**Article**

**Bioleaching of Iron, Copper, Lead, and Zinc from the Sludge Mining Sediment at Different Particle Sizes, pH, and Pulp Density Using *Acidithiobacillus ferrooxidans***

Dana Rouchalova, Kamila Rouchalova, Iva Janakova *, Vladimir Cablik and Sarah Janstova

Faculty of Mining and Geology, VSB—Technical University of Ostrava, 70800 Ostrava, Czech Republic; dana.rouchalova.st@vsb.cz (D.R.); kamila.rouchalova.st@vsb.cz (K.R.); vladimir.cablik@vsb.cz (V.C.); sarah.janstova@vsb.cz (S.J.)

* Correspondence: iva.janakova@vsb.cz; Tel.: +420-5969-935-75

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**Abstract:** Globally, the amounts of metal ore deposits have been declining, so the research directions investigating the extraction of metals from materials that are classified as waste are gaining more importance every year. High concentrations of Cu, Pb, Zn, and Fe were analyzed in the sludge sediment (Zlaté Hory, Czech Republic), which is a waste product of the mining industry. In the bioleaching process, bacterial cells have been established as being able to convert metals from solid to liquid phase. However, the most important parameters of bioleaching are particle size, pH, and pulp density, thus our research focused on their optimization. The acidophilic and mesophilic bacteria *Acidithiobacillus ferrooxidans* were applied due to the high Fe content in the sample. The recovery of metals in the leachate was determined by F-AAS and the residual metal concentrations in the waste fraction were analyzed by XRF. The grain size fractions <40 µm – 200 µm were investigated. The atomic absorption spectrometry (AAS) results show that the highest Fe (76.48%), Cu (82.01%), and Pb (88.90%) recoveries were obtained at particle size of 71–100 µm. Zn was dissolved for all fractions above 90%. Experiments with different pH values were performed at a pH of 1.6–2.0. The highest dissolution rates of Zn, Fe, and Cu were achieved with a suspension pH of 1.8, where 98.73% of Zn, 85.42% of Fe, and 96.44% of Cu were recovered. Due to the high percentage dissolution of metals, experiments were performed under pilot conditions in a bioreactor at a pulp density of 2.5% and 4.2% (w/v). From an economic point of view, the leaching time of 28 days was evaluated as sufficient.

**Keywords:** bacterial leaching; particle size; pH; *Acidithiobacillus ferrooxidans*; lead; zinc; copper; iron

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**1. Introduction**

Bio-leaching is often used in the case of wastes that contain low concentrations of elements for which other separation methods cannot be used or would not be sufficiently efficient. Even in the 21st century, research has dealt with other possibilities of applying bioleaching in practice using various bacterial cultures [1]. Ore deposits with high concentrations of metals are becoming smaller and in the future rocks with a lower percentage of metals or waste will be used more and more often [2,3]. Globally, up to 10% of copper is obtained by leaching with the help of microorganisms. The application of bioleaching in practice includes many advantages when compared to other processes. The formation of inorganic gaseous pollutants, such as sulfur oxides, is eliminated. If arsenic is present in the ore, the formation of its compounds is prevented, as in the case of ore processing by pyrometallurgical processes. These products must be further disposed of as hazardous waste [4].
From an economic point of view, the bioleaching process is very advantageous, not only due to the elimination of flue gas cleaning, but also due to its simple application [5–7]. The process of dissolving metals in solution is influenced by several parameters that are related to the properties of microorganisms, chemical reactions (formation of precipitates), or the degree of friction in the suspension [8]. These include pH, particle size, pulp density, stirring frequency (rpm), temperature, nutrient concentration, oxygen content, and total bioleaching time [9–11]. These factors have a significant effect on the overall success of the transition of metals to the liquid phase and it is important to investigate their effect in order to achieve the highest possible yield of metals [10,12].

Deveci’s research [13] looked at the effect of solid material on the viability of a mixture of bacterial cultures. Their amount decreased in proportion to the decreasing radius of the particles to a value of approximately 45 µm. Particles that were <45 µm no longer had such a significant effect on the population of microorganisms and the particle effect on the decrease of bacteria decreased.

Nemati et al. [14] investigated the effect of different grain sizes on populations of microorganisms during leaching and its overall effectiveness. In the finest fraction <25 µm, a significant decrease in the amount of microorganisms in the medium was found in a short period of time from the start of the experiments. It has been shown that different size fractions have an effect on the recovery leaching of metals. Not only in terms of the effect of particles on bacteria, but also due to the formation of clots. These form on the surface of the grains as leaching products, and their amount often depends on the mineralogical composition of the sample, which may vary slightly from fraction to fraction. [8,15]. The pH value is one of the determining parameters of the metabolic activity of A. ferrooxidans cells. However, the optimal pH for growth and cellular oxidation, as well as the pH cut-off at which the bacterium is able to perform basic biological functions, varies in many sources [4,15–18]. In previous studies, the dependence of pH value and extraction efficiency was investigated, but, for other bacterial species [19], for non-stationary systems [20–22], or the research focused only on iron [23]. In our study, the polymetallic waste of A. ferrooxidans was leached at different pH values, which were kept limit-stationary during dissolution.

Our research deals with the effect of particle size, pH, and pulp density due to the importance of these factors and its goal is to intensify the overall gain of metals. There is much work on bioleaching using different types of microorganisms, mixtures of bacterial cultures, fungi, and archaea [1,24–28], but the effect of pH is not sufficiently studied. Different size fractions and pulp densities are among the major aspects of the process and the results should clarify the choice of conditions for further studies of bioleaching and their subsequent use in practice. In addition, the comparison of the effect of pulp density was carried out in pilot plant conditions, which was to simulate the situation in industrial use.

2. Materials and Methods

2.1. Ore Samples

The sample was obtained from the Jeseníky-Zlaté Hory mountains in the Czech Republic. The owner of the waste material is the state enterprise Diamo, specifically the enterprise GEAM in Dolní Rožínka. In the past, Au and sulphide ores containing Cu, Zn, and Pb were mined in the area [29]. The sludge sediment was dried for 24 h in a Memmert UF 110PLUS (Memmert GmbH + Co. KG, Schwabach, Germany) oven (108 L) at 90 °C to constant weight due to the high humidity of the sample. The sample was crushed while using a VIPO VMA - 386, VIPO vibrating mill, and it was sufficiently homogenized. Due to experiments with different particle sizes (<40 µm, 40–71 µm, 71–100 µm and 100–200 µm), a part of the sample was mechanically separated while using a Retsch AS 200 digit cA (Retsch GmbH, Haan, Germany) analytical sieving machine. XRD confirmed the presence of FeS₂, ZnS, SiO₂, CuFeS₂, and PbS in the sediment. A representative sample was obtained, its element content was determined by XRF analysis, and it is shown in Table 1.
Table 1. Elemental chemistry of sludge sediment determined by XRF spectrometry.

| Elements | Mass Fraction [%] | Elements | Mass Fraction [%] |
|----------|-------------------|----------|-------------------|
|          | Average           | SD       | Average           | SD       |
| Cu       | 1.743             | 0.023    | Pb                | 0.342    |
|          | 0.013             |          |                   |          |
| Fe       | 20.309            | 0.064    | S                 | 0.840    |
|          | 0.011             |          |                   | 0.118    |
| P        | 0.822             | 0.011    | Zn                | 3.878    |
|          |                   |          |                   | 0.118    |

2.2. Microorganism and Preparation of Media

Mesophilic and acidophilic bacteria *Acidithiobacillus ferrooxidans* were applied on all of the experiments. The inoculum with bacteria was sent from the workplace of the Czech Collection of Microorganisms (Brno). The bacteria were cultured in 9K medium, the composition of which was described in 1959 by Silverman and Lundgren. The medium was prepared by dissolving 2 g (NH₄)₂SO₄, 0.5 g K₂HPO₄, 0.5 g MgSO₄·7H₂O, 0.1 g KCl, and 0.01 g Ca(NO₃)₂ in 700 mL distilled water. Next, 40 g of FeSO₄·7H₂O were dissolved in 300 mL of distilled water, and the two solutions were mixed in order to eliminate the formation of undesired precipitates [30]. All of the chemicals used were supplied by Penta. The main parameters of *A. ferrooxidans* culture include pH and temperature. The acidophilic conditions of the solution (range 1.8–2.2) were stabilized by the addition of 5M H₂SO₄. A constant temperature of 30 °C was maintained by a laboratory incubator and a thermostat LP BT 120 M. A 9K solution was prepared 14 days before the start of the experiments due to the optimal growth of bacteria in the medium.

2.3. Chemical Leaching

A blank experiment was performed to compare the efficiency of abiotic and bacterial extraction. The sample was dissolved in sulfuric acid pH 2.0, which was kept stationary during extraction. The parameters of the sterile experiments were particle size <40 µm and suspension density 6.0% (w/v). Acid oxidation was performed in Erlenmeyer flasks. The system temperature was not regulated due to the absence of the microbial cells. All of the experiments were performed in triplicate.

2.4. Bioleaching Experiments

All of the experiments were performed in triplicate and the error bars were calculated as the standard deviations.

2.4.1. Particle Size Experiments

Grain size fractions were tested in order to compare the effect of different particle sizes: <40 µm, 40–71 µm, 71–100 µm, and 100–200 µm. The experiments were performed in Erlenmeyer flasks at pH 2.0 and a pulp of density of 6.7% (w/v). The constant pH value was stabilized while using 5M H₂SO₄ in the case of exceeding the pH by a solution of 0.4M NaOH. The suspension was placed in an incubated INFORS HT Multitron II (Infors LTD., Reigate, UK) shaker, which maintained a constant temperature of 30 °C and agitation rates of 100 rpm (Figure 1). The experiments were performed for 42 days in order to determine the optimal leaching time after the end of the experiments.
2.4.2. pH Experiments

The sludge sediment was extracted in 9K inoculated medium at stationary pH values of 1.6, 1.8, 2.0, and 2.2 in an INFORS HT Minifors 2.5 LTV (Infors LTD., Reigate, UK) bioreactor/incubator shaker. The bioreactor allows for automatic pH regulation only in the range of values 2–12. In accordance with the technological parameters of the device, the samples were leached in the fermenter at pH 2.0 and 2.2 (Figure 2). At pH 1.6 and 1.8, extraction was performed in 2 L Erlenmeyer flasks that were placed in an incubator shaker. Identical parameters of all experiments were solid concentration 5.6% (w/v), particle size <40 µm, temperature 30 °C, and stirring speed 150 rpm.
2.4.3. Pulp Density Experiments

The experiments under pilot conditions were performed in a New Brunswick Scientific Bioflo & CelliGen 310 (Eppendorf, Hamburg, Germany) bioreactor. The bioreactor was equipped with its own software, which recorded the temperature (30 °C), suspension pH (2.0), and kept them constant throughout the experiments (Figure 3). The samples with pulp densities of 2.5% and 4.2% (w/v) were tested. The suspension was agitated with a mechanical stirrer, which ensured an intensive suspension of the solid fraction in the biomedia, thus ensuring effective contact of the grains and the solution with the microorganisms and preventing the sample from settling at the bottom of the bioreactor.

Figure 3. Bioleaching of samples in pilot plant conditions in a Bioflo & CelliGen 310 bioreactor.

2.4.4. Solid and Liquid Sampling

The solid and liquid phases were collected and separated by filtration under reduced pressure (KNF LIQUIDPORT NF 300 KT.18S, KNF Neuberger GmbH, Schmachtl, Germany). All of the samples were taken in triplicate. The solid sample was washed with 40–50 mL of 0.1 M HCl and then poured into distilled water until the acidity of the solution dropped to 5.0–5.3. The sample was filtered again, and the solid fractions were dried in an oven [Memmert, UF 110PLUS, Memmert GmbH + Co. KG, Schwabach, Germany (108 L)] at 60 °C after 4–5 h to constant weight. Subsequently, the sample was homogenized, and a representative sample was obtained from the total amount, which was placed in plastic containers with screw caps. The total leaching time was 42 days and the solid (and liquid) phases were collected at weekly intervals.

2.4.5. Chemical Analysis

Heavy metal concentrations in the leaching solutions were determined by flame atomic absorption spectrometry (F-AAS; VARIAN AA 280FS, Agilent, Santa Clara, California, USA). The methods of calibration curves that were prepared by the operator before the actual determination using certified standards were used for the analyses. Evaluation and concentration calculations were performed while using instrument software (SpectrAA Base, PRO and CFR software versions) from Varian Australia Pty Ltd. Quantitative analysis of metals in waste solid fractions was performed by X-ray fluorescence spectrometry. Analyte concentrations were determined on powder compressed tablets while using a BRUKER S8 TIGER (Brukner Co., Billerica, MA, USA) equipped with an automatic crystal changer (XS-55, PET, LiF 200) and a very powerful 4 kW Rh tube. The
concentrations of Fe, Pb, Cu, and Zn were determined by the QUANT T calibration method, which consists in the application of reference materials (CRM). The calibration of the elements was performed within one software solution, which eliminated the necessity of creating a self-calibration by the operator.

The pH values were measured while using a pH meter WTW 330i/Set (Xylem Analytics Germany Sales GmbH & Co. KG, WTW, Weilheim, Germany) (accuracy ± 0.005). The pH values in the bioreactors were automatically regulated. The relative amounts of metals in the leachates RA [%] were calculated while using the following equations [31]:

\[ RA = \frac{M_1}{M_s} \times 100 \]  
\[ M_1 = C_e \times V_e \times f \]  
\[ f = \sum_{e=1}^{n} C_{e-1} + V_{e-1} \]  
\[ M_s = C_s \times M_s \]  

In these equations, \( M_1 \) represents the amount of heavy metal in solution after chemical or biological extraction [mg]; \( M_s \) represents the weight of heavy metal in the mine sediment sludge [mg]; \( C_e \) represents the concentration of the dissolved element in the extract at the time of sampling \( e \) [mg L\(^{-1}\)]; \( V_e \) represents the volume of leachate at the relevant sampling time [l]; \( f \) represents the total correlation factor of previous samples; \( V_{e-1} \) represents the volume of leachate that was taken from the extraction system at time \( e-1 \) [l]; \( C_s \) represents the metal concentration in the mine sediment before the extraction process [mg kg\(^{-1}\)]; and, \( m \) represents the weight of dried sediment [kg].

3. Results and Discussion

3.1. Interaction of Sample, Medium and Microorganisms

From the chemical point of view, the following reactions took place during the experiments. During direct leaching, the sample containing the sulphides of the metals in question reacted with sulfuric acid (or water) in the presence of oxygen in order to form metal sulphates. Sulphate metals are readily soluble and they can be subsequently separated from the liquid phase (Equations (5)–(8)). Acidophilic mesophilic bacteria \( A. \) ferrooxidans participated in all reactions [32–36].

**Pyrite:** FeS\(_2\) + 7 O\(_2\) + 2 H\(_2\)O \(\rightarrow\) \( A. \) ferrooxidans \(\rightarrow\) 2 FeSO\(_4\) + 2 H\(_2\)SO\(_4\)  

**Sphalerite:** ZnS + \( \frac{3}{2} \) O\(_2\) + H\(_2\)SO\(_4\) \(\rightarrow\) \( A. \) ferrooxidans \(\rightarrow\) ZnSO\(_4\) + H\(_2\)O + S  

**Chalcopyrite:** CuFeS\(_2\) + O\(_2\) + 2 H\(_2\)SO\(_4\) \(\rightarrow\) \( A. \) ferrooxidans \(\rightarrow\) CuSO\(_4\) + FeSO\(_4\) + 2 H\(_2\)O + 2 S  

**Galenite:** PbS + \( \frac{1}{2} \) O\(_2\) + H\(_2\)SO\(_4\) \(\rightarrow\) \( A. \) ferrooxidans \(\rightarrow\) PbSO\(_4\) + H\(_2\)O + S  

At the same time, due to indirect leaching, the sulphide ores of a given metal oxidized with ferric sulphate, which is described by Equation (9) [1].

\[(Zn, Cu, Fe, Pb)S + Fe(\text{SO}_4)\_2 \rightarrow (Zn, Cu, Fe, Pb)SO\(_4\) + 2 FeSO\(_4\) + S\]  

The resulting ferrous sulfate was further oxidized in the presence of sulfuric acid and microorganisms to ferrous sulfate (Equation (10)), which re-entered into reaction No. 9 [37].

\[4 \text{FeSO}_4 + 2 \text{H}_2\text{SO}_4 + \text{O}_2 \rightarrow \text{Fe}^{2+}(\text{SO}_4)\_2 + 2 \text{H}_2\text{O}\]  

Elemental sulfur reacted with water in the presence of oxygen to form sulfuric acid (Equation (11)), which is essential for both bioleaching mechanisms [38–40].

\[S + \frac{1}{2} \text{O}_2 + \text{H}_2\text{O} \rightarrow \text{Fe}^{2+}(\text{SO}_4)\_2 + 2 \text{H}^+\]  

Chemical reaction No. 11 returned sulfuric acid to the system, which had a positive effect on the equilibrium state in the leaching process. This process was accompanied by a negative value of the
reaction enthalpy, heat was released, which resulted in a faster course of the surrounding reactions [41]. According to Equation (5), the metal sulfides were converted to sulfates that have completely different ion arrangements in their crystal lattice. If we compare the ion radii of these two chemical structures, the sulfates show larger values, which resulted in better availability for *A. ferrooxidans* bacteria [1]. In order for leaching in the presence of microorganisms to proceed as efficiently as possible, an important supply of oxygen was an important factor. This is necessary for the proper activity of bacteria. Not only in the case of the conversion of metal sulphides into sulphates, but also of ferrous iron to trivalent (Equation (10)) [42]. The sufficient agitation of the suspension was ensured during all of the experiments.

The bacterium *A. ferrooxidans* was chosen for the research due to the composition of the sample, which draws energy from oxidation-reduction reactions of inorganic substances (divalent Fe, reduced form S) and macroergic compounds. The ATP (adenosine triphosphate) molecule provides energy by converting it to ADP (adenosine diphosphate) and releasing phosphate. The bacterium is cultured in an acidic environment, but inside the cell the pH has a weakly acidic character (around 6.5) [43,44]. During bioleaching, the cell is attached to the leached grain and it receives O₂, CO₂, N₂, and H₂ from the air [4]. Airborne CO₂ is processed through the Calvin cycle, with ribulose-1,5-bisphosphate carboxylase/oxygenase being the major biocatalyst for these reactions. During the process, the sulphate anion SO₄²⁻ is formed, which, in the case of controlled leaching in the bioreactor, contributes to a more efficient extraction of metals. A longer exposure of test samples and biomedia is required for bioleaching of sulphide ores [45]. Therefore, the tests were performed for 42 days in order to determine the most economically advantageous leaching time after the end of the experiments.

3.2. Chemical Leaching

The test sample containing 7 mg·kg⁻¹ Ag. *A. ferrooxidans* cells is not inhibited by the silver content of the leached material, as has been shown in thermophilic bacteria [45]. Leaching with *A. ferrooxidans* is economically advantageous due to lower temperatures and no significant corrosive processes as in the case of thermophiles [10]. Akcil et al. [28] have shown a direct relationship between the efficiency of metal extraction and the amount of bacterial content in the medium. During the tests, the optimal conditions were maintained for the sufficient multiplication of microorganisms in order to ensure the most efficient dissolution of metals.
Figure 4 shows that the curves expressing the dependences of chemical metal recoveries on times showed a completely different trend in the case of Fe, Pb, and Zn than the solubilization curves of elements in biological leaching. The majority of heavy metals were chemically extracted after 14 days, and the prolongation of the extraction time did not lead to an increase in metal removal yields. After 14 days, 4.71% Fe, 9.01% Pb, 12.98% Cu, and 31.88% Zn were chemically leached. The increase in the percentage of dissolved Fe, Pb, Cu, and Zn was only 2.21%, 1.61%, 13.36%, and 5.31%, respectively, between days 14 and 42. Only for Cu, the optimal extraction time was 21 days, when 22.01% of the metal was extracted. Consistently, as observed by Ye et al., dissolution in H$_2$SO$_4$ (pH 2.0) was the least effective for Fe [20]. After seven days, only 2.67% Fe was transferred to the solution, which was the lowest efficiency that was achieved in the study. Extending the extraction time to 42 days increased the Fe content in the acid to 6.92%, while, at pH 2.0 and 1.8, the Fe recovery efficiencies were 60.57% and 85.42% in Figure 13a.

Figure 4, 13 show that chemical extraction resulted in lower dissolution efficiencies for all elements than in biological phase enriched systems. After 42 days, H$_2$SO$_4$ dissolved only 37.19% Zn, 26.34% Cu, and 10.62% Pb, with the relative increase in metal ions in the biological extract (pH 2.0) being 57.12% Zn, 64.0% Cu, and 86.4% Pb. The presented results correlate with the theoretical assumption that the total concentration of metals in biological leachate is given by the sum of ions that are released by metabolic activity of living cells and elements dissolved by acid, while the concentration of sulfate anions in solution increases due to bacterial oxidation [46]. However, in the blank experiment, there were higher extraction efficiencies than those that were determined by Ye et al. [20].

The pH dependence as a function of time was almost linear in chemical extraction. The values of pH deviations from the required pH value 2.0 did not exceed 0.05 degrees during leaching. Ye et al. noted the same low rate of fluctuation of pH values during the leaching of H$_2$SO$_4$ [20]. Significant fluctuations in pH values were observed during bioleaching, while the oxidation of crystalline sulfides to sulfate anions and the activity of *A. ferrooxidans* cells were confirmed by decreases in pH. The interfacial transition of metals due to sulfuric acid oxidation was evaluated as significantly limiting.
3.3. Influence of Particle Sizes on Metal Extraction Efficiency

The success of bioleaching is influenced by many parameters that have been chosen with respect to other scientific studies. In the study of Olubambi et al. [10], the effect of agitation on the percentage dissolution of metals was monitored and the best results were obtained at a stirring speed of 150 rpm. The experiments were performed in a closed bioreactor. In the case of our research, the experiments were performed in Erlenmeyer flasks and, in order to avoid loss of suspension, the agitation rate of 100 rpm was evaluated to be sufficient. The solid fraction was suspended during leaching and it did not settle to the bottom of the flasks. The pH value was chosen, not only with regard to the use of acidophilic microorganisms, but also with the assumption of reducing the probability of the formation of jarosite precipitates. Zhao et al. [47] confirmed that at a pH value exceeding 2.0, there is a higher probability of the formation of jarosite-type compounds. Another factor for jarosite formation is the total concentration of ferrous and ferric iron in the biomed. In a study by Tipre et al. [48] the same metals were extracted as in our experiments using a mixed culture of bacteria (including *A. ferrooxidans*). The pH was 2.0. In the study of Acevedo et al. [42], Fe was the major element in the sample. The thermophilic bacterium *Sulfolobus metallicus* at pH 2.0 was used in order to obtain it. Because of the given studies, the pH value was maintained by the addition of 5M H₂SO₄ or a solution of 0.4M NaOH. In order to have a sufficient number of samples for sampling (six samples), 100 g of sample was leached for each grain fraction.

3.3.1. Iron

Liquid samples were determined by atomic absorption spectrometry (AAS) analysis and the dependence of the percentage dissolution of Fe on the leaching time for individual particle sizes was determined (Figure 5). In solution, Fe was present in ionic form in oxidation stages II and III. It was found that Fe most readily passed into the liquid phase in the grain fraction 71–100 µm during the whole course of the experiments, when it was extracted from 76.48% (Fe concentration in the extract 11.558 g·L⁻¹). At a particle size of 40–71 µm, it was dissolved from 74.32% and, at a finest fraction, <40 µm from 66.48%. The worst results were achieved with the largest particle size 100–200 µm, when it was leached from 61.24%. The percentage difference in metal dissolution between the best and worst rated fractions was 15.26%. It is evident from the course of the curves that there was no significant inhibition of Fe leaching due to the formation of jarosite-type precipitates. These products were prevented by keeping the pH low, which was around 2.0.
In a study conducted by Conić et al. [1] Fe was leached from 68% at particle size <10 µm, agitation rate 150 rpm, pH 1.6, and pulp density of 8% (w/v) and the use of mixed culture microorganisms (including *A. ferrooxidans*). In a study by Acevedo et al. [42], the highest percentage dissolution was achieved with the finest fraction <38 µm and the least efficient extraction was demonstrated, as in our experiments with the largest particle size 106–150 µm. Nevertheless, over 80% of the Fe was leached in all of the experiments performed. It is probable that, in the case of the largest grains (100–200 µm), there was probably no sufficient contact of microorganisms and particles due to the smaller specific surface area. Olubambi et al. [49] monitored the dissolution of Fe at pH 2.0 and the application of a mixed culture of bacteria (including *A. ferrooxidans*). Even at low pH, the trend of the curves was first increasing (extraction from 48–50%), but it then decreased (extraction from 37–44%) for 21 days. It was found that the cause was the formation of secondary iron precipitates, which prevented further leaching. The best Fe extraction results were obtained with a grain size (75–106 µm). Despite maintaining a low pH value in our experiments, the formation of a very small amount of precipitates of the jarosite type is not ruled out, due to which the Fe extraction did not exceed 80%. These compounds have been shown not only to prevent further dissolution, but also to prevent sufficient O$_2$ access [50].

The concentration losses of Fe from solid waste material were determined by XRF analysis (Figure 6). For comparison, a blank is displayed for all subscriptions. Of all the analysed metals and of all elements in general, Fe was the most represented in the sludge sediment at a concentration of 203,085 mg·kg$^{-1}$. After 42 days, the highest decrease in metal concentration was found in the grain fraction 71–100 µm (47,564 mg·kg$^{-1}$). The XRF results correspond exactly to the liquid fraction analyses that were determined by AAS.
3.3.2. Copper

Copper is one of the predominant elements, in which leaching with the help of microorganisms is applied in practice. Worldwide, up to 10% of total metal production is obtained by this microbial technology [4]. Figure 7 shows the increasing course of the Cu leaching curves in the range of 42 days. From day 9, there was a more significant percentage increase in particles 40–71 µm, which was evaluated as the second best extractable in the end (79.15%). The largest grain fraction of 100–200 µm was dissolved from 75.37%. From day 20 until the end of the tests, the least willingness to transfer metal to solution was demonstrated for the finest particle size <40 µm (73.34%). The best results were obtained with a grain size of 71–100 µm, when the metal was released from chalcopyrite into the liquid phase from 82.01% (Cu concentration in the extract 1.116 g·L⁻¹). The percentage difference in Cu extraction between the best and worst rated fractions was 8.67%.

Figure 6. Concentration loss of Fe in waste material for individual grain fractions.
Figure 7. Influence of particle size on Cu recovery (pH 2, stirring speed 100 rpm, and 6.67% (w/v) pulp density).

In a study conducted by Conić et al. [1], Cu was extracted from 83% under similar conditions. In the case of our experiments, it was shown that the worst results were obtained with the finest fraction. Deveci [13] investigated the effect of grain radii on the microbial activity of bacteria. It has been shown that shrinking particles have a negative effect on the cellular structure of *A. ferrooxidans* (as well as *L. ferrooxidans*), which are damaged by friction. In a study by Nemati et al. [14], when *Sulfolobus metallicus* was applied, requiring an acidic and thermophilic environment, the finest particles <25 µm showed a loss of microorganisms in the medium during the experiments. By the impact of fine particles on the bacterial walls, the microorganisms were inhibited, and further did not participate in the oxidation of sulfides and ferrous sulfate. Agitation is also an important factor, which, in the case of very fine grains, promotes damage to cellular structures. On the contrary, in the case of larger particles, it accelerates the overall course of the process and keeps the grains in sufficient buoyancy. Consequently, they are easily accessible to multiplied colonies of microorganisms in solution. These studies confirm the results of our experiments.

The best percentage dissolution of Cu was achieved in the fraction 71–100 µm. The particle size in the range of 71–100 µm was evaluated as the most effective, even in the case of Fe leaching. In a study by Olubambi et al. [10] of all monitored fractions (<33 µm, 53–75 µm, 75–106 µm, >106 µm), the highest metal dissolution was achieved with a particle size of 75–106 µm, which corresponds to our conclusions. Nevertheless, only 14% of Cu was leached. It was found that, in some cases, copper-ferrous sulphide showed higher resistance to dissolution and insufficient kinetics of chemical reactions were demonstrated during the process. This phenomenon could be prevented by higher temperature (the use of thermophilic bacteria) or by prolonging the exposure time of the biomedia and the sample. In the case of our experiments, Cu was gradually released from chalcopyrite, and the temperature of 30 °C was sufficient. After 28 days from the start of the experiments, there were no significant changes in the transition of the metal to the solution.

The decreasing Cu concentrations in the waste fraction were analyzed by XRF and they are shown together with a blank sample in Figure 8. Prior to the experiments, the sample contained
17,434 mg·kg\(^{-1}\) Cu. The highest concentration loss of metal was determined at a particle size of 71–100 µm, when it decreased to 3,105 mg·kg\(^{-1}\).

Figure 8. Concentration loss of Cu in waste material for individual grain fractions.

3.3.3. Zinc

Figure 9 shows the dependence of the percentage dissolution of Zn on the leaching time for different grain fractions.

Figure 9. Influence of particle size on Zn recovery (pH 2, stirring speed 100 rpm, and 6.67% (w/v) pulp density).
In the case of Zn, the smallest differences in percentage leaching between different particle sizes were demonstrated. Until day 30, the fraction 71–100 µm was evaluated to be the most effective with a final extraction of 97.69% (Zn concentration in the extract 2.634 g-L⁻¹). For grains in the range of 40–71 µm, the resulting percentage extractability was 98.18%. These values do not differ much from the results of the previous fraction. Particles that were in the range of 100–200 µm were leached from 94.66% and the finest grains from 92.91%. The percentage difference between the most effective and the least soluble particle size was 5.27%. Zn was evaluated as the best extractable of all monitored metals, when, for all grains, the final leachability exceeded 90%. On the twenty-first day after the start of the experiments, three grains (<40 µm, 40–71 µm, 100–200 µm) showed almost identical dissolution results. In the study of Conić et al. [1], Zn was 89% soluble. In a study by Olubambi et al. [10], the best results were obtained with a grain size between 75–106 µm (65.2%) and 53–75 µm (62.5%), with a difference of only 2.7%. It was shown that, as in the case of Cu, the worst rated grain size was <40 µm. Makita et al. [51] found that very fine particles can form more complicated solutions, which has a negative effect on the overall metal leaching process.

The waste solid material was determined by XRF and the concentration loss of Zn in the solid fraction is shown together with the blank sample presented in Figure 10. In the original sample, the Zn content was determined to be 38,777 mg·kg⁻¹. The best results were obtained with a particle size of 40–71 µm, when the decrease in metal concentration to 716 mg·kg⁻¹ was analyzed.

![Figure 10](image-url)  
Figure 10. Concentration loss of Zn in waste material for the different grain fractions.

3.3.4. Lead

Figure 11 shows the dependence of the percentage dissolution of Pb on the exposure time of the biomedia to the solid sample. The best results were obtained with a grain size of 71–100 µm, where Pb was extracted from 88.90% (Pb concentration in the extract 223.670 mg·L⁻¹). In the case of the 40–71 µm fraction, Pb was leached from 85.83% and, in the case of the largest particle size, 100–200 µm from 78.84%. An unexpected course of leaching was found for the finest grains <40 µm, which was initially the worst leached fraction, but, from day 22, there was a significant increase in extractability.
until the dissolution of Pb amounting to 86.68%. The percentage difference between the best and least soluble particle size was 10.06%.

![Figure 11. Influence of particle size on Pb recovery (pH 2, stirring speed 100 rpm, and 6.67% (w/v) pulp density).](image)

It has been confirmed in several studies that, in the case of Pb bioleaching, the process may be inhibited due to the formation of precipitates. Under certain conditions, sulfate ions in the medium can react with divalent Pb ions to form lead sulfate, which settles on the surface of the particles in insoluble form. If the grains are significantly coated with this precipitate, the access of bacteria is prevented, and the extractability of the metal is gradually slowed down. Another negative phenomenon is the insufficient supply of oxygen, which is needed for the proper functioning of microorganisms [49]. These phenomena were confirmed in a study conducted by Keeling et al. [52], where the dependence of the formation of precipitates and insufficient dissolution of metals was demonstrated. Due to the effect of these compounds, the action of trivalent iron ions on sulphides in the grains is prevented, thus reducing the solubility of the elements. The trend of the curves of our experiments shows that there was no inhibition of the leachability of the metal into solution due to precipitates.

The best evaluated fraction was the particle range of 71–100 µm, not only in the case of Pb, but also in other monitored metals: Cu and Fe. In the case of Zn, there were slight differences in the leachability for the first two best extractable grain sizes (40–71 µm and 71–100 µm). In a study conducted by Makita et al. [51], it has been shown that the most efficient grain size corresponds to particles around 75 µm. This was also confirmed in a study by Olubambi et al. [10], where the best results were obtained for the 75–106 µm fraction (63.5%). The trend of all the curves was initially sharply increasing, but, from day 13, it gradually decreased due to the formation of a precipitate of lead sulphate. The insoluble compound gradually settled on the surface of the grains, thus preventing further extraction. The 100–200 µm fraction was evaluated as the worst leached. In a study by Nemati et al. [14], the largest particles in the range of 150–180 µm were evaluated as the worst extractable. The cause was insufficient access of microorganisms to metals in the form of sulfides in the leached sample.
The Pb content in the sludge sediment was determined by XRF to be $3,422 \text{ mg kg}^{-1}$. The dependence of the concentration loss of metal during the leaching period of 42 days is shown, together with the blank sample in Figure 12. The highest decrease of the Pb concentration was determined in the fraction 71–100 µm when 391 mg kg$^{-1}$ was analyzed.

![Figure 12. Concentration loss of Pb in waste material for the different grain fractions.](image)

3.4. Influence of pH Value on Metal Extraction Efficiency

The studied pH values were selected on the basis of previous studies [20,23]. Kocadagistan et al. [23] determined the lowest extraction efficiency of *A. ferrooxidans* cells across the entire range of pH values of 1.5–2.5 in a leaching system with a constant pH of 1.5. The study also evaluated the total Fe recovery efficiencies in suspensions with pH values of 2.25 and 2.5 as relatively similar.

An increase in the pH of the leaching solution above the limit value of 2.5 is associated with undesired precipitation of Fe$^{3+}$ ions and the subsequent adhesion of bacterial cells to the formed precipitate. The formation of the jarosite phase, and the associated decrease in the concentration of Fe$^{3+}$ ions, are a significant inhibitory factor in the oxidation of mineral grains [53]. The mentioned mechanism basically falls into the so-called passivation, when during bioleaching a substance is formed, which is concentrated on the surface of solid particles. The formation of an interfacial barrier disrupts the supply of nutrients and dissolved oxygen to microorganisms and, at the same time, prevents the material flow of metals. CuFeS$_2$, a CuS$_{In}$, ammonium jarosite, and polysulfide were detected as the main passivation layers in the leaching of Cu minerals by *A. ferrooxidans* [54].

The sludge sample was leached at pH 1.6, 1.8, 2.0, and 2.2. The yields of heavy metals were calculated from Equations (1)–(4) based on F-AAS analyses. The optimal pH value of bacterial leaching was evaluated on the basis of solubilization curves shown in Figure 13.
Figure 13. Dependence of the percentage of dissolved metal on time at differential pH values for Fe (a), Pb (b), Zn (c), and Cu (d).

Figure 13a shows that the bacterial dissolution efficiency of Fe was the lowest of all metals. When initial pH values were 1.6, 1.8, 2.0, and 2.2, it resulted in Fe recovery efficiencies of 24.10%, 28.65%, 16.54%, and 11.18%; Pb recovery efficiencies of 62.96%, 60.64%, 67.67%, and 72.26%; Zn recovery
efficiencies of 77.16%, 84.93%, 74.38%, and 73.30%; and, Cu recovery efficiencies of 49.76%, 61.36%, 56.62%, and 46.93%, respectively, after seven days (Figure 13). From the absolute values of the differences of all extraction yields of metals, which were related to the same extraction time, the strongest dependence between Fe extraction efficiency and pH value was derived for Fe. The difference between the percentage of Fe in the extract (day 35) at pH 1.8 and 2.2 was 32.49%. Throughout the bacterial leaching, higher amounts of Fe were analyzed in suspensions with pH values of less than 2.0. Removal yields of Fe (day 42) decreased in the following order: 1.8 (85.42%) > 1.6 (79.53%) > 2.0 (60.57%) > 2.2 (52.29%). The decrease in Fe contents in extracts with a pH value ≥ 2.0 could be caused by different types of agitation in extraction devices or by precipitation of dissociated forms of Fe. In 2 L Erlenmeyer flasks, the suspension was agitated in the orbital direction, while, in the bioreactor, the particles were dispersed by vertical flow. Qui et al. determined a significant increase in the formation of iron precipitates in the leaching suspension with a pH value that was higher than 2.0 [22]. In contrast to Cu and Zn, in which the percentage of ions in the solution exceeded 90% after only 21 days (Figure 13c,d) and from an economic point of view it would be possible to terminate the extraction by this time, the Fe concentration in the extract increased up to 35 days (Figure 13a). Because the dependences of total Fe recoveries on time were almost linear, a longer extraction time (35 days) was evaluated to be optimal for Fe.

Figure 13b shows that Pb was the second best biologically extractable metal. In the initial phase of bioleaching (by day 21), most Pb was released into the pH 2.2 solution. When stationary pH values were 1.6, 1.8, 2.0, and 2.2, it resulted in Pb recovery efficiencies of 67.58%, 72.29%, 82.94%, and 91.41%, after 21 days; 79.03%, 79.00%, 96.23%, and 93.65%, after 28 days; and, 87.79%, 92.01%, 96.83%, and 95.49, after 35 days. The achieved yields of Fe show that the rate of dependence of total Pb recovery on pH decreased with increasing bioleaching time. From an economic point of view, a leaching time of 28 days was evaluated to be sufficient at pH 2.0; however, in a leachate of pH 1.8 (optimum for other metals), it would be necessary to extend the process time to 35 days. At relatively analogous leaching parameters, i.e., initial pH 2.0, stirring speed 160 rpm, constant temperature 30 °C bacterial species *A. ferrooxidans*, and suspension density 5% (w/v), Ye et al. [20] extracted 4.13% Pb, 85.45% Fe, and 97.77% Zn after 50 days. By reducing the pulp density to 2% (w/v), a maximum Pb extraction efficiency of 9.08% was achieved [20]. Figure 13 shows that, after 42 days, at a stationary pH value of 2.0, agitation rate of 150 rpm, constant temperature of 30 °C, and pulp density 5.6% (w/v), 97.02% Pb, 60.57% Fe, 94.31% Zn, and 90.34% Cu were dissolved.

Figure 13c shows that Zn extraction due to the integration cooperation of chemical and biological dissolution, was evaluated as the highest. Zn was the only metal for which the removal efficiencies exceeded 70% after seven days and 90% after 42 days over the entire pH range. The dependence between the amount of dissolved metal and the pH value was evaluated as the weakest for Zn, because Zn underwent cellular dissolution easily in the whole range of pH values. After 42 days, 95.85%, 98.73%, 94.31%, and 92.30% of Zn were dissolved at pH 1.6, 1.8, 2.0, and 2.2.

Figure 13d shows that the Cu dissolution curves showed a general trend in which the maximum concentration increase of Cu in the solution, which was limited to day 28, was determined during the first three weeks of bioleaching. During the whole extraction, the highest percentage of Cu was always determined in a solution with a pH value of 1.8. After 42 days, 95.46%, 96.44%, 90.34%, and 81.17% Cu were recovered at pH 1.6, 1.8, 2.2, and 2.2.

Figure 13 shows that, in relation to the oxidative activity of *A. ferrooxidans*, a pH value of 1.8 was evaluated as the optimal pH for the recovery of Fe, Zn, and Cu. The efficiency of bacterial extraction at pH 1.8 (day 42 of leaching) decreased in the following order: Zn (98.73%) > Cu (96.44%) > Pb (93.17%) > Fe (85.42%). The final concentrations of heavy metals in the leachate at pH 1.8 were 9.671 g L⁻¹ Fe, 1.965 g L⁻¹ Zn, 0.948 g L⁻¹ Cu, and 179.559 mg L⁻¹ Pb. For Pb only, the optimum pH was 2.0. When stationary pH values were 1.8 and 2.0, it resulted in Pb recovery efficiencies of 93.17% and 97.02%, after 42 days. The quantitative difference of the stated values was only ~3.85%. Because Pb was the second best biologically extractable metal and high yields were achieved over the entire range of pH values, a value of 1.8 was evaluated as the optimal pH for the bioleaching of polymetallic sludge sediment.
The division of bacterial cells is negatively affected by too low, but also high, pH values [55]. Hydrogen cations are essential for the metabolism of bacteria. In order to oxidize the divalent form of iron to the trivalent form, the microbial cell takes up hydrogen ions from the surrounding solution [23]. The interaction of H⁺ with the bacterium is made possible by the negatively charged functions that are part of its outer cell wall. The cell wall is a carrier of carboxyl, hydroxyl, and phosphate groups, which dissociate in an aqueous medium [18,56]. However, too high concentrations of H⁺ in solution alter the electrical properties of cell membranes. Excess H⁺ ions cause a decrease in the activity of bacterial enzymes, which ultimately causes a reduction in the oxidation of mineral grains [55].

Waste material from biological leaching is characterized by concentration losses of metals in solid samples presented in Figure 14.
Pb concentration [mg∙kg$^{-1}$]

Time [days]

Blank  pH 1.6  pH 1.8  pH 2.0  pH 2.2

Zn concentration [mg∙kg$^{-1}$]

Time [days]

Blank  pH 1.6  pH 1.8  pH 2.0  pH 2.2
Figure 14. Metal concentrations in solid samples taken during bioleaching at differential pH values for Fe (a), Pb (b), Zn (c), and Cu (d).

Table 1 shows that in the original sludge sediment, an average of 203,085 mg·kg⁻¹ Fe, 38,777 mg·kg⁻¹ Zn, 17,434 mg·kg⁻¹ Cu, and 3,422 mg·kg⁻¹ Pb were analyzed by XRF spectroscopy. A blank experiment presented in Figure 4 is shown to compare the contributions of bacterial oxidation to the total amount of metals removed. After 42 days, the waste fraction from chemical extraction contained 189,619 mg·kg⁻¹ Fe, 25,020 mg·kg⁻¹ Zn, 12,990 mg·kg⁻¹ Cu, and 3,163 mg·kg⁻¹ Pb. After 42 days of bioleaching at a stationary pH value of 1.8 (optimum), the concentration of metals in the waste was reduced to 29,710 mg·kg⁻¹ Fe, 507 mg·kg⁻¹ Zn, 627 mg·kg⁻¹ Cu, and 241 mg·kg⁻¹ Pb. From the concentration losses of elements in the sludge sediment, which were correlated to the individual sampling times, the kinetics of Zn dissolution were evaluated as the highest and the dissolution rate of Fe as the lowest.

3.5. Influence of Pulp Density in Pilot Plant Conditions

Before starting the experiments, the bioleaching parameters were selected on the basis of research [42,47,48]. The advantage of the bioreactor was maintaining constant values throughout the leaching process: temperature (30 °C), pH (2), and agitation (150 rpm). Steady pH values were achieved with an automatic dispenser of 5M H₂SO₄ and in the case of exceeding the value with an alkaline solution of 0.4M NaOH. Olubambi et al. [10] evaluated the most effective agitation to be 150 rpm for all three leached metals (Zn, Cu, Pb) from the investigated mixing speeds (100, 150, and 200 rpm). The same mixing speed was set in our experiments. The total grain size of the sample after milling treatment without separation on analytical sieves (<40–200 µm) was used.

3.5.1. Bioleaching of Iron and Zinc

Figure 15 shows the dependence of the percentage leaching of Fe and Zn for different pulp densities. From day 24, more efficient Zn dissolution was achieved at a pulp density of 2.5% (w/v), with Zn extracted from 97.08% and 4.2% (w/v) from 95.03%. Zn was the best leachable metal of all the monitored elements. Fe was leached at a pulp density of 2.5% (w/v) of 58.75% and at a pulp
density of 4.2% (w/v) of 47.08%. For both metals, better results were obtained with a lower pulp density. The percentage difference was 11.67% for Fe and 2.05% for Zn.

Figure 15. Influence of pulp density on Fe and Zn recovery (pH 2, stirring speed 100 rpm, and particle size < 40–200 µm).

Olubambi et al. [35] studied the effect of pulp density of 5%, 10%, 15%, and 20% (w/v) on the bioleaching of sulphide ores. In the case of Zn, it was found that, the lower the weight of sulfide rock used, the more successful the total metal extraction. For pulp density of 5% (w/v), it was 83.1% and, for 20% (w/v), it was only 45.0%. In a study conducted by Acevedo et al. [42], it was found that a higher weight fraction of the solid phase had a negative effect on the total amount of multiplied bacteria (Sulfolobus metallicus) in the medium and it caused a lower content of leached Fe. This was also due to less oxygen access to the grains, which had a negative effect on the proper activity of the bacteria. Ghassa et al. [57] confirmed the negative effect of increasing pulp density on Zn leaching. Tipre et al. [40] found that increasing the weight of the sample in solution resulted in lower bacterial activity. A mixed culture of microorganisms (including A. ferrooxidans) was applied and it was determined that increasing the pulp density of 5% (w/v) reduced the solubility of Zn and Cu by 0.2 g·L⁻¹ per day.

3.5.2. Bioleaching of Copper and Lead

Figure 16 shows the percentage dissolution of Cu and Pb for different pulp densities of the sample. In the case of Cu, the most significant concentration increase in the solution was observed until day 14 from the start of the experiments, when extraction was achieved from 65.38% for pulp density of 2.5% (w/v) and from 62.43% for 4.2% (w/v). From day 12, a pulp density of 2.5% (w/v) was evaluated as more effective, with a final leaching of 79.11%. For a pulp density of 4.2% (w/v), the metal dissolution was determined to be 73.03%. In the case of Pb, the metal was dissolved from 89.35% for pulp density of 2.5% (w/v) and from 80.69% for pulp density of 4.2% (w/v). The percentage difference was 6.08% for Cu and 8.66% for Pb.
In a study conducted by Olubambi et al. [10] for all monitored metals, it was shown that at a higher value of pulp density the percentage dissolution of elements decreased. In the case of Cu, an extraction of 15.9% and 20% (w/v) g of 12.5% were found at a pulp density of 5% (w/v). In the case of Pb, metal dissolution was determined to be 64.8% for 5% (w/v) and 30.9% for 20% (w/v). This was also confirmed in the studies of Makita et al. [51] and Abhilash et al. [58], when a higher percentage of Cu was achieved with a smaller amount of sample. The conclusions of the studies correspond to our experiments, where increasing the value of pulp density resulted in a decrease in metal leaching.

This section may be divided by subheadings. It should provide a concise and precise description of the experimental results, their interpretation, as well as the experimental conclusions that can be drawn.

3.6. Processing of the Pregnant Leach Solution

After converting the metals to the liquid phase, it is economically advantageous to reduce the volume of the acidic solution with metal ions and, thus, increase their concentration. Ion exchangers or organic solvent extractions are used for this. In the case of ion exchangers, the ions from the acidic extract are exchanged for an equivalent proportion of ions in the solid ion exchanger. During the extraction, the metals are transferred to the organic solvent due to their higher affinity, and subsequent re-extraction yields a much more concentrated solution. Once the volume of the liquid phase is reduced, the metals are most often precipitated by cementation or are obtained by electrolysis. In the case of our experiments, their separation by cementation would be recommended, when metals with a more positive electrode potential are displaced from solution by a metal with a more negative potential, which, in turn, passes into solution. Acidic extracts that formed during the bioloeaching process must be subjected to neutralization, e.g., CaOH$^2$ [59–64].
4. Conclusions

In this study, the feasibility of recovering heavy metals from sludge mine sediment by bacterial extraction was investigated and the results can be summarized, as follows:

- There is great potential for recovery of Fe, Zn, Cu, and Pb from mining wastes by biological leaching (hydrometallurgical).
- High extraction efficiencies of heavy metals were achieved by leaching Acidithiobacillus ferrooxidans bacteria under laboratory and pilot conditions.
- Chemical extraction with sulfuric acid was achieved lower metal yields than biological leaching, which integrates the principle of microbial dissolution and acid oxidation.
- Particle size, pH, and pulp density significantly influenced metal recovery efficiencies.
- The optimal parameters for the bioleaching of polymetallic waste were pH 1.8, particle size 71–100 µm, extraction time 35 days, and orbital direction of agitation.
- It was found that increasing the pulp density reduced the amount of regenerated metals.
- Bacterial leaching under optimized conditions could be applicable to waste materials with similar total elemental chemistry.
- The main benefits of the proposed methodology are the reduction of sludge sediment weight, metal recovery, and partial removal of permanently stored mining waste, which is a local environmental risk due to the high concentration of heavy metals.
- The practical disadvantage of the method is the low kinetics of the process.

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References

1. Conić, V.T.; Rajčić Vujasinović, M.M.; Trujić, V.K.; Cvetkovski, V.B. Copper, zinc, and iron bioleaching from polymetallic sulphide concentrate. Trans. Nonferrous Met. Soc. China 2014, 24, 3688–3695, doi:10.1016/S1003-6326(14)63516-0.
2. Akeil, A. Potential bioleaching developments towards commercial reality: Turkish metal mining’s future. Miner. Eng. 2004, 17, 477–480, doi:10.1016/j.mineng.2003.10.016.
3. Guezenne, A.-G.; Bru, K.; Jacob, J.; d’Hugues, P. Co-processing of sulfidic mining wastes and metal-rich post-consumer wastes by biohydrometallurgy. Miner. Eng. 2015, 75, 45–53, doi:10.1016/j.mineng.2014.12.033.
4. Valdès, J.; Pedroso, I.; Quatrini, R.; Dodson, R.J.; Tettelin, H.; Blake, R.; Eisen, J.A.; Holmes, D.S. Acidithiobacillus ferrooxidans metabolism: From genome sequence to industrial applications. BMC Genom. 2008, 9, 597, doi:10.1186/1471-2164-9-597.
5. Mishra, D.; Kim, D.J.; Ralph, D.E.; Ahn, J.G.; Rhee, Y.H. Bioleaching of spent hydro-processing catalyst using acidophilic bacteria and its kinetics aspect. J. Hazard. Mater. 2008, 152, 1082–1091, doi:10.1016/j.jhazmat.2007.07.083.
6. Wang, J.; Zhao, H.-B.; Zhuang, T.; Qin, W.-Q.; Zhu, S.; Qiu, G.-Z. Bioleaching of Pb–Zn–Sn chalcopyrite concentrate in tank bioreactor and microbial community succession analysis. Trans. Nonferrous Met. Soc. China 2013, 23, 3758–3762, doi:10.1016/s1003-6326(13)62926-x.
7. Giaveno, A.; Lavalle, L.; Chiacchiarini, P.; Donati, E. Bioleaching of zinc from low-grade complex sulfide ores in an airlift by isolated Leptospirillum ferrooxidans. Hydrometallurgy 2007, 89, 117–126, doi:10.1016/j.hydromet.2007.07.002.
8. Olubambi, P.A.; Ndlovu, S.; Potgieter, J.H.; Borode, J.O. Effects of ore mineralogy on the microbial leaching of low grade complex sulphide ores. *Hydrometallurgy* 2007, 86, 96–104, doi:10.1016/j.hydromet.2006.10.008.
9. Amiri, F.; Mousavi, S.M.; Yaghmaei, S. Enhancement of bioleaching of a spent Ni/Mo hydrotreating catalyst by Penicillium simplicissimum. *Sep. Purif. Technol.* 2011, 80, 556–576, doi:10.1016/j.seppur.2011.06.012.
10. Olubambi, P.A.; Ndlovu, S.; Potgieter, J.H.; Borode, J.O. Role of ore mineralogy in optimizing conditions for bioleaching low-grade complex sulphide ores. *Trans. Nonferrous Met. Soc. China* 2008, 18, 1234–1246, doi:10.1016/s1003-6326(08)60210-1.
11. Deveci, H.; Akcil, A.; Alp, I. Bioleaching of complex zinc sulphides using mesophilic and thermophilic bacteria: Comparative importance of pH and iron. *Hydrometallurgy* 2004, 73, 293–303, doi:10.1016/j.hydromet.2003.12.001.
12. Chen, S.-Y.; Lin, J.-G. Bioleaching of heavy metals from contaminated sediment by indigenous sulfur-oxidizing bacteria in an air-lift bio reactor: Effects of sulfur concentration. *Water Res.* 2004, 38, 3205–3214, doi:10.1016/j.watres.2004.02.050.
13. Deveci, H. Effect of particle size and shape of solids on the viability of acidophilic bacteria during mixing in stirred tank reactors. *Hydrometallurgy* 2004, 71, 385–396, doi:10.1016/S0304-386X(03)00112-9.
14. Nemati, M.; Lowenadler, J.; Harrison, S.T.L. Particle size effects in bioleaching of pyrite by acidophilic thermophile *Sulfobolus metallicus* (BC). *Appl. Microbiol. Biotechnol.* 2000, 53, 173–179, doi:10.1007/s002530050005.
15. Bosecker, K. Bioleaching: Metal solubilization by microorganisms. *FEMS Microbiol. Rev.* 1997, 20, 591–604, doi:10.1016/S0168-6445(97)00036-3.
16. Amaro, A.M.; Chamorro, D.; Seeger, M.; Arredondo, R.; Peirano, I.; Jerez, C.A. Effect of external pH perturbations on in vivo protein synthesis by the acidophilic bacterium Thiobacillus ferrooxidans. *J. Bacteriol.* 1991, 173, 910–915, doi:10.1128/jb.173.2.910-915.1991.
17. Chen, P.; Yan, L.; Wu, Z.; Xu, R.; Li, S.; Wang, N.; Liang, N.; Li, H. Draft genome sequence of extremely acidophilic bacterium Acidithiobacillus ferrooxidans DLC-5 isolated from acid mine drainage in Northeast China. *Genom. Data* 2015, 6, 267–268, doi:10.1016/j.jgdata.2015.10.018.
18. Zhang, S.; Yan, L.; Xing, W.; Chen, P.; Zhang, Y.; Wang, W. Acidithiobacillus ferrooxidans and its potential application. *Extremophiles* 2018, 22, 563–579, doi:10.1007/s00792-018-1024-9.
19. Mikoda, B.; Potysz, A.; Kniecik, E. Bacterial leaching of critical metal values from Polish copper metallurgical slags using Acidithiobacillus thioxidans. *J. Environ. Manag.* 2019, 236, 436–445, doi:10.1016/j.jenvman.2019.02.032.
20. Ye, M.; Yan, P.; Sun, S.; Han, D.; Xiao, X.; Zheng, L.; Huang, S.; Chen, Y.; Zhuang, S. Bioleaching combined brine leaching of heavy metals from lead-zinc mine tailings: Transformations during the leaching process. *Chemosphere* 2017, 168, 1115–1125, doi:10.1016/j.chemosphere.2016.10.095.
21. Ahmadi, S.; Vafaie Sefidi, M.; Shadman, M.M.; Azimi Dijvejin, Z.; Hosseini, H. The optimization of Cu and Fe bioleaching from converter slag using Acidithiobacillus ferroxidans. *J. Adv. Environ. Health Res.* 2017, 5, doi:10.22102/jaehr.2017.81512.2013.
22. Qiu, M.; Wang, G.; Zhang, W.; Xiong, S. Optimizing conditions for bacterial leaching of copper from discarded mines. *J. Univ. Sci. Technol. Beijing Miner. Metall. Mater.* 2006, 13, 108–111, doi:10.1016/S1005-8850(06)60214-8.
23. Kocadagistan, M.E.; Bayhan, Y.K.; Çakici, A. Fe Extraction from Çayeli Copper Ores by Bioleaching with Eco Friendly Acidithiobacillus ferrooxidans. *J. Chem. Soc. Pak.* 2017, 39, 13.
24. Gerayeli, F.; Ghojavand, F.; Mousavi, S.M.; Yaghmaei, S.; Amiri, F. Screening and optimization of effective parameters in biological extraction of heavy metals from refinery spent catalysts using a thermophilic bacterium. *Sep. Purif. Technol.* 2013, 118, 151–161, doi:10.1016/j.seppur.2013.06.033.
25. Chen, S.-Y.; Lin, J.-G. Enhancement of metal bioleaching from contaminated sediment using silver ion. *J. Hazard. Mater.* 2009, 161, 893–899, doi:10.1016/j.jhazmat.2008.04.035.
26. Schippers, A.; Breuker, A.; Blazeka, A.; Bosecker, K.; Kock, D.; Wright, T.L. The biogeochemistry and microbiology of sulfidic mine waste and bioleaching dumps and heaps, and novel Fe(II)-oxidizing bacteria. *Hydrometallurgy* 2010, 104, 342–350, doi:10.1016/j.hydromet.2010.01.012.
27. Yang, J.; Wang, Q.; Wang, Q.; Wu, T. Heavy metals extraction from municipal solid waste incineration fly ash using adapted metal tolerant Aspergillus niger. *Bioresour. Technol.* 2009, 100, 254–260, doi:10.1016/j.biortech.2008.05.026.
28. Akcil, A.; Ciftci, H.; Deveci, H. Role and contribution of pure and mixed cultures of mesophiles in bioleaching of a pyritic chalcopyrite concentrate. *Miner. Eng.* 2007, 20, 310–318, doi:10.1016/j.mineng.2006.10.016.
29. Úvod | DIAMO, State Enterprise. Available online: https://www.diamo.cz/en (accessed on 14 October 2020).
30. Silverman’, M.P.; Lundgren, D.G. Studies on the chemolithotrophic iron bacterium ferrooxidans i. An improved medium and a harvesting procedure for securing high cell yields. J. Bacteriol. 1959, 77, 642–647.
31. Nguyen, V.K.; Lee, M.H.; Park, H.J.; Lee, J.-U. Bioleaching of arsenic and heavy metals from mine tailings by pure and mixed cultures of Acidithiobacillus spp. J. Ind. Eng. Chem. 2015, 21, 451–458, doi:10.1016/j.jiec.2014.03.004.
32. Rohwerder, T.; Gehrke, T.; Kinzler, K.; Sand, W. Bioleaching review part A: Progress in bioleaching: Fundamentals and mechanisms of bacterial metal sulfide oxidation. Appl. Microbiol. Biotechnol. 2003, 63, 239–248, doi:10.1007/s00253-003-1448-7.
33. Lottermoser, B.G. Mine Wastes: Characterization, Treatment and Environmental Impacts, 3rd ed.; Springer: New York, NY, USA, 2010; ISBN 978-3-642-12418-1.
34. Guler, E. Pressure acid leaching of sphalerite concentrate. Modeling and optimization by response surface methodology. Physicochem. Probl. Miner. Process. 2016, 52, 479–496, doi:10.5277/PPMP160139.
35. Bogdanović, G.; Stanković, V.; Trumić, M.; Antić, D.; Trumić, M. Leaching of low-grade copper ores: A case study for “Kraču Bugaresku-Cementacija” deposits (Eastern Serbia). J. Min. Metall. Min. 2016, 52, 45–56, doi:10.5937/jmma1601045b.
36. Mejia, E.R.; Ospina, J.D.; Márquez, M.A.; Morales, A.L. Bioleaching of Galena (PbS). In Fourier Transform—Materials Analysis; Salih, S.M., Ed.; BoD—Books on Demand: Norderstedt, Germany, 2012; pp. 185–206. ISBN 978-953-51-0594-7.
37. Rezvani Pour, H.; Mostafavi, A.; Shams Pur, T.; Ebadi Pour, G.; Haji Zadeh Omran, A. Removal of sulfur and phosphorus from iron ore concentrate by leaching. Physicochem. Probl. Miner. Process. 2016, 52, 845–854, doi:10.5277/PPMP160226.
38. Fomchenko, N.V.; Muravyov, M.I. Selective leaching of zinc from copper-zinc concentrate. Appl. Biochem. Microbiol. 2017, 53, 73–77, doi:10.1134/S0003683817010197.
39. Ghassa, S.; Noaparast, M.; Shafaei, S.Z.; Abdollahi, H.; Gharabaghi, M.; Boruomand, Z. A study on the zinc sulfide dissolution kinetics with biological and chemical ferric reagents. Hydrometallurgy 2017, 171, 362–373, doi:10.1016/j.hydromet.2017.06.012.
40. Esparza, M.; Cárdenas, J.; Bowien, B.; Jedlicki, E.; Holmes, D.S. Genes and pathways for CO₂ fixation in the obligate, chemolithoautotrophic acidophile, Acidithiobacillus ferrooxidans, Carbon fixation in A. ferrooxidans. BMC Microbiol. 2010, 10, 229, doi:10.1186/1471-2180-10-229.
41. Robertson, S.; Vercuil, A.; van Staden, P.; Craven, P.A. Bacterial heap leaching approach for the treatment of low grade primary copper sulphide ore. In The Third Southern African Conference Base Metals: “Southern Africa’s Response to Changing Global Base Metals Market Dynamics”; S39; Southern African Institute of Mining and Metallurgy: Johannesburg, South Africa, 2005; pp. 471–484. ISBN 978-1-919783-74-1.
42. Acevedo, F.; Gentina, J.C.; Valencia, P. Optimization of pulp density and particle size in the biooxidation of a pyritic gold concentrate by Sulfolobus metallicus. World J. Microbiol. Biotechnol. 2004, 20, 865–869, doi:10.1007/s11274-004-1006-1.
43. Grundwell, F.K. The kinetics of the chemiosmotic proton circuit of the iron-oxidizing bacterium Thiobacillus ferrooxidans. Bioelectrochem. Bioenerg. 1997, 43, 115–122, doi:10.1007/S0302-4598(96)05175-6.
44. Quatrini, R.; Johnson, D.B. Acidithiobacillus ferrooxidans. Trends Microbiol. 2019, 27, 282–283, doi:10.1016/j.tim.2018.11.009.
45. Neale, J.W.; Gericke, M.; Ramcharan, K. The application of bioleaching to base metal sulfides in southern africa: Prospects and opportunities. S. Afr. Inst. Min. Metall. 2011, 367–389, doi:10.13140/2.1.2461.6323.
46. Fontì, V.; Dell’Anno, A.; Beolchini, F. Does bioleaching represent a biotechnological strategy for remediation of contaminated sediments? Sci. Total Environ. 2016, 563–564, 302–319, doi:10.1016/j.scitotenv.2016.04.094.
47. Zhao, K.; Gu, G.; Wang, X.; Yan, W.; Qiu, G. Study on the jarosite mediated by bioleaching of pyrrhotite using Acidithiobacillus ferrooxidans. BioSci. J. 2017, 33, 721–729, doi:10.14399/bj-v33n3-33824.
48. Tipre, D.R.; Dave, S.R. Bioleaching process for Cu–Pb–Zn bulk concentrate at high pulp density. Hydrometallurgy 2004, 75, 37–43, doi:10.1016/j.hydromet.2004.06.002.
49. Olubambi, P.A.; Potgieter, J.H.; Ndlovu, S.; Borode, J.O. Electrochemical studies on interplay of mineralogical variation and particle size on bioleaching low grade complex sulphide ores. Trans. Nonferrous Met. Soc. China 2009, 19, 1312–1325, doi:10.1016/S1003-6326(08)60443-4.
50. Kodali, B.; Rao, M.B.; Narasu, M.L.; Pogaku, R. Effect of biochemical reactions in enhancement of rate of leaching. *Chem. Eng. Sci.* **2004**, *59*, 5069–5073, doi:10.1016/j.ces.2004.09.004.

51. Makita, M.; Esperón, M.; Pereyra, B.; López, A.; Orrantia, E. Reduction of arsenic content in a complex galena concentrate by Acidithiobacillus ferrooxidans. *BMC Biotechnol.* **2004**, *4*, 22, doi:10.1186/1472-6750-4-22.

52. Keeling, S.E.; Palmer, M.-L.; Caracatsanis, F.C.; Johnson, J.A.; Watling, H.R. Leaching of chalcopyrite and sphalerite using bacteria enriched from a spent chalcocite heap. *Miner. Eng.* **2005**, *18*, 1289–1296, doi:10.1016/j.mineng.2005.05.015.

53. Nemati, M.; Harrison, S.T.L. Effects of solid particles on thermophilic bioleaching of sulphide minerals. In *Process Metallurgy*; Amils, R., Ballester, A., Eds.; Biohydrometallurgy and the Environment toward the Mining of the 21 Century—Proceedings of the International Biohydrometallurgy Symposium; Elsevier: Amsterdam, The Netherlands, 1999; Volume 9, pp. 473–482.

54. Dong, Y.; Lin, H.; Xu, X.; Zhang, Y.; Gao, Y.; Zhou, S. Comparative study on the bioleaching, biosorption and passivation of copper sulfide minerals. *Int. Biodeterior. Biodegrad.* **2013**, *84*, 29–34, doi:10.1016/j.ibiod.2013.05.024.

55. Chien, S.-Y.; Lin, J.-G. Bioleaching of heavy metals from sediment: Significance of pH. *Chemosphere* **2001**, *44*, 1093–1102, doi:10.1016/S0045-6535(00)00334-9.

56. Fombal, M.; Gadd, G.M. Biosorption: Current perspectives on concept, definition and application. *Bioresour. Technol.* **2014**, *160*, 3–14, doi:10.1016/j.biortech.2013.12.102.

57. Ghassa, S.; Boroumand, Z.; Abdollahi, H.; Moradian, M.; Akcil, A. Bioleaching of high grade Zn–Pb bearing ore by mixed moderate thermophilic microorganisms. *Sep. Purif. Technol.* **2014**, *136*, 241–249, doi:10.1016/j.seppur.2014.08.029.

58. Abhilash; Mehta, K.D.; Pandey, B.D. Bacterial leaching kinetics for copper dissolution from a lowgrade Indian chalcopyrite ore. *Rem Rev. Esc. Minas* **2013**, *66*, 245–250, doi:10.1590/S0370-44672013000200017.

59. Hong, Y.; Valix, M. Bioleaching of electronic waste using acidophilic sulfur oxidising bacteria. *J. Clean. Prod.* **2014**, *65*, 465–472, doi:10.1016/j.jclepro.2013.08.043.

60. Schnell, H.A. Bioleaching of Copper. In *Biomining: Theory, Microbes and Industrial Processes*; Springer: Berlin, Germany, 1997; pp. 21–43. ISBN 978-3-662-0613-8.

61. Acevedo, F.; Gentina, J.C.; Bustos, S. Bioleaching of minerals—A valid alternative for developing countries. *J. Biotechnol.* **1993**, *31*, 115–123, doi:10.1016/0168-1656(93)90141-9.

62. Zhu, N.; Xiang, Y.; Zhang, T.; Wu, P.; Dang, Z.; Li, P.; Wu, J. Bioleaching of metal concentrates of waste printed circuit boards by mixed culture of acidophilic bacteria. *J. Hazard. Mater.* **2011**, *192*, 614–619, doi:10.1016/j.jhazmat.2011.05.062.

63. Lan, Z.; Hu, Y.; Liu, J.; Wang, J. Solvent extraction of copper and zinc from bioleaching solutions with LIX984 and D2EHPA. *J. Cent. South Univ. Technol.* **2005**, *12*, 45–49.

64. Choi, J.-W.; Song, M.-H.; Bediako, J.K.; Yun, Y.-S. Sequential recovery of gold and copper from bioleached wastewater using ion exchange resins. *Environ. Pollut.* **2020**, *266*, 115167, doi:10.1016/j.envpol.2020.115167.

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