THE EFFECTS OF ACIDITY, TEMPERATURES AND METALLIC IONS ON THE OXIDATIVE ACTIVITY OF THE ACIDOPHILIC HETEROTROPHIC BACTERIA, PRESENT IN MINING EFFLUENTS FROM ASECARE MINE

CIŞMAŞIU Carmen Mădălina

Abstract. The structural and physiological characterization of the species from acidophilic microbial communities may lead to the discovery of new species of acidophilic heterotrophic bacteria. These bacteria play an important role in bioremediation processes, due to their oxidative activity in environments polluted with metallic ions. The experiments prove the highest oxidative activity was found at 28°C and pH 2.5, meaning that these would be the best conditions in the natural environment for maximum activity of the genus *Acidiphilium*. The decrease of the pH to 2.0, as well as its increase to 3.5 determined the reduction of the oxidative activity to 25% from the values obtained at an optimum pH.

Keywords: *Acidiphilium* sp., mining effluent, metallic ions, biosorption.

Rezumat. Efectele acidităţii, temperaturilor şi ionilor metalici asupra activităţii oxidative a bacteriilor heterotrofe acidofile prezente în efluenţii minieri de la mina Asecare. Caracterizarea structurală şi fiziologică a speciilor din comunitatea microbiană acidofilă poate duce la descoperirea de noi specii de bacterii heterotrofe acidofile. Aceste bacterii joacă un rol important în procesele de bioremediere, datorită activităţii lor oxidative în medii poluate cu ioni metalici. Experimentele au arătat că cea mai bună activitate oxidativă s-a realizat la 28^0C şi pH 2,5, însemnând că acestea pot fi condiţii bune pentru activitatea maximă a genului *Acidiphilium* în mediile naturale. Scăderea pH-ului la 2,0, de asemenea creşterea la 3,5 determină reducerea activităţii oxidative cu 25% faţă de valorile obţinute la pH-ul optim.

Cuvinte cheie: *Acidiphilium* sp., efluenţi minieri, ioni metalici, biosorpţie.

INTRODUCTION

Acid mine drainage in a watershed can be a consequence of mining coal or mineral deposits. A significant amount of scientific research has been conducted to determine the chemical reactions that create acidity and lead to the precipitation of dissolved metals, but despite improvements in prediction and prevention methods, acid mine drainage problems persist.

After applying the classical procedure of ores manufacture and from the natural bacterial solubilization of the ores residual waters, there result metallic ions. The presence of metallic ions in the residual waters resulting from the ores processing stations in higher concentrations raises an important problem about depolluting the environment (BRUHN et al., 1999; DOPSON et al., 2003; KOZLOV & ZVEREVA, 2007).

The study of acidophilic microorganisms has gained increasing importance due to their adaptations to cope with extreme environments (BAKER & BANFIELD, 2003; JOHNSON, 1999) but also due to their biotechnological applications in bioremediation of polluted areas or metallic ions recovery from low-grade ores and wastewater mine drainage. The ability of these microorganisms to adapt to various environmental conditions is extremely useful also for their use in the biosorption of metals from acidic mining effluents (CARLSON, 1998; CIŞMAŞIU et al., 2004; RAKESH, 1990).

Obligate acidophilic heterotrophic bacteria were isolated from weakly acidic environments such as sewage and soil, as well as from acidic mine drainage. However, the characteristics of the bacteria from weakly acidic environments differed from the genus *Acidiphilium* in the pH growth range and sensitivity to organic substrates (JOHNSON, 2003; KISHIMOTO & TANO, 1987).

One of the effects is the increase in heavy metal solubility, which results in the accumulation of these toxic elements in the environment. Consequently, these sites become inhospitable and only those microorganisms able to tolerate the acidity and the high concentration of heavy metals can survive (CIŞMAŞIU, 2004; HIRAISHI & SHIMADA, 2001; RODRIGUEZ & DIAZ, 2009).

The preferences of acidophilic bacteria for the low pH level are the results of their adaptation to the environment under the influence of essential factors of their natural habitat. Therefore, a high acidity of medium indicates a high developmental and activity level for these microorganisms. Thus, the bioprospection of these natural selected organisms represents an important strategy in order to obtain agents for bioremediation processes (CIŞMAŞIU, 2001; HAWKSWORTH, 1992; LEDIN & PEDERSE, 1996).

The activity and selectivity of the acidophilic heterotrophic bacteria in the accumulation of chemical elements and the synthesis of organic compounds are determined by the physiological properties of the microorganisms, the characteristics of the elements and by a series of physical and chemical factors (pH, temperature, metal concentration, nutritional medium composition, the quantity and quality of the organic and inorganic substances (CIŞMAŞIU, 2008; ZARNEA, 1994).

The heterotrophic acidophilic bacteria of the *Acidiphilium* genus are the most numerous and have the greatest importance in the acid media. Taking into consideration the importance of knowing the optimum development

conditions of the heterotrophic acidophilic bacteria of the genus *Acidiphilium*, our research has tried to establish the effects of acidity in different experimental conditions (temperature, airing) on their growth and activity (CIŞMAŞIU, 2004; HARRISON, 1984; LOPEZ-ARCHILIA et al., 1995).

The activity of acidophilic heterotrophic bacteria is influenced by the acidity and temperature levels, although these microorganisms are adapted to grow in conditions of a low pH. Respecting the optimum values of the pH and temperature is necessary in laboratory experiments for testing the behavior of the acidophilic heterotrophic bacteria in the presence of raised concentrations of metal ions in the environment (CIŞMAŞIU et al., 2004; JOHNSON, 2003).

After isolating more strains from the acid effluents of the Ilba mining area (Maramureş, Romania), identified according to the morphological and physiological characteristics as being part of *Acidiphilium* genus (CIŞMAŞIU, 2008), the study focused on their oxidative activity.

Since *Acidiphilium* is both a mesophilic and acidophilic microorganism, the influence of temperature and pH on its enzymatic activity was investigated. This paper presents our results regarding the effects of acidity, temperatures, and metallic ions, in various experimental conditions on the oxidative activity of the acidophilic heterotrophic bacteria belonging to the genus *Acidiphilium*.

MATERIALS AND METHODS

Types of the analyzed microorganisms

In different perimeters from our country, mining activities cause serious problems of pollution to aquatic and terrestrial environment. In order to obtain a large game of acidophilic heterotrophic bacteria and with potential in biotechnological processes, there have been sampled waters (4) and sediments (4) mining from Ilba mining sites, like sources to obtain populations on selective medium.

For the identification of bacterial strains they were inoculated into GYE solid medium with glucose as source of carbon and pH=3.0 (KARAVAIKO, 1988). There were taken into consideration the following features: morphology of colonies, rate of growth, morphology and size of microscopically structures involved in growth, potential of growth into nutrient medium containing different concentration of metallic ions. The main criteria for the isolation of bacterial pure cultures were rate of growth into specific nutrient media.

The isolated strains were characterized by means of Gram staining, cellular morphology, biochemical features (oxidase and catalase activity, the production of pigments in selective media). The most representative structures and colonies were included in photographic database. We aimed at appreciating the influence of acidity on the development and activity of the populations of the *Acidiphilium* genus (Plate 1).

PLATE 1 / PLANŞA 1

Photo 1 Photo 2

Acidiphilium sp. (pink colony - photo 1, white colony - photo 2) isolated from mining effluents of Ilba mining area (Maramureş county)
Acidiphilium sp. (colonie roz - foto 1, colonie albă – foto 2) izolate din efluenți minieri de la mina Ilba (județul Maramureş)

Experimenting techniques

In the experiments of testing the action of acidity on the growth and activity of the heterotrophic acidophilic bacteria, the *Acidiphilium* populations were grown on media having the pH values of 2.0, 2.5, 3.0, 3.5 incubated at 28^0C and 37^0C in stationary and agitation conditions (150 rpm) for 20 days.

The bacterial cultures were made in Erlenmeyer flasks of 100 ml with 30 ml culture medium and 3 ml bacterial inoculums represented by 7 days old cultures.

The oxidative activity of these populations was studied through the determination of the pH value, as a result of producing organic acid, at 4-day intervals, as a reaction catalyzed by these bacteria.

The influence of Cu^{2+} and Zn^{2+} concentrations between 1,000-5,000 ppm on the acidophilic heterotrophic microorganisms was studied in conditions of continuous stirring (150 rpm) at optimum temperature (28^0C).

In the experiments of testing the bacterial cultures development in the presence of higher concentrations of metallic ions their growth was estimated through measuring the optical density (OD) to 660 nm (UV spectrophotometer), at 2-day intervals in a 12 days incubation period.

RESULTS AND DISCUSSION

In accordance with various essential parameters of their natural habitat (temperature, pH, soil humidity, O_2 and CO_2 concentration, Eh, metallic ions) the morphological and physiological changes of the indigenous acidophilic heterotrophic bacteria are produced. In the present study, there are rendered some results about the influence of acidity, temperatures, and metallic ions on the growth and activity of the heterotrophic acidophilic bacteria in an optimum growth medium.

The results referring to the influence of acidity (2.0, 2.5, 3.0, 3.5) and temperature (28^0C, 37^0C) on the growth and activity of *Acidiphilium* populations, cultivated on selective GYE medium, are represented in Figs. 1, 2.

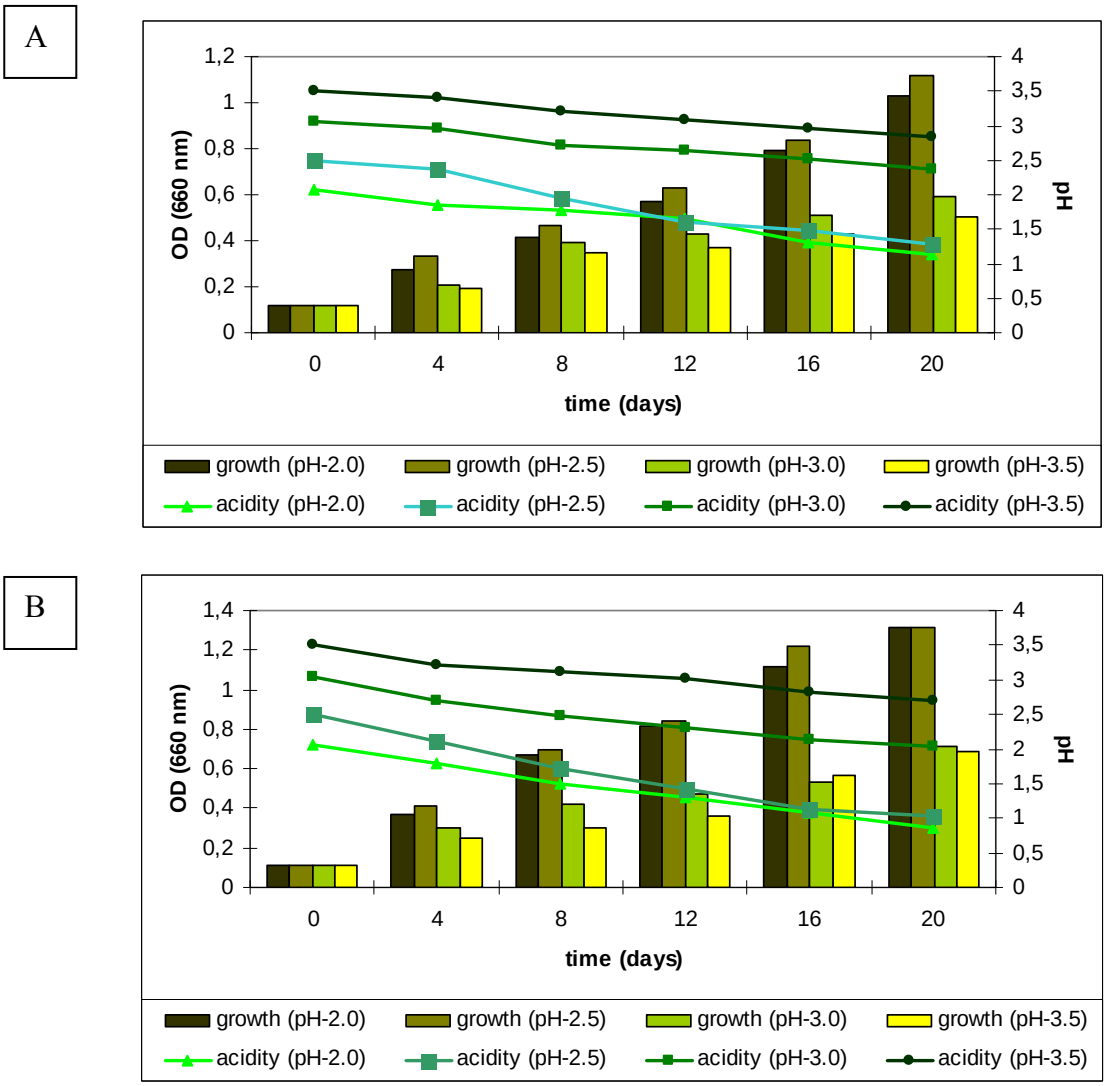
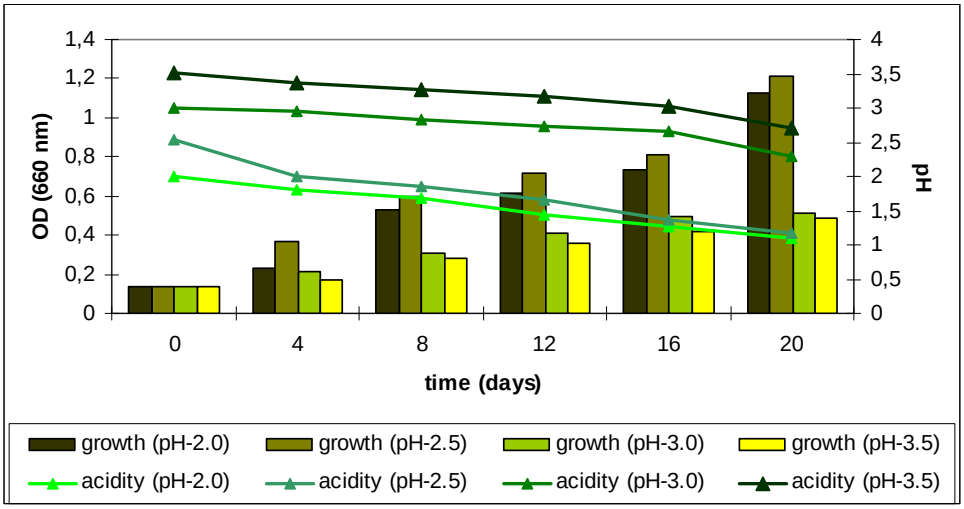


Figure 1. The dynamics of the growth and oxidative activity of acidophilic heterotrophic bacteria isolated from Ilba mining area at 28^0C (A - static conditions; B - stirring conditions).
Figura 1. Dinamica creşterii şi activităţii oxidative a bacteriilor heterotrofe acidofile izolate de la mina Ilba la 28^0C (A - condiţii statice; B - condiţii de agitare).

A



B

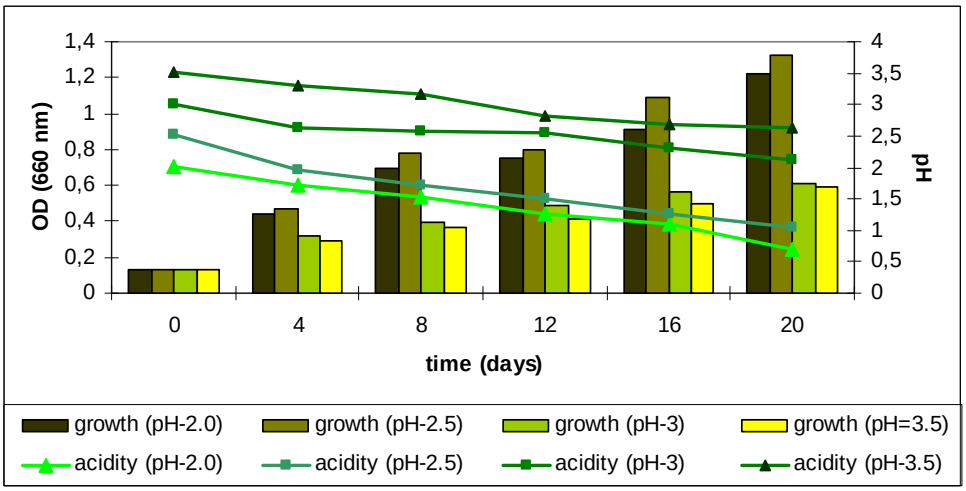


Figure 2. The dynamics of the growth and oxidative activity of acidophilic heterotrophic bacteria isolated from Ilba mining area at 37^0C (A - static conditions; B - stirring conditions).
Figura 2. Dinamica creşterii şi activităţii oxidative a bacteriilor heterotrofe acidofile izolate de la mina Ilba la 37^0C (A - condiţii statice; B - condiţii de agitare).

The influence of acidity on the growth and activity of *Acidiphilium* populations have indicated that the optimal pH is 2.5, though a variation of pH within the range 2–3 keeps these two parameters within 75-80% of the maximum values. We have also observed a directly correlation between the oxidation activity of the *Acidiphilium* population and the growth evolution of bacterial culture (Figs. 1, 2).

Increasing the temperature (37^0C) and the pH (3.5) of the solution furthermore negatively influences the growth and metabolic activity of acidophilic heterotrophic bacteria, in both static and stirring conditions. These results could help us to improve the growing conditions for the acidophilic heterotrophic bacteria belong to *Acidiphilium* genus.

The experiments comparing the effect of acidity on the acidophilic heterotrophic bacteria, isolated from Ilba mining area, offered strong evidence that the stirring conditions are more adequate for growing these bacterial strains in lab (Figs. 1, 2).

In case of testing the resistance of *Acidiphilium* populations at higher copper concentrations between 1.000-5.000 ppm, it was found that raising the copper concentration in the culture medium determined the slowing growth of bacterial culture through extending the lag period to 4 - 6 days, depending on the copper sulfate concentration (Fig. 3).

The comparative analysis of the results obtained in this study, illustrated in figure 3 demonstrated that the population P_7 are less sensitive to the concentrations tested, compared to the strain I_4, on the whole incubation period. So, after 12 incubation days at a concentration of 3.000 ppm Cu^{2+}, the population P_7 had an optical density of 1.141, compared to the strain I_4 for which the optical density had only values of 0.717.

Comparing the influence of the higher copper concentrations on the growth of the populations of *Acidiphilium* sp., isolated from Ilba mining area, it was noticed that the raise of the copper concentration in culture medium determines the slowing down of the bacterial cultures growth depending on the copper sulfate concentration in the

medium. It was also established that the strain I_4 is the most sensitive at concentrations of 3.000-5.000 ppm Cu^{2+}, compared to the populations tested in the same experimental conditions (Fig. 3).

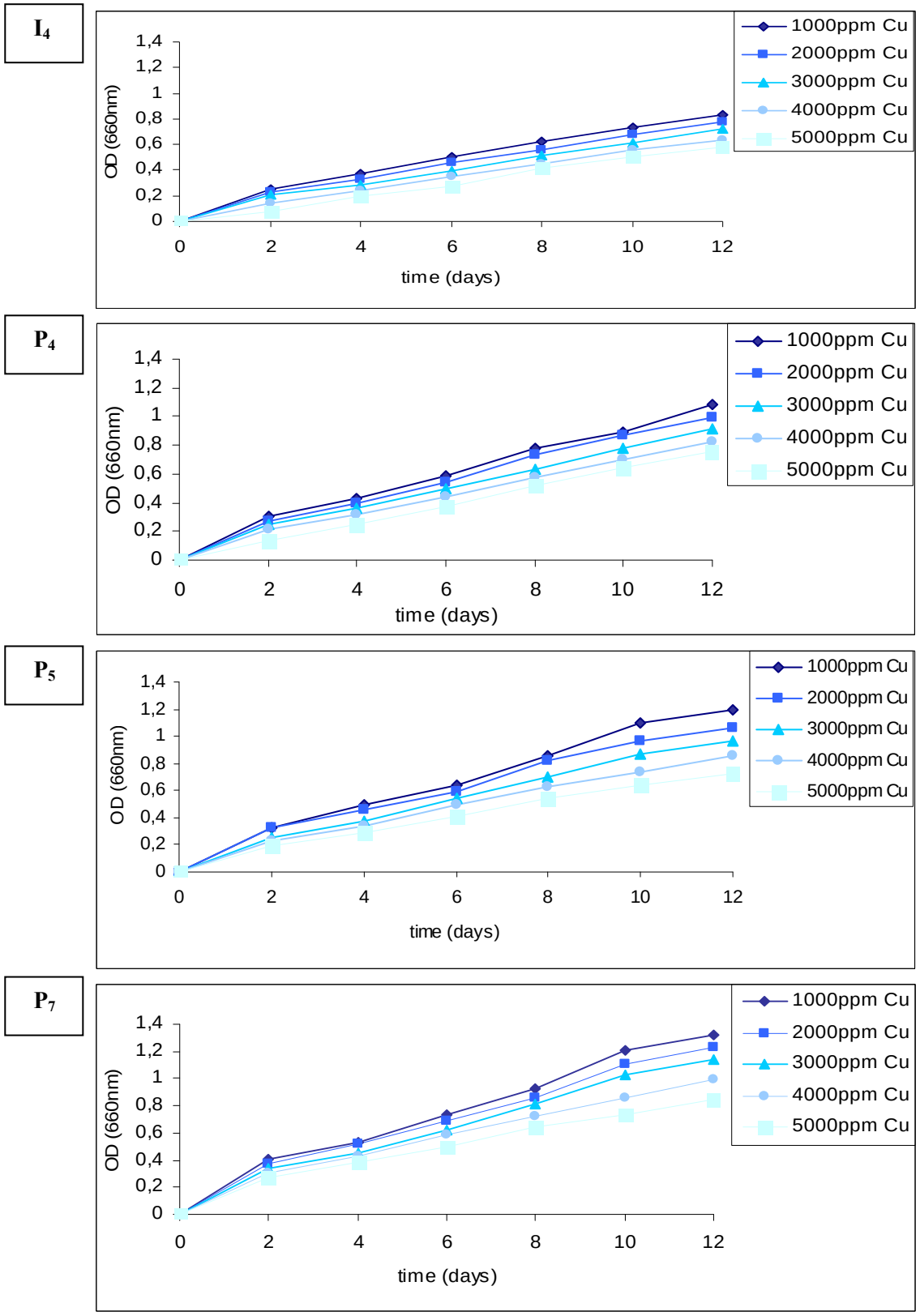


Figure 3. Testing the tolerance of *Acidiphilium* populations under increased copper concentrations.
Figura 3. Testarea toleranței populațiilor de *Acidiphilium* în prezența concentrațiilor crescute de cupru.

In the case of testing the resistance of the *Acidiphilium* population at concentrations of 1.000-5.000 ppm Zn^{2+} it was noticed that the higher zinc concentration in the culture medium determined the diminution of the growth of the

bacterial cultures by extending the lag period to 3-5 days, depending on the zinc sulfate concentration. So, in case of P_1, it was established that the cultures grown in the presence of a concentration 3.000 ppm Zn^{2+} have an optical density of 1.236, and those grown in the presence of 5.000 ppm Zn^{2+} concentration, the optical density reached only values of 0.901 (Fig. 4).

The research demonstrated the higher resistance of the *Acidiphilium* sp. populations to higher concentrations of zinc by showing a more intense growth to concentrations between 1.000-3.000 ppm Zn^{2+}. Raising the concentration of metallic ions over 3.000 ppm Zn^{2+} has a negative influence on the growth of the acidophilic heterotrophic bacteria from *Acidiphilium* genus (Fig. 4).

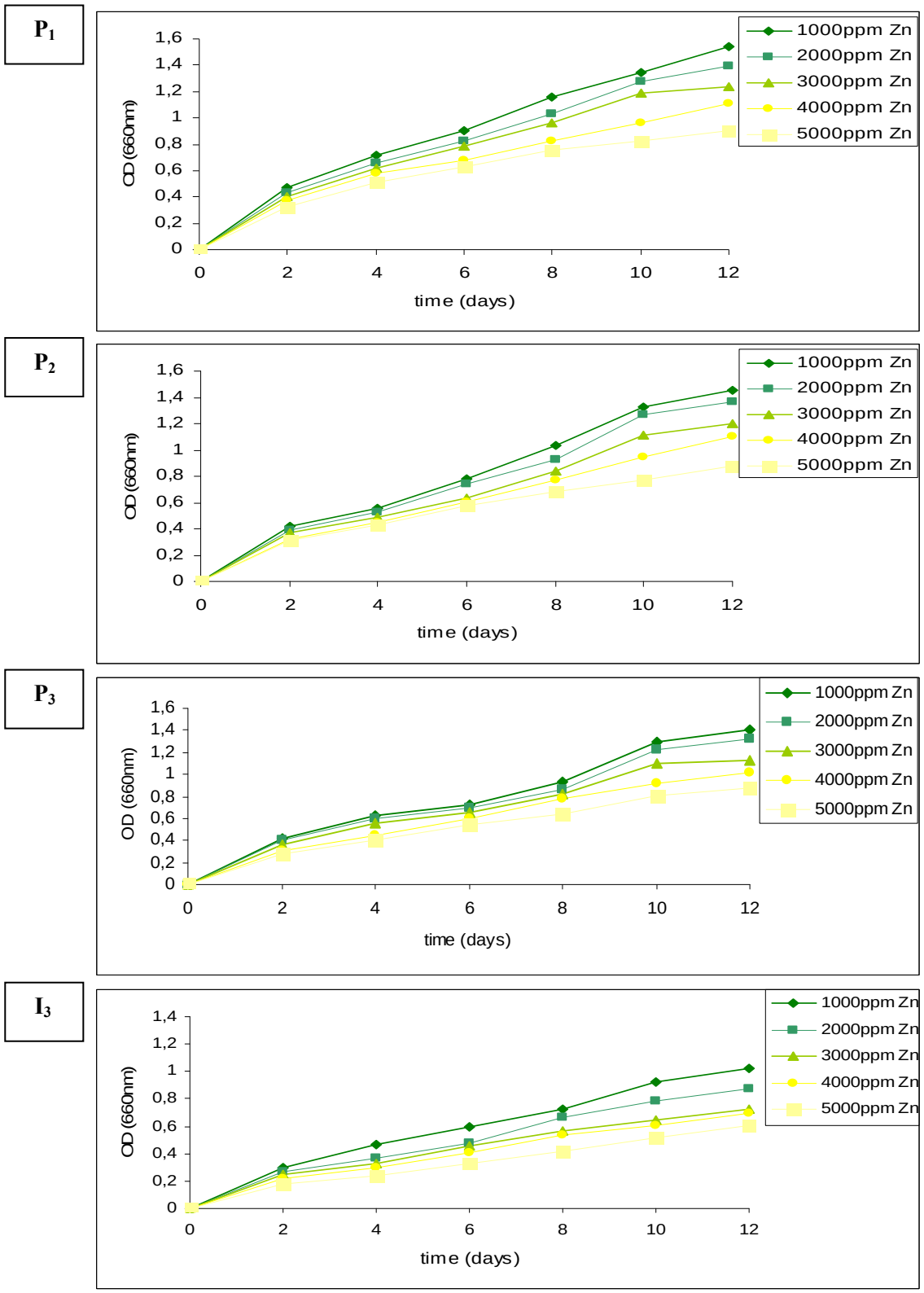


Figure 4. Testing the tolerance of *Acidiphilium* populations under increased zinc concentrations.
Figura 4. Testarea toleranței populațiilor de *Acidiphilium* în prezența concentrațiilor crescute de zinc.

When comparing the effects of the concentrations 3.000-5.000 ppm Zn^{2+} on the populations of *Acidiphilium* sp., isolated from Ilba mining area, it resulted that P_1 is the most tolerant to the tested zinc concentrations comparatively to the other populations (P_2 and P_3) in an interval of 8 days.

The highest sensitivity to the presence of zinc in culture medium was determined by the strain I_3, whose growth was strongly inhibited by all tested zinc concentrations. So, after 12 incubation days at a concentration of 5,000 ppm, the optical density reached values of only 0.607, compared to P_1, which at the same metal concentrations had the optical density 0.901 (Fig. 4).

The results of this study showed that heavy metal resistance among bacteria is widespread. The populations isolated from mining effluents of Ilba mining area, showed an extreme tolerance (up to 3.000 ppm) to the tested metals, which is in agreement with the results of other studies (CIŞMAŞIU, 2004; RAKESH, 1990).

The results of the comparative research on the tolerance to high concentration of cooper and zinc of the bacterial populations and strains isolated from mining sites of Ilba confirm the data from the specialty literature about the high abilities of the population to adapt to extreme medium conditions compared to the bacterial strains (DOPSON et al., 2003).

The results regarding the influence of the metallic ions on the growth of the acidophilic heterotrophic bacteria from *Acidiphilium* sp. permitted the selection of some bacterial strains and populations, with a higher resistance to the presence of these ions in the medium regarding the improvement of the biosorption processes (Figs. 3,4).

The acidophilic heterotrophic bacteria belonging to *Acidiphilium* genus, which proved to be efficient at high Cu^{2+} and Zn^{2+} levels, are of interest for biotechnological utilization for the biosorption of metallic ions. The high incidence of heavy metal resistance detected in this work indicates the potential of these microorganisms as bioremediation agents.

CONCLUSIONS

The comparative studies regarding the influence of temperature on the activity *Acidiphilium* populations revealed that their metabolic activity is developed with a maximum intensity at 28^0C.

Our results show that a continuous exposure to higher temperatures, about 37^0C, may induce a certain degree of adaptation of bacterial populations to the thermal conditions.

The level of growth and metabolic activity of acidophilic heterotrophic bacteria is higher at a pH - 2.5, though a variation of the pH within the range 2.0-3.5 affects bacterial growth with less than 25%.

Comparing the resistance of the *Acidiphilium* sp., isolated from Ilba mining area, at concentrations of 3.000-5.000 ppm Cu^{2+} in the medium, it is noticed that P_7 is the most tolerant one to the concentrations tested.

The comparative research regarding the behavior of the *Acidiphilium* populations at higher concentrations of Zn^{2+} showed the fact that P_1 are the most tolerant to the concentrations of 1.000-3.000 ppm.

The experimental results permitted the selection of microbial cultures, which can offer a higher efficiency to the biosorption processes, having a maximum oxidizing activity even in the presence of some high metal concentrations.

REFERENCES

BAKER B. J. & BANFIELD J. F. 2003. *Microbial communities in acid mine drainage*. FEMS Microbiology Ecology. **44**: 139-152.

BRUHN D. F., THOMPSON D. N., NOAH K. S. 1999. *Microbial ecology assessment of a mixed copper oxide/sulfide dump leach operation*. In: Biohydrometallurgy and the Environment Toward the Mining of the 21st Century, Part B – Molecular Biology, Biosorption, Bioremediation, R. Amils and A. Ballester eds.: 559-567.

CARLSON C. 1998. *Influences of Sorption on Microbial Degradation of Organic Substances*. Introductory paper. University of Lund, Sweden. URL: http://www.ecotox.lu.se/STAFF/CK/Introduc.htm [accessed October 2001].

CIŞMAŞIU CARMEN. 2001. *The influence of acidity on the growth and activity of acidophilic heterotrophic bacteria, isolated from mining effluents*. Revue Romaine of Biology. Biol. Végét. Bucharest. **46**(1): 19-27.

CIŞMAŞIU CARMEN MĂDĂLINA. 2004. *The study of acidophilic microbiota from industrial effluents with acid pH (2.0-4.0) and high concentrations of metallic ions*. PhD Thesis, Institute of Biology Romanian Academy: 330 pp.

CIŞMAŞIU CARMEN MĂDĂLINA, BORDEIANU MARIA, STANCU RODICA. 2004. *Removing the metallic ions from the industrial residual waters using the aerobe heterotrophic bacterial cultures*. Proceedings of the Institute of Biology, Romanian Academy. Annual Scientific Session. Bucharest. **6**: 199-211.

CIŞMAŞIU CARMEN. 2008. *The acidophilic microorganisms diversity present in habitats with acidic extreme conditions, with implication in the environmental bioremediation of high concentrations of metallic ions*. Argesis. Studii şi Comunicări. Seria Ştiinţele Naturii. Muzeul Judeţean Argeş. Piteşti. **15**: 41-52.

DOPSON M., AUSTIN C. B., KOPPINEEDI P. R., BOND P. L. 2003. *Growth in sulfidic mineral environments. Metal resistance mechanisms in acidophilic micro-organisms*. Microbiology. **149**: 1959-1970.

HARRISON A. P. 1984. *The acidophilic thiobacilli and other acidophilic bacteria that their habitat*. Ann. Rev. Microbial. **38**: 265-292.

HAWKSWORTH D. L. 1992. *Biodiversity and Global Change*. Solbrig, O.T., Van Emdem, H.M., Van Oordt eds., Paris. Int. Union of Biological Sciences: 88-93.

HIRAISHI A. & SHIMADA K. 2001. *Aerobic anoxygenic photosynthetic bacteria with zinc-bacteriochlorophyll*. J. Gen. Appl. Microbiol. **47**: 161-180.

JOHNSON D. B. 1999. *Importance of microbial ecology in the development of new mineral technologies*. In: Biohydrometallurgy and the Environment Toward the Mining of the 21st Century - Part B, Proceedings of the International Biohydrometallurgy Symposium IBS'99, edited by R. Amils and A. Ballester. Madrid. Spain: 645-656.

JOHNSON D. B. 2003. *Chemical and microbiological characteristics of mineral spoils and drainage waters at abandoned coal and metal mines*. Water Air Soil Pollut: Focus. **3**: 47-66.

KARAVAIKO G. I. 1988. *Methods of isolation, evaluation and studying of microorganisms*. In*:* Biotechnology of Metals Manual Center of Int. Projects GKNT, Moscow, G. I. Karaivaiko, G. Rossi, D. A. Agate, N. S. Groudev and A. I. Avakyan eds.: 47-86.

KISHIMOTO N. & TANO T. 1987. *Acidophilic heterotrophic bacteria isolated from acidic mine drainage, sewage and soils*. J. Gen. Appl. Microbiol. **33**: 11-25.

KOZLOV M. V. & ZVEREVA E. L. 2007. *Industrial barrens: extreme habitats created by non-ferrous metallurgy*. Rev. in Environmental Science and Biotechnology. **6**: 231-259.

LEDIN M. & PEDERSE N. K. 1996. *The environmental impact of mine wastes – roles of microorganisms and their significance in treatment of mine wastes*. Earth-Sci. Rev. **41**: 67-108.

LOPEZ-ARCHILIA A. I., MARIN I., AMILS R. 1995. *Microbial ecology of an acidic river: Biotechnological applications*. In: Biohydrometallurgical processing, Biochemistry, Genetics and Molecular Ecology of Bioleaching Microorganisms, Biosorption, Bioaccumulation and Treatment of Coal, Oil and Effluents, C. A. Jerez, T., Vargas, H., Toledo and J.V. Wiertz eds. Univ of Chile: 132-145.

RAKESH K. J. 1990. *Copper-resistent microorganisms and their role in the environment*. World Journal of Microbiology and Biotechnology. **6**: 356-365.

RODRIGUEZ R. & DIAZ M. 2009. *Analysis of the utilization of mine galleries as geothermal heat exchangers by means of a semi-empirical prediction method*. Renewable energy. **34**: 1716-1725.

ZARNEA G. 1994. *Influenţa condiţiilor de mediu. Microorganisme extremofile*. In: Tratat de microbiologie general. Edit. Academiei Române Bucureşti. **5**: 169-178.

Cişmaşiu Carmen Mădălina
Institute of Biology of Romanian Academy of Sciences
Spl. Independentei no.296, sect.6, 060031, Bucharest
E-mail: madalinabio@yahoo.com, carmen.cismasiu@ibiol.ro.

Received: February 12, 2010
Accepted: July 14, 2010