THE INFLUENCE OF PHYSICO-CHEMICAL PARAMETERS ON EXTRACELLULAR HYDROLASES FROM *Acidiphilium* SPECIES, ISOLATED FROM ACID MINE DRAINAGE

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Abstract. Amylases are one of the most important and oldest industrial enzymes. The capability of the acidophilic heterotrophic bacteria to product amylases represents an adaptation to the physico-chemical conditions from mining biotopes. In our study, the investigations focused on acidophilic heterotrophic bacteria belong to the genus *Acidiphilium* due to its ability to develop in such polluted area and the potential bioremediation application. The influence of physico-chemical parameters on extracellular hydrolase's production is related to the bacterial growth of the genus *Acidiphilium*. The comparative study of the amylolitic activity of *Acidiphilium* sp. at different growth temperatures conditions revealed the highest activity at 28°C and pH 3, meaning that these would be the best conditions in the natural environment for maximum extracellular enzymatic activity of the genus *Acidiphilium*. Also, the optimum yeast extract concentration was 0.2%, while values lower than 0.1% inhibited the extracellular hydrolase's activity.

Keywords: extremophiles, *Acidiphilium* sp., acidophilic protein, bioremediation.

Rezumat. Influenţa parametrilor fizico-chimici asupra hidrolazelor extracelulare de la speciile de *Acidiphilium* izolate din drenaje miniere acide. Amilaza este una din cele mai importante şi vechi enzime industriale. Capacitatea bacteriilor heterotrofe acidofile de a produce amilaze reprezintă o adaptare la condiţiile fizico-chimice din biotopurile miniere. În acest studiu, investigaţiile s-au axat pe bacterii heterotrofe acidofile ce aparţin genului *Acidiphilium* datorită abilităţii lor de a se dezvolta în zone poluate cu metale grele şi potenţialului de aplicare în bioremediere. Influenţa parametrilor fizico-chimici asupra producerii de hidrolaze extracelulare este corelată cu creşterea bacteriilor din genul *Acidiphilium*. Studiile comparative ale activităţii amilolitice a speciilor de *Acidiphilium* în diferite condiţii de temperatură arată că cea mai crescută este la 28^{0}C şi pH 3, însemnând că acestea pot fi cele mai bune condiţii naturale de mediu pentru activitatea enzimatică extracelulară maximă a genului *Acidiphilium*. De asemenea, concentraţia optimă de extract de drojdie a fost 0,2%, în timp ce valori mai mici de 0,1% au inhibat activitatea hidrolitică extracelulară.

Cuvinte cheie: extremofile, *Acidiphilium* sp., proteine acidofile, bioremediere.

INTRODUCTION

The environment is polluted by numerous organic and inorganic compounds, heavy metals in particular. Rapid industrialization has led to increased disposal of heavy metals and radionuclides into the environment. Heavy metal resistance is a widespread attribute among acidophilic heterotrophic bacteria isolated from mining environments. The high incidence of heavy metal resistance detected indicates the potential of these bacteria as bioremediation agents (DJUKIE & MANDIC, 2006; TAPIA et al., 2009; RAMPELOTTO, 2010).

Water pollution is a major global problem. Physical-chemical processes in use for heavy metal removal from wastewater include precipitation, coagulation, reduction processes, ion exchange, membrane processes and adsorption. The unique properties of acidophilic heterotrophic bacteria are their metabolic activity in highly acidic environments and their heavy metal resistance. Therefore, acidophilic heterotrophic bacteria could be used to degrade organic pollutants in acidic wastewaters (RODRIGUEZ & DIAZ, 2009).

Microorganisms that live in extreme environments are resource for industrial biotechnology, either as intact and active cells, in pure culture and consortia, or as sources of extremozymes. The interest in these enzymes is not limited by their industrial application they can be also used as a model system for the study of stabilization and enzyme activation mechanisms research of structure functional protein properties, which provide catalysis under extreme condition (KOZLOV & ZVEREVA, 2007; MOROZKINA et al., 2010).

Extracellular enzymes are those enzymes that are completely dissociated from the cell and found free in the surrounding medium. Among the physical parameters, the pH of the growth medium plays an important role by inducing morphological change in the organism and in enzyme secretion. The use of acid-stable amylases is an actively developed modern field of extremophilic bacteria application (VAN DER MAAREL et al., 2002; GUPTA et al., 2003).

The acid mine drainage is one of the main problems associated with mining activities. One of the effects is the increase in heavy metal solubility, which results in the accumulation of these toxic elements in the environment. In consequence these sites become inhospitable and just acidophilic heterotrophic bacteria able to tolerate the acidity and the high concentration of heavy metals can survive (SILVER & PHUNG, 2009; CIŞMAŞIU, 2004; JOHNSON & HALLBERG, 2005).

Amylases are among the most important enzymes with a wide variety of industrial applications. These enzymes display highest specificity towards starch followed by amylase, amylopectin, cyclodextrin, glycogen and maltotiose. α-Amylase is an inducible enzyme and is generally induced in the presence of starch or its hydrolytic product. Higher concentrations of glucose or complex organic supplements, which are commonly used in most heterotrophic media, completely inhibited growth (PANDEY et al., 2000; MOROZKINA et al., 2010).

The ability of heterotrophic bacteria to adapt to acidic environments is highly exploited in bioremediation of mining effluents containing high concentrations of heavy metals. Among the extracellular enzymes involved in metal biosorption, extracellular amylases are one of the most important and widely used in biotechnologies. Most of amylases are known to be metal ion-dependent, namely divalent ions like Ca^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+}, Fe^{2+} (SIVARAMAKRISHNAN et al., 2006; HAFERBURG & KOTHE, 2007; GADD, 2010).

The capacity of *Acidiphilium* species to utilize a variety of organic substrates and to reduce Fe^{3+} indicates that they might be of ecological significance in bioleaching environment. Furthermore, *Acidiphilium* species are used in many commercial bioleaching operation sites and they are believed to play a crucial role in the bioleaching processes. Additionally, *Acidiphilium* sp. can remove the organic materials produced by acidophilic chemolithotrophic bacteria and thus enhance the leaching efficiency of ores (HIRAISHI & IMHOFF, 2005).

Acidophilic bacteria have effective mechanisms to maintain intracellular ionic homeostasis and keep pH values close to neutral inside cells. Therefore, a high acidity of medium indicates a high developmental and activity level for these microorganisms. The active process of metal removal by living cells is referred to as bioaccumulation and the passive sorption of the dead cell walls is considered biosorption (JOHNSON & HALLBERG, 2005; RAMPELOTTO, 2010).

A special importance has been given to the microbial diversity which represents an ecological source for selecting adequate microorganisms for the environment bioremediation. Recently, we isolated several strains from mining effluents of two different areas from Rosia Poieni (Alba dept.) and Ilba (Maramures dept.) sulphidic mines in Romania (CIŞMAŞIU, 2004).

In our study, the investigations focused on the heterotrophic *Acidiphilium* sp. due to its ability to develop in such polluted area and the potential bioremediation application. Strains isolated in previous studies were used to investigate its extracellular hydrolases, this being the first attempt in Romania (CIŞMAŞIU, 2010).

Acidophilic heterotrophic bacteria, identified after the morphological aspects and physiological characteristics as being part of the genus *Acidiphilium*, were highly adapted to these polluted environments, the study of extracellular hydrolases became more necessary. This current work focused on the study of extracellular hydrolysis from two of the *Acidiphilium* species isolated from acidic mining effluents, considering their putative applications in bioremediation.

MATERIAL AND METHODS

Types of the analysed microorganisms

This study presents the influence of yeast extract concentrations, contact time, temperature on extracellular hydrolases of mesophilic and acidophilic *Acidiphilium* strains isolated from mining effluents of Roşia Poieni and Ilba areas. On the NP-starch medium (pH 3.0), it was revealed the existence in analysed samples of the heterotrophic acidophilic bacteria belonging to the genus *Acidipihilium* (LOBOS et al., 1986). It is obvious the influence of the increased pH values of the samples analysed on the presence and numerical density of the acidophilic heterotrophic bacteria of the *Acidiphilium* genus (CIŞMAŞIU, 2008; HIRAISHI & IMHOFF, 2005; TAPIA et al., 2009).

The strains and populations of acidophilic heterotrophic bacteria belong to *Acidiphilium* sp*.,* were analysed having in view the growth of the bacterial cultures in the specific culture media. The presence of acidophilic heterotrophic bacteria was revealed after the incubation for 21 days at 28^0C on the liquid GYE medium by the appearance of sediment, by the medium turbidity and the decrease under 2.0 of the initial pH value of the medium (pH 3).

Cellular and colony morphology of the isolated strains were characterized using a phase contrast microscope. The isolated strains were characterized regarding the Gram staining, cellular morphology and biochemical features (oxidase and catalase activity, biosynthesis of organic acids). The determination of catalase production by the isolated strains was performed by emulsified of a loopful of the culture in few drops of 3% hydrogen peroxide. If the hydrogen peroxide effervesces, the bacteria tested are catalase positive.

Depending on their physiological features, acidophilic heterotrophic bacteria were grown in specific media. For selection acidophilic heterotrophic bacteria were cultivated in two types of the NP-starch medium (LOBOS et al., 1986) with 0.1% yeast extract (medium I) and 0.2% yeast extract (medium II) with pH 3, with incubation at 28^0C for 21 days.

Experimental methods

To establish the best conditions of producing α-amylase the following physic-chemical parameters were tested: the incubation temperature (20^0C, 24^0C and 28^0C), pH value of culture medium (3.0, 4.0 and 5.0) and yeast extract concentration (1-2 g/l).

The temperatures were considered 20°C, 24°C and 28°C, in accordance with the variations of this parameter in the effluents' environment. Bacterial growth was carried out at different temperatures and pH for up to 21 days, under stirring or static conditions, and in the presence of various concentrations of yeast extract. The optical density of the bacterial cultures has been determined at 7 days periods, at spectrophotometer (660 nm).

The amylolitic activity of the extracellular hydrolyzing enzymes in the culture media was measured spectrophotometrically, using Wohlgemuth method (GUPTA et al., 2003), by monitoring the starch-iodine complex hydrolysis. These are based on decrease in starch-iodine intensity colour, increase in reducing sugars, degradation of colour-complexed substrate and decrease in viscosity of the starch suspension.

The quantitative test of the hydrolysis starch based on determination the activity of amylase in 1% starch presence and reading spectrophotometer at 580 mm at a 7 days interval. The enzymatic activity was expressed in units Wohlgemuth/ml (GUPTA et al., 2003). In the experiment of establishing the best starch concentration, the best parameters previously determined were observed: pH 3 and incubation temperature 28^0C.

RESULTS AND DISCUSSION

Heavy metal resistance is a widespread attribute among bacteria isolated from mining effluents of two different areas of the county (Rosia Poieni-Alba dept. and Ilba-Maramures dept.), acidophilic heterotrophic bacteria are associated with *Thiobacillus ferrooxidans* strains isolated from a wide variety of sources. Certain conditions which support the growth of *T. ferrooxidans*, such as acidic, mesophilic, aerobic, ferrous iron or other mineral sulfide substrate, are suitable for the perpetuation of some heterotrophic consorts. It has been shown that *T. ferrooxidans* cells excrete organic compounds, such as pyruvate, glutamate, aspartate, serine, glycine and other amino acids, which these heterotrophs can use as substrates for the bacterial growth (CIŞMAŞIU, 2008; HIRAISHI & IMHOFF, 2005).

According to other scientists α-amylases are active at the optimal growth temperature of the microorganism which has produced them. Agitation intensity influence the oxygen transfer rates in many bacterial fermentations and thus influences bacterial morphology and product formation. It has been reported that a higher agitation speed is sometimes benefited to bacterial growth and thus may increase enzyme production. However, it is reported that the variations in bacterial morphology as a consequence of changes in agitation rate do not affect enzyme production at a constant specific growth rate (CIŞMAŞIU, 2004; GUPTA et al., 2003).

In order to test the physico-chemical parameters on the dynamics starch hydrolysis, there were selected *Acidiphilium* populations, isolated from mining acidic water and sediments at Roşia Poieni and Ilba areas, having a heavy metabolic activity in NP-starch medium. The results referring to the influence of yeast extract concentrations (1-2g/l), contact time (7, 14, 21days) and acidity (3.0, 4.0; 5.0), and on the growth and extracellular enzymatic activity of two different *Acidiphilium* populations, cultivated on selective NP-starch medium, are represented in figures 1 and 4.

Among the physical parameters, the pH of the growth medium plays an important role by inducing morphological change in the organism and in enzyme secretion. The pH change observed during the growth of the organism also affects product stability in the medium. The pH values serves as a valuable indicator of the initiation and end of enzyme synthesis. The comparative study of the extracellular hydrolytic activity of *Acidiphilium* sp. at different growth pH conditions revealed the highest activity at pH 3, confirming the acidophilic characteristics of this genus (Figs. 1; 2).

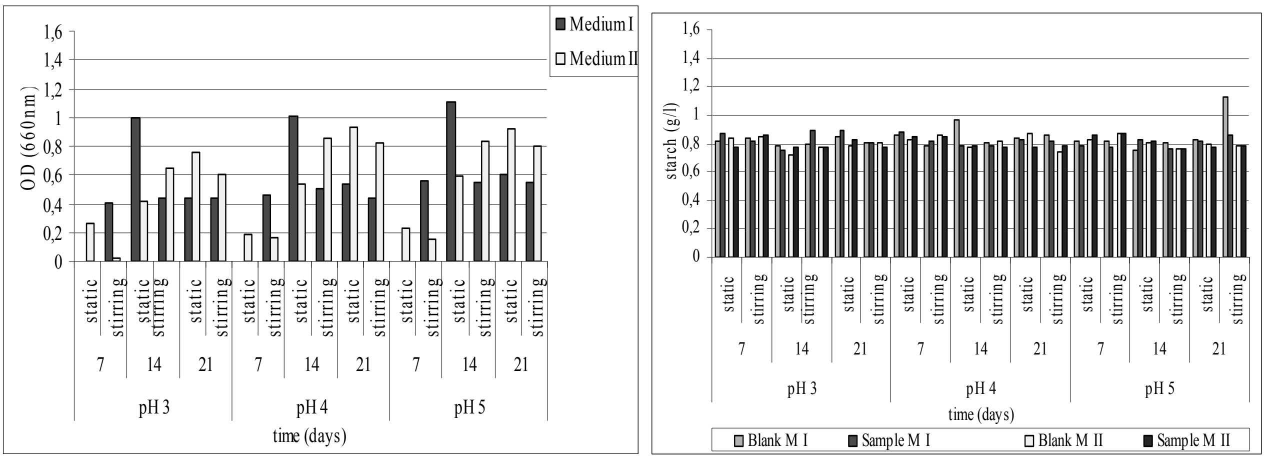

Figure1. The dynamics of bacterial and chemical starch hydrolysis in presence of the *Acidiphilium* P_4 population in medium with pH (3; 4; 5) and yeast extracts concentrations (1-2g/l) for several incubation periods (7, 14, 21 days).
Figura 1. Dinamica hidrolizei bacteriene şi chimice a amidonului în prezenţa populaţiei de *Acidiphilium* P_4 în mediu cu pH (3; 4; 5) şi concentraţii diferite de extract de drojdie (1-2g/l) la diverse perioade de incubare (7, 14, 21 zile).

Due to the increasing pH values from 3.0 to 4.0 it can be noticed the intensification of the extracellular hydrolytic activity of *Acidiphilium* cultures, emphasized by the quantity diminution of the starch in medium, used by acidophilic heterotrophic bacteria as a source of carbon and energy. On the other hand, simultaneously with the pH increase there is a significant diminution of the starch chemical hydrolysis (Fig. 2).

The enzymatic activity increased when cells were cultivated under stirring conditions as compared to static conditions. Stirring conditions determine a higher oxidation activity of bacterial populations, correlated with a higher growth level. The experiments evidenced that the P_7 population had the most hydrolytic activity (Figs. 2-4).

Under these conditions, the highest extracellular hydrolytic activity of both *Acidiphilium* populations was obtained at 24°C and pH 3, while at 20^0C the microbial hydrolysis was negligible, as expected for a mesophilic species. The tests realized under optimal conditions of temperature (24^0C) and pH (3) evidenced the presence of a significant activity at yeast extract 1 g/l, in comparison with the chemical control. Therefore, at the initial concentration higher than 1g/l, the starch concentrations remained in culture liquids are close to the one determined at the chemical control at the end of the experiment (Figs. 1-4).

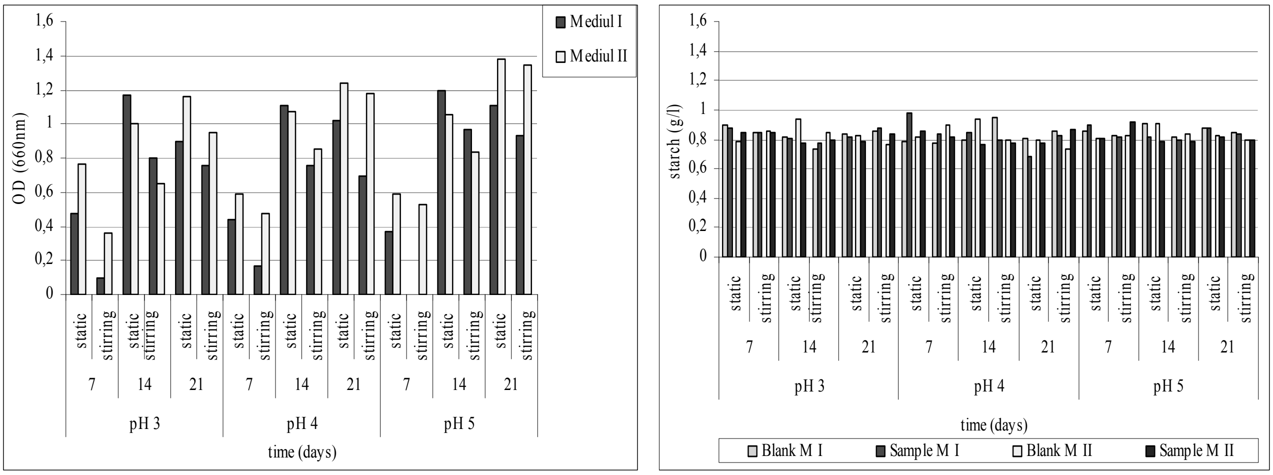

Figure 2. The dynamics of bacterial and chemical starch hydrolysis in presence of the *Acidiphilium* P_7 population in medium with pH (3; 4; 5) and yeast extracts concentrations (1-2g/l) for several incubation periods (7, 14, 21 days).
Figura 2. Dinamica hidrolizei bacteriene şi chimice a amidonului în prezența populației de *Acidiphilium* P_7 în mediu cu pH (3; 4; 5) şi concentrații diferite de extract de drojdie (1-2g/l) la diverse perioade de incubare (7, 14, 21 zile).

At the temperature of 24^0C, from the tested pH values, at pH 3 the two bacterial populations showed a heavier enzymatic activity in the 21 days of testing, in comparison with the cultures with a pH 5.0. In this way, after 21 days of testing the microbial cultures hydrolyzed 15.3g/l starch, comparatively with 7.3g/l at pH 5.0. After 14 days of incubation the extracellular hydrolytic activity of microbial cultures maintained a lower level than in the first part of the experiment (Figs. 3; 4).

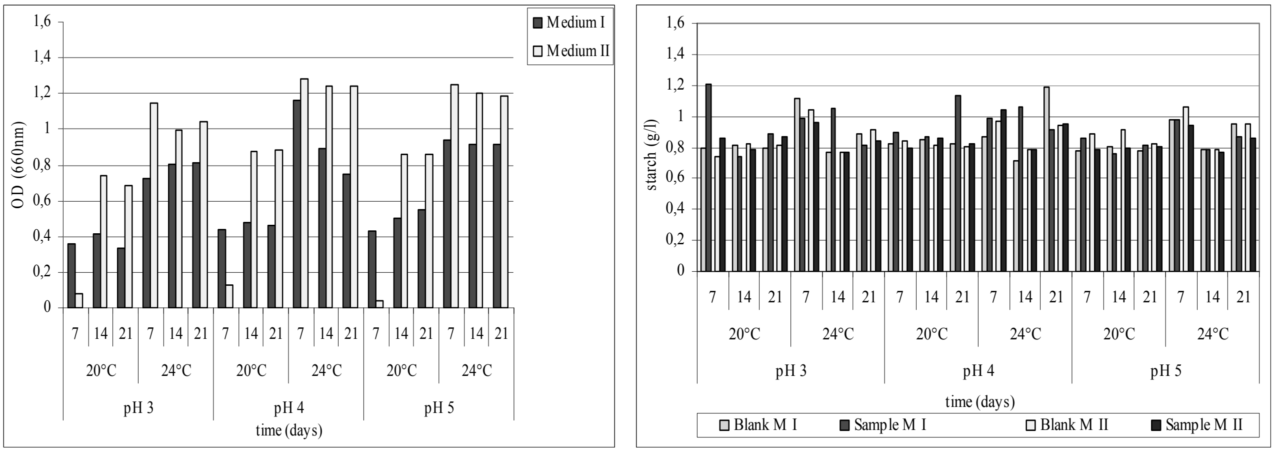

Figure 3. The growth and the starch hydrolyzing activity of the *Acidiphilium* P_4 population under increased yeast extracts concentrations at different temperature and pH values for several incubation periods.
Figura 3. Creşterea şi activitatea de hidroliză a amidonului a populației de *Acidiphilium* P_4 în prezența concentrațiilor crescute de extract de drojdie la diferite valori de temperatură, pH şi perioade de incubare.

Though as a result of temperature increase, the chemical hydrolysis of starch intensifies the difference between these and the one catalyzed by microbial cultures is obviously at 28^0C, due to the much heavier metabolic activity of *Acidiphilium* cultures during the same testing period. Another noticed factor was the pH value of the culture medium, whose influence on starch hydrolysis is shown in figures 1-4.

According to other scientists, generally, α-amylases are active at the optimal growth temperature of the bacteria which has produced them (PANDEY et al., 2000; SIVARAMAKRISHMA et al., 2006; MOROZKINA et al., 2010). Previous studies have proved that the optimal growth temperature for the tested strains in this paper is still the one of 28^0C (CIŞMAŞIU, 2008).

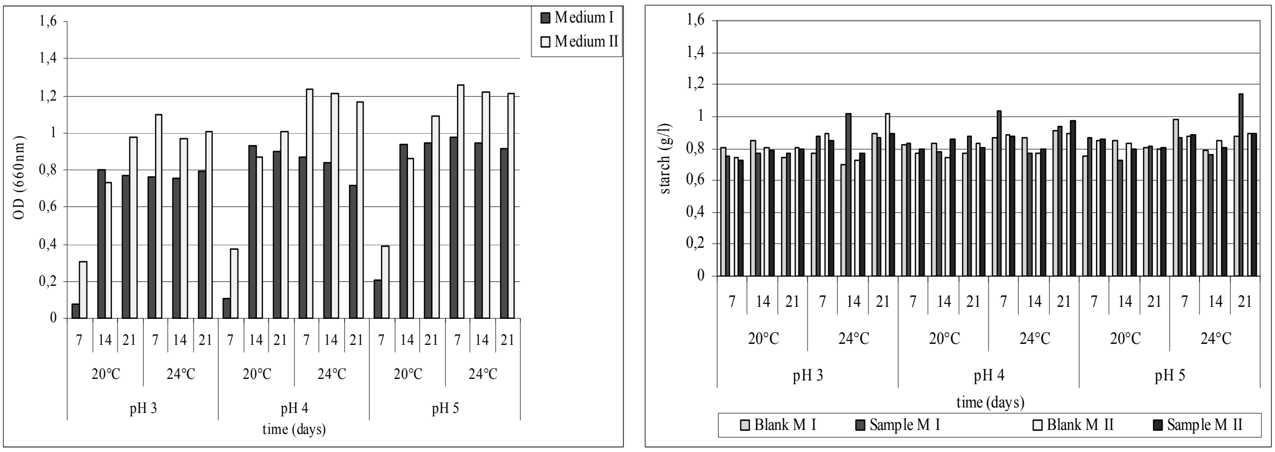

Figure 4. The growth and the starch hydrolyzing activity of the *Acidiphilium* P_7 population under increased yeast extracts concentrations at different temperature values and pH values for several incubation periods.
Figura 4. Creşterea şi activitatea de hidroliză a amidonului de către populaţia P_7 de *Acidiphilium* în prezenţa concentraţiilor crescute de extract de drojdie la diferite valori de temperatură, pH şi perioade de incubare.

CONCLUSIONS

In order to test the physico-chemical parameters on the dynamics starch hydrolysis, there were selected *Acidiphilium* populations, isolated from mining effluents of Roşia Poieni and Ilba areas, having a heavy metabolic activity in NP-starch medium.

The extracellular amylases are active in a wide range of pH with its maximum activity at pH values 2.5-3.0. The pH change observed during the growth of the organism also affects product stability in the medium. The acid production activity by acidophilic heterotrophic bacteria increased with cellular growth, the lowest pH values being obtained after 14 days of incubation.

This enzymatic activity was dependant on the yeast extract concentration, with an optimum at 0.2%, while for values lower than 0.1% the extracellular hydrolysis activity was absent. Amino acids in conjunction with minerals have also been reported to affect α-amylase production.

Physico-chemical characteristics and the diversity of the acidophilic heterotrophic bacteria in the acidic water samples suggested that the biogeochemical properties, pH and temperature, could be key factors in controlling the structure of the bacterial population.

There is a strong relationship between the acidity of the medium and the amylolitic activity of the acidophilic heterotrophic bacteria. Results showed that hydrolyzing activity detected in this work indicates the potential of these bacteria as bioremediation agents.

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