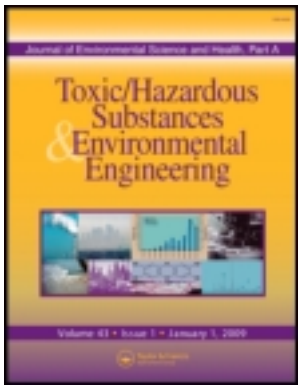

Journal of Environmental Science and Health, Part A: Toxic/Hazardous Substances and Environmental Engineering

Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/lesa20

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Published online: 10 Jan 2014.

To cite this article: Jigisha Panda & Priyabrata Sarkar (2014) Biosensing and bioremediation of Cr(VI) by cell free extract of Enterobacter aerogenes T2, Journal of Environmental Science and Health, Part A: Toxic/Hazardous Substances and Environmental Engineering, 49:5, 600-608, DOI: 10.1080/10934529.2014.859466

To link to this article: http://dx.doi.org/10.1080/10934529.2014.859466

Journal of Environmental Science and Health, Part A (2014) **49**, 600–608

ISSN: 1093-4529 (Print); 1532-4117 (Online)
DOI: 10.1080/10934529.2014.859466

Biosensing and bioremediation of Cr(VI) by cell free extract of *Enterobacter aerogenes* T2

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Hexavalent chromium or Cr(VI) enters the environment through several anthropogenic activities and it is highly toxic and carcinogenic. Hence it is required to be detected and remediated from the environment. In this study, low-cost and environment-friendly methods of biosensing and bioremediation of Cr(VI) have been proposed. Crude cell free extract (CFE) of previously isolated *Enterobacter aerogenes* T2 (GU265554; NII 1111) was prepared and exploited to develop a stable biosensor for direct estimation of Cr(VI) in waste water, by using three electrodes via cyclic voltammetry. For bioremediation studies, a homogeneous solution of commercially available sodium alginate and CFE was added dropwise in a continuously stirred calcium chloride solution. Biologically modified calcium alginate beads were produced and these were further utilized for bioremediation studies. The proposed sensor showed linear response in the range of 10–40 $\mu g\ L^{-1}$ Cr(VI) and the limit of detection was found to be 6.6 $\mu g\ L^{-1}$ Cr(VI). No interference was observed in presence of metal ions, e.g., lead, cadmium, arsenic, tin etc., except for insignificant interference with molybdenum and manganese. In bioremediation studies, modified calcium alginate beads showed encouraging removal rate 900 mg Cr(VI)/m^3 water per day with a removal efficiency of 90%, much above than reported in literature. The proposed sensing system could be a viable alternative to costly measurement procedures. Calcium alginate beads, modified with CFE of *E. aerogenes*, could be used in bioremediation of Cr(VI) since it could work in real conditions with extraordinarily high capacity.

Keywords: Chromium-resistant bacteria, *Enterobacter aerogenes* T2, biosensor, cyclic voltammetry, alginate beads, bioremediation.

Introduction

The name "Chromium" is derived from the Greek word "chroma" (χρώμα), meaning color, since it forms intensely colored compounds. It was discovered by LouisNicolas-Vauquelin in the mineral *leadchromate*) in 1797. In the Earth's crust, chromium is concentrated (> 200 mg Kg^{-1} or mg L^{-1}) in ultramafic rocks and serpentinities of ophiolite complexes that constitute ∼1% of the terrestrial landscape, primarily within populated areas of Circum-Pacific and Mediterranean region.[1] Chromium (a heavy metal belonging to group VIB in the periodic table, having atomic number 24) in its hexavalent form is widely used in industrial operations (leather tanning, electroplating, paints/pigment production, steel manufacture, etc.) because of its hardness and stability in natural environment.[2] As industrial effluents, chromium is released in the environment in concentrations as high as 250–300 mg L^{-1},[3] which is substantially above the threshold limit (50 $\mu g\ L^{-1}$) permitted by the World Health Organization (WHO). Because Cr(VI) is carcinogenic in nature, several health hazards are associated with Cr(VI) exposure in humans, including nasal irritation and ulceration, skin irritation, eardrum perforation and lung carcinoma. Furthermore, Cr(VI) can accumulate in placenta, impairing foetal development in mammals.[4] Hence, detection or sensing along with quantification and subsequent remediation of Cr(VI) from industrial effluent, drinking water is necessary.

Conventional analytical methods for estimation of chromium ions consist of spectrophotometry, atomic emission spectrometry, graphite furnace atomic absorption spectrometry, mass spectrometry and X-ray fluorescence. However, these sophisticated analytical techniques are costly and time consuming and hence cannot be employed for routine field analysis. To meet this requirement, biosensors having high sensitivity, low cost, inherent simplicity, miniaturization and rapid detection time can be used for estimation of chromium.[5]

A biosensor is a device incorporating a biological sensing element (bacterial whole-cells, enzymes, immunogens, chemoreceptors, tissue organelles, etc.) connected to a transducer. A transducer (electrodes, transistors, thermistors, optical fibres, piezoelectric crystals etc.) converts an observed change (physical or chemical) into a measurable

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Received April 15, 2013.
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signal (via an amplifier), usually an electronic signal whose magnitude is proportional to the concentration of a specific analyte. Various authors have previously reported Cr(VI)-resistant bacteria that are known to have chromate reductase enzyme (that converts toxic Cr(VI) to relatively non-toxic Cr(III))[6] and hence their extracted crude cell free extract or extracted/partially-purified enzyme could be used for the purpose of biosensing and quantification of Cr(VI) in real sample. Biosensing of chromium from bacterial crude cell free extract has not been reported before in literature.

Most of the researchers have tried to estimate Cr(VI) using indirect methods. Zeng et al.[7] determined trace amount of Cr(VI) by an inhibition based enzyme (glucose oxidase) biosensor by incorporating an electron transfer mediator, and subsequently studied the mechanism of the redox reaction. The sensor was reported to suffer from interference by mercury and silver while detecting chromium. The limit of detection of the sensor was 0.49 μg L^{-1} Cr(VI). However, sensitivity, reproducibility and cost of production of the sensor were not reported. Michel et al.[8] have developed biosensor for chromate determination with the help of cytochrome C_3 (isolated from sulphate-reducing bacterium *Desulfomicrobium norvegicum*) by virtue of its chromate reductase activity. The sensitivity of the sensor was reported to be 1.31 μA L μg^{-1} Cr(VI) with a limit of detection of 200 μg L^{-1} Cr(VI).

The sensor exhibited interference due to the presence of dissolved oxygen, bicarbonates, sulphates, molybdenum and manganese in the analyte. Reproducibility and cost of production of the sensor were not mentioned. Nepomuscene et al.[9] have developed a urease (extracted from *Dolichos uniflorus*) based biosensor for the determination of chromium ions in wastewater. The sensor had reproducibility of 40–60% for the obtained response. The limit of detection of the sensor was 20 μg L^{-1} Cr(VI). We proposed to develop a very simple electrochemical technique for direct estimation of Cr(VI) (below the permissible limit of WHO) in wastewater. A three-electrode system involving standard electrodes was envisaged for this purpose.

There exist a number of conventional methods for removing toxic chromium ion such as chemical reduction followed by precipitation, ion-exchange and *adsorption* on activated coal, alum, kaolinite and ash.[10] However, the costs of the required equipment and the operational costs are prohibitively high for large-scale treatment. Microbial uptake and reduction of toxic Cr(VI) has practical importance, because biological strategies provide green technology, which is also cost effective.[11] *Adsorption* is the most frequently applied technique due to its advantages such as variety of adsorbent materials and high efficiency at a relatively low cost.[12]

Alginate is a biodegradable, biocompatible and non-toxic biopolymer, which is known to be an efficient heavy metal scavenger. Alginates are produced by a number of organisms and are constituents in the cell walls of brown algae. It (often commercially available as sodium alginate etc.) is a linear polysaccharide of (1→4)-linked α-L-guluronate (G) and β–D-mannuronate (M) residues arranged in an irregular block-wise pattern along the linear chain. Because of its biodegradability and ability to form gels with a variety of cross-linking agents/ divalent cations (thereby producing thermally irreversible, water insoluble gels), it is widely used for metal ion removal from aqueous solutions. Alginates interact with a variety of metallic species through several mechanisms, including electrostatic and ionic interactions, covalent-like bonding, coordination, and redox reactions. This ability to bind and adsorb metals prompted scientists to use alginate for recovery of both toxic pollutants and precious metals from aqueous effluents.[13]

In the present study, sodium alginate was converted calcium alginate polymeric beads in a controlled manner using calcium chloride solution and was further modified with cell-free extract of *Enterobacter aerogenes* T2 for bioremediation of Cr(VI). Reduction of Cr(VI) to Cr(III) is a redox reaction and requires supply of electrons. In microorganisms, bacterial enzyme e.g. chromate reductase is responsible for direct reduction of chromate. *Enterobacter aerogenes* T2 is an aerobic bacterium and in aerobic organisms, this reductase activity is mostly localized in the cytosolic fraction of the cell.[14] Hence, in this study, bacterial cell-free extract was used to modify calcium alginate beads, for bioremediation of Cr(VI).

Materials and methods

Materials used

Tryptone (Hi-media, India), yeast extract (E. Merck, Germany), sodium chloride or NaCl (E. Merck), potassium dichromate or $K_2Cr_2O_7$ (E. Merck), 1,5- diphenylcarbazide (E. Merck), sodium carbonate or Na_2CO_3 (E. Merck), copper sulfate or $CuSO_4$ (E. Merck), disodium hydrogen phosphate or Na_2HPO_4 (E. Merck), sodium dihydrogen phosphate or NaH_2PO_4 (E. Merck), calcium chloride or $CaCl_2$ (E. Merck), dipotassium hydrogen phosphate or K_2HPO_4 (E. Merck), potassium dihydrogen phosphate or KH_2PO_4 (E. Merck), magnesium sulfate or $MgSO_4$ (E. Merck), potassium nitrate or KNO_3 (E. Merck), sodium potassium tartarate (E. Merck), sodium hydroxide or NaOH (E. Merck), sodium arsenate or $NaH_2AsO_4{\cdot}H_2O$ (E. Merck), sodium arsenite or $NaAsO_2$ (E. Merck), copper chloride or $CuCl_2$ (E. Merck), mercuric chloride or $HgCl_2$ (E. Merck), nickel sulphate or $NiSO_4(H_2O)_6$ (E. Merck), lead nitrate or $Pb(NO_3)_2$ (E. Merck), tin chloride or $SnCl_2$ (E. Merck), commercially available Folin-Ciocalteau reagent (E. Merck), lysozyme (3X crystallized ext. egg white extrapure for biochemistry, supplied by SRL, India), sodium salt of ethylene diamine tetra acetic acid or EDTA (E. Merck), sodium alginate (E. Merck), silver-silver chloride

(Ag-AgCl) reference electrode (MF-2052), platinum coil counter electrodes (BASi Analytical Instruments, West Lafayette, IN, USA), microtips (of 2–10 μL, 10–200 μL, up to 1000 μL capacities), gamma irradiation sterilized petriplates (Tarson, India), all glasswares (Borosil, India).

Preparation of cell-free extract (CFE) from Enterobacter aerogenes T2

Hexavalent chromium [Cr(VI)] resistant bacteria *Enterobacter aerogenes* T2 was isolated (from tannery effluent), characterized and exploited in lab-level bioremediation experiments in our previous work.[15] *E. aerogenes* T2 (NCBI GenBank Accession No.GU265554) was deposited at the *Microbial Culture Collection of National Institute for Interdisciplinary Science and Technology* (NIICC, NIIST, Kerala, India) and depository number was received as NII 1111.[16] Cell-free extract (CFE) from *Enterobacter aerogenes* T2 was prepared for modification of polymeric beads to enhance bioremediation of Cr(VI). For this purpose, Luria-Bertani (L.B.) broth medium (tryptone 10 g/L, yeast extract 5 g L^{-1}, sodium chloride 5 g L^{-1}; pH 7) having 10 mg L^{-1} Cr(VI) was prepared and inoculated with 5% inoculum of *Enterobacter aerogenes* T2 and cultured in a flask on shaker incubator for 24 h. The culture suspension was centrifuged (5415 D Eppendorf Centrifuge, Germany) at 5,000 *g* for 5 min at 4°C, supernatant discarded and pellets primarily washed with sterile normal saline (0.89% of NaCl) a couple of times and then finally washed with Tris-HCl buffer (pH 7; 100 mM).

The pellet was treated with lysozyme (10 μg mL^{-1}; pH 8.2) and EDTA (1 mM), kept at room temperature (37°C) for 20 min and then frozen at −20°C in a freezer (Blue Star, Germany).[16] The frozen pellet was thawed, and this freeze-thaw procedure was repeated three times. The suspension was sonicated (Vibra-Cel, Vibracell Sonics, Newtown, CT, USA) in ice water bath with ultra-sonic probe (amplitude of 35%) for 10 min (30 sec of sonication at 1-min interval cycles) with 750W power supply. The suspension (optical density 0.54 at 600 nm absorbance) was then centrifuged at 10,000 *g* for 15 min at 4°C. The supernatant thus formed was decanted and the CFE thus obtained was lyophilized in a freeze-drier (Cool Safe, Scanvac, Germany) and the lyophilized powder was stored at −20°C for both biosensing and bioremediation experiments.

Chromate reductase activity in CFE of Enterobacter aerogenes T2

It was observed in our previous work [15,16] that *Enterobacter aerogenes* T2 could accumulate and transform Cr(VI) to its non-toxic form Cr(III). Hence reducing activity should have been present in the cell cytosol of the isolate. To assay for Cr(VI) reduction activity in CFE of *Enterobacter aerogenes* T2, a reaction mixture (0.8 mL) was prepared with 200 μM of Cr(VI) in 50 mM phosphate buffer (Na_2HPO_4; NaH_2PO_4) (pH 7). An aliquot of 0.2 mL of CFE was added to the mixture to initiate the reaction. Reduction of Cr(VI) was measured by estimating the depletion of Cr(VI) in the reaction mixture after 30 min of incubation at 37°C.

One unit of Cr(VI) reductase activity is defined as the amount of enzyme that converts 1.0 μM Cr(VI) per min at 37°C. Cr(VI) was quantified by spectrophotometry (at 540 nm) using 1, 5- diphenylcarbazide as the complexing reagent.[17] Protein was estimated by Folin-Ciocalteau using bovine serum albumin (BSA) as the standard.[18] The extracted, lyophilized CFE suspension of *Enterobacter aerogenes* T2 was used for development of Cr(VI) biosensor. The same lyophilized CFE was also used to enhance metal uptake and reduction capacity of calcium alginate bioploymeric beads and applied for bioremediation of Cr(VI).

Instrumentation for biosensing Cr(VI)

Due to redox nature of the catalytic reaction, cyclic voltammetric sensing system has been chosen to monitor the reduction of Cr(VI) to Cr(III) with varying applied voltage. All cyclic voltammetric responses were observed by an AUTOLAB (Ecochemie B.V., The Netherlands) electrochemical analyzer (PGSTAT 12). The terminals of the working, reference and counter electrodes of the AUTOLAB analyzer were connected to the respective terminals of the electrochemical cell via standard connectors and experimental input parameters such as scan rate, potential, time of observation, etc. were supplied through general purpose electrochemical software (GPES) from a computer interfaced with the analyzer. The CFE of *Enterobacter aerogenes* T2 served as the biological detector element for detection and quantification of Cr(VI).

Cyclic voltammetry

Cyclic voltammetry was performed at an optimized scan rate of 10 mV/s (by varying scan rates from 10–50 mV/s) from −1.0 to +0.3 V range of potential versus silver-silver chloride (Ag/AgCl) reference, Platinum (Pt) coil counter and Platinum (Pt) sheet (6 mm × 7.5 mm) working electrode. As evident from enzyme assay (Fig. 1), the CFE contained chromate reductase property that reduced Cr(VI) to Cr(III), with the loss of 3 electrons. This catalytic reaction was used in the present study and Cr(VI) was measured via the following irreversible reduction reaction:

$$\text{Cr(VI)} + \text{chromate reductase enzyme (present in cell free extract)} = \text{Cr(III)} - 3e^{-} \quad (1)$$

A working solution (3 mL) of Cr(VI) was prepared in various concentrations in phosphate buffer (50 mM, pH 7) from a stock solution of Cr(VI) and 20 μL of T2-CFE was added into the working solution having bare electrodes. Scan rates affect the results in case of biological reactions.

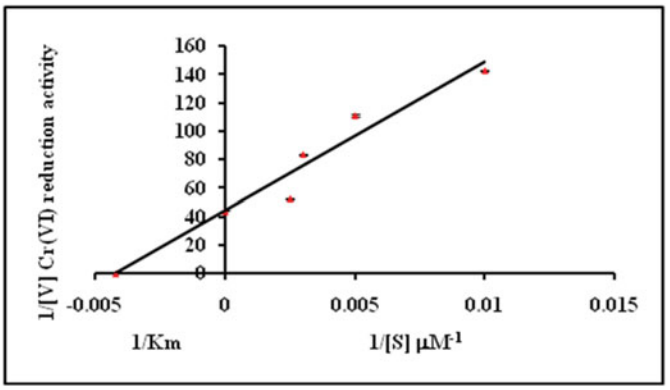

Fig. 1. Lineweaver-Burk plot for chromium reduction capacity of *Enterobacter aerogenes*.

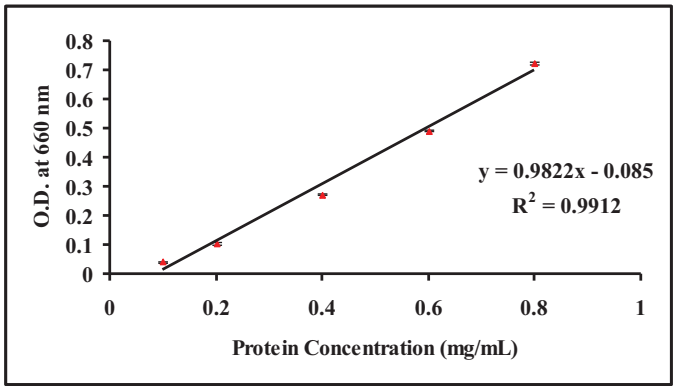

Fig. 2. Standard curve for protein estimation by the Folin–Ciocalteau method.

A very fast rate (20–50 mV/s) rendered the desired peaks invisible due to slow biological reactions and a scan rate of 10 mV/s gave the highest peak in terms of current response.[9]

All the experiments were performed in triplicates and only the representative results reported. Unknown concentration of Cr(VI) could be estimated from the equation of standard graph (plotted from varying concentrations of Cr(VI)). It might be noted that the sole biodegradable material (cell free extract) was not immobilized on the surface of the working electrode, and it was preserved in freeze-dried form in refrigerator. The sample was in contact with the CFE only at the time of experiment and thus stability of the CFE could be considered as stability of the sensor.

Various heavy metals/metalloids are generally found in tannery/sewage waste effluents. Hence, their interfering effects were investigated in presence of Cr(VI) (40 μg L^{-1}) with their concentrations two times the statutory limits laid down by World Health Organization (WHO). Alumina powder was used for polishing and sonication in 1:1 ethanol-water solution was used for cleansing of counter and working electrodes before any experiment. All the major operations were carried out at ambient temperature of 37°C.

Preparation of CFE modified calcium alginate beads

Calcium alginate beads were prepared by dispensing 3% (w/v) sodium alginate aqueous solution (along with lyophilized powder of CFE of *Enterobacter aerogenes* T2) dropwise to a continuously-stirred (at 250 rpm) 2% (w/v) $CaCl_2$ solution.[19] Beads were kept in $CaCl_2$ solution overnight and before any experiment, these were washed three times with distilled water and the excess water was absorbed on filter paper. These modified alginate beads were used to remediate 10 mg/L Cr(VI) from synthetic M2 solution[2] having K_2HPO_4 1.06 g/L, KH_2PO_4 0.2 g L^{-1}, $MgSO_4.7H_2O$ 0.5 g L^{-1}, $CaCl_2$ 0.05 g L^{-1}, KNO_3 2 g L^{-1}, NaCl 1 g L^{-1} (pH 7) and varying dosages of beads with respect to M2 media volume (1:100, 1:50, 1:25, 3:25, 5:25 or 1:5) were used in 25 mL of M2 solution. All these operations were carried out at 37°C.

Characterization of modified alginate-T2 beads

The fresh and used modified alginate beads were dried and characterized by Scanning Electron Microscopic or SEM (S3400N, Hitachi, Japan), Energy Dispersive X-ray Spectroscopic or EDS (FEI, Quanta 200, Hillsboro, OR, USA) microanalysis, X-ray Diffraction analysis or XRD (PW 3040/60, X'Pert PRO, PANanalytical, The Netherlands), Fourier Transform Infra-Red or FTIR spectroscopy (NicolateiS10, ThermoScientific, The Netherlands) to investigate how these beads interacted with Cr(VI) and subsequently remediated it via bio-adsorption and bioaccumulation. Encouraging results were obtained when compared with reported bioremediation of other strains. This process being superior in all respects, raised a strong hope for being used in the removal of Cr(VI) from industrial effluents.

Results and discussion

Assay results of Cr(VI) reduction activity of CFE

Unknown concentrations of total protein were measured by the Folin-Ciocalteau method [18] and the values were estimated from the calibration curve ($R^2 = 0.991$) for protein, using Bovine Serum Albumin as the standard protein (Fig. 2). A mixture of 2% sodium carbonate, 0.1 N NaOH solution, 1.56% copper sulfate and 2.37% sodium potassium tartarate was used along with 1N commercially available Folin-Ciocalteau reagent for this estimation procedure. From the standard curve equation, y = 0.9895x - 0.072, unknown concentrations of protein was calculated. This protein value was used to calculate V_{max}.

The kinetics of Cr(VI) reductase activity (over a concentration range i.e., 0–500 μM of Cr(VI)) fitted well with the linearized Lineweaver-Burk plot (Fig. 1), and K_m and V_{max}

values obtained from the plot were 0.023 μM Cr(VI) and 237 μmol Cr(VI)/mg protein/min, respectively. The maximal velocity (V_{max}) was found to be very high, denoting that a moderately high amount of cell free extract was required for reduction of small amount of Cr(VI) (as evident from the substrate concentration or K_m value). However, these results confirm the presence of soluble enzymatic mechanism (chromate reductase) in the cytoplasmic fraction of the crude cell-free extract of the strain.

Development of Cr(VI) biosensor with CFE of Enterobacter aerogenes T2

Evaluation of optimum pH. pH of buffer was varied over a range of 5–9 and cyclic voltammetry was performed to find out optimum pH at which the sensor could show highest sensitivity to 40 μg/L of Cr(VI) in working solution. The absolute values of current response for pH 5, 6, 7, 8 were found to be 22.3, 25.6, 25.2, 20.4 μA respectively and for pH 9 it was 18.8 μA. Hence, the required optimum pH was evaluated to be 6, at which the highest peak for response current was observed at ambient temperature of 37°C, and all subsequent experiments were therefore conducted at pH 6.0.

Cyclic voltammogram and construction of calibration curve. Cyclic voltammetry for standard solutions of Cr(VI) was performed as described in section 2 and are presented in Figure 3. It could be seen that the reduction peak increased steadily with increase in concentration of Cr(VI). The response in current was evaluated from these peaks after baseline correction and plotted against concentration of Cr(VI) (10–40 μg L^{-1}) to construct the calibration curve (Fig. 4) for the sensory system. The corresponding responses for 10, 20, 30, 40 μg L^{-1} were found to be 25.6, 33, 37, 43.3 μA, respectively. Current responses were calculated in terms of peak heights as obtained from the cyclic voltammogram. The peak height was calculated from the voltammogram (with the help of GPES), by drawing a baseline from the rising point of the peak upto the decreasing point and then a perpendicular was drawn upon this baseline from the highest point of the peak. This perpendicular line gave the value of the peak height expressed as current response. This peak appeared due to the irreversible reduction of Cr(VI) to Cr(III) via loss of three electrons (catalyzed by chromate reductase enzyme present in CFE) in the working solution.

An unknown concentration of Cr(VI) in tannery effluent (with suitable dilution) was detected by cyclic voltammetry and the concentration of the Cr(VI) in the effluent was calculated from the equation of standard graph and folds of dilution. This was found to be 2.1 mg/L of Cr(VI), which was in accordance with colorimetric estimation. Five tannery effluent samples were collected from various industrial waste sites for quantification by our proposed sensor as well as for comparing with regular colorimetric procedure (by DPC). Only one representative data has been reported here.

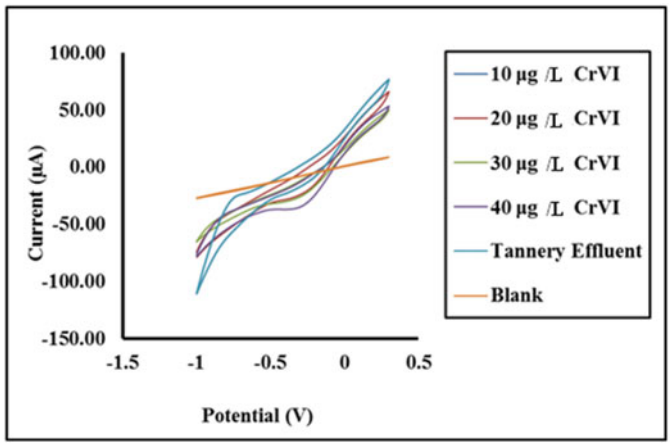

Fig. 3. Cyclic voltammogram of various concentrations of Cr(VI) (scan rate = 10 mV/s, potential = −1.0 to +0.3 V; the corresponding responses for 10, 20, 30, 40 μg L^{-1} being 150, 320, 476.66, 580 μA, respectively).

When efficiency of this sensor was compared to the regular colorimetric procedure (by DPC), the latter was found to have faulty estimations of known sample concentrations by a range of −3 to +0.1, whereas our sensor estimated perfectly (Fig. 5). All the major operations were carried out at ambient temperature of 37°C.

It might be noted that the colorimetric estimation by standard protocol of APHA or any other commercially available detection kit has detection capacity limited to 100 μg L^{-1} only, whereas detection limit of the present method is lower.

Study on interference. Tests were conducted (Fig. 6) to see if there was any interference of various metals/metalloids by measuring the response in presence of the two-fold concentrations of threshold limits of interfering substances such as arsenic (AsIII and AsV, 20 μg L^{-1}), cadmium (Cd II, 1 mg L^{-1}), copper (Cu II, 2.6 mg L^{-1}), mercury (Hg II,

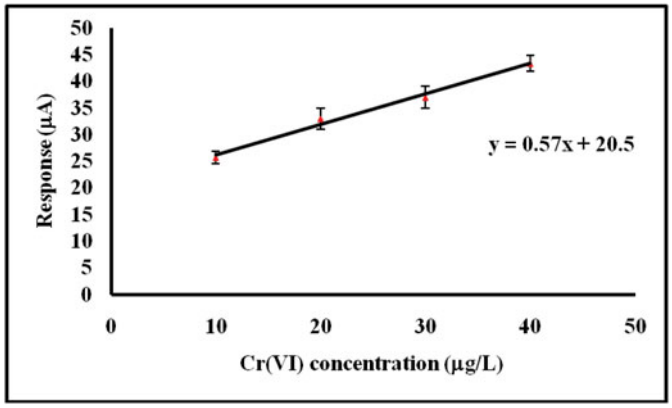

Fig. 4. Calibration curve for various standard concentrations of Cr(VI) (10–40 μg L^{-1}), $n = 3$.

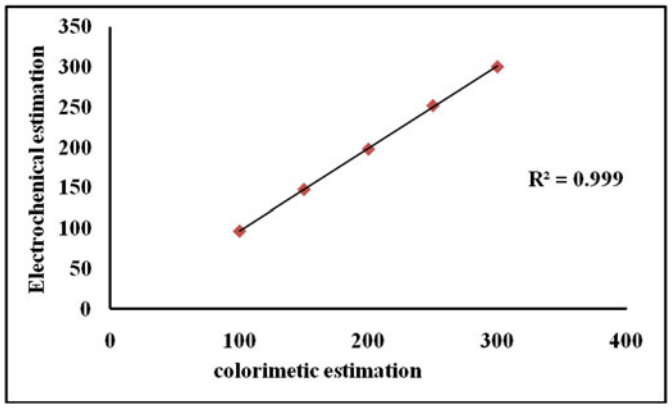

Fig. 5. Detection and estimation by developed biosensor (electrochemical sensing) versus colorimetric estimation by D.P.C at 540 nm.

40 μg L^{-1}), nickel (Ni II, 1 mg L^{-1}), lead (Pb II, 200 μg L^{-1}), tin (Sn II, 500 μg L^{-1}) and sulfate (SO_4^{2-}, 1 mg L^{-1}), which might be present in the chromium-contaminated effluent samples. No interference was observed in presence of these various ions due to the probability of CFE having chromate reductase enzyme specific for Cr(VI) reduction only. Though there were some interference due to presence of molybdenum (Mo) and manganese (Mn), these were not significant and not included in Figure 6. The interference studies of molybdenum and manganese were conducted with a concentration of 600 μg L^{-1} and 20 mg L^{-1}, respectively (being double their threshold values) and the interference was 3–5% of that obtained using Cr(VI).

Comparisons with reported Cr(VI) biosensors. Most of the reported works (Table 1) used indirect quantifications of Cr(VI) based on inhibition method in urea-urease interaction,[9] glucose-glucose oxidase (GOx) interaction,[7] etc.

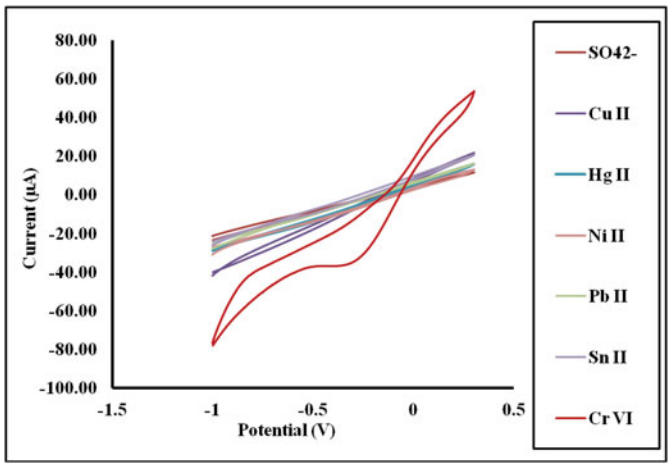

Fig. 6. Cyclic voltammogram of various interfering ions while detecting Cr(VI).

The ranges of detection of these procedures were much above WHO's prescribed limit of 50 μg L^{-1} Cr(VI) in water. One direct estimation by cytC3 enzyme-based [7] sensor from sulphate reducing bacteria could detect in mg/L concentration only. Our linear range of detection (Fig. 4) was below WHO's prescribed limit, i.e., 10–40 μg L^{-1} of Cr(VI). Limit of blank or LoB is the highest apparent analyte concentration expected to be found when replicates of a blank sample containing no analyte are tested. It was found to be 0.0412 [$LoB = mean_{blank} + 1.645(SD_{blank}) = 0.0327 + 1.6645(0.00513)$]. Limit of detection, LoD (a calculated value of lowest analyte concentration at which detection is feasible), was found to be 6.6 μg L^{-1} Cr(VI) [$3.3(SD/S) = 3.3 \times 1.14/0.57$]. As evident from the linear standard curve (Fig. 4), the Limit of quantification, or LoQ (a value in the form of smallest concentration of a measure that can be reliably measured by an analytical procedure), was found to be 20 μg L^{-1} Cr(VI)[20][$10(SD/S) = 10 \times 1.14/0.57$].

The sensitivity of the sensor was found (slope of the standard calibration curve) to be 0.57 μA L μg^{-1} Cr(VI) and the reproducibility (coefficient of variance) was 3.20 to 6.25% of the obtained response. It is well understood that a laboratory setup with electrochemical analyzer and electrode-system would be required for measurement of Cr(VI). Excluding the fixed cost for equipment and electrodes (for innumerable measurements), the running cost for each measurement will be less than a cent ($0.01). No interference was observed in the presence of metal ions, e.g., lead, cadmium, arsenic, tin etc. Thus the proposed sensing system could be a viable alternative to costly measurement procedures.

Bioremediation of Cr(VI) by alginate-T2 beads

The modified alginate beads were used to remediate 10 mg L^{-1} Cr(VI) from synthetic M2 solution having K_2HPO_4 1.06 g L^{-1}, KH_2PO_4 0.2 g L^{-1}, $MgSO_4.7H_2O$ 0.5 g L^{-1}, $CaCl_2$ 0.05 g L^{-1}, KNO_3 2 g L^{-1}, NaCl 1 g L^{-1} (pH 7) and it was observed that the optimum beads dosage to amount of water could be 1:5 (other dosages showed slower remedial rates, e.g., the rates were 270, 350, 580, and 750 mg Cr(VI)/m^3/day for dosages 1:100, 1:50, 1:25 and 3:25 respectively). This optimized ratio was used in a volume of 500 mL M2 media stirred continuously at room temperature (37°C) and at 300 rpm. The following equation was used to calculate the% removal of Cr(VI) = $(C_o - C_i)/C_o \times 100$ where C_o = initial concentration, C_i = final concentration. The initial concentration (C_o) was the amount of Cr(VI) added to the medium before starting the remedial process and final concentration (C_i) of the Cr(VI) was the amount left in the medium after a certain time of performing the remedial process. The residual Cr(VI) in the medium was quantified by spectrophotometry (at 540 nm) using 1, 5- diphenylcarbazide as the complexing reagent. The average *Remedial Rate (R)* was found to be 900 mg Cr(VI)/m^3 of water/day [R = (amount of chromium removed in mg

Table 1. Comparative study on performance of microbial biosensors.

Serial no.	*Sensor*	*Features*
1.	Biosensor based on inhibitory effect of Cr(VI) on urease - urea reaction (Nepomuscene et al.[9])	Modified sol-gel technique used; Indirect estimation; Linear response range: 10–50 mg L^{-1} Cr(VI)
2.	Biosensor based on inhibitory effect of Cr(VI) on GOx-glucose reaction (Zeng et al.[7])	Electropolymerized aniline membrane used; Ferrocene used as electron transfer mediator; Indirect estimation; Linear response range: 0.49 μg L^{-1} to 8.05 mg L^{-1} Cr(VI)
3.	Cytochrome C3-based biosensor (Michel et al.[8])	Glassy carbon electrode used as working electrode; Cellulose dialysis membrane used for enzyme entrapment; Direct estimation; Limit of Detection: 200 μg L^{-1}
4.	Biosensor developed by our protocol	Platinum electrode used as working electrode; Direct estimation; Linear response range: 10–40 μg L^{-1}; Limit of Detection: 6.6 μg L^{-1} Cr(VI)

from 1 m^3 of water)/number of days = (9 mg L^{-1}) × 1000 L/m^3/10 days = 900] as evident from the graph (Fig. 7).

It was observed that the remediation rate (R) was very high, up to 20 h, moderate from 20–120 h and low from 120–240 h (Fig. 7). The rapid phase involved physical adsorption, i.e., *biosorption* and slow phase involved active metabolism-dependent transport of metal into the bacterial cells or *bioaccumulation*. These modified alginate beads were used to remediate 10 mg L^{-1} Cr(VI) from synthetic M2 solution having K^+, H^+, PO_4^{3-}, Mg^{2+}, SO_4^{2-}, Ca^{2+}, Cl^-, Na^+ and nitrate ions, which were probably co-adsorbed with chromate ions to some extent but that did not diminish the efficiency of the bioremedial capacity of the beads. This was proved by their moderately high bioremedial rate of 900 mg Cr(VI)/m^3 of water/day. Though there might be some inhibition of coexisting ions, bioremediation supersedes it. Our strain gave encouraging% removal (99.7%)[16] when compared to the strains isolated by Shakoori et al.,[21] i.e., CMB Cr1 remediating Cr(VI) up to 87.17% and by Zhiguo et al.[22] (CSCr-3) up to 80%. Thus the proposed process might be useful for removal of chromium from contaminated water.

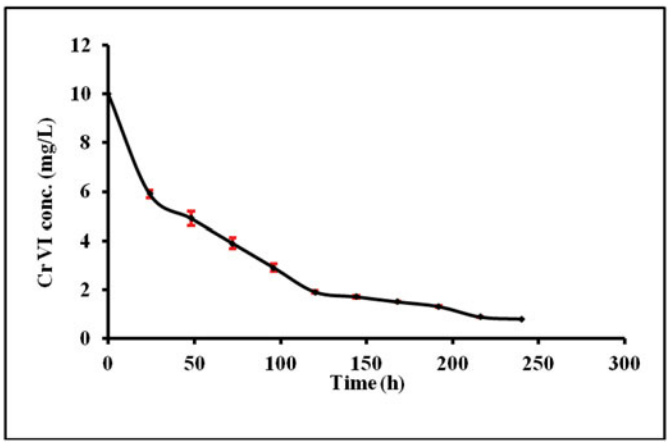

Fig. 7. Bioremediation of 10 mg L^{-1} Cr(VI) by alginate-T2 beads.

Characterization of modified alginate-T2 beads used for bioremediation

Scanning Electron Microscopic or SEM (S3400N, Hitachi, Japan) images of freshly prepared beads (Fig. 8a) show its surface topography (formed by the change in intensity of the secondary electrons emitted from the surface of beads, due to incident electron beam) and give a good representation of the 3D structure of the beads. The shape of the prepared alginate beads was found to be predominantly ellipsoidal spheres, with 3–5 mm average diameter. In Energy Dispersive X-ray Spectroscopic or EDS (FEI, Quanta 200) microanalysis, X-rays emitted from the beads due to incident electron beams gave elemental compositions of the samples.

Figure 8b depicts presence of potassium (K), phosphorus (P), magnesium (Mg), sodium (Na), sulfur (S) and chromium Cr. This proves that chromium was accumulated in the calcium alginate beads modified with CFE of T2, whose peak was not present in the fresh beads (not shown within the figure). Phase identification of samples was carried out with the help of X-ray diffraction analysis or XRD (PW 3040/60, X'Pert PRO, PANanalytical, The Netherlands) at 40 kV voltage and 30 mA current and calibrated with 0.15406 nm radiation, λ; α a standard silicon sample, using Ni-filtered Cu K at a rate of 4° per minute, using 2θ value range of 0 to 55° (along the x-axis) versus intensity count (in a.u.) along the y-axis. The peaks of used beads at 20.6488°, 28.1830° of 2θ values (as seen in the rounded area of the combined graph, Fig. 8c) had intensities of 86.20, 60.33 a.u. counts, respectively, which were absent in the graph of fresh beads.

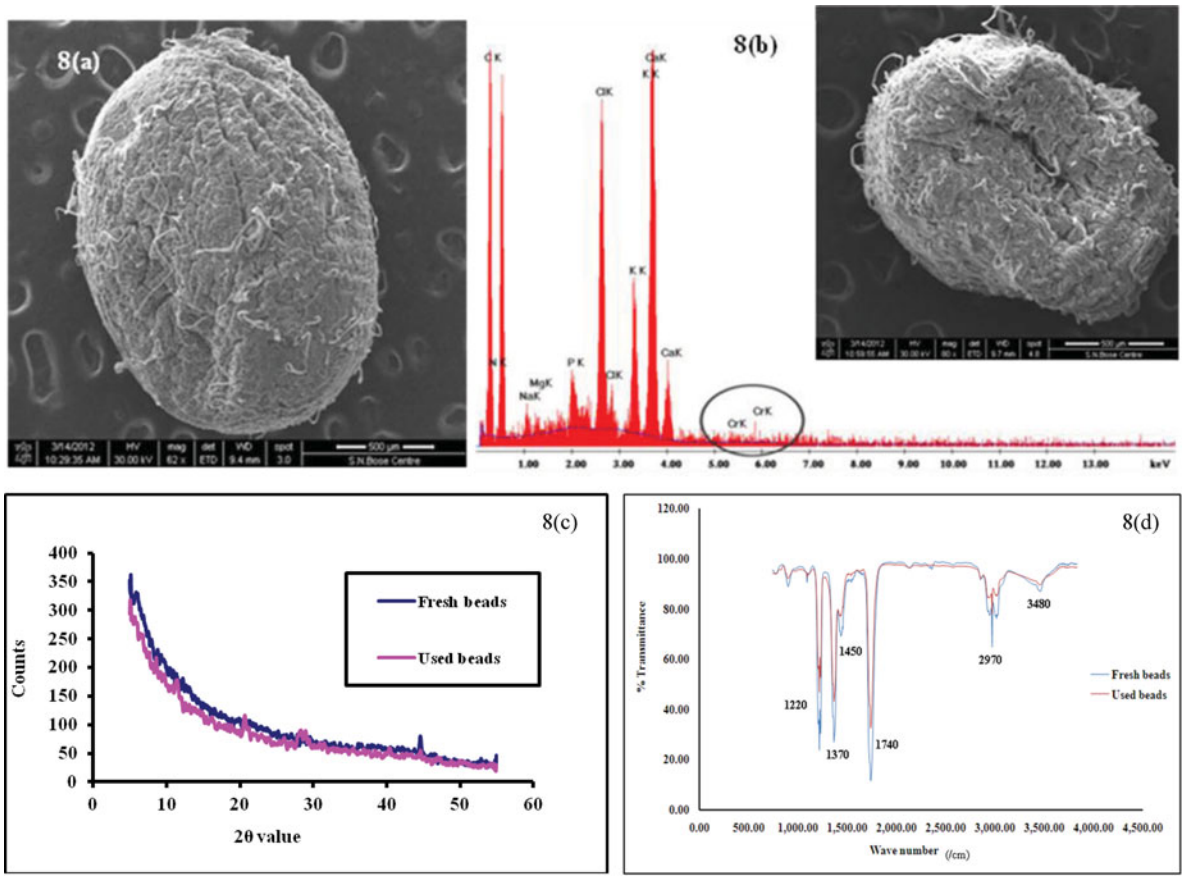

Fig. 8. Various characterizations of alginate-T2 beads: (a) Scanning Electron Microscopic image of fresh whole alginate-T2 beads; resolution = 500 μm; (b) Energy Dispersive X-ray Spectroscopic microanalysis of used beads showing Cr(VI) accumulation; (c) X-Ray Diffraction graphs of used and unused beads; (d) Fourier Transform Infrared spectra of fresh and used alginate-T2 beads.

The formation of crystalline end product could be Cr(III), since chromate anions should not bind to electronegative surface functional groups found on Gram-negative bacteria *E. aerogenes* T2 [14,15], i.e., bioremediation might have occurred primarily by biosorption, followed by bioaccumulation and finally bioconversion of toxic amorphous Cr(VI) to relatively non-toxic, crystalline Cr(III). Fourier Transform Infra-Red or FTIR spectroscopy (Nicolet S10, ThermoScientific, The Netherlands) of powdered form of calcium alginate beads (modified with lyophilized powder of *Enterobacter aerogenes* T2 bacteria)—both fresh and used was performed to acquire information regarding wave number changes (peak-shifting) of the functional groups (Fig. 8d). Spectra were obtained from 4200 to 700 cm^{-1}.

For both samples, peaks were found at 1220, 1370, 1450, 1740, 2970 and 3480 cm^{-1}. The peaks found for used beads were diminished in intensity than those of unused beads, probably due to interaction of functional groups with Cr(VI). The broad adsorption peak (ranging from 3200 upto 3700 cm^{-1}) at 3480 cm^{-1} revealed vibrational band of O-H, probably indicating the presence of surface hydroxyl groups.[19] Amide (-NH) groups might also be present within this range, as a part of bacterial protein-biomass, and were responsible for biosorption of Cr(VI) in the loaded biosorbents. Similarly, $>CH_2$ was indicated by peak at 2970 cm^{-1}. Peaks at 1220, 1370, 1450 and 1740 cm^{-1} were due to carboxylate (−COO), carbonyl (−CO) and carbon-carbon double (C=C) bonds. The peak broadening at 1450 cm^{-1} might be due to symmetric stretches of carboxylate (−COO) group, present in the adsorbent, which formed O−Cr−O (of $Cr_2O_7^{2-}$ ions).

Conclusion

A very simple technique was developed for estimation of Cr(VI) in wastewater using three- electrode system, via cyclic voltammetry. This sensor proved to be a sensitive one, since it could predict chromium concentration in the concentration range below the threshold limit of WHO, i.e., 50 μg L^{-1}. The sensor was low cost, reliable, sensitive

(detection limit 6.6 μg L^{-1}) and had the advantage of working at normal environmental conditions. No interference was observed in the presence of metal ions, e.g., lead, cadmium, arsenic, tin, etc. Moreover, the freeze-dried CFE could be used at any time and no modification of WE was required. Thus the proposed sensing system could be a viable alternative to costly measurement procedures.

The bioremediation study with CFE-modified alginate beads could achieve high bioremediation rate with 90% removal of Cr(VI). Thus, the proposed process might be useful for removal of chromium from contaminated water. The comparison with published literature confirmed highest remediation rate in the proposed process. The characterization experiments of the fresh and used CFE-modified calcium alginate beads revealed that the remediation could be by biosorption and subsequent reduction of Cr(VI).

Funding

The author JP gratefully acknowledges the fellowship provided by the University Grants Commission (UGC), India. The author PS acknowledges a research fund from the UGC.

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