Available online at www.sciencedirect.com

ScienceDirect

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

Short Communication

The effect of Cu (I) and Cu (II) ions' low concentrations on growth, biohydrogen production and the F_oF_1-ATPase activity of *Rhodobacter sphaeroides*

Lilit Hakobyan [a], *Harutyun Sargsyan* [a], *Lilit Gabrielyan* [a,b], *Armen Trchounian* [a,b,*]

[a] *Research Institute of Biology, Biology Faculty, Yerevan State University, 1 A. Manoukian Str., 0025 Yerevan, Armenia*

[b] *Department of Microbiology & Plants and Microbes Biotechnology, Biology Faculty, Yerevan State University, 1 A. Manoukian Str., 0025 Yerevan, Armenia*

ARTICLE INFO

Article history:
Received 5 December 2015
Received in revised form
3 June 2016
Accepted 15 July 2016
Available online 30 July 2016

Keywords:
Cu ions
Redox potential
Biohydrogen
ATPase activity
Rhodobacter sphaeroides

ABSTRACT

Various metal ions play a key role in growth and biohydrogen (H_2) production by phototrophic bacteria through incorporation into or stimulation of the responsible enzymes and/or related metabolic pathways. This study shows, that Cu (I) ions, at low concentration (<5 μM), suppressed the growth of *Rhodobacter sphaeroides* strain MDC6521, isolated from mineral springs in Armenia, but enhanced the H_2 yield by this bacterium. In contrast, Cu (II) ions with the same concentrations inhibited H_2 yield. These ions also affected the medium redox potential during *R. sphaeroides* growth. *N,N′*-dicyclohexylcarbodiimide-sensitive ATPase activity of *R. sphaeroides* membrane vesicles was increased in the presence of both ions. Cu ions at low concentrations are suggested to affect biohydrogen production pathways or responsible enzymes in *R. sphaeroides*, proton F_oF_1-ATPase of which could be a target for these ions.

Introduction

Growth and molecular hydrogen (H_2) production by different bacteria are strongly dependent on various external factors such as nature and character of substrates utilized, light intensity, pH, temperature, and growth medium content, specifically presence of heavy metal ions [1–3]. The distinguishing effects of different metal ions on H_2 production by bacteria during dark- and light-fermentation have been obtained, having concentration-dependent manners [3–6]. Heavy metals have been reported to demonstrate stimulatory, inhibitory, or

* *Corresponding author*. Department of Microbiology & Plants and Microbes Biotechnology, Biology Faculty, 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.ijhydene.2016.07.103
0360-3199/

toxic effects on biochemical reactions, depending on their concentrations [7–9]. However, mechanisms of these effects are not clear, although it is suggested to be result of influence of the metal ions on photosynthetic system formation processes and responsible enzyme activity. Understanding the appropriate mechanisms of heavy metals effects can improve H_2 production biotechnology development and application.

Purple non-sulfur *Rhodobacter sphaeroides* is a promising object for biotechnological research, and this bacterium was found to be suitable to perform a photo-fermentation of various carbon-containing organic substrates and wastes, leading to H_2 production [10–12]. In our previous studies, the stimulating effect of various metal ions on growth and H_2 production have been shown for *R. sphaeroides* from Armenian mineral springs [4,5]. The highest growth and H_2 yield was obtained for bacterial cells in the presence of Fe^{2+}, Mo^{6+} and Mg^{2+}. *R. sphaeroides* was unable to grow in medium without Mg^{2+}, indicating that Mg^{2+} is important for growth of purple bacteria [4]. H_2 production was not observed in the absence of Fe^{2+}, indicating that Fe^{2+} is required for H_2 production [5]. Moreover, the obtained results indicated a relationship between H_2 yield and the activity of proton F_oF_1-ATPase, a key enzyme of bioenergetics relevance [5].

The aim of the current research is the study of the influence of copper (Cu) ions in low concentrations on growth and H_2 production by photo-fermentative purple bacteria. The role of Cu ions in the metabolism of different bacteria has been explored by many researchers [3,7–9,13–16]. For example, Cu ions in low concentration (<0.05 mM) have been established to be required for *Escherichia coli* growth [13–15]. However, in a higher concentration (>0.1 mM), they are toxic, disturbing the membrane permeability and inhibiting enzymes activity [15,16]. Cu ions easily interact with radicals; their toxicity is based on interaction with the cell membrane and on the production of hydroperoxide radicals [7]. Moreover, H_2 production by *E. coli* was also inhibited by Cu ions [15]. The inhibitory effect of Cu ions was also reported for *Enterococcus hirae*, the disruption of disulfide-dithiol interchange between the F_oF_1-ATPase and the other membrane proteins was suggested as one of the possible action mechanisms [17,18]. Cu ions are important for several processes in photosynthetic organisms; however the inhibitory effects were shown [8,19,20]. But the effect of Cu ions at low concentrations on *R. sphaeroides* has not been investigated yet. These effects are also of an interest, since many chemical compounds affect cellular processes at low and ultra-low concentrations [21,22] including H_2 production by bacteria [23]. Moreover, these effects can be distinguishing factors between their low and moderate concentrations as shown for H_2 production with oxidizers and reducers [23,24]. For explaining these effects, it is necessary to understand the influence of low and ultra-low concentrations of compounds on the processes of structure formation, and change in aqueous solutions, and set up the relationship of properties of aqueous solutions with their biological effects [21,22]. It is interesting how the effects of compounds at low concentrations differ from those of relatively high concentrations.

This paper presents the influence of Cu ions' low concentrations on *R. sphaeroides* growth and H_2 production. Direct effect of these ions on the F_oF_1-ATPase activity was identified.

Materials and methods

Bacterial strain and growth conditions

R. sphaeroides strain MDC6521 (Microbial Depository Center, National Academy of Sciences of Armenia, Yerevan, Armenia, WDCM803), which was isolated from the Arzni mineral spring in the Armenian mountains, was used [4,5,24]. The bacterium was grown in batch culture (150 mL flasks) anaerobically upon illumination with a light intensity of ~36 W/m^2 in Ormerod medium with carbon source – succinate (3.54 g L^{-1}) and nitrogen source – yeast extract (0.2%), as described previously [23–25]. Halogen lamp (60 W) was used for illumination. Light intensity was measured by a lux-meter LM37 (Carl Roth, Germany). The growth of bacterial culture was recorded by changes in optical density (OD_{660}), using a Spectro UV–Vis Auto spectrophotometer (Labomed, USA), and by determining dry weight (DW) of bacterial biomass (g L^{-1}), which was correlated with OD_{660} according the equation: $DW = 0.50 \times OD_{660}$. Specific growth rate was evaluated as ln2/doubling time of OD within an interval, where the logarithm of culture OD increased with time in a linear manner (logarithmic growth phase), and it was expressed as h^{-1} [5,23–25].

The concentration of Cu^+ and Cu^{2+} in the growth medium ranged from 1 μM to 5 μM. Cu^{2+} and Cu^+ were added to the appropriate concentration from freshly prepared sterile solutions of $Cu_2Cl_2 \cdot 2H_2O$ and $CuCl_2 \cdot 2H_2O$.

The medium pH, redox potential determinations and H_2 assay

The initial pH of the bacterial growth medium was maintained at 7.0 ± 0.1 by 0.1 M NaOH or 0.1 M HCl and determined at certain time intervals (from 0 h to 96 h) by a pH-meter (HANNA Instruments, Portugal) with selective pH electrode, as described [5,24].

The medium redox potential (E_h) was determined during *R. sphaeroides* growth using a pair of redox (platinum (Pt) and titanium-silicate (Ti–Si)) electrodes, as described [5,23–25]; reference (Ag/AgCl) electrode was employed. Ti–Si electrode measured the overall E_h, whereas Pt electrode (sensitive to O_2 and H_2) under anaerobic conditions detected only H_2 [24]. E_h of both electrodes were tested in the control solution as described [24]: E_h at 25 °C was of 245 ± 10 mV. E_h kinetics determined using pair of redox electrodes during culture growth gives information about main redox processes and also H_2 generation [23–25]. The H_2 yield was evaluated by the drop of E_h to low negative values during bacterial growth as described and expressed in mmol/g DW [5,24,25]. Note that this determination of H_2 is close to the method with Clark-type electrode employed by other authors [26]. In addition, H_2 generation was confirmed by the chemical method, as described [5,27].

ATPase activity assay

ATPase activity was measured by amount of inorganic phosphate (P_i), liberated after adding 5 mM ATP (Tris salt) to membrane vesicles of *R. sphaeroides* [5,23,24]. Membrane

vesicles were prepared as before [5,24]; P_i was determined by the spectrophotometric method, as described [5,24], corrections were made for blanks without ATP or membrane vesicles. ATPase activity was expressed in nmol P_i per μg protein per min. The assay mixture was 50 mM Tris–HCl buffer (pH 8.0), containing 0.4 mM $MgSO_4$ was used. When used, membrane vesicles were pre-incubated with Cu^+, Cu^{2+} or *N,N′*-dicyclohexylcarbodiimide (DCCD) for 10 min. Note, DCCD is known as inhibitor for the proton F_oF_1-ATPase in bacteria, including *R. sphaeroides* [1,2,5,28].

Reagents, data processing and others

DCCD, ATP, sodium succinate from Sigma Aldrich (USA); yeast extract, $Cu_2Cl_2 \cdot 2H_2O$, $CuCl_2 \cdot 2H_2O$ from Carl Roths GmbH (Germany) and other reagents of analytical grade were used. Each experiment was repeated three times; error bars were presented on the figures. Standard errors such as standard deviation were calculated using appropriate function of Microsoft Excel 2013. Student criteria (p) were employed to validate the difference in average data between various series of experiments and controls, as described previously [23–25].

Results and discussion

Bacterial growth in the presence of Cu (I) and Cu (II) ions' low concentrations

Different ways to enhance H_2 production by photosynthetic bacteria include not only choosing effective and cheap substrates, but also exploring novel pathways in H_2 metabolism and regulation of electrons transfer within the cell membrane and of membrane-associated enzymes activity [1]. This could engineer new methods of regulating H_2 production by different bacteria.

The bacterial growth characteristics were monitored during phototrophic growth of *R. sphaeroides* MDC6521 in Cu^+ and Cu^{2+} containing media. Addition of low concentrations (1–5 μM) of Cu^+ and Cu^{2+} caused a delayed inhibition of *R. sphaeroides* growth. In fact, Cu ions support bacterial growth during first 72 h; however the further measurements detected inhibition of growth in Cu^+ and Cu^{2+} containing media, in contrast to the control, which reached the maximum after 96 h of growth (Fig. 1). In the presence of 5 μM Cu^+, the specific growth rate was ~2.5-fold lower than that of the control (~60% inhibition), whereas bacterial growth rate decreased ~4-fold (~74% inhibition) by an addition of 5 μM Cu^{2+} (Table 1). The suppression of *R. sphaeroides* growth in the presence of Cu ions may be caused by action of the reagent on E_h determining bacterial growth, as suggested [15,17], or by direct effect of these ions on bacterial membrane or key enzymes.

Effects of Cu (I) and Cu (II) ions' low concentrations on the redox potential kinetics and its relationship with pH change

Low concentrations of Cu ions were discovered to affect the E_h drop of the growth medium of *R. sphaeroides* during the anaerobic growth upon illumination (Fig. 2). The E_h of growth medium, measured by a Pt electrode, gradually decreased

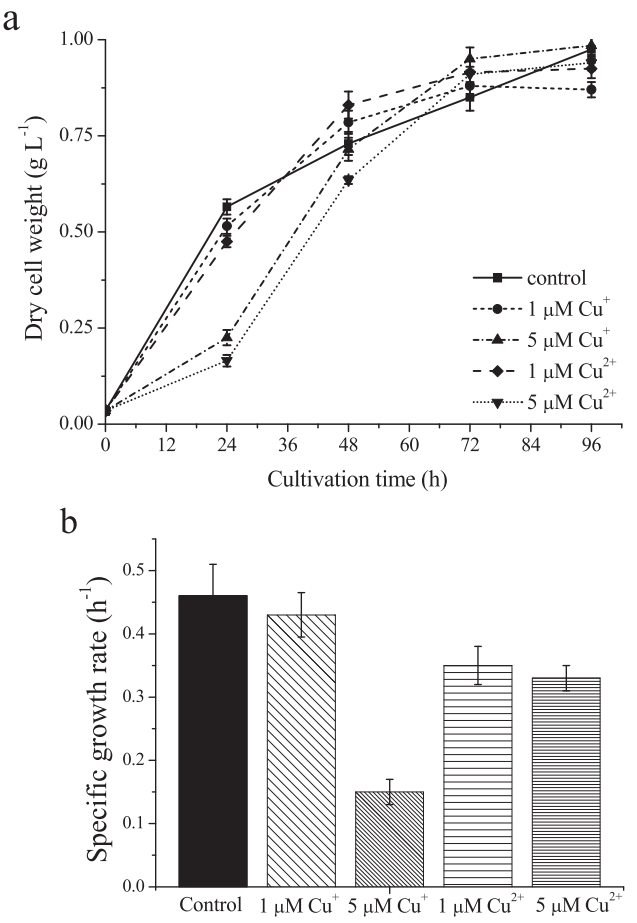

Fig. 1 – Effects of Cu^+ and Cu^{2+} ions' low concentrations on the *R. sphaeroides* MDC6521 cell growth properties: dry cell weight (a) and specific growth rate (b). 1 μM or 5 μM of Cu^+ and Cu^{2+} were added to the growth medium before inoculation of bacteria; control was the bacterial growth in the medium without any additions. For the others, see "Materials and methods" section.

during the anaerobic growth of *R. sphaeroides* (0–96 h): the intensity of the E_h drop depends on the growth conditions. Low concentrations of Cu ions were shown to have an enhancing effect on the E_h drop after 72 h of bacterial growth (Fig. 2a). The increase of concentration from 1 μM to 5 μM reduced the stimulatory effects of both ions, but the decrease was found to be more significant for Cu^{2+}. Further growth of bacteria in the presence of Cu ions resulted in the decrease of E_h drop.

The relationship of E_h kinetics and the change of external pH has previously been shown [5,23,24]. The results obtained for the effects of Cu ions low concentrations on external pH variation during bacterial growth (Fig. 2b), pointed out that the final pH of medium was higher than the initial pH, which was an important factor for the bacterial growth, as suggested before [5,24]. During the growth in the presence of Cu^+ and Cu^{2+} up to 96 h, external pH increased from 7.0 (initial pH) to 8.80–9.15 (see Fig. 2b). In comparison with control, the difference between initial pH and value of pH after 96 h of bacterial growth was more pronounced in the presence of Cu^+ ions (see Fig. 2b). This difference between Cu^+ and Cu^{2+} ions'

Table 1 – The changes of cell growth rate, H_2 yield and ATPase activity in *R. sphaeroides* MDC6521 during growth in anaerobic conditions in the presence of Cu ions.

Growth conditions	Growth rate, %	H_2 yield, %	ATPase activity, %
Control (no addition)	100	100	100
1 μM Cu^+	93.5 ± 1.5 (p* < 0.05)	138.0 ± 2.1 (p < 0.05)	140.5 ± 2.0 (p < 0.05)
5 μM Cu^+	39.0 ± 1.0 (p < 0.01)	125.0 ± 2.0 (p < 0.05)	154.0 ± 2.5 (p < 0.05)
1 μM Cu^{2+}	76.0 ± 1.5 (p < 0.05)	115.0 ± 2.0 (p < 0.01)	119.0 ± 2.0 (p < 0.01)
5 μM Cu^{2+}	26.0 ± 1.0 (p < 0.01)	81.0 ± 1.5 (p < 0.01)	135.0 ± 2.2 (p < 0.05)

*p was calculated for the difference between the values of experimental samples and appropriate control.

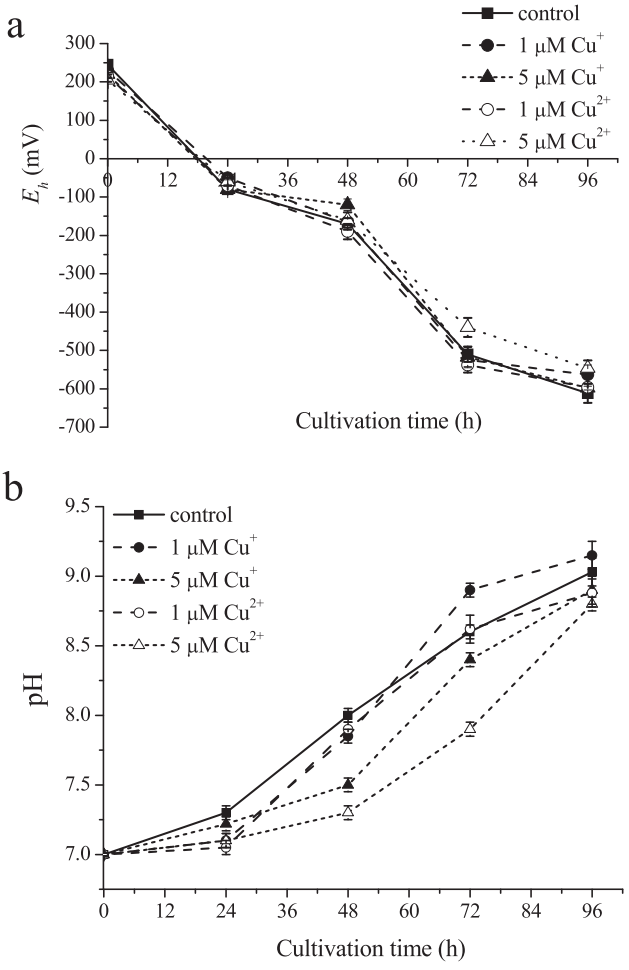

Fig. 2 – Effects of Cu^+ and Cu^{2+} ions' low concentrations on E_h kinetics (a) and pH change (b) during *R. sphaeroides* MDC6521 anaerobic growth in batch culture upon illumination.

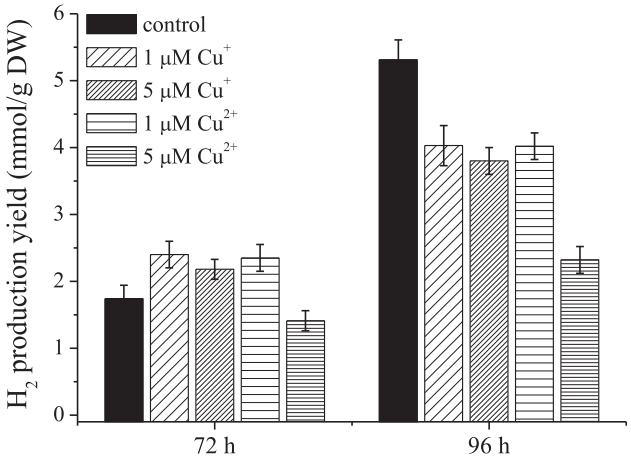

Fig. 3 – Effects of Cu^+ and Cu^{2+} ions' low concentrations on H_2 photoproduction during *R. sphaeroides* MDC6521 anaerobic growth in batch culture.

effects on medium pH may be coupled with the reducing and oxidizing properties of copper.

Effects of Cu (I) and Cu (II) ions' low concentrations on H_2 production and membrane associated F_oF_1-ATPase activity

Cu ions might affect H_2 production by *R. sphaeroides*. In fact, H_2 production by this bacterium after 72 h of growth was enhanced ~1.4- and 1.25-folds (~38% and ~25%) with 1 and 5 μM Cu^+, respectively (Fig. 3, see Table 1). Such an enhancement (~15%) was observed with 1 μM Cu^{2+} either, although the concentration dependent manner of such an effect was more dramatic in this case (see Fig. 3 and Table 1). Lin and Shei [3] have also shown the stimulating effect of Cu^{2+} low concentrations on fermentative H_2 production. However, 5 μM Cu^{2+} inhibited H_2 yield by ~20% (see Fig. 3 and Table 1). After 96 h of growth H_2 production was inhibited, this could be a result of the increased external pH. A comparison of the effects of Cu ions on the growth and H_2 production by *R. sphaeroides* can suggest the importance of H_2 production in mechanisms of copper-resistance in these bacteria, in addition to those reviewed [7].

The effect of low concentrations of Cu ions on H_2 production could be explained by a direct influence of these ions on the F_oF_1-ATPase. As known, H_2 production in purple bacteria is mediated by nitrogenase, requiring the energy of ATP, which is produced by the F_oF_1-ATPase [2,23]. The H^+-translocating ATPase of purple bacteria belongs to F-type ATPase [23]. Some conformational change in the F_oF_1-ATPase, leading to modulation of activity by Cu^{2+}, is possible [28]. Cu^+ can act as reducer and affect the process by breaking down the disulfides in membrane proteins. Indeed, DCCD-sensitive ATPase activity of membranes isolated from bacteria after 72 h of growth has been increased by addition of Cu ions; these effects had a concentration dependent manner, and different values for Cu^+ and Cu^{2+} (Fig. 4, see Table 1). The results suggest an interaction between Cu^+ and the F_oF_1-ATPase.

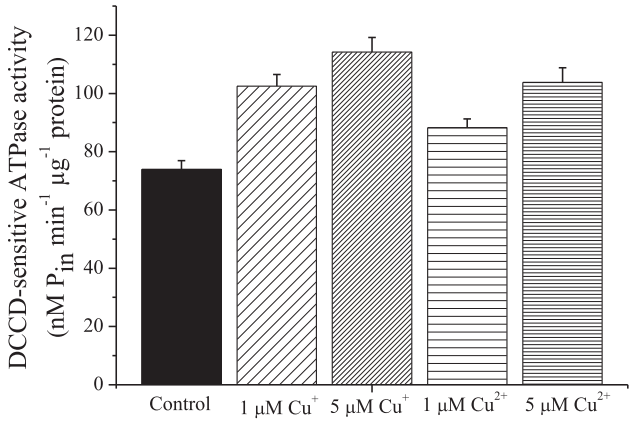

Fig. 4 – Effects of Cu^{+} and Cu^{2+} ions' low concentrations on DCCD-sensitive ATPase activity of membrane vesicles of *R. sphaeroides* MDC6521.

Conclusions and consequences

H_2 photoproduction by *R. sphaeroides* MDC6521 from the Arzni mineral spring in Armenia, at the presence of Cu^{+} and Cu^{2+} low concentrations, have been investigated. Cu^{+} at low concentration (5 μM) inhibits bacterial growth, but stimulates H_2 production by *R. sphaeroides*, whereas Cu^{2+} at the same concentration inhibits both processes. Such effects on the growth were suggested to be intermediated through E_h and supported by concentration-dependent effects observed. The difference between Cu^{+} and Cu^{2+} effects may be coupled with reducer and oxidizer properties of copper. Cu ions were likely to directly affect the bacterial membrane F_oF_1-ATPase, which can be a target for various heavy metals ions in bacterial cells.

The use of oxidizers and reducers to modify the redox environment is a simple way to affect bacterial metabolism and interfere with the biotechnological processes. Understanding of appropriate mechanisms could lead to directed regulation of cellular metabolism.

Acknowledgments

This study was supported by Research Grants from the State Committee of Science, Ministry of Education and Science of Armenia, to AT (#13-1F002 and #15T-1F123), from Armenian National Science & Education Fund based in USA (ANSEF) to LH (#NS-Biotech-3921).

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] Gabrielyan L, Trchounian A. Purple bacteria and cyanobacteria as potential producers of molecular hydrogen: an electrochemical and bioenergetic approach. In: Trchounian A, editor. Bacterial membranes. Kerala: Research Signpost; 2009. p. 233–73.
[3] Lin Ch-Y, Shei Sh-H. Heavy metal effects on fermentative hydrogen production using natural mixed microflora. Int J Hydrogen Energy 2008;33:587–93.
[4] 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;37:7482–6.
[5] Hakobyan L, Gabrielyan L, Trchounian A. Bio-hydrogen production and the F_oF_1-ATPase activity of *Rhodobacter sphaeroides*: effects of various heavy metal ions. Int J Hydrogen Energy 2012;37:17794–800.
[6] Liu BF, Ren NQI, Ding J, Xie GJ, Guo WQ. The effect of Ni^{2+}, Fe^{2+} and Mg^{2+} concentration on photo-hydrogen production by *Rhodopseudomonas faecalis* RLD-53. Int J Hydrogen Energy 2009;34:721–6.
[7] Nies DH. Microbial heavy-metal resistance. Appl Microbiol Biotechnol 1999;51:730–50.
[8] Giotta L, Agostiano A, Italiano F, Milano F, Trotta M. Heavy metal ion influence on the photosynthetic growth of *Rhodobacter sphaeroides*. Chemosphere 2006;62:1490–9.
[9] Mudhoo A, Kumar S. Effects of heavy metals as stress factors on anaerobic digestion processes and biogas production from biomass. Int J Environ Sci Technol 2013;10:1383–98.
[10] Sargsyan H, Gabrielyan L, Trchounian A. The distillers grains with solubles as a perspective substrate for obtaining biomass and producing bio-hydrogen by Rhodobacter Sphaeroides. Biomass Bioenergy 2016;90:90–4.
[11] Budiman PM, Wu TY. Ultrasonication pre-treatment of combined effluents from palm oil, pulp and paper mills for improving photofermentative biohydrogen production. Energy Convers Manag 2016;119:142–50.
[12] Budiman PM, Wu TY, Ramanan RN, Md Jahim J. Improvement of biohydrogen production through combined reuses of palm oil mill effluent together with pulp and paper mill effluent in photofermentation. Energy Fuels 2015;29:5816–24.
[13] Jun Y, Yi L, Peng L, Huigang L, Ming S, Songsheng Q, et al. Study of the thermokinetic properties of copper (II) on *Escherichia coli* growth. Biol Trace Elem Res 2003;92:61–70.
[14] Rensing C, Grass G. *Escherichia coli* mechanisms of copper homeostasis in a changing environment. FEMS Microbiol Rev 2003;27:197–213.
[15] Kirakosyan G, Trchounian A. Redox sensing by *Escherichia coli*: effects of copper ions as oxidizers on proton-coupled membrane transport. Bioelectrochemistry 2007;2007(70):58–63.
[16] Lebedev VS, Volodina LA, Deinega EY, Fedorov YI. Structural changes in the surface of *Escherichia coli* bacteria and the copper-induced permeability of the plasma membrane. Biophysics 2005;50:100–6.
[17] Vardanyan Z, Trchounian A. The effects of copper (II) ions on *Enterococcus hirae* cell growth and the proton-translocating F_oF_1 ATPase activity. Cell Biochem Biophys 2010;57:19–26.
[18] Trchounian A, Kobayashi H. Relationship of K^{+}-uptaking system with H^{+}-translocating ATPase in *Enterococcus hirae*, growth at a high or low alkaline pH. Curr Microbiol 1998;36:114–8.
[19] Osman MEH, Elnaggar A, Yanni YG, Wiessner W. Possible inhibition sites of Cu^{2+} in photosynthetic electron transport of cyanobacteria. Cytobios 1996;87:89–98.
[20] Mallick N. Copper-induced oxidative stress in the chlorophycean microalga *Chlorella vulgaris*: response of theantioxidant system. J Plant Physiol 2004;161:591–7.
[21] Konovalov AI, Ryzhkina IS. Highly diluted aqueous solutions: formation of nano-sized molecular assemblies (nanoassociates). Geochem Int (Moscow) 2014;52:1207–26.

[22] Konovalov AI, Ryzhkina IS, Murtazina LI, Kiseleva Iu V. Forming the nanosized molecular assemblies (nanoassociates) is a key to understand the properties of highly diluted aqueous solutions. Biophysics (Moscow) 2014;59:341–6.
[23] Gabrielyan L, Sargsyan H, Trchounian A. Novel properties of photofermentative biohydrogen production by purple bacteria *Rhodobacter sphaeroides*: effects of protonophores and inhibitors of responsible enzymes. Microb Cell Fact 2015;14:131–40.
[24] 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.
[25] Sargsyan H, Gabrielyan L, Hakobyan L, Trchounian A. Light-dark duration alternation effects on *Rhodobacter sphaeroides* growth, membrane properties and bio-hydrogen production in batch culture. Int J Hydrogen Energy 2015;40:4084–91.
[26] Eltsova ZA, Vasilieva LG, Tsygankov AA. Hydrogen production by recombinant strains of *Rhodobacter sphaeroides* using a modified photosynthetic apparatus. Appl Biochem Microbiol 2010;46:487–91.
[27] Maeda T, Wood TK. Formate detection by potassium permanganate for enhanced hydrogen production in *Escherichia coli*. Int J Hydrogen Energy 2008;33:2409–12.
[28] Trchounian A. *Escherichia coli* proton-translocating F_0F_1-ATP synthase and its association with solute secondary transporters and/or enzymes of anaerobic oxidation-reduction under fermentation. Biochem Biophys Res Commun 2004;315:1051–7.