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Long-term chromate reduction by immobilized fungus in continuous column

Rashmi Sanghi*, Ashish Srivastava

302 Southern Laboratories, Facility for Ecological and Analytical Testing, Indian Institute of Technology Kanpur, Kanpur, UP 208016, India

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ABSTRACT

The immobilized fungus *Coriolus versicolor* was examined in a continuous fixed bed column for long-term Cr(VI) reduction at its physiological pH. The effects of operating parameters like flow rate, glucose concentration in the influent feed, COD, initial Cr(VI) concentration on the Cr(VI) reduction were investigated. Increase in the inlet Cr(VI) concentration and flow rate through the column led to a higher breakthrough of the Cr(VI) ions in the effluent. Cr(VI) reduction rate increased with increase in initial Cr(VI) concentration of up to 60 mg/L and thereafter showed a gradual decline. A Fourier transform infrared spectra were employed to elucidate the possible biosorption mechanism as well. The readiness of the thiol group of the fungal protein to interact with the Cr(VI) ion in addition to its strong reducing ability makes it a particularly important entity in the metabolism of Cr(VI). The possible role of thiol in the Cr(VI) reduction via the formation of Cr(VI) thioester is discussed. The study clearly exhibits the usage of live fungus for the long-term continuous removal of Cr(VI) as well as recovery of the metal ions from wastewater.

1. Introduction

Chromium has been widely recognized as a toxic mutagen [1] and a carcinogen yet is an important metal, which is used in a variety of industrial applications. Chromium is a metal that can exist in oxidation states from −2 to +6, The trivalent oxidation state is the most stable form of chromium In biological systems, chromium is naturally found in its trivalent state at very variable levels, whereas the hexavalent form is generally a derivative of man's activities. Cr(VI) tends to associate with oxygen generating the powerful oxidants chromate (CrO_4^{2-}) and dichromate ($Cr_2O_7^{2-}$). The biological effects of chromium are highly dependent on the oxidation state. Derivatives of Cr(III) are water insoluble compared to Cr(VI) derivative compounds that are highly soluble [2].

Requirements of large quantity of chemicals or energy can be a limitation for the application of physicochemical methods for removing Cr(VI). Removal of heavy metal ions using biosorption could be a promising technology and has received more and more attention in recent years [3,4,5]. Microorganisms, which are capable of transforming metals from one oxidation state to another, facilitate detoxification and/or the removal of chromium, and have thus received recognition [6]. In the concept of biosorption, several chemical processes may be involved, such as bioaccumulation, bioadsorption, precipitation by H_2S production, ion exchange, and covalent binding with the biosorptive sites, including carboxyl, hydroxyl, sulphydryl, amino and phosphate groups of the microorganisms [7].

The surfaces of fungal cells appear to act as ion exchange resins [8]. From the quantitative point of view, the surface sorption usually can contribute the larger proportion to total metal uptake, and thus binding to cell walls appears to be the most significant mechanism of sorption. Since it is energy independent, it occurs in both living and dead microbial biomass, including fungal mycelium [9].

The aim of the present work is to assess the long-term performance of thiol containing live fungus *Coriolus versicolor* for the continuous reduction of Cr(VI) in upflow fixed bed columns in a growth-supportive medium at physiological pH. The effects of some operating parameters, such as inlet Cr(VI) concentration, media composition, and flow rate were examined and optimized for the long-term Cr(VI)-reduction performance in the column. In this study we have used white-rot fungus *C. versicolor* as a bioreductant since it has a high growth rate and can grow under a variety of environmental conditions including low pH, high pollutant concentration. The possible mechanism for the reduction of Cr(VI) to Cr(III) by the fungus is also discussed.

2. Materials and methods

2.1. Reagents

A stock solution of Cr(VI) was prepared containing 18.6736 g $K_2Cr_2O_7$ per litre of deionized water. Sterilized stock Cr(VI) solution was added to sterile medium to a desired concentration of Cr(VI) with minimal dilution of the medium. All chemicals used were of AR grade.

* Corresponding author. Fax: +91 512 2597866.
E-mail address: rsanghi@gmail.com (R. Sanghi).

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2.2. Microorganism and media

A white-rot fungal strain, *Coriolus versicolor*, was obtained from Institute of Microbial Technology, Chandigarh, India. The strain was maintained at 4 °C on malt agar slants. The liquid growth medium used for inoculating the fungus, consisted of 10 g/L glucose and 5 g/L malt extract (S.D Fine Chemicals, Mumbai). The growth medium used was always autoclaved (WidWo Cat. AVD 500 horizontal autoclave) at 15 psi for 30 min and cooled to room temperature before use. The inlet pH of feed containing Cr(VI) was 7.2. No adjustments in pH were made. In case of continuous flow column study, the media prepared every third day, had the following ingredients per litre of tap water: glucose 2 g, malt extract 1 g, peptone 0.5 g, KH_2PO_4 2 g, $MgSO_4$ 1.023 g, $CaCl_2$ 0.1325 g and $MnCl_2$ 0.099 g.

2.3. Immobilization of fungus in column

The columns were made of borosilicate glass with 2.2 cm ID, 31 cm height, and 22.5 cm bed length. The glass column was packed with ceramic beads on which the fungus was immobilized. To avoid channel effects the Cr(VI) solutions (10–80 mg/L) were continuously pumped upward through the column by a peristaltic pump (Mclin). The flow rates of the solutions were varied from 20 to 50 mL/h. The hydraulic residence time for the column reactor was 54.8 min.

Initially the columns were conditioned by recycling fungus-containing growth media for a week followed by the feeding of fresh growth media until the COD reduction reached a steady state. Thereafter the influent growth medium was supplied with known concentration of metal solution. The conditions for maximum Cr(VI) reduction like influent Cr(VI) concentration, flow rate and glucose concentration in growth medium, were investigated and then subsequent analyses were performed under these optimized condition. Column effluent samples were collected at regular time intervals and COD, pH, Cr(total), Cr(VI), Cr(III), concentration were monitored. The COD measurements were made by closed reflux method according to the standard APHA procedure [12]. All experiments were performed in duplicates at room temperature.

2.4. Instrumental analysis

Infrared (IR) spectra were recorded on a BRUCKER, VERTEX-70, and Infrared spectrophotometer making KBr pellets in reflectance mode. The pH of solutions was measured using a Digital pH-meter (MK VI Systronic). Spectrophotometric analysis was carried out on a Perkin Elmer, lambda-40 UV-VIS spectrophotometer with a 1 cm path length. For the SEM studies, samples of fungal biomass were coated under vacuum with a thin layer of gold and examined by scanning electron microscopy [FEI (QANTA 200)] at 10–17.5 kV with a tilt angle 45°.

2.5. Chromium analysis

The analysis of Cr(VI) in solution was carried out by the diphenylcarbazide colorimetric method [10]. Diphenylcarbazide forms a red-violet complex selectively with Cr(VI). The color was fully developed after 15 min and the sample solutions were analysed using the UV-VIS spectrophotometer and the absorbance of the color was measured at 540 nm. The total chromium concentration was determined by oxidizing any trivalent chromium with potassium permanganate, followed by analysis as hexavalent chromium. Cr(III) was determined from the difference between total chromium and Cr(VI) concentrations. The instrument response was periodically checked with metal ion standard solutions.

Table 1
Reduction of Cr(VI) ion at different flow rates (initial metal ion concentration 30 mg/L).

Flow rate (mL/h)	mg Cr(VI) reduced/L h
20	11.51 ± 0.4
25	11.13 ± 0.5
30	11.55 ± 0.5
35	11.72 ± 0.7
40	8.88 ± 0.5
45	6.67 ± 0.4
50	5.41 ± 0.2

2.6. Glucose analysis

The determination of reducing sugar was done using 3,5-dinitrosalicylic acid method [11]. The effect of glucose concentration on % Cr(VI) reduction was studied by varying the concentration from 0 to 5 g/L. Once the glucose concentration was optimized to 2 g/L, further experiments were carried out at this concentration only. The glucose consumed with time was also monitored and equated with the Cr(VI) reduction with time.

3. Results and discussion

3.1. Monitoring of column conditions

The columns were run in duplicates. The results revealed the presence of both Cr(VI) and Cr(III) in the effluent solution after sorption of Cr(VI) on the biomass whereas on the fungal biomass, only Cr(III) was found to be present. A decrease in pH from 7.2 to 3.2 was observed during each Cr(VI) reduction cycle. In the first hour itself the pH reduced to 4.6 and thereafter the pH decrease was slow. Reduction in pH of medium is possibly due to the accumulation of organic acid metabolites [12]. With the decrease in solution pH, protonation of amine sites (NH_2) of fungus increased favouring more electrostatic attraction of negative $HCrO_4^-$ ion yielding high removal of Cr(VI). Perusals of the literature had also reported the reduction of Cr(VI) to Cr(III) at acidic pH along with protons been consumed supported by Eq. (1) [13]:

$$HCrO_4^- + 7H^+ + 3e^- \rightarrow Cr^{3+} + 4H_2O \quad E^0 = 1.33\,V \tag{1}$$

Variation of flow rate though the column (shown in Table 1) revealed that maximum reduction occurs up to the range of 35 mL/h, above this flow rate chromate reduction decreases gradually due to lesser contact time between the metal ions and the biomass.

The reduction rate of Cr(VI) was very fast initially; about 65% of the starting Cr(VI) (30 mg/L) was reduced within the first 2.8 h of the reaction. However, the residual concentration of Cr(VI) reached its minimum in 24 h. This rapid rate of Cr uptake by the immobilized fungus has a significant practical importance for applications in small reactor volumes, thus ensuring efficiency as well as economy.

3.2. Effect of glucose concentration on Cr(VI) reduction

Glucose concentration was varied from 0 to 5 g/L and it was observed that the reduction rate significantly increased from 0 to 2 g/L but became almost constant after 2 g/L. In the absence of glucose only sorption process takes place which results in poor reduction (Table 2). A glucose concentration of 2 g/L and above in media resulted in the best Cr(VI) reduction. These results suggested that glucose plays a vital role of carbon source in the reduction process. A comparison of quantitative uptake of glucose reveals that the rate and extent of metal uptake is significantly enhanced by the presence of glucose (Fig. 1). This enhanced metal removal capability may be related to an increase in the availability of energy and cel-

Table 2
Reduction of Cr(VI) ion at different glucose concentrations in growth medium (initial metal ion concentration 30 mg/L, flow rate 35 mL/h).

Glucose (g/L)	mg Cr(VI) reduced/L h
0	5.4 ± 0.5
0.5	9.6 ± 0.2
2	12.6 ± 0.2
3	9.3 ± 0.5
4	9.2 ± 0.4
5	8.4 ± 0.4

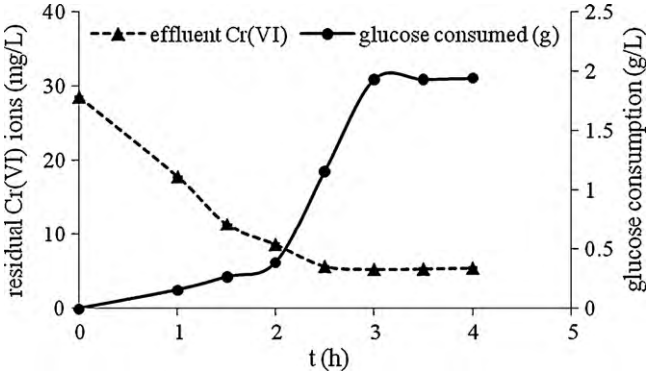

Fig. 1. Time-course profiles for Cr(VI) reduction and glucose consumption by fungal biomass.

lular activities, including metal adsorption activity, in the presence of glucose.

The glucose used was almost completely consumed in 3.2 h evidencing the role of glucose in the chromium reduction which also reached its maximum in the first 2.8 h and thereafter became constant as in Fig. 1. Clearly the glucose consumption rate was proportional to the Cr(VI) reduction. But even after the glucose is almost fully consumed in 3.2 h, the chromium reduction remains constant till a fresh Cr(VI) is supplied, evidencing the role of fungal enzymes in the whole metabolic process.

3.3. Effect of Cr(VI) concentration on Cr(VI) reduction

To evaluate the effect of the influent Cr(VI) concentration on its reduction by the fungus in the continuous packed bed column, the influent concentration was slowly increased in the range of 10–80 mg/L under the same operating conditions.

The reduction rate for Cr(VI) increased apparently from 4.5 to 16.6 mg Cr(VI)/(L h) in 2.4 h when the initial Cr(VI) concentration was increased from 10 to 30 mg/L respectively, and remained constant thereafter upto 80 mg/L initial Cr(VI) concentration (Fig. 2). Maximum 96% removal at 10 mg/L and 42% removal at 80 mg/L

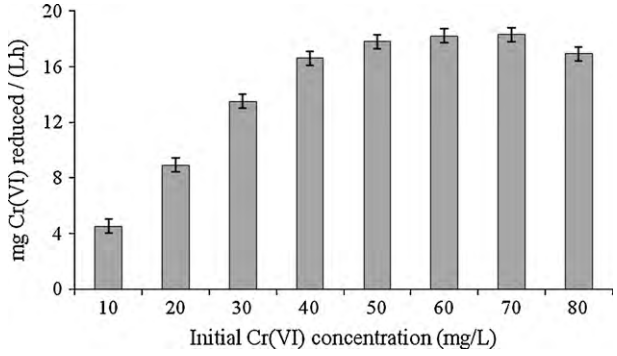

Fig. 2. Effect of initial Cr(VI) concentration on Cr(VI) reduction (flow rate and glucose concentrations were 35 mL/h and 2 g/L respectively).

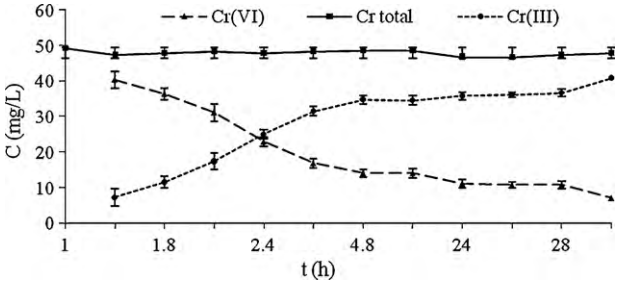

Fig. 3. Change in Cr(VI), Cr(III) and total Cr concentration in effluent with time in one cycle (*C*: concentration, *t*: time).

were achieved. The % removal of Cr(VI) decreased on increasing influent metal ion concentration further. At low influent concentration (10–30 mg/L), all Cr(VI) ions present in solution could interact with binding sites present on fungal cell wall and hence the high Cr(VI) removal. However with increase in concentration (40–80 mg/L) due to saturation of binding site on the fungal mass, some Cr(VI) ions are left unabsorbed in solution leading to decreased reduction rate. The influent Cr(VI) concentration was not further increased as it could inhibit Cr(VI) uptake and growth of fungus.

Taking into consideration that the stable forms of chromium are the trivalent and the hexavalent, it seems most likely that the C. *versicolor* strain was capable of transforming the highly toxic and soluble Cr(VI) to the much less toxic and less mobile trivalent form. Clearly, the decrease in the Cr(VI) led to a simultaneous increase of Cr(III) in solution (Fig. 3), however total chromium in solution remained virtually constant and chromium did not accumulate in the fungal biomass. On the fungal surface chromium was found to be present only in the trivalent state.

3.4. Fourier transform infrared (FTIR) spectra analysis

To elucidate the biosorption of Cr(VI), the FTIR spectra of chromium-loaded biomass were compared with the control (protonated biomass) (Fig. 4). The broad adsorption peak around 3400 cm^{-1} is indicative of the existence of OH and NH stretching, thus showing the presence of hydroxyl and amine groups on the fungal cell wall. Medium absorption band at 2921 cm^{-1} could be assigned to –CH stretching vibration of C–CH_3, meanwhile the peak at 1380 cm^{-1} could be attributed to –CH_3 wagging (umbrella deformation). The absorption peaks at 1647 cm^{-1} and around 1537 cm^{-1} representing the amide I and amide II bands of amide bond, respectively, are due to functional groups in the protein peptide bond [14,15]. The peaks at 2337 cm^{-1}, 1230 cm^{-1}, 1058 cm^{-1} and 540 cm^{-1} represented the NH_2 stretch, C–O–C stretch, C–O stretch and C–C–O–C bend.

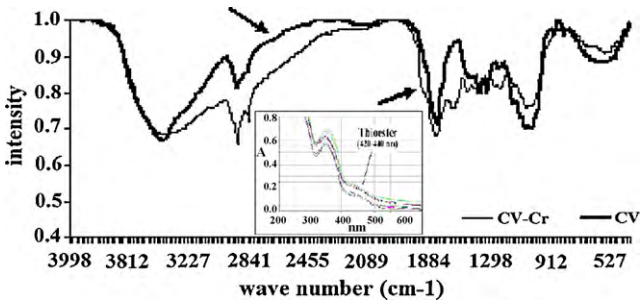

Fig. 4. FTIR spectra of — control (CV) and — Cr(VI)-adsorbed biomass (CV–Cr). In the inset is the UV–vis spectra of Cr (VI) containing fungal effluent showing thioester formation in 420–440 nm range.

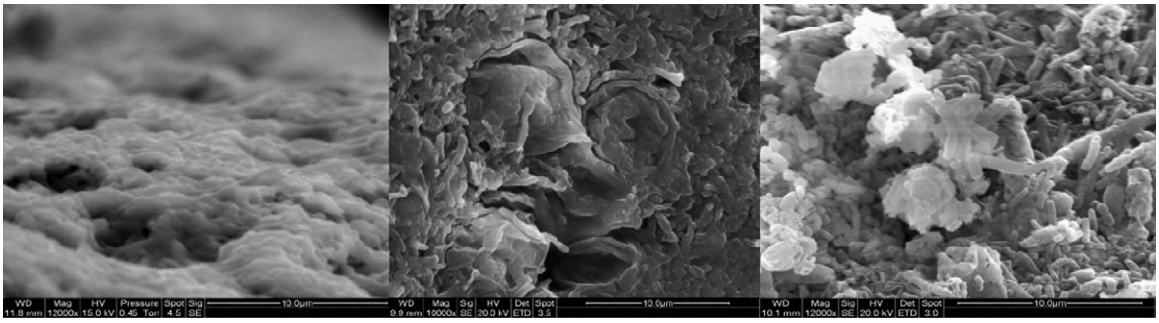

Fig. 5. SEM micrographs of newly grown fungus, immobilized fungus in column and Chromium laden fungus.

The changes in the fungal FTIR after coming in contact with Cr(VI) indicated some chemical interaction between the metal ions and functional groups in cell wall. The peaks at 3400 cm^{-1}, 2920 cm^{-1} and 2335 cm^{-1} from the chromium-loaded biomass were less intense compared with the control. Results suggested that the protonated amide is involved in the irreversible binding of chromium during Cr(VI) biosorption.

The readiness of the thiol group to interact with the chromate ion in addition to its strong reducing ability makes it a particularly important entity in the metabolism of Cr(V1) [15,16]. This is clearly evidenced by the disappearance of the peak at 2576 cm^{-1} indicating depletion of free S–H groups and involvement of the thiol groups in the reduction process. The reduction of Cr(VI) species proceeds via the formation of transient Cr(VI) thioester as evident by the appearance of a shoulder (CO of the carboxylic groups of amino acids) at 1726 cm^{-1}.

The reaction between Cr(VI) and thiol protein proceeds with the formation of a Cr(VI)-thioester intermediate, which then decays to the Cr(III) product with time. The presence of thioester is further evidenced by the absorbance band with its characteristic low absorbance at 420–440 nm [17] in the absorption spectrum (Fig. 4) which decreases with time. With increase in time, the absorption band at 370 nm representing Cr(VI) decreased along with the thioester band.

3.5. SEM analysis

Assessment of morphological changes in response to chromium accumulated on the fungal strain, *Coriolus versicolor* was performed by Scanning Electron Microscopy (SEM) analysis. SEM analysis of fungal mycelium (*C. versicolor*) was shown at 48 h incubation without Cr(VI) and Cr(VI) loaded biomass (Fig. 5). The biosorbed chromium on the fungal surface was Cr(III) as Cr(VI) is reduced to Cr(III) which in turn is free to bind to these sites. The fungal biomass of the column was much denser showing heavy chromium accumulation on its surface.

3.6. Mechanism

The molecular mechanisms of heavy metals accumulation by white-rot fungi have not been studied extensively. In living cells the sorption mechanisms include both metabolism dependent and independent processes. Metabolism independent uptake process essentially involves cell surface binding through ionic and chemical interaction, while dependent process deals with the binding of both the surfaces followed by intracellular accumulation [18]. The uptake of Cr(VI) may include biosorption, diminished accumulation, precipitation and reduction of Cr(VI) to Cr(III). Metal ions are bound to the surface of fungal cell wall, composed mostly of polysaccharides. Cr(VI) toxicity is believed to be caused by the negatively charged chromate oxyanion, which can be easily transported into microbial cells by the sulphate transport system. Inside the cell, chromate can react with a variety of low and high molecular weight cellular components in order to produce reactive intermediates to finally form Cr(III) species. Most of the studies of Cr(VI) reduction in the literature have been carried out in aqueous solution using several reductants, either at highly acidic pH values (1–1.7) using HClO, or at physiological pH using chelating buffer such as Tris–HCl [19]. One electron or two electron reduction paths have been proposed in the literature for the reaction of Cr(VI) with thiols [15] ascorbic acid [16]. The reaction of thiols and ascorbic acid with Cr(VI) were carried out without adjusting the pH externally and it was found that the intrinsic pH of the reaction mixture lies in the range of 6.8–7.5 during the progress of the reaction.

The literature also reveals that thiol could play an important role in reduction of Cr(VI) at neutral pH by forming thioester species [20]. The Cr(VI)–thiol reactions can be written schematically as:

$$[Cr_2O_7]^{2-} + 2RSH \rightarrow 2[RSCrO_3]^- + H_2O$$

where $RSCrO_3^-$ is the relatively stable Cr(VI)–thioester intermediate (RSH standing for the corresponding thiol). The –SH of the thiol is an important group in the reaction of Cr(VI) with biological reductants. The reductant reacts with Cr(VI) at a significant rate only when protonated or that the reductant must supply Cr(VI) with a proton. The stoichiometry for reaction of Cr(VI) with thiol was ~3 thiol molecules per Cr(VI) and can be represented as:

$$nH^+ + 2Cr^{VI} + 6RSH \rightarrow 2Cr^{III} + 3RSSR$$

The Cr(III) species formed gets trapped in the fungal matrix and the ligating amino groups are responsible for its chelation. The formation of Cr(VI) thioester intermediate was apparent (Fig. 4) with increase in absorbance at 420–440 nm observed immediately after initiating the reaction. Change in absorbance at 350 nm with time during the reaction of Cr(VI) with fungal thiols has been described in Fig. 4. The factors controlling both the initial reactivity of chromate and the generation of reactive intermediate are, as yet, far from fully clarified.

The columns are continuously running for more than a year now under physiological conditions in growth-supportive medium. The natural weather conditions ranged from 15 °C in winters to 44 °C in summers. Other than providing the growth medium, no alterations were made in the running of the columns under natural conditions. Though there is no way to quantify the fungal mass, the consistency in COD reduction is indicative of the sustainable growth and viability of the fungus. Initially, the Cr(III) in the form of green precipitate sticking on the fungal biomass was visible in the column. With continuous spiking of Cr(VI), after about three months the column was saturated with the dirty green Cr(III) precipitate. It could be easily collected and recovered from the effluent.

4. Conclusions

Use of *C. versicolor* for the very long-term Cr(VI) reduction ability in continuous upflow column represents a very potential successful strategy to bioremediate Cr(VI) toxic wastewaters in their natural habitat with extremes of weather conditions. Due to the surface immobilization of fungal hyphal biomass, the solute could easily pass through the highly porous matrix of ceramic beads for reaching the functional groups of the biomass. The main purpose of this paper is to establish the optimization parameters for the reduction of Cr(VI) with *C. versicolor* such that the process is sustainable for a very long term. The long-term potential of the column packed with fungal biomass for Cr(VI) detoxification was demonstrated very effectively. The fungus clearly is able to thrive, grow and successfully reduce toxic Cr(VI) to its less toxic form even under repeated exposure of Cr(VI) for more than a year. The results indicated that the packed columns with the immobilized living cells is more convenient in operation and economic in treatment compared with traditional methods as it could resolve the problem of blockage as well as recovery of the metal ions from wastewater.

Acknowledgments

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