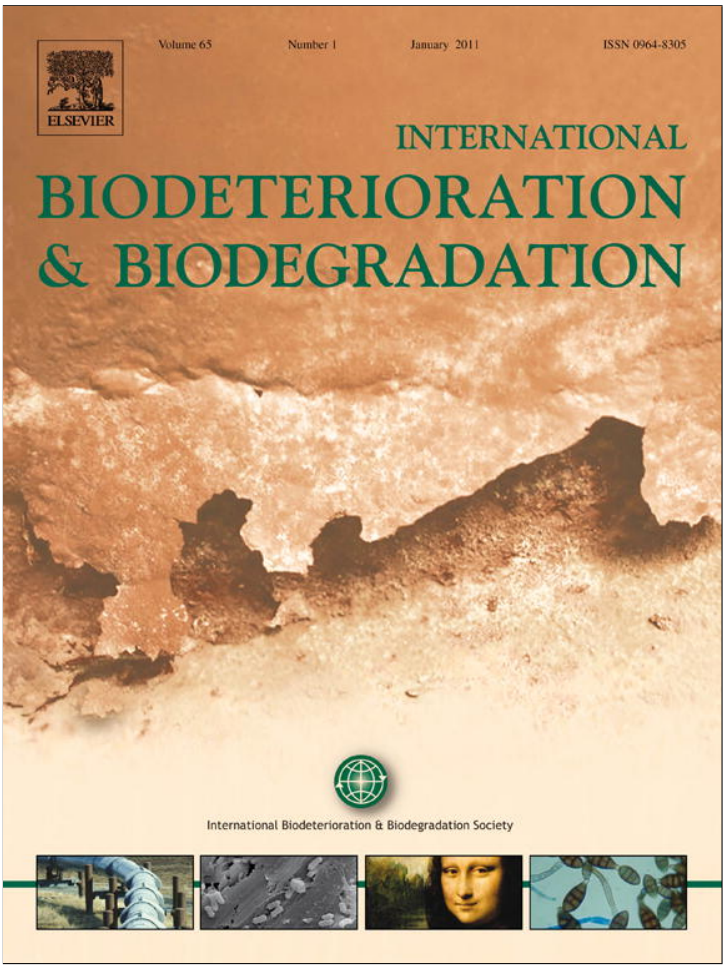

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Review

Indigenous microorganisms as potential bioremediators for environments contaminated with heavy metals

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ABSTRACT

Heavy metal pollution is one the most serious environmental problems facing our planet today, and immediate solutions are needed. Heavy metals such as copper (Cu) and chromium (Cr) play an important role as trace elements in biochemical reactions, but these metals are toxic at higher concentrations. In our region, mining and industrial activities have led to large-scale copper contamination in the environment. All organisms have homeostasis mechanisms for this metal, but when these controls fail or are exceeded several toxicological processes can develop.

Problems involving Cr contamination are related to the fact that Argentina is an important world producer of leather. A chromium compound is used as a tanning agent, which has resulted in severe contamination near tanneries, with a mix of Cr(III) and Cr(VI). At present, the conventional technologies used to remove heavy metals from the environment involve physicochemical processes, which are costly and require large amounts of energy and specialized equipment. However, microbe-based removal is now considered to be an effective alternative method to the conventional processes and is receiving greater levels of interest for potential uses in bioremediation.

This review discusses the toxic effects of Cu and Cr on the environment and on human health, as well as possible approaches for bioremediation of these metals using native microbes from our region.

Contents

1. Introduction

Industrial and mining activities are important for economic development, especially in many developing countries. However,

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these activities also represent the main sources of heavy metal contamination.

The term "heavy metals" is often used to refer to a group of metals and semi-metals that have been associated with contamination as well as potential human toxicity or ecotoxicity. However, the use of this term is inconsistently applied in the scientific literature, which can lead to general confusion, because of a tendency to assume that all "heavy metals" have highly toxic or ecotoxic properties. Furthermore, the term has never been formally defined by any authoritative body such as the IUPAC (International Union of Pure and Applied Chemistry). The most longstanding definition of heavy metals is based upon the density of the elemental form of the metal, with heavy metals classified as those elementals which have metallic characteristics and densities above 7 g ml^{-1}. Over the years, this definition has been modified by various authors, but these new definitions remain relatively inconsistent since no relationship can be found between density and any of the various physicochemical concepts that have been used to define "heavy metals", or the toxicity or ecotoxicity attributed to them. Semi-metals such as selenium (Se) and arsenic (As) also belong to this group. These are often found in the environment with metals, and appearances and physical properties similar to metals but with chemical behavior that is similar to non-metals (Duffus, 2002).

The fact remains that some heavy metals can be toxic to a variety of life forms. Others are considered essential in certain amounts, but they can also become toxic at higher doses. For that reason, some researchers have proposed that heavy metals be split into two categories, based upon a physiological point of view: essential, but harmful at high concentrations (e.g., Fe, Zn, Cu, Mn, Co, Ni, Cr) and non-essential or toxic (e.g. Hg, Cd, Pb) (Valls and Lorenzo, 2002). Heavy metal toxicity is also highly dependent upon the particular form in which the element is present in the system, known as its species: electronic or oxidation state, and/or complex or molecular structure (Ramírez-Ramírez et al., 2004). It is important to note that the accumulation of high concentrations of heavy metals, even those that are essential, also affects biological diversity and can cause serious ecological problems including negative impacts on soil fertility (Ashraf and Tasneem, 2007). When such metals accumulate in aquifers or surface waters, they can cause disease and even death of livestock along with associated economic losses. Exposure can also travel up the food chain, exposing human populations to routine consumption of unknown quantities of pollutants, thereby putting human health at risk as well (Soares et al., 2003; Malik, 2004; Machado et al., 2010).

Development of suitable methods for cleaning up contaminated environments continues to be an important topic in terms of environmental restoration and protection. In many developing countries, the removal of heavy metals is performed mainly through the use of physicochemical processes, which are very expensive and require large amounts of energy and specialized equipment (Beleza et al., 2001). The overall situation has become even more critical in developing countries, where legislation tends to be weak and water treatment facilities poor. As a result, it has become critical to search for new techniques to reduce heavy metal concentrations to acceptable environmental levels at manageable costs, in order to protect human health. Some microorganism-based bioremediation techniques have been developed to exploit the potential of certain taxa to degrade and detoxify particular contaminants. These biological systems are less affected by environmental extremes than physicochemical methods, and they also have the perceived advantage of being more cost-effective.

In northwestern Argentina, contamination with Cu and Cr in particular constitutes one the region's most serious environmental problems, and immediate solutions are therefore demanded. Both of these metals have been implicated in the pathogenesis of a number of degenerative diseases, including those in which excessive formation of reactive oxygen species (ROS) has been suggested (Colin, 2012). This review first summarizes our current understanding regarding the toxic effects of heavy metals, particularly Cu and Cr, and then focuses on potential approaches for microbial remediation of these metals using native microbes from our region.

2. Biological functions and copper toxicity

Copper is a very versatile heavy metal, able to cycle between two redox states, Cu(II) and Cu(I), with Cu(I) being highly unstable. Copper performs a myriad of functions in biological systems, making it an element that is essential for the existence of all currently known life forms. In humans, for example, copper is third in abundance amongst the essential heavy metals, after iron and zinc (Barceloux, 1999). All living organisms require copper as a catalytic cofactor for basic biological process such as respiration (Puig and Thiele, 2002). Some landscapes have been found to be Cu deficient, limiting the survival and reproduction of plants and/or animals. However, the same chemistry that makes copper essential also makes it potent cytotoxic when homeostatic controls on Cu fail (Georgopoulus et al., 2002a). Homeostasis is the property of a system that regulates its internal environment and tends to maintain a stable, constant condition. Both prokaryotic and eukaryotic cells possess homeostatic mechanisms to regulate the concentration of copper ions and minimize the toxic effects produced by excessive levels. Therefore, when this mechanism becomes saturated, the toxic effects are produced by the metal. Noticeable effects have been reported for exposure to higher concentrations of copper, including gastrointestinal effects (nausea, vomiting, abdominal pain), which tends to occur in patients with whole blood Cu levels above 2.9 mg ml^{-1}. Whole blood copper levels in excess of 7.9 mg ml^{-1} have been associated with jaundice, renal dysfunctions, and toxic shock (Georgopoulus et al., 2002b). Furthermore, redox reactions arising from transition metals such as Cu are the principal chemical origin of radicals (often referred to as free radicals) and reactive oxygen species (ROS). These chemically reactive species have been implicated in the pathogenesis of neurological conditions such as Alzheimer's disease (Manton et al., 2004; Leutner et al., 2005; Dumont and Beal, 2010), which is a degenerative pathology characterized by accumulation of an amyloid in the brain, specifically a copper zinc-metalloprotein that aggregates and becomes redox-active in the presence of excessive amounts of these metals. Heavy metals also play a key role in atherosclerosis (Arslan et al., 2010), which constitutes the primary cause of cardiovascular disease. In this case, ROS generation causes oxidation of low density lipoproteins (LDLs), damaging the vascular wall and stimulating macrophage uptake and the formation of foam cells, which in turn results in plaque formation in the arteries (Colin, 2012).

The many uses of Cu in industrial applications have led to its widespread presence in the environment, including in soils, silts, water sources, and wastewater. Copper cannot be destroyed and therefore tends to accumulate in soils, plants, and animals, potentially increasing its concentrations in higher levels of various food chains (Georgopoulus et al., 2002a). In Argentina, the legal limit permissible for Cu in drinking water is 1 mg l^{-1}, whereas the European Union limit is significantly higher at 3 mg l^{-1} (Georgopoulus et al., 2002b). In our region (Tucumán, Argentina) the main hydrographic feature is the Salí River, which crosses the entire province. The Salí River has an influence area of 60,000 ha and also receives effluents from several local industrial sources. Analysis of sediment samples collected from the river has indicated the presence of inorganic pollution as Cu and other heavy metals as

well as organic pollution (Romero et al., 1997). Moreover, there is a Filter Plant for Cu processing from a copper mine near the river. Its effluents are discharged in a drainage channel that provides water used for sugarcane irrigation on the sides of the channel. The remaining water flow ends up in the Frontal Hondo dam reservoir in a neighboring province (Benimeli et al., 2003). Because of these processes, the problem of Cu contamination is currently one the most serious environmental problems facing our region, and diverse approaches for cleaning up copper-contaminated environments are being evaluated.

3. Biological functions and chromium toxicity

Chromium is the seventh most abundant element on earth and occurs in diverse oxidation states, occurring naturally in soils, rocks, and living organisms (Poljsak et al., 2010). As mentioned above, biological effects associated with Cr exposure are diverse, since the nature of these effects depends upon the metal speciation and the particular organism tested. Cr(VI) and Cr(III) are ecologically important because they are the most stable oxidation species in the natural environment (Cefalu and Hu, 2004). Cr(III) is also an essential trace element that is well-known for its specific role in the maintenance of normal carbohydrate metabolism in mammals and yeasts (Debski et al., 2004). A large body of literature related to research involving both experimental animals and human subjects also indicates that chromium is an essential element involved in the normal action of insulin (Ghosh et al., 2002; Althuis et al., 2002; Khamaisi et al., 2003; Rajpathak et al., 2004). It has also been suggested that this ion is involved in the tertiary structure of proteins and the composition of cellular RNA and DNA (Gulan Zetic et al., 2001; Zayed and Terry, 2003). However, at high concentrations, Cr(III) has been shown to have negative effects on cellular structures. Cr(VI), on the other hand, is more mobile and more soluble in water than Cr(III). It is also less chemically stable and more bioavailable, due to its high permeability of biomembranes (Megharaj et al., 2003). Cr(VI) is always toxic to living organisms and has been listed as a priority pollutant and a human carcinogen by the US Environmental Protection Agency. In vivo studies have revealed that Cr(VI) is approximately 100 times more toxic (Beleza et al., 2001) and 1000 times more mutagenic than Cr(III) (Czakó-Vér et al., 1999).

Chromium is also one of the most widely used metals in a variety of industrial processes, such as steel production, wood preservation, leather tanning, metal corrosion inhibition, and production of paints and pigments (Baldi et al., 1990). Industrial effluents containing Cr compounds are released directly or indirectly into natural water sources, usually without proper treatment, resulting in anthropogenic contamination of natural environments (Viti et al., 2003; Cefalu and Hu, 2004; Cheung and Gu, 2007). Conventional treatment processes for Cr detoxification generally involve aqueous reduction of Cr(VI) by a reductant and subsequent pH adjustment to the neutral range in order to precipitate the less soluble Cr(III). However, this process requires large amounts of chemicals and energy and therefore is often not economically feasible. Because of this, new alternatives for Cr(VI) removal from contaminated environments are being continually evaluated.

4. Microbial remediation as an emerging technology

In more economically developed countries, removal of heavy metals is typically performed using traditional physicochemical methods such as oxidation and reduction, chemical precipitation, filtration, electrochemical treatment, evaporation, ion-exchange, and reverse osmosis. As mentioned above, these processes are very expensive and may also have several other disadvantages, such as unpredictable levels of metal ion removal, high reagent requirements, and the generation of toxic sludge, which requires extreme caution in disposal because of the risk of secondary environmental pollution. These disadvantages are more pronounced when the metal concentrations being removed are low. Moreover, conventional remediation technologies require that treatments for removal of contaminants take place at a separate treatment facility (ex-situ technologies). As a result, for better protection of human health it remains important to develop new techniques for reduction of heavy metal concentrations to acceptable environmental levels, but at more manageable costs.

The remediation of contaminated environments using biological methods, known as bioremediation, offers high specificity in the removal of particular heavy metals of interest while also offering operational flexibility. Bioremediation technologies also sometimes involve in situ treatment of contaminants at the actual location of the contamination. These approaches are currently classified into three categories: (i) bioattenuation, which is the method of monitoring the natural progress of degradation to ensure that contaminant concentration decreases with time; (ii) biostimulation, where natural biodegradation or biotransformation is stimulated with nutrients, electron acceptors, or substrates; and (iii) bioaugmentation, which is a way to enhance the biodegradative or biotransforming capacities of contaminated sites by inoculation with microorganisms that possess the desired catalytic capabilities (Iwamoto and Nasu, 2001). Microorganisms in general can degrade numerous environmental pollutants without producing toxic intermediates (Kothe et al., 2005). In the case of heavy metals, main mechanisms of microbial remediation can include active transport, mediated by efflux pumps, intra- and extracellular sequestration, enzymatic transformation to other, less toxic chemical species by redox reactions, methylation, or alkylation/dealkylation, and/or reduction in the sensitivity of cellular targets to metal ions (Gadd, 2001). Soil bioremediation constitutes a special challenge because of the heterogeneity of soils and sediments, and also because well-adapted microorganisms are needed to bioremediate in specific environments (Tabak et al., 2005). It is generally assumed that exposure to metals leads to the establishment of a tolerant or resistant microbial population (Viti and Giovannetti, 2001).

It is clear that a more lucid understanding of the mechanisms of metal toxicity on living cells may lead to the development of novel technologies to mitigate such toxicity. To this end, microorganisms are useful models for the study of various aspects of oxidative stress at the biochemical, molecular, and cellular levels. This is because the natural stress factors, as well as the damage caused by oxidative stress to nucleic acids, proteins, lipids, and other cell components, are very similar in all types of organisms. This is also true because at all levels of cell organization, the principles of cellular defense against oxidative stress are similar, for example, the nature and role of antioxidants and antioxidative enzymes that act to decrease ROS concentrations, the repair of damaged macromolecules, and the elimination of irreparable proteins (Poljsak et al., 2010). Many microorganisms have also evolved other complex mechanisms to counteract the toxic effects of metals (Silver and Phung, 1996). Thus bacteria, yeasts, algae, and fungi have been located in and isolated from sites contaminated with heavy metals, and are now under study as possible bioremediators of environmental contamination (Machado et al., 2008; Sharma and Fulekar, 2009; Ray and Ray, 2009; Ruta et al., 2010). The next section places particular emphasis on the potential use of metal-resistant microorganisms that are indigenous to contaminated areas for the development of Cu and Cr bioremediation strategies:

4.1. Copper-resistant Amycolatopsis sp.

The screening and characterization of metal-resistant microorganisms is important for the development of novel bioremediation processes. Actinobacteria are Gram-positive, free-living saprophytes found predominantly in soils (Kavitha and Vijayalakshmi, 2007; Mohan and Vijayakumar 2008), and many of these have been identified as possible heavy metal bioremediators. Because of their filamentous nature, actinobacteria are considered as an intermediate group between bacteria and fungi (Pandey, 2004). Their metabolic diversity, particular growth characteristics, mycelial form, and relatively rapid ability to colonize selective substrates, make them well-suited for use as agents for bioremediation of inorganic and organic compounds. Amoroso et al. (1998) have also reported that metal resistance and biosorption ability may be widespread among actinobacteria growing in contaminated environments.

There is an abundance of information available on Cu-resistance mechanisms in Gram-negative bacteria (Munson et al., 2000), but there is still insufficient information on these mechanisms in actinobateria. Our research group has therefore worked on this subject, focusing our efforts on Cu-resistant actinobateria populations. Initially, sediment samples were collected from the El Cadillal water reservoir (non-contaminated area), and from a drainage channel that receives effluents from a copper filter plant (contaminated area) (Albarracín et al., 2005). Both sites are located in Tucuman, Argentina. Qualitative screening assays (Fig. 1) showed that 100% of the isolated microorganisms from the contaminated area were resistant to up to 80 mg l^{-1} of $CuSO_4$, while actinobacteria strains isolated from non-contaminated areas were sensitive. Subsequent semi-quantitative assays in agar culture showed that all but one of the strains isolated from the contaminated sediments in the drainage channel were resistant up to the highest Cu concentration tested (1000 mg l^{-1}). However, the strains isolated from the non-contaminated site showed tolerance only up to $CuSO_4$ concentrations of 200 mg l^{-1} or 400 mg l^{-1} (Fig. 2). Based upon these results, Albarracín et al. (2005) selected the most resistant actinobacteria strains, initially named AB0. Measurement of residual Cu in the supernatant of minimum medium (MM) revealed a useful diminution of copper by this strain.

Later AB0, taxonomically named as *Amycolatopsis tucumanensis* DSM 45259 (Albarracín et al., 2010a), showed a growth inhibition of 32% in batch culture during 6 days of incubation in the presence of 39 mg l^{-1} Cu. Measurement of residual Cu indicated a useful reduction of 71.2% at this time (Albarracín et al., 2005). Subcellular fractioning assays also showed that 40% of the retained Cu was associated with a produced exopolymer (Albarracín, 2007) (Fig. 3),

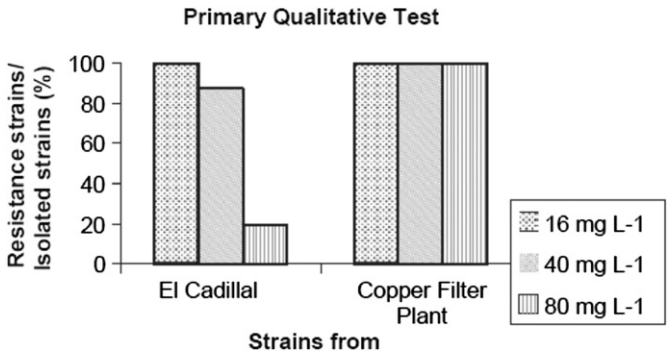

Fig. 1. Qualitative copper resistance of actinomycete strains isolated from drainage channel (19 strains) and a water reservoir "El Cadillal" (31 strains) (Albarracín et al., 2005).

but mainly within the cells (Albarracín et al., 2008) (Fig. 4). These last authors hypothesized that the Cu-bioaccumulation displayed by the *A. tucumanensis* DSM 45259 phenotype may be related to the presence of a copper P-type ATPase, as has been observed in other resistant microorganisms (Solioz and Vulpe, 1996). In order to verify this, specific oligonucleotides for targeting genes coding for Cu P-Type ATPases that could be involved in the Cu uptake ability of this strain were constructed. A 607 bp DNA fragment was amplified and sequenced from *A. tucumanensis* DSM 45259. Interestingly, BLAST search analysis showed 71% protein homology of the deduced sequence with a putative cation-transporting ATPase from *Nocardia farcinica,* and 65% with a Cu-translocating ATPase from *Mycobacterium flavescens*. This was the first report of the presence of Cu P-type ATPase genes in the genus *Amycolotopsis*.

Monitoring was also performed of the growth and viability of *A. tucumanensis* DSM 45259 inoculated in sterile soil microcosms (SM) with 80 mg of Cu kg^{-1} of soil (SM80b). Efficiency of the bioremediation process was confirmed using *Zea mays* as a bioindicator (Albarracín et al., 2010b). Soil microcosms not experimentally polluted with Cu were used as a control (SM20b). In both cases, maximum growth (2.5×10^9 CFU g^{-1} of soil) was obtained after 7 days of incubation. Moreover, the Cu bioimmobilization ability of *A. tucumanensis* DSM 45259 in soils was assessed by measuring the bioavailable Cu in the soil solution extracted from polluted soil using chemical and physical methods. Copper levels in this soil solution were found to be 31% lower compared to the solution from the non-bioaugmented soil.

Since Cu is a redox-active metal, which acts as a catalyst in the formation of ROS, Dávila Costa et al. (2011) compared the effects of Cu on the growth, viability, morphology, and antioxidation ability of the novel Cu-resistant strain *A. tucumanensis* DSM 45259 vs the Cu-sensitive *Amycolatopsis eurytherma*. Interestingly, the increase in ROS production in the former, from the basal level to the stress conditions, was found to be less than in the Cu-sensitive strain. Also, in the presence of Cu, *A. eurytherma* suffered inexorable morphological alteration while *A. tucumanensis* DSM 45259 remained unaffected. Also the levels of the antioxidant enzymes superoxide dismutase (SOD) and catalase (CAT), as well as the levels of metallothioneins (MT), were all found to be greater in *A. tucumanensis* DSM 45259 than in *A. eurytherma*.

Finally, Achín Vera (2008) reported that *A. tucumanensis* DSM 45259, in the presence of Cu(II), increased their reduction ability of Cr(VI) from a liquid bimetal system [Cu(II) + Cr(VI)]. In this connection, studies performed with cell-free extracts of *Bacillus* sp. showed that Cr(VI) was reduced by a chromate reductase which was stimulated by the presence of Cu(II) under aerobic conditions using NADH as an electron donor (Camargo et al., 2003). Soils and sediments typically present problems of co-contamination with various heavy metals; therefore these results suggest that this strain could be highly effective for development of a microbial remediation process for sites co-contaminated with both metals. However, further studies may still be required to confirm the potential of *A. tucumanensis* DSM 45259 for Cr(VI) removal.

4.2. Chromium-resistant Streptomyces sp.

In bacteria, Cr(VI) rapidly enters the cytoplasm where it may exert its toxic effects. In the cytoplasm, Cr(VI) toxicity is mainly related to the process of reduction in which, like copper, free radicals may be formed. Bacterial Cr(VI) reduction has been demonstrated in several Gram-negative bacteria (Park et al., 2000; Myers et al., 2000; Pattanapipitpaisal et al., 2001; Mabbett and Macaskie, 2001; Ganguli and Tripathi, 2002; Vaimajala et al., 2002; Camargo et al., 2004; Asatiani et al., 2004; Bae et al., 2005;

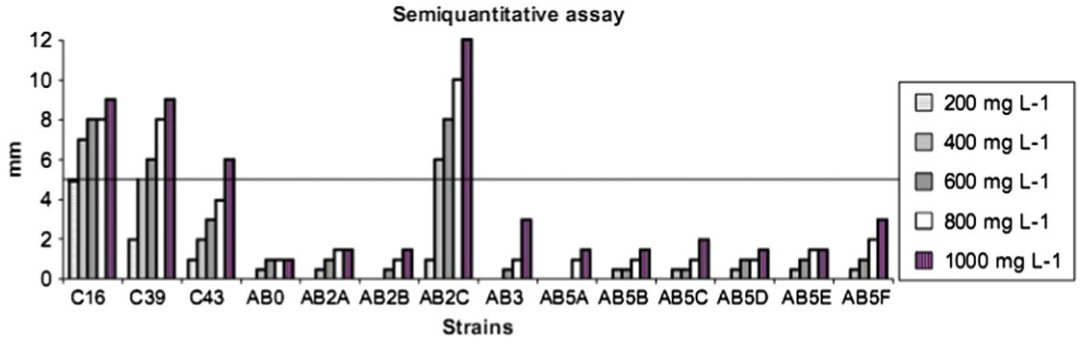

Fig. 2. Semi-quantitative resistance at 200, 400, 600, 800 and 1000 mg l^{-1} of Cu(II) concentrations measured as inhibition zone in mm. The horizontal line indicates the arbitrary limit used to consider copper-resistant (below) and non-resistant (up) strains. Fifty microliters of various concentrations were placed in a well in the MM agar medium. AB-strains – from sediment of the drainage channel. C-strains from "El Cadillal" (Albarracín et al., 2005).

Liu et al., 2006). Gram-positive bacteria also have been shown to possess Cr(VI) reduction ability. Among actinobacteria, the genus *Streptomycetes* represents up to 20% of the bacteria in soils (Kothe et al., 2005). Our research group has isolated nine strains of Cr(VI)-resistant actinobacteria from contaminated sites in Tucumán, which were identified as members of the genus *Streptomyces* (Polti et al., 2007). Among these strains, *Streptomyces* sp. MC1, isolated from sugarcane, has demonstrated a notable ability to reduce Cr(VI) in liquid MM, as well as in soil extracts (SE) and soil sample (SS) (Polti et al., 2009). In liquid MM, the growth profiles observed both with and without addition of 5 mg l^{-1} of Cr(VI) were almost identical, while Cr(VI) removal was close to 100% after 48 h of incubation. In the presence of 50 mg l^{-1} of Cr(VI), this strain showed 70% growth inhibition and only 50% Cr(VI) removal under non-adapted conditions. Chromate-pre-adapted cells inoculated with 50 mg l^{-1} of Cr(VI) showed, however, lower levels of growth inhibition, while Cr(VI) removal reached almost 75%. The growth and Cr(VI) removal abilities shown by this strain in SE samples were also highly promising, with a 30% reduction in Cr(VI) after 96 h of incubation. Moreover, Polti et al. (2009) reported an absence in growth inhibition in the presence of 50 mg kg^{-1} Cr(VI) after 1 week of incubation in sterile SS, and a reduction of Cr(VI) from 50 to 5 mg kg^{-1} during the exponential growth phase.

It is known that biotransformation of Cr(VI) to Cr(III) using various species of bacteria is currently the most pragmatic approach to reducing Cr(VI), with a well-established feasibility for bioremediation (Park et al., 2000; Myers et al., 2000; Pattanapipitpaisal et al., 2001; Mabbett and Macaskie, 2001; Ganguli and Tripathi, 2002; Vaimajala et al., 2002; Megharaj et al., 2003; Camargo et al., 2004; Asatiani et al., 2004; Bae et al., 2005; Liu et al., 2006). In this connection, Polti et al. (2010) detected and quantified chromate reductase activity in all cell fractions of *Streptomyces* sp. MC1, finding the highest levels of activity in their cell-free extract and whole cells fraction, followed by lower values in cell wall and supernatant fractions. These findings may prove to be of great relevance, since there are only a few existing studies on Cr(VI) bioreduction by actinomycetes.

On the other hand, Pereira (2010) demonstrated that the ability of *Streptomyces* sp. MC1 to reduce Cr(VI) was enhanced by the presence of sulfate ions in the culture medium. Some authors have reported that in a variety of cells (bacteria and yeast), chromate actively crosses biological membranes by means of the sulfate uptake pathway, which reflects the chemical analogy between these two oxyanions. Guillén-Jiménez et al. (2008) reported that sulfate plays an important role in tolerance to Cr(VI) as well as Cr(VI) reduction in *Candida* sp. FGSFEP. However, these results differ from other reports where no inhibitory or stimulating effects were found for sulfate in terms of Cr(VI) reduction in aerobic microbial cultures in *Pseudomonas putida* or in strains of *Bacillus* sp. In *Streptomyces* sp. MC1 plasmids have not been found and Cr(VI) resistance was due to the reduction of Cr(VI) to Cr(III), which does not cross the membrane, rather than to chromium bioaccumulation. More studies are required in order to elucidate the mechanism of chromium resistance in this strain and how the sulfate is involved.

Finally, in our research group, studies of the effects of Cr(VI) on this strain's morphological characteristics have also been performed (Pereira, 2010; Polti et al., 2011). Intra-cellular Cr accumulation was initially detected used cytochemical staining, with control cells (Fig. 5A) showing lower intra-cellular electrodensity than cells grown in the presence of metal (Polti et al., 2011) (Fig. 5B). On the other hand, scanning electron microscopy (SEM)

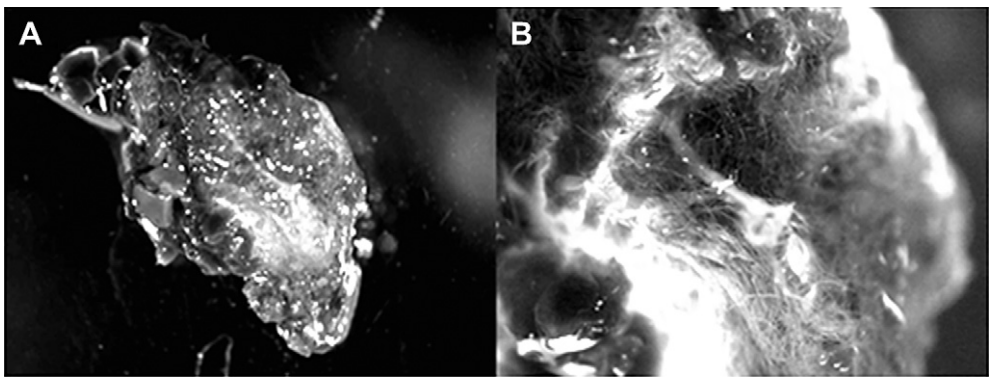

Fig. 3. Stereoscopic viewing through magnifying glass exopolymer obtained from a culture of *Amycolatopsis tucumanensis* in MM supplemented with copper. (A) 0,8× and (B) 3× (Albarracín, 2007).

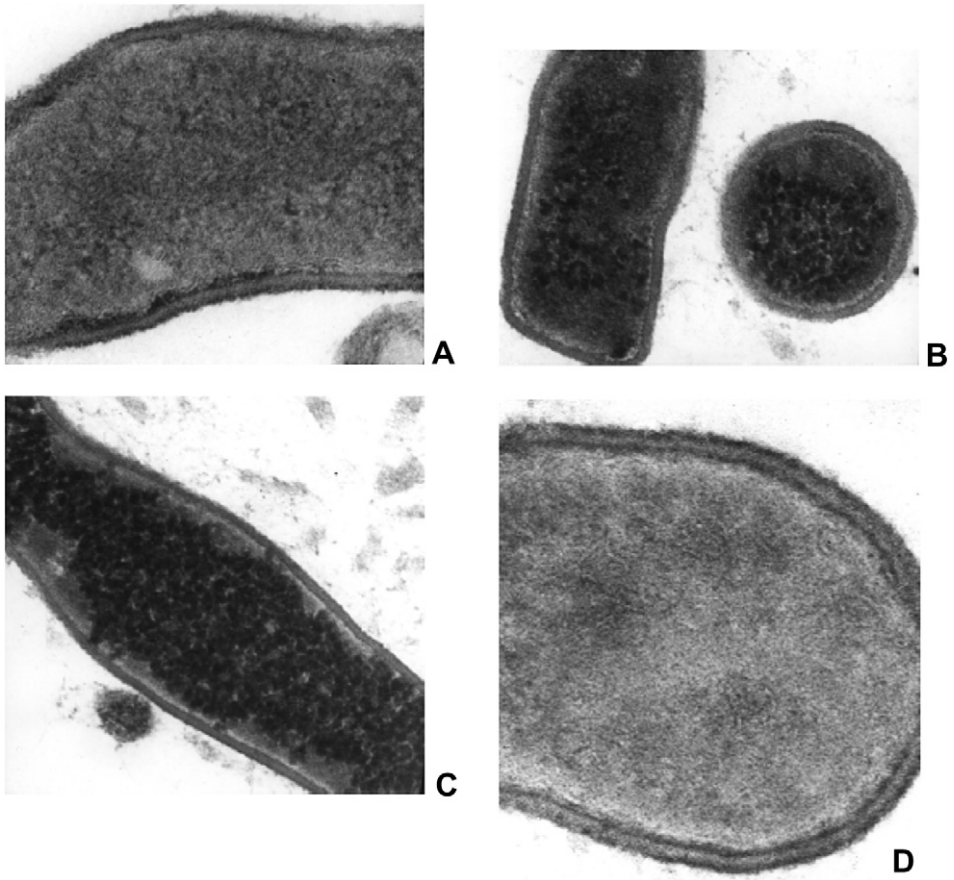

Fig. 4. Micrographs obtained from electron microscopy of *Amycolatopsis* sp. AB0 grown with Cu (II), 32 mg l^{-1}, in the MM at 3 (A; 140,600×), 5 (B; 82,640×) and 7 days (C; 82,640×) using Timm's reagent staining method (Kodama 1993). Arrows indicate copper deposits at the cell wall. D. Control cell cultivated in MM without copper (234,300×) (Albarracín et al., 2008).

showed that Cr(VI)-exposed *Streptomyces* sp. MC1(Fig. 6B) were rounder and shorter than non-exposed specimens (Fig. 6A). Interestingly, Pereira (2010) reported that degree of cell branching in this strain decreased by 31% in the presence of Cr(VI) compared to control cells grown without Cr(VI). This could represent an adaptive response to metal-dependent stress conditions.

4.3. Metal-resistant yeasts

Although in recent decades great importance has been given to the study of diversity in biological systems, and especially the different forms of life adapted to specific ecosystems, there is still very little such information available regarding metal-

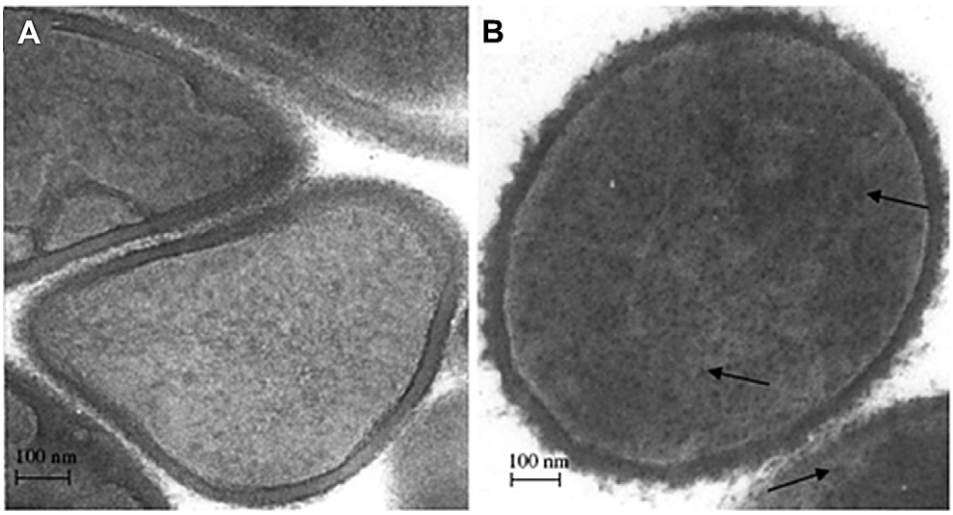

Fig. 5. TEM micrographs of *Streptomyces* sp. MC1, after 7 days of growth in MM: (A) control, without Cr(VI). (B) Supplemented with Cr(VI) 50 mg^{-1}, using Timm's reagent staining method. *Arrows* indicate intra-cellular aggregates (Polti et al., 2011).

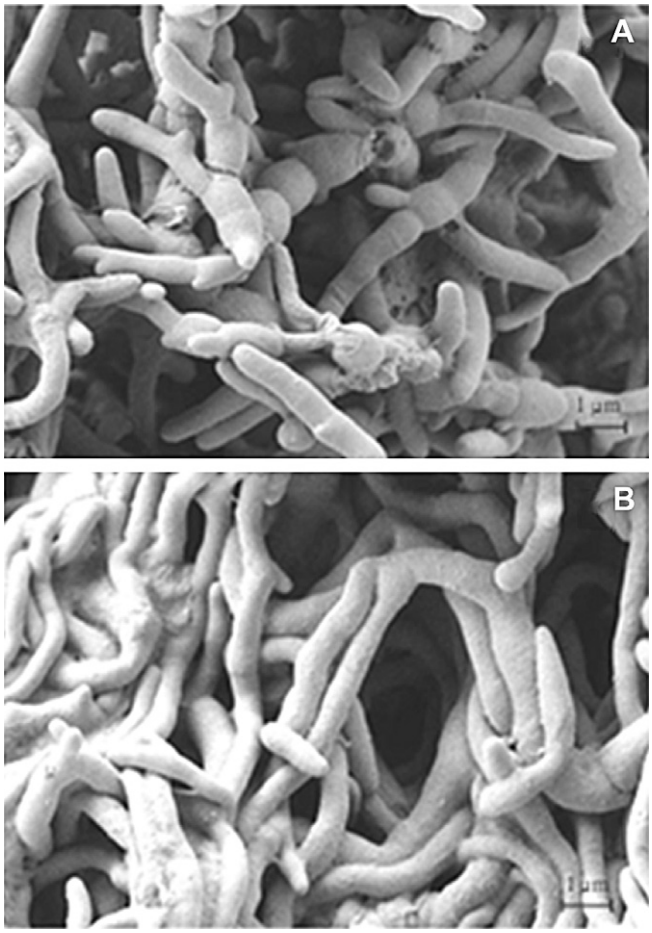

Fig. 6. Scanning electron micrograph of *Streptomyces* sp. MC1, after 7 days of growth in MM: (A) control, without Cr(VI); (B) Supplemented with Cr(VI) 50 mg^{-1}, showing filamentous mycelium (Polti et al., 2011).

resistant yeast populations in Argentina. Therefore, a variety of studies focused upon on Cu- and Cr-resistant yeast populations have conducted (Villegas et al., 2005, 2008; Villegas, 2006). To this end, samples were collected and analyzed from three contaminated contexts in Argentina: wastewater sediment from a copper filter plant located in Tucumán province; tannery ponds located in Nonogasta, La Rioja province; and sediment from a nickel-copper mine in Virorco, San Luis province (Villegas, 2006). Assays of metal tolerances were performed according to the method of agar diffusion described by Villegas et al. (2004). Based on these results, five isolates were selected and characterized for use in further Cr(VI) or Cu(II) removal assays (Villegas et al., 2005; Villegas, 2006). Only three of five isolates were identified to species level: *Candida fukuyamaensis*, *Rhodotorula mucilaginosa,* and *Aureobasidium pullulans.* Others two isolates were assumed to correspond to the genera *Lecythophora* and *Candida* (Villegas et al., 2005). In the next two sections, studies on Cu and Cr tolerance for these indigenous yeasts are reviewed:

4.3.1. Copper-resistant yeasts

The effects of Cu(II) concentration on biomass production, specific growth rate, and glucose consumption in the yeasts *C. fukuyamaensis* RCL-3 and *R. mucilaginosa* RCL-11 were evaluated in a YNB-glucose liquid medium at 30 °C (Villegas et al., 2005; Villegas, 2006). YNB-glucose was used because complex media, as YEPD, are inadequate due to the high concentration of organic components that absorb the metal ions (Villegas et al., 2012). It was observed that the growth of *C. fukuyamaensis* RCL-3 and *R. mucilaginosa* RCL-11 decreased in accordance with the increase of Cu(II), with *R. mucilaginosa* RCL-11 being the most strongly affected. In regard to specific glucose consumption, this strain showed values with an almost linear relationship to increases in the concentration of Cu(II) in the culture medium. Villegas et al. (2005) have also reported on the ability of *C. fukuyamaensis* RCL-3 and *R. mucilaginosa* RCL-11 to uptake Cu(II) from culture media with different profiles in terms of metal bioaccumulation kinetics. The maximum values of Cu(II) uptake were achieved by *C. fukuyamaensis* RCL-3, which was maintained inside the cells and released later into the supernatant of the culture. On the contrary, *R. mucilaginosa* RCL-11 stored the accumulated metal up until the end of the experiment. It is interesting to note that *C. fukuyamaensis* RCL-3 belongs to an ascomycetous genus, while *R. mucilaginosa* RCL-11 belongs to a basidiomycetous one. Since the existence of differences between ascomycetous and basidiomycetous yeasts in terms of cell wall structure, cytology, physiology, protein and enzyme contents, etc., has been well-established (Moore, 1998), it can be assumed that the fact that *C. fukuyamaensis* RCL-3 released the Cu to the extracellular space, while *R. mucilaginous* RCL-11 retained the metal inside the cell, is related to these phylogenetic differences or the presence of copper pump in the first yeast. This may be a protective mechanism to prevent the toxic effects of excess Cu. This mechanism was first described in mammalian and plant cells (Clemens et al., 1999; Linder, 2001). However, a pump was found in *Candida albicans*, by methods of molecular biology, that would expel Cu out of the cell. This pump was identified in *Saccaromyces cerevisiae* in the Golgi membrane, while in *C. albicans* is was also localized in the plasmatic membrane (Weissman et al., 2000). In a later work, other authors suggested that although a high Cu concentration induces an efflux mechanism in *Yarrowia lipolytica* (which also belongs to an ascomycetes genus), the released Cu becomes entrapped in the periplasm and in other parts of the cell wall (Ito et al., 2007).

In regard to intra-cellular Cu localization, Villegas et al. (2009a) studied the sequential effects on Cu(II) at 0.5 mM on cellular structures and the possible locations in *C. fukuyamaensis* RCL-3 and *R. mucilaginosa* RCL-11 after 72 h of cultivation with control cell comparisons. At 24 h both yeasts showed cytoplasmatic lysis; however, *C. fukuyamaensis* RCL-3 also showed evidence of cell wall degradation and irregular shapes. At 48 h, the authors observed significant changes in cell interior contrast and density, with intracellular deposition of the metal in the cytoplasm appearing as large dark anomalies (grains) (Fig. 7A and Fig. 8A). At 72 h of incubation, these grains were more heavily represented in the cytoplasm of *R. mucilaginosa* RCL-11. Few *C. fukuyamaensis* RCL-3 cells possessed these dark bodies, with those existing located mainly located next to the cell wall and with abundant broken cells present (Fig. 7B and Fig. 8B). The authors suggested that electron-dense bodies found in both yeast would correspond to intra-cellular Cu deposits.

Finally, Villegas et al. (2009a) have also reported on an increase in endogenous SOD and CAT activity in both yeasts, which could be related to ROS formation by exposure to high Cu(II) concentrations. *R. mucilaginosa* RCL-11, which has the capacity to retain Cu inside of the cell (Villegas et al., 2005), presented higher CAT activity than *C. fukuyamaensis* RCL-3. Interestingly, when *R. mucilaginosa* RCL-11 cells pre-adapted to Cu concentrations were used, total SOD and CAT activity was lower than the results obtained with non-adapted cells of the same strain. The authors suggested that the cytoplasm of adapted cells possesses a larger number of proteins that sequester Cu, which prevents the free ions from producing ROS, thereby decreasing oxidative stress. As a result, adapted cells were

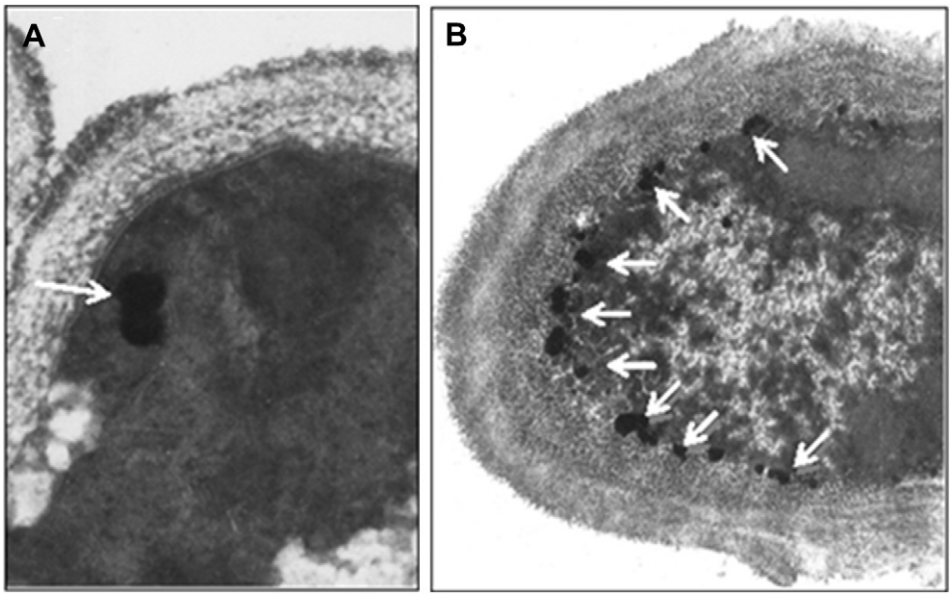

Fig. 7. Transmission electron images (TEM) of *C. fukuyamaensis* RCL-3; grown in the presence of Cu(II) 0.5 mM: (A) at 48 h and (B) at 72 h. Arrows indicate bodies electron-dense. 33.300× (Villegas et al., 2009a).

able to grow at higher Cu(II) concentrations than non-adapted cells (Villegas et al., 2009b).

4.3.2. Chromium-resistant yeasts

Cr(VI) resistance mechanisms in yeasts are not well understood. Earlier studies involving certain species of *Candida* and *Rhodosporidium* have shown that, under aerobic conditions, chromate resistance is related to reduced ion uptake rather than to biological reduction of Cr(VI) to Cr(III) species, and very little accumulation of Cr was observed (Baldi et al., 1990; Pepi and Baldi, 1992; Krauter et al., 1996). In contrast, more recent studies have demonstrated that *Candida utilis* and another Cr(VI)-resistant yeast identified as *Candida maltose* RR1, had the ability to partially reduce Cr(VI) to Cr(III) and to accumulate Cr into the cells (Muter et al., 2002; Ramírez-Ramírez et al., 2004). In our research group, studies involving two yeasts isolated from tannery ponds, *Lecythophora* sp. NGV-1 and *Candida* sp. NGV-9, indicated that Cr(VI) concentration decreased without changes in total extra- and intra-cellular Cr (Villegas et al., 2008). Considering that Cr(III) is the only stable and soluble Cr component formed by Cr(VI) reduction, it could be inferred that the Cr remaining in the supernatant in this study was Cr(III). When *A. pollulans* VR-8 was incubated with 0.5 mM Cr(VI) was found to remove 100% of the Cr(VI) after 45 h of incubation, and 68% of the total Cr after 100 h of incubation. However, when this strain was incubated with 1 mM Cr(VI), only 60% of the Cr(VI) and 15% of the total Cr were removed. According to Villegas et al. (2008), the order of Cr(VI) removal efficiency for these strains was as follows: *A. pullulans* VR-8 > *Lecythophora* sp. NGV-1 > *Candida* sp. NGV-9. Similar results were obtained for these strains under different culture conditions by Fernández et al. (2010).

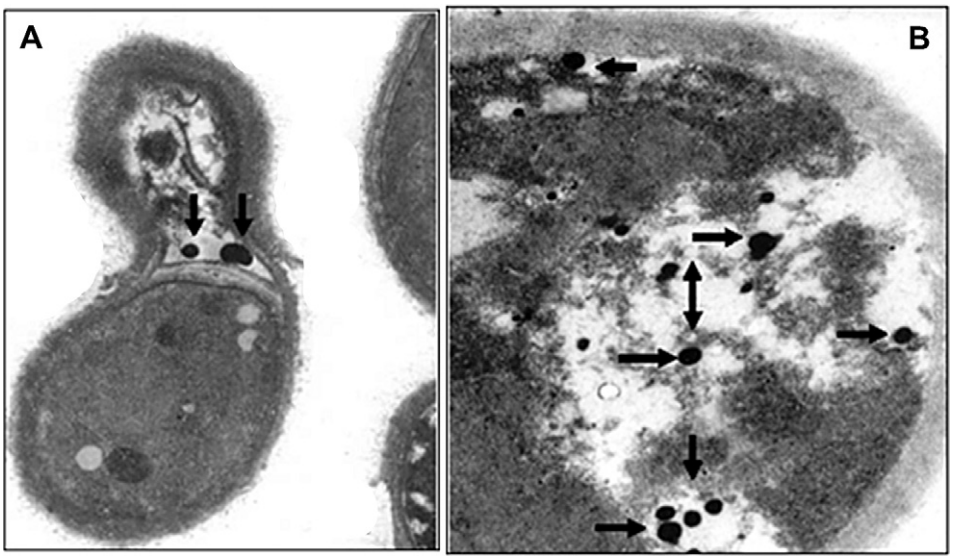

Fig. 8. Transmission electron images (TEM) of *R. mucilaginosa* RCL-11 grown in the presence of Cu(II) 0.5 mM: (A) at 48 h and (B) at 72 h. Arrows indicate bodies electron-dense. 33.300× (Villegas et al., 2009a).

5. Conclusions

As shown throughout this review, populations of both actinobacteria and yeasts that can survive and even flourish in environments contaminated with heavy metals have been isolated and identified by our research group. It appears that these microorganisms indigenous to our region have evolved a variety of mechanisms for metal uptake and homeostasis. Thus, their metal processing capabilities could be used to concentrate, remove and recover metals from contaminated sites and thereby enhance the efficiency of treatment processes. These studies seem to be highly promising in terms of the creation of platforms that encourage the development of bioremediation processes using native microorganisms. The most important characteristic of this approach is that the system is environment friendly. However, more studies are required to develop and improve future applications of metal-resistant microbes for cleaning up polluted environments, and we are continuing to work on this subject.

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