The adaptation of gram-negative bacteria to acidic environmental conditions with implication in heavy metals removal processes

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Abstract
Bacteria that live in acidic environments are very important for industrial biotechnology. The physiological, biochemical and ecological studies, including the isolation of new acidophilic heterotrophic bacteria, will allow a better understanding of their signification for biodiversity and bioremediation. The aim of the present paper was to study the biosorption of heavy metallic ions by 8 strains and 3 populations of aerobe heterotrophic bacteria. They presented a maximum activity of acidophilic heterotrophic populations at acidity and temperature values close to the physico-chemical properties of habitats from which they were taken. The use of silicagel, zeolite and molecular sieve was proved efficient in the immobilization of neutrophilic and acidophilic heterotrophic bacteria by accentually decreasing of the optical density of cultures. Also, higher percentages biosorption of several metallic ions were obtained using populations of acidophilic bacteria compared with purified strains, which confirms the adaptation of populations to higher concentrations of Cu²⁺ (68%), Zn²⁺ (80%) and Ni²⁺ (84%). In the experiments of the chromium removal from industrial waste water by the acidophilic heterotrophic bacterial strains an extraction of the hexavalent chrome of 86.78 - 99.95% was noticed. The same experiments evidenced the fact that Cr³⁺ and Cr⁶⁺ were removed in bigger percentages (73.33% and 94.07%) after 48 hours contact between neutrophilic bacterial population and industrial waste water.

Keywords: genus Acidiphilium, biosorption, heavy metals

Introduction
The environment is polluted by numerous organic and inorganic compounds, heavy metals in particular. Rapid industrialization has led to increased disposal of heavy metals into the environment. A problem regarding the presence of metallic ions in the environment is their accumulation in the trophic chains and persistence in nature. Heavy metal resistance is a widespread attribute among bacteria isolated from industrial effluents. The high incidence of heavy metal resistance detected indicates the potential of these bacteria as bioremediation agents (BRUINS & al. [2], HAFERBURG & KOTHE [7], KOZLOV & ZVEREVA [12]). The acid mine drainage is an ideal ecosystem to study microbial ecology of the main concepts: (1) abiotic factors affecting the presence and distribution of microorganisms; (2) biotic factors affect, can change, abiotic conditions of the habitat; (3) interactions biotic occur between different groups of microorganisms and biological characteristics affecting the ecosystems; (4) the microorganisms that live and grow in extreme environments can reveal unique mechanisms of adaptations to environmental stress, such as acidic pH, high concentrations of heavy metals (BAKER & BANFIELD [1], JOHNSON [9]). These considerations have led to interest in the mechanisms of microbial diversity and the biotechnology for microbial sorption of heavy metals. This method can be used to control pollution and to recover these metals with important economical consequences (GADD [6], JOHNSON [10]). Among the most efficient biotechnological processes of recovering/removing the metal ions from the industrial effluents are the adsorption and
bioaccumulation of the metals by aerobe heterotrophic bacteria through two mechanisms: (1) linking the metal ions to some sites situated on the surface of the cellular wall, characteristics to the biosorption; (2) the intracellular accumulation of the metal achieved through the active and passive transport of the metal cations inside the cellular wall and membrane, characteristics to be the bioaccumulation processes (DOPSON & al. [5], JOHNSON & HALLBERG [11]). A special importance in using the aerobe heterotrophic bacteria in metal biosorption and bioaccumulation processes from industrial residual waters is represented by the resistance of these bacteria to higher concentrations of metallic ions existent in those media. The metal resistance mechanisms identified in neutrophilic are also present in acidophilic bacteria, in some cases utilizing homologous proteins, but in many cases the degree of resistance is greater in acidophiles (MOROZKINA & al. [13], RAKESH [14]). Microbiological technology of removal by metallic ions from industrial waste waters appear to present a low cost application, not an additional factor of environmental pollution and allows recovery of heavy metals from industrial waste waters. Advantages microbiological method for removing heavy metals led to increased laboratory studies to improve the removal efficiency of metals from industrial waste waters by microbiological processes (CISMASIU [4], RAMPELOTT [15], PATTANAPIPITPAISAL & al. [17]). The adsorption of the heavy metals ions can be made using different biomasses of bacteria, as an adsorbing agent for different metallic ions. The selection of the adsorption process of the metals are influenced by the physiological characteristics of the microorganisms, nutritive medium composition, the physical-chemical factors in the medium, all of them being important elements in understanding and controlling the microbiological of the metal removal process from industrial waste water (RAKESH [14], SILVER & PHUNG [16]). Due to the influence of the physical-chemical factors on the development and the metabolic activity of the aerobe heterotrophic bacteria present in the industrial effluents, the study of these parameters was imposed for raising the efficiency of the processes of adsorption and bioaccumulation of the metallic ions. In this study, the investigation focused on the Acidiphilium sp. due to its ability to develop in such polluted area and its potential bioremediation application (CISMASIU [3], HIRAISHI & IMHOFF [8]).

In this context, it became necessary studying the capacity of metallic ions removing from industrial waste waters by aerobe heterotrophic bacteria, neutrophilic (isolated from industrial waste water) and acidophilic (isolated from mining effluents), in different experimental conditions (pH, oxygen concentration in solutions, the biomass type, the heavy metals concentration, the contact time between the biomass and industrial waste waters).

**Materials and Methods**

The analyzed samples of industrial waste waters came from processing of metallic surfaces (economic washing after chromation) and from lather process (solution exhausted in chromium, rechromation and basification). The residual water from metallic surfaces covering had pH values from acidic (pH 2.75, 5.01) to neutral (pH 6.82) and alkaline (pH 8.65).

In the experiments of metallic ions biosorption from industrial residual waters there were used neutrophilic heterotrophic bacteria, isolated from analyzed industrial water samples and acidophilic heterotrophic bacteria, isolated from mine waters and sediments collected from two different sites (Rosia Poieni – Alba dept. and Ilba – Maramures dept.).

The presence of the neutrophilic heterotrophic bacteria in industrial water samples was determined using a culture medium having the following composition: bullion-1000ml, agarose-20g with a pH of 7.6. The work technique consisted in getting colonies isolated on medium distributed in Petri plates and incubated at 28°C for 48 hours. The selection of
neutrophilic heterotrophic bacteria with raised capacity to adsorb of metallic ions was based on the ability of methyl violet sorption by 21 strains of these bacteria isolated from the industrial waste water.

In a view to obtaining populations of acidophilic heterotrophic bacteria were got using isolated colonies on agarized selective GYE culture media, having glucose as source of carbon. Isolated colonies obtained are colored pink and white (Cismasiu [4]). Among acidophilic heterotrophic bacteria were identified bacteria belonging to genus *Acidiphilium*. The bacteria identification was done based on their morpho-physiological characteristics: non-spore forming, Gram-negative rod-shaped, strict aerobe, mesophilic, chemoorganotrophic, extremely acidophilic, motile with flagella.

**The experiments of biosorption**

The isolated heterotrophic bacterial strains were firstly selected based on MICs (minimum inhibitory concentrations) to several heavy metals, such as Cu$^{2+}$, Zn$^{2+}$, Ni$^{2+}$ and Cr$^{6+}$, and further characterized by rate of biosorption of these ions in different culture conditions.

In the present study it was followed the influence of biological, chemical and physical factors, such as: type of biomass, heavy metals concentrations in the solution, pH, oxygen concentration in solutions, the contact period between biomass and industrial waste waters.

To select the aerobe heterotrophic bacteria with a high capacity of heavy metals removing from industrial waste water, the biosorption capacity of 8 heterotrophic bacterial strains was tested under the form of living biomass cultivated in specific medium prepared with industrial water and as immobilized biomass in contact with industrial water.

In order to raise the efficiency of the biosorption process it was used an immobilized biomass on 3 adsorbent materials (silicagel, zeolite and molecular sieve) to treat the industrial waste water. In the biosorption experiments were used populations of aerobic heterotrophic bacteria: (1) neutrophilic bacterial population, isolated from industrial waste waters; (2) acidophilic bacterial population, isolated from acidic mining effluents from Rosia Poieni (Alba dept.).

The working technique consisted in: (1) making the inoculums by growing the aerobe heterotrophic bacteria on selective liquid medium, mentioned above; (2) in order to immobilize the bacteria to the adsorbent material then passing the bacterial culture through adsorbent material column; (3) flooding the column with culture medium and keeping it for 24 hours at 28°C; (4) passing the industrial water through the adsorbing material column; (5) separation of the biomass from the industrial water through centrifugation at 12,000rpm for 15 minutes; (6) determination of the optical density of the heterotrophic bacterial culture at 660nm and passing through adsorbent material support; (7) determination the metal concentration at the end of the experiments.

The cell growth was measured by the spectrophotometer method and the concentration of heavy metals remaining in the industrial water was determined by atomic adsorption spectrometry method.

**Results and discussion**

The use of silicagel, zeolite and molecular sieve was proved efficient in the immobilization of acidophilic heterotrophic bacterial strains, isolated from mining effluents, by accentually decreasing of the optical density of cultures after passing through adsorbent support column. Thus, the bacterial strain with initial density of 0.469 after passing through silicagel had an optical density of 0.050. In case of zeolite the initial optical density of the bacterial strain decreased from 0.513 to 0.041 and on the molecular sieves from 0.501 to 0.061 (fig. 1).
Figure 1. The immobilization step of acidophilic heterotrophic bacterial strains using different adsorbent material (silicagel, zeolite, molecular sieve)

Figure 2 showed the results obtained in the experiments of metallic ions biosorption from industrial water using acidophilic heterotrophic strains immobilized on silicagel, zeolite and molecular sieve. The results proved that the best removal for Zn$^{2+}$ (35.83-54.71%) followed Cu$^{2+}$ (15.36-26.24%) and Ni$^{2+}$ (8-44%). The biosorption experiments from industrial water with pH 5.01 using acidophilic heterotrophic strains showed that these bacteria have a high tolerance to zinc (54.71%) in comparison to nickel (44%) and copper (16.8%) (fig. 2).

After successively passing the acidophilic heterotrophic bacterial populations through the three immobilization materials (silicagel, zeolite and molecular sieves), it was noticed that the culture density has lowered clearly. Thus, after three passing through the acidophilic heterotrophic bacterial population on zeolite the initial optical density of 0.761 got to 0.046. Also, a higher adsorption of the acidophilic heterotrophic population on the molecular sieve was noticed from 0.721 to 0.041 (fig. 3).
The adaptation of gram-negative bacteria to acidic environmental conditions with implication in heavy metals removal processes

Fig. 3. The immobilization step of acidophilic heterotrophic bacterial populations using different adsorbent material (silicagel, zeolite, molecular sieve)

The metallic ions biosorption from industrial waste water with pH 5.01 by acidophilic heterotrophic bacterial populations was realized in high and similar percentages for nickel and zinc (over 70%) in case of all adsorbent materials. Obvious differences were observed in the copper biosorption by acidophilic heterotrophic bacterial populations immobilized, the best percentages being obtained on silicagel. Thus, acidophilic heterotrophic populations removed these three metals in the following order: nickel (76-84%) > zinc (70-80%) > copper (61.6-68%) (fig. 4).

The experiments showed the fact that the percentages of heavy metals removed are not influenced by the initial pH value (pH 5.01), but mostly by the adsorbing capacity of the acidophilic heterotrophic bacterial population on the adsorbing material (silicagel, zeolite and molecular sieve) (fig. 3-4).

Fig. 4. Metallic ions removal process from industrial waste water (pH 5.01) by acidophilic heterotrophic bacterial populations immobilized on different adsorbent supports
Using silicagel and zeolite as adsorbent supports for the heavy metals biosorption permitted nickel (84%) and zinc (80%) removal at close values, only in the case of copper the biomass of acidophilic heterotrophic bacterial populations immobilized on silicagel removed high percentages of Cu^{2+} (68%) from industrial waste water with pH 5.01 (fig. 4).

Utilizing three adsorbent supports (silicagel, zeolite and molecular sieve) for immobilization of neutrophilic heterotrophic bacterial populations, isolated from industrial waste waters, permitted a good fixation of these bacteria in the case of molecular sieve. Thus, after the bacterial population passing through the molecular sieve, the initial optical density decreased from 0.711 to 0.067. In case of zeolite the initial optical density of the bacterial population lowered from 0.531 to 0.071 (fig. 5).

**Fig.5.** The immobilization step of neutrophilic heterotrophic bacterial populations using different adsorbent material (silicagel, zeolite, molecular sieve)

In the experiments of metallic ions biosorption from industrial waste water at pH 8.65 with a biomass of neutrophilic heterotrophic bacterial populations it was noticed that Zn^{2+} was extracted in the biggest percentages on all the adsorbing materials used, followed by Cu^{2+} and Ni^{2+}. Thus, Zn^{2+} was removed in 80% on molecular sieve, 70% on zeolite and 48% on silicagel. The highest removal percentages of Cu^{2+} were obtained on zeolite (68%) followed by molecular sieve (52%) and silicagel (36%) and Ni^{2+} was removed in 60% on silicagel (fig. 6).

**Fig.6.** Metallic ions removal process from industrial waste water (pH 8.65) by neutrophilic heterotrophic bacterial populations immobilized on different adsorbent supports
The adaptation of gram-negative bacteria to acidic environmental conditions with implication in heavy metals removal processes

In testing experiments of neutrophilic heterotrophic bacteria growing in the presence of hexavalent chrome, this group of bacteria presented an appreciable tolerance to this metal proved by high values of optical density at interval of 24 and 48 hours to cultures in a medium prepared with chrome waste waters with pH 6.82 (fig. 7).

After the experiments of chrome removal from industrial waste water using neutrophilic heterotrophic bacterial strains, it was found that there is a high correlation between the bacterial growth and their activity. Thus, the most active strains in the hexavalent chrome biosorption were T-31, which removed 20% of metallic ions and presented the best growing with optical density of 1.701 after 48 hour of cultivation (fig. 7-8).

The biosorption experiments had evidenced the high capacity of aerobe heterotrophic bacteria to reduction of Cr$^{6+}$. These had removed chromium in 74.04-99.95 percentages varying the pH value, the bacteria used in waste treatment, the contact period between cells and metallic ions (fig. 9-10).

**Fig. 7.** The growth of neutrophilic heterotrophic bacterial strains in medium prepared with chrome industrial waste water (pH 6.82)

**Fig. 8.** Hexavalent chrome removal from industrial waste water (pH 6.82) treated with neutrophilic heterotrophic bacterial strains
Conclusions

Purified heterotrophic bacterial strains were less resistant to the concentration of metallic ions used, this fact being evidenced through a weak metabolic activity appreciated through the lower oxidation/reduction level in the medium of these metals. The experiments of heavy metals biosorption from industrial water using acidophilic heterotrophic strains showed that these have a high tolerance to zinc (54.71%) in comparison to nickel (28%) and copper (16.8%). In the experiments of metallic ions biosorption by acidophilic bacterial populations was noticed an extraction of 68% Cu$^{2+}$, 80% Zn$^{2+}$ and 84% Ni$^{2+}$. Also, high percentages biosorption of several metallic ions were obtained using populations of acidophilic heterotrophic bacteria compared with purified strains, which confirms the adaptation of populations to higher concentrations of Cu$^{2+}$, Zn$^{2+}$ and Ni$^{2+}$. In the experiments of metallic ions biosorption with immobilized neutrophilic heterotrophic bacterial population from the three materials used for immobilization, the biggest percentages of metallic ions extracted were got with the molecular sieves (80% Zn$^{2+}$ and 60% Ni$^{2+}$) and zeolite (70% Zn$^{2+}$
The adaptation of gram-negative bacteria to acidic environmental conditions
with implication in heavy metals removal processes

and 68% Cu^{2+}). The experiments of Cr^{6+} extracted using acidophilic heterotrophic bacterial strains showed these have a high tolerance to chromium (86.78-99.95%). High percentages of heavy metals removed from industrial waste waters demonstrated the efficiency of microbiological processes for remediation of environments contaminated with these metals.

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References