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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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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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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+}