THE TAXONOMIC AND PHYSIOLOGIC DIVERSITY OF THE ACIDOPHILIC CHEMOLITHOTROPHIC BACTERIA OF THE GENUS *THIOBACILLUS* USED IN ORES SOLUBILIZATION PROCESSES

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Abstract. The development of biotechnological processes, based mainly on the activity of the acidophilic chemolithotrophic, proved their efficiency in recovering metals from sulphides ores and mining drains and in bioremediation of the polluted environment with residual inorganic substances, like the heavy metals ions and their compounds.

Due to the influence of the physical-chemical factors on the development and the metabolic activity of the microorganism's present in the industrial effluents, the study of these parameters was imposed for raising the efficiency of the processes of adsorption and biosolubilization of the metallic ions.

A special importance for using bacteria of the genus *Acidithiobacillus* in the biosolubilization processes of heavy metals from acid mine tailings is represented by the resistance of these bacteria to high concentrations of metal ions.

The experiments prove a strong relationship between the acidity of the medium and the behaviour of the acidophilic chemolithotrophic bacteria. The comparative analyses regarding the influence of metallic ions (Cu^{2+}, Zn^{2+} and Fe^{2+}) on the physiologic diversity of the *Acidithiobacillus* populations, isolated from the mining sites, demonstrated the higher resistance of these bacteria to higher concentrations of metallic ions.

Key words: chemolithotrophic bacteria, metallic ions, resistance, *Acidithiobacillus* genus, biosolubilization, ores.

1. INTRODUCTION

Bioremediation of environments polluted with metallic ions has gained importance, with the development of the mining industry, as an ecological process as compared to conventional methods. The adaptative ability of acidophilic chemolithotrophic bacteria to various environmental conditions is very important for their use in the solubilization of metals from coal, sulphides ores and acidic mining effluents. This feature offers new perspectives for their use in biotechnological processes (HARRISON, 1984; JOHNSON, 1999)

Bacterial leaching of metals sulphides has developed rapidly in the course of the last decade. The recovery of heavy metals by applying microorganisms is now an established biotechnological technique. The mobilization of metal cations from insoluble ores by biological oxidation and complexation processes is referred to as bioleaching (LAZAR *et al.,* 1993; KARAVIKO *et al*., 1994; BOSECKER, 1999).

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Bioleaching is an economical method for the recovery of metals from minerals, especially from low grade ores. The acidophilic bacteria involved in the biooxidation of minerals cause natural leaching of sulphide minerals direct or indirect mechanism. The unique properties of acidophilic chemolithotrophic bacteria are their metabolic activity in highly acidic environments and their heavy metal resistance (DOPSON *et al.,* 2003; SUZUKI, 2001; OKIBE *et al*., 2004).

Low metal concentrations are essential elements for the metabolic processes, while the high concentrations are toxic. Acidophilic bacteria frequently adapt to the concentrations of toxic compounds such as metallic ions, developing protective mechanisms with role of protecting those bacteria in an unfriendly environment (RAKESH, 1990; SCHRENK *et al*., 1988).

The activity of the acidophilic bacteria at ecosystem level is very complex: a) in the trophic chains and nets; b) in limitating the development of some organism's populations; c) in forming and preserving the soil structure, essential for water, air and nutrients circulation; d) bioindicators for the "health" of the ecosystems (ZARNEA G., 1994; MARIEKIE *et al*., 2003).

The structural and physiological characterization of the species from microbial communities may lead to the discovery of new species of acidophilic bacteria, which could play an important role in bioremediation processes, due to their metabolic activity in environments polluted with metallic ions (HIRAISHI *et al*., 1998, RAWLINGS *at al.,* 1995; CISMASIU *et al*., 2002).

The acidophilic chemolithotrophic bacteria are present as populations, both in natural media and in those created by man. In many cases, their presence is evidenced through their metabolically products or the biomass accumulation (HAWSKSWORTH, 1992).

The economic implications of ecological effects are identified to the extent they can be determined within acceptable limits. A variety of ecological processes are affected and altered by air pollution. Such processes include community succession and retrogression, nutrient biogeochemical cycling, photosynthetic activity, primary and secondary productivity, species diversity and community stability (BAKER *et al*., 2003; NICOMRAT *et al.,* 2008).

The natural habitats of acidophilic bacteria are acidic mineral environments. Many of the acidophilic oxidize sulphur or reduced inorganic sulphur compounds to sulphuric acid which acidifies volcanic solfataras, effluents of sulphides ores or coal mines. The predominant bacteria in such environments are *Acidithiobacillus ferrooxidans* and *Acidithiobacillus thiooxidans*. Both species are obligate chemolithoautotrophs and play a crucial role in leaching of heavy metals from sulphides ores (GROUDEV *et al.,* 1993; NORRIS *et al.,* 1998; GOMEZ *et al.,* 1999; ROHWERDER *et al.,* 2003).

The increasing pollution of the environment raised the interest for the resistance of the acidophilic bacteria to metals and there is the potential recovery, but also in detoxifying the media polluted by heavy metals. They have a major contribution to the circuit of biogenic elements in the nature and prevent the accumulation of residual materials of different environmental contaminants (JOHNSON, 1998; JOHNSON *et al*., 1998; RAWLINGS, 2005).

The activity of acidophilic chemolithotrophic bacteria is influenced by the acidity and temperature levels, although these microorganisms are adapted to grow in conditions of low pH (CISMASIU *et al*., 2000). This paper represents a contribution to the knowledge of the taxonomic and physiologic diversity of the acidophilic chemolithotrophic for the improvement of the biosolubilization of metallic ions processes.

2. MATERIAL AND METHODS

2.1. PHYSIOLOGICAL GROUPS OF MICROORGANISMS OF INTEREST FOR METALLIC IONS SOLUBILIZATION

In different perimeters from our country, mining activities causes serious problem of pollution to aquatic and terrestrial environment. In order to obtain a large game of acidophilic chemolithotrophic bacteria, with potential in biosolubilization processes, have been recolted samples of mining effluents from Ilba mining areas, Asecare mine, like sources to obtain populations on selective medium.

In order to obtain the strains and populations of *Acidithiobacillus ferrooxidans* from these samples were used selective liquid culture medium 9K (mineral medium with a pH of 2.5), in which the energetic substratum was represented by the ferrous sulphate in the optimum concentration of 43.22g/l. To isolate strains it was used the 9K solid medium (KARAVAIKO, 1988).

The growing of the sulphur-oxidizing bacteria, *Acidithiobacillus thiooxidans*, in liquid Waksman medium (pH=4.0) is revealed after 21 days by the lowering under 2.0 of the initial pH value.

In a view to obtaining populations of acidophilic chemolithotrophic bacteria were got using isolated colonies on agarized selective culture media, following in dynamics the physiological activity in inorganic media specific. Isolated colonies obtained are coloured brownish red. Using these technique 10 populations of *Acidithiobacillus ferrooxidans* were isolated.

2.2. EXPERIMENTAL CONDITIONS AND PARAMETERS UNDER INVESTIGATION

The bacterial cultures have been incubated, for 21 days, in various experimental conditions:

– temperature: 28°C and 37°C;

- acidity: pH – 2.0, 2.5, 3.0, 3.5;
- static and stirring (150 rpm) conditions.

The bacterial cultures have been grown in 100ml Erlenmeyer glasses containing 30 ml growth medium and 3 ml inoculum (bacterial culture 7 days old).

In investigating the influence of acidity on the metabolically activities of the analysed bacteria the following aspects were pursued: (1) the concentration of the Fe^{2+} biologically oxidated; (2) the concentration of Fe^{3+} resulted after the biological oxidation; (3) the acidity (assessing the pH value of the culture liquids).

The growth of *Acidithiobacillus thiooxidans* cultures has been quantified by measuring the optical density (OD) at the wavelength of 660 nm (spectrophotometer UV), while the activity has been appreciated through the decrease of the pH (pH meter type PHM 26) of the culture solutions at every 4 days.

The populations of acidophilic chemolithotrophic bacteria were analyzed regarding their resistance to different concentrations of metallic ions, having in view the growth of the bacterial cultures in the specific culture media.

To evaluate the tolerance of *Acidithiobacillus ferrooxidans* isolated to high concentrations of Fe^{2+} in the medium (8–16g/l) were used the 10 strains and 10 populations selected on the basis of high capacity of oxidation of 8g/l Fe^{2+} in the medium after 8 days of incubation at 28°C. From them, there were selected 2 strains and 2 populations which oxidated the biggest amount of Fe^{2+} in 8 days.

The influence of Cu^{2+} and Zn^{2+} concentrations between 1000–5000ppm on the acidophilic chemolitotrophic bacteria was studied in conditions of continuous stirring (150rpm) at optimum temperature (28°C). In Erlenmeyer flask (100ml) were introduced 30 ml selective medium, which contains the molar solutions of Cu^{2+} and Zn^{2+}, 3 ml bacterial inoculum (7 days culture).

In the experiments of testing the bacterial cultures development in the presence of higher concentrations of metallic ions their growth was intended through measuring the optical density (OD) to 660nm (spectrophotometer UV), at 2 days intervals in 14 days.

The flacon test was inoculated with bacteria incubated at 28°C in agitation conditions (150 rpm) for 14 days. The concentration of ferrous iron biooxidized was determined at a 2 days interval volumetrically, by titration with a solution of potassium dichromate in the presence of sulphur diphenylamine as indicator.

3. RESULTS AND DISCUSSION

The experiments have indicated that the influence of acidity on bacterial growth and activity at 28°C and 37°C is stronger for the population isolated from mining waters than for the population isolated from sediments, both in static and dynamic conditions. We have also found that the optimal pH for the bacterial growth and activity is 2.5, though a variation of pH within the range 2–3.5 keeps these two parameters within 60–70% of the maximum values (Figs. 1–2).

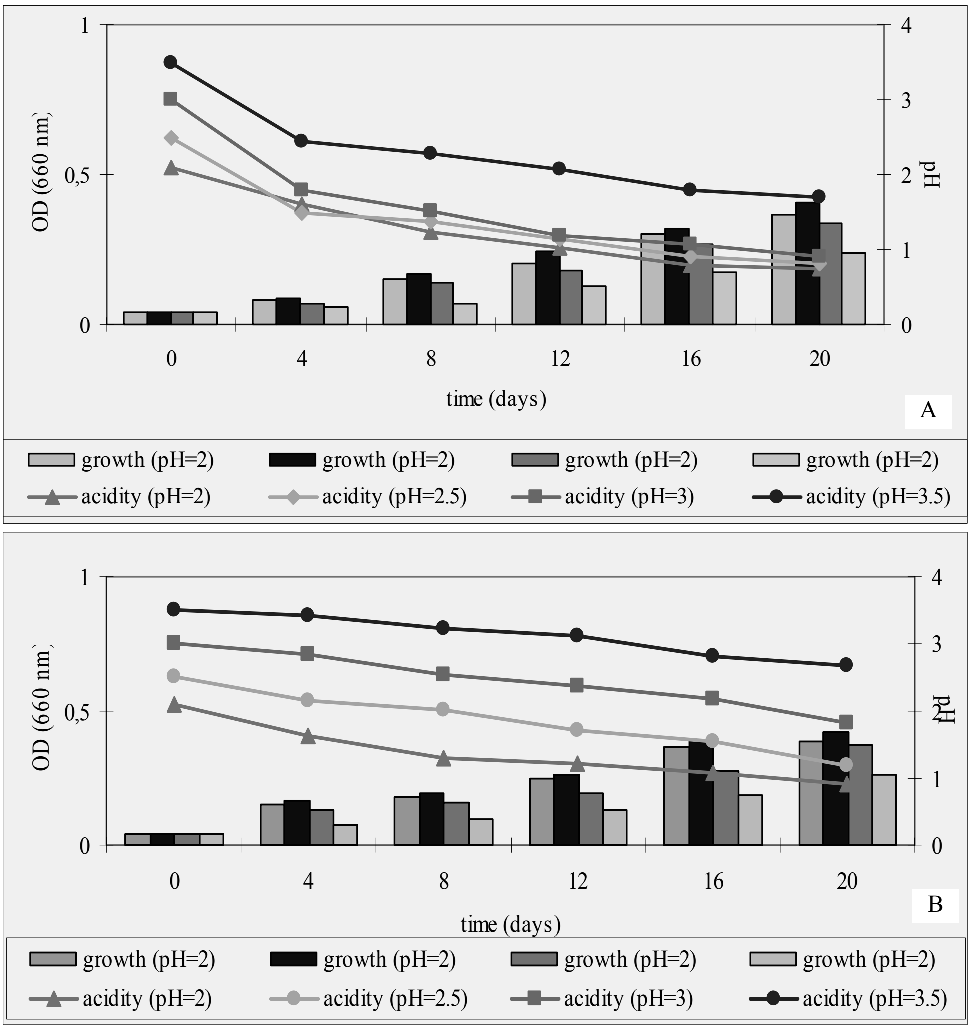

Fig. 1 – The dynamics of the growth and metabolic activity of the *Acidithioobacillus thioxidans* population isolated from Ilba at 28°C (A – static conditions; B – stirring conditions).

Comparative analysis indicates that these populations of acidophilic sulphur-oxidizing bacteria have similar optimal conditions for growth and activity, but *Acidithiobacillus thiooxidans* isolated from mining waters is more efficient in sulphur oxidation evidenced by stronger decreasing the final pH value of culture medium than *Acidithiobacillus thiooxidans* isolated from sediments. We have also observed a direct correlation between the sulphur oxidation activity of the *Acidithiobacillus thiooxidans* and growth evolution of bacterial culture.

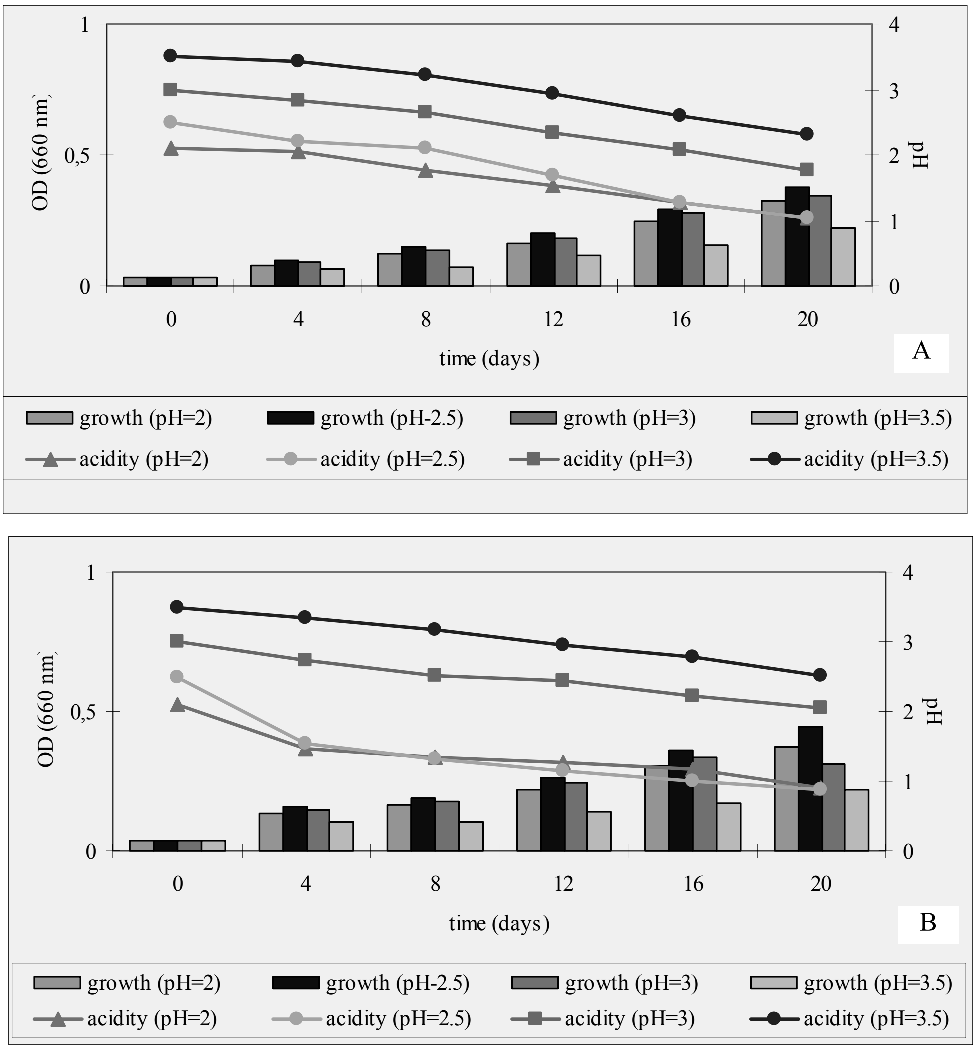

Fig. 2 – The dynamics of the growth and metabolic activity of the *Acidithioobacillus thiooxidans* population isolated from Ilba at 37^0C (A – static conditions; B – stirring conditions).

The study of physico-chemical factors evidenced that the 28°C temperature, the 9K medium with pH 2.5 and the agitation of cultures represent the optimum conditions for the oxidative activity of the population of *Acidithiobacillus ferrooxidans*.

The comparative studies, on populations of sulphur-oxidizing bacteria of *Acidithiobacillus thiooxidans*, show that the populations isolated from mining waters has a better growth than the populations isolated from sediments in similar conditions. At the temperature of 37°C there is a smaller difference between the growth levels of the populations.

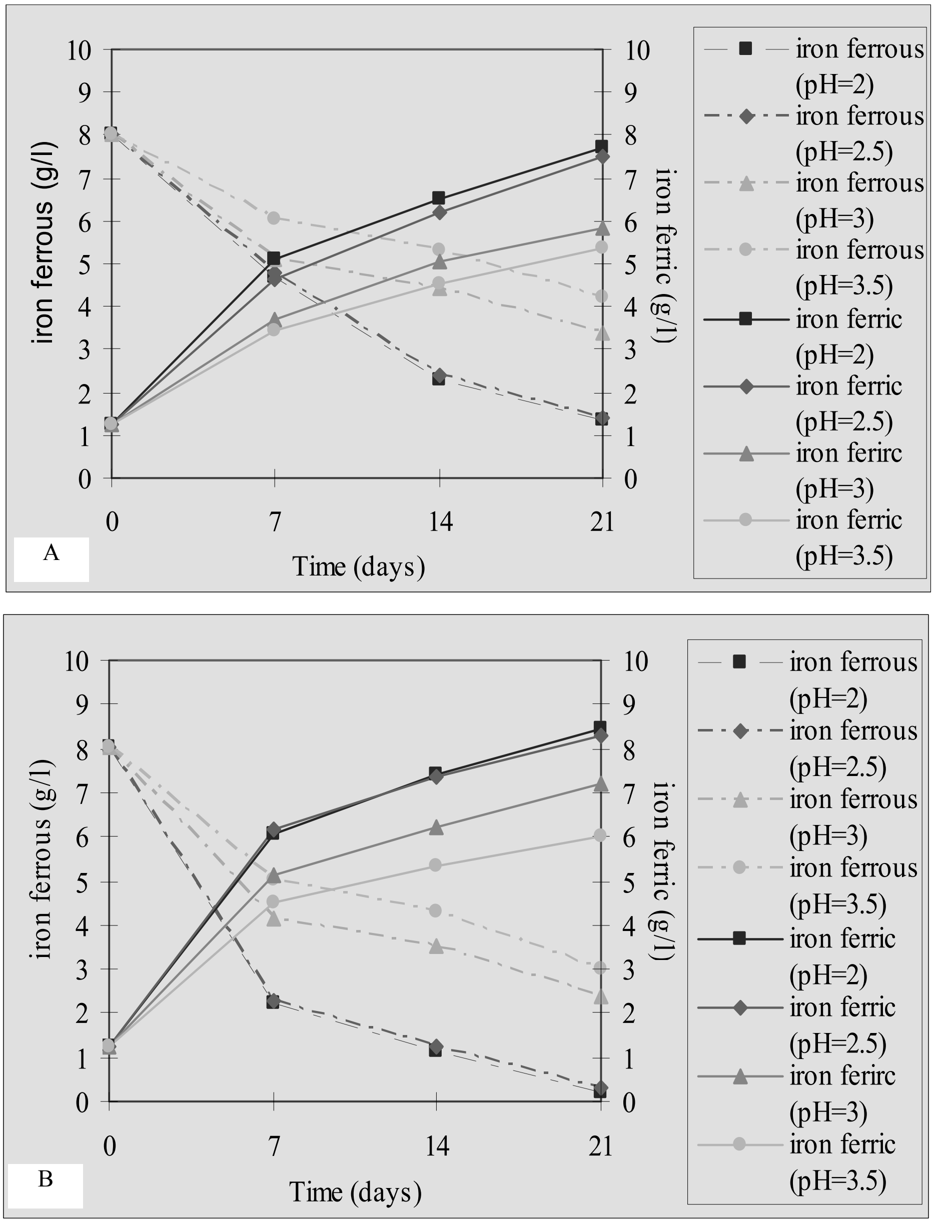

Fig. 3 – The influence of acidity on the dynamics of oxidating the Fe^{2+} by the *Acidithiobacillus ferrooxidans* population isolated at Ilba at 28°C (A-static conditions, B-agitated conditions).

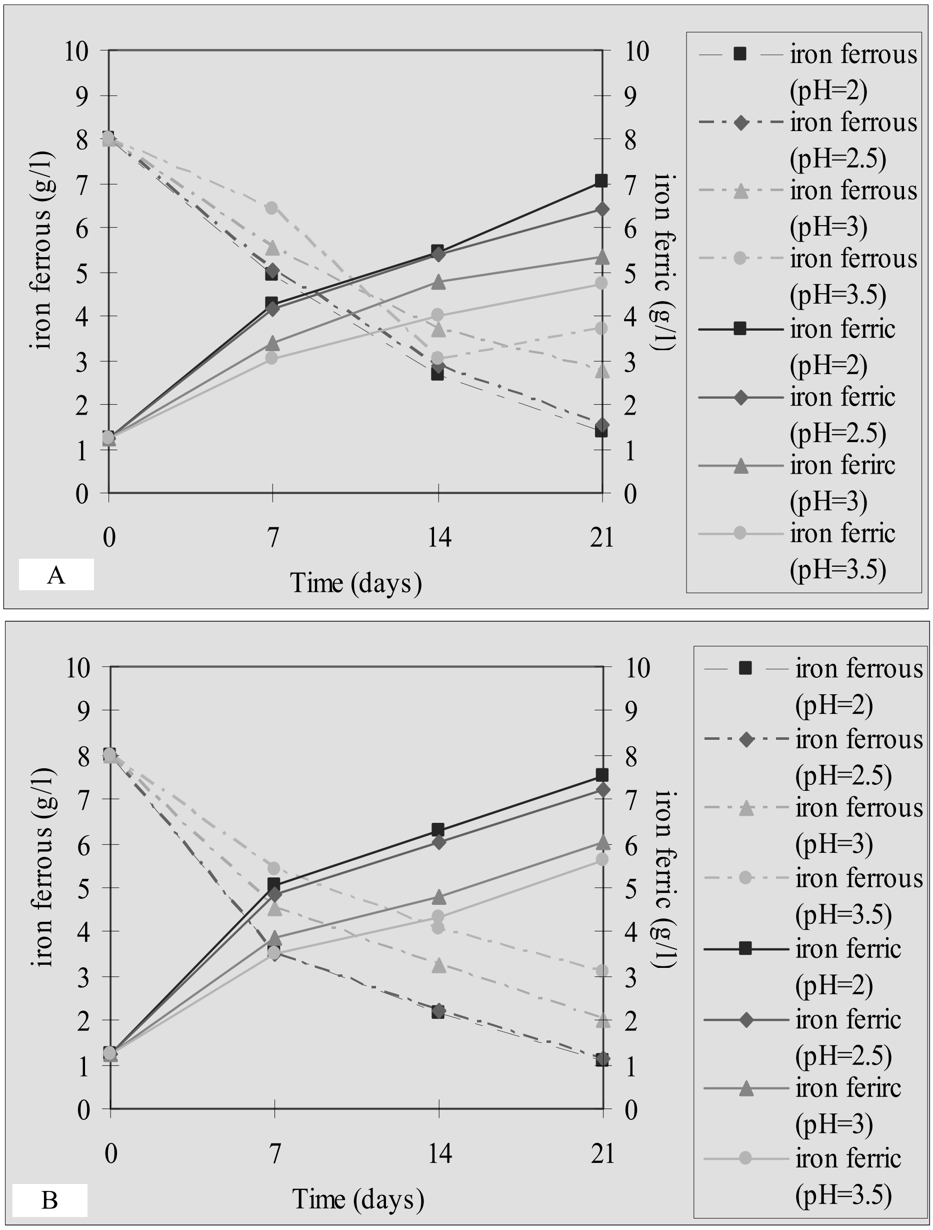

Fig. 4 – The influence of acidity on the dynamics of oxidating the Fe^{2+} by the *Acidithiobacillus ferrooxidans* population isolated at Ilba at 37°C (A-static conditions, B-agitated conditions).

The results obtained showed that these microorganisms are efficient in the oxidation of Fe^{2+} at 28°C and the superior limit of the biological oxidation of Fe^{2+} is situated around the value of 37°C.

The experimental results comparing the effect of temperature on acidophilic chemolitotrophic bacteria of the type *Acidithiobacillus thiooxidans* and *Acidithiobacillus ferrooxidans* have shown that under conditions of continuous stirring bacterial growth and metabolic activity run at higher rates than under static conditions.

The comparative studies on the influence of temperature on the physiological diversity of acidophilic chemolithotrophic bacteria showed that their peak development and metabolic activity occurs at 28°C.

The experimental results indicate a higher activity level of bacterial populations, correlated with a higher growth level, in stirring conditions as compared to static conditions. On the other hand, the *Acidithiobacillus ferrooxidans* population is more sensitive to variations of pH level, because the bacterial growth has a stronger decrease when the pH varies from 2.5 and 3.5 (Figs. 1–4).

After the experiments of testing the influence of different pH values (2–3.5) on the growth and metabolically activity of the bacterial population of *Acidithiobacillus ferrooxidans*, it was noticed that they present a growth and maximum activity of the cultures at pH values close to the pH of the habitats from which they were isolated. A continuous and longer exposure (21 days) at higher temperatures, of approximately 37°C can induce the adaptation of the bacterial metabolism to the respective thermal conditions.

Comparative studies regarding the resistance of the strains and populations of *Acidithiobacillus ferrooxidans* isolated from mining effluents from Ilba mining area, at increased concentrations of Fe^{2+}, Cu^{2+}, Zn^{2+} are presented in figures 5, 6, 7.

The comparative research regarding the resistance of the *Acidithiobacillus ferrooxidans* populations isolated from mining waters and sediments from Ilba site, at higher Fe^{2+} concentrations (8–16g/l Fe^{2+}), are presented in figure 5.

In the case of testing the resistance of the bacterial *Acidithiobacillus ferrooxidans* at high concentration of metallic ions, using the 9K medium with 43.22g/l $FeSO_4$ allowed the selection of populations and strains, which oxidized in 8 days of experiments the biggest amount of Fe^{2+}.

The comparative analyses regarding the behaviour of the bacterial strains (A_1 and A_2) isolated from the two mining sites illustrated that the P_1 population is the most tolerant to increased concentrations of Fe^{2+} (16 g/l Fe^{2+}). Also, it was observed that the A_2 strain is the most sensitive to high concentrations of Fe^{2+} (12–16 g/l Fe^{2+}).

In the case of testing the bacterial population at the concentrations of 10 g/l Fe^{2+} and 12 g/l Fe^{2+}, the populations had an intense metabolically activity, oxidation the whole quality of ferrous iron in six days. The experiments evidenced that the consequence of increasing the concentration of ferrous iron in culture medium over 14 g/l Fe^{2+} was the slow development of the bacterial populations, through making the lag period longer 2–4 days depending on the concentration of ferrous sulphate.

Compared to the *Acidithiobacillus ferrooxidans* populations, the bacterial strains were less resistant to the concentrations of iron used, this fact being evidenced through a weak metabolic activity appreciated through the lower oxidation level in the medium of Fe^{2+} (Fig. 5).

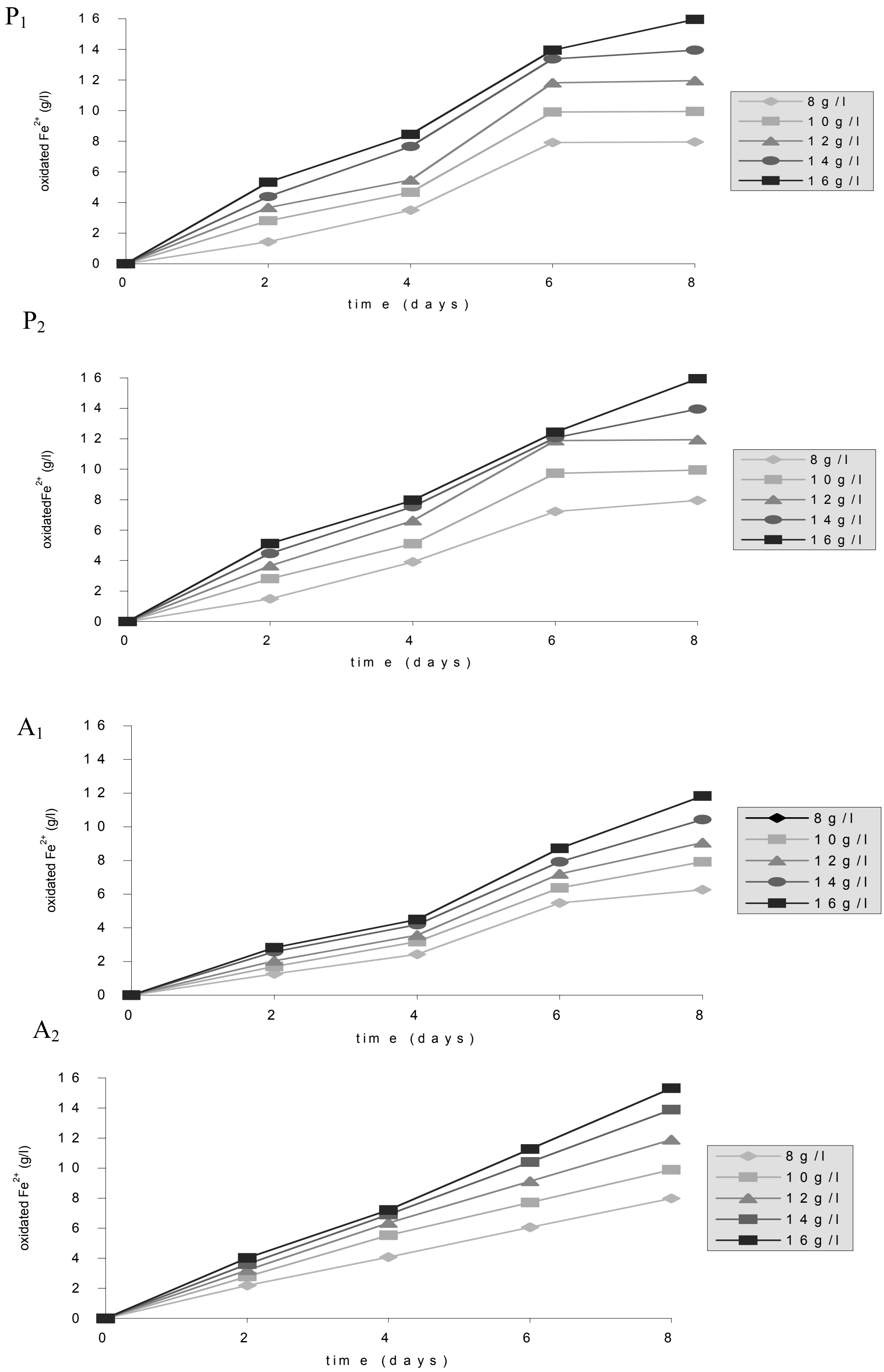

Fig. 5 – The dynamics of Fe^{2+} oxidation by *Acidithiobacillus ferrooxidans* strains (A_1, A_2) and populations (P_1, P_2) in 9K medium with different concentrations of ferrous sulphate.

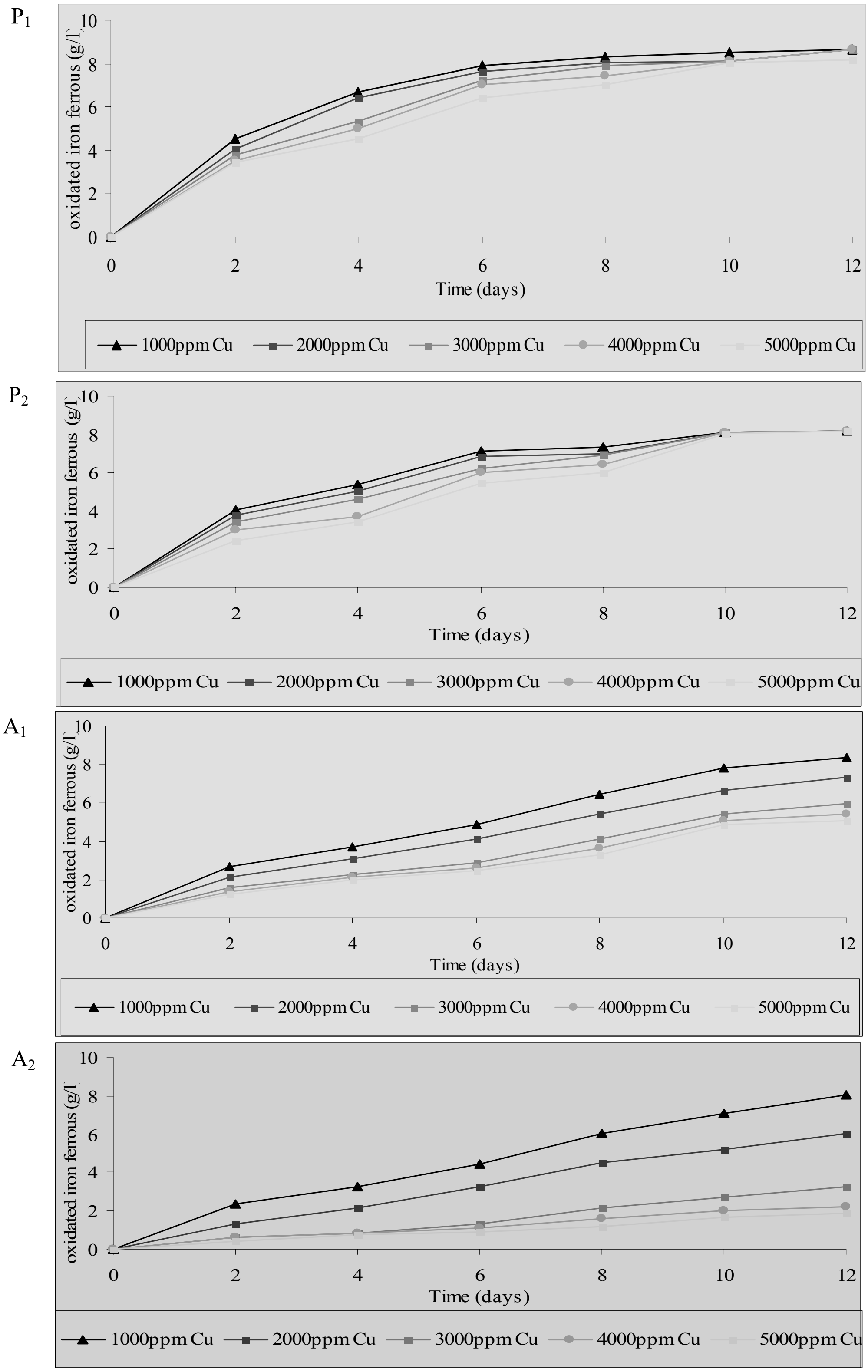

Fig. 6. – The dynamics of Fe^{2+} oxidation by *Acidithiobacillus ferrooxidans* strains (A_1, A_2) and populations (P_1, P_2) in 9K medium with different concentrations of Cu^{2+}

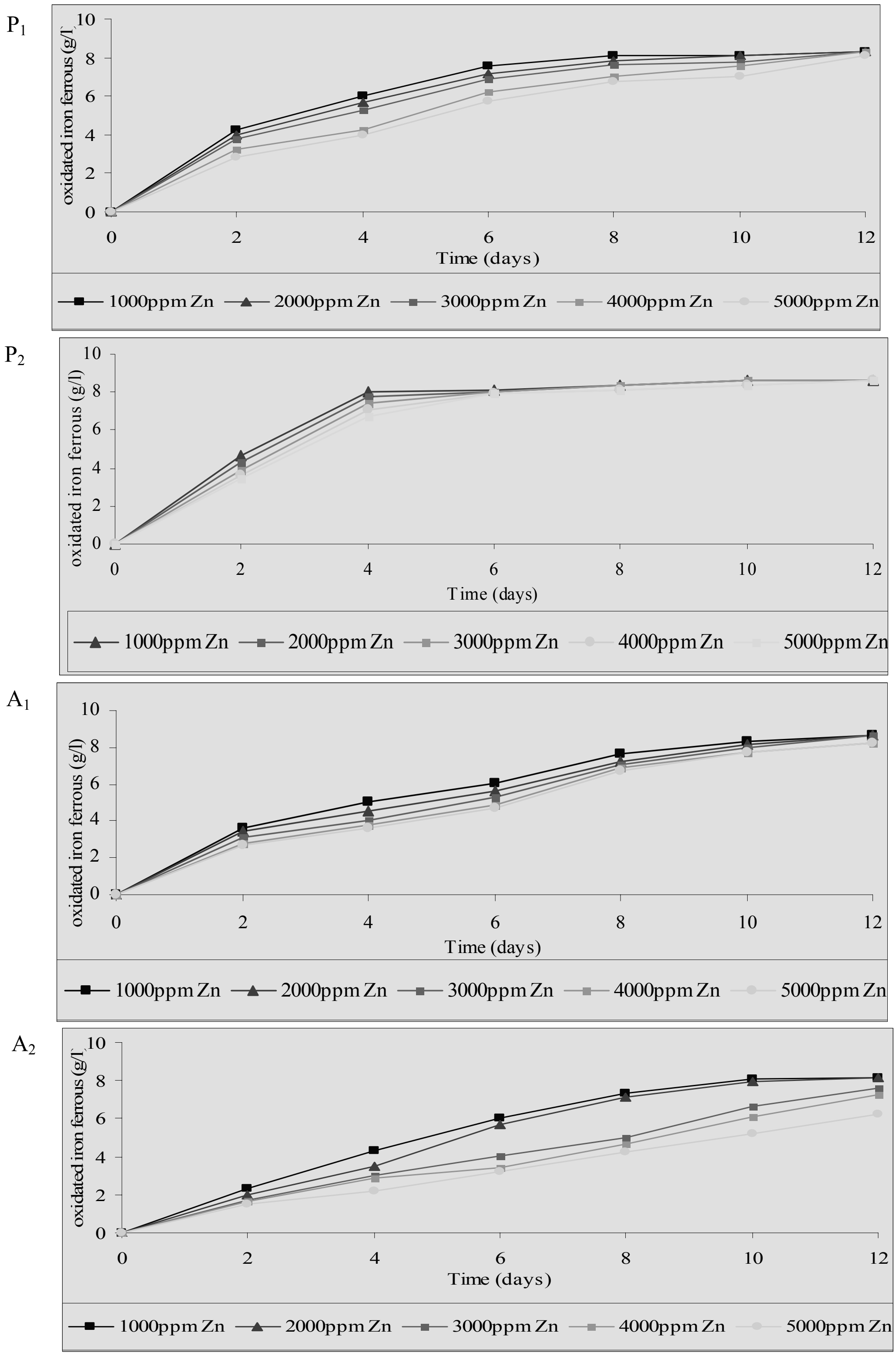

Fig. 7 – The dynamics of Fe^{2+} oxidation by *Acidithiobacillus ferrooxidans* populations (P_1 P_2) and strains (A_1, A_2) in 9K medium with different concentrations of Zn^{2+}.

Comparative studies regarding the resistance of *Acidithiobacillus ferrooxidans* cultures, isolated from mining effluents and sediments from Ilba site, at different concentrations of Cu^{2+} and Zn^{2+}, are presented in figure 6 and 7.

The comparative results regarding the behaviour of the *Acidithiobacillus ferrooxidans* populations, isolated at Asecare mine, illustrated that the P_1 population present a more intense metabolically activity compared to other populations at the concentrations of 5000ppm Cu^{2+}, having an compared to the A_1 strain.

Our results indicate the fact that A_1 strain, isolated from Ilba site, presents an intense growth and metabolically activity compared to the other strains, in the same experimental conditions. Thus, this strain oxidized the whole amount of Fe^{2+} in the medium in the presence of the 5 Cu^{2+} concentrations in a 12 days period.

The experimental results in which were compared the effects of the concentrations 1000–5000ppm Zn^{2+} on the populations of *Acidithiobacillus ferrooxidans* showed that the higher Cu^{2+} concentration in the culture medium determined diminishing the growth of the bacterial cultures by extending the lag period to 3–5 days, depending on the copper sulfate concentration.

As for the populations of *Acidithiobacillus ferrooxidans* isolated from Ilba site, it was found that P_2 population has a more intense metabolically activity compared to P_1 population, in the same experimenting conditions, because this strain oxidized the whole amount of Fe^{2+} in the presence of 5000ppm Zn^{2+} concentrations, in a 6 days experiment.

The experimental results revealed the fact that the increase of the Zn^{2+} concentration in the culture medium lead to the lowered growth of the bacterial populations, by increasing the lag period to 3–4 days according to the zinc sulphate concentration.

The A_1 strain isolated from mining effluents, at Asecare mine, presented a higher sensitivity to the concentration of 5000ppm Zn^{2+} compared to the A_2 strain isolated from sediments. This was demonstrated by the oxidizing of a smaller amount of Fe^{2+}, in the period of 12 days experimenting, a longer lag phase and a reduced ratio of oxidizing Fe^{2+}.

The experimental results in which was compared the resistance of the *A. ferrooxidans* populations at different Zn^{2+} concentrations showed that the bacterial population isolated from mining waters presented a higher metabolically activity, being more resistant to the higher concentration of Zn^{2+} than the populations isolated from mining sediments at Asecare mine.

The research made demonstrated the higher resistance of the P_2 population to higher concentrations of Zn^{2+} by showing a more intense metabolically activity to concentrations between 3000–5000ppm (Fig. 7).

The results of the comparative research regarding the tolerance to higher concentrations of Cu^{2+}, Zn^{2+} and Fe^{2+} of the *Acidithiobacillus* sp. from the Asecare mine confirm the data from special literature (RAKESH, 1990, CISMASIU *et al.*,

2000) about the higher abilities of the bacterial populations to adapt to extreme medium conditions compared to the bacterial strains. So, in the presence of concentrations of 3000–5000ppm Cu^{2+} and Zn^{2+}, the bacterial populations P_1 and P_2 had a more intense growth and metabolically activity, compared to bacterial strains. It was established that the growth of the populations is similar to other bacterial strains tested in the same experimenting conditions.

Screening experiments showed that there were differences in the metal uptake behavior of the isolates. The results regarding establishing the influence of metallic ions on the growth of the acidophilic chemolithotrophic bacteria from *Acidithiobacillus* 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 biosolubilization processes (Figs. 4–7).

The research regarding tolerance to high Fe^{2+}, Zn^{2+}, Cu^{2+} concentrations of the populations and strains of *Acidithiobacillus ferrooxidans*, confirms the data in the speciality literature (RAKESH,1990) regarding the increased capacities of the bacterial population to adapt to the extreme medium conditions (acid pH and high concentrations of metal ions) (Figs. 4–6).

4. CONCLUSIONS

The processing of manufacture ores modifies the chemical composition and the pH value of above waters from the industrial manufacture ores. Decreasing the pH value influence the taxonomic and physiologic diversity of the acidophilic chemolithotrophic bacteria belonging to *Acidithiobacillus* genus.

The comparative results about the effects of acidity on the oxidative activity of the bacteria *Acidithiobacillus ferrooxidans* at 28°C and 37°C evidenced a more intense oxidative activity at pH values between 2–2.5 at a temperature of 28°C.

At the concentrations of 10 g/l Fe^{2+} and 12 g/l Fe^{2+} the bacterial populations had an intense metabolically activity, oxidating the whole amount of Fe^{2+} in 6 days and at concentrations of 14 g/l Fe^{2+}, 16 g/l Fe^{2+} the Fe^{2+} in the medium was oxidized in a longer period of time.

For some bacterial strains, the presence of higher Cu^{2+} and Zn^{2+} levels (3000 to 5000ppm) in the environment enhanced biomass development, such as was observed in the strains of *Acidithiobacillus ferrooxidans* and *Acidithiobacillus thiooxidans*.

The increased resistance of acidophilic chemolithotrophic bacteria to metallic ions is presumably due to their adaptation to polluted environments since they were mainly isolated from mining effluents and sediments with Fe^{2+}, Cu^{2+} and Zn^{2+} ions.

The comparative results regarding the influence of the high concentrations of Cu^{2+} and Zn^{2+} on the metabolically activity of the *Acidithiobacillus ferrooxidans* populations illustrated the fact that the populations isolated from mining water are less sensitive than those from sediments.

Compared to the *Acidithiobacillus ferrooxidans* populations, the bacterial strains belonging to this type were less resistance to the Cu^{2+} and Zn^{2+} concentrations used, pointed out by weak metabolically activity appreciated through the oxidizing of a small amount of Fe^{2+}.

The experimental results confirm the existence of a close correlation between the conditions of temperature, acidity and metabolically activity of acidophilic chemolithotrophic bacteria. This gives indications on the optimal experimental conditions for laboratory investigations on these bacteria.

The present study permitted knowing the resistance to the bacteria *Acidithiobacillus ferrooxidans* to selecting strains and populations resistant to high concentrations of Fe^{2+}, which can offer an increased efficiency to the processes of oxidizing pyrite, a process that is used before recuperating previous metals.

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