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ORIGINAL RESEARCH

Effect of aluminum in *Bacillus megaterium* nickel resistance and removal capability

Efecto del Aluminio sobre la Resistencia y la capacidad de remoción de Ni de *Bacillus megaterium*

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ABSTRACT

The increasing water pollution by heavy metals is considered an alarming situation worldwide, due to the adverse impact they cause in ecosystems and human health. Although conventional techniques are available to diminish the metal concentration present in water bodies, they offer disadvantages, like inefficient metal removal, toxic sludge generation, and high operating costs. In contrast, biotechnological approaches may render a viable alternative, since they offer lower environmental impacts and operating costs, and also higher removal efficiencies when metals are present in small concentrations. It has been shown that the simultaneous presence of more than one metal can generate synergistic, additive or antagonistic effects, thus affecting their removal, and it has been previously demonstrated that *B. megaterium* strain MNSH1-9K-1 possesses the ability to remove metals present in liquid and solid wastes. Therefore, the goal of the present work was to study *B. megaterium* MNSH1-9K-1 Ni resistance and removal properties in liquid medium, and to evaluate the variation of these abilities in the presence of another toxic metal, namely Al, which is also commonly found in liquid wastes. To this end, *B. megaterium* was grown in LB medium with the addition of Ni and/or Al at diverse concentrations, and both metal resistance and Ni removal capabilities were assayed by viable count, and Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES), respectively. The results obtained strongly suggest that *B. megaterium* MNSH1-9K-1 presents more susceptibility to Ni than to Al, and that Ni removal is enhanced by the presence of Al.

Keywords: *B. megaterium*, Biotreatment, Metal resistance, Ni removal.

RESUMEN

El incremento en la contaminación de agua por metales pesados es considerada una situación alarmante a nivel mundial, debido al impacto adverso que éstos generan en los ecosistemas y en la salud humana. Las técnicas convencionales para disminuir la carga de metales presentan desventajas, como son: la incompleta remoción de los metales, la producción de lodos tóxicos y los altos costos de operación. En contraste, los enfoques biotecnológicos representan una alternativa, ya que se consideran más amigables con el ambiente, tienen costos de operación menores y la eficiencia de remoción de metales es mayor cuando éstos se presentan en bajas concentraciones. Anteriormente se ha demostrado que la presencia simultánea de más de un metal puede generar efectos sinérgicos, aditivos o antagónicos, afectando así su remoción, y también ha sido reportada la habilidad de *B. megaterium* MNSH1-9K-1 para remover metales. Por ello, el objetivo del presente trabajo fue analizar las propiedades de resistencia y remoción de Ni de la cepa MNSH1-9K-1 en medio líquido, así como evaluar la variación de dichas propiedades en presencia de Al, también considerado como un metal tóxico comúnmente encontrado en los residuos industriales. Para ello, *B. megaterium* fue crecida en medio LB con la adición de Ni y/o Al en diversas concentraciones, analizándose tanto la resistencia como la capacidad de remoción de Ni, por medio de cuenta viable y Espectrometría de Emisión Óptica por Plasma Acoplado Inductivamentente (ICP-OES), de forma respectiva. Los resultados obtenidos sugieren fuertemente que *B. megaterium* MNSH1-9K-1 presenta una mayor susceptibilidad a Ni que a Al, y que la remoción de Ni es promovida por la presencia de Al.

Palabras clave: *B. megaterium*, Biotratamiento, Resistencia a metales, Remoción de Ni.

1. INTRODUCTION

Heavy metals occur as natural constituents of the earth crust. In rocks, they exist as their ores in different chemical forms, from which they are recovered as minerals (Rubinstein & Barsky 2002). However, the increasing contamination of heavy metals into the soil and waters due to human activities has created an environmental alarming situation and a major health concern worldwide, as they cannot be broken down to non-toxic forms, and therefore have long-lasting effects on ecosystems (Hernández *et al*., 1998; Ceribasi & Yetis 2004; Monachese *et al*., 2012). Anthropogenic metal sources include: wastewater arising from informal settlements (Jackson *et al*., 2009), leachates from domestic and industrial landfill sites (Moodley *et al*., 2007), industrial wastes (Kamaldeep *et al*., 2011), disposal of metal-containing industrial effluents (Phuong *et al*., 1998), mining activities (Eisler 1998; Duruibe *et al*., 2007), effluent from storage batteries and automobile exhaust (Verma & Dubey 2003; Dogan *et al*., 2009), and the manufacturing and inadequate use of fertilizers, pesticides, etc. (Tsezos & Volesky 1981; McConnell & Edwards 2008). Through rivers and streams, metals are transported as either dissolved species in water or as an integral part of suspended sediments, and may then contaminate superficial water bodies or seep into the

underground contaminating water from underground sources, particularly wells (Duruibe *et al*., 2007).

Hence, it is necessary to establish efficient methods to minimize or even eliminate metals prior to its discharge to the environment. Conventional approaches to reduce heavy metal contamination in water include: 1) chemical precipitation or flocculation, followed by sedimentation and disposal of the resulting sludge; 2) ion exchange; 3) adsorption by minerals, industrial byproducts, polymeric materials or agricultural wastes; 4) membrane filtration; 5) electrotreatments; 6) evaporation; and 7) photocatalytic processes (Barakat 2011). However, these processes present significant disadvantages, as incomplete removal, production of toxic sludge, high costs due to high-energy requirements, and the need of skilled technicians (Eccles 1999).

Thus, biotechnological processes are an innovative and promising technology available for the removal of heavy metals and its recovery from polluted water and lands, proved as an effective eco-friendly and economically feasible technology (Ameer Basha & Rajaganesh 2014; Coelho *et al*., 2015), due to their low costs and higher efficiency at low metal concentrations, where physicochemical removal methods fail (Mejáre & Bülow 2001; Perpetuo *et al*., 2011).

Among the heavy metals found as water pollutants, Nickel (Ni) and Aluminum (Al) are considered as highly valuable metals, used in numerous industrial processes. Specifically, Ni is employed for the fabrication of alloys, coatings, batteries, and some other uses, such as kitchen wares, mobile phones, jewelry, medical equipment, transport, buildings construction, and power generation (Keim 1990; Cempel & Nikel 2006). Although Ni has biological activity in bacteria at nanomolar concentrations, being an essential component of the enzymes particularly involved in ureolysis, hydrogen metabolism, methane biogenesis and acetogenesis (Bartha & Ordal 1965; Olson *et al*., 2001; Mulrooney & Hausinger 2003), it is considered as a highly toxic metal (Cempel & Nikel 2006), which treatment is important due to their hazardous nature, and even more, its presence in solid and liquid residues represents a valuable source for metal recovery.

It is known that essential and non-essential heavy metal ions at toxic levels bind to cellular structures, causing destabilization of these structures and of biomolecules (cell wall enzymes, DNA and RNA), thus inducing replication defects and cellular malfunctioning (Perpetuo *et al*., 2011). Microorganisms have developed diverse strategies for their survival in heavy metal-polluted habitats, including detoxifying mechanisms such as biosorption, bioaccumulation, biotransformation and biomineralization. Several factors may influence metal uptake, such as type of metal, their concentration and availability, degree of exposure, temperature, nature of the medium, salinity, and the specific metal removal capabilities of the microorganism used (Coelho *et al*., 2015; Dixit *et al*., 2015). Although metal combined effects has been scarcely studied (Crafford & Avenant-Oldewage 2010; Fierros-Romero *et al*., 2016 b), synergistic or additive joint actions of metals have been documented (Enserink *et al*., 1991; Palaniappan & Karthikeyan 2009), and antagonistic interactions may also occur (Kwong & Niyogi 2009).

Previous studies show that the members of the genus *Bacillus* present high tolerance to heavy metals (Yilmaz 2003; Singh *et al*., 2010, 2013; Elsilk *et al*., 2014). However, although *Bacillus* metal resistance has been documented for different species (Kamala-Kannan & Jae Lee 2008), few experiments have been performed regarding the effect on resistance of a combination of metals in liquid medium (Margaryan *et al*., 2013; Fierros-Romero *et al*., 2016 b). In particular, it has been reported that *B. megaterium* possesses an

intrinsically high level of resistance to hostile conditions (Salgaonkar *et al*., 2013; Pal *et al*., 2014) and metal exposition (e.g. Hg and Ni) (Narita *et al*., 2003; Rajkumar *et al*., 2013), among other preeminent properties like its morphological and molecular characteristics, which confer this microorganism with the capacity to produce metabolites of industrial relevance (Vary *et al*., 2007), and the ability to accumulate and chelate metals (Sharma *et al*., 2016). Because of this latter capacity, *B. megaterium* has been used for the biological treatment of high metal content wastes like phosphogypsum waste (Stefanescu 2015) and spent catalysts (Gómez-Ramírez *et al*., 2014; Arenas-Isaac *et al*., 2016).

Among the few previous reports regarding *Bacillus* resistance in the presence of metal mixtures, results have shown that *Bacillus megaterium* strain MNSH1-9K-1 possesses the ability to tolerate up to 200 ppm of each Ni and V contained together in liquid media (Fierros-Romero *et al*., 2016 b). Also, it was observed that this strain presents the capability of removing 15.38 ± 1.30 ppm of Ni, and 32.06 ± 1.08 ppm of V from PHGII liquid medium with 200 ppm of each Ni and V, corresponding to a removal of 7.69 ± 0.65 % of Ni and 16.03 ± 0.54 % of V (Fierros-Romero *et al*., 2016 b).

Thus, the current study analyzed the potential of *B. megaterium* strain MNSH1-9K-1 to remove Ni from liquid medium at different concentrations, as well as the changes in Ni resistance and removal ability of this strain in the presence of another toxic metal: Al. The results presented may contribute to the knowledge of how the metal resistance and removal abilities of a microorganism may vary in the presence of more than one metal, which may be a common situation in the case of contaminated waters.

2. MATERIALS AND METHODS

2.1. Bacterial strain and growth conditions

Bacillus megaterium strain MNSH1-9K-1 (GenBank accession number KM654562.1) was used for this study, which isolation from a high metal content site has been previously described (Arenas-Isaac *et al*., 2016). LB liquid medium was used for microbial growth at 200 rpm and 37°C. Inoculums were prepared overnight, and microbial density was adjusted to an $O.D._{600\ nm} = 0.1$ to begin experimentation.

2.2. Metal resistance

Cultures were grown for 48 hours in 125 ml Erlenmeyer flasks containing 10 ml of LB liquid medium and different concentrations of Al, Ni, or both metals, in the ranges of 25 to 200 ppm, provided as $AlCl_3$ and $Ni(NO_3)_2 \cdot 6H_2O$, respectively, as specified for each experiment. Also, samples without metals were included, as controls. Viability was assessed by taking a 100 µl aliquot from each culture, and serial 10-fold dilutions were prepared in phosphate-buffered saline (PBS) buffer (Nicholson & Setlow 1990), and plated on LB solid medium. Finally, LB plates were incubated for 24-48 h at 37 °C to perform colony counting.

2.3. Metal removal from liquid medium

Experimental sets were prepared in 125 ml Erlenmeyer flasks containing LB liquid medium supplemented with different concentrations of one or both metals [provided as $AlCl_3$ and $Ni(NO_3)_2 \cdot 6H_2O$], to 10 ml final volume. Also, samples without metals were included, as controls. After 48 hours of growth at 37°C and 200 rpm, the liquid phase of each sample was filtered using a cellulose acetate syringe filter (Alltech, Deerfield, IL, USA). Subsequently, the samples were acid digested. For this purpose, 1 ml samples were placed in cylindrical silicon carbide vials, and 6 ml of concentrated HNO_3 and 2 ml of concentrated HCl were added, and samples were digested in a microwave reaction system (Multiwave PRO, Anton Paar), using an HF100 rotor. Digestion conditions were: 600 W for 6 vessels, 40 bar, 210-240°C, with pRate of 0.3 bar sec^{-1}, ramp 15 min, hold 15 min, and cooling 15 min. Afterwards, 20 ml of deionized water was added to the cylindrical vial, and the supernatant was collected and filled up to 100 ml with deionized water. Metal analysis was performed at 231.604 nm for Ni and 396.152 nm for Al by ICP-OES (Varian Model 710-ES). Metal concentrations were calculated based on a calibration curve covering 0.1-10 mg kg^{-1}, using a commercial standard (High-Purity, cat. # ICP-200-7-6) (Fierros-Romero *et al*., 2016 b).

2.4. Statistical analysis

Basic statistical parameters and analysis of variance (ANOVA) were performed using the commercial statistical software OriginPro 9.0. Differences with P values of ≤ 0.05 were considered statistically significant.

3. RESULTS

3.1. Effect of Al and Ni on cell resistance

To investigate the resistance of *B. megaterium* MNSH1-9K-1 to different concentrations of Ni and Al, the strain was grown at 37 °C and 200 rpm for 48 hours in 125 ml Erlenmeyer flasks containing 10 ml of LB liquid medium, and diverse Al or Ni concentrations. As it can be observed in Fig. 1, the median lethal concentration (LC_{50}) and the 90 lethal concentration (LC_{90}) for Al were found when adding 147.25 ± 1.77 ppm and 150.50 ± 0.71 ppm, respectively. In the other hand, the LC_{50} for Ni is achieved with 75.5 ± 3.53 ppm, and LC_{90} is caused by 96.25 ± 1.77 ppm of the metal.

To investigate the resistance of *B. megaterium* MNSH1-9K-1 to different concentrations of a Ni-Al mixture, the strain was exposed to diverse concentrations of both metals, in the mixtures indicated in Fig. 2. As could be expected, cell viability loss was observed in every condition tested, in comparison to the control without metals. A 20-fold decrement in resistance was observed when the strain was exposed to mixtures were Al prevailed in proportions of 125/5 (25:1) and 100/10 (10:1) Al/Ni ppm, respectively, or when Ni concentration increased to 75 ppm, in the mixture of 25/75 Al/Ni ppm. Also, it was shown that a mixture of 50/50 Al/Ni ppm caused a similar fold decrement in cell viability. When cells were exposed to 75/25 Al/Ni ppm, cell viability presented an 80-fold decrement, and the highest cellular death was obtained in the cultures that were exposed to the combined effect Al/Ni of 10/100 ppm (1:10), and 5/125 ppm (1:25), exhibiting up to 170-fold and

500-fold viability loss, respectively. Samples with only Al or Ni in 175 ppm were also included in Fig. 2 to compare cell viability, showing a 22-fold, and 167-fold lessening in this parameter, respectively.

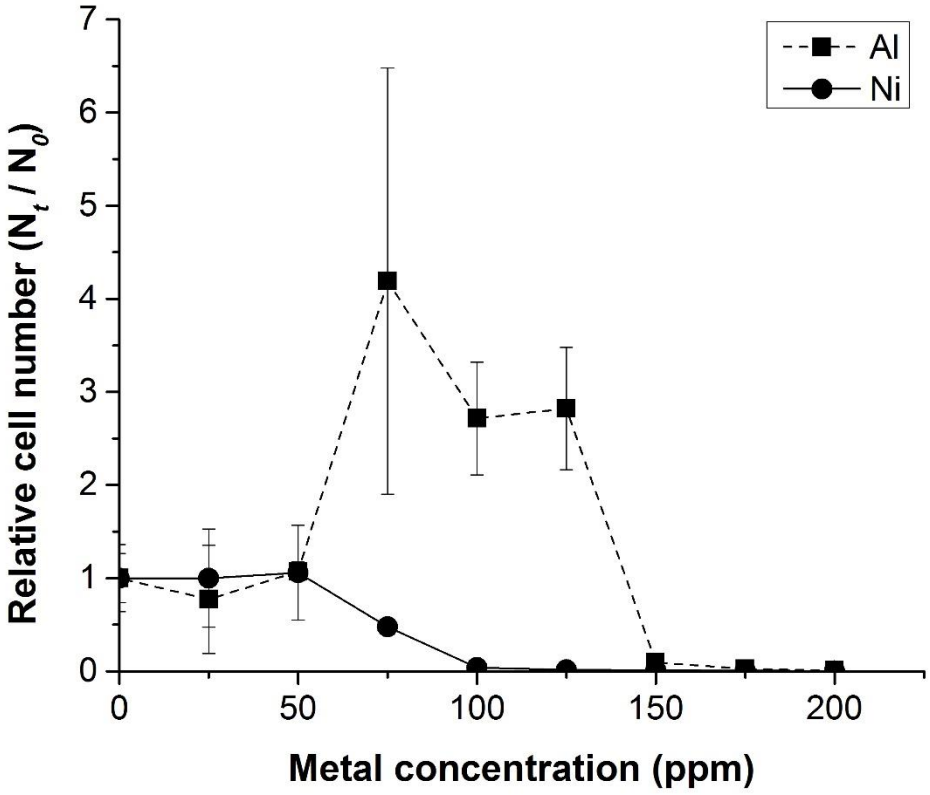

Fig. 1. Resistance of *B. megaterium* MNSH1-9K-1 to Al and Ni. The strain was grown in LB liquid medium in the presence of Al or Ni at the concentrations indicated (0 to 200 ppm), and the results were normalized to those from the control (without metal). Data are presented as averages ± standard deviations (n = 4).

3.2. Assessment of *B. megaterium* metal removal ability

In order to evaluate the ability of *B. megaterium* to remove Ni from liquid medium containing different concentrations of this metal, the strain was grown in LB liquid medium under the conditions described in Materials and Methods, and diverse Ni concentrations were added to the samples. The results showed that *B. megaterium* MNSH1-9K-1 was unable to remove Ni under any of the conditions tested (data not shown).

It has been documented that Al and Ni may appear together as contaminants of liquid and solid wastes from the metal-mechanic sector, automotive industry, petroleum refining processes, among other anthropogenic activities. So, to further investigate if MNSH1-9K-1 was able to remove Ni in the presence of another toxic metal, namely Al, the strain was grown in the presence of both metals, at the same mixture concentrations used to evaluate resistance (Fig. 2). The data shown are the results of the subtraction of the concentration

(ppm) of each element at t_0 minus the remained Ni concentration (ppm) at the end of experimentation. Also, Ni removal in the abiotic control (0.139 ± 0.094 ppm) was subtracted from the data to obtain final removal at each condition tested.

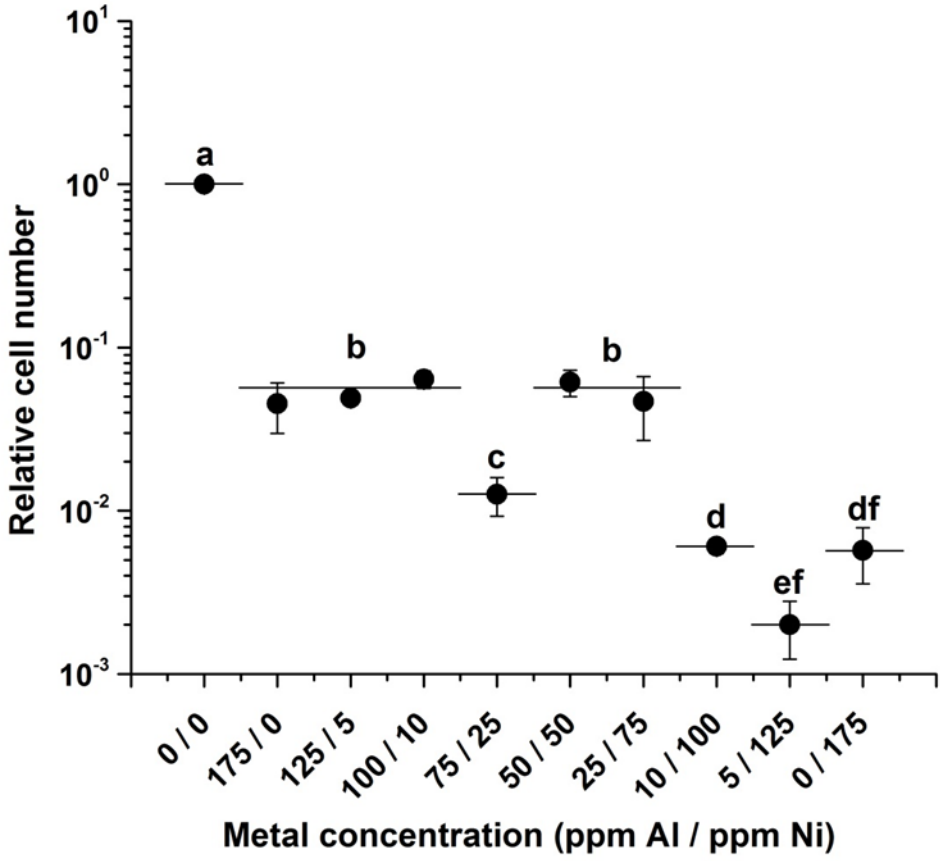

Fig. 2. Resistance of *B. megaterium* MNSH1-9K-1 to Al/Ni mixtures. The strain was grown in LB liquid medium in the presence of Al and Ni, and the results were normalized to those from the control (without metals). Data are presented as averages ± standard deviations (n = 4).

Surprisingly, *B. megaterium* was able to remove Ni when Al was present (Fig. 3), and this Ni removal extent tended to increase with the diminishing concentration of Al, coupled with Ni increase, from 1.65 ± 0.15 in 125/5 (25:1) to 7.12 ± 0.85 in 25/75 (1:3) Al/Ni ppm, until the highest Ni uptake of 10.75 ± 1.91 ppm was reached in the mixture of 10/100 (1:10) Al/Ni ppm, and then lowering again to 4.20 ± 1.24 in 5/125 (1:25) Al/Ni ppm.

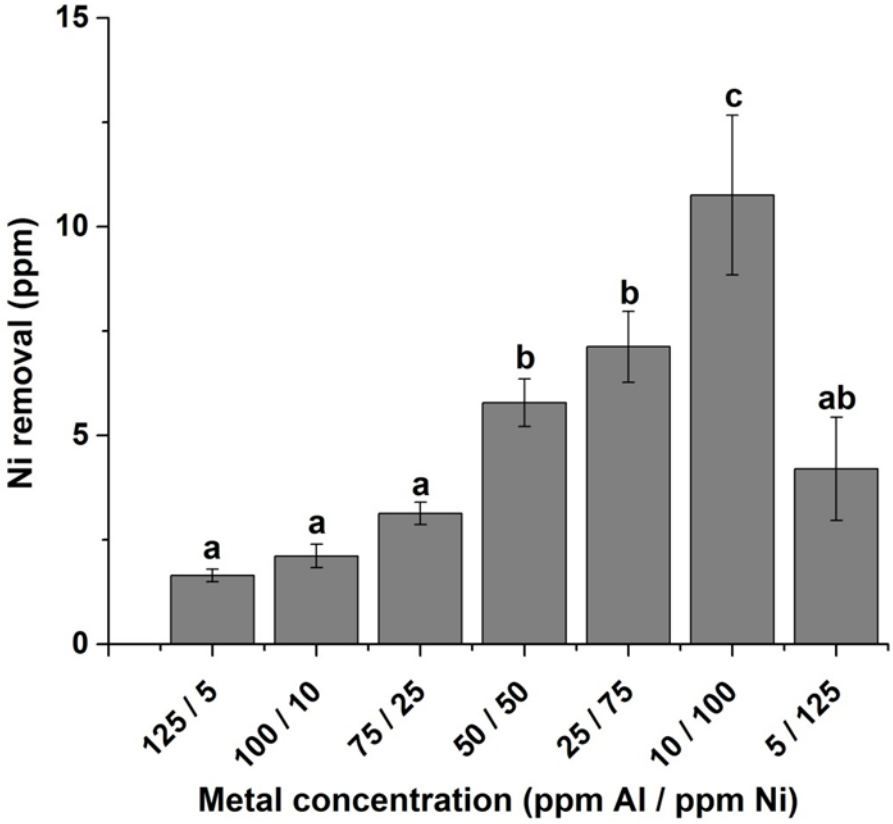

Fig. 3. Removal of Ni in the presence of Al. *B. megaterium* MNSH1-9K-1 was grown in LB liquid medium in the presence of different Al/Ni mixtures. Data are presented as averages ± standard deviations (n = 2), and lowercase letters indicate groups of data that were not significantly different by ANOVA ($P > 0.05$).

4. DISCUSSION

The results obtained in this study strongly suggest that *B. megaterium* is more susceptible to Ni than to Al (Fig. 1), as can be observed by comparing LC_{50} and LC_{90} data, which demonstrate a 2-times more sensitivity of the microorganism to Ni. Evenmore, when cells were exposed to 175 ppm of each metal, MNSH1-9K-1 showed a 7.5-fold decrement in cell viability with Ni, compared to the survival observed with Al. This Ni > Al toxicity is in accordance with previous reports that show that Ni may be more toxic for biological systems than Al (Caicedo *et al*., 2008), since Ni is considered a hazardous metal (Amer 2002), as this metal has been related to 362 alterations in DNA repair mechanisms, epigenetic effects, and carcinogenesis (Macomber & Hausinger 2011).

In the case of Al toxicity, its ions may bind to diverse cellular components, altering lipid-protein interactions, modifying the cellular transport activity, and blocking surface potential. Also, once inside the cell, Al ions can alter the metabolism by binding to enzymes or to enzyme substrates (Garcidueñas-Piña & Cervantes 1996).

So when strain MNSH1-9K-1 was exposed to Ni/Al mixtures, it was also evident a more pronounced viability loss as Ni concentration increased in samples (Fig. 2), showing MNSH1-9K-1 up to 24-times more sensitivity to a 5/125 (1:25) Al/Ni ppm than to a 125/5 (25:1) Al/Ni ppm mixture. It has been documented that the *B. megaterium* genome contains open reading frames of genes involved in stress responses, including oxidative stress, osmotic stress, heat shock, and detoxification (Liu *et al*., 2011), which activations, at least in its most studied partner *B. subtilis*, overlap between general stress responses and more specific pathways (Young *et al*., 2013), and are highly intertwined under the control of central regulators like σ^B, PerR, and extracytoplasmic sigma factors (Helmann 2002; Helmann *et al*., 2003; van der Steen & Hellingwerf 2015).

The differences in cell survival when *B. megaterium* is exposed to diverse Ni/Al mixtures may be due to the diverse molecular mechanisms activated under certain metal stress conditions, directly related to the specific combination of Ni and Al concentrations present in the samples. As has been previously suggested, *Bacillus* cells may regulate their responsive systems depending on the rate and type of stress they are suffering, and even more, the microorganism may activate diverse pathways in response to stress increase or continuity (Young *et al*., 2013). For example, it was recently demonstrated that σ^B response is overlaid on the more specific stress responses, with a magnitude that increases with the speed at which salt and ethanol stress levels increase (Young *et al*., 2013).

With respect to *B. megaterium* Ni removal capability, reports have shown that this microorganism possesses high metal sorption capacity (Monachese *et al*., 2012), and Ni specific response genes have been identified in MNSH1-9K-1 by a polymerase chain reaction (PCR) approach (Fierros-Romero *et al*., 2016 a). However, these both mechanisms may not explain the results shown in the current study, since Ni uptake was only observed when Al was also present in the system. Also, it is very interesting to note that higher Ni removal efficiencies took place when Ni was present at 5 and 10 ppm, showing 25% and 20% removal efficiencies, respectively. At higher Ni concentrations, these efficiencies diminished to around 10%, but nevertheless, the highest removal concentration was observed when Al/Ni mixture was 10/100 ppm, where also the specific removal rate was calculated to be of 14,190 mg of removed Ni per mg of dry cell weight.

Some metals, like Fe, Zn, Cu, and Mg, Mn, and Ca, are known to be widely used in biochemical processes in trace concentrations, being estimated that at least one-third of all proteins require metals (Waldron & Robinson 2009); whereas other metals like Ni, Co, Se and Mo are only used by some organisms (Solioz *et al*., 2011). In the other hand, for toxic metals without a known function in biology, as Pb, Ag, Cd, Al, As, Cs, Cr, Hg, or Pb (Tchounwou *et al*., 2012), specialized defense mechanisms have evolved in many bacterial species (Waldron & Robinson 2009). Although it is known that metal removal systems encountered in microorganisms are of varying specificity (Gadd 1990), the interaction of specific and non-specific heavy metal homeostasis mechanisms in bacteria are not clearly understood yet. Thus, it is possible that the observed Ni uptake ability may be the result of a non-specific metal removal mechanism that is enhanced in *B. megaterium* by Al.

In conclusion, this study shows that *B. megaterium* MNSH1-9K-1 presents a more accentuated susceptibility to Ni than to Al, and it is possible that besides the specific Ni removal mechanisms previously reported in this microorganism (Eppinger *et al*., 2011; Fierros-Romero *et al*., 2016 a, b), that may be activated under other precise conditions different from the ones tested during the current analysis, the strain MNSH1-9K-1 possesses interesting non-specific mechanisms that promote Ni uptake under stress

conditions promoted by the presence of Al. In this regard, further –omic studies may be important to elucidate the Ni uptake mechanisms of *B. megaterium*, and even more, identify the bacterial pathways implicated in its capability to remove metals when present in mixtures. Also, further analysis may be important to evaluate the combination of physicochemical and biotechnological methods using *B. megaterium*, in order to improve the efficiency of current conventional waste water heavy metal removal techniques, for example, when low metal concentrations are present.

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CONFLICT OF INTEREST

All the researchers listed as authors of the current study declare that there is no conflict of interests regarding the publication of this manuscript.

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