Uptake and removal of toxic Cr(VI) by *Pseudomonas aeruginosa*: physico-chemical and biological evaluation

Suparna Chatterjee, Indranil Ghosh and Kalyan K. Mukherjea*

Department of Chemistry, Jadavpur University, Kolkata 700 032, India

The present study evaluates the biosorption of Cr(VI) by *Pseudomonas aeruginosa* from synthetic solution and tannery effluents. The absorption was studied under different initial Cr(VI) concentrations at different pH values and in the presence of other metals. The Cr(VI) concentration in the effluent, sludge and soil of tannery industries was measured. A maximum absorption was found at 30 mg/l of Cr(VI) at pH 8, which decreased in the presence of cadmium. Cyclic voltammogram confirmed the reduction of Cr(VI). FTIR analysis showed that the carboxyl and amino groups on the bacterial surface bind chromium. SEM and EDX revealed that Cr(VI) is reduced to Cr(III).

Keywords: Bioremediation, metal adsorption, *Pseudomonas aeruginosa*, tannery effluents.

CHROMIUM compounds are being widely used in leather tanning, steel production, as metal corrosion inhibitor, alloy formation, in paints as pigments and various other applications[1]. Chromium thus is a contaminant in the soil, sediment, surface and groundwater in the trivalent and hexavalent forms[2]. Hence chromium-associated pollution is a cause of great concern. Chromium is an essential trace element for living organisms[3]. Of all the oxidation states of chromium, Cr(III) and Cr(VI) occur most commonly. Cr(VI) induces toxicity as it causes mutation[4] and cancer[5] in animals and mutation in bacteria. It is toxic even at a concentration of 20 μg/l (ref. 6). Heavy metal removal by chemical precipitation[7], ion exchange, reverse osmosis and solvent extraction has disadvantages due to high cost and energy of complex processes[8]. On the other hand, bioremediation has advantages like the possibility of metal recovery, easy waste disposal of the incineration process and low cost. Metal uptake by microorganisms is an environment-friendly alternative[9] of heavy metal remediation. Both living and dead microbial mass are capable of taking up metal ions from aqueous solution[10]. Microorganisms take up metal ions either actively (bioaccumulation) and/or passively (biosorption)[11]. Biosorption is the ability of biological materials to accumulate heavy metals from waste water through metabolically mediated or physico-chemical pathways of uptake[12]. It is based on mechanisms such as complexation, ion exchange, adsorption, chelation and micro precipitation[10]. Removal of heavy metals using different biosorbents has been found to be highly selective depending on the typical binding profile of the biosorbents[13]. Successful application of biosorption depends on the parameters like initial metal concentration and contact time[14].

In this present study a Gram-negative, ubiquitous, aerobic rod, *Pseudomonas aeruginosa*, has been used to assess the removal of chromium with a view to provide environment-friendly methods of removal of toxic chromium Cr(VI) from industrial effluents. The cell wall of this bacterium is composed of peptidoglycan, teichoic acids along with carboxyl, phosphoryl, hydroxyl and amino functional groups at the surface[15]. Fourier transform infrared (FTIR) analysis was carried out to determine the involvement of the type of functional groups in metal adsorption. Fein *et al.*[16] reported that some components of the cell wall serve as electron donors for the reduction reaction while metal binding[16].

Materials and methods

All the chemicals were either AR or GR grade. All-glass triple-distilled water (TDW) was used throughout the study.

Biosorption studies

The parameters responsible for removal such as time of contact, initial metal concentration, pH of culture media and other interfering metal ions like cadmium and iron[17] which are normally present in tannery effluents have been studied. This study has been performed both by supplementing synthetic solution of Cr(VI) and also treating Cr(VI) from the effluents of tannery industries in and around Kolkata, India.

P. aeruginosa was cultured and maintained in Luria Bertani (LB) agar plates and slants in our laboratory. The organism was grown and cultured aerobically with agitation at 37°C in the presence of Cr(VI) in LB broth

*For correspondence. (e-mail: k_mukherjea@yahoo.com)

(consisting of 10 g tryptone, 10 g NaCl and 5 g yeast extract in 1 l of TDW, and pH adjusted to ~ 7.0). Five per cent of the culture was used as inoculum after reaching the mid exponential growth phase of cells (OD_{600nm} = 1) measured using a UV–Vis Spectrophotometer (Hitachi U-3410 Spectrophotometer). The growth of the organism was observed in media containing different concentrations of Cr(VI) solution ranging from 1 to 40 mg/l of hexavalent chromium (supplied in the form of potassium dichromate solution in deionized water and filter-sterilized before use). The four initial metal ion concentrations that were studied during the 7-day culture period were 10, 20, 30 and 40 mg/l. Microorganisms grown in different concentrations of metal solution were harvested by centrifugation at 6000 rpm for 15 min at 4°C. The supernatant was separated after the cells were pelleted down and the pellets were washed thrice with deionized water to remove chromium not sorbed by the bacteria. The supernatant was then subjected to acid digestion using conc. HNO_3 (five parts), conc. H_2SO_4 (five parts) and H_2O_2 (three parts). The Cr(VI) ion concentration was determined spectrophotometrically (Hitachi U-3410 Spectrophotometer) at 540 nm after complexation with 1,5-diphenylcarbazide[18]. All the tests were performed in triplicate. Control experiments without the organism were also performed with LB medium containing the same initial concentration of Cr(VI) as potassium dichromate to ensure that removal was due to the microorganism and not due to any other abiotic reason or precipitation.

Other conditions that influence the metal removal efficiency by the bacterial strain such as pH and presence of other metals were also studied. The metal removal efficiency was studied at six different pH conditions, i.e. 3, 4, 5, 6, 8 and 9. The study was also performed in the presence of other metals like cadmium and iron, which are normally found in tannery effluents. Initial Cr(VI) concentration was 30 mg/l, since maximum absorption occurred at this concentration. Initial Cd and Fe concentration added was 0.076 and 0.75 mg/l respectively at pH ~ 7 (ref. 17). All the tests were performed in triplicate.

Cyclic voltammetric study

Cyclic voltammetric (CV) study was performed with an ECO-CHEMIE Cyclic Voltameter Model AutoLab 302. Voltammetric experiments were carried out in a one-compartment cell using a Pt working electrode of surface area 1.2 cm^2, a large Pt foil counter electrode, and a saturated calomel reference electrode (SCE). The voltammetric experiments were carried out between the potential range –0.5 and 1.5 V (versus SCE). The study was performed under the same conditions for media containing 30 mg/l Cr(VI) as the control and solution after treatment with *P. aeruginosa* for 72 h as the experiment. The voltammetric peak current was obtained from which the current density calculated was used to show the changes of electrochemical responses to the reaction system.

FTIR analysis

The bacteria pelleted by centrifugation of 50 ml cultures at 6000 rpm for 15 min at 4°C were lyophilized using an Ice dryer (Eyela, Tokyo Rikakikai Co Ltd, FD-1). Chromium-loaded biomass was washed, dried and powdered after biosorption of chromium ions under the same conditions. One milligram of finely crushed biomass was then mixed with 400 mg potassium bromide. The mixture was ground into fine powder and translucent sample disks obtained by using a manual hydraulic press at a pressure of 100 kg cm^{-2} for 10 min. The disk was then fixed in a FTIR Spectrometer (FTIR 8400S SHIMADZU). FTIR spectrum of the biomass unexposed (control) and exposed to Cr(VI) at concentration of 30 mg/l was obtained from 400 to 4000 cm^{-1}.

Scanning electron microscopy

Scanning electron microscopic (SEM) studies were carried out, which revealed the surface changes after treatment with chromium, and the precipitation products of hexavalent chromium was substantiated by energy-dispersive X-ray spectroscopy (EDX). The surface structure of the untreated *P. aeruginosa* as well as that treated with Cr(VI) at a concentration of 30 mg/l was analysed by SEM. The existence of metal ion on the surface of the bacterial biomass was determined using EDX. The pellet from the experiment was washed thoroughly with sterilized TDW, immersed in glutaraldehyde (2.5% v/v, Fluka) for 2 h at room temperature and washed thoroughly with sterilized TDW. The pellet was then subjected to osmium tetraoxide staining (2% v/v, Fluka) for 1 h and washed thoroughly with sterilized TDW. Next, the pellet was dehydrated by transferring it into a series of 25, 50, 70, 90 and 100% (v/v) of ethanol (Fluka) for 5 min. The final dehydration in 100% ethanol was carried out for 10 min. The dehydrated pellet was then dried overnight in an oven and mounted on a glass slide 120 stab with a double-stick carbon tab followed by coating with a thin layer of gold under vacuum to increase the electron conduction and to improve the quality of the micrographs[19]. SEM–EDX studies were performed using a JEOL JSM-6700F Field Emission SEM.

Study area

The East Kolkata wetland and waste recycling region covers about 12,500 ha and is surrounded by tannery industries[20]. A canal that carries only untreated tannery

wastes of this region was selected for collection of effluent and sludge samples at distances of 0.05, 0.25, 0.5 and 0.75 km from the last discharge point of the tannery industry. Soil samples were collected from two different depths of 15 and 30 cm from the agricultural land about 1 km away from the tannery industry. The reference site (control) was selected 10 km away, which is not exposed to any sewage or industrial effluent.

Sampling and sample analysis

Samples were collected in sterile glass bottles, transported to the laboratory on ice and processed within 6 h of collection. Water samples were filtered with 0.22 μm membrane after collection. Wet sludge was collected and kept in the laboratory for drying, of which 0.5 g was taken as sample and suspended in TDW. Soil samples of 1 kg were collected. These were kept in sterilized glass container for drying. Dried soil sample of 0.5 g was taken for digestion. The Cr(VI) ion concentration was determined spectrophotometrically at 540 nm after complexation with 1,5-diphenylcarbazide[18]. All the tests were performed in triplicate. The pH of the effluent was measured using a pH meter (model CT No. CL46, Toshniwal, India).

Biosorption study of the effluent

The same experiment was repeated taking the tannery effluent of Calcutta Metropolitan Area after filter sterilization using 0.22 μm membrane under aseptic conditions for performing the microbial removal of Cr(VI), as was done for the synthetic solution. Control experiments without the organism were also performed with LB medium containing the same initial concentration of Cr(VI) as potassium dichromate to ensure that the removal was due to the microorganism.

Results

Microbial study

It was observed that so far as the absorption of chromium from synthetic solution was concerned, there occurs a maximum absorption of Cr(VI) on the third day following slight decrease from the fourth day onwards till the seventh day (Figure 1). Maximum absorption of the metal was found at alkaline pH (i.e. at pH 8), whereas absorption at acidic pH was slightly low (Figure 2). It was found that cadmium induces a decrease in absorption, while iron was found to be indifferent (Figure 3).

CV study

The approximate $E^0(a)$ for Cr(III)/Cr(VI) couple corresponds to 1.36 V with respect to the standard hydrogen electrode (SHE) for the solution containing only Cr(VI) (Figure 4 *a*). Similarly, the approximate $E^0(b)$ corresponds to 1.34 V for the solution containing Cr(VI) after treatment with *P. aeruginosa* for 72 h (Figure 4 *b*). The standard electrode potential for the electrode reaction converting $Cr_2O_7^{-2}$ to Cr^{+3} was 1.33 V with respect to the SHE[21,22]. The peak current density calculated was 0.32 and 0.1 mA/cm^2 for profiles a and b respectively. This suggests that the concentration of Cr(VI) left in the solution is 9.38 mg/l after treatment with *P. aeruginosa*. Thus, the CV study indicated that 68.75% of Cr(VI) was being reduced to Cr(III).

FTIR study

Surface characterization of *P. aeruginosa* by FTIR analysis without and with adsorbed chromium is shown in Figure 5. The complex nature of the biomass examined is

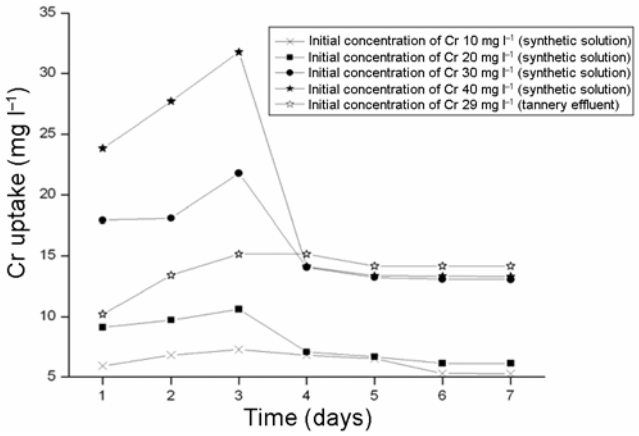


Figure 1. Concentration of chromium (VI) uptake by *Pseudomonas aeruginosa*.

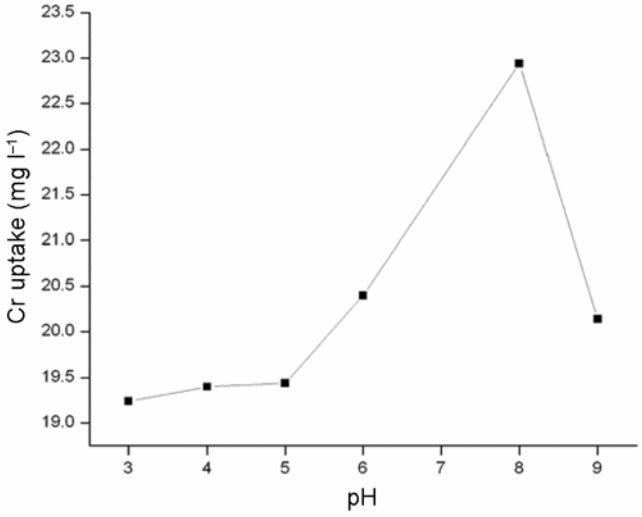


Figure 2. Chromium uptake by *P. aeruginosa* on the third day at different pH values and at initial concentration of 30 mg/l.

indicated by a number of absorption peaks in the control sample (Figure 5 *a*). FTIR study of the biomass shows that the band observed at 3421 cm^{-1} results from $–NH_2$ asymmetric stretching mode of amines, which is slightly broad indicating overlapping of amines and hydroxyl stretching on the bacterial surface[23]. The absorption peak at 3290 cm^{-1} indicates the hydroxyl groups and amine groups of proteins. The bands at 2927 and 2680 cm^{-1} can be assigned to the –CH stretching[24]. The absorption band at 1649 cm^{-1} is a result of C=O stretching mainly conjugated to a –NH deformation mode, and can be attributed to the amide I bands of the amide bond due to the protein peptide bonds[25]. The band at 1445 cm^{-1} indicates symmetric bending of $–CH_3$ of the acetyl-moiety. The 1400 cm^{-1} band is due to the vibration of O–H carboxylate ions[26]. A band at about 1232 cm^{-1} represents $–SO_3$ stretching[27]. A trough observed at 1068 cm^{-1} represents –CN stretching due to the vibration of the protein fractions[28]. In the Cr(VI)-treated sample (Figure 5 *b*), there is shifting of the band to 3407 cm^{-1} from 3421 cm^{-1} with substantial decrease in intensity. There is also change of the band at 3290 cm^{-1}. The shift in the band to 2875 cm^{-1} from 2927 cm^{-1} can also observed. Slight depression of the band at 1234 cm^{-1} and decrease in the band intensity at 1652 is also noticed. There occurred a shift of the band to 1398 cm^{-1} from 1400 cm^{-1}, with a decrease in intensity. Shift of the band at 1074 cm^{-1} from 1068 cm^{-1} is also observed.

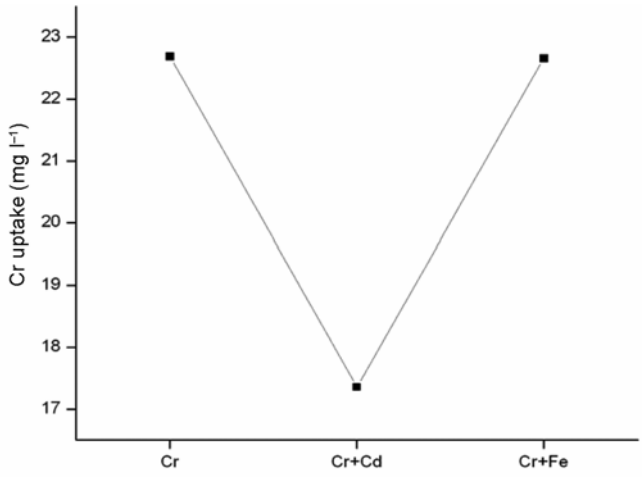


Figure 3. Chromium uptake by *P. aeruginosa* on the third day in the presence of Cd and Fe.

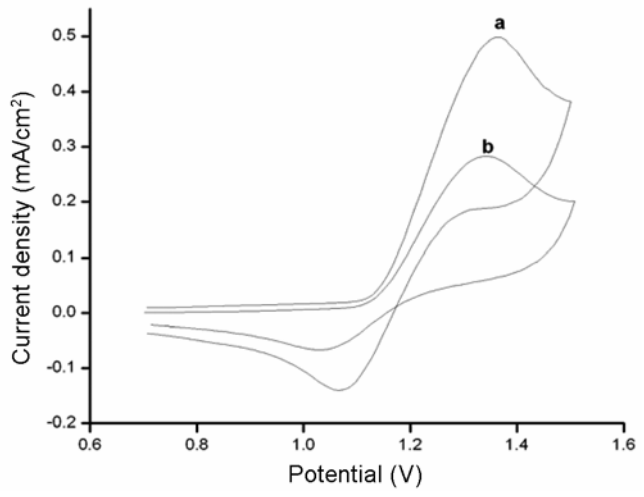


Figure 4. Cyclic voltammogram of LB medium containing 30 mg/l Cr(VI) (a) before and (b) after *P. aeruginosa* treatment. Rest time, 5 s and scan rate, 10 mV/s.

SEM study

SEM photomicrographs of *P. aeruginosa* taken before and after Cr(VI) biosorption are presented in Figure 6 *a* and *b* respectively. SEM studies reveal that before Cr(VI) biosorption, the cells appeared to be plump having smooth surfaces in a loosely-bound form. After interaction with Cr(VI), precipitates in the form of round globules and amorphous substances aggregated all over the cell surface of *P. aeruginosa*. Such substances were also found dispersed in the culture medium containing Cr(VI). The Cr(VI)-treated *P. aeruginosa* cell wall becomes rough. The bulging of cell wall along the uneven cell surface after treatment with 30 mg/l Cr(VI) for 72 h was also noticed. EDX was performed at 20 kV for confirming the sorption products on the bacterial cell surface, since X-ray absorption provides information on the electronic and structural state of an element. EDX of the control and treated samples is presented in Figure 7 *a* and *b* respectively.

Evaluation study

The contamination of Cr in tannery effluent and sludge at different distances from the discharge point is shown in

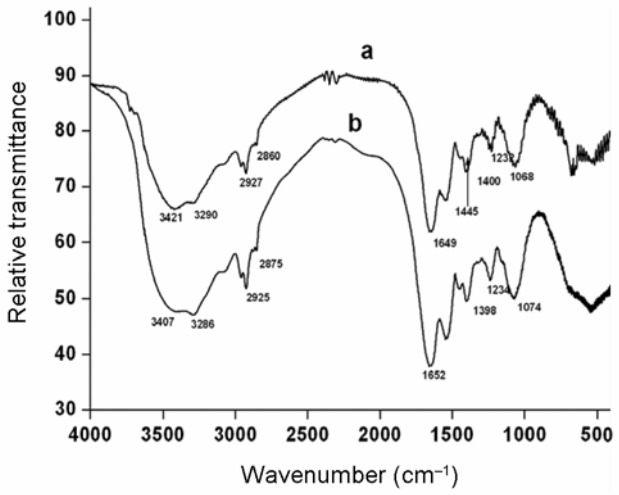


Figure 5. FTIR spectra of *P. aeruginosa* prepared in KBr disks: (a) control and (b) Cr(VI)-treated.

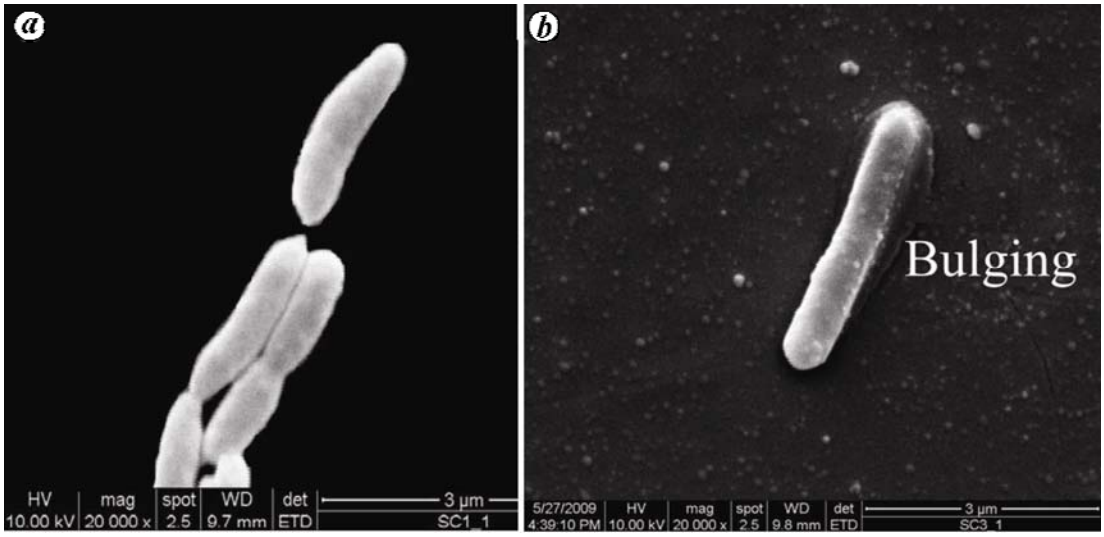


Figure 6. SEM photomicrographs of *P. aeruginosa*. (***a***) Control and (***b***) Cr(VI)-treated.

Table 1. Concentration of Cr(VI) in effluent and sludge

Distance from discharge point (km)	Concentration of Cr(VI) in effluent (mg/l)	Concentration of Cr(VI) in sludge (mg/l)
0.05	29.12	2530.08
0.25	17.00	2676.96
0.5	10.08	2800.00
0.75	8.08	2829.20

Table 2. Concentration of Cr(VI) in soil

Type of sample	Concentration of Cr(VI) at 15 cm depth (mg/l)	Concentration of Cr(VI) at 30 cm depth (mg/l)
Non-contaminated (control)	30.14	18.74
Contaminated (test sample)	106.74	74.20

Table 1. It can be observed that the concentration of Cr(VI) decreases in the effluent and increases in the sludge respectively from the last discharge point of the tannery industry. The concentration of Cr in controlled and test samples at 15 and 30 cm depth is presented in Table 2. It is evident that the concentration of chromium is more on the surface of the soil than at a depth. The pH value of the tannery effluent was found to be 6.7.

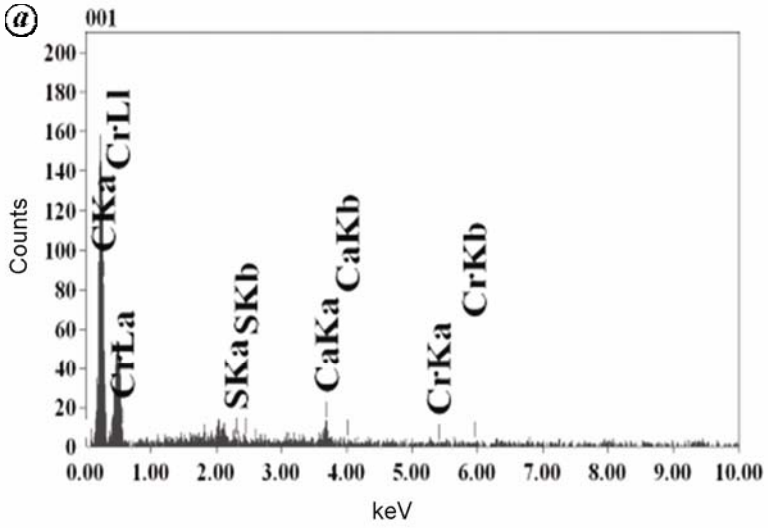
Biosorption study of the effluent

The maximum removal of Cr(VI) from tannery effluent of industrial area by *P. aeruginosa* was found to be 52.26% (Figure 1). It showed maximum absorption on the third day and remained the same till the seventh day.

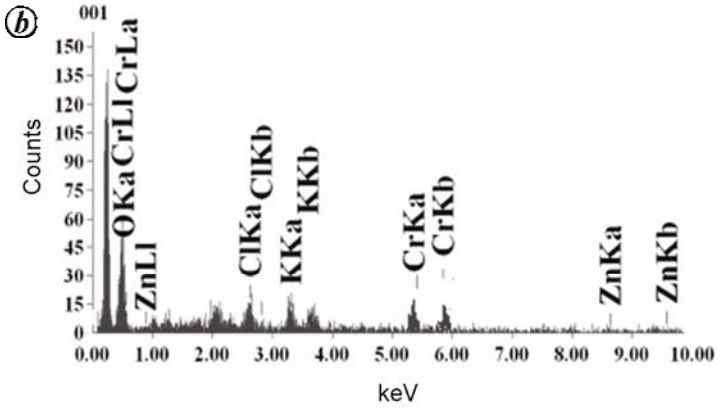
Discussion

The present study shows that the metal-removal capacity of the bacterial strain increases with time, with maximum removal on the third day being 72.56% of the initial concentration in the case of synthetic solution. From the fourth day onwards there occurred a decrease in the metal uptake capacity of the organism in the present experimental set-up. The removal efficiency was found to be marginally better with increase in the initial concentration of the metal up to 30 mg/l of Cr load.

Due to higher affinity of the sorbent for the sorbate species, the latter is attracted and bound there by different mechanisms. The process continues till equilibrium is established between the metal species sorbed by the bacteria and the metal remaining in the solution[14]. The degree of affinity between the bacteria and the metal determines its absorption. The complex structure of the bacteria implies that there are many ways for the metal to be taken by the microbial cell depending on the location of the metal to be bound. In the present study it was found that under stressed condition, i.e. in the presence of toxic heavy metal, the efficiency of absorption slowly increased with increasing time till a certain period, and then the absorption decreased and became static from the



Ele...	Cl...	(keV)	mass%	Error%	At%	Comp...	mass%	Cation	K
C K		0.277	91.48	0.81	98.23	C	91.48	0.00	91.9053
O			3.32						
S K		2.307	1.13	2.20	0.45	SO3	2.82	4.07	1.8373
Ca K		3.690	4.08	2.17	1.31	CaO	5.71	11.78	6.2574
Cr K									
Total			100.00		100.00		100.00	15.85	



Ele...	Cl...	(keV)	mass%	Error%	At%	Comp...	mass%	Cation	K
O			13.67						
Cl K		2.621	30.12	6.19	52.86	Cl	30.12	0.00	38.3980
K K		3.312	29.35	10.86	23.35	K2O	35.36	21.09	32.6857
Cr K		5.411	5.02	24.79	3.01	Cr2O3	7.34	2.72	5.2688
Zn K		8.630	21.83	59.87	20.78	ZnO	27.17	9.38	23.6476
Total			100.00		100.00		100.00	33.19	

Figure 7. EDX of *P. aeruginosa*. (***a***) Control and (***b***) Cr(VI)-treated.

fourth day onwards. This probably happens because the healthy microbial population is not maintained due to metal toxicity or other unsuitable environmental factors. A major source of chromate toxicity is the generation of reactive oxygen species (ROS) during its reduction and also that different enzymes differ in the amount of ROS they generate[29]. One of the most important parameters in the metal absorption process is pH, since it affects the solution chemistry of the metal. In aqueous waste, Cr(VI) is present either as dichromate ($Cr_2O_7^{2-}$) in acidic environment or as chromate (CrO_4^-) in alkaline environment. At pH 3, there is a decrease in the growth of the microorganisms which might have affected the absorption of the metal ions. It is interesting to note that metal uptake is

favoured at alkaline pH and it is retarded in presence of another toxic metal, cadmium. Possibly, this decreases chromium removal from tannery effluent since it contains many other toxic substances.

The CV study also confirmed the reduction and 68.75% removal of Cr(VI) by *P. aeruginosa.* IR spectroscopic study of the cell surface of *P. aeruginosa* was done to understand and interpret the mechanism involved in metal-ion binding. Shifting of the IR band to 3407 cm^{-1} with substantial decrease in intensity indicates the role of the amine group in metal binding. Changes of the band at 3290 cm^{-1} indicate the involvement of hydroxyl and amine stretching of the protein. Shift in the band to 2875 cm^{-1} can be assigned to the –CH stretching after metal binding. Decrease in the band intensity at 1652 cm^{-1} may be due to the amide group taking part in metal absorption. The shifting of the band to 1398 cm^{-1} with a decrease in intensity corresponds to O–H bending after metal binding to the carboxylate ions. Shift of band at 1074 cm^{-1} may be due to the possible role of C–O polysaccharide in the biosorption process. Amorphous substances bound to the terminals of *Pannonibacter phragmitetus* BB were found, which were presumed to be precipitates of Cr(III) compounds as a result of Cr(VI) reduction[30]. The electronegative functional groups like hydroxyl, carboxyl and phosphate on the Gram-negative bacterial surface help in the binding of these reduced products[31]. The electronegative groups inhibit chromate binding but not Cr(III) as the precipitation products of Cr(VI)[32].

The unused Cr from the tannery comes out with the effluent and gets sedimented as sludge. The upper part of soil of agricultural land is contaminated by the heavy metal due to long-term application of raw effluent and sludge. The sludge containing fleshing from tanneries is also used as food for fish in different wetlands and ponds[33]. Genotoxicity of the fish *Labeo bata* grown in the sewage-fed fish farms of East Kolkata wetlands has been detected[21]. This heavy metal is bioaccumulated in different parts of the body of vegetables and fishes, and is then transferred to higher trophic level causing a great threat, as finally it enters the human system through the food chain. These results indicate a decline in chromium concentration in effluent as a function of distance, whereas a reverse trend is observed in the case of sludge. This shows that the rate of accumulation of Cr at the top of the soil is greater compared to the deep soil and therefore, hazardous to plant biomass[34]. Raw sewage water is used for the irrigation of agricultural land and for aquaculture with special reference to pisciculture[20]. The untreated effluent of the leather tanning industry contains 29 mg/l of chromium (VI), which is much higher than the permissible limit of 0.05–1 mg/l (ref. 35). Since the Cr(VI) concentration in most of the tannery effluents was reported to be within 10 mg/l, 7.21 mg/l (ref. 17) and 247.2 μg/l (ref. 36), so this study was carried out with a maximum limit of 40 mg/l. The maximum removal of Cr(VI) from tannery effluent of industrial area by *P. aeruginosa* was found to be 52.26%. Thus bioaccumulation can be a useful alternative to the conventional system for the removal of toxic metals from industrial effluents.

Conclusion

The present study reveals the role of *P. aeruginosa* in bioremediation of Cr(VI). Maximum removal occurred on the third day at an initial concentration of 30 mg/l. Alkaline pH increases and cadmium decreases the biosorption of Cr(VI). FTIR spectrophotometry indicated the involvement of two major functional groups, the carboxyl and the amines on the cell surface, which dominated the process of biosorption. The deposition of Cr(III) by the reduction of Cr(VI), on the bacterial surfaces is suggested from SEM–EDX studies, which have also been confirmed from the CV study. The low cost, high efficiency and possibility of metal recovery envisage the potentiality of the technique for commercial exploitation.

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