Indian Journal of Biotechnology
Vol 16, July 2017, pp 370-379

Novel microorganisms for the treatment of Ni and V as spent catalysts

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Received 24 October 2014; revised 29 October 2015; accepted 26 November 2015

Oil refining industry has used a great amount of catalysts, which once exhausted due to metal and hydrocarbon poisoning are disposed in special confining sites. Catalyst disposal represents a serious environmental problem due to the potential risk of metal leaching by natural events. The necessity to treat great amounts of catalysts and the existence of microorganisms that coexist with metals suggest that microorganisms can be used for the treatment of hazardous wastes such as spent catalysts. The aim of the present study was to isolate microorganisms from rich metal sites, mines, soils and water from rivers close to mines and then evaluate their ability to remove Ni and V from spent catalyst. Twenty-six isolates were obtained from samples using 9K liquid media and from them, only twelve isolates presented a minimum inhibitory concentration (MIC) higher than 200 ppm of Ni and V, then were evaluated for their ability to remove Ni and V in 9K liquid media added with 16% (w/v) pulp density of the catalyst. Results showed that isolates MNSH1-9K-1 and PRGSd-9K-4 had the highest removal for Ni and V corresponding to 149.5 mg Kg^{-1} and 920.5 mg Kg^{-1}, respectively and were identified by sequencing of 16S ribosomal RNA gene as *Bacillus megaterium* and *Bacillus subtilis*, respectively.

Keywords: Metals, spent catalyst, microbial removal, *Bacillus subtilis*, *Bacillus megaterium.*

Introduction

Operations of petroleum refining, specifically the hydrotreating process, have increased the discharge of spent catalysts, consequently increasing the problem of hazardous waste disposal. Worldwide, the quantity of spent catalysts discarded is in the range of 150,000–170,000 ton/year[1]. The catalysts are mainly composed by Al_2O_3, Pb, Mo, Co and Fe; some of them are present as part of the catalytic matrix but others like Ni and V also may be accumulated from the cracking of hydrocarbons, causing poisoning of the catalysts[2-3].

Bioleaching has been found to be a novel approach for the recovery of metals from various solid industrial wastes[4]; this process is based on the ability of some microorganisms to transform solid compounds to extractable entities[5]. During the growth process of bacteria and fungi, metabolites are formed; they are able to extract valuable metals from waste materials due to their acidic nature or their ability for complex formation[6-7].

The ability to remove metals by microbial pathway can be carried out by several factors: 1) production of organic and inorganic acids during microbial growth, 2) redox reactions[8], 3) production of acid metabolites or chelating agents in the culture medium[9] 4) metal biosorption processes to the cell wall and 5) formation of inclusion bodies in cytoplasm[10]. Bioleaching process involves that organisms interact with the insoluble metal in a liquid medium resulting in soluble and easily removable elements[11-12].

Currently, spent catalysts are disposed in landfills, though, metals as Ni and V can be recovered[13]. Conventional technologies for metal recovery from spent catalysts have been conducted by using a combination of pyro and hydrotechniques[14], metallurgical processes such as incineration that emit SO_2 into the atmosphere[15]. Chemical leaching using acid or caustic solutions generate liquid wastes that represent also a serious concern to the environment and human health[16] although the recovered metals can be used again in different processes[17-18]. The aim of the present study was to obtain microorganisms with the ability for removing Ni and V from spent catalysts with high concentrations of target metals in liquid media.

Materials and Methods

Culture Medium

Culture medium used for isolation and studies of metal removal in spent catalyst was 9K, with a composition in g/L: KH_2PO_4, 0.4; $CaCl_2$ $2H_2O$, 0.2;

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$MgSO_4$ $7H_2O$, 0.4; $(NH_4)_2SO_4$, 0.4; $FeSO_4$ $7H_2O$, 33.3; pH was adjusted to 2.0, using H_2SO_4 at 10%. PHGII solid medium was used for the determination of viable count with a composition in g/L: casein peptone, 4; dextrose, 2; yeast extract, 1.0; agar-agar at a concentration of 15 g/L, pH was adjusted to 7.0.

Microorganism Source

Microorganisms were obtained from six samples at sites located in Guanajuato, Mexico; from 3 different locations: Valenciana mine, Nopal mine and Guanajuato river water. In the same, way samples of water, two types of soil and sediment were stored at 4°C until use. Table 1 shows the codification of samples used as source of microorganisms.

Spent Catalyst Source

The catalyst impregnated with Ni and V was provided by Mexican Petroleum Institute (IMP) located in Mexico City. Table 2 shows its metal content, which was determined by ICP-OES following the methodology explained later. The catalyst was stored under environmental conditions.

Isolation of Ni and V Resistant Microorganisms

In order to obtain Ni and V resistant microorganisms, enriched cultures were prepared from six samples. Two grams or 2 mL of samples were placed in 125 mL Erlenmeyer flasks containing 30 mL of 9K medium that was supplemented with Ni^{2+} and V^{5+} at 10 to 100 ppm each. Nickel and vanadium were provided as salts of Ni $(NO_3)_2.6H_2O$ and $NaVO_3$. Flasks were incubated at 30ºC, 140 rpm for 7 days or until turbidity was developed, which was related to microbial growth, approximately every 7 days, 3 mL samples were transferred to fresh medium increasing metal concentration up to 100 ppm of Ni^{2+} and V^{5+}. Finally, from enriched populations, decimal dilutions were prepared and 100 µL aliquots were spread on PHGII solid medium containing plates supplemented with Ni and V at 20 ppm. Petri dishes were incubated at 30°C for 7 days or until observing microbial growth. Isolated colonies were selected based on a different colonial morphology and plated on fresh PHGII medium to obtain pure cultures. Polarized light microscopy (Axio Scope.A1) was used to determine purity of the colonies by means of Gram staining and pure microbial cultures were stored in 1.6 × 12.50 cm tubes containing PHGII medium supplemented with Ni and V at 20 ppm, each at 4°C.

Table 1 — Sampling sites and coding of samples that were used as microorganism source.

Source of microorganism	Codification
Valenciana mine	MV
Nopal mine wet soil 1	MNSH1
Nopal mine wet soil 2	MNSH2
Nopal mine dry soil 2	MNSS
Guanajuato river water	PRGL
Guanajuato river sediment	PRGSd

Table 2 — Metal content in the catalyst used in this study.

Metals	ppm
Al	103071.58 ±5468.26
As	821.58 ±30.52
Cr	66.43 ±15.26
Fe	3994.08 ±286.76
Mg	525.61 ±45.93
Mo	18.31 ±0.38
Ni	427.51 ±29.53
P	75.57 ±5.40
V	2164.85 ±76.61
Zn	53.73 ±4.11

Determination of Minimum Inhibitory Concentration (MIC)

The minimum inhibitory concentration (MIC) of Ni and V was determined. Firstly, an inoculum was prepared as follows: isolates were grown at 24 to 48 h in 9K liquid media without metals at 150 rpm, 30°C; microbial density of each isolate was adjusted to 2×10^6 CFU mL^{-1}. An inoculum at 10% (3 mL) was added to experimental sets, which were prepared in 125 mL Erlenmeyer flasks containing 30 mL of 9K medium plus the mixture of Ni and V, at the following concentrations each: 100, 120, 140, 160, 180, and 200 ppm. Sets were prepared in duplicate. An inoculated control but without metals was also included. Flasks were incubated at 30°C, 150 rpm for 7 days. The MIC was defined as the highest concentration of the metals in which the isolates failed to grow[19-20].

Microbial Growth and Metal Removal

The microorganisms with a MIC higher than 200 ppm were selected and evaluated for their ability to grow and remove target metals (Ni and V); in 125 mL Erlenmeyer flasks were added 30 mL of culture medium 9K; after that two treatments were prepared; first treatment: a concentration of 200 ppm Ni and 200 ppm V in salt form was added; second treatment was added with 16% w/v of catalyst; inoculum in both treatments was adjusted to a density of 3 x 10^7 CFUmL^{-1} by cell counting with Neubauer chamber and used to inoculate experimental sets, treatments were

incubated at 30°C, 150 rpm for 7 days, each culture was prepared in duplicate and non-inoculated controls were included, after the incubation period, microbial growth was determined by viable plate count, taking 1 ml of the liquid medium; the pH was determined according to the NMX-AA-008-SCFI-2000 method[21]. Residual concentration of metal in catalyst was determined by ICP-ES, the catalyst was separated from culture medium by a filtering process; the solid and filtered liquids were used for metal quantification following the methodology referred below.

Digestion and Analysis of Metals

All samples were subjected to metal analysis by using an inductively coupled plasma optical emission spectrometer (ICP-OES, Varian Model 710-OES) after acid digestion. 100 mg or 1000 µL of samples were placed in 100 mL sintered silicon carbide digestion vessels, adding 6 mL of HCl and 2 mL of HNO_3, vessels were placed in a microwave reaction system (Microwave Pro Anton Paar) with rotor HF100 using the next condition for 8 vessels: power 800 W; pressure 40 bar, temperature 210-240°C for 20 min. Afterwards, using a 10 mL syringe and filtering in a 0.2 µm cellulose filter, the filtrate was collected in a 100 mL volumetric flask and set with deionized water; from the flask, 30 mL were withdrawn into plastic tubes at 4°C until analysis. The metal analysis was performed by ICP-OES at the following wavelengths (nm): Ni (231.604) and V (292.401) in which metal concentrations were calculated based on different calibration curves: 0.1 to 10 ppm for Ni and V, using a commercial standard (High-Purity).

Microbial Identification

The isolated microorganisms were subjected to microbial identification as follows: total genomic DNA was extracted from 30 mL of fresh microbial culture of MNSH1-9K-1 and PRGSd-9K-4; cells were collected by centrifugation and cell lysis was achieved using 200 µL of buffer (10 mM Tris HCl, pH 8.0, 1 mM EDTA, 10 mM NaCl, 1% SDS); 0.1 g of glass beads and 200 µL of a 25:24:1 mixture of phenol: chloroform: isoamyl alcohol were mixed in a vortex with 30 sec pulses and incubating during 30 sec on ice, after centrifugation, the aqueous phase was collected; DNA was precipitated using 2 volumes of ethanol and finally, DNA quality was estimated by 1% agarose gel electrophoresis in 1X TAE buffer and staining with GelRed® (Biotium Inc., Hayward, CA, USA). PCR amplifications of the 16S rRNA gene were performed with a Techne TC- 3000 Thermal Cycler (Barloworld Scientific, USA) using universal bacterial primers. The reactions were done in 50 µL mixtures containing 5 µL of 10X PCR buffer, 0.4 µM of each of the primers 16S ribosomal DNA: 16SrF 5´-GCG GAT CCG CGG CCG CTG CAG AGT TTG ATC CTG GCT CAG- 3´ and 16SrR 5´- GGC TCG AGC GGC CGC CCG GGT TAC CTT GTT ACG ACT T-3´[22], 0.2 mM of each of the four dNTPs, 2 mM MgCl2, and 2 U of *Taq* polymerase (Fermentas, Thermo Fisher Scientific, Inc.).

Amplifications were done by using an initial denaturation step of 94°C for 5 min, followed by 35 cycles of 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C, and a final extension at 72°C for 10 min. PCR products ~1575 bp were checked in 1% agarose gels stained with GelRed® and purified with QIAquick gel extraction kit (QIAGEN N.V., Netherlands), pyrosequencing 16S amplicons at MACROGEN, Korea; the MNSH1-9K-1 and PRGSd-9K-4 sequences of 1318 and 1115, nucleotides respectively were queried against a taxonomic database of high quality sequences derived from NCBI using BLASTN[23] to explore the taxonomic hierarchy. A collection of taxonomically related sequences was obtained from the NCBI Taxonomy Homepage and used for performing a multiple alignment analysis with T-coffee[24]. Only common 16S rRNA gene regions were included in the phylogenetic tree, and similarity analyses using the Jukes-Cantor model were done with the MEGA 5[25]. The phylogenetic trees were constructed using the neighbor-joining method, and 1000 bootstrap replications were assessed to support internal branches[26]. The genus and species limits were 95% and 97%, respectively, according to previously suggested criteria[27].

Results and Discussion

Isolation of Ni and V Resistant Microorganisms

Twenty six isolates were obtained from six environmental samples (tailing mines and the river next to mines, Table 3) using 9K medium supplemented with 100 ppm of Ni and 100 ppm of V salts. Sherameti *et al*[28], reported that cells of microorganisms from habitats with metal may develop metal resistance systems in an attempt to protect sensitive cellular components. Several factors determine the extent of resistance in a microorganism: the type and number of mechanisms for metal uptake, the role each metal plays in normal metabolism, and the presence of genes located on plasmids, chromosomes, or

Table 3 — Number of isolates obtained from different samples.

Samples	Number of isolates	Coded isolates
MV	4	MV-9K-1, MV-9K-2, MV-9K-3, MV-9K-4
MNSH1	4	MNSH1-9K-1, MNSH1-9K-2, MNSH1-9K-3, MNSH1-9K-4
MNSH2	6	MNSH2-9K-1, MNSH2-9K-2, MNSH2-9K-3, MNSH2-9K-4, MNSH2-9K-5, MNSH2-9K-6
MNSS	3	MNSS-9K-1, MNSS-9K-2, MNSS-9K-4
PRGL	3	PRGL-9K-1, PRGL-9K-2, PRGL-9K-3
PRGSd	6	PRGSD-9K-1, PRGSD-9K-2, PRGSD-9K-3, PRGSD-9K-4, PRGSD-9K-5, PRGSD-9K-6

transposons that control metal resistance exposition may generate resistance and activate metabolic activities that allow them to grow in the presence of heavy metals with the possibility to produce metabolites such as biosurfactants[29], chelating agents like siderophores[30] or organic and inorganic acids[31], thus, detoxify the medium in which they are changing the phase or oxidation state in which the metal is found.

From the twenty six isolates obtained, predominant group corresponded to Gram negative population with 15 isolates (57.7%), 10 Gram-positive (38.5%) and 1 yeast (3.8%). The influence of cellular wall in both microbial groups to bind metals has been studied[32-35]. A Gram positive cell wall is composted by 40 – 90% peptidoglycan. On the surface of this layer, it can be embedded in polysaccharides acids called teichoic and teichuronic acids which are covalently bonded to the peptidoglycan layer and have been studied in metal tripping, since the phosphoryl groups confer a negative charge to the cell wall, these negatively charges can serve as cation-sequestering mechanisms. Whereas in Gram negative bacteria the phosphate groups are in the lipopolysaccharides (LPS's), in the case of phospholipids, they are on the surface of the outer membrane which provide the negative charge on the cell wall promoting the free interaction with the metal ions[36-37]; the microscopic analysis showed that 23 isolates have a shape of rod (bacillus), 2 shaped spherical (coccus) and one yeast. Studies conducted about environmental contaminated with heavy metals, mentioned some species of bacilli found in these environments and they have been studied for the treatment of soils contaminated with metals such as *Bacillus subtilis*, *Bacillus simplex*, *E. coli* K12 and *Saccharomyces cerevisiae* that have been studied to remove Cu, Co, Ni, Zn, Cd and Sr, from aqueous solutions as biosorbent materials[38-40].

Bacterial Resistance to Metals (MIC)

The Minimum Inhibitory Concentration allows knowing the concentration of the metal that the microorganism is able to resist. From twenty six isolates only twelve had a MIC higher than 200 ppm for Ni and V (Table 4) representing 44% of total isolates, this result may indicate that although the isolates came from environmental samples containing Ni and V, the concentration and oxidation state of metals, influences the development of resistance or tolerance skills of microorganisms to survive in the presence of metals. In the case of *Bacillus circulans*, it has been reported with MIC values of 0.5 ppm for Ni[41], *Enterobacter cloacae* with values of 0.3 and 1 ppm to V and Ni respectively[42], *E. coli*, *Staphylococcus* sp., *Pseudomonas aeruginosa*, *Bacillus subtilis*, *Ralstonia solanacearum*, *Rhodococcus opacus* presented a MIC higher than 5 ppm[28,38,43]. The above data suggests that isolates which had the MIC higher than 200 ppm could produce metabolites that favor or enhance a metal removal process from wastes with high metal content (> 200 ppm). Figure 1 shows the photomicrographs of the twelve isolates that showed a MIC higher than 200 ppm of Ni-V.

Ni and V Removal by Isolated Microorganisms

The treatment in liquid media with 200 ppm of Ni-V. The removal of Ni and V was performed with 12 isolates that showed a MIC higher than 200 ppm. Figure 2a,b shows the results obtained in 9K medium with 200 ppm of Ni and V. The twelve strains tested were able to remove the metals with values from 0.95 to 61.1 ppm (0.5 to 36%) to Ni and from 0.37 to 90.99 ppm (0.2 to 49%) for V. The isolates MNSH1-9K-2, MNSH2-9K-1 and PRGSd-9K-1 showed percentages greater than 20%; the isolate MNSH1-9K-2 removed 61.10 ppm Ni and 90.99 ppm for V, isolate MNSH2-9K-1 36.2 ppm Ni and 53 ppm V, isolate PRGSd-9K-1 removed 62.17 ppm Ni and 81.05 ppm for V.

Treatments in liquid media with catalyst at 16%. In the treatment with catalyst at 16% (Figure 3a and 3b) removal was observed from 9.7 to 149.5 $mgKg^{-1}$ (2 to 33%) for Ni and from 0.75 to 920 $mgKg^{-1}$ (0.06 to 32%) for V, isolates MNSH1-9K-1 removed 149.5 $mgKg^{-1}$ of Ni and 127.5 $mgKg^{-1}$ of V. Isolate PRGSd-9K-4 removed 116 $mgKg^{-1}$ of Ni and 923 $mgKg^{-1}$ of V.

Table 4 — Minimum Inhibitory Concentration of twenty-six isolates in liquid culture.

		After 72 h at 30°C						
Isolates	Gram	100	120	140	160	180	200	MIC ppm
MNSH1-9K-1	Positive	+	+	+	+	+	+	>200
MNSH1-9K-2	Negative	+	+	+	+	+	+	>200
MNSH1-9K-3	Negative	+	+	+	-	-	-	160
MNSH1-9K-4	Negative	+	+	+	-	-	-	160
MNSH2-9K-1	Positive	+	+	+	+	+	+	>200
MNSH2-9K-2	Positive	+	+	+	+	+	+	>200
MNSH2-9K-3	Positive	+	+	+	+	-	-	180
MNSH2-9K-4	Negative	+	+	-	-	-	-	140
MNSH2-9K-5	Negative	+	+	-	-	-	-	140
MNSH2-9K-6	Negative	+	-	-	-	-	-	120
MNSS-9K-1	Positive	+	+	+	+	-	-	180
MNSS-9K-2	Negative	+	+	+	+	-	-	180
MNSS-9K-4	Negative	+	-	-	-	-	-	120
PRGSd-9K-1	Positive	+	+	+	+	+	+	>200
PRGSD-9K-2	Negative	+	+	+	-	-	-	160
PRGSD-9K-3	Negative	+	+	+	-	-	-	160
PRGSD-9K-4	Negative	+	+	+	+	+	+	>200
PRGSD-9K-5	Negative	+	+	+	+	-	-	180
PRGSD-9K-6	Yeast	+	+	+	+	+	+	>200
PRGL-9K-1	Positive	+	+	+	+	+	+	>200
PRGL-9K-2	Negative	+	+	+	+	+	+	>200
PRGL-9K-3	Negative	+	+	-	-	-	-	140
MV-9K-1	Positive	+	-	-	-	-	-	120
MV-9K-2	Positive	+	+	+	+	+	+	>200
MV-9K-3	Positive	+	+	+	+	+	+	>200
MV-9K-4	Negative	+	+	+	+	+	+	>200

+ : microbial growth, -: no microbial growth, MIC: Minimum inhibitory concentration.

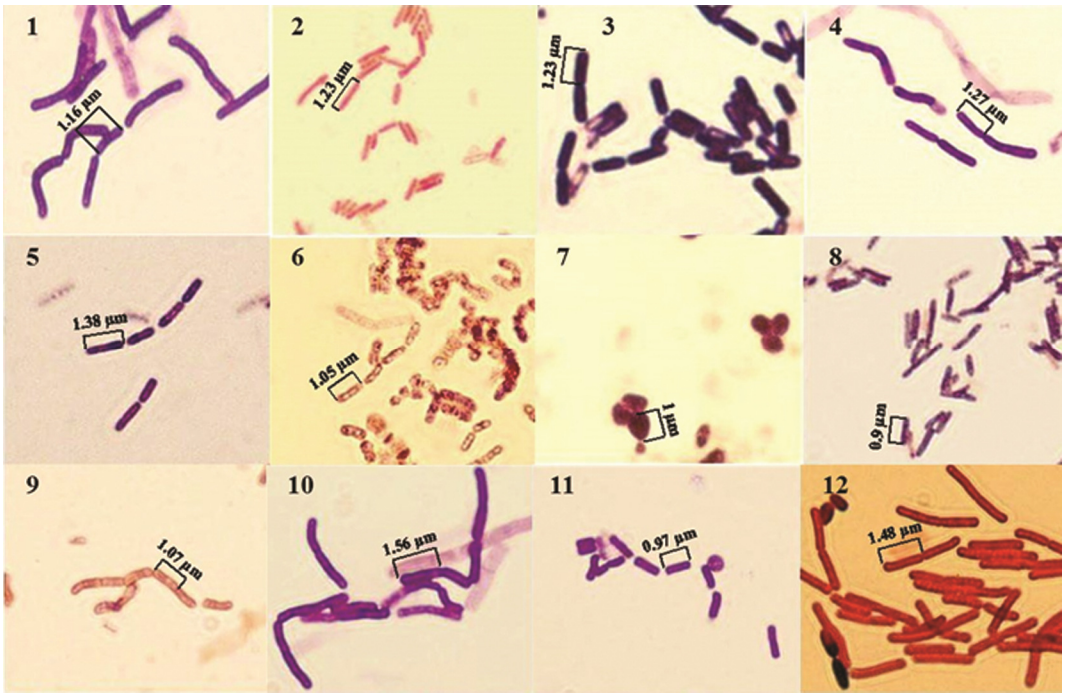

Fig. 1 — Photomicrographs of the twelve isolates (Gram stain): 1) MNSH1-9K-1, 2) MNSH1-9K-2, 3) MNSH2-9K-1, 4) MNSH2-9K-2, 5) PRGSd-9K-1, 6) PRGSd-9K-4, 7) PRGSd-9K-6, 8) PRGL-9K-1, 9) PRGL-9K-2, 10) MV-9K-2, 11) MV-9K-3, 12) MV-9K-4.

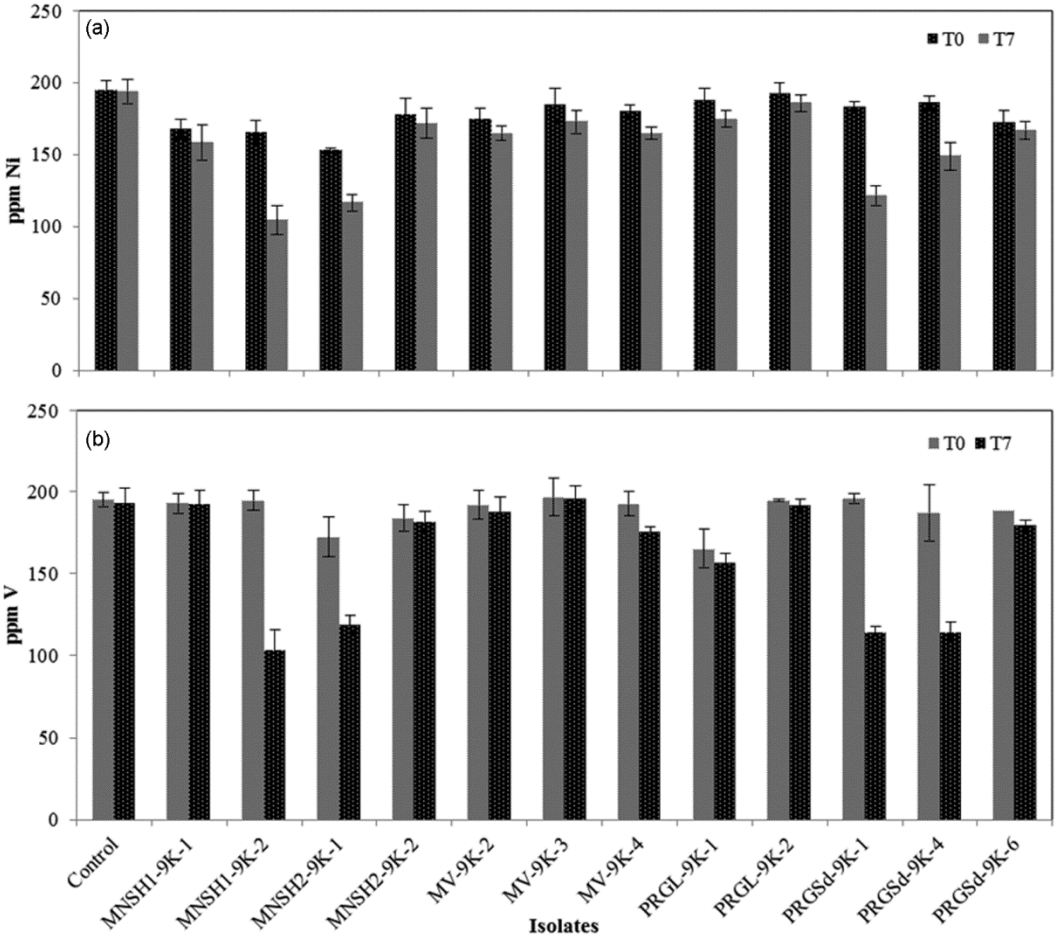

Fig. 2 — Content of metal (ppm) in the liquid medium with 200 ppm of Ni-V at the beginning (0 days) and after the treatment (7 days) by the twelve isolates. a) Ni removal; b) V removal; incubation 30º C, 150 rpm.

The comparison of treatment with salts of Ni and V and the treatment with 16% of catalyst can be observed that in the second, the microorganisms were able to increase the removal of Ni and V with respect to the treatment of salts of Ni and V. This may be due to the catalyst composition since it contains other metals like Fe, Mo (Table 2), which once in the medium could function as promoters for expression of enzymes that require metal cofactors to be expressed (metalloenzymes), so the removal was efficient in presence of spent catalyst[44-45], but more studies are needed to confirm it.

Figure 4a and 4b show the microbial growth curve of the different isolates in presence of 200 ppm of Ni-V. Two different graphics are shown due to the difference in microbial population density. The initial pH was 2.0 in the liquid media, but it was observed that adding the catalyst, increased the pH from 2.0 to 3.65 and remained unchanged along the test, with respect to treatment with salts of Ni and V, pH increased from 2.0 to 2.16 by the addition of salts, though as in the previous treatment, the pH was unchanged along the test. Considering the composition of 9K medium, which does not contain any organic carbon source and due to the pH remained unchanged, even in treatments with the isolates showing the highest removal, discarding the production of secondary metabolites that assist in the chelation of metals, or acids for leaching[46-49].

Isolates coded as MNSH1-9K-1 and PRGSd-9K-4 showed the highest removal percentages of Ni and V in the catalyst, 149.5 $mgKg^{-1}$ Ni and 920.5 $mgKg^{-1}$ V respectively, they corresponded to Gram-positive bacillus. For acid pH, cell walls would have a negative net charge, which promotes attractions between positively charged cation such as Ni^{2+} and negatively charged binding sites based on electrostatic forces[50]. Although the identification and the

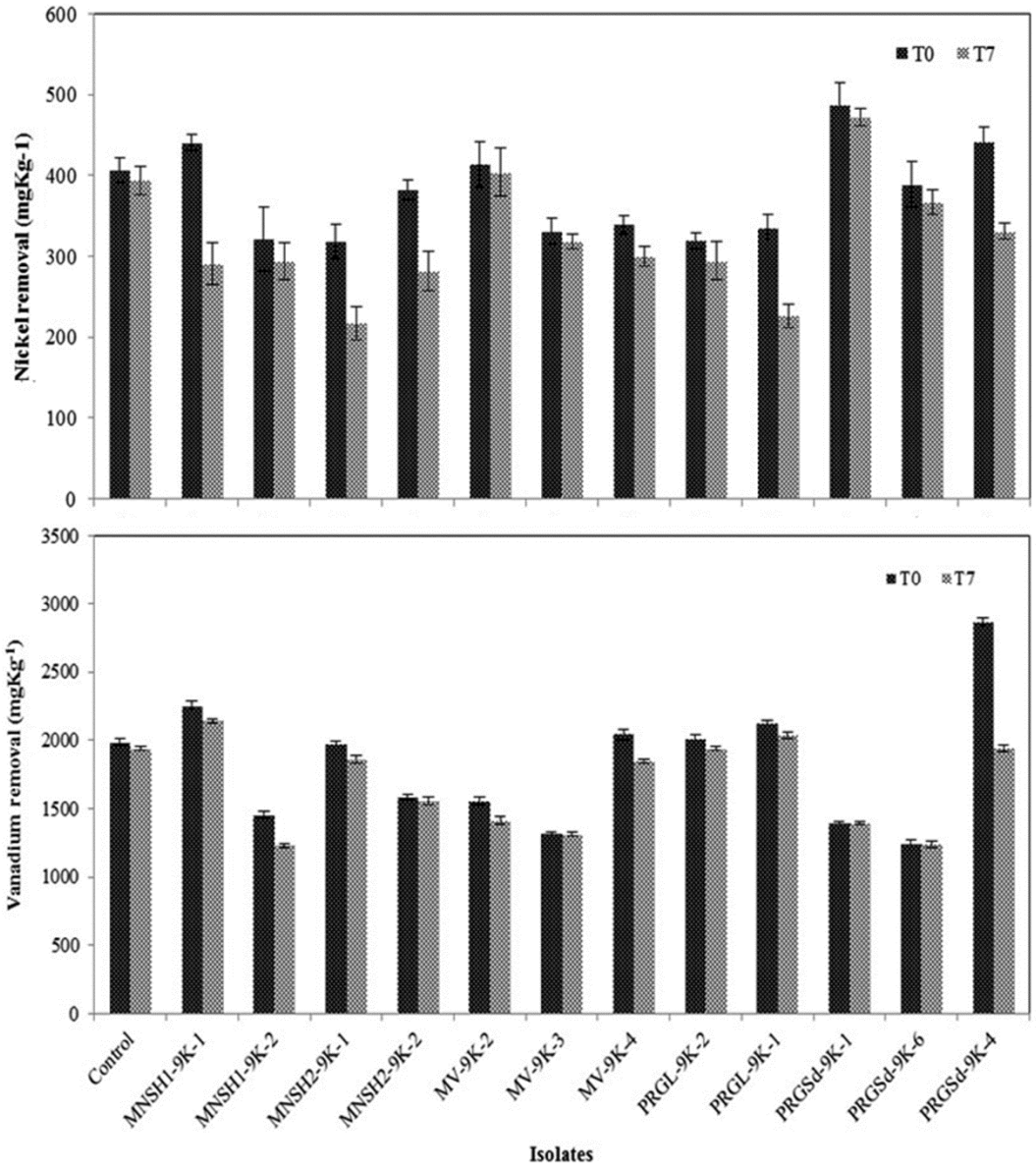

Fig. 3 — Content of metal ($mgKg^{-1}$) in the catalyst at the beginning (0 days) and after the treatments (7 days) by the twelve isolates. a) Ni removal; b) V removal; incubation 30º C, 150 rpm.

mechanisms involved in the removal of Ni and V by strains MNSH1-9K-1 and PRGSd-9K-4 is unknown yet, the strains were able to resist and work in a solid waste with more than 200 ppm of Ni and V.

Microbial Identification

Based on the comparative analysis of the 16S rDNA sequence with those available in the database, it was concluded that the MNSH1-9K-1 and PRGSd-9K-4 strains were related to the genus *Bacillus* (Figure 5). Because the 16S rDNA gene sequence did not allow solving the PRGSd-9K-4 isolate to species through different bioinformatics analysis used, which only allowed us to classify within the genus *Bacillus*, closely related to *Bacillus subtilis* according to the Rossello-Mora criteria[27]. It has been reported that *Bacillus subtilis* is able to accumulate different metals as V, Ni, Pb, Zn and Cu[19, 51].

The MNSHI-9K-1 isolate was identified as *Bacillus megaterium*, this organism is often found in contaminated environments, because of its location, such bacteria can metabolize a variety of substrates and intends to use in bioremediation and waste treatment[52]. In these species have been identified numerous specific genes relating to environmental interactions, cell envelope, transport, signal

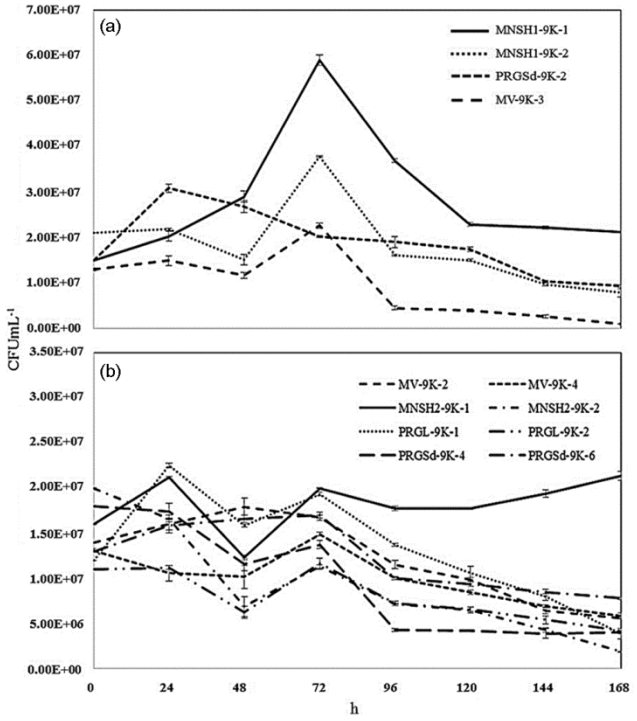

Fig. 4 — Microbial growth of the twelve isolates in 9K medium during 7 days (168 h) during Ni and V removal (200 ppm) at 30º C, 150 rpm.

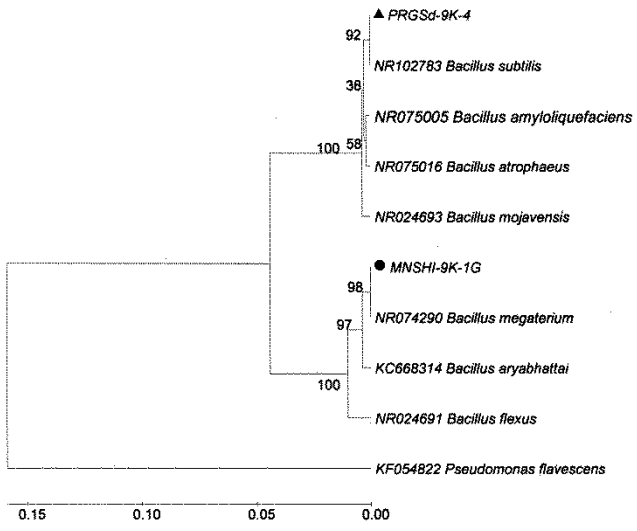

Fig. 5 — The cladogram was constructed by using MEGA 5 software[25]. Evolutionary relationships were estimated by UPGMA method with 1000 bootstrap value to obtain the consensus tree[55-56]. Evolutionary distances were calculated using the Jukes-Cantor method[57].

transduction and gene regulation involved in cell detoxification[53]. The ability for metal removal of isolates from a spent catalyst containing Ni and V can be attributed to different mechanisms already described in bacteria included genera *Bacillus* like bioaccumulation and detoxification[54].

Conclusion

Twenty six bacteria were isolated from samples mines Guanajuato, Mexico. Twelve isolates showed a MIC of 200 ppm for Ni and V. Isolates MNSH1-9K-1 showed the highest removal for Ni (149.5 ppm) and PRGSd-9K-4 for V (920.5 ppm) compared to other isolates in 9K medium from spent catalyst. MNSH1-9K-1 and PRGSd-9K-4 were identified as *Bacillus megaterium* and *Bacillus subtilis* respectively. There is not specific information in literature for the catalysts treated by *Bacillus* species; however, this could be an interesting aspect for futures studies.

Acknowledgement

This project was supported from CONACYT Grant 131203 and Instituto Politécnico Nacional, Mexico.

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