The influence of pH and temperature on the enzymatic activity of acidophilic heterotrophic microorganisms of the genus Acidiphilium

CARMEN MADALINA CISMASIU[1]

Keywords: extremophiles, acidophilic bacteria, bioremediation, acid mine drainage

1 Introduction

The study of acidophilic microorganisms has gained increasing importance due to their adaptations to cope with extreme environments (Baker & Banfield 2003, Johnson & Hallberg 2005) but also due to their biotechnological applications in bioremediation of polluted areas or metallic ions recovery from low-grade ores and wastewater mine drainage (Johnson 2003, Johnson & Hallberg 2003). The ability of these microorganisms to adapt to various environmental conditions is extremely useful also for their use in the biosorption of metals from acidic mining effluents (Carlson 1998, Johnson 1998).

The tolerance of acidophilic bacteria to low pH (1-4) is the result of their adaptation, these species being able to maintain their cytoplasm pH near the neutral value (Matzke et al., 1997). Their enzymatic activity is influenced by the acidity and temperature levels (Norris & Johnson 1998, Gupta et al. 2003); on their turn, through their metabolic activity, the acidophilic bacteria modify the oxide-reduction potential of the environment (Cismasiu 2001, 2004, Cismasiu et al. 2004)

Acidiphilium is a genus, belonging to Eubacteria, phylogenetic division α-Proteobacteria, order Acetobacteraceae; its physiology is close to *Acidocella*, the two genera being frequently isolated from the same acidic habitats (Cismasiu 2004). Due to its heterotrophic nutrition, *Acidiphilium* has the possibility to develop in organic media (with different concentrations of proteins or sugars). As carbon source it uses many different carbohydrates such as: fructose, glucose, sucrose, maltose, cellobiose, xylose, starch and glycogen (Cismasiu et al. 2000, 2002, Dopson et al. 2003).

The aim of the study was to identify new organisms able to cope with the extreme environmental conditions from the mining areas and to isolate them in order to study their bioremediation potential. After isolating more strains from the acid effluents of the Ilba mining area (Maramureş, Romania), identified according to the morphological and physiological characteristics as being part of *Acidiphilium* genus (Cismasiu 2008), the study focused on their metabolic activity.

Since *Acidiphilium* is both a mesophilic and acidophilic microorganism, the influence of temperature and pH on its enzymatic activity was investigated. This paper presents the influence of these two parameters on the amylolitic activity of *Acidiphilium*; starch was selected as a substrate, representing both the carbon and the energetic source.

2 Material and Methods

Ilba mining area is located in the Northern part of Romania, near the border with Hungary and Ukraine, in Maramures county (Fig.1); although the mine was closed in 2001, acidic wastewaters still represent an environmental threat, especially due to their low pH and high heavy metals content, leading to the degradation of local terrestrial and aquatic habitats.

1 Institute of Biology, Romanian Academy, Splaiul Independentei no. 296 sect.6, RO-060031 Bucharest, Romania. e-mail: madalinabio@yahoo.com

Figure 1. *Location of Ilba mining area (white rectangle) (modified from www.google.earth.com)*

The samples were collected in spring 2005 from several effluents and the *Acidiphilium* strains presenting the highest metabolic activity were isolated for future studies (Cismasiu, 2008). The strains (Fig.2), were cultivated in GYE (glucose yeast extract) medium with 0.1% starch, pH 3.0, and incubation at 28^0C for 21 days.

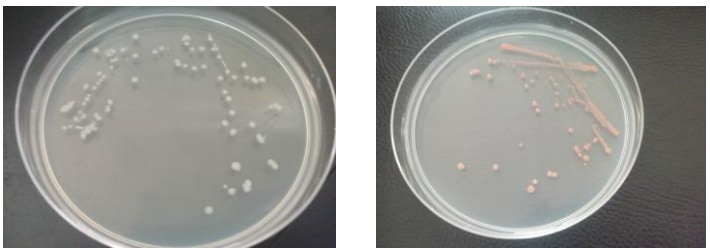

Figure 2. *Acidiphilium sp. (white and red colonies) isolated from mining effluents of Ilba area*

In order to establish the best conditions for the amylolitic activity of the *Acidiphilium* strains, several growth temperatures (15°C, 28°C, 37°C and 42°C) and pH - values (1.5, 2.0, 2.5, 3.0 and 3.5) were tested at different concentrations of starch substrate (1, 2, 3 g/l). These experiments were realized in 100 ml Erlenmeyer flasks, with 30 ml GYE medium and 10% inoculum. The microbial cultures were incubated for 7, 14 and 21 days under continuous agitation.

Starch hydrolysis was assessed by Wohlgemuth method (Gupta et al., 2003): the amylase hydrolyzes starch to simpler sugars which do not give the same color reaction with iodine. 3.5 ml starch solution was added to 0.25 ml triplicate samples and incubated 30 minutes at 37 °C; the reaction was stopped with HCl 1N. After adding a solution of iodine – potassium iodide 0.1 N, the hydrolysis intensity was assessed through spectrophotometer determination at 580 nm of the blue compounds formed between starch and iodine.

3 Results and Discussion

The coal and metal ore mines, and the neighboring areas, are known as extreme habitats (Kozlov & Zvereva, 2007); in the mine galleries, with the increase in depth, the temperature of the surrounding rocks keep rising: for instance, by 2000, in the Chinese coal mines the average depth was about 650 m and the temperature ranged between 35.9 – 36.8 °C (He, 2009). A similar situation is in Europe, where many mine galleries are now considered as geothermal heat exchangers, with the potential of being used as a renewable source of energy (Rodriguez & Diaz, 2009). Besides the high temperatures, the mining areas present usually high concentrations of heavy metals and very low pH (Johnson & Hallberg 2005).

Due to their adaptations, extremophile bacteria are able to cope with such environmental conditions. In our study, the investigations focused on the heterotrophic *Acidiphilium* sp. due to its ability to develop in such polluted areas and the potential bioremediation application. Strains isolated in previous studies (Cismasiu, 2008) were used to investigate its metabolic activity, this being the first attempt of its kind in Romania.

The comparative study of the amylolitic activity of *Acidiphilium* sp. at different growth temperatures and pH conditions revealed the highest activity at 28 and 37 °C, confirming the mesophilic characteristics of this genus. Even at these temperatures, their activity was high only in the first two weeks, decreasing in the third week, possibly due to the released metabolites; however, this hypothesis should be further corroborated.

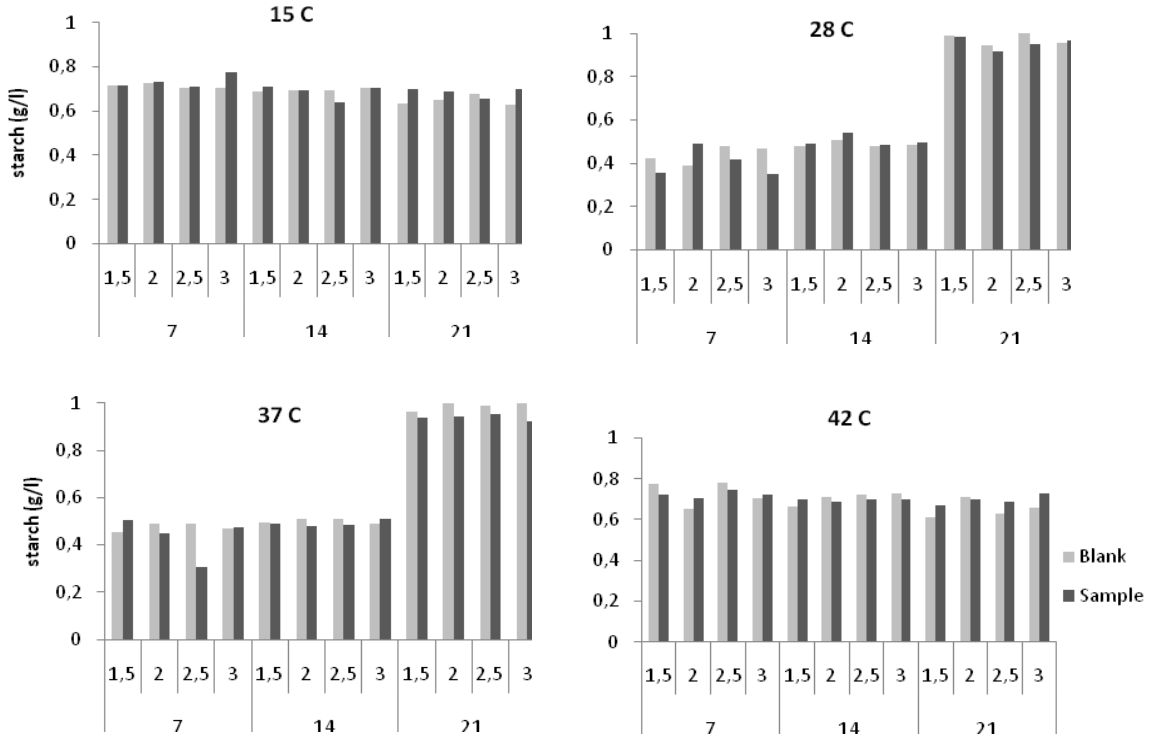

Figure 3. *Starch hydrolysing activity of Acidiphilium at different temperatures (15, 28, 37, 42 °C) and pH (1,5; 2; 2,5; 3), for several incubation periods (7, 14, 21 days). The highest activity was noticed between 28 and 37 °C. Substrate concentration: 1 g/l starch.*

As 28 and 37 °C were proven to be the best temperatures for the amylolitic activity of *Acidiphilium*, they were selected for further experiments, aiming to find the optimum substrate concentration; starch concentration was raised to 2 and 3 g/l, while pH was kept at 3 and 3.5 (Fig.4).

Although the substrate concentration increased, and an increased amount of hydrolyzed products was expected, the results indicated 1g/l as the optimum starch concentration (Fig. 3, 4), confirming previous studies (Popea 2002).

Previous studies of the *Acidiphilium* sp. isolated from different samples have shown that the bacteria isolated from the mine wastewater are more active than the lab cultures probably as a consequence of their adaptations to the high acidic conditions from the mine (Cismasiu, 2004).

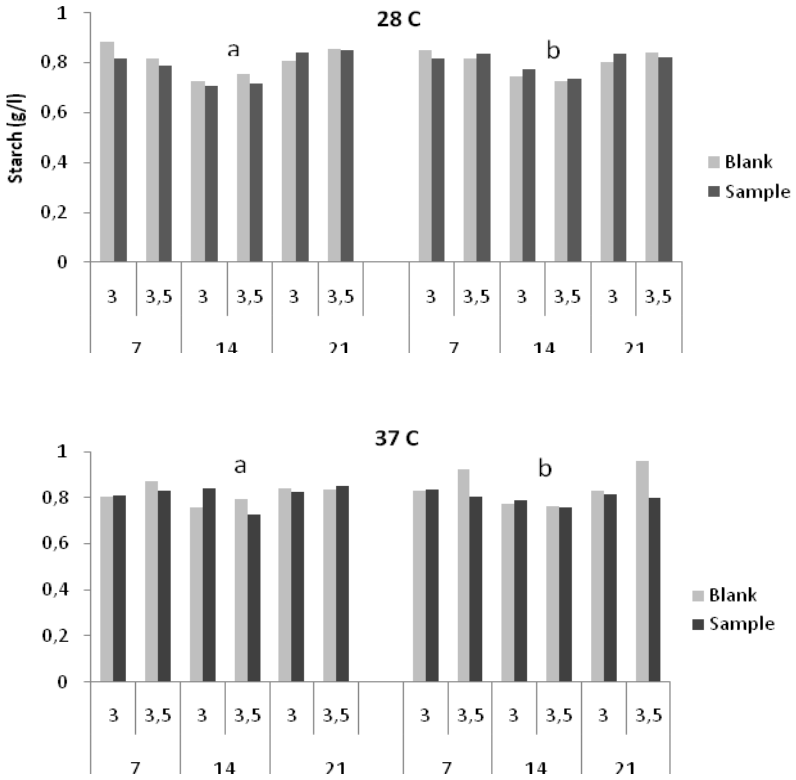

Figure 4. *Starch hydrolyzing activity of Acidiphilium sp. at 28 and 37°C, pH 3 and 3,5 with different substrate amount (a = 2 g starch, b = 3 g starch) and different incubation periods (7, 14, 21 days)*

4 Conclusions

The *Acidiphilium* strains isolated from the acid mine drainage of Ilba mining area exhibited the highest amylolitic activity at 28^{0}C and 37^{0}C, at pH 2,5 and 3. At 15^{0}C and 42^{0}C the microbial hydrolysis was not significant, proving the mesophilic character of this genus; these values are similar with those recorded at the sampling site (water temperature: 28 ^{0}C, pH = 2.5). The optimum substrate concentration was 0.1 % starch, while values higher than 0.2 % inhibited the metabolic activity.

Unlike other extremophiles, whose maximum metabolic activity occurs at higher temperatures and bigger depths, *Acidiphilium* sp. are able to cope with low pH even at 28 – 30 ^{0}C, the surface temperature of the acid mine drainage in Ilba area; their potential for bioremediation (e.g. retention of heavy metals) seems high and could be further investigated in a local pilot study. For instance, the mine wastewater could be passed through a column with absorbent material synthesized from yeasts and *Acidiphilium* cultures; the biomass could be further separated by filtration or centrifugation and used to recover the retained heavy metals.

Future studies will focus on identifying a narrower range of the optimum temperature where *Acidiphilium* presents its highest enzymatic activity, as well on the implementation of these results in the ecological reconstruction of Ilba mining area.

Acknowledgements

The study was financed by the Romanian Academy, project no. 5: Investigation of acidostable enzymes of heterotrophic acidophilic bacteria belonging to the genus Acidiphilium.

References

Baker, B.J., Banfield, J.F. (2003): Microbial communities in acid mine drainage, FEMS Microbiology Ecology, 44:139–152.

Carlson, C. (1998): Influences of Sorption on Microbial Degradation of Organic Substances. Introductory paper.University of Lund, Sweden. URL: http://www.ecotox.lu.se/STAFF/CK/Introduc.htm [accessed October 2001].

Cişmaşiu, C.M., Voicu, A., Lăzăroaie, M. M., Lazăr, I. (2000): The effect of temperature of the several heterotrophic acidophilic and chemolitotrophic sulfur oxidizing bacteria, isolated from mine effluents, in various experimental conditions. In: Proceedings of the Institute of Biology, Romanian Academy, Annual Scientific Session, Bucharest, III: 325-333.

Cişmaşiu**,** C. (2001): The influence of acidity on the growth and activity of acidophilic heterotrophic bacteria, isolated from mining effluents. Revue Roumaine de Biology, Serie Biol Végét, Académie Roumaine, Bucharest, 46 (1): 19-27.

Cişmaşiu, C., Toniuc, M., Popea, F. (2002): Physiological microorganisms groups present in the mining residual waters. Proceedings of the Institute of Biology, Romanian Academy, Annual Scientific Session, Bucharest , IV: 235-242.

Cismasiu C.M. (2004): The study of acidophilic microbiota from industrial effluents with acid pH (2.0-4.0) and high concentrations of metallic ions. PhD Thesis, Institute of Biology, Romanian Academy, 330 p.

Cişmaşiu C. M., Bordeianu M., Stancu R. (2004): Removing the metallic ions from the industrial residual waters using the aerobe heterotrophic bacterial cultures. Proceedings of the Institute of Biology, Romanian Academy, Annual Scientific Session, Bucharest, VI: 199-211.

Cismasiu C. (2008): The acidophilic microorganisms diversity present in habitats with acidic extreme conditions, with implication in the environmental bioremediation of high concentrations of metallic ions. ARGESIS – Studii şi Comunicări, Seria Ştiinţele Naturii, Muzeul Judeţean Argeş, Piteşti, România, XV: 41-52.

Dopson, M., Austin, C.B., Koppineedi, P.R., Bond, P.L. (2003): Growth in sulfidic mineral environments. Metal resistance mechanisms in acidophilic micro-organisms. Microbiology. 149**:** 1959–1970.

Gupta, R., Gigras, P., Mohapatra, H., Goswami, K. V., Chauhan, B. (2003): Microbial α-amylases- a biotechnological perspective. Process Biochemistry, 00: 1-18

He, M.C. (2009): Application of HEMS cooling technology in deep mine heat hazard control. Mining Science and Technology, 19: 269-275.

Johnson, B.D. (1998): Biodiversity and ecology of acidophilic microorganisms, FEMS Microbiology Ecology, 27: 307-317.

Johnson D.B. (2003): Chemical and microbiological characteristics of mineral spoils and drainage waters at abandoned coal and metal mines. Water Air Soil Pollut: Focus, 3: 47– 66.

Johnson D.B., Hallberg K.B. (2003): The microbiology of acidic mine waters. Res Microbiol., 154: 466– 473.

Johnson, B.D., Hallberg, K.B. (2005): Acid mine drainage remediation options: a review. Science of the Total Environment, 338: 3– 14.

Kozlov, M.V., Zvereva, E.L., (2007): Industrial barrens: extreme habitats created by non-ferrous metallurgy. Rev. in Environmental Science and Biotechnology, 6(1-3): 231-259.

Matzke, J., Schwermann, B., Bakker, E.P. (1997): Acidostable and acidophilic proteins: The example of the α-amylase from *Alicyclobacillus acidocaldarius*. Comp Biochem Physiol., 118(3): 475-479.

Norris, P.R., Johnson, D.B. (1998): Acidophilic microorganisms. In: Horikoshi, K., Grant, W.D (eds): Extremophiles. Microbial Life in Extreme Environments, Wiley, New York, p. 133-154.

Popea, F., 2002: The action of some physical-chemical factors on the starch hydrolyzed by microorganisms which belong to S*ulfurococcus* genus. Proceedings of the Institute of Biology, IV: 327-334.

Rodriguez, R., Diaz, M. (2009): Analysis of the utilization of mine galleries as geothermal heat exchangers by means of a semi-empirical prediction method. Renewable energy, 34: 1716 – 1725.