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# Comparative Studies on the Bioremediation of Hexavalent and Trivalent Chromium using *Citrobacter freundii*: Part I-Effect of parameters controlling Biosorption

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**ABSTRACT:** The potential of *Citrobacter freundii*, a Gram negative bacteria for the remediation of hexavalent chromium (Cr(VI)) and trivalent chromium (Cr(III)) from aqueous solutions was investigated. Bioremediation of Cr(VI) involved both biosorption and bioreduction processes, as compared to only biosorption process observed with respect to Cr(III) bioremediation. In the case of Cr(VI) bioremediation studies, about 59 % biosorption was achieved at an equilibrium time of 2 h, initial Cr(VI) concentration of 4 mg/L, pH 1 and a biomass loading of $5x10^{11}$ cells/mL. The remainder, 41 %, was found to be in the form of Cr(III) ions owing to bioreduction of Cr(VI) by the bacteria resulting in the absence of Cr(VI) ions in the residue, there by meeting the USEPA specifications. Similar studies were carried out using Cr(III) solution for an equilibrium time of 2 h, Cr(III) concentration of 4 mg/L, pH 3 and a biomass loading of $6.3x10^{11}$ cells/mL, wherein a maximum biosorption of about 30 % was achieved.

**Key words:** Biosorption, Bioreduction, Hexavalent chromium, Trivalent chromium, *Citrobacter freundii*

## INTRODUCTION

Chromium is a heavy metal of commercial importance and a substantial quantity of it is released into the environment as waste in hexavalent and trivalent forms. The World Health Organisation (WHO), the International Agency for Research on Cancer and the Environmental Protection Agency (EPA) have established hexavalent chromium (Cr(VI)) compounds as human carcinogens (Pattanapipitpaisal *et al*., 2001; Das and Mishra, 2010). Anthropogenic activities such as mining, production of steel and nonferrous alloy in metallurgical industries, leather tanning, chrome plating, paint manufacturing and the use of chromium as a dye in textile and ceramic industries releases considerable amount of Cr(VI) into the environment (Gallios and Vaclavikova, 2008; Dhal *et al*., 2013; Sillerova *et al*., 2014) posing a threat to all living beings and surroundings. On the other hand, trivalent chromium Cr(III) is considered as a micronutrient, essential for maintaining normal blood sugar levels, in regulating carbohydrate and lipid metabolism and in enhancing insulin signaling (Gallios and Vaclavikova, 2008; Vaiopoulo and Gikas, 2012). The maximum contaminant level (MCL) for Cr(VI) as per the United States Environmental Protection Agency (USEPA) regulations in domestic water supplies should be 0.05 mg/L, while total Cr composing of Cr(III) and Cr(VI) should be below 2 mg/L (Saha and Orvig, 2010, Tekerlekopoulou *et al*., 2010).

The various conventional methods used for mitigation of several Cr polluted sites, such as reduction followed by chemical precipitation, electrochemical precipitation, solvent extraction, membrane separation and ion exchange suffer from other secondary problems (Owlad *et al*., 2009). As an alternative to these techniques, bioremediation has gained increasing attention (Hou *et al*., 2012). Biosorption, a bioremediation process, is defined as the passive uptake of toxicants by dead/inactive biological materials or by materials obtained from biological sources. The process apart from being non-polluting is found to be highly selective, more efficient, easy to operate and hence cost-effective for treatment of large volumes of wastewater containing low metal ion concentrations (Al-Garni, 2005; Saha and Orvig, 2010). The various biosorbents used for the metal and dye removal and their biosorption efficiencies have been comprehensively reviewed (Vijayaraghavan and Yun, 2008; Oliveira *et al*., 2011).

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Numerous bacteria are being researched upon for their ability to biosorb heavy metals (Badar *et al.*, 2000) especially for Cr remediation purposes (Murugavelh and Mohanty, 2013) due to their small size, ubiquity, ability to grow under controlled conditions, resilience to a wide range of environmental conditions (Silva *et al*., 2008) and more importantly the presence of different functional groups on their cell wall that act as binding sites for the contaminant Cr ions (Tekerlekopoulou *et al*., 2013) . *Citrobacter freundii* has been reported to accumulate various elements such as lead, uranium (Macaskie *et al*., 1992), lanthanum and thorium (Tolley *et al*., 1995; Yong and Macaskie, 1997). *C. freundii* has also been used for the biosorption of lead, cadmium and zinc (Puranik and Paknikar, 1999). Bonthrone *et al*. (2000) have assessed the metal binding behavior of an extrapolymeric material of *Citrobacter* sp. for uranyl ion. *Citrobacter* sp. was isolated among other microorganisms from an aerospace industrial sludge and was used in consortia to model Cr(VI) removal in draw-fill reactors in both suspended and attached growth systems (Tekerlekopoulou *et al*., 2013). There is a paucity of literature on the utility of *Citrobacter* sp. for the bioremediation of chromium. In the present investigation, the bioremediation of Cr(VI) and Cr(III) has been studied using *Citrobacter freundii*. The effects of contact time, pH, biomass loading and metal concentration on the bioremediation process have been ascertained to elucidate the possible mechanisms of bioremediation.

## MATERIALS & METHODS

Analytical grade $K_2Cr_2O_7$ and $CrCl_3.6H_2O$, sources for Cr(VI) and Cr(III) respectively, were obtained from Merck, Germany and Loba Chemie, India. Other reagents used for various experiments such as NaOH, HCl, $H_2SO_4$ and acetone were all of analytical grade. Analytical grade 1,5 diphenyl carbazide, a Cr(VI) complexing agent was purchased from Merck, Germany for spectrophotometric analysis. Deionised water of conductivity 18.2 MΩ cm from a MilliQ system was used in all the experiments.

A pure strain of *Citrobacter freundii* (MTCC No. 8128) was procured from Institute of Microbial Technology (IMTECH), Chandigarh, India. Nutrient broth (NB) of pH 7, the constituents of which are 5 g/L peptone, 2 g/L yeast extract, 1 g/L beef extract and 5 g/L NaCl was used as growth medium for the bacteria. The medium containing 10 % of the freshly inoculated bacteria was agitated in a orbital Orbitek shaker at a temperature of 30°C for 24 h. The growth was monitored by bacterial cell counting using Petroff Hausser counter in conjunction with Leitz phase contrast microscope (Laborlux K Wild MPS12).

For the biosorption experiments, appropriate volume of 24 h bacterial culture grown in NB medium was centrifuged at 10,000 rpm for 10 min using a Remi refrigerated centrifuge. Subsequently the obtained pellet was washed thoroughly with deionised water and used for the experiments. The biosorption test procedure was standardized as follows: A known amount of the bacterial pellet of desired cell count was dispersed in Cr solution of desired concentration and the pH adjusted to a chosen value using a Systronics digital pH meter. The samples were made upto to a final volume of 100 ml in 250 mL Erlenmeyer flask and were then agitated in a Orbitek rotary shaker at 30°C for a desired period of time. The suspensions were then centrifuged in a Remi refrigerated centrifuge at 10,000 rpm for 10 min. The chromium concentration in the supernatant was determined and the percentage of biosorption was estimated. Total chromium in the samples was estimated using Thermo Electron Corporation M Series Atomic Absorption Spectrometer (AAS).

The concentration of Cr(VI) was estimated by 1, 5 diphenylcarbazide (DPC) method using a Labomed Inc. UV-VIS spectrophotometer at the wavelength of 540 nm. The concentration of Cr(III) present in the sample was calculated by subtracting Cr(VI) concentration obtained using DPC method from the total Cr concentration obtained using AAS.

All the above experiments were carried out in duplicate and the standard deviation was determined.

## RESULTS & DISCUSSION

The growth curve for C. *freundii* was initially determined and is portrayed in Figure 1. It is evident that the bacterial count increases exponentially upto 10 h from $1.5x10^9$ cells/mL to $8x10^{10}$ cells/mL after which the stationary phase is attained. Simultaneously, the change in pH over the period of bacterial growth was also monitored. The pH of the medium is found to increase from 7.0 to about 8.4 and this can be attributed to deamination reaction as a result of metabolic activity of the bacteria during growth. On Gram staining, the bacterium is found to be Gram negative. In order to examine the effect of contact time of the Cr ions with the biomass,

bacterial cells of count $1.4x10^9$ cells/mL were subjected to Cr(VI) and Cr(III) solutions of concentration 4 mg/Leach at pH 2 for varying time intervals at 30°C. From Fig. 2(a), it is clear that total chromium biosorption increases with increase in contact time upto 1 h after which a plateau is attained. About 17 % total Cr biosorption is achieved. In further experiments, an equilibrium time of 2 h was used. In order to ascertain whether the bioreduction process is also occurring simultaneously to biosorption, the Cr(VI) concentration was independently determined by the DPC method. It is noteworthy that bioreduction of Cr(VI) results in the increase in Cr(III) concentration in solution as a function of time (Fig. 2(a)). Figure 2(b) depicts the kinetics of Cr(III) biosorption, which shows a similar trend as that observed for Cr(VI). The biosorption of Cr(III) increases with time upto about 1 h and subsequently attains saturation. In further experiments with Cr(III), the equilibrium time was fixed at 2 h.

To find out the effect of pH on biosorption studies, the pelleted fully grown bacterial cells of count $1.4x10^9$ cells/mL were dispersed in Cr(VI) and Cr(III) synthetic solutions of concentration 4 mg/L each at varying pH from 1 to 6 and allowed to equilibrate for 2 h. From Fig. 3(a) it is evident that Cr(VI) is stable over the entire

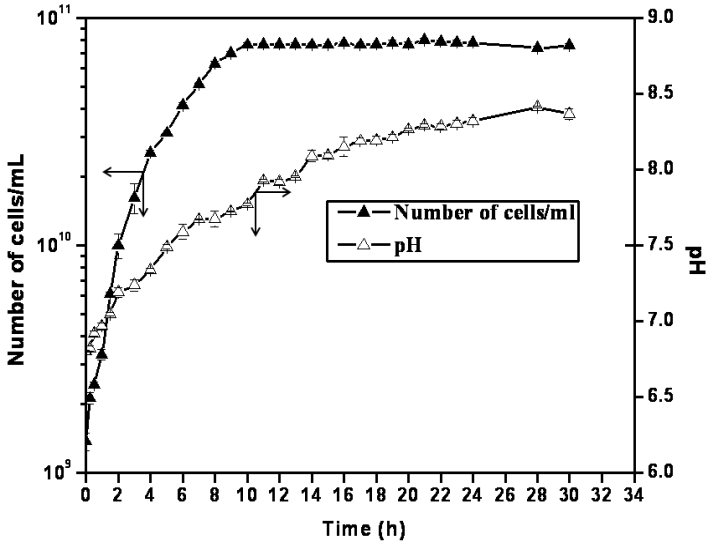


**Fig. 1. Growth curve of *C. freundii* along with change in pH with growth**

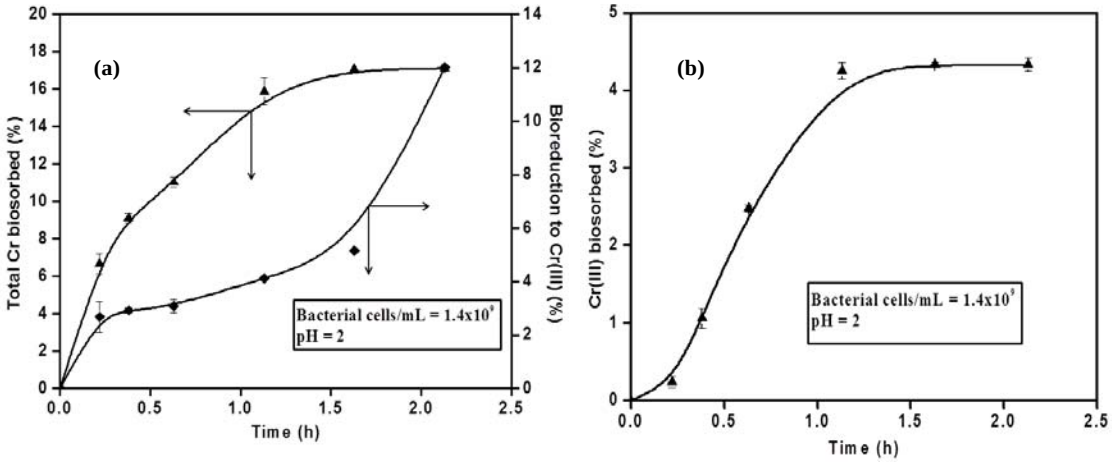


**Fig. 2. Effect of contact time on biosorption of (a) Cr(VI) and (b) Cr(III); [Cr(VI) and Cr(III) = 4 mg/L, pH 2, $1.4x10^9$ cells/mL and 30°C]**

pH range studied in the absence of bacteria. In the presence of the cells, the Cr(VI) concentration is found to decrease from 4 mg/L to about 3.5 mg/L in the pH range of 1 to 3, while there is not much change in Cr(VI) concentration between pH 4 to 6 (Fig. 3(a)). The percentage of total Cr biosorbed is found to decrease from 14 % to about 1 % as the pH is increased from 1 to 6 indicating the repulsion of negatively charged oxyanion of Cr(VI) with the negatively charged functional groups present on the bacteria as shown in Fig. 3(b). At highly acidic pH, the bacterial cell surface becomes protonated attaining a positive charge, facilitating the biosorption of Cr(VI) to a greater extent. Figure 3(b) also shows that bioreduction of Cr(VI) to Cr(III) is maximum at pH 1 (36 %) and decreases sharply to about 4 % at pH 3. From Fig. 3(c) it is evident that Cr(III) is stable upto pH 3 and thereafter its concentration decreases in solution due to precipitation. However, in the presence of the bacterial cells, the Cr(III) concentration continuously decreases from about 4 mg/L to less than 1 mg/L at pH 6, due to both biosorption and precipitation processes. Hence, taking into consideration removal of Cr(III) only by biosorption, pH 3 was chosen for the further studies.

In order to study the effect of biomass loading on Cr biosorption, the cell concentration was varied from $1.4x10^9$- $7.5x10^{11}$ cells/mL. Cr(VI) biosorption experiments were conducted at pH 1, an equilibration time of 2 h, and the initial Cr(VI) concentration of 4 mg/L. From Fig. 4(a) it is observed that percentage

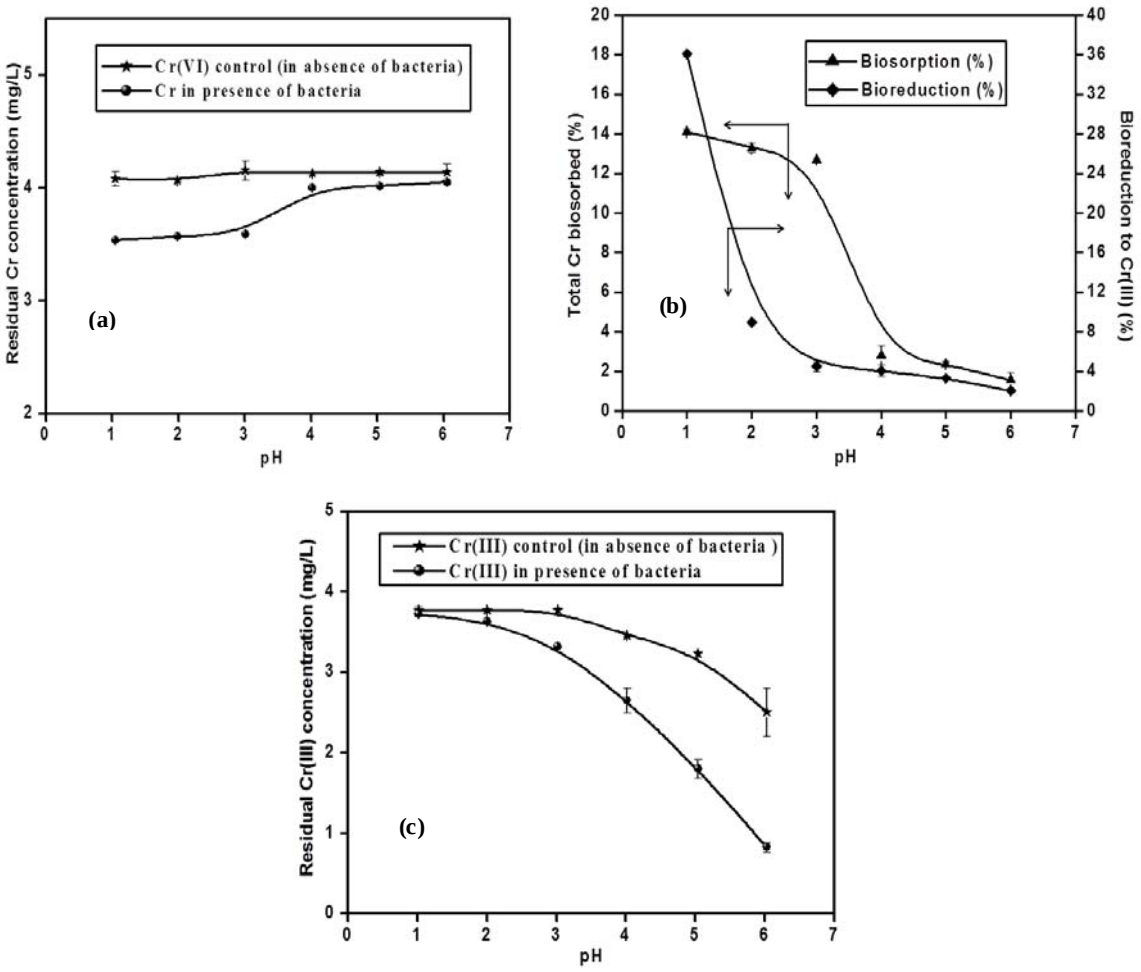


**Fig. 3. Effect of pH on Cr biosorption: (a) Cr(VI) stability and biosorption [Cr(VI) = 4 mg/L, $1.4x10^9$ cells/mL, equilibrium time ($t_e$) = 2 h and 30°C ]; (b) Residual Cr(VI) analysis after Cr(VI) biosorption experiment; (c) Cr(III) stability and biosorption [Cr(III) = 4 mg/L, $1.4x10^9$ cells/mL, $t_e$ = 2 h and 30°C ]**

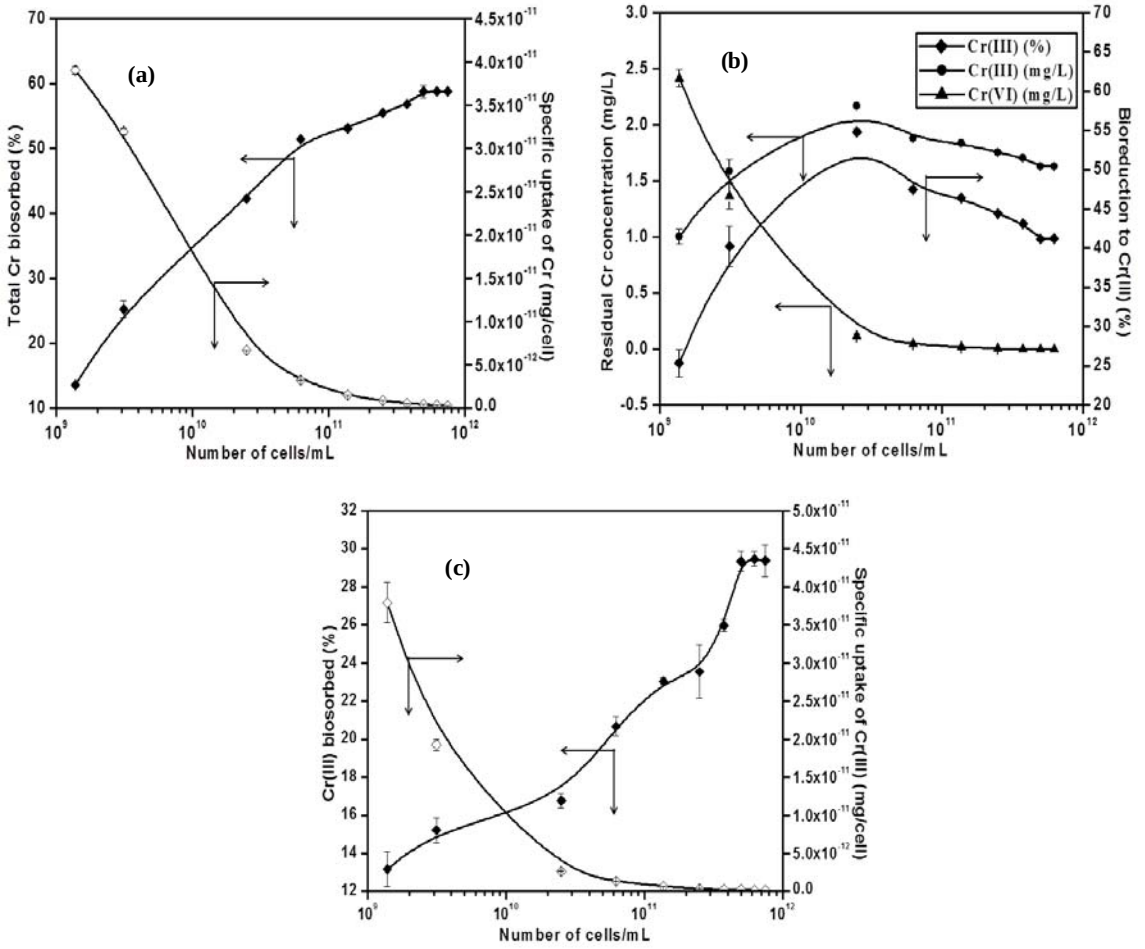


**Fig. 4. Effect of biomass loading on Cr biosorption: (a) Total Cr biosorption (%) and specific uptake (mg/cell) [Cr(VI) = 4 mg/L, pH = 1, $t_e$ = 2 h and 30°C]; (b) Residual Cr(VI) concentration and % bioreduction; (c) Cr (III) biosorption (%) and specific uptake (mg/cell) [Cr(III) = 4 mg/L, pH = 3, $t_e$ = 2 h and 30°C]**

biosorption increases with increase in cell concentration, which is attributable to the increase in surface binding sites. A maximum of 58.8 % biosorption was obtained for a biomass loading of $5x10^{11}$ cells/mL, and this was fixed as the biomass concentration for further studies. The decrease in the specific uptake of Cr(VI) with increase in biomass loading shown in Fig. 4(a), arises due to the crowding of cells resulting in the reduction of exposure of functional groups per cell. This phenomenon is known as 'screen effect' and has been reported in other works (Sekhar *et al*., 1998). Bioreduction process is observed to increase with increase in biomass as shown in Figure 4(b), justifying the requirement of more electron donor functional groups. At a higher biomass loading, it is also noticed that a complete bioreduction of Cr(VI) to Cr(III) occurs with the Cr(VI) concentration in solution tending to zero (Fig. 4(b)). This resulted in achieving complete removal of Cr(VI) ions, adhering to the USEPA regulations. A concomitant increase in the Cr(III) concentration in solution is observed in Fig. 4(b). Biosorption experiments of Cr(III) conducted at pH 3 resulted in a maximum biosorption of 29.5 % of Cr(III) at a biomass loading of $6.3x10^{11}$ cells/mL as depicted in Fig. 4(c). The specific uptake of Cr(III) shows a similar behaviour to that observed in the case of Cr(VI) (Fig. 4(c) ).

For the biosorption isotherm experiments, bacterial cells ($5x10^{11}$ cells/mL corresponding to 0.2 g dry weight) were allowed to react with varying initial concentrations of Cr(VI) from 2 mg/L to 10 mg/L maintaining other conditions the same. The amount of

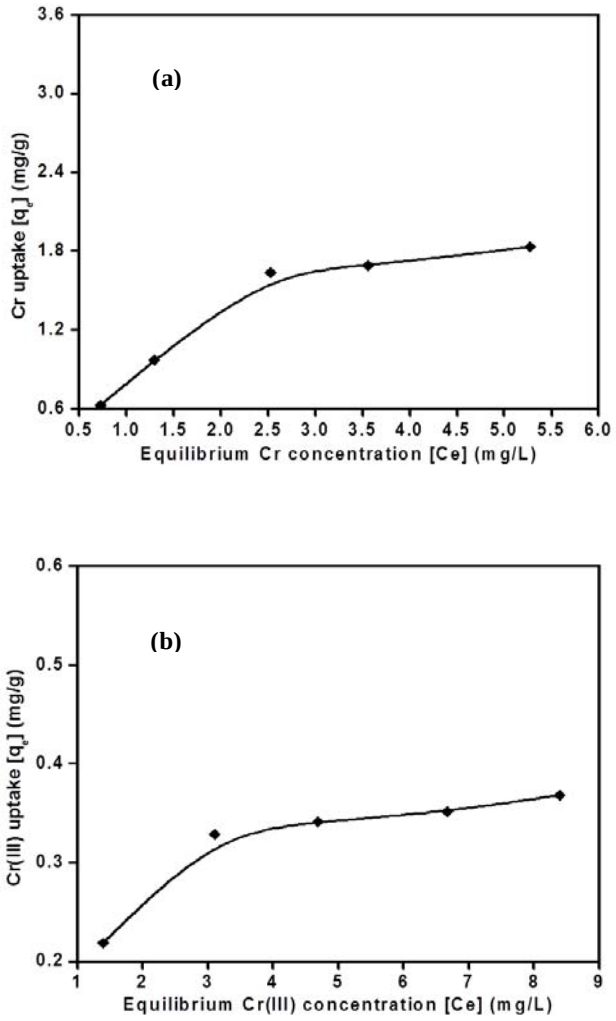


**Fig. 5. Biosorption isotherm of Cr (VI) and Cr(III): (a) Cr(VI) biosorption [pH = 1, 5x10$^{11}$ cells/mL (dry wt. = 0.2 g), $t_e$ = 2 h and 30$^o$C]; (b) Cr(III) biosorption [pH = 3, 6.3x10$^{11}$ cells/mL (dry wt. = 0.3 g), $t_e$ = 2 h and 30$^o$C]**

Cr biosorbed is found to increase initially upto about 2.5 mg/L equilibrium concentration and thereby attains a saturation value, indicative of Langmuirian behavior (Fig. 5(a). In the case of Cr(III) biosorption, the initial cell count was 6.3x10$^{11}$ cells/mL corresponding to 0.3 g dry weight. The other conditions were akin to those chosen for Cr(VI). Fig. 5(b) shows the isotherm obtained for Cr(III) biosorption. In this case also, the shape of the isotherm is similar to that observed for Cr(VI). It becomes of interest to elucidate the mechanism involved in the bioremediation of Cr by *C. freundii*. Towards this, the data obtained from the biosorption studies have been fitted to thermodynamic and kinetic models. ATR-FTIR, XPS and electrokinetic studies have been carried out to identify the mode of interaction of the chromium species with the functional groups of the bacteria. The results are discussed in part II of this paper.

## CONCLUSIONS

Almost complete bioremediation of Cr (VI) could be achieved from a feed solution of 4 mg/L at an optimum pH of 1 with a biomass loading of 5x10$^{11}$ cells/mL utilizing *Citrobacter freundii*. Both biosorption (59%) and bioreduction to Cr(III) (41%) are found to play a role in the bioremediation process. Further, about 30 % biosorption of Cr(III) could be accomplished from an aqueous solution containing 4 mg/L at an optimum pH of 3 with a biomass loading of 6.3x10$^{11}$ cells/mL.

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