

# The Microbial Oxidation of Iron and Sulphur in an Acid Solution from an Auriferous Sulphides Mine from the South-West of Romania

VASILE-DANIEL GHERMAN<sup>1\*</sup>, JEAN-GABRIEL BRÉHERET<sup>2</sup>, ANDREI MOTOC<sup>3</sup>, CORINA SAMULĂ<sup>4</sup>,  
MARIOARA NICOLETA FILIMON<sup>5</sup>, AURICA - BREICA BOROZAN<sup>6</sup>

<sup>1</sup> University of Polytechnic, Faculty of Hydrotechnic, 1 George Enescu Str., 300022, Timișoara, Romania

<sup>2</sup> University of François-Rabelais, GÉEAC Laboratory, Science and Technical Faculty, Grandmont Parc F-37200, Tours, France

<sup>3</sup> "Victor Babes" University of Medicine and Pharmacy, Department of Anatomy and Embriology, 2 Piata Eftimie Murgu, 300041 Timisoara, Romania

<sup>4</sup> "Victor Babes" University of Medicine and Pharmacy, Department of Biochemistry, 2 Piata Eftimie Murgu, 300041 Timisoara, Romania

<sup>5</sup> West University from Timisoara, Faculty of Chemistry-Biology-Geography, Department of Biology, 16 Pestalozzi Street, Timisoara, Romania

<sup>6</sup> Banat University of Agricultural Sciences and Veterinary Medicine from Timisoara, Faculty of Horticulture, 119 Calea Aradului, Timisoara, Romania

*The rock layers from above the cavity are slowly crossed, through micro cracks, by the rain water. The rain water arrives on the cavity wall as a concentrated solution with a high acidity level (pH 1-1.5). The percolating acid solution is crowded by acidophilic microorganisms with a dominating population of chemo-autotrophic bacteria *Acidithiobacillus ferrooxidans* provides the primary organic substance by oxidation of pyrite from the cavity walls and with six strains of *Penicillium* representing the heterotrophic organisms that use the organic substance from the primary producer. Between the *A. ferrooxidans* population and the *Penicillium* strain populations a close trophic relationship is established and the microorganisms number depends on the acid solution concentration. The ferric hydroxide formed through the oxidation of pyrite by *A. ferrooxidans* is deposited at the *Penicillium* hyphae exterior and forms minerals structures.*

*Keywords: acid solution, acidithiobacillus ferrooxidans, autotrophe, ferric hydroxide, heterotrophs, penicillium, pyrite*

Highly acid medium limits the access of the organisms that are unable to adapt to this stress, and are considered as "extreme life medium" [1].

The artificial or natural cavities from volcanic or metamorphic rocks that are rich in iron, sulphur or other reduced ions, represent a totally different system from the ones in the calcareous rocks. The studies on the acid medium have revealed the great diversity of organisms that are part of three systematic fields: Eubacteria, Archaea and Eucaria. Between the acidophilic organisms populations there are many interactions, especially trophic kind connections.

The most numerous microbiological studies have been done on the mine drainage waters: in Iron Mountain, California [2, 3]; in Scandinavia and the United Kingdom [4]; Alaskan and Canadian drainages gold mines [5]; in Norwegian copper mine [6]; these waters represent an important pollution source.

This study took place in an old mine (the Kiesberg Mine), abandoned at the end of the XIX<sup>th</sup> century, located in the South-West of Romania, near Oravita town.

This mine is located in the Banat Mountain and it was dug in the metamorphic rocks of auriferous sulphides of Policarpus ore. The thickness of the metamorphic rocks above the cavity is at least 8 meters; they are covered by several meters of limestones and marls.

Policarpus ore mineralization is mainly made of magnetic pyrite and pyrite, and secondary of chalcopyrite, cobaltine and marcasite.

The rain water crosses these layers throughout the rocks' micro cracks and arrives on the cavity vault as a high acidity concentrated solution (pH 1-1.5).

The flowing period through micro cracks is of 2 weeks. During this period, the rainwater dissolves a series of

minerals: silicates and iron sulphides. Various kinds of crusts are forming in this acid medium. As the oxidative activity is intimately linked to the deposit of some minerals in their structure, so arise the question of the contribution of organisms to the leaching of the minerals from the source rock and 2 to the new mineral deposits.

The extremely particular medium from the Kiesberg cavity induces the presence of characteristic microorganisms associations that are physiologically and ecologically very interesting. In this study the identification, in two different hydrological seasons, a wet one and a dry one, during the years of 2004 and 2005, of characteristic microorganisms associations which populate the walls of this old mine. the oxidative microbial metabolisms from the acid solution characterization and characterization of mineral deposits (crusts) that are based on the metabolism of this microorganisms associations were proposed.

## Experimental part

### Materials and methods

On the acid solution both chemical and microbiological analysis have been done and electronic microscopy has been applied. The chemical analysis have been done using the atomic absorption spectrometry (GBC 932 plus). The followed parameters were (in mg/L<sup>1</sup>): total Fe; Na; K; Cr; Cd; Ni; Pb; Zn; Mn; Cu.

Microbiological analysis established all the microorganisms groups from the percolation solution.

The isolation of acidophilic chemo-autotrophic bacteria was performed by inoculating the growth medium (9K - pH 2.5) [7] for the isolation of *Acidithiobacillus ferrooxidans* [8] (*Thiobacillus ferrooxidans*), the Winogradski medium (W<sub>6</sub> - pH 6) [9] for other ferrous-bacteria, and the Starkey medium (S<sub>6</sub> - pH 6) [10] for *Acidithiobacillus thiooxidans*.

\* email: ghermanvasile2000@yahoo.com

The isolation of acidophilic heterotrophic microorganisms has been performed through enriched growing on the March medium- pH-2.5 [11], and on the G.Y.E. at pH 3 [1].

The isolation and identification of fungus [12] has been realized on the solid Czapeck medium [12, 13], starting from the serial dilutions of the enriched growing realized from samples in the liquid March medium.

The indirect determination of *Acidithiobacillus* strains by the measurement of pH medium was achieved by seeding the test samples on the 9K, S<sub>5</sub> and S<sub>6</sub>, and also on the W<sub>6</sub> medium. The growing was determined by the quantity of oxidized thiosulphate. The *Acidithiobacillus* is estimated by the final pH value after 28 days [14].

The determination of the most probable number of the microorganisms was done by performing decimal or serial dilution, with 3 repetitions/dilution, while the observation of the number of positive tubes, the numerical value, was established from the table [15]. The method of counting the colonies on the solid medium was used also for the numerical estimates.

The biofilm and the isolated crusts from the Kiesberg cavity vault have been subjected also to the electronic microscopy in S.E.M. and T.E.M. The samples for the electronic microscopy have been done by the standard method [16].

In (SEM), for the dehydration of the samples, both the method of critical point and the hexametil-disilazan method were used. The apparatus used was a Zeiss Gemini 982 microscop. The observation took place at an acceleration tension of 15 kV and at a work distance of 16mm. The obtained images are digitals with a resolution of 1021 . 1024 pixels.

In (TEM), the apparatus used was an ultramicrotom Ultracut E Reichert and the observations were done on 2 apparatus: JEOL 1010 and JEOL 1230 at an acceleration tension of 80 kV.

## Results and discussion

The chemical analysis was the first analysis that was made on the percolation solution. The pH of this solution is between 0.5 and 2, and the analysis of the elements led to the following results (mg/L<sup>-1</sup>): total Fe >15; Na 30; K 22; Cr 0.227; Cd 0.08; Ni 1.18; Pb 0.04; Zn 2.9; Mn >7.2; Cu >10. The chemical analysis on this solution was difficult to make because of the very small quantities that could be gathered as samples.

The results of the microbiological analysis performed on this acid solution are presented in table 1.

As it is indicated on the table 1, seven strains of microorganisms forming a consortium, and thus functionally interconnected, were determined in the acid solution, both in 2004 and 2005. *Acidithiobacillus ferrooxidans* is the only bacterial strain, the rest being represented by six strains of *Penicillium* fungus, which are a part of Moniliacea Family.

In 2004 and 2005, a clear domination of *Acidithiobacillus ferrooxidans* (80 %) is observed during both seasons; the remaining 20 % corresponds to the *Penicillium* strains. *A. ferrooxidans* is a chemo-autotrophic bacteria that oxidizes the pyrite, the Fe<sup>2+</sup> and the sulphur compounds (stemming from the oxidation of pyrite), providing the primary production of organic substance; the six strains of *Penicillium* represent the heterotrophs that use the organic substance produced by *A. ferrooxidans* (fig.1.).

*Acidithiobacillus ferrooxidans* is usually isolated strain, known since 1950 as a metallic sulphide oxidizer [17, 18]. This strain was found in the mine water drainage, with high acidity. The fungus species from the *Penicillium* strain are heterotrophs eukaryotes that are often found on the organic substances from the acid waters [12, 19, 20].

In the acid solution studied by us at the Kiesberg Mine, the association of the *Acidithiobacillus ferrooxidans* with the six strains of *Penicillium* into a continuous biofilm on the cavity vault and the forming of ferric hydroxide crusts

**Table 1**  
MICROORGANISMS IDENTIFIED IN THE ACID SOLUTION AND THEIR MOST PROBABLE NUMBER DURING THE TWO SEASONS OF 2004 AND 2005

Strain	2004 (n°. of cells/ml)		2005 (n°. of cells/ml)	
	Wet	Dry	Wet	Dry
<i>Acidithiobacillus ferrooxidans</i>	9500	4500	9500	8000
<i>Penicillium expansum</i>	600	500	600	600
<i>Penicillium cyclopium</i>	200	150	250	150
<i>Penicillium frequentans</i>	300	200	500	300
<i>Penicillium griseo-azureum</i>	0	200	0	200
<i>Penicillium citreo-viride</i>	200	0	300	200
<i>Penicillium lividum</i>	200	200	300	300

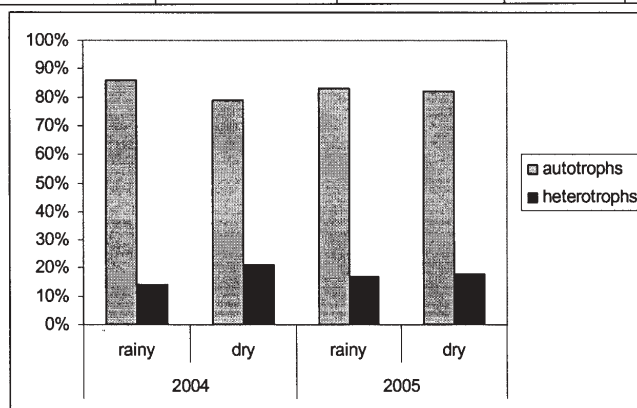


Fig. 1. The ratio of physiological groups of microorganisms from the acid solution, during the rainy and dry seasons of 2004 and 2005

is characteristic to this mine. Close trophic type relationships are established between the organisms of this consortium.

In the formation areas of the crusts, the percolation water which crosses the cavity vault throughout fine cracks is full of dissolved salts. Because of this high concentration of elements, the solution has a very high superficial tension. Thus, the solution forms a continuous 0.5 mm thick biofilm on the cavity vault surface.

The working of this alive system, formed by chemo-autotrophic bacteria as *Acidithiobacillus ferrooxidans* and the six strains of *Penicillium* and heterotrophs, is quite simple.

This system is based on the metallic sulphides oxidation from the metamorphic rock layer from the vault of the cavity.

In the Kiesberg mine, beside the acid solution, a strong oxidative metabolism has been observed also in the case of some microbial gelatinous formation. Results of this metabolism are big quantities of ferric hydroxide and sulfate [21].

The permanently filled with water micro cracks from the Kiesberg cavity vault are habited with chemo-autotrophic bacteria. Some of these bacteria are attached to pyrite, calcopyrite, marcasite minerals found in the rock mass, others are free in the solution found in micro cracks. Thus, the percolation solution represents a continuous biofilm of chemo-syntetized bacteria in the micro cracks from the rock in the cavity vault and of *Penicillium* in the biofilms from the cavity vault and in the crusts.

The metals biosolubilization in the micro cracks from the rock mass can be done, as it is well known, in two ways: direct biosolubilization and indirect biosolubilization.

The direct biosolubilization is done enzymatically and the microorganisms are in an intimate contact with the pyrite crystal [22].

The microbial oxidation of pyrite by the bacteria that are attached to the crystal is best described by the following reaction (1):



The generated  $Fe^{2+}$  from this reaction is further away oxidized by the unattached to pyrite bacteria that are found in the whole percolation solution mass (2). This solution arrives on the cavity vault and a consortium of *A. ferrooxidans* and *Penicillium* is formed within it [22]:



Beck's data (1960) [3] concerning the molar ratio of  $Fe^{2+}$  oxidized at assimilated  $CO_2$ , show that approximately 100 mols  $Fe^{2+}$  must be oxidized in order to settle 1 mol of  $CO_2$  by *A. ferrooxidans*.

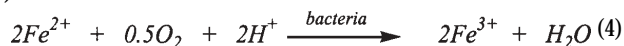
In S.E.M., a great number of chemo-autotrophic bacteria were observed (fig. 2, b.). Thus, in order to maintain this consortium metabolism that leads to the forming and the growth of crusts on the cavity vault, big quantities of  $Fe^{2+}$  from the rock mass from above the cavity are being consumed.

The  $Fe^{3+}$  resulted from the oxidation of  $Fe^{2+}$  by the unattached bacteria from the cavity micro cracks, then causing the chemical oxidation of residual pyrite (3), growing even more the oxidized pyrite quantity and the quantity of  $Fe^{2+}$  that is available to the chemo-autotrophic bacteria:

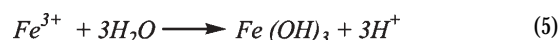


This process represents the indirect bio oxidation of pyrite [22].

The  $Fe^{2+}$  resulted by direct and indirect biosolubilization from pyrite, is oxidized to  $Fe^{3+}$  by the *A. ferrooxidans* cells that are associated to the *Penicillium* hyphae in the biofilm (4).



The  $Fe^{3+}$  can form the ferric hydroxide which precipitates, mineralizing the consortium hyphae (5):



Due to the fact that the biofilm is pretty stable being extremely slowly supplied by the percolation water and because of the high concentration of dissolved substances, these solutions deposit a continuous layer of ferric hydroxide at the air-solution interface. This layer is between few tens and few hundreds microns thick. The layer is being formed in a slowly, discontinuous way (in a period of time of 30 days or more) and it has a micro layers structure (fig. 2. a, b).

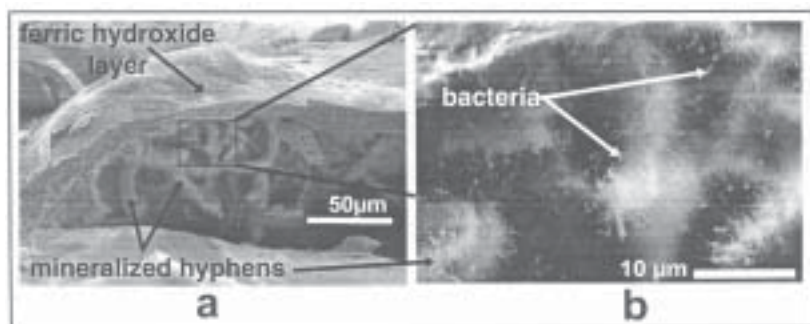


Fig. 2. a., b. General view of ferric hydroxide layer, mineralized hyphae and bacteria

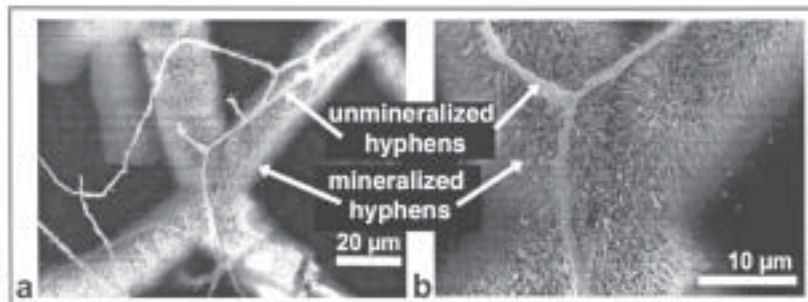


Fig. 3. a. General view of mineralized hyphae and alive hyphae; b. Alive hyphae, mineralized hyphae and bacteria

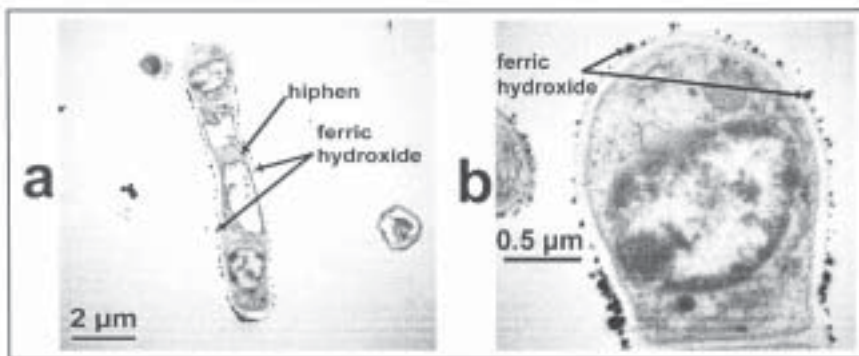


Fig. 4. a., b. Alive hyphae sectioned with globular ferric hydroxide that it is deposited at the cells exterior

While this layer at the biofilm exterior is being formed, in the interior of the biofilm the deposit of ferric hydroxide at the hyphae exterior takes place. These hyphae give the crusts interior a curly aspect.

In S.E.M. it can be observed a significant difference between the mineralized and not mineralized hyphae from the biofilm (fig. 3. a., b.).

Penicillium hyphae have been isolated, in Trump fixer, from the biofilms on the crusts and samples in T.E.M. have been done. Thus, at the first examination of the sections the deposition of the ferric hydroxide at the exterior of the cellular wall was noticed. The ferric hydroxide is in globular shape and it covers the exterior of the hyphae (fig. 4. a., b.).

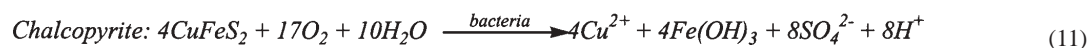
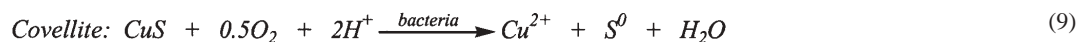
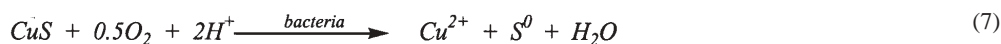
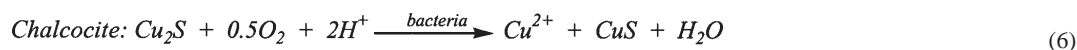
The intimate association between bacteria and fungus is being done by the hyphae' extensions, by which the hyphae take over the metabolic bacterial organic products.

It can be said that between the chemo-autotrophic bacteria from the biofilm and the heterotrophs fungus there is a mutual relationship. A. ferrooxidans can use  $\text{NH}_4^+$  and some amino-acids as nitrogen source from fungus and thus it is very irresolute during the attack of metallic sulphides.

Besides the  $\text{Fe}^{2+}$  from pyrite, the chimio-sintetized bacteria such as A. ferrooxidans from the consortium, they use also the sulphur from the sulphides in the cavity vault rock as energy source. The metabolism of  $\text{S}^0$  brings a bigger quantity of energy than the  $\text{Fe}^{2+}$  [22].

It is probable that the iron and sulphur atoms from pyrite to be concomitantly oxidized, but only after the sulphides are dissociated from the minerals' crystal network [23].

During our chemical analysis we established important quantities of copper on the percolation solution. Thus, A. ferrooxidans oxidize also a various range of copper sulphides, beside the pyrite, in the microcracks from the cavity vault (6, 7, 8, 9, 10, 11):



## Conclusions

The acid overburden percolating solution (pH 1.21) from the Kiesberg mine, houses a rich acidophilic community of microorganisms. This community is formed by a numerous population of Acidithiobacillus ferrooxidans (80%), that represents the primary producer of organic substance which oxidizes the pyrite from the cavity walls and by the populations of six strains of Penicillium (20%); these heterotrophic organisms use the organic substances provided by the primary producer.

As oxidative reactions, A. ferrooxidans oxidizes: 1) the metallic sulphides, specially the pyrite from the cavity rock (1, 3). From this pyrite the ferric hydroxide results (5) and 2)  $\text{S}^0$  from which the sulphuric acid results and this acid lowers the pH of the environment (8, 10) (pH 1.21). The ferric hydroxide formed is deposited at the Penicillium hyphae exterior and forms minerals structures.

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