

Article

Enrichment Cultures of Extreme Acidophiles with Biotechnological Potential

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Abstract

The purpose of this work was to obtain specialized enrichment cultures from an original extreme acidophilic consortium of extremely acidophilic microorganisms and to study their microbial community composition and biotechnological potential. At temperatures of 25, 35, 40 and 50 °C, distinct enrichments of extremely acidophilic microorganisms used in the processes of bioleaching sulfide ores were obtained using nutrient media containing ferrous sulfate, elemental sulfur and a copper sulfide concentrate as nutrient inorganic substrates, with and without the addition of 0.02% yeast extract. The microbial community composition was studied using the sequencing of the V3–V4 hypervariable region of the 16S rRNA genes. The different growth conditions led to changes in the microbial composition and relative abundance of mesophilic and moderately thermophilic, strict autotrophic and mixotrophic microorganisms in members of the genera *Acidithiobacillus*, *Sulfobacillus*, *Leptospirillum*, *Acidibacillus*, *Ferroplasma* and *Cuniculiplasma*. The dynamics of the oxidation of ferrous iron, sulfur, and sulfide minerals (pyrite and chalcopyrite) by the enrichments was also studied in the temperature range of 25 to 50 °C. The study of enrichment cultures using the molecular biological method using the metabarcoding method of variable V3–24 V4 fragments of 16S rRNA genes showed that enrichment cultures obtained under different conditions differed in composition, which can be explained by differences in the physiological properties of the identified microorganisms. Regarding the dynamics of the oxidation of ferrous ions, sulfur, and sulfide minerals (pyrite and chalcopyrite), each enrichment culture was studied at a temperature range of 25 to 50 °C and indicated that all obtained enrichments were capable of oxidizing ferrous iron, sulfur and minerals at different rates. The obtained enrichment cultures may be used in further work to increase bioleaching by using the suitable inoculum for the temperature and process conditions.

Keywords: bioleaching; sulfide minerals; acidophilic microorganisms; pyrite; chalcopyrite; metabarcoding



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1. Introduction

Currently, biohydrometallurgical technologies are widely used on an industrial scale for the production of non-ferrous and precious metals from sulfide ores and concentrates. The most used biohydrometallurgical technologies are based on the microbial oxidation of sulfide minerals contained in ores by acidophilic microorganisms oxidizing ferrous iron and

sulfur as an energy substrate [1–3]. Microorganisms used in biohydrometallurgy have been intensively studied due to their great practical importance [4]. The most well-studied bacterium is *Acidithiobacillus ferrooxidans* (formerly *Thiobacillus*), which is capable of oxidizing both ferrous iron and sulfur [5]. Currently, it is known that biohydrometallurgical processes are often dominated by other species of microorganisms (about 30 species) [4]. These microorganisms belong to various groups of procaryotes; however, they have common physiological properties that allow us to use these microorganisms in biohydrometallurgical processes: they are acidophiles (the optimal pH for their life activity is below 3) [4,6]; they utilize inorganic compounds (ferrous iron, sulfur and its reduced compounds, and sulfide minerals) to obtain energy [4,7]; and they are resistant to heavy metal ions that accumulate in their habitats as a result of the dissolution of sulfide minerals [7–9]. These microorganisms differ in their optimal growth temperature: some of them (*Leptospirillum* spp. and *Acidithiobacillus* spp.) are mesophiles (i.e., the optimal temperatures for them are below 40 °C), others (*Sulfobacillus* spp., *Acidimicrobium* spp., *Acidithiobacillus caldus*, and *Acidiplasma* spp.) are moderate thermophiles (the optimal temperatures for them are 40–60 °C), and the third (archaea of the order *Sulfolobales*) are thermophiles (the optimal temperatures for them are above 60 °C). They also differ in their ability to oxidize various energy substrates: some microorganisms oxidize only ferrous iron (*Ferroplasma* spp. and *Leptospirillum* spp.), and others are capable of oxidizing only sulfur and its soluble reduced compounds (*Acidithiobacillus caldus* and *A. thiooxidans*), while others oxidize both ferrous iron and sulfur compounds (*A. ferrooxidans* and *Sulfobacillus* spp.). According to the ability to assimilate various carbon sources (organic and inorganic (CO₂)), some of them are autotrophs, i.e., capable of assimilating CO₂ (*Leptospirillum* spp. and *Acidithiobacillus* spp.), others are heterotrophs, i.e., require organic carbon sources for growth (*Ferroplasma* and *Acidiplasma* spp.), and some of them are mixotrophs, capable of assimilating both CO₂ and organic substrates, but, as a rule, requiring organic substances for stable growth (*Sulfobacillus* spp.) [4]. For some groups, the possibility of CO₂ fixation has been poorly understood. For example, weak carbon dioxide fixation was demonstrated for some strains of the species *F. acidiphilum* and the putative “chimaeric pathway” for CO₂ fixation was assumed in *F. acidarmanus*, despite the fact that all known representatives of the genus *Ferroplasma* require the presence of organic nutrients in the medium for growth [4,10,11].

Technological processes in practice are carried out not by pure cultures of microorganisms but by their communities in which microorganisms interact. For example, it is known that mixotrophs and heterotrophs consume organic substances secreted by autotrophs [12,13]. In technological processes, succession (i.e., a change in the composition of the microbial population) occurs, caused by changing conditions due to sulfide oxidation (for example, in bio-oxidation heaps, there is a gradual increase in temperature and the displacement of mesophiles by thermophiles) [14,15].

Microbial communities (or populations) that carry out the processes of the heap and reactor bio-oxidation of sulfide ores and concentrates, and the patterns of their formation, have been actively studied (Table 1). It has been shown that their composition can change during the bioleaching process and depends on factors such as the composition of mineral raw materials, temperature, etc.

The data presented in Table 1 indicate that the composition of microbial communities that carry out the processes of the bio-oxidation of sulfide ores and concentrates is not distinguished by great species diversity, which is obviously due to the extreme operating conditions of such communities, which are unfavorable for most known living organisms. At the same time, microbial communities of acidophilic microorganisms that carry out bio-oxidation processes are able to adapt to different environmental factors (temperature and the composition of oxidized ores and concentrates), while adaptive changes occur in

the communities, leading to a change in their composition and a change in the dominant groups of microorganisms, which, in turn, allows processes to be carried out in a wide range of conditions.

Since acidophilic microorganisms that oxidize sulfide minerals, as well as their consortia, are widely used for processing mineral raw materials, their isolation and a study of their biotechnological potential and adaptation for the bio-oxidation of various mineral raw materials are of great interest. Research has been conducted to isolate new strains and obtain consortia of acidophilic microorganisms and study them, including determining their composition and their ability to oxidize sulfide minerals [16–25]. Such strains and microbial consortia can be used to carry out technological processes, in particular to create active microbial consortia for inoculation [26–29].

Table 1. Composition of microbial population of bioleach heaps and reactors.

Source	Main Minerals of Ore/Concentrate	T, °C	Microbial Population	Reference
Heap bioleaching	Pyrite, arsenopyrite	Gradual increase from ambient temperature to 60–80 °C	<i>A. ferrooxidans</i> , <i>Leptospirillum</i> spp., <i>Sulfobacillus</i> spp. Temperature increase led to the predominance of the genera <i>Acidianus</i> , <i>Metallosphaera</i> , <i>Sulfolobus</i>	[14]
	Pyrrhotite, pyrite, sphalerite, pentlandite, violarite, chalcopyrite and graphite	Gradual increase from 0 to 30 and 90 °C	<i>A. ferrooxidans</i> , <i>A. caldus</i> and <i>L. ferrooxidans</i> , <i>S. thermosulfidooxidans</i>	[15]
	Pyrite, chalcocite, covellite, enargite	No data	<i>Acidithiobacillus</i> , <i>Leptospirillum</i>	[30]
	Pyrite, chalcopyrite, molybdenite, sphalerite, galena	No data	<i>Acidithiobacillus ferrovorans</i> , <i>A. ferrooxidans</i> , <i>Rhodanobacter</i> , <i>Thiobacillus</i> , <i>Leptospirillum</i> , <i>Acidiphilium</i>	[31]
	Pyrite, chalcocite, covellite, enargite	No data	<i>Acidithiobacillus</i> , <i>Sulfobacillus</i> , <i>Acidiferrobacter</i>	[20]
Bioleach reactor	Pyrite	42	<i>Leptospirillum ferriphilum</i> , <i>A. caldus</i> , <i>Ferroplasma acidiphilum</i> , <i>Sulfobacillus benefaciens</i>	[32]
	Pyrite, chalcopyrite, sphalerite	45	<i>A. caldus</i> , <i>L. ferriphilum</i> , <i>Sulfobacillus</i> sp., <i>Ferroplasma</i> sp.	[33]
	Pyrite, arsenopyrite, chalcopyrite	45	<i>A. caldus</i> , <i>Sulfobacillus thermosulfidooxidans</i> , “ <i>Sulfobacillus montserratensis</i> ”	[34]
	Pyrite, arsenopyrite	40–50	<i>Sulfobacillus</i> sp., <i>A. caldus</i> , <i>L. ferriphilum</i> , <i>Ferroplasma</i> sp., <i>Acidiplasma</i> sp.	[35]
	Pyrite, arsenopyrite	40–50	<i>Acidithiobacillus</i> , <i>Leptospirillum</i> , <i>Sulfobacillus</i> , archaea <i>Ferroplasma</i> , <i>Acidiplasma</i> , <i>Cuniculiplasma</i> , “ <i>Ca.Carboxiplasma ferriphilum</i> ” (A-plasma)	[36]
	Pyrite, arsenopyrite	40–50	<i>Acidithiobacillus</i> , <i>Leptospirillum</i> , <i>Sulfobacillus</i> , archaea <i>Ferroplasma</i>	[37]

The purpose of this work was to obtain enrichments of extremely acidophilic microorganisms and study their species composition and biotechnological potential, conducting the following tasks:

- To obtain enrichments of extremely acidophilic microorganisms used in the processes of bioleaching sulfide ores, using nutrient media containing ferrous sulfate, elemental sulfur and copper sulfide concentrate as nutrient substrates at wide temperature range 25–50 °C;
- To determine the microbial composition of the enrichment cultures using the metabarcoding of variable V3–V4 fragments of 16S rRNA genes and compare enrichments grown under different conditions;
- To study the dynamics of the oxidation of ferrous iron ions, sulfur, and sulfide minerals (pyrite and chalcopyrite) by the obtained enrichments in the temperature range of 25–50 °C to establish the effect of temperature on the activity of enrichments.
- Based on the data obtained, to make a conclusion about the possibility of using the obtained enrichment cultures in biotechnological processes.

2. Materials and Methods

2.1. Obtaining the Enrichments

As an inoculum for obtaining enrichment cultures, we used an enrichment culture from a sample of ore dressing wastes (old pyrite tailings), which was maintained under constant cultivation conditions (at ambient temperature, 20–25 °C) in the Research Center of Biotechnology, the Russian Academy of Sciences (Moscow, Russian Federation) [38], which was used for the bioleaching of a sample of old tailings of polymetallic ore flotation [39]. Since, within the framework of the research objectives, it was necessary to obtain enrichments with the greatest possible diversity of acidophilic microorganisms, a nutrient medium that contained several nutrient substrates (ferrous sulfate, elemental sulfur, sulfide and copper–zinc concentrate) was used to obtain enrichments. We used 9KS medium, which contained (g/L) $(\text{NH}_4)_2\text{SO}_4$ —3.0, KCl—0.2, $\text{MgSO}_4 \times 7\text{H}_2\text{O}$ —0.5, K_2HPO_4 —0.5, and $\text{FeSO}_4 \times 7\text{H}_2\text{O}$ —9.82 [40]. To set the initial pH at 1.5, 1.5 mL/L sulfuric acid was added to the medium. In some experimental variants, 0.02% yeast extract (YE) was added to the medium. Elemental sulfur S^0 and copper–zinc concentrate, which contained 24.4% Fe, 6.2% Cu, 7.3% Zn, and 1.7% As, the main minerals of which were chalcopyrite (CuFeS_2), tennantite ($\text{Cu}_{12}\text{As}_4\text{S}_{13}$), sphalerite (ZnS), and pyrite (FeS_2) [41], were also added into the medium (10 g/L). To obtain the enrichments, the medium was poured (100 mL each) into 250 mL Erlenmeyer flasks, 2 mL of the inoculum was added, and then the cultures were incubated at the appropriate temperature (25, 35, 40, and 50 °C) in thermostats using KS 260 basic shakers (IKA, Staufen, Germany) at 200 rpm.

To assess the growth of enrichments, medium samples were periodically collected, and parameters that indicate the growth of microbial cultures were measured: pH, redox potential (Eh) (using a pH-150MI pH meter (Izmerytelnaya Tekhnika, Moscow, Russian Federation), divalent and trivalent iron content. The content of iron ions was determined using trilonometric titration [42]. A quantitative assessment of microorganisms was carried out by direct counts using an Amplival (Carl Zeiss, Jena, Germany) microscope equipped with a phase-contrast device.

2.2. Microbial Population Analysis

The compositions of the resulting enrichments, as well as the inoculum, were determined by the method of metabarcoding V3–V4 fragments of the 16S rRNA gene. The metabarcoding of V3–V4 fragments of the 16S rRNA gene is widely used for the analysis of microbial populations of various habitats, as it ensures the identification of prokaryotes of

various groups due to the sufficiently long length of the resulting amplifier for analysis [43]. In the present work, the metabarcoding of V3–V4 fragments of the 16S rRNA gene made it possible to compare the composition of the obtained enrichment cultures and reveal microbial groups prevalent under different conditions.

To isolate DNA, the biomass was separated from the solid and liquid components of the medium by centrifugation using an Eppendorf 5804 centrifuge (Eppendorf, Wesseling-Berzdorf, Germany). Samples were first centrifuged at 1000 rpm for 5 min to separate the solid components of the medium. The supernatant was used to sediment the biomass by centrifugation at 10,000 rpm within 15 min. The resulting sediment of the biomass of acidophilic microorganisms was washed, resuspending the pellets in the mineral medium of the following composition (g/L), $(\text{NH}_4)_2\text{SO}_4$ —3.0, KCl—0.2, $\text{MgSO}_4 \times 7\text{H}_2\text{O}$ —0.5, K_2HPO_4 —0.5, H_2SO_4 —6 mL/L, and centrifuging the suspension at 10,000 rpm within 15 min to remove precipitates of iron compounds and then performing the same procedure using the same solution with a neutral pH (which did not contain H_2SO_4) to remove the acid. The sample was resuspended in 500 μL of lysis buffer (0.15 M NaCl, 0.1 M $\text{Na}_2\text{-EDTA}$, pH 8.0). Before DNA extraction, the buffer with lysed biomass was stored at -20°C in plastic tubes with screw caps.

The composition of microbial populations that formed during the experiments under different conditions was determined by the metabarcoding of V3–V4 variable 16S rRNA gene fragments using the MiSeq system (Illumina, San Diego, CA, USA). Procedures of biomass sampling, DNA extraction, amplification, sequencing, and bioinformatic analysis were described in detail in our previous work [36]. In short, 16S rRNA gene fragment reads of all samples were clustered together into OTUs (operational taxonomic units) at 97% identity, and the relative abundance of each OTU at each sample was calculated by mapping reads to the OTUs. Low-quality reads, singleton OTUs and chimeric sequences were removed during the process.

The 16S rRNA gene V3–V4 fragment sequences were deposited in the NCBI Sequence Read Archive and are available via the BioProject accession number PRJNA1267857.

2.3. Study of Dynamics of Oxidation of Ferrous Iron Ions, Sulfur, and Sulfide Minerals (Pyrite and Chalcopyrite) by Obtained Enrichments

To conduct experiments on the bio-oxidation of sulfide minerals (pyrite FeS_2 and chalcopyrite CuFeS_2) and sulfur, a liquid nutrient medium containing mineral salts was used. The experiments were carried out in flasks with 100 mL of nutrient medium and 0.5 g of minerals or sulfur S^0 using KS 260 basic shakers (IKA, Germany) (200 rpm) for 10 days in experiments with minerals and 5 days in experiments with elemental sulfur. The experiments were carried out at temperatures at which enrichment cultures were obtained; when conducting experiments with cultures obtained on a medium containing YE, 0.02% (w/v) YE was also added to the medium. Cultures in all experiments were inoculated so that the initial total microbial cell count was approximately 0.5×10^7 cells/mL.

The experiments on iron bio-oxidation were carried out in a similar way using 9KS medium [40] for 1–5 days, depending on the rate of iron bio-oxidation.

To analyze the process of the bioleaching of minerals, samples of the liquid phase were collected. In all selected samples, pH, oxidation/reduction potential (Eh), and ferrous and ferric iron ion concentration were measured; in experiments on the bioleaching of chalcopyrite, copper concentration was also measured.

The parameters were used as they characterized microbial activity during the bio-oxidation of sulfide minerals [1]. The abiotic acid leaching of sulfide minerals leads to proton consumption, a pH increasing and the dissolution of metal cations (including Fe^{2+} and Cu^{2+}). The activity of acidophilic microorganisms oxidizing sulfide minerals results

in Fe^{2+} oxidation and Fe^{3+} accumulation in the medium and a pH decrease due to the oxidation of the elemental sulfur and sulfur moiety of sulfide minerals.

The concentration of iron ions in the medium was determined spectrophotometrically using the thiocyanate method [42]. The copper content in the medium was measured using a KVANT-2mt atomic absorption spectrometer (Kortek, Moscow, Russia). The degree of the leaching of metals from minerals was assessed by the concentration of iron and copper ions.

3. Results

3.1. Obtaining and Conducting Analysis of Enrichments

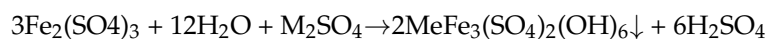
The results of the obtaining of enrichments are presented in Table 2.

Table 2. Changes in physico-chemical parameters of medium during the growth of the acidophilic enrichment cultures.

Cultivation Conditions			pH		Eh, mV		Fe^{3+} , g/L		Fe^{2+} , g/L		Microbial Cell Number, $\times 10^7$ cell/mL	
T, °C	YE	Incubation, Days	Initial	Final	Initial	Final	Initial	Final	Initial	Final	Initial	Final
25	-	20	2.05	1.16	325	872	0.28	2.8	1.96	0	0.2	42.3
35	-	20	2.05	1.54	325	858	0.28	1.4	1.96	0	0.2	26.6
40	-	30	2.05	1.73	325	771	0.28	0.56	1.96	0	0.2	1.2
40 *	-	7	1.73	1.12	771	795	0.56	1.89	1.96	0	2.2	11.3
40	+	5	2.05	1.85	325	757	0.28	1.33	1.96	0	0.2	4.7
50	-	30	2.05	1.73	325	680	0.28	0.56	1.96	0.14	0.2	0.2
50 *	-	7	1.73	1.55	680	728	0.28	0.84	1.96	0	1.2	1.8
50	+	5	2.05	1.57	325	803	0.28	1.19	1.96	0	0.2	7.8

* The inoculation of the *A. caldus* MBC-1 strain into the enrichment.

The data presented show that the active growth of microorganisms was observed at temperatures of 25 and 35 °C. Over 20 days of incubation, the number of microbial cells increased by two orders of magnitude (up to 42.3 and 26.6×10^7 cells/mL, respectively). Parameters that indirectly characterize the activity of acidophilic microorganisms that oxidize sulfide minerals also indicated the active growth of target groups of microorganisms in these enrichments: pH decreased significantly during incubation, Eh values rose above 800 mV, and ferrous ions were completely oxidized to Fe^{3+} . This indicated the active oxidation of sulfur and ferrous ions. At higher temperatures, the growth of microorganisms was less active. In cultures that were grown at 40 and 50 °C in a medium containing YE, growth was also quite active. At the end of the incubation, the total number of microbial cells reached 4.7 and 7.8×10^7 cells/mL, respectively, which was 3–10 times lower than at 25 and 35 °C but an order of magnitude higher than at the beginning of the incubation after inoculation. In these cultures, Fe^{2+} ions were also completely oxidized, and the pH decreased compared to the initial value. It should be noted that the total concentration of iron ions in cultures grown at 40 and 50 °C was significantly lower than at the beginning of the incubation. This may be explained by the fact that at elevated temperatures, insoluble jarosite is formed at a high rate, which leads to a decrease in the concentration of iron ions [44]:



where Me represents ions K^+ , Na^+ , NH_4^+ , and H_3O^+ .

The active growth of cultures was not observed at 40 and 50 °C in a medium without YE. If, at 40 °C and under these conditions, weak growth was observed, and the number of cells increased several times compared to the initial one (up to 1.2×10^7 cells/mL), at 50 °C, there was virtually no growth of microorganisms, and after 30 days of incubation, the number of cells in the culture did not exceed that immediately after inoculation. At the same time, at 40 °C, Fe^{2+} ions were completely oxidized in the medium, and at 50 °C, after long-term incubation, sufficiently high concentrations of Fe^{2+} (0.14 g/L) remained in the medium.

It should be noted that at the same temperatures in a medium with YE, the growth of cultures was quite rapid, and within 5 days, the complete oxidation of Fe^{2+} ions in the medium occurred.

Therefore, to obtain active cultures growing at high temperatures, strain *A. caldus* MBC-1 was additionally used. This strain was deposited in the collection of the Federal Research Center of Biotechnology of the Russian Academy of Sciences of the Center for Collective Use “Collection of unique and extremophilic microorganisms of various physiological groups for biotechnological purposes UNIQEM”. This strain was isolated from the pulp of a bio-oxidation reactor and is an autotrophic, moderately thermophilic, sulfur oxidizer [45]. Previous studies have shown that this strain in a mixed culture can support the activity of mixotrophic bacteria of the genus *Sulfobacillus* [46]. These mixotrophic bacteria are usually not capable of the active growth and oxidation of minerals in a medium without an organic carbon source, such as YE. Moreover, in a mixed culture with the *A. caldus* strain MBC-1, the *S. thermosulfidooxidans* VKVM1269^T strain was able to actively oxidize pyrite in a medium without YE. Thus, it was shown that the autotrophic strain *A. caldus* MBC-1 is capable of supplying mixotrophic microorganisms with its exometabolites, maintaining their activity. As expected, the inoculation ($\sim 1.0 \times 10^7$ cell/mL) of *A. caldus* MBC-1 in the enrichments at 40 °C and 50 °C in the medium without YE led to an increase in the growth and activity of the enrichment cultures. After 7 days of incubation, changes in the monitored parameters indicated a relatively high oxidative activity of the cultures: the pH values of the medium decreased in both cultures, the concentrations of Fe^{3+} ions and Eh values increased, and Fe^{2+} ions were completely oxidized. At 40 °C, the number of microorganisms increased by an order of magnitude (up to 11.3×10^7 cells/mL), while at 50 °C, the growth was less active, and the total number of microorganisms increased only to 1.8×10^7 cells/mL. Thus, the use of the *A. caldus* MBC-1 strain made it possible to obtain enrichments of acidophilic microorganisms growing at 40 and 50 °C in a medium without organic nutrients.

Thus, it was possible to obtain enrichments of acidophilic microorganisms that oxidize Fe^{2+} , S^0 and sulfide minerals in the temperature range of 25–50 °C.

The composition of the obtained enrichments was determined using the method of metabarcoding V3–V4 fragments of the 16S rRNA gene (Table 3). The properties of acidophilic microorganisms identified in the enrichments are shown in Table 3.

It should be noted that in most enrichments, the vast majority of sequences (94–100%) belonged to acidophilic microorganisms typically found in microbial communities that oxidize sulfide ores and minerals. Most of the identified acidophilic microorganisms, except for the archaea of the g. *Cuniculiplasma*, are capable of oxidizing Fe^{2+} and/or sulfur compounds, i.e., capable of oxidizing sulfide minerals. Moreover, in all cultures, the presence of heterotrophic neutrophilic microorganisms was observed, for which everything about the ability to oxidize sulfide minerals is unknown. These microorganisms were likely present in the ore concentrate used to obtain the enrichments.

Table 3. Composition and relative abundance of microbial groups in original inoculum and enrichment cultures.

Genus	Enrichment						
	Inoculum	25 °C	35 °C	40 °C	40 °C + YE	50 °C	50 °C + YE
<i>Acidithiobacillus</i>	22.97	37.74	26.83	93.08	0.00	7.01	0.00
<i>Acidiferrobacter</i>	0.00	0.00	0.00	0.00	0.00	0.00	0.00
<i>Acidiphilum</i>	0.00	0.00	0.00	0.00	0.00	0.00	0.00
<i>Sulfobacillus</i>	4.31	12.71	6.21	5.44	39.09	2.04	94.59
<i>Leptospirillum</i>	5.74	42.88	39.15	0.00	56.89	0.00	0.01
<i>Acidibacillus</i>	0.83	6.52	0.00	0.00	0.00	0.00	0.00
<i>Ferropasma</i>	66.03	0.07	0.79	1.48	0.00	13.54	0.00
<i>Acidiplasma</i>	0.00	0.00	0.00	0.00	0.00	0.00	0.00
<i>Cuniculiplasma</i>	0.00	0.00	26.94	0.00	0.01	0.00	0.00
A-plasma	0.00	0.00	0.01	0.00	0.00	0.00	0.00
E-plasma	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Acidophiles	99.88	99.92	99.93	100.00	96.00	22.59	94.60
<i>Escherichia-Shigella</i>	0.06	0.02	0.02	0.00	1.23	0.00	3.93
<i>Sphingomonas</i>	0.01	0.00	0.01	0.00	0.10	0.19	0.54
<i>Faecalibacterium</i>	0.00	0.00	0.00	0.00	0.45	0.00	0.00
<i>Limnochordaceae</i>	0.00	0.00	0.00	0.00	0.02	11.79	0.00
<i>Halobacteroidaceae</i>	0.00	0.00	0.00	0.00	0.02	2.08	0.00
<i>Methylococcobium</i>	0.00	0.00	0.00	0.00	0.01	2.48	0.00
<i>Dehalococcoidia</i>	0.00	0.00	0.00	0.00	0.00	3.41	0.00
<i>Anaerolineaceae</i>	0.00	0.00	0.00	0.00	0.01	3.45	0.06
<i>Aminicenantes</i>	0.00	0.00	0.00	0.00	0.00	3.10	0.00
<i>Cyanobium</i>	0.00	0.00	0.00	0.00	0.00	0.53	0.00
<i>Bathyarchaeia</i>	0.00	0.00	0.00	0.00	0.00	4.63	0.00
<i>Methanosaeta</i>	0.00	0.00	0.00	0.00	0.00	3.85	0.00
<i>Woesearchaeales</i>	0.01	0.00	0.00	0.00	0.01	17.01	0.00
Candidatus Aenigmarchaeum	0.00	0.00	0.00	0.00	0.00	8.53	0.00
<i>Methanoregula</i>	0.00	0.00	0.00	0.00	0.00	3.96	0.00
<i>Methanofastidiosales</i>	0.00	0.00	0.00	0.00	0.00	0.55	0.00
Other microbial groups	0.05	0.04	0.04	0.00	2.18	11.85	0.87
Total	100	100	100	100	100	100	100

The composition of the enrichments corresponded to the data on the physiological properties of the identified microorganisms (Table 4).

In the inoculum that was cultivated at ambient temperature, bacteria of the genus *Acidithiobacillus* and archaea of the genus *Ferropasma* were predominant (89% of the relative abundance), and bacteria of the genera *Sulfobacillus* and *Leptospirillum* were also detected.

Table 4. Physiological properties of microorganisms identified in the enrichments.

Genus	Properties						Reference
	Relation to Temperature	Topt (Tmin–Tmax), °C	pHopt (pHmin–pHmax)	Oxidized Inorganic Substrate	Carbon Nutrition	Consumed Organic Matter	
<i>Acidithiobacillus</i>	Mesophiles, moderate thermophiles	25–45 (5–52)	2.0–2.3 (1.0–3.5)	Fe ²⁺ and S	Autotrophs, mixotrophs	YE	[4]
<i>Sulfobacillus</i>	Thermotolerant microorganism	38–39 (30–47)	1.5 (0.8–2.2)	Fe ²⁺ and S	Mixotroph	YE, glucose, fructose, arabinose, mannose, glycerin, mannitol, citric acid	
<i>Leptospirillum</i>	Thermotolerant microorganism	30–37 (10–45)	1.3–1.8 (no data)	Fe ²⁺	Autotroph	-	
‘ <i>Acidibacillus</i> ’	Mesophiles, moderate thermophiles	30–43 (20–50)	1.7–2.8 (no data)	Fe ²⁺ and S	Heterotrophs	YE	
<i>Ferroplasma</i>	Thermotolerant microorganisms	35 (15–45)	1.7 (1.3–2.2)	Fe ²⁺	Mixotroph	YE	
<i>Cuniculiplasma</i>	Thermotolerant microorganism	37–40 (10–48)	1.0–1.2 (0.5–4.0)	-	Heterotroph	YE and meat extract, tryptone	
A-plasma	-	-	-	Presumably Fe ²⁺	Heterotroph	-	[34]

Incubation at 25 and 35 °C led to changes in the composition of microbial populations (Table 3). In both cases, the elimination of archaea of the genus *Ferroplasma* occurred, and the culture was dominated by bacteria of the genera *Acidithiobacillus* and *Leptospirillum*, (80.9 and 65.9% of the relative abundance, respectively) and bacteria of the genus *Sulfobacillus* (Table 3). Iron-oxidizing bacteria of the genus ‘*Acidibacillus*’ (valid studies on the genus have not been published as of yet [47]) and archaea of the genus *Cuniculiplasma* were revealed at 25 °C and 35 °C, respectively. The predominance of bacteria of the genera *Acidithiobacillus* and *Leptospirillum* in cultures obtained at 25 and 35 °C corresponded to their physiological properties. Bacteria of the genus *Leptospirillum* are mesophilic iron-oxidizing autotrophs. Sequences of representatives of the genus *Acidithiobacillus* identified in the cultures were closely related to the species *A. thiooxidans*, a mesophilic autotrophic sulfur oxidizer.

In the enrichment obtained at 40 °C in a medium without YE, sequences of representatives of the genus *Acidithiobacillus* were predominant, most closely related to the species *A. caldus*, a moderate thermophilic sulfur oxidizer, among the strains of which are both autotrophs and mixotrophs (Tables 3 and 4). Considering that cells of the *A. caldus* MBC-1 strain were inoculated into this culture, the predominance of sequences from this strain is expected. Sequences of bacteria of the genus *Sulfobacillus* and archaea of the genus *Ferroplasma* were also identified in the enrichment. These microorganisms are able to interact in mixed cultures with autotrophs, including strains of *A. caldus* consuming its exometabolites [46,48]. Thus, obtaining an enrichment at 40 °C using the *A. caldus* MBC-1 strain made it possible to obtain a mixed culture of autotrophic sulfur-oxidizing and mixotrophic iron-oxidizing microorganisms capable of stable active growth at 40 °C.

In the enrichment obtained at 40 °C in a medium with YE, bacteria of the genera *Sulfobacillus* and *Leptospirillum* predominated (Table 3). Bacteria of the genus *Sulfobacillus* are moderately thermophilic and thermotolerant iron and sulfur-oxidizing mixotrophic microorganisms (Table 4). Therefore, their predominance in a culture obtained at 40 °C in a medium with YE is expected. At the same time, bacteria of the genus *Leptospirillum* were also predominant. These bacteria are autotrophic thermotolerant iron oxidizers that can dominate populations of acidophilic microorganisms at temperatures around 40 °C (Table 4). There are no data on the dependence of their growth on the presence of organic substances

in the medium. Therefore, the predominance of these bacteria in the culture obtained in the medium with YE cannot be directly explained based on their known properties; however, a temperature of 40 °C is close to the optimum for these microorganisms.

In the enrichment obtained at 50 °C in a medium with YE, bacteria of the genus *Sulfobacillus* were highly dominant (94.6%) (Table 3), which corresponds to their physiological properties (moderate thermophilic and thermotolerant iron and sulfur-oxidizing mixotrophs) (Table 4).

In the enrichment obtained at 50 °C in a medium without YE, bacteria of the genera *Acidithiobacillus* and *Sulfobacillus*, as well as archaea of the genus *Ferroplasma*, predominated among acidophilic microorganisms (Table 3). The predominance of the bacteria of the genera *Acidithiobacillus* and *Sulfobacillus* can be explained by the cultivation temperature and inoculation of cells of the strain *A. caldus* MBC-1 into the enrichment, as well as the high temperature of cultivation (Table 4). At the same time, the archaea of the genus *Ferroplasma* tend to grow actively at lower temperatures. In this case, thermotolerant representatives of this genus were probably present in the culture [49]. This enrichment also had a high proportion of various neutrophilic heterotrophic microorganisms. In fact, their high proportion could be explained by the low total number of microorganisms in the culture, which led to an increase in the proportion of contaminating heterotrophic microorganisms during the analysis.

Thus, the analysis of the obtained enrichments showed the presence of acidophilic microorganisms that participate in the bio-oxidation of sulfide minerals, while the composition of the cultures depended on the isolation conditions.

3.2. Study of Dynamics of Oxidation of Ferrous Iron, Sulfur, and Sulfide Minerals by Obtained Enrichments

A study of the dynamics of the oxidation of Fe^{2+} ions (Figure 1) showed that the highest rate of the oxidation of ferrous iron ions was in experiments with enrichments at 40 and 50 °C in a medium with YE. This may be explained by the predominance of the bacterial genus *Sulfobacillus*, which is capable of the rapid oxidation of Fe^{2+} ions [40].

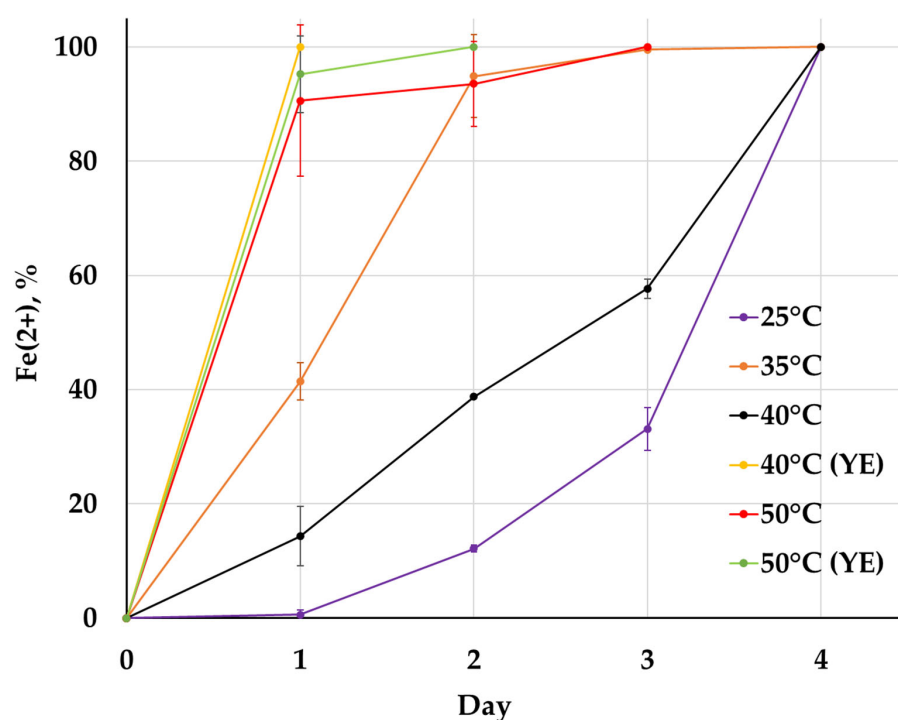


Figure 1. Dynamics of ferrous iron oxidation by the enrichments in nutrient medium 9KS ($n = 2$).

The study of the dynamics of S^0 oxidation (Figure 2) showed that the highest oxidation rate of sulfur was in experiments with the enrichment obtained at 40° without YE. This can be explained by the predominance of *A. caldus* bacteria in the culture, which are capable of the rapid oxidation of sulfur compounds, the growth optimum of which is close to a temperature of 40 °C.

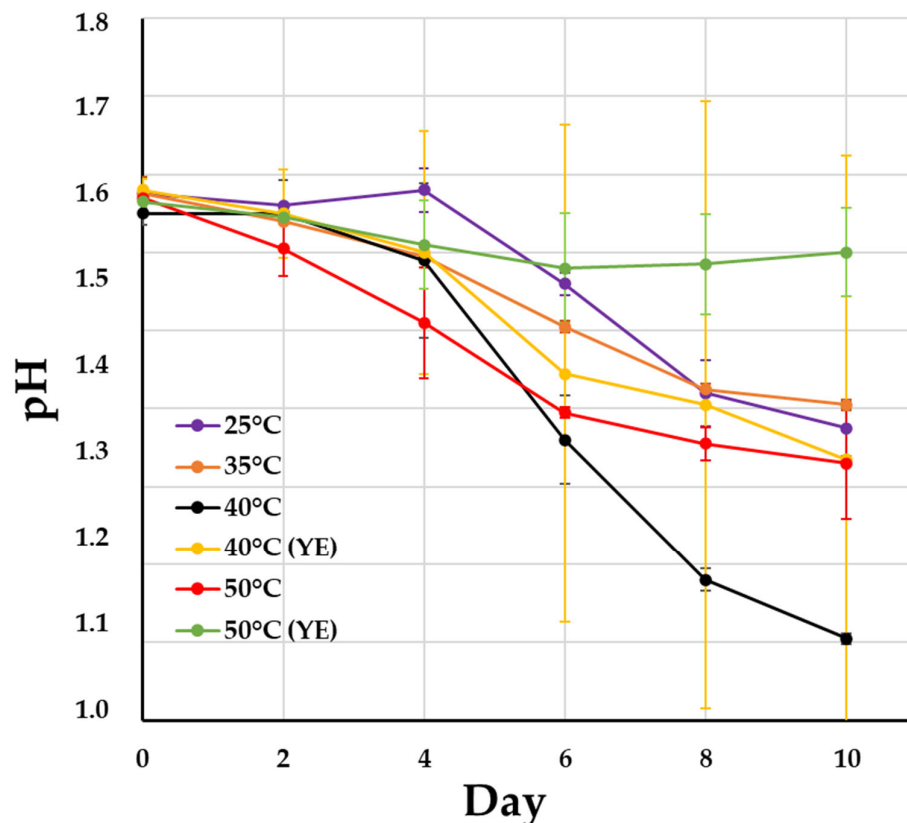


Figure 2. Changes in the pH values during the bio-oxidation of S^0 by the enrichments ($n = 2$).

A study of the dynamics of pyrite leaching (Figures 3 and 4) showed that during the bio-oxidation of pyrite, there were no significant differences in changes in pH and Eh values (despite a significant decrease in the pH level in the experiment with sulfur (Figure 2)), with the exception of a more active decrease in pH in the experiment with the enrichment at 50 °C without YE. The Eh value in all variants of the experiment reached high values (above 750 mV), which was confirmed by low (trace) concentrations of Fe^{2+} ions in the medium after 4 days of oxidation. At the same time, the dynamics of changes in the concentrations of Fe^{3+} ions in the medium differed in different versions of the experiment. This may be explained by the reprecipitation of iron ions in the form of jarosite [44]. The degree of pyrite leaching, which was calculated from the maximum concentration of iron ions in the medium during the experiment, was the lowest in experiments with cultures at 25 °C and 50 °C (medium with YE). In other cases, the degree of pyrite leaching was comparable and reached 11–14%, which was comparable to pyrite leaching reached in flask experiments in our previous study [46]. At 35 and 50 °C, the degree of leaching was maximum at the end of the experiment, and at 40 °C, the maximum was reached on day 4.

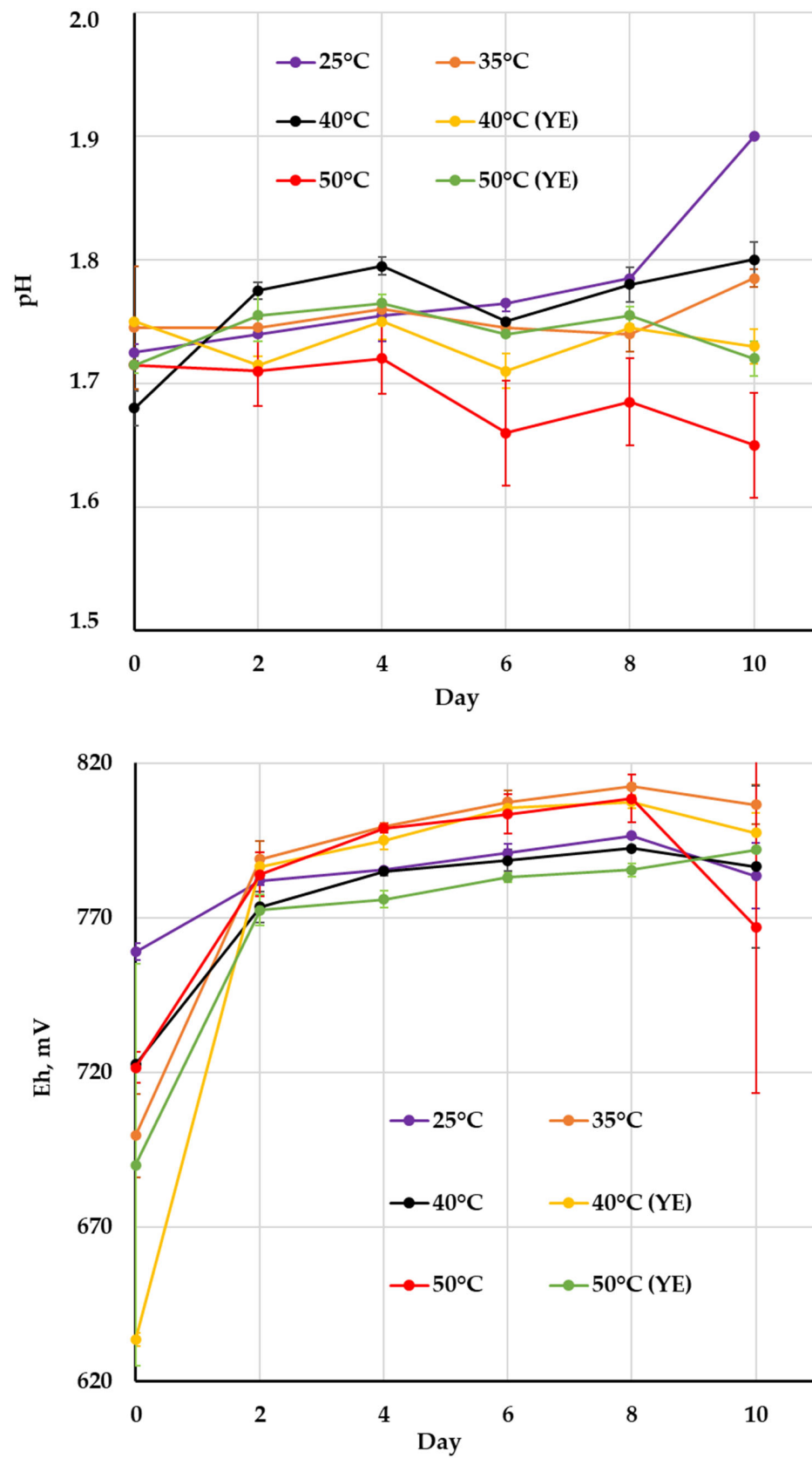


Figure 3. Cont.

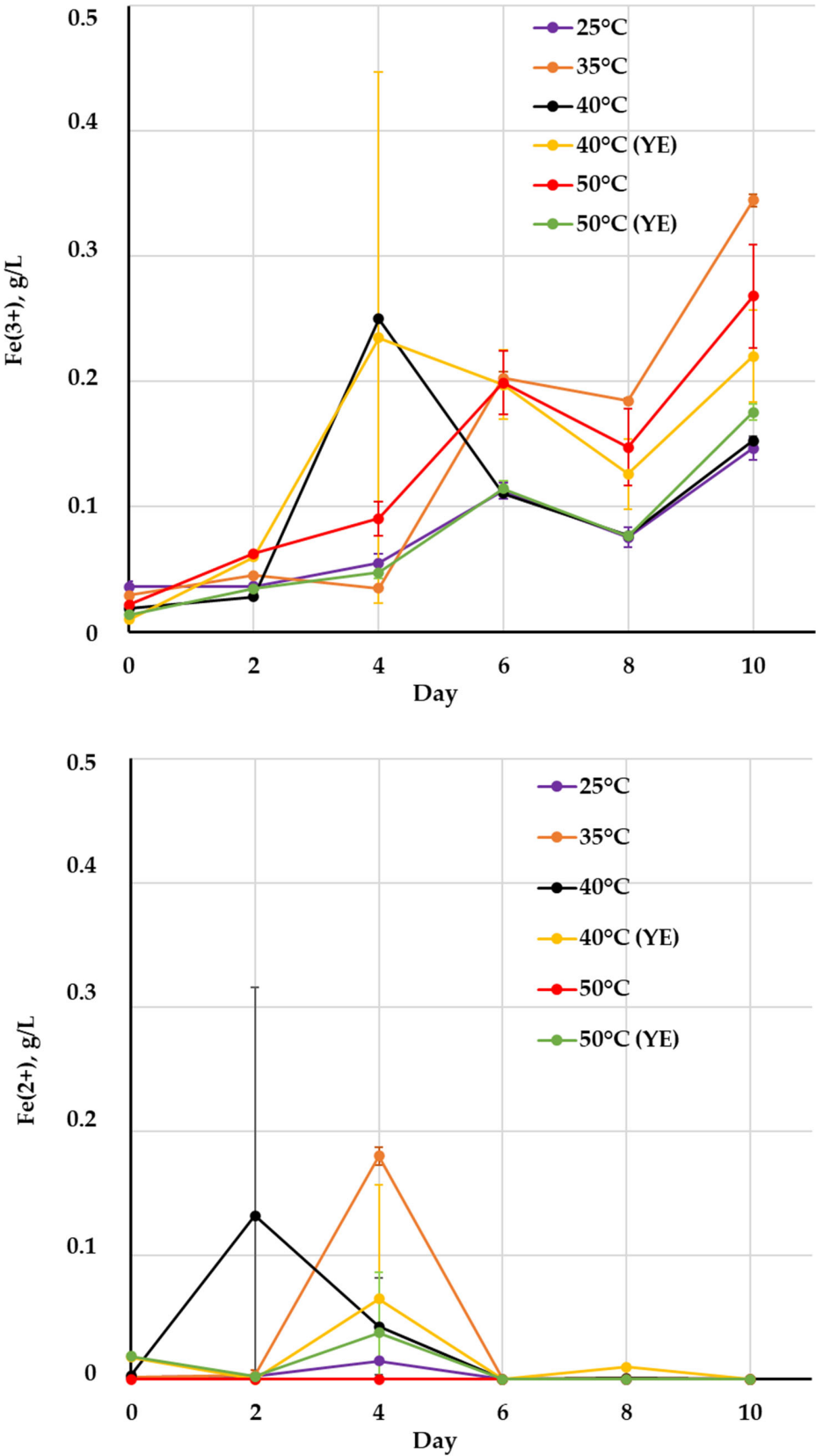


Figure 3. Changes in the medium parameters during the oxidation of pyrite by the enrichments ($n = 2$).

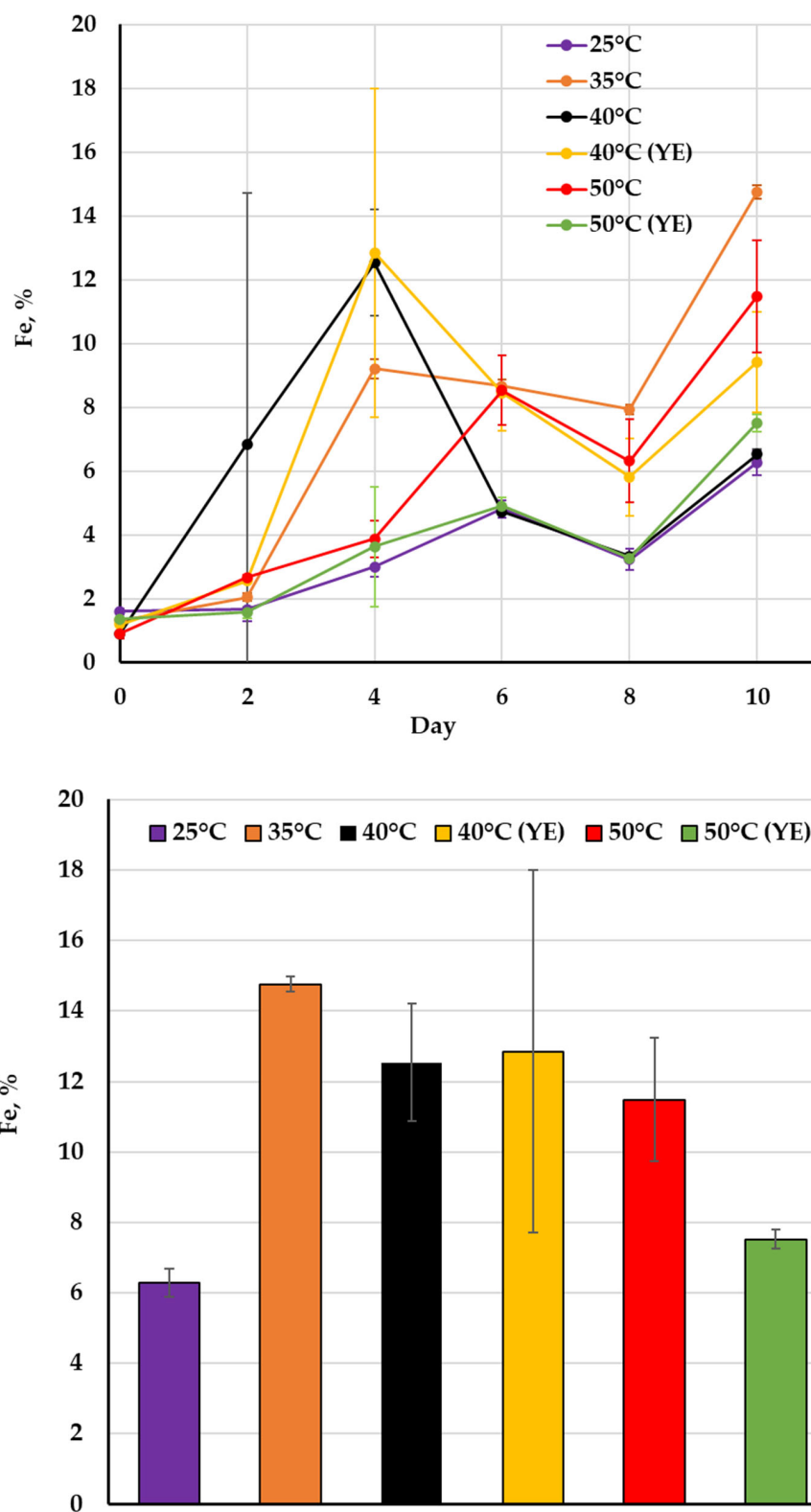


Figure 4. The degree of iron leaching in the process of pyrite oxidation by enrichments ($n = 2$).

The dynamics of chalcopyrite bioleaching are presented in Figures 5–7.

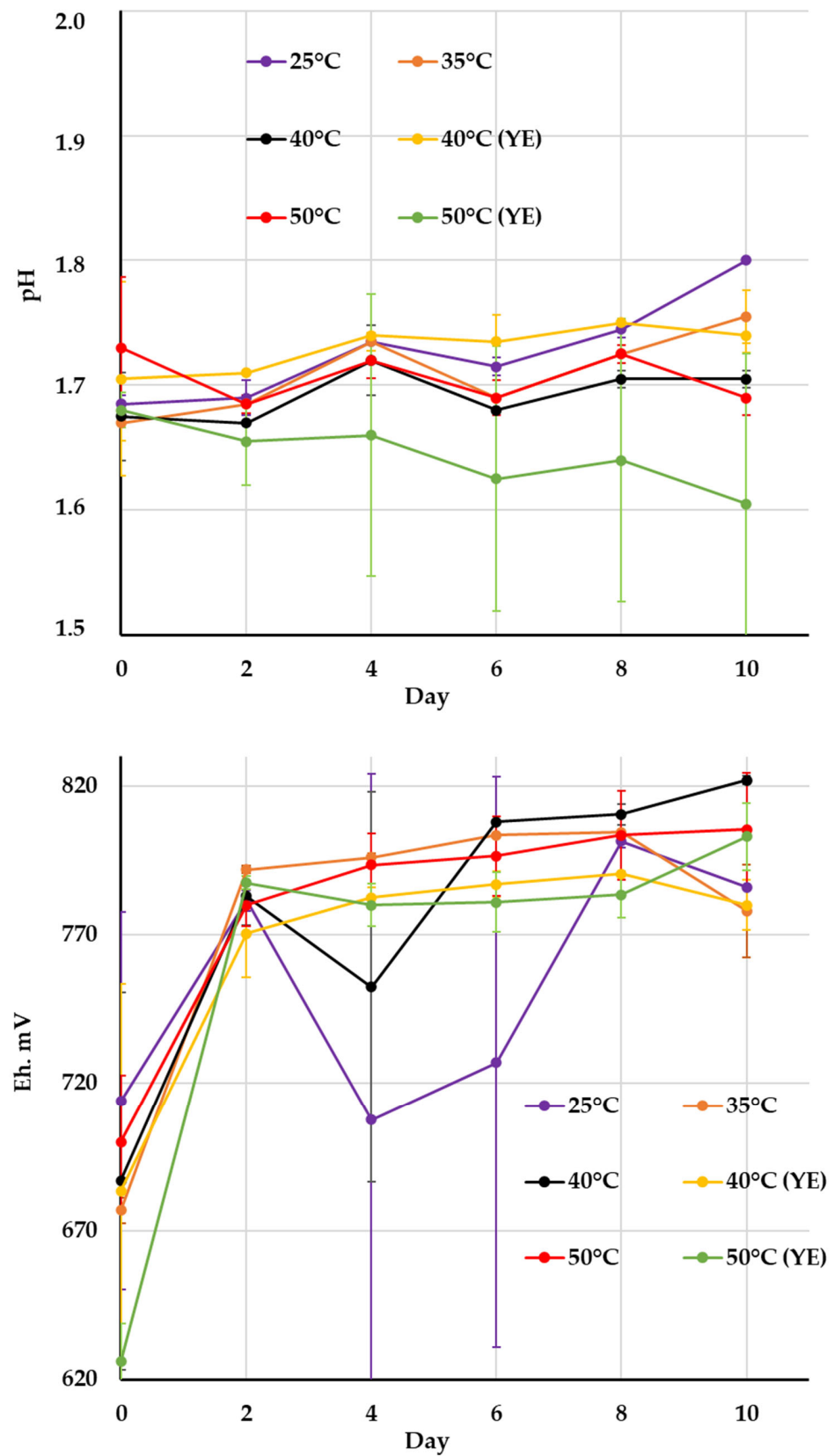


Figure 5. Cont.

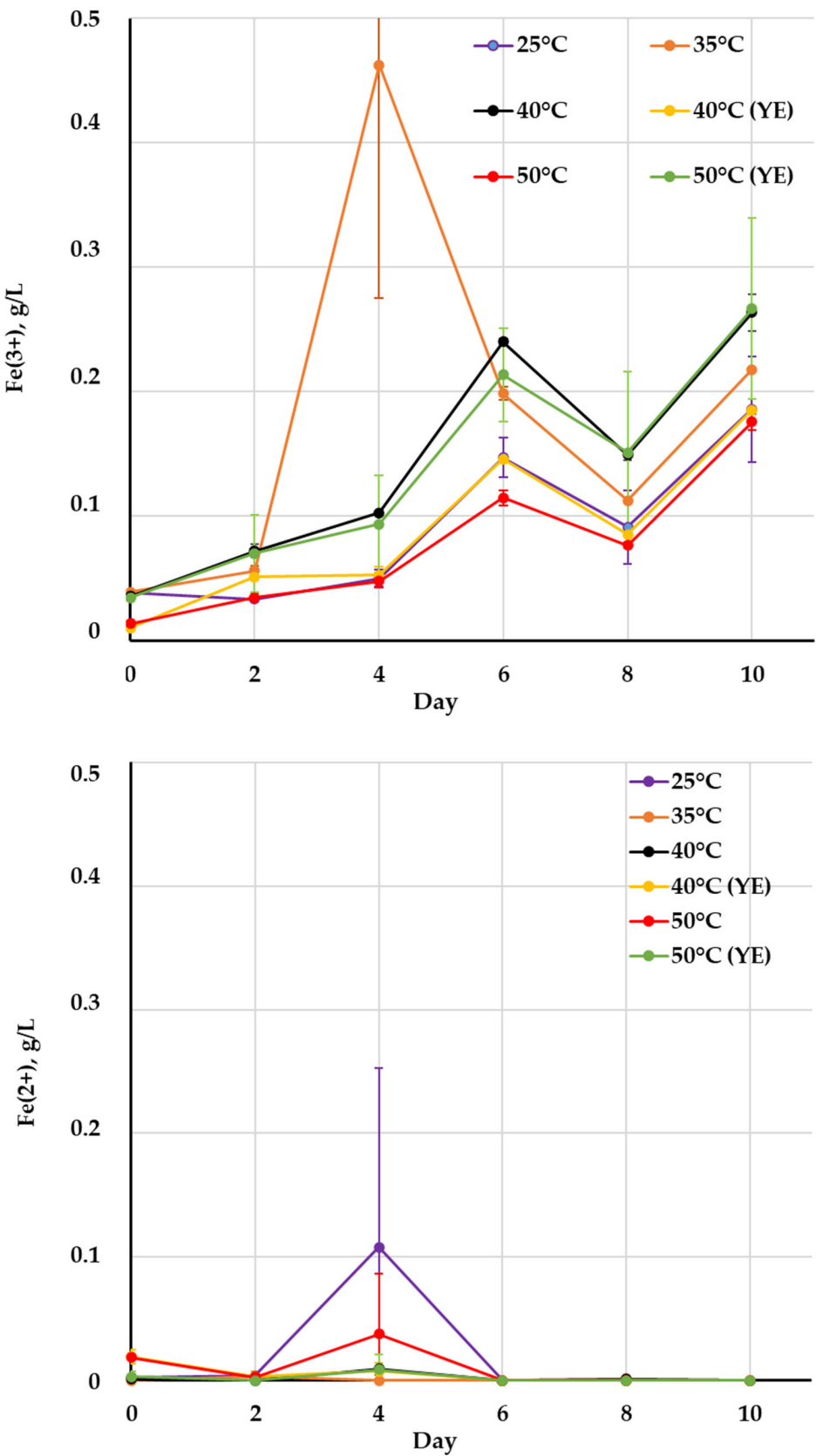


Figure 5. Changes in the medium parameters during the oxidation of chalcopyrite by the enrichments ($n = 2$).

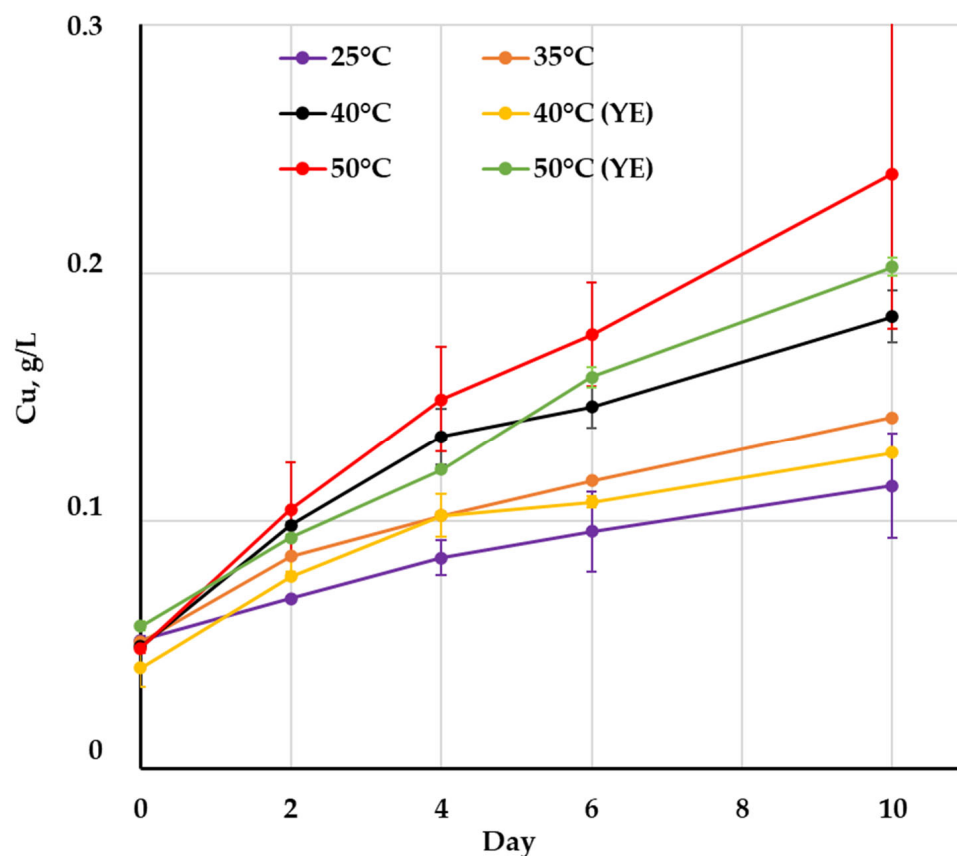


Figure 6. Dynamics of changes in copper concentration in the medium during the bio-oxidation of chalcopyrite by the enrichments ($n = 2$).

Changes in the pH values did not differ significantly despite a significant decrease in the pH level in the experiment with sulfur (Figure 2), with the exception of a more active decrease in pH in the experiment with a culture grown at 50 °C with YE. Eh in all variants of the experiment reached high values (above 750 mV), which was confirmed by low (trace) concentrations of Fe^{2+} ions. The exception was the experiment at 25 °C, where the concentration of Fe^{2+} ions increased on day 4, and a decrease in the Eh of the medium was also observed.

At the same time, the dynamics of changes in the concentrations of Fe^{3+} ions in the medium differed in different versions of the experiment. This may be explained by the reprecipitation of iron ions in the form of jarosite [44]. Thus, at 35 °C on day 4, a peak in the concentration of Fe^{3+} ions was observed, which then decreased.

In contrast to the concentrations of iron ions, the concentration of copper ions in the medium gradually increased, and no fluctuations in concentrations were observed (Figure 7). This may be due to the fact that copper is not reprecipitated during the bioleaching process. The degrees of the leaching of iron and copper differed for the same reason.

The degree of copper leaching was maximum at 50 °C (8–10%). This is consistent with the data available in the literature that state that the rate of chalcopyrite bioleaching depends on temperature and increases with increasing temperature [50–53].

Thus, it was shown that all obtained enrichment cultures are capable of oxidizing sulfide minerals, which are common components of sulfide ores. Moreover, the degree of copper leaching depended more on temperature than pyrite bioleaching.

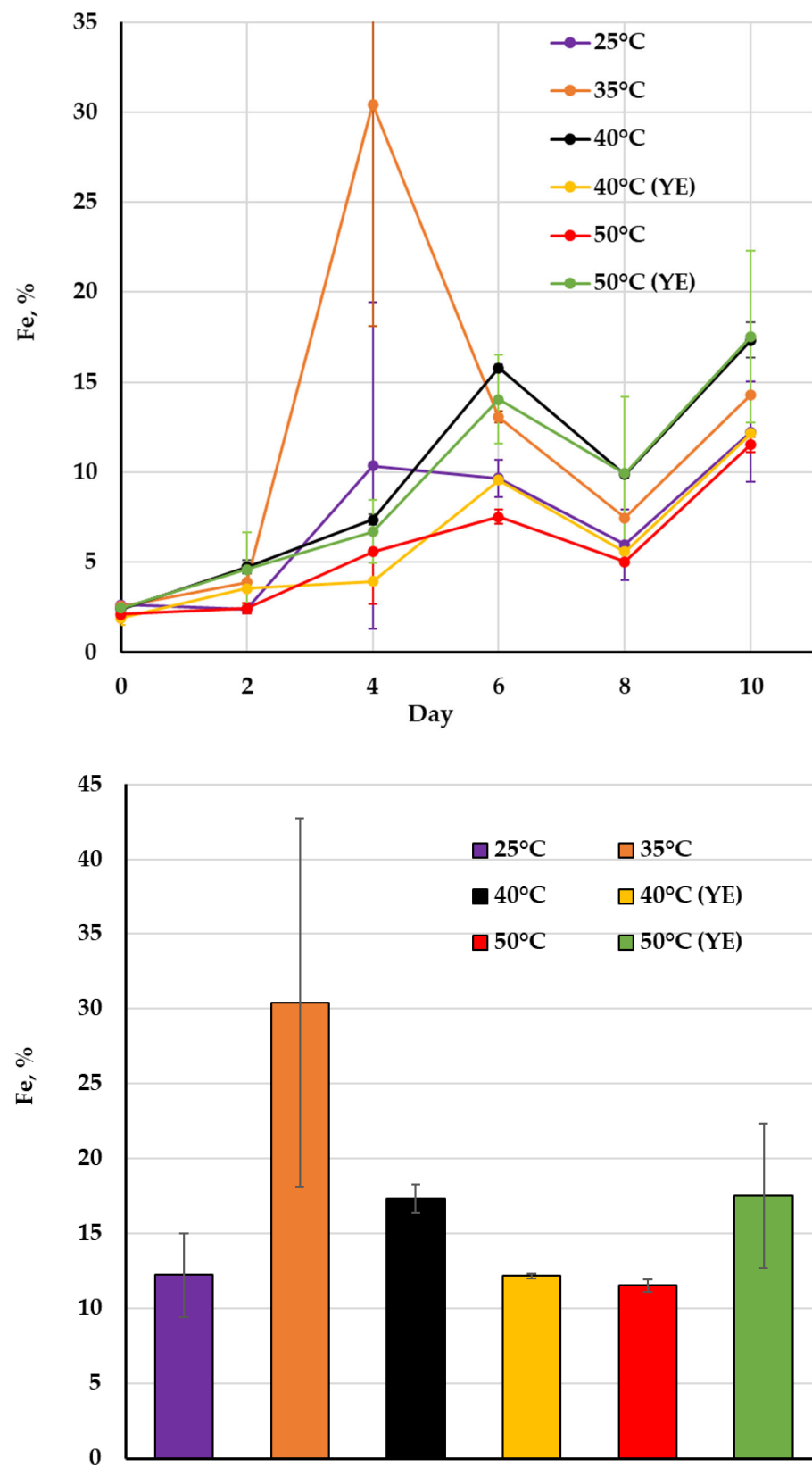


Figure 7. Cont.

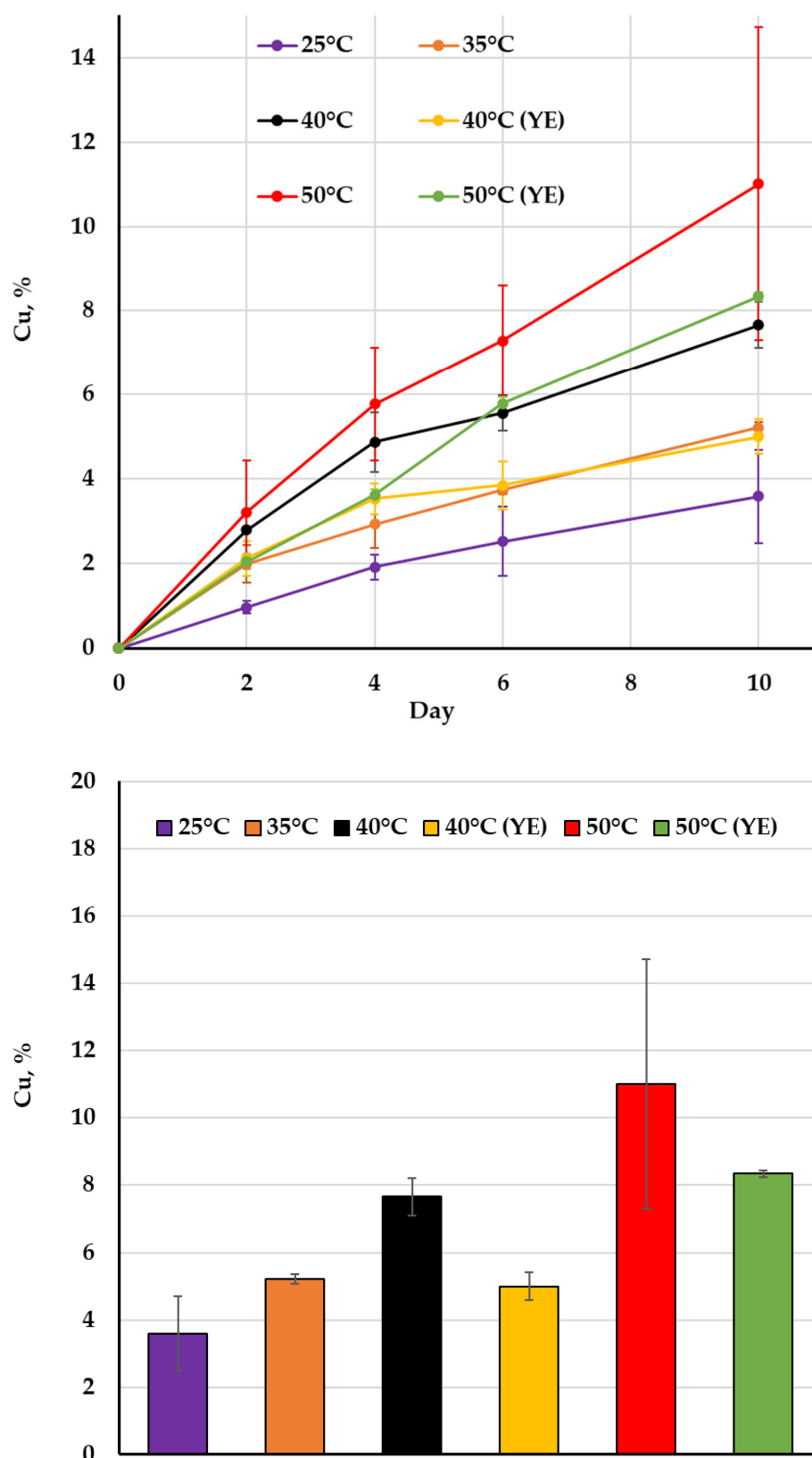


Figure 7. Changes in iron and copper concentrations in the medium during the bio-oxidation of chalcopyrite by the enrichments ($n = 2$).

4. Discussion

In the present work, enrichments with different properties were obtained at different temperatures using the same inoculum and nutrient medium, with and without YE. The

enrichments obtained differed in microbial composition in accordance with the temperature characteristics of their isolated counterparts and were able to oxidize sulfide minerals with at different rates. In recent years, indigenous acidophilic strains and enrichments have been actively isolated and tested as inocula for bioleaching. For example, an autochthonous *A. ferrooxidans* metapopulation was isolated from a sample of pregnant bioleach solution and was used for the leaching of pyrite tailings containing Au and Ag at 30 °C [16]. Strain *Acidiphilium cryptum* isolated from hot spring water in Japan was used in the experiments for the bioleaching of sphalerite, galena, pyrite, and chalcopyrite at 32 °C [17]. Strain *A. ferrooxidans* YQ-N3 isolated from a sample of acid mine drainage (AMD) in China was successfully used in a coal biodesulfurization experiment at 30 °C [20]. A strain of *Leptospirillum* sp. CC isolated from a sample of polymetallic ore in Armenia was used for the bioleaching of pyrite and chalcopyrite at 40 °C. It was shown that this strain was able to leach pyrite and chalcopyrite both in the experiments as a pure culture and in a mixed culture with other autotrophic and heterotrophic acidophiles; the mixed cultures leached sulfide minerals more actively in comparison to the pure culture [21]. In another study [24], a consortium was enriched from AMD water samples at 37 °C dominated by the genera of *Leptospirillum* and *Acidithiobacillus* in Armenia. This consortium extracted 50% Cu from copper concentrate after 20 days of bioleaching at 5% pulp density and 40 °C. Casas-Vargas et al. [22] obtained several enrichments and microbial isolates from mining samples of the Amolanas Mine (Chile), cultured at 30, 37, and 50 °C. Under mesophilic conditions, representatives of the genera *Leptospirillum* and *Acidithiobacillus* were predominant, while at 50 °C, representatives of the genus *Sulfobacillus* were the main microorganisms in the composition of the enrichments.

In the present work, we obtained several enrichment cultures with different compositions and mineral oxidizing capacities that may allow these enrichments to serve as inoculum for the bioleaching of various mineral raw materials at different temperatures. For example, we showed that the bioleaching of chalcopyrite was more efficient at 50 °C, which may be explained by the properties of this mineral. It is known that chalcopyrite bioleaching rates depend on temperature and oxidation/reduction potential (ORP). The rate of copper bioleaching is increased at low ORP [54] and high temperature [52–54]. Thus, its bioleaching rate may be regulated by temperature and ORP control, which in turn may be regulated by the composition of the inoculum [52–54].

Therefore, the obtained enrichments may be used in further work as suitable inoculum to perform bioleaching processes under their optimal temperatures and growth conditions. The use of the inocula adapted to various conditions may provide an increase in the bioleaching rate [26].

5. Conclusions

In the present work, enrichments of acidophilic microorganisms that were active in the temperature range of 25–50 °C were obtained, which was determined by analyzing parameters of the medium reflecting the activity of microorganisms that oxidize Fe²⁺ ions, sulfur and sulfide minerals. The study of the enrichments using the metabarcoding of variable V3–V4 fragments of 16S rRNA genes showed differences in composition between the enrichment cultures obtained under different conditions, which can be explained by differences in the physiological properties of the identified microorganisms.

A study of the dynamics of the oxidation of ferrous iron, sulfur, and sulfide minerals (pyrite and chalcopyrite) by the enrichments in the temperature range of 25–50 °C allowed us to establish that all cultures were capable of oxidizing the studied inorganic substrates.

Based on the data obtained, we can conclude that the obtained enrichments can be used in further research, conducting laboratory tests on the biotechnological processing of mineral raw materials.

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Data Availability Statement: The raw data generated from the 16S rRNA gene profiling are accessible via the BioProject accession number PRJNA1267857.

Conflicts of Interest: Author Elena Latyuk was employed by the company RIVS Group of Companies. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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