Journal of Photochemistry & Photobiology, B: Biology 164 (2016) 271–275

Contents lists available at ScienceDirect

Journal of Photochemistry & Photobiology, B: Biology

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

Comparative effects of Ni(II) and Cu(II) ions and their combinations on redox potential and hydrogen photoproduction by *Rhodobacter sphaeroides*

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ARTICLE INFO

Article history:
Received 3 September 2016
Received in revised form 25 September 2016
Accepted 30 September 2016
Available online 01 October 2016

Keywords:
Rhodobacter sphaeroides
Heavy metal ions
Redox potential
H_2 photoproduction
ATPase activity

ABSTRACT

The aim of the present work was the study of comparative effects of Cu(II) and Ni(II) and their mixture on growth, redox potential, hydrogen (H_2) yield and ATPase activity in phototrophic purple bacteria *R. sphaeroides* MDC6522 from Jermuk mineral spring in Armenia. It was ascertained, that Cu^{2+} and Ni^{2+} have different effects on bacterial specific growth rate: in the presence of 5 μM Cu^{2+} growth rate was ~3.2-fold lower in comparison with control (no addition), and increased ~1.5-fold in medium with 5 μM Ni^{2+}. These changes may be resulted by action of the ions on redox potential (E_h). Low concentrations of Ni^{2+} had an enhancing effect on the E_h drop and H_2 production. The increase of concentration from 1 to 5 μM enhanced the stimulatory effect of Ni^{2+}. H_2 yield in *R. sphaeroides* (72 h of growth) was enhanced ~3-fold with 5 μM Ni^{2+}, whereas in the presence of 5 μM Cu^{2+} H_2 yield was ~1.2 fold lower in comparison with control. Cu^{2+} + Ni^{2+} combinations effects were differed from the effect when ions used separately. When Cu^{2+} and Ni^{2+} were added together, the Ni^{2+} stimulatory effect disappeared, which indicated that heavy metal ions mixture may have different action mechanisms. Moreover, *N,N′*-dicyclohexylcarbodiimide-sensitive ATPase activity of *R. sphaeroides* membrane vesicles has been increased in the presence of both ions, but in the presence of Cu^{2+} the influence was feebly marked in comparison with Ni^{2+}. The results suggest an interaction between these ions and the F_OF_1-ATPase. Thus, the results obtained point out discrimination between Cu^{2+} and Ni^{2+} and their combinations effects and reveal new regulatory pathways to enhance H_2 yield in *R. sphaeroides*.

1. Introduction

In the recent years the production of hydrogen (H_2) by microorganisms (known as biohydrogen) has a great interest in the biotechnology of biofuels [1–3]. H_2 production is considered as one of the promising ways to generate effective, ecologically clean and renewable energy sources from various organic substrates and wastes and can make a significant role in alternative H_2 energetics [3–5]. Biological H_2 production has several advantages in comparison with other especially thermochemical methods, among which — using cheap substrates, processing at low temperature and lower costs of production, especially H_2 production by photosynthetic organisms, which use sunlight as an energy source.

It is well known that two types of enzymes, nitrogenase and hydrogenase, are involved in H_2 metabolism in photosynthetic organisms, and H_2 production rate and yield depend on the various factors such as type of microbial culture, carbon and nitrogen sources, anaerobic conditions, temperature, pH, light intensity, metal ions and others [3,6–9]. By manipulating these factors it will be possible to significantly enhance H_2 production.

Different heavy metals such as iron (Fe), calcium (Ca), manganese (Mn), molybdenum (Mo), magnesium (Mg), and others are involved as "essential" elements in metabolism of microorganisms through stimulating the responsible enzymes and related metabolic pathways [10,11]. Some heavy metals such as cobalt (Co), copper (Cu), nickel (Ni), and zinc (Zn) are toxic for microorganisms at high concentrations [11–14]. Presence of heavy metals in the bacterial growth media can lead to changes in growth characteristics and fermentative activity of bacteria [11,14,15].

Photosynthetic bacteria can grow in various conditions, including metal-containing environments. Metal ions play key roles in metabolism of purple bacteria. Fe, Ni and Mo are the component of several enzymes, and the photosynthetic electron transfer chain carriers contain Fe [10,16–18]. Mg ion is an activator of various enzymes, and it is also a component of cell walls and cell membranes, and many photosynthetic electron carriers contain Mg such as chlorophyll and bacteriochlorophyll [11,17,19]. Mn is an important element in photosynthetic organisms: in purple bacteria it involves in regulation of nitrogenase

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http://dx.doi.org/10.1016/j.jphotobiol.2016.09.040
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activity [11,20]. Cu ions as trace element are present in photosynthetic organisms' growth media in low concentration [7,21,22].

In our previous works, the stimulatory effects of various metals ions were demonstrated on growth and H_2 production by purple bacteria *Rhodobacter sphaeroides* MDC6521 isolated from Arzni mineral springs in Armenia [8,23]. The highest H_2 yield in these bacteria was obtained in the presence of Fe^{2+}, Ni^{2+}, and Mo^{6+} [8,23]. Moreover, the other results indicated a relationship between H_2 production and the F_OF_1-ATPase activity [8].

Ni ion is a basic component of enzyme involved in H_2 metabolism such as [Ni–Fe] hydrogenase; it affects the H_2 production: a high concentration of Ni can inhibit the [Ni–Fe] hydrogenase activity, but a low concentration of Ni is required for activation of [Ni–Fe] hydrogenase [18]. Cu and Ni ions in high concentration (>0.1 mM) are toxic, disturbing the membrane permeability and inhibiting enzymes activity in *Escherichia coli* and *Enterococcus hirae* [12,24,25]. Sapra with co-workers have shown the inhibitory effect of Cu ions on hydrogenase activity in archaebacteria [26]. The inhibitory effect of these ions was reported for various bacteria, but the effects of Cu and Ni ions low concentrations and, especially, their combinations on photofermentative H_2 production by *R. sphaeroides* MDC6522 (isolated from Jermuk mineral springs) have not been investigated yet.

The investigation of Cu and Ni ions and their combinations effects on the H_2 production ability of purple bacteria is very important, because nowadays heavy metal pollution of the environment becomes a serious problem for microorganisms, living in various ecological niches. Understanding the mechanisms of heavy metals and their combinations effects can be useful to improve H_2 production by photosynthetic bacteria and its application in biotechnology.

In the present work the effects of Cu and Ni ions and their combinations on the growth properties, redox potential and H_2 production by *R. sphaeroides* have been studied. In addition, the effects of these ions on the F_OF_1-ATPase activity were determined.

2. Materials and Methods

2.1. Bacterial Strain and Cultivation Conditions

R. sphaeroides strain MDC6522 (Microbial Depository Center, National Academy of Sciences of Armenia, Yerevan, Armenia, WDCM803), isolated from the Jermuk mineral spring in the Armenian mountain (altitude above sea level 2100 m) [6,7], was used in this study.

The bacterial culture was grown anaerobically in batch culture (150 mL flasks) upon illumination (~36 W m^{-2}) at initial pH 7.0 in Ormerod medium as described earlier [6]. Halogen lamp (60 W) was used for illumination. Light intensity was measured by a lux-meter LM37 (Carl Roth, Germany). The growth of bacteria was monitored by changes in the optical density (OD_{660}) using a Spectro UV–Vis Auto spectrophotometer (Labomed, USA).

Growth characteristics as lag phase duration was determined graphically (intersection of tangent to growth curves) as time interval, during which cell number remains relatively constant (time before doubling of OD), and specific growth rate was calculated as the quotient of ln2 division on doubling time of OD over the interval, when the logarithm of OD of the culture at 660 nm increased with time linearly (logarithmic growth phase), and it was expressed as h^{-1} as described [5,6].

The concentration of Ni^{2+} and Cu^{2+} in the growth medium ranged from 1 μM to 5 μM. Ni^{2+} and Cu^{2+} and their combinations were supplemented with the appropriate concentrations from freshly prepared sterile solutions of $NiCl_2$ and $CuCl_2 \cdot 2H_2O$ into the growth medium before bacterial inoculation.

2.2. Redox Potential and Medium pH Determination

The redox potential (E_h) was measured using a pair of redox (platinum (Pt) and titanium-silicate (Ti–Si)) electrodes during *R. sphaeroides* anaerobic growth, as described before [5,6]. Pt electrode (sensitive to O_2 and H_2) under anaerobic conditions detected only H_2, whereas Ti–Si electrode measured the overall E_h. E_h of both electrodes were tested in the control solution, as described [6,27]: E_h at 25 °C was of 245 ± 10 mV.

The pH values were measured by a pH-meter (HANNA Instruments, Portugal) with selective pH electrode (HJ1131B) during bacterial growth, as described [5,27]. The initial pH was maintained at 7.0 ± 0.1 by 0.1 M NaOH or 0.1 M HCl.

2.3. The H_2 Yield Assay

The H_2 yield by bacteria was calculated by the decrease of E_h to low negative values during bacterial growth, as described [23,27] and expressed in mmol L^{-1}. In addition, H_2evaluation in bacterial suspension was visualized by the appearance of gas bubbles using Durham tubes and was confirmed by the chemical assay based on the bleaching of solution of potassium permanganate in H_2SO_4 in the presence of H_2 [28].

2.4. ATPase Assay

Membrane vesicles were isolated by the Kaback method, as described earlier [8,27]. The ATPase activity of membrane vesicles was determined by the amount of liberated inorganic phosphate (P_i) after adding 3 mM ATP to membrane vesicles by the spectrophotometric method using a Spectro UV–Vis Auto spectrophotometer (Labomed, USA), as described before [27]. Corrections were made for blanks without ATP or membrane vesicles. The assay mixture was 50 mM Tris–HCl buffer (pH 8.0); containing 0.4 mM $MgSO_4$ was used. When necessary, membrane vesicles were pre-incubated with 0.2 and 0.5 mM *N*,*N*′-dicyclohexylcarbodiimide (DCCD)and heavy metals ions for 10 min. Note, DCCD is known as inhibitor for the H^+-translocatingF_OF_1-ATPase in bacteria, including *R. sphaeroides* [8,27].

2.5. Reagents, Data Processing and Others

ATP (Tris salt), DCCD, sodium succinate from Sigma Aldrich (USA); yeast extract, Tris (amino-methane), $CuCl_2 \cdot 2H_2O$ and $NiCl_2$ from Carl Roth GmbH (Germany), and other reagents of analytical grade were used. The average data are presented from three independent measurements; error bars were presented on figures. Standard errors were calculated using appropriate function of Microsoft Excel 2013, as described [5,27]. The Student's validity criteria (P) were calculated to show the reliability of difference between experimental data and control.

3. Results and Discussion

3.1. *R. sphaeroides* Growth Properties, Redox Potential and pH Changes in the Presence of Cu^{2+} and Ni^{2+} and Their Combinations

As there is a mixture of various heavy metals in environment, it is interesting to examine the effects of heavy metal combination on growth properties of *R. sphaeroides* MDC6522. The bacterial growth characteristics were studied during phototrophic growth of *R. sphaeroides* in 1–5 μM Cu^{2+} and Ni^{2+} and their combination containing media. It was shown, that growth lag phase was prolonged when Cu^{2+} was added separately and in the presence of Cu^{2+} + Ni^{2+} mixture, but not Ni^{2+} (Fig. 1a). Specific growth rate by addition of Cu^{2+} and Ni^{2+} also changed in differed manner: in the presence of 5 μM Cu^{2+} growth rate was ~3.2-fold ($p < 0.001$) lower in comparison with control (no addition), and has increased ~1.5-fold ($p < 0.01$) in medium with 5 μM Ni^{2+} (Fig. 1b). The similar result was obtained with *R. sphaeroides* other strain MDC6521, isolated from Arzni mineral spring, in the presence of Ni^{2+} [23].

In the presence of 5 μM Cu^{2+} + Ni^{2+} mixture specific growth rate has decreased ~2.7-fold ($p < 0.001$). It is interesting, that when Cu^{2+}

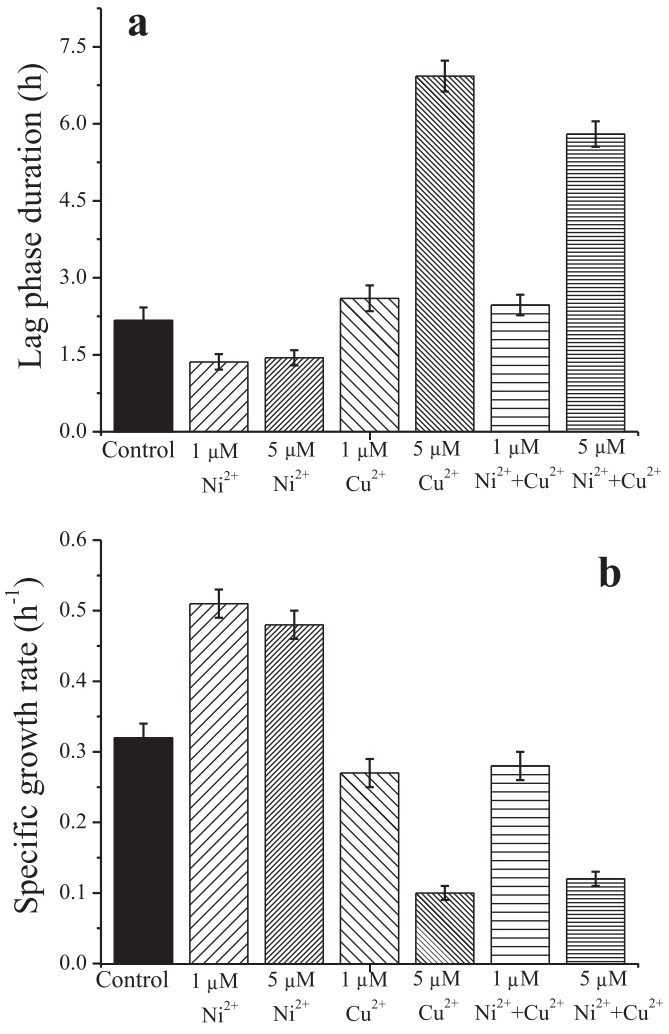

Fig. 1. *R. sphaeroides* MDC6522 growth characteristics: lag phase duration (a) and specific growth rate (b) in the presence of Cu^{2+} and Ni^{2+} and their combinations. Control was without heavy metal addition. For the other, see Materials and Methods section.

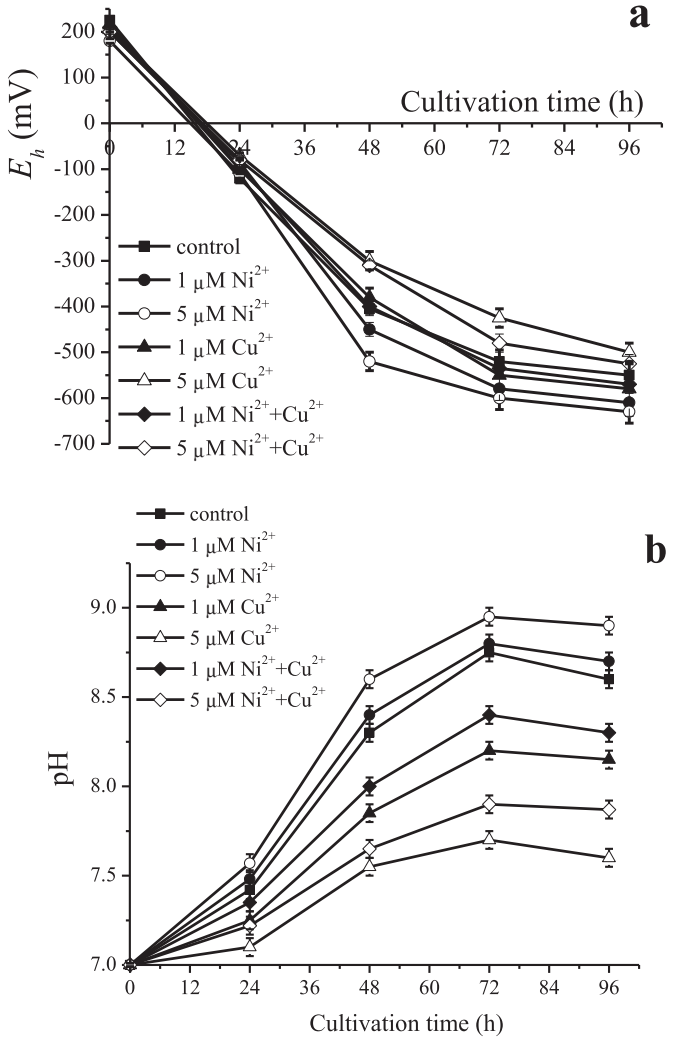

Fig. 2. E_h (a) and pH (b) changes during *R. sphaeroides* MDC6522 anaerobic growth in batch culture upon illumination in the presence of Cu^{2+} and Ni^{2+} and their combinations. For the other, see Materials and Methods section.

and Ni^{2+} were added together, the stimulatory effect of Ni^{2+} disappeared, which indicated that heavy metal ions combinations have different action mechanisms. This effect is similar to the effect obtained in *E. hirae* with $Cu^{2+} + Fe^{3+}$ mixture, when stimulatory effect of Fe^{3+} on bacterial growth disappeared in the presence of Cu^{2+} [24]. Unfortunately, the mechanisms linked with the neutralization of stimulatory effects are not clear yet.

These changes may be resulted by action of the ions on E_h or by direct effect of these ions on bacterial membrane. Bacterial growth medium E_h is considered as important factor of the environment, which can be defined as biological system ability to reduce or oxidize various compounds [24,27]. Change in E_h value was observed during *R. sphaeroides* MDC6522 growth. As shown in Fig. 2a, the initial positive E_h value of *R. sphaeroides* cells gradually dropped to negative ones during 96 h of bacterial growth. These results indicate various redox reactions observed during bacterial anaerobic growth. The initial value of E_h of *R. sphaeroides* control cells (in the metals ions absence) was 225 ± 5 mV, which decreased to −520 ± 25 mV during *R. sphaeroides* growth up to 72 h (Fig. 2a). Low concentrations of Cu and Ni ions were discovered to affect the E_h drop of the *R. sphaeroides* growth medium in a concentration dependent manner. In the presence of 1 μM Ni^{2+} E_h decreased to −580 ± 25 mV. The increase of concentration from 1 μM to 5 μM reduced the stimulatory effects of Ni^{2+} (E_h decreased up to −600 ± 25 mV) (Fig. 2a). In contrast, in the presence of 5 μM Cu^{2+} E_h only declined to −425 ± 20 mV. Addition of 1 μM $Cu^{2+} + Ni^{2+}$ mixture into the bacterial growth medium did not led to any changes in E_h in comparison with control, whereas in the presence of 5 μM $Cu^{2+} + Ni^{2+}$ mixture E_h dropped to–480 ± 20 mV (Fig. 2a). These changes may be coupled with the electrochemical potential of Cu^{2+}/Cu^{+}, which is equal −268 mV [11]. It is known, that Cu easily interacts with radicals, and this ion toxicity is based on the hydroperoxide radicals production and on interaction with cell membrane [11,13]. Different effects of Cu^{2+} and Ni^{2+} on E_h value may be coupled with their action mechanisms as oxidizer and reducer [24].

The other factor is medium pH, because it can affect the activity of H_2-producing enzymes, such as nitrogenase and hydrogenase, as well as the H_2 metabolic pathways [6,27]. During the anaerobic growth of *R. sphaeroides* MDC6522 control cells up to 72 h, the pH of medium has risen from 7.0 ± 0.01 (initial pH) up to 8.75 (Fig. 2b). After then pH decreased, which can be caused by the production of photofermentative end-products, particularly acids, which could decay with H_2 production and CO_2 co-evolution, which can also moderate pH change [5,6].

In the presence of 1 μM Ni^{2+} pH change during bacterial growth was similar to the control; the pH value increased to ~9.0 in the presence of 5 μM Ni^{2+} (Fig. 2b). The other kinetics of pH was observed in the presence of Cu^{2+}: pH increased to ~7.7 in the presence of 5 μM Cu^{2+},

whereas in the presence of 5 μM Cu^{2+} + Ni^{2+} mixture pH of medium increased to ~8 (Fig. 2b).

3.2. H_2 production yield by R. sphaeroides in the presence of Cu^{2+} and Ni^{2+} and their combinations

The negative value of E_h is connected with H_2 production, because for the reaction of $2H^+ + 2e^- \rightarrow H_2$ E_h equals to −414 mV [27]. A relationship between dropping of E_h and H_2 yield was shown for various bacteria: the electron flow can be shifted toward the reduction of protons to H_2 under strong reducing conditions [25,27].

Heavy metal ions might affect H_2 production by *R. sphaeroides*. The effect of Cu and Ni ions on H_2 yield in *R. sphaeroides* presented a different picture (Fig. 3). H_2 yield in *R. sphaeroides* after 72 h of growth was enhanced ~1.5-fold ($p < 0.05$) and ~2.5-fold ($p < 0.01$) with 1 μM Cu^{2+} and Ni^{2+}, respectively. However, 5 μM Cu^{2+} inhibited H_2 yield ~1.2-fold ($p < 0.05$), whereas in the presence of 5 μM Ni^{2+} H_2 yield was ~3.4-fold ($p < 0.001$) higher in comparison with control. These data were similar to the results on Ni^{2+} effects obtained for the other strain *R. sphaeroides* MDC6521 isolated from Arzni mineral spring; but in the medium with the same concentration Ni^{2+} H_2 production by str. MDC6521 was ~3-fold ($p < 0.01$) higher in comparison with control [23]. Mineral springs of Jermuk and Arzni differ from each other by their physicochemical properties: water of Jermuk mineral spring (altitude above sea level 2100 m) is sulfate-chloride type with temperature of 11–58 °C and pH of 6.0–7.6; while water of Arzni spring (altitude above sea level 1250 m) is sodium-chloride type with temperature of 13.3–22.5 °C and pH 6.3–6.6; total mineralization of Jermuk is higher than of Arzni [29]. Physicochemical properties of these springs can be a reason of some differences in the metabolism of investigated strains.

It is interesting, that when Cu^{2+} and Ni^{2+} were added together, the stimulatory effect of Ni^{2+} on H_2 production also disappeared: in the presence of 1 μM Cu^{2+} + Ni^{2+} mixture H_2 yield was similar to the control after 72 h bacterial growth, and was increased ~1.6-fold ($p < 0.01$) after 96 h bacterial growth (Fig. 3). By addition of 5 μM Cu^{2+} + Ni^{2+} mixture H_2 yield was decreased ~1.4-fold ($p < 0.025$) after 96 h bacterial growth (Fig. 3). The results obtained suggest that heavy metal ions combination have different action mechanisms. It is possible that Cu ions have negative effect on the nickel uptake in this bacterium. Ni transfer systems in purple bacteria have not been investigated well, but two mechanisms for Ni uptake have been identified in various photosynthetic bacteria: Ni-specific ABC transporters and HupE/UreJ families of secondary systems [30].

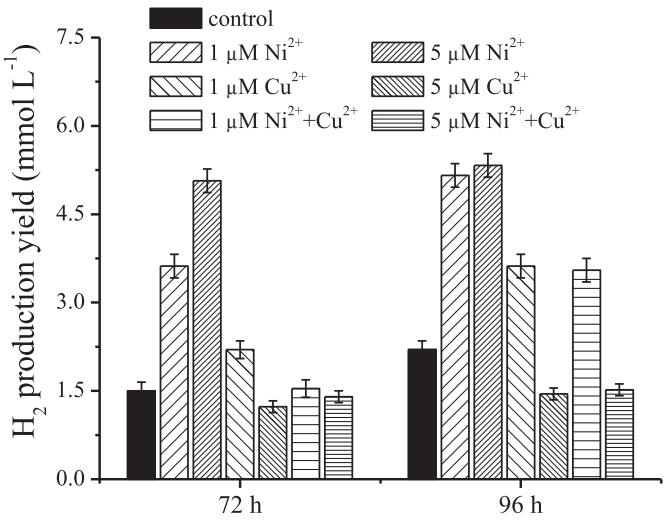

Fig. 3. H_2 yield during *R. sphaeroides* MDC6522 anaerobic growth in batch culture in the presence of Cu^{2+} and Ni^{2+} and their combinations. For the other, see Materials and Methods section.

3.3. Effects of Cu^{2+} and Ni^{2+} and Their Combinations on Membrane-associated F_OF_1-ATPase Activity

To explain the possible mechanisms of heavy metal ions and their mixture, membrane-associated F_OF_1-ATPase activity was determined. H_2 production in photosynthetic bacteria is catalyzed by nitrogenase, which requires the energy from ATP produced by F_OF_1-ATPase [27]. ATPase activity was determined in the presence of DCCD, an inhibitor of proton-translocation transport systems [8,27]. DCCD affects the ATPase activity of membrane vesicles, isolated after 72 h of bacterial growth, in concentration dependent manner (Fig. 4). Indeed, DCCD-sensitive ATPase activity has been increased by addition of both ions; these effects had the different values for Ni^{2+} and Cu^{2+} (Fig. 4). In the presence of Cu^{2+} ATPase activity was feebly marked in comparison with Ni^{2+}. By addition of Cu^{2+} + Ni^{2+} mixture ATPase activity was similar to data obtained when Cu^{2+} added separately (not shown). The results suggest an interaction between these ions and the F_OF_1-ATPase. Cu^{2+} and Ni^{2+} may directly affect the F_OF_1-ATPase, but this effect can be intermediated by E_h. As known, the ATPases of bacteria are redox-regulated enzymes [31].

4. Conclusion

Thus, the results point out discrimination between Cu^{2+} and Ni^{2+} and their combination effects and reveal new regulatory pathways to enhance H_2 yield in *R. sphaeroides*. It was observed that the effects of heavy metal mixture differed from the effects, when metal ions were used separately. Ni^{2+} at low concentration (5 μM) stimulates bacterial growth, redox potential decrease and H_2 production by *R. sphaeroides*, whereas Cu^{2+} at the same concentration inhibits these processes (Table 1). It is interestingly, when both ions were added simultaneously, the effect of Ni^{2+} disappeared, and only inhibitory effect of Cu^{2+} was detected (see Table 1). Such effects were suggested to be intermediated through E_h and supported by concentration-dependent effects observed. The data obtained indicate the metal ions used don't only act as oxidizer and reducer, but show specific mechanisms of influence.

DCCD-sensitive ATPase activity has been enhanced in the presence of both ions, but in the presence of Cu^{2+} the influence was feebly marked in comparison with Ni^{2+} (see Table 1). In the presence of Cu^{2+} + Ni^{2+} combination F_OF_1-ATPase activity was similar to data

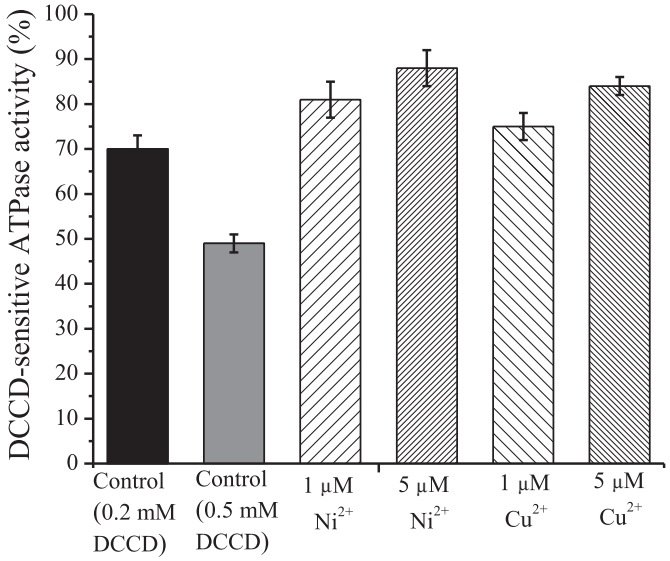

Fig. 4. DCCD-sensitive ATPase activity of *R. sphaeroides* MDC6522 membrane vesicles in the presence of Cu^{2+} and Ni^{2+} and their combinations. Control without heavy metal addition was in % from total activity, 0.2 mM and 0.5 mM DCCD were present in the assay medium, when indicated. For the other, see Materials and Methods section.

Table 1
The changes of *R. sphaeroides* MDC6522 specific growth rate, H_2 production yield and ATPase activity by addition of Cu and Ni ions and their mixture.

Conditions	Specific growth rate, %	H_2 yield (after 72 h), %	ATPase activity, %
Control (no additions)	100	100	100
1 μM Ni^{2+}	160	240	116
5 μM Ni^{2+}	150	336	125
1 μM Cu^{2+}	84.4	146	107
5 μM Cu^{2+}	31.3	81.5	120
1 μM Ni^{2+} + 1 μM Cu^{2+}	87.5	103	108.6
5 μM Ni^{2+} + 5 μM Cu^{2+}	37.5	93	117

obtained, when Cu^{2+} added separately. The results suggest an interaction between these ions and the F_OF_1-ATPase. The similar results on heavy metal mixture effects on growth and ATPase activity were obtained for other bacteria such as *E. coli* and *E. hirae* [24,25].

The results obtained are novel and might reveal the new approaches in regulation of growth and hydrogen metabolism in photosynthetic bacteria in metal-containing environments.

Acknowledgment

This study was supported by Research Grant from the State Committee of Science, Ministry of Education and Science of Armenia, to AT (#15T-1F123).

References

[1] S.I. Allakhverdiev, V. Thavasi, V.D. Kreslavski, S.K. Zharmukhamedov, V.V. Klimov, S. Ramakrishna, D.A. Los, M. Mimuro, H. Nishihara, R. Carpentier, Photosynthetic hydrogen production, J. Photochem. Photobiol. 11 (2010) 101–113.
[2] N. Basak, A.K. Jana, D. Das, D. Saiki, Photofermentative molecular biohydrogen production by purple-non-sulfur (PNS) bacteria in various modes: the present progress and future perspective, Int. J. Hydrog. Energy 39 (2014) 6853–6871.
[3] A. Trchounian, Mechanisms for hydrogen production by different bacteria during mixed-acid and photo-fermentation and perspectives of hydrogen production biotechnology, Crit. Rev. Biotechnol. 35 (2015) 103–113.
[4] K. Trchounian, A. Trchounian, Hydrogen production from glycerol by *Escherichia coli* and other bacteria: an overview and perspectives, Appl. Energy 156 (2015) 174–184.
[5] H. Sargsyan, L. Gabrielyan, A. Trchounian, The distillers grains with solubles as a perspective substrate for obtaining biomass and producing bio-hydrogen by *Rhodobacter sphaeroides*, Biomass Bioenergy 90 (2016) 90–94.
[6] H. Sargsyan, L. Gabrielyan, L. Hakobyan, A. Trchounian, Light-dark duration alternation effects on *Rhodobacter sphaeroides* growth, membrane properties and bio-hydrogen production in batch culture, Int. J. Hydrog. Energy 40 (2015) 4084–4091.
[7] L. Gabrielyan, H. Torgomyan, A. Trchounian, Growth characteristics and hydrogen production by *Rhodobacter sphaeroides* using various amino acids as nitrogen sources and their combinations with carbon sources, Int. J. Hydrog. Energy 35 (2010) 12201–12207.
[8] L. Hakobyan, L. Gabrielyan, A. Trchounian, The effect of various metal ions on biohydrogen production and the F_OF_1-ATPase activity of *Rhodobacter sphaeroides*, in: A. Veziroglu, M. Tsitskishvili (Eds.), Black Sea Energy Resource Development and Hydrogen Energy Problems, NATO Science for Peace and Security Series C: Environmental Security, Springer, Netherlands 2013, pp. 165–177, http://dx.doi.org/10.1007/978-94-007-6152-0_15.
[9] N. Rashid, K. Lee, J.-In Han, M. Gross, Hydrogen production by immobilized *Chlorella vulgaris*: optimizing pH, carbon source and light, Bioprocess Biosyst. Eng. 36 (2013) 867–872.
[10] M.J. Huertas, L. López-Maury, J. Giner-Lamia, A.M. Sánchez-Riego, F.J. Florencio, Metals in cyanobacteria: analysis of the copper, nickel, cobalt and arsenic homeostasis mechanisms, Life 4 (2014) 865–886.
[11] D.H. Nies, Microbial heavy-metal resistance, Appl. Microbiol. Biotechnol. 51 (1999) 730–750.
[12] V.S. Lebedev, L.A. Volodina, E.Y. Deinega, Y.I. Fedorov, Structural changes in the surface of *Escherichia coli* bacteria and the copper-induced permeability of the plasma membrane, Biophysics 50 (2005) 100–106.
[13] N. Mallick, Copper-induced oxidative stress in the chlorophycean microalga *Chlorella vulgaris*: response of the antioxidant system, J. Plant Physiol. 161 (2004) 591–597.
[14] A. Mudhoo, S. Kumar, Effects of heavy metals as stress factors on anaerobic digestion processes and biogas production from biomass, Int. J. Environ. Sci. Technol. 10 (2013) 1383–1398.
[15] Ch.-Y. Lin, Sh.-H. Shei, Heavy metal effects on fermentative hydrogen production using natural mixed microflora, Int. J. Hydrog. Energy 33 (2008) 587–593.
[16] D. Carrieri, G. Ananyev, A.M. Garcia Costas, D.A. Bryant, G.Ch. Dismukes, Renewable hydrogen production by cyanobacteria: nickel requirements for optimal hydrogenase activity, Int. J. Hydrog. Energy 33 (2008) 2014–2022.
[17] B.-F. Liu, N.-Qi. Ren, J. Ding, G.-J. Xie, W.-Q. Guo, The effect of Ni^{2+}, Fe^{2+} and Mg^{2+} concentration on photo-hydrogen production by *Rhodopseudomonas faecalis* RLD-53, Int. J. Hydrog. Energy 34 (2009) 721–726.
[18] J. Wang, W. Wan, Influence of Ni^{2+} concentration on biohydrogen production, Bioresour. Technol. 99 (2008) 8864–8868.
[19] H. Alshiyab, M.S. Kalil, A.A. Hamid, W.M.W. Yusoff, Trace metal effect on hydrogen production using *C. acetobutylicum*, J. Biol Sci. 8 (2008) 1–9 OnLine.
[20] D. Yoch, Manganese, an essential trace element for N_2 fixation by *Rhodospirillum rubrum* and *Rhodopseudomonas capsulata*: role in nitrogenase regulation, J. Bacteriol. 40 (1979) 987–995.
[21] J. Cheng, M. Zhang, W. Song, A. Xia, J. Zhou, K. Cen, Cogeneration of hydrogen and methane from *Arthrospira maxima* biomass with bacteria domestication and enzymatic hydrolysis, Int. J. Hydrog. Energy 36 (2011) 1474–1481.
[22] B. Xu, P. Cheng, Ch. Yan, H. Pei, W. Hu, The effect of varying LED light sources and influent carbon/nitrogen ratios on treatment of synthetic sanitary sewage using *Chlorella vulgaris*, World J. Microbiol. Biotechnol. 29 (2013) 1289–1300.
[23] L. Hakobyan, L. Gabrielyan, A. Trchounian, Ni (II) and Mg (II) ions as factors enhancing biohydrogen production by *Rhodobacter sphaeroides* from mineral springs, Int. J. Hydrog. Energy 37 (2012) 7482–7486.
[24] Z. Vardanyan, A. Trchounian, Cu(II), Fe(III) and Mn(II) combinations as environmental stress factors have distinguishing effects on *Enterococcus hirae*, J. Environ. Sci. 28 (2015) 95–100.
[25] K. Trchounian, A. Poladyan, A. Trchounian, Optimizing strategy for *Escherichia coli* growth and hydrogen production during glycerol fermentation in batch culture: effects of some heavy metal ions and their mixtures, Appl. Energy 177 (2016) 335–340.
[26] R. Sapra, K. Bagramyan, M.W.W. Adams, A simple energy-conserving system: proton reduction coupled to proton translocation, Proc. Natl. Acad. Sci. U. S. A. 100 (2003) 7545–7550.
[27] L. Gabrielyan, H. Sargsyan, A. Trchounian, Novel properties of photofermentative biohydrogen production by purple bacteria *Rhodobacter sphaeroides*: effects of protonophores and inhibitors of responsible enzymes, Microb. Cell Factories 14 (2015) 131–140.
[28] T. Maeda, T.K. Wood, Formate detection by potassium permanganate for enhanced hydrogen production in *Escherichia coli*, Int. J. Hydrog. Energy 33 (2008) 2409–2412.
[29] A.Kh. Paronyan, Ecology and biodiversity of phototrophic bacteria of various ecosystems of Armenia, Biol. J. Armenia 54 (2002) 91–98 in Russian.
[30] T. Eitinger, J. Suhr, L. Moore, J.A. Smith, Secondary transporters for nickel and cobalt ions: theme and variations, Biometals 18 (2005) 399–405.
[31] D. Bald, H. Noji, Y. Hirono-Hara, T. Hisabori, Redox regulation of the rotation of F1-ATP synthase, J. Biol. Chem. 276 (2001) 39505–39507.