

**METHOD OF THERMODYNAMIC PROGNOSIS FOR NEW ENVIRONMENTAL
BIOTECHNOLOGIES DEVELOPMENT**

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Abstract

New environmental biotechnology was developed on the base of thermodynamic prognosis method. Thermodynamic prognosis method allows predict the way of microorganisms and xenobiotics interaction. Biotechnology allows simultaneous to destroy environmentally hazardous solid food waste and purify the metal-containing waste water. Application of this biotechnology allows to reduce the mass of waste in dozens times for 10 - 12 days and get a commercially valuable products - metal concentrate and technically clean water for closed production cycles.

Key words: *microbial biotechnology, thermodynamic prognosis, processing of organic waste; metal resistant microorganisms, metal wastewater purification.*

1. INRODUCTION

The development of industry and agriculture naturally leads to an increase of the total number of environmentally hazardous organic and inorganic waste. Today there is a wide range of physical, chemical, and biological technologies of waste treatment and processing. However, the tendency to a catastrophic increase of waste clearly indicates the failure of environmental technologies' effectiveness. Therefore, the primary task of global importance is the development of new technologies that will effectively process the accumulated astronomical amounts of organic solid waste and industrial waste water.

Obviously, new technologies need to be deprived of the modern technology disadvantages, such as the unprofitability and low efficiency. In our opinion, biotechnologies are the most promising ones for the environmental protection, and above all - microbial biotechnology. The variety of microbial metabolic pathways allows simultaneous realize all theoretically possible mechanisms for the physical, chemical and biological treatment of xenobiotics. Environmental microbial technology of the new generation must have the following properties.

1. Efficiency - technologies should ensure the full and fast processing of the major types of waste. These include, first of all, organic (food and agriculture) waste and industrial waste water (e.g., metal containing).
2. Universality - simultaneous processing and detoxification of all classes of xenobiotics.
3. Manufacturability - the ability to scale and replicate typical industrial biotechnologies.
4. Profitability
5. Liquidity - the technologies should provide a high turnover of investment and as a consequence - the entrepreneurs' interest in large-scale implementation of environmental biotechnologies.

The attractiveness of our biotechnologies is based on their high efficiency and on minimizing the cost of waste fermentation. The principal novelty of our biotechnology is in possibility to get commercially

valuable products out of solid and liquid waste. We developed new theoretical approaches to create such universal biotechnology. We developed method of thermodynamic prognosis of microorganisms and xenobiotics interaction. This method makes theoretical foundation for biotechnology development that processes effectively the major classes of waste (xenobiotics).

The major classes of waste are:

- concentrated natural organic compounds (solid food and agricultural waste);
- synthetic organic xenobiotics (nitrochloroaromatic, etc.);
- inorganic xenobiotics (toxic metals, etc.).

Theoretically designed biotechnologies allow us to neutralize these xenobiotics quickly and effectively and receive a wide range of commercially valuable products at the same time. Thus, while fermentation of solid food waste three types of energy produced, such as:

- molecular hydrogen or methane,
- biofuels (alcohols),
- solid fuel (unfermented lignin cellulose).

Microbial polymetallic waste water purification provides concentration of valuable non-ferrous metals. Finally, due to industrial waste water from the synthetic organic compounds and toxic metals treatment, and toxic leachate from food waste purification we can get the main commercial product - ecologically clean water.

We used the method of thermodynamic prognosis of microorganisms and xenobiotics interaction to develop the theoretical foundations of new environmental biotechnology. The method is based on the Pourbaix diagrams. Necessary conditions for the most efficient disposal of xenobiotics by microorganisms were theoretically calculated according to the Pourbaix diagrams that define the state (stability) of organic and inorganic compounds (in the coordinates of pH and Eh). For example, it is theoretically possible to substantiate the optimal simultaneous destruction of organic xenobiotics and removal metals from solutions by microbial technology. Further optimum dissimilative microbial metabolism path and the appropriate physiological groups of microorganisms are selected for realization of thermodynamic direction of xenobiotics degradation.

2. MATERIALS AND METHODS

2.1. Thermodynamic prognosis

For thermodynamic prognosis of microorganisms and xenobiotics interaction Pourbaix diagrams were used. These diagrams are represented by plots of the electrode potentials on the pH of the solution for the real establishing equilibrium. Electrode potentials (Eh) are expressed in millivolts (mV) according to the hydrogen scale ([Pourbaix 1963](#)).

2.2. Preparation of metals solutions

Mercury(II), chromium(VI) and copper(II) containing soluble salts were used to study the interaction of microorganisms with metals.

Standard solution of Hg^{2+} at 1000 ppm was prepared by dissolving metallic mercury in nitric acid. Metallic mercury (1000 mg) and diluted (1:1) HNO_3 (5 ml) were added into a heat-resistant 100 ml flask. The solution was evaporated on a water bath (100 °C) to form a "wet crystals" of $\text{Hg}(\text{NO}_3)_2$. The crystals were dissolved in 50 ml of distilled water and transferred to 1.0 liter Mohr's flask. Flask with

residues of mercury nitrate was washed twice with distilled water and this solution was added to the Mohr's flask. Then the volume of $\text{Hg}(\text{NO}_3)_2$ solution was adjusted to 1 liter.

Chromate standard solution contained 100000 ppm of cation Cr(VI). To prepare the solution 37,3 g of K_2CrO_4 was dissolved in 100 ml of distilled water.

Standard solution of Cu^{2+} contained 20000 ppm of copper cations. To prepare the solution 7,8 g of $\text{CuSO}_4 \times 5\text{H}_2\text{O}$ was dissolved in 100 ml of distilled water.

Standard solutions of metals (except mercury) were sterilized in a 100 ml hermetically sealed flask on water bath (100 °C) for 15 min.

2.3. Analytical determination of metals

The concentration of mercury in solution was determined by atomic absorption on spectrophotometer "Saturn 3" (Magos 1971).

The concentration of Cr (VI) was determined colorimetrically using 1,5-diphenylcarbazide (DPC). Two ml of the sample, 0,5 ml of H_2SO_4 (1:1 diluted), 0,5 ml of DPC (0,5% solution in ethanol) were added to the analytical tube. Measurement range is 0,1 – 20,0 ppm of metal ions. The concentration of Cr(VI) was determined by calibration graph using photoelectrocolorimeter KFK-2MP. Wavelength $\lambda = 540$ nm, the optical cuvette step 0,5 cm.

Concentration of Cu^{2+} was determined by titration a complex of cupric cation (Cu^{2+}) and *p*-aminorezorcine (PAR) with solution of ethylene diamine tetraacetate (EDTA). To the culture medium containing Cu^{2+} was inoculated with 0,1 ml of 0,1% PAR solution. The red complex of PAR copper formed. During the titration of the complex with EDTA solution (2500 ppm) complex Cu^{2+} -PAR was destroyed, which was evidenced by the change of red color to yellow. Concentration of Cu^{2+} was determined by the calibration graph. The amount EDTA solution used for the complex destruction is directly proportional to the content of Cu^{2+} in the concentration range 0,1-1500 ppm.

2.4. Indicators of metal accumulation (R, Kd)

The effectiveness of solution purification was determined by the coefficient of metal extraction by granules (R, %) and the coefficient of redistribution (Kd):

$$R = \frac{\Delta C}{C_0} \times 100\% \quad K = \frac{\Delta C}{C_0} \times \frac{m_1}{m_2} \quad \text{where } \Delta C \text{ is a difference between the initial } (C_0) \text{ and final}$$

concentration of metal (C_p); m_1 - the mass of solution; m_2 - mass of the microbial biocatalyst (MBC) granules.

2.5. Maximum allowable concentrations of metals

The maximum permissible concentrations of metals (MPC) for microorganisms were determined by inoculation cultures on an agar medium in a concentration gradient of metal. For this purpose agar medium (Nutrient Agar, HIMEDIA) with increasing concentrations of toxic metal ions was poured into a series of Petri dishes.

The next sterile aqueous solutions of metal salts were used for concentration gradient of metal in the medium preparation: $\text{Hg}(\text{NO}_3)_2$, $\text{CoCl}_2 \times 6\text{H}_2\text{O}$, $\text{NiCl}_2 \times 6\text{H}_2\text{O}$, K_2CrO_4 , $\text{CuSO}_4 \times 5\text{H}_2\text{O}$. The metal salts solutions were added to a flask with 25 ml of melted and cooled to 50 °C agar medium and then poured into Petri dishes.

Suspended in physiological solution microorganisms were inoculated with the loop on the surface of the medium. Cultivated at 25 °C. The maximum concentration of metals where growth was observed was taken as MPC, i.e. "maximum permissible concentration".

2.6. Microorganisms (pure cultures and associations)

For the metal wastewater purification and the organic waste degradation preparate of microbial biocatalyst (MBC) and isolated from the Antarctic ecosystem metal resistant culture *Brevibacterium antarcticum* were used. Granules of MBC consist of a wide range of microbial communities - digested sludge, activated sludge, bird droppings and cow dung. *B. antarcticum* is resistant to copper at 1000 ppm Cu²⁺.

2.7. Technique of pure cultures isolation

The isolated strains were obtained by exhausting dispersion on Nutrient agar HIMEDIA. To isolate metal resistant pure cultures aqueous solutions of metal salts were added to the agar medium: CuSO₄×5H₂O, Hg(NO₃)₂, CoCl₂×6H₂O, NiCl₂×6H₂O, K₂CrO₄.

2.8. Identification of cultures

Metal resistant cultures were identified by classical methods, according to the determinant Bergey's manual of systematic bacteriology ([Bergey's Manual of Systematic Bacteriology](#) 2012).

2.9. Measuring of biomass growth (optical density)

The increasing of microbial biomass in an aqueous phase was determined colorimetrically by the change of optical density. The optical density was measured at $\lambda = 540$ nm and cuvette optical step 0.5 cm (Gerhardt 1981, Ntaikou et al 2010).

2.10. Determination of the gas phase composition

Composition of the gas (H₂, CO₂) synthesized during the organic substances fermentation was determined to characterize the metabolic activity of microorganisms. For gas and culture liquid sampling a sterile plastic syringes with a rubber seal on the piston of 2,5; 11, 50 and 100 ml were used (manufactured «Bayer» and «BogMark»). Samples were withdrawn by piercing the rubber plugs with a syringe needle. Synthesized gas volume was measured on a syringe scale while indentation piston with overpressure gas. The amount and composition of the gas was determined daily.

The gas composition was determined by a standard procedure by heat conduction in the katharometer on gas chromatograph LHM-8-MD. Two steel columns were used - one (I) for the analysis of H₂, O₂, N₂ and CH₄, the second (II) for the analysis of CO₂. Columns parameters: I - l = 3 m, d = 3 mm, the sorbent 13X (NaX); II - l = 2 m, d = 3 mm, the sorbent Porapak-Q. The temperature of column is +60 °C, evaporator + 75 °C, detector + 60 °C, the detector current is 50 mA. Carrier gas is argon with the flow rate 30 ml/min. The gas concentration (H₂, O₂, N₂ and CO₂ in %) was calculated by peak areas registered by recorder.

2.11. Obligate anaerobes cultivation technique

Anaerobic microorganisms were cultivated by Hungate's method (Hungate 1969). Tubes, flasks and culture medium were bubbled by argon (O₂ concentration not higher than 0.01%). The intensity of the flow was adjusted so that through the cultivator passed argon volume equal to the thirty cultivator volumes. Rezazurine was used as an indicator of anaerobiosis and H₂S was used as reducing agent (0,1 ml per 10 ml of medium) while *Clostridium lituseburense* cultivating. After air removing cultivators were sealed with rubber plugs and hydrogen sulfide was added by syringe. Hydrogen sulfide created Eh = - 120 ... -130 mV (pH = 7,0 ... 7,2).

2.12. Anaerobic redox-cultivator

We developed anaerobic redox-cultivator to investigate patterns of microbial waste processing (Chernishenko et al 1990).

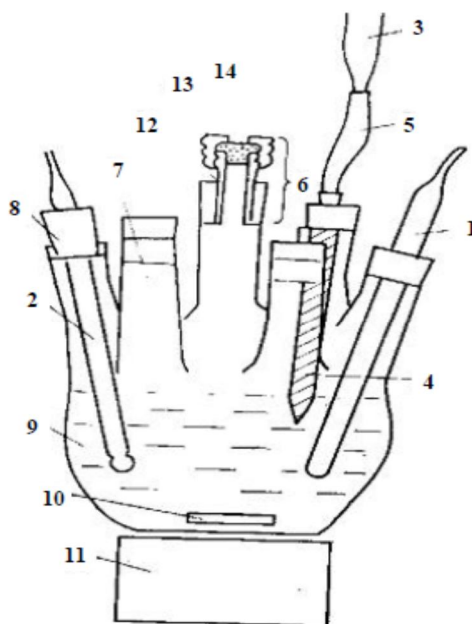


Fig. 1. Scheme of redox-cultivator. Measuring electrodes: 1 – platinum for redox-potential, 2 – for pH, 3 – reference chlorine-silver electrode; 4, 7, 8, 14 – conical rubber plug; 5 – silicone tube; 6 – sampler; 9 – cultivator; 10 – glass capsule; 11 – magnetic stirrer; 12 – glass tube; 13 – metal clip.

The cultivator allows continuously pH and Eh measuring. The cultivator ensures sterility and sealing. The measuring cell is a flask made of heat-resistant glass "Pyrex". Scheme of redox-cultivator is shown on Fig. 1. Its volume (0,2 - 2,0) and the number of external tubes (3 - 5 pcs.) depends on the objectives of the experiment. Measuring (1 and 2) and reference (3) electrodes fixed in the impermeable to O₂ rubber tube.

The sterility of the redox-cultivator was provided by a so-called electrolyte bridge. Electrolyte bridge contains 2% of agar saturated by KCl. Electrolyte bridge was sterilized in an autoclave at 1.0 atmospheres. Trimmed pipette 10 cm long was sterilized separately. Pipette segment was inserted into the conical rubber plug (4). Thin layer of basalt wool was inserted in the pipette nozzle. Melted 2% agar saturated by KCl was poured into trimmed pipette and a thin silicone tube (5) was immersed in the agar. The tube with KCl (saturated solution) was worn on the end of the chlorine-silver electrode (3). Layer of basalt wool held saturated with KCl agar in the pipette. Dipping rubber plug into the agar at a distance of 0,5 – 1,0 cm from the end of the pipette excluded the possibility of free-flowing KCl solution from the reference electrode in the cultivator. These operations provided sterile measuring of pH and Eh, as well as free electrolytic connection of electrode pairs for the pH and Eh measurement.

Measuring electrodes and rubber cover were sterilized by immersing them in perhydrol (33% H₂O₂ solution) for 10 - 15 minutes. The electrodes were wiped with sterile filter paper and dried in a stream of warm air around the gas burner after getting out of perhydrol. Cultivator with electrolyte bridge was sterilized by autoclaving before the experiment. Thereafter sterile medium was added to cultivator (9) and heated to remove O₂. Without stopping cultivator boiling was injected with long needle which supplied with sterile argon current (into a needle cannula sterile layer of basalt wool is inserted). The medium was cooled on a water bath. Measuring electrodes were consistently introduced into the cultivator. Cultivator was injected through rubber plug of the sampler (14) with a thin needle which flow argon at a pressure of 0,5 atm through a layer of sterile basalt wool.

External tubes were sealed with samplers (6) or conical rubber plugs (7). The sampler is a glass tube (12) threaded at the throat. Metal clip (13) pressed tightly a flexible plug to the throat of sampler.

Cultivator with a liquid medium was held for one day in a thermostat (t = 36 °C) for sterility check. Then the inoculum and was added to the medium by injection through a plug. Mass transfer in the cultivator was provided with a magnetic stirrer (11) which rotated ferromagnetic in a glass capsule (10) in the medium. The gas phase composition, optical density of the culture liquid was determined every hour. The measuring cell provided anaerobic growth conditions for at least 14 days.

2.12. Module pilot plant

The dynamics of microbial metal waste water purification was investigated in a "module pilot plant" (MPP) in a flow mode (Figure 8). The MPP consists of the following units: centrifugal pump for supplying waste water in the storage tank (1 cub. m), flow dispenser, module 10 cub. dm (5 kg). The module is divided by partitions with holes into 10 sections.

3. RESULTS AND DISSCISSION

3.1. Thermodynamic prognosis of microbial chromate reduction

We considered the example of thermodynamic prognosis of the new microbial biotechnology, which allows simultaneously the environmentally hazardous polymeric organic waste destroying and the metal-containing wastewater cleaning. The technology is based on coupled reaction of glucose oxidation and chromate reduction by microorganisms.

In anaerobic conditions microorganisms hydrolyse organic polymers compounds to carbohydrates and ethanol. Further carbohydrates are fermented to H₂ and CO₂. Fermentation of carbohydrates occurs with low potential catabolic enzymes (hydrogenases and dehydrogenases), which creates in a culture liquid very low redox potential – 400 ... – 420 mV (Gottschalk 1986). We shown the falling down of redox potential to – 400 mV in the process of glucose fermentation by culture of *Clostridium lituseburens* (Fig. 2).

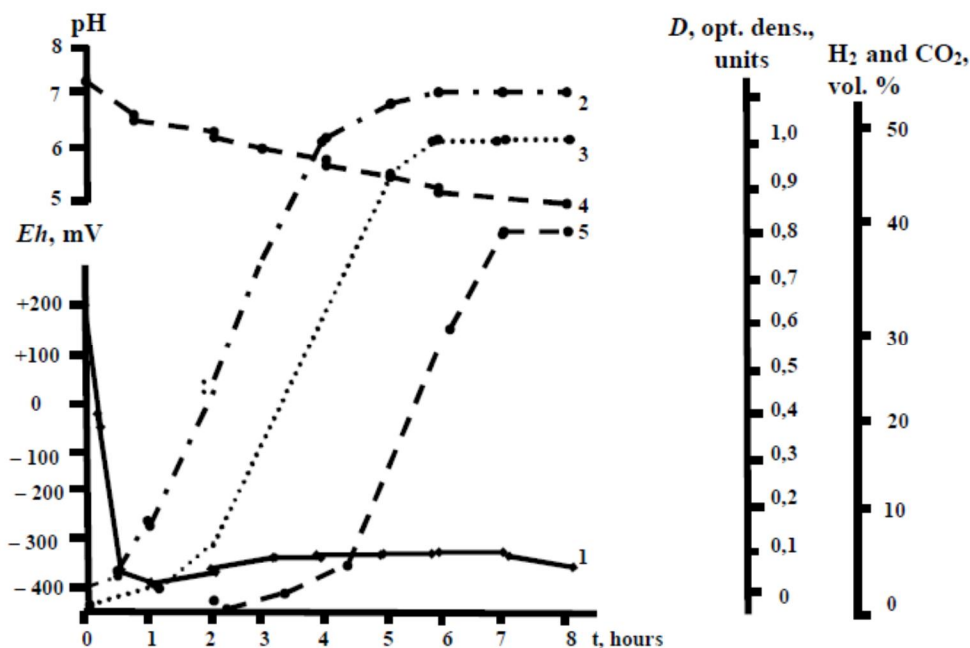


Fig.2. Glucose fermentation by *Clostridium lituseburens*: H₂ synthesis, Eh reduction to – 400 mV, 1 – Eh, mV; 2 – D, optical density units; 3 – H₂ volume, %; 4 – pH; 5 – CO₂ volume, %.

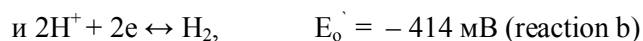
Redox potential of reducing chromate to chromium hydroxide(III) is +555 mV at pH 7,0 (Figure 3, Equation 5). Obviously, chromate is a high-potential oxidizing agent towards dehydrogenases and hydrogenases.

Potentials difference between the donor and acceptor reaction in 100 mV leads to nonspecific reducing of acceptor. Difference of potentials in the 955 mV is to ensure fast and efficient reducing of chromate by anaerobic microorganisms (Gottschalk 1986).

Suggested model of microbial chromate reduction can be quantitatively substantiated by the method of thermodynamic prognosis.

Lets consider the thermodynamic prognosis of microorganisms' metabolic pathways and their interaction with chromium compounds. For this purpose we used the diagrams of elements stability in aqueous solutions in the coordinates "pH-Eh" by Pourbaix (Pourbaix 1963) (Fig. 3).

There are determined by thermodynamic properties of water limitations for energy metabolism of the microorganisms. Thermodynamic stability of the water zone is in the range of Eh = – 414 ... +814 mV (pH = 7,0). This zone is limited by two redox reactions:



Water is a reducing agent and is oxidized to O₂ in the first case (reaction a). In the second case the proton of water is an oxidizing agent and is reduced to H₂ (reaction b).

It follows that the energy metabolism of microorganisms is possible only in the zone of the thermodynamic stability of water (Fig. 3. Zone 2). Microorganisms can interact only with those compounds that are in the zone of water thermodynamic stability.

Consider the thermodynamic properties of chromium compounds and determine which of its redox forms are in the zone of water thermodynamic stability and available for the transformation by microorganisms (Fig. 3). In aqueous solution the chromium compounds can be stable in the following redox forms: Cr(VI), Cr(III), Cr(II) и Cr⁰. The redox potentials of chromium compounds reduction are both in the zone of sustainability of water (Zone 2) and outside of it (Zone 1 and Zone 3). High-potential compounds of Cr(VI), such as H₂CrO₄, HCrO₄⁻ are strong oxidants. Their potentials are located in Zone 1, i.e. outside of zone of water thermodynamic stability (Figure 3, reaction of 1 and 2).

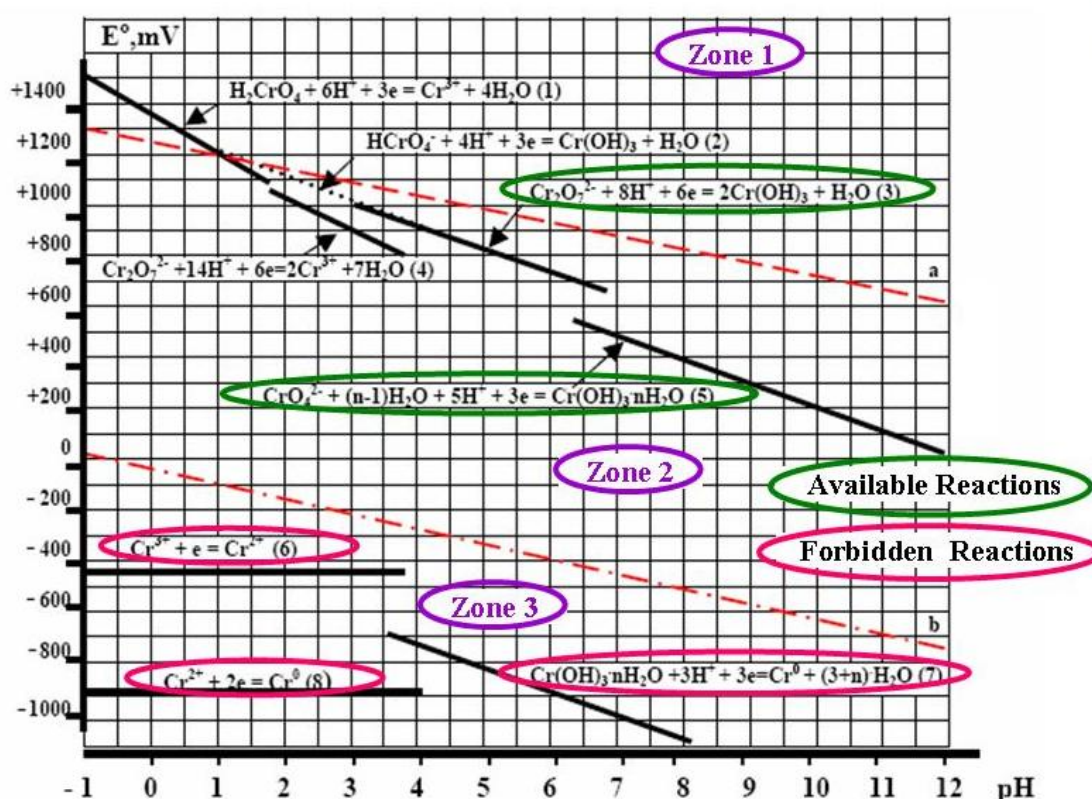


Figure 3. Thermodynamic prognosis of microbial reduction of chromium compounds

Note: a (upper) and b (lower) - limits of the thermodynamic stability of water;

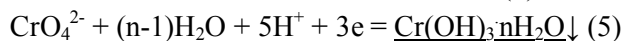
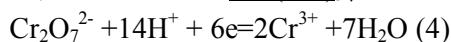
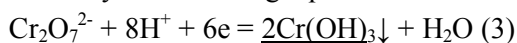
$$a: \text{O}_2 + 4\text{H}^+ = 2\text{H}_2\text{O} \quad \text{та} \quad E_h = 1,228 - 0,0591 \text{ pH} - 0,0295 \lg \text{PH}_2;$$

$$b: 2\text{H}^+ + 2\text{e} = \text{H}_2 \quad \text{та} \quad E_h = 0,000 - 0,0591 \text{ pH} - 0,0591 \lg \text{PH}_2$$

Obviously, they are powerful oxidants and disinfectants towards microorganisms, and lead to irreversible oxidation of enzymes and structural components of cells. Therefore H₂CrO₄ and HCrO₄⁻ are actually unavailable for energy metabolism of microorganisms. Contact of microorganisms with

these compounds leads to the inevitable, immediate death of microorganisms. Unable to be reduced by microorganisms chromium compounds (according to the thermodynamic prognosis) are in zone 3. Redox potentials for reduction of Cr(III) compounds are in the range $E_h = -420 \dots -910$ mV, i.e. far outside the zone of water stability (Figure 3, reactions 6-8). For most low potential catabolic enzymes (hydrogenases and dehydrogenases) E_h is in diapason $-400 \dots -420$ mV (Gottschalk 1986). It is clear that the electron transfer from the dehydrogenases with potential ≈ -400 mV to $\text{Cr(OH)}_3 \cdot n\text{H}_2\text{O}$ with the potential of reduction ≈ -900 mV is impossible. Therefore microorganisms can not reduce the Cr(III) to Cr(II), and furthermore – to Cr^0 . According to this, microorganisms can interact only with those compounds of chrome, whose potentials are in zone 2, i.e. inside zone of thermodynamic water stability. Thermodynamic properties of chromium compounds (Fig. 3) give the possibility to choose the optimal reaction, which leads to chromate removing from solution by microbial metabolism.

Inside zone of water stability there are three reactions of Cr(VI) compounds reductions (Figure 3, reaction 3, 4, 5). They are described by the following equations:



The main task of purification is removing chromium compounds from a solution (i.e., transfer chromium compounds to an insoluble form). Therefore, the reaction 4 can not be used for removing chromium from solution because in this case chromate reduces to soluble cation Cr^{3+} . As it shown on Figure 3, Cr^{3+} cation is stable in acidic conditions ($\text{pH} = -1,0 \dots 4,0$). It follows that the microbial reduction of chromium compounds ($\text{Cr}_2\text{O}_7^{2-}$ and CrO_4^{2-}) should be carried out at $\text{pH} > 5,0$. Under these conditions the chromate is removed from solution in accordance with equations 3 and 5 with formation of insoluble hydrated chromium hydroxide(III), – $\text{Cr(OH)}_3 \cdot n\text{H}_2\text{O}\downarrow$. Microorganisms during substrate (electron donor) oxidation form a flow of reducing equivalents which reduce chromate to insoluble chromium hydroxide(III). Therefore, the metabolic active microorganisms serve as an electron donor and chromate as an acceptor. Obviously, the reduction will occur with maximum efficiency and rate since maximum potential difference between the donor and the electron acceptor. Lowest redox values are known for the anaerobic fermentation of carbohydrates (polymers and monomers). During the starch and glucose fermentation E_h decreases almost to the theoretical allowable values: $-350 \dots -400$ mV at $\text{pH} 6,2$ (Figure 2). For the acceptor reaction $\text{CrO}_4^{2-} + (n-1)\text{H}_2\text{O} + 5\text{H}^+ + 3\text{e}^- = \text{Cr(OH)}_3 \cdot n\text{H}_2\text{O}\downarrow$, potential $E_h = +555$ mV ($\text{pH} = 7,0$). Potential difference between the donor and acceptor reactions $\Delta E_h = E_{h_{\text{acceptor}}} - E_{h_{\text{donor}}} = +555 \text{ mV} - (-400) \text{ mV} = 955 \text{ mV}$. Hence fast and effective chromate removal from solution is available at the neutral pH. Thus, we have shown that the thermodynamic prognosis allows the simultaneous occurrence of two processes. The first one is fermentation of polymeric organic compounds. The second is the reduction of chromate to insoluble compounds. These two processes are presented in the form of an integrated scheme:

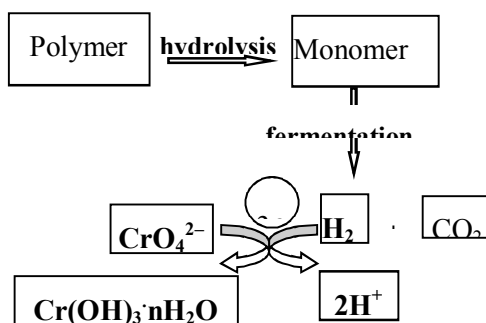
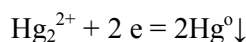


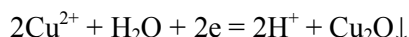
Fig. 4. Scheme of simultaneous polymer fermentation and chromate reduction.

The combination of the two processes can both destroy environmentally hazardous organic compounds and remove toxic chromate from the solution simultaneously. This approach is universal. Redox reaction also ensures removing from the solutions other high potential metal-oxidizers such as copper and mercury. Thus, for the reaction



redox potential $E_h = +790 \text{ mV}$ ($\text{pH} = 2,0$).

For the reaction of Cu^{2+} reduction to insoluble Cu(I) ↓



redox potential $E_h = +390 \text{ mV}$ ($\text{pH} = 7,0$) (Pourbaix 1963).

Therefore, creating the potential – 300...– 400 mV at the organic fermentation leads to a thermodynamically inevitable reduction of both Hg_2^{2+} and Cu^{2+} to insoluble compounds. There are three obligate conditions for the powerful synthesis of reducing equivalents.

Here is the list of these conditions:

1. high substrate concentrations;
2. high concentration of microorganisms;
3. variety of physiological groups of microorganisms.

The substrates serve as electron donors for reducing of metals-oxidizers to insoluble compounds. Water-insoluble organic polymers must be used as the substrate according the following reasons:

1. Polymers insoluble in water, so they, in contradiction to soluble substrates (e.g., glucose), do not inhibit the growth of microorganisms at the high concentrations.
2. Insoluble polymers provide prolonged metabolic activity of association as they are hydrolyzed by microorganisms gradually according to their consumption.
3. Polymers serve as substrates for the microorganisms' immobilization, which promotes the microbial biomass accumulation and improves the metals reduction.

Concentrated biomass of microorganisms creates a strong flow of reducing equivalents during the substrate fermentation. A variety of physiological groups of microorganisms ensures simultaneous fermentation of various classes of organic compounds in the form of monomers, oligomers and polymers. Finally, the physiological diversity of microorganisms allows providing the majority of theoretical mechanisms of microbial metals removing from aqueous solutions. These include the physical and chemical sorption, reduction to insoluble forms, sorption by the cell walls, accumulation, and precipitation of metals by microbial exometabolites.

All these necessary conditions occur in our granular microbial preparation - microbial biocatalyst (MBC). The MBC consist of microorganisms, carbon and energy sources, phosphorus, nitrogen, etc., as well as compounds which keep the granules of MBC in the stable form in water solutions (commercial "know-how"). Biocatalyst microorganisms are presented by mixed microbial communities (MMC). The composition of MMC includes communities of digested sludge, activated sludge, poultry and cow manure. Obviously, integration of microbial associations provides simultaneous fermentation of wide range of organic compounds (protein and carbohydrate polymers). Simultaneous fermentation of a wide range of organic compounds creates a powerful flow of reducing equivalents required for the effective microbial reduction of metals-oxidizers (CrO_4^{2-} , Hg_2^{2+} , Cu^{2+}). The removal efficiency of metal-oxidizers also increases due to the high concentration of

metabolically active cells in the MBC granules. The total cell number in MBC granules is $n \times 10^6 \dots n \times 10^7$ per 1 gram of absolutely dry weight (Table 1).

№	Group of microorganism	Electron donor	Electron acceptor	Cell quantity in 1 gram of MBC
1	ammonifying	protein	O ₂	$7,0 \times 10^7 - 8,1 \times 10^7$
2	spore-forming	protein	O ₂	$8,6 \times 10^5 - 1,2 \times 10^6$
3	denitrifying	organic acid	NO ₃ ⁻	$3,0 \times 10^4 - 5,0 \times 10^4$
4	sulfate-reducing	organic acid	SO ₄ ²⁻	$6,4 \times 10^5 - 1,0 \times 10^6$
5	cellulosolytic	cellulose	–	$2,5 \times 10^6 - 3,9 \times 10^6$

Tab. 1. The total cell number of different physiological groups of microorganisms in the MBC and electron donors and acceptors characteristics.

Thus, we have theoretically grounded the necessary and sufficient conditions for the universal microbial technology of environmentally hazardous organic waste fermentation and purification of metal containing waste water. We tested the effectiveness of biotechnology on model and industrial wastewater containing Hg²⁺, CrO₄²⁻ and Cu²⁺.

3.2. Purification of mercury-containing waste water by MBC preparation.

To investigate the interaction patterns of MBC with mercury compounds, we used a standard solution of Hg(NO₃)₂ with 1000 ppm Hg²⁺. Redox potential Eh = +920 mV at pH = 2,0 in the standard solution of mercury. Therefore, to obtain parameters of MBC metabolic activity in the control, i.e. without Hg(NO₃)₂, acidic condition were created as well (pH = 2,0) using dilute HNO₃. In the experiment and in the control the weight ratio of MBC and the solution was 1:40. In the control microorganisms carried out fast correction (auto regulation) of redox potential and pH in the medium (Fig. 5).

During the first six hours of cultivation redox potential decreased from +500 to – 300 mV and further practically did not change. The pH in two hours increased from 2,0 to 6,0 and hereinafter was practically constant. After increasing pH to 6,0 microorganisms started to grow. The optical density of the solution increased from 0,0 to 0,3 optical density units in the period from 6 to 36 hours of cultivation. The findings suggest the potential use of the MBC in the highly acidic conditions.

The interaction of MBC microorganisms with Hg²⁺ at 1000 ppm is shown at Figure 6. First of all it should be noted that the MBC microorganisms capable to proliferate in a concentrated solution of mercury. Growth of microorganisms was observed in the period from 24 to 72 hours of cultivation. The change of culture optical density (biomass increase) shows three phases of growth. The first (A) is lag phase, the second (B) is phase of logarithmic growth, and the third (C) one is stationary phase. Accumulation of mercury by microorganisms was observed in all three phases of growth.

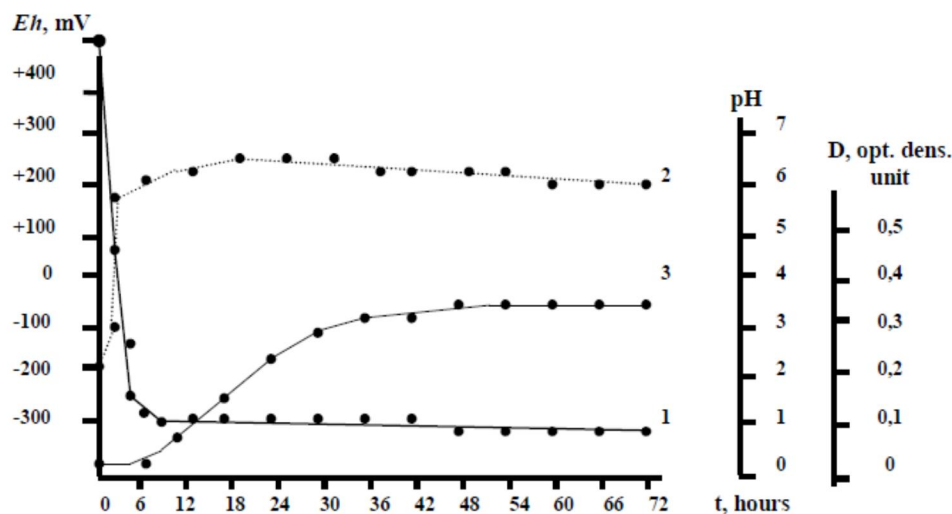


Fig. 5. Metabolic activity of MBC without Hg^{2+} (control). 1 – Eh, mV; 2 – pH; 3 – D, optical density units.

In the A phase microorganisms removed from a solution 74% of Hg^{2+} , in phase B – 88%, and finally in phase C – 100% mercury. Obviously, the mercury recovery in phase A took place due to physical-chemical adsorption, since there was no growth of microorganisms. However, further removal of the mercury was due to the metabolic activity of microorganisms. This was indicated by biomass growth and reducing the redox potential in the phases B and C. Expressed microbial auto regulation effect of physical-chemical parameters in phase A should be noted. Thus, microbial growth started only after reducing the redox potential to +240 mV and the pH increase from 2,0 to 6,0. Thus, granular MBC can be used to purify highly toxic metal wastewater. The final concentration of mercury in solution was 1 ppm. Accordingly, the degree of extraction of mercury was $R = 99,9\%$, and $K_d = 35664$. These data demonstrate the high efficiency of purification of the concentrated mercury-containing solution by granular MBC.

3.3 Purification of the model chromate-containing water by MBC preparation

Next, we have demonstrated the effectiveness of applying MBC for model chromate-containing wastewater purification. The results are shown in Fig. 7. In the experiment we used a solution containing 200 ppm of CrO_4^{2-} in distilled water. Thermodynamic calculations (Fig. 3) show that the efficiency of chromate reduction to insoluble chromium hydroxide(III) is proportional to the potential difference between the donor and acceptor (CrO_4^{2-}) systems (Pourbaix 1963). Metabolically active bacteria (electron donors) provide very low values of the redox potential ($E_h = -300 \dots -400$ mV). Therefore, to create a maximum potential gradient chromate was added into the solution already containing metabolically active microorganisms. Granules of MBC were filled with distilled water to start the metabolism of microorganisms. During the first four hours E_h decreased to $-390 \dots -400$ mV. Furthermore, in the fourth hour of cultivation H_2 concentration in gas phase was 40%. Thus, after 4 hours MBC microorganisms were in a phase of metabolic activity, and created low redox potential and flow of reducing agents.

Therefore chromate solution was added to the cultivator to a final concentration of 200 ppm of CrO_4^{2-} . As it was expected, the high-potential anion CrO_4^{2-} immediately began to reduce by microorganisms. Redox enzyme systems actively reduced CrO_4^{2-} and redox potential rose to +190 mV. Naturally, the synthesis of hydrogen at such a high potential stopped. Metabolically active bacteria completely

reduced chromate in two hours after its injection into the culture fluid. Reducing the chromate concentration was followed by a correlative redox potential reduction. After full reducing of chromate to insoluble $\text{Cr}(\text{OH})_3 \times n\text{H}_2\text{O}$ and decreasing the potential to -390 mV hydrogen syntheses resumed. So metabolically-active MBC quickly and efficiently reduces chromate in high concentrations to the insoluble chromium hydroxide(III) and thus completely purified the model waste water.

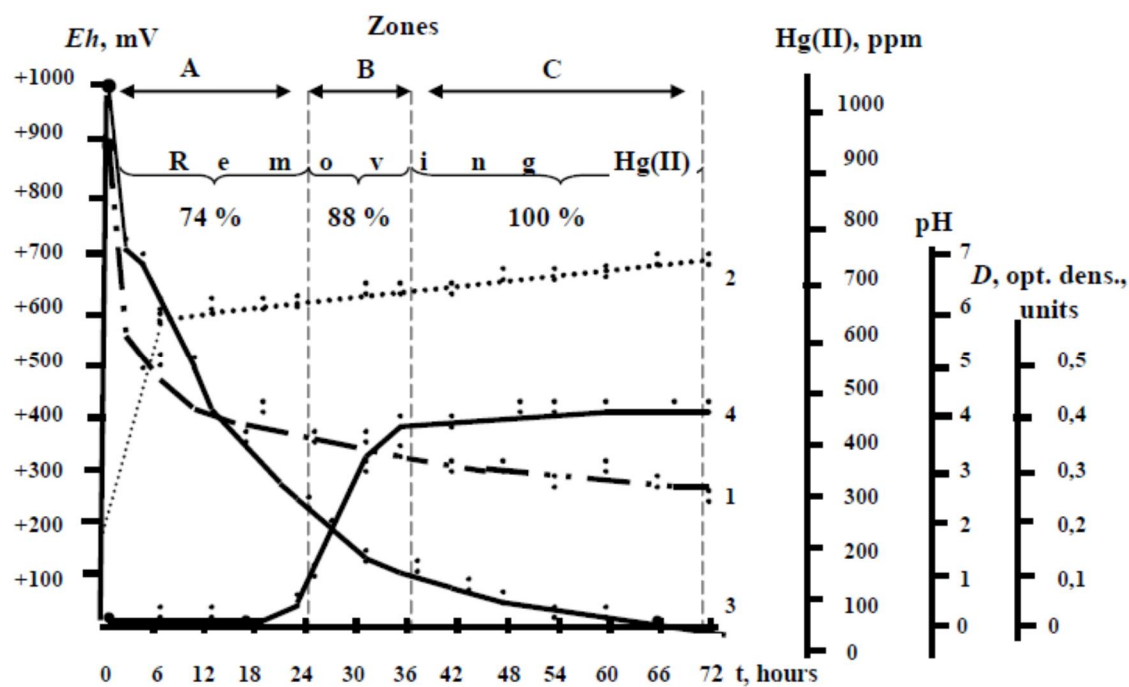


Fig. 6. Metabolic activity of the MBC while removing Hg^{2+} from solution. 1 – Eh, mV; 2 – pH; 3 – concentration of Hg^{2+} in solution, ppm; 4 – D, optical density units (biomass growth). A, B, C – zones of MBC microorganisms' metabolic activity.

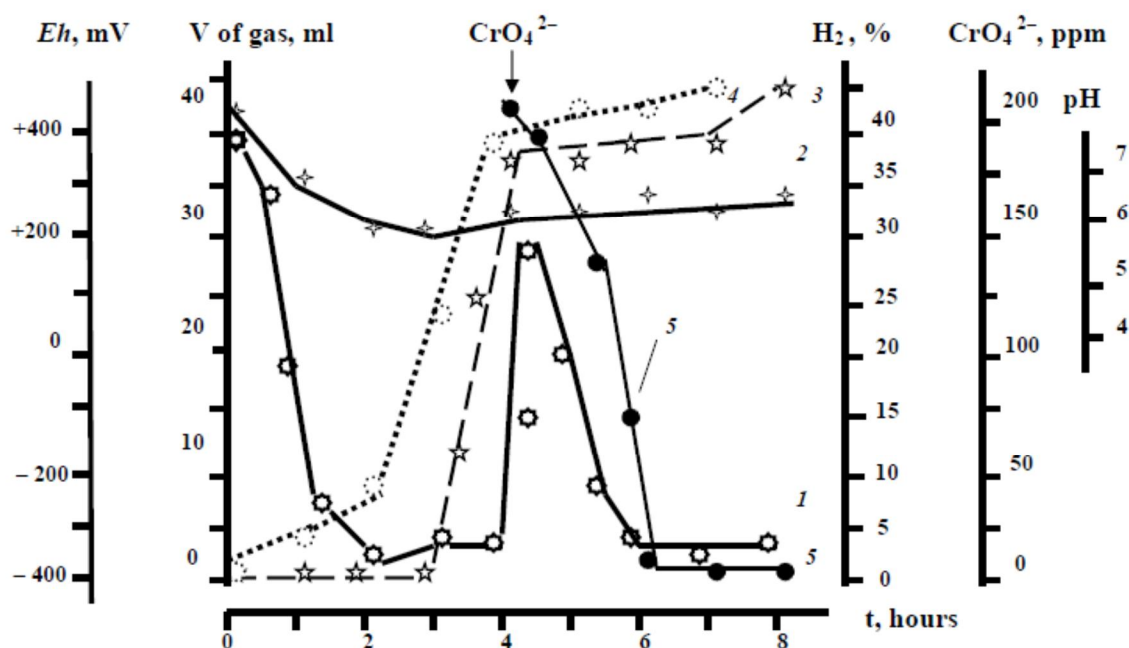


Fig. 7. Reduction of CrO_4^{2-} by MBC and metabolic parameters of the microorganism during the chromate removing from the model wastewater; 1 – Eh, mV; 2 – pH; 3 – H_2 , %; 4 – gas volume, ml; 5 – CrO_4^{2-} concentration, ppm.

3.4. Purification of galvanic wastewater by MBC preparation

Furthermore, we showed the efficiency of MBC for the industrial chromate waste water (CWW) at galvanic manufactory treatment. Pilot-scale tests were carried out at the "Municipal Engineering" plant (Kyiv, Ukraine). These CWW are typical for galvanic production. They contain chromate (50-70 ppm CrO_4^{2-}), trace concentrations of Zn^{2+} , Cu^{2+} , NaOH , Na_2CO_3 , Na_3PO_4 and others. The pH is 6,8...7,2, $\text{Eh} = +450...+470$ mB. Purification of CWW by microbial biocatalyst was performed on a module pilot plant (MPP). The MPP consists of the following units: centrifugal pump for supplying waste water in the storage tank (1 cub. m), flow dispenser, module 10 cub. dm (5 kg). The module is divided by partitions with holes on 10 sections. One cub. dm of MBC was in each section.

The effectiveness of wastewater treatment was determined by flow rate, concentration of CrO_4^{2-} , value of the redox potential along the length of the module, and "coordinate of analytical zero" (CAZ). Index CAZ represents a linear coordinate along the length of module, wherein the chromate concentration is zero. Purification of CWW was performed in steady state and flow modes. In steady state mode module with dry granules was poured with waste water. Microorganisms adapted to CrO_4^{2-} and reduced it to an insoluble hydroxide chromium(III). Time of adaptation was four days at 50 ppm of chromate. After the reduction of the chromate concentration to analytical zero, the module was switched to a flow mode. The flow rate was gradually increased, but only after chromate concentration decreased to zero at least in the ninth section. In this case, CAZ is 0,9 meters by 1,0 meters length of the module. Initial flow rate was 6 liters/day. Flow rate gradually increased to 200 liters/day.

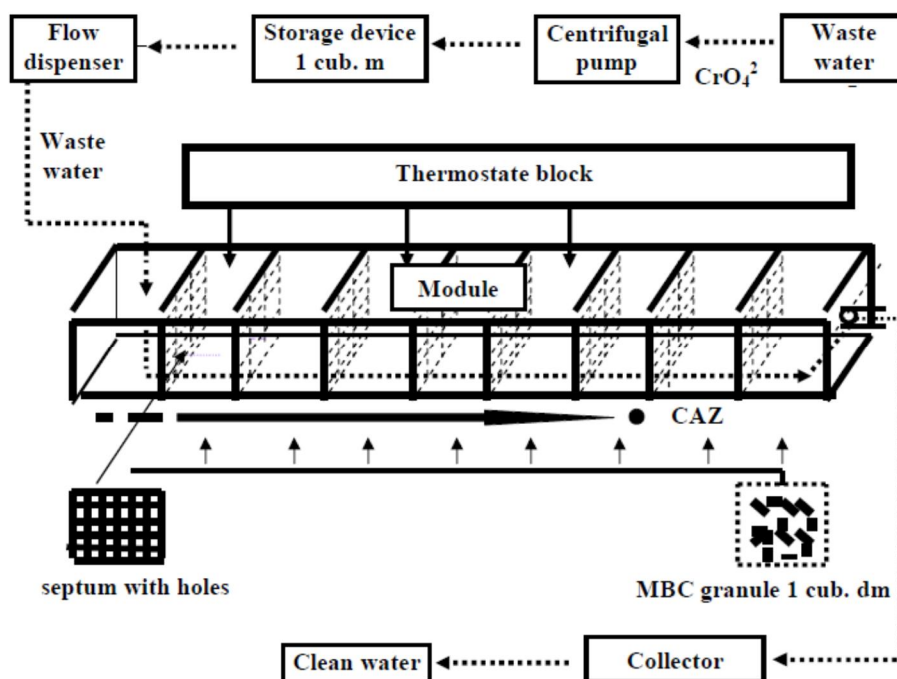


Fig. 8. Scheme of the module pilot plant (MPP)

Figure 9 shows the relation of the chromate concentration along the length of the module with the Eh. Chromate concentration is zero at the point A (CAZ = 0,6 m) when Eh = - 100 mV.

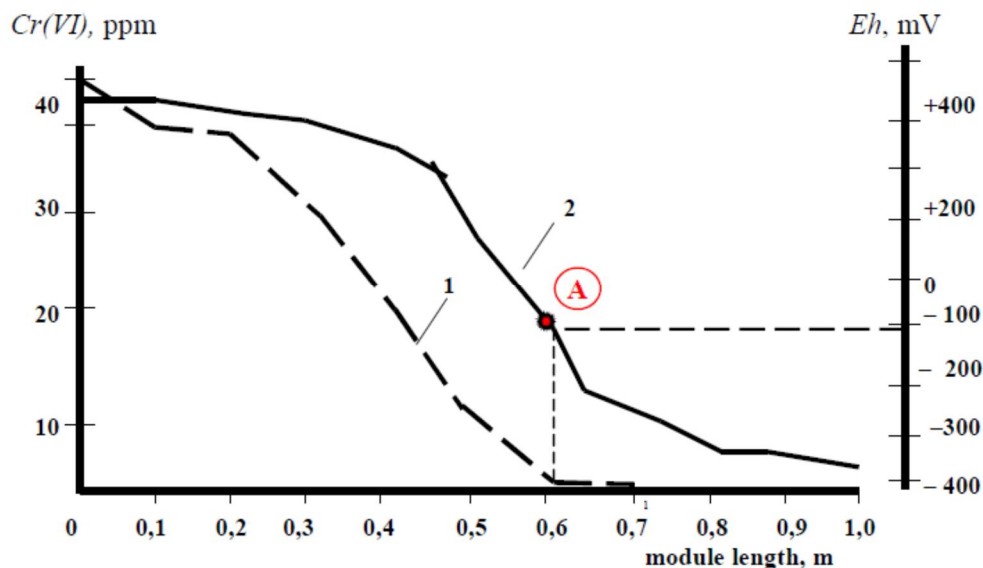


Fig. 9. Change of the redox potential and the chromate concentration by the module length; 1 - concentration of Cr(VI), ppm; 2 - Eh, mV (pH 6,5 – 7,6); 3 – 0,1 ... 0,3 ... - distance from the point of flow supply in the module.

Data show that decreasing of the redox potential correlates with a decreasing of chromate in solution. Chromate is absent in solution when $Eh \leq -100$ mV (analytical zero by DPC). Thus, the gradient of the redox potential along the length of the module is a reliable quantitative index of CWW treatment. Chromate is absent when $Eh = -100$ mV. The main technological parameters of CWW treatment are following. Calculated integral biocatalyst sorption capacity of MBC is $500 \text{ mg CrO}_4^{2-}$ for dry weight granulated microbial preparation. Thus, the efficiency of technology is close to 50%. Formation of bright blue precipitate of chromium hydroxide(III) on the surface of MBC granules shows the effectiveness of CWW treatment by microbial biocatalyst as well. Microorganisms so intensely reduced chromate that on MBC granules a bright blue thick crust of chromium hydroxide formed (Figure 10). The maximal precipitation of chromium hydroxide(III) took place in the first section of module.

This data show the possibility of valuable non-ferric metals obtaining with our biotechnology.

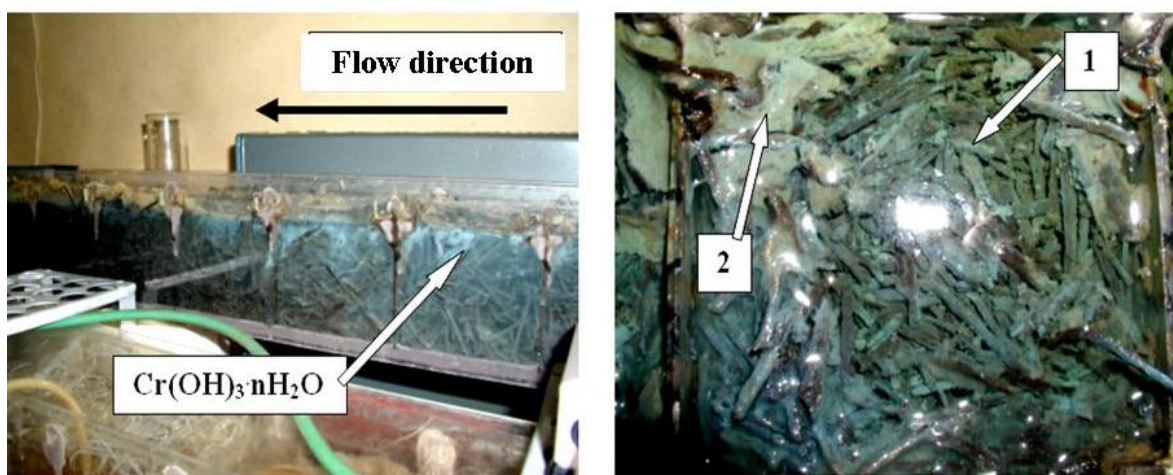


Fig 10. The precipitation of blue chromium hydroxide (III) on microbial granules; 1 – MBC granules; 2 – bright blue thick crust of chromium hydroxide.

3.5 Copper waste water treatment by metal resistant culture *Brevibacterium antarcticum*

These data suggest that unadapted microorganisms can provide effective purification of metal containing waste water. However, it is obvious that the efficiency of the process can be significantly improved by using resistant to ultra-high concentrations of toxic metals microorganisms. During the systemic investigation of the structure and function of Antarctica microbial communities we isolated dozens of resistant to high concentrations of the most toxic metals strains (Tashyrev 2009). For example, metal resistant culture *Brevibacterium antarcticum* was isolated. *B. antarcticum* is resistant to Cu^{2+} , Co^{2+} , Ni^{2+} , CrO_4^{2-} in the concentration range of 300 ... 1000 ppm of metal ions. This culture is hemoorganotrophic and during the substrate fermentation reduces metal-oxidizers to insoluble compounds. Potato waste was used as a substrate and Cu^{2+} was used as representative toxic metal for modeling the biotechnological process of wastewater treatment. Model waste water was purified in MPP (Fig. 8). Initial copper concentration was 325 ppm of Cu^{2+} . Potato cubes were added in sections 1-7. In sections 8 – 10 was placed capron fiber. Both potato and capron fiber served as substrates for *B. antarcticum* cells immobilization. Plant was converted into a flow regime after 24 hours of adaptation. The flow rate of the waste water was 1,2 liter/hour. During purification the copper

concentration was reduced in 3250 times (from 325 to 0,1 ppm of Cu^{2+}). Therefore, metal resistant microorganisms are the most suitable for purification of metal containing waste water from toxic metals.

The development of our technology can simultaneously achieve four positive effects. The first positive effect is the destruction of hazardous organic solid waste (potato). The second one is the toxic waste water purification. The third is valuable copper concentrate ($\text{Cu}_2\text{O}\downarrow$) obtaining. And finally, the fourth effect is clean water for technically closed production cycles obtaining.

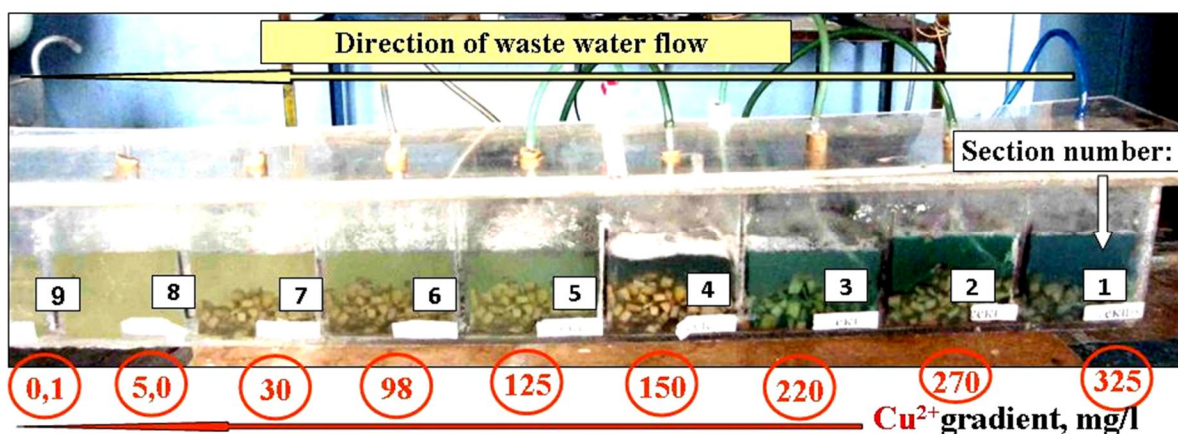


Fig. 11. Module pilot plant for copper wastewater purification by culture *B. antarcticum*.

3.6. Bioprospecting: extreme ecosystems as a source of metal resistant microorganisms for environmental biotechnologies

On the example of metal resistant microorganisms from Antarctic ecosystems we have shown that bioprospecting (isolation of metal resistant microorganisms) is a very promising direction for the environmental biotechnologies development. Hence systematic screening of microorganisms in extreme ecosystems should be provided for the technologically perspective strains isolation. So we investigated the presence of metal resistant microorganisms in the soil of Negev desert (Israel) and salt marshes of Pomorie (Bulgaria). To isolate metal resistant microorganisms we found the optimal concentration of carbon sources and the maximum permissible concentration (MPC) of toxic metals. We tested the toxic metals with known mechanisms of microbial damage as a representative. They are:

- metals-oxidizers (CrO_4^{2-}),
- metals-substitutes (Ni^{2+} , Co^{2+}),
- metals with combined effect (that have the properties of metals-oxidizers and metals-substitutes such as Hg^{2+} , Cu^{2+}).

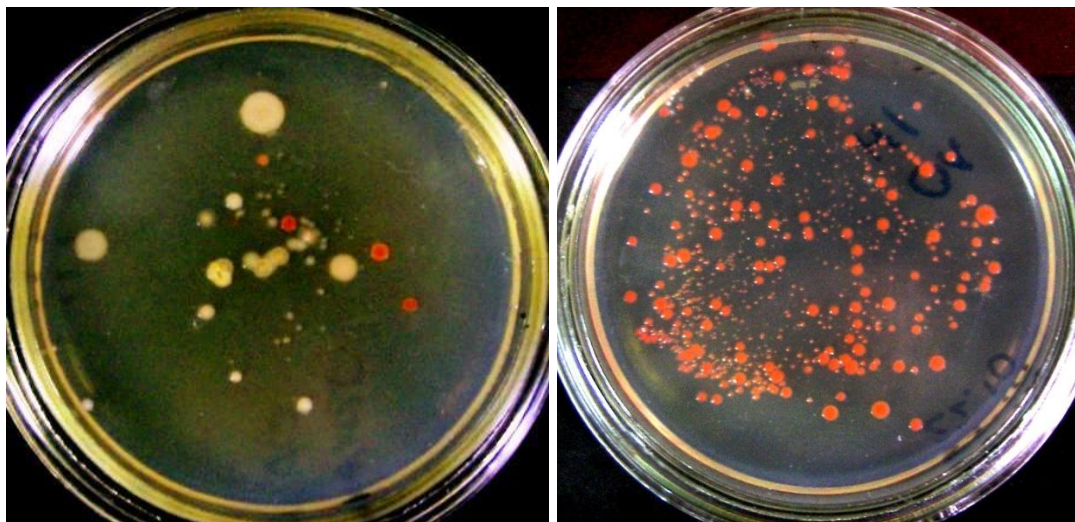


Fig.12. Representative strains from Negev desert soil (Israel).

For hemoorganotrophic microorganisms of soil Negev the following MPC were found: 1300 ppm Cu^{2+} (1 strain), 1000 ppm CrO_4^{2-} (5 strains), 20 ppm Ni^{2+} (6 strains), 15 ppm Co^{2+} (5 strains) and 10 ppm Hg^{2+} (4 strains).

Metal polyresistant strains were isolated from the soil of the Negev desert. Thus, the three representative strains, i.e. typical for the soil Negev ecosystem, are able to grow at 1300 ppm Cu^{2+} , two – at 500 ppm Ni^{2+} , two – at 700 ppm CrO_4^{2-} and two strains grow even at 40 ppm Hg^{2+} .

Metal resistant microorganisms were isolated from Pomorie salt marshes as well. For these strains MPC were estimated: 300 ppm Cu^{2+} , 500 ppm CrO_4^{2-} , 100 ppm Ni^{2+} and 10 ppm Hg^{2+} . A representative of the salt marshes *Bacillus subtilis* is resistant to 500 ppm Cu^{2+} .

So it is obvious that resistant to the most toxic metals microorganisms are widespread in extreme ecosystems. That's why extreme ecosystems are the reliable source of microorganisms perspective for metal wastewater purification biotechnologies.

4. CONCLUSION

1. We developed a method of thermodynamic prognosis of microorganisms with xenobiotics interaction. This method creates a theoretical basis for the maximally effective environmental biotechnologies.
2. The confirmation of two processes was experimentally demonstrated: the destruction of environmentally hazardous organic waste and withdrawing of the most toxic metals from model and industrial wastewater.
3. Due to these biotechnologies it possible to obtain commercially valuable products - concentrate of non-feric metals and ecologically clean water.
4. Extreme ecosystems serve as the reliable source of metal resistant microorganisms for development of new high efficient environmental biotechnologies.

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