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MICROBIOLOGY

# Reduction in concentration of chromium (VI) by *Lysinibacillus macroides* isolated from sediments of the Chapala Lake, Mexico

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**Abstract:** The Chapala Lake is one of the most polluted lakes in Mexico, due to the in flow of effluents from several industrial plants, the lake accumulates pollutants such as chromium(VI) which is considered important for aquatic ecosystem. This study aimed was to evaluate the ability to decrease the concentration of chromium (VI) by *Lysinibacillus macroides* 2(1B)104A, isolated from sediments of the Chapala Lake. The strain was identified through 16S rRNA sequencing and phylogenetic analysis. Results showed that this strain grows in concentrations of 50, 100, 200 and 300 $mgL^{-1}$ Cr(VI), in pH ranging 6 to 7, showing 79.508% reduction in concentration 50 $mgL^{-1}$, determining that the reduction occurs extracellularly. Likewise, it was observed that *Lysinibacillus macroides* reduced the concentration of Cr(IV) in the broth, it was not observed that the bacteria could sequester Cr(VI) in the membrane or intracellularly. However, it reduced the concentration of Cr(VI) in the broth. *Lysinibacillus macroides* 2(1B)104A isolate showed having the ability that decrease the concentration of Cr(VI), which makes it a viable options for bioremediation of water polluted with this metal.

**Key words:** 16S rRNA, Chapala Lake, *Lysinibacillus macroides,* reduction Cr(VI).

## INTRODUCTION

The Chapala Lake represents the final part of the Lerma-Chapala basin, located in the states of Jalisco and Michoacán, Mexico. This lake is an example of the disconnection between people who pollute upstream, and those who suffer the effects of pollution downstream of the basin, which flows into the Chapala Lake. The consequence of this water pollution is the generation of imbalances in ecosystems related to this Lake, affecting several human activities, such as: fishing, agriculture, ranching and recreative activities (Sandoval-Moreno & Ochoa-Ocaña 2010). The main problem associated with poor water quality in the Lerma-Chapala system is the presence of heavy metals, *i.e.* zinc, cadmium, mercury, lead and chromium, which have been detected since two decades ago (Hansen 1992). The pollution caused by heavy metals in water has always been a major problem, since these are not biodegradable and accumulates in live tissues. One of the most important heavy metal observed in the Lerma-Chapala system is chromium, its presence is associated to many industries like leather, tanning and textiles, wood conservation, and aluminum anodization industry and cooling with water (Panigatti et al. 2012). Thus, solid and liquid wastes of chromium are released into surrounding areas and water

bodies, affecting the Lerma-Chapala basin with a chromium concentration from 68 to 96 μg $g^{-1}$ sediment (Hansen 1992), with maximum values of 25 μg $L^{-1}$ water (Zarazúa et al. 2013).

Chromium species in this basin may be found in different oxidation states, trivalent [Cr(III)] and hexavalent [Cr(VI)]. These two species are more stable and common states. Chromium(III) may be found as oxides, hydroxides or sulfates, which are linked mainly to organic matter in soil and water. On the other hand, Cr(VI) is generally associated to oxygen as chromate ($CrO_4^{-2}$), or dichromate ($Cr_2O_7^{-2}$) ions. Hexavalent chromium [Cr(VI)] is extremely toxic, inducing mutagenic and carcinogenic effects in biological systems, due to its strong oxidative property. It has been reported that Cr(VI) is metabolized by a network of mechanisms leads to the generation of reduced chromium species and reactive oxygen species, which will result either in activation or detoxification depending on the site of the intracellular reduction and its proximity to DNA, 42.9% incidence have been reported in experimental laboratory animal which are linked to lung cancer mainly (De Flora 2000). However, Cr(III) is less toxic and bioavailable than Cr(VI) (Martin et al. 1994, Poornima et al. 2010, Ramírez-Diaz et al. 2009).

Chromium contamination and damages caused by its input to several ecosystems have generated studies on bioremediation, using the ability of some microorganisms to concentrate metals diluted in aqueous solutions, storing them in their cellular structure, *i.e.* yeast, fungi, algae and bacteria (Basu et al. 2014, Duca et al. 2012). Ilias et al. (2011) reported that isolates of *Staphylococcus aureu*s and *Pediococcus pentosaceus* can grow on 2,000 $mgL^{-1}$ Cr(VI) (as $K_2Cr_2O_7$) in Luria-Bertani (LB) medium; Pal & Paul (2004) described a group of 34 chromium-resistant bacteria isolated from naturally occurring chromium percolated serpentine soil able to reduce chromate under aerobic conditions, and one of this isolates *Bacillus sphaericus* was tolerant to 800 $mgL^{-1}$ Cr(VI) and reduced 80% Cr(VI) during growth; Duca et al. (2012) evaluated the ability of *Aspergillus niger* BM-56 to remediate Cr(VI) solutions obtaining a removal efficiency of 100% at different metal concentrations (0.1; 0.5 and 1.0 $mgL^{-1}$) and pH (2.5; 4.5; 5.5 and 7.0). This research has been reported in the reduction of chromium, transforming Cr(VI) to Cr(III) under aerobic and anaerobic conditions.

Some microorganisms can grow under high concentrations of chromium. Recent advances in bioremediation focused on the capability of the microorganisms to adapt and grow in the polluted environment have been developed. Besides, most of surface-water bodies are currently facing this type of contamination. Knowledge of the resistance of microorganisms to chromate and other heavy metals is important for identifying the reduction potential of these in soil and water pollution, and designing of bioremediation treatment with these microorganisms. Therefore, this study aimed to identify and to evaluate the ability of a bacterial strain 2(1B)104A isolated from the Chapala Lake to reduce Cr(VI).

## MATERIALS AND METHODS

*Microbial isolation.* The sediment samples were collected from the Chapala Lake (20°08'34.6"N and 102°46'24.7"W). A composite sample was made, for which sediments were taken from 3 separate points (20 m), then homogenized and stored in 50 mL sterile Falcon vials. These samples were stored at 4°C and transported to the Genomic Laboratory of Universidad de la Ciénega del Estado de Michoacán de Ocampo for their analysis (De Anda et al. 2013). To 150

mL of nutritive broth were added 10 g of each sediment sample and incubated at 37°C at 200 rpm for 21 days. Microbial biomass aliquots were collected at 0, 3, 7, 14 and 21 days of incubation, which were diluted up to $10^6$ using sterile water, and the last three dilutions were inoculated on Petri dishes containing nutritive agar. The Petri dishes inoculated were incubated at 37°C for 5 days (Soto-Padilla et al. 2014). After this period of incubation, the bacterial population and diversity were quantified, based on the microscopic and macroscopic characteristics of the colonies.

*Bacterial resistance to Cr(VI).* This assay was conducted by inoculating 0.8 O.D. in 600 nm of the studied strains in nutritive broth supplemented with several increased concentrations of potassium dichromate ($K_2Cr_2O_7$), from 50 to 700 $mgL^{-1}$. After 48 h of incubation, the bacterial growth was quantified by turbidimetry at a wavelength of 600 nm in the UV -Vis spectrophotometer (Lambda 2) (Guo et al. 2010).

*Growth kinetics*. Bacterial growth kinetics were conducted under the four concentrations where bacterial resistance was detected. Bacteria were grown in nutritive broth and incubated at 37°C, with continuous shaking at 200 rpm for 24 h. Then, 10% of pre-inoculum was inoculated in Erlenmeyer flasks containing a nutrient broth supplemented with increased concentration of $K_2Cr_2O_7$ as 50, 100, 200 and 300 $mgL^{-1}$ of Cr(VI). These flasks were previously sterilized in autoclave for 15 minutes at 121°C and 15 psi. Inoculated flasks were incubated under continuous agitation at 37°C and 200 rpm (Thacker et al. 2007). The quantification of microbial growth was conducted by turbidimetry; thus 3 mL aliquots were used from each concentration at different intervals of time (0, 2, 5, 19.5, 24.5, 29, 44, 53, 68, 78, 96 h). Optical density was measured on each sample at a wavelength of 600 nm using a UV-Vis spectrophotometer (Lambda2) (Soto-Padilla et al. 2014). Also, pH variation was measured during the experiment for 96 h. All experiments were conducted in triplicate.

*Reduction kinetics of Cr(VI).* The reduction of Cr(VI) was determined using a nutritive broth supplemented with $K_2Cr_2O_7$ at concentration of 50 $mgL^{-1}$ of Cr (VI), which was inoculated at 37°C and 200 rpm for 96 h and the 0.8 O.D. The reduction of Cr(VI) was evaluated by taking 3 mL aliquots of cellular suspension at different time intervals (0, 2, 4, 6, 10, 26, 48, 96 h). Samples were centrifuged at 3000 rpm during 15 min. The supernatant was recovered to determine the broth concentration of Cr(VI) using Cr(VI)-specific colourimetric reagent S-diphenyl carbazide (DPC) 0.25% (w/v) prepared by acetone (Joutey et al. 2014). The evaluation of extracellular adsorption and intracellular accumulation of Cr(VI) was evaluated according to Huang (2014). The determination of the Cr(VI) concentration was determined using the photometric diphenylcarbazide method, measuring the optical density at 540 nm by using a UV-Vis spectrophotometer (Lambda2) (Thacker et al. 2007). The determination of total chromium ($Cr_{tot}$) was performed using flame atomic absorption spectrophotometer (AAS) (Perkin-Elmer, Canada) (Oyetibo et al. 2013).

*Bacterial molecular Identification.* Bacterial Genomic DNA was extracted according to the Phenol-Chloroform-Alcohol isoamyl protocol (Guo et al. 1997). The amplification of 16S rRNA gene was conducted by Polymerase Chain Reaction (PCR), using the universal primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'TACGGYTACCTTGTTACGACTT-3'), amplifying an expected fragment length of 1.5kb. The PCR was carried out using a Master mix (PROMEGA) with a final volume of 50 µL, and the following amplification conditions: 1 cycle at 95°C for 1 min, 35 cycles at 95°C for 30 s, 54°C for 30 s, 72°C

for 1 min, with extension at 72°C for 5 min. The amplicons were visualized with electrophoresis in 1% agarose gel, using ethidium bromide as dye. Purification of the fragment was conducted with UltraClean® 15 DNA Purification Kit (From Agarose Gels and Solutions from Mo Bio laboratories following manufactures indications). DNA sequencing was conducted at Laboratorio Nacional de Genómica para la Biodiversidad (LANGEBIO) from CINVESTAV Irapuato, using a Sanger platform. The phylogenetic analysis was carried out using the FinchTV, SeaView, ClustalX2, Mega6 and Excel 2010 (similarity index) software.

## RESULTS

*Isolation and selection of the bacterial strain.* twenty-six bacterial strains resistant heavy metals from sediments collected in Chapala Lake were isolated. This bacterial collection was studied, observing that some strains could grow in presence of chromium, the strain 2(1B)104A showed the greatest growth capacity in the presence of this metal. The microscopic characterization showed that strain 2(1B)104A is a sporulated bacillus Gram positive. The macroscopic morphology of this strain on nutritive agar, showed amorphous colonies of 3-5 mm diameter with a convex elevation, as well as a curved edge, with a light-yellow color, opaque, and humid.

*Resistance to Cr (VI).* The resistance of strain 2(1B)104A to Cr(VI) was evaluated in concentrations that ranged from 50 to 700 $mgL^{-1}$ of Cr(VI) (Figure 1), quantifying a greater bacterial growth at 50 $mgL^{-1}$ of Cr(VI). Cellular growth under concentrations of 100, 200 and 300 $mgL^{-1}$ of Cr(VI) did not show statistical differences. The bacterial growth was diminished at concentrations greater than 300 $mgL^{-1}$ of Cr(VI).

*Kinetics for bacterial growth and reduction of Cr(VI).* Growth kinetics were evaluated at concentrations of 50, 100, 200 and 300 $mgL^{-1}$ Cr(VI), since this is where bacterial growth occurred in the resistance assay. Figure 2 shows the growth kinetics in concentrations of 50, 100, 200 and 300 $mgL^{-1}$ of Cr(VI), where the higer growth was observed in the concentration of 50 $mgL^{-1}$ of Cr(VI), so this concentration was used to perform the reduction kinetics of Cr(VI) and to determine if the Cr(VI) is being sequestered by the bacteria in the membrane or intracellularly, or if there is an extracellular reduction in the culture medium. Also, in the nutrient broth there was a reduction in the concentration of Cr(VI), however, the total Cr in the broth remained close to 50 $mgL^{-1}$, whereas the concentration of Cr(VI) intracellularly it is not found and only very little is found in the membrane (approximately 4-5 $mgL^{-1}$) (in figure 3).

The pH during Cr(VI) reduction did not show significant variation at the different concentrations evaluated, compared to the initial values (Figure 4), with variation which ranged between 0.24 and 0.29 in the different concentrations.

*Molecular identification of bacteria.* The strain 2(1B)104A showed resistance to Cr, consequently, there was an interest for its taxonomic identification using molecular techniques. Taxonomic identification showed that strain 2(1B)104A belongs to the genus *Lysinibacillus* (Figure 5).

Using data generated from the Neighbor Joining method, the similarity index was calculated. The specie identified was *Lysinibacillus macroides,* showing a similarity index of 97.86%.

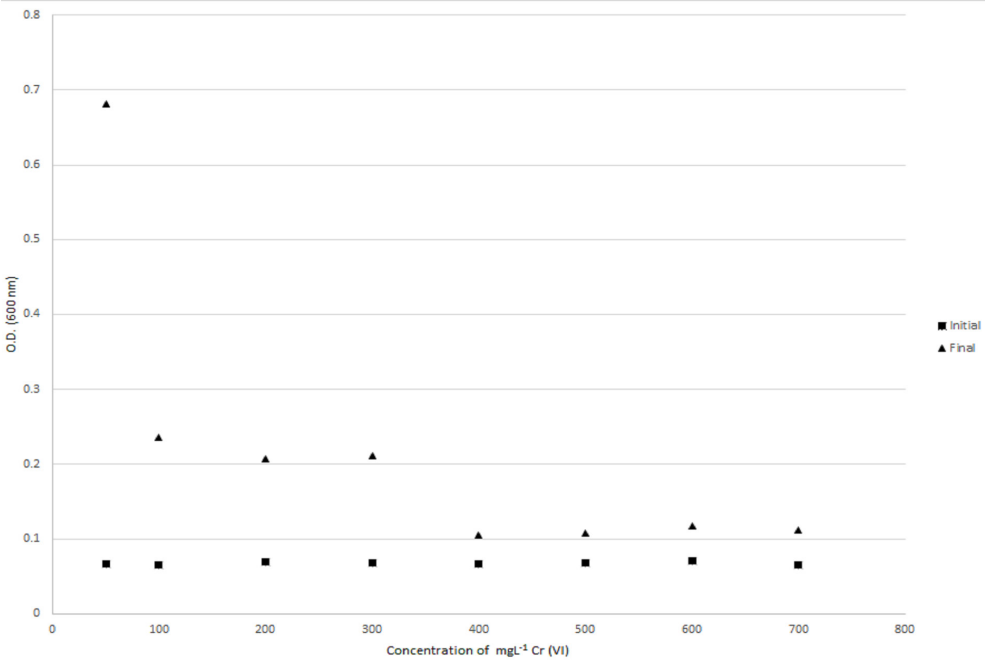


**Figure 1.** Resistance of the strain 2(1B)104A to different concentrations of Cr after 48 h of growth.

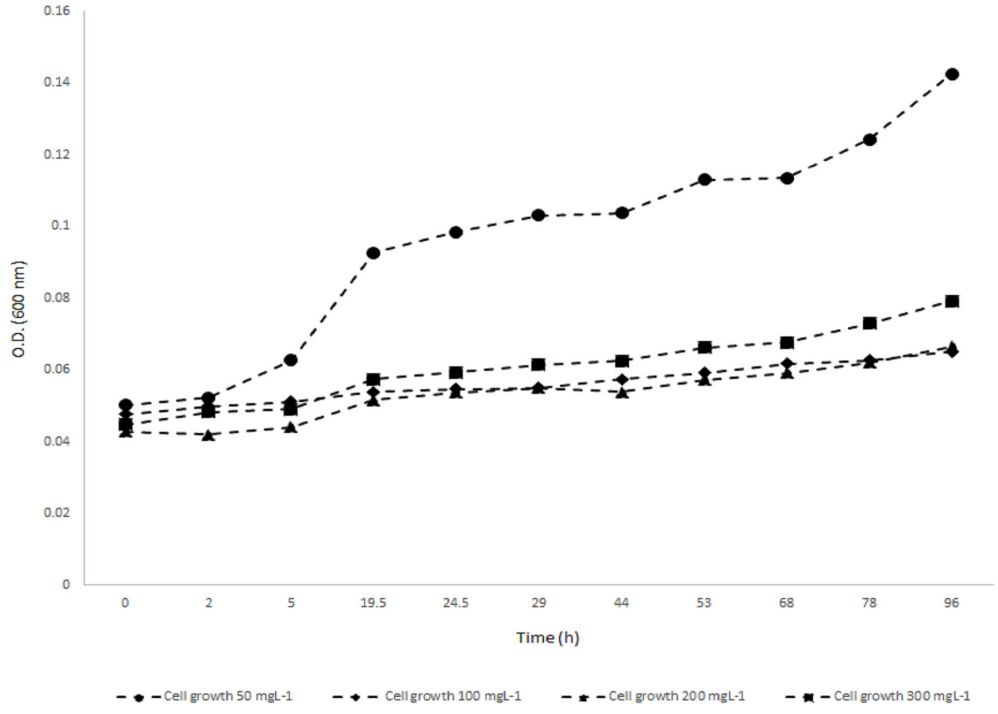


**Figure 2.** Kinetic cellular growth of the strain 2(1B)104A.

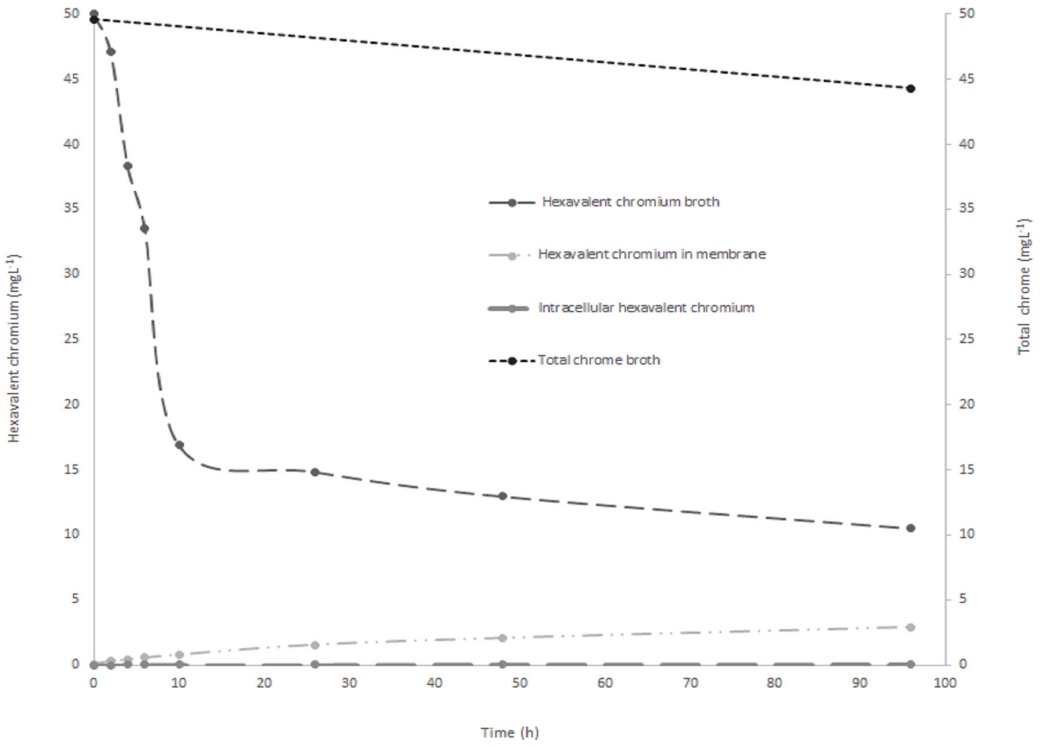


**Figure 3.** Kinetics of the reduction of Cr(VI) to 50 $mgL^{-1}$ by the strain 2(1B)104A.

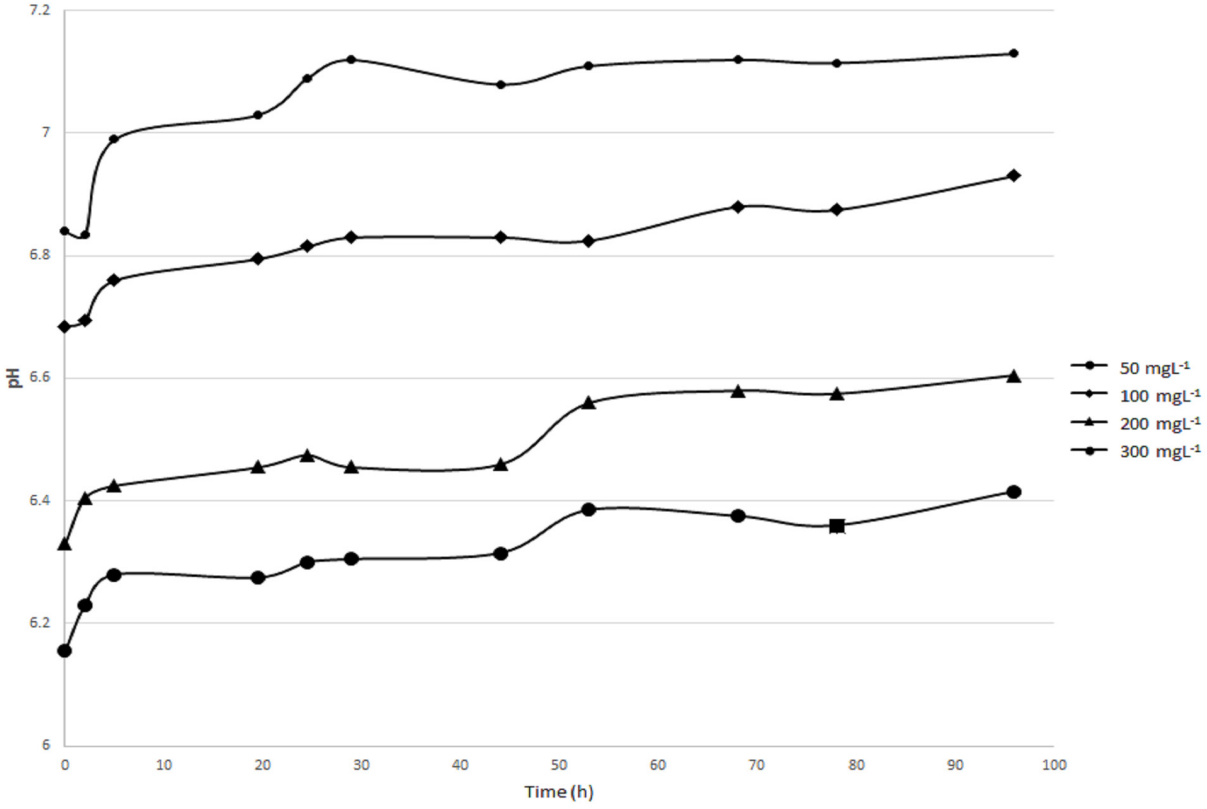


**Figure 4.** Variation of pH during reduction of Cr(VI) by the strain 2(1B)104A.

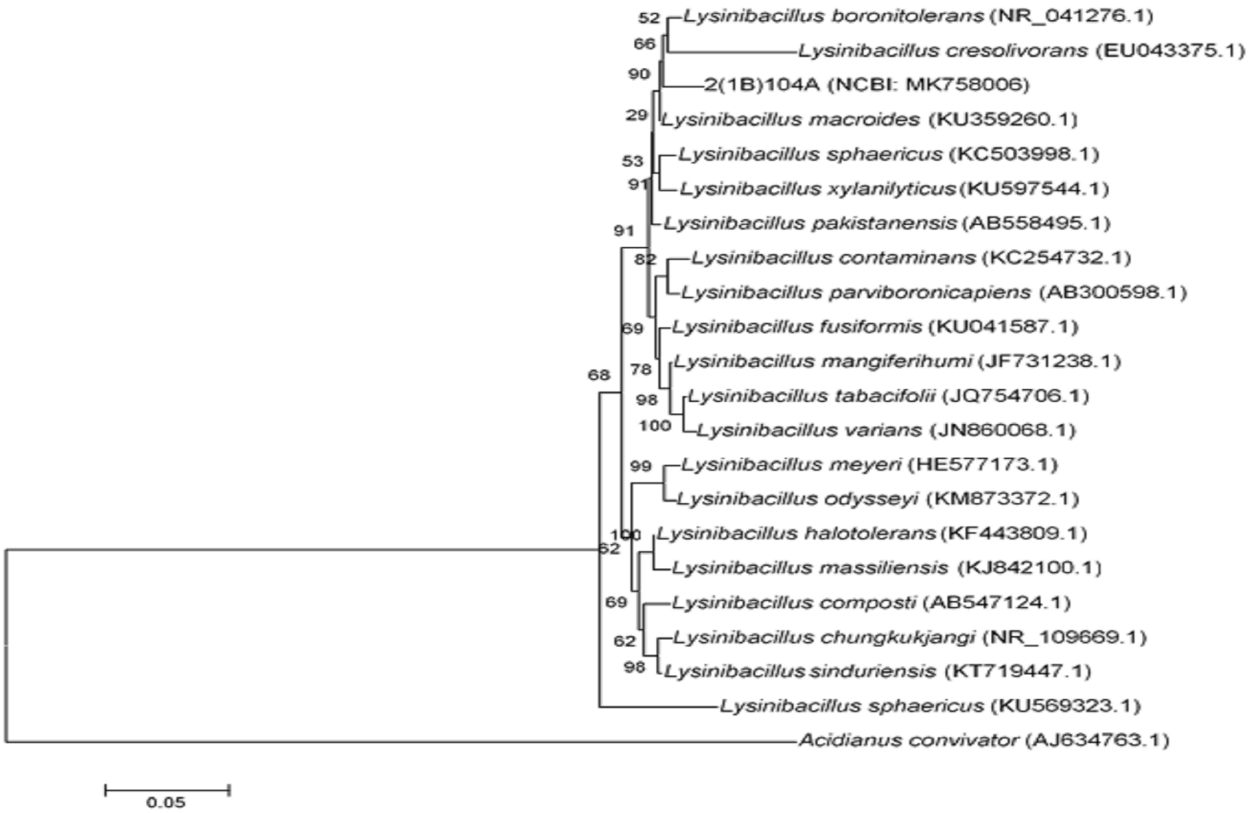


**Figure 5. Phylogenetic tree of the strain 2(1B)104A with respect to the species of *Lysinibacillus* conducted with the Neighbor Joining method, the accession number registered on the NCBI for the species utilized in the analysis is shown in parenthesis.**

## DISCUSSION

Several studies have reported the presence of heavy metals in the Chapala Lake (such as chromium, cyanide, lead, mercury, cadmium, copper and zinc). These metals have also been reported in plants growing around this Lake (Sandoval-Moreno & Ochoa-Ocaña 2010, Zarazúa et al. 2013). Cr(VI) is a significant pollutant due to the potential negative effects on human health, Trujillo-Cárdenas et al. (2010) reported Cr concentrations of 44.7 and 42.1 $mgKg^{-1}$ in two sediment sampling sites at the Chapala Lake. These high chromium content conditions induce microbial mechanisms to decrease the toxic effects of this metal, although chromium is an essential micronutrient required for the growth of many organisms, in high concentration is toxic, carcinogenic and teratogenic, thus chromium has been designated by EPA as the main pollutant. It is well known that Cr(VI) in solution is commonly found as hydro chromate oxyanions ($HCrO_4^-$), chromate ($CrO_4^{-2}$) or dichromate ($Cr_2O_7^{-2}$) forms, the latter is one of the chemical species that remains in the environment for long periods (Poornima et al. 2010).

The growth capability showed by *Lysinibacillus macroides* under a concentration of 300 $mgL^{-1}$ (Figure 1) proves a promising trait as remediation agent in environments polluted by this metal; like *Bacillus subtilis* which was reported to grow at 2.5-7 $mgL^{-1}$ of Cr(VI) (Basu et al. 2014), and *Escherihia coli* at 200 $mgL^{-1}$ (Pannigatti et al. 2012). Also, Numrah & Hafsa (2014) reported the isolation of two *Pseudomonas* strains that grew under concentrations of 50 $mgL^{-1}$ of Cr(VI).

Thus, it is important to mention that the isolated strains from the Chapala Lake in this study have higher resistance compared to strains isolated in different studies. *Lysinibacillus macroides* growth at concentration of 50 $mgL^{-1}$ of Cr(VI) showed a reduction of Cr(VI) at 79.508% in 96 h (Figure 3). Ramírez-Díaz et al. (2009) explains that the extracellular reduction Cr(VI) can be considered as a mechanism of resistance to chromates, being part of a system of resistance to a chromate that has been related to the extracellular reduction of Cr(VI). The potential use of strain () in the Chapala Lake are promising due to the chromium concentrations present in sediments are within these ranges, it is assumed that Cr(VI) reduction is an important strategy for resistance to chromate in bacteria, besides Cr(VI) suggests that the activity of reducing this ion in microorganisms is an adaptation mechanism to enzymatic activity caused by exposure to chromate (Ramirez-Díaz et al. 2009). Several studies have reported the diversity of bacterial capable of reducing Cr(VI), and the variation in the range for their degradation capability. Ilias et al. (2011) reported that *Staphylococcus aureus* and *Pediococcus pentosaceus* can grown under concentrations of 2000 $mgL^{-1}$ of Cr(VI) in contaminated effluents from the leather tanning industry in Hazaribagh Tannery area. These strains reduced 90% in 5 h and 90% in 10 h in a solution with 20 $mgL^{-1}$, respectively. Studies such as Pal & Paul (2004) reported that 34 strains resistant to chromium, show different ways to reduce this pollutant; however, *Bacillus sphaericus* showed tolerance up to 800 $mgL^{-1}$ of Cr(VI) and a reduction 80% after 48 h of growth. Ahirwar et al. (2015) isolated three strains from soil identifying *Pseudomonas fluorescence*, *Bacillus cereus* and *Bacillus decolorationis*, and reported that *P. fluorescence* could remove 87%, 73%, 65% and 60% of chromium from the medium in 48 h with initial concentrations of 5, 10, 25 and 50 $mgL^{-1}$, respectively. Numrah & Hafsa (2014) found that *Pseudomonas putida* and *Pseudomonas plecoglossicida* from sewage mud collected in Hudiera showed a reduction rate for Cr of 65% and 50% in a medium with 20 $mgL^{-1}$ Cr(VI) in 12 h. Although these studies report a large variety of chromium reducing microorganisms, there are no reports of bacteria that reduce chromium in the Chapala Lake. Therefore, this study focuses on the isolation of autochthonous chromium reducing bacteria from this aquatic system capable of reducing the toxicity this metal.

It is important to mention that researchers are currently looking for innovative technology to bioremediate polluted areas with different heavy metals, such as chromium. Thus, the metabolic potential of microorganisms to remove this metal is a promising alternative way to clean those zones contaminated with chromium (He et al. 2011). The strain *Lysinibacullis macroides* which was isolated and characterized in the Chapala Lake possess the ability for growth in high concentration of Cr(VI), where it has concentrations of 44.7 to 42.1 $\mu g\ Kg^{-1}$ in sediment (Trujillo-Cárdenas et al. 2010) and concentration reaching up to 25 $\mu g\ L^{-1}$ in the water (Zarazúa et al. 2013). This information suggests that this strain is adapted and has several mechanisms to utilize chromium. The identification of strain 2(1B)104A (accession number in the NCBI: MK758006) was conducted through the comparison of amplified sequences of the 16S rRNA gene published in the NCBI gene bank, allowing to determine that strain 2(1B)104A belongs to the genus *Lysinibacillus*. A more detailed analysis of the similarity index of all species of this genus from data found in the NCBI, identified this species as Lysinibacillus macroides with a similarity index of 97.86% with the reference for *Lysinibacillus macroides* (accession number in the NCBI: KU359260.1).

Despite that this species has not been reported as capable of reducing chromium, other species from this genus such as *Lysinibacillus fusiformis* had been isolate from contaminated waters and showing a minimum inhibitory concentration of 60 mM of Cr(VI) in an R2A medium (He et al. 2011). Likewise, it was reported that *Lysinibacillus mangiferihumi* has been isolated from Lake Lonar in Maharashtra, India, showing the capability to reduce chromium up to 84% in 96 h in a nutritive broth supplemented with 100 µgmL-1 of K2Cr2O7 (Tambekar et al. 2015). Study reported by Molokwane & Chirwa (2009) indicated that genus *Bacillus* and *Lysinibacillus* are the predominant microorganisms involved in Cr(VI) reduction in an in-situ remediation by a microcosm reactor operated at 40 mgL-1 of Cr(VI) after 17 days of operation, concluding that the predominance of *Bacillus* and *Lysinibacillus* species was either due to resilience against toxicity or adaptation to the changing conditions in the reactor. These reports suggest that the genus *Lysinibacillus* has several mechanisms for the reduction of Cr(VI), since it is common to find them in soil and water contaminated with Cr(VI), as in Chapala Lake. This study shows that *Lysinibacillus macroides* isolated from Chapala Lake presents extracellular mechanisms to reduce the concentrations of Cr(VI) to Cr(III), making it impermeable to the cell membrane of the bacteria and causing the chromium to remain in the culture medium, making these bacteria an option to be used in waters contaminated with this heavy metal.

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## Author contributions

Study conception: HPCC, SPMY; Data gathering: HPCC, CSA, FTE, SPMY; Data analyses: HPCC, CSA, SPMY; Material contribution: HPCC, LVF, DSVS, EAMI, SPMY; Manuscript main writing: HPCC; Manuscript editing and review: HPCC, LVF, DSVS, EAMI, CSA, FTE, SPMY.