ISSN 0026-2617, Microbiology, 2012, Vol. 81, No. 4, pp. 397–404. © Pleiades Publishing, Ltd., 2012
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EXPERIMENTAL ARTICLES

Rates of Sulfide Mineral Oxidation by Acidophilic Chemolithotrophic Microbial Communities from Various Sources

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Received December 1, 2011

Abstract—A correlation was observed between the rate of oxidation of pure sulfide minerals (pyrite, pyrrhotite, and arsenopyrite) by communities of acidophilic chemolithotrophic microorganisms (ACM) and the mineral substrate where these communities were formed. The ACM community formed during continuous oxidation of the pyrite–arsenopyrite ore concentrate (Kyuchus deposit) exhibited the highest rate of pyrite oxidation. The highest rate of pyrrhotite oxidation was observed for the ACM community developed during semicontinuous oxidation of the pyrrhotite-containing pyrite–arsenopyrite ore concentrate (Olympiadinskoe deposit), by the communities isolated from the pyrrhotite concentrate, and ore of the Shanuch deposit. In the case of arsenopyrite oxidation, the ACM community isolated during oxidation of the Olympiadinskoe ore concentrate grew without a lag phase. Other communities commenced arsenopyrite oxidation at various rates only after a two-day lag phase. The similarity of the mineralogical characteristics of pure sulfide minerals with those of the minerals in the substrates where the ACM communities developed may affect the rates of oxidation.

Keywords: pyrite, pyrrhotite, arsenopyrite, acidophilic chemolithotrophic microorganisms, microbial communities, oxidation of sulfide minerals

DOI: 10.1134/S0026261712040030

In natural ecosystems and in biotechnological processes, oxidation of sulfide minerals, components of the ores of nonferrous and noble metals, is carried out by communities of acidophilic chemolithotrophic microorganisms (ACM), which are able to oxidize ferrous iron, elemental sulfur, and reduced sulfur compounds [1]. The species composition of these communities depends on such factors as temperature, pH, and the concentration of the ions of toxic elements. The role of some of these factors, such as temperature, has been studied in detail. *Acidithiobacillus ferrooxidans, At. thiooxidans, Leptospirillum ferrooxidans*, and *L. ferriphilum* dominate under mesophilic conditions, while *At. caldus, Sulfobacillus* species, and *Acidimicrobium ferrooxidans* prevail under moderately thermophilic conditions. Archaea of the genera *Acidianus, Sulfolobus*, and *Metallosphaera* predominate at the temperatures above 60°C [2].

The composition of the oxidized substrate is an important but insufficiently studied factor affecting the growth of acidophilic chemolithotrophs. Sulfide ores of different deposits differ in their mineralogical composition and the ratio of chemical elements. Sulfide minerals contain iron and sulfur (pyrite, marcasite, and pyrrhotite), nonferrous metals: copper (chalcopyrite, chalckosite, and covellite), zinc (sphalerite), and nickel (pentlandite), and metalloids: antimony (antimonite) and arsenic (arsenopyrite).

An attempt to determine the effect of the composition of the oxidized mineral substrate on the structure of microbial communities was made in [3]. The flasks with mineral medium and various mineral concentrates were inoculated with 12 strains of moderately thermophilic ACM. After 30 days of bioleaching at 45°C, the structure of microbial communities was investigated. In each case, not more than 5 strains of the 12 inoculated ones remained in the community, with one or two strains dominating in some cases. No clear correlation between the species composition of the communities and the composition of the oxidized substrate was observed.

In some cases, the species composition of the community formed during bioleaching was shown to differ from that of the inoculum [4], with the composition of the community varying during a prolonged process [5]. This may result from the fact that bioleaching of sulfide ores in prolonged continuous processes results in gradual changes in the composition of the mineral substrate, since a single deposit may contain the zones with ores of different composition. Thus, adaptive changes in the composition of microbial communities may occur during continuous biooxidation.

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Table 1. ACM communities used in the present work

Mineral substrate for community formation	Mineral substrate composition	Biooxidation conditions	Community composition	Methods for determination of community structure	Reference
Olympiadinskoe ore concentrate (Northern Krasnoyarsk krai, Russia)	Pyrrhotite, pyrite, arsenopyrite, antimonite	Continuous biooxidation in industrial reactors, Olympiadinskoe gold-recovering factory, 40°C	*Acidiferrobacter thiooxidans, L. ferriphilum*, *Sb. thermosulfidooxidans*, *F. acidiphilum*, *At. ferrooxidans Alicyclobacillus tolerans*, *Acidiphilum cryptum*	Cultural techniques, construction and analysis of the 16S rRNA clone libraries	[9]
Olympiadinskoe ore concentrate (Northern Krasnoyarsk krai, Russia)	Pyrrhotite, pyrite, arsenopyrite, antimonite	Semicontinuous biooxidation in laboratory reactors at 50°C with 0.02% of yeast extract in the medium	*Sb. thermosulfidooxidans* HT-1, *Sb. thermosulfidooxidans* HT-3	Cultural techniques	[10]
Kyuchus ore concentrate (Northern Sakha Republic, Russia)	Pyrite, arsenopyrite, antimonite	Continuous biooxidation in laboratory reactors under a two-stage mode (sampling from the second-stage reactor at 40°C)	*L. ferriphilum, Sb. thermosulfidooxidans, Sb. thermotolerans*, *Sb. benefaciens*	Construction and analysis of the 16S rRNA clone libraries	[11]

Apart from the mineralogical composition of the substrate, electrophysical properties of the minerals within this substrate also affect the process of biooxidation of sulfide minerals. This phenomenon was investigated in the case of pyrite [6]. The conductivity type of pyrite affected the oxidation kinetics of this semiconductor. Pyrite varieties are semiconductors with different types of conductivity: n-type (electron) and p-type (hole). The p-type pyrite was oxidized more rapidly than the n-type pyrite. ACM of phylogenetically distant groups (*At. ferrooxidans, Sb. thermotolerans*, and *Ferroplasma acidiphilum*) had different rates of adaptation to pyrites differing in their electrophysical properties and different rates of oxidation of these substrates [7, 8]. The highest capacity for adaptation to all the pyrite types studied was observed in *At. ferrooxidans*, while no adaptation to the pyrites with hole or mixed type conductivity was observed in the case of an archaeon *F. acidiphilum*. The highest and the lowest rates of oxidation of p-type pyrite were found in *Sb. thermotolerans* Kt1^T and *F. acidiphilum* Y^T, respectively. Among two *At. ferrooxidans* strains, TFBk and TFV-1, strain TFBk isolated from the substrate of a more complex mineralogical composition exhibited higher growth rates.

The goal of the present work was to determine the effect of the mineral substrates on which moderately thermophilic and thermotolerant microbial communities were formed on the rates of oxidation of pure sulfide minerals. For this purpose, the mineral medium with one of the sulfide minerals (pyrite, pyrrhotite, or arsenopyrite) as an energy source was inoculated with the biomass of different ACM communities and the oxidation dynamics was determined.

MATERIALS AND METHODS

Microbial cultures and mineral substrates. The biomass from several ACM communities and enrichment cultures were used as inocula. Some communities with the previously studied composition [9–11] from the pulp of the reactors carrying out the oxidation of various sulfide minerals were used (Table 1). Enrichment cultures of moderately thermophilic and thermotolerant ACM obtained in the course of the present work on mineral substrates listed in Table 2 were also used. Pure sulfide minerals (pyrite, pyrrhotite, and arsenopyrite) from the collection of the Laboratory of Chemolithotrophic Microorganisms, Institute of Microbiology, Russian Academy of Sciences were used as sole energy sources for biooxidation.

Isolation of enrichment cultures. To obtain enrichment cultures of microbial communities, 5-g portions of the mineral substrates were placed in sterile flasks with 100 mL of the 9KS mineral medium [12] containing 2 g ferrous iron and 1 g elemental sulfur as energy sources and incubated at 45°C. During the incubation, pH was maintained at ≤2 by adding, if necessary, 10 N H_2SO_4. ACM development was monitored by ferrous iron oxidation and pH decrease. The species composition of the enrichments was determined by molecular genetic techniques, i.e., by analysis of the libraries of the 16S rRNA gene clones obtained from the total DNA of the biomass using the

Table 2. Mineral substrates used for the isolation of the ACM enrichment cultures

Substrate, deposit	Mineral composition	Deposit location
Shanuch deposit ore concentrate	Pyrrhotite, pentlandite, bravoite	Kamchatka peninsula, Russia
Shanuch deposit oxidized ore	Ferric iron minerals, pentlandite, chalcopyrite	Kamchatka peninsula, Russia
Poputninskoe deposit ore	Pyrite, quartz, mica	Krasnoyarsk krai, Russia

universal bacterial and archaeal primers as was previously described [9].

Oxidation of sulfide minerals. Sulfide minerals were oxidized in flasks with 100 mL of the 9K salt base (without iron sulfate) and 1 g of a mineral, which were inoculated with the biomass of one of the communities. In some experimental variants, the medium was supplemented with 0.02% yeast extract (YE). The mineral substrates used for the cultivation of microorganisms were removed from the inoculum by centrifugation for 1 min at 3000 *g*. Microbial cells were then precipitated by centrifugation (10000 *g*, 15 min), washed twice with the 9K mineral salt solution, and resuspended in the same solution. Microbial suspensions were introduced to the flasks in order to achieve the initial cell number of 1×10^6 cell/mL. The cultivation was carried out on a rotary shaker (170 rpm) at 40°C.

The microorganisms were enumerated by direct count under a phase contrast Amplival light microscope (Carl Zeiss, Germany). The concentrations of ferrous and ferric iron ions were determined by chelatometric titration [13]. Decrease in pH of the medium was an indicator of sulfur oxidation.

The growth parameters presented in this work were obtained in three independent experiments carried out in two repeats. The experimental results were statistically treated using the Microsoft Excel software package.

RESULTS

Isolation of Enrichment Cultures and Determination of Their Species Composition

Analysis of the library of the 16S rRNA genes isolated from the total DNA using the universal bacterial and archaeal primers revealed the species composition of the ACM enrichment cultures isolated from the Shanuch deposit oxidized ore, Shanuch deposit ore concentrate, and the Poputninskoe deposit ore (Table 2). Each of the enrichments was shown to contain only one *Sb. thermosulfidooxidans* strain. According to 16S rRNA gene sequencing, the strains from the Shanuch deposit ore concentrate and from the oxidized ore of this deposit designated as *Sb. thermosulfidooxidans* Sh10-1 and *Sb. thermosulfidooxidans* Sh10-2, respectively, were phylogenetically identical. One strain *Sb. thermosulfidooxidans* Poput10 was isolated from the ore of the Poputninskoe deposit. The 16S rRNA sequences of these microorganisms were deposited in the GenBank database under accession numbers JN180088–180090.

Comparative Oxidation Rates of Sulfide Minerals as Sole Energy Sources by Microbial Communities Isolated from Different Substrates

The biomass of three previously studied ACM communities [9–11] and three enrichment cultures obtained in the present work was used as inocula. The designations of the microbial communities are presented in Table 3.

Pyrite oxidation. Changes in the parameters of the medium during pyrite oxidation by various microbial communities are shown on Fig. 1. At the beginning of the process, in all experimental variants a pH increase to 1.95 was observed, followed by its gradual decrease, resulting probably from the oxidation of sulfur compounds, intermediates in the oxidation of this sulfide mineral. The pH decrease was most pronounced (to 1.45–1.50) in the variants *3–6*, while in the variants *2* and *7* pH decreased only to 1.8. In the variants *2* and *7*, the rates of changes in other parameters (Eh, ferric iron concentration, and cell number) were also lower. Development of the community *2* probably depends on the presence of available organic compounds in the medium. This suggestion is supported by the fact that the variant *1*, with the same community used as inoculum, but with YE in the medium, was closer to the other experimental variants in all the parameters, with the cell number (7×10 cell/mL) being the second only to variant *5*. The variant *5* was ahead of other variants in all parameters, especially in the cell number, which reached 12×10^7 cell/mL by the 12th day of cultivation. This may indicate that the microorganisms of this community (formed on the Kyuchus pyrite–arsenopyrite concentrate) were adapted to pyrite, which had the mineralogical properties similar to the energy substrate used in our experiments. On the contrary, variant *7* inoculated with the community from the

Table 3. Designations for the experiments on mineral oxidation by microbial communities

Designation	Inoculum
1*	Community from semicontinuous biooxidation of the Olympiadinskoe ore concentrate in laboratory reactors at 50°C
2	Community from semicontinuous biooxidation of the Olympiadinskoe ore concentrate in laboratory reactors at 50°C
3	Community from continuous biooxidation in industrial reactors, Olympiadinskoe gold-recovering factory, 40°C
4	Enrichment culture from Shanuch oxidized ore, 45°C
5	Community from continuous biooxidation in laboratory reactors, Kyuchus ore concentrate, 40°C
6	Enrichment culture from Shanuch ore concentrate, 45°C
7	Enrichment culture from Poputninskoe ore, 45°C

Note: The medium was supplemented with YE (0.02%).

Poputninskoe pyrite ore lagged behind in all the parameters, since the pyrite of this ore had mineralogical characteristics significantly different from that used in our experiments.

Thus, in this series of experiments, the community formed during the oxidation of a pyrite-containing concentrate (variant *5*) had the highest values of all the parameters of pyrite oxidation.

Pyrrhotite oxidation. Dynamics of the parameters of pyrrhotite oxidation is shown on Fig. 2. Unlike pyrite, pyrrhotite is an acid-soluble sulfide mineral, and the dynamics of its oxidation had the following specific features. Since pyrrhotite dissolution with consumption of the acid occurred at the initial stage of the process, followed by the oxidation of elemental sulfur, pH increased significantly in all the variants at the onset of biooxidation (from 1.5 to 2.3–2.6) and then decreased gradually. In this series of experiments, the highest rates of pyrrhotite oxidation were maintained by the communities formed on pyrrhotite-containing substrates. In variant *1*, pH began to decrease two days earlier than in the other variants, the rate of iron oxidation was higher, and more cells were accumulated (6.7×10^7 cell/mL by day 4). In variant *2*, all the parameters changed more slowly, probably as a result of dependence of the community on organic carbon, similar to the previous series. In variants *4* and *6*, with the microbial communities isolated from pyrrhotite-containing substrates (concentrate and ore of the Shanuch deposit), the mineral was also oxidized relatively rapidly. Although the ferric iron concentration in these variants was somewhat higher than in variant *1* (1.74 and 1.6 g/L, respectively), the maximum cell concentration was almost three times lower than in variant *1* ($1.4–1.7 \times 10^7$ cell/mL), probably also as a result of the absence of organic compounds, which is necessary for constructive carbon metabolism of sulfobacilli (the only components of these cultures). Variant *5*, inoculated with the community formed on a substrate not containing pyrrhotite, had lower rates of all the parameters than the communities formed on pyrrhotite-containing substrates. Variant *7* (community from the Poputninskoe pyrite ore) had a relatively high rate of pyrrhotite oxidation. This was possibly the result of the enrichment procedure in the medium with elemental sulfur, which probably favored accumulation of sulfur-oxidizing microorganisms. The latter are crucially important for pyrrhotite oxidation, since during dissolution in acidic environment, the pyrrhotite particles become covered with a layer of insoluble elemental sulfur, inhibiting further oxidation. Variant *3* was slow in respect to all the parameters, probably because this community was isolated from the Olympiadinskoe ore concentrate, where the activity of sulfur-oxidizing bacteria was low.

Thus, in this series of experiments the community (variant *1*) and enrichment cultures (variants *4* and *6*) isolated from pyrrhotite-containing substrates outperformed the other communities in all parameters of pyrrhotite oxidation.

Arsenopyrite oxidation. Arsenopyrite is one of the sulfide minerals most easily oxidized by microorganisms due to its low electrode potential. Thus, even the microorganisms isolated from the substrates not containing arsenopyrite were able to oxidize it after a short lag phase. The changes in the process parameters by different communities are shown on Fig. 3. Unlike pyrite and pyrrhotite oxidation, experiments with arsenopyrite revealed no significant differences in the dynamics of the parameters of its oxidation by different communities, apart from the cell numbers. In variants *1* and *5*, the highest cell numbers (5.7×10^7 and 5.3×10^7 cell/mL, respectively) were almost three times higher than in other experimental variants ($1.5–2.0 \times 10^7$ cell/mL). In variant *1* (with YE) inoculated with the community containing only sulfobacilli isolated from an arsenopyrite-containing substrate, active growth commenced immediately, without a lag phase, and ceased rapidly due to consumption of the energy source, resulting in massive cell lysis. In variant *5*, leptospirilli were probably responsible for the major contribution to the cell number during the

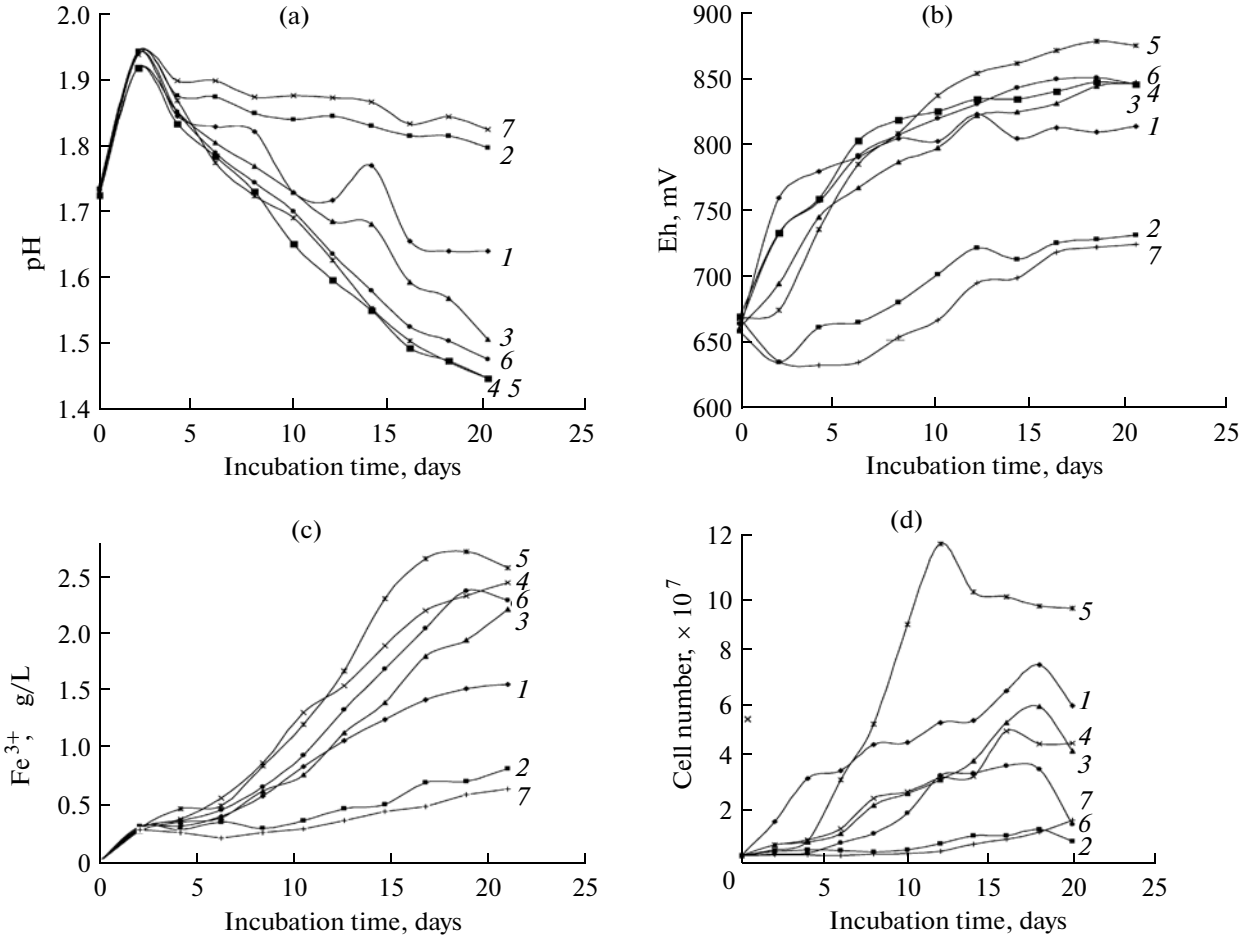

Fig. 1. Dynamics of pyrite oxidation by microbial communities *1–7*: pH (a), Eh (b), Fe^{3+} (c), and cell numbers (d). The designations for microbial communities and enrichment cultures *1–7* are given in Table 3.

exponential growth phase, since their population densities are usually higher than those of sulfobacilli, especially under autotrophic conditions. After 15 days of growth, a drastic pH increase was observed in this variant, resulting in massive cell precipitation with jarosite. Importantly, the ferric iron concentrations in the medium did not differ significantly from those in other experimental variants.

Thus, no significant differences in the oxidation parameters between communities of different origin were observed in this experimental series.

DISCUSSION

Oxidation of sulfide ores by ACM communities is based on the ability of microorganisms to oxidize iron and sulfur, the components of these minerals. Sulfides may be oxidized via two different pathways with formation of different intermediate compounds [14]. Pyrite, molybdenite, and tungstenite are decomposed via the thiosulfate pathway, where thiosulfate is the main intermediate oxidized to sulfate, while sulfur is a by-product. The net reactions of the thiosulfate mechanism follow the equations:

$$FeS_2 + 6Fe^{3+} + 3H_2O = S_2O_3^{2-} + 7Fe^{2+} + 6H^+,$$

$$S_2O_3^{2-} + 8Fe^{3+} + 5H_2O = 2SO_4^{2-} + 8Fe^{2+} + 10H^+.$$

Pyrrhotite, galenite, sphalerite, and most other sulfide minerals are oxidized with formation of polysulfide and elemental sulfur as the major intermediate products. Under acidic conditions, polysulfide is decomposed to elemental sulfur S_8. The net reactions are described by the following equations:

$$MS + Fe^{3+} + H^+ = M^{2+} + 0.5H_2S_n + Fe^{2+},$$

$$0.5H_2S_n + Fe^{3+} = 0.125S_8 + Fe^{2+} + H^+,$$

$$0.125S_8 + 1.5O_2 + H_2O = SO_4^{2-} + 2H^+.$$

In the first case, Fe^{3+} ion is the only oxidizing agent and the oxidation is carried out only by the microorganisms capable of Fe^{2+} oxidation. In the second case, both Fe^{3+} and H^+ participate in the reactions, so that

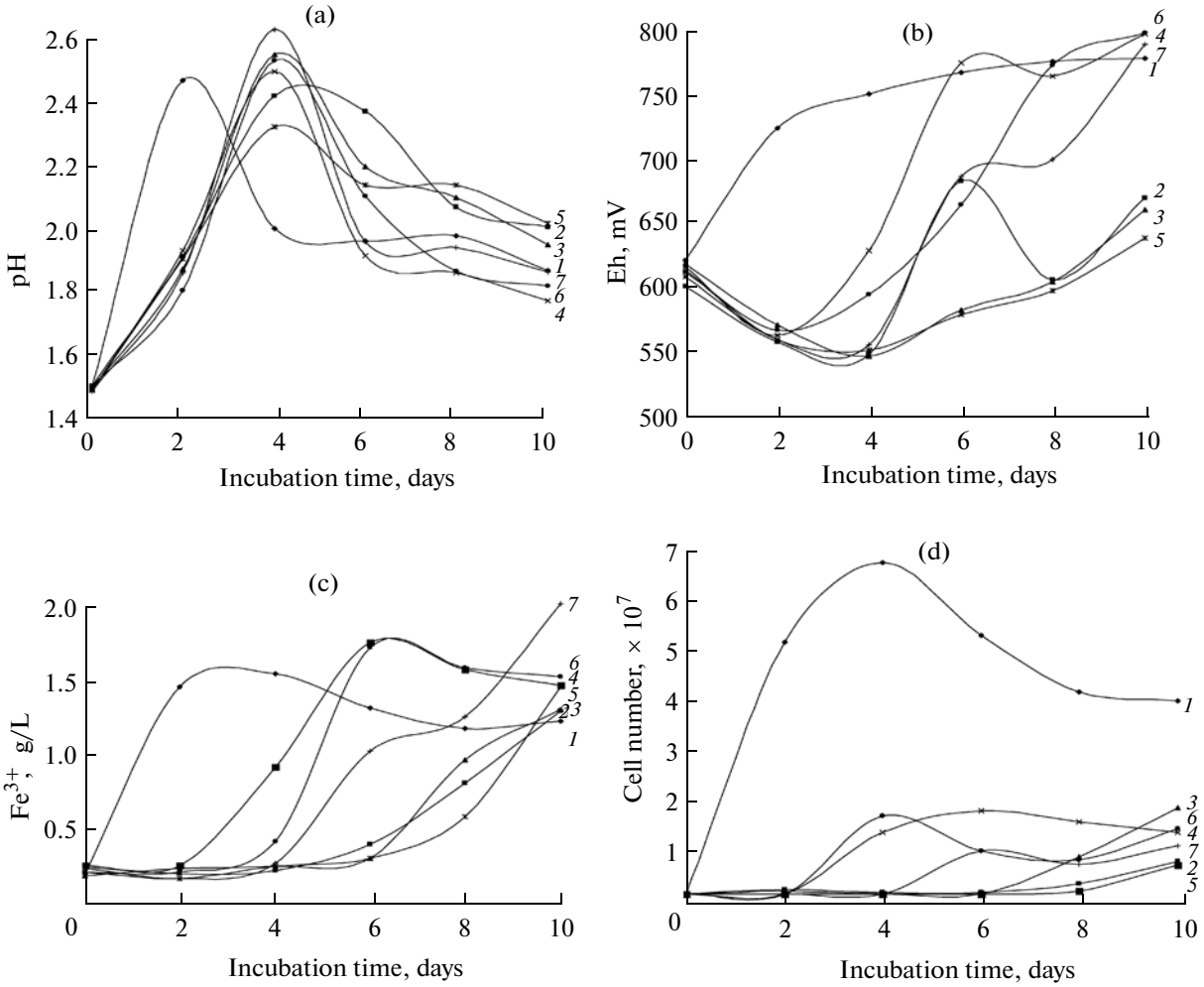

Fig. 2. Dynamics of pyrrhotite oxidation by microbial communities. The designations are as on Fig. 1.

this process may be carried out by microorganisms oxidizing only sulfur [14].

Two main hypotheses exist concerning microbial oxidation of sulfide minerals. According to the first one, the "direct contact mechanism" hypothesis, the oxidation occurs during the interaction of the cellular enzymatic systems with the substrate. The second one is the "indirect mechanism" hypothesis, suggesting that the microorganisms simply oxidize the dissolved ferrous iron ions producing an oxidizer (ferric iron ions). Existence of the direct mechanism is supported by the observations on bacterial attachment to mineral surfaces. The enzymatic systems used by the microorganisms for direct oxidation of sulfide minerals, have not, however, been revealed [15]. The "indirect contact" model of sulfide oxidation, with the microorganisms acting as producers of an oxidizing agent Fe^{3+} and oxidizers of sulfur compounds, is presently universally accepted. Cell adhesion to the mineral surface is an important factor, providing for efficient interaction between the crystalline structure of a mineral and microbial oxidative systems [16]. Due to the differences in the composition of the cell wall lipopolysaccharides and proteins, the charge of the cell surface differs in the cells grown on solid substrates (sulfur or pyrite) and those growing on dissolved ferrous iron [17]. Involvement of the extracellular polymeric substances (EPS) in the oxidation of sulfide minerals was shown. The cells with removed EPS lost their capacity for attachment [18], while the EPS composition varied depending on the oxidized substrate, with hydrophobic fatty acids or uronic acids and carbohydrates predominant in the cells growing on sulfur and pyrite, respectively [19]. Formation of complexes with uronic acids results in Fe^{3+} accumulation in the EPS, facilitating the oxidative attack on sulfides [20]. During the oxidation of mineral substrates, most of the cells attach to the surface rapidly [21], with selective attachment to sulfides, rather than to silicates [22]. The differences between the microorganisms in their capacity for adhesion to different substrates are probably the main cause for the effect of the composition of mineral substrates on the composition of the ACM

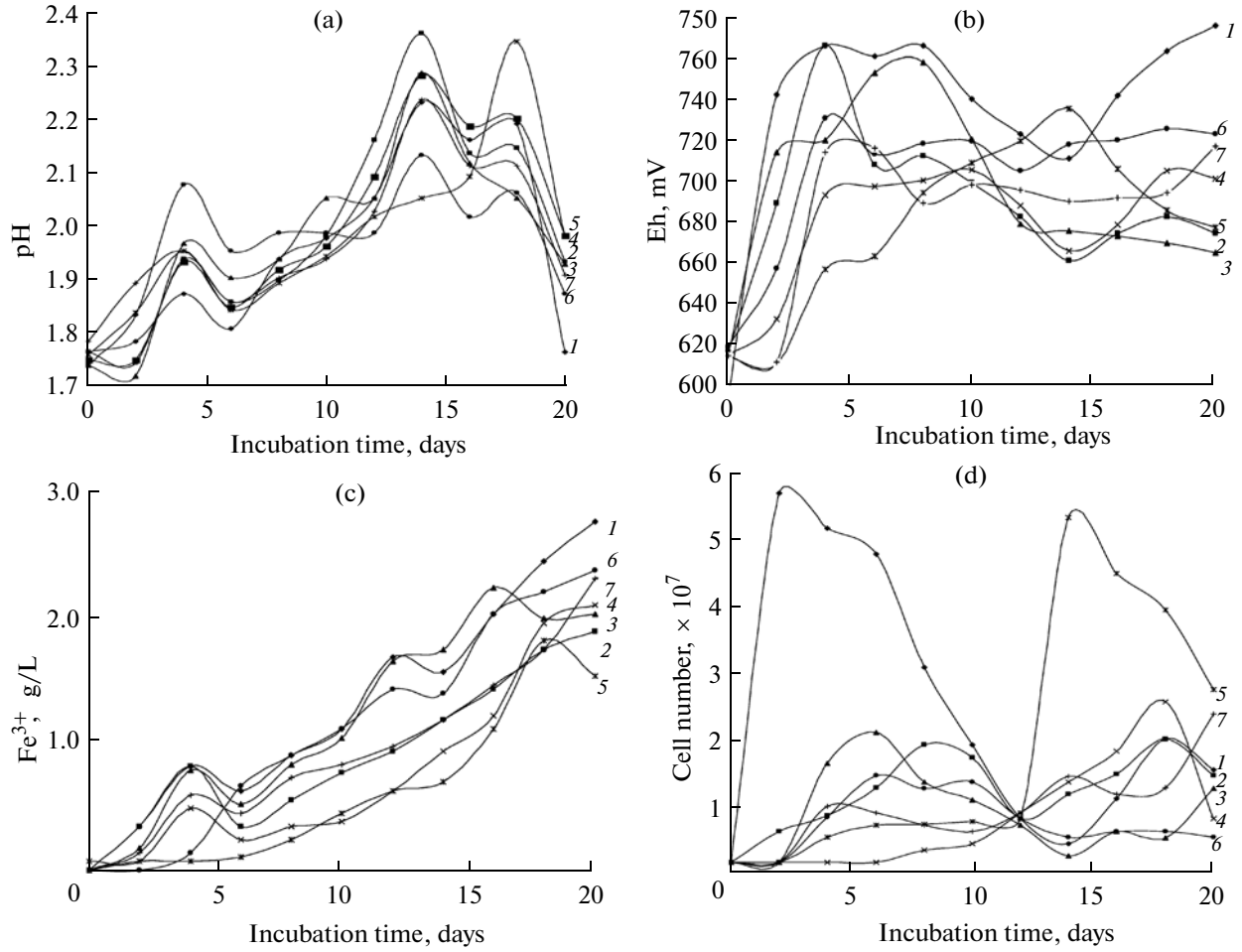

Fig. 3. Dynamics of arsenopyrite oxidation by microbial communities. The designations are as on Fig. 1.

community forming during their oxidation and on their ability to oxidize a specified mineral substrate.

At. ferrooxidans is known to attach to pyrite and jarosite precipitates [23], while chalcopyrite-growing sulfur-oxidizing archaeon *S. metallicus* attaches to the substrate only after formation of a film of elemental sulfur on its surface [24]. Capacity of several strains of mesophilic and moderately thermophilic microorganisms for adhesion to various solid substrates was studied [25, 26]. Capacity for attachment to different substrates (pyrite, quartz, sulfur, or glass) was shown to vary in different species and even in different strains within a species. This may be an explanation for the differences in the dynamics of oxidation of different sulfide minerals by microbial communities formed on different mineral substrates. During the oxidation of a mineral substrate in the course of formation of a microbial community, enrichment of the strains possessing the surface structures providing for adhesion to specific minerals probably occurs. The knowledge of the mechanism of oxidation of a mineral is therefore important. The oxidation by the polysulfide mechanism (e.g., in the case of pyrrhotite) results in abundant formation of elemental sulfur on its surface [27], while significantly less sulfur is formed during the oxidation by the thiosulfate mechanism [28]. Thus, the differences in the cellular surface structures are important for microbial adaptation to the oxidation of sulfide minerals oxidized by different mechanisms. As was mentioned above, growth on sulfur and pyrite resulted in significant differences in the EPS composition.

ACKNOWLEDGMENTS

The work was supported by the Russian Foundation for Basic Research, project no. 10-04-00589 and carried out using the unique equipment of the Biotechnology Center, Russian Academy of Sciences.

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