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# Phylogenetic analysis of strains isolated from mine tailings and evaluation of their resistance to As and Zn

# Análisis filogenético de cepas aisladas de jales mineros y evaluación de su resistencia a As y Zn

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## Abstract

Heavy metals (HM) pollution is a global concern as mining activity is a major source of those hazardous compounds. In this research, five microorganisms were isolated from mine tailings associated with a high concentration of heavy metals and identified by molecular techniques. Two different samples of mine tailings were the source of culturable strains resistant to HM, native heterotrophic bacterial and fungal microorganisms were isolated and tested for As and Zn resistance (15 mM of $ZnCl_2$ and 20 mM $NaAsO_2$). Results showed a strain compatible with *Bacillus cereus* as the most resistant bacterial strain (7 mM of $NaAsO_2$ and 5 mM of $ZnCl_2$), and the fungus *Exophiala oligosperma* as the most tolerant (20 mM for As and 15 mM for Zn). This performance proposes that the strains isolated from the mine tailings are usable tools for the biotreatment of sites contaminated with HM and that they can also have diverse and innovative applications, economically competent, and friendly with the environment. Some of the isolated strains showed a lower tolerance to HM tested; however, their molecular characterization suggests them as profitable strains for health and industrial purposes, so they should be subjected to a more in-depth analysis.

*Keywords*: *Bacillus cereus*, *Exophiala oligosperma*, *Talaromyces*, heavy metals, phylogeny, biotechnology.

## Resumen

La contaminación por metales pesados (MP) es una preocupación mundial, ya que la actividad minera es una fuente importante de estos compuestos peligrosos. En esta investigación se aislaron cinco microorganismos de jales mineros asociados a una alta concentración de MP los cuales fueron identificados molecularmente. Dos muestras diferentes de jales mineros fueron la fuente de cepas cultivables resistentes a MP, aislándose microorganismos fúngicos y bacterianos heterótrofos y se les determinó su resistencia al As y Zn (15 mM of $ZnCl_2$ y 20 mM $NaAsO_2$). Se encontró una cepa bacteriana compatible con *Bacillus cereus* como la más resistente (7 mM de $NaAsO_2$ y 5 mM de $ZnCl_2$) y el hongo *Exophiala oligosperma* como el más tolerante (20 mM para As y 15 mM para Zn). Por lo anterior, las cepas aisladas de los jales mineros pueden ser utilizados en el tratamiento de sitios contaminados con MP y que también pueden tener aplicaciones diversas e innovadoras, económicamente competentes y amigables con el medio ambiente. Algunas de las cepas aisladas mostraron una tolerancia más baja a los MP; sin embargo, su caracterización molecular las sugiere como rentables para fines sanitarios e industriales, por lo que se sugiere someterlas a un análisis más profundo.

*Palabras clave*: *Bacillus cereus*, *Exophiala oligosperma*, *Talaromyces*, metales pesados, filogenia, biotecnología.

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# 1 Introduction

Heavy metal (HM) pollution of the environment is a common issue worldwide, the different compartments of the Earth (soil, air, and water) are exposed to natural and anthropogenic sources of these pollutants (Wolterbeek and Verburg 2001; Li *et al*., 2013; Khalid *et al*., 2017) that influence the concentration in areas where they are deposited, leading to toxic levels for any taxon (Butler *et al*., 2009; Oves *et al*., 2012).

There are four phyla mostly present in mining environments: *Firmicutes*, *Acidobacteria*, *Proteobacteria*, and *Bacteroidetes*, also fungal and bacterial endophytic microorganisms that can be found in these environments and potentially be used in heavy metals bioremediation (Biró *et al*., 1993; Mendez *et al*., 2008; Martino and Perotto 2010; Pires *et al*., 2017; Thavamani *et al*., 2017; Ji *et al*., 2018; Wu *et al*., 2018). The biochemical pathways and the biomolecules from native microorganisms of heavy metal polluted zones are interesting alternatives for nanoparticles synthesis, remediation techniques, and even waste valorization (Nair *et al*., 2007; Hashim *et al*., 2011; Núñez-Ramírez *et al*., 2011; Saha *et al*., 2015; Yadav *et al*., 2017; Liu *et al*., 2018; Ponce *et al*., 2018; Sun *et al*., 2018). The treatment of tailings to obtain valuable metals as an alternative for wastes reducing and economic viable source of precious metals involves the effective extraction of those remain materials, the interaction of complex matrixes with microbial substances and direct contact can be employed as an effective pretreatment for metal recovery from mine tailings (Ndlovu *et al*., 2017).

Regarding the recent use of microorganisms resistant to heavy metals, a strain of *Acidithiobacillus thiooxidans* was tested in bioleaching of industrial waste, highlighting the ability of the DSM 26636 strain isolated from a Mexican soil with high S content to degrade industrial waste enriched with metal pollutants, in this case steel (Gómez-Ramírez *et al*., 2021). Kinetic and stochiometric parameters of sulfide oxidation process by a bacterial culture isolated from sulfidogenic sludge were studied using in situ pulse respirometry and ex situ microrespirometry approach. In order to achieve the optimization of biotechnological processes such as sulfide oxidation this respirometry study explored a novel approach; however, the results showed some throwbacks in the estimation of the kinetic parameters (Keb-Fonseca *et al*., 2022).

It is a fact that human activity for manufacture and development of technology and goods has disastrous consequences; a study of diesel degradation in the presence of heavy metals was carried out using autochthonous Antarctic strains, due to the presence of this compounds as co-pollutants with petroleum hydrocarbons where the greatest diesel degradation was found in the presence of Hg (Zakaria *et al*., 2020). On the other hand, the use of microorganisms from extreme or hostile conditions, such as high heavy metal content, has demonstrated the efficacy of native marine bacteria, specifically a strain of *Serratia* spp, to produce silver nanoparticles with application in cosmetics, medicine, food, and agriculture fields (De Silva *et al*., 2020).

Heavy metal toxicity may lead to malfunction in the homeostasis of essential elements in the human body, indirect and direct cell damage in form of structural changes in biomolecules and oxidative stress, respectively (Briffa *et al*., 2020). The natural presence of heavy metals in soil and water, such as As and Zn by the mining industry is a major concern, specifically in Mexico, where leaching from old and new tailings mobilizes their toxic compounds towards surrounding areas, increasing the concentration of heavy metals to critical levels, for environments directly related to communities (Espinosa *et al*., 2009; de la Peña-Torres *et al*., 2012; García-Arreola *et al*., 2015). As is a metalloid well known for its ability to affect health and for being a non-essential element; on the other hand, Zn is an essential micronutrient that also becomes toxic at high concentrations (Espinosa *et al*., 2009; Plum *et al*., 2010; Briffa *et al*., 2020). In addition to environmental and health problems, Zn and As are present in the form of refractory minerals with valuable metals. The interaction of microorganisms with the refractory mineral as a pretreatment for greater recovery and less expensive processes stands out the importance and interest for the isolation and study of native species in areas contaminated by heavy metals (Méndez and Armienta 2003; Van Zyl *et al*., 2008; Núñez-Ramírez *et al*., 2011; Abba *et al*., 2020). Mechanisms of microorganisms such as biosorption, expulsion (resistance to heavy metal through expulsion pumps), detoxification or even energy production from metalloids, As for example, are profitable tools in heavy metals attenuation applications (Meléndez-Sánchez *et al*., 2021).

The isolation, characterization, and evaluation of the resistance to heavy metals (Zn and As) of native microorganisms of metal-rich mine tailings was the main objective of this research to propose

the isolated strains as usable for the bioremediation of heavy metals. The characteristic growth curves of each microorganism isolated from two mine tailings in Mexico were constructed, the phylogenetic identification, and tolerance to $ZnCl_2$ and $NaAsO_2$ is also presented in this work. Most of the studies reported for heavy metal resistant strains from mine tailings focusses on autotrophic bacteria; however, organotrophic microorganisms grow faster and in the presence of higher organic content, together with a larger size and higher biomass production, it is what makes the research interesting to explore organotrophic organisms for the remediation of heavy metals.

## 2 Methodology

### *2.1 Reactants*

The following chemicals were purchased from the Solbiosa Company (Guadalajara, Mexico): Casein peptone (batch 2001181), Yeast extract (batch 1907228), Bacteriologic agar (batch 3232890) from Bioxon BD Company, $ZnCl_2$ (batch J0317144) from Meyer Company (Ciudad de Mexico, Mexico); the Tris ultrapure molecular grade (batch 18B1015) was obtained from IBI Scientific (Iowa, USA).

Primers LR5, NS1, 63f, and 1492r were synthesized and provided by Integrated DNA Technologies (IDT, USA). The PCR reagents (Taq DNA polymerase, 5x colorless and 5x green buffers, 25 mM $MgCl_2$, 10 mM dNTP´s mix and nuclease free water) were purchased from Promega Company as well as the Wizard® SV Gel and PCR Clean-Up System and the 1kb DNA ladder.

### *2.2 Mine tailing samples description and characterization*

Three different samples were used in this experimentation (MAMPE19-A, MAMPE19-B, and MAMPE19-C); MAMPE-19-A and MAMPE19-B were mine tailings from a Pb-Zn mining operation in Hidalgo, México (20°43'36", 99°23'57") and sample MAMPE19-C came from an Ag extraction mine in Coahuila de Zaragoza, México (28°21'32.76", 102°34'30.36"). As these samples leave the industrial process under a specific particle size of 200 mesh (0.074 mm), both samples only required to be stored in polyethylene sealed bags at room temperature until use. Samples were characterized by X-Ray Diffraction analysis (XRD) using a D8 Advance (Drucker) diffractometer with a Cu K$\alpha$ ($\lambda$ = 15.41 nm) radiation at 50 kV, 150 mA and with an angle 2$\theta$ of 10-90°. A step of 0.02° with a ramp of 1°/min (Akhgar and Pourghahramani 2016), each sample was homogenized and crushed manually with mortar and pestle, after homogenization 20 g were stored in polyethylene bags and sent to a specialized laboratory at the Universidad Nacional Autónoma de México (UNAM) to perform the analysis. Due to the nature of all samples, high levels of heavy metals were expected, especially As and Zn. An ICP (Inductively coupled plasma) analysis was carried out using ICP - OES (iCAP 7000, Thermo Scientific, USA); the analysis was carried out by the SGS company to determine the concentration As and Zn in the tailings used in the experimentation by digesting the samples with aqua regia.

### *2.3 Isolation and morphological characterization of microorganisms*

#### *2.3.1 Bacteria: Culture medium, growth conditions, and staining*

Luria-Bertani (LB), 9K, and ATCC 125 were selected as the growth media for bacteria; LB is a rich medium with an organic carbon source, whereas 9K and ATCC 125 are "selective medium" for autotrophic-lithotrophic bacteria capable of surviving in extreme acid conditions (Silverman and Lundgren 1959; Shang *et al*., 2015), Table 1 provides the composition for each culture medium used. For enrichment of mine tailings bacteria, each sample was added in 10 w/v to 100 mL of media, incubated at 30°C and 160 rpm for 5 days in rich media, and 21 days for selective media according to daily monitoring of growing by fresh smear at 40X (Pires *et al*., 2017; Núñez Ramírez *et al*., 2018). After enrichment, each culture was serial diluted to $10^{-7}$ and reinoculated on the respective solid media. The colonies after 24 h of incubation at 28°C were selected according to occurrence and shape, selecting only individual isolated colonies. At least 7 cross-streaking steps were carried out to obtain a pure culture. After continuous cross-streak culture, Gram staining was performed (Smith and Hussey 2005) and a complementary Ziehl-Neelsen staining was also used for the characterization of microorganisms.

Table 1. Composition of the culture medium used for the isolation of native microorganisms resistant to heavy metals from the mine tailings (g/L).

| 9K | LB | ATCC 125 |
|---|---|---|
| $(NH_4)_2SO_3 = 3$ | $Casein\ peptone\ =\ 10$ | $(NH_4)_2SO_4\ =\ 0.2$ |
| $K_2HPO_4 = 0.5$ | $Yeast\ extract =\ 5$ | $MgSO_4 \cdot 7H_2O = 0.5$ |
| $MgSO_4 \cdot 7H_2O = 0.5$ | $NaCl\ =\ 10$ | $CaCl_2 = 0.25$ |
| $KCl = 0.1$ | | $KH_2PO_4 = 3$ |
| $Ca(NO_3)_2 = 0.01$ | | $FeSO_4 = 0.005$ |
| $FeSO_4 \cdot 7H_2O = 44.22$ | | |

For the macroscopic description of the colonies, the strains were cross streaked in solid LB medium and incubated at 36°C for 24 h.

#### 2.3.2 *Fungi: Culture medium, growth conditions, and staining*

After incubation for bacteria enrichment, cultures exhibited fungi-like structures, so the enrichment process was again performed to discard cross-contamination in the experimentation. Once the same fungi-like structures prevail in cell enrichment, 1 mL of culture was serially diluted up to $10^{-5}$ and inoculated in Potato Dextrose Agar (PDA) and Yeast extract Peptone Dextrose Agar (YPD), Petri dishes were incubated at 28°C for 5-8 days (Cisneros de la cueva *et al*., 2016). After characteristic individual fungal colonies appeared, these were selected and purified according to macro and microscopic differentiation, after 5 reseeding steps, the mycelium was stained with lactophenol blue and analyzed at 40X. The macroscopic description of the colonies was made in PDA Petri dishes after 14 days of incubation at 28°C.

### 2.4 *Molecular characterization of isolated microorganisms*

#### 2.4.1 *Bacterial DNA extraction*

For DNA extraction, MAMPE19-BBM and MAMPE19-BCG strains were incubated in LB liquid medium, at 160 rpm and 30°C for 24 h, and the total DNA was extracted employing lysozyme to perform the lysis. To the resulting DNA elution, 5 $\mu$L of cell resuspension solution was added and incubated for 1 h at 36°C for DNA purification, the DNA extraction was corroborated on 1% agarose gel stained with ethidium bromide.

#### 2.4.2 *Fungal DNA extraction*

For DNA extraction, MAMPE19-BHV, MAMPE19-BHN, and MAMPE19-CHG fungal strains were incubated in YPD liquid medium at 28°C without mixing for 10 days, the total DNA was extracted according to Harju *et al*., (2004). The DNA elution was purified with 5 $\mu$L of cell resuspension solution for RNA removal in samples, DNA extraction was observed on 1% agarose gel stained with ethidium bromide.

#### 2.4.3 *16S rRNA amplification*

After confirmation of DNA integrity for bacteria on agarose gel, the 16S rRNA gene amplification was carried out following the subsequent reaction mix for a total volume of 25 $\mu$L with 63f and 1492r primers (5'- CAGGCCTAACACATGCAAGTC-3' and 5'-GGTTACCTTGTTACGACTT-3') (Pires *et al*., 2017); 0.5 $\mu$L dNTPs (10 mM), 5 $\mu$L of Gotaq buffer 5x, 1.25 $\mu$L of each primer, 0.125 $\mu$L of DNA polymerase, 5 $\mu$L of $MgCl_2$ (25 mM), 10.375 $\mu$L of nuclease free water and 1.5 $\mu$L of DNA sample. The mix was performed in a container with ice. After temperature gradient for Tm primers election on a thermal cycler (T100-BioRad, Irvene, CA, USA), the PCR conditions were, first step of denaturation of 2 min at 95°C followed by 35 denaturation cycles of 30 s at 95°C, annealing step for 30 s at 54.6°C (the best band pattern obtained in temperature gradient assay) and an extension step of 1.5 min at 72°C, and a final

extension at 72°C for 5 min. Positive PCR amplicons were tested on 1% agarose gel stained with 0.3 $\mu$g/mL of ethidium bromide using a 1kb molecular marker for size comparison and 5x green buffer as a sample loading solution.

#### *2.4.4 ITS Intergenic region amplification*

The fungal DNA amplification was carried out with primers NS1 and LR5 (5'- GTAGTCATATGCTTGTC TC-3' and 5'- TCCTGAGGGAAACTTCG-3') for the target hypervariable intergenic region of fungi, the reaction mix was the same as in Section 2.4.3. The PCR conditions were a first step of 5 min of denaturation, a second step of 10 cycles of denaturation for 30 s at 95°C, annealing at 65°C for 30 s and an extension step of 1 min at 72°C; after this complete cycle, 20 cycles of denaturation at 95°C, a second annealing step for 30 s and extension for 1 min at 72°C, finishing the PCR with a 7 min final extension at 72°C, the amplicons were analyzed on 1% agarose gel.

#### *2.4.5 Phylogenetic analysis*

After PCR product purification according to PCR purification kit supplier instructions, the amplicons were sent to perform bidirectional Sanger sequencing in the Instituto de Biotecnología - UNAM (Cuernavaca, Morelos, Mexico). The sequences were cured in Bioedit (2020) and the consensus sequence employed to select homologous sequences displayed in the BLAST tool from National Center for Biotechnology Information (NCBI 2021), the phylogenetic trees were built up in MEGA X by the Neighbor Joining method (Hall 2013; Newman *et al*., 2016).

### *2.5 Growth curves and preservation of cultures*

The characteristic curve of the isolated strains, MAMPE19-BCG and MAMPE19-BBM, for a 24 h period vs optical density (OD) and cells/mL plot was constructed, determined with analysis in spectrophotometer G10S (UV-Vis BIO Thermo Fisher Scientific) and plate count, respectively; samples of 1 mL of media was used to evaluate growth as a function of the OD change using sterile media as a control.

Isolated bacteria (MAMPE19-BCG and MAMPE19-BBM) were reproduced in 500 mL batch reactors at the already established conditions (200 mL of culture medium, 30°C, and 160 rpm) and harvested at 4 h, corresponding to the exponential phase. All batch reactors contents were transferred to 1.5 mL microcentrifuge tubes and ultracentrifuged (Microlite microcentrifuge Thermo Electron Corporation) for 5 min and 13,500 rpm to concentrate cells 4 times to reduce the 200 mL in 64 tubes. Samples of concentrated cells were preserved in 2 mL cryogenic tubes (Nalgene Company, Rochester, NY) adding 800 $\mu$L of concentrated cells and 70 $\mu$L of sterile dimethyl sulfoxide (DMSO) as a cryoprotective agent to preserve cells and perform all resistance tests from the same batch and eliminate possible errors. When needed, preserved cells are thawed at room temperature and rinsed 3 times with sterile media solution before inoculating culture in fresh medium to reactivate cells for resistance tests (Kim *et al*., 2002).

Every experiment started from reactivated culture (cryogenic samples) and a 12 h pre-inoculum with theoretical initial OD adjusted to 0.01 as shown in equation 1.

$$OD_1 v_1 = OD_2 v_2 \tag{1}$$

Where $OD_1$ was the optical density of pre-inoculum and $OD_2$ the target initial density, $v_1$ was the volume needed to adjust the OD of the new culture. Once known the characteristic curve of each strain, doubling time ($T_d$) was calculated in h as shown in equation 2 (Singh *et al*., 2008), where $\mu$ was the specific growth rate (1/h) obtained from the tendency line of the exponential phase of the growth curve.

$$T_d = \frac{\ln 2}{\mu} \tag{2}$$

For the fungal strains, OD measurement did not apply because the growth of the isolated strains was not homogeneous in the liquid medium used in the experiments, MAMPE19-CHG, MAMPE19-BHV, and MAMPE19-BHN, Petri dishes with PDA with each strain were incubated for 15 days at 28°C, mycelium was then collected cutting the entire colony with a scalpel and resuspended in 100 mL of sterile distilled water for conidia elution, each strain eluted was tested by triplicate for growth. The characteristic curves were constructed using 15-day PDA Petri dishes, with an initial inoculum of 20 $\mu$L of conidia suspension and incubated at 28°C, the radial growth was measured every 24 h (Nam *et al*., 2019; Rose and Devi, 2018), the number of cells was estimated as colony forming units (CFU)/mL by direct count on PDA medium. The mycelium was collected from

sacrificed Petri dishes and resuspended in 100 mL sterile distilled water, after ten minutes of elution homogenization, serial dilutions were conducted up to $10^{-5}$, and 5 $\mu$L of each dilution were transferred to PDA Petri plates (by triplicate) to be incubated at 28°C for 6-8 days (Cisneros de la cueva *et al*. 2016), CFU/mL were calculated as shown in equation 3.

$$\frac{CFU}{mL} = \frac{\text{(Average number of colonies on plate)} * \left(\frac{1}{Dilution}\right)}{\text{mL inoculum}} \quad (3)$$

## 2.6 As and Zn resistance tests

### 2.6.1 Screening resistance tests

To determine the concentration range of heavy metals to evaluate in the resistance test a screening preliminary test was conducted. Petri dishes containing concentrations from 0.5 to 50 mM of sodium arsenite and from 0.5 to 20 mM of zinc chloride were inoculated with each strain for the range adjustment for bacteria and fungi organisms.

### 2.6.2 Resistance tests for bacterial strains

To evaluate the tolerance of isolated strains, LB liquid and solid media was supplemented with different Zn as $ZnCl_2$ and As as $NaAsO_2$ millimolar concentrations. In solid media, the growth of strains was estimated as CFU/mL and in liquid media by the change in optical density ($OD_{600}$), each experiment was carried out by triplicate. To construct curves with liquid medium, 96-well sterile microplates (Corning 3361) were used with a working volume of 200 $\mu$L per well, each sample was diluted to 1 mL with distilled water and change in OD monitored with time every h using the spectrophotometric method. The specific growth rate for the exponential phase was calculated using equation 2 by fitting an exponential trend line to the data. On the solid supplemented media, a 12 h pre-inoculum was inoculated by triplicate (for reproducibility) in serial dilutions up to $10^{-7}$, the colonies were counted and compared with control of not supplemented medium, after incubation at 36°C the number of viable cells was calculated according to equation 3. Table 2 resumes the concentration range used for the resistance tests.

### 2.6.3 Resistance tests for fungal strains

To evaluate the tolerance of isolated fungal strains, PDA medium was supplemented with different millimolar concentrations, of Zn ($ZnCl_2$) and As ($NaAsO_2$), the growth was measured as diameter in mm using a vernier caliper and CFU/mL, each experiment was carried out by triplicate for reproducibility. A concentrated conidia elution of known concentration was used as inoculum for each test, this was inoculated in serial dilutions up to $10^{-5}$ in supplemented Petri dishes with $NaAsO_2$, $ZnCl_2$, and no supplemented LB as a control. A tolerance index (TI) was calculated as shown in equation 4, where stressed mycelium refers to fungal growth in supplemented plates (Rose and Devi 2018; Nam *et al*., 2019); finally, a count of CFU was performed from stock conidia solution at different As and Zn concentration. Table 2 resumes the concentration range used for the resistance tests.

$$TI = \frac{\text{Radial growth of stressed mycelium}}{\text{Radial growht of unstressed mycelium}} \quad (4)$$

## 2.7 Statistical analysis

Minitab 18.1 software was used to conduct the statistical analysis of results with a one-way analysis of variance (ANOVA) and Dunnett comparison between controls and supplemented media to test for significant differences at 95% confidence interval (CI).

Table 2. Concentration range of As and Zn used in resistance tests.

| Heavy Metal-Metalloid | Bacteria | | Fungi | |
|---|---|---|---|---|
| | mmol/L | mg/L | mmol/L | mg/L |
| Zn | 1 - 2 | 136.32 - 272.64 | 1-15 | 136.32 - 2,044.00 |
| | 1 - 7 | 136.32 - 954.24 | | |
| As | 1 - 2 | 129.91 - 259.82 | 1-20 | 129.91 - 2,598.20 |
| | 1 - 10 | 129.91 - 1,299.10 | | |

Table 3. X-Ray Diffraction (XRD) mineralogical analysis of the tailing samples subjected to evaluation.

| Sample | Major (<30% wt) | Minor (2% -10% wt) | Trace (<2% wt) |
|---|---|---|---|
| MAMPE19-A | Calcium sulfate dihydrate | Potassium sulfate and iron hexahydrate | Silicates |
| MAMPE19-B | Silicon oxide | Aluminum potassium silicate<br>Calcite<br>Iron and zinc sulfate<br>Sodium and Calcium silicates | Zinc and silver minerals |
| MAMPE19-C | Calcite | Quartz, hematite, goethite, loseyite, kaolunite, nepheline | Plumbojarosite, dolomite, cerussite, rhodochrosite, oligonite, ilmenite, pyrolusite, fluorite, quenselite, mimetite, blixite, hemimorphite |

Table 4. ICP results for sample MAMPE19-C and MAMPE19-B from Coahuila de Zaragoza and Hidalgo mine tailings, respectively.

| Analyte | | Ag | As | Cd | Cr | Mn | Pb | Zn | Sb | Cu |
|---|---|---|---|---|---|---|---|---|---|---|
| mg/Kg (ppm) | B | 3 | 6,258 | 10 | 6 | 228 | 665 | 2,510 | 277 | 151 |
| | C | 2 | 2,192 | 103 | <1 | >10,000 | >10,000 | >10,000 | 281 | 390 |

# 3 Results and discussion

## *3.1 Characterization of mine tailings*

The results after XRD assays for samples MAMPE19-A, MAMPE19-B, and MAMPE19-C are summarized in Table 3, the three samples exhibits a polycrystallinity and contain $SiO_2$ despite their different origin as silicon dioxide is the most common mineral found in nature; sample A showed less mineral diversity in XDR analysis, and for B the more diverse, the presence of minerals with Zn as Fe-Zn sulphate was an important starting point to select the samples to conduct further experimentation, due to the interest in this study for isolation of As and Zn resistant strains. Even though sample C did not show crystal structures of As and Zn it was not discarded, a more sensitive analysis showed concentrations >2,000 ppm for As and >10,000 ppm for Zn, as presented (sample B included) in Table 4.

## *3.2 Macro and microscopic morphological characterization*

### *3.2.1 Bacteria*

From sample MAMPE19-B two strains were isolated in medium LB, both strains were gram-positive rod-shaped cells, showing in some staining the presence of spores (MAMPE19B-BCG and MAMPE19-BBM). Both strains were considered as no acid-alcohol resistant after Ziehl Neelsen staining. MAMPE19-BBM colonies were circular between 1 and 2 mm in diameter, with convex elevation and whitish yellow color, while MAMPE19-BGM were larger irregular colonies between 2 and 4 mm of diameter with raised elevation and whitish yellow color (Figure 1). The morphology of two strains after isolation was constant between every re-inoculation cycle in solid media. The *Firmicutes* phylotype is commonly found in mine tailings from Pb-Zn operations, around 37.5% of the total diversity belonging to this phylotype under slightly acid pH (Mendez *et al*. 2008) and ≥ 90% of the population of strains isolated from As and other heavy metal polluted areas with mine tailing were found as gram-positive (Ponce *et al*., 2018).

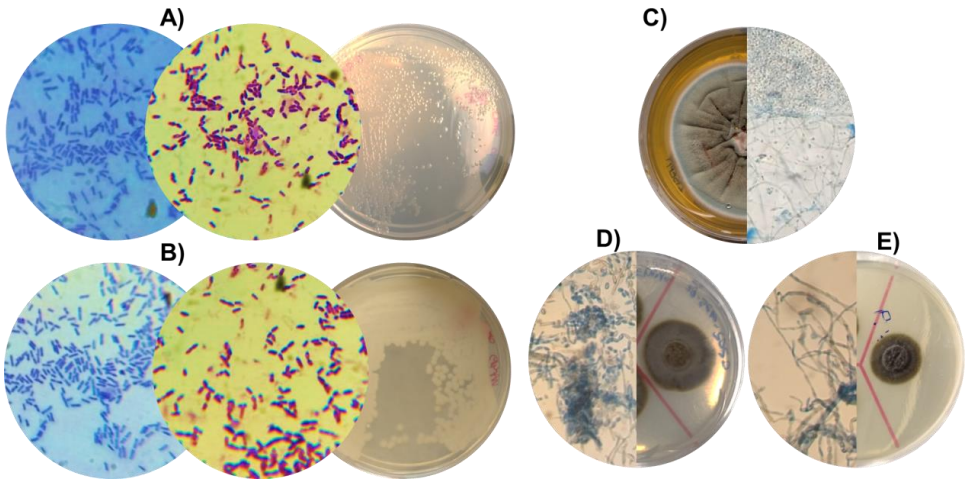


Fig. 1. Macro and microscopic characterization of isolated strains from mine tailings MAMPE19-B and MAMPE19-C. A) Ziehl Neelsen, Gram staining, and solid media growth for MAMPE19-BBM. B) Ziehl Neelsen, Gram staining, and solid media growth for MAMPE19-BCG. C) Lactophenol staining and solid media growth for MAMPE19-CHG. D) Lactophenol staining and solid media growth for MAMPE19-BHV. D) Lactophenol staining and solid media growth for MAMPE19-BHN.

#### *3.2.2 Fungi*

Strain MAMPE19-CHG was isolated from sample MAMPE19-C, and strains MAMPE19-BHN and MAMPE19-BHV were isolated from sample MAMPE19-B. Morphology of colonies from strain -CHG was green colour with an orange reverse, the colony was flat with a woolly appearance, in the lactophenol blue staining the presence of non-septate hyphae and ascospores were observed (Pitt 2014). Both strains BHV and BHN were dark-grey color with aerial mycelium with no pigmentation at the reverse, general velvety appearance (Bossler *et al*., 2003) and under 40X objective, there was pseudo septate hyphae presence (Figure 1). Since the morphological identification of fungal strains may be subjective, this approach presents only a complement for DNA analysis performed in molecular characterization.

### *3.3 Molecular characterization*

Results for all five strain sequences were aligned, and individual trees were constructed to know the identity of each strain: two bacterial and three fungal microorganisms as described below.

#### *3.3.1 Bacteria*

Figure 2A shows the phylogenetic tree for strain MAMPE19-BBM, which has high (97.38%) similarity with *Lysinibacillus sphaericus*, a strain belonging to *Firmicutes* phylotype, one of the most common bacteria phyla found in the rhizosphere. The genus *Lysinibacillus* is a symbiotic bacterium of plants and animals, a plant-growth-promoting (PGP) genus as well, and in many cases found in heavy metal polluted environments (Hashim *et al*., 2011; Pires *et al*., 2017; Aguilar *et al*., 2020); the bootstrap coefficients of each branch with high values represent the reproducibility in rebuilding the tree.

Strain MAMPE19-BCG phylogeny in Figure 2B indicates high similarity (98.62%) with *Bacillus cereus* strain, another *Firmicutes* member and a pathogenic bacterium, *B. cereus* is also a PGP strain capable of bioaccumulating heavy metals and possesses active mechanisms in response to HM stress (Behera *et al*., 2014; Egidi *et al*., 2016; Huang *et al*., 2018; Aguilar *et al*., 2020). Genomic analysis of a *Bacillus cereus* strain highlighted its ability to produce a biosurfactant with proven efficacy for Pb, Cd, and Cr removal (Ayangbenro & Babalola, 2020).

#### *3.3.2 Fungi*

Results were subjected to phylogenetic analysis and after alignment, there were two genera of fungal microorganisms (Figures 2C and D), strain MAMPE19-CHG corresponded to *Talaromyces* sp, another name for those species is as a subgenus of *Penicillium*, the *Biverticillium* (Pitt 2014); the isolated strain has the maximum identity to *Talaromyces*

*verruculosus*, an excellent cellulase and antimicrobial extrolite producer, a secondary metabolite secreted to the extracellular medium (Hu *et al*., 2016). Nam *et al*., (2019) reported a study of a *Talaromyces* sp strain with the ability to absorb As in form of As (III) and As (V), experiments of mycelium coated with hematite nanoparticles also showed promising results about the use of chemical and biological assisted technologies for HM remediation.

The other genus was *Exophiala* for strains MAMPE19-BHV (*Exophiala* sp) and MAMPE19-BHN (*Exophiala oligosperma*), this result was expected due to the high similarity between the two colonies isolated from sample MAMPE19-B. The *Exophiala* genus is a member of dark septate endophytic (DSE) type of fungi with genera as *Phialophora*, *Phialocephala*, and *Eptodontidium* as the major representatives of this group. Frequently, their presence is related to an environment with high metal levels, and in a symbiotic relationship with plants (Kumar *et al*., 2018), the *Exophiala pisciphila* strain showed that its interaction with maize roots enhanced their growth under heavy metal stress by Pb, Zn, and Cd (Li *et al*., 2011) and there are reports of *Phialocephala fortinii* also a DSE specie for promoting plant growth by improving K-uptake and also reducing the heavy metal absorption in roots plant (Yamaji *et al*., 2016).

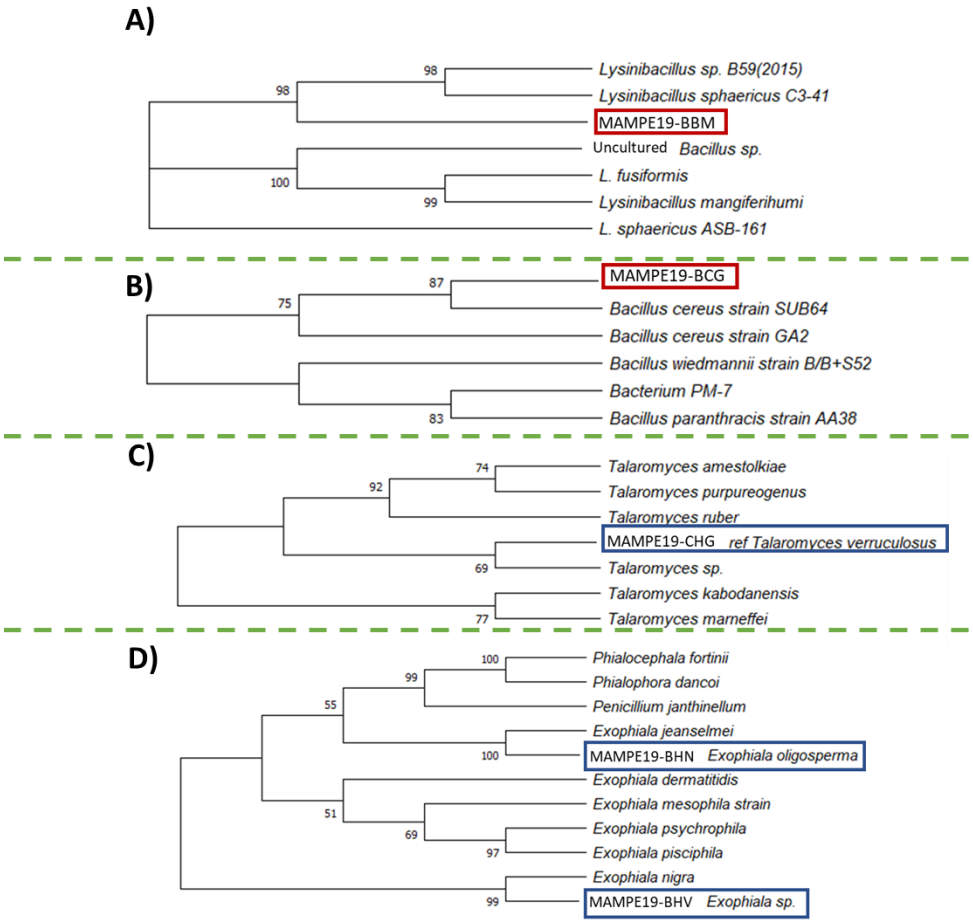


Fig. 2. Phylogenetic trees for strains isolated from two different mine tailing samples. A) Strain MAMPE19-BBM isolated from Pb-Zn mine tailings (Hidalgo). B) Strain MAMPE19-BCG isolated from Pb-Zn mine tailings (Hidalgo). C) Strain MAMPE19-CHG isolated from Ag mine tailing (Coahuila de Zaragoza). D) Strains MAMPE19-BHN and MAMPE19-BHV isolated from Pb-Zn mine tailing (Hidalgo).

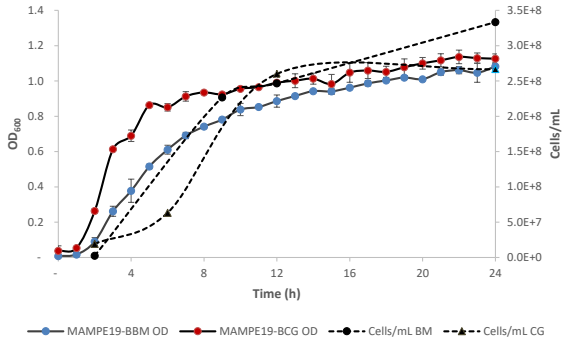


Fig. 3. Growth curves in optical density and cell/mL versus time for the two isolated bacteria *Bacillus cereus* and *Lysinibacillus sphaericus* (MAMPE19-BCG and MAMPE19-BBM) from mine tailing samples MAMPE19-B (Pb-Zn mine tailings) and MAMPE19-C (Ag mine tailings). Values presented are means ± SD ($n$ = 3).

## *3.4 Characteristic growth curve and resistance test*

### *3.4.1 Bacteria*

The curve from $OD_{600}$ values and cell/mL for strains MAMPE19-BCG and MAMPE19-BCG shown in Figure 3 presents two specific different characteristic curves for each strain, the doubling time for BCG was 0.57 h and 0.51 h for BBM, characteristic short periods of heterotrophic bacteria, for BBM the maximum number of cell/mL was 3.33 $\times 10^8$ and for BCG 2.67 $\times 10^8$, these values of generation time and cell count specifically for LB media, 30°C and 160 rpm.

The specific growth rates ($\mu$) obtained for well-microplates curves are shown in Table 5 where the specific growth rate showed a decrease as a function of the presence of heavy metals for the MAMPE19-BCG strain, showing a decrease in the slope for the exponential phase and an extension in the lag phase as the concentration of $NaAsO_2$ and $ZnCl_2$ in the medium increased (Figure 4A and B). For MAMPE19-BBM almost the same tendency was shown as inhibition effects in the microbial growth as concentrations of $NaAsO_2$ and $ZnCl_2$ increased (Figure 4C and D). As was the stronger inhibitor for specific growth and only the 1 mM concentration of $NaAsO_2$ enhanced the $\mu$ of BBM (Table 5).

Table 5. Specific growth of MAMPE19-BCG and MAMPE19-BBM strains.

| ***MAMPE19-BCG*** | | | |
|---|---|---|---|
| **$NaAsO_2$ (mM)** | **μ (1/h)** | **$ZnCl_2$ (mM)** | **μ (1/h)** |
| 1 | 0.8309 | 1 | 0.9016 |
| 5 | 0.8486 | 3 | 0.8965 |
| 7 | 0.6251 | 5 | 0.5639 |
| 10 | 0 | 7 | 0 |
| Control | | 1.1625 | |
| ***MAMPE19-BBM*** | | | |
| 1.0 | 0.9671 | 1.00 | 0.7509 |
| 1.25 | 0.2505 | 1.25 | 0.6538 |
| 1.5 | 0.2867 | 1.5 | 0.2419 |
| 2.0 | 0 | 2.00 | 0.3355 |
| Control | | 0.8443 | |

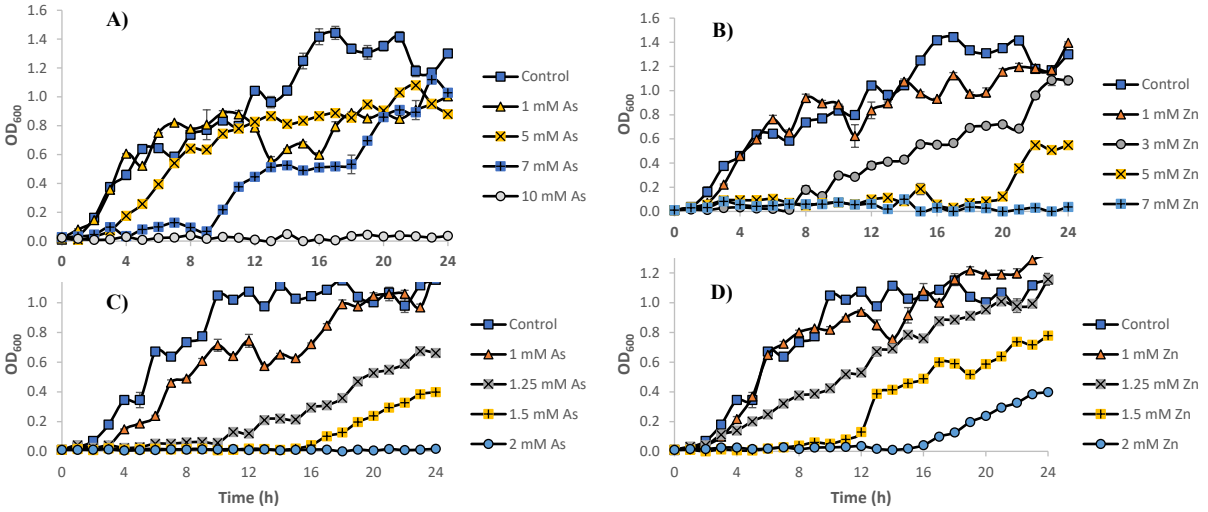


Fig. 4. A) Microbial growth as a function of $OD_{600}$ in the presence of $NaAsO_2$ for *Bacillus cereus* strain (MAMPE19-BCG). B) Growth of *Bacillis cereus* (MAMPE19-BCG) in the presence of $ZnCl_2$. C) Microbial growth as a function of $OD_{600}$ in the presence of $NaAsO_2$ for *Lysinibacillus sphaericus* strain (MAMPE19-BBM). D) Growth of *Lysinibacillus sphaericus* (MAMPE19-BBM) in the presence of $ZnCl_2$. Values presented are means ± SD ($n$ = 3).

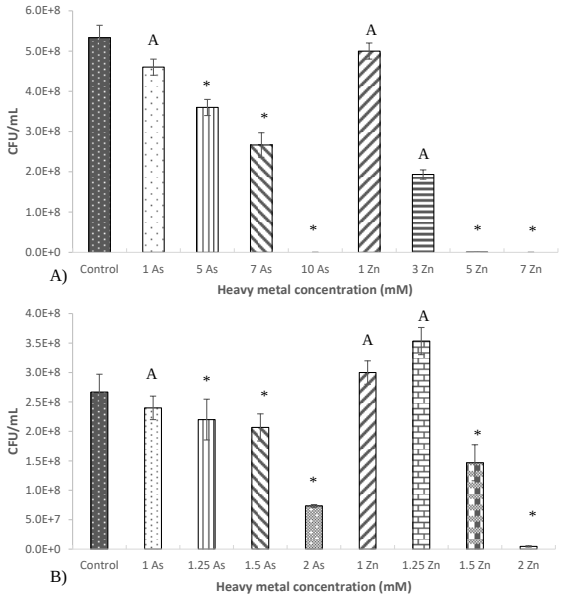


Fig. 5. CFU/mL of bacteria isolates from mine tailings in the presence of $NaAsO_2$ and $ZnCl_2$. A) CFU/mL of *B. cereus* strain (MAMPE19-BCG) isolated from Pb-Zn mine tailings sample MAMPE19-B. B) CFU/mL of strain *Lysinibacillus sphaericus* (MAMPE19-BBM) isolated from Pb-Zn mine tailings sample MAMPE19-B. Values presented are means ± SD (n = 3). A Represents no differences between control and treatment ($p > 0.05$). *Represents statistical differences with control ($p < 0.05$).

The results in cell/mL have a similar tendency as those of $OD_{600}$, in Figure 5A the number of cells/mL for MAMPE19-BCG in presence of arsenite, tend to decrease as the concentration of arsenite increases. For $ZnCl_2$, the tendency was the same but with an accentuated decrease in the number of cells for concentrations 5 mM and 7 mM. In comparison with the same concentration $NaAsO_2$, as observed in OD curves, an interesting result, because even though strain MAMPE19-BBM in Figure 5B resisted lower As and Zn concentrations, the first two concentrations of Zn enhanced the number of total cells. There are reports of strains of *Bacillus cereus* and *Lysinibacillus* capable of reducing the concentration of arsenite and arsenate in the medium by intracellular accumulation (Aguilar *et al*., 2020). The bacterial strains of the present study can be evaluated for their ability to bioaccumulate As and Zn species, or in another case, their plant growth effect on heavy metal phytoremediation. The difference in the range of tolerance to As and Zn between the species isolated from the same samples is not accidental, since their biochemical machinery can allow the cell to survive and adapt better to heavy metal stress, and it is for this reason that molecular studies for the identification of genes involved in resistance to heavy metals are carried out in the same way to suggest mechanisms to resist or tolerate the presence of HM (Wu *et al*., 2018; Han *et al*., 2019).

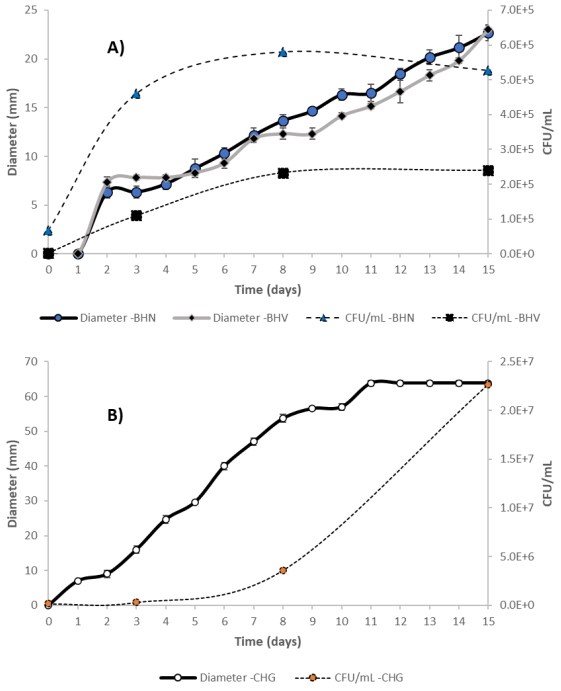


Fig. 6. A) Characteristic curves of fungi strains *Exophiala oligosperma* (MAMPE19-BHN) and *Exophiala* sp (MAMPE19-BHV) isolated from Pb-Zn mine tailings sample MAMPE19-B. B) Characteristic curve of *Talaromyces verruculosus* strain (MAMPE19-CHG) isolated from an Ag mine tailings sample MAMPE19-C. Values presented are means ± SD (n = 3).

There is a study of the improvement of growth rate and tolerance to HM with a *Bacillus cereus* and *Lysinibacillus macrolides* strains in which ZnO nanoparticles in low concentration and it suggest a synergy dynamic between nanoparticles and the two bacteria, as in the present study the lowest concentration evaluated did not affect the growth of the microorganisms (Akhtar *et al*., 2021).

### *3.4.2 Fungi*

The characteristic curve of change in diameter ($\Delta D$) vs time for fungal isolated strains and an approximation in the number of CFU/mL is presented in Figure 6. The two strains isolated from sample MAMPE19-B showed a highly similar growth curve on solid media as a $\Delta D$ function; however, the cell count corresponding to each point of the curve showed a greater difference, corresponding with the visual observation of apparently slow and poor growth of strain MAMPE19-BHV in comparison with MAMPE19-BHN. For strain MAMPE19-CHG isolated from tailing sample MAMPE19-C, the growth was larger in diameter and in CFU/mL.

The *Talaromyces* genera, closely related to *Penicillium* may be identified as an opportunistic microorganism, mostly present in industrial food processing or food as cereal; however, there are also reports of this genus found under heavy metal stress condition, in As-contaminated soils from mining activity (Kumar *et al*., 2018; Nam *et al*., 2019). In both cases the strains isolated from sample MAMPE19-B presented a stationary phase tendency in the curve of CFU (Figure 6A), contrasting with diameter variation, this result may suggest the presence of dead cells at the center of the circular colony and the growth of external cells at a lower ratio. The other case is strain MAMPE19-CHG where the Petri dishes were completely saturated with the colony growth and even after constant diameter lectures, the number of CFU/mL had an exponential tendency probably due to a greater capacity of the *Talaromyces* strain to sporulate as observed in lactophenol blue staining, where observation at 40X the number of spores was apparently higher in this strain. The tolerance index was calculated (equation 4), the graphs obtained showed the characteristic behavior for growth under stress by the presence of heavy metal using a tolerance index versus time graph (Anahid *et al*., 2011). The graphs comprise five phases: lag phase (I), rapid growth (II), retarded growth (III), similar growth (IV), and enhanced growth (V), as shown in Figure 7.

Despite a faster growth of strain CHG, their performance in presence of sodium arsenite only reflected a tolerance up to 5 mM, the strains MAMPE19-BHN and MAMPE19-BHV isolated from the same tailing sample had a higher resistant index for Zn (BHV) and the highest tolerance range in presence of As (up to 20 mM for strain MAMPE19-BHN).

The selection of practically identical colonies from sample MAMPE19-B raised the hypothesis that both could be the same isolated microorganism; but slight differences in color, growth rate, and the incapability to grow in the presence of $NaAsO_2$ (the reason because of no As tolerance index for strain MAMPE19-BHV), is related to *Exophiala* genera, in which the melanin is mainly present in the cell wall and it is responsible for reducing heavy metal stress by forming ionic bonds with metallic species (Zhan *et al*., 2011; Kumar *et al*., 2018); this *Exophiala* strain isolated from the two, was selected as a green colony.

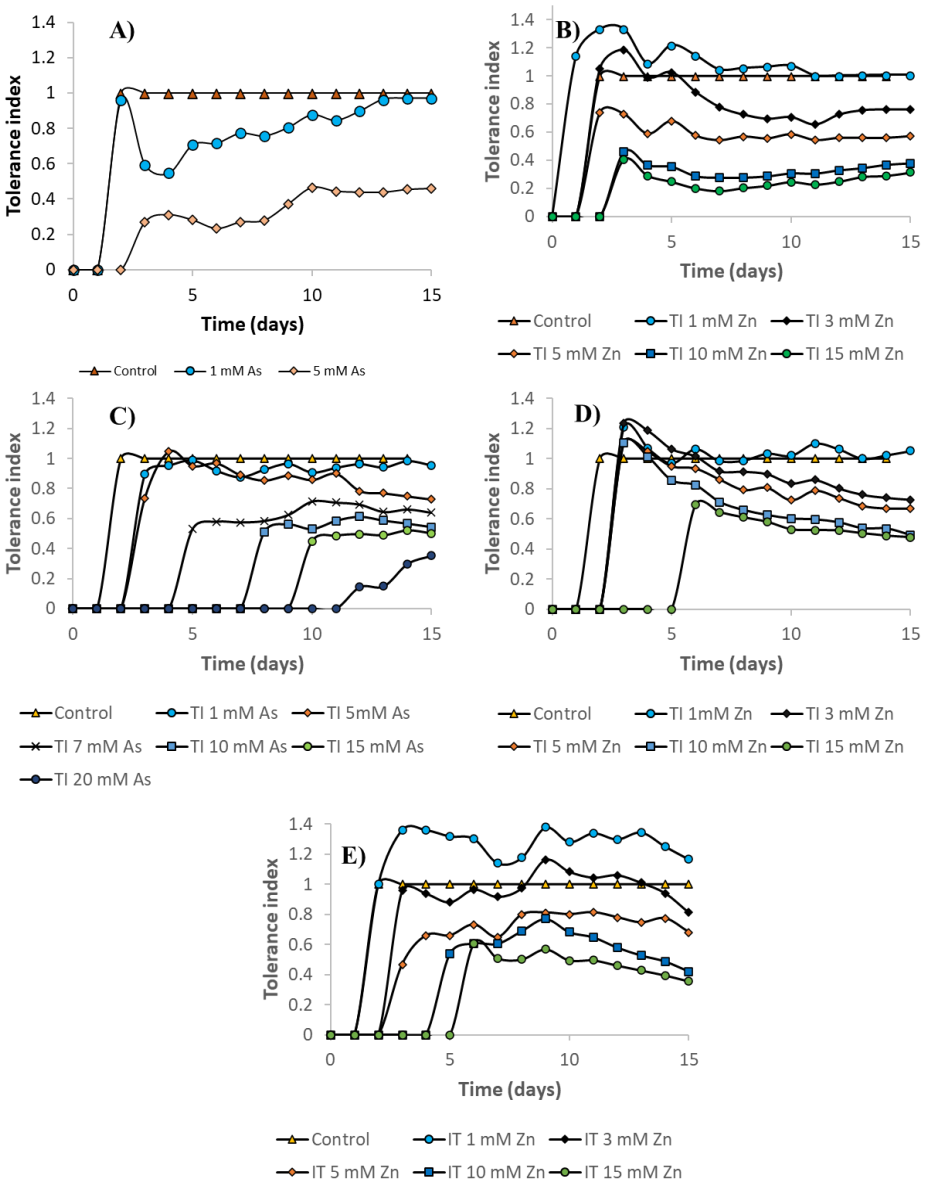


Fig. 7. Tolerance index (TI) of fungal isolates from mine tailing samples MAMPE19-C (Ag mine tailing) and MAMPE19-B (Pb-Zn mine tailing) to $NaAsO_2$ and $ZnCl_2$. A) Tolerance to As for *Talaromyces verruculosus* strain (MAMPE19-CHG). B) Tolerance to Zn for *Talaromyces verruculosus* strain (MAMPE19-CHG). C) Tolerance to As for *Exophiala oligosperma* strain (MAMPE19-BHN). D) Tolerance to Zn for *Exophiala oligosperma* (MAMPE19-BHN). E) Tolerance to Zn for *Exophiala* sp (MAMPE19-BHV).

From experiments carried out in Petri dishes supplemented with As and Zn, the two *Exophiala* strains exhibited a major black pigmentation with the increase in Zn concentrations; for *Talaromyces* strain, the characteristic green colony changed to a white lumpy colony with orange reverse in concentrations from 5 mM $ZnCl_2$, this genus has also been investigated for its pigments and production of metabolites (Venkatachalam *et al.*, 2018). The differences between CFU due to the presence of heavy metals are presented in Figure 8 for the three fungal isolated strains. In Figures 7 and 8 the growth enhancement in presence of small concentrations of Zn can be visualized, from 1-3 mM $ZnCl_2$ presented

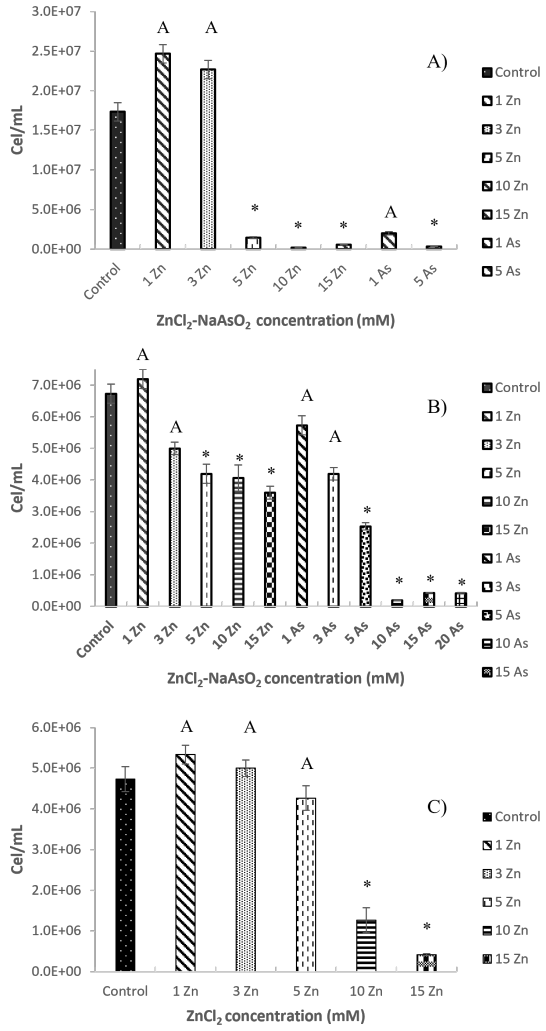


Fig. 8. Variation in the number of CFU/mL for fungi strains isolated from mine tailings. A) Strain *Talaromyces verruculosus* (MAMPE19-CHG) isolated from Ag mine tailings sample MAMPE19-C. B) Strain *Exophiala oligosperma* (MAMPE19-BHN) isolated from Pb-Zn mine tailings. C) Strain *Exophiala* sp (MAMPE19-BHV) isolated from Pb-Zn mine tailings. Values presented are means ± SD ($n$ = 3). A Represents no differences between control and treatment ($p > 0.05$). * Represents statistical differences with control ($p < 0.05$).

as a higher number of cells/mL and the same for the tolerance index shown as index patterns over the control curve, since Zn is an essential micronutrient, low concentrations of it may enhance the microbial growth of a tolerant strain, or as shown in other investigations, low $ZnCl_2$ concentration may not affect the growth of an evaluated strain (Wei *et al*., 2009).

For fungal strains isolated in the present work, a *Talaromyces* strain isolated from an As-contaminated area exhibited tolerance up to 1,000 mg/L of arsenite and arsenate (Nam *et al*., 2019) a higher but similar value than the reported in this study (5 mM or 649 mg/L $NaAsO_2$). The possibility to train the strain exists due to its natural tolerance and rapid reproduction among the other fungal strains isolated here for future experimentation, as shown by Romero *et al*., (2006) with a trained strain of *Talaromyces* to bioremediate a co-contaminated environment with copper and organic compounds. The effect of growth, adsorption, and accumulation of HM in *Triticum aestivum* in the presence of *Talaromyces pinophilus* was addressed by El-Shahir *et al*. (2021), where the benefit of inoculation with *T. pinophilus* acting as a barrier for the adsorption of HM was demonstrated and allowing good growth of *T. aestivum* in soil amended with sewage sludge. *Exophiala* genus is found in stressing environmental conditions as HM in soils, in direct interaction with plants in symbiotic relationships as a dark septate endophytic fungus (Kumar *et al*., 2018), the melanin pigmented fungi are more resistant to a stressful condition as the presence of heavy metals, and the melanin has been considered because of their antioxidant properties in the hyphae and also in their host (Li *et al*., 2011; Mandyam & Jumpponen, 2005); the melanin production as a protective agent against environmental stress in presence of Cd as a heavy metal has also been addressed, as the observation in this study of darker colonies in increasing Zn concentrations. There are also reports of *Exophiala psychrophile* strain present in As-rich technosols in Slovakia (Šimonovičová *et al*., 2016). The study of melatonin inference on direct heavy metal resistance in *Exophiala pisciphila* proved a metal-induced stress tolerance in the presence of exogenous melatonin and also enhanced the production of this molecule in the presence of Cd, Pb, and Zn (Yu *et al*. 2021).

After resistance test experimentation, the results of statistical analysis and differences among each control strain (grown without heavy metal presence) and strain in presence of the different levels of $ZnCl_2$ and $NaAsO_2$ are summarized in supplemental information (Table 6). The statistical analysis of supplemented-heavy metal experiments compared to each control did not show significant differences for the lower concentration of $ZnCl_2$ and $NaAsO_2$ (1 mM) except for strain MAMPE19-BHV which was unable to grow in presence of arsenite; the higher concentration with

no significant difference as compared to the control was 5 mM of $ZnCl_2$ for strain MAMPE19-BHV (100% homology with *Exophiala* sp.).

Table 6. Statistical differences on heavy metal exposure vs growth of isolated strains from mine tailings.

| Strain | MAMPE19-BCG | | MAMPE19-BBM | | MAMPE19-BHN | | MAMPE19 BHV | | MAMPE19-CHG | |
|---|---|---|---|---|---|---|---|---|---|---|
| HM (mM) | $NaAsO_2$ | $ZnCl_2$ | $NaAsO_2$ | $ZnCl_2$ | $NaAsO_2$ | $ZnCl_2$ | $NaAsO_2$ | $ZnCl_2$ | $NaAsO_2$ | $ZnCl_2$ |
| 1 | A | A | A | A | A | A | - | A | A | A |
| 1.25 | - | - | * | A | - | - | - | - | - | - |
| 1.5 | - | - | * | * | - | - | - | - | - | - |
| 2 | - | - | * | * | - | - | - | - | - | - |
| 3 | - | A | - | - | A | A | - | A | - | A |
| 5 | * | * | - | - | * | * | - | A | * | * |
| 7 | * | * | - | - | - | - | - | - | - | - |
| 10 | * | - | - | - | * | * | - | * | - | * |
| 15 | - | - | - | - | * | * | - | * | - | * |
| 20 | - | - | - | - | * | - | - | - | - | - |

HM = Heavy metal  Confidence interval = 95% & α = 0.05

A Represents no differences between control and treatment ($p > 0.05$)  * Represents statistical differences with control ($p < 0.05$)  - No evaluated concentration or no growth for strain

The highest value with growth for bacteria in presence of $NaAsO_2$ and $ZnCl_2$ were 7 mM and 5 mM for strain MAMPE19-BCG (98.62% homology with *Bacillus cereus*) respectively, and for fungal strains 20 mM of $NaAsO_2$ and 15 mM $ZnCl_2$ (for strain MAMPE19-BHN with 97% of homology with *Exophiala oligosperma*). The rest of the isolated strains showed lower resistance as presented in Table 6.

The isolation of heterotrophic culturable microorganisms from heavy metal polluted areas, such as mine tailings, for potential application in bioremediation and phytoremediation of contaminated environments has been studied to take advantage of the ability of these organisms to survive and coexist in extreme and variated environments, and as a part of their physiological activities and interaction with other species decrease the level of contamination caused by heavy metals (Wei *et al*., 2009; Govarthanan *et al*., 2013; Teng *et al*., 2017; Fan *et al*., 2018; Rose and Devi 2018; Wu *et al*., 2018; Nam *et al*., 2019; Aguilar *et al*., 2020; Seitkamal *et al*., 2020).

## Conclusions

Two members of the *Firmicutes* phylotype were isolated, the molecular characterization of these strains identifies them as plant growth promoting bacteria, so this quality, together with its tolerance capacity, opens the field to its use in bioremediation technologies but also in phytoremediation. These possible applications give important information to scale up the experimentation that can be conducted since it involves the potential of the plant to attenuate HM pollution with the symbiotic enhance that PGP-bacteria can provide (Li *et al*., 2019). The little or no effect on microbial growth by low doses of heavy metals, as presented in this work, has also been seen in other investigations (Wei *et al*., 2009; Huang *et al*., 2018).

In this investigation, it was possible to isolate native bacterial and fungal microorganisms from both mine tailings. According to our results, fungal strains were the most resistant species, and their tolerance capacity makes them candidates for future studies with real samples and on a larger scale. It is necessary to carry out a more specialized and in-depth investigation, such as experiments with isolated species in a bioleaching process with samples of mine tailings to verify their efficiency to consider them as possible prospects with applications in biometallurgical processes, bioremediation, and phytoremediation, and perhaps in nanobioremediation and synthesis of nanoparticles. However, most of the strains in the present study exhibited high levels of tolerance to heavy metals.

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