

Identification and Analysis of the Bio-Reduction of Chromium (VI) by *Pseudomonas Fluorescens*

Carlos Andres Pena Guzman^a, Jenny Natalia Ramirez Esquivel^a, Robert Johan Quimbay Moreno^a, Duvan Javier Mesa Fernandez^b

^aUniversidad Autónoma de Colombia, Programa de Ingeniería Ambiental, Calle 12B No. 4–31, Bogotá, Colombia

^bUniversidad Santo Tomás, Programa de Ingeniería Ambiental, Carrera 9 No. 51-11, Bogotá, Colombia
carpeguz@gmail.com

This paper focuses on the identification and analysis of the ability of *Pseudomonas fluorescens* to reduce chromium (VI) in aqueous media. To develop this bio-reduction capability, microorganisms which potentially can grow in a medium containing up to 100 mg/L of chromium, were selected and used. In order to determine the extension of the reduction of hexavalent chromium (VI) in lab water, samples of 20 and 50 mg/L were added with 5, 10 and 15 % of final inoculum, respectively to the volume of the samples. Inoculum had a population of approximately 12×10^8 CFU/mL. Results suggest the occurrence of reduction in all the samples studied. This reduction occurred significantly in the tests P-20.5, P-20.10 and P-20.15, which yielded a reduction of chromium (VI) of 3.6, 1.88 and 5.95 mg/L respectively, for samples P-50.5, P-50.10 and P-50.15 were obtained reductions of 2.36, 4.19 and 4.47 mg/L. The highest levels of bio-reduction were obtained in P-20.15 and P-50.15 tests.

1. Introduction

The water resources in Bogotá, Colombia, are affected by a large number of industrial and residential discharges, most of them without any treatment. In some rivers it has been found an association between Total and Hexavalent Chromium with tannery, metallurgy, dyeing, textile and other industries, which cause serious environmental and health problems (Silgado et al., 2014) due to the ecological occurrence and carcinogenic properties of chromium (VI). (Chirwa and Smit, 2010).

The environmental District Department of Bogotá has conducted studies that show high concentrations of Total Chromium in industrial discharges, reaching up to 9 mg/L. These values exceed the permissible threshold of 1 mg/L for Total chromium and 0.5 mg/L for chromium (VI) following national regulations like Resolutions 3956/09 and 3957/09.

Due to the aforementioned factors, it is really important to develop biological methods for reductions of pollutants, in order to mitigate its associated problems in water (Nikazar et al., 2008). It has been reported that the use of microorganisms for reduction of heavy metals is a highly efficient and cost effective technology (Di Nanno, et al, 2003, Meli et al, 2007).

One of these microorganisms is the *Pseudomonas fluorescens*, which has been studied in many fields of biotechnology on an experimental scale in order to determine its maximum bio-reduction capacity and environmental restoration of areas with presence of high levels of Chromium (VI) in water and soil; This ability is due to its metabolic versatility, its great adaptability to almost any type of pollutant and fast population growth (Chirwa and Wang, 1997).

2. Materials and methods

2.1 Isolation and potentiation of bacteria

1 mL of a commercial product (PC) used in agriculture (Fosforris®) was spreaded on petri dishes with King B agar solid medium in triplicate. The media was then incubated for 72 h.

To determine the growth and the potentiation of the bacteria at different concentrations of chromium (VI), three steps were carried out:

Phase I: Concentration of chromium (VI) was increased in the bacterial culture in the range from 5 mg/L to 50 mg/L by addition of a solution of 5 mg/L each time. Each addition was made by triplicate (27 samples total). Growth of bacteria was observed in all Petri dishes after 72 h of incubation.

Phase II: Concentration of chromium (VI) was increased by additions of 10 mg/L from 60 to 100 mg/L. Each experiment was performed by triplicate (15 samples). It was observed the growth of bacteria in all Petri dishes after 72 h of incubation.

Phase III: Concentration of chromium (VI) was increased by additions of 20 mg/L from 100 mg/L to 200 mg/L by triplicate (18 samples). In this test was observed the growth inhibition for all the strains.

2.2 Preparation of Inoculum from *Pseudomonas fluorescens* used in the in-vitro tests of bio-reduction of chromium (VI).

200 mL containing 10 % of inoculum was prepared by mixing 540 ml of nutrient broth and 60 mL of inoculum as prepared initially. Solution was then incubated at 35 °C for 10 h. Afterwards, the incubate was measured by spectrophotometry showing an absorbance of 0.518 in the McFarland standard, corresponding to an approximate concentration of 12×10^8 CFU/mL. The number of "Colony Forming Units" UFC/ml was determined by the method of McFarland.

2.3 Determination of the reduction of chromium (VI)

Six in-vitro tests were carried out. First three samples with a concentration of 20 mg/L of chromium (VI) and 5, 10 and 15 % of the final inoculum (%V/V). Referred as P-20.5, P-20.10 and P-20.15 respectively. Three other tests were ran using a concentration of 50 mg/L of chromium (VI) and 5, 10 and 15 % of the final inoculum (P-50.5, P-50.10 and P-50.15), each test was made by triplicate and at two levels of chromium (VI) 20 mg/L and 50 mg/L respectively, for a total of 20 experimental samples. Table 1 reviews the methodology used. The analysis of samples was made with a X-ray Fluorescence Spectrometer (Shimadzu EDX-720) an a calibration curve for concentrations between 5 mg/L and 105 mg/L of chromium (VI). In each session 20 samples for duplicate were analyzed, for a total of 240 analyses.

Table 1: Tests used for In-vitro determination of chromium (VI) bio-reduction using *Pseudomonas fluorescens*

mg/L of Chromium (+6)	ml Inoculum	Repetitions	Target
20	10 - 20 - 30	3	1
50	10 -20- 30	3	1

3. Results and discussion

3.1 Chromium (VI) resistance.

Table 2 shows the results from the three phases of potentiation of the *Pseudomonas fluorescens*. It is observed that in the first two phases corresponding to 42 tests (70 % of the total tests), a bacterial growth occurred until 100 mg/L of chromium (VI). The third phase corresponding to 18 tests (30 % of the total tests) which had concentrations greater than 100 mg/L of chromium (VI) showed bacterial inhibition growth. To verify that at concentrations greater than 100 mg/L of chromium (VI) the bacteria does not survive, 4 more tests were performed using concentrations of chromium (VI) in a narrower range (105, 110, 115 and 120 mg/L) when compared with those applied in Phase III. Results indicate a clear growth inhibition of the bacteria at all 4 concentrations. This Result shows that at concentrations above 100 mg/L of chromium (VI) *Pseudomonas fluorescens* does not survive.

Table2: Potentiation Bacterial Testing

Phase	Number of test	Growth (%)	Inhibition Growth (%)
Phase I	27	100	0
Phase II	15	100	0
Phase III	18	0	100

However, several authors reported the evaluation of the Chromium (VI) reduction at higher concentrations. Nevertheless, the assessment methodologies used were different, for example De Leo et al. (1994) achieved bio-reductions with initial concentrations of 112.5, 200 and 314 mg/L of chromium (VI) by using Batch cultures tests which contained 100 mL of a sterile medium plus the desired concentration of chromium. Besides, Hussein et al. (2004) conducted stress tests of the bacteria found a tolerance to 208 mg/L (4 mM) conducted in casamino acid agar plates (Narayani and Shetty, 2013).

3.2 Bio-reductions test

To evaluate the reduction of chromium, first a set of three averaged values for each target was obtained, in order to verify its behavior. In addition to the average data, it was calculated an overall average for a full result demonstrating reduction of chromium (VI). However, the trend of data per session did not represent the expected reduction, because some of the data analyzed by different teams showed significant differences between them. For example, for the test P-50.5 the average value for Test 1 and Test 3 were greater than the target (figure 1).

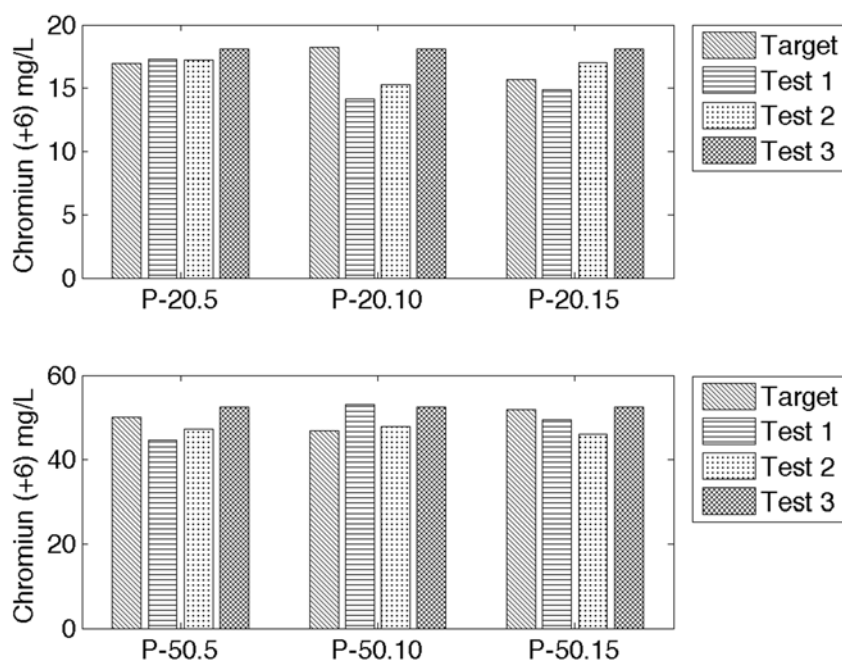


Figure 1: Analysis of the average data session 1

Due to the high variation the median values of all tests per session were used to verify the removal of chromium (VI). In table 3 values obtained per session are presented as well as the total removal concentrations from session 1 to 6.

It can be seen that the tests P-20.15 and P-50.15 produced the highest reduction with values of 5.9 and 4.5 mg/L, respectively. Moreover, for the concentration of 50 mg/L it was observed a direct relationship between the amount of inoculums and the reduction capacity, higher amount of inoculums produced higher reduction. However, that behavior was not followed for the test with 20 mg/L, which showed an asymmetrical relationship.

Additionally, it can be seen that the ability of chromium (VI) reduction by *Pseudomonas fluorescens* was higher in test with 50 mg/L than in the test with 20 mg/L. A direct relationship between the sessions and the reduction of Cr (VI) was observed and is shown in Figure 2. For test P-20.5 the highest levels of reduction were presented in sessions 2 and 5 with a decrease of 1.06 and 2.03 mg/L of chromium (VI), for P-20.10 the highest level of reduction were obtained in sessions 4 and 6 with a decrease of 0.53 and 0.87 mg/L. In test P-20.15 the highest level of reduction was presented in sessions 2 and 4 with a decrease of 2.5 and 1.82 mg/L of chromium (VI).

Table 3: Concentration of chromium (+6) in sessions 1 to 6.

Session	P-20.5	P-20.10	P-20.15	P-50.5	P-50.10	P-50.15
1	16.2	15.2	17.2	49.1	48.5	46.9
2	15.2	15.1	14.7	49.6	48.5	46.7
3	14.7	14.9	14.5	48.4	48.3	44.3
4	14.6	14.3	12.7	47	45.9	43.9
5	12.6	14.2	12	46.8	45.6	43.8
6	12.6	13.3	11.3	46.7	44.4	42.4
Total Remove (mg/L)	3.6	1.9	5.9	2.4	4.1	4.5
Total percentage (%)	22.21	12.35	34.55	4.81	8.63	9.53

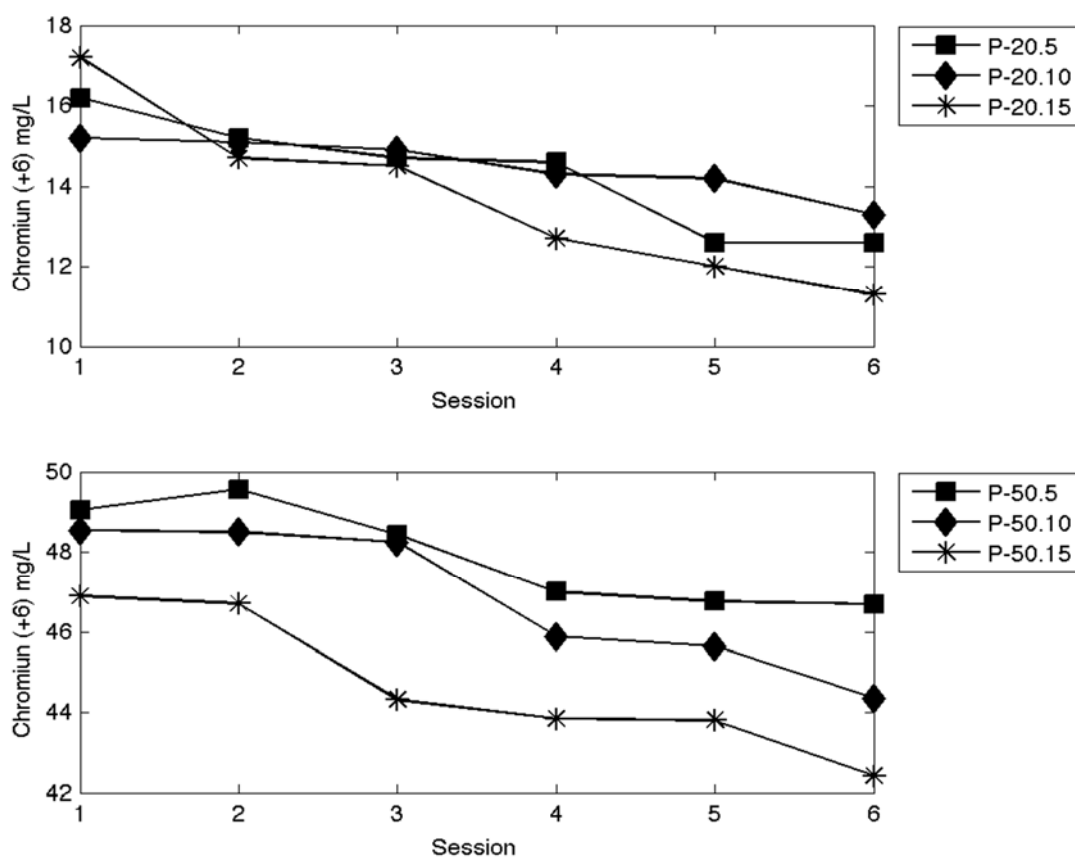


Figure 2: Behaviour in the reduction of Cr +6, after the bacterial inoculation at 20 and 50 mg / L.

For tests containing 50 mg/L of chromium (VI) in P-50.5 with the inoculums at 10 and 15 % the highest levels of reduction were presented in sessions 3 and 4 with a decrease of 1.13 and 1.43 mg/L. Those values represented almost half of chromium (VI) reduction capacity. In P-50.10 the greatest reduction was found in sessions 4 and 6 with a decrease of 2.36 and 1.29 mg/L. Finally, for test P-50.15 was found the highest reduction with respect to the other tests (session 3 and 6) with a decrease of 2.38 and 1.39 mg/L.

Finally, the reduction of chromium (VI) to chromium (III) by the bacterium was identified also by the precipitate formed at the bottom of the erlenmeyer employed in the in-vitro tests. This is an evidence of the internal biochemical process of the bacteria, in which the chromium (+3) is precipitated as the hydroxide compound.

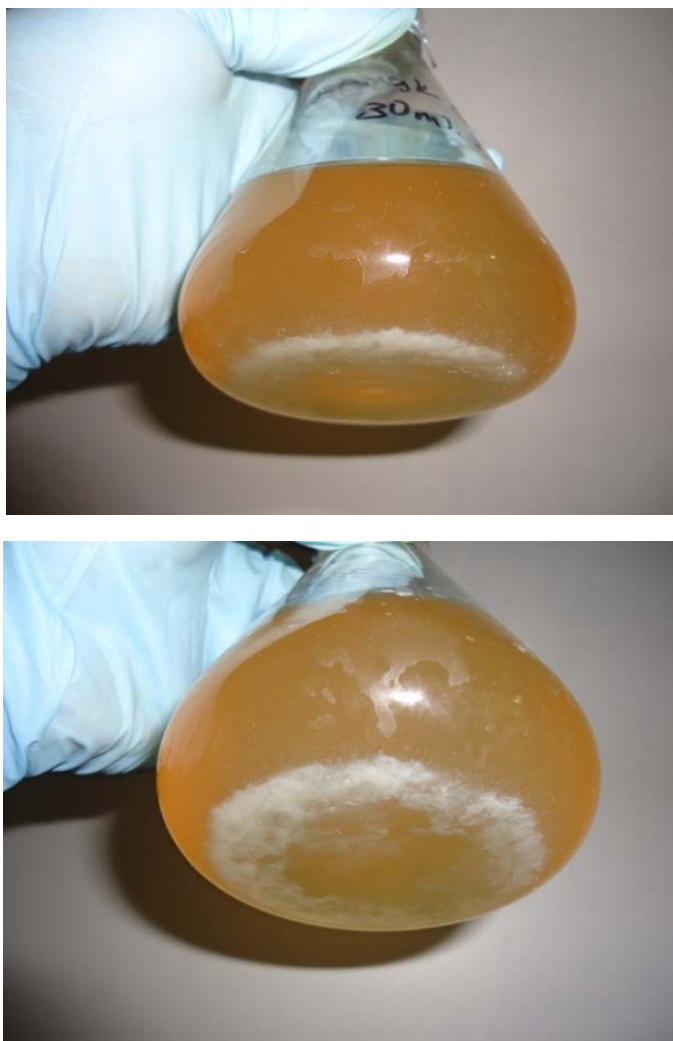


Figure 3. Chromium (III) trivalent precipitated in vitro tests using *Pseudomonas fluorescens*.

4. Conclusions

We verified that by using the proposed method of potentiation, *Pseudomonas fluorescens* can grow in culture media containing high concentrations of chromium (VI). It was observed bacterial growth up to 100 mg/L of Cr (VI) above which inhibition took place and *Pseudomonas* do not survive

It was found that the most efficient amount of inoculum was 30 ml, equivalent to 15% in volume of each sample. Using that amount of inoculum the greatest bio-reduction of chromium (VI) to Cr (III) was observed for all tests performed..

It was observed a direct relationship between reduction rate and percentage of inoculum at 50 mg/L Cr (VI), where a higher inoculums percentage represents a higher reduction capability.

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