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EXPERIMENTAL ARTICLES

Physiological Properties of *Acidithiobacillus ferrooxidans* Strains Isolated from Sulfide Ore Deposits in Kazakhstan

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Abstract—*Acidithiobacillus ferrooxidans* strains were isolated from acidophilic microbial communities of Kazakhstan sulfide ore deposits. Their biotechnologically important properties (optimal and maximal growth temperatures and resistance to NaCl) were determined. While temperature optima of the strains were the same (30–32°C), temperature ranges were different. Thus, strain TFBK oxidized iron very poorly at 37°C, while for strain TFV the iron oxidation rate at this temperature was insignificantly lower than at lesser temperatures. NaCl inhibited the oxidative activity of both strains. Iron oxidation by strain TFV was inhibited at 5 g/L NaCl and was suppressed almost completely at 20 g/L. Iron oxidation by strain TFBK was inhibited by NaCl to a lesser degree, so iron oxidation rate was relatively high at 10 g/L, while at 20 g/L NaCl the process was not suppressed completely, although the oxidation rate was low. Sulfur oxidation by these strains was less affected by NaCl than oxidation of ferrous iron. Sulfur oxidation by strain TFV was inhibited considerably, albeit not suppressed completely, only at 20 g/L NaCl, but was not suppressed completely. Sulfur oxidation by strain TFBK was more affected by NaCl. At 10 g/L NaCl the oxidation rate was much lower than at lower NaCl concentrations (sulfate concentrations after 6 days of oxidation at 5 and 10 g/L NaCl were ~130 and ~100 mM, respectively). While sulfur oxidation by strain TFBK was considerably inhibited at 10 and 20 g/L NaCl, similar to strain TFV it was not suppressed completely. Our results indicate the adaptation of the species *A. ferrooxidans* to a broad range of growth conditions.

Keywords: acidophilic microorganisms, *Acidithiobacillus ferrooxidans,* iron oxidation, sulfur oxidation, salt tolerance

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Acidithiobacillus ferrooxidans is the first described and the best-studied species of acidophilic microorganisms oxidizing sulfide minerals [1, 2]. It is a mesophile, obligate autotroph, and a facultative aerobe, deriving energy from oxidation of Fe^{2+}, sulfur and sulfur compounds, or sulfide minerals. It is also capable of anaerobic oxidation of sulfur compounds and hydrogen with Fe^{3+} as an electron acceptor. The species is known to be phylogenetically heterogeneous, and the physiological properties of its strains may vary significantly [3–7]. A number of isolates initially described as *A. ferrooxidans* have been reclassified as belonging to new species *A. ferrivorans* [8] and *A. ferridurans* [9] based on their genetic, biochemical, and physiological characteristics.

Numerous works on microbial ecology revealed that due to the physiological diversity of *A. ferrooxidans* strains, they are ubiquitous at the sites of sulfide mineral oxidation and are often the dominant components of acidophilic microbial communities [10].

Capacity of acidophilic microorganisms for oxidation of sulfide minerals is widely used in biohydrometallurgical technologies for recovery of nonferrous and noble metals from sulfide ores [11]. *A. ferrooxidans* strains and other mesophilic microorganisms often predominate in the processes of heap leaching, when oxidation is not rapid and self-heating of the material does not occur [12]. Investigation of biodiversity of acidophilic microorganisms is important for biohydrometallurgical applications. Thus, monitoring of occurrence of *A. ferrooxidans* strains in the ecosystems of sulfide ore deposits and investigation of their physiological properties attracts attention of researchers in various countries [7, 12].

While the hydrometallurgical technologies for ore processing require large amounts of water, metal deposits are often located in areas with a freshwater shortage, such as Chilean or Australian deserts. Water containing high concentrations of various ions is

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therefore often used for biotechnological operations [14]. Hard water may contain cations of alkaline and alkaline earth metals, as well as anions (sulfate and chloride), which suppress the growth and oxidizing activity of acidophilic microorganisms. Chloride was shown to exhibit a stronger inhibitory effect than other anions (sulfate and phosphate) [14, 15].

The Republic of Kazakhstan has a rich mineral resources base, with its reserves of many metals among the greatest in the world [16]. Introduction of biohydrometallurgical technologies in the republic is promising due to a number of sulfide ore deposits. Many deposits are, however, located in areas experiencing freshwater shortages, so introduction of biohydrometallurgical technologies may require using hard water. Investigation of extreme acidophiles resistant to the ions present in hard water, and specifically to chloride, is important for both biotechnology and microbial ecology.

The goal of the present work was to isolate *A. ferrooxidans* strains from acidophilic microbial communities of Kazakhstan sulfide ore deposits and to investigate their biotechnologically important physiological properties, namely, temperature range for growth and NaCl concentration range in the medium.

MATERIALS AND METHODS

Isolation of pure microbial cultures and cultivation conditions. Samples of acid mine drainage water were from off-balance uranium–molybdenum ores of the Vostok uranium deposit and of the pyrite–arsenopyrite ore of the Bakyrchik deposit. *A. ferrooxidans* strains were isolated at 30°C in 9K medium with $FeSO_4 \cdot 7H_2O$ as an energy source, which is used for cultivation of iron-oxidizing acidithiobacilli [17]. Enrichment cultures of iron-oxidizing mesophilic acidophilic organisms were obtained. Pure cultures were isolated by tenfold terminal dilutions under the same conditions. Bacteria were grown in 250-mL Erlenmeyer flasks with 100 mL of the medium on an orbital shaker (200 rpm) at 30°C.

Phylogenetic analysis by the 16S rRNA gene sequencing. The 16S rRNA genes were amplified and sequenced using the primers universal for most prokaryotes [18]. Amplification was carried out on a Cetus 480 (Perkin Elmer, Sweden) with BioTaq heat-stable DNA polymerase (Dialat Ltd., Moscow, Russia), according to the manufacturer's recommendations. After purification of the PCR products on low-melt agarose and on columns (Promega, United States), the 16S rRNA genes were sequenced using the Silver Sequencing kit (Promega, United States) according to the manufacturer's recommendations. Preliminary analysis of the similarity between the 16S rRNA gene sequences was carried out using the BLAST server (http://www.ncbi.nlm.nih.gov/blast). Aligning of the sequences with those of the most closely related bacterial species and construction of the phylogenetic tree were carried out using the ClustalW2 and TreeDyn software packages [19].

Light microscopy. Quantitative enumeration of microbial cells was carried out under an Amplival phase contrast microscope (Carl Zeiss, GDR) in 20 fields of view.

Physiological properties of the microorganisms. Effect of temperature on microbial growth was studied in the 9K medium. Oxidation of elemental sulfur was studied in the same medium with elemental sulfur (10 g/L) replacing ferrous iron as an energy source. Prior to inoculation, the medium with elemental sulfur was supplemented (1 mL/L) with the trace element solution containing the following (g/L): $FeCl_3 \cdot 6H_2O$, 1.1; $CuSO_4 \cdot 5H_2O$, 0.05; H_3BO_3, 0.2; $MnSO_4 \cdot H_2O$, 0.2; $NaMoO_4 \cdot 2H_2O$, 0.08; $CoCl_2 \cdot 6H_2O$, 0.06; $ZnSO_4 \cdot 7H_2O$, 0.09. The final pH of the medium with S^0 and Fe^{2+} was adjusted with 10 N H_2SO_4 to 2.4–2.5 or 1.7–1.8, respectively. For investigation of resistance of the organism to different NaCl concentrations, it was added to the medium in desired amounts. The concentrations of ferric and ferrous iron ions were determined by complexometric titration [20]. Sulfate concentration was determined turbidimetrically [21].

All presented values for the growth parameters were obtained in two independent experiments carried out in two repeats. The results of typical experiments are presented on the figures.

RESULTS AND DISCUSSION

Isolation of Pure Microbial Cultures and Determination of Their Phylogenetic Position

Pure cultures of acidophilic bacteria were isolated from enrichment cultures of mesophilic acidophilic iron-oxidizing microorganisms obtained from the samples of acid mine drainage water from off-balance ores of the Vostok deposit and of the Bakyrchik deposit ore by tenfold terminal dilutions. Based on their morphological and physiological properties, the strains were tentatively classified as members of the genus *Acidithiobacillus* and designated TFV and TFBK.

Sequencing of the 16S rRNA genes of these strains and comparison of the obtained sequences with those from the GenBank database (http://www.ncbi.nlm.nih.gov/nuccore) was used to determine the phylogenetic position of the strains. The strains TFV and TFBK were assigned to the species *A. ferrooxidans* (Fig. 1), with 98.5–99% similarity with the 16S rRNA gene sequences of *A. ferrooxidans* strains. The gene sequences of strains TFBK and TFV were deposited to GenBank under accession nos. KM192210 and KM192211.

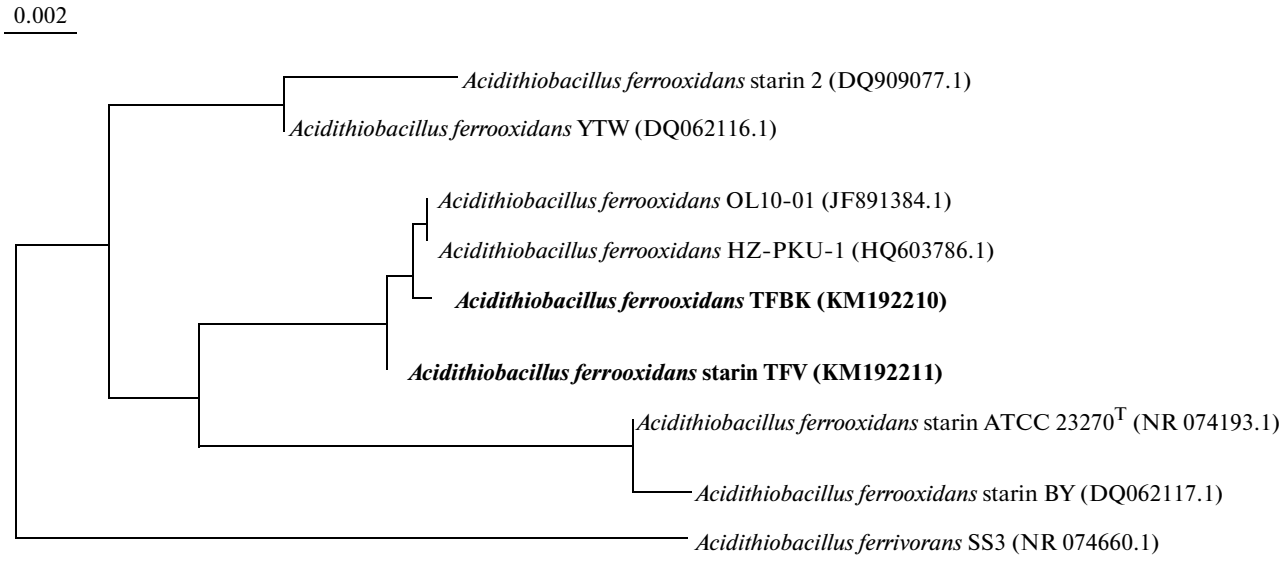

Fig. 1. Phylogenetic tree of the 16S rRNA gene sequences within the genus *Acidithiobacillus* with positions of the new isolates, *A. ferrooxidans* strains TFBK and TFV. The bootstrap support level was above 70%. The sequences obtained in the present work are marked by boldface. The evolutionary distance scale is shown in the upper left. The neighbor joining algorithm was used. The 16S rRNA gene sequence of *A. ferrivorans* SS3 (NR 074660.1) was used as an outgroup.

Physiological Properties of the Isolates

Effect of temperature on the oxidation of ferrous iron. Since *A. ferrooxidans* is an obligate autotroph capable of using ferrous iron as a sole energy source, the rates of Fe^{2+} oxidation by strains TFV and TFBK were used as criteria of the effect of temperature on the physiological activity of these strains. The initial cell concentration was ~10^7 cells/mL.

Effect of temperature on iron oxidation by the studied *A. ferrooxidans* strains is shown on Fig. 2. It can be seen that the stains have almost the same temperature optima for growth (30–32°C), corresponding to the highest rates of iron oxidation. The rates of iron oxidation were slightly lower at 25 and 35°C, while no strain exhibited significant Fe^{2+} oxidation at 40°C. Interestingly, the rate of iron oxidation by strain TFBK at 37°C was extremely low (Fe^{2+} concentration decreased from 7.7 to 6.7 g/L during 48 h of the experiment), while iron oxidation by strain TFV at the same temperature was only slightly lower than at 25°C (after 48 h of the experiment, Fe^{2+} concentration decreased to 1.4 g/L).

These results are in agreement with the previously obtained data on the differences in the highest growth temperature between *A. ferrooxidans* strains, with some strains being thermotolerant and growing even at 40°C, while the others grow at temperatures not exceeding 35–37°C [7]. Ability to survive elevated temperatures may be of high adaptive value to the mesophilic *A. ferrooxidans* strains. Oxidation of sulfide minerals is exothermal, and biological oxidation of such minerals in nature or in the technological processes often results in the temperature increasing to values unfavorable for mesophilic microorganisms (40–70°C).

Effect of NaCl concentrations on Fe^{2+} and S^0 oxidation. The effect of NaCl concentration in the medium on iron oxidation by the studied *A. ferrooxidans* strains is shown on Fig. 3. It can be seen that NaCl inhibited iron oxidation by both strains. Iron oxidation was suppressed insignificantly at 0.5 and 1 g/L NaCl. At 5 g/L NaCl, active iron oxidation by strain TFV began after a 24-h lag phase, and the oxidation rate was considerably less than at lower NaCl concentrations (after six days of cultivation, 1.8 g/L residual Fe^{2+} was present in the medium, compared to trace concentrations at 0.5 or 1 g/L NaCl). The rate of iron oxidation at NaCl 10 g/L was still lower (5 g/L residual Fe^{2+} after six days of cultivation). At NaCl 20 g/L, the rate of iron oxidation was insignificant (~0.5 g/L in six days).

Iron oxidation by strain TFBK was less inhibited by NaCl. At 5 g/L NaCl, the oxidation rate was slightly lower than at smaller NaCl concentrations (trace amounts of Fe^{2+} were present in the medium after three days of oxidation at 0, 0.5, and 5 g/L NaCl). At 10 g/L NaCl, the rate of iron oxidation, although considerably lower than at smaller NaCl concentrations, still remained relatively high (~0.5 g/L residual Fe^{2+} after three days of oxidation). At 20 g/L NaCl, the rate of iron oxidation decreased drastically, although it remained much higher than in the case of strain TFV (~2 g/L was oxidized in six days).

Effect of NaCl on sulfur oxidation by the studied *A. ferrooxidans* strains is shown in Fig. 4. It may be

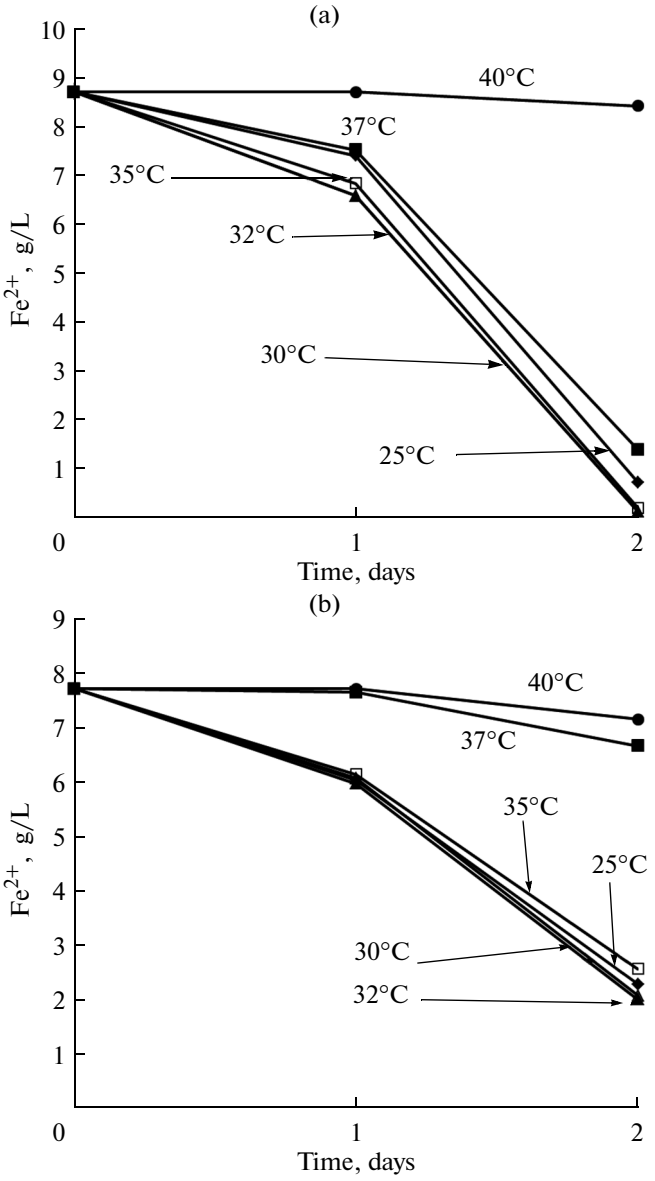

Fig. 2. Oxidation of ferrous iron (Fe^{2+} concentrations) by *A. ferrooxidans* strains TFV (a) and TFBK (b) at different temperatures.

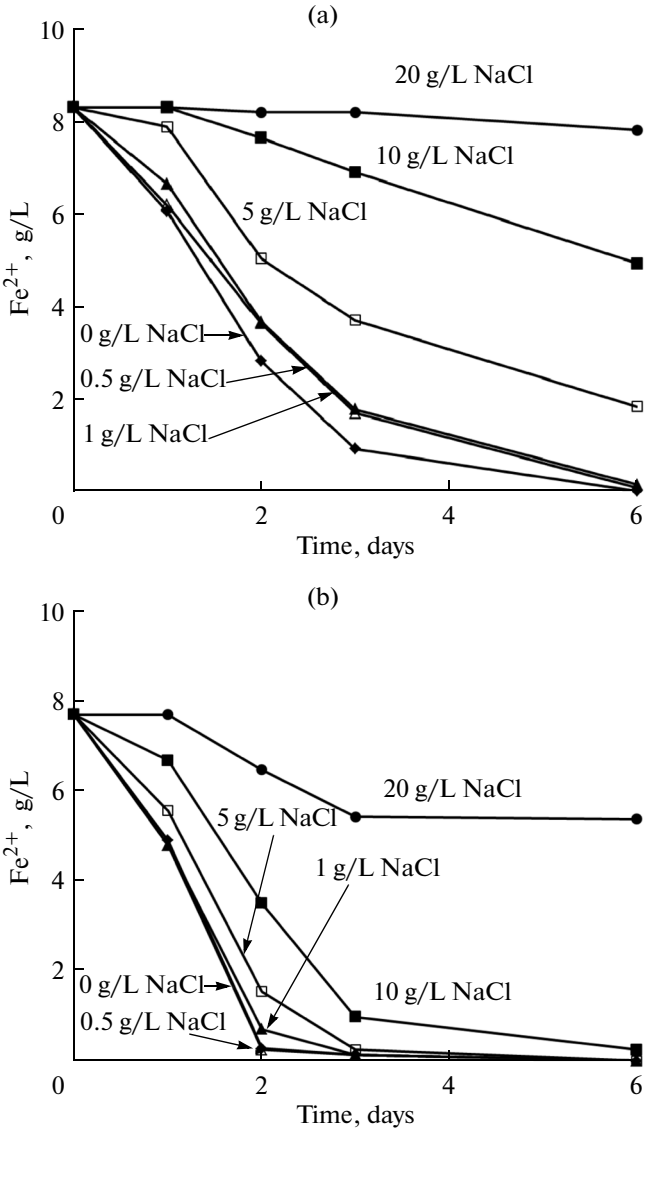

Fig. 3. Oxidation of ferrous iron (Fe^{2+} concentrations) by *A. ferrooxidans* strains TFV (a) and TFBK (b) in the presence of different NaCl concentrations.

seen that at high concentrations (10–20 g/L) NaCl inhibited sulfur oxidation by both strains. However, at 0.5 or 1 g/L NaCl both strains oxidized sulfur more rapidly than in the absence of NaCl in the medium. Sulfur oxidation by strain TFV became noticeably inhibited only at 20 g/L NaCl (while the oxidation rate was considerably less than at lower NaCl concentrations, oxidation was not completely suppressed, and sulfate concentration increased from 40 to 80 mM during six days). At 10 g/L NaCl, the rate of sulfur oxidation was slightly lower than at 5 g/L or in the absence of NaCl in the medium.

Sulfur oxidation by strain TFBK was more pronouncedly inhibited by NaCl. At 10 g/L NaCl, the oxidation rate was considerably lower than at smaller NaCl concentrations (sulfate concentrations after six days of oxidation were 130 mM at 0 and 5 g/L NaCl and ~100 mM at 10 g/L NaCl). While at 20 g/L NaCl, sulfur oxidation by strain TFBK was inhibited considerably; similar to strain TFV, it was not suppressed completely (after six days, sulfate concentration increased from 40 to 80 mM).

Thus, sulfur oxidation by *A. ferrooxidans* strains TFBK and TFV was less suppressed by NaCl than the oxidation of ferrous iron. This result agrees with the earlier data [22] on iron oxidation by *A. ferrooxidans* strains being suppressed by lower chloride concentrations than sulfur oxidation. In general, our results

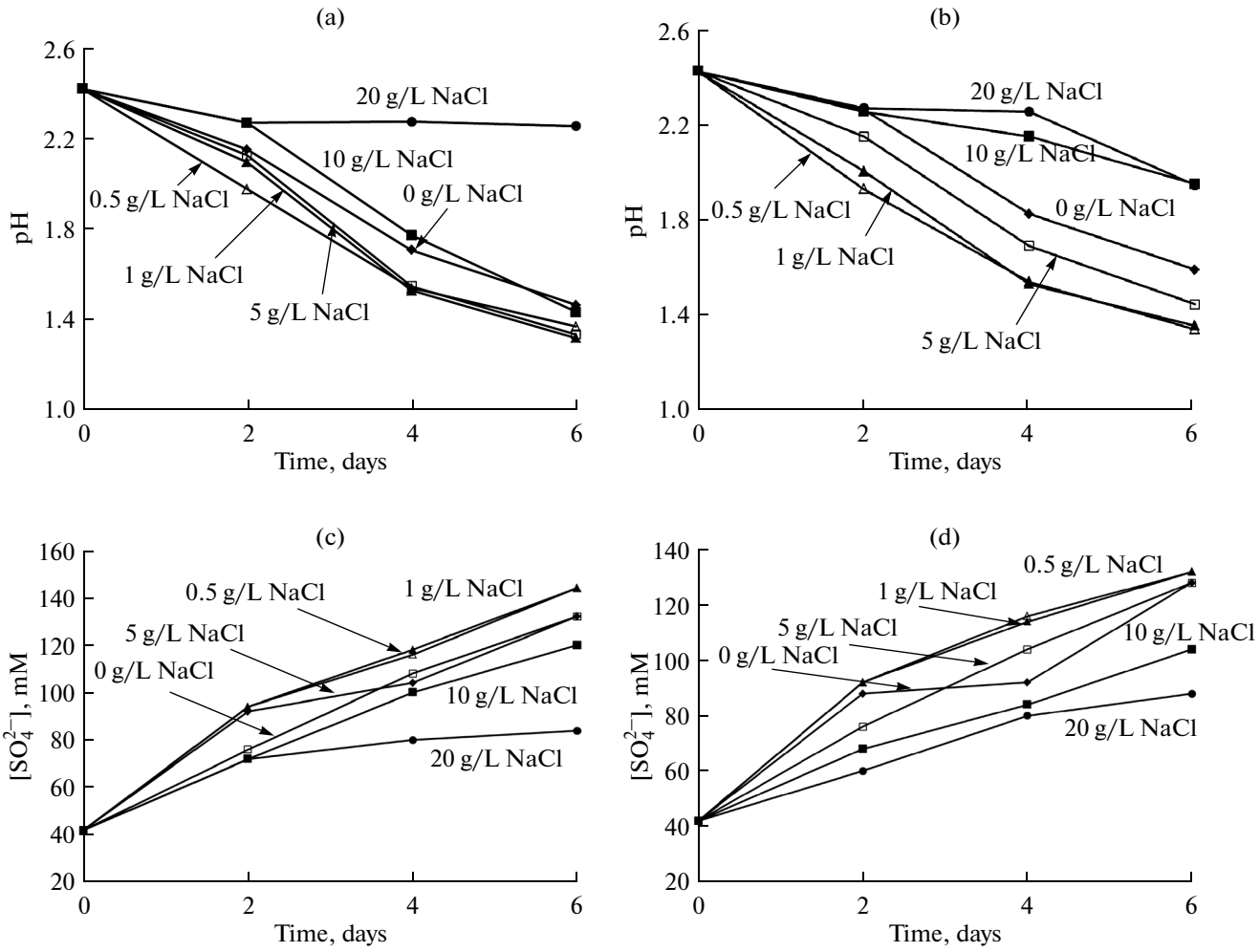

Fig. 4. Oxidation of elemental sulfur by *A. ferrooxidans* strains TFV and TFBK in the presence of different NaCl concentrations: pH in the course of sulfur oxidation by strain TFV (a), pH in the course of sulfur oxidation by strain TFBK (b), sulfates in the medium in the course of sulfur oxidation by strain TFV (c), and sulfates in the medium in the course of sulfur oxidation by strain TFBK (d).

Resistance of *A. ferrooxidans* strains to chloride, mM

Strain	Fe^{2+} oxidation		S^0 oxidation		Salt used	Reference
	optimum	complete suppression	optimum	complete suppression		
TFBK	2–11	Above 347	11–19	Above 347	NaCl	–
TFV	2	347	11–19	Above 347	NaCl	–
DSM 14882^T	1	121	ND*	ND*	NaCl	[14]
SM-4	50	Above 500	50	Above 500	KCl	[22]

* The data are not available in the cited source.

showed that the studied strains were somewhat more resistant to NaCl than some of the previously studied strains of this species. For instance, growth of the type strain *A. ferrooxidans* DSM 14882^T was considerably inhibited by 3.5 g/L NaCl and became completely suppressed at 7 g/L NaCl [14] (table). The differences in the inhibition of oxidation of various substrates by different strains in the presence of different NaCl concentrations may be due to the differences in the composition and structure of the membrane proteinaceous ETC components involved in the oxidation of inorganic substrates (the ETC responsible for sulfur and iron oxidation by *A. ferrooxidans* are known to consist of different components). *Acidithiobacillus* strains, although closely related phylogenetically, were shown to differ in the structure of their ETC components [23].

Analysis of the results of the present work and their comparison to the data obtained by other researchers suggest a conclusion that *A. ferrooxidans* is a species adjusted to a broad range of growth conditions (table). The strains TFBK and TFV isolated in the course of the present work and exhibiting resistance to high NaCl concentrations may be promising for metal recovery from sulfide ores in the case of freshwater shortage.

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