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Bioleaching of Chalcopyrite by a New Strain *Leptospirillum ferrodiazotrophum* Ksh-L Isolated from a Dump-Bioleaching System of Kashen Copper-Molybdenum Mine

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Abstract: A new strain of *Leptospirillum* sp. Ksh-L was isolated from a dump-bioleaching system of the Kashen copper-molybdenum mine (South Caucasus). Ksh-L is an obligate chemolithoautotroph, capable of oxidizing ferrous iron (Fe^{2+}). Cells are Gram-negative and vibrio- or spirillum-shaped of a 0.5–3 µm size. The optimal conditions for the growth are 35 °C and pH 1.6–1.8. Cu^{2+} and Zn^{2+} have different effects on the oxidizing ability of the *Leptospirillum* sp. Ksh-L culture depending on the phase of growth and concentration of Fe^{2+}. Under the conditions of gradually increasing the concentration of copper in the medium, during 4–5 successive subculturing experiments, it was possible to obtain an adapted culture of *Leptospirillum* sp. Ksh-L, capable of growing in the medium in the presence of up to 400 mM Cu^{2+}. A bioleaching experiment indicates that Ksh-L can efficiently oxidize chalcopyrite. However, the bioleaching of copper from chalcopyrite by *Leptospirillum ferrodiazotropum* Ksh-L increased about 1.8 times in association with *At. thiooxidans* ATCC 19377. Phylogenetic analysis based on 16S rRNA gene sequences (GenBank ID ON226845) shows that strain Ksh-L forms a single cluster into Group III. The strain possesses 99.59%, 99.52%, and 96.60% sequence similarity with the strains YTW-96-06, YTW-66-06, and *Leptospirillum ferrodiazotrophum* 5C in Group III, respectively.

Keywords: *Leptospirillum ferrodiazotrophum*; isolation; characterization; phylogenetic analysis; bioleaching of sulfide minerals

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1. Introduction

Bioleaching is a technique that uses microorganisms to remove metals from ore where traditional extraction methods are not economically viable. This method is frequently applied to sulfide mineral ores, which are the source of many valuable and precious metals, including copper, gold, and silver. Traditional methods of metal extraction from sulfide minerals, such as pyrometallurgy, are costly, energy-intensive, and environmentally damaging [1,2]. Bioleaching, using microbial metabolisms to break down metal ores, provides a low-cost solution to this issue. Microbes produce energy during the bioleaching process by oxidizing iron and sulfur from sulfide minerals.

Sulfide ores can be dissolved by ferrous-oxidizing acidophiles to create ferric iron, which then attacks minerals to form sulfur or polysulfide on the ore surface [3,4]. The target metals are released as a result of the oxidants' attack on the sulfide minerals [2]. Due to the

intimate connections between functional oxidizers, environmental conditions, and bioleaching performances, many researchers have recently focused on microbial communities [5,6].

Approximately 70% of the copper reserves in the world come from chalcopyrite [7,8]. It is one of the most resistant ores in hydrometallurgical processing and the most common copper ore. Modern research on the possibility of increasing total recoveries of metal values from such mineral resources plays a significant role in the bio-hydrometallurgical processing of complex low-grade non-ferrous metal concentrates [9–13].

Mesoacidophiles are the most frequently used microorganisms in industrial low-grade chalcopyrite heap-bioleaching operations because of their ambient temperature optimum and ranges, which have been shown to have a significant impact on the rate of copper extraction from chalcopyrite [14–16].

Leptospirilla was discovered to be the predominant iron-oxidizing bacteria in gold-arsenopyrite and pyrite bio-oxidation reactors working at 40 °C, although *Acidithiobacillus ferrooxidans* had long been thought to be the most significant microorganism in the bioleaching of metals [17–22].

Leptospirillum spp. bacteria are vibrio- and spiral-shaped chemolithotrophic organisms. They have been formally recognized as coherent bacteria since they fix carbon utilizing the Benson–Calvin cycle, employing oxygen as their only electron acceptor and ferrous iron as their sole electron donor [20,23–26]. The genus *Leptospirillum* has been classified into three groups, I, II, and III based on the 16S rRNA gene phylogeny [27]. *Leptospirillum ferrooxidans* is a representative of group I [28], *Leptospirillum ferriphilum* and *Leptospirillum rubrum* are representatives of group II, and *Leptospirillum ferrodiazotrophum* is a representative of group III [29–31]. In addition, microbial community genomics has identified further species, "*Leptospirillum* sp. group IV UBA BS" [30,31].

Notably, proteomics research has revealed that *L. ferrodiazotrophum* (group III) carbohydrate metabolism is significantly better than that of *Leptospirillum* group II and *Ferroplasma* type II [25]. Also, because the nif genes were only found in this bacterium's genome sequence, it is thought to be a minor member of the genus and the only one that is capable of fixing nitrogen [30–33]. Based on this, one representative was isolated in a nitrogen-free liquid medium from AMD biofilm in which *Leptospirillum* group III was abundant [31]. This Fe(II)-oxidizing, free-living diazotroph was tentatively named "*L. ferrodiazotrophum*". Hence, despite its low quantity in the microbial community, *L. ferrodiazotrophum* played a central role in biofilm formation and nitrogen fixation.

Gene complement variations highlight significant physiological variances that may have played a crucial role in the *Leptospirillum* groups' likely sympatric separation. *Leptospirillum* group II is more capable of producing potentially important polymers for the establishment of floating biofilms, such as cellulose, cellobiose, and starch/amylose, than *Leptospirillum* group III in dealing with the osmotic challenges brought on by the near-molar $FeSO_4$ solutions. With the potential completion of the glycolysis and TCA pathways, *Leptospirillum* group III appears to be more suited for energy production and nitrogen fixation. These results are in line with the descriptions of *Leptospirillum* group II as an early colonist and *Leptospirillum* group III as a component of late-stage biofilms [34,35]. Interestingly, the complements of signal transduction and chemotaxis genes in *Leptospirillum* groups II and III are quite different, as are many regulatory genes, pointing to adaptation to various microenvironments (such as those with particular levels of oxygen, redox potential, and availability of fixed nitrogen). Signal transduction, motility, and chemotaxis appear to be more crucial in *Leptospirillum* group III than in group II, according to genomic and proteomic evidence [33,35]. *Leptospirillum* group III is identified by biofilm characterization investigations as distributed cells and microcolonies in interior biofilm zones [34], where geochemical gradients are predicted to be prominent. Combining this distribution with the presumed metabolic traits may suggest that *Leptospirillum* group III is a microaerophile that prefers to grow in nutrient-poor areas of biofilms, where its capacity to fix nitrogen may be essential. Where oxygen availability is certainly low and *Leptospirillum* group II is present, a

few studies on anaerobic metabolism (for example, making use of a within-biofilm nitrogen cycle) are available [33].

Most metals are soluble in acidic pH, and extremely acidophilic microorganisms should be tolerant to high concentrations of metals. Acidophiles are metal-tolerant by both active and passive mechanisms. The passive metal tolerance mechanism is based on the internal positive membrane potential by creating a chemiosmotic gradient that the cations should travel against to enter the cytoplasm [36,37]. Active systems include the efflux of metals from the cytoplasm to the periplasmic space, carried out by ATPases located in the internal membrane of the bacteria [38]. Some microorganisms may pump metal from the cytoplasm directly to the extracellular space by systems of the RND (resistance nodulation cell division) family of carriers, the Cus system of *Escherichia coli* being the best known of this kind of detoxification organization [39]. The capacity of some species to bind the metal in the periplasmic space using metal chaperones has also been reported for copper [40].

It should be noted that bacterial growth in the form of biofilms significantly increases the resistance of bacteria to metals [41–43]. Acidophiles attached to surfaces, such as sulfide minerals, form biofilms that usually include extracellular polymeric substances (EPSs) that can sorb metals to provide a further degree of metal tolerance [44,45].

Several mechanisms of resistance to Mo (VI) have been identified in acidophiles, including the putative Mo (V) resistance mechanism in *At. ferrooxidans* strain Funis 2–1 [46]. Mo (VI) is chemically reduced by Fe(II), and Mo(V) forms binds to the plasma membrane, probably to the cytochrome-c oxidase (lowering its activity), inhibiting Fe(II) oxidation and consequently growth. Resistance is based on a combination of a cytochrome-c oxidase that is tolerant to higher concentrations of Mo (V) and on Mo (V)-oxidizing activity six-fold greater than that detected in the sensitive *At. ferrooxidans* strain AP19-3 [46].

With the development of sequencing technology, many genomic-analysis-based studies have investigated the mechanisms underlying heavy metal resistance under extremely acidic conditions [47–50]. Several genes associated with EPS formation have been identified thus far [51]. Genome/transcriptome analyses showed the presence of genes involved in biofilm formation in *Leptospirillum* spp. [51,52]. In *Acidithiobacillus* and *Leptospirillum* spp. a membrane efflux pump encoded by the czcCBA cluster is responsible for resistance to cadmium, zinc, and cobalt [48,52–54]. The resistance of bacteria to copper is very important from the point of view of their application in biotechnological processes, where the concentration of copper ions can vary in the range from 15 to 100 mM $CuSO_4$ [55]. Copper resistance systems in some acidophiles include a copper P-Type transporter identified in *L. ferriphilum* ML-04 [48]. In addition, earlier descriptions of acidophile copper resistance and the potential role of inorganic polyphosphates in metal resistance are also available [56,57].

This study addressed the characterization and identification of *Leptospirillum* Ksh-L, isolated from an acid mining drainage of copper ore in Armenia, its ability to degrade sulfide minerals, and its tolerance to metal ions.

2. Materials and Methods

2.1. Culture Conditions and Isolation

To obtain an enrichment culture of iron-oxidizing bacteria, 9K medium [58] with ferrous iron as a source of energy was inoculated with a sample of ore material from a dump-bioleaching system of Kashen copper-molybdenum mine (South Caucasus) and incubated at 35 °C and 150 rpm. The serial dilution method was used to obtain a pure culture of *Leptospirillum* spp.

2.2. Morphology Studies

Gram staining was performed using the Huker method [59]. The morphology of cells was studied with Motic BA310 trinocular (×1000) microscope supplied by Moticam A16 Camera (Barcelona, Spain, MoticIncorporation Ltd.).

2.3. Optimal pH and Temperature for Growth

Studies of the influence of temperature and pH on the growth of strain Ksh-L were carried out in 100 mL flasks containing 50 mL of 9K medium with ferrous iron (Fe^{2+}) and 10% inoculum. Cultivation was performed on the orbital shaker-incubator ES-20/60 (Biosan, Riga, Latvia) at 150 rpm. The growth ranges for temperature and pH were set as 25–50 °C and 1.6 to 3.0, respectively.

2.4. Influence of Copper and Zinc

The influence of copper and zinc ions on oxidation of Fe^{2+} by *Leptospirillum* sp. Ksh-L was studied in Mackintosh (MAC) medium [60] in the concentration range from 10 to 300 mM and at different concentrations of the source of energy (Fe^{2+}). The data presented in the text are formed on the average from repeated experiments with ±2% variation of Fe^{2+}.

2.5. EPS Analysis, Extraction, and Determination

2.5.1. EPS Extraction

For EPS extraction experiments, culture *Leptospirillum* Ksh-L was grown in 10 L MAC medium supplemented with $FeSO_4 \times 7H_2O$ as a source of energy at 35 °C with shaking at 160 rpm. In the stationary growth phase, cells were collected by centrifugation (10,000 rpm, 10 min) at 4 °C. To get rid of any remaining bacteria, the supernatant was collected and filtered through 0.2 m pore size filters under sterile conditions. Colloidal EPS was present in the fraction obtained. The pellet was re-suspended in 10 mL of 20 mM EDTA at pH 7 and centrifuged for 10 min at 7500 rpm (9900× *g*) and 4 °C to release the bound EPS. The washed fraction was applied to the supernatant. The fresh pellet was re-dissolved in 10 mL of 20 mM EDTA at pH 7, and the suspension was incubated at 4 °C for 1 h while being shaken. The remaining cells were then removed by centrifuging the bacterial suspension and extracting agent mixtures for 10 min at 7500 rpm (9900× *g*) and 4 °C [61].

2.5.2. Determination of EPS Composition

Total carbohydrate values were determined by spectrophotometry using the phenol-sulfuric acid method with D-glucose as the standard, as described by [62]. Total protein quantification was conducted by spectrophotometry using the Bradford procedure [63]. A calibration curve was developed using a series of bovine serum albumin (BSA) standards. Uronic acids were quantified using the protocol of Blumenkrantz and Asboe-Hansen [64]. Meta-hydroxydiphenyl solution in 0.5% NaOH, ortho-hydroxydiphenyl, H_2SO_4/sodium tetraborate solutions, and cetyltrimethylammonium bromide solution were used as a reaction mixture. Absorbance measurements were performed at 520 nm. The content of proteins, carbohydrates, and uronic acids in EPS was expressed in μg/mL culture medium.

2.6. DNA Extraction, PCR of 16s rRNA, Sequencing, and Phylogenetic Analysis

2.6.1. DNA Extraction

The identification of isolated strain Ksh-L was performed based on 16S rRNA gene nucleotide sequence analysis. DNA extraction was carried out according to the Macherey-Nagel™ (NucleoSpin™) protocol. Bacterial cultures in the logarithmic phase were centrifuged to obtain the bacterial biomass and frozen for DNA purification and taxonomic classification of the strain based on 16S rRNA gene sequences. For DNA extraction, the following reagents were used: BE buffer (Tris-HCl pH-8.0), proteinase K, MG-lysozyme solution, BS salt buffer, 96% ethanol, and distilled water.

2.6.2. Extraction and Purification of PCR Product

A 1.5% agarose gel Tris-HCl buffer solution was prepared. The agarose aqueous solution was heated in a microwave oven for 1–2 min before boiling and then cooled to 45–60 °C. A 1% 10 mM Tris-HCl, 1 mM EDTA aqueous solution (1 μL per 10 mL agarose solution) was added to the cooled agarose solution. A total of 5 μL of the test DNA solution

was mixed with a 3 µL 6 µL 6X Thermo Scientific TriTrack DNA Loading Dye and 5 µL Thermo Scientific GeneRuler buffer containing 1 kb DNA Ladder markers. Electrophoresis was performed on GE Healthcare/Amersham Pharmacia EPS-601 Electrophoresis Power Supply for 25 min at a voltage of 104 V at 100 mA. The electropherograms and images were obtained using the GeneMarkerHID® Spectrum GeneMarkerHID® and Spectrum ProView™ Sequencing Software TM625 package (Promega Corporation 2800, Fitchburg, MA, USA).

The PCR amplification was carried out according to the Macherey-Nagel™ (NucleoSpin™) Extract II protocol. PCR amplification was carried out in 50 µL reaction mixture, which contained 10 mg of DNA sample, 1 µL universal bacterial PCR primers a-fD1 (27F) (AGAGTTTGATC-CTGGCTCAG) and rP2 (ACGGCTACCTTGTTACGAG), b- 908 fwd and 796 rev, 5 µL Taq-DNA polymerase, 1 µL dNTPs, and 40 µL double distilled water. PCR temperature–time process is as follows: 1st cycle 95 °C × 5 min, further 30 cycles of denaturation −94 °C × 40 s, connection 50/53 °C × 55–60 s, synthesis 72 °C × 1.5 min, final cycle −72 °C × 3 min, and then standby at 8–10 °C. PCR products were tested by 1.5% agarose gel electrophoresis and sequenced with primers 908fwd (16Sfwd) (GTGCCAGCAGCCGCG) and 796rev (16Srev) (GGGTTGCGCTCGTTG) by Microsynth AG (Balgach, Switzerland). PCR product purification was performed using the QIAquick PCR purification kit following the manufacturer's instructions. PCR fragments were sequenced by a 454 GS-FLX Titanium sequencer, using the Sanger method [65].

2.6.3. Construction of Phylogenetic Tree

Close relative and phylogenetic affiliation of the obtained 16S rRNA sequences were determined by submitting to the NCBI 16S ribosomal RNA GenBank database using NCBI blastn search analyses (www.ncbi.nlm.nih.gov) performed with Geneious prime 2022.0.2. (https://www.geneious.com) and the 16S Biodiversity tool (RDP tool version 2.12) [66,67]. The construction of phylogenetic trees was performed with MEGA 11 software using the neighbor-joining method [68,69].

2.7. Leaching Experiments

Chalcopyrite ($CuFeS_2$) from Shamlugh ore deposit (Armenia) was tested in the bioleaching experiments. The chemical composition of minerals is presented in Table 1. Feed minerals were ground to a particle size ≤ 63 µm.

Table 1. Chemical composition of the analyzed chalcopyrite (wt%).

Sample	Fe	Cu	S
Chalcopyrite	29.7	30.2	33.8

Bioleaching of chalcopyrite was performed using a pure culture of *L. ferrodiazotropum* Ksh-L as well as its associations with *At. thiooxidans* ATCC 19377 obtained from DSMZ. Bioleaching experiments were carried out in 250 mL Erlenmeyer flasks containing 100 mL of MAC medium without iron at 30 °C. Pulp density (PD) was 4% and pH 1.8. The inoculum of used cultures was 10%, and all experiments were carried out in triplicate. Chemical control with the same conditions and without inoculum was included. Copper, total iron, ferric (Fe(III)), and ferrous (Fe(II)) ions in leachate were analyzed for 30 days. pH was measured using Hi2211-01 Benchtop pH/mV Meter (Hanna Instruments, Vöhringen, Germany). The value of oxidation/reduction potential (ORP) was measured using a standard hydrogen electrode (SHE) in relation to an Ag/AgCl reference electrode (mV vs. Ag/AgCl). Copper and total iron were determined using atomic absorption spectrophotometer AAS SP-IAA1800H (Bioevopeak, Qingdao, China). Concentrations of ferric (Fe(III)) and ferrous (Fe(II)) ions were determined using the complexometric method with EDTA [70].

Consumption of Fe(II) was calculated as a difference between initial Fe(II) and concentration of Fe(II) determined at certain times of experiment (Equation (1)). Consumption is expressed by g/L and %.

$$[Fe(II)]_c = [Fe(II)_i - [Fe(II)]_t / [Fe(II)]_i \times 100\% \quad (1)$$

where $[Fe(II)]_c$ is the consumption of Fe(II); $[Fe(II)_i$– is initial concentration of Fe(II) in the medium; and $[Fe(II)]_t$—concentration of Fe(II) determined at certain point of time.

Inhibition of iron oxidation in the presence of metal ions was determined according to Equation (2).

$$\text{Inhibition } (\%) = Fe(II)_{cm} / Fe(II)_c \times 100 \quad (2)$$

where $Fe(II)_{cm}$ is consumption of Fe in the presence of metal ions (g/L), and $Fe(II)_c$ is consumption of Fe in the absence of metal ions (g/L).

3. Results

3.1. Isolation of Strain Ksh-L

The selected sample from a dump-bioleaching system of the Kashen copper-molybdenum mine was transferred in a 9K liquid medium and incubated at 35 °C at 150 rpm for 5–7 days. As a result of the active growth of bacterial cells, the medium color changes from pale green to orange-red due to iron oxidation.

3.2. Morphology

Cells of *Leptospirillum* sp. Ksh-L are Gram-negative, motile, and vibrio- or spiral-shaped. Cells have a diameter of 0.5 µm and a length of 1.0–3.0 µm (Supplementary Figure S1). This is typical for the genus of *Leptospirillum*. The morphology results show that strain Ksh-L seems to be consistent with previously described *Leptospirillum* species [71].

3.3. Optimal pH and Temperature for Growth

The growth of the newly isolated Khs-L strain was evaluated according to the increase in the number of cells and the results of their biological activity, increasing the amounts of Fe^{3+} in the medium. As shown in Figure 1, the growth curves of the *Leptospirillum* sp. Ksh-L strain in the initial pH range of 1.6–2.0 are S-shaped. The duration of the lag phase was about 48 h, but the growth of bacteria was more active at pH values of 1.6 and 1.8 (Figure 1).

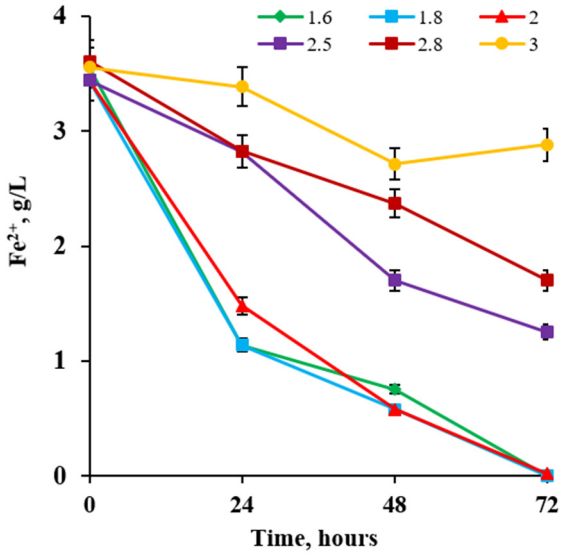

Figure 1. Dynamics of ferrous iron oxidation by *Leptospirillum* sp. Ksh-L at different pH values (Fe^{2+}—4 g/L, temperature 35 °C, 160 rpm).

The most intensive oxidation of Fe^{2+} was observed at pH values of 1.6 and 1.8. As can be seen from the presented data, at the initial pH values of 2.8 and 3.0, Fe^{2+} oxidation was sharply suppressed, and the duration of the lag phase was increased to 20 and 48 h, respectively (Figure 1). It can be explained that the pH variation affects enzyme activity because changes in ionization affect the system components. This indicates that the Ksh-L strain is sensitive to pH, and a much higher pH value will inhibit the activity of bacteria.

As can be seen from Figure 2, the optimum growth temperature of the Ksh-L strain was observed at 35 °C. Thus, optimal conditions for the growth of isolated strain *Leptospirillum* sp. were observed at 35 °C and a pH range from pH 1.6 to pH 2.0.

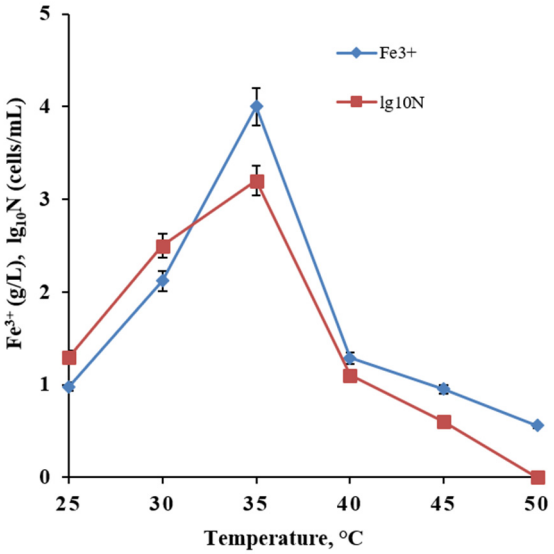

Figure 2. Effect of temperature on growth and iron oxidation by *Leptospirillum* sp. Ksh-L (pH 2.0, duration 48 h). N represents cell number (cells/mL).

3.4. Influence of Copper and Zinc

Like most bioleaching microorganisms, strain *Leptospirillum* sp. Ksh-L has been isolated from an environment (dump-bioleaching system) that has unusually high concentrations of potentially toxic metals (e.g., copper and iron) as well as high concentrations of heavy metals (e.g., arsenic and silver). These metals can exert harmful effects on microorganisms. Their toxic effects include the blocking of biologically important functional groups and the denaturation of enzymes [72].

Cu (II) inhibition of growth and Fe(II) oxidation have also been demonstrated in *Sulfobacillus thermosulfidooxidans* subsp. *asporogenes* via competitive inhibition of Fe(II) oxidation [73]. The influence of Cu^{2+} and Zn^{2+} ions on the oxidation of Fe^{2+} by strain Ksh-L was studied in a concentration range from 25 to 200–300 mM. As can be seen from Figure 3a, copper in all tested concentrations inhibits the oxidation of Fe^{2+}.

Furthermore, the higher the copper concentration, the higher the extent of inhibition of iron oxidation. Thus, iron oxidation by *Leptospirillum* sp. Ksh-L for 24 h was suppressed by 36.7, 65.3, and 89.8% at copper concentrations of 50, 100, and 150 mM, respectively, with a content of 3.2 g/L Fe^{2+} in the medium. Cu^{2+} in the concentration of 200–300 mM almost completely (90%–96%) inhibits iron oxidation by *Leptospirillum* sp. Ksh-L (Figure 3b). It can be noted that the extent of inhibition of iron oxidation by Cu^{2+} ion reduces for 48 h along with bacterial growth and is 17.4, 44.9, and 71.5%, respectively (Figure 3b).

The effect of copper on oxidation of Fe^{2+} *Leptospirillum* sp. Ksh-L was studied depending on substrate concentrations in the medium (Figure 4a,b). As shown in Figure 4, the degree of oxidation of Fe^{2+} by *Leptospirillum* sp. Ksh-L in the presence of tested copper concentrations was slightly higher when the content of Fe^{2+} in the medium increased from 4.0 g/L (71.4 mM) to 7.0 g/L (125 mM). Thus, the increase in the concentration of substrate

from 4.0 to 7.0 g/L leads to the enhancement of the amount of oxidized iron by bacteria in the presence of the tested concentration of copper.

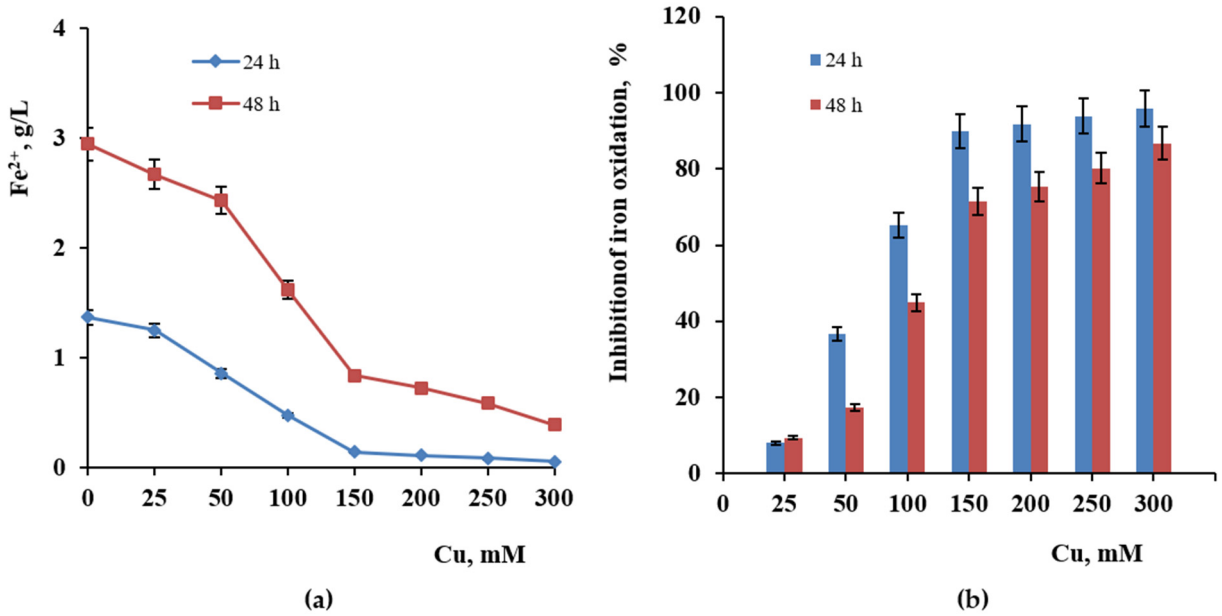

Figure 3. Oxidation of Fe^{2+} g/L (**a**) and inhibition of iron oxidation % (**b**) by *Leptospirillum* sp. Ksh-L at different concentrations of copper during 24 and 48 h of growth.

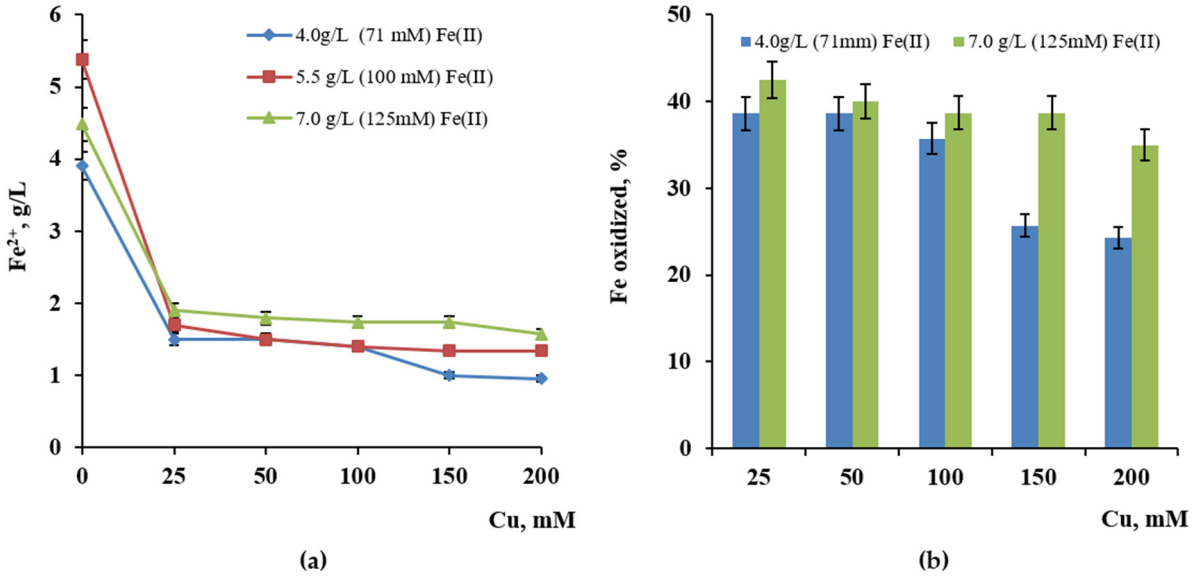

Figure 4. Effect of Cu on oxidation of Fe^{2+} g/L (**a**) and % (**b**) by *Leptospirillum* sp. Ksh-L at different initial concentrations of Fe^{2+} in the medium (pH 1.95, temperature 35 °C, 180 rpm, duration—43 h).

From the data presented, it can be seen that the oxidation of Fe^{2+} by *Leptospirillum* sp. Ksh-L is suppressed by about 25% at 25 mM of Zn^{2+} and is sharply inhibited (up to 65–77%) at zinc concentrations in the range from 50 to 200 mM (Figure 5a,b).

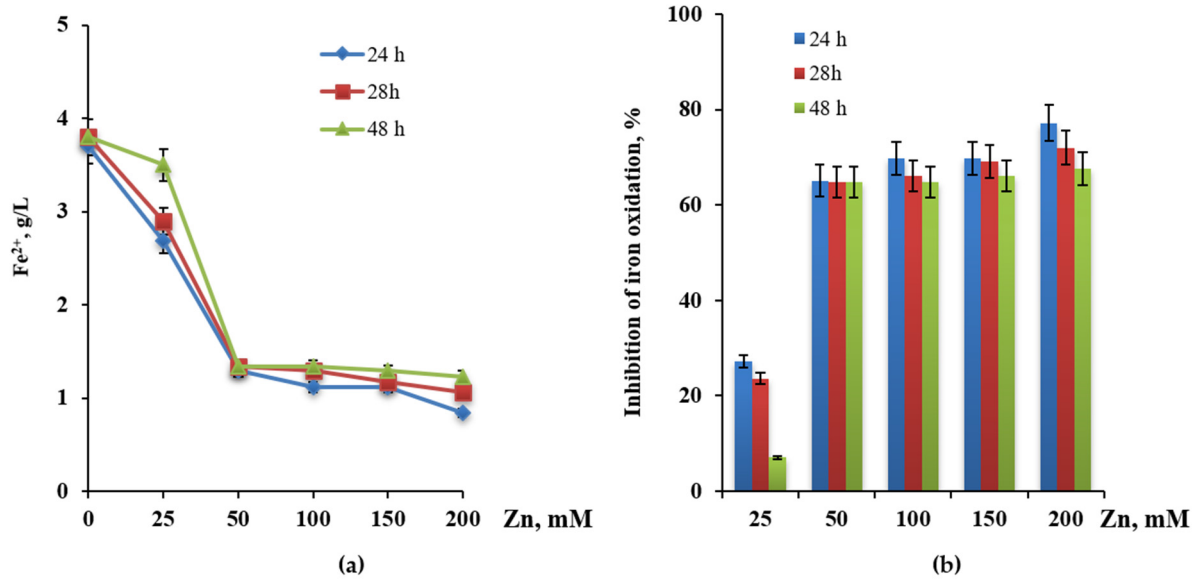

Figure 5. Influence of different concentrations of Zn^{2+} ions on oxidation of Fe^{2+} (**a**) and % (**b**) by *Leptospirillum* sp. Ksh-L depending on cultivation period (Fe^{2+}—3.8 g/L, pH 1.95, temperature 35 °C, 180 rpm).

As shown in Figure 6, an increase in the substrate concentration led to the enhancement of the amount of oxidized iron by Ksh-L in the presence of 25 mM Zn^{2+}, while it did not facilitate iron oxidation by bacteria in concentrations of Zn above 100 mM. Moreover, no decrease in the inhibitory effect of zinc ions was observed along with the growth of bacteria, as shown in the case of copper (Figure 5).

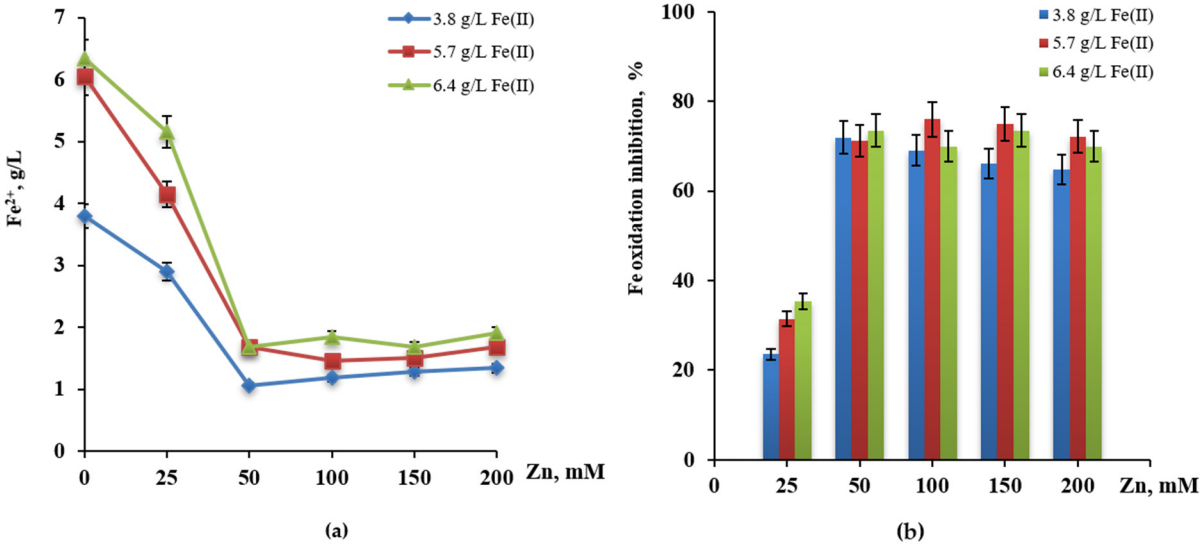

Figure 6. Influence of Zn^{2+} on oxidation of Fe^{2+} g/L (**a**) and Fe oxidation inhibition % (**b**) by *Leptospirillum* sp. Ksh-L at different concentrations of substrate (Fe^{2+}) (pH 1.95, temperature 35 °C, 180 rpm, 24 h).

Along with the growth of *Leptospirillum* sp. Ksh-L, the amount of oxidized iron in the presence of 50 mM Cu^{2+} increased. It is assumed that with the growth of bacteria, the cells form EPSs and create accordingly a less toxic and more favorable environment for the growth of cells in the presence of copper.

3.5. Adaptation of *L. ferrodiazotrophum* Ksh-L

Although bacteria do not react well to sudden and significant changes in heavy metal ion concentrations, they can be adapted to gradually increased concentrations over a while, to increase their tolerance to such metals. In the next series of the experiment under conditions of gradually increasing the concentration of copper in the medium, during 4–5 successive subculturing experiments, it was possible to obtain an adapted culture of *Leptospirillum* sp. Ksh-L capable of growing in the medium in the presence of up to 400 mM Cu^{2+} (Figure 7).

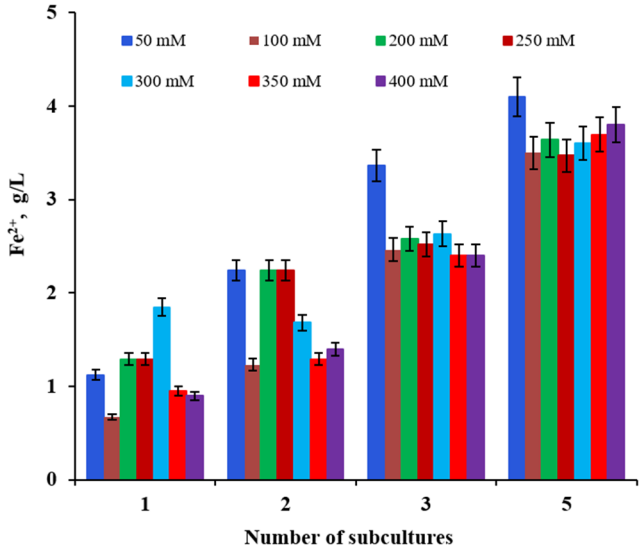

Figure 7. Oxidation of Fe^{2+} during an adaptation of *Leptospirillum* sp. Ksh-L under conditions of gradually increased concentrations of Cu in the medium (50–400 mM Cu^{2+}).

3.6. EPS Analysis

EPSs play an essential role in the formation of a biofilm, which mediates the adhesion of cells to the mineral surface and forms a cohesive three-dimensional polymer, interconnecting and immobilizing cells in the process of bioleaching by iron- and sulfur-oxidizing bacteria [74–76]. An important role of capsular polysaccharides as a fundamental structural element of the EPS, determining the mechanical stability of biofilm was disclosed. One of the objectives of the present study was to investigate the chemical composition of a colloidal polysaccharide of the newly isolated iron-oxidizing chemolithotrophic bacteria *L. ferrodiazotrophum* Ksh-L. The studies carried out showed that the total amounts of colloidal and capsular EPSs are 474.4 and 208.58 µg/mL, respectively.

As shown in Table 2, the amount of carbohydrates in the capsular EPS is considerably higher than that in the colloidal EPS. The amounts of protein in colloidal and capsular EPSs are approximately the same, 24.41 µg/mL and 27.33 µg/mL, respectively. Uronic acids were not detected in both EPSs. The obtained data are comparable and agree with the corresponding data of other species of the genus *Leptospirillum*, *L. ferriphilum* CC and *L. ferrooxidans* ZC studied by us previously [77,78].

Table 2. EPS composition of *L.ferrodiazotrophum* Ksh-L grown on ferrous iron (Fe (II)) as a source of energy (pH 1.95, temperature 35 °C, 180 rpm, cultivation 72 h).

EPS Composition	Protein (µg/L)	Carbohydrates (µg/L)	Uronic Acids
Capsular	16.3	6.0	BDL *
Colloidal	8.3	3.9	BDL *

* BDL: below the detection limit.

3.7. Phylogenetic Analysis of 16S rRNA

A PCR-amplified 16S rRNA product was detected by 1% agarose gel electrophoresis (Supplementary Figure S2). The length of the 16S rRNA of *Leptospirillum* sp. Ksh-L is about 1.5 kb and is sequenced sequentially. The sequence of the 16S rRNA of *Leptospirillum* sp. Ksh-L was submitted to GenBank, and the accession number ON226845 was obtained. The length of sequenced fragments of the gene encoding 16S rRNA is 1469 bp. The nucleotide sequence of the strain *Leptospirillum* sp. Ksh-L was phylogenetically compared with the *Leptospirillum* species (Figure 8).

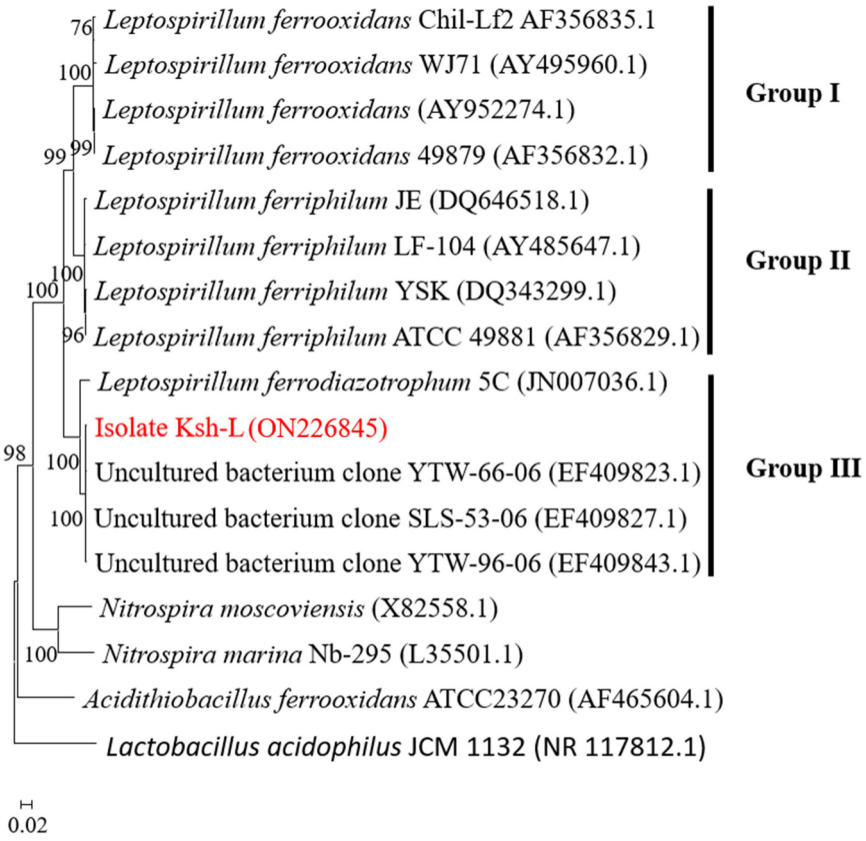

Figure 8. Phylogenetic position of strain *Leptospirillum* sp. Ksh-L. The evolutionary history was inferred using the neighbor-joining method [79]. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (10,000 replicates) are shown next to the branches [80]. New isolate highlighted in red.

Preliminary screening of the GenBank database was performed using BLAST (http:www.ncbi.nlm.nih.gov/blast). Based on the homology of 16S rRNA, the phylogenetic development tree was built as shown in Figure 8. The sequence was divided into three groups: Group I—*L. ferrooxidans*; Group II—*L. ferriphilum*; and Group III—*Leptospirillum ferrodiazotrophum* and uncultured clones. The isolated *Leptospirillum* sp. Ksh-L strain formed a single cluster into Group III and possessed 99.66% sequence similarity with uncultured bacterium clone SLS-53-06 (Table 3). As shown in Figure 8, the isolate Ksh-L compared to other strains in Group III with strains YTW-96-06, YTW-66-06, and *Leptospirillum ferrodiazotrophum* 5C and possessed 99.59%, 99.52%, and 96.60% sequence similarity, respectively (Table 3).

In Figure 8, the *Lactobacillus acidophilus* JCM 1132 strain is used as an out-group to root the tree, and the database accession numbers of the gene sequences used are given in parentheses.

Thus, the newly iron-oxidizing strain Ksh-L obtained from a dump-bioleaching system of the Kashen copper-molybdenum mine was identified as *Leptospirillum ferroodiazotrophum*. Type strain: *Leptospirillum ferroodiazotrophum* Ksh-L.

Table 3. Identity of 16S rRNA gene of isolated *Leptospirillum* sp. Ksh-L with other strains.

Isolated Strain	Strain Name, GenBank (Accession Number)	Identity, %	Reference
L. ferrodiazotrophum Ksh-L	Uncultured bacterium clone YTW-66-06 (EF409823.1)	99.52	[81]
	Uncultured bacterium clone SLS-53-06 (EF409827.1)	99.66	[81]
	Uncultured bacterium clone YTW-96-06 (EF409843.1)	99.59	[81]
	Leptospirillum ferrodiazotrophum 5C (JN007036.1)	96.60	[82]
	Leptospirillum ferriphilum ATCC 49,881 (AF356829.1)	91.02	[20]
	Leptospirillum ferrooxidans WJ71 (AY495960.1)	89.60	[83]
	Nitrospira moscoviensis (X82558.1)	81.22	[84]

3.8. Bioleaching of Chalcopyrite

A comparative study was carried out on the bioleaching of chalcopyrite by a pure culture *L. ferrodiazotrophum* Ksh-L and its association with sulfur-oxidizing bacteria *At. thiooxidans* ATCC 19377 at 30 °C.

The data presented in Figure 9a,b show that compared to uninoculated control, isolated bacterium *L. ferrodiazotropum* Ksh-L stimulated the extraction of copper and iron by 1.7 and 2.3 times, respectively. However, in association with sulfur-oxidizing *At. thiooxidans*, *L. ferrodiazotropum* Ksh-L oxidizes chalcopyrite much more actively than in pure culture. This finding corresponds with the literature data from similar studies conducted using *Leptospirillum ferooxidans* with the association of *At. thiooxidnas* or *At. caldus*. According to some researchers, mixed cultures of mesophiles have been reported to oxidize sulfide minerals more efficiently than pure cultures [85–87].

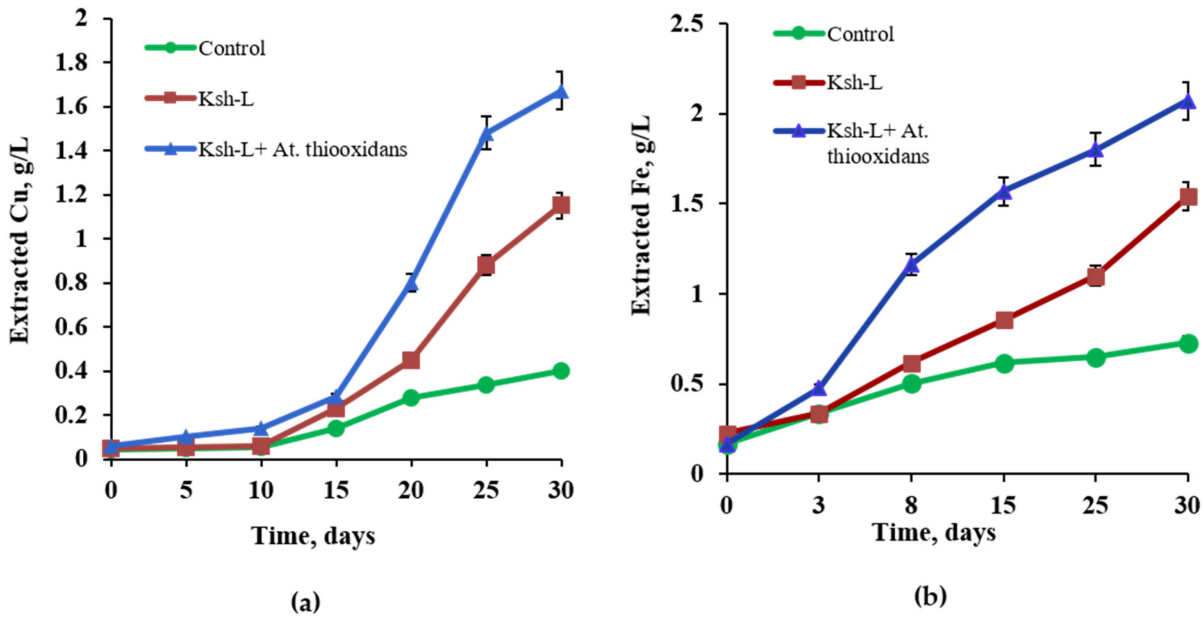

Figure 9. Bioleaching of copper (**a**) and iron (**b**) from chalcopyrite by pure-culture *L. ferrodiazotrophum* Ksh-L and association with *At. thiooxidans* ATCC 19377 ($CuFeS_2$—4%, pH 1.8, temperature 30 °C, 180 rpm).

Thus, in the presence of *At. thiooxidans*, the extraction of copper and iron from chalcopyrite by *L. ferrodiazotropum* Ksh-L for 30 days increases approximately 1.8 and 1.9 times, respectively (Figure 9a,b, Table 4).

Table 4. Leaching of iron and copper from chalcopyrite by *L. ferrodiazotropum* Ksh-L and association with sulfur-oxidizing bacteria *At. thiooxidans* ATCC 19377.

Bacteria	Extraction of Fe 30 Days				Extraction of Cu		Final	
	g/L			%	g/L	%	pH	ORP, mV
	Fe^{3+}	Fe^{2+}	Fe Total	Fe Total				
Control (uninoculated)	0	0.672	0.672	5.6	0.52	4.1	1.8	520
L. ferrodiazotropum Ksh-L	1.096	0.448	1.544	12.8	0.884	6.9	1.7	600
L. ferrodiazotropum Ksh-L + *At. thiooxidans*	2.016	0.616	2.744	23.0	1.67	13.4	1.5	720

The data presented in Table 1 show that chalcopyrite leaching is correlated with the pH and ORP of the solution. When *L. ferrodiazotropum* Ksh-L was used in monoculture, the final pH was 1.7, and the ORP was 600 mV, while in the *L. ferrodiazotropum* Ksh-L variant with *At. thiooxidans* ATCC 19377, the pH value was comparatively lower (1.5), and the ORP was significantly higher (720 mV) (Table 4).

Chalcopyrite is an acid-soluble sulfide mineral and is therefore subject to attack by both ferric iron (Fe^{3+}) and protons (H^+) (Equations (3) and (4)) [55,88].

$$CuFeS_2 + 2Fe_2(SO_4)_3 \rightarrow CuSO_4 + 5FeSO_4 + 2S^0 \quad (3)$$

$$CuFeS_2 + 4H^+ \rightarrow Fe^{2+} + Cu^{2+} + 2H_2S \quad (4)$$

$$0.125S_8 + 1.5\,O_2 + H_2O \xrightarrow{At.thiooxidans\ \text{ATCC 19377}} {SO_4}^{2-} + 2H^+ \quad (5)$$

At. thiooxidans ATCC 19377 in a mixed culture oxidizes sulfide and sulfur to sulfuric acid and contributes to the decrease in pH (pH 1.5), thereby preventing the formation of jarosite and a hydrophobic layer of sulfur on the surface of chalcopyrite (Equation (5)), removes the effect of passivation of the mineral, and promotes intense oxidation of chalcopyrite. According to Christel and Dopson, 2016, sulfur-oxidizing *A. caldus* seemed to have a supporting role in the early stages of mineral dissolution [89]. The attached sulfur-oxidizing bacteria on the mineral surface utilized the reduced inorganic sulfur compounds (RISCs) released from the mineral by a direct mechanism [90]. In a study conducted by Tao et al. (2021), six artificial communities with varying functions or biodiversity were recreated using six common bioleaching species for chalcopyrite leaching. Communities with low diversity also performed somewhat poorly in bioleaching, and the absence of sulfur oxidizers greatly decreased copper extraction rates in those communities [91].

4. Conclusions

A new strain of iron-oxidizing strain *Leptospirillum* sp. Ksh-L was isolated from a dump-bioleaching system of the Kashen copper-molybdenum mine. The cells of the strain Ksh-L are Gram-negative, motile, and vibrio- or spiral-shaped, with a 0.5 μm width and a 1.0–3.0 μm length.

The optimal temperature for the growth of *Leptospirillum* sp. Ksh-L is 35 °C, and the optimal pH is 1.6–1.8. The current study showed how different concentrations of commercially important metals (Cu^{2+}, Zn^{2+}) have various effects on the oxidizing ability of the strain *Leptospirillum* sp. Ksh-L, depending on the phase of growth and concentration of ferrous iron as a source of energy.

Based on the homology of 16S rRNA, the isolated strain *Leptospirillum* sp. Ksh-L formed a single cluster into Group III and, compared to other strains in Group III, possessed 99.59%, 99.52%, and 96.60% sequence similarity with strains YTW-96-06, YTW-66-06, and *Leptospirillum ferrodiazotrophum* 5C, respectively. Thus, the newly iron-oxidizing strain Ksh-L obtained from a dump-bioleaching system of the Kashen copper-molybdenum mine was identified as *Leptospirillum ferroodiazotrophum*. Type strain: *Leptospirillum ferroodiazotrophum* Ksh-L.

It was also shown that the bioleaching of copper and iron from chalcopyrite by the association of *L. ferrodiazotropum* Ksh-L and *At. thiooxidans* ATCC 19377 in comparison

with pure-culture *L. ferrodiazotropum* Ksh-L for 30 days increases about 1.8 and 1.9 times, respectively. Thus, it is supposed that the association of isolated *L. ferrodiazotropum* Ksh-L with sulfur-oxidizing *At. thiooxidans* ATCC 19377 can be successfully used to enhance the efficiency of copper extraction from chalcopyrite.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/min14010026/s1, Figure S1: Microphotography of iron-oxidized bacteria (Motic BA 310 trinocular (×1000) microscope with Digital Camera Moticam A16 (×0.5)). Figure S2: Gel electrophoresis of PCR amplification of 16S rRNA amplified product of 16S rDNA for bacterial isolate Leptospirillum sp. Ksh-L.

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