3+} on Hyd activity is proposed. Interestingly, Fe^{2+} has been shown to be required for H_2 production by *R. sphaeroides* [24], suggesting different mechanisms of H_2 production [2]. Non-direct effect of heavy metals on Hyd activity should not be ruled out: they can affect ORP mediating changes in enzymes activity and redox processes [34]. Moreover, Fe^{3+} in combination with Mo^{6+} was significant for H_2 production.

Bacteria have developed multiple ways for accumulation of those heavy metals, such as primary and secondary transport systems, membrane channels, operating together with siderophores [18,20,32,33,36]. But in considerably higher concentrations (>0.1 mM) heavy metals might cause inhibition of bacterial growth

and Hyd activity and change proton F_OF_1-ATPase, as shown with *E. coli* [25,31].

These results are of interest too, since *E. coli* has been recently used in the mixed culture with *R. sphaeroides* to develop H_2 production biotechnology [37] or two stage technology with these bacteria developed instead of one [2,38] for which effects of heavy metals and their mixtures might be considered as optimization strategy for enhanced biohydrogen production. Furthermore, effects of metal ions and their mixtures supplemented into the growth medium to obtain bacterial biomass and to increase H_2 production will be studied while using different substrates or wastes and at different pHs. This will enlarge the applications of metals and their mixtures in biotechnology and energy production by bacteria.

4. Conclusions and consequences

Co-supplementation of some heavy metal ions and their mixtures in low concentrations can markedly stimulate *E. coli* growth on glycerol and significantly enhance H_2 production yield in batch culture. Discrimination between Fe^{2+} and Fe^{3+} was important for biomass yield and H_2 production. Some interaction of Ni^{2+} with Fe^{3+} and role of Fe^{3+} in combination with Mo^{6+} were suggested to determine the activity of H_2 producing Hyd enzymes. These results were novel and significant for H_2 production by *E. coli* .

E. coli growth and H_2 production from glycerol as a waste and a cheap substrate is not industrially practical so far, and these novel findings about the effects of some heavy metals and their mixtures might have advantageous application to increase bacterial biomass, as well as to enhance bio-hydrogen production. These will be important for construction of new strains by metabolic engineering and optimization of technology conditions, especially bacterial growth medium composition and nutrient content paying attention to the effect of the mixture of Ni^{2+} + Fe^{2+}. Bio-hydrogen, as applied energy source with no impact on the environment, would become more advantaged and economically effective.

Conflict of interest

The authors have no conflict of interest.

Acknowledgements

The authors thank to Ms. Astghik Vardanyan for help in some measurements and Dr. Lilit Hakobyan (Research Institute of Biology, Yerevan State University, Armenia) for help in improving English. This work was supported by Armenian National Science and Education Fund (USA) Grants to KT (NS-Biotech-3460) and AP (NS-Biotech-3212), Research grant of the State Committee of Science, Ministry of Education and Science of Armenia, to AT (#13-F002 and #15T-1F123).

References

[1] Trchounian A. Mechanisms for hydrogen production by different bacteria during mixed-acid and photo-fermentation and perspectives of hydrogen production biotechnology. Crit Rev Biotechnol 2015;35:103–13.
[2] Trchounian K, Trchounian A. Hydrogen production from glycerol *by Escherichia coli* and other bacteria: an overview and perspectives. Appl Energy 2015;156:174–84.
[3] Mattam AJ, Clomburg JM, Gonzalez R, Yazdani SS. Fermentation of glycerol and production of valuable chemical and biofuel molecules. Biotechnol Lett 2013;35:831–42.
[4] Dharmadi Y, Murarka A, Gonzalez R. Anaerobic fermentation of glycerol by *Escherichia coli*: a new platform for metabolic engineering. Biotechnol Bioeng 2006;94:821–9.
[5] Murarka A, Dharmadi Y, Yazmandi SS, Gonzalez R. Fermentative utilization of glycerol by *Escherichia coli* and its implications for the production of fuels and chemicals. Appl Environ Microbiol 2008;74:1124–35.
[6] Ghimire A, Frunzo L, Pirozzi F, Trably E, Escudie R, Lens PNL, et al. A review on dark fermentative biohydrogen production from organic biomass: process parameters and use of by-products. Appl Energy 2015;144:73–95.
[7] Trchounian K, Sanchez-Torres V, Wood TK, Trchounian A. *Escherichia coli* hydrogenase activity and H_2 production under glycerol fermentation at a low pH. Int J Hydrogen Energy 2011;36:4323–31.
[8] Poladyan A, Avagyan A, Vassilian A, Trchounian A. Oxidative and reductive routes of glycerol and glucose fermentation by *Escherichia coli* batch cultures and their regulation by oxidizing and reducing reagents at different pHs. Curr Microbiol 2013;66:49–55.
[9] Trchounian K, Poladyan A, Vassilian A, Trchounian A. Multiple and reversible hydrogenases for hydrogen production by *Escherichia coli*: dependence on fermentation substrate, pH and the F_0F_1-ATPase. Crit Rev Biochem Mol Biol 2012;47:236–49.
[10] McDowall JS, Murphy BJ, Haumann M, Palmer T, Armstrong AF, Sargent F. Bacterial formate hydrogenlyase complex. Proc Nat Acad Sci USA 2014;111: E3948–56.
[11] Andrews SC, Berks BC, Mc Clay J, Ambler A, Quail MA, Golby P, et al. A 12-cistron *Escherichia coli* operon (*hyf*) encoding a putative proton-translocating formate-hydrogenlyase system. Microbiology 1997;143:3633–47.
[12] Trchounian K, Pinske C, Sawers G, Trchounian A. Characterization of *Escherichia coli* [NiFe]-hydrogenase distribution during fermentative growth at different pHs. Cell Biochem Biophys 2012;62:433–40.
[13] Trchounian K, Soboh B, Sawers G, Trchounian A. Contribution of hydrogenase 2 to stationary phase H_2 production by *Escherichia coli* during fermentation of glycerol. Cell Biochem Biophys 2013;66:103–8.
[14] Trchounian K, Trchounian A. *Escherichia coli* hydrogenase 4 (*hyf*) and hydrogenase 2 (*hyb*) contribution in H_2 production mixed carbon (glucose and glycerol) fermentation at pH 7.5 and pH 5.5. Int J Hydrogen Energy 2013;38:3921–9.
[15] Sargent F. The model [NiFe]-hydrogenases of *Escherichia coli*. Adv Microb Physiol 2016;68:433–507.
[16] Trchounian A, Sawers RG. Novel insights into the bioenergetics of mixed-acid fermentation: can hydrogen and proton cycles combine to help maintain a proton motive force? Int Union Biochem Mol Biol, Life 2014;66:1–7.
[17] Forzi L, Sawers RG. Maturation of [NiFe]-hydrogenases in *Escherichia coli*. Biometals 2007;20:565–78.
[18] Pinske C, Sawers G. The role of the ferric-uptake regulator Fur and iron homeostasis in controlling levels of the [NiFe]-hydrogenases in *Escherichia coli*. Int J Hydrogen Energy 2010;35:8938–44.
[19] Pinske C, Sawers G. The importance of iron in the biosynthesis and assembly of [NiFe]-hydrogenases. Biomolecular concepts 2014;5:55–70.
[20] Andreini C, Bertini I, Cavallaro G, Holliday GL, Thornton JM. Metal ions in biological catalysis: from enzyme databases to general principles. J Biol Inorg Chem 2008;13:1205–18.
[21] Ogata H, Nishikawa K, Lubitz W. Hydrogens detected by subatomic resolution protein crystallography in a [NiFe] hydrogenase. Nature 2015;520(7548):571–4.
[22] Karadag D, Puhakka JA. Enhancement of anaerobic hydrogen production by iron and nickel. Int J Hydrogen Energy 2010;35:8554–60.
[23] Hakobyan L, Gabrielyan L, Trchounian A. Ni (II) and Mg (II) ions as factors enhancing biohydrogen production by *Rhodobacter sphaeroides* from mineral springs. Int J Hydrogen Energy 2012;37:7486–91.
[24] Hakobyan L, Gabrielyan L, Trchounian A. Bio-hydrogen production and the FoF_1-ATPase activity of *Rhodobacter sphaeroides*: effects of various heavy metal ions. Int J Hydrogen Energy 2012;37:17794–800.
[25] Kirakosyan G, Trchounian A. Redox sensing by *Escherichia coli*: effects of copper ions as oxidizers on proton-coupled membrane transport. Bioelectrochemistry 2007;70:58–63.
[26] Sapra R, Bagramyan K, Adams MW. A simple energy-conserving system: proton reduction coupled to proton translocation. Proc Nat Acad Sci USA 2003;100:7545–50.
[27] Bagramyan K, Mnatskanyan N, Poladian A, Vassilian A, Trchounian A. The roles of hydrogenases 3 and 4, and the F_0F_1-ATPase, in H_2 production by *Escherichia coli* at alkaline and acidic pH. FEBS Lett 2002;516:172–8.
[28] Gabrielyan L, Sargsyan H, Hakobyan L, Trchounian A. Regulation of hydrogen photoproduction in *Rhodobacter sphaeroides* batch culture by external oxidizers and reducers. Appl Energy 2014;131:20–5.
[29] Poladyan A, Trchounian K, Sawers G, Trchounian A. Hydrogen-oxidizing hydrogenases 1 and 2 of *Escherichia coli* regulate the onset of hydrogen evolution and ATPase activity, respectively, during glucose fermentation at alkaline pH. FEMS Microbiol Lett 2013;348:143–8.
[30] Piskarev M, Ushkanov VA, Aristova NA, Likhachev PP, Myslivets TS. Establishment of the redox potential of water saturated with hydrogen. Biophysics 2010;55:19–24.
[31] Kirakosyan G, Trchounian K, Vardanyan Z, Trchounian A. Copper (II) ions affect *Escherichia coli* membrane vesicle's SH-groups and a disulfide–dithiol interchange between membrane proteins. Cell Biochem Biophys 2008;51:45–50.
[32] Vardanyan Z, Trchounian A. The effects of manganese (II) but not nickel (II) ions on *Enterococcus hirae* cell growth, redox potential decrease, and proton-coupled membrane transport. Cell Biochem Biophys 2013;67:1301–6.
[33] Vardanyan Z, Trchounian A. Cu(II), Fe(III) and Mn(II) combinations as environment stress factors have distinguishing effects on *Enterococcus hirae*. J Environ Sci 2015;28:95–100.
[34] Vassilian A, Trchounian A. Effect of the medium redox potential on the growth and metabolism of anaerobic bacteria. Biophysics 2008;53:281–93 [in Russian].

[35] Trchounian K, Abrahamyan V, Poladyan A, Trchounian A. *Escherichia coli* growth and hydrogen production in batch culture upon formate alone and with glycerol co-fermentation at different pHs. Int J Hydrogen Energy 2015;40:9935–41.
[36] Ouyang Z, Isaacson R. Identification and characterization of a novel ABC iron transport system in *Escherichia coli*. Infect Immun 2006;74:6949–56.
[37] Sargsyan H, Trchounian K, Gabrielyan L, Trchounian A. Novel approach of ethanol waste utilization: biohydrogen production by mixed cultures of dark- and photo-fermentative bacteria using distillers grains. Int J Hydrogen Energy 2016;41:2377–82.
[38] Schievano A, Tenca A, Lonati S, Manzini E, Adani F. Can two-stage instead of one-stage anaerobic digestion really increase energy recovery from biomass? Appl Energy 2014;124:335–42.