CHEMISTRY JOURNAL OF MOLDOVA. General, Industrial and Ecological Chemistry. *2017, 12(1), 110-114*
ISSN (p) 1857-1727 ISSN (e) 2345-1688
http://cjm.asm.md
http://dx.doi.org/10.19261/cjm.2017.404

EFFECT OF COPPER CONCENTRATION ON THE GROWTH OF *METHYLOCOCCUS CAPSULATUS* (STRAIN M)

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Abstract. Growth of cells *Methylococcus capsulatus* (strain M) under deficiency and exposure to various concentration of copper (concentration range 0-100 μM) in the culture media was studied. Morphocytological analysis has shown that the excess of copper ions is accumulated as granules concentrated near the cell surface and in the cytoplasm. Growth of *M. capsulatus* (M) cells under copper-excess (60-100 μM) growth conditions is also accompanied by increase of methanobactin secretion into the growth medium which binding excess of copper. Another maximum methanobactin secretion into the growth medium is observed when copper is present at low concentrations in the growth medium (up to 10 μM) which provides the cell with the essential copper.

Keywords: copper, biosorption, *Methylococcus capsulatus*, methanobactin.

Received: 31 March 2017/ Revised final: 12 May 2017/ Accepted: 15 May 2017

Introduction

Microorganisms are known to accumulate and assimilate toxic, radioactive, and noble metals from solutions. Depending on metabolism and specificity of certain chemical species, this process can also lead to the synthesis of nanoparticles. In particular, cyanobacteria *Spirulina platensis* and *Nostoc linckia* are known for their ability to synthesize silver nanoparticles [1]. In order to establish the promising strains for environmental remediation of metal ions, the ability of microorganisms to accumulate heavy metals should be investigated and, also, the studies of toxic effects of metal ions on living microorganisms should be considered.

Methane-oxidizing bacteria, also known as methanotrophs, may be promising bacteria for environmental bioremediation. Methanotrophs can exist in a variety of habitats and found in a wide range of pH, salinity, temperature, oxygen concentrations, heavy metal concentrations, and radiation [2,3]. Methanotrophs activate the tough C-H bond in methane which they use as a carbon and energy source. This reaction is catalyzed by a unique enzyme – methane monooxygenase (*MMO*), which is known to exist in two forms: soluble (*sMMO*) and particulate (*pMMO*), both of which are multi-component enzymes.

Methanotrophs are of scientific interest due to their potential applications in bioconversion and processes of biosorption, bioaccumulation and bioremediation of various heavy metals. It has been suggested that methanotrophs also influence the speciation and bioavailability of metals in the environment [3,4]. It is known about the reductive transformation of soluble and more toxic Cr(VI) into a less toxic insoluble Cr(III) species by well characterized model of methanotroph *Methylococcus capsulatus* (Bath) [5]. Another reason to employ methanotrophs for bioremediation of contaminated ecosystems is their ability to degrade several toxic compounds, which they use as a carbon source. For example, *MMO* synthesized by *Methylosinus trichosporium OB3b*, can oxidize not only methane, but also a wide range of aliphatic, aromatic and alicyclic hydrocarbons, and their chloride derivatives [6].

Copper has a central place in the metabolism of methanotrophs. In order to satisfy their high requirements for copper, methanotrophs synthesize methanobactins (*Mbs*) that are the new type of metal-binding peptides. *Mbs* are low-molecular-weight (<1200 Da) chalkophores, expressed and secreted in response to copper decrease in the growth medium [7]. *Mbs* extracted from several methanotrophs, consist of nitrogen-containing heterocycles conjugated with the neighboring thioamide groups, thus forming copper-binding ligands [8]. All *Mbs* exhibit a high affinity for copper, which could find practical implementation [7]. It was shown that *Mb* synthesized by *Methylosinus trichosporium OB3b*

can bind metals other than Cu(I,II), such as Ag(I), Au(III), Co(II), Cd(II), Fe(III), Hg(II), Mn(II), Ni(II), Pb(II), U(VI), and Zn(II), however, with the lower binding constants as compared to Cu(II) [9]. At the present time, *Mb* isolated from *M. capsulatus* (M) is known to bind Fe(III) and Zn(II), as well as Cu(II) [10]. Therefore, *Mb* could potentially play a critical role for the successful use of methanotrophs in detoxification of metal-polluted site. In this regard, we aimed to study the effects of different copper concentrations on the growth of methane-oxidizing bacteria, *Methylococcus capsulatus* (M), and on expression of *Mb* in order to highlight the bioremediation potential of methanotrophs.

Experimental

M. capsulatus (M) cells were cultivated under continuous flow conditions in the *Ankum 2M* fermenter (Russia) in a standard mineral medium, at 42ºC, pH=5.6, containing different $CuSO_4$ concentrations, as was described before [11]. Growth kinetics was assessed through measuring changes in the values of absorbance at 600 nm using a spectrophotometer KFK-3-01-ZOMZ (Russia).

Mb concentration in the supernatant fraction, obtained after cells precipitation by centrifugation at 6000 g for 30 min using an OPN-8 centrifuge (Kyrgyzstan), was measured spectrophotometrically at 394 nm (which is one of the *Mb*'s absorption maxima). *Mb* concentration was also determined using gravimetric methods [12]. The supernatant fluid remaining after cells precipitation was concentrated 10-20 times using rotor vaporizer Buchi (Switzerland). *Mbs* were extracted through liquid chromatography on Diaion HP20 [13]. The samples were then lyophilized and weighed.

Biomass was determined by drying a fixed volume of cells culture to constant mass.

Sample preparation for electron microscopy (JEM-100B, Japan) and the analysis itself were completed as described before [14]. Previously concentrated cells suspension was incubated in sodium sulphide at pH 7.5 for 20-30 min (control – incubation in the absence of sodium sulphide). The cells were washed in 0.25 M sodium phosphate buffer (pH 7.2), dehydrated in ethanol, and then embedded in epoxy resin.

Results and discussion

We studied the effect of copper ions on *M. capsulatus* (M) growth kinetics using continous flow cultivation under different $CuSO_4$ concentrations in nutrient medium. The experiments showed that the bacterial specific growth rate is dependent on the copper content in the growth medium. Different growth rates of *M. capsulatus* (M) cells under different copper conditions are observed even in the initial sections of the growth curves (Figure 1).

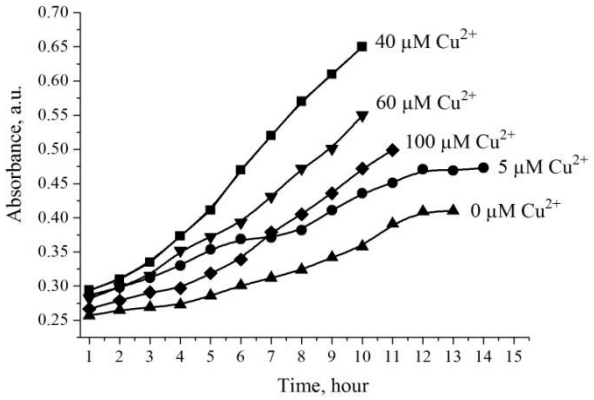

Figure 1. Growth curves of *M. capsulatus* (M) at different copper concentrations in the growth medium.

At low-copper content in growth medium (0÷5 μM) the stationary growth phase occurs in several hours. The increase of copper concentration in growth medium up to 40 μM induces a brisk growth of the cellular culture, these are the optimal copper concentrations. Further increase of copper concentration above 40 μM leads to inhibition of the *M. capsulatus* (M) cells growth. For *M. capsulatus* (M) cells, copper LD_{50} value is 4.9 mg/L (copper concentration in growth medium within which specific growth rate is decreased by the factor 2 - ½μ). Copper concentration of 12 μg/L, which corresponds to 0.19 μM, is widely used in ecotoxicological experiments aimed to assess this metal genotoxicity [15,16]. It can be suggested, that bacteria *M. capsulatus* (M) possess copper-tolerance.

Microorganisms have evolved various mechanisms of metal-resistance such as active efflux pumping of the toxic metal out of the cell, a decrease in metal uptake due to changes in cell permeability, intracellular and extracellular metal binding with its further detoxification, enzymatic conversion to a less toxic form [17].

In order to study the *M. capsulatus* (M) metal-resistance mechanism, a series of electron microscopic examinations of the said bacteria culture was conducted to determine cells morphology at different copper concentrations in growth medium (Figure 2).

At the copper concentration that favours fastest specific growth rate of the studied culture and those below it (≤ 40 μM) no structural changes in *M. capsulatus* (M) cells are observed (Figure 2a). Cells are cocci (within 1 μm) and diplococci. Prevalence of the latter is observed in cultures growing in conditions close to optimal, whereas the former, having dark cytoplasm (as displayed by phase contrast microscopy) dominate (up to 95%) under nonsterile conditions. A well-developed network of internal cytoplasmic membranes with localized pMMO is present. At copper concentrations 1.5 times higher than optimal, electron-dense intracellular inclusions are found, which are nonmetabolized copper spread throughout cytoplasm in nanoscale granules (Figure 2b). Metal ions entering the bacteria cell cytoplasm can be complexed by inorganic anions. It is possible, that copper is accumulated in sulphides, as it happens in *Mycobacterium scrofulaceum* cells [18]. After further increase in copper concentration in the medium (up to 100 μM), electronic microphotographs show copper sorption on the cell wall with copper at the same time being present in cells cytoplasm (Figure 2c). Copper adsorption on the surface of *M. capsulatus* (M) occurs by binding with the cell wall and cytoplasmic membrane. Changes taking place in *M. capsulatus* (M) cells at elevated copper concentrations (≥100 μM) are mostly concerned with the cells morphology. During *M. capsulatus* (M) cultivation, an increase in cell size is observed (Figure 2c), which is concerned with the suppression of cell division. *M. capsulatus* (M) metal-resistance is provided by sorption and intracellular copper accumulation through complexation with microorganisms' biomass. Accumulation of Cu(II) in the periplasmic space and outer membrane was, also, observed in bacteria *Pseudomonas syringae* [19].

Generally, heavy metal ions are transferred into the cells through the active transport mechanisms. For copper uptake, some methanotrophs synthesize a low-molecular copper-binding peptide - *Mb* [7]. It is known, that *Mb* contributes to the growth of methane-oxidizing bacteria, and its biosynthesis depends on the concentration of copper in the growth medium. However, not all methanotrophs can synthesize *Mb* [20]. We extracted such low-molecular peptide from *M. capsulatus* (M) [11,13].

Maximum *Mb* (extracted from *M. capsulatus* (M) as well as another studied bacteria culture) secretion into the growth medium is observed when copper is present in the nutrient medium in low concentrations (up to 10 μM) (Figure 3), in this conditions, *Mb* molecules exist in copper-free form.

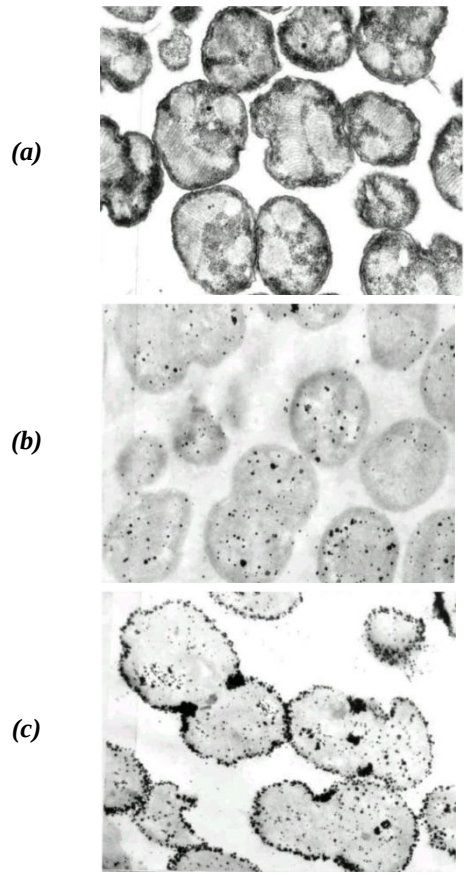

Figure 2. Morphocytological features of *M. capsulatus* (M) cells at different copper concentrations in growth medium: *(a)* – 40 μM, (*b*) – 60 μM, (*c*) – 100 μM.

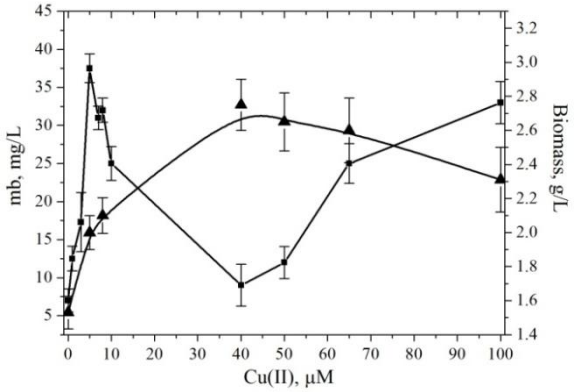

Figure 3. Extracellular methanobactin content (■) and *M. capsulatus* (M) biomass level (▲) at different copper concentrations in growth medium.

At optimal copper concentrations (40 μM), there is a decrease in *Mb* content in the growth medium. At this point, intracellular *Mb* concentration is 23-35 mg/L. However, in case of copper concentrations above the optimal concentrations, *Mb* secretion into the growth

medium is increased; in this case, *Mb* exist in copper-complexed form. Copper detoxification occurs through the metal binding, which allows the growth of *M. capsulatus* (M) biomass (biomass level is maintained). At 60-100 μM concentrations of copper in the growth medium, were identified intracellular inclusions in the cytoplasm of *M. capsulatus* (M) cells (Figure 2b), and the binding with cytoplasmic membrane is triggered (Figure 2c). Production of high concentrations of *Mb* protects the methanotrophs as well as other microorganisms from copper and most transition metals by binding them. Also, metal complexation by *Mb* provides the cell with the essential metals (lack of which the cell may currently experience) but, moreover, neutralizes excess amounts of these metals in the nutrient medium. In addition, solubilisation of copper and other metals by *Mb* decreases their toxicity with regard to other ecosystem components.

Thus, one of the mechanisms of interactions between heavy metal ions and methanotrophs are biosorption of heavy metals and/or binding by *Mb*, as in the case of copper. The study of the influence of other heavy metals on methanotrophs is the object of further research.

Conclusions

The bioremediation of metal ions using methanotrophs is still at initial stage of study. Microbial-based bioremediation of heavy metals can be performed by detoxification of metals through their conversion to less toxic or less soluble form. Our results showed that higher concentrations of copper (60-100 μM) lead to copper biosorption by the bacteria *M. capsulatus* (M) in the cytoplasm and on the cell surface from the environments. It was shown for the first time that at high concentrations of copper ions in the growth medium of *M. capsulatus* (M) an increase of *Mb* expression in the growth medium is observed. Thus, the use of methanotrophs in bioremediation of copper and other metals from polluted site could be an emerging biotechnology tool.

Acknowledgments

This work was performed according to state task of Federal Agency for Scientific Organizations of Russian Federation (No. 0089-2014-0006).

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