

Biosorption Potency of *Aspergillus niger* for Removal of Chromium (VI)

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Abstract. *Aspergillus niger* isolated from soil and effluent of leather tanning mills had higher activity to remove chromium. The potency of *Aspergillus niger* was evaluated in shake flask culture by absorption of chromium at pH 6 and temperature 30°C. The results of the study indicated removal of more than 75% chromium by *Aspergillus niger* determined by diphenylcarbazide colorimetric assay and atomic absorption spectrophotometry after 7 days. Study of microbial Cr(VI) reduction and identification of reduction intermediates has been hindered by the lack of analytical techniques that can identify the oxidation state with subcellular spatial resolution. Therefore, removal of chromium was further substantiated by transmission electron microscopy (TEM), scanning electron microscopy (SEM), and energy-dispersive X-ray spectroscopy (EDX), which indicated an accumulation of chromium in the fungal mycelium.

Heavy metals are one of the major water pollutants present in industrial effluent [12]. Water pollution resulting from an increased concentration of heavy metals is causing serious ecological problems in many parts of the world. Heavy metals are generally deposited in liver, muscles, kidneys, spleen, skin, bone, and soft tissues of human beings [16]. Pollution by chromium is of considerable concern as the metal has found widespread use in electroplating, leather tanning, metal finishing, and chromate preparation. Chromium occurs in the aqueous system as both trivalent and hexavalent forms, the latter being of particular concern because of its greater toxicity [17, 18]. Tanneries are mainly responsible for the release of huge amounts of chromium (as chromium sulfate), which has a higher tendency to convert into Cr(VI) in the effluent. The concentration of chromium in soil and effluent of leather tanning mills varies from 500 to 7000 ppm [19]. It is difficult to remove higher amounts of chromium present in tannery and industrial effluent. Physicochemical methods have been practiced for several decades for the removal of toxic heavy metals from industrial wastewaters. These include precipitation of metals such as hydroxide, car-

bonates, and sulfides, adsorption on the activated carbon, and use of ion exchange resin and membrane separation process. Biotransformation and biosorption are emerging technologies that utilize the potential of microorganisms to transform or adsorb metal. Microbial viability is essential for biotransformation as these reactions are enzyme mediated [4, 9, 13]. Genetically potential microorganisms including fungi can be isolated from highly contaminated sites due to their continuous enrichment and adaptation in polluted environments [1]. Generally, metal ions are converted into insoluble form by specific enzyme-mediated reactions and are removed from the aqueous phase. Cr(VI) (chromate) is a widespread environmental contaminant [5, 11]. The study of microbial Cr(VI) reduction such as identification of reduced intermediates, has been hindered by the lack of analytical techniques that can identify oxidation states with subcellular spatial resolution [17]. The most common method for measuring Cr(VI) reduced in bacterial cultures is the diphenylcarbazide colorimetric assay in which the Cr(VI) concentration is determined by oxidation products of the diphenylcarbazide reagent. However, this bulk technique cannot provide the sub-micron-scale information necessary for understanding microbial reduction processes. Transmission electron microscopy (TEM) and scanning electron microscopy (SEM) have sufficient resolution to study the spatial

relationship between cells and reduction products, as well as their chemistry [16]. In addition energy-dispersive X-ray spectroscopy (EDX) has been used to identify elements present in reduced products associated with microorganisms and wetland plants [15, 23]. Therefore, fungal strains are isolated from the chromium contaminated sites and methods are optimized for analysis of effective removal of chromium for evaluation of bioremediation of chromium from contaminated sites.

Materials and Methods

Sampling site and isolation of fungal strain. The sediment sample and liquid effluent (1:10w/v) were collected from three sites of a main channel of tanneries located in Jazmau, Kanpur, Uttar Pradesh, India towards Lucknow Road. Samples were stored at 4°C in a refrigerator. A fungus strain (*Aspergillus niger*) isolated from sediment flooded with the tannery effluent was used for removal of chromium (VI) as described by Srivastava and Thakur [20]. Fungal inoculum in the form of pellets prepared for removal of chromium was grown and cultured on potato dextrose agar plates. Mycelia discs (1 cm diameter) were cut from the active growth zone of fungal mycelia of the agar plates. Erlenmeyer flasks containing potato dextrose broth and streptopenicillin (100 ppm) were taken and inoculated. The flasks were incubated at 30°C for 4 days under shaking condition in orbital shaker. After growth of mycelium, it was filtered by cheesecloth and placed on Petri plates. Water evaporated, and fungal discs prepared by cutting in approximately 2.0-mm size were used in the removal of chromium.

Culture condition and removal of chromium. The fungal isolate, *Aspergillus niger*, grew in minimal salt medium containing (g/L): Na₂HPO₄·2H₂O, 7.8; KH₂PO₄, 6.8; MgSO₄, 0.2; Fe (CH₃COO)₄NH₄, 0.01; Ca (NO₃)₂·4H₂O, 0.05; NaNO₃, 0.085; and trace element solution, 1 mL/L [20, 21]. The salt of potassium chromate (500 ppm) was used as a source of hexavalent chromium. The pH was maintained at 6.0 incubated at 30°C in a rotatory shaker for 7 days. Chromium was measured at an interval of 0, 1, 3, 5, and 7 days. On the basis of chromium analysis, percentage removal was studied by the isolates along with the control.

Analysis of chromium from the effluent. A sample from each experiment flask(s) was drawn on the following 0, 1, 3, 5, and 7th days. The sample was centrifuged at 10,000 rpm for 10 min at 4°C. The presence of chromium was determined in supernatant and fungal mycelium. Supernatant (50 mL of sample) was mixed with concentrated HNO₃ (5 mL) and boiling chips. The content was boiled and evaporated to 16–20 mL on a hot plate. Concentrated HCl (5 mL) was added and boiled again. The solution was boiled until the sample became clear and brownish fumes were evident. Finally, it was cooled and diluted up to 50 mL with distilled water. An aliquot of this solution was used for determination of the concentration of total chromium with the help of a flame atomic absorption spectrophotometer (GBC, Avanta–Sigma) [8].

The presence of chromium in fungal biomass was determined by transferring the pellet in the known weight sterile crucible, and pellets were dried overnight at 60°C in the oven. The weight of dried pellets was calculated, which indicated a dry ash form of fungal mycelium. Dry ash of fungal mycelium (1 g) was taken and crushed in a pestle and mortar. The ground material was placed in a conical flask (50 mL) and 20 mL of acid mixture (Tri acid mixture HNO₃: H₂SO₄: HClO₃ 10:1:4) was added. The content of the flask was mixed properly. Initially, the

flask was placed on a slow heating hot plate in a digestion chamber and then the flask was heated at a higher temperature until the production of red NO₂ fumes ceased. The contents were further evaporated until the volume was reduced to about 3 to 5 mL but not to dryness. The completion of digestion was confirmed when the liquid became colourless and, finally, chromium was determined by the method of Greenberg et al. [8]. The concentration of chromium ions (ppm) in the respective samples, pellets as well as supernatants, was analyzed with the help of a flame atomic absorption spectrophotometer at 357.9 nm wavelength and having an optimum working range 0.2 to 10 ppm. The flame type used was air-acetylene (oxidizing) with a lamp current of 4 mA.

Scanning electron microscope (sem) and energy dispersive X-ray spectrometer (EDX). Cells fixed as described above were smeared over the coverslip coated with poly-L-lysine for 30 min in wet condition [1]. The specimen was washed with buffer, dehydrated in a series of ethanol-water solution (30, 50, 70, and 90% ethanol, 5 min each), and critical point dried under a CO₂ atmosphere for 20 min. Mounting was done on aluminium stubs, and cells were coated with 90-Å thick gold-palladium coating in a polaron Sc 7640 sputter coater (VG Microtech, East Sussex, TN22, England) for 30 min. Coated cells were viewed at 15 kV with scanning electron microscopy (Leo Electron Microscopy Ltd., Cambridge). Dx4 Prime Energy Dispersive X-ray spectrometer (EDAX) was performed at 20 kV for confirmation of the chromium accumulation in the fungal mycelium.

Transmission electron microscopy (TEM). Transmission electron microscopy was performed in fungal cells fixed in glutaraldehyde (1% solution) and paraformaldehyde (2%) buffered with sodium phosphate buffer saline (0.15M, pH 6.8). Fixation was for 12–18 hours at 4°C temperature, after which the cells were washed in fresh buffer, and post-fixed for 2 hours in osmium tetroxide (1%) in the same buffer at 4°C. After several washes in buffer, the specimens were dehydrated in graded acetone solutions and embedded in CY 212 araldite. Ultrathin sections of 60–80 nm thickness were cut using an ultracut E, Ultramicrotome, and the sections were stained in alcoholic uranyl acetate (10 min) and lead citrate (10 min) before examining the grids in a transmission electron microscope (Morgagni 268 D TEM, Fei Company, The Netherlands) operated at 60–80 kV [6].

Results and Discussion

Isolation and characterization of fungal strain. The serial dilution technique was adopted for the isolation of fungal strains from the tannery effluent enriched soil and sediment. The colonies of fungus that appeared on a PDA agar plate were isolated and further purified on a potato dextrose agar plate by the process of spot inoculation. Five isolates (FK1 to FK5) were isolated and tested for their ability for bioaccumulation of chromium from the mineral salt medium (MSM) containing potassium chromate solution [20]. *Aspergillus niger* has been used for the bioaccumulation of chromium in batch culture containing MSM and potassium chromate. The results presented in Figure 1 show the maximum removal of chromium, 90% and 86% (68% and 55%) at 50 ppm and 100 ppm chromate at the 7th and 3rd days, respectively. In the case of the 500 ppm chromate, the removal of

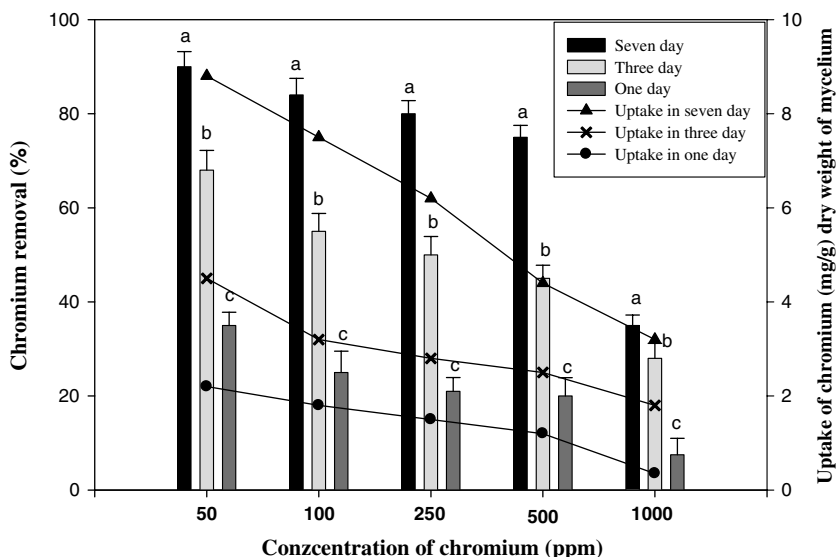


Fig. 1. Percent removal and uptake of chromium (potassium chromate) by *Aspergillus niger*. Within each group, values not followed by the same letter are significantly different at $P \leq 0.05$ (error bars are standard deviation).

chromate was 75% and 45% at the 7th and 3rd days, respectively. The data from this study indicated a significant removal of chromium up to 500 ppm.

The bioaccumulation of chromium that was determined after digestion of fungal mycelium is presented in Figure 1. Uptake of chromium in fungal mycelium was maximum at a low concentration of chromate at 50 ppm, i.e., 8.9 and 4.5 mg/g dry wt of mycelium, while it was minimum at 1000 ppm chromate, i.e., 3.3 and 1.8 mg/g dry wt of mycelium at the 7th and 3rd day, respectively. Despite the toxicity of the effluent, the microbial flora of tannery wastes was relatively rich with the *Aspergillus niger* group.

Uptake of heavy metal ions by fungal microorganisms may offer an alternative method for their removal from wastewater. Living and dead cells of fungi are able to remove heavy metal ions from aqueous solutions [24, 25]. For such an application, fungal biomass would have to be easily available in substantial quantities. Fungi are used in a variety of industrial fermentation processes, which could serve as an economic and constant supply source of biomass for the removal of metal ions. Fungi can also be easily grown in substantial amounts using unsophisticated fermentation techniques and inexpensive growth media. They are highly robust and tolerant to contaminants; therefore, a fungal biomass could serve as an economical means for removal/recovery of metal ions from aqueous solutions [24]. Fungi belonging to the genera *Rhizopus* and *Penicillium* have already been studied as a potential biomass for the removal of heavy metals from aqueous solution [22, 24, 24]. But little is known about the removal of heavy metals such as lead, cadmium, copper, and nickel from aqueous solutions using the fungus. Huang and

Huang demonstrated that *Aspergillus oryzae* can remove cadmium and copper ions from aqueous solution [10].

Evaluation of chromium absorption by scanning electron microscopy and energy dispersive X-ray spectrometry. Assessment of morphological changes in response to chromium accumulated in the fungal strain, *Aspergillus niger* and quantification of chromium within fungal strains was performed by Scanning Electron Microscopy (SEM) and Energy Dispersive X-ray Analysis (EDX) analysis. Scanning Electron Microscopy (SEM) analysis of fungi (*Aspergillus niger*) was shown at 48 h incubation without chromate exposure (Fig. 2a). The hyphae of fungi were cylindrical, septate, and branched and there was no peak of chromate at 5.4 keV after 48 h incubation determined by Energy Dispersive X-ray Analysis (EDX) (Fig. 2a). However, when chromate (500 ppm) containing mycelium was applied for SEM-EDX, it revealed that chromium was uniformly bound to the fungal mycelium and a higher chromate absorption together with flocculation in mycelium was observed (Fig. 2b). The biosorbed chromate was assumed to be Cr(III), as Cr(VI) is reduced to Cr(III) that is free to bind to these sites and, once bound, acts as a template for further heterogeneous nucleation and crystal growth [3]. Quantification of chromium within fungal strains performed by SEM and EDX analysis gave confirmation of chromate accumulation within the fungal strain *Aspergillus niger*.

Evaluation of chromium absorption by transmission electron microscopy. Transmission Electron Microscopy (TEM) was performed for the identification of chromate accumulation within cells of microorganisms.

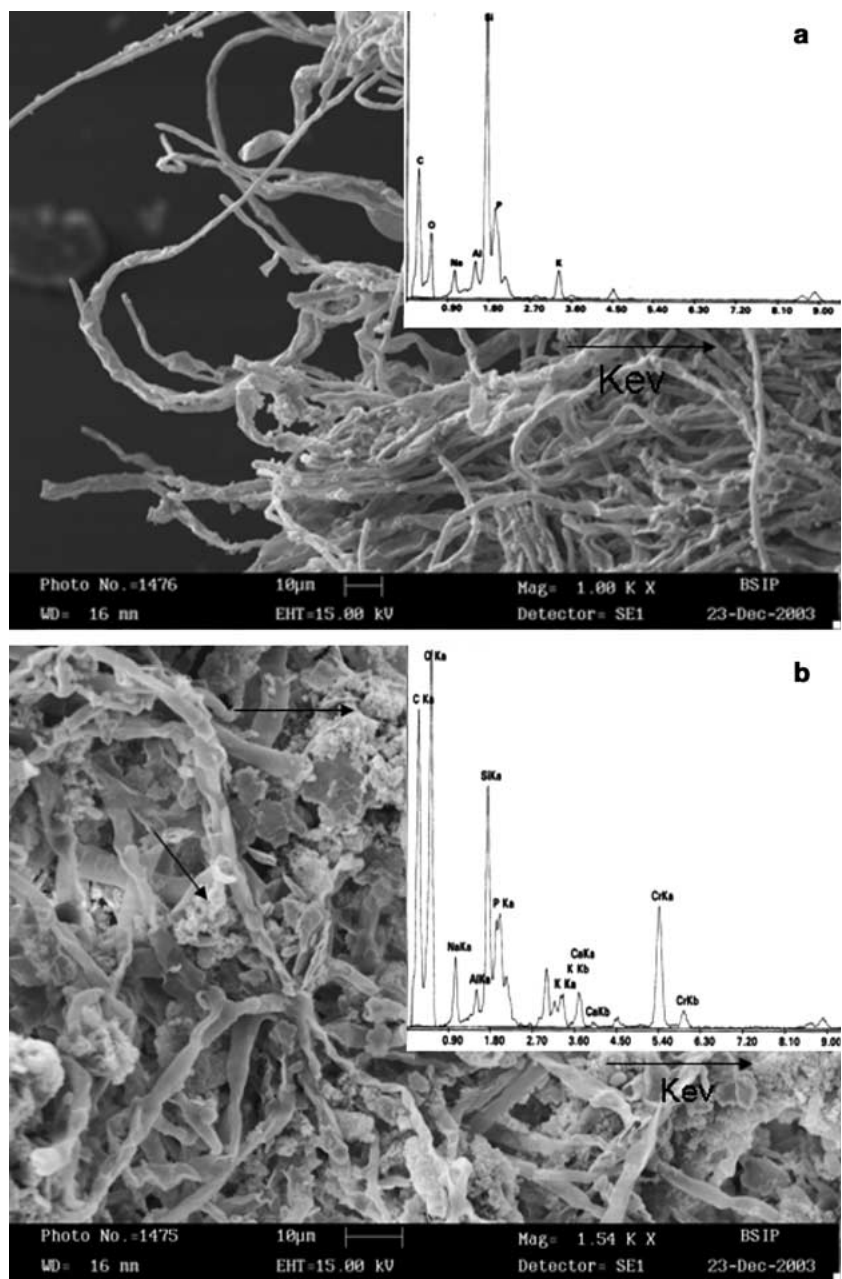


Fig. 2. Scanning electron microscopy and energy dispersive X-ray analysis of fungal mycelium, *Aspergillus niger* (a), and biosorption of chromium in the mycelium (b).

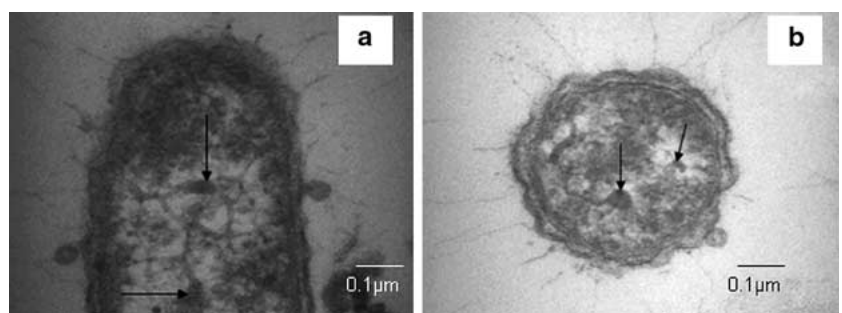


Fig. 3. Transmission electron microscopy of chromium (potassium chromate) absorption in *Aspergillus niger*. (a) indicated longitudinal section and (b) transverse section of fungi.

Electron micrograph of fungi showed an electron-dense area around the cell wall. It was observed that the cell wall of fungi was the main part of the cell involved in the accumulation of chromate from the solution. TEM revealed that Cr(III) was uniformly adsorbed to the surface mycelium and precipitated. At higher chromate concentrations, the density of chromate sequestered on the cell wall and also in the cell interior, indicating its (chromate) penetration into the cell. This was revealed by the electron-dense area within the cell (Fig. 3a and 3b).

The mechanisms of metal binding are not well understood due to the complex nature of the microbial biomass, which is not readily amenable to instrumental analysis [11]. However, localization of metals has been carried out using electron microscopic and X-ray energy dispersive analysis studies. X-ray photoelectron spectroscopy for chemical analysis studies is a relatively new technique for determining the binding energy of electrons in atoms/molecules, which depends on the distribution of valence charges and thus gives information about the oxidation state of an atom/ion [11]. Electron microscopic observation carried out by Mullen et al. revealed the presence of Ag^{2+} as discrete particles at or near the cell wall of both gram-positive and gram-negative bacteria and the presence of silver was confirmed by energy dispersive X-ray analysis (EDX) [16]. Large particles containing gold were localized in *Sargassum natans* cells by EDX carried out in conjunction with scanning electron microscopy [12].

Leusch et al., using the X-ray photoelectron spectroscopy, observed that iron was present in two oxidation states when brown seaweed *Sargassum fluitans* was exposed to Fe^{2+} , while only Fe^{3+} was present when the biomass was exposed to ferric ions [13]. TEM and EDX analysis revealed that Cr(VI) is reduced to Cr(III) that was uniformly distributed and adsorbed to the surface of the cells initially, and then it was bound and precipitated inside the cells in the form of a hydroxyl and carboxyl group [15]. The binding of chromate to the surface of the cell wall was due to the presence of electronegative functional groups, i.e., carboxyl, hydroxyl, and phosphoryl. McLean and Beveridge have reported that *Pseudomonas* (CRB5) reduced toxic chromate [Cr(VI)] to insoluble Cr(III) precipitation under aerobic and anaerobic conditions [15]. DeLeo and Ehrlich reported that formation of Cr(III) precipitates was not evident in batch and continuous cultures of *P. fluorescens* LB300 whereas *Pseudomonas chromatophilica*, *P. ambigua*, and *Pseudomonas maltophilia* all induce the formation of precipitates [7]. TEM and EDS revealed that Cr(III) was adsorbed and precipitated on the surface of the

microbial cells and finally dispersed inside the cells in bulk amount. Microorganisms have excellent nucleation sites for grained mineral formation due to their high surface area and volume ratio and the presence of an electronegative charge [2]. Surface functional groups (e.g., carboxyl, phosphoryl, and hydroxyl) play a major role in the bioaccumulation of metals and significantly reduced chromate, which is toxic. The information available so far on the biosorption of heavy metal including chromium is limited to a few fungal strains. The data of this study may be useful for the decontamination of soil and effluent polluted by chromium. The reclamation of chromium-contaminated soil has yet to be investigated in the industrial environment. More technical and feasibility data are required for a better understanding and effective use of the fungal bioabsorption and protective mechanisms of *Aspergillus niger* to detoxify the effects of chromium. The detoxification of chromium by *Aspergillus niger* may be mediated either by the enzymatic antioxidant defense system such as peroxidase, catalase, and ascorbate peroxide or phytochelatin, which sequester metals inside the cells by binding through thiol coordination and limit damage to the metabolic processes by reducing cytotoxic free metal ions [14, 22].

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