THE EFFECT OF SALTS COMPOSITIONS ON THE EXTRACELLULAR AMYLASES ACTIVITY FROM *ACIDIPHILIUM* POPULATIONS ISOLATED FROM MINING EFFLUENTS

CISMAŞIU Carmen Mădălina

Abstract. Increased environmental pollution as a result of mining activities generated an increase of the interest for understanding the mechanisms of the adaptation of microorganisms to these environments and especially for their potential biotechnological application. We isolated two populations of acidophilic heterotrophic bacteria of the *Acidiphilium* genus found in water and sediment samples from the mining sites at Roşia Poieni (Alba county) and Baia (Tulcea county) with increased resistance to metallic ion (Fe^{2+}, Cu^{2+}, Zn^{2+}, Ni^{2+}). These bacteria were tested for the influence of chloride and sulphate salts on their growth profile and for the extracellular starch degradation activity in the presence of environmental cations (Ca^{2+}, Na^{+}) and anions (Cl^{-}, SO_4^{2-}). Our results indicate significant differences on the adaptation of the two bacterial populations to anions and cations in medium culture, differences correlated with specific conditions in the original sites. The population isolated from Baia has an increased efficiency of starch degradation in the presence of the above-mentioned salts, indicated by a decrease of pH in the culture medium in a relatively short period of time (7-14 days of incubation at 28°C) as a result of organic acids production, efficiency correlated with an increased bacterial growth. Our study illustrates the efficiency of degradation of organic substances under the influence of acidophilic heterotrophic bacteria in the presence of the above mentioned salts.

Keywords: mining effluents, anions, organic substances, amylases.

Rezumat. Efectul compoziţiei sărurilor asupra activităţii amilolitice extracelulare a populaţiilor de *Acidiphilium* izolate din efluenţi minieri. Accentuarea gradului de poluare a mediului în urma exploatării miniere a determinat creşterea interesului pentru mecanismele de adaptare a microorganismelor şi mai ales pentru extinderea potenţialului de aplicaţii biotehnologice a acestora. Probele de ape şi sedimente miniere de la Roşia Poieni (jud. Alba) şi Baia (jud. Tulcea) au fost folosite pentru izolarea de populaţii de bacterii heterotrofe acidofile din genul *Acidiphilium* cu toleranţa crescută la ioni metalici (Fe^{2+}, Cu^{2+}, Zn^{2+}, Ni^{2+}). Acestea au fost testate cu privire la influenţa clorurilor şi sulfaţilor asupra profilului de creştere şi a activităţii de degradare extracelulară a amidonului în prezenţa cationilor (Ca^{2+}, Na^{+}) şi anionilor (Cl^{-}, SO_4^{2-}) existenţi în situsurile de prelevare a acestora. Rezultatele obţinute au evidenţiat diferenţe semnificative privind adaptarea celor două populaţii bacteriene la prezenţa în mediul de cultură a anionilor şi cationilor, fapt corelat cu condiţiile specifice situsului de origine al probelor. Populaţia izolată de la Baia prezintă o eficienţă sporită a degradării amidonului în prezenţa sărurilor menţionate anterior, observată prin scăderea valorii pH-ului în mediul de cultură într-un timp relativ scurt (7-14 zile de incubare la temperatura de 28°C) ca urmare a producerii de acizi organici, eficienţă corelată cu creşterea celulară a bacteriilor heterotrofe acidofile. Studiul de faţă a evidenţiat creşterea eficienţei procesului de degradare a substanţelor organice sub acţiunea bacteriilor heterotrofe acidofile, în prezenţa sărurilor existente în situsurile menţionate anterior.

Cuvinte cheie: efluenţi minieri, anioni, substanţe organice, amilaze.

INTRODUCTION

Research on ecology of extreme acidic environments revealed significant / considerable diversity among acidophilic microorganisms. These encompass acidophilic bacteria and archaea that perform the oxidation of sulphide ore as well as other microorganisms having synergistic or antagonistic effects on the process. The study of the interaction between acidophilic microorganisms is crucial for the development of mining technologies. Also, ecological studies have opened new horizons for understanding the biochemical processes that occur in the minerals leaching environments (AL-AZKI, 2006; 2010-2011; GONZALES TORIL et al., 2003).

Acidophilic bacteria are present as mixed populations both in natural and man-made environments. In many cases, their presence is indicated by their metabolic products or by biomass accumulation. These bacteria are widespread in the mining areas: ore and acid mine drainages, as well as the neighbouring soil. Acidophilic bacteria from the above-mentioned habitats are either heterotrophic or chemolithotrophic, being involved in the bioleaching, bioaccumulation, bioprecipitation and biofixation of metals. Their presence indicates negative changes in ecosystems, resulting from the activity of pollutants or other disturbing factors before these changes affect more evolved, higher organisms (CISMAŞIU, 2004; GIANFREDA & RAO, 2004; JOHNSON, 2003; SHAFAAT et al., 2011).

During the last decade, the discovery of acidophilic heterotrophic bacteria ability to degrade a variety of organic compounds from the inorganic synthesis products contributed to the development of bioremediation technologies. In order to estimate the contaminants biodegradability and to specify the result of a bioremediation strategy, as well as the potential risks to human health associated with the transport of contaminants in soil, it is crucial to understand the various processes affecting organic substances (CARLSON, 1998; DAS et al., 2010; JOHNSON, 2012).

It is also very important to understand the mechanisms through which acidophilic microorganisms are involved in metal accumulation, especially for the development of these microbial processes of concentration, removal or recovery of metals from aqueous solutions. Therefore, understanding the chemical and physiological reactions that take

place during the accumulation of metal ions may lead to a more effective efficient control of the process parameters in order to increase the retention rate and specificity of metals accumulation (BOND et al., 2000; SINGH et al., 2011).

It is widely accepted that the chemical and physiological reactions during retention of metal ions depend on the physiological conditions of microbial cells, the metals chemical state during interaction with cells and these are strongly influenced by the environment (JOHNSON, 2012; RAJVINDER et al., 2012).

Understanding the metabolic activity of the acidophilic heterotrophic bacteria represents a landmark in establishing the influence of physico-chemical conditions on the processes of metals sorption through their action on the degradation of organic substances in the environments polluted with metallic ions. Depending on the variation of essential parameters in their natural habitat (temperature, pH, degree of humidity concentration of O_2 and CO_2, Eh and metallic ions), morphological and physiological changes of indigenous acidophilic bacteria occur (CISMAŞIU, 2004; GIANFREDA & RAO, 2004).

In this context, due to the influence of physico-chemical factors on the development of acidophilic heterotrophic bacteria from the *Acidiphilium* genus, our study presents the effects of cations and anions on the starch extracellular degradation activity in the presence of these microorganisms.

MATERIAL AND METHODS

1. Microbial population and media

The investigated bacterial populations in this study (noted P_4 and P_7) were isolated from waters and mining sediment samples resulted from industrial activity in Baia (P_4) and Rosia Poieni (P_7) areas. Baia village is located in Tulcea county, situated approximately 300 km east of Bucharest and Rosia Poieni village is located in Alba county, approximately 280 km north-west of Bucharest. The investigated sites are characterized by high concentrations of metallic ions and isolated populations containing strains belonging to the *Acidiphilium* genus. The populations were isolated on GYE solidified culture media having pH value 3.0 before autoclaving. The medium has been solidified by using 20 g/l agar. When the liquid form of culture medium was used in experiments the growth of heterotrophic population of the *Acidiphilium* genus was recorded after three weeks of incubation at 28^0C. The growth of population has been demonstrated also by decreasing of pH value of GYE medium if compare with the initial value of 3.0 (HIRAISHI & IMHOFF, 2005).

2. Physical parameters and experimental conditions

The experiments have been conducted in 100ml volume Erlenmeyer flask, containing 50ml of culture medium related to each experimental variants (described in Table 1) and five ml of inoculums.

Table 1. Experimental variants.

Exp. no.	Medium composition
1.	GYE medium + 3g/l starch + 0.1% $CuCl_2$ + 0.1% $MgCl_2$
2.	GYE medium + 3g/l starch + 0.1% $CuCl_2$ + 0.1% $MgCl_2$ + 0.1% $CaCl_2$
3.	GYE medium + 3g/l starch + 0.1% $CuCl_2$ + 0.1% $MgCl_2$ + 0.1% NaCl
4.	GYE medium + 3g/l starch + 0.1% $CuSO_4$ + 0.1% $MgSO_4$
5.	GYE medium + 3g/l starch + 0.1% $CuSO_4$ + 0.1% $MgSO_4$ + 0.1% Ca SO_4
6.	GYE medium + 3g/l starch + 0.1% $CuSO_4$ + 0.1% $MgSO_4$ + 0.1% Na_2SO_4

The culture growth has been conducted for seven, 14 and 21 day. The temperature conditions during all experiments were 28^0C and the microbial culture were shacked at moderately value of 150rpm. In order to estimate the microbial growth, the optical density at 660 nm has been recorded spectrophotometrically.

3. Amylases activity assay

The starch degrading activity has been evaluated following the Wohlgemuth method (GUPTA et al., 2003). Regarding the study of raising the efficiency of the starch degradation process, the experiments were accompanied by chemical controls and biological controls (the P_4 population with low resistance to metallic ions).

The starch degradation by the extracellular enzymatic activity is influenced by the physiological characteristics of the acidophilic heterotrophic bacteria and was measured spectrophotometrically at 580nm using an Analytic Jena equipment.

RESULTS

To better understand the degradation mechanisms of organic materials from environments subject to metal ions contamination, we performed a comparative analysis of the influence that salts composition in mining effluents have on the amylolitic activity of heterotrophic acidophilic bacteria belonging to genus *Acidiphilium*. To this end, we isolated two bacterial populations from waste waters and mining sediments from Baia and Rosia Poieni, two highly polluted industrial regions where the metal ions concentration is much higher than the current accepted international standards.

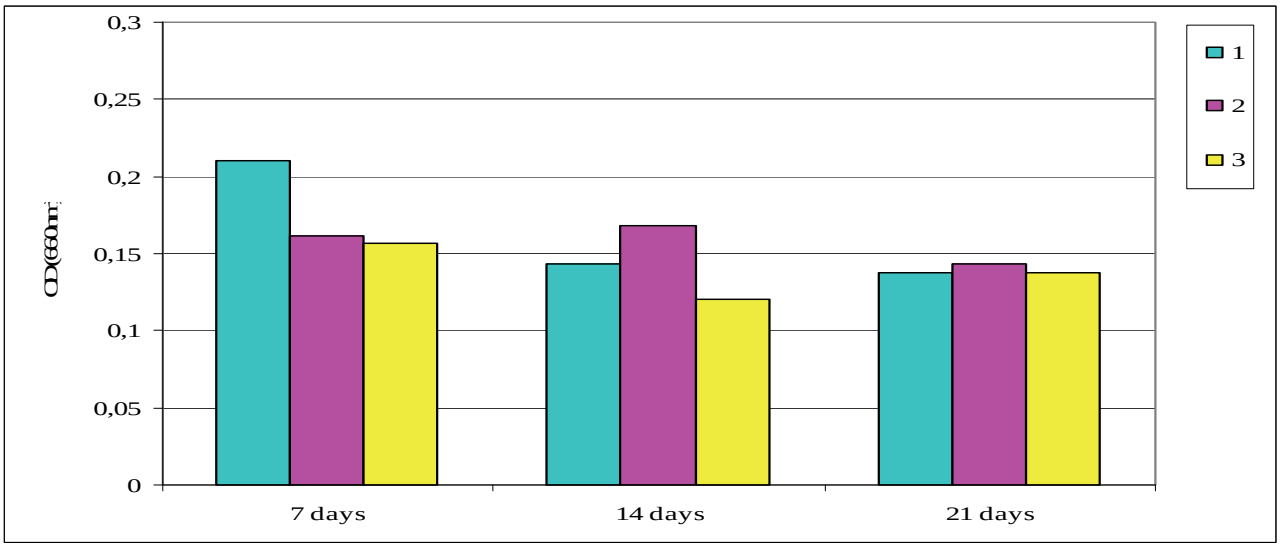

Figure 1. The influence of chloride from salts compositions on the growth profile of P_4 population.
The experimental conditions 1, 2, 3 are described in Table 1.

Data shown in figure 1 indicated that the growth of the microbial population P_4 from Baia site was less sensitive to the cations from salts (chloride) in culture medium. Thus, after seven days of incubation, in condition 1 (presence of Cu^{2+} and Mg) we recorded an optical density around 0.2 at 660 nm. A relatively similar value was observed in conditions 2 (containing calcium ion additionally to condition 1) and 3 (containing sodium), namely around 0.15 at 660 nm.

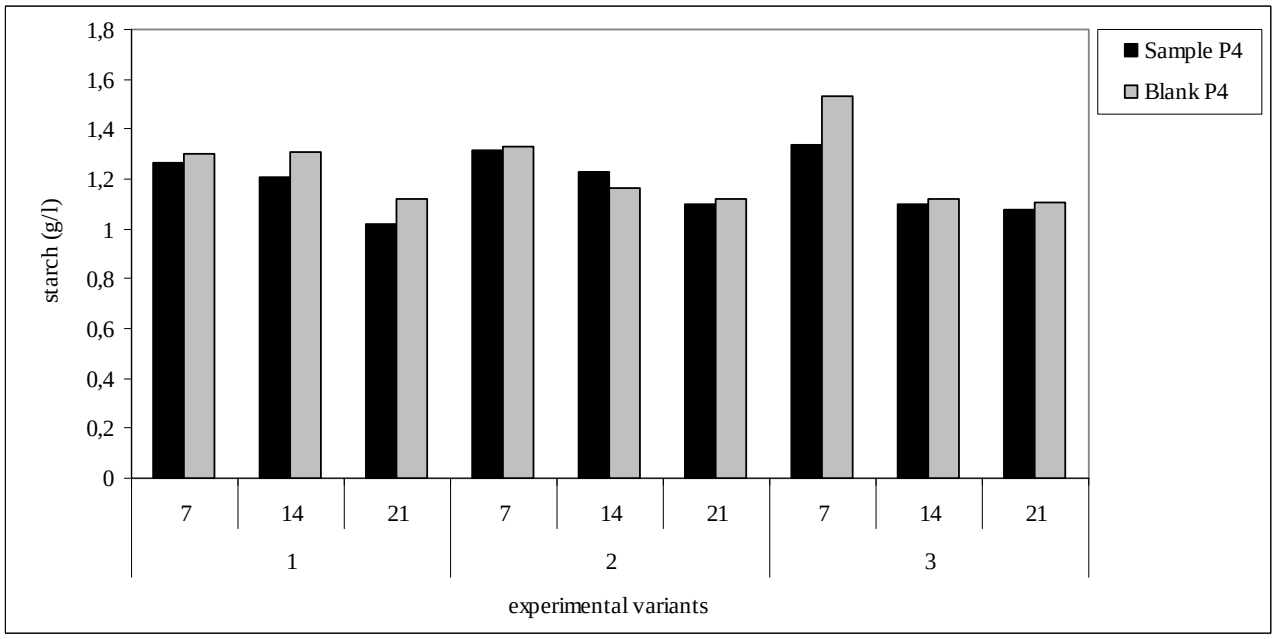

Figure 2. The influence of chloride from salts compositions on the extracellular amylases activity of the P_4 population.
The experimental conditions 1, 2, 3 are described in Table 1.

We further observed slightly differential effects induced by the presence of sodium and calcium on the growth kinetics of the microbial population P4: while calcium stimulated bacterial growth during the first 14 days, sodium had an adverse effect. On the contrary, in condition 1 and 3 the growth was diminished. After 21 days of growth, similar profiles were recorded under all conditions, with values close to the data obtained at 14 days. One exception was condition 3 where the presence of sodium appears to slightly stimulate the microbial population growth (Fig. 1).

Data showed in figure 2 revealed that extracellular amylase activity of the microbial population P_4 has a similar behaviour at seven, 14 and 21 days of cultivation. In condition 1 (absence of sodium and calcium chlorides), the activity values were higher and the presence of calcium (condition 2) and sodium (condition 3) induced a slight decrease in activity (Fig. 2).

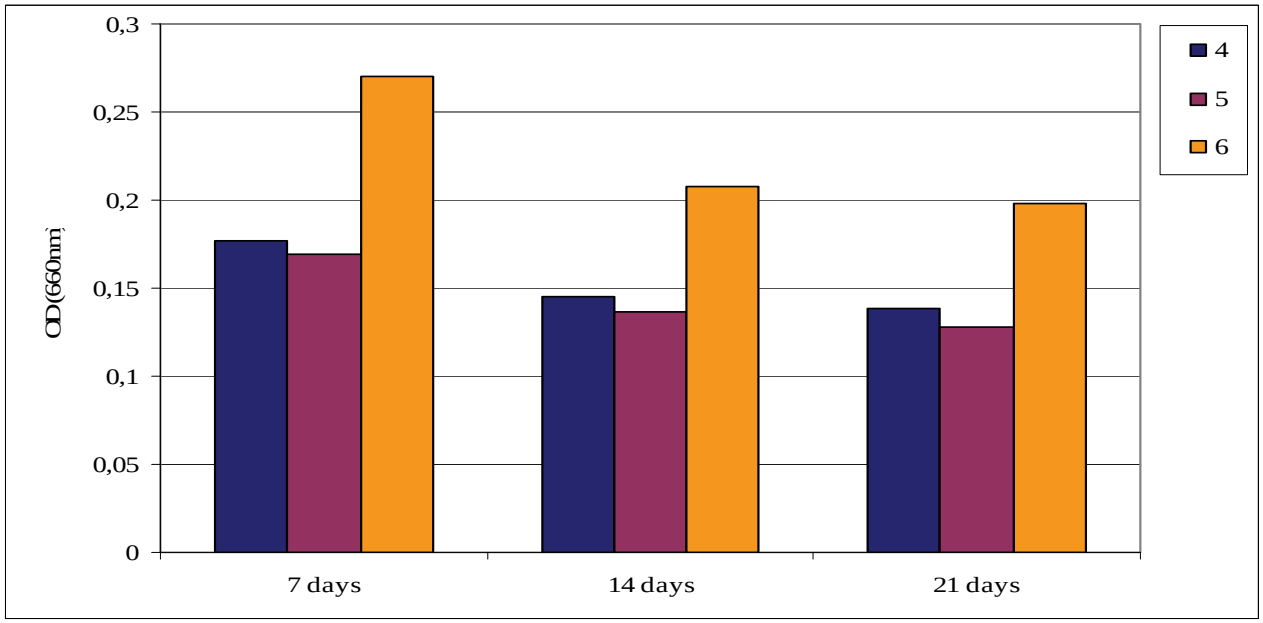

Figure 3. The influence of sulphates from salts compositions on the profile growth of the P_4 population.
The experimental conditions 4, 5, 6 are described in Table 1.

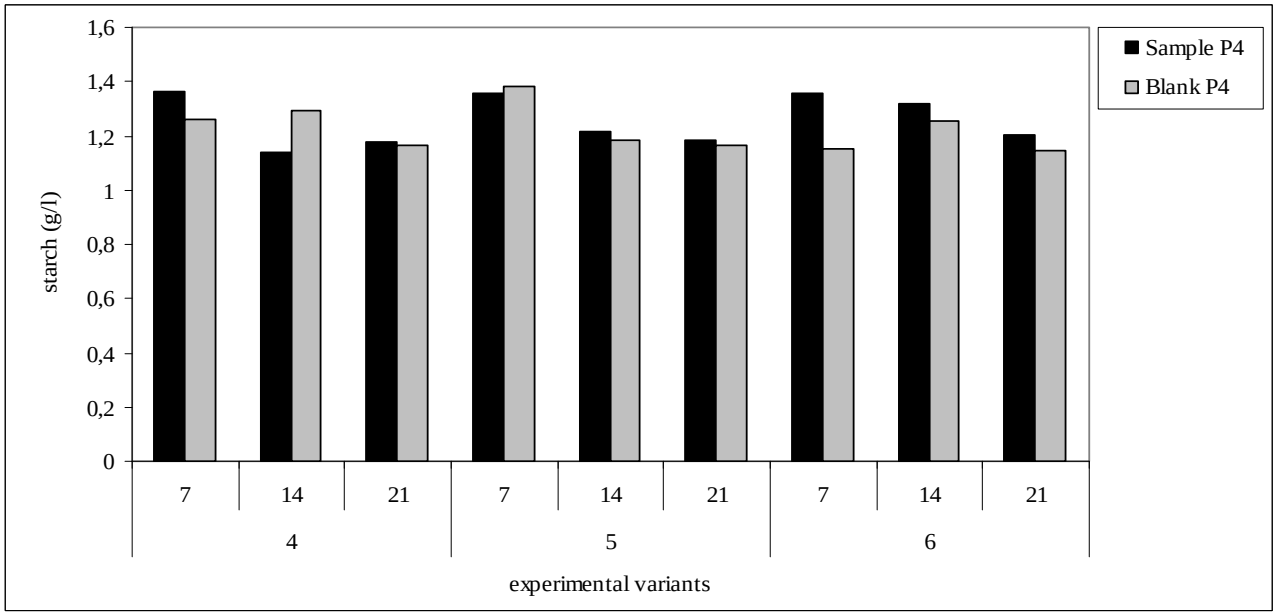

Figure 4. The influence of sulphates from salts compositions on the extracellular amylases activity of the P_4 population.
The experimental conditions 4, 5, 6 are described in Table 1.

Figure 3 shows how the presence of cations as sulphates salt in culture medium influences the growth of the microbial population P_4. Thus, the best growth was obtained in the presence of sodium sulphate (condition 6) after seven days of incubation.

When culture medium contained sodium sulphate, after 14 and 21 days of cultivation the growth of population P_4 decreased. Similar behaviours were observed in other experimental conditions, namely the presence of calcium sulphates (5) and absence of sodium and calcium sulphates (4).

The extracellular amylases activity measured in the presence of sulphates in culture medium (Fig. 4) of the microbial population P_4 revealed a similar profile compared to the activity in the presence of chlorides (Fig. 2). Thus, the maximum values are recorded in the absence of sodium and calcium at seven days of growth.

The data from figure 5 reveals that growth of microbial population P_7 (isolated from Roșia Poieni) is more strongly affected by the chlorides in culture medium. Similarly to the population P4, the most effective growth was observed in the absence of calcium and sodium chloride at seven days of cultivation (Fig. 5). After this period, the growth is diminished excepting the growth condition 3 (presence of sodium chloride) at 14 days of cultivation (Fig. 5).

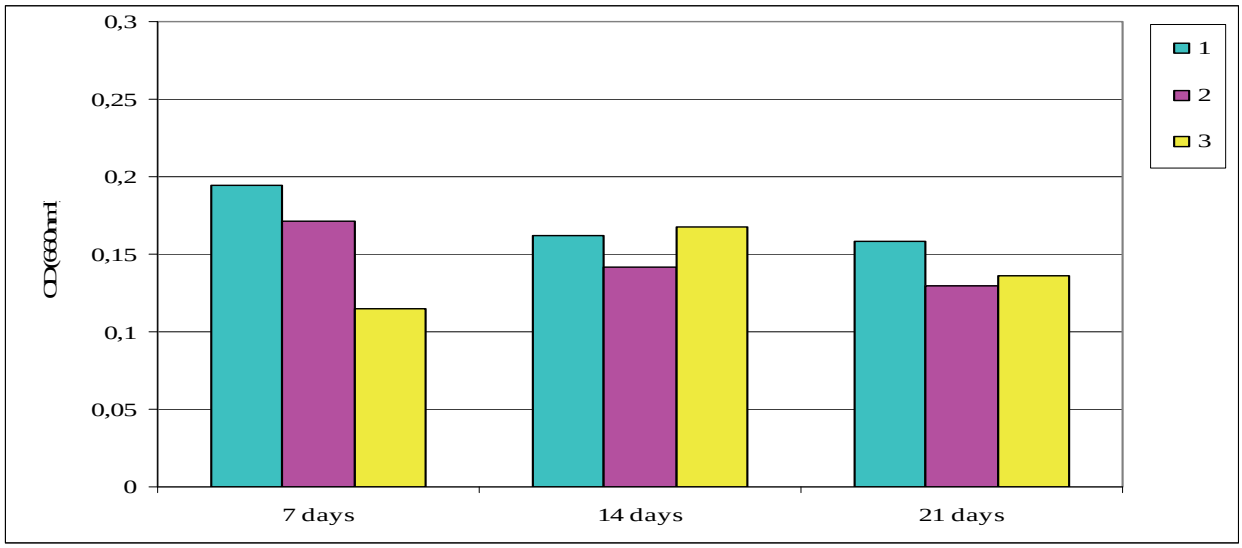

Figure 5. The influence of chloride from salts compositions on the profile growth of the P_7 population. The experimental conditions 1, 2, 3 are described in Table 1.

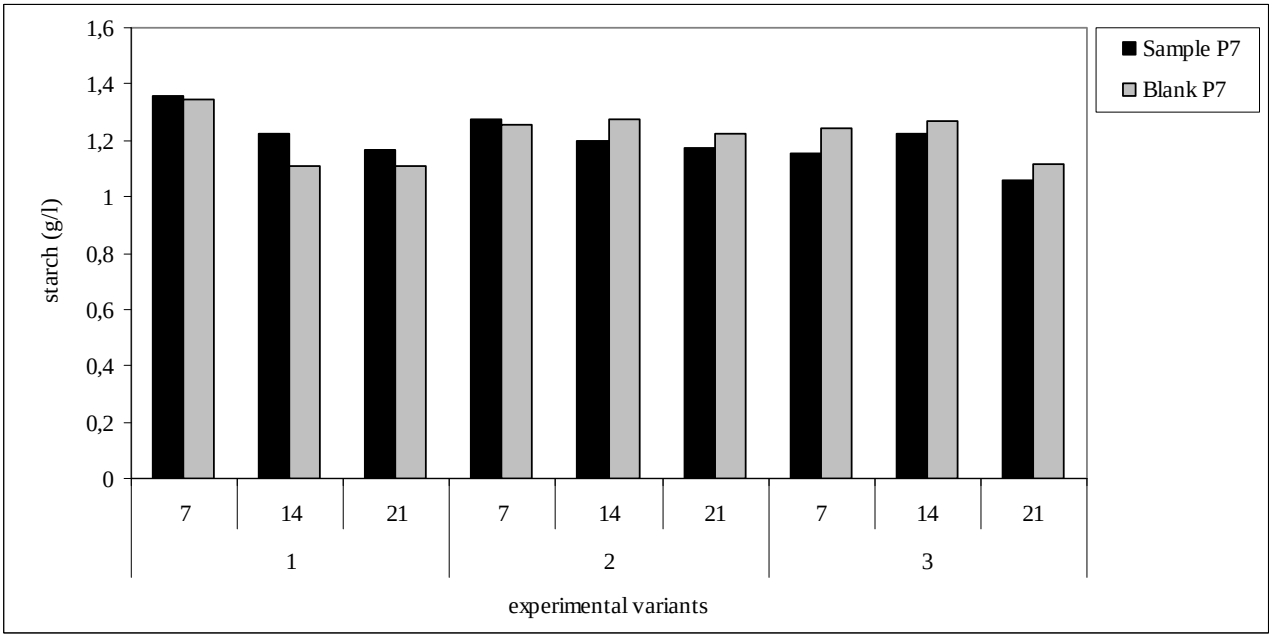

Figure 6. The influence of chloride from salts compositions on the extracellular amylases activity of the P_7 population. The experimental conditions 1, 2, 3 are described in Table 1.

Similar results were observed for the amylase activity in the presence of chlorides in culture medium for the microbial population P_7 if compare with data from figures 2 and 4. The high values are noted in the absence of sodium and calcium and the activity decreases with incubation time (Fig. 6). One exception could be observed at 14 days of incubation in the presence of sodium chlorides.

The results showed in figure 7 reveal that sulphates have a different effect on the growth of the microbial population P_7 if compared to the microbial population P_4 (Fig. 3). Thus, comparative studies about the profile growth of the microbial population P_7 is good in the absence of sodium taking into account that no influence was observed when comparing with data recorded for the experimental conditions 4, 5 and 6 in figure 7. A slight influence of sodium sulphates on the growth of the microbial population P_7 could be regarded as an exception (Fig. 7).

The extracellular amylase activities of the microbial population P_7 follow the behaviour recorded in the case of the presence of chlorides in culture medium (Fig. 6) or similar with the population P_4 (Figs. 2 and 4). Similar behaviours of amylase activity in the presence of various ratios between sodium and magnesium have been previously reported from halophytic archaea isolated from various natural salt lakes (ENACHE et al., 2009).

The literature data revealed that such kind of microorganisms survive in harsh environments where they exist developing a variety of strategies (ENACHE et al., 2001; ENACHE & FAGHI, 1999; GIANFREDA & RAO, 2004).

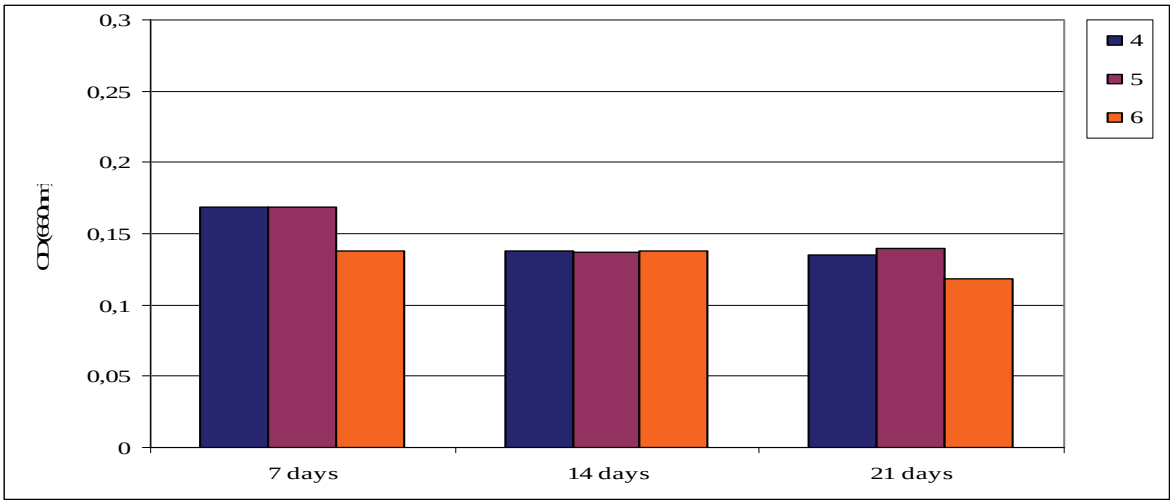

Figure 7. The influence of sulphates from salts compositions on the profile growth of the P_7 population.
The experimental conditions 4, 5, 6 are described in Table 1.

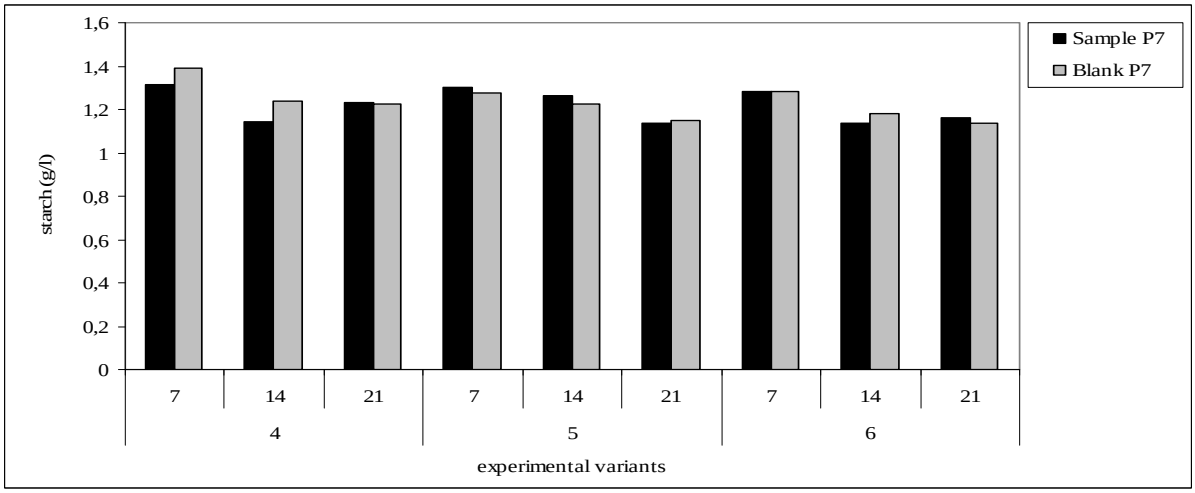

Figure 8. The influence of sulphates from salts compositions on the extracellular amylases activity of the P_7 population.
The experimental conditions 4, 5, 6 are described in Table 1.

The enzyme of these types of extremophilic microorganisms are able to cope with high ionic strength in the presence of salt mixtures, and their stability in harsh pH conditions could constitute an advantage for exploitation in several biotechnologies destined to environmental protection and historically polluted area decontamination (AL-AZKI, 2010-2011; DAS et al., 2010; ENACHE et al., 2001; GIANFREDA & RAO, 2004).

CONCLUSIONS

Our results bring an important contribution to understanding the correlation between specific environmental conditions and corresponding bacterial strains.

The two bacterial strains have different growth profiles and different sensitivity to chloride and sulphate from salts. Sulphate salts stimulates growth of the microbial population from Baia site (P_4) from an initial 70% increase to 50% after longer periods of time (21 days).

On the contrary, chloride has a moderate influence on bacterial growth in the case of P_4 population (25-30% after 7 days) and the effect is not maintained after 21 days. Also, our results show a different growth pattern of P_4 population in environments with different cations: while $CaCl_2$ slightly stimulates growth after 14 days, NaCl has an inhibitory effect.

On the other hand, P_7 population from Roşia Poieni site has an improved growth in the presence of chloride compared to sulphates although the effect is lost after longer times (21 days). Comparative analysis regarding the influence of cations on the P_7 population growth profile shows a stimulative effect of Na^+, especially after 14 days (30%).

Continuous agitation conditions in the presence of 3g/l starch induce an increased efficiency of extracellular amylases from P_4 population when chloride is present in the environment, while sulphate salts have a moderately

inhibitory effect. In case of chloride, extracellular amylolitic activity is stimulated by the presence of calcium ions in the culture medium, when compared to sodium ions.

When studying the influence of salts (sulphates and chlorides) on starch degradation, we observed a stimulation of enzymatic activity in the presence of P_7 population, which reflects the adaptation of these bacteria to the culture conditions.

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

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