Journal of Geoscience and Environment Protection, 2024, 12, 109-121
https://www.scirp.org/journal/gep
ISSN Online: 2327-4344
ISSN Print: 2327-4336

Microbial Community with Potential for Metal Release Isolated from Palca Mine Tailings Pond in Peru

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How to cite this paper: Moreno, C. W. G., Karolski, B., Utimura, S. K., Cabrera, R. C., Torres, J. A. C., Cotrina, D. M. C., de Santis Braga, E., Fermandez, D. S. G., Espinosa, D. C. R., & Sigolo, J. B. (2024). Microbial Community with Potential for Metal Release Isolated from Palca Mine Tailings Pond in Peru. *Journal of Geoscience and Environment Protection, 12,* 109-121.
https://doi.org/10.4236/gep.2024.127007

Received: June 6, 2024
Accepted: July 22, 2024
Published: July 25, 2024

Abstract

The improvement of microbial characterization has increased the comprehension of microbial population and their ability in the microbiological metal dissolution. Bioleaching processes have been expanded to use microorganisms for the recovery of metals from ores and wastes. This study introduces Palca mine tailings pond in Peru which turned into acid mine drainage (AMD). AMD is a source of microbial communities whose microorganisms may support the aqueous extractive metallurgy for metal recovery. Four samples of AMD were collected from different locations and the elemental characterization showed concentrations of metals, such as Cu, Zn, Al, Mn, and Fe. The pH of the samples showed variation between 1.9 - 6.8. Twenty-one microorganisms were isolated and selected according the cell morphology. 16s rRNA gene sequences identified five species of which three belong to the bacterial kingdom and two to the Fungi kingdom. Two of the bacterial species were ferrous ion oxidizing bacteria, identified as *Acidithiobacillus ferriphilus* and *Acidithiobacillus ferridurans*; and the other one a ferric ion reducing bacteria identified as *Acidiphilium acidophilum*. The fungi species identified were *Rhodotorula sinensis* and *Exophiala xenobiotica*, a filamentous fungus isolated for the first time from an AMD.

Keywords

Acidophilic Microorganisms, Acid Mine Drainage—AMD, Bioleaching Process, Metals Recovery, *Acidithiobacillus*

1. Introduction

The mining industry plays a crucial role in global economic development by supplying essential raw materials for various industrial processes. However, mining activities often result in the generation of vast quantities of mine tailings, which pose significant environmental challenges due to their high metal content and acidic nature, leading to the production of acid mine drainage (AMD) (Menzel et al., 2021; Dong et al., 2023). In response to these challenges, biotechnological approaches such as microbial leaching, or bioleaching, have gained attention for their potential to recover metals from low-grade ores and mine wastes under environmentally benign conditions (Srichandan et al., 2019).

Bioleaching involves the use of acidophilic microorganisms capable of oxidizing sulfide minerals and solubilizing metals into solution, offering a sustainable alternative to conventional metallurgical processes (Kaksonen et al., 2018). This microbial-mediated process has been successfully applied in mining operations globally, including prominent sites in Chile and Peru, highlighting its feasibility and economic viability in metal extraction (Naseeri et al., 2019).

The Palca mine in Peru presents a notable case study due to its acidic tailings ponds enriched with various metals. These environments harbor diverse microbial communities adapted to extreme conditions, including acidophilic bacteria and fungi with specialized metabolic pathways for metal oxidation and solubilization (Tayebi-Khorami et al., 2019; Hu et al., 2020). Understanding the composition and metabolic potential of these microbial communities is essential for optimizing bioleaching strategies and enhancing metal recovery efficiency from mining wastes.

Recent studies emphasize the significance of exploring microbial diversity in extreme environments like mine tailings for their biotechnological applications in metal bioleaching and environmental remediation (Chen et al., 2020). These environments not only provide a habitat for microorganisms capable of surviving under harsh conditions but also offer potential solutions to mitigate the environmental impacts associated with mining activities.

In this context, the present study aimed to isolate, identify, and evaluate microbial species from mining tailings in Palca, specifically assessing their potential for biolixiviation applications. Bacteria and acidophilic heterotrophic fungi present in these tailings were investigated, demonstrating the ability to oxidize ferrous ions and produce extracellular polymeric substances (EPS) that facilitate metal solubilization (Bernadelli et al., 2021). These findings contribute to expanding our understanding of microbial ecology in mining environments and underscore the role of microbial consortia in sustainable mining practices and environmental management.

2. Methodology and Study Area

2.1. Mine Tailing Pond Samples

The samples were collected in an abandoned mine located in the district of Palca (Province of Tacna, Peru), at 17˚46'S-17˚79'S latitude and 69˚51'W-69˚87'W longitude in **Figure 1**. The pH (Hinotek—Champ) of each water sample was determined *in situ* and the samples were stored in flasks under refrigeration at 4˚C.

Figure 1. Location of the sampling area: (a) and (b) Geographic location of Peru and the province of Tacna. (c) Sampling area in Palca district (Google EarthTM Mapping Service).

2.2. Isolation of Iron Oxidizing Microorganisms

Aliquots of 2.5 mL was inoculated into 125 ml Erlenmeyer flask containing 25 mL of culture medium for iron-oxidizing, acidophilic microorganisms (Hallberg & Jhonson, 2003; Johnson & Hallberg, 2007) and incubated at 30˚C maintained in lightly agitation (150 rpm) for 15 days. Once grown, 20 µL of the culture was transferred to Petri dishes containing modified solid enrichment medium (Johnson, Macvicar, & Rolfe, 1987, Johnson, 1995; Johnson & Hallberg, 2007; Johnson &

Hallberg, 2008). The plates were incubated at 30˚C for 30 days. The number of microorganisms in the supernatant was monitored by microscopic counting on Neubauer Chamber. The culture of microorganisms was stored at 4˚C.

2.3. Cell Morphology

Morphological analysis of the isolated microorganisms was conducted using a ZEISS® microscope with a magnification of 100x. Cells from 10 ml of culture medium were concentrated by centrifugation at 10,000 g for 5 minutes and subsequently resuspended in 1 ml of fresh culture medium. Following this procedure, Gram staining was carried out to examine the cellular morphology microscopically.

2.4. Identification of Isolated Microorganisms

The isolated microorganisms were subjected to MALDI-TOF® Biotyper mass spectrometry (Singhal et al., 2015). For this, 1 ml of culture was removed for protein extraction using 300 µl of H_2O and 900 µl of ethanol and the mixture was homogenized and centrifuged at 18,000 g for 2 minutes. The pellet was resuspended in 50 µl of formic acid (70%) and 50 µl of acetonitrile. The suspension was homogenized and centrifuged at 18,000 g for 2 min. 1 µl of the supernatant was deposited on the mass spectrometer plate and 1 µl of matrix solution was added (alpha-cyano-4-hydroxynaminic acid in a saturated solution with 50% acetonitrile and 2.5% trifluoroacetic acid).

For the extraction of genomic DNA, the isolated strains were cultivated in 50 ml of isolation medium and the biomass was recovered by centrifugation (10,000 g × 10 min.). Genomic DNA was extracted from the recovered biomass using the DNeasy PowerSoil kit (Qiagen), following the manufacturer's recommendations. The 16S rRNA gene was amplified by PRC, using primers 515F (5'-GTGCCA GCMGCCGCGGTAA-3') and 806R (5'-GGACTACHVG GGTWTCTAAT-3'). Amplification was performed in a total volume of 25 µL containing 12.5 µl of ReadyMix Taq PCR (Sigma); 1 µL of 515 F primer (10 pmol/µL) and primer 806R (10 pmol/µL); 5.5 µL of sterile milli-Q water and 5 µL of DNA (30 to 60 ng/µL). The amplification program consisted of initial denaturation for 3 min. at 94˚C, followed by 30 cycles with denaturation of 1 min. at 94˚C, 1 min. at 53˚C for annealing, 2 min. at 72˚C for extension and a final extension of 7 min. at 72˚C. DNA quality was checked on a 0.8% agarose gel (stained with 0.01% SYBR Green) and quantified with a Nanodrop spectrophotometer. The PCR products were sent for sequencing to the DNA Sequencing Service (SSDNA) of the Human Genome and Stem Cell Research Center at the University of São Paulo. The sequences obtained were compared with 16S rRNA sequences deposited in the public GenBank database.

3. Results

3.1. Characterization of the Mine Tailings Pond

The water samples were collected from the abandoned mine tailings pond in

Chulluncane Hill, Canaura Area, Palca district, province of Tacna, Peru (Figure 2). This mine is located in 3000 meters above sea level. The results of pH and elemental analysis of the samples are shown in Table 1 and Table 2.


(a) **(b)**

Figure 2. Aspects of Abandoned mine in Palca. Sampled points: (a) TAC-P1 and TAC-P2, (b) two points in the acid drainage flow where TAC-P3 (arrow in the top) and TAC-P4 (arrow in the bottom).

Table 1. Coordinates and pH data of the sampled points.

Samples	Coordinates		pH
	Latitude	Longitude	
TAC-P1	17˚46'57.1"	69˚51'49.5"	5.89
TAC-P2	17˚46'57.6"	69˚51'48.6"	6.88
TAC-P3	17˚46'56.9"	69˚51'48.6"	1.94
TAC-P4	17˚47'02.9"	69˚51'51.5"	2.34

Table 2. Elemental composition of the mine tailings pond samples (mg·L^{-1}).

Element	Concentration (mg/L)			
	TAC-P1	TAC-P2	TAC-P3	TA-P4
Al	38.1	10.5	241	117
Cu	193	154	253	479
Zn	8.98	38.8	58.7	111
Fe	42.7	14.3	911	90.6
Mg	57.4	46.8	137	231
Mn	8.56	4.83	49.4	46.6
Na	15.7	12.3	n.d	22.2
Ni	<1.11	<1.11	<1.11	<1.11
Co	<0.572	<0.572	<0.572	<0.572
SO_4	8863.9	5685.5	4125.2	6167.1

n.d. not determined.

The samples showed pH between 1.9 - 6.8 (**Table 1**) and the most abundant metals were Al, Cu, Fe, and Zn. The samples contained considerable amounts of copper (154 - 479 mg·L^{-1}) and zinc (8.98 - 111.0 mg·L^{-1}) showed in **Table 2**. The most interesting metals including Cu were supposedly concentrated within the sulfide fraction. It is presumed that pyrite is present in these tailings pond samples. This allows the chance and availability to use these microbial communities for bioleaching processes, since pyrite supplies as a source of energy substrate for microorganisms (Zheng et al., 2020).

The bacterial colonies were observed on Petri dishes containing different culture media. These colonies were selected based on colony characteristics and cellular morphology. Hendrich et al., (2021). A total of nineteen microbial colonies were selected and grouped into ferrous ion oxidizing or non-ferrous ion-oxidizing isolates.

The oxidation of the ferrous ion by the selected isolates can be observed by the presence of a reddish precipitate (ferric ion) on the colony (**Figure 3**). The nineteen selected bacterial isolates, nine of them initially grew on iron-oxidizing culture medium with pH > 4.0 (moderate acidophiles) and the other ten initially grew on iron-oxidizing culture medium with pH < 2.5 (extreme acidophiles) (Johnson & Mcginness,1991; Zheng et al., 2020; Hedrich & Schippers, 2021).

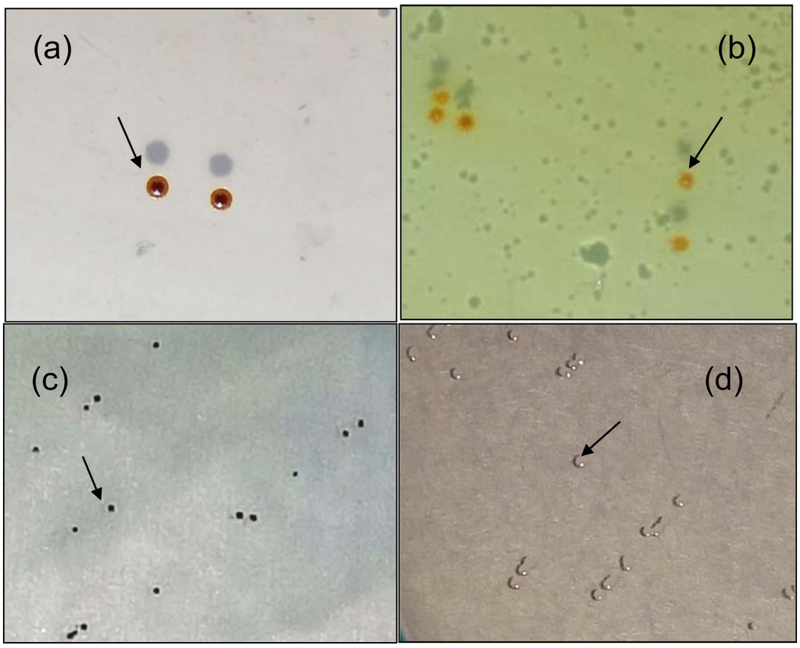

Figure 3. Microbial colonies grown on different culture media (100×): (a) Colonies oxidizing Fe^{2+} isolate in iron-oxidizing culture medium (pH < 2.5), (b) Colonies non-oxidizing Fe^{2+} isolate in iron-oxidizing culture medium (pH > 4.0), (c) Colonies non-oxidizing Fe^{2+} isolate in iron-oxidizing culture medium (pH > 4.0), (d) Colonies non-oxidizing Fe^{2+} isolate in iron-oxidizing culture medium (pH < 2.5).

3.2. Determination of Cell Morphology

The Gram stain method was performed on the isolate microorganisms to determine the presence of two types of cellular morphologies as bacilli and coccobacilli (Barahona et al., 2014). The size of the isolated bacterial cells varied between 2 and 5 µm (**Figure 4**). As shown in **Figure 4**, two types of cell morphologies were seen under the optical microscope (100×) (ZEISS), rod-shaped cells and oval yeast-like cells. The microorganism (in bacillary format) adhered to the surface of the precipitated iron present in the culture medium. The precipitated iron was added in reduced form (Fe^{2+}) and used as an energy source for the microorganism.

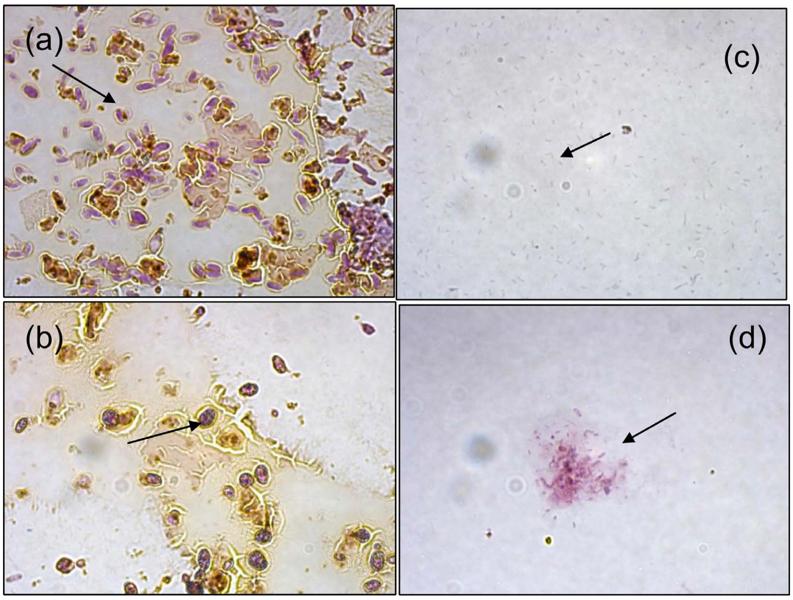

Figure 4. Bacterial images in optical microscopy (100×) for morphological identification. (a) bacillus ≅ 5 µm Gram (+); (b) coccobacillus ≅ 5 µm Gram (+); (c) bacillus ≅ 2.5 µm Gram (−); 100×. (d) bacillus ≅ 2.5 µm Gram (−).

3.3. Molecular Identification of the Isolated Strains

MALDI-TOF was used to identify the species of the isolated bacteria, however, when comparing the protein peaks obtained from the isolated bacteria with the protein peaks of the bacteria in the database, it was not possible to identify either the genus or the species of the bacterial isolated groups. However, two different types of protein profiles are observed, this could indicate the presence of two different bacterial species (or groups). It was not possible to create the protein profiles of the extreme acidophilic isolates. The maximum value of the scores obtained from the profiles when comparing the peaks was 1.5; that is, a value below the value required for the grouping of bacteria within a microbial genus.

3.4. Genomic DNA Extraction and 16S rRNA Gene Amplification

The 16S rRNA gene sequences of the isolated microorganisms were compared with sequences deposited in the GenBank database to determine the identification of the microorganisms (Caporaso et al., 2011). **Table 3** shows the hits of the blast search against the entire nucleotide collection of the GenBank using the obtained sequence as a query.

Table 3. First five hits of the blast search from the GenBank.

Lengh (pb)	(Blast) Best Hits	Access Code	% Identity
390	*Rhodotorula sinensis*	KJ708403.1	98
206	*Exophiala xenobiotica*	FJ225746.1	97
242	*Acidithiobacillus ferriphilus*	NR_147744.1	97
240	*Acidithiobacillus ferridurans*	NR_117036.1	98
233	*Acidiphilium acidophilum*	NR_036837.1	95

3.5. Effects of Temperature and pH on the Growth of Ferrous Ion Oxidizing Bacteria

A. ferridurans and *A. ferriphilus* strains were cultivated in basal salt medium with 25 mM ferrous ion at different temperatures and pH to determine the optimum growth temperature and pH. The specific growth rate of microorganisms was determined during the exponential growth phase by the semi-log graph of ferric ion concentration against time (**Table 4**). The ideal growth pH of the isolated strains is shown in **Table 5** as a function of the best growth rate.

Table 4. Bacterial growth in different temperatures and cultivated in medium with ferrous ion as an energy source.

Bacteria	Growth Temperature (˚C)	Growth Rate (μ)	Time (hours)	R^2
Acidithiobacillus ferrooxidans	15	0.027	25.8	0.996
	20	0.030	22.9	0.997
	25	0.040	17.3	0.996
	30	0.060	11.5	0.999
	35	0.066	10.5	0.999
Acidithiobacillus ferriphilus	15	0.025	29.6	0.996
	20	0.035	19.8	0.996
	25	0.046	15.0	0.998
	30	0.069	10.3	0.998
	35	0.067	10.4	0.999
Acidithiobacillus ferridurans	15	0.016	48.1	0.996
	20	0.032	22.0	0.996
	25	0.039	17.9	0.999
	30	0.061	11.3	0.996
	35	0.059	11.6	0.999

*A. ferroxidans**: was used as a control. A: under evaluation.

Table 5. Ideal pH for growth of isolated bacterial strains.

Bacteria	Growth pH	Growth Speed (µ)	R^2
Acidithiobacillus ferriphilus	1.7	0.047	0.999
	2.0	0.069	0.998
	2.4	0.057	0.999
	2.8	0.035	0.997

4. Discussions

The findings of this study reveal a diverse microbial community in the tailings ponds of the Palca mine in Peru, with significant potential for metal solubilization and recovery. Molecular identification of isolated microorganisms, through 16S rRNA gene sequencing, indicated the presence of species belonging to the genera *Rhodotorula, Exophiala, Acidithiobacillus*, and *Acidiphilium*. A notable discovery was the isolation of the black yeast *Exophiala xenobiotica* from the mine's acid drainage, marking its first report in such extreme environments. *E. xenobiotica* is known for its tolerance to adverse conditions and toxic compounds, commonly found in habitats rich in monoaromatic hydrocarbons and alkanes (Ide-Pérez et al., 2020).

The presence of species such as *Rhodotorula sinensis* and *Exophiala xenobiotica*, both acidophilic heterotrophs, suggests their potential contribution to the biodegradation of toxic organic compounds present in mining tailings. Previous studies have demonstrated that incorporating acid-tolerant heterotrophic microorganisms into bioleaching systems can significantly enhance process efficiency by degrading low molecular weight organic acids and dissolved organic matter, which are toxic to *Acidithiobacillus* species (Wang et al., 2020). Additionally, it has been shown that *Rhodotorula mucilaginosa* has the capability to bind Fe (III) via extracellular polymeric substances (EPS), potentially enhancing bioleaching efficiency by maintaining elevated Fe (III) levels in solution (Wang et al., 2010; Zheng & Li, 2016).

The identification of autotrophic acidophilic species such as *Acidithiobacillus ferriphilus* and *Acidithiobacillus ferridurans* is particularly relevant due to their ability to oxidize ferrous ions and derive energy from inorganic compounds. These bacteria play a crucial role in metal solubilization from sulfide minerals in bioleaching processes (Bernadelli et al., 2021; Dong et al., 2023). *A. ferriphilus* and *A. ferridurans* exhibit optimal growth temperatures between 25˚C and 30˚C and an optimal pH range of 1.7 to 2.4, conditions commonly found in acidic mining tailings, indicating their suitability for bioleaching applications.

The presence of *Acidiphilium acidophilum*, an acidophilic heterotrophic species, suggests its potential role as a growth stimulant for other acidophilic autotrophic bacteria in bioleaching processes, as previously reported (Zhang et al., 2013). Such interactions can enhance the efficiency of bioleaching processes by improving environmental conditions and promoting the activity of key microorganisms involved in metal solubilization.

It is important to note that MALDI-TOF mass spectrometry did not allow conclusive identification of the isolated species, likely due to the lack of specific protein profiles in the database used (Bou et al., 2011). This underscores the need to complement identification methods with molecular techniques such as 16S rRNA gene sequencing to achieve more precise identification of microorganisms in complex environments like mining tailings.

Collectively, the results of this study, along with previous scientific literature, suggest that the microbial community isolated from Palca mine tailings holds promising potential for applications in the bioremediation and bioleaching of mining tailings. The presence of acidophilic heterotrophic and autotrophic species capable of degrading toxic organic compounds, oxidizing ferrous ions, and solubilizing metals from sulfide minerals opens new avenues for the development of more sustainable and environmentally friendly technologies.

However, further research is needed to better understand the metabolic pathways and molecular mechanisms involved in metal solubilization by these microorganisms. Avdalović et al. (2015) emphasize the importance of studying phosphorus solubilization processes by acidophilic microorganisms, which could have applications in the sustainable management of soils and the production of natural fertilizers.

Another relevant aspect is the ability of isolated microorganisms to tolerate high concentrations of metals, a crucial characteristic for the development of efficient microbial consortia in the bioremediation of environments contaminated with heavy metals. In summary, the results of this study highlight the microbial diversity in extreme environments such as mining tailings, suggesting a pivotal role in enhancing bioleaching processes by optimizing the capabilities of microorganisms for metal solubilization and the degradation of toxic organic compounds.

5. Conclusion

The study reports the isolation of acidophilic bacteria and yeasts present in the acid tailings ponds of the Palca mine in Peru. Species such as *Rhodotorula, Exophiala, Acidithiobacillus* and *Acidiphilium* were identified, emphasizing specially the isolation of *Exophiala xenobiotica*, a yeast isolated for the first time from acid mine drainage. These microbial communities, with heterotrophic species such as *Rhodotorula sinensis* and *Exophiala xenobiotica*, as well as autotrophic species such as *Acidithiobacillus ferriphilus* and *Acidithiobacillus ferridurans*, show promising potential for optimizing bioleaching and metal solubilization in acid mine environments. Furthermore, this report highlights the importance of the study of this extreme environment for the detection of microorganisms with metabolic characteristics that can be used specially in bioremediation and bioleaching processes.

Acknowledgements

The authors wish to thank the Fundação de Amparo a Pesquisa do Estado de São Paulo (FAPESP), the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), São Paulo, Brazil for PhD scholarship (141086/2015-7), financial support (870243/1997-7) and the Coordination for Improvement of Graduate Education (CAPES).

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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