THERMODYNAMIC SUBSTANTIATION OF INTEGRAL MECHANISMS OF MICROBIAL INTERACTION WITH METALS

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Abstract. Despite the promising application of microorganisms for purification of metal-containing wastewater such biotechnologies have not been widely used since their empirical basis. Therefore, the aim of our work was to apply thermodynamic prognosis to determine the optimal pathway of microbial removal of toxic metals (CrO_4^{2-} , Cu^{2+}) and its experimental justification. There was theoretically substantiated on the base of thermodynamic prognosis and experimentally confirmed integration of microbial reduction of metals, and their precipitation by microorganisms. Developed approach is the methodological basis for fast and effective purification of metal-containing wastewater.

Key words: thermodynamic prognosis, toxic metals, wastewater, metal reduction, metal precipitation.

INTRODUCTION

The level of environment pollution with heavy metals is constantly increasing. The global use of metal-containing raw materials in industry, medicine and agriculture has led to wide pollution of environment with toxic metals [37]. That is why the ecological safety of the environment is one of the prior directions of modern science. Sewage water from industrial enterprises is one of the most polluted with toxic metals in the environment. There are metallurgical engineering, electroplating, tanning and textile industry, preservation of wood, water-cooling and chromate preparation [12]. The routine methods for industrial waste purification from heavy metal are cementation, precipitation, ion exchange, electro-dialysis, electro-winning, electro-coagulation and reverse osmosis [1]. These technologies are often low-efficiency, expensive and also can't access necessary level of wastewater purification from heavy metals in high concentration. According to these disadvantages development of novel more efficient technologies is absolutely necessary [8]. Immobilization of heavy metals by microorganisms and reduction of metals to insoluble compounds are the most effective, low-cost and ecologically friendly methods of sewage treatment. There are various biotechnological methods for purification of industrial wastewater from Cr(VI) and Cu^{2+} , which are based on their accumulation and reduction to insoluble compounds by microorganisms [7, 17, 30, 25].

Determination of mechanisms of microbial interaction with toxic metals has both fundamental and practical importance. The fundamental one is thermodynamic justification of optimal pathway of microbial interaction with metals that leads to their extraction from water solution (wastewater). The practical importance is development of novel effective environmental biotechnologies for purification of sewage from toxic metals. Such metals as chromium and copper are widespread toxic environmental pollutants [19, 11]. Anion of CrO_4^{2-} is a metal-oxidizer

[36]. It irreversibly oxidizes components of microbial cell and blocks its electron transporting chain. Cation of Cu^{2+} is a metal of combined action. It oxidizes microbial cell components and also it substitutes macroelements in cell wall and in the active centers of enzymes [36]. That is why they are very toxic for microorganisms and dangerous for environment generally [32, 3, 18].

Thus, the aim of our work was to apply thermodynamic prognosis to determine the optimal pathway of microbial removal of toxic metals (CrO_4^{2-} , Cu^{2+}) and its experimental justification.

MATERIALS AND METHODS

Thermodynamic prognosis of microbial interaction with compounds of chromium and copper.

Prognosis of interaction of microorganisms with chromium and copper compounds was carried out on the basis of Pourbaix diagrams of the state of elements in the pH-Eh coordinates [28]. On its basis, the stability fields of metals in aqueous solutions were calculated, and the optimal metabolic pathways of microbial interaction with metals, leading to their rapid and efficient extraction from solutions, were determined.

Cultivation of microorganisms in the presence of toxic metals.

Preparation of the inoculum.

The community of hydrogen-synthesizing soil microorganisms was used as an inoculum. To obtain it, 50 grams of diced potatoes (the length of cube rib – 1 cm), 5 g of soil and 100 ml of boiled and cooled tap water were added to a 250 ml vial. The mixture was pasteurized for 10 minutes in a water bath at temperature of 75°C. After that, the vial was hermetically sealed with an elastic rubber stopper and a metal seal. Microorganisms were cultured during 5 days ($t = 30^\circ\text{C}$). Increase in the volume of gas and appearance of H_2 in the gas phase evidenced the development of hydrogen-synthesizing microorganisms. Further, community of hydrogen-synthesizing microorganisms was used as an inoculum to study the patterns of microbial interaction with metals.

Study of the patterns of microbial growth in the presence of toxic metals.

The vials (volume 500 ml) were filled with 300 ml of tap water, 50 g of model mixture of food waste (diced raw potatoes and meat in an equal weight ratio) and 5 ml of inoculum of anaerobic hydrogen-synthesizing microorganisms. Toxic metals were added at the following concentrations: CrO_4^{2-} - 5000 ppm, Cu^{2+} - 10000 ppm. Cultivation was carried out during 15 days ($t = 30^\circ\text{C}$). The growth of microorganisms was determined by decrease in the redox potential (E_h) of the medium, change the pH, increase in the optical density of the medium, decrease in the concentration of CrO_4^{2-} and Cu^{2+} , and also increase in the concentration of CuCO_3 .

The pH and E_h of the culture liquid was measured by the potentiometric method with the pH-meter-millivoltmeter "pH-150 MA". The measuring electrode ЭСК-10603/4 was used to determine the pH. Redox-potential was measured using platinum measuring electrode ЭПБ-1. The silver chloride electrode ЭБЛ-1М3 was used as reference.

Biomass growth was determined by optical density of the cell suspension by the photoelectrocolorimeter (PEC) at $\lambda=540$ nm and 0,5 cm of an optical path length.

Determination of CrO_4^{2-} concentration was carried out by the colorimetric method, which is based on the appearance of crimson color interacting with diphenylcarbazide (DPC). A centrifuged culture liquid (2 ml), 0.5 ml of H_2SO_4 (1:3) and 0.5 ml of DPC were added into the tube. The sensitivity of the method is 1 ppm. Optical density of solution was measured by the photoelectrocolorimeter (PEC) at $\lambda=500$ nm and 0.5 cm of an optical path length [15].

To determine the concentration of Cu^{2+} , PAR (4-(2-pyridylazo)-resorcinol) was used. The sensitivity of the method is 1 ppm. Optical density of solution was measured by the photoelectrocolorimeter (PEC) at $\lambda=500$ nm and 0.5 cm of an optical path length [29].

To determine the concentration of CuCO_3 , 3 ml of a suspension (culture liquid) containing microbial biomass and insoluble CuCO_3 was taken from the vial with a syringe through a stopper and transferred to an analytical tube. Then 0.1 ml of 1N HCl was added to the tube to dissolve copper carbonate to form Cu^{2+} cation. Further, the concentration of Cu^{2+} was determined using PAR [29].

RESULTS AND DISCUSSION

The high values of the redox potential of the oxidation-reducing systems they form are the factors determining toxicity of metals [36]. In neutral conditions (pH=7.0) water is stable in the range of

standard values of the redox potential E_o' from -414 to +814 mV (Fig. 1). The upper limit of water stability is determined by the reversible oxidation reaction of water oxygen to molecular oxygen: $2\text{H}_2\text{O}=\text{O}_2 + 4\text{H}^+ + 4\text{e}^-$; $E_o' = +814$ mV. The lower limit of water stability is determined by the reversible reaction of proton reduction to molecular hydrogen: $2\text{H}^+ + 2\text{e}^-=\text{H}_2$; $E_o' = -414$ mV [36]. It follows that water has the properties of a binary redox buffer that is stable in the range of the standard redox potentials from -414 to +814 mV.

Consequently, all redox reactions of energy and constructive metabolism of microorganisms are possible only in this range. Fig. 1a shows the redox potentials of seven reactions of chromium compounds reduction. Reaction No. 1 is outside the upper limit of the stability of water, and therefore it cannot be carried out by microorganisms. It is obvious that addition of HCrO_4^- into the culture medium with an extremely high redox potential, $E_h = +1200$ mV (pH = 2.0), will lead to irreversible oxidation of the structural components and enzymes of microorganisms. This will lead to the death of microorganisms.

Reactions No. 5, 6, and 7 have redox potentials, more electro negative than the lower limit of water stability. Therefore, redox enzymes of microorganisms with higher potentials cannot reduce Cr^{3+} , Cr^{2+} and $\text{Cr}(\text{OH})_3$. Moreover, these chromium compounds are reducing agents regarding to the proton of water, that inevitably leads to the reduction of the proton to molecular hydrogen.

Hence it follows that reactions No. 2, 3 and 4 are thermodynamically acceptable for microorganisms, since they are inside the field of thermodynamic stability of water. The reaction No. 4 is the most suitable for biotechnologies since only this reaction leads to insoluble product of microbial reduction of chromate – chromium(III) hydroxide - $\text{Cr}(\text{OH})_3 \cdot n\text{H}_2\text{O}$. This reaction provides the removal of chromium compounds from solution.

Similar regularities are also inherent in oxidized copper compounds (Fig. 1b). The potentials of Cu^{2+} reduction reactions (reactions No. 8-14) are inside the water stability zone, and therefore it is theoretically permissible to reduce copper compounds by microorganisms.

Thermodynamic prognosis allows determining the conditions for reduction of copper by microorganisms, as well as the final products of the reactions. Thus, in the range of acidic values, pH = 0.0-5.0, Cu^{2+} cation will be reduced by microorganisms to insoluble compounds (Cu_2O and CuO). These reactions lead to positive environmental effect – microbial removal of copper from solutions (sewage).

Fig. 1. Thermodynamic prognosis of microbial interaction with metals:
a – chromium compounds; b – copper compounds

Removal of copper from solutions is possible not only due to the reduction reactions, but also due to biologically mediated alkalisation of the medium. Thus, at $\text{pH} > 5.0$ (Fig. 1b), Cu^{2+} cation is transformed into insoluble copper hydroxide – Cu(OH)_2 . Increase in pH of culture medium leading to the formation of Cu(OH)_2 is possible, at least due to the two metabolic pathways. The first one is dissimilatory denitrification, in which Nitrogen of nitrate is reduced to N_2 , and the alkaline metal cation is accumulated in the medium ($\text{Na}^+ + \text{NO}_3^- \rightarrow \text{Na}^+ + \text{N}_2$), and the pH shifts to the alkaline conditions [20]. The second pathway is the cleavage of amino groups in the proteins by ammonifying bacteria, followed by the accumulation of alkaline Nitrogen compounds ($\text{NH}_2\text{-CH}_2\text{-COOH} \rightarrow \text{CH}_3\text{-COOH}^- + \text{NH}_4^+$, NH_4OH and NH_3) [38, 33]. These two metabolic pathways lead to alkalization of the medium to pH values of 7.8-8.2. Obviously, under such alkaline conditions, the cation Cu^{2+} will inevitably precipitate – Cu(OH)_2 . It is necessary to mention the third biologically mediated pathway of copper precipitation – the formation of insoluble copper carbonate – CuCO_3 . Carbon dioxide is common end product of the metabolism of all chemoorganotrophic microorganisms [14]. Dissolved CO_2 dominates under acidic conditions, a mixture of CO_2 and HCO_3^- in neutral conditions, and CO_3^{2-} in the alkaline ones [14]. Insoluble copper carbonate CuCO_3 is formed during the interaction of Cu^{2+} cation with CO_3^{2-} anion.

Thus, thermodynamic prognosis allows theoretically substantiating the determination of any type of microbial interaction with the cation Cu^{2+} ,

leading to the removal of copper compounds from solutions (sewage).

Prognosis also makes it possible to significantly improve the efficiency of microbial copper removal by integrating at least two types of interaction. For example, when microorganisms use protein compounds (amino acids) as carbon and energy sources, the redox potential will be decreased to negative values ($-100 \dots -300 \text{ mV}$), as well as alkaline cations will be accumulated due to deamination of amino acids. Thus, the efficiency of removal of Cu^{2+} cation will be increased both due to its reduction to insoluble Cu_2O and Cu^0 , and due to the formation of insoluble Cu(OH)_2 and CuCO_3 due to alkalization of the medium (Fig. 1b).

It is obvious that in binary reactions of reduction of oxidized compounds of copper and chromium, metals function as electron acceptors. Organic compounds (sources of carbon and energy) are donors of electrons. Microorganisms act as biocatalysts. In our opinion, it is advisable to consider the sources of carbon (energy) and microorganisms as a donor system.

All the dissimilative biochemical reactions carried out by microorganisms have a redox potential specific for them.

For example, for nitrate reduction ($\text{C}_6\text{H}_{12}\text{O}_6 + 4.8 \text{NO}_3^- + 4.8 \text{H}^+ = 6\text{CO}_2 + 2.4 \text{N}_2 + 8.4 \text{H}_2\text{O}$ [2]) the redox potential determined by cytochromes is about $+200 \dots +300 \text{ mV}$ [33]. The redox potential is more electro negative, $\text{Eh} = -300 \dots -414 \text{ mV}$ during hydrogen fermentation [6, 34] ($\text{C}_6\text{H}_{12}\text{O}_6 + 2\text{H}_2\text{O} = 2\text{CH}_3\text{COOH} + 2\text{CO}_2 + 4\text{H}_2$ [10, 6]).

The efficiency of redox reactions is determined by the potential difference between donor and acceptor systems. Low-potential hydrogen fermentation is more effective for reduction of both CrO_4^{2-} and Cu^{2+} (Fig. 2). The potential difference between cytochromes and chromate is 355 mV, and between hydrogenases and chromate is 969 mV. Similar patterns are for the reduction of Cu^{2+} by microorganisms. Thus, the potential difference between cytochromes and Cu^{2+} is 300 mV, and between hydrogenase and Cu^{2+} is 914 mV.

Hence, it is obvious that hydrogen dark fermentation is the most effective metabolic pathway for the biotechnology of purification of industrial

wastewater containing CrO_4^{2-} and Cu^{2+} . Of course, the use of glucose as a donor substrate is unprofitable and impossible. However, food and other waste containing polymeric carbohydrates (for example, starch, cellulose, etc.) are suitable for biotechnology. Hydrolysis of such waste under anaerobic conditions leads to the formation of soluble carbohydrates (including glucose) and further to the rapid and effective reduction of chromium and copper ions to insoluble compounds:

Fig. 2. Effectiveness of microbial reduction of toxic metals

The efficiency of metal removal can be increased by the presence of protein compounds. For hydrogen-synthesizing anaerobes, proteins are additional electron donors, as well as precursors of alkaline compounds, which increase copper deposition efficiency due to the formation of $\text{Cu}(\text{OH})_2$ and CuCO_3 . Multi component food waste of megacities are almost unlimited sources of polymeric compounds of carbohydrates and proteins. Given the fact that anaerobic fermentation of polymers leads to their hydrolysis (i.e. destruction), it is obvious that two positive environmental processes can be combined in industrial biotechnologies: destruction of environmentally hazardous organic compounds and removal of toxic metals from wastewater. The third

positive effect of biotechnology is the formation of environmentally friendly energy carrier – molecular hydrogen, as well as a valuable product – metal concentrate.

It should be emphasized that, at first glance, the growth of low-potential hydrogen-synthesizing bacteria ($E_h = -400 \dots -414$ mV) is not possible in the presence of high-potential CrO_4^{2-} ($E_h = +555$ mV) and Cu^{2+} ($E_h = +500$ mV). High-potential metals must inevitably suppress the growth of obligate anaerobic microorganisms. Nevertheless, the simultaneous occurrence of these processes is possible creating a stereometric gradient of the redox potential in a heterophase system (Fig. 3).

Fig. 3. Combination of hydrogen fermentation of organics and reduction of CrO_4^{2-}

Solid particles of food waste at the bottom of the cultivator with facultative and obligate anaerobic hydrogen-synthesizing microorganisms immobilized on them create an anaerobic zone with a local redox potential in the solid phase $-300\ldots-400$ mV. This anaerobic zone is not available for the solution of high-potential chromium due to diffusion difficulties, and also due to the "flow" of reduced low-potential exometabolites formed during the hydrolysis of solid organic compounds. The gas bubbles formed during hydrogen fermentation facilitate the rapid transport of reductants into the chromate solution, and its rapid and unavoidable reduction to insoluble chromium(III) hydroxide. Such regularities are similar to Cu^{2+} .

Fig. 3 shows that a stereometric gradient of the redox potential from -400 mV in the bottom zone to $+555$ mV in the upper zone should be created in the heterophase redox system. It is obvious that rapid and effective reduction of chromates by the products of anaerobic metabolism will occur in the middle zone with an assumed integral redox potential about $-150\ldots-200$ mV.

Thus, on the basis of the thermodynamic prognosis of microbial interaction with toxic metal-oxidizers CrO_4^{2-} and Cu^{2+} , we have determined the necessary and sufficient conditions for effective biotechnology of industrial sewage treatment.

1. Reduction of metals to insoluble forms is carried out by obligate anaerobic microorganisms.

2. Multi component food waste are the most suitable substrates for this process.

3. The maximum efficiency of metal removal is achieved through the creation of a heterophase system.

We have experimentally confirmed the above mentioned theoretical positions (Fig. 4).

Fig. 4a shows the dynamics of fermentation of the model substrate and the conjugate reduction of chromate. During the first 2.5 days the growth of microorganisms and reduction of chromate are absent. At the same time, there is an adaptive decrease in the redox potential of medium from $+525$ mV to $+360$ mV. Apparently, at this time facultative anaerobic microorganisms reduce dissolved oxygen and release the reduced exometabolites into the medium. After 2.5 days of cultivation, the growth of microorganisms begins simultaneously with the decrease of redox potential and concentration of Cr(VI). Apparently, the growth of microorganisms at a high redox potential in the solution ($+360$ mV) is explained by the fact that anaerobic microorganisms created a local redox about $-300\ldots-400$ mV during 2.5 days in the solid phase at the bottom of the vial. Then, in accordance with the above theoretical model, the reduced exometabolites entered the upper zone, into the chromate solution, and quickly reduce it. It is evidenced by decrease in the redox potential from $+360$ mV to -300 mV correlated with a decrease in the chromate concentration. After 6 days of cultivation, the concentration of Cr(VI) decreased from 5000 ppm to an analytical zero [15].

Fig. 4. Effectiveness of microbial removal of CrO_4^{2-} and Cu^{2+} from solution

Similar patterns of interaction of anaerobic microorganisms with Cu^{2+} in the heterophase system are shown in Fig. 4b. So, for the first 5 days there is an adaptation of microorganisms and a gradual decrease in the redox potential of the medium from $+480$ mV to $+350$ mV. After 5 days of cultivation, a

sharp decrease in the redox potential from $+350$ mV to -50 mV leads to a correlative rapid decrease in the concentration of Cu^{2+} . During 12 days of cultivation, the concentration of Cu^{2+} decreases from 10000 ppm to zero.

The thermodynamic prognosis indicates inevitable reduction of Cu^{2+} to Cu_2O under anaerobic conditions. However, in this heterophase system, a direct analytical determination of reduced by microorganisms Cu(I) is impossible, since insoluble Cu_2O compound is formed at the bottom of the vial. Indirect evidence of Cu^{2+} removal from the solution due to microbial reduction is the decrease of the redox potential to -50 mV, as well as the formation of a loose layer of brown precipitate specific to Cu_2O at the bottom of the vial.

Unlike chromates, the interaction of microorganisms with Cu^{2+} consists not only of the reduction of Cu^{2+} , but also of the formation of insoluble CuCO_3 . So, the concentration of CuCO_3 increases in the medium after 5 days, and at the end of cultivation it reaches 5800 ppm. An increase in the concentration of CuCO_3 correlated with an increase in the pH of the medium. Consequently, we can assume that Cu^{2+} at the concentration of 4200 ppm microorganisms removed from the medium due to formation of insoluble Cu_2O , and the remaining part by precipitation in the form of CuCO_3 .

It should be emphasized that in galvanic and industrial wastewater the concentration of Cr(VI) is not more than 25-150 ppm [9, 21, 23]. Thus, the concentration of Cr(VI) in sewage of galvanic enterprises of Bangkok was determined to be 25 ppm [39]. In our experiments, the initial concentration of Cr(VI) is 33...200 times higher, and yet obligate anaerobic microorganisms completely reduced chromate and removed it from the solution.

Similar patterns are also characteristic for copper containing wastewater. The concentration of copper in industrial sewage, as a rule, does not exceed 50-100 ppm [23].

At present, there are no microbial technologies that provide effective treatment of industrial sewage from copper in the concentration range of 200-500 ppm Cu^{2+} [24, 13, 4]. The maximum resistance to copper is shown on the example of the strain *Pseudomonas* spp, which is resistant to 300 ppm Cu(II) [31] and three strains isolated from the "metal-bearing" river Mogpog, resistant to 15-390 ppm Cu^{2+} [26]. There are data about possibility of effective purification of industrial wastewater from Cu^{2+} compounds due to mechanisms of sorption by microbial cells [17, 30, 25]. We showed that precipitation of Cu^{2+} by microorganisms in the form of CuCO_3 and reduction to insoluble Cu_2O are also very effective.

Chromium resistant microbial strains capable to accumulate and reduce chromium compounds were also isolated. Thus, soluble forms of Cr(VI) are converted into insoluble Cr(III) hydroxide, which is

not harmful to both living organisms and the environment. Reduction is carried out by soluble reductases. Recently, two soluble Cr(VI) reductases, *ChrR* and *YieF*, have been purified from *Pseudomonas putida* MK1 and *Escherichia coli*, respectively. Direct application of Cr(VI) reductases may be a promising biotechnological approach for bioremediation of a wide range of contaminated environments [7]. Using initial Cr(VI) concentrations of 2 to 8 ppm immobilized intact cells and the cell-free extracts *Bacillus* sp. ES 29 reduced 84 to 98% of the influent Cr(VI) [5]. Strain *Vogococcus fluvialis* isolated from contaminated industrial effluent was shown to carry out high efficiency in detoxification of dichromate by reducing it. These cells had high potential to reduce chromate to its non toxic trivalent form and could tolerate a maximum of 25 mM chromate [22]. Also chromate reducing bacteria *Pseudomonas aeruginosa* strain MIE3 was isolated. It reduced 52% of 150 ppm potassium dichromate (Cr(VI)) in the nutrient broth after 24 hours incubation [16].

It is clear from this data that it becomes necessary to isolate significantly more resistant microorganisms for the purification of industrial sewage containing chromium and copper compounds in significantly higher concentrations [36].

In addition, despite the advent of modern environmental technologies, heavy metals are continuously accumulated in the environment, including chromium and copper. Our methodological approach, based on thermodynamic prognosis of microbial interaction with metals, allows not only to effectively neutralize their toxic forms, but also to obtain valuable concentrate of metals.

CONCLUSIONS

The biotechnology developed by us can ensure the purification of sewage from the compounds of chromium and copper with high efficiency. It can be assumed that the proposed biotechnology will also be effective for the purification of industrial wastewater from other toxic metals-oxidizers, for example, Hg(II) , V(V) , U(VI) , etc. It will serve as the subject of our further research.

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ТЕРМОДИНАМИЧНО ДОКАЗВАНЕ НА ИНТЕГРАЛНИ МЕХАНИЗМИ ЗА МИКРОБИАЛНО ВЗАИМОДЕЙСТВИЕ С МЕТАЛИ

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Резюме: Въпреки нарастващия интерес към приложението на микроорганизмите за пречистване на отпадъчни води, съдържащи метали, досега микробните биотехнологии в тази област не са широко използвани поради тяхната емпирична основа. Ето защо целта на нашата работа е насочена към разработване на оптималните условия за отстраняване на токсични метали (CrO_4^{2-} , Cu^{2+}) с микроорганизми на базата на термодинамична прогноза и нейната експериментална обосновка. Получени са теоретични доказателства за експериментално потвърдено взаимодействие между микроорганизмите и редукцията или утаяването на металите. Разработеният подход е методологична основа за бързо и ефективно пречистване на отпадъчните води, съдържащи метали.

Ключови думи: термодинамична прогноза, токсични метали, отпадъчни води, редукцията на метали, утаяване на метали

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