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Research article

Genotoxicity evaluation of tannery effluent treated with newly isolated hexavalent chromium reducing *Bacillus cereus*

Vineeta Kumari [a], Ashutosh Yadav [b], Izharul Haq [a], Sharad Kumar [a], Ram Naresh Bharagava [b], Sudheer Kumar Singh [c], Abhay Raj [a, *]

[a] *Environmental Microbiology Laboratory, Environmental Toxicology Group, CSIR-Indian Institute of Toxicology Research (CSIR-IITR), MG Marg, Lucknow, Uttar Pradesh, 226001, India*

[b] *Department of Environmental Microbiology, Babasaheb Bhimrao Ambedkar University (A Central University), Vidya Vihar, Raebareli Road, Lucknow, Uttar Pradesh, 226025, India*

[c] *Microbiology Division, CSIR-Central Drug Research Institute (CSIR-CDRI), Sector 10, Jankipuram Extension, Sitapur Road, Lucknow, Uttar Pradesh, 226031, India*

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ABSTRACT

In this study, the efficiency of free and immobilized cells of newly isolated hexavalent chromium [Cr(VI)] reducing *Bacillus cereus* strain Cr1 (accession no. KJ162160) was studied in the treatment of tannery effluent. The analysis of effluents revealed high chemical oxygen demand (COD-1260 mg/L), biological oxygen demand (BOD_5-660 mg/L), total dissolved solids (TDS-14000 mg/L), electrical conductivity (EC-21.5 mS/cm) and total chromium (TC-2.4 mg/L). The effluents also showed genotoxic effects to *Allium cepa*. Treatment of tannery effluent with isolated *B. cereus* strain led to considerable reduction of pollutant load. The pollutant load reduction was studied with both immobilized and free cells and immobilized cells were more effective in reducing COD (65%), BOD (80%), TDS (67%), EC (65%) and TC (92%) after 48 h. GC-MS analysis of pre and post-treatment tannery effluent samples revealed reduction of organic load after treatment with free and immobilized cells. An improvement in mitotic index and reduction in chromosomal aberrations was also observed in *A. cepa* grown with post-treatement effluent samples compared to untreated sample. Results demonstrate that both methods of bacterial treatment (free and immobilized) were efficient in reducing the pollutant load of tannery effluent as well as in reducing genotoxic effects, however, treatment with immobilized cells was more effective.

1. Introduction

Environmental pollution by effluents of tannery industries is a major concern throughout the world. Plethora of chemicals such as acids, alkalis, chromium salts, tannins, biocides, solvents, sulfides, dyes etc. are used during leather processing and remain in the effluent. These are difficult to treat by conventional activated sludge process due to high toxicity. Their discharge in to water bodies poses a serious threat to the living organisms inhabiting respective ecosystem and also tends to be accumulated in food chain. There are about 3000 major tanneries in India, located in different parts of the country. Most of them (nearly 80%) are engaged in chrome tanning process (Shukla et al., 2009). The effluent discharged from these tanneries after treatments is still left with high level of BOD, COD, TDS and other specific pollutants such as chromium [Cr(III) & Cr(VI)], pentachlorophenol, surfactant, synthetic tannins, azo dyes, chloride, sulphate and oil and grease (Singh et al., 2013; Thakur and Srivastava, 2011). The discharge of inadequately treated tannery effluent causes water and soil pollution and affects plant and animal health. The genotoxic and mutagenic effect of tannery effluents from leather processing industry is well documented (Raj et al., 2014; Gupta et al., 2012; Alam et al., 2009).

Over the years, various physical and chemical processes including advance oxidation processes using ozone, electrochemical treatment, fenton, photocatalysis (UV/TiO2) and membrane processes have been developed to enhance pollutants removal from effluent (Lofrano et al., 2013). Most of these processes removed the majority of colloidal organic substances and suspended materials; but, refractory compounds still remained in the effluent. To over-come these problems bio-based remediation

* Corresponding author.
E-mail addresses: araj@iitr.res.in, abhayraj_1@rediffmail.com (A. Raj).

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strategies have evolved as a promising tool. Bioremediation of tannery effluents is an environment friendly, safe, cost effective alternative to traditional physical and chemical methods. Bioremediation of chromium in tannery effluents using bacteria or fungal mycelia as bioabsorbent has been studied earlier (Saranraj and Sujitha, 2013).

Immobilization of microbial cells has received increasing interest in the field of waste treatment (Martins et al., 2013) as it offers advantages such as high biomass, high metabolic activity, stability and strong resistance to toxic chemicals. Immobilized microorganisms can be reused several times without significant loss of activity, making them cost effective. The present study was undertaken to isolate potential Cr(VI) reducing bacteria and study the bioremediation potential of free with immobilized cells for tannery effluent treatment.

2. Materials and methods

2.1. Collection of tannery effluents and soil samples

Effluent samples were collected from a common effluent treatment plant (CETP) at district Unnao, Uttar Pradesh, India. It is an activated sludge process-based common effluent treatment plant receiving effluent from 21 tanneries with an inflow of 2.15 million liters per day (MLD). Effluent samples were collected from outlet point of CETP in plastic bottles of 0.5 lit capacity. Samples were brought to laboratory in icebox and stored at 4 °C. Similarly, soil samples were collected from effluent receiving drain in pre-sterilized collection tubes.

2.2. Isolation and screening of chromium (VI) reducing bacteria

Isolation of chromium resistant bacteria from soil sample was done by an enrichment culture technique in mineral salt medium (MSM) supplemented with 100 mg/L of Cr(VI). The MSM contained (g/L): NaCl: 1; $CaCl_2.2H_2O$: 0.1; $MgSO_4.7H_2O$: 0.5; KH_2PO_4: 1; Na_2HPO_4: 1, yeast extract: 4 and pH = 6.0 (Mahmood et al., 2013). Erlenmeyer flasks containing autoclaved MSM (99 mL) were supplemented with filter-sterilized Cr(VI) as $K_2Cr_2O_7$ @ 100 mg/L and were inoculated with 1 g of soil. The flasks were incubated at 37 °C under shaking condition (120 rpm) in an orbital shaker (Innova-4230, New Brunswick, USA). After 7 days of enrichment, culture broth was serially diluted and plated on MSM agar plate containing 100 mg/L of Cr(VI). The plates were incubated at 37 °C for 48 h. Potential isolates were tested for Cr(VI) reduction in MSM.

2.3. Growth tolerance and chromium (VI) reduction

Growth tolerance and Cr(VI) reduction assay was performed by growing the isolate in 50 mL LB broth supplemented with 0, 25, 50, 100, 150, 200, 250, 300, 350 and 400 mg/L of Cr(VI). The flasks were incubated at 35 °C under shaking (120 rpm). After 24 h of growth, culture broth from each test concentration was centrifuged (8000 rpm for 15 min) and pellet was re-suspended in same volume of distilled water. The optical density (OD_{600}) of bacterial suspension was measured spectrophotometrically (Techcomp, Korea). Cr(VI) concentration of centrifuged supernatant was measured by 1, 5-diphenylcarbazide (DPC) method at 540 nm (APHA, 2005) and was quantified using a standard plot prepared from $K_2Cr_2O_7$ in the range of 1–10 mg/L. The percent reduction of Cr(VI) was calculated using the formula:[(Ci-Cf)/(Cix100), where, Ci = initial Cr(VI) conc. (mg/L) and Cf = final Cr(VI) conc. (mg/L)].

2.4. Characterization of culture supernatant, cell free extract and membrane fraction for the localization of chromium reduction and chromium reductase activity

Isolate was grown in LB broth containing 80 mg/L Cr(VI) at 35 °C and 120 rpm for 24 h. Cells (30 mL) were harvested by centrifugation (5000 rpm for 30 min) and supernatant was stored at 4 °C. Cell pellet was re-suspended in 30 mL phosphate buffer (100 mM, pH = 7.0) and cells were disrupted by sonication (Sonics VCX 750, USA) with 15-s pulses at 15-s interval for 30 min. The resultant homogenate was centrifuged at 15,000 rpm for 15 min at 4 °C and cell free extract was collected and stored. The remaining fraction containing cell wall fraction was re-suspended in 30 mL phosphate buffer. Total chromium, Cr(VI), chromium reductase activity and total protein content of all three fractions (culture supernatant, cell free extract and cell wall fraction) was estimated.

2.5. Identification of selected bacterial isolate

Morphological and biochemical tests were conducted as per standard method (Barrow and Feltham, 1993). The molecular characterization of the isolate was done by 16S rRNA gene sequencing. DNA was extracted using UltraClean Microbial DNA isolation Kit (MO BIO, USA) according to the manufacturer's instructions. PCR amplification of the 16S rRNA gene was performed with 16S rRNA universal Primers: 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-TACGGTTACCTTGTTAC G ACTT-3′)at annealing temperature of 56 °C (35 cycles). The PCR product was purified by gel extraction (Gel extraction Kit, Qiagen) and was sequenced in an ABI 3130 genetic analyzer using Big Dye Terminator version 3.1 cycle sequencing kit. The nucleotide sequences of 16S rRNA gene were compared with available sequences using NCBI-BLAST.

2.6. Scanning elcelctron microscopy (SEM) analysis

SEM analysis was carried out to observe the morphological changes on the cell surface of isolate exposed to Cr(VI). Cells grown in LB broth (with and without 100 mg/L of Cr(VI)) at 120 rpm, 35 °C for 24 h were harvested by centrifugation at 5000 rpm for 30 min. The pellets were washed thrice with phosphate buffered saline (PBS) and pre-fixed with 2.5% glutaraldehyde for 2 h at 4 °C. The pre-fixed cells were washed with PBS twice and post-fixed with 1% osmium tetraoxide for 1 h. After washing with PBS thrice dehydration process was performed with 15, 30, 60, 90 and 100% (v/v) of acetone. The fixed cells were dried and gold-coated using Mini sputter coater (Model- SC7620, Quorum, Technologies, UK) and then examined with a field emission of SEM (Quanta 450 FEG, FEI, Netherland).

2.7. Preparation of bacterial cell suspension

Bacterial cells were grown in MSM (200 mL) at 35 °C under shaking (120 rpm) for 24 h and afterwards were separated by centrifugation (8000 rpm for 15 min). The pellet was re-suspended in 20 mL of 0.1 M phosphate buffer (pH = 7.0) at a final concentration of 215 mg wet cells/mL and used in bioremediation studies.

2.8. Immobilization of cells

Ten mL of bacterial cell suspension was mixed with 10 mL of 4% sterile sodium alginate solution (final alginate concentration of 2%). The alginate-bacterial mixture was then dropped gently in calcium chloride (0.1 M) solution using a sterile syringe to get equal sized beads. The beads were kept in the same solution for 30 min at 4 °C

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