Bioleaching of pyrite and chalcopyrite by new isolated thermotolerate sulfur-oxidizing bacteria Acidithiobacillus tandzuti sp.nov.

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Abstract— New gramnegative bacterium was isolated from acid drainage water of Tandzut gold-polymetallic ore deposit of Armenia. Bacteria are capable of oxidizing sulfur, thiosulfate and tetrathionate as energy sources. The growth of bacteria is possible in the range 25-40 with optimal temperature 37 °C. The optimum pH is 2.8 for the growth on sulfur and tetrathionate and 4.0-4.5 for thiosulfate. The G+C contents in DNA is 54.6 mol /%. Analysis of 16S r RNA sequence of isolated bacteria indicates high level of similarity with analogous sequence of *Acidithiobacillus thiooxidans.* However, DNA–DNA hybridization value between genomic DNA of isolated bacteria and *At. thiooxidans* was only 51%. On the basis of the above mentioned genotypic peculiarities as well as phenotypic differences (higher optimal temperature for growth and greater size of cells), the isolated bacteria was identified as a new species of sulfur-oxidizing bacteria *Acidithiobacillus tandzuti* strain 5 (type strain – *At. tandzuti* INMI-6966). *At.tandzuti* in mixed culture with moderate thermophilic sulfobacilli allows to conduct the oxidation of pyrite and chalcopyrite in autotrophic condition with intensity observed during mixotrophic growth of sulfobacilli in the presence of yeast extract.

Keywords— *Acidithiobacillus tandzuti*, bioleaching of pyrite and chalcopyrite, DNA –hybridization, mixed cultures, sulfur-oxidizing bacteria.

1 Introduction

Sulfuric acid generated from the oxidation of sulfur and reduced inorganic sulfur compounds (RISCs) is responsible for the low pH environment where mineral oxidation take place. Sulfur and RISCs serve as electron donor for the wide variety of iron- and sulfur-oxidizing bacteria [14]. It is considered that extremely acidophillic bacterium *Acidithiobacillus thiooxidans* plays a leading role in the oxidation of sulfur and RISCs both in natural and technogenic conditions [7], [12]. However a number of other autotrophic acidophilic bacteria capable of oxidizing elemental sulfur is described [2], [6], [8]. Among them only *Thiobacillus albertensis* [2] and *Acidithiobacillus caldus* [5], [6] are represented as separate species whereas the other bacteria because of the lack of essential differences from *At. thiooxidans* in the last publication of Bergey's Manual (Bergey's Manual of Systematic Bacteriology, 2001) were related to synonyms of *At. thiooxidans*. Investigations carried out recently show that *At. caldus* is the main sulfur oxidizing bacteria in the continuous biooxidation process functioning at 40 degrees [4], [5]. It is assumed that the role of *At. caldus* in association with chemolithotrophic bacteria in biooxidation of arcenopyrite is to remove inhibiting sulfur layer formed on the surface of mineral or to supplement mixothrophic and heterotrophic growth of other sulfur- and iron-oxidizing bacteria.

The aim of the this work was the investigation of morpho-physiological, genetic and other peculiarities of new thermotolerant sulfur- oxidizing bacteria isolated from Tandzut gold-bearing ore deposit in Armenia.

2 Materials and Methods

2.1 Culture condition and isolation

Strain 5 sulfur oxidizing bacteria was isolated from Tandzut gold-bearing polymetallic ore deposit in Armenia. For isolation of bacteria 9K medium [16] containing elemental sulfur as the source of energy was used. Sulfur was sterilized separately by flowing stream and was added to the medium immediately before using. pH of the medium was adjusted to 3.0-3.5 with 10 N H_2SO_4. Enrichment culture was obtained by incubation of 9K medium inoculated with acid drainage water sample at 37 °C. Pure culture was obtained by streaking out the enrichment culture of sulfur- oxidizing bacteria on solid medium, containing 0.6% agarose (Agarose, Type I, SIGMA) and sodium tetrathionate ($Na_2S_4O_6x2H_2O$) as the source of energy [11]. The purity of culture obtained was checked by its sowing on the same medium, containing 0.05 and 0.1 % yeast extract or glucose as the only source of energy (instead of sulfur).

2.2 Microscopic studies

Total preparations for electron microscopy were contrasted by 1% phosphorous volframic acid. In order to obtain ultrathin sections bacterial cells were fixed in 1% osmium tetraoxide according to Ryter-Kellenberger method. After dehydration in a graded series of ethanol the preparations were embedded in epon-812. The ultrathin sections obtained by ultramicrotom LKB-4800, were post-stained with uranil acetate and lead citrate. The thin sections were examined by electron microscope CM10 Crio Philips Electron optics Eindhoven (Netherland).

2.3 Optimal pH and temperature for growth

The study of the effect of temperature and pH on the growth of isolated sulfur oxidizing bacteria was carried out in 100 mL flasks containing 50 mL of 9K medium with bacterial suspension and incubated on stationary conditions. To determine the optimal temperature, the growth of bacteria and sulfur oxidation were tested in the range of 25-45 °C. Optimal pH for sulfur- oxidizing activity of isolated strain was studied in the range of pH from 2.0 to 7.0.

Bacterial growth was estimated by decreasing of pH of the medium and by the amount of SO_4^{2-} resulted in the oxidation of elemental sulfur or other reduced sulfur compounds. SO_4^{2-} ions were determined spectrofotometrically [3].

2.4 DNA extraction, PCR of 16S rRNA and phylogenetic analysis

DNA was extracted from bacteria by method described [1]. Two independent preparations of DNA were obtained for each studying strains. The G + C content was determined by melting point analysis (method of thermal denaturation) [13]. The extend of DNA - DNA hybridization of bacteria was determined by the method of optical reassociation [10].

16S rRNA gene sequencing. The polymeric chain reaction (PCR) and further sequencing of PCR fragments of 16S rRNA gene were carried out using universal primer system [9].

Analysis of PCR products was done by mean of electrophoresis in 2 % gel of agarose.

The extraction and purification of PCR products, corresponding to different areas of gene were carried out using set of reagents wizard PCR Presp Promega, USA, according to producer's recommendation.

Sequencing of amplification products was done using the method of Senger [15] by means of set of reagents of Big Dye terminator v.3.1. (Applied Biosystems, Inc, USA) in automatic sequenator ABI PRIZM 3730 (Applied Biosystems, Inc, USA) according to producer r's ecommendation.

Analysis of 16S rRNA sequences. The 16S rRNA gene nucleotide sequences of isolated strains were analyzed using BLAST (http://ncbi.nlm.nih.gov/BLAST). Construction of phylogenetic tree was done by MEGA 6.06 software using neighbour-joining method. Bootstrap analysis was carried out on 1000 replicate input data sets.

2.5 Leaching experiments

For purpose to study the ability of isolated bacteria for oxidation of pyrite consists of 43.8 % Fe, 49 % S and chalcopyrite containing 30.2 % Cu, 29.7 % Fe, 38 % S from Shamlugh ore deposit (Armenia) were used. The minerals were ground to a size fraction of between 60-100 μm and sterilized at 112 °C for 20 min. Experiments were conducted using 50 mL 9K medium and 4 % of minerals in 250 mL Erlenmeyer flasks that were shaken at 150 rev/ min in a water bath. The temperature of a water bath was maintained at 37 °C. According to the experiment active cultures of *At.tandzuti* str. 5 or *S. thermosulfidooxidans* str. 6 [18]. and their mixed cultures were added to flasks. 1 mL of leaching medium was periodically removed to determine the cell number, pH of the medium and extracted metal concentration. Intensity of pyrite and chalcopyrite oxidation was estimated by the amount of iron and copper ions transferred into the medium. Fe^{2+} and Fe^{2+} ions were determined compleximetrically using EDTA. Soluble copper and total iron were determined by atomic-absorbtion spectrophotometer AAS 1N (Carl Zeiss).

3 Results and Discussion

3.1 The isolation of sulfur oxidizing

For the isolation of sulfur- oxidizing bacteria 9K medium with elemental sulfur as the source of energy was infected with acid drainage water of Tandzut gold-polymetallic ore and incubated at 37 °C for 7-10 days. Pure cultures were obtained using Manning solid media.

3.2 Morphology

Cells in logarithmic phase of growth represent straight rods 0.5-0.7 μm in diameter and 1.2-4.0 μm in length with oval edges (Fig. 1 a, b). Cells show a negative Gram reaction and have typical for Gram-negative bacteria cell wall structure, as viewed in ultrathin sections. Cells are motile in young culture.

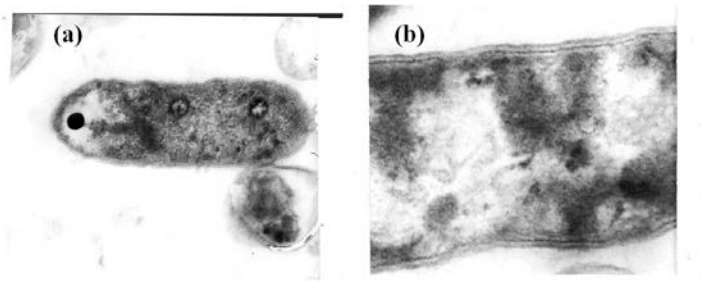

Fig. 1. Electron Microscope photographs of strain 5 (a), x38500 (b)

3.3 The influence of temperature and pH

As shown in Figure 2a the optimum temperature for the growth of strain 5 is 37 °C. The growth of bacteria is possible at the range of 25 - 40 °C (Fig. 2a). Thus, the isolated bacteria in comparison with *At.thiooxidans* have higher growth tempera-

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ture and are considered as thermotolerant.

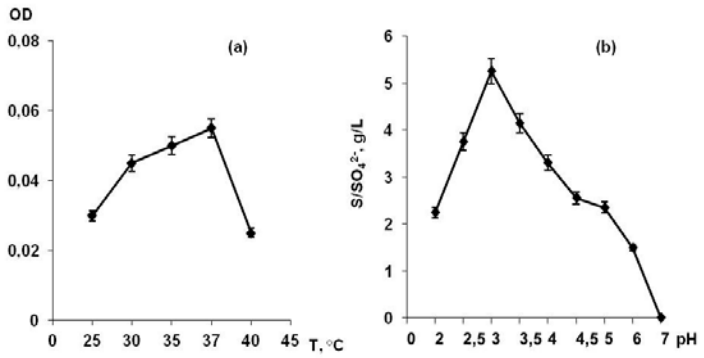

Fig. 2 Growth of str. 5 and oxidation of elemental sulfur at different temperatures (a) and pH (b)

The investigations have shown that the maximum rate of oxidation of elemental sulfur by isolated bacteria was observed at pH 3.0 (Fig. 2 b). The growth of bacteria is possible at pH 2.0-6.0. pH values higher than 5.0 inhibit growth of bacteria which are completely stopped at pH 7.0. The optimum pH for the growth on tetrathionate ($S_4O_6^{2-}$) is 2.7; on thiosulfate ($S_2O_3^{2-}$) is 4.0-4.5.

During the growth of strain 5 on the medium with elemental sulfur the increase of the optical density of the medium and its rapid acidity were observed as a result of active formation of sulphuric acid in 4 - 5 days (Fig. 2). While growing on the medium with thiosulfate, the amount of $S_2O_3^{2-}$ decreases. Instead, the optical density of the medium increases and pH reduces from 5.0 to 3.1.

3.4 Phylogenetic analysis of 16S rRNA

Content of G+C in DNA for isolated strain 5 is 54.6 mol/% that is close to *At. thiooxidans* (51.4 mol/%) and considerably lower that of *At. albertensis* (61.5 %) and *At. caldus* (64 mol/%). But the investigation of DNA - DNA homology has shown that the level of similarity of genomes of isolated strain and *At.thioxidans* is only 51 %.

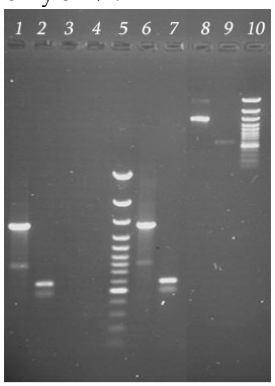

Fig. 3 Obtaining of PCR fragments of 16S rRNA gene
5, 10 – Molecular mass markers GeneRuler 100 bp, fermentas, #SM0321;
1, 6 – PCR-products, corresponding to polymerase coppies of 16S rRNA gene, independent preparations of DNA;
2, 7 - PCR-products, cooresponding to the first 570 nucleotides of 16S rRNA gene, independent preparation of DNA;
3, 4 – the reactions in the absence of DNA- matrix with primers, corresponding to whole scale copies (3, 8) and the first 570 nucleotides of the gene (4, 9) – the control of quality of reaction mixture;
8 – purified PCR –product for whole scale copping (copies) of the gene;
9 – purified PCR product for the first 570 nucleotides of the gene

While making preliminary analysis of sequence fragments on the basis of more detailed comparative philogenetic analysis with using close sequences of 16S rRNA genes represented in the database GenBank we have the following results: strain 5 clustered with different strains of *At. ferrooxidans* and *At. thiooxidans* (Fig. 3, 4).

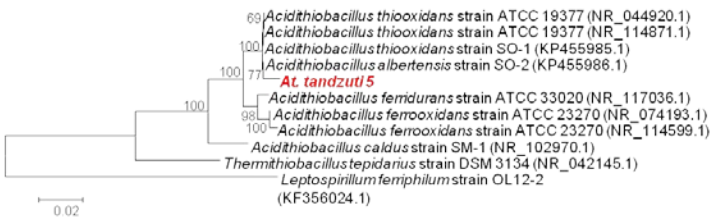

Fig. 4 Phylogenetic position of isolated strain 5

In that heterogeneous cluster the investigated strain with high level of authenticity is included in the philogenetic subcluster formed by strains of species of *At. thiooxidans* from database (including sample strain of type species). The 16S rRNA sequence from studied strain indicates high level of homology with analogous sequences from other strains of that species. Besides, the level of similarity of the 16Sr RNA gene sequence of the investigated strain from different subclusters of the strains *At. ferrooxidans* was noticeably lower- 97.0-98.1 % and with another strains of this genus *At. caldus* was much lower- 95.1-95.3 % (Fig. 4). The level of similarity of the 16S rRNA sequence discovered between the studied strain and the strain of *At . thooxidans* corresponds to intraspecies.

3.5 Leaching results

The ability of isolated strain to oxidize sulfide minerals was studied regarding pyrite and chalcopyrite. Dynamics of oxidation of pyrite (FeS_2) by thermotolerant sulfur- oxidizing bacteria *At.tandzuti* 5 and *S. thermosulfidooxidans* 6 as well as their mixed culture is shown in Fig. 5.

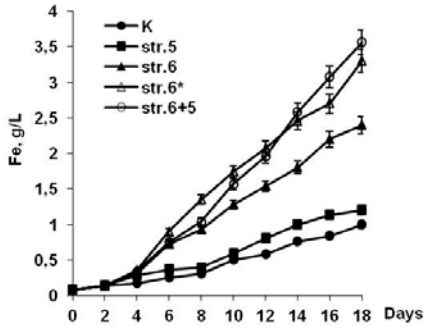

Fig.6 Bioleaching of pyrite by *At.tandzuti* strain 5, *S.thermosulfidooxidans* 6 and their mixed culture (t 37°C, pH 1.9; PD 4%)
* *S.thermosulfidooxidans* 6 grown mixotrophically with 0.05% yeast extract

As shown in Figure 5, during 16 days the cultivation of strain 5 was leached 1.2 g/L Fe. During mixotrophic growth of *S. thermosulfidooxidans* 6 the amount of leached iron was 3.1 g/L whereas during its autotrophic growth it was 2.4 g/L iron (Fig. 5).

The cultivation of *S.thermosulfidooxidans* 6 in mixed culture with sulfur- oxidizing bacteria *At. tandzuti* 5 without adding yeast extract resulted in increasing of leached iron up to 3.56 g/L which is close to that observed during mixotrophic growth of *S thermosulfidooxidans* 6.

Chalcopyrite is one of the most refractory sulfide minerals for both chemical and microbiological oxidation. As shown in Figure 7 the leaching rates of copper and iron during oxidation of $CuFeS_2$ by sulfur- oxidizing bacteria strain 5 is very low and does not differ from that of uninoculated control. Poor oxidation of $CuFeS_2$ was observed during autotrophic growth of *S. thermosulfidooxidans* 6. Studies carried out have shown that the activity of *S. thermosulfidooxidans* 6 increases when grows together with *At. tandzuti* 5. Thus, for 8 days of cultivation intensity of mixed culture by the amount of dissolved copper reached those of *S. thermosulfidooxidans* 6 growing mixotrophically (0.94 g/L) (Fig. 6b). However, the concentration of leached iron during 18 days by mixed culture considerably (2.16 g/L) exceeds *S. thermosulfidooxidans* 6 growing mixotrophically (1.6 g/L) (Fig. 6a).

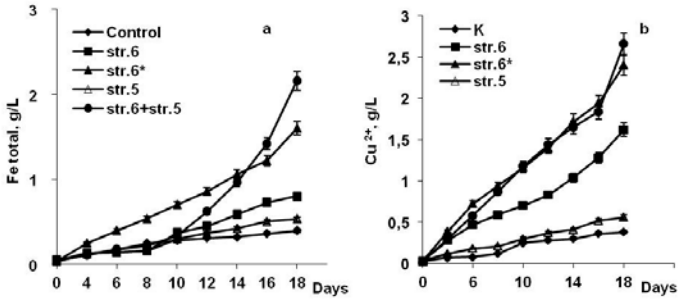

Fig. 6 Bioleaching of iron (a) and copper (b) during oxidation of chalcopyrite by *At.tandzuti* 5, *S.thermosulfidooxidans* 6 and their mixed culture (T 37 °C; pH 1.9; PD 4%)

CONCLUSION

At present for the identification of the species a polyphase approaches are used where the dominating role is given to approximately 70 % of reassociation of DNA. Regarding the analysis of the 16S rRNA it should be mentioned that in many cases the insufficiency of the comparative application of the sequence analysis of that conservative gene and genetic product in the identification of the bacteria has been observed. However, it should be noted that all strains of *At.thiooxidans* are mesophiles. The principle difference of the isolated str.5 from the strains of cluster V is its thermotolerance, the ability to grow in the range of temperatures 25 - 40 with optimal temperature of 37 °C.

At the same time the optimal temperature of the isolated strain is noticeably lower than that of *At. caldus* (45 °C).

The strain 5 differs from phylogenetically familiar microorganisms *At. thiooxidans* by greater size of cells which vary from 0.5 to 0.7 μm in diameter and from 1.2 to 4.0 μm in length. According to the literature data the size of the cells *At. thiooxidans* should not exceed 1.5 μm (Stackebrandt and Goebel, 1994).

Taking into account the above mentioned on the basis of essential phenotypic differences from *At. thiooxidans* (higher optimal temperature for growth and, greater size of the cells), as well as G + C content in the DNA and the level of homology (51 %) the isolated strain was identified as distinguished from *At. thiooxidans*, *At. albertensis* and *At. caldus* a new species of sulfur- oxidizing bacteria and by the name of ore deposit was called *Acidithiobacillus tandzuti* strain 5. The strain is deposited in the Microbial Depositary Center of SPC "Armbiotechnology" of NAS of Armenia under the number INMIA-6966. 16S rRNA sequence was submitted to the GenBank and the accession number DQ991232 was obtained.

Description of *Acidithiobacillus tandzuti* sp.nov.

Cells are motile in young culture. Cells are Gram negative rods 0.5 - 0.7μm in diameter and 1.2 - 4.0 μm in length. Strictly autotroph, oxidizing S, $S_2O_3^{2-}$ and $S_4O_6^{2-}$. Thermotolerant: the temperature range is 25 - 45°C and the optimum is 37 °C. Acidophilic: the pH range for the growth is 2.0 - 6.0 and the optimum is pH 3.0 for sulfur, pH 2.7 for tetrathionate and pH 5.0 for thiosulfate. The G + C content is 54.6 mol /%.

The type strain, 5 (=INMIA-6966), was isolated from Tandzut polymetallic ore drainage water.

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tion.

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