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ENZYMATIC POTENTIAL OF ACIDOPHILIC HETEROTROPHIC BACTERIA FOR REMOVAL AND RECOVERY OF METAL IONS FROM ACID MINE DRAINAGE

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Abstract. The most efficient biotechnological processes for the recovery/removal of metal ions from industrial effluents include biosorption and bioaccumulation of heavy metals by acidophilic heterotrophic bacteria of the genus *Acidiphilium*. Enzymes activated by different metal ions have an exacerbation of catalytic function in their presence due to the formation of enzyme-metal complexes more active or due to the formation of enzyme-metal complexes, due to the formation of substrate-complexes or accumulation of these ions. In case of metalloenzymes, metal ion is bound and incorporated into higher order structures of protein providing an active catalytic structured and/or functional role. Acidophilic heterotrophic bacteria tested have been represented by populations of *Acidiphilium* species. In all experiments performed, the hydrolytic activity of *Acidiphilium* population isolated from Roşia Poieni led to sharp decrease of starch in culture medium. In experiments testing of the hydrolytic activity at different concentrations of starch it was observed a more accentuated hydrolysis to its initial concentration of 0.2% in culture medium. Also, the presence of NaCl and $CaSO_4$ concentration of 0.1% in organic medium with starch stimulates the development of acidophilic heterotrophic bacteria belonging to genus *Acidiphilium*, as well as their extracellular amylolitic activity.

Keywords: genus *Acidiphilium*, growth, glycosyl hydrolases, biosorption.

Rezumat. Potenţialul enzimatic al bacteriilor heterotrofe acidofile pentru îndepărtarea şi recuperarea ionilor metalici din ape acide de mină. Printre cele mai eficiente procese biotehnologice de recuperare/îndepărtare a ionilor metalici din efluenţi industriali se numără biosorbţia şi bioacumularea metalelor grele de către bacteriile heterotrofe acidofile din genul *Acidiphilium.* Enzimele activate de diferiţi ioni metalici prezintă o exacerbare a funcţiei catalitice în prezenţa acestora fie datorită formării unor complecşi mai activi enzimă-metal, fie datorită formării unor complecşi substrat-metal sau acumulării acestor ioni. În cazul metaloenzimelor, ionul metalic este legat şi înglobat în structurile de ordin superior ale proteinei catalitice active îndeplinind un rol structural şi/sau funcţional. Bacteriile heterotrofe acidofile testate au fost reprezentate de populaţii de *Acidiphilium.* În toate experienţele efectuate, activitatea hidrolitică a populaţiei de *Acidiphilium* izolată de la Roşia Poieni a determinat scăderea accentuată a concentraţiei amidonului în mediul de cultură. În experienţele de testare a activităţii hidrolitice la diferite concentraţii de amidon s-a observat o hidroliză mai accentuată a acestuia la concentraţia iniţială de 0,2% în mediu de cultură. De asemenea, prezenţa NaCl şi $CaSO_4$ în concentraţie de 0,1% în mediu organic cu amidon stimulează dezvoltarea bacteriilor heterotrofe acidofile din genul *Acidiphilium*, precum şi activitatea amilolitică extracelulară a acestora.

Cuvinte cheie: genul *Acidiphilium*, creştere, glicozid hidrolaze, biosorbţie.

INTRODUCTION

In the last 10-15 years, the ability of acidophilic microorganisms to degrade a variety of synthetic organic compounds in inorganic products contributed to the development of bioremediation technologies. Accountable to estimated biodegradation of contaminants and indicated potential risks to human health associated contaminants transport in soil it is necessary to know the different processes that affect organic substances.

Microbial degradation is the main process through that contaminates are eliminated. Acidophilic microorganisms include heterotrophic bacteria that catalyse the reduction of organic substances. Since these reduction reactions can generate alkalinity, they have potential in biological remediation in AMD. The rate and level of microbial degradation are controlled by biotic and abiotic factors among which absorption has important consequences. An instable organic compound can be absorbed according to the pH environment. (CARLSON, 1998; JOHNSON, 1998, 2003; BENYEHUDA *et al*., 2003; ŞTEFĂNUŢ, 2012).

The use of acidophilic heterotrophic bacteria to remove heavy metals from polluted environments is an unconventional method, but it provides an economic approach and perspective for their decontamination. When the biosorption phenomenon was discovered, it opened the path for some biotechnologies meant to ensure the removal of metals from used industrial waters. This method can be used to control pollution, to recover the contaminated surface and phreatic waters and to recover these metals with important economic consequences. Bacterial properties, as well as cell surface charge, hydrofobicity, extracellular surfaces such as polysaccharides and proteins, influence the bacterial uptake (BRODA, 1992; JOHNSON, 1995; AHLUWALIA & GOYAL, 2007; ZEYAULLAH *et al*., 2009).

Acidophilic heterotrophic bacteria are able to mobilize metals by (1) the formation of organic and inorganic acids (protons); (2) reactions of oxidation and reduction; (3) the excretion of complexion agents. A series of organic acids are formed by bacterial metabolism, which results in complex and chelate formation. The biosorbent contains a variety of functional sites including carboxyl, imidazole, sulfhydryl, amino, phosphate, sulphate, thioether, phenol, carbonyl, amide and hydroxyl moieties (IGWE & ABIA, 2006; WANG & CHEN, 2009; CISMAŞIU, 2011).

Understanding the mechanisms of those microorganisms that are involved in metal accumulation is very important, especially from the point of view of microbial processes that bring to the removal of metals and their recovery from watery solutions. Knowledge of physico-chemical reactions taking place during the accumulation of metal ions may lead to the achievement of an efficient control of the process parameters in order to increase the retention rate and specificity of metal accumulation (MURALEADHARAN *et al*., 1991; MACEK *et al*., 2009; GADD, 2010).

Among the most efficient biotechnological processes of metals recovery/removal from industrial effluents we mention heavy metals bioaccumulation and biosorption by the acidophilic heterotrophic bacteria belonging to genus *Acidiphilium*. The inhibitory or toxic effect of heavy metals on the microorganisms imposed to explain experimentally the adaptation aspects of acidophilic heterotrophic bacteria belonging to the genus *Acidiphilium* to elevated levels of metal ions. Thus, it was found that among the extracellular enzymes, which attach to heavy metals, there are proteins, such as mettalothionein. (NORRIS *et al*., 2000; REDDY *et al*., 2003; MONTEIRO DE SOUZA & MAGALHAES, 2010).

Enzymes activated by different metal ions have a catalytic function exacerbation in their presence due to the formation of enzyme-metal complex active either because of the formation of substrate-metal complex or accumulation of these cofactors. In the case of the metalloenzymes, metallic ions are bound and concealed in a higher order structures of protein catalytic activities providing an active catalytic structural or/and functional. The removal of the cofactor leads to the loss of enzyme activity, caused by structural changes induced by this phenomenon (SPARKS, 2005; VIJAYARAGHAVAN & YUN, 2008).

A key feature of metalloenzymes is high stability of the complex formed between metallic ions and protein side, known as thermodynamic stability. Metallic ions can affect the catalytic function of the enzyme by acting: (1) substrate binding and/or enzyme cofactors; (2) activation of enzyme-substrate complex after its formation; (3) induce certain changes in the conformation of the enzyme; (4) participation in some redox reactions. Metallic ions can make two links between two across advanced amino acids. The relationship of calcium ions produces an increase of the α-helix structure. (SARIKAYA & CIRAKOCLU, 1989; SPAIN & ALM, 2003).

Particular importance to use acidophilic heterotrophic bacteria of genus *Acidiphilium* in the process of biosorption and bioaccumulation of heavy metals from industrial wastewater is held by the resistance of these bacteria to metal ions existing in the environment. This fact offers the possibility of efficient use in biotechnology processes (JOHNSON, 2001; CISMAŞIU, 2011; SINGH *et al*., 2011).

On the other side, the resistance study of acidophilic heterotrophic bacteria belonging to genus *Acidiphilium* to elevated levels of metal ions contribute to their removing from industrial wastewater. In this context, the influence of metal ions was tested on extracellular hydrolytic activity of acidophilic heterotrophic bacteria isolated from acidic water/sediment by Roşia Poieni area (Alba County) and Baia area (Tulcea County).

MATERIAL AND METHODS

Types of bacteria

The studied bacteria were isolated from two types of mining effluents with high concentrations of metallic ions: sample 1 was isolated from Roşia Poieni area (Alba county) with pH of 1.45 to 2.31 and high content of Fe, Cu, Zn, Pb, Al, Ni, Cr, Cd; sample 2 was isolated from Baia area (Tulcea county) with pH of 1.50 to 2.47 and high content of Fe, Cu, Mg, Al, Zn, Ca.

The acidophilic heterotrophic bacteria were represented by *Acidiphilium* populations marked P_4, isolated from Roşia Poieni area, and marked P_7, isolated from Baia area, obtained on the GYE medium with pH=3 (CISMAŞIU, 2004).

Bacterial cultivations

In order to increase the efficiency of the starch enzymatic hydrolysis using *Acidiphilium* populations in the presence of metallic ions, experiments were accompanied by chemical controls (metallic ions and selective culture medium) and biological controls (population with a low resistance to metallic ions).

Bacterial cultures were grown in 12 Erlenmeyer flask with the selective medium, which contains solutions of $CaSO_4$ and NaCl and culture inoculums represented 7 days periods. Chemical controls were made in two Erlenmeyer flask with selective medium, which contains solutions of $CaSO_4$ and NaCl respectively.

The effects of metallic ions on extracellular hydrolytic activity of acidophilic heterotrophic bacteria have been studied under continuous shaking conditions (150rpm). Incubation period was 21 days. Observations are carried out initially, after 7 days, 14 days and 21 days.

Enzymatic assays

Acidophilic heterotrophic bacteria of the genus *Acidiphilium* are involved in metal biosorption processes based on biosorbent materials from decantation ponds, allowing their use for the waste water treatment by extraction and recovery of metal ions. In investigating the influence of metallic ions on extracellular hydrolytic activity of the analysed bacteria it was followed the growth of acidophilic heterotrophic bacteria (measuring spectrophotometer turbidity at a wavelength of 660 nm) and extracellular enzymatic activity (the spectrophotometric determination of starch at 580 nm) by Wohlgemuth method (CISMAŞIU, 2011).

These bacteria use organic substances and molecular hydrogen as donor's electron, the acceptors electrons being sulphates, elemental sulphur and some partially oxidized sulphur compounds (SO_3^{2-}, $S_2O_3^{2-}$) that are reduced to the sulphides. Experimental variants of medium have been analysed: V_1 = GYE + 0.1% $CaSO_4$;

V_2 = GYE + 1g starch + 0.1% $CaSO_4$;
V_3 = GYE + 2g starch + 0.1% $CaSO_4$;
V_4 = GYE + 0.1% $CaSO_4$ + 0.1% NaCl;
V_5 = GYE + 1g starch + 0.1% $CaSO_4$ + 0.1% NaCl;
V_6 = GYE + 2g starch + 0.1% $CaSO_4$ + 0.1% NaCl.

RESULTS

In this study it has been tested the extracellular hydrolytic capacity of acidophilic heterotrophic bacterial populations in different experimental conditions represented in figures 1-4. For the identification of the heterotrophic bacterial populations of the genus *Acidiphilium* in an acid mine drainage sample from Roşia Poieni and Baia areas, there were analysed, having in view the inoculation into selective solid media, and taken into consideration the following features: morphology of colonies, rate of growth, morphology and size of microscopical structures, potential of growth into nutrient medium containing different concentration of substratum, acid tolerance.

Experimental tests concerning the influence of Ca^{2+} ion as $CaSO_4$ and Na^+ ion as NaCl on growth of two bacterial populations, noted P_4 and P_7, belonging to the genus *Acidiphilium*, incubated for 6 experimental variants of culture medium, at various incubation period are rendered in figures 1 and 2.

Comparative studies regarding the influence of metallic ions on the populations of heterotrophic bacteria belonging to the genus *Acidiphilium* indicates that the population growth is better in case of P_4 population compared with the P_7 population in similar conditions of growing (pH=3.0, stirring conditions). After 14 days of incubation period there is a lower difference between bacterial growths of two bacterial populations. This difference is due to adaptation of P_4 population to the physical conditions existing in the respective habitat, characterized by higher acidity values than those of the habitat from Baia (Figs. 1, 2).

Experiments have shown that in the case of acidophilic heterotrophic bacteria isolated from Roşia Poieni area the effect of metal ions on the growth is more obvious under stirring conditions. It was also found that their growths are carried out with maximum intensity at 7 days. A continuous and extended exposure of P_4 population, during 21 days, in GYE medium with 0.1% $CaSO_4$, 0.1% NaCl and different concentrations of starch may induce a relative adaptation of bacterial extracellular hydrolytic activities to such conditions.

It was observed that in case of an incubation period of 14 - 21 days both bacterial growths are reduced. Also, at 7 days of incubation the growth and extracellular hydrolytic activity is high, optimal incubation period ranging between 7 and 14 days. Thus, agitated cultures have reached an optical density of 1.125 to 0.192 in case of V_3 experimental variant.

There is a significant increase in the development of the two bacterial populations; OD is 6 times higher in case of the population P_4 under stirring conditions, which provides hints on the optimal conditions for the cultivation of these species under laboratory conditions (Figs. 1-3).

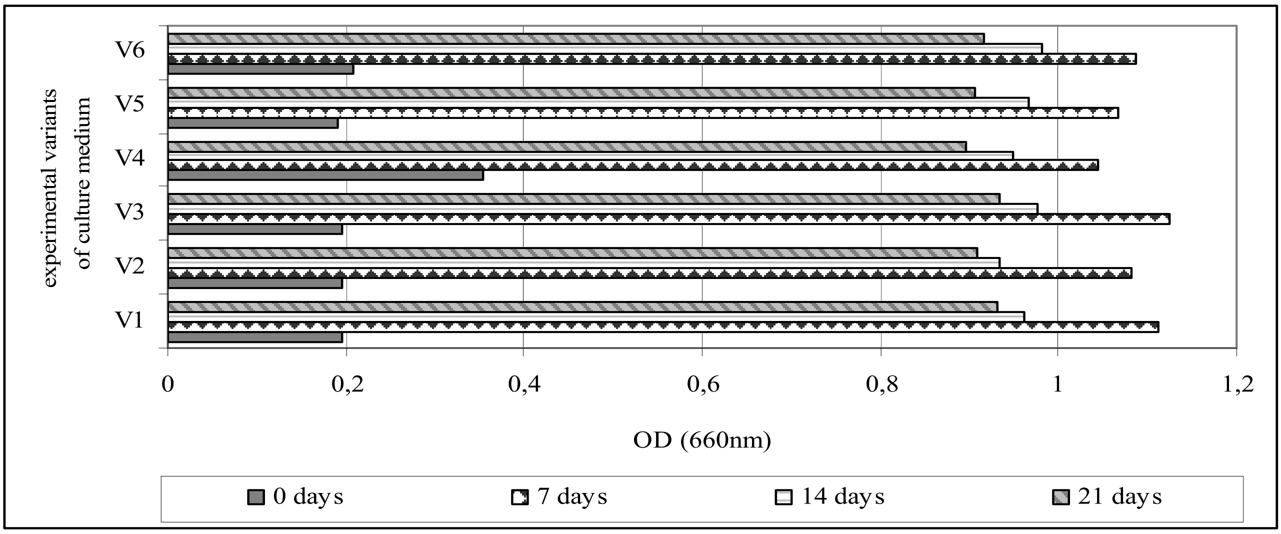

Figure 1. The development of acidophilic heterotrophic bacterial population P_4 from the genus *Acidiphilium* in GYE medium with 0.1% $CaSO_4$, 0.1% NaCl and different concentrations of starch at intervals of 7 days incubation.
Figura 1. Dezvoltarea populaţiei P_4 de bacterii heterotrofe acidofile din genul *Acidiphilium* in mediu GYE cu 0,1% $CaSO_4$, 0,1% NaCl şi diferite concentraţii de amidon la intervale de incubare de 7 zile.

Comparative studies regarding the extracellular hydrolytic activity of *Acidiphilium* populations, isolated from Roşia Poieni and Baia mining effluents, in presence of different starch concentrations, 0.1% $CaSO_4$ and 0.1% NaCl are illustrated in figures 3 and 4.

Comparative analyses regarding the effects of 0.1% $CaSO_4$ and NaCl concentrations on the growth of *Acidiphilium* populations, isolated from Roşia Poieni and Baia areas, have allowed the selection of bacterial populations showing intense extracellular hydrolytic activity in the presence of these concentrations.

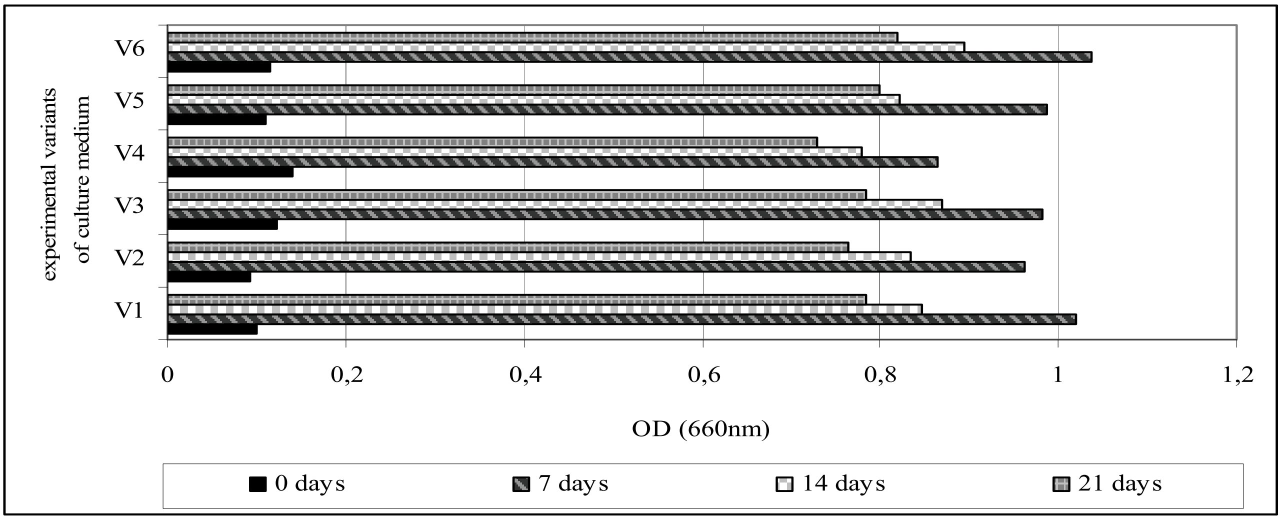

Figure 2. The development of acidophilic heterotrophic bacterial population P_7 from genus *Acidiphilium* in GYE medium with 0.1% $CaSO_4$, 0.1% NaCl and different concentrations of starch at intervals of 7 days incubation.
Figura 2. Dezvoltarea populaţiei P_7 de bacterii heterotrofe acidofile din genul *Acidiphilium* in mediu GYE cu 0,1% $CaSO_4$, 0,1% NaCl şi diferite concentraţii de amidon la intervale de incubare de 7 zile.

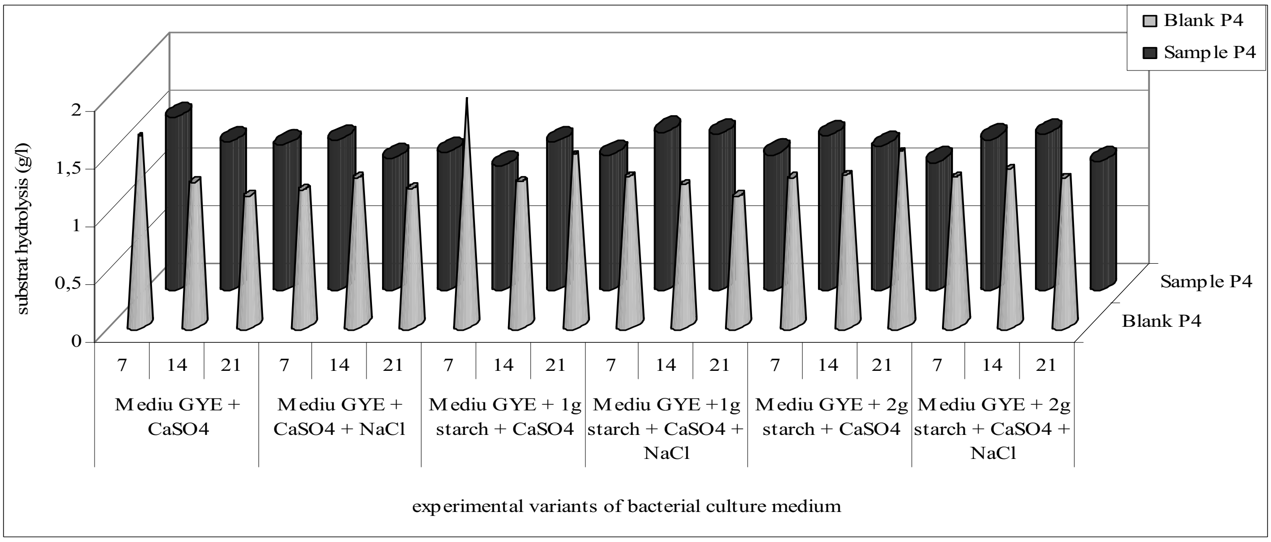

Figure 3. The dynamics of bacterial and chemical starch hydrolysis in the presence of the *Acidiphilium* P_4 population in GYE medium with 0.1% $CaSO_4$, 0.1% NaCl and different starch concentrations at intervals of 7 days incubation.
Figura 3. Dinamica hidrolizei bacteriene şi chimice a amidonului în prezenţa populaţiei de *Acidiphilium* P_4 în mediu GYE cu 0,1% $CaSO_4$, 0,1% NaCl şi concentraţii diferite de amidon la intervale de incubare de 7 zile.

As a result of starch concentrations increase, the chemical hydrolysis of starch intensifies the difference between these and the one catalysed by microbial cultures, which becomes obvious at 2g/l starch. Another noticed factor was the NaCl value of the culture medium, the influence of which on extracellular starch hydrolysis is shown in figure 4.

The comparative study of the extracellular hydrolytic activity of *Acidiphilium* populations, isolated from Roşia Poieni and Baia areas, at different starch and metallic ions concentrations revealed the highest activity in GYE medium with 0.2% starch, 0.1% $CaSO_4$ and 0.1% NaCl, confirming the physical-chemical characteristics of this genus. Further increase of the incubation period has a negative influence on the growth and extracellular starch hydrolysis, emphasizing their strong decrease after 21 days.

The results of the comparative research on the effects of 0.1% $CaSO_4$ and NaCl concentrations in different experimental variants concerning the extracellular hydrolytic activity of *Acidiphilium* populations confirm the data from specialized literature about the abilities of these populations to adapt to extreme medium conditions. These bacterial populations will grow on selective media with different concentrations of metallic ions for testing their resistance at increased concentrates of metallic ions (Figs. 1-4).

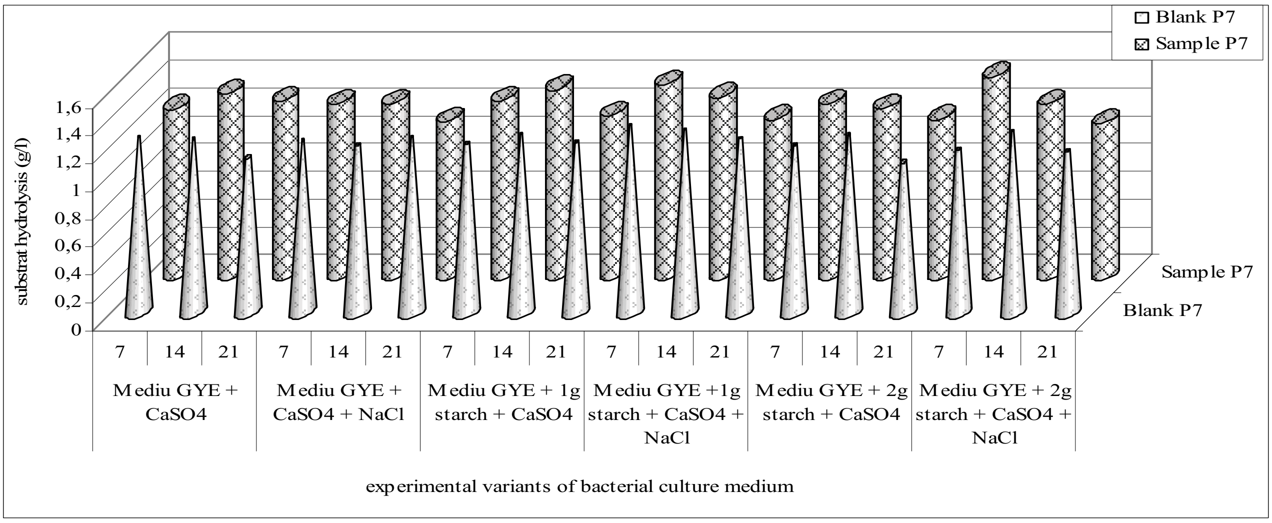

Figure 4. The dynamics of bacterial and chemical starch hydrolysis in the presence of the *Acidiphilium* P_7 population in GYE medium with 0.1% $CaSO_4$, 0.1% NaCl and different concentrations of starch at intervals of 7 days incubation.
Figura 4. Dinamica hidrolizei bacteriene şi chimice a amidonului în prezenţa populaţiei de *Acidiphilium* P_7 în mediu GYE cu 0,1% $CaSO_4$, 0,1% NaCl şi concentraţii diferite de amidon la intervale de incubare de 7 zile.

CONCLUSIONS

The acidophilic heterotrophic bacteria, in optimal developmental conditions at 0.1% $CaSO_4$ and 2g/l starch, may have an active contribution to biosorption processes in presence of high concentrations of metallic ions.

Therefore, an acidity of culture medium indicates a high developmental and extracellular hydrolytic activity level for these bacteria.

There is a strong relationship between the chemical characteristics of the culture medium and the extracellular hydrolytic activity of the acidophilic heterotrophic bacteria.

The experimental results indicate a higher extracellular hydrolytic activity level of bacterial populations correlated with a higher growth level in stirring conditions.

The level of growth and extracellular starch hydrolysis of acidophilic heterotrophic bacteria is maximal at 0.2% starch, though a variation of starch within the range 0.1% – 0.2% affects bacterial growth with less than 20%.

As 0.1% $CaSO_4$ and 0.1% NaCl were proven to be the best metallic ions concentrations for the extracellular amylolitic activity of *Acidiphilium* populations, they were selected for further experiments, aiming to find the optimum substrate concentration; starch concentration was raised to 1 and 2 g/l, while pH was kept at 3.

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