THE ACIDOPHILIC BACTERIA ABILITY TO PRODUCE METALLOENZYMES RESPONSIBLE FOR THE STARCH DEGRADATION IN THE PRESENCE OF HEAVY METAL IONS

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Abstract. An action of removing heavy metal ions from aqueous solutions consists in their biosorption using the acidophilic heterotrophic bacteria of the *Acidiphilium* genus present in aquatics and ground environments. The assimilative capacity of heavy metals by the aerobe bacterial biomass has been investigated and compared to different types of biomass, grown in laboratory conditions by exploiting synthetic wastewater. Some of these types of biomass have a high potential for biosorption, appreciated as competitive in the biotechnological detoxification of industrial effluents. Efficiency of the extracellular enzymatic activity of the acidophilic heterotrophic bacteria is correlated in direct proportion to the amount of organic substances from the environment, the concentration of oxygen and heavy metallic ions. In this context, this paper presents the influence of Cu^{2+}, Zn^{2+} and Ni^{2+} on the enzymatic activity of the extracellular starch degradation in the presence of acidophilic heterotrophic bacterial populations of the *Acidiphilium* genus in order to increase the efficiency of organic substances deterioration ability for contaminated environments with heavy metal ions. The obtained results showed a stimulation of starch degradation by the action of extracellular amylolitic enzymes synthesized by acidophilic bacterial populations in the presence of heavy metal ions. In addition, the performed studies allowed the selection of bacterial cultures with increased degradation capacity of organic substances under extreme environmental conditions for removal of heavy metallic ions from industrial waste waters.

Keywords: metalophiles, biocatalytic application, starch hydrolyzing, enzymes.

Rezumat. Capacitatea bacteriilor acidofile de a produce metaloenzime responsabile cu degradarea amidonului în prezenţa ionilor de metale grele. O metodă de îndepărtare a ionilor de metale grele din soluţii apoase o reprezintă biosorbţia acestora folosind bacterii heterotrofe acidofile din genul *Acidiphilium* prezente în mediile acvatice şi terestre. Capacitatea de asimilare a metalelor grele de către biomasa aerobă de bacterii a fost investigată şi comparată la diferite tipuri de biomasă, crescută în condiţii de laborator utilizând ape reziduale sintetice. Unele din aceste tipuri de biomasă au un potenţial ridicat de biosorbţie, apreciate ca biotehnologii competitive în detoxifierea efluenţilor industriali. Eficienţa activităţii enzimatice extracelulară a bacteriilor heterotrofe acidofile este corelată direct proporţional cu cantitatea de substanţă organică din mediu, concentraţia de oxigen şi ioni metalici. In acest context, studiul de faţă prezintă influenţa Cu^{2+}, Zn^{2+} şi Ni^{2+} asupra activităţii enzimatice de degradare a amidonului în prezenţa populaţiilor de bacterii heterotrofe acidofile din genul *Acidiphilium* în vederea creşterii eficienţei capacităţii de degradare a substantelor organice din medii poluate. Rezultatele obţinute au evidenţiat o stimulare a hidrolizei amidonului sub acţiunea enzimelor amilolitice extracelulare sintetizate de populaţiile bacteriene în prezenţa ionilor de metale grele. De asemenea, studiile efectuate au permis selectarea unor culturi bacteriene cu capacităţi crescute de degradare a substanţelor organice în condiţii extreme de mediu pentru îndepartarea ionilor metalici din ape reziduale industriale.

Cuvinte cheie: metalofile, aplicaţie biocatalitică, hidrolizarea amidonului, enzime.

INTRODUCTION

The uncontrolled discharge of industrial waste waters into the river systems containing metallic ions induce profound changes of water quality which are translated in disrupting the ecosystems due to perturbation processes in the trophic chain, with inhibiting mineralization and accumulating of heavy metals in elevated concentrations in certain aquatic organisms. The presence of metallic ions in the solution and the need for their removal has opened two important ways to approach research, namely: their extraction in order to recover heavy metal ions and industrial waste water treatment to reduce the concentrations of heavy metal ions to values mentioned in international standards (VOLESKY, 1994; GUPTA & MOHAPATRA, 2003; SUD et al., 2008; SINGH et al., 2011).

In polluted habitats, the most sensitive species removed environmental pollutants, reduce the competitive interactions and also promote the proliferation of stress-tolerant species; therefore, biodiversity is greatly reduced in the proximity of the discharges of industrial waste water and the acidophilic bacteria are reduced to several dominant species. The mechanism of the action to chemical agents is realized by their effect on one of the significant structures of the cell, namely cell membranes, proteins, the cytoplasmic enzyme or the nuclear apparatus, whose normal operating mode is absolutely necessary to acidophilic heterotrophic bacteria for adaptation at elevated concentrations of metal ions from the surrounding environment (LEUNG et al., 2001; KIRK et al., 2002; GOMES & STEINER, 2004; ENACHE & KAMEKURA, 2010; LU et al., 2010; ALMEIDA et al., 2011).

Acidophilic heterotrophic bacterial populations participate in the mineralization of organic substances from the soil, in these ways vegetal and animal residues shall be brought in a form that is useable in the nutrition of plants or other microorganisms. They are involved in the soil solubilisation of mineral stocks, facilitating their use by acidophilic heterotrophic bacteria with implication in reduced environmental pollution by industrial mining activities. It is considered that the chemical and physiological reactions taking part in the retention of heavy metal ions was depended on the microbial cell of physiological requirements, the chemical status of the heavy metal ions in the cells of impact with the products secreted by the cells. As a whole, all of these are substantially influenced by surrounding environment (VIEIRA & VOLESKY, 2000; AGRAWAL et al., 2005; AIYER 2005; GAYRIVRILESCU & CHISTI, 2005).

The populations of acidophilic heterotrophic bacteria isolated from acid mine waters shall establish a series of relationships leading to the formation of community structures that ensure an optimum use of energy flow in extreme environments and a given acid homeostasis. Their diversity shall be amended under the influence of the stress factors from the acidic environment (GOYAL et al., 2003; LENTZEN & SCHWARZ, 2006; JOHNSON & HALLBERG, 2008).

The heterogeneity of physical-chemical factors of the surrounding environment stimulates the ecosystem stability, the diverse nature of acidophilic heterotrophic bacterial populations present in extremes habitats. The environmental disturbance as frequency and intensity, correlated to the degree of heterogeneity has determined the speed and range of progressive flexibility in a continuous ecosystem. The heterogeneity of physico-chemical requirements, such as concentration and type of organic and inorganic substances, conditions of pH, Eh, degree of illumination, humidity, osmotic pressure, the concentration of dissolved gases (mainly O_2 and CO_2), of toxic or inhibitory substances create physico-chemical conditions favourable to certain species and unacceptable for others. As a consequence, the environmental compartments show a discontinuous spatial distribution of acidophilic heterotrophic bacterial populations (TILMAN et al., 1997; KONSULA & LIAKOPOULOU-KYRIAKIDES, 2004; KIMURA et al., 2011; SHARMA et al., 2012).

The acidophilic heterotrophic bacteria have metabolism products of the type that can be synthetically chemically used as a carbon source of organic compounds but behave differently regarding the nitrogen of the source nature, as some of them still maintain the capacity to use inorganic chemicals, while others require amino acids and use just the organic part of nitrogen compounds and of the chemical complex. They are also called organotrophic bacteria because they use the organic substances as a carbon source for the biosynthesis and energy production. They show a large adaptability to the environmental conditions regarding the temperature and pressure and the mineralization. (BERTOLDO & ANTRANIKIAN, 2002; HAKI & RAKSHIT, 2003; REDDY et al., 2003; GUDASZ et al., 2010; KRISHNANI et al., 2012).

The biodegradation of the carbon compounds by acidophilic heterotrophic bacterial populations is very different in aerobic and anaerobic niches. Thus, in aerobic environments, by natural synthesis, organic chemicals are completely degraded, whereas in anaerobic environments they are incorporated as organic connections of the carbon substances becoming recalcitrant to biodegradation. They may be converse to the other recalcitrant forms or deposited in the layer and the exposure to physical and chemical processes determine the digenesis of fossil fuels under the impact of the complex community of acidophilic heterotrophic populations (HOOPER & VITOUSEK, 1997; GIANFREDA & RAO, 2004; BERGGREN et al., 2010; PANDEY & FULEKAR, 2012).

The data in specialized literature have pointed out that the submissions of calcareous rocks reduce the amount of carbon stock available for the biological systems from the surrounding environment. This may return to circulation following the erosion processes or the indirect action of acidophilic heterotrophic bacterial populations. Furthermore, coals contain sulphur compounds in quantities varying between 0.5 to 11% in the form of organic or inorganic complexes (sulphides of lead, iron, zinc, cooper or as $CaSO_4$ in the water and only very rarely as sulfur content) (YOUNGER, 2004; SAJEDI et al., 2005; AZKI, 2008; 2009; SINGH et al., 2011; AXINI, 2012).

It is well known that the physiological group of heterotrophic bacteria present in the acid mining drainage has an ecological importance, as well as a practical importance, being a source of new bacteria with biotechnological potential. The study of the interactions between acidophilic heterotrophic bacteria and heavy metal ions can be achieved through active processes, involving the metabolic sequences of living microorganisms or passive processes, independent of cellular metabolism. The residual biomasses of heterotrophic bacteria belonging to the *Acidiphilium* genus proved an affinity for a wide variety of heavy metal ions such as Cr^{6+}, Cr^{3+}, Cu^{2+}, Zn^{2+} and Ni^{2+} (ATKINSON et al., 1998; BOUKHIFI & BENCHEIKH, 2000; AZKI, 2003; WANG & CHEN, 2009; FAROOQ et al., 2010).

In view of the above, the present study is aimed at evaluating the extracellular starch degradation enzyme activity of the *Acidiphilium* populations, isolated from representative mining sites in Romania, in the presence of the heavy metallic ion solution and at selecting some bacterial cultures with high starch degradation process in the presence of these ions in the environment.

MATERIAL AND METHODS

To cultivate the acidophilic heterotrophic bacteria from the *Acidiphilium* genus it was used a selective medium, organic medium with pH=3.0, in which the source of energy is the glucose (CISMAŞIU et al., 2010). From samples of complex ores, mining waters and sediments (Baia and Rosia Poieni County) bacterial strains and populations belonging to this genus were obtained after 21-day incubation at 28^0C. The isolated colonies obtained on agar medium are cultivated in GYE liquid medium in continuous agitation conditions at a temperature of 28^0C for 7 days.

In order to increase the efficiency of the starch degradation process using *Acidiphilium* populations in the presence of the metallic ions solution, experiments were accompanied by chemical controls (heavy metallic ions and selective culture medium). Chemical controls were made in six Erlenmeyer flask with GYE medium at the starch concentration of 2.0g/l as an optimum substrate, which contains solutions of $CuSO_4$ and $CuCl_2$ respectively, $ZnSO_4$ and $ZnCl_2$ respectively, $NiSO_4$ and $NiCl_2$ respectively. The tests about the influence of $CuSO_4$-$CuCl_2$ solutions, $ZnSO_4$-$ZnCl_2$ solutions and $NiSO_4$-$NiCl_2$ solutions on the starch degradation enzymatic activity of bacterial populations were performed in GYE medium with 2.0 g/l starch at a temperature of 28^0C in stirring incubation conditions. Growth experiments were carried out at different species type, heavy metal ion type and contact times between cell and these ions. Regarding the influence of metallic ions solution on extracellular starch degradation process of the acidophilic heterotrophic bacteria it was followed the bacterial density of *Acidiphilium* populations (measuring spectrophotometer turbidity at a wavelength of 660nm) and extracellular starch enzymatic activity of bacterial populations (the spectrophotometer determination of starch at 580nm) by Wohlgemuth method (CISMAŞIU, 2012).

RESULTS

Comparative studies made on the *Acidiphilium* populations cultivated in GYE medium with 2.0g/l starch in the presence of 0.1% $CuSO_4$ and $CuCl_2$ solution, of 0.1% $ZnSO_4$ and $ZnCl_2$ solution and of 0.1% $NiSO_4$ and $NiCl_2$ solution are illustrated in figures 1-6. Regarding the influence of 0.1% $CuSO_4$ solution, respectively $CuCl_2$, on the growth of two bacterial populations, it is shown that the P_4 population, isolated from Roşia Poieni area, is more sensitive to 0.1% $CuSO_4$ compared to 0.1% $CuCl_2$ in the same experimenting conditions after 7 days of incubation. It was also established that, during the period, they present a reduced growth, although they are cultivated in the same experimental conditions. Thus, in an organic medium with 0.1% $CuSO_4$ solution, the P_4 population had an optical density of only 0.213, compared to the P_7 population in the presence of 0.1% $CuCl_2$, for which the values reached 0.235 after 7 incubation days (Figs. 1-2).

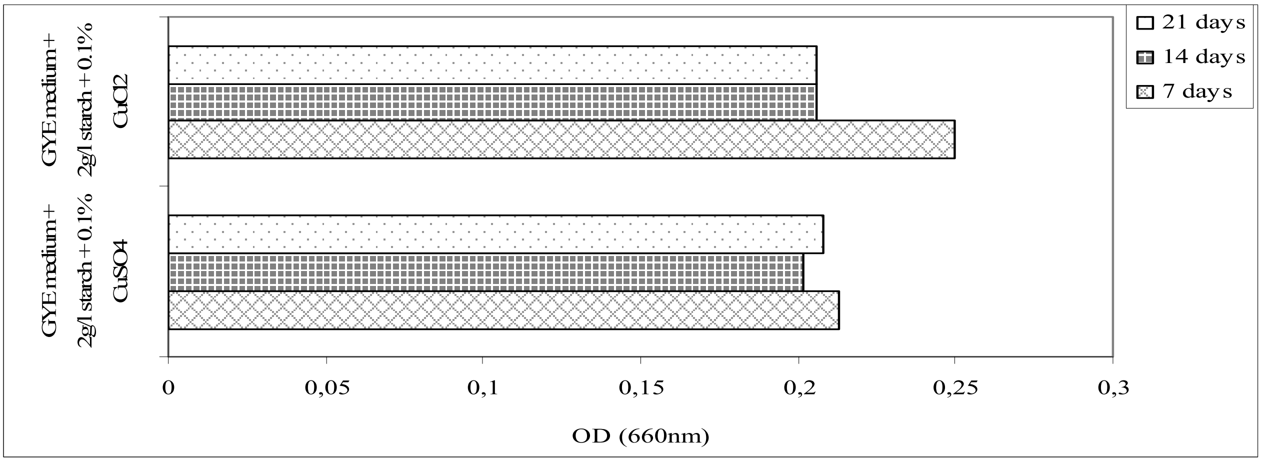

Figure 1. The bacterial density of the P_4 population from the *Acidiphilium* genus in the presence of 0.1% $CuCl_2$, respectively 0.1% $CuSO_4$, in the GYE medium with 2g/l starch, at intervals of 7 days incubation.

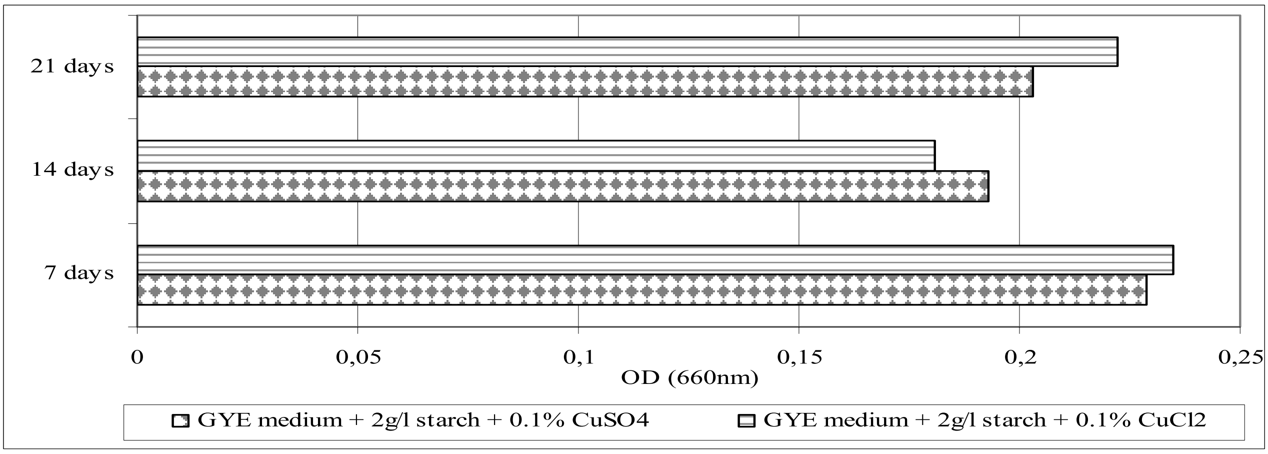

Figure 2. The bacterial density of the P_7 population from the *Acidiphilium* genus in the presence of 0.1% $CuSO_4$, respectively 0.1% $CuCl_2$, in the GYE medium with 2g/l starch.

The comparative results regarding the bacterial density of the two *Acidiphilium* populations in the presence of 0.1% $ZnSO_4$, $ZnCl_2$ respectively, proved to be efficient in the extracellular starch degradation processes in the GYE medium with 2g/l starch. It was also demonstrated that the final pH values of treated organic medium with 2g/l starch were lower than the initial values of 3.0, a fact correlated with the higher bacterial density of the acidophilic heterotrophic population isolated from mining effluents in different experimental conditions after 21 days of incubation (Figs. 3-4).

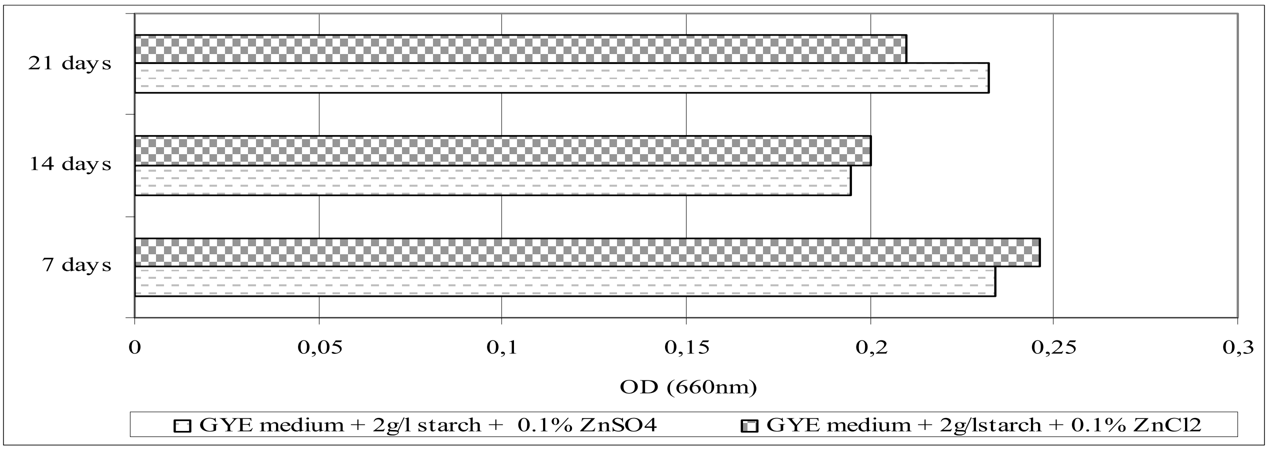

Figure 3. The bacterial density of the P_4 population from the *Acidiphilium* genus in the presence of 0.1% $ZnSO_4$, respectively 0.1% $ZnCl_2$, in the GYE medium with 2g/l starch.

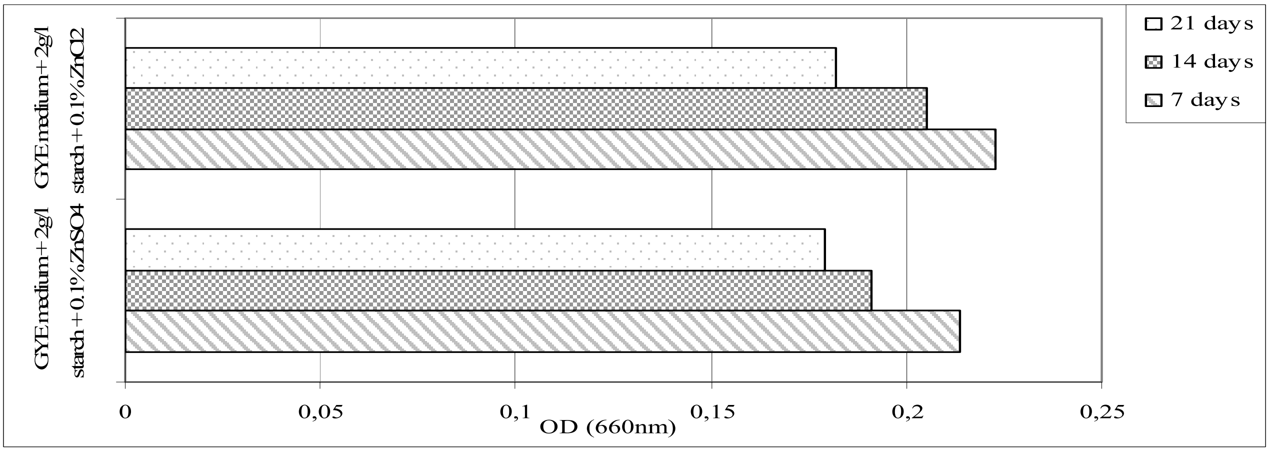

Figure 4. The bacterial density of the P_7 population from the *Acidiphilium* genus in the presence of 0.1% $ZnCl_2$, respectively 0.1% $ZnSO_4$, in the GYE medium with 2g/l starch.

Treating the GYE medium with 2g/l starch with biomass of the two *Acidiphilium* populations in the presence of 0.1% $NiSO_4$ and $NiCl_2$ solutions proved that the highest optical density of the bacterial culture was got at pH=3.0 by up to 7 and 21 days of incubation periods at the same heavy metal ion concentration (Figs. 5-6).

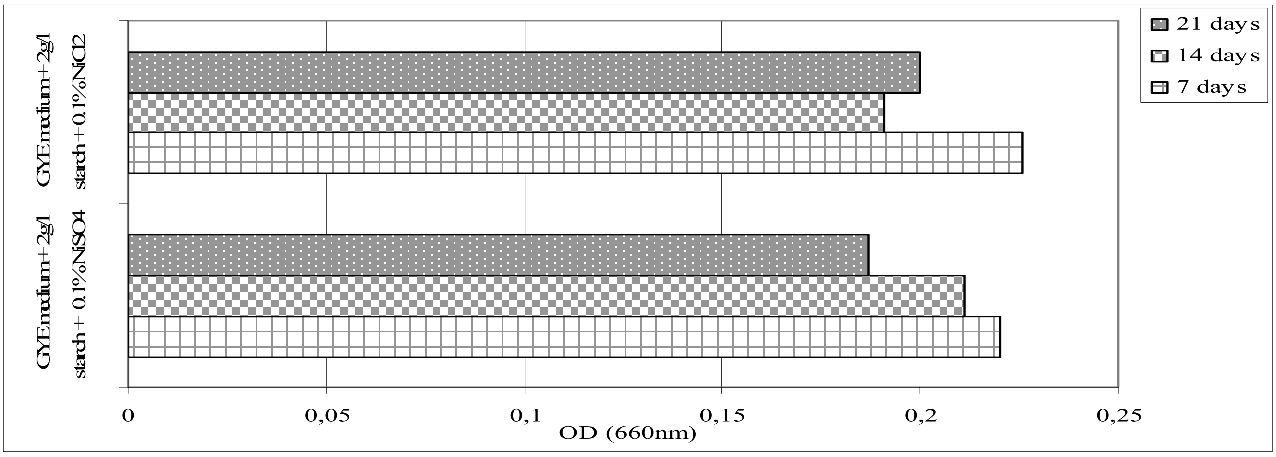

Figure 5. The bacterial density of the P_4 population from the *Acidiphilium* genus in the presence of 0.1% $NiCl_2$, respectively 0.1% $NiSO_4$, in the GYE medium with 2g/l starch.

The most important physical and chemical parameters that influence the growth of the *Acidiphilium* populations, isolated from water mining effluents of Baia and Roşia Poieni areas, in the presence of 0.1% $NiSO_4$-$NiCl_2$ solution are the contact time between biomass and the GYE medium with 2g/l starch as energy source, the type of heavy metal ions and the metal ion concentration from this selective medium in optimal experimental conditions. This fact is correlated with the high fixation capacity of acidophilic heterotrophic bacterial populations in order to establish their distribution in polluted environments with higher content of heavy metallic ions, namely Cu^{2+}, Ni^{2+} and Zn^{2+} acting as enzyme cofactors in the oxidation-reduction reactions (Figs. 1-6).

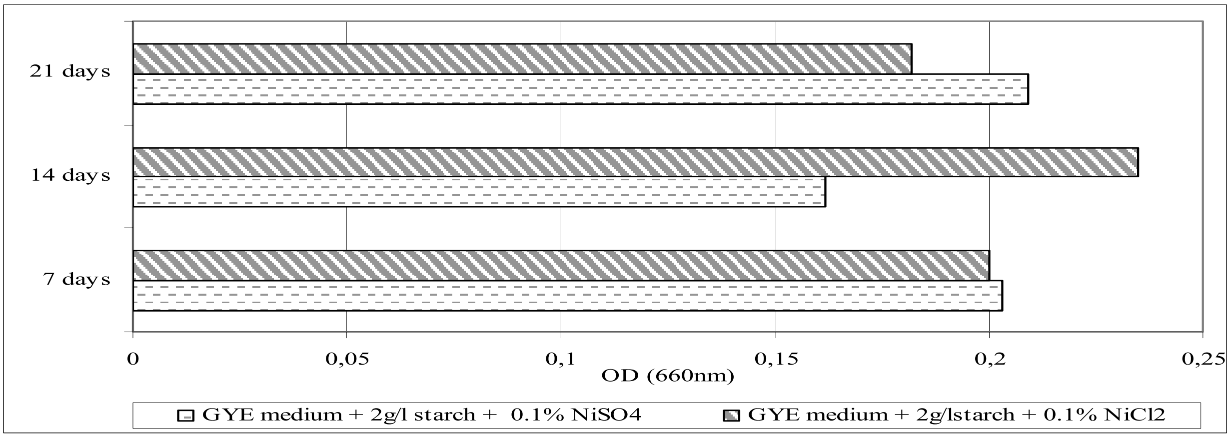

Figure 6. The bacterial density of the P_7 population from the *Acidiphilium* genus in the presence of 0.1% $NiSO_4$, respectively 0.1% $NiCl_2$, in the GYE medium with 2g/l starch.

The compared data within studying the extracellular starch degradation enzymatic activity of the two bacterial populations in the same optimum growth conditions with the 0.1% $CuSO_4$ and $CuCl_2$ solutions, the 0.1% $ZnSO_4$ and $ZnCl_2$ solutions and the 0.1% $NiSO_4$ and $NiCl_2$ solutions are illustrated in figures 7-9. The results regarding the influence of the 0.1% $CuSO_4$, $NiSO_4$ and $ZnSO_4$ concentration, in different experimental variants, concerning the extracellular hydrolytic activity of the acidophilic heterotrophic bacteria from *Acidiphilium* sp., isolated from the two mining sites mentioned above, permitted the selection of some bacterial strains and populations with a higher resistance to the presence of these ions in the medium regarding the development of the biosorption processes (Figs. 7-8).

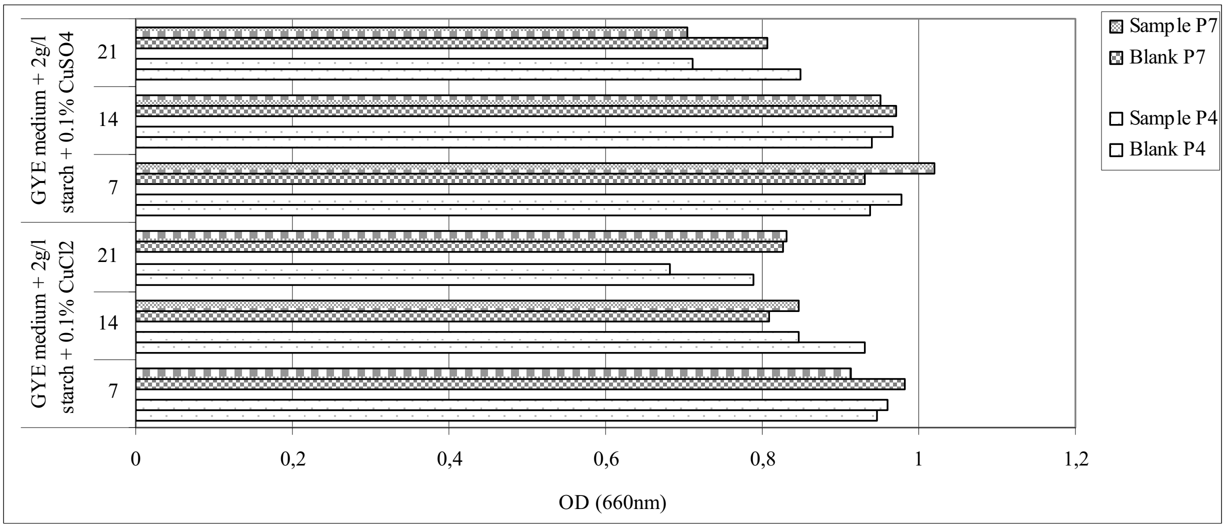

Figure 7. The extracellular starch degradation enzyme activity of the *Acidiphilium* populations in the presence of 0.1% $CuSO_4$, respectively 0.1% $CuCl_2$, at 2g/l starch in GYE medium.

In all performed experiments the extracellular activity of the starch degradation by the *Acidiphilium* populations isolated from water and sediment samples from Baia and Rosia Poieni areas was stimulated by the presence of Cu^{2+} in the form of $CuSO_4$, Zn^{2+} in the form of $ZnSO_4$, Ni^{2+} in the form of $NiSO_4$, acting as cofactors, in the concentration of 0.1% on the selective medium with 2g/l starch (Figs. 7-9). The comparative analysis of the results obtained in this study, illustrated in figure 9 demonstrated that the P_4 population (isolated from Roşia Poieni) are less sensitive to the tested concentration, compared to the P_7 population (isolated from Baia) on the whole incubation period. The comparative studies regarding the influence of the metallic ions solution on the growth and the starch degradation activity are developed with a maximum intensity up to 14 days of incubation periods at the same substrate concentration. High percentage degradation of the substrate obtained by using populations of heterotrophic bacteria compared with purified strains, which confirms the adaptation of the two *Acidiphilium* populations to the used concentrations of heavy metal ions, are illustrated in figures 7-9.

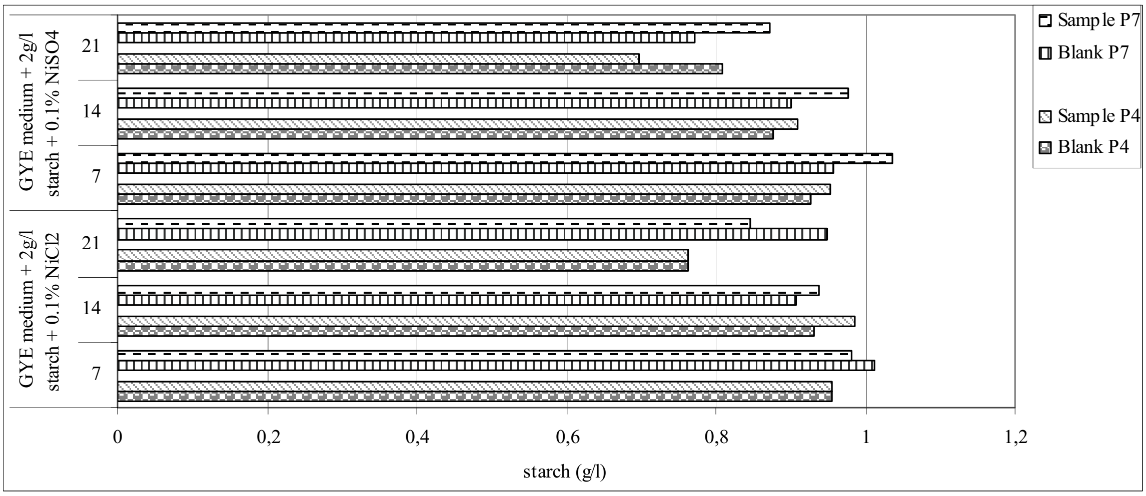

Figure 8. The extracellular starch degradation enzyme activity of the *Acidiphilium* populations in the presence of 0.1% Ni SO_4, respectively 0.1% Ni Cl_2, at 2g/l starch in GYE medium.

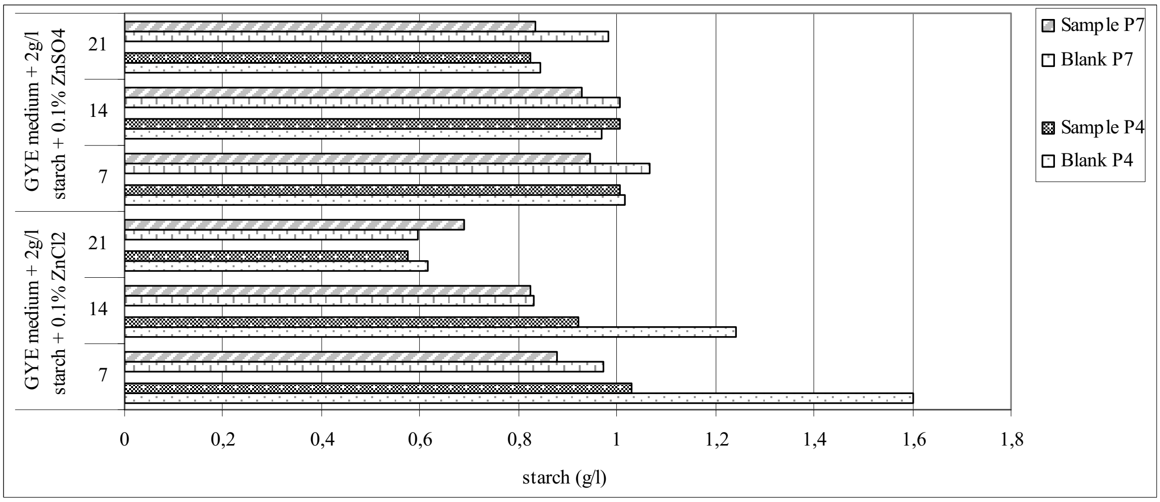

Figure 9. The extracellular starch degradation enzyme activity of the *Acidiphilium* populations in the presence of 0.1% $ZnSO_4$, respectively 0.1% $ZnCl_2$, at 2g/l starch in GYE medium.

The comparative analyses regarding the influence of the heavy metallic ions (Cu^{2+}, Zn^{2+} and Ni^{2+}) on the extracellular starch degradation activity using acidophilic heterotrophic bacterial biomass evidenced the fact that the cultures grown in the presence of 0.1% $CuCl_2$, $ZnCl_2$ and $NiCl_2$ solutions have a slower enzymatic activity at a substrate concentration established at 2g/l starch. The comparative studies made on the influence of heavy metallic ion solutions on the growth and the extracellular enzymatic activity of the *Acidiphilium* populations cultivated in GYE medium with starch, as carbon and energy source, proved that the influence occurs at a great intensity up to 7 days of incubation periods at the same heavy metal ion concentration in continuous agitation conditions. The comparative results of the research regarding extracellular starch degradation enzyme activities of the bacterial populations in the presence of 0.1% $CuSO_4$, $ZnSO_4$ and $NiSO_4$ solutions evidenced the bacterial density in the GYE medium with 2g/l starch was raised, a fact correlated with higher percentages of the hydrolytic activity, thus confirming the data from the specialty literature on the higher abilities of the mixed populations to adapt to acidic conditions. In accordance with the specialty literature, an advantage in the extracellular hydrolysis process of starch is the fact that the value of the pH and the optimum temperature coincide with the optimum values to increase the bacterial density of acidophilic heterotrophic bacterial populations concerned to the decontamination of polluted environments with high concentrations of metal ions (GOYAL et al., 2003; GIANFREDA & RAO, 2004; AIYER, 2005; WANG & CHEN, 2009; CISMAŞIU et al., 2010).

CONCLUSIONS

The extracellular starch degrading activity of acidophilic heterotrophic bacterial populations in the presence of 0.1% $CuSO_4$, $ZnSO_4$ and $NiSO_4$ solutions revealed a correlation among the bacterial density and the chemical valence of heavy metallic ions that act as cofactors. Through their extracellular hydrolytic activities, they lead to changes in the values of the oxidation-reduction potential by modifying the medium composition correlated to the decrease of the initial pH value of the organic medium. High percentage degradation of the substrate was observed by using acidophilic heterotrophic bacterial populations, which modify the medium composition correlated to the increase of the bacterial density after 14 days at 28^0C, which confirms the adaptation of these bacteria to the used concentration of heavy metal ions. The study on metabolism of the acidophilic heterotrophic bacteria is of great importance for the selection of some bacterial strains and populations, which can offer a higher efficiency to the biosorption processes when they are used, having a significant increase of bacterial density and a maximum extracellular starch degradation enzymatic activity even in the presence of some heavy metallic ions concentrations. Regarding the dynamics of the physiological conditions in the organic medium specific to the *Acidiphilium* genus, it was proved that the ability to secrete extracellular substances involved in the starch degradation activities of bacterial populations depends on the chemical valence of the heavy metallic ions, the physical-chemical factors in the selective medium, all of them being important in the removal processes of carbon compounds from industrial waste waters and explanation of biodiversity in extreme environments.

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