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In Vitro Evaluation Of Bio reduction Of Hexavalent Chrome By Marine Microorganisms Isolated In The Cartagena Bay For Wastewater Treatment

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Contamination problems by heavy metals and specifically by hexavalent chromium generated by different production processes impact aquifers worldwide and require effective remediation methods for their control. The exploration of microorganisms in saline environments with the capacity to bio reduce hexavalent chromium (Cr^{6+}) to trivalent chromium (Cr^{3+}), allows an alternative to the use of biotechnological processes, reducing its toxicity. In the present study, marine microorganisms were isolated from water and sediments, adapted to high concentrations of hexavalent chromium, from 300ppm to 1000ppm with bio reductive potential in wastewater. Bio reduction bioassays were carried out in selective liquid and solid culture media, to which potassium dichromate ($K_2Cr_2O_7$) was added. Morphological and biochemical identification was carried out with API, preserving colonies. Spectrophotometric validation was developed to evaluate (Cr^{6+}), verifying the bio reduction efficiency in laboratory bioassays with King broth and 300 ppm of potassium dichromate. The different broths were evaluated for enrichment, being the nutritive broth and King the best, showing high turbidity and growth in a short time. Among bacteria isolated from water and sediment, the latter showed rapid growth from 18 to 24 h. Gram positive and negative bacilli (*Bacillus subtilis, Bacillus brevis, Bacillus megaterium, Escherichia coli and Citrobacter kosseri*) were found at 500/1000 ppm and biochemically characterized. Bio reduction percentages greater than 91% were obtained at 96 h, in concentrations of 300ppm of hexavalent chromium. Thanks to the selective isolation, tolerance and resistance to hexavalent chromium, these microorganisms proved to be bio reductive of this metal. Therefore, the use of these microorganisms on a full scale can be considered as a result for wastewater treatment where hexavalent chromium is used. Likewise, the use of microorganisms used in the bio reduction process is an alternative to Environmental Microbial Biotechnology that will bring benefits by reducing contamination.

1. Introduction

Chromium is one of the most toxic and abundant metals, that in its hexavalent form causes contamination of surface and groundwater, due to its usual industrial application, such as steel production, electroplating, leather tanning, metal processing such as chrome plating, manufacture of pigments for paints and dyes (Horsfall et al.,2006).

Chromium exists in a wide range of oxidation states from -2 to +6, the dominant species in nature being the hexavalent (Cr^{6+}) and trivalent (Cr^{3+}) (Busto et al., 2016). Hexavalent chromium usually present as chromate (CrO_4^{2-}) and dichromate ($Cr_2O_7^{2-}$), has higher levels of toxicity compared to other states From Valencia". In addition to being highly harmful, it is mobile and has a high residence time in surface and groundwater, which poses a risk to the health of humans and animals, also affecting the development and growth of plants (Lívia et al., 2013).

To reduce or eliminate hexavalent chromium present in solid and liquid waste, various physicochemical methods have been carried out or developed such as chemical reduction and precipitation, adsorption, ion exchange, reverse osmosis, electrodialysis (Shanker et all., 2005), among others, but these methods have the disadvantage high costs, incomplete metal removal, high energy demand, and the generation of secondary

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waste. Currently, the study of alternatives with biological methods, using microorganisms, being a field is emerging and can treat wastewater with metals, which could reduce the costs of chemicals and energy consumption (Panigatti et al., 2012;Muñoz et al., 2015).
Therefore, the search for and obtaining bacteria with a high potential for reducing heavy metals is essential for the development of efficient biological methods in wastewater treatment (Abskaron et al, 2009). The foregoing allows considering hexavalent chromium bioreductive bacteria as a biotechnological tool, where it is proposed to isolate them from marine waters and sediments of an industrial zone in the Bay of Cartagena, showing their tolerance or resistance, for later use in treatment. of sewage.

2. Material and Methods

2.1 Chemical Reagents

For the in vitro evaluation of hexavalent chromium reduction, a 2000 ppm potassium dichromate stock solution (Merck Brand) and a potassium chromate stock solution (Merck Brand) were prepared, which would be used in the microorganism selection dilutions. and bioreduction potential. Additionally, for the culture media, salts, carbohydrate sources and proteins were used, as described in the following section on culture media.

2.2 Microbiological culture media assay

In order to evaluate the growth of the microorganisms and thus be able to select a medium for the chromium reduction bioassay, the following media were used in 1000 ml:
• Nutritive Broth: Yeast extract - 0.3g; Peptone - 0.5g; Sodium chloride (NaCl) - 2g (Revelo et all, 2015)
• King Broth: Peptone - 2g; Dipotassium hydrogen phosphate (K_2HPO_4) - 0.15g; Magnesium sulfate heptahydrate ($MgSO_4$ - $7H_2O$) - 2g (Oves et all, 2013)
• Lee Broth: Glucose - 2.5g; Dipotassium hydrogen phosphate (K_2HPO_4) - 2.5g; Magnesium sulfate heptahydrate (MgSO4-7H2O) - 2g; Ammonium sulfate (NH_4) 2SO4 - 5g; Sodium Chloride (NaCl) - 5g (Coreño-Alonso et all, 2014)
• EG Broth: Ammonium Chloride (NH_4Cl) - 5mg; Dipotassium hydrogen phosphate (K2HPO4) - 5mg; Potassium dihydrogen phosphate (KH_2PO4) - 5mg; Sodium chloride (NaCl) - 1mg; Magnesium sulfate heptahydrate ($MgSO_4$-$7H_2O$) - 1mg; Ammonium Acetate (NH_4COOCH_3) - 200mg; Yeast extract - 15mg; Peptone - 50mg (Teles et al, 2018)

2.3 Sampling

Samples of marine surface sediments and water were taken in Cartagena Bay, Colombia, in 9 stations:
E1 (Latitude 10.390262 /Longitude -75.533290), E2 (Latitude 10.319929 / Longitude -75.541060), E3 (Latitude 10.318369 / Longitude -75.526882), E4 (Latitude 10.325081 / Longitude -75.517751), E5 (Latitude 10.36755 / Longitude -75.510444), E6 (Latitude 10.375544 / Longitude -75.516585), E7 (Latitude 10.382154 / Longitude -75.532465), E8 (Latitude 10.391596 / Longitude -75.551964) y E9 (Latitude 10.397261 / Longitude -75.540941), taking into account a distance of 300-500 m from sources of pollution.
To collect the samples, the selected land was divided into rectangular sections of 10 m each, crossing each section diagonally according to the methodology proposed by Hudson (2006), and taking samples of water and sediment in the corners of the rectangles. Imaginaries raised, as shown in figure 1. The samples from each point (500 ml water bottles and 300 g sediment carrier) were stored by joining them in composite samples of water and sediment respectively, storing in the laboratory at a temperature of 4 ° C.

2.4 Selective isolation of microorganisms in water and sediments of Bahia

2.4.1. Recovery of microorganisms.

Composite samples of water and sediment were prepared respectively, which were placed in nutrient broth: 1 ml of liquid sample + 9 ml of medium and 2 mg of sediment sample + 8 ml of distilled water, subsequently taking 1 ml + 9 ml of broth; homogenizing by continuous shaking and incubating at 28 ° C. These suspensions were subsequently enriched after 24 hours in the culture media previously prepared for 24 hours at a temperature of 28 ° C.

2.4.2. Primary selection of microorganisms at 50 and 150 ppm Cr^{6+}.

From the chromate and dichromate stock solution, 250 microliters equivalent to 50 ppm were extracted, 750 microliters equivalent to 150 ppm were also taken, which were added to the King and Nutritive Broths previously prepared, 1 ml of enriched culture was added to 9 ml of chromed broth and incubated at room temperature (28°C).

2.4.3. Final selection in King medium with 300, 500 and 1000 ppm of Cr +6.
The potential chromium-reducing microorganisms were seeded in liquid and solid King media, verifying growth at 18, 24, 48 and 72 h, the plate isolation was carried out by the striae method.

2.4.4. Phenotypic characterization and conservation of pure morphotypes
GRAM staining was performed on 16 marine microorganisms with the original agar with the type of sample, the hexavalent chromium salt and where it was cultured, fixing the samples in glass plates with 2% saline solution, and observing the cell morphology in the microscope, after staining and for the identification of the resistant bacteria selected in the chromium bioassay, the biochemical characterization was carried out by means of assays through the API gallery of Biomerieux.

2.5. Calibration standard curve diphenyl carbazide

The reference method applied is the Standard Method for the Analysis of Drinking and Waste Waters 3500-Cr. A Genesys 10 UV Spectrophotometer was used to work at 540 nm absorbance, using the modified method of Sierra and García, 2012 with diphenyl carbazide solution.
Solutions with concentrations of 0.9, 1.9, 3.9, 7.8, 15.6, 31.2, 60.5, 250 ppm were prepared in test tubes; which were measured in the spectrophotometer with the blank at 540 nm obtaining a curve with a linear correlation of 0.8945 due to the interference provided by the color of the diphenylcarbazide (Severiche and Gonzalez, 2013), which was subsequently corrected with broth enriched with chromium at 300 ppm, with dilutions at the same concentrations, in order to evaluate the difference presented by the color of the chromed solution, observing that the reaction of chromium with diphenylcarbazide stabilizes the color of the sample and therefore increases the linear correlation of the curve obtained at 0.9329.

2.5 2.6 Bioassay of Marine Microorganisms

The 16 strains of marine microorganisms were activated by ringing in King broth with 300 ppm of Cr + 6; of which 4 strains were taken for the preparation of the inoculum for the bioassays:
• MM-001-Cr2 Inoculum: 9mL of King broth were taken at 300 ppm with 1ml of Strain MM-001.
• MM-005-Cr2 Inoculum: 9mL of King broth were taken at 300 ppm with 1ml of Strain MM-005.
• MM-007-Cr2 Inoculum: 9mL of King broth were taken at 300 ppm with 1ml of Strain MM-007.
• MM-009-Cr2 Inoculum: 9mL of King broth were taken at 300 ppm with 1ml of Strain MM-009.
• MIX-Cr2 inoculum: 9mL of King broth were taken at 300 ppm with 0.25ml of strain MM-001, 0.25ml of strain MM-005, 0.25ml of strain MM-007 and 0.25ml of strain MM-009, to complete the volume of 1ml.

3. Results and discussion

3.1. Testing with microbiological cultivation media
When enriching the microorganisms in the culture media, the King medium presented better growth results, so this was taken for the continuation of the tests, verifying growth at 18, 24, 48 and 72 h, in isolation by the stretch mark method as in the research carried out by Oves et al., 2013, which presented favorable results for the growth of *Pseudomonas aeruginosa* in this medium, for chromium reduction.
In addition to the different broths worked on in this research (Nutritive, King, Lee and EG), other culture media can be found that according to the antecedents, LB (Luria-Bertani) and BHI (Brain Heart Infusion) where this yields percentages of 100% reduction, however, these results are for low concentrations, respectively (Soto Rueda et all, 2017)

3.2 Selective isolation of microorganisms in water and sediments of Bay

Regarding the enrichment of marine microorganisms, using different means found in the bibliography, better results were presented in Nutritive and King Broths, using chromate and dichromate salts at 50 ppm.
When moving to nutritive and King agar medium with 50 and 150 ppm of these salts, growth results were obtained at 18 h with sediments in King broth, but at 24 hours both in water and in sediments. For marine microorganisms with greater tolerance to hexavalent chromium, King agar medium was prepared with concentrations of chromate and potassium dichromate at 300, 500 and 1000 ppm, of which for the first concentration, growth of colonies was achieved at 18 h with sediments and dichromate salt, at 24 hours for both types of samples; for the second concentration, growth was obtained at 24 hours in a sediment sample with chromate and dichromate salts, and at 48 hours in both samples, finally, for 1000 ppm growth was noted after 72 hours with both samples and salts.
When comparing the diversity of microorganisms that grew in nutritive and King Broth, negative bacilli growth was observed in all, except for King Broth with a water sample, being able to determine a greater load of microorganisms in sediments. The selective isolation of bacteria from the water and sediments found in the

Bay of Cartagena made in different broths, showed better results in Nutritive and King broth, where there was greater biomass and diversity, finding Gram positive and negative bacilli, such as Gram positive cocci in the sediments. , as well as a faster growth from 18 to 24 hours, as can be seen in Table 1.. The biochemical characterization of the chosen morphotypes were, MM-001 (*Bacillus subtilis*), MM-005 (*Bacillus megaterium*), MM-007 (*Escherichia coli*), MM-009 (*Citrobacter kosseri*).

Table 1: Phenotypic characteristics of isolated bacteria

Strain	Strain code	Culture medium	[ppm]	Source	Phenotypic	Gram stain
1	MM-001	Nutritive Agar	150	Sediment		C+
2	MM-002	Nutritive Agar	150	Sediment		Mix
3	MM-003	King's Agar	300	Sediment		B-
4	MM-004	King's Agar	300	Sediment		Mix
5	MM-005	King's Agar	300	Sediment		C+
6	MM-006	King's Agar	300	Sediment		C-
7	MM-007	King's Agar	300	Water		B-
8	MM-008	King's Agar	500	Water		Mix
9	MM-009	King's Agar	1000	Sediment		CB-
10	MM-010	King's Agar	1000	Sediment		Mix
11	MM-011	King's Agar	1000	Sediment		C+
12	MM-012	King's Agar	1000	Sediment		C+
13	MM-013	King's Agar	1000	Sediment		C+
14	MM-014	King's Agar	1000	Sediment		Mix
15	MM-015	King's Agar	1000	Sediment		Mix
16	MM-016	King's Agar	1000	Sediment		Mix

3.3 Bioassay of Marine Microorganisms

Of the 16 isolated strains, the 4 with the highest growth in agar and the highest turbidity in less than 24 h in chromium-enriched liquid medium were selected, due to their greater tolerance to chromium, exposing them in bioprocesses with synthetic residual water, with a Cr + concentration. 6 of 300 ppm, determined the percentage of reduction and the final concentration of Cr + 6 for 96 hours, obtaining the results presented in Figure 1.

By performing API biochemical behavior tests on the four cultures with the highest tolerance to chromium, the following microorganisms were identified: *Bacillus subtilis* (M-001), *Bacillus megaterium* (M-005), *Escherichia coli* (M-007) and *Citrobacter kosseri* (M-009), and the reductions found were 87.5%, 94.2%, 95.2%, and 87.1% respectively. Being the strain M-007 (*E. coli*), that presented a greater percentage of reduction of Chromium in 96 hours, according to the results obtained in similar studies by Panigatti et all, 2009 and Abskharon et all, 2009.

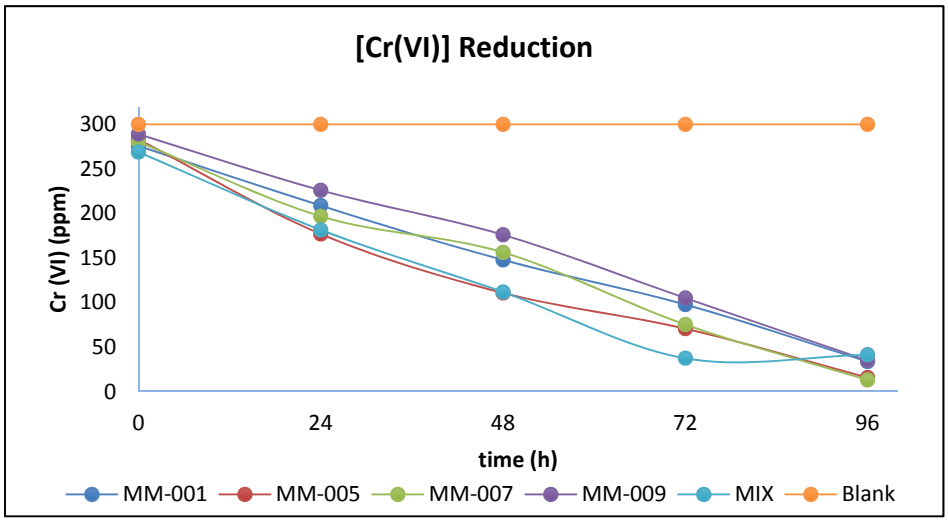

Figure 1: Chromium reduction bioassay

The results obtained indicate that the marine microorganisms recovered in the area around the bay of Cartagena have a great capacity to reduce hexavalent chromium, being that the sediment samples obtained better results than with the water samples, because in the sediments there is greater accumulation of microorganisms.
When analyzing the results obtained by means of statistical analysis by ANOVA, the response variable being the concentration of hexavalent chromium and the categorical factor being the absorbances obtained by spectrophotometry, different trend lines can be noted, which the green and gray lines indicate predictions regarding the limit values that can be obtained from said equation, by means of the graph of the adjusted model through a simple regression.

4. Conclusions

From the evaluation carried out for marine microorganisms, it can be inferred that the selective isolation of microorganisms from the water and sediments found in the Bay of Cartagena, testing different broths for enrichment, showed better results in King broth, using chromate and dichromate as sources in concentrations of 50, 150, 300, 500 and 1000 ppm, where there was greater biomass and diversity, such as Gram positive and negative bacilli, Gram positive cocci in the sediments of King broth with dichromate, as well as a faster growth between 18 to 24h.
Marine microorganisms isolated and tolerant to hexavalent chromium were shown to be potentially reducing agents of this metal. The marine microorganisms with considerable bioreduction potential were strains MM-001 and MM-007 and MM-009 (positive cocci, bacilli and negative cocci) with a bioreduction percentage of 92% at a concentration of 300 ppm with potassium dichromate; MM-005 strains (positive cocci) had a high bioreduction percentage of 96%. The mixed consortium prepared (MIX) was tolerant to a concentration of 300ppm of potassium dichromate, with a reduction percentage of 91% being considered an optimal consortium.

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