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BIOREMEDIATION OF HEXAVALENT CHROMIUM IN WASTEWATER EFFLUENT BY *PSEUDOMONAS PUTIDA* (MTCC 102)

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ABSTARCT

The advantage of utilizing microbes is that they can biodegrade the organic compounds easily. The strain of Pseudomonas putida (MTCC 102) was purchased from IMTECH Chandigarh. The Pseudomonas putida removed 88% Cr (VI) in wastewater effluent after 96 hrs. The removal of Cr (VI) with Pseudomonas putida species was determined chemically by using Diphenylcarbazide method with UV – Vis spectrophotometer at 540 nm. The optimum pH for removal of Cr (VI) in wastewater effluent was 5.2. At this pH about 88% bioremediation of Cr (VI) was done.

Keywords: Pseudomonas putida, Diphenylcarbazide, UV – Vis Spectrophotometer, Bioremediation.

1. INTRODUCTION

Chromium is a major contaminate compound found in effluent. It causes many human health problems. It causes physical discomfort and some life threatening illness including damage to body metabolisms [1]. Chromium exists in nature in their valence states from -2 to +6 [2]. Mostly Chromium used in two common forms Cr (III) or Cr (VI). Cr (VI) is highly reactive and is strong oxidizing agent. Cr (VI) is so called carcinogen and is soil surface water and ground water contaminant [3]. For public water supply the maximum tolerance of total Chromium has been 0.05 mg/lt according to Indian Standards. In water bodies the permissible level of Cr 50 µg/dm³ and in drinking water as 3 µg/dm³ and that Cr (III) as 100 µg/dm³ [4].

Initially the various physical and chemical methods were used for treatments [5]. Many types of conventional methods which were used to remove chromium in water includes pumping or excavation of the contaminated material, addition of chemical reductant, precipitation, sedimentation, or ion exchange and/or adsorption [6]. These physicochemical methods are very costly because they require high energy, pumping, excavation, chemical addition.

Bioremediation uses microorganisms to break down toxic and hazardous compounds in the environment. Bioremediation has already proven itself to be a cost-effective and eco-friendly method [7].The various types of microbes which were used for bioremediation includes *Philodina roseola* which biosorb 40% Cr (VI). The *Notommata copeus* removes Cr (VI) 82% after two days [8]. The strain *Bacillus cereus* FA-3 was removes a maximum of 72% Cr (VI) at 1000 µg/ml chromate concentration (Singh N., Verma T. and Gaur R., 2013). But *Pseudomonas putida* removes maximum of 90% Cr (VI). *Pseudomonas* sp. used by Murugesan and Maheswari for the removal of Cr (VI) [9].

The objective of present study was to bioremediate Cr (VI) in industrial effluent by using *Pseudomonas putida* (MTCC 102).

2. MATERIAL AND METHODS

For the bioremediation of Cr (VI) the lyophilized culture of *Pseudomonas putida* (MTCC 102) was inoculated in a nutrient broth for proper growth development. After 24 hrs the culture was grown on nutrient agar at 28°C for 24 – 48 hrs. The chemicals used for this study were of Himedia, Loba, Fisher, Nice and Thomas.

Sample collection and Characterization

The wastewater effluent was collected from the chromium deposited and contaminated site located at industrial area of Punjab and was examined to estimate the amount of pollutants in the water samples. For water sample characterization Biological Oxygen Demand (BOD), Chemical Oxygen Demand (COD), Dissolved oxygen (DO), parameters were determined.

Preparation of standard curve of chromium

Diphenylcarbazide assay for measurement of Cr^{6+} was the standard method used from the document of "Guide manual for water & wastewater analysis" used by the central pollution control board (CPCB). By this procedure only hexavalent chromium (Cr^{6+}) was measured. The hexavalent chromium was determined spectrophotometrically by reaction with diphenylcarbazide in acid solution.

Preparation of reagents

Reagents and distilled water free from Chromium contamination were used.

i. Stock chromium solution: Dissolved 141.4 mg $K_2Cr_2O_7$ in water and diluted to 100mL.
ii. Standard chromium solution: Diluted 1mL stock chromium solution to 100mL.
iii. Nitric acid: HNO_3, conc.
iv. Sulphuric acid: conc. H_2SO_4, 1+1 (18N); H_2SO_4 (6N)
v. Sulphuric acid: 0.2N diluted 17ml 6N H_2SO_4 to 500ml with water.
vi. Phosphoric acid: conc. H_3PO_4.
vii. Diphenylcarbazide solution: Dissolved 250mg 1, 5-diphenylcarbazide (1, 5 diphenylcarbohydrazide) in 50 mL acetone. Stored in a brown bottle.
viii. Sodium hydroxide 1N: Dissolved 40g NaOH in 1L water. Stored in plastic bottle.

Procedure

Prepared stock solution of chromium of 500 ppm. Pipette measured volumes of standard chromium solution 50 – 500 ppm ranging from 2 to 20mL, to give standards for 10 to 100 µg Cr, into 250mL beakers or conical flasks.

1. Added 0.25mL (5 drops) H_3PO_4 in standard chromium solution.
2. The pH of solution was adjusted with the 0.2N H_2SO_4 and pH meter to pH ± 0.5.
3. Transferred solution to a 100mL volumetric flask, diluted to 100mL and mixed.
4. Added 2mL diphenylcarbazide solution, mixed and kept for 5 to 10 min for full colour development.
5. Transferred an appropriate portion to 1 cm absorption cell and measured its absorbance at 540nm, using reagent water as reference.
6. Constructed a standard curve by plotting absorbance values against chromium concentration (ppm).

Preparation of cell biomass for biosorption

Biosorption was done by using *Pseudomonas putida.* The media for growth was prepared with 100 ppm of chromium and that media was inoculated with 18 hrs old culture. After inoculation, the flasks were kept on a rotary shaker at 120 rpm for 24 hrs for incubation. After 24 hrs, flask containing cells with Cr (VI) were harvested by centrifuging them in cold centrifuge to recover the cells. The cells were washed two times with distilled water. The biomass was estimated by using dry weight method.

Biosorption of Cr (VI) using biomass of bacteria

A known weight of the biomass diluted to 100 ml. The flasks were incubated on a rotary shaker at 80 rpm for 48 hrs. After every half an hour, the sample was taken, centrifuged and the supernatant was analyzed for Cr (VI) concentration.

Estimation of Cr (VI)

Hexavalent chromium was estimated by using diphenyl carbazide reagent with spectrophotometer [10]. Added 0.43ml of 3M H_2SO_4 to 0.1 ml of sample followed by 1 ml of 2, 5 diphenyl carbazide solution. It was mixed and made total volume 25 ml. After 10 minutes reading was observed against reagent blank at 540 nm in a spectrophotometer.

Metal sorption studies

The 100 mg Cr (VI)/l were used as working solution and its pH was adjusted to 4.0 with 0.1 M sodium hydroxide and 0.1 M nitric acid. The cells were added to chromium solution of triplicate 25 ml volume of in 150 ml conical flasks. For 1 h the flasks were shaken at 150 rpm at ambient temperature (28 ± 3°C).Both metal-free and biosorbent-free solutions were used as controls. The cells were separated by centrifugation at 10,000 rpm for 10 min at 4 °C and washed twice with deionized water. Then the biomass was taken and used to determine the Chromium biosorption. The biomass was then dried at 80°C in an oven, before treated with the acid mixture. Then the amount of chromium biosorbed with biomass was calculated as mg Cr/g dry weight and determined the standard deviation.

Cr (VI) reduction experiments

For hexavalent chromium reduction, three $K_2Cr_2O_7$ concentrations i.e. 100, 500, 1000µg/ml & two cell concentrations 0.5ml, 1ml were used. For reduction Deleo-Ehrlich medium was used. The cultures were incubated at 150 rpm at 37°C.

At regular intervals 24, 48, 72, 96 hrs samples were taken aseptically and Cr (VI) reduction was analyzed by DPC method [11].

Effect of bacterial concentration on Cr (VI) removal

Two different concentrates of bacterial culture 5 and10µl was inoculated in 100 ml sample and kept in shaker incubator at 30°C for 96 hours at 120 rpm. 10 ml sample was taken using sterilized pipette at regular intervals of 24 hours and all the samples were stored at 4°C for 6 hours before analysis of Cr (VI). After that Cr concentration was analyzed by DPC method at 540 nm [12] [13].

Effect of time on Cr (VI) removal

The free biomass cultures of same concentration (5, 10µl/100ml sample) were used to remove Cr (VI) within 96 hrs. The bacterial culture was incubated at 30°C at 120 rpm. The 10 ml sample was taken at different time interval of 24 hrs and the sample was stored at 4°C for 6hrs before Cr (VI) analysis. Cr concentration was observed after 6 hrs using DPC method.

Effect of pH on Cr (VI) removal

The bacterial culture was inoculated into a 250 ml conical flask containing 100 mg/lt of Chromium. The pH was varied from 3 to 11. The pH of the culture medium was adjusted using dilute HCl or NaOH. The culture was shaken on a rotary shaker at 120 rpm in a temperature controlled water bath. After 24 hrs, DPC method was used to measure Cr removal.

3. RESULT AND DISCUSSION

Characteristics of Collected Samples

Chromium contaminated water samples were collected in screw capped sterilized bottles from industrial area of Punjab State in India.

Table 1. Physiological Characterization of collected waste water

Sr. No.	Parameters	Values
1.	pH	7.4-7.8
2.	BOD	560-406
3.	COD	1794-1831
4.	DO	50-100

Standard curve of chromium

Diphenylcarbazide method was used for preparation of standard curve of chromium. The results of standard curve for chromium concentration are given below for chromium from 50 to 500 ppm concentration. Because the reaction with diphenylcarbazide was specific for chromium. Optical density was measured at 540 nm.

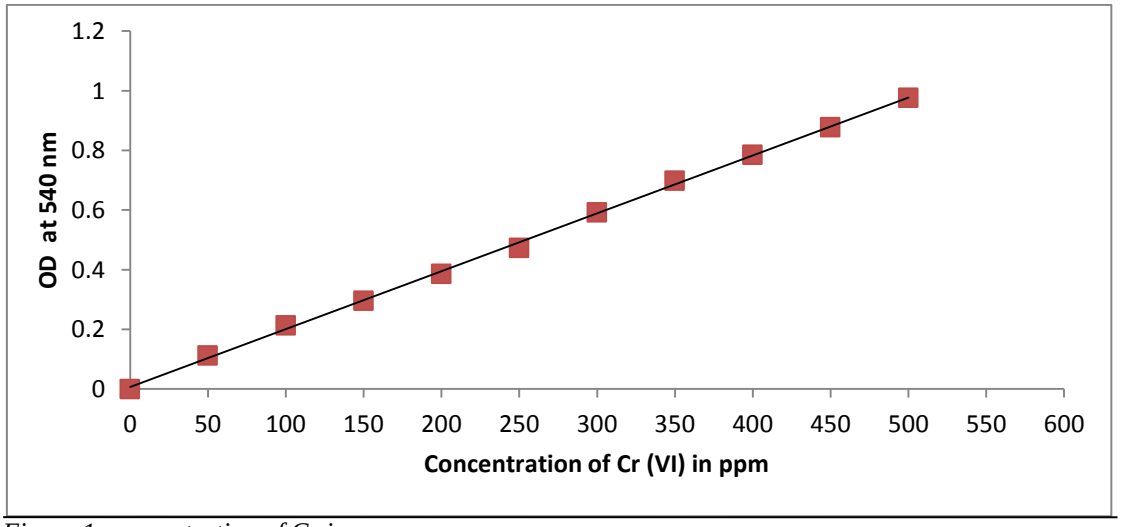

Figure 1. concentration of Cr in ppm.

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Biomass growth

The active biomass culture of *Pseudomonas putida* was obtained by growing culture on a nutrient agar by streaking method and incubated at 28°C for 24 to 48 hrs for the proper growth development

Figure 2. Pseudomonas putida

***Biosorption of Cr (VI) using biomass of bacteria*:**

The bacterial culture was utilized the Cr (VI) up to 100 ppm without affecting other metabolic activities. The biosorption of chromium by bacterial culture was slowly 8.7% in initial stage and it was maximum increased to the 55% at 240 minutes.

Graph showing biosorption of chromium in medium by using free *Pseudomonas putida*

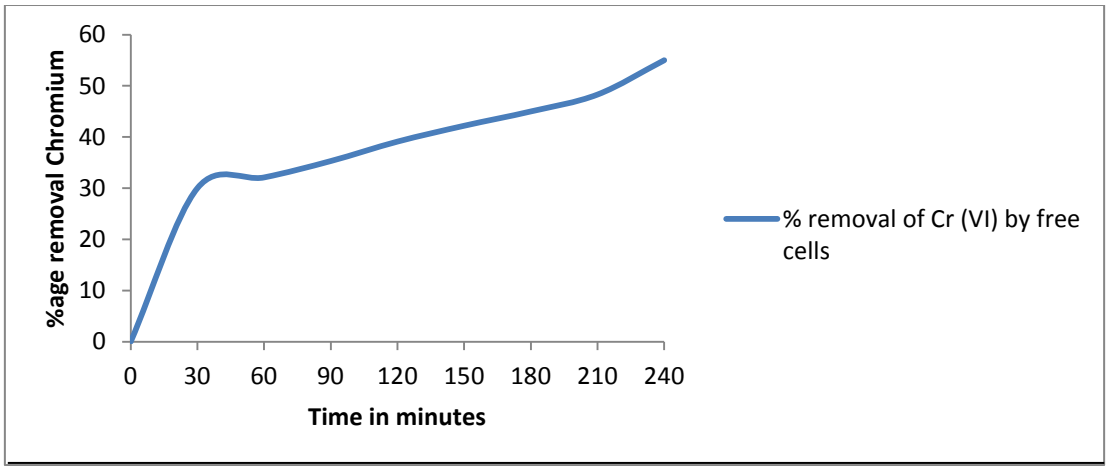

Figure 3. Removal of Cr by free cells

Metal sorption studies

The strain biosorbed Cr of less than 10 mg Cr/g dry weight, showing a MIC in the range of 60 mg Cr (VI)/l. The strain biosorbed 18, 20, 21 mg Cr/g dry weight at 50, 100, 150 Cr concentration respectively. The standard deviation is 9.912.

Graph showing comparative biosorption of chromium relation between tolerance and biosorption of free cells

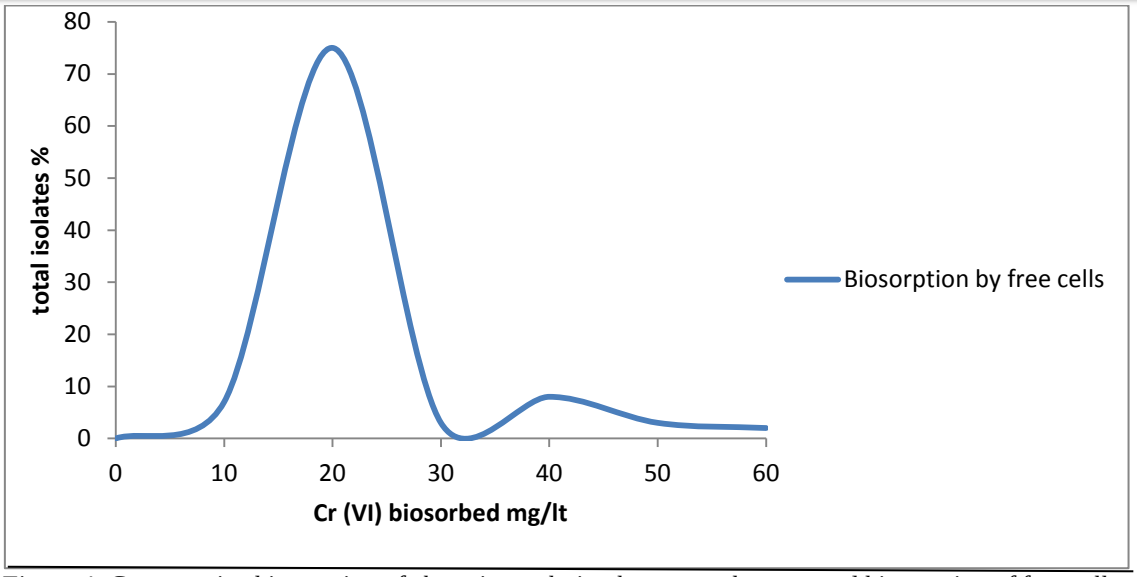

Figure 4. Comparative biosorption of chromium relation between tolerance and biosorption of free cells

Cr (VI) reduction experiments

Hexavalent chromium reduction was carried out with initial three $K_2Cr_2O_7$ concentrations i.e. 100, 500, 1000µg/ml & two cell concentrations 0.5 & 1ml. After 72 hr the chromium was completely reduced in100 µg/ml by the strain. At 1000 µg/ml concentration the strain reduced of Cr (VI) 96 hr respectively.

Graph showing reduction of Cr (VI) by free cells.

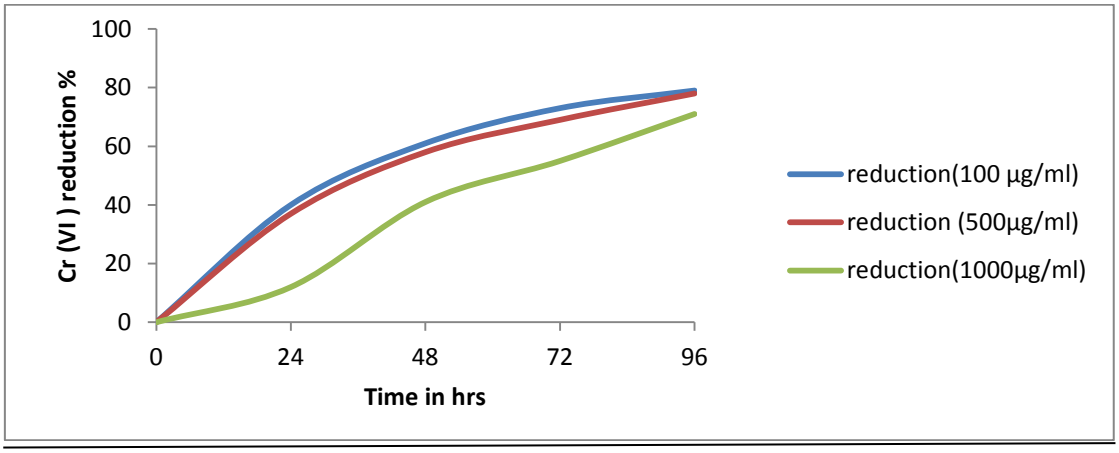

Figure5 . Removal of Cr by free cells

Effect of Time on Removal of Cr (VI)

The metal removal efficiency increased with time. A maximum Cr (VI) removal was estimated 90 % by *Pseudomonas putida* in industrial effluent, during 96 hours. Because *Pseudomonas putida* remove highest Cr (VI), corresponding to increase in time and reached a maximum value at a particular time; that is equilibrium time that was 96 hours. After 96 hrs, removal efficiency constant because adsorption and desorption becomes equal to each other [14].

***Effect of microbial concentration on Removal of Cr (VI) in industrial effluent*:**

Most of the hexavalent chromium (90%) absorbed by *Pseudomonas putida* from industrial effluent during 96 hours of incubation period and it was increased with increase in amount of culture. Thus, metal biosorption increased with increase in concentration as long as binding sites were available. Initially Cr binding was rapidly, but also reaches capacity or equilibrium after 96 hrs [15] [16].

Graph showing the effect of different time & concentration of free biomass on Cr (VI) removal

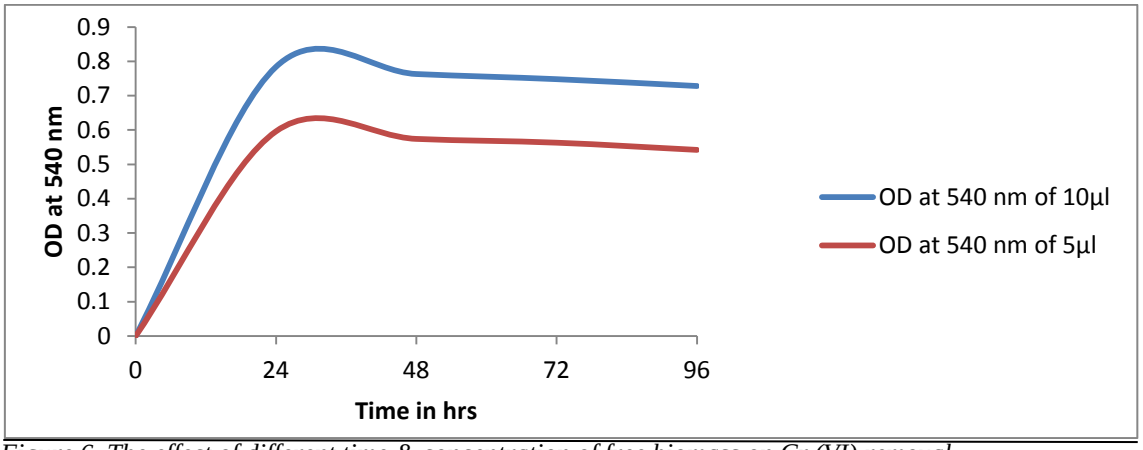

Figure 6. The effect of different time & concentration of free biomass on Cr (VI) removal

Effect of pH on Cr (VI) removal

The optimum pH for chromium removal in wastewater effluent by free cells was 5.2. The bioaccumulation capacity was low at pH values below five were due to the competition of hydrogen ion with metal ion for the sorption site. Because at lower pH, the protonation of binding site resulting from high concentration of proton, negative charge intensity decreased on the site which results in decreasing the binding of metal ion. Most of the microbial surfaces were negatively charged due to the functional group ionization, which contributing to metal binding.

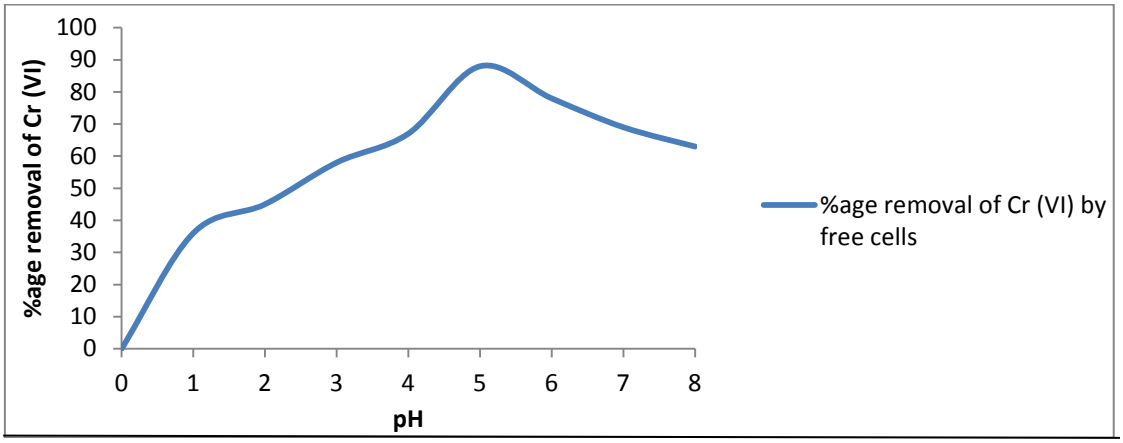

Figure 7. The effect of different pH on Cr (VI) removal

4. CONCLUSION

We can conclude, that at optimum pH for chromium removal in wastewater effluent by free cells were 5.2 and most of the hexavalent chromium (90%) absorbed by *Pseudomonas putida* from industrial effluent during 96 hours of incubation period and it was increased with increase in amount of culture. The bioaccumulation capacity was low at pH values below five were due to the competition of hydrogen ion with metal ion for the sorption site. A maximum Cr (VI) removal was estimated 90 % by *Pseudomonas putida* in industrial effluent, during 96 hours. Because *Pseudomonas putida* remove highest Cr (VI), corresponding to increase in time and reached a maximum value at a particular time; that is equilibrium time that was 96 hours. Finally, we can conclude that Cr was dissociated from effluent. Future studies, perhaps using alternative solution that includes modification in used microbial species for optimized best performance. This will determine an ultimate cure for the

complete bioremediation of this hazardous metal from environment.

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