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BIOREMEDIATION AND BIOCONVERSION OF CHROMIUM AND PENTACHLOROPHENOL IN TANNERY EFFLUENT BY MICROORGANISMS

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ABSTRACT

Chromium sulphate Cr(III) and pentachlorophenol (PCP) widely used as tanning and biocide respectively in leather preparation are highly toxic and recalcitrant. Biosorption of chromium by *Aspergillus niger* FIST1 was evaluated, and process parameters were optimized in presence of carbon, nitrogen, carbon:nitrogen, pH, temperature, different concentration of chromium. The potency of *Acinetobacter* sp.IST3 for degradation of pentachlorophenol was determined by HPLC after formation of tetrachlrohydroquinone and chlrorohydroquinone. Bioremediation of chromium and PCP were tested in bioreactors in sequential way where bacterium treated effluent subsequently treated by fungus showed reduction of chromium (82%) and PCP (85%) after 120 hrs. Biosorption of chromium was determined by transmission electron microscopy (TEM), scanning electron microscopy (SEM) and energy-dispersive X-ray spectroscopy (EDX). Recovery of chromium in tannery effluent was initially obtained by CaO:MgO (2:1) and pH adjusted to 7.0-7.6, and chromium absorbed by fungus and bacteria, was further used for tanning of the leather. Results of the study indicated that quality of the leather prepared by absorbed chromium of fungus and bacteria was better than chromium recovered by CaO:MgO determined by SEM. In view of above results 'tanning cake' was prepared which is better substitute of raw chromium used for tanning in leather mills.

Keywords: Bioconversion; Bioremediation; Chromium; Pentachlorophenol; Tannery

1. INTRODUCTION

Tanneries are responsible for environmental pollution as they use huge amounts of chromium sulphate Cr(III) and pentachlorophenol (PCP) in the leather tanning processes and biocides to inhibit the growth of microorganism (Ackerley et al., 2004). Effluent contaminated by metals and chlorinated organic compounds are difficult to remediate. These compounds are toxic and recalcitrant, persist longer in the environment, and cause adverse effects to flora and fauna. The concentration of Cr(III) varies from 500 to 7000 ppm in tannery effluent which may be converted into Cr(VI) in the environment in free form that is toxic. Pentachlorophenol and its substitutes' concentration also varies from 10 to 90 ppm in tannery effluent that are highly toxic and recalcitrant. Environmental pollution by chromium may be severe. The electroplating, metal finishing, chromate preparation and leather-tanning industries contribute to environmental contamination with Cr(VI) and Cr(III) (Ackerley et al., 2004). Cr(VI) is soluble, toxic, and carcinogenic, whereas Cr(III) is less soluble and less toxic.

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Chromate reductases are a group of enzymes that catalyze the reduction of toxic and carcinogenic Cr(VI) to the less soluble and less toxic Cr(III). Thus, it is desirable to change Cr(VI) into Cr(III) in the environment (Horitsu et al., 1987; Park et al., 2000).

The tannery industries are considered as polluting due to the inherent manufacturing processes as well as type of technology employed in the manufacture of hides and skin into leather. During the tanning process at least 300 kg of chemicals are added per ton of hides. Physicochemical methods are employed for removal of heavy metals from the effluent such as precipitation with hydroxide, carbonates and sulphides, adsorption on the activated carbon, use of ion exchange resins and membrane separation processes (Park et al., 2000). These methods are responsible for generation of pollution, and the treatment processes are expensive. Biotransformation and biosorption are emerging technologies, which utilize the potential of microorganisms to transform or to adsorb metal. Intact microbial cell, live or dead, and their products can be highly efficient for bioaccumulation of both soluble and particulate forms of metals (Kovacevic et al., 2000). Higher fungi (mushrooms), bacteria, yeast, seaweed and plant bark materials are abundantly available in nature and can be used a source of low cost biosorbents (Kovacevic et al., 2000; Blake et al., 1993). The use of microbial cells as biosorbents of heavy metals is a potential alternative to conventional methods used to decontaminate liquid waste water.

PCP is widely used as herbicide, insecticide, fungicide, algaecide, disinfectants and as an ingredient in antifouling paints (Edgehill & Finn, 1983). Pentachlorophenol and its biotransformation products manifest toxic effect. The PCP gets access in the body system through inhalation and skin. It has been observed that lipoproteins have high affinity with PCP and the bonding between blood carriers. Pentachlorophenol may cause irritation of the skin, conjunctiva and upper respiratory tract, demonstrable systemic absorption and impairs oxidative phosphorylation pathway (Xun & Orser, 1991; Thakur, 1995). The problem of waste treatment can be approached by getting rid of the pollution by proper effluent treatment, controlling pollution occurring at different stages of leather manufacturing and bioconversion processes by absorbing and reusing the toxic compounds for commercial application. Biotechnology plays an important role in tannery effluent treatment. It is believed that a functionally and genetically potential microorganisms having potency to remove contaminants, absorb shock load of toxicants during treatment, and potency to associate with indigenous microbes with mutual interaction in the bioreactor, would be useful for treatment of tannery effluent and bioconversion and product recovery. Fungal strains have significant potency to adsorb chromium but it is refractive to grow on chlorinated phenols. Therefore, potential fungal and bacterial strains are isolated and process parameters are optimized for biosorption of chromium and pentachlorophenol from tannery effluent for tanning of leather. The quality of leather was evaluated by Scanning Electron Microscopy.

2. MATERIALS AND METHODS

2.1. Isolation of fungal strains and fungal inoculums

The soil sample was collected from the sediment core of main channel of tannery effluent located at Jazmau, Kanpur, U.P. The soil was serially diluted in 10 fold, and diluted sample (0.1 ml) was spread on the potato dextrose agar (PDA) plate. The plates were incubated at 30^{o}C for 4 days. The microbial colonies (fungi) appeared on the PDA plates were isolated, purified and characterized based on their morphological structures as colour, texture, and diameter of the mycelia, and microscopic observation of spore formation.

2.2. Source of bacterial community and enrichment

Bacterial population was obtained from sediment samples together with effluent (1:10 w/v) collected from sites of Century pulp and paper mill Ltd., Lalkuan, Uttaranchal, India, and main channel of tannery effluent located at Jazmau, Kanpur, U.P. India. The microbial population was enriched in mineral salt medium (MSM) containing (L^{-1}): $Na_2HPO_4.2H_2O$ 7.8 g; KH_2PO_4 6.8 g; $MgSO_4$ 0.02 g; $Fe(CH_3COO)_3NH_4$ 0.01 g; $Ca(NO_3)_2.4H_2O$ 0.05 g; $NaNO_3$ 0.085 g; trace element solution as described by Pfenning and Lippert 1 ml, and pentachlorophenol (0.1g/L) in the chemostat (Thakur, 1995). The morphologically distinct isolates were identified biochemically, Biolog test method and 16S rDNA analysis (Sharma et al., 2009).

2.3. Screening of potential microbial strain for removal of chromium

The fungal and bacterial isolates were screened for their chromium removal potentiality under minimal salt medium containing (g/l): $Na_2HPO_4.2H_2O$ 7.8; KH_2PO_4 6.8; $MgSO_4$ 0.2; $Fe(CH_3COO)_3NH_4$ 0.01; $Ca(NO_3)_2.4H_2O$ 0.05, and potassium chromate (500 mg/L), pH adjusted to 6.0 as described by Thakur (Thakur, 1995). The effluent was inoculated in an Erlenmeyer flask with individual fungal and bacterial isolates and incubated at 30^oC in a rotary shaker for 7 days. Chromium was measured at an interval of 0, 1, 3 and 7 days. On the basis of commiserative analysis and percentage reduction was studied by the individual isolates along with the control and the most potential strains were selected for further analysis (Volesky & Holzen, 1995; Srivastava & Thakur, 2006).

2.4. Bioreactor and chromium removal in tannery effluent

A laboratory scale sequential bioreactor was fabricated from 2 L glass column connected with another 2 liter glass fractionation column in a sequential way as described earlier (Srivastava & Thakur, 2006). The column was equipped with stirring (250 rev/min) and aeration (500 mL/min). The bacterial strain (*Acinetobacter* sp. IST3) was inoculated in step I reactor, and effluent was subsequently treated by fungal strain FK1 (*Aspergillus niger* FIST1) in step II. In another set of bioreactor initially tannery effluent was treated by fungi and subsequently by bacteria. The flow inlet-outlet rate, 20.8 mL/h, was maintained in bioreactor. The pH was maintained between 5-6.5 throughout the course of treatment.

2.5. Scanning Electron Microscope (SEM)-Energy Dispersive X-ray Spectrometer (EDX) and Transmission Electron Microscopy (TEM)

Assessment of morphological changes in response to chromium accumulated in fungal strain, *Aspergillus niger*, and quantification of chromium within fungal strains was performed by SEM and EDX analysis. The sample was prepared as described earlier (Srivastava & Thakur, 2007) Transmission Electron Microscopy (TEM) was performed for the identification of chromate accumulation within cells of microorganism. The sample was prepared as described earlier (Srivastava & Thakur, 2007).

2.6. Analysis of chromium and Pentachlorophenol

The percent reduction of chromium was determined using flame atomic absorption spectrophotometer as described by Greenberg et al. (1995). Pentachlorophenol was extracted by acidifying 10 mL effluent sample with 5N HCl. PCP from the sample was extracted in dichloromethane and used for the determination of PCP by High Performance Liquid Chromatography and GC-MS (Sharma et al., 2009; Thakur et al., 2001).

2.7. Recovery of chromium and leather preparation

Chromium absorbed in microorganism (fungus and bacteria) was collected from the bioreactors. The microbial cells including mycelium of fungus were removed by filtration, and dried in the oven at 80^oC overnight named as “chromium cake”. Chromium was also recovered from tannery effluent by addition of CaO:MgO (2:1) and adjusting pH 7.0-7.6.

For preparation of leather freshly flayed goat skins of 1kg was collected from a local slaughter house. It was cut into two halves. The two halves were taken for the two different experimental purposes. One is for the chemical treatment and another for the biological treatment. For chemical treatment 500kg of raw skin were taken in a tray and were treated with 2% sodium sulfide and 10% calcium oxide (CaO) to destroy the hairs on the skins and dehairing purposes. After completion of dehairing, the skins were washed and the dehairing skins were collected. The dehaired skin obtained from the above processes was cut into two halves of 300gm and 200gm each. The dehaired skins were treated with 3 percent ammonium nitrate for deliming and tanning was performed by chromium precipitated by CaO:MgO (2:1) and absorbed by fungus, *Aspergillus niger* FIST1, and bacterium, *Acinetobacter* sp. IST3. Samples from the experimental and control crust leathers were cut into small pieces. The specimens were cut with uniform thickness without any pre-treatment. Samples were dehydrated and SEM analysis was performed (Srivastava & Thakur, 2007).

3. RESULTS AND DISCUSSION

3.1 Isolation and characterization of fungal isolates

Five different types of colonies of the fungus were isolated, and further purified on potato dextrose agar plates. The fungal isolates were identified based on morphological characteristics and microscopic observations. In this study FK1 was identified as *Aspergillus niger* and FK2 as *Hirsutella* sp. The data of the study indicated that chromium removed by FK1 was 75% and FK2 fungus was able to remove 60 per cent chromium at day 7.

3.2. Isolation and characterization of bacterial isolates

Morphological characterization of stabilized PCP consortium of tannery and pulp and paper mill was evaluated. The result of the study indicated the presence of four bacterial isolates in tannery consortium (TE1, TE2, TE3 and TE4) and three in pulp and paper mill (PCP1, PCP2 and PCP3). PCP1, PCP2 and PCP3 were identified as *E.coli*, *Pseudomonas aeruginosa* and *Acinetobacter* sp. by biochemical, Biolog and 16S rDNA analysis. Fifteen carbon sources were used for the screening of potential strain on MSM agar plates containing chlorinated organic compounds. PCP3 strains had higher growth on pentachlorophenol and its degradation product i.e. trichlorohydroquinone (TCHQ) and chlorohydroquinone (CHQ) (Sharma et al., 2009). Batch culture study for the chromate biosorption by bacterial strain was performed for the period of 7 days. Results of comparative study of all three strains for biosorption of chromate show significant reduction of chromate from potassium chromate solution. Percent chromium reduction by PCP3 strain was 86% at day 7, while PCP1 and PCP2 reduced 45% and 55% respectively. Therefore, PCP3 (*Acinetobacter* sp. IST3) was selected with the fungal strain FK1 *Aspergillus niger* (FIST1) for treatment and recycling of chromium and PCP of tannery effluent in sequential bioreactor.

3.3. Evaluation of chromium removal in bioreactor

The bioreactor was designed for studying the biotreatability of chromium in tannery effluent by *Aspergillus niger* (FIST1) and *Acinetobacter* sp. IST3 (PCP3). In first step of sequential bioreactor where initially tannery effluent was treated by bacteria, and bacteria treated effluent subsequently treated by fungi showed reduction of chromate (90%) and PCP (67%), after 15 days. In second set of sequential bioreactor effluent was treated initially by fungus and then followed by bacteria remove chromate (64.7%) and PCP (58%) after 15 days (Figure 1). The data of the study indicated higher reduction of chromium in bioreactor where effluent initially treated by bacteria followed by fungi. This may be due to utilization of pentachlorophenol in step 1 reactor by *Acinetobacter* sp. IST3 that has not inhibited the growth of fungi in step II.

However, in second set of reactor fungal growth was inhibited by pentachlorophenol, which prevented the bioaccumulation of chromium in mycelium (Thakur et al., 2001; Shah & Thakur, 2002).

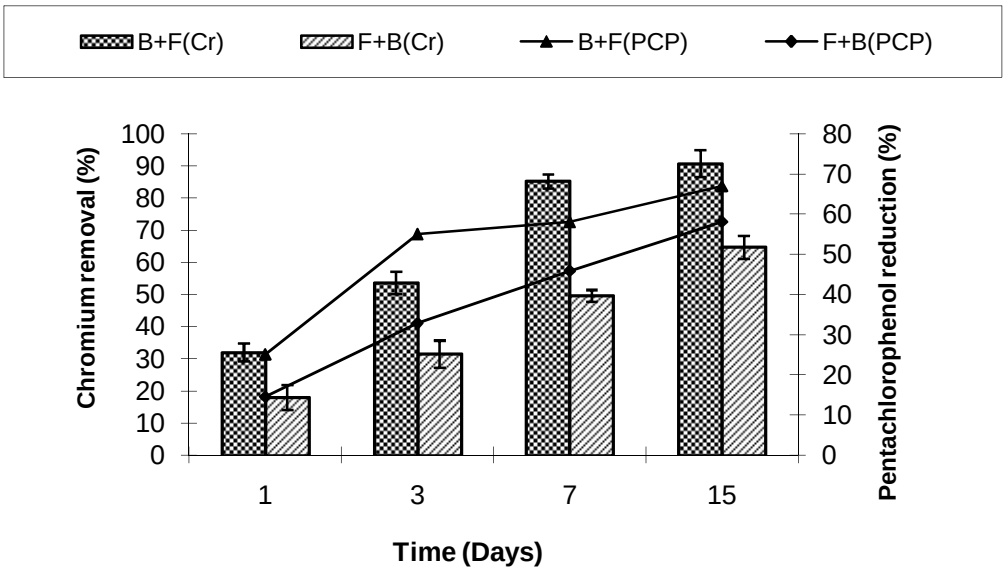

Figure 1 Changes in chromium and pentachlorophenol content in two set of sequential bioreactor in which tannery effluent initially treated by bacteria (*Acinetobacter* sp. IST3) followed by fungi (*Aspergillus niger* FIST1) (set I, B+F) and another set where effluent initially treated by fungi (*Aspergillus niger* FIST1) and then bacteria (*Acinetobacter* sp. IST3) (Set 2, F+B) (Error bars are standard deviation)

Growth of fungal strain in bioreactor was determined by measuring biomass. It is known that the microorganism thrive in extreme conditions, therefore, a sequential bioreactor was used in this study to sustain the shock load in the reactor. The proper aeration made the reactor aerobic suitable for the fungal growth.

The degradation of pentachlorophenol (PCP) by bacterial strain in two-step bioreactor was determined by HPLC analysis. It was observed that the formation of intermediary metabolite in 3 days sample and it confirmed by standard run of respective compounds (Figures 2(a), 2(b)). Retention time and peak area of PCP and tetrachlorohydroquinone (TECHQ) in HPLC profile was 9.386 min and 3.904 min and area size 223038 and 84982 at 0 h respectively. Peak of TECHQ was increased in 3 day sample (peak area 1050143; RT 3.924) and subsequently decreased in 7 day sample, (peak area 950143; RT 3.932) indicating conversion of PCP in TECHQ. PCP concentration was decreased in 3 day sample, respectively (Figure 2(b)). These metabolite peaks were slowly reduced but have not completely disappeared at 96 hour.

This suggests that Aceinetobacter sp. IST3 was able to degrade the chlorinated compounds present in the effluent but had not been completely eliminated. Edgehill and Finn, 1983 isolated a bacterial strain of *Arthrobacter* sp. capable of utilizing PCP as a sole carbon source and energy between 10 and 135 mg/l. The PCP is degraded to tetrachlorohydroquinone by PCP-4 mono-oxygenease by Flavobacterium. The second enzyme involved in PCP degradation is tetrachlorohydroquinone dehalogenase which catalyze sequential dehalogenation of tetrachlorohydroquinone to 2, 3, 6-trichloro-p-hydroquinone and then to 2, 6 dichloro-p-hydroquinone (2, 6 DCHQ) (Sharma et al., 2009; Thakur et al., 2001; Shah & Thakur 2002).

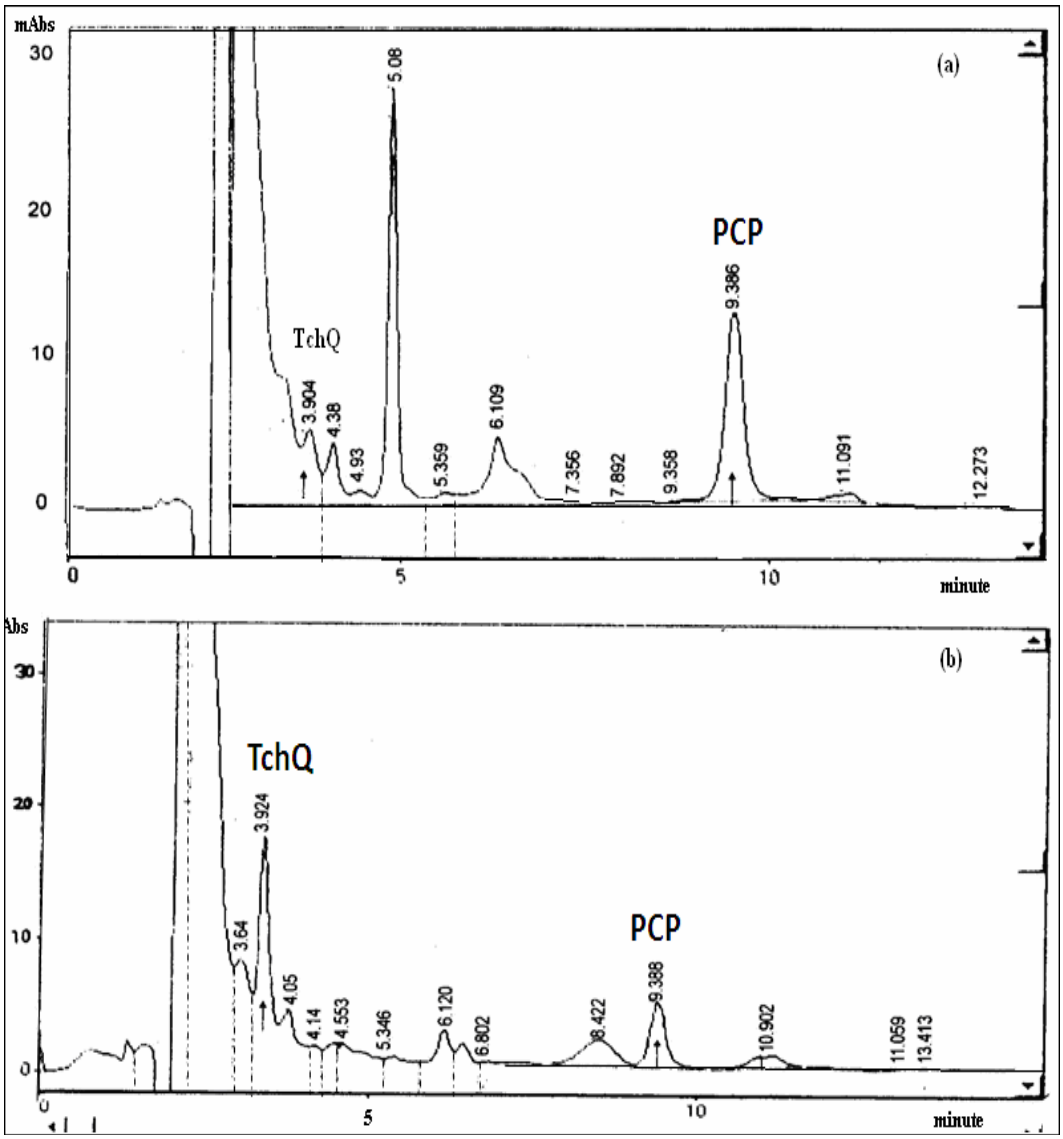

Figure 2 HPLC profile of PCP degradation and metabolites formed in two-step sequential bioreactor by bacteria (*Acinetobacter* sp. IST3) followed by fungi (*Aspergillus niger* FIST1) (PCP, pentachlorophenol, TchQ, tetrachlorohydroquinone). In Figure (a) is HPLC profile at 0 day (untreated tannery effluent), and Figure (b) HPLC profile at 3 day of treated tannery effluent.

3.4. Evaluation of chromium biosorption by SEM-EDX and TEM analysis

SEM-EDX analysis of fungi (*Aspergillus niger* FIST1) and bacterium (*Acinetobacter* sp. IST3) was determined at 24h incubation. The hyphae of fungi were cylindrical, septate and branched and there was no peak of chromium at 5.4 keV in control. However, samples of chromium treated in bioreactor have shown that chromium was uniformly bound to the fungal mycelium and there was chromium peak level at 5.4 keV as determined by Energy Dispersive X-ray Analysis (EDX)(Figures 3(a) and 3(b)).

TEM analysis of fungal and bacterium isolate revealed that Cr(III) was uniformly adsorbed to the surface of the fungus mycelium and precipitated. At higher chromate concentration the density of chromate sequestered on the cell wall, and also in the cell interior indicating chromate penetration in to the cell. This was revealed by the electron dense area within the cells of fungi and bacterium both (Figures 4(A) and (B)).

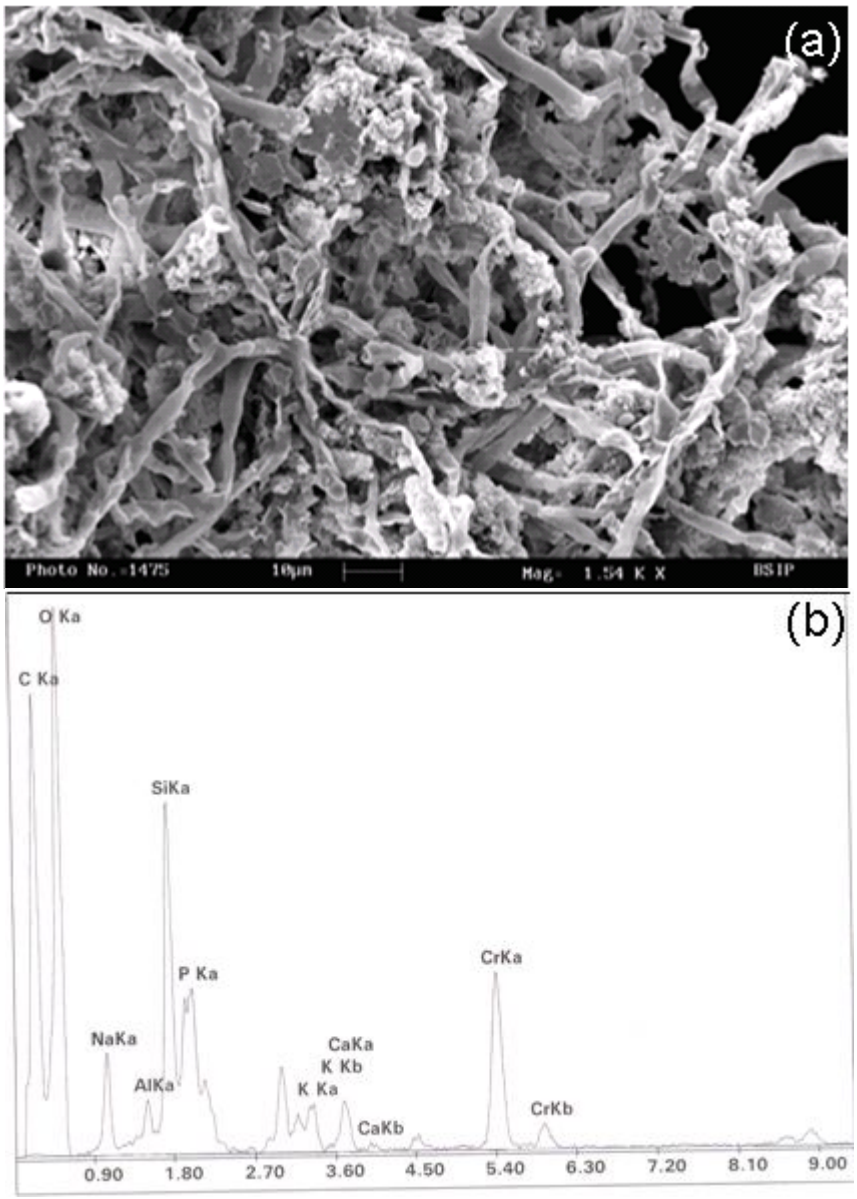

Figure 3 Scanning Electron Microscopy (a) and Energy Dispersive X-Ray analysis (b) of fungal mycelium, *Aspergillus niger* FIST1 after biosorption of chromium in the mycelium after 24 hour incubation. Precipitates of chromium in mycelium are indicated as white clump formation in figure

Microorganisms have excellent nucleation sites for grained mineral formation, due to their high surface area and volume ratio and the presence of electronegative charges on the cell wall (Baldi et al., 1990; David et al., 1973; Radehaus & Schmidt, 1992; Beveridge, 1988) Surface functional groups (e.g. carboxyl, phosphoryl and hydroxyl) play major role in bioaccumulation of metals and significantly removed chromate which is toxic (David et al., 1973; Radehaus & Schmidt, 1992; Beveridge, 1988; Leusch et al., 1995).The biosorbed chromate was assumed to be Cr(III), as Cr(VI) is reduced to Cr(III) in the living cells due to reducing environment and enzymes present inside the cell.

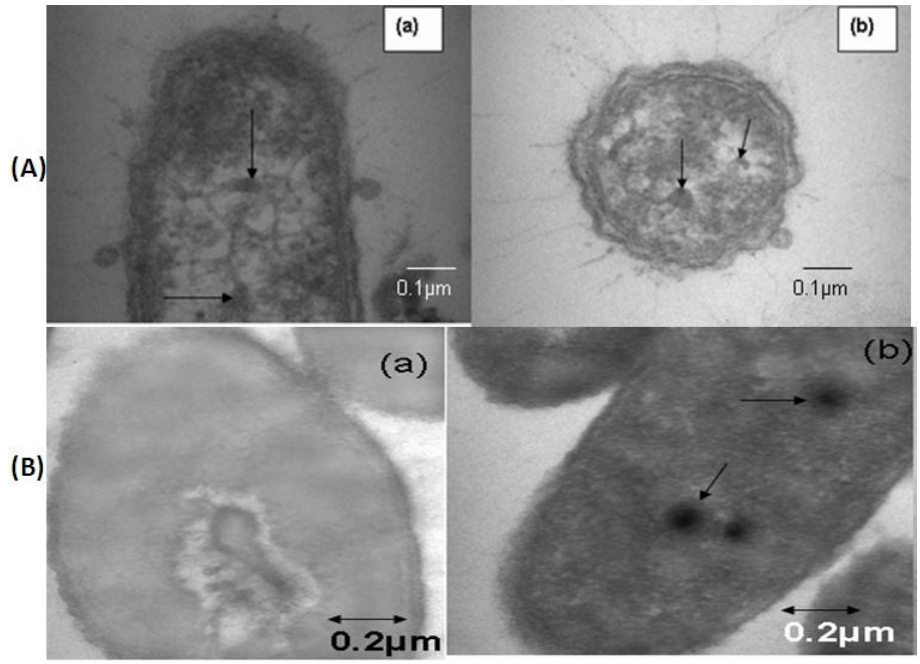

Figure 4 Transmission Electron Microscopy of chromium (tannery chromate) biosorption in *Aspergillus niger* FIST1 (A) indicated longitudinal section (a) and (b) transverse (b), and TEM of *Acinetobacter* sp. IST3 (B) chromium (tannery chromium) uptake by *Acinetobacter* sp., no chromium in control [B(a)] and (b) indicated longitudinal section of *Acinetobacter* sp. IST3 cells (0.2µm). Arrow indicated intracellular accumulation of chromium

3.5. Preparation and analysis of tanning cake and leather

Chromium absorbed in microorganism (fungus and bacteria) was collected from the bioreactors. The microbial cells including mycelium of fungus were removed by filtration, and dried in the oven at 80OC overnight. Chromium was recovered from tannery effluent by addition of CaO:MgO (2:1) and adjusting pH 7.0-7.6. Result of the study indicated biosorption of chromium in the fungal spore balls and mycelium (Figure 5) which was prepared in cake form (Figure 5(b)) named as “tanning cake”. Chromium absorbed by fungus and bacteria and chromium recovered by CaO:MgO was further used for tanning of the leather. Result of the study indicated that quality of the leather prepared by chromium absorbed by fungus and bacteria was better. In view of above result ‘tanning cake’ was prepared which is a better substitute of raw chromium used for tanning in leather mills.

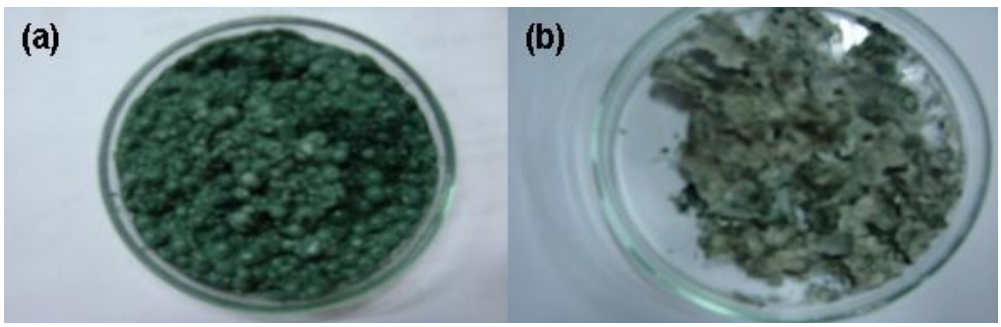

Figure 5 Biosorption of chromium by spores of *Aspergillus niger* FIST1 during treatment in the bioreactor (a). Tanning cake prepared from the fungal biomass (*Aspergillus niger* FIST1) during treatment in the bioreactor (b)

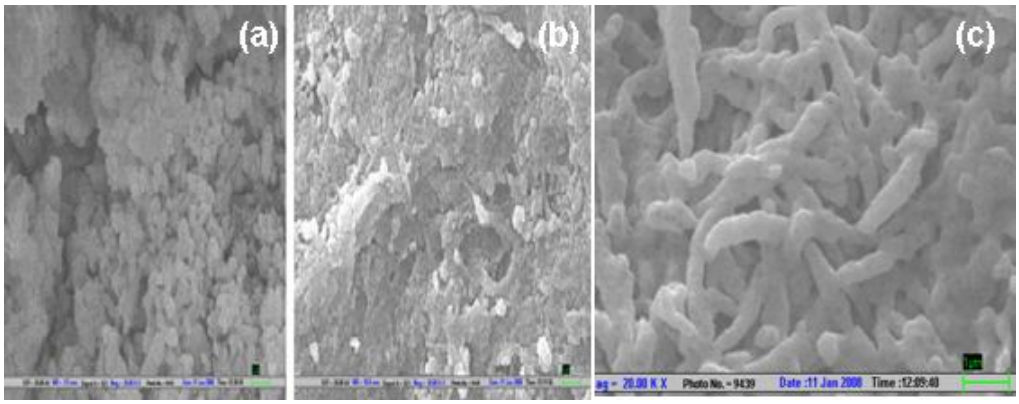

Figure 6 Scanning Electron Microscopy (SEM) of leather preparation after tanning with CaO and MgO (a, b) and chromium absorbed by fungi, *Aspergillus niger* FIST1(c)

The quality of leather was determined by Scanning Electron Microscopy. Figure 6 shows the Scanning Electron Microphotographs (SEM) of leather samples after treatment of CaO:MgO (2:1) and adjusting pH 7.0-7.6, and chromium absorbed by the microorganisms. As can be seen in Figure 6a and b, fibres of the leather coagulated and holes appeared in leather, however, leather treated by chromium absorbed by microorganisms (chromium cake) showed a clean grain surface which indicated that there was no damage of grain. More over there was clear separation of fibres, which indicated a quality of leather for commercial applications.

4. CONCLUSION

In this study fungal and bacterial strains were isolated and identified for the removal of chromium and pentachlorophenol. The two step sequential bioreactor was used for the treatment of tannery effluent. The best combination of sequential bioreactor treatment was bacteria treated effluent followed by fungi. The Aceinetobacter sp. IST3 was able to degrade the chlorinated compounds, pentachlorophenol, present in the effluent. The fungal strain *Aspergillus niger* was efficient for removal of chromium from the tannery waste. SEM-EDX analysis of fungi (*Aspergillus niger* FIST1) and bacterium (*Acinetobacter* sp. IST3) showed the presence of chromium within the cell. TEM analysis of fungal and bacterium isolate revealed that Cr(III) was uniformly adsorbed to the surface of the fungus mycelium and precipitated. Chromium absorbed by fungus and bacteria and chromium recovered by CaO:MgO was further used for tanning of the leather. Tanning cake was prepared which is a better substitute of raw chromium used for tanning in leather mills.

5. ACKNOWLEDGEMENTS

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