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Microbial communities of acidic post-mining environments and their use in laterite bioreduction

Agnieszka PAWLOWSKA[1], Zygmunt SADOWSKI[2] and Katarzyna WINIARSKA[3]*

Authors' affiliations and addresses:
[1] Department of Process Engineering and Technology of Polymer and Carbon Materials, Wrocław University of Science and Technology, Wybrzeże Wyspiańskiego 27, 50-370 Wrocław, Poland
e-mail: agnieszka.pawlowska@pwr.edu.pl

[2] Department of Process Engineering and Technology of Polymer and Carbon Materials, Wrocław University of Science and Technology, Wybrzeże Wyspiańskiego 27, 50-370 Wrocław, Poland
e-mail: zygmunt.sadowski@pwr.edu.pl

[3]Department of Analytical Chemistry and Chemical Metallurgy, Wrocław University of Science and Technology, Wybrzeże Wyspiańskiego 27, 50-370 Wrocław, Poland
e-mail: katarzyna.winiarska@pwr.edu.pl

***Correspondence:**
Agnieszka Pawlowska, Department of Process Engineering and Technology of Polymer and Carbon Materials, Wrocław University of Science and Technology, Wybrzeże Wyspiańskiego 27, 50-370 Wrocław, Poland
e-mail: agnieszka.pawlowska@pwr.edu.pl

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Abstract
The biogeochemistry of post-mining areas is important because of their impact on the environment and the possibility of remediating such areas. Such a hostile environment is also a source of acidophilic microorganisms that can dissolve minerals that might be applied in the extractive metallurgy of low-grade oxide ores. Therefore, the study used autochthonic microorganisms isolated from water and sediment samples taken from places affected by acid mine drainage. The acidophiles were isolated under aerobic and anaerobic conditions. Genomic analysis revealed low prokaryotic diversity. The genera with a relative abundance greater than 1% were *Acidithiobacillus* (39.8-84.2%), *Ferrimicrobium* (26.4-34.4%), *Acidiphilium* (8.31-28.8%), and *Leptospirillum* (9.6-32.1%). Aerobic bacteria were adapted to reduce iron(III) under a nitrogen atmosphere and the presence of elemental sulfur as an electron donor. It was shown that increasing the amount of sulfur (1-5 g) had little effect on the process kinetics. In anaerobic bottom sediments, *Sulfobacillus* (45.7%) and *Acidisphaera* (32.7%) predominated. Microorganisms showed the fastest reduction activity when glycerol was used as an electron donor. Four weeks of anaerobic-controlled bioreduction of laterites yielded nickel and magnesium at 33.3% and 77.3%, respectively, which could have potential applications in the processing of laterites.

Keywords
anoxic leaching, anaerobic growth, bioreductive leaching, laterite ore, Wiśniówka Quarry

Introduction

According to the literature, many acidophilic microorganisms have been isolated from natural resources such as ores, rocks, minerals, river water, tap water, or acid mine drainage (AMD). The microbial diversity at the AMD sites examined to date includes organisms primarily belonging to Bacteria, Archaea, and, to a lesser extent, Eukarya (mainly fungi and algae) (Méndez-García et al., 2015). The bacteria that are most commonly used in the heap bioleaching process are *Acidithiobacillus*, *Acidiphilium*, and *Leptospirillum* (Bond et al., 2000; Olson et al., 2003; Xingyu et al., 2010). The latter is the most relevant genus within the phylum *Nitrospirae,* which inhabits AMD systems. These chemolithoautotrophic bacteria obtain energy from ferrous iron oxidation and have optimal growth temperatures of 26-30°C (Méndez-García et al., 2015). Furthermore, heterotrophic iron-oxidising *Actinobacteria* are found in acidic environments (Bond et al., 2000), such as *Ferrimicrobium acidiphilum,* which allows the development of autotrophic microorganisms by removing dissolved organic components (lysates and exudates), inhibiting their growth (Bacelar-Nicolau & Johnson, 1999).

In most cases, the isolation of acidophilic microorganisms includes a 9K medium or its modifications, with ferrous salt as the main component. For example, bacteria that oxidise elemental sulphur were isolated from Kazakhstan's Bakyrchik sulphide ore deposits using 9K medium supplemented with $FeSO_4 \cdot 7H_2O$ as a ferrous iron source at 30°C (Kanaev et al., 2015). 9K medium (pH 3-3.5) with different proportions of $FeSO_4 \cdot 7H_2O$ and glucose was also used to isolate *Acidiphilium* sp. DX1-1. The mixotrophic bacterium was obtained from liquid samples (pH 3.0) taken from the Yangtao Wu reservoir belonging to the Dexing copper mine located in Jiangxi province in China. Flasks were incubated at 30°C (200 r/min.) under aerobic conditions (Zhang et al., 2013). Another acidophilic microorganism, *Acidithiobacillus ferrooxidans* FJ1, FJ2, and FJ3, was isolated from water samples of three hot sulfur springs in Ramsar (Iran). 9K medium (pH 2.0) was used and Starkey medium composed of (per 1 litre of deionised water): $(NH_4)_2SO_4$ 1.0 g, $CaCl_2 \cdot 2H_2O$ 0.14 g, KH_2PO_4 3.0 g, $MgCl_2 \cdot 6H_2O$ 0.1 g and 10 g of sulfur (180 rpm, 30°C). For purification, a culture medium supplemented with 1.5% agar was implemented (Jahani et al., 2015). In turn, Zhang et al. (2013) used a 9K basal salt supplemented with ferrous sulfate or elemental sulfur (pH 2.0, 30°C, 200 rpm) and modified 9K–Fe-agarose solid medium (pH between 3.0 and 3.5). The liquid samples for the isolation of *A. ferrooxidans* strain QXS-1 were also collected from an acid mine drainage from the Qixiashan Pb-Zn-Ag mine area in China's Jiangsu province (pH 3.0). In the work of Sugio et al. (2008), *A. ferrooxidans* strain D3-2 with high copper bioleaching activity was isolated from a low-grade sulfide ore dump at a copper mine in Chile. The cultivation medium was as follows: $FeSO_4 \cdot 7H_2O$ (3%), $(NH_4)_2SO_4$ (0.3%), K_2HPO_4 (0.05%), $MgSO_4 \cdot 7H_2O$ (0.05%), KCl (0.01%), $Ca(NO_3)_2$ (0.001%), and pH 2.5.

Microorganisms commonly applied to the oxidative extraction of valuable metals from ores can also be used for the reductive dissolution of minerals. This process was called "biomining in reverse gear" (Johnson, 2012; Johnson & Du Plessis, 2015). It is well known that *A. ferrooxidans* can aerobically respire using both Fe and S as the electron donor (Dockrey et al., 2014; Ohmura et al., 2002). However, reports from the literature show that mesophilic and acidophilic bacteria, such as *Acidithiobacillus ferrivorans* and *A. ferridurans*, can also oxidise sulfur under anaerobic conditions (Das et al., 1992; Gargarello et al., 2010; Kucera et al., 2016; Pronk et al., 1992). The two-stage adaptation method was used by Osorio (2013). At first, *A. ferrooxidans* was grown aerobically in an elemental sulfur medium at pH 2.5. The cultures were then inoculated in an anaerobic medium containing 1% (w/v) of elemental sulfur and 20 mM of ferric salts (pH 2.0, 30°C) and cultivated under a nitrogen atmosphere. *A. ferrooxidans* was also taken as a model bacterium to test the bioreduction process in a putative Mars subsurface habitat (Bauermeister et al., 2014). The results showed that acidophiles could grow on the nutrients provided by Mars' minerals under anaerobic conditions using the redox cycling of iron. In addition to the genus *Acidithiobacillus,* bacteria of the genus *Sulfobacillus* are facultative chemolithotrophs with an optimal mixotrophic growth type in the presence of organic matter. They can also utilise yeast extract and glucose as carbon and energy sources under heterotrophic and a combination of autotrophic and heterotrophic (mixotrophic) conditions (Karavaiko et al., 2001; Zhuravleva et al., 2009). Furthermore, the moderately thermophilic species *S. thermosulfidooxidans* (strain TH1) and *S. acidophilus* (strains ALV, THWX and YTF1) can grow heterotrophically under oxygen-limited conditions when yeast extract and glycerol are present in the culture medium (Bridge & Johnson, 2000). As they are facultative anaerobes capable of ferric iron respiration (Hedrich & Johnson, 2013), they can have potential applications in oxide ore leaching.

The nickel content of laterite deposits is relatively low compared to that of sulfur ores. Therefore, the recovery techniques must be economical and efficient. Biological leaching with organic acids produced by fungi does not yield the expected results due to biomass's strong nickel ion adsorption (Valix et al., 2001), and directed studies on laterite leaching toward the utilisation of chemolithotrophic bacteria (Marrero et al., 2015, 2017). Recently, bioleaching of limonite ore by *Acidithiobacillus* spp. under anaerobic conditions was reported (Johnson et al., 2021). The use of acidophilic microorganisms for the reductive dissolution of oxide ores is still being investigated. Furthermore, the Polish post-mining areas affected by AMD have not been fully examined. Therefore, this work aimed to isolate species belonging to mixed communities in areas affected by acid mine drainage for their possible application in the bioreduction of laterites.

Materials and Methods

Sample origin

Samples used for the isolation of bacteria were taken from Purple Lake, an artificial pond formed in the place of an abandoned pyrite mine in the Rudawy Janowickie, south-west Poland (water sample, PL), Wiśniówka Quarry in south-central Poland (water sample, W) and from the post-mining lakes located in Łęknica, on the territory of the Muscau Bend (water samples MB1, MB2, MB3, solid sample MB4). The temperature of the water samples collected was 10°C, and the pH was 3.2, 3.32, 3.5, 3.0 and 2.47 for PL, W, MB1, MB2, and MB3, respectively. The coordinates of the sampling locations are as follows: PL 50.828012552037954, 15.973872499386953, W 50.93272982772665, 20.68585969520077, MB1 51.53792250878743, 14.77477590716071, MB2 51.531357158445665, 14.767340529662743, MB3 and MB4 51.529004741503, 14.762916080141897.

The bioreduction process was performed using low-grade laterite ore from the Szklary deposit. The elemental analysis identified by XRF (ARL QUANT'X EDXRF analyser, Thermo Fisher Scientific) showed the presence of 30.09 Si, 8.68 Fe, 8.68 Ca, 7.68 Mg, 2.02 P, 1.98 Ni, 0.682 Al, 0.499 Cr, 0.091 Co, 0.096 Mn (m/m%). It contains fine grains of silica minerals (mainly chalcedony), magnesite, and fragments of pimelite, vermiculite, and garnierite. The main ore mineral was magnetite. Small quantities of goethite and hematite were also present (Pawlowska & Sadowski, 2017).

Microorganisms isolation procedure

The isolation procedure was performed separately under aerobic and anaerobic conditions. The bacteria were isolated from the water samples (MB1, MB2, MB3, PL, W) using a 9K medium, pH 2.0, composed of 3 g $(NH_4)_2SO_4$, 0.1 g KCl, 0.5 g KH_2PO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.01 g $Ca(NO_3)_2$, 44.8 g $FeSO_4 \cdot 7H_2O$ per litre of deionised water. The flasks were incubated at 30°C, 150 rpm. The mineral salt solution was sterilised by autoclaving at 121°C for 20 min, and the iron sulfate solution was sterilised by filtration using a 0.22 μm filter. The MB4 bottom sediment, protected from air, was used to isolate anaerobic bacteria. The basal salt solution composed of 0.5 g $MgSO_4 \cdot 7H_2O$, 0.15 g $(NH_4)_2SO_4$, 0.05 g KCl, 0.05 g KH_2PO_4, 0.01 g $Ca(NO_3)$ per litre of deionised water and 10.85 g of $Fe_2(SO_4)_3$ was used, supplemented with glucose (3-10 g/l), glycerol (10 g/l), and yeast extract (5 mg/l). During isolation, pH and redox potential (Eh) were monitored. Microbial oxidation/reduction was followed by ferrous and ferric iron analysis. The titration method was used. 1 ml of sample was mixed with 20 ml of sulphosalicylic acid as an indicator and titrated with a 0.025 M ethylenediaminetetraacetic acid (EDTA) solution. The indicator changed from deep purple to an endpoint that was pale yellow. Then an oxidiser $(NH_4)_2S_2O_8$ was added to the solution. If a colour turned purple, the EDTA solution was titrated again until the pale yellow colour returned. The first titration result allowed the estimation of Fe^{3+} and the second Fe^{2+} ions. The final concentration was calculated using Eq. 1, where V_{EDTA} is the volume in ml of EDTA used in the titration, and C_{Fe} is the ferrous and ferric iron concentration in [g/l].

$$C_{Fe} = V_{EDTA} \cdot 1.396 \quad (1)$$

All samples were subjected to genomic analysis.

Microbiome analysis

Genomic DNA was isolated using the GeneMatrix Environmental DNA/RNA Extraction Kit (EURx, Gdańsk, Poland). The quality of the material obtained was checked by electrophoresis on 1% agarose gel. Measurements were performed using a Qubit 3.0 fluorimeter (Thermo Scientific). A dedicated Qubit High Sensitivity DNA kit reagent was used to assess the amount of matrix supplied. Specific primers were used to amplify the 16SrRNA gene fragment in the samples: 6S_V3-F 357F: 5-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-CCTACGGGNGGCWGCAG-3 and 16S_V4-R 785R: 5-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG -GACTACHVGGGTATCTAATCC-3. The PCR primer sequences from the work of Klindworth et al. (2013) were used. The amplification was carried out in an ABI 9700 thermocycler (Life Technologies) using the thermostable polymerase Kapa HiFi PCR Mix (Roche). The following procedure was used: initial holding at 95°C (2 min) followed by 25 cycles at 95°C for 15 s, 60°C for 10 s, 72°C for the 30s, and final extension for 7 min at 72°C. The process was completed by cooling to a temperature of 10°C. The PCR products were purified using Ampure XP (Beckman Coulter). Cleared PCR products were prepared for sequestration by adding specific adaptors P5 and P7 at the 5' ends of the amplicons. The NGS libraries were sequenced using the MiSeq device (Illumina) and the MiSeq -v3 600 cycle reagent kit. The EzBioCloud service and the 16S-based MTP microbiome taxonomic profiling tools analysed the obtained sequestration data.

Adaptation of aerobic acidophiles to anaerobic conditions

The consortium isolated from MB3 water samples was used for adaptation. Initially, microorganisms were cultured in a 9 K medium under aerobic conditions at a pH of 1.5. After achieving maximum cell growth (approximately three days), the inoculum was transferred to a new medium composed as follows (per 1 litre of

deionised water): $(NH_4)_2SO_4$ 132 mg, $MgSO_4 \cdot 7H2O$ 490 mg, K_2HPO_4 41 mg, $CaCl_2 \cdot 2H_2O$ 9 mg, KCl 52 mg, supplemented with 5 mg/l of yeast extract, glycerol (10 g/l), and 10.85 g iron(III) sulfate. The microbial reduction was monitored by analysing ferrous and ferric iron concentrations. The bacteria were cultivated until the pH medium was close to 1.0.

Reductive bioleaching of laterite ore

Reductive bioleaching was performed using a 0.2 - 0.5 mm fraction of laterite ore. The process was carried out in one litre stirred reactors (30°C), with 10% (w/v) of solid sample placed in a plastic chamber filled with nitrogen to obtain anaerobic conditions. Consortium MB4 and basal salt solution supplemented with glycerol and yeast extract were used. The total volume was 850 ml, including 100 ml of inoculum. The pH of 1.0 was maintained throughout the process using concentrated H_2SO_4. The Ni and Mg content was determined using the ICP-OES method (ICP-OES 5110 Agilent).

Results and discussion

Isolation of aerobic acidophilic microorganisms

Water samples were taken to isolate microorganisms from various mining activity sites. These locations were chosen because the chemical composition of the waters was characterised by the presence of dissolved iron, which resulted from the oxidation of minerals such as pyrite. The isolation of acidophiles was carried out for 20 days, monitoring the concentration of ferrous and ferric iron, pH, and changes in the redox potential. The results are presented in Tab. 1. The isolation parameters of the water samples from the anthropogenic reservoirs of the former lignite mine in Muscau Bend (MB1, MB2) were similar in terms of iron concentration and pH value. The redox potential reflects the degree of oxidation and, therefore, the microbial activity. The higher the oxidation-reduction potential, the more iron(II) ions have been oxidised. The redox potential at the end of the process was higher for MB1 than for MB2 and was 689 and 630 mV, respectively. In the case of MB3, ferrous iron was completely oxidised to ferric iron after two weeks. A high Fe^{2+}/Fe^{3+} ratio was supported by a high value of the redox potential (682 mV). Microbial bio-oxidation activity was also observed in a sample isolated from Wiśniówka (W), where Eh on day 10 was 598 mV, while 356 mV, 354 mV, 439 mV and 484 mV for MB1, MB2, MB3 and PL, respectively. The final pH was comparable for these samples (2.11-2.33) and slightly lower for W (1.98).

Tab. 1 Ferric and ferrous iron concentration, pH and Eh of leachate during isolation

Time [days]	0	5	10	14	20
MB1					
Fe(II) [g/l]	8.93	8.24	7.47	6.84	0.00
Fe(III) [g/l]	0.35	0.42	0.49	0.98	6.28
pH	2.00	2.02	2.04	2.07	2.25
Eh [mV]	340	349	356	391	689
MB2					
Fe(II) [g/l]	8.93	8.52	8.10	7.12	0.00
Fe(III) [g/l]	0.35	0.42	0.42	0.98	6.70
pH	2.00	2.01	2.05	2.1	2.21
Eh [mV]	340	345	354	397	630
MB3					
Fe(II) [g/l]	8.93	6.56	5.30	0.00	0.00
Fe(III) [g/l]	0.42	1.26	1.68	6.70	6.42
pH	2.00	2.02	2.04	2.18	2.11
Eh [mV]	340	389	439	682	678
PL					
Fe(II) [g/l]	8.93	6.70	6.28	0.00	0.00
Fe(III) [g/l]	0.42	0.42	0.98	6.28	5.72
pH	2.00	2.18	2.30	2.33	2.33
Eh [mV]	340	426	484	530	643
W					
Fe(II) [g/l]	6.70	6.28	0.00	0.00	0.00

Fe(III) [g/l]	0.42	0.73	6.28	5.17	4.89
pH	2.01	2.08	2.27	2.12	1.98
Eh [mV]	360	387	598	635	658

Isolation of anaerobic microorganisms

Electron donors, such as organic compounds, play a key role in bioreduction. Isolation of anaerobic bacteria from the bottom sediments of MB4 was carried out for seven weeks. The culture medium was supplemented with glucose or glycerol as electron donors. The process was monitored by measuring the concentrations of ferrous and ferric ions at certain intervals, as shown in Fig. 1. The iron(III) ion bioreduction rate was the fastest when glycerol was used. After four weeks, all ferric iron was reduced. Therefore, a glycerol-based medium was taken for further experiments. This is confirmed by the media used so far in the literature for the isolation and culture of reducing bacteria from acidic environments, containing glycerol and yeast as a secondary source of carbon (Hedrich & Johnson, 2013; Johnson, 2012; Ňancucheo et al., 2016).

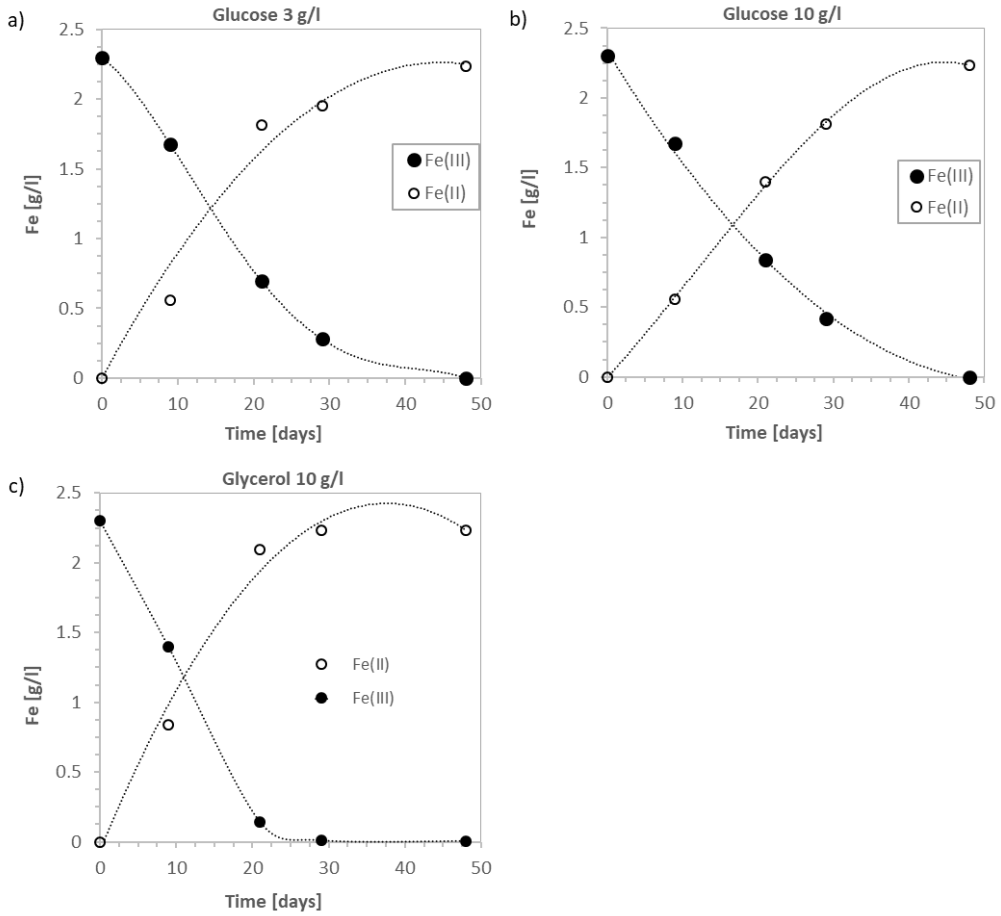

Fig. 1 Changes of ferrous and ferric iron concentrations during bacteria isolation in the presence of various organic substances, (a, b) glucose, (c) glycerol

Analysis of microbial population

According to Tab. 2, for microorganisms isolated from water samples, *Acidithiobacillus* spp. dominated in all samples (39.8% MB1, 53.8% MB2, 50.9 MB3, 70.8 PL and 84.2% W). The species most widely detected was *A. ferrooxidans* (Tab. 3), which commonly occurs in strongly acidic environments and can oxidise iron and sulfur under oxic conditions (Méndez-García et al., 2015). Other microorganisms with relative abundances higher than 1% originated from the genera *Ferrimicrobium* (34.4% MB1, 26.4 MB2), *Acidiphilium* (15.5% MB1, 8.31% MB2, 15.7% MB3, 28.8% PL, 14.0% W), *Leptospirillum* (9.6% MB1, 10.6% MB2, 32.1% MB3).

Tab. 2 Results of the sequential analysis of isolated microorganisms - genus (amount in [%] of total reads)

Taxon name (genus)	MB1	MB2	MB3	MB4	PL	W
Acidithiobacillus	39.8	53.8	50.9	16.7	70.8	84.2
Ferrimicrobium	34.4	26.4	0.761	0.004	0	0
Acidiphilium	15.5	8.31	15.7	0.092	28.8	14.0
Leptospirillum	9.60	10.6	32.1	0.004	0	0.839
Sulfobacillus	0.169	0.39	0	45.7	0	0.0273
Acidisphaera	0.007	0.001	0.002	32.7	0	0
Mesorhizobium	0	0	0	0	0.316	0.374
Edaphobacter	0	0	0.0017	3.44	0	0
Aciditerrimonas	0.0021	0	0	0.792	0	0
Others	0.522	0.499	0.535	0.568	0.084	0.560

Analysis of microbial population composition shows differences between water and sediment samples. MB4, isolated from bottom sediments under anoxic conditions, showed that the main genera are *Sulfobacillus* (45.7% of total reads), followed by *Acidisphaera* (32.7%), *Acidithiobacillus* (16.7%), and *Edaphobacter* (3.44%). *Sulfobacillus* spp. (phylum Firmicutes, order Clostridiales) are common inhabitants of extremely acidic, metal-rich ecological niches. Bacteria of this genus are sulfur-oxidisers and can oxidise or reduce iron. They are also used successfully in the bioleaching or bio-oxidation of sulfide ores and ore concentrates.

Johnson and Hallberg (2003) analysed the distribution of different genera of acidophiles in acid mine waters. In environments where pH was between 2.2 and 3.4, *A. ferrooxidans*, *L. ferrooxidans*, and *Acidiphilium* spp. were present, with a predominance of the first. Similarly to the tested samples, iron oxidisers such as *Ferrimicrobium* spp. were found.

Leptospirillum was not present in the MB4 sediments, as it has a strictly aerobic metabolism. These ferrous iron-oxidising bacteria were shown to dominate in an acidic environment. Therefore, it was surprising that less than 1% of the reads related to these genera were found in the PL and W samples. It follows that factors other than those studied, e.g., the change in the ferrous iron concentration, affect the biodiversity of post-mine reservoirs.

According to the geomicrobiological model of the Rio Tinto sediments (Sánchez-Andrea et al., 2011), the upper parts near the surface are inhabited mainly by *A. ferrooxidans*, *Sulfobacillus*, *Acidiphilium*, *Alicyclobacillus*, and *Ferroplasma*. This is in line with the analysis of MB4 sediment, where similar strains were detected.

Tab. 3 Results of the sequential analysis of isolated microorganisms -species (amount in [%] of total reads)

Taxon name (species)	MB1	MB2	MB3	MB4	PL	W
Acidithiobacillus ferrooxidans	38.9	52.9	50.1	15.3	70.8	84.2
Acidithiobacillus_uc	0.591	0.595	0.729	0.326	0.046	0.0168
Acidithiobacillus ferriphilus	0.206	0.200	0.0051	0.0121	0	0
PAC002241_s	0	0	0	0.956	0	0
Ferrimicrobium acidiphilum	34.4	26.4	0.761	0.004	0	0
Acidiphilium cryptum group	15.4	8.24	15.6	0.003	28.8	13.92
Leptospirillum ferrooxidans	9.39	10.56	32.1	0.004	0.0144	0.839
Sulfobacillus thermotolerans	0.001	0.001	0	45.69	0	0.027
JF766485_s (Sulfobacillus)	0.168	0.389	0	0		
Acidisphaera_uc	0	0	0	0.309	0	0
DQ469200_s	0.007	0.001	0.002	32.36	0	0
Mesorhizobium huakuii group	0	0	0	0	0.316	0.360
Granulicella acidiphila	0	0	0.0017	3.440	0	0
PAC002285_s	0	0	0	0.780	0	0
Others	0.937	0.714	0.7012	0.8159	0.0236	0.6372

Adaptation of aerobic acidophiles to anoxic conditions

The adaptation of isolated aerobic acidophiles to anaerobic conditions was carried out in two stages for the MB3 water microflora. The microorganisms were first grown under aerobic conditions in a 9K medium. After seven days, the medium was changed and supplemented with sulfur, and the conditions became anaerobic by filling the bottle with nitrogen. The anaerobic process was continued for the next 21 days. The process was monitored by analysing ferrous and ferric ion concentrations (Fig. 2).

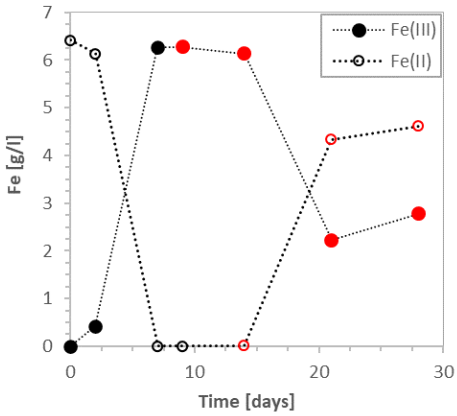

Fig. 2 Changes in Fe^{3+} and Fe^{2+} concentration during the two stages of adaptation of the MB3 consortium (black points, aerobic growth, red points, anaerobic growth)

Consortium MB3 was able to reduce Fe(III) to Fe(II). During the first seven days of incubation, the concentration of ferrous rapidly decreased, and microorganisms fully oxidised it. After one week, when the process was switched to anaerobic, a rapid reduction in Fe(III) to Fe(II) was observed, and at the end of the process, the soluble ferrous iron concentration was 4.5 g/l and ferric 2.8 g/l. A lower oxidation-reduction rate might result from lower bacterial activity after a longer cultivation time or partial precipitation of iron compounds in the form of jarosite.

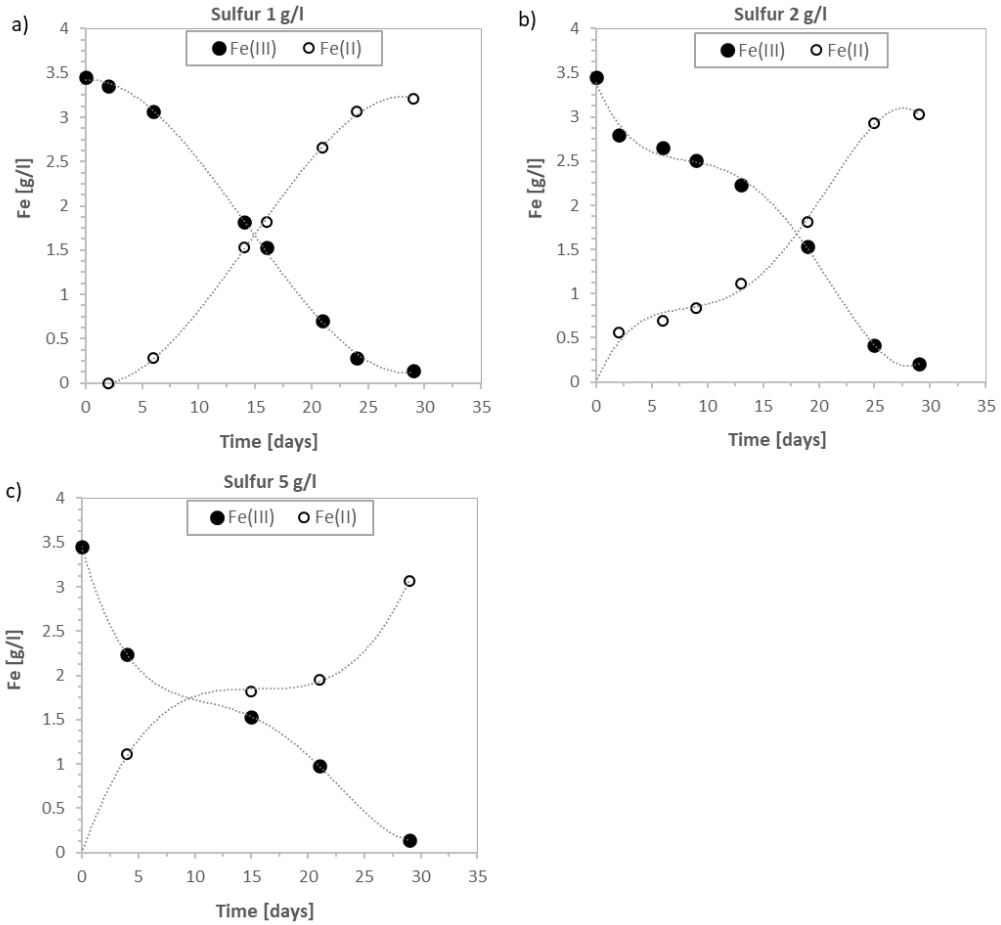

Fig. 3 Changes in ferrous and ferric iron concentrations during adaptation of anaerobic acidophiles to sulfur

Under anoxic conditions, some acidophilic bacteria, including *A. ferrooxidans*, can reduce ferric iron by growing on elemental sulfur as an energy source. Due to its relatively low cost and acid consumption compared to organic electron donors, tests were performed with a systematic increase in sulfur (Fig. 3). It was shown that its increasing amount in the medium had no significant effect on the bioreduction rate. It might suggest that the tested consortium can reduce ferric iron irrespective of the electron donor (Johnson et al., 2021).

Reductive dissolution of laterite ore

Laterites are characterised by a low metal content in the ore, a heterogeneous mineralogical composition, and a high elemental dispersion in the ore material. These factors make them difficult to enrich. A suitable method of processing the nickel deposits is needed to exploit the potential of Poland's nickel deposits. One possible solution is using acidophilic bacteria for the bioreductive dissolution of low-grade oxide ores. Initial reductive dissolution experiments were carried out using sample MB3, as it contained a high percentage of *A. ferrooxidans* (50.1%) and genius *Acidiphilium* (15.6%), which are capable of reducing iron under microaerophilic and anaerobic conditions. However, the MB3 microflora adapted to anaerobic conditions could not effectively dissolve nickel from laterite ore (data not shown). It is probable that, with the presence of mineral material, bacteria eliminated the need to use ferric iron as a terminated electron acceptor as they utilised a fermentative pathway (Johnson & McGinness, 1991). Bioreduction was possible when the MB4 microflora, isolated from the bottom sediments, was used. The ability of MB4 to carry out the reduction process was probably due to differences in the composition of the consortium, predominating species *Sulfobacillus* capable of ferric iron respiration. During the process, pH 1.0 was maintained. Such a low pH enhances acid attack during mineral dissolution and reduces metal precipitation (Johnson et al., 2013). The Mg and Ni ions were measured at specific intervals. The results obtained are depicted in Fig. 4.

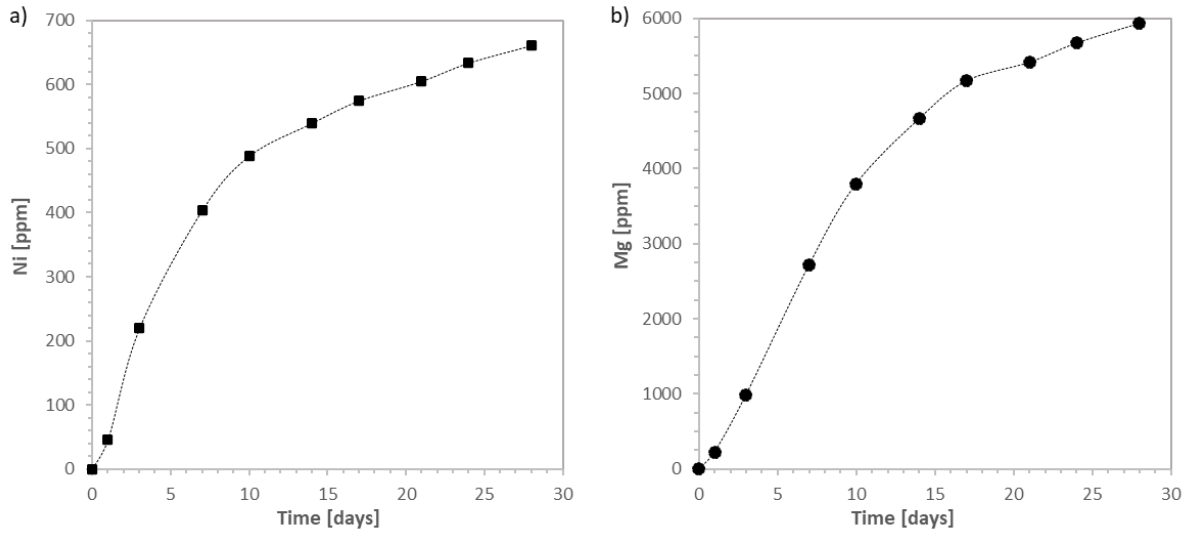

Fig. 4 Concentration of nickel (a) and magnesium (b) versus time as a result of the bioreductive dissolution of laterites

After 28 days, the nickel concentration was 660 ppm and the magnesium 5935 ppm, corresponding to 33.3% and 77.3% for Ni and Mg, respectively, with a solid content of 10% w/v. The recovery of metals depends on many factors, such as the mineral sample (its composition, particle size, pulp density), the type of microorganism and the process conditions (pH, temperature). Microorganisms reduce ferric iron minerals and, therefore, accelerate the dissolution of valuable metals. The phenomenon of reductive bioleaching of nickel is complex, with biological and chemical extraction taking place at the same time. This process can be described as presented in Eq. 2. The resulting electrons are consumed by bacterial cells to reduce ferric irons. Further, ferrous ions assist nickel reduction under acidic conditions, as presented in Eq. 3 and 4. The overall net reaction is represented by Eq. 5.

$$C_3H_8O_3 + 3H_2O \rightarrow 3CO_2 + 14H^+ + 14e^- \quad (2)$$

$$2Fe^{2+} \rightarrow 2Fe^{3+} + 2e^- \quad (3)$$

$$NiO_2 + 4H^+_{(aq)} + 2e^- \rightarrow Ni^{2+}_{(aq)} + 2H_2O \quad (4)$$

$$NiO_2 + 4H^+_{(aq)} + 2Fe^{2+} \rightarrow Ni^{2+}_{(aq)} + 2Fe^{3+} + 2H_2O \quad (5)$$

As the main mineral present in the ore is magnetite, biocatalytic reduction with glycerol as an electron donor can also be described as follows (Eq. 6):

$$7Fe_3O_4 + C_3H_8O_3 + 42H^+ = 3CO_2 + 21Fe^{2+} + 25H_2O \quad (6)$$

Providing external electron donors should not be problematic, as glycerol is highly available since the biofuel industry generates a large amount of crude glycerol as a by-product (Mattam et al., 2013).

Polish laterite ore has previously been subjected to chemical and biogenic leaching. Ochromowicz and Leśniewicz (2018) showed that using sulfuric acid at 20°C and a 1/10 solid-to-liquid ratio, 35% Ni and 20% Mg could be leached from Polish laterites in 6 h. The highest nickel recovery was achieved for 3N sulfuric acid at 90°C (~93%). Pawlowska and Sadowski (2017) used citric acid as a reagent for nickel extraction. The highest metal extraction yielded 67% for 1 N citric acid and 2.5% (w/v) of solid after 50 days of the process. The reported recovery of nickel for *A. niger* broth did not exceed 20% after two weeks and was affected by a decrease in nickel concentration due to biosorption in biomass, suggesting that it was ineffective as a lixiviant. Therefore, from an economic point of view, bacteria have an advantage in the applicability of reductive extraction due to lower biomass production.

Regarding global oxide ores, Johnson et al. (2021) conducted anoxic bioleaching of Shevchenko limonite from Kazakhstan using a consortium of mesophilic bacteria and obtained similar results. Extraction yielded approximately 30% Ni and 70% Mg (temperature 35°C, solid concentration 5% w/v). Hallberg et al. (2011) used a strain such as *A. ferrooxidans* to leach low-grade nickel laterite from a mine in Western Australia in the anaerobic bioreactor with a nickel extraction yield of over 80% and manganese of 100% after 30 days of the process. Reactors with a 2-liter working volume were supplied with 112.5 g of laterite ore.

Conclusions

The diversity of microbes detected in Polish acid mine waters was not fully investigated. The study of the biogeochemistry of AMD is important due to its impact on the environment. A better understanding of microbial diversity can help inhibit the AMD effect by achieving the desired effect of remediation. Acidophilic bacteria were isolated from environmental samples collected from Poland, such as Wiśniówka Quarry, Purple Lake (an old pyrite mine) and post-mine lakes in the eastern part of the Muskau Arch (Western Poland). The gene sequence analysis showed that the acid water samples from Purple Lake and Wiśniówka were *Acidithiobacillus* and *Acidiphilium*. At the same time, for MB1 and MB2, the main genera detected were *Acidithiobacillus, Ferrimicrobium, Acidiphilium*, and *Leptospirillum*. In the case of MB3, the *Ferrimicrobium* content was less than 1% of the total reads. The consortium MB4 isolated from sediment samples under a nitrogen atmosphere was mainly composed of *Sulfobacillus*, *Acidisphaera*, and *Acidithiobacillus*.

Reductive leaching with microorganisms is still a challenge to apply on an industrial scale. Preliminary research has shown that aerobic acidophiles MB3 adapted to anaerobic conditions reduced ferric ions. However, only the MB4 consortium could reductively leach laterites, yielding 33.3% Ni and 77.3% Mg. It provides future opportunities to use new techniques to exploit low-grade nickel deposits, but more research is needed. The possible flow chart of the process is presented in Fig. 5.

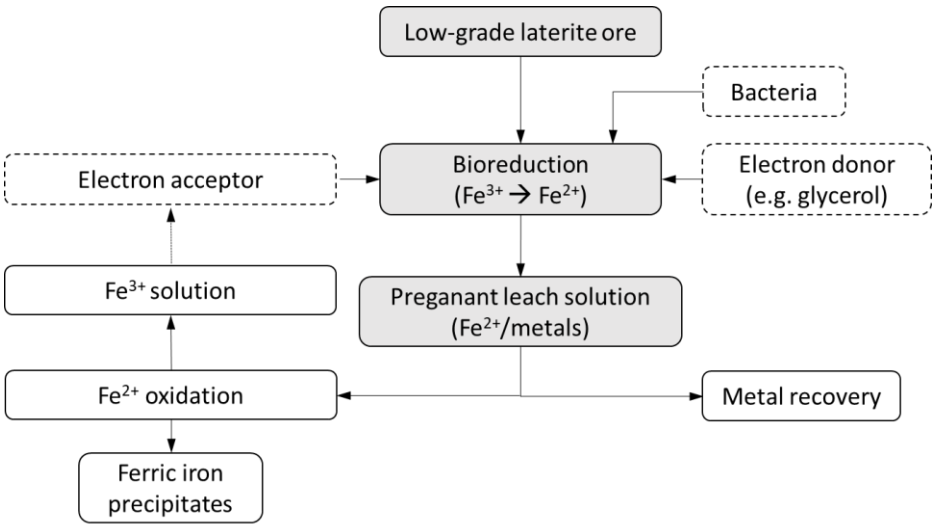

Fig. 5 Conceptual diagram of the laterites bioreduction

Several elements limit the implementation of bioreduction on an industrial scale, such as the process kinetics, its efficiency, and the use of an additional carbon source. However, the use of waste glycerol from biodiesel production or targeted microorganisms gives the potential for future application.

Authors Contributions

Agnieszka Pawlowska: conceptualisation, methodology, investigation, formal analysis, writing—original, draft preparation, writing—review and editing, funding acquisition. Zygmunt Sadowski: methodology, investigation, writing—review and editing, funding acquisition. Katarzyna Winiarska: methodology, investigation, writing—review and editing. All authors read and approved the final manuscript.

References

Bacelar-Nicolau, P., & Johnson, D. B. (1999). Leaching of pyrite by acidophilic heterotrophic iron-oxidizing bacteria in pure and mixed cultures. Applied and Environmental Microbiology, 65(2). https://doi.org/10.1128/aem.65.2.585-590.1999

Bauermeister, A., Rettberg, P., & Flemming, H. C. (2014). Growth of the acidophilic iron-sulfur bacterium Acidithiobacillus ferrooxidans under Mars-like geochemical conditions. Planetary and Space Science, 98. https://doi.org/10.1016/j.pss.2013.09.009

Bond, P. L., Druschel, G. K., & Banfield, J. F. (2000). Comparison of acid mine drainage microbial communities in physically and geochemically distinct ecosystems. Applied and Environmental Microbiology, 66(11). https://doi.org/10.1128/AEM.66.11.4962-4971.2000

Bridge, T., & Johnson, D. B. (2000). Reductive dissolution of ferric iron minerals by Acidiphilium SJH. Geomicrobiology Journal, 17(3). https://doi.org/10.1080/01490450050121161

Das, A., Mishra, A. K., & Roy, P. (1992). Anaerobic growth on elemental sulfur using dissimilar iron reduction by autotrophic Thiobacillus ferrooxidans. FEMS Microbiology Letters, 97(1–2). https://doi.org/10.1016/0378-1097(92)90381-W

Dockrey, J. W., Lindsay, M. B. J., Mayer, K. U., Beckie, R. D., Norlund, K. L. I., Warren, L. A., & Southam, G. (2014). Acidic microenvironments in waste rock characterized by neutral drainage: Bacteria–mineral interactions at sulfide surfaces. Minerals, 4(1). https://doi.org/10.3390/min4010170

Gargarello, R. M., Di Gregorio, D., Huck, H., Fernandez Niello, J., & Curutchet, G. (2010). Reduction of uranium(VI) by Acidithiobacillus thiooxidans and Acidithiobacillus ferrooxidans. Hydrometallurgy, 104(3–4). https://doi.org/10.1016/j.hydromet.2010.03.032

Hallberg, K. B., Grail, B. M., Plessis, C. A. D., & Johnson, D. B. (2011). Reductive dissolution of ferric iron minerals: A new approach for bio-processing nickel laterites. Minerals Engineering, 24(7). https://doi.org/10.1016/j.mineng.2010.09.005

Hedrich, S., & Johnson, D. B. (2013). Aerobic and anaerobic oxidation of hydrogen by acidophilic bacteria. In FEMS Microbiology Letters (Vol. 349, Issue 1). https://doi.org/10.1111/1574-6968.12290

Jahani, S., Fatemi, F., Firoz-E-Zare, M. A., & Zolfaghari, M. R. (2015). Isolation and Characterization of Acidithiobacillus ferrooxidans Strain FJS from Ramsar, Iran. Electronic Journal of Biology, 11(4).

Johnson, D. B. (2012). Reductive dissolution of minerals and selective recovery of metals using acidophilic iron- and sulfate-reducing acidophiles. Hydrometallurgy, 127–128. https://doi.org/10.1016/j.hydromet.2012.07.015

Johnson, D. B., & Du Plessis, C. A. (2015). Biomining in reverse gear: Using bacteria to extract metals from oxidised ores. Minerals Engineering, 75. https://doi.org/10.1016/j.mineng.2014.09.024

Johnson, D. B., Grail, B. M., & Hallberg, K. B. (2013). A new direction for biomining: Extraction of metals by reductive dissolution of oxidized ores. Minerals, 3(1). https://doi.org/10.3390/min3010049

Johnson, D. B., & Hallberg, K. B. (2003). The microbiology of acidic mine waters. In Research in Microbiology (Vol. 154, Issue 7). https://doi.org/10.1016/S0923-2508(03)00114-1

Johnson, D. B., & McGinness, S. (1991). Ferric iron reduction by acidophilic heterotrophic bacteria. Applied and Environmental Microbiology, 57(1). https://doi.org/10.1128/aem.57.1.207-211.1991

Johnson, D. B., Smith, S. L., & Santos, A. L. (2021). Bioleaching of Transition Metals From Limonitic Laterite Deposits and Reassessment of the Multiple Roles of Sulfur-Oxidizing Acidophiles in the Process. Frontiers in Microbiology, 12. https://doi.org/10.3389/fmicb.2021.703177

Kanaev, Z. K., Bulaev, A. G., Kanaev, A. T., & Kondrat'eva, T. F. (2015). Physiological properties of Acidithiobacillus ferrooxidans strains isolated from sulfide ore deposits in Kazakhstan. In Microbiology (Russian Federation) (Vol. 84, Issue 3). https://doi.org/10.1134/S0026261715030091

Karavaiko, G. I., Krasil'nikova, E. N., Tsaplina, I. A., Bogdanova, T. I., & Zakharchuk, L. M. (2001). Growth and carbohydrate metabolism of sulfobacilli. Microbiology, 70(3). https://doi.org/10.1023/A:1010463007138

Klindworth, A., Pruesse, E., Schweer, T., Peplies, J., Quast, C., Horn, M., & Glöckner, F. O. (2013). Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. Nucleic Acids Research, 41(1). https://doi.org/10.1093/nar/gks808

Kucera, J., Pakostova, E., Lochman, J., Janiczek, O., & Mandl, M. (2016). Are there multiple mechanisms of anaerobic sulfur oxidation with ferric iron in Acidithiobacillus ferrooxidans? Research in Microbiology, 167(5). https://doi.org/10.1016/j.resmic.2016.02.004

Marrero, J., Coto, O., Goldmann, S., Graupner, T., & Schippers, A. (2015). Recovery of nickel and cobalt from laterite tailings by reductive dissolution under aerobic conditions using acidithiobacillus species. Environmental Science and Technology, 49(11). https://doi.org/10.1021/acs.est.5b00944

Marrero, J., Coto, O., & Schippers, A. (2017). Anaerobic and aerobic reductive dissolutions of iron-rich nickel laterite overburden by Acidithiobacillus. Hydrometallurgy, 168. https://doi.org/10.1016/j.hydromet.2016.08.012

Mattam, A. J., Clomburg, J. M., Gonzalez, R., & Yazdani, S. S. (2013). Fermentation of glycerol and production of valuable chemical and biofuel molecules. In Biotechnology Letters (Vol. 35, Issue 6). https://doi.org/10.1007/s10529-013-1240-4

Méndez-García, C., Peláez, A. I., Mesa, V., Sánchez, J., Golyshina, O. V., & Ferrer, M. (2015). Microbial diversity and metabolic networks in acid mine drainage habitats. In Frontiers in Microbiology (Vol. 6, Issue MAY). https://doi.org/10.3389/fmicb.2015.00475

Ňancucheo, I., Rowe, O. F., Hedrich, S., & Johnson, D. B. (2016). Solid and liquid media for isolating and cultivating acidophilic and acid-tolerant sulfate-reducing bacteria. FEMS Microbiology Letters, 363(10). https://doi.org/10.1093/femsle/fnw083

Ochromowicz, K., & Leśniewicz, T. (2018). Optimization of laterites leaching by application of sequential design of experiments. Physicochemical Problems of Mineral Processing, 54(2). https://doi.org/10.5277/ppmp1828

Ohmura, N., Sasaki, K., Matsumoto, N., & Saiki, H. (2002). Anaerobic respiration using Fe3+, S0, and H2 in the chemolithoautotrophic bacterium Acidithiobacillus ferrooxidans. Journal of Bacteriology, 184(8). https://doi.org/10.1128/JB.184.8.2081-2087.2002

Olson, G. J., Brierley, J. A., & Brierley, C. L. (2003). Bioleaching review part B: Progress in bioleaching: Applications of microbial processes by the minerals industries. In Applied Microbiology and Biotechnology (Vol. 63, Issue 3). https://doi.org/10.1007/s00253-003-1404-6

Osorio, H., Mangold, S., Denis, Y., Ñancucheo, I., Esparza, M., Johnson, D. B., Bonnefoy, V., Dopson, M., & Holmesa, D. S. (2013). Anaerobic sulfur metabolism coupled to dissimilatory iron reduction in the extremophile Acidithiobacillus ferrooxidans. Applied and Environmental Microbiology, 79(7). https://doi.org/10.1128/AEM.03057-12

Pawlowska, A., & Sadowski, Z. (2017). Influence of chemical and biogenic leaching on surface area and particle size of laterite ore. Physicochemical Problems of Mineral Processing, 53(2). https://doi.org/10.5277/ppmp170216

Pronk, J. T., De Bruyn, J. C., Bos, P., & Kuenen, J. G. (1992). Anaerobic growth of Thiobacillus ferrooxidans. Applied and Environmental Microbiology, 58(7). https://doi.org/10.1128/aem.58.7.2227-2230.1992

Sánchez-Andrea, I., Rodríguez, N., Amils, R., & Sanz, J. L. (2011). Microbial diversity in anaerobic sediments at Río Tinto, a naturally acidic environment with a high heavy metal content. Applied and Environmental Microbiology, 77(17). https://doi.org/10.1128/AEM.00654-11

Sugio, T., Wakabayashi, M., Kanao, T., & Takeuchi, F. (2008). Isolation and characterization of Acidithiobacillus ferrooxidans strain D3-2 active in copper bioleaching from a copper mine in Chile. Bioscience, Biotechnology and Biochemistry, 72(4). https://doi.org/10.1271/bbb.70743

Valix, M., Usai, F., & Malik, R. (2001). Fungal bio-leaching of low grade laterite ores. Minerals Engineering, 14(2). https://doi.org/10.1016/S0892-6875(00)00175-8

Xingyu, L., Bowei, C., Jiankang, W., & Renman, R. (2010). Leptospirillum forms a minor portion of the population in Zijinshan commercial non-aeration copper bioleaching heap identified by 16S rRNA clone libraries and real-time PCR. Hydrometallurgy, 104(3–4). https://doi.org/10.1016/j.hydromet.2010.03.024

Zhang, Y., Yang, Y., Liu, J., & Qiu, G. (2013). Isolation and characterization of acidithiobacillus ferrooxidans strain QXS-1 capable of unusual ferrous iron and sulfur utilization. Hydrometallurgy, 136. https://doi.org/10.1016/j.hydromet.2013.03.005

Zhuravleva, A. E., Ismailov, A. D., & Tsaplina, I. A. (2009). Electron donors at oxidative phosphorylation in bacteria of the genus Sulfobacillus. Microbiology, 78(6). https://doi.org/10.1134/S0026261709060228