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The Effect of Metal Ions on the Growth and Ferrous IronOxidation by *Leptospirillum ferriphilum* CC Isolated from Armenia Mine Sites

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Abstract: The aim of this study is to investigate the potential of newly isolated strain *Leptospirillum* (*L.*) *ferriphilum* CC for bioleaching of pyrite and chalcopyrite in pure or mixed culture with other iron- and/or sulfur-oxidizing bacteria. In this paper, kinetics of ferrous iron (Fe^{2+}) oxidation by newly isolated strain *Leptospirillum* (*L.*) *ferriphilum* CC was studied. The effect of initial Fe^{2+} in the concentration range of 50–400 mM on bacterial growth and iron oxidation was studied. It was shown that microbial Fe^{2+} oxidation was competitively inhibited by Fe^{3+}. The influence of copper, zinc, nickel and cobalt ions on the oxidation of Fe^{2+} by *L. ferriphilum* CC was also studied. Minimal inhibitory concentrations (MIC) for each metal ion were determined. The toxicity of the ions was found to be as follows: Co > Zn > Ni > Cu. The comparison of iron oxidation kinetic parameters of *L. ferriphilum CC* with other strains of *L. ferriphilum* indicates the high potential of strain *L. ferriphilum* CC for biogenic regeneration of concentrated ferric iron (Fe^{3+}) in bioleaching processes of ores and ore concentrates. Bioleaching tests indicated that the newly isolated *L. ferriphilum* CC can be a prospective strain for the bioleaching of sulfide minerals in pure culture or in association with other iron- and/or sulfur-oxidizing bacteria.

Keywords: *leptospirillum ferriphilum*; iron-oxidizing kinetics; specific grow rate; saturation constant; tolerance to metal ions

Citation: Khachatryan, A.; Vardanyan, N.; Vardanyan, A.; Zhang, R.; Castro, L. The Effect of Metal Ions on the Growth and Ferrous IronOxidation by *Leptospirillum ferriphilum* CC Isolated from Armenia Mine Sites. *Metals* **2021**, *11*, 425. https://doi.org/10.3390/met11030425

Academic Editors: Anna H. Kaksonen and Jean-Francois Blais

Received: 20 January 2021
Accepted: 27 February 2021
Published: 5 March 2021

1. Introduction

Bioleaching is an environmentally friendly technology that is increasingly applied worldwide for processing of mineral raw materials and for recovery of copper, uranium and gold from low-grade ores and waste materials [1]. Bioleaching technology converts an insoluble valuable metal sulfide into a soluble form by means of microorganisms or destroys the lattice of the sulfide minerals to make the gold available for further extraction by cyanidation [2,3]. Studies have shown that *Leptospirillum* sp. (mainly *Leptospirillum ferriphilum*) are the dominant iron-oxidizing bacteria in gold-bearing arsenopyrite (FeAsS) and pyrite (FeS_2) in biooxidation reactors functioning at or over 40 °C [4,5]. A high Fe^{3+}/Fe^{2+} ratio, elevated temperatures (40 °C), as well as extremely low pH values (pH 1.0) are the most favorable conditions for the growth of the bacteria of the genus *Leptospirillum*.

It is considered that the main mechanism of microbial attack on the metal sulfides is an indirect contact mechanism. Metal sulfide is oxidized by Fe^{3+} and the role of microorgan-

isms refers to the oxidation of Fe^{2+} and regeneration of Fe^{3+}. The importance of microbial Fe^{2+} oxidation in bioleaching of sulfide minerals is well known, and widely reported in the literature [6,7]. During bioleaching processes, the sulfide minerals are chemically oxidized by Fe^{3+} (Equation (1)). The resulting Fe^{2+} is then regenerated biologically by microorganisms (Equation (2)) [7,8].

$$2MS + 4Fe^{3+} \rightarrow 2M^{2+} + 4Fe^{2+} + 2S^0 \quad (1)$$

$$4Fe^{2+} + 4H^+ + O_2 \rightarrow 4Fe^{3+} + 2H_2O \quad (2)$$

Therefore, the influence of Fe^{3+} and Fe^{2+} ions on the growth of bacteria and Fe^{2+} oxidation activity often determines the intensity of metal bioleaching processes [9,10].

The most important microorganisms involved in the regeneration of Fe^{3+} iron, responsible for the oxidation of exposed sulfide minerals are Fe^{2+} oxidizing bacteria *Acidithiobacillus (At.) ferrooxidans*, *Leptospirillum (L.) ferrooxidans*, *L. ferriphilum*, *Sulfobacillus (Sb.) thermosulfidooxidans*, *Acidimicrobium ferroooxidans*. Currently, the mechanism and kinetics of Fe^{2+} oxidation is well studied in *At. ferrooxidans*. However, during recent decades, *Leptospirillum* species have increasingly attracted the attention of researchers as alternative iron oxidizers. This interest can be attributed to the fact that compared to the acidithiobacilli, leptospirilli are more tolerant to lower pH and higher cultivation temperature [7,11], besides leptospirilli possess higher iron oxidation activity, compared with *Acidithiobacillus* sp. and *Sulfobacillus* sp. bacteria [12]. These properties make the species of genera *Leptospirillum* potential candidates for the development of new technologies based on the biological Fe^{2+} oxidation.

Dissolution of metal sulfides results in low pH environments containing highly elevated concentrations of transition and non-transition metals. Metals at high concentrations disrupt cell membranes, alter enzymatic specificity, hinder cellular functions, and damage DNA [13]. The study of the resistance of acidophilic bacteria to metal ions is limited only to the Gram-negative bacterium *At. ferrooxidans*. *At. ferrooxidans* is resistant to high concentrations of copper (up to 800 mM $CuSO_4$) and other metals [14,15]. *L. ferrooxidans* is known to be resistant to 5 mM Cu^{2+} in the medium [16]. There are no data on the resistance of *L. ferriphilum* to copper. 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 mM to 100 mM [17].

Gram-negative bacteria have a mechanism for active removal of copper from the cytoplasm into the periplasmic space with the help of P-type ATP-ases, localized in the inner membrane [18]. Some microorganisms can pump copper from the cytoplasm directly into the extracellular space using a system of resistant nodule cell division (RND). This type of detoxification is well known and described in *E. coli* [19]. It has also been reported about the ability of certain bacteria to bind copper in the periplasmic space with copper chaperones [14,20].

It is shown that *At. ferrooxidans* ATCC 23270 can function at high copper concentrations due to approximately 10 genes in the chromosome, which are directly related to its resistance to copper. They include three genes encoding P-type ATP-ases that are related to its resistance to copper transport (copA1Af, copA2Af, and copBAf), three RND-related genes responsible for removing copper from the cell (*cusAAf*, *cusBAf*, and *cusCAf*) and two genes, encoding periplasmic chaperones for copper (cusFAf and copCAf) [15,21].

Some of these genes associated with copper resistance are also found in *Leptospirillum* spp. bacteria using metagenomic analyzes of nucleotide sequences [22].

The toxicity of zinc is associated with its ability to form complexes with cellular components [17]. It is shown that zinc ions competitively inhibit the oxidation of Fe^{2+} in *Sb. thermosulfidooxidans* [23]. The toxicity of zinc to *At. ferrooxidans* depends on the substrate used. *At. ferrooxidans* resistant to 153 mM Zn^{2+} whilst growing on Fe^{2+} is sensitive to 92 mM when growing on thiosulfate [24]. Kondrateva et al. reported the adaptation of *At. ferrooxidans* to 1 M of Zn^{2+} [25].

An example of high tolerance to Fe^{3+} is the competition between iron-oxidizing bacteria in leaching solutions, which according to Rawlings et al. occurs in favor of *L. ferrooxidans* and *Sb. themosulfidooxidans* [26]. It was shown that Fe^{3+} ions competitively inhibit the oxidation of Fe^{2+} in *At. ferrooxidans*, *L. ferrooxidans*, and *Sb. themosulfidooxidans* [23,26,27].

At. ferrooxidans isolates from a Cu^{2+}/Ni^{2+} tailings environment were inhibited by >320 mM Ni^{2+} and adapted strains can tolerate up to 1 M Ni^{2+} [28]. Sampson and Phillips studied the influence of Ni^{2+} in the range of 0 to 150 mM on Fe^{2+} oxidation of a mixed culture of *At. ferrooxidans* and *L. ferrooxidans* [29]. They showed that the oxidizing ability of the mixed culture was unaffected up to 80 mM Ni^{2+}. Increasing the concentration of Ni^{2+} from 80 to 150 mM, the rate of oxidation was found to be only reduced slightly.

Acidophiles are metal-tolerant by both passive and active mechanisms. Passive mechanisms include an internal positive membrane potential that creates a chemiosmotic gradient against which metal cations must move, as well as the formation of metal sulfate complexes reducing the concentration of the free metal ion. Active systems include efflux proteins that pump metals out of the cytoplasm and conversion of the metal to a less toxic form. An important strategy used by all microorganisms to survive a toxic flux of metals is the formation of biofilms. Metal sequestration takes place in biofilms since extracellular polymers, cell membranes and cell walls of the microbial community provide many cationic and anionic sites for the interaction with metal ions and their biosorption [30].

Inhibition kinetics of Fe^{2+} oxidation by *L. ferriphilum* in the presence of ferric, nickel and zinc ions were studied by Nurmi et al. 2009 [31]. The results of their study demonstrate that *L. ferriphilum* is tolerant to high levels of metal ions in ferrous sulfate medium. The low pH value of 1.0 prevents metal precipitation and thus the metal additions represented the bio-available levels. Precipitation of Fe^{3+} in the form of ferric hydroxysulfates occurs at higher pH (>1.5).

The study of kinetics of Fe^{2+} oxidation by *L. ferriphilum* is of great interest from the point of their great potential in bioleaching of metals from mineral raw materials at elevated temperature.

In this paper, the influence of Fe^{2+}, Fe^{3+}, as well as copper, zinc, nickel and cobalt ions on the kinetics of Fe^{2+} oxidation by isolated *L. ferriphilum* CC was comparatively studied. Microbial Fe^{2+} oxidation by *L. ferriphilum* CC was competitively inhibited by Fe^{3+}. The toxicity of the studied ions was found to be as follows: Co > Zn > Ni > Cu. The iron oxidation kinetic studies indicate that *L. ferriphilum* CC possesses high capacity for biogenic regeneration of concentrated Fe^{3+} in bioleaching processes of ores and ore concentrates.

2. Materials and Methods

2.1. Microorganisms and Cultivation

In this study, the iron-oxidizing bacterium *L. ferriphilum* CC isolated from bioleaching pulp of copper concentrate in Armenia was used [12,32]. *L. ferriphilum* CC is deposited at the Microbial Depository Center (MDC) of the SPC "Armbiotechnology" of National Academy of Sciences (NAS) of Armenia under the number MDC 7047.

L. ferriphilum CC and *At. ferrooxidans* Ks [33] were grown in Mackintosh (MAC) medium [34], containing 20 g/L of $FeSO_4 \times 7H_2O$ (analytical grade, VWR Chemicals, Radnor, PA, USA, GPR RECTAPUR®) as an energy source [32]. In the logarithmic phase of the growth the bacterial cells were collected by centrifugation at 6000 g for 10 min using a centrifuge (Biosan LMC-56, Riga, Latvia). Biomass collected was resuspended in the same medium without Fe^{2+}. Extracellular polymeric substances (EPS)-free cells were prepared according to Castro et al. 2014 [35].

2.2. Bioleaching of Sulfide Minerals

Pyrite (FeS_2) consists of 43.8% Fe, 49% S and chalcopyrite ($CuFeS_2$) containing 30.2% Cu, 29.7% Fe, 38% S from Shamlugh ore deposit (Armenia) ground to 43-63 µm were used for leaching experiment. Leaching experiments were carried out in 250 mL conical flasks. A volume of 50 mL Mackintosh medium without Fe^{2+}, adjusted to pH 1.7

by H_2SO_4 and a bacterial suspension with 5 g of mineral grains (pyrite, chalcopyrite), was added to the flasks. Both pure cultures of *L. ferriphilum* CC and *At. ferrooxidans* Ksh and mixed cultures of both species were used in the bioleaching experiments. The initial cell number was 1.5-2 × 10^8 cells/mL. The bioleaching experiments were carried out at 37 °C with shaking at 180 rpm in an incubator (Biosan, Latvia). Sampling was performed every 3rd day over a period of 16 days. The intensity of pyrite and chalcopyrite oxidation was estimated by the quantity of the dissolved Fe^{3+} and Fe^{2+} and Cu^{2+} ions in the medium.

2.3. The Influence of Fe^{2+}/Fe^{3+} and Other Metal Ions on Growth and Fe^{2+} Oxidation

The kinetics of Fe^{2+} oxidation by *L. ferriphilum* CC was studied in Mackintosh medium with Fe^{2+} in the concentration range of 50–400 mM. The influence of Fe^{3+} ions on the growth rate and Fe^{2+} oxidation by *L. ferriphilum* CC was studied at the concentrations from 2 to 100 mM.

For metal ions Co^{2+}, Zn^{2+}, Ni^{2+}, and Cu^{2+}, appropriate sulfates (analytical grade, VWR) were used. Ferric sulphate from Puratronic® (analytical grade, Alfa Aesar, Haverhill, MA, USA) was used to prepare Fe^{3+} solutions. The concentration range of Cu^{2+}, Zn^{2+} and Ni^{2+} on Fe^{2+} oxidation by *L. ferriphilum* was 10–150 mM.

Number of viable cells was determined by the method of tenfold dilution. The most probable number (MPN) of cells was calculated using the Mac-Credy tables with three replicates [36]. The maximum specific growth rate (μ_{max}) of bacteria was determined using Monod equation, which gives the relationship between the specific growth rate (μ) and the limiting substrate concentration:

$$\mu = \mu_{max} \times S/(K_S + S) \quad (3)$$

where μ is the specific grow rate (1/h), μ_{max} is a constant (the maximum specific growth rate), K_S is a constant (substrate saturation constant, mM or Monod constant) which equals the substrate concentration that supports half of the maximum growth rate, S is the concentration (mM) of the growth rate-limiting substrate. The equation of Michaelis–Menten was used to determine V_{max}.

$$V_{max} = [S]/K_M + [S] \quad (4)$$

where V_{max} represents the maximum oxidation rate achieved, happening at saturating substrate concentration. The value of the Michaelis constant K_M is numerically equal to the substrate concentration at which the reaction rate is half of V_{max}. The Lineweaver–Burk plot was used to determine K_M and V_{max}. The Lineweaver–Burk plot puts 1/[S] on the x-axis and 1/V on the y-axis.

The inhibition constant (K_i) was calculated from the Equation (5) [37]:

$$K_i = i/(K_p/K_m - 1) \quad (5)$$

where K_i is inhibitory constant (mM), I is concentration of inhibitor ($Fe_2(SO_4)_3$) (mM), K_M is Michaelis constant. K_M (mM) is that concentration of substrate at which $V = V_{max}$. K_P is Michaelis constant in the presence of Fe^{3+} in the medium (mM).

Influence of copper, zinc, nickel and cobalt ions on oxidation of iron by *L. ferriphilum* CC was studied in MAC medium with 100 mM Fe^{2+} in the concentration range from 10 mM to 150 mM. We investigated iron oxidation dynamics in the presence of metals for 3–4 days.

2.4. Physico–Chemical Analysis

Concentrations of Fe^{3+} and Fe^{2+} were determined by the complexometric method with EDTA [36]. Total iron ions were determined by atomic-absorption spectrophotometry AAS 1N (Carl Zeiss, Jena, Germany) using an air–propane–butane flame.

The redox potential was measured with an oxidation/reduction potentials (ORP)-electrode met BNC-connector (Pt/Ag/AgCl) of Hi2211-01 Benchtop pH/mV Meter (Hanna Instruments, Vöhringen, Germany). pH was determined with a Hi2211-01 Benchtop pH/mV Meter equipped with an Ag/AgCl electrode.

Experiments were performed in triplicate. The data were analyzed statistically by Excel using student *t*-test and the presented data in the text are the average values from repeated experiments with ±2% variation (in standard deviation).

3. Results and Discussion

3.1. Bioleaching of Sulfide Minerals

The newly isolated iron-oxidizing bacterium *L. ferriphilum* CC was studied for bioleaching of sulfide minerals in comparison with iron- and sulfur-oxidizing bacteria *At. ferrooxidans* Ksh [37]. Results obtained showed that newly isolated strain *L. ferriphilum* CC is capable of oxidizing pyrite; however, *At. ferrooxidans* Ksh significantly exceeded *L. ferriphilum* in the activity of pyrite oxidation (Table 1). As has been shown previously, the oxidation of pyrite by iron-oxidizing *Leptospirillum* spp. bacteria is accompanied by the accumulation of sulfur on the surface of the mineral, which in turn prevents further oxidation of pyrite [38]. Taking into consideration the results mentioned above, we tested the association of *L. ferriphilum* with *At. ferrooxidans* Ksh in bioleaching of pyrite. The data given in the Table 1 show that the efficiency *At. ferrooxidans* Ksh in pyrite oxidation increases 1.8 times when co-cultivated with *L. ferriphilum* CC. It should be noted that when using the *At. ferrooxidans* Ksh with *L. ferriphilum* CC due to the high iron-oxidizing activity of *L. ferriphilum* CC, the extracted iron was found exclusively in the form of Fe^{3+}, which ensured the highest ORP value (775 mV) and, therefore, a high oxidizing property of the leaching solution (Table 1).

Table 1. Bioleaching of iron from pyrite by *L. ferriphilum* and *At. ferrooxidans* Ksh and their association.

Bacteria	Extracted Fe for 20 Days				pH, Initial/Final	Final ORP mV
	mg/L			%		
	Fe^{3+}	Fe^{2+}	Fe Total			
Control	0	840	840	4.8	1.7/1.6	600
L. ferriphilum	784	1456	2240	12.8	1.7/1.3	625
At. ferrooxidans Ksh	1624	1792	3416	19.5	1.7/1.2	635
At. ferrooxidans Ksh + *L. ferriphilum CC*	5824	616	6440	36.8	1.7/1.3	775

As shown in Figure 1, isolated iron- and sulfur-oxidizing bacterium *At. ferrooxidans* Ksh oxidize chalcopyrite more actively than *L. ferriphilum* CC. However, the extraction of copper (Figure 1a) and iron (Figure 1b) from chalcopyrite by *At. ferrooxidans* Ksh enhances by about 1.3 and 1.2 times, respectively, in association with *L. ferriphilum* CC (Figure 1). It is concluded that the presence of *L. ferriphilum* CC in association resulted in rapid oxidation of Fe^{2+} and regeneration of oxidative agent Fe^{3+}, which in turn accelerates chalcopyrite oxidation.

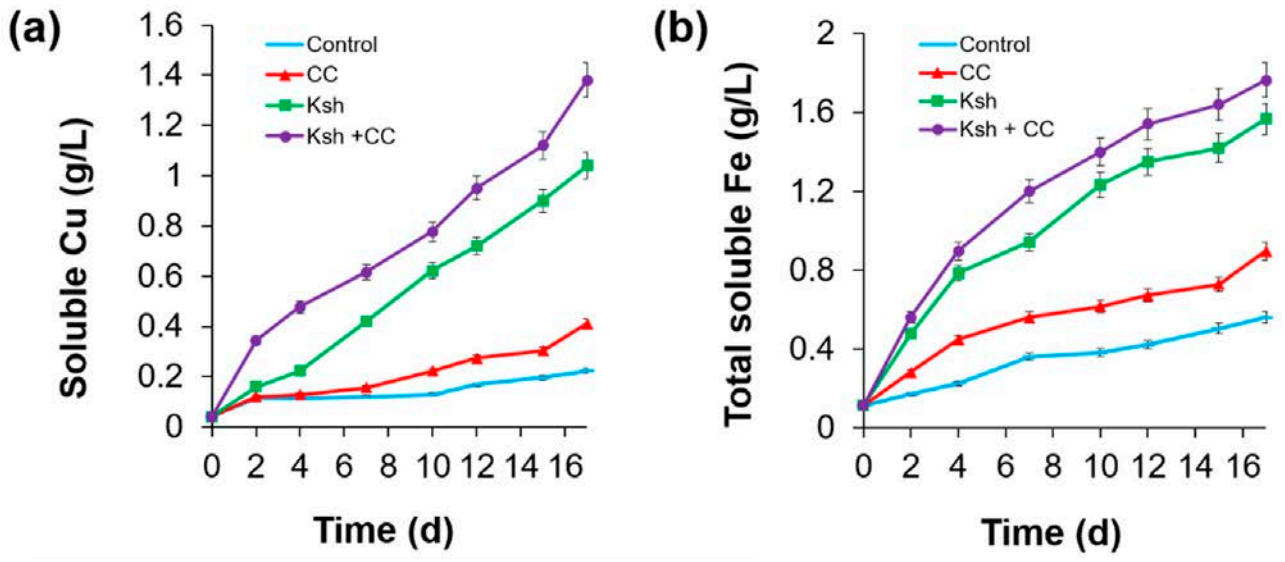

Figure 1. Bioleaching of Cu (**a**) and Fe (**b**) from chalcopyrite by iron- and sulfur-oxidizing bacteria *At. ferrooxidans* Ksh and iron-oxidizing *L. ferriphilum* CC.

It is concluded, that newly isolated *L. ferriphilum* CC can be a prospective strain for the bioleaching of sulfide minerals in pure culture or in association with other iron- and/or sulfur-oxidizing bacteria.

3.2. The Effect of Substrate Concentration

Fe^{2+} oxidation by *L. ferriphilum* CC was carried out on the rotary shaker. Kinetics of bacterial growth and iron oxidation is well described by Monod equation. Applying the Monod equation to the data obtained (Figure 2), growth and Fe^{2+} biooxidation parameters μ_{max} and V_{max} were determined as 0.48/h and 6.2 mM/h, respectively. Studies carried out showed that the growth of *L. ferriphilum* CC and the activity of Fe^{2+} oxidation depend on the concentration of the latter in the medium. Below is a quantitative characteristic of bacterial growth and Fe^{2+} oxidation depending on the initial concentration of Fe^{2+} in the medium (Table 2).

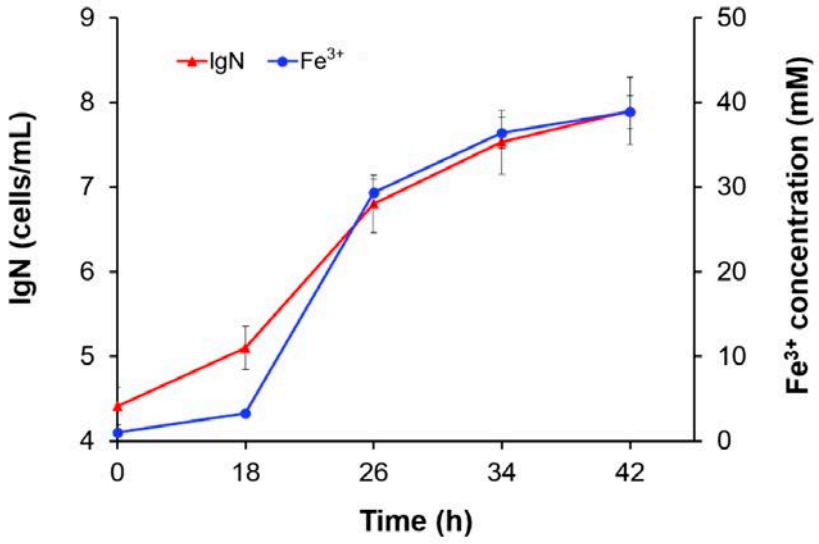

Figure 2. Dynamics of growth and iron oxidation by *L. ferriphilum* CC. Cells were grown in Mackintosh (MAC) medium with 70 mM Fe^{2+} and an initial pH of 2 at 37 °C and 200 rpm. N represents cell number (cells/mL).

Table 2. Growth and iron oxidation characteristics of *L. ferriphilum* CC at different initial concentrations of Fe^{2+}.

Fe^{2+} Concentration (mM)	μ_{max} (1/h)	V_{max} (mM/h)
50	0.31 ± 0.024	1.7
100	0.48 ± 0.018	6.2
200	0.41 ± 0.017	6.0
300	0.28 ± 0.026	4.6
400	0.090 ± 0.00082	3.7

As can be seen from Table 2, maximum values of specific growth (0.41–0.48/h) and Fe^{2+} oxidation rates (6.0–6.2 mM/h) were detected at Fe^{2+} concentrations of 100–200 mM. At higher concentrations, a slow growth of bacteria and Fe^{2+} oxidation suppression was observed, reaching maximum values at 400 mM Fe^{2+}. At a concentration of 50 mM Fe^{2+} in the medium, limitation by the substrate occurred. Thus, the optimal concentrations of Fe^{2+} for the growth of *L. ferriphilum* CC are 100–200 mM.

3.3. The Effect of Fe^{3+} Concentration

The tolerance of metal leaching bacteria to high Fe^{3+} concentrations is important in tank leaching applications. Fe^{3+}, being the product of Fe^{2+} oxidation, accumulates in the medium as iron-oxidizing chemolithotrophic bacteria grow. Therefore, the effect of the initial concentrations of Fe^{3+} on the oxidation of Fe^{2+} by bacteria can be studied only during the first hours (10–17 h) of cultivation, as the generated amount of Fe^{3+} by oxidizing Fe^{2+} is insignificant. Results obtained are presented in Table 3.

Table 3. Growth of *L. ferriphilum* CC on 100 mM Fe^{2+} with different initial concentrations of Fe^{3+}.

Fe^{3+} Concentration (mM)	μ_{max} (1/h)	V_{max} (mM/h)
2.0	0.35 ± 0.025	6.5
20.0	0.32 ± 0.026	6.1
50.0	0.26 ± 0.014	5.5
75.0	0.19 ± 0.010	3.2
100.0	0.16 ± 0.011	1.9

As the presented data show, in the presence of elevated concentrations of Fe^{3+} ions, inhibition of the growth of *L. ferriphilum CC* and Fe^{2+} oxidation was observed. Inhibition is expressed in the decrease in the specific growth rate and Fe^{2+} ion oxidation rate (Table 3, Figure 3.). Furthermore, the inhibition degree increases with increased concentrations of Fe^{3+}. The correlation between Fe^{2+} oxidation rate by *L. ferriphilum* CC and dynamics of changes in pH of the medium was detected (consumption of H^+ ions and increase in pH) (Figure 3c). At low values of Fe^{3+}, more active consumption of H^+ protons have been observed, which are necessary for the oxidation of Fe^{2+} (Equation (2)).

The study of the kinetic parameters of the oxidation of Fe^{2+} has shown that the affinity of *L. ferriphilum* CC to Fe^{2+} increases in the presence of Fe^{3+} ions in the medium. Thus, the saturation constant (K_m) for Fe^{2+} oxidation in *L. ferriphilum* CC was 0.83 mM $FeSO_4$ in the absence of Fe^{3+}, while it increased to 1.5 mM $FeSO_4$ at the initial concentration of 50.0 mM $Fe_2(SO_4)_3$ in the medium (Figure 4). It is concluded that like *Sb. thermosulfidooxidans* [23] and *At. ferrooxidans* [11], Fe^{3+} ions competitively inhibit the oxidation of Fe^{2+} in *L. ferriphilum* CC. The inhibition constant (K_i) was 61.95 mM $Fe_2(SO_4)_3$.

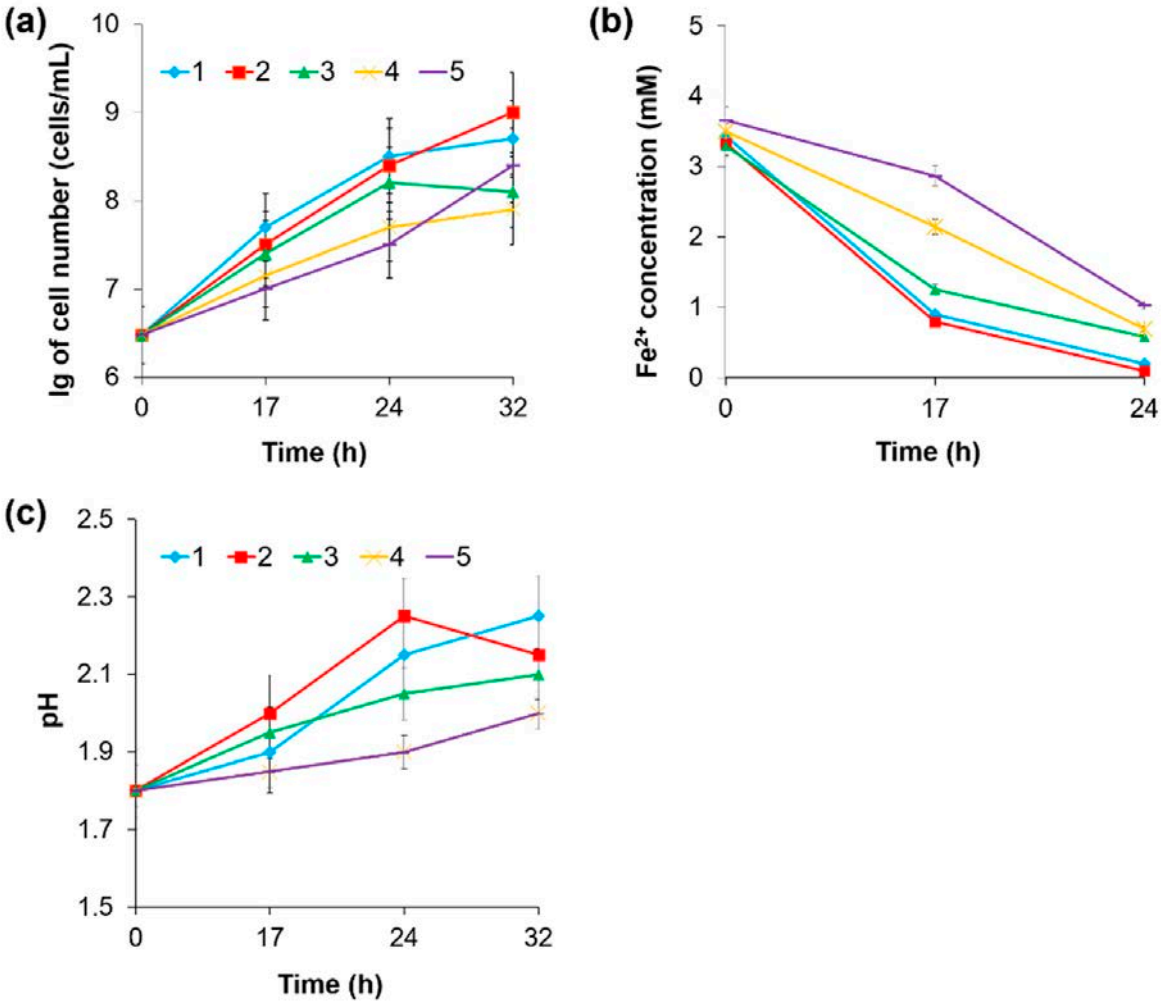

Figure 3. Effect of Fe^{3+} on dynamics of growth of *L. ferriphilum* CC (**a**), oxidation of Fe^{2+} (**b**) and pH changes (**c**) at concentrations: 2 mM (1), 20 mM (2), 50 mM (3), 75 mM (4) and 100 mM Fe^{3+} (5).

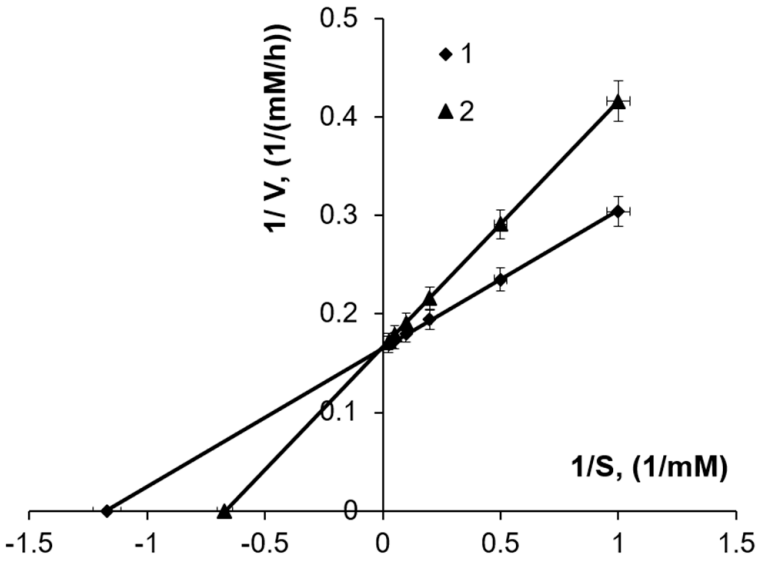

Figure 4. Determination of K_m according to Lineweaver–Burk; 1—in the absence of Fe^{3+}, 2—in the presence of 50 mM Fe^{3+}.

Based on the results, the optimal concentrations of Fe^{2+} for the growth of *L. ferriphilum* CC were in the range of 100–200 mM. Higher concentrations of Fe^{2+} inhibited the growth of bacteria and the consequent oxidation of Fe^{2+}. For cells of *L. ferriphilum* CC, the saturation constant for Fe^{2+} was less than that of *Sb. thermosulfidooxidans* (3.4–4.1 mM Fe^{2+}) [23] and *At. ferrooxidans* (1.34 mM Fe^{2+}) [39,40]. Thus, by the affinity for Fe^{2+}, *L. ferriphilum* CC considerably exceeds *At. ferrooxidans*.

Fe^{3+} ions competitively inhibit the growth of *L. ferriphilum* CC and Fe^{2+} oxidation. This has been reported for other bacteria like *At. ferrooxidans* [27,39,41]. It should be noted that Fe^{3+} at a concentration of 280 mM inhibits the growth and Fe^{2+} oxidation activity of *At. ferrooxidans* [42].

Fe^{2+} oxidation proceeds in the presence of 200 mM Fe^{3+} (data not shown) allows the successful use of *L. ferriphilum* CC in biogenic regeneration of concentrated Fe^{3+} solutions for biohydrometallurgical applications.

3.4. The Influence of Other Metal Ions

The influence of copper, zinc, nickel and cobalt ions on the oxidation of Fe^{2+} by *L. ferriphilum* CC was studied. The ions concentration varied from 10 to 150 mM. The studies showed that the extent of Fe^{2+} oxidation by *L. ferriphilum* CC was 58.4%, 42.8%, 53.3% and 31.3% at 20 mM copper, zinc, nickel and cobalt, respectively (Table 4). As can be seen from the presented data, zinc and cobalt ions at a concentration of 10 mM inhibited the oxidation of Fe^{2+} in *L. ferriphilum* CC by about 40% and 60%, respectively. Minimal inhibitory concentrations were 10 mM for Zn and Co and 20 mM for Cu and Ni. The toxicity of the ions was found to be as follows: Co > Zn > Ni > Cu.

Table 4. The influence of metal ions on Fe^{2+} oxidation by *L. ferriphilum* CC.

Metal ions (mM)	**Fe, Oxidized in 46–48 h**							
	Cu^{2+}		**Zn^{2+}**		**Ni^{2+}**		**Co^{2+}**	
	g/L	%	g/L	%	g/L	%	g/L	%
0	3.2 ± 0.19	100	1.6 × 0.023	100	3.4 ± 0.028	100	3.8 ± 0.024	100
10	–	–	1.0 ± 0.036	64.3	–	–	1.5 ± 0.0048	38.8
20	1.9 ± 0.049	58.4	0.67 ± 0.015	42.8	1.8 ± 0.0036	53.3	1.2 ± 0.0017	31.3
50	1.6 ± 0.014	50.0	0.56 ± 0.013	35.7	1.5 ± 0.018	43.3	0.85 ± 0.013	22.6
100	1.3 ± 0.0049	39.6	0.50 ± 0.0050	17.6	1.1 ± 0.049	31.7	0.78 ± 0.0086	20.9
150	1.2 ± 0.0080	36.9	–	–	0.84 ± 0.011	25.0	0.74 ± 0.0029	19.7

Cells were grown at 37 °C and 200 rpm with an initial pH 1.8.

Simultaneously, it has been established that as bacteria grow, the decrease in the inhibitory effect of copper ions is observed (Figure 5). Thus, with the growth of bacteria, the extent of inhibition of iron oxidation by *L. ferriphilum* CC decreases. Overcoming of the inhibitory effect of copper in *L. ferriphilum* CC was observed only at copper concentrations of 20 mM and 50 mM. At higher concentrations of copper, iron oxidation was inhibited at about the same extent and did not depend on the duration of cultivation of bacteria (Figure 5).

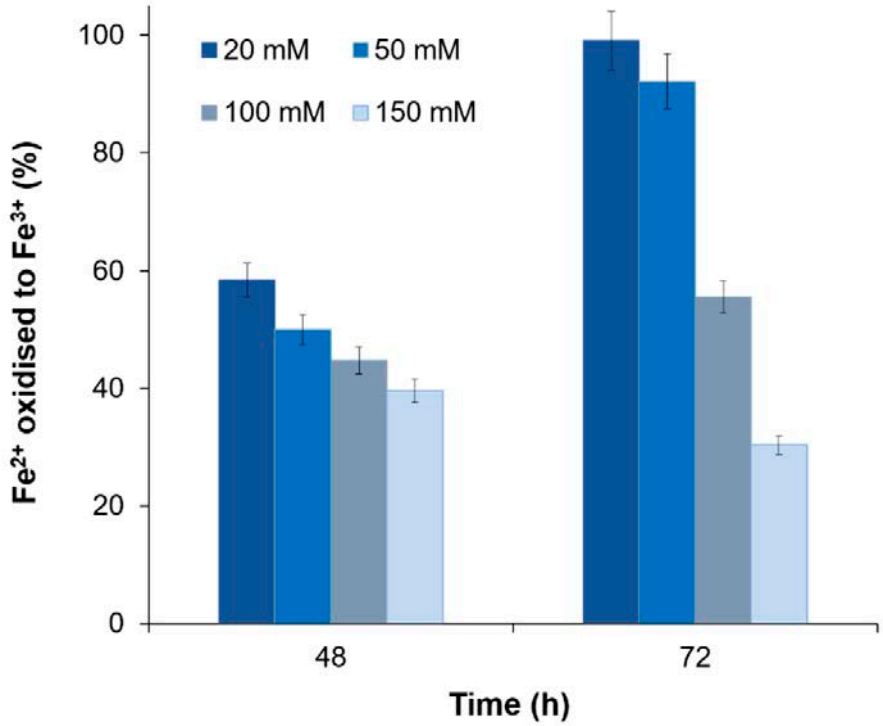

Figure 5. Effect of copper ions at different concentrations on Fe^{2+} oxidation by *L. ferriphilum* CC. Cells were grown in MAC medium with an initial pH of 2 at 37 °C and 200 rpm.

It is assumed that with the growth of bacteria, cells form EPS and accordingly create a less toxic and more favorable environment for their growth in the presence of copper ions. To test this hypothesis, we conducted studies with bacterial cells devoid of EPS. Properties and composition of EPS produced by *L. ferriphilum* CC were described previously [32,33]. The studies have shown that cells of *L. ferriphilum* CC devoid of EPS resulted from centrifugation at 6000 *g* are not able to grow and oxidize Fe^{2+} even in the presence of low concentrations of metals (Figure 6).

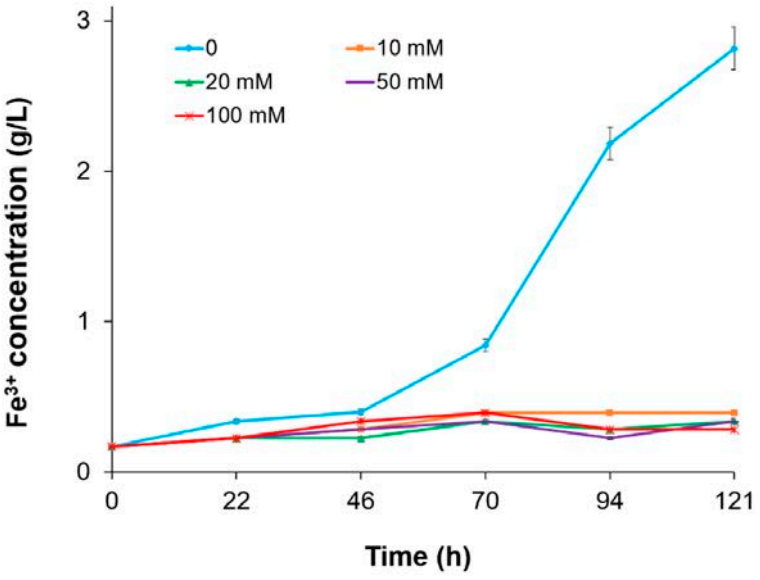

Figure 6. Dynamics of iron oxidation by cells of *L. ferriphilum* CC at different concentrations of Zn. The inoculum was extracellular polymeric substances (EPS)-free cells prepared according to Castro et al. 2014 [35].

EPS produced by microorganisms make up the intercellular space and form the structure of the biofilm matrix. The main role of EPS is the mediation of the initial attachment of cells to solid substrates (sulfide minerals, etc.) and protection against undesirable environmental factors. It is assumed that during their growth, bacterial cells form biofilm consisting of EPS which significantly increases the resistance of bacteria to heavy metals. The toxicity of the ions was found to be as follows: Co > Zn > Ni > Cu.

4. Conclusions

The effect of initial Fe^{2+} and Fe^{3+} concentrations on growth and Fe^{2+} oxidation by a newly isolated strain *L. ferriphilum* CC from Armenia mine sites was studied. The highest specific growth and Fe^{2+} oxidation rates were detected at Fe^{2+} concentrations of 100–200 mM. At higher concentrations (above 200 mM), the growth of bacteria and Fe^{2+} oxidation suppression was observed. The maximum specific growth rate (μ_{max}) of bacteria and half saturation constant (K_S) were 6.2 mM/h, and 0.83/h, respectively. For cells of *L. ferriphilum* CC, the saturation constant for Fe^{2+} was less than that of *Sb. thermosulfidooxidans* (3.4–4.1 mM Fe^{2+}) [23] and *At. ferrooxidans* (1.34 mM Fe^{2+}) [39,40]. Thus, by the affinity for Fe^{2+}, *L. ferriphilum* CC considerably exceeds *At. ferrooxidans*. It was shown that Fe^{2+} oxidation was competitively inhibited by Fe^{3+}. The toxicity of the metal ions was found to be as follows: Co > Zn > Ni > Cu. Comparing the obtained results with the data in the literature, it can be concluded that *L. ferriphilum* CC studied is generally not inferior in their resistance to copper, zinc, nickel [26,28]. Along with the growth of bacteria the decrease in inhibitory effect of metal ions was observed. It is assumed that during their growth, bacterial cells form biofilm consisting of EPS which significantly increases the resistance of bacteria to heavy metals. The comparison of kinetic parameters obtained for *L. ferriphilum* with other bacteria indicates the high potential of *L. ferriphilum* in leaching processes of ores and concentrates for biogenic regeneration of concentrated Fe^{3+}.

Author Contributions: Conceptualization, N.V., A.V. and R.Z.; methodology, N.V., A.V.; software, N.V.; validation, N.V., and A.V.; formal analysis, A.K. and A.V.; investigation, A.K. and A.V.; resources, N.V.; data curation, A.V. and N.V.; writing—original draft preparation, A.V., N.V. and R.Z.; writing—review and editing, R.Z. and L.C.; visualization, A.K. and A.V.; supervision, N.V. and R.Z.; project administration, N.V.; funding acquisition, R.Z. and L.C. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Spanish Ministry of Economy and Competitiveness (project MAT2014-59222R).

Data Availability Statement: Not applicable.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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