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Bioremoval of trivalent chromium using *Bacillus* biofilms through continuous flow reactor

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ABSTRACT

Present study deals with the applicability of bacterial biofilms for the bioremoval of trivalent chromium from tannery effluents. A continuous flow reactor was designed for the development of biofilms on different substrates like glass beads, pebbles and coarse sand. The parameters for the continuous flow reactor were 20 ml/min flow rate at 30 °C, pH4. Biofilm biomass on the substrates was in the following sequence: coarse sand > pebbles > glass beads (4.8×10^7, 4.5×10^7 and 3.5×10^5 CFU/cm^2), which was confirmed by CLSM. Biofilms developed using consortium of *Bacillus subtilis* and *Bacillus cereus* on coarse sand had more surface area and was able to remove 98% of Cr(III), SEM-EDX proved 92.60% Cr(III) adsorption on biofilms supported by coarse sand. Utilization of *Bacillus* biofilms for effective bioremoval of Cr(III) from chrome tanning effluent could be a better option for tannery industry, especially during post chrome tanning operation.

1. Introduction

Vellore district is mushroomed with many tanneries. There is lot of unorganized tanning industries; which forms the livelihood of Vellore population. Cr(III) is used as a tanning agent in the leather industries; the wastewater resulting from chrome tanning processes contains high amount of chromium metal, which is harmful for the environment and human health [1]. Toxicological studies of Cr(III) compounds reported skeletal and neurological disorders [2]. Cr(III) compounds are cytotoxic and forms DNA adduct [3].

Though there are many methods for effluent treatment like precipitation, chemical oxidation or reduction, lime neutralization, ion exchange, filtration, electrochemical treatment, reverse osmosis, membrane technologies and evaporation recovery. All these methods are expensive and will produce solid sludge containing toxic compounds [4]. And also chromium at low concentrations in the effluent waters cannot be removed by conventional methods [5]. Thus an alternate treatment strategy is required, which would be environment friendly. Indigenous chromium tolerant bacterial strains might be a better choice for the development of biofilms towards chromium removal from tannery effluent.

The purpose of the present study was to isolate Cr(III) tolerant bacterial species from tannery effluent polluted sites in Palar river basin and to develop a bacterial biofilm on different substrates like glass beads, pebbles, coarse sand for bioremoval of Cr(III). The study focuses on the bioremoval of Cr(III) using indigenous chromium tolerant bacterial biofilms through continuous flow reactor. The biofilm bioreactor would be a better choice for tanneries to alleviate Cr(III) pollution in the effluent waters. This technology could be adopted in an industrial scale for environmental problems of tanneries.

2. Materials and methods

2.1. Isolation, screening and characterization of effective strains

Soil and water samples were collected from chromium polluted sites in the Palar river basin of Vellore district, Tamilnadu, India. Samples were processed for the isolation of Cr(III) tolerant bacterial strains as per APHA [6]. Trivalent chromium tolerant bacteria were isolated using nutrient agar plates amended with $Cr(NO_3)3{\cdot}9H_2O$ at pH 4 and incubated at 30 °C for 3–5 days.

The effective strains were screened based on maximum tolerable concentration (MTC), Cr(III) bioremoval ability and exopolysaccharide (EPS) production [7]. The selected strains were characterized morphologically, biochemically and physiologically following Gerhardt et al. [8]. The taxonomical identifications of the selected bacterial strains were confirmed by 16S rRNA gene sequencing.

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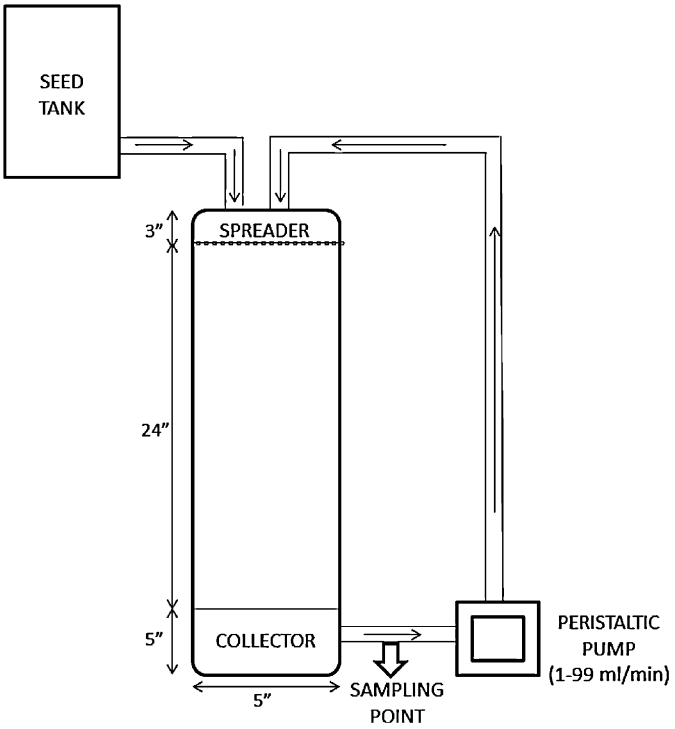

Fig. 1. Schematic diagram of continuous flow biofilm cultivation reactor.

2.2. Biofilm formation by tissue culture plate assay

The bacterial biofilm estimation was carried out using standard tissue culture plate (TCP) assay as described by Christensen et al. [9]. In the present study, two bacterial strains (*Bacillus subtilis* VITSCCr 01 and *Bacillus cereus* VITSCCr 02) were observed for their ability to form biofilm on tissue culture plate. Overnight cultures of *Bacillus* strains were diluted to 1×10^6 CFU/mL in fresh Nutrient Broth (NB) with 0.5% glucose after adjusting the OD at 600 nm. Aliquots (200 μL) of various concentrations of Cr(III) (10, 25, 50, 75, and 100 mg/l) was transferred onto 96-well flat-bottom tissue culture plates, uninoculated broth served as control. Plates were incubated in static and dynamic conditions for 48 h at 30 °C. After incubation, the content of each well was gently removed by tapping the plates. The wells were washed twice with 200 μL of deionized water to remove free-floating 'planktonic' bacteria. Biofilms in plates were dried at 60 °C for 1 h and adherent bacteria were stained with 200 μL of 0.1% crystal violet for 5 min. The plates were rinsed twice with deionized water to remove excess stain and dried at 37 °C for 2 h. Stained adherent cells were detached from the plates using 200 μL of 30% (w/v) glacial acetic acid for 10 min with shaking at 300 rpm. The OD of stained biofilm was determined at 492 nm (Power Wave XS2, Biotek Corp, Microplate reader). The mean OD value obtained from the media control well was deducted from all the test OD values.

2.3. Continuous flow reactor design for biofilm development

Simplified continuous flow reactor was designed for the cultivation of biofilm and for the treatment of chrome tanning effluent. The continuous flow reactor was fabricated using silicate glass material attached with a two channel peristaltic pump (Ravel Hiteks, RH-P 100 S–100–2H) to enable the uninterrupted flow of culture. The description of the reactor set up is given in Fig. 1.

The overnight culture of *B. subtilis* VITSCCr 01 and *B. cereus* VITSCCr 02 and their consortium were used as seed inoculum for biofilm development. The reactor was packed with sterile glass beads (5 mm)/pebbles10–15 mm)/coarse sand (3–5 mm) up to 2/3 of the column length. The bacterial inoculum was fed through the substrates at different flow rates of 10, 20, 30, 40 and 50 ml/min, up to 72 h at 30 °C. Intermittent samples were collected at 24, 48 and 72 h and were further characterized.

2.4. Characterization of developed biofilms

2.4.1. Biomass estimation

The developed biofilms were estimated for total bacterial biomass by viable plate count method. 1 cm^2 biofilm samples were scraped from the substrates using a sterile scalpel and dispersed in 10 ml of sterile water. The dispersed biofilm was plated on nutrient agar plates and incubated at 30 °C for 24 h. The NA plates were calculated for colony forming units and there by biomass per cm^2 were calculated.

2.4.2. Stability of biofilms

Stability of biofilm on the substrates was studied to check the irreversible biofilm formation. After the biofilm development, sterile water was passed through the column at 50 ml/min flow rate for 24 h. Stable biomass was calculated by detecting biomass adhered on the substrate/cm^2 after water flow from initial biomass calculated as in Section 2.4.1.

2.4.3. Microscopic examination

Morphological characterization of biofilms was done by Scanning Electron Microscope (SEM), Atomic force microscope (AFM) and Confocal laser scanning microscopy (CLSM). For SEM the biofilms samples were fixed with 2.5% glutaraldehyde, ethanol (dehydrated) and coated with gold under vacuum in an argon atmosphere. The surface morphology of the gold coated samples was visualized by a Scanning Electron Microscope (Hitachi S4000). SEM allowed the identification of any interesting structural features on the morphology of biofilms.

For AFM imaging the biofilms were fixed with 0.4% paraformaldehyde for 5 min and fixed cells were imaged using Atomic Force Microscopy (Non contact mode) (Nanosurf Easy Scan2, Nanosurf Inc.; USA). This mode of AFM enhances the topography of biofilm by scanning the tip above the surface without damaging the biofilm.

For CSLM biofilm samples from all substrates were thoroughly washed thrice with 0.05 M phosphate buffer, pH 7.4 and immersed in 1 ml solution of phosphate buffered-saline for 5 min. Samples were stained with acridine orange (0.01%, w/v) for 10 min at room temperature in the dark. Then, samples were washed with PBS and fixed with 4% glutaraldehyde for 1 h. Laser confocal scanning microscope (Leica SP2; Leica Microsystems, Heidelberg, Germany) was used for the imaging of samples. The Leica confocal software was used for analysis of biofilm images, which allowed for collection of *z*-stacks three-dimensional (3D) reconstruction. Excitation and emission wavelength were set to 460 and 650 nm respectively by adjustable spectrum slit.

2.5. Cr(III) bioremoval studies in continuous flow reactor using Bacillus biofilms

The chromium uptake studies were performed with standard chromium solutions (25, 50 and 100 mg/l) and chrome tanning effluents (1:10 and 1:100 diluted with distilled water). After biofilm formation, the biofilm bed was washed out and the Cr(III) solutions or the sterile chrome tanning effluents were passed continuously through the column with a flow rate of 20 ml/min. Cr concentration at the inlet (initial concentration) as well as at the outlet (final concentration) of the column was measured by Atomic