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Enterococci and Bacilli from surface water: assessment of their resistance to copper and antibiotics

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ABSTRACT. Heavy metal-resistant bacteria can be efficient bioremediators of metals and might provide an alternative method for metal removal in contaminated environments. The present study aims to isolate bacteria from the aquatic environment and evaluate their potential tolerance to copper metal, aiming at bioremediation processes. Also, compare co-resistance to heavy metal and antibiotics. The morphology of isolates was observed, and sequence analysis (16S ribosomal DNA) revealed that isolated strains were closely related to species belonging to the genera *Enterococcus* and *Bacillus*. Bacterial isolates were resistant to $CuSO_4$, with a minimum inhibitory concentration of 0.78 mg ml^{-1}. *Enterococcus lactis* was resistant to a combination of copper and tetracycline. The other tested isolates were sensitive to the tested antimicrobials. The metal removal ability of these isolates was assayed using atomic absorption spectroscopy, and the strains 27, 23, and *E. lactis* were best at removing heavy metals, at 87.7%. *Enterococcus casseliflavus* EC55 was 62%, followed by *Bacillus aerius* (18.4%), *E. casseliflavus* EC70 (10%) and *Bacillus licheniformis* (10%). Based on our findings, *Enterococcus* sp and *Bacillus* sp. have potential applications in enhanced remediation of contaminated environments.

Keywords: heavy metal-resistant; *Enterococcus* sp; *Bacillus* sp; $CuSO_4$.

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Introduction

It is known that the increased use of metals and chemicals in the process industries has resulted in the generation of large amounts of effluents containing toxic heavy metals and these effluents can accumulate in the environment, due to their non-degradable nature (Gautam, Gautam, Banerjee, Chattopadhyaya, & Pandey, 2016). Toxic metals can accumulate along the food chain, causing chronic toxicity to the aquatic environment and in humans (Pugazhendhi, Ranganathan, & Kaliannan, 2018).

Several microorganisms that live in these environments adopt different mechanisms to adapt to these heavy metal and antibiotics stresses. This occurs because some mechanisms for heavy metal resistance function in a similar way of those for resistance to antibiotics. It is very important to elucidate the bacterial resistance to both in detail for further understanding the bacterial cross-resistance and its ecological risk (Zhou et al., 2015).

On the other hand, this co-resistance can facilitate the bioremediation of heavy metal by the microorganism (Selvi et al., 2012). Many living bacteria have been reported to transform toxic contaminants into their less toxic forms (Karthik et al., 2017).

Bioremediation processes have attracted much attention as part of the search for new, economically viable technologies for the removal of toxic metals from the environment. Bioremediation is advantageous because conventional methods (e.g., chemical precipitation, membrane separation, ion exchange, reverse osmosis, chemical oxidation, electrochemical treatment, and adsorption) are relatively costly and eco-friendly (Peng et al., 2010; Weng et al., 2014; Gautam et al., 2016; Karthik et al., 2017).

However, the first step for a bioremediation process project, it is suggested to determine the microorganisms existing in contaminated areas, since these native microorganisms, in addition to tolerating metals, are also adapted to the environmental conditions of temperature, humidity and pH (Muñoz-Silva, Olivera-Gonzales, Torres, & Tamariz-Angeles, 2019).

Gram-positive bacteria constitute a morphologically diverse group with high metabolic versatility. As reported, they are the good bioremediation, include metal-complex formation, reduction of certain metals to less toxic species, or efflux of the metals from the cell (Zampieri, Pinto, Schultz, Oliveira, & Oliveira, 2016).

Because these microorganisms are able to transform metals into less toxic or non-toxic, they are potentially useful in bioremediation of environmental contaminated (Karthik et al., 2017). Such application is especially useful with bacteria adapted to the aquatic environment and can be used *in situ*. Microorganisms are capable of capturing heavy metals in aqueous solution, trapping the ions in the cell wall structure, being able to internalize the cell (Selvi et al., 2012).

The present study, we screened and isolated bacteria strains from the aquatic environment and to evaluate co-resistance to antimicrobial and copper, potentially useful microorganisms for application in bioremediation processes.

Material and methods

Area and water sampling

The present study was conducted in six different aquatic regions of two rivers in the catchment area of Apucarana City (Paraná State, Brazil) during October-November 2014 and February-March 2015. These rivers (Pirapó and Ivaí) are among the primary sources of drinking water in the city.

All water samples were collected in sterile bottles and stored at 4°C. Each sampling site was recorded using GPS (global positioning system) technology (Figure 1).

Isolation of cu-resistant bacteria

Cu-resistant bacteria were isolated by the serial dilution method in saline water (0.85% w v^{-1}). Then, a 100 µl aliquot of each dilution was spread onto Luria-Bertani agar (LB) (HiMedia, Mumbai, India) containing filter-sterilized $CuSO_4$ (12 mg L^{-1}) (Sigma-Aldich-USA). The plates were incubated at 37°C for 24 hours. Morphologically different colonies were picked and purified by repeated sub-culturing on the same medium. All bacterial isolates were categorized based on the Gram staining reaction. These isolates were stored in LB broth (HiMedia Laboratories Pvt. Ltd, Mumbai, India) and 20% (v v^{-1}) glycerol stocks at -20°C for further analysis.

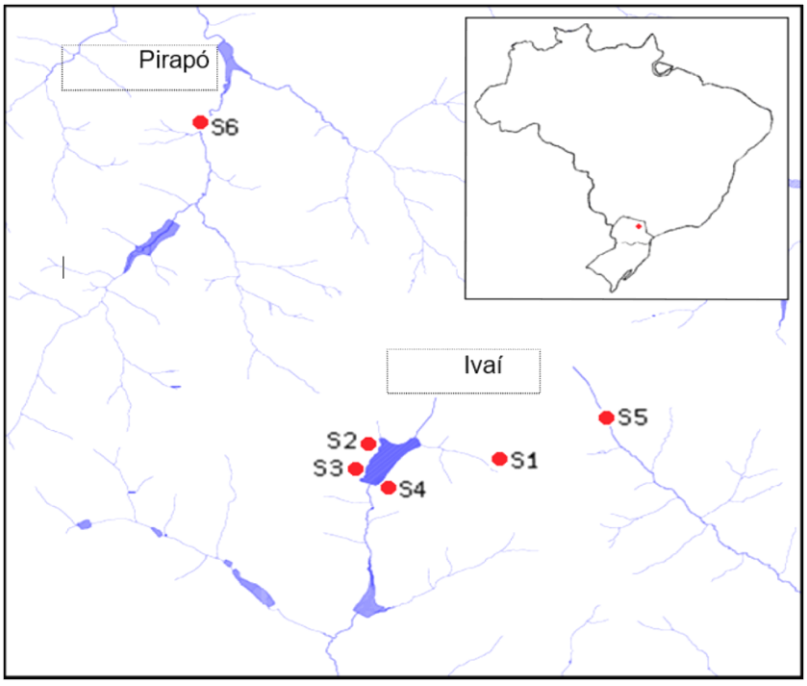

Figure 1. Geographical positions of sampling in North of Paraná (Brasil). Sign represents different sampling sites in Pirapó river catchment área. Code/GPS coordinates: S1/ 23º 34'3.83" S 51º 27' 46.7" W; S2/ 23º 34' 9.95" S 51º 28' 25.54" W; S3/ 23º 34' 2.36" S 51º 28' 31.2" W; S4/ 23º 33' 52.92" S 51º 28' 25.81" W; S5/ 23º 31' 13.97" S 51º 29' 22.32" W; S6/ 23º 33' 31.54" S 51º 27' 10.57" W.

Genomic DNA extraction

Genomic DNA was extracted using the simple boiling assay. Briefly, the bacterial pellets were suspended in 200 µL of TE buffer (Invitrogen™ byLife Technologies, EUA) and subjected to 30 min. of boiling (90°C). Immediately after boiling, the microfuge tubes were placed in an ice bath for 15 min. and then centrifuged at 8000 g for 10 min. at room temperature (SL-700 Solab-BR). The genomic DNA (50 µl supernatant) was collected and stored at -20°C.

Detection of the *tcrB* gene by PCR

The presence of the *tcrB* gene was detected by PCR, using primers tcrBF (5′-CAT CACGGTAGCTTTAAGGAGATTTTC-3′) and tcrBR (5′-AGAGGACGCCGCCACCATTG-3′), with an expected amplification product of 663 bp. The following cycling parameters were used for all PCR: 10 min. at 95°C, then 30 cycles of 30 s at 94°C, 30 s at 55°C, and 1 min. at 72°C, with a final step of 10 min. at 72°C and holding at 10°C (Thermal Cycler, Applied Biosystems-USA). The amplification products were separated by 1% agarose gel (Invitrogen™ electrophoresis byLife Technologies, EUA), stained with ethidium bromide (1 µg mL^{-1}), and observed under ultraviolet (UV) light (Super-Bright-Brasil). The size of the amplified product was compared to a molecular weight marker (1 kb DNA plus, Invitrogen™ byLife Technologies- EUA).

Identification of the selected strain by PCR

For the amplification of 16S ribosomal RNA (rRNA) of the isolated bacterial strains, we used oligonucleotide primers (5´-3´) F 27 (AGAGTTTGATCCTGGCTCAG) and R 1492 (GGTTACCTTGTTACGACTT) (Wang, Li, Xiang, & Zhai, 2007). Thermal cycling was performed by initial denaturation at 94°C for 2 min., 35 cycles of denaturation at 94°C for 1 min., annealing at 50°C for 1 min., and elongation at 72°C for 1 min., with a final elongation step of 72°C for 10 min. The amplified fragments were purified with the QIAquick PCR Purification Kit (Qiagen-USA) according to the manufacturer's instructions. The PCR product was sequenced using a Ludwig Biotecnologia (Brasil) sequencer. The generated gene sequences were compared with sequences available in GenBank by using the BLASTn program (Basic Local Alignment Search Tools/ http://www.ncbi.nih.gov). The phylogenetic analysis was done using MEGA (version 7). A phylogenetic tree was made from unambiguously arranged nucleotides (Saitou & Nei, 1987).

Minimum inhibitory concentrations of copper

The minimum inhibitory concentrations (MICs) of $CuSO_4$ were determined for all isolates (Wiegand, Hilpert, & Hancock, 2008) with modifications. The assays were performed in a microtiter plate containing a total volume of 100 µL of Muller Hinton broth (HiMedia Laboratories Pvt. Ltd, Mumbai, India), metal concentrations between 0.012 and 1.2 mg L^{-1}, and bacterial inoculum (1×10^6 CFU mL^{-1}). The plates were incubated at 24 and 37°C for 24 hours, and the OD was measured at 600 nm (Bio-TeK EL 808). The lowest concentration of the heavy metal that did not favor microbe growth was considered the MIC. Optic density (OD) values were converted to percentage of bacterial growth.

Antibiotic sensitivity assay

The antibiotic sensitivity of the strains was tested using the disc diffusion method (Clinical and Laboratory Standart Institute [CLSI], 2014). To perform the disc diffusion test, cultures were grown in Muller Hinton broth and incubated overnight at 37ºC for 24 hours. The culture suspension was then used to inoculate Muller Hinton agar plates, and an antibiotic disc was placed on the agar surface. The antibiotic disc includes ampicillin (10 µg), ciprofloxacin (5 µg), erythromycin (15 µg), gentamicin (10 µg), norfloxacin (10 µg), teicoplanin (30 µg), tetracycline (30 µg), and vancomycin (30 µg) (Laborclin-Brasil). After incubating the plates at 37ºC for 24 hours, the zone of inhibition was recorded. *Staphylococcus aureus* ATCC 25923 samples (American Type Culture Collection) were used as a control during the susceptibility tests.

Determination of heavy metals degradation

Heavy metal concentrations were estimated by atomic absorption (AA) spectroscopy (Solaar S Series - Thermo Scientific-USA) (Manisha, Dinesh, & Arun, 2011) following the standard method recommended by American Public Health Association (Apha, 1998). Heavy metal content in the culture sample was estimated before and after treatment of the culture medium by metal-resistant bacteria. The experiments were carried out in 250 mL flasks containing 100 mL of LB broth (HiMedia Laboratories Pvt. Ltd, Mumbai, India)

containing 0.78 mg L^{-1} $CuSO_4$ and 1 × 10^6 UFC mL^{-1} bacterial isolate. The flasks were kept in a shaker at 150 rpm at 37°C for 24 hours. Next, the cells were separated by centrifugation, and the metal concentration in the culture medium was determined. The percentage of degradation was calculated as described by Basha and Rajaganesh (2014).

Statistical analysis

Data were evaluated using one-way ANOVA and Tukey's test considering $p < 0.05$ as significant to establish significant differences between bacteria growth and temperature of incubation. All the experiments were carried out in triplicates.

Result and discussion

The city of Apucarana is an industrial center, including textile and furniture industries that use toxic metals during production. Much of this material is disposed of in industrial effluent for treatment and then sent to the rivers in the region. Toxic metal pollution (e.g., Cu, Cd, Ni, Cr, Zn, Pb, As, and Hg) from anthropogenic activities and domestic and industrial waste discharges are increasing (Wu, Cui, Li, & Sun, 2015).

A total of 72 isolates that grew under a high concentration of $CuSO_4$ were randomly selected for genotypic verification for the *tcr*B gene, which confers resistance to copper. Of these, the *tcr*B gene was amplified from seven isolates (27, 23, EL, BA, BL, EC55, and EC70) (Table 1). Two of these isolates (EC55 and EC70) produced an amplification product of the expected size (663 bp), and the others presented non-specific amplification (Figure 2A). All seven isolates were included in the copper resistance analyses.

Although the World Health Organization drinking water guidelines suggest a Cu (II) limit of 2.0 mg L^{-1} (World Health Organization [WHO], 2004), in humans, constant consumption of this metal can cause mucosal corrosion, hepatic renal damage, gastrointestinal damage, nervous system disorder, and pancreas and heart disease (Ghosh & Saha, 2013; Yargıç, Şahin, Özbay, & Önal, 2015). In our work, we calculated a MIC of 0.78 mg L^{-1}, but this can reach higher values depending on the selective pressure that this metal exerts on bacterial cells.

Bacterial strains were identified taxonomically by 16S rDNA gene sequencing (Table 1). Comparing these 16S rDNA sequences to those in NCBI/GenBank, the isolated strains were found to belong to the genera *Enterococcus* and *Bacillus*, sharing more than 90% similarity to their closest relatives. The sequences of strains 23 and 27 did not align with any microorganisms deposited in the DNA database.

To determine their phylogenetic positions, the 16S rDNA gene sequences of all isolates were analyzed, and a phylogenetic tree was constructed (Figure 2). Phylogenetic analysis showed that isolate 27 is associated with BL, and isolate 23 is strongly associated with BA.

The cell growth of the seven evaluated isolates differed considerably in relation to the incubation temperature; strains 23, 27, EL, and EC55 presented almost 100% growth at 37°C; already at the incubation temperature at 28°C, the best results were presented by the isolates *B. licheniformis* BL and *E. casseliflavus* EC70, although these isolates did not show significant growth in this copper concentration (Figure 3). For the other isolates, the temperature of 37°C showed the best bacterial growth.

At 28°C, the microbial growth under the $CuSO_4$ condition was slow, and this increased with increasing temperature (up to 37°C). Biosorption between biomass and metal Cu involves a combined chemical and physical sorption, with a rise in temperature from 28 to 37°C, the pores on the bacteria enlarge which increases the surface area. This leads to high sorption, diffusion, and penetration of metal into the cell (Saleem, Pirzada, & Qadeer, 2007).

Table 1. Identification of isolated bacterial strains based on 16S rRNA gene sequence analysis and their close relative published in DNA databases.

Strain	Closely related taxa identified by using the BLAST database	Sequence similarity (%) of 16S rRNA gene with closely related taxa	Accession number of 16S rRNA gene
23	-	-	-
27	-	-	-
EL	*Enterococcus lactis*	94 %	NR_117562.1
BA	*Bacillus aerius*	90%	NR_118439.1
BL	*Bacillus licheniformis*	94%	KR999957.1
EC55	*Enterococcus casseliflavus*	97%	NR_119280.1
EC70	*Enterococcus casseliflavus*	95%	NR_104560.1

(-) Not identified.

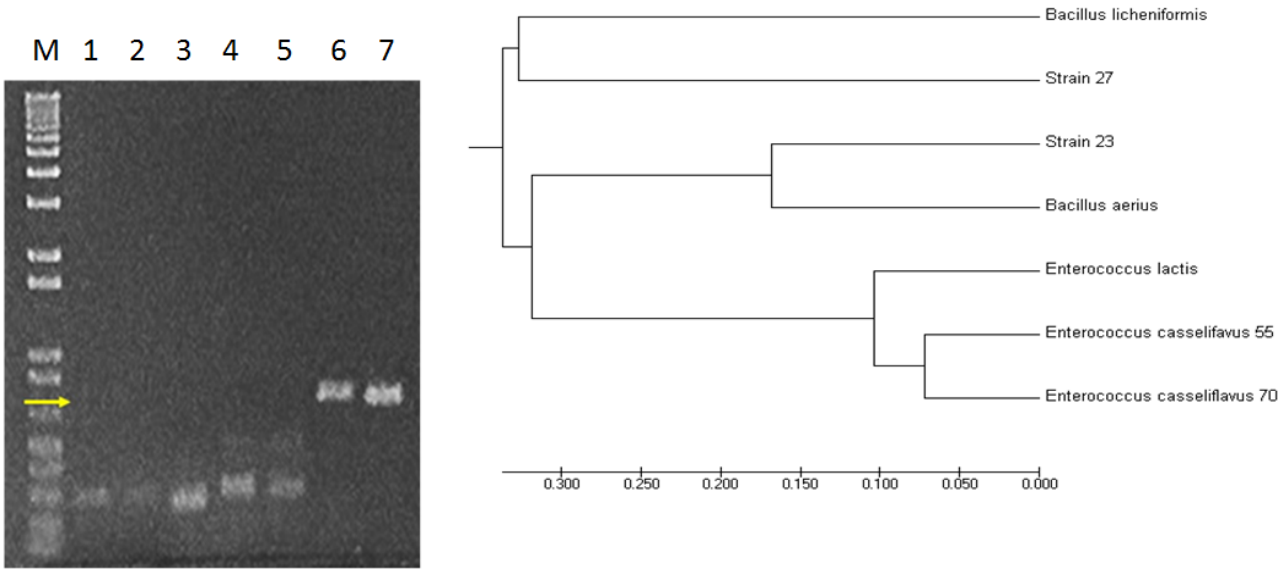

Figure 2. (A) PCR amplified products of tcrB gene (663 pb; yellow arrow) of isolated bacterial: Isolates 27 and 23 (lines 1 and 2), *Enterococcus lactis* EL (line 3), *Bacillus aerius* BA (line 4), *Bacillus licheniformis* BL (line 5), *Enterococcus casseliflavus* EC55 (line 6) and EC70 (line 7), respectively. M: marker 1 Kb DNAplus; (B) Phylogenetic tree showing the interrelationships of isolated bacterial strains based on 16SrDNA gene sequence. The rooted tree was constructed using the MEGA7 software.

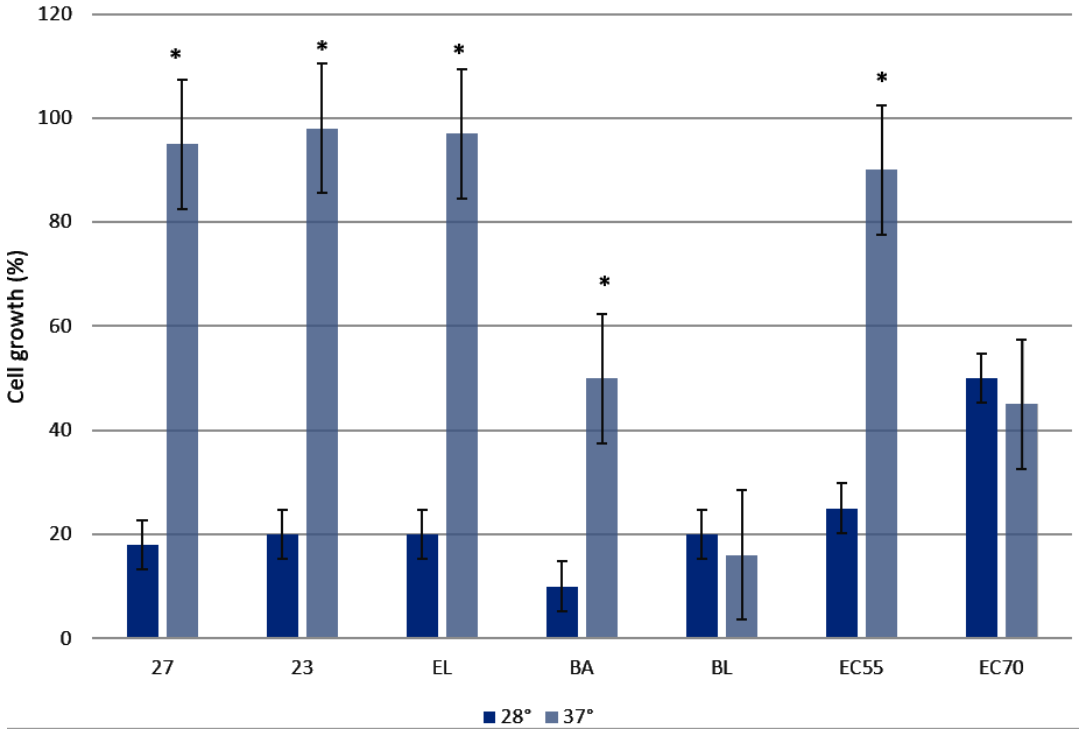

Figure 3. Cell growth of the isolates in 0.78 mg L^{-1} CuSO4 and incubation at 28 and 37°C. Isolates 27 and 23, *Enterococcus lactis* EL, *Bacillus aerius* BA, *Bacillus licheniformis* BL, *Enterococcus casseliflavus* EC55 and EC70. (*) $p < 0.05$. Bars represent the mean and standard deviations of three independent experiments.

Temperature plays a major role in the biosorption of several metals since it alters the rate of sorption and the interaction between adsorbate and adsorbent. The increase in uptake with a rise in temperature indicates that this is an endothermic process (Alkan, Kalay, Doğan, & Demirbaş, 2008).

Metal-resistant bacteria can play an important role in the clean-up of contaminated water because resistance to the metals is a general approach that occurs in the biological management of contaminated surroundings (Zampieri et al., 2016). Based on the ability of some isolated strains to grow in the presence of copper (Figure 2B), these were further tested for their potential used in biosorption (analyzed by AA). Among the tested strains, 23, 27, and EL were best at heavy metal removal (87.7%), followed by EC55 (62%), BA, EC70 and BL (18.4 and 10% removal, respectively).

The biosorption of heavy metals by microorganisms has received much attention in recent years, being a low-cost and cheaper alternative to cleaning contaminated environments (Banerjee, Pandey, Ray, & Kumar, 2015; Naz et al., 2016).

Matyar, Akkan, Uçak, and Eraslan (2010) and Rahman and Singh (2018) isolated copper-resistant bacteria from water. Bacterial resistance to toxic metals is an important factor in the study of the remediation of contaminated areas since this resistance is directly related to the survival capacity and bacterial growth in high concentrations of toxic metals (Kang, Kwon, & So, 2016).

Alam, Ahmad, and Malik (2011) analyzed bacterial resistance and found that 23% of the isolates were classified as Gram-positive and resistant to up to 800 μg mL^{-1} of copper. These data corroborate our study, since 100% of our isolates identified by the molecular analyzes were Gram-positive, in addition to presenting high resistance to copper.

Although several studies have reported that metal-resistant bacteria might also be resistant to antibiotics (Kimiran-Erdem et al., 2006; Matyar, Kaya, & Dinçer, 2008), this was not observed in our study, since only the isolated *E. lactis* was resistant to both copper and tetracycline. The other isolates presented sensitivity to the tested antimicrobials.

Resistance to antimicrobials and toxic metals is important for the survival of bacteria in contaminated environments, as most of the time bacteria can share elements of genetic mobility and as a consequence, these genes can be exchanged between bacteria via horizontal gene transfer or prolonged exposure to metals and antimicrobials (Zampieri et al., 2016). The principal mechanisms of bacterial resistance to antibiotics are efflux pumps, spontaneous chromosomal mutations, and conjugative plasmids, which can also account for the reported co-resistance of some bacteria to antibiotics and metals (Kimiran-Erdem et al., 2006; Zhou et al., 2015).

The results of this present study demonstrate the potential of isolates 23, 27, and EL for use in bioremediation processes; these isolates grew under high concentrations of the tested metal.

A clear understanding of the mechanisms that bacteria use to tolerate metals will likely contribute to the development of novel, efficient, and environmentally-friendly technologies. In our study we identified microorganisms from the environment that are well-adapted to low water quality and adaptable to diverse conditions, and thus having the potential for use in clean technologies.

Conclusion

We found that genera *Bacillus* and *Enterococcus* are effective, tolerant, economical, and environment-friendly for removing $CuSO_4$. The most growth and removal of copper (62 and 87.7%) was observed for the *Enterococcus* strains. The best temperature was 37°C at 0.78 mg L^{-1}, at a contact time of 24 hours. All of the tested metal-resistant bacterial isolates have the potential for use in the removal of Cu, suggesting that bacteria can play an important role in the removal of heavy metals from industrial effluent during enhanced bioremediation of contaminated environments.

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