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ORIGINAL ARTICLE

# Molecular techniques for the assessment of Cr (VI) reduction by *Bacillus thuringiensis*

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## Abstract

Effluent pollution with Cr (VI) is a worldwide environmental problem. In the Pasto River (southeastern, Colombia), previous studies reported contamination with this metal at points near tanneries. To establish the role of *Bacillus thuringiensis* in Cr (VI) reduction in water from Pasto River, experiments were carried out with untreated Pasto River water (treatment 1), sterile Pasto River water inoculated with *B. thuringiensis* (treatment 2), and unsterilized Pasto River water inoculated with *B. thuringiensis* (treatment 3). All experiments were conducted in bioreactors with a controlled temperature of 20 °C and constant agitation for 156 h. Samples of 20 mL were taken every 12 h from each treatment to track Cr (VI) reduction levels and to confirm microorganism identity via molecular methods involving denaturing gradient gel electrophoresis (DGGE), restriction enzyme digestion profiles (RFLP), and bioinformatic analysis. Cr (VI) reduction was higher in treatment 3 (99.42 %) as opposed to treatment 2 (76.12 %) and treatment 1 (74.46 %). The molecular identity of *B. thuringiensis* was determined via sequencing of the 16SrRNA gene, and RFLP assessments in all three treatments revealed *B. thuringiensis* profiles. Since *B. thuringiensis* was present in all three treatments trough time, Cr (VI) reduction can be attributed to this bacterium.

**Keywords:** Heavy metals; Chromium reduction; Cr reducing bacteria; DGGE (DeCS).

## 1. Introduction

Chromium (Cr) is used in several industrial, metallurgical, textile, and fertilizer activities, resulting in direct and indirect discharges to main effluents. Often, the amount of chromium discharged exceeds the maximum concentration established by local or national Environmental Protection Agencies (EPA) in drinking water (100 $\mu g\, L^{-1}$) [1]. There are two states of stability of this metal: trivalent chromium [Cr (III)] and hexavalent chromium [Cr (VI)]. Although the first state is not toxic, it has been described that oxidation-reduction processes can transform it into Cr (VI), a highly toxic, carcinogenic, and mutagenic state. Cr (VI) has properties of a high degree of corrosion, solubility in water, and ease of crossing biological membranes, thus causing alterations in nucleic acids [2].

There are different alternatives for the recovery of Cr (VI) from contaminated effluents, among which bioremediation stands out for its efficiency and low cost compared to conventional technologies used for heavy metal removal [3]. One of the biological systems to be highlighted is mediated by wild bacteria isolated from contaminated environments, which have great adaptability at physiological and metabolic levels, wide functional diversity, and greater efficiency

when implemented in contaminated effluent treatment systems [4]. In this sense, Oves *et al.* [5] evaluated *Pseudomonas aeruginosa* OSG41 strain tolerance to and reduction capacity of Cr (VI); the strain was isolated from water contaminated with heavy metals and the effect of its inoculation was measured on the growth of a chickpea culture subjected to high concentrations of Cr (VI). The authors determined that the strain had a high potential for tolerance (up to 1800 mg $mL^{-1}$) and reduction of Cr (VI) (up to 100 %) depending on physicochemical factors, also reducing the cytotoxic effect of the metal in chickpea plants.

Consecutively, Hossan *et al.* [6] studied the efficiency of reduction and biosorption of Cr (VI) by chromate resistant bacteria isolated from tannery effluents and established that of the total of isolated strains, the strain SH-1 characterized as *Klebsiella* sp. presented Cr (VI) reduction efficiencies of 95.0 % and 63.1 % in the laboratory and directly in the effluent, respectively. Similarly, Mishra *et al.* [7] investigated the Cr (VI) reduction efficiency of the *Microbacterium paraoxydans* CRB 19 strain, isolated from chrome-contaminated tannery wastewater from a local common effluent treatment plant and determined that the bacterium revealed a wide tolerance to Cr (VI) ($\leq$ 1000 mg $L^{-1}$) and a reduction potential that ranges between 39.2 % and 93.5 %.

On the other hand, plant growth promoting rhizobacteria (PGPR) can increase host plant tolerance to cope up with heavy metal induced stress, which can improve plant growth. A study carried out by Karthik *et al.* [8] isolated a Cr (VI) tolerant PGPR strain and evaluated its plant growth promoting (PGP) properties under Cr (VI) stress. The rhizobacterial strain AR6 was isolated from the rhizosphere of *Phaseolus vulgaris*, this was specifically selected due to its high Cr (VI) tolerance (1200 µg $mL^{-1}$) and substantial production of PGP substances. Inoculation of strain AR6 significantly increased the root length of test crops in the presence of Cr (VI) and produced a considerable number of colonizes on the root of versatile dicot and monocot plants. Moreover, strain AR6 exhibited strong antagonistic activity against the phytopathogen *Aspergillus niger*. Thus, this study suggests that metal tolerant and PGP activities of the rhizobacterial strain AR6 could be exploited for environmental and agricultural challenges [8].

In the department of Nariño, Colombia, there is a high degree of contamination of effluents by heavy metals, mainly Cr (VI) generated from leather treatment in tanneries. Chromium concentrations of 59 mg $L^{-1}$ have been reported in the Pasto River [9]. A previous study evaluated the efficiency of the reduction of Cr (VI) by the wild bacterium *Bacillus thuringiensis* in different treatments using water from the Pasto River as a substrate [10]. The results indicated reduction levels between 74 % and 92 %. In this study, however, the presence and dynamics of the bacteria during the entire fermentation process were not determined experimentally with respect to the bacterial load of the substrate used. Nevertheless, the results are promising in the context of bioremediation and can be taken as a basis for implementing decontamination strategies on a large scale.

Molecular techniques and markers aid the characterization of microbial communities and the detection of microorganisms. One example is denaturing gradient gel electrophoresis (DGGE). DGGE is a sensitive and relatively reproducible method central to preliminary studies of microbial diversity, and its results could be used as a reference for further investigations where information is complemented in more detail [11]. The objective of the present investigation was to determine the presence of the *B. thuringiensis* bacteria during the Cr (VI) reduction process in a batch treatment using residual contaminated water from the Pasto River as a substrate.

## 2. Materials and methods

The microorganisms used in this study were obtained from the bank of isolates of the Microbial Biotechnology research group at University of Nariño, which are preserved in 30 % glycerol (GenBank: MK561610). The isolates were recovered in Luria Bertani (LB) culture medium at 37 °C for 24 h. An experimental design was carried out using three treatments with three biological replicates.

The experimental units were bioreactors of 60 cm in height and 11.40 cm in diameter. Batch-type fermentation was carried out with a controlled temperature of 20 °C and constant agitation at 50 vvm (volume of air per volume of medium per minute). The following treatments were used:

- **Treatment 1:** Water from the Pasto River without sterilization (control without inoculation).
- **Treatment 2:** Sterile water from the Pasto River inoculated with *B. thuringiensis*.
- **Treatment 3:** Unsterilized water from the Pasto River inoculated with *B. thuringiensis*.

The bioreactors were operated for 156 h, during which 20 mL samples were taken in sterile 50 mL falcon tubes every 12 h for total DNA extraction. To quantify the Cr (VI) reduction of the bacterial isolates in the water samples, a standard curve was made using the 1,5-diphenylcarbazide method proposed in the 1999 Standards Methods for the examination of water and wastewater [12]. Later the samples were analyzed taking this method into account, namely an aliquot of 5 mL of diluted sample (dilution factor 1/100) was taken every 12 h, $H_2SO_4$, $H_3PO_4$, and 1.5 diphenylcarbazide were added, homogenized and allowed to stand until the development of the red-violet color characteristic of complex formation and absorbance at 543 nm was measured. Consecutively, the concentration of Cr (VI) and the reduction percentage were determined, using the equation of the line obtained from the standard curve [12].

### 2.1. Chromosomal DNA extraction

Total DNA extraction was carried out using the protocol of Burbano and colleagues. [13] with modifications in terms of reagent concentrations, incubation times, and sample volume. First, 20 mL aliquots of water were taken from each treatment at three-time intervals (0 h, 72 h and 156 h). These were centrifuged for 5 min at 6461 *g*. The supernatant was discarded, and the samples were washed three times with 500 µL of TE 1X buffer solution, followed by 400 µL of SET buffer solution [20 % sucrose, 50 mM EDTA, and 50 mM Tris-HCl, pH 7.6]. The tubes were homogenized with a vortex, and 80 µL of lysozyme were added, followed by incubation at 37 °C for 1 h and 30 min (with shaking every 10 min).

Next, 20 µL of 10 % sodium dodecyl sulfate (SDS) solution and 5 µL of proteinase K (20 mg $mL^{-1}$) were added. The mixture was incubated at 37 °C for 2 h with constant shaking. After that, 80 µL of 5 M NaCl and 60 µL of 10 % hexadecyl trimethyl ammonium bromide (CTAB) were added and homogenized with a vortex. The mixture was then incubated in a water bath at 65 °C for 20 min with constant agitation. Then, ~ 600 µL of phenol-chloroform-isoamyl alcohol (25:24:1) were added and mixed with a vortex, and the samples were centrifuged at 6461 *g* for 10 min at room temperature.

The aqueous phase was separated into a new sterile centrifuge tube, and 1 µL of RNAse (20 mg $mL^{-1}$) was added. The mixture was incubated for 1 h at 37 °C. Additionally, an equal volume of chloroform-isoamyl alcohol (24:1) was added, a vortex was applied, and the tubes were centrifuged for 10 min at 6461 *g* at room temperature. The aqueous phase was removed and

carefully transferred to another new Eppendorf tube. To achieve DNA precipitation, 0.60 volumes of isopropanol were added to the tube walls. Once the formation of a precipitate was observed, the tubes were centrifuged for 10 min at 8283 *g*.

The DNA pellet was washed twice with 200 µL of 70 % ethanol and dried in an extraction chamber for 15 min. Finally, the DNA was resuspended in 30 µL of ultra-pure water and refrigerated at −20 °C. The integrity of the DNA obtained was verified on 1 % agarose gels (2 µL of TE 1X, 1 µL of bromophenol blue, and 2 µL of DNA from each sample) [14]. *Lambda HindIII* (Promega; Madison, Wisconsin, USA) was used as the molecular size marker, and the operating conditions were 70 V for 1 h and 30 min. The gel was visualized on a Benchtop3UV Transilluminator photo-documentator at a wavelength of 302 nm.

### 2.2. PCR amplification of the 16S rRNA gene for DGGE

The primers 341F-GC + glue GC 5 '(CCTACGGGAGGCAGCAG) 3' [15] and 907R 5 '(CCGTCAATTCMTTTGAGTTT) 3' [15] were used for amplification. PCR reactions were performed at a volume of 50 µL, a final concentration of 0.40 µM of primers, a mix of dNTPs at 0.05 mM, Taq DNA Polymerase at 0.025 U $\mu L^{-1}$, 1 mM $MgCl_2$, 10 µL of colorless buffer (Promega; Madison, Wisconsin, USA), and 32.75 µL of sterile ultrapure water. A DNA template was added for each of the samples at a concentration of 15 ng $\mu L^{-1}$. Water was used as a negative control, and *E. coli* DNA was used as a positive control.

The PCR reaction was performed in a Multigene Labnet thermocycler with the following thermocycling program: 94 °C for 10 min; 35 cycles of 94 °C for 1 min, 54 °C for 1 min, and 72 celsius for 2 min; and a final extension of 10 min at 72 °C.

The PCR products were visualized on 1 % agarose gels treated with ethidium bromide (5 µg $mL^{-1}$). The gels were run in 1X TAE buffer for 1 h at 70 V using 100 bp as a molecular size marker (Promega; Madison, Wisconsin, USA). The gels were visualized on a Benchtop 3UV Transilluminator photo-documentator at a wavelength of 302 nm.

### 2.3. DGGE

PCR products were separated by DGGE. First, a stock solution of acrylamide - bis acrylamide at a 19: 1 ratio was made from which denaturant gradients of 40 % and 60 % were formed (**Table 1**). Polyacrylamide gels were prepared using a TV400-DGGE chamber filling system (Scie-Plas Ltd.; Cambridge, UK), and then the TAE 1X run buffer was preheated to 60 °C for 1 h. A pre-run was carried out for 15 min at 100 V. To avoid interference with polyacrylamide residues, gel wells were washed with run buffer, and then 10 µL of the sample were loaded from each time interval (0 h, 72 h and 156 h) with 5 µL of loading buffer.

An amplicon of the *B. thuringiensis* 16S rRNA gene was used as comparison standard, and a 100-bp ladder (Promega; Madison, Wisconsin, USA) was used as the molecular size marker. The running conditions were 100 V and 60 °C for 5 h. After this time, the gel was treated with a solution of ethidium bromide (15 µL of 1 % ethidium bromide in 100 mL of TAE 1X) for 40 min in the dark, washed with sterile distilled water, and visualized on a Benchtop 3UV Transilluminator photodocumentator at a wavelength of 302 nm.

**Table 1.** Standard solutions for denaturing gradient formation.

| **Solution of Acrilamida–Bis acrilamida 19:1 (stock)** | |
|---|---|
| **Reagents** | **Quantitity** |
| Acrilamide | 5.47 mol |
| Bis acrilamide | 0.06 mol |
| Sterile distilled water | Fill to 100 mL |
| **Solution 0 %** | |
| Stock solution | 15 mL |
| Buffer TAE 50X | 2 mL |
| Sterile distilled water | Fill to 100 mL |
| **Solution 100 % (incubation to 37 °C)** | |
| Stock solution | 15 mL |
| Buffer TAE 50X | 2 mL |
| Deionized Formamide | 40 mL |
| Urea | 6.99 mol |
| Sterile distilled water | Fill to 100 mL |
| **Denaturing gradient 40 %** | |
| Solution 0 % | 9.6 mL |
| Solution 100 % | 6.4 mL |
| TEMED | 12 µL |
| Ammonium persulfate 10 % (APS) | 120 µL |
| **Denaturing gradient 60 %** | |
| Solution 0 % | 6.4 mL |
| Solution 100 % | 9.6 mL |
| TEMED | 12 µL |
| Ammonium persulfate 10 % (APS) | 120 µL |

### 2.4. Band selection and purification

DNA bands without degradation characteristics and with the same position with respect to the comparison pattern (DNA of *B. thuringiensis*) were selected. To elute the DNA from each of the bands, they were cut and individually deposited in separate 1.5 mL Eppendorf tubes containing 20 µL of sterile ultrapure water. The tubes were incubated overnight at 4 °C. Next, an amplification of the 16SrRNA gene was performed using 10 µL of the eluate as a template, and the generated amplicons were used for a second PCR using the same volume of the template (10 µL).

Additionally, purification of the obtained PCR product was carried out using an *Illustra GFX PCR DNA* and *Gel Band Purification Kit* (General Electric; Colombia) according to the manufacturer's specifications. The product was visualized in a 1 % agarose gel treated with ethidium bromide at 5 µg $mL^{-1}$ with the following running conditions: 60 V for 1 h, TAE 1X buffer, and 100 bp as the molecular size marker (Promega; Madison, Wisconsin, USA). The gel was visualized on a Benchtop 3UV Transilluminator photo-documentator at a wavelength of 302 nm.

### 2.5. Restriction enzyme analysis (RFLP)

In order to confirm the presence of *B. thuringiensis* during the reduction process, the amplicons obtained in the second PCR were digested with three restriction enzymes: *DraI*, *HindIII,* and *HinfI* (Promega; Madison, Wisconsin, USA). To this end, we worked with a final volume of 20 µL containing 14.30 µL of sterile deionized water, 2 µL of 10X buffer, 0.20 µL of BSA (10 µg µL$^{-1}$), 3 µL of the corresponding 16SrRNA gene amplifier (1 µg µL$^{-1}$), and 0.50 µL of the restriction enzyme (10 U µL$^{-1}$). Water was added as a negative control, and *E. coli* DNA was used as a positive control.

Digestion was carried out for 3 h at 37 °C, and the product obtained after digestion was visualized in a 1 % agarose gel treated with ethidium bromide at 5 µg mL$^{-1}$ using the following run conditions: 60 V for 1 h, TAE 1X buffer, and 100 bp as a molecular size marker (Promega; Madison, Wisconsin, USA). The result was verified by in silico tests in the bioinformatics program pDRAW32 version 1.1.130. The tests were done using sequences of fragments of the *B. thuringiensis* 16SrRNA gene with a size similar to the study amplicon (566 bp) from the National Center for Biotechnology Information (NCBI) database.

### 2.6. Bioinformatic analysis of diversity and similarity

An analysis was performed using the program Photocaptw version 6.0 to calculate the approximate size of the bands located in the same position with respect to *B. thuringiensis* and those that were located at different heights. Through this analysis, diversity and similarity indices (Shannon, Simpson, and maximum likelihood) were established among the different treatments (Estimates 9.1.0 and Ntsys 2.1). A dendrogram was generated using the program Ntsys version 2.1 (License UH3071IX) according to the coefficient of similarity (maximum likelihood).

## 3. Results

Chromium reductions of 74.46 %, 76.12 %, and 99.42 % were observed for treatments 1, 2, and 3, respectively. Notably, these results were obtained in a previous study. With the modified DNA extraction protocol, it was possible to obtain samples with quality and concentration greater than 48 ng µL$^{-1}$. **Figure 1** shows the bands corresponding to the DNA obtained in each of the treatments with their respective replicates.

### 3.1. Amplification of the 16SrRNA gene for DGGE

**Figure 2** shows the PCR products of the samples from each treatment (72 h of the Cr (VI) reduction process), which are free of contamination. This is also reflected in the negative control used in the process. By comparison with the 100-bp marker, it was established that the product obtained was approximately 566 bp.

#### *3.1.1. DGGE*

Considering the modifications made in the protocol (mainly in the amounts of tetramethyl ethylene diamine and ammonium persulfate, adequate polymerization and runs of the different amplification samples of the 16S rRNA gene were achieved. **Figure 3** shows the DNA profiles of each of the treatments analyzed with their respective replicates.

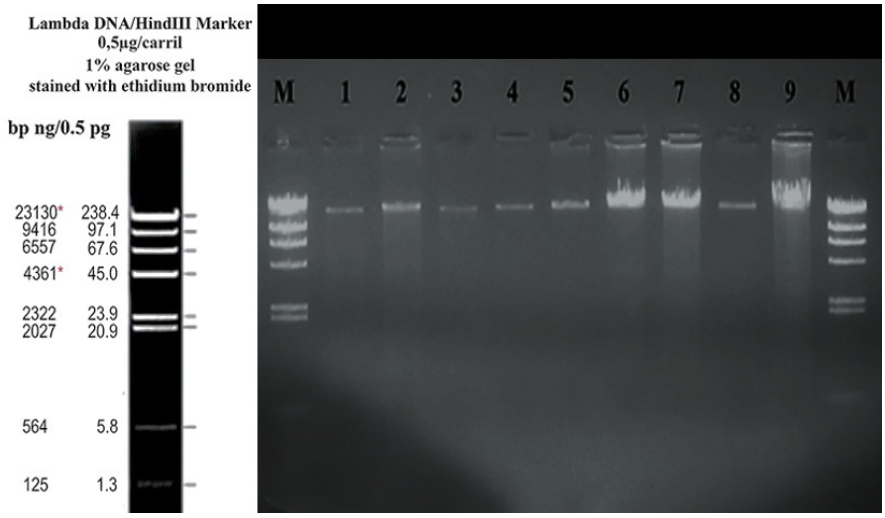


**Figure 1.** Total DNA extraction (Time 0 of the reduction process). The molecular marker (M) Lambda *Hind III* (Promega, Madison,Wisconsin,USA) is observed in the first and last lane. First treatment (raw water from Río Pasto) lanes 1 -3; second treatment (sterile water from Río Pasto inoculated with *Bacillus thuringiensis*) lanes 4-6 and third treatment (unsterilized water from Río Pasto inoculated with *B. thuringiensis*) lanes 7-9. Run conditions: 1 % agarose gel, run in 1X TAE buffer at 70 V for 1 h and 30 min, treated with 5 µg $mL^{-1}$ solution of ethidium bromide and photodocumented in the Benchtop3UV Transilluminator kit at a wavelength of 302 nm.

### *3.1.2. RFLP*

The *DraI* and *HindIII* enzymes did not generate fragments of differential sizes, despite *HinfI* generating two fragments of approximately 650 bp and 100 bp. Therefore, an *in-silico* verification was carried out with the program Pdraw 32, version 1.1.130. The results confirmed that the *DraI* and *HindIII* do not generate any type of cut in *B. thuringiensis* sequences (obtained from the base of NCBI data). For this reason, only *HinfI* was used as reference point (**Figure 4**).

## 3.2. Analysis of diversity and similarity

All the bands that were in the gel at the same height of that corresponding to *B. thuringiensis* had a size of 566 bp. Regarding the Shannon and Simpson indices (**Table 2**), a low diversity among samples was evidenced. It should be noted that each treatment presented the same diversity index at the three times evaluated, which indicates that the present bacterial populations probably managed to remain stable in the face of environmental conditions.

**Table 2.** Diversity indices of the treatments in three-time intervals: first treatment (water from the Río Pasto without sterilizing); second treatment (sterile water from Río Pasto + *B. thuringiensis*) and third treatment (unsterilized water from Río Pasto + *B. thuringiensis).* Tra: treatment and T: time.

| Indices | Tra.1 T0 | Tra. 2 T0 | Tra.3 T0 | Tra.1 T4 | Tra.2 T4 | Tra.3 T4 | Tra.1 T7 | Tra.2 T7 | Tra.3 T7 |
|---|---|---|---|---|---|---|---|---|---|
| **Taxa** | 3 | 1 | 2 | 3 | 1 | 3 | 3 | 1 | 2 |
| **Individuals** | 3 | 1 | 2 | 3 | 1 | 3 | 3 | 1 | 2 |
| **Dominance** | 0.3333 | 1 | 0.5 | 0.3333 | 1 | 0.3333 | 0.3333 | 1 | 0.5 |
| **Simpson** | 0.6667 | 0 | 0.5 | 0.6667 | 0 | 0.6667 | 0.6667 | 0 | 0.5 |
| **Shannon** | 1.099 | 0 | 0.6931 | 1.099 | 0 | 1.099 | 1.099 | 0 | 0.6931 |

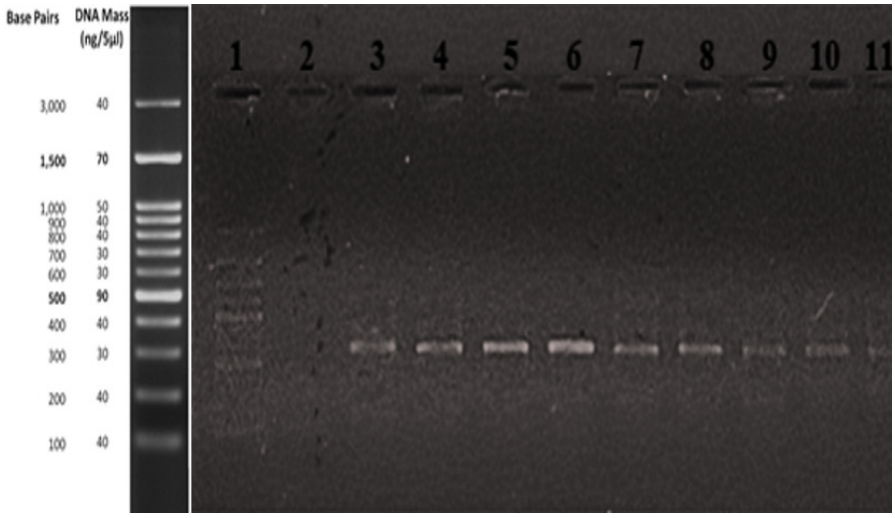


**Figure 2.** Amplification of the 16SrRNA gene 72 h of the Cr (VI) reduction process. Lane 2: negative control. First treatment (unsterilized water from Río Pasto), lanes 9-11; second treatment (sterile water from the Río Pasto inoculated with *Bacillus thuringiensis*), lanes 3-5; and third treatment (unsterilized water from the Río Pasto inoculated with *B. thuringiensis*), lanes 6-8. Size marker is observed in the first lane, molecular mass 100 bp (Promega, Madison, Wisconsin, USA). Run conditions: 1 % agarose gel, run in 1X TAE buffer at 70 V for 1 h, treated with a solution of 5 µg $mL^{-1}$ of ethidium bromide and photodocumented in the Benchtop3UV Transilluminator kit at a wavelength of 302 nm.

**Figure 5** shows the dendrogram generated by Ntsys version 2.1 (License UH3071IX). The grouping of the different treatments was established at each time according to the obtained, maximum likelihood-based coefficients of similarity. The shared similarity between the treatments was 75 %, indicating the prevalence of *B. thuringiensis* during the fermentation process. As previously reported, the dendrogram also reflects similarity levels of 100 % and 76 % between the three-time intervals in the second and third treatments, respectively. However, the first treatment presented a similarity of 46 %.

## 4. Discussion

The obtained DNA samples showed small traces of contaminants, which are possibly associated with the fact that the extraction processes in residual water usually presents interference due to the number of substances dissolved in the medium, such as organic matter and heavy metals. According to the physical, chemical, and biological characteristics of the sample for bacterial DNA extraction, there are different types of inhibitors that can restrict the adequate recovery of DNA [16,17]. However, the analyzed samples presented adequate characteristics to be processed with different molecular techniques.

It was verified that all the treatments presented higher DNA concentrations at the first time. In the first treatment, the bands obtained at 72 h and 156 h had lower concentrations. This was possibly caused by the high proportion of Cr (VI) and low concentration of nutrients from the treatment, which prevented normal growth of the microorganisms, resulting in population decline [18]. The obtained PCR product (566 bp) was that expected for the 16S rRNA gene with the 341F and 907R primers, as found in studies carried out by Faissal *et al.* [19]. They reported amplicons between 500 bp and 550 bp for the 16S rRNA gene with the same primers [19].

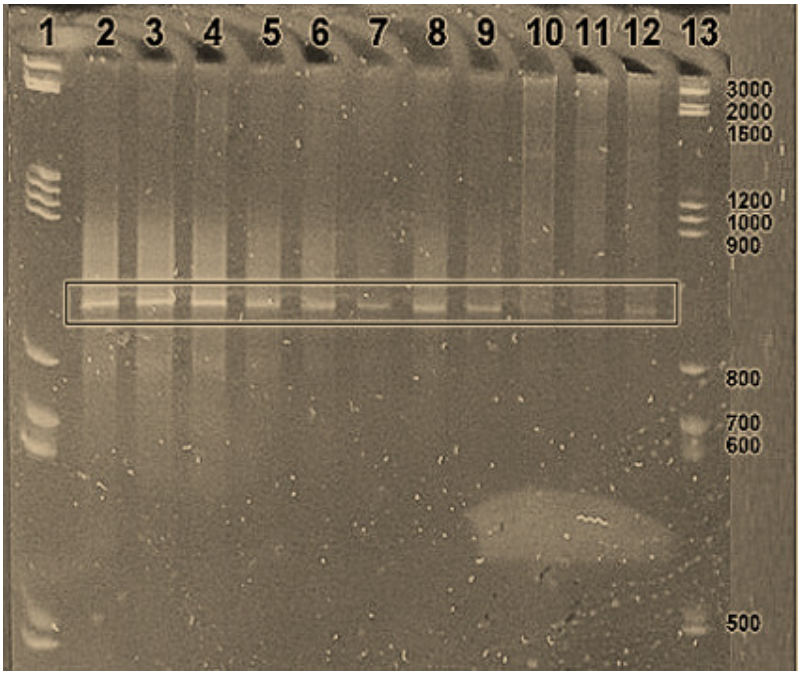


**Figure 3.** Verification of the denaturing gradient electrophoresis process (DGGE) at 156 h of the Cr (VI) reduction process. Lanes 1 and 13 show the marker of molecular size 100 bp (Promega, Madison,Wisconsin,USA). First treatment (unsterilized water from the Pasto River) lanes 2, 4, and 5; second treatment (sterile water from the Río Pasto inoculated with *B. thuringiensis*) lanes 10-12; third treatment (raw water from Río Pasto inoculated with *B. thuringiensis*) lanes 6-8; lanes 3 and 9 DNA of *B. thuringiensis*. Run conditions: polyacrylamide gel in 40 % denaturing gradient, run in TAE 1X buffer at 100 V for 5 h at 60 °C, treated with a solution of ethidium bromide (15 µL of 1 % ethidium bromide diluted in 100 mL of TAE 1X) and photodocumented on a Benchtop3UV Transilluminator equipment at a wavelength of 302 nm.

The bands generated in each of the treatments were positioned at a similar height with respect to *B. thuringiensis*, which probably indicates that they are the same bacterial species. However, some studies exposed the difficulty in separating fragments that differ by 2 or 3 bases due to the high degree of homogeneity in the sequences of some genes, which occurs with the 16S rRNA gene sequences [20-22]. It is possible that a microorganism is represented by several bands. It is also possible that two different DNA fragments migrate to the same position in the gel, so that the DGGE profiles are misinterpreted.

It has been determined that errors in the stages prior to the development of the DGGE technique (DNA extraction and PCR) can introduce biases in the generated profiles, such as preferential amplifications and the formation of chimeric molecules and heteroduplex molecules [21, 22]. In this sense, it was necessary to complement and verify the results obtained with cutting with restriction enzymes. In this way, the restriction enzyme *HinfI* generated two fragments in the study source samples, similar to those generated in *B. thuringiensis* (see Figure 4). Thus, it could be established that the isolated study source was present during the fermentation process. For this reason, it is responsible for the increase in the percentage of reduction of Cr (VI) in the treatments.

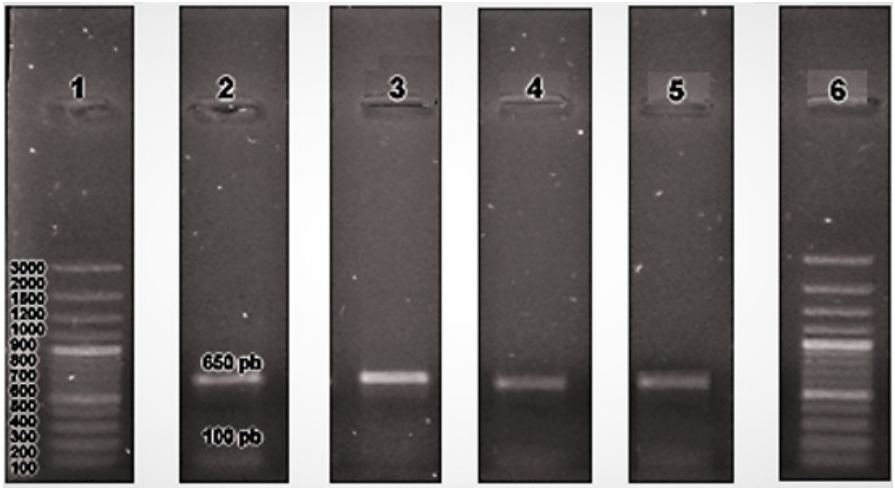


**Figure 4.** Digestion profile with the endonuclease *HinfI* on selected study samples at of the Cr (VI) reduction process. Lane 1 shows the marker of molecular size 100 bp (Promega, Madison,Wisconsin,USA) (M). First treatment (raw water from Río Pasto) lanes 4; second treatment (sterile water from Río Pasto inoculated with *B. thuringiensis*) lane 2 and third treatment (raw water from Río Pasto inoculated with *B. thuringiensis*) lane 3; lane 5 *B. thuringiensis* DNA 1 % agarose gel, run in 1X TAE buffer at 60 V for 1 h, treated with 1 % ethidium bromide solution and photodocumented on a Benchtop3UV Transilluminator kit at a wavelength of 302 nm.

This approach may be a promising alternative for the bioremediation of contaminated effluents since bacteria isolated from contaminated environments can present a wide variety of strategies to survive in such environments [23]. *Bacillus cereus* was isolated from a coal mine, and it exhibited different types of stress responses in a medium supplemented with Cr (VI) [23]. The results support the process of chromium reduction through the synthesis of chromoreductases.

Restriction enzymes recognize and cut palindromic sequences, so profiles generated are specific to a given nucleotide sequence. However, to ensure the reproducibility of the results, it is necessary to use several restriction enzymes since there are different critical factors that can affect and inhibit endonuclease activity. These factors include DNA purity, temperature, and pH with respect to stability, contaminants with (−) charge, DNA contaminated with other types of DNA, and the degree of methylation, among others [24, 25, 26].

The results obtained in this study are based on two types of molecular techniques (DGGE and RFLP). The need to use complementary molecular markers follows the inherent limitations and specific advantages of the individual approaches (DGGE and RFLP). These methods cannot fully encompass the complexity of the analysis and study of biological diversity on their own [26]. Similar investigations were proposed by Chai *et al.* [27], they evaluated the reducing activity of Cr (VI) of *Pannonibacter phragmitetus* in bioremediation processes for contaminated soil and the dynamics of the bacterial population over time using molecular techniques such as RFLP and real-time PCR. They concluded that the bacterium presents a potential for application in bioremediation processes in relation to its population abundance [27].

The Shannon and Simpson indices revealed low diversity between the samples, which is mainly due to the influence of the high concentration of Cr (VI) in the medium and the limiting factors. According to Pineda and Rodríguez [3], a low diversity is attributed to a high concentration of heavy metals that influence the prevalence of bacterial species capable of tolerating adverse conditions. In addition, factors such as nutrient limitation, aeration, pH, and temperature can reduce the number of taxa [3]. The low level of similarity in the first treatment may be related to the

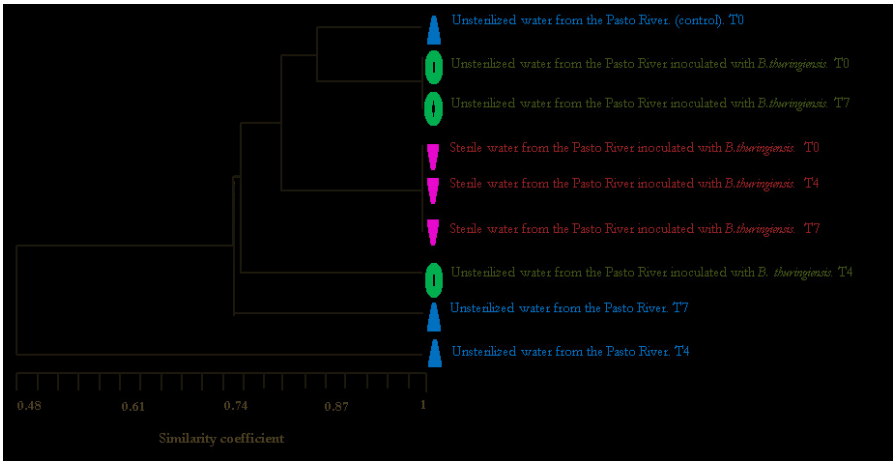


**Figure 5.** Dendrogram of the treatments analyzed in three stages (maximum likelihood coefficient): first treatment (water from the Río Pasto without sterilizing); second treatment (sterile water from the Río Pasto inoculated with *B. thuringiensis*); and third treatment (unsterilized water from the Río Pasto inoculated with *B. thuringiensis*). Initial concentration of approximately 59 mg $L^{-1}$ of this metal; T0: time 0, T6: time 6 and T13: time 13 (0 h, 72 h and 156 h). The difference in each of the times is 12 h for a total of 156 h of reduction.

dynamics of bacterial populations since the ecosystem in which they were found may be affected by intra- and interspecific interactions between microorganisms, the presence of contaminating substances, the amount of available nutrients, oxygen concentration, pH, and temperature [3]. These factors cause each bacterial population to respond in a different way to the stimuli generated by its microenvironment [17, 18].

Treatment 2 presented constant the diversity indices and similarity coefficients through time. This occurred because a single bacterial species (*i.e., B. thuringiensis*) was predominant throughout the Cr (VI) reduction process, thus reflecting the axenic conditions in which the treatment was maintained. The opposite situation occurred in the group generated in the other treatments since their substrate was unsterilized, Pasto River water. Therefore, the bacterial communities present in the water samples differ significantly depending on the interaction and medium in which they developed [17,18].

Similar studies were proposed by Ma *et al.* [28], who evaluated the reduction of Cr (VI) by a mixed bacterial consortium and the variation in microbial diversity. In the study done by Ma *et al.* [28], it was possible to determine that this heavy metal significantly influences the establishment of bacterial communities [28]. This was due to the fact that the Shanon and Simpson indices decreased after the addition of Cr (VI) in the medium. In addition, it was evident that the genera *Aeromonas*, *Pseudogracilibacillus,* and *Macellibacteroides* predominated throughout the fermentation process since they expressed specific functional genes for the elimination of chromium, which allows them to overcome the environmental conditions and to be selected by the environment. Similarly, Lin *et al.* performed a characterization of microbial communities in different wetland substrates to treat Cr (VI)-contaminated water using molecular techniques such as DGGE and sequencing. This research used band analysis and diversity indices to establish that under the stress of Cr (VI), bacterial diversity decreases significantly, and sensitive bacteria tend to die and become replaced by bacteria that are tolerant or sensitive to this metal [29].

On the other hand, Yin *et al.* [30] evaluated the effect of cadmium stress on the diversity of a soil microbial community. They concluded that cadmium pollution significantly changed the structure of bacterial communities and promoted the growth and development of some tolerant bacterial species at a low level of cadmium stress of 0.5 mg $kg^{-1}$. High levels of cadmium-caused stress significantly inhibited the growth of *Bacillus thermoamylovorans* and *Bacillus foraminis*. These preliminary results revealed the response of the soil microbial community structure to heavy metal pollution and provided a theoretical reference for early warnings of trends in soil quality changes.

The different studies cited indicate the importance of characterizing and determining the dynamics of native bacterial species in environments contaminated with heavy metals. The ultimate goal is to generate bioremediation strategies from bacteria that are resistant to these metals. By means of the molecular markers used, *B. thuringiensis* was found to be present during the fermentation process. For this reason, it may be the main responsible for the Cr (VI) reduction process and is a promising alternative for metal decontamination processes.

## 5. Conclusions

The analyzed treatments presented low diversity indices and high similarity coefficients (Shannon and Simpson), which is mainly due to the influence of the high concentration of Cr (VI) in the medium and the limiting factors such as nutrient limitation, pH and temperature. The low level of similarity observed in the first treatment may be related to the dynamics of bacterial populations. This indicates that the concentration of Cr (VI) can influence the establishment of different bacterial species. The molecular tools used (DGGE and RFLP) in this study confirmed that *B. thuringiensis* was present during the Cr (VI) reduction process in the treatments where it was inoculated, thus supporting the notion that Cr (VI) reduction is mainly carried out by this microorganism.

For the technique RFLP, it is advisable to use several restriction enzymes because different factors can cause the loss of restriction sites, preventing the generation of the fragments expected by the cuts. It is important to note that for treatment 2, diversity indices and similarity coefficient remained constant trough time, reflecting the axenic conditions in which the treatment was maintained. This approach may be a promising alternative for the bioremediation of contaminated effluents since bacteria isolated can present a wide variety of strategies to survive in such environments.

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## 7. Conflict of interest

The authors declare that they have no affiliations with or involvement in any organization or entity with any financial interest (such as honoraria; educational grants; participation in speaker membership, employment, consultancies, stock ownership, or other equity interest; and expert

testimony or patent arrangements) or non-financial interest (such as personal or professional relationships, affiliations, knowledge, or beliefs) in the subject matter or materials discussed in this manuscript. The authors declare that this work does not entail any conflict of interest.

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## Técnicas moleculares para la determinación de la reducción de Cr (VI) por *Bacillus thuringiensis*

**Resumen:** La contaminación de efluentes con Cr (VI) es un problema ambiental global. En el rio Pasto (sureste de Colombia), estudios previos han reportado contaminación con este metal en sitios cercanos a curtiembres. Para establecer el papel de *Bacillus thuringiensis* en la reducción de Cr (VI) en el agua del río Pasto, se llevaron a cabo experimentos con aguas no tratadas del rio Pasto (tratamiento 1), agua estéril del rio Pasto inoculada con *B. thuringiensis* (tratamiento 2) y agua no esterilizada del rio Pasto inoculada con *B. thuringiensis* (tratamiento 3). Todos los experimentos se condujeron en biorreactores con temperatura controlada de 20 °C y agitación constante por 156 h. Se tomaron muestras de 20 ml cada 12 horas de cada tratamiento para registrar los niveles de reducción de Cr (VI) y confirmar la identidad de los microorganismos por métodos moleculares que involucraron electroforesis en gel con gradiente denaturante (DGGE), perfiles de digestión de enzimas de restricción (RFLP) y análisis bioinformáticos. La reducción de Cr (VI) fue mayor en el tratamiento 3 (99.42 %) en oposición al tratamiento 2 (76.12 %) y al tratamiento 1 (74.46 %). La identidad molecular de *B. thuringiensis* se determinó por medio de secuenciación del gen 16SrRNA y la determinación de RFLPs en los tres tratamientos revelaron los perfiles de *B. thuringiensis*. Dado que *B. thuringiensis* estuvo presente en los tres tratamientos a lo largo del tiempo, la reducción de Cr (VI) puede atribuirse a esta bacteria.

**Palabras Clave:** Metales pesados; reducción de cromo; bacterias reductoras de Cr, DGGE (DeCS).

## Técnicas moleculares para avaliar redução de Cr (VI) por *Bacillus thuringiensis*

**Resumo:** A poluição por efluentes de Cr (VI) é um problema ambiental mundial. Estudos anteriores relataram contaminação com este metal em pontos próximos a curtumes no rio Pasto (sudeste da Colômbia). Para estabelecer o papel do *Bacillus thuringiensis* na redução de Cr (VI) da água do rio Pasto, realizamos experimentos com água do rio Pasto não tratada (tratamento 1), água do rio Pasto esterilizada e inoculada com *B. thuringiensis* (tratamento 2) e água do rio Pasto não esterilizada e inoculada com *B. thuringiensis* (tratamento 3). Todos os experimentos foram realizados em reatores biológicos com temperatura controlada de 20 °C e agitação constante por 156 h. Amostras de 20 mL de cada tratamento foram tomadas cada 12 h para rastrear a redução nos níveis de Cr (VI) e para confirmar a identidade do microrganismos presentes através de métodos moleculares como electroforese em gel com gradiente de desnaturação (DGGE), perfis de digestão de enzimas de restrição (RFLP), e análises bioinformáticas. A Redução de Cr (VI) foi maior no tratamento 3 (99.42 %) do que nos tratamentos 2 (76.12 %) e 1 (74.46 %). A identidade molecular de *B. thuringiensis* foi determinada por sequenciamento do gene 16SrRNA. Os estudos RFLP dos três tratamentos mostraram perfis de *B. thuringiensis*. Uma vez que *B. thuringiensis* esteve presente nos três tratamentos ao longo do tempo, a redução de Cr (VI) pode ser atribuída a esta bactéria.

**Palavras-chave:** Metais pesados; Redução de cromo; Bactérias redutoras de Cr; DGGE (DeCS).

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