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Bioremoval of zinc and manganese by bacterial biofilm: A bioreactor-based approach

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ABSTRACT

Industrialization has led to the disposal of a massive amount of heavy metals every year, showing perilous effects on humans, marine life and agricultural products. There are numerous chemical and biological approaches available but their implementation is limited due to high cost and low efficiency. Therefore, the present study was focused on the biofilm- based bioremoval of heavy metals (zinc and manganese) using indigenous bacteria isolated from the tannery sludge obtained from Ranipet, Tamil Nadu. The effective isolate was capable of tolerating up to 2000 mg/L of zinc and manganese and was further used for development of biofilm on the peels of *Cucumis sativus*. The isolate was found to be a close neighbour of *Pseudomonas beteli* by 16S rRNA gene sequencing. The uptake efficiency of the biofilm formed on the substrate was estimated to be 69.9% for zinc and 78.4% for manganese by atomic absorption spectroscopy. The scanning electron microscopy showed the biofilm formation on the substrate and also revealed the presence of heavy metal ions adsorbed on the biofilm. The adsorption kinetics of the substrate followed a heterogeneous mode of adsorption of the heavy metals as it showed a higher R^2 value for the Freundlich isotherm kinetics as compared to that of Langmuir. Thus, the peels of *Cucumis sativus* was assessed to be effective for the bioremoval of zinc and manganese.

1. Introduction

Environmental pollution has created a snag through industrial disposals [1]. The increase in the toxicity level by heavy metals deposition has become a threat to all the living forms. Ranipet has been considered as India's fourth largest urban area filled with numerous leather and chemical industries. Industries have contributed to a huge amount of metal deposition like zinc, chromium, copper, manganese, mercury, lead, nickel, etc., in and around Vellore, India, the most concerned metals as declared by the World Health Organization [2].

Zinc (Zn) is a micronutrient at lower concentrations and plays a vital role as an anti-inflammatory and antioxidant agent in chronic disorders. It also acts as an enhancer for mRNA synthesis and in the regulation of NK-kB activation [3]. But, at higher concentrations, zinc leads to neurosensory and neuropsychiatric disorders [4]. It could lead to anemia, impaired iron mobilization, increased plasma cholesterol and abnormal cardiac function [5]. A single report of fatal zinc phosphide poisoning from a tertiary care hospital in the state of Tamil Nadu, India has been reported in 2013 [6]. Research has reported that the role of zinc in regulation of cell apoptosis is quite equivocal as its role depends on its concentration [7].

Manganese (Mn) plays a vital role in bone building and metabolism which includes bone mineralization and cellular protection from free radical species [8] at its minimal dosage but when it exceeds the permissible limit, it results in clinical disorders [9]. The major targets, of Mn exposures, are the industrial and agrochemical workers. Some of the clinical neurotoxicity in patients with long-term exposure leads to renal failure or chronic liver dysfunction which is mainly due to the inability of Mn excretion from blood [10]. Exposure to manganese (Mn) at higher concentration may result in Dystonia, Hypokinesia, and Neurotoxicity [11]. The contribution of zinc by industries has reached to about 20 mg/L in water and 2000 to 1,80,000 mg/L in soil [12]. The maximum permissible limits of secondary drinking water declared by WHO for Zn and Mn is 5 mg/L and 0.05 mg/L respectively [13]. Excessive discharge of heavy metals may end in biomagnifications leading to a great loss in marine, agriculture and other ecosystems.

There are several reports on the application of bacteria for the remediation of various heavy metals [14,15]. The microorganisms play a vital role in biogeochemical cycling of the heavy metals thereby helping in the cleaning up of the environment [16]. Bacteria such as *Pseudomonas putida*, *Klebsiella variicola*, *Enterobacter cloacae*, *Salmonella enterica* are reported to detoxify heavy metals [15,17]. The key role of this uptake mechanism is played by the bacterial biofilms and extracellular polymeric substances (EPS). A study by Sundar et al. showed the

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effective removal of Chromium (III) using a biofilm developed by the consortium of *B. subtilis* and *B. cereus* in a bioreactor [18]. A study by Characklis et al. reveals that due to the polyelectrolyte nature of EPS, the microbial biofilms gets highly absorptive to available heavy metals [19]. The negatively charged functional groups present in EPS helps in immobilization of the heavy metal ions [20].

There are few studies implemented for bioremoval of Zn and Mn by a bioreactor-based approach were substrates such as polypyrrole, modified nanoparticles and zeolite have been used [21,22]. All the substrates have shown to possess the removal capacity of toxic compounds but lack behind due to cost effectiveness and release of hazardous chemicals in the environment which leads to more destruction. In this study, there has been no use of any such chemicals that can further interfere which nature's geological balance. Rather, we have used an ecofriendly biosystem product for the treatment of the heavy metals. Even the secondary biological treatment is always ecologically and economically preferable when compared to the tertiary chemical treatment.

In this study, the indigenous bacterium isolated from the tannery sludge of Common Effluent Treatment Plant, Ranipet, India was used for the bioremoval of Zn and Mn. A pilot scale bioreactor was designed and the peels of *Cucumis sativus* was used for the substrate for the development of biofilm with the bacterium. Cucumber has been shown to uptake higher concentrations of lead (60–600 ppm) in hydroponic condition [23]. Despite being a worthy transporter of metals, cucumber is mostly used as its saps are easy to collect. Isotherm kinetics was studied for the adsorption of heavy metals by the substrate. The toxicity study of the sorbate was performed by seed germination assay before and after the treatment with synthetic zinc and manganese solution. The effective bacterial strain was characterized by morphological, biochemical and molecular methods.

2. Materials and Methods

2.1. Sample Collection

The tannery sludge was collected from the Common Effluent Treatment Plant (CETP), Ranipet (12.9275° N, 79.3302° E) at Vellore, Tamil Nadu, India. The sludge sample was collected using sterile polyethylene bags and was processed in the laboratory within 2 h for the isolation of heavy metal resistant bacteria.

2.2. Isolation, Identification and Characterization of Multi-heavy Metal Resistant Bacteria

The sludge samples were inoculated in Luria-Bertani (LB) broth amended with 100 mg/L Zn and Mn in the form of zinc chloride and manganese sulfate salts in Erlenmeyer flask. The flasks were incubated for 5 to 7 d in a rotary shaker at 120 rpm at 28 ± 2 °C to enrich the cultures. Upon incubation, 0.1 mL of the enriched culture was spread on LB agar plate supplemented with 100 mg/L of Zn and Mn. The isolates obtained were assessed for biochemical analyses, Gram staining and motility [24].

2.3. Determination of Maximum Tolerance Concentration (MTC) and the Growth Kinetics of the Isolates

For the determination of maximum tolerance of the isolates, 5 mL LB broth was prepared with zinc and manganese concentration ranging from 100 to 3000 mg/L. The seed culture was inoculated and incubated for 24 to 72 h in the shaker at 120 rpm. Uninoculated broth served as control. After the incubation period, the drop-plate method was performed to confirm the viability of cells in the nutrient agar plate and was incubated for 24 h [25].

The influence of Zn and Mn together on the isolates was studied by comparing the growth pattern of the isolates in the presence and absence of metals. Zn and Mn were simultaneously used in the media for all the experiments. The concentration of the metals was maintained 50 mg/L. A volume of 2 mL of bacterial culture with 0.5 OD (1.5×10^8 CFU/mL cell count) was used for inoculation in LB broth and the flasks were incubated in a rotary shaker at 28 ± 2 °C. Growth was monitored by assessing the cell density by UV Spectroscopy at every 2 h interval until the cells reached the decline phase [26].

2.4. Biofilm Assay for the Isolates

The qualitative estimation of biofilm producing bacteria was performed using the test tube method. An overnight seed culture of the effective bacterium was inoculated in LB broth and was kept for 24 h in shaking condition. The test tubes were decanted and washed with deionized water followed by phosphate buffer solution. The tubes were stained with 0.1% crystal violet and kept still for 2 min. The tubes were rinsed with distilled water and dried at 60 °C for 1 h. A violet film formation on the walls of the test tube indicates the development of biofilm [27].

2.5. Scanning Electron Microscopy (SEM) of the Substrate

SEM was performed to observe the presence of the biofilm on the substrate. The samples were placed in 1 cm × 1 cm glass slide and were fixed with 4% glutaraldehyde for 2 h and air dried. The specimen was sputter coated with gold and was viewed on the Carl Zeiss SUPRA 55 Field Emission SEM at an accelerating voltage of 5 kV with different magnifications [28]. The quantification of the amount of metal uptake by the substrate was determined by OXFORD Energy-dispersive X-ray spectroscopy (EDX).

2.6. Experimental Set-up

A bioreactor was designed using bottles made of polyethylene terephthalate. The dimension of the bioreactor was 72 × 18 cm with an outlet and inlet. A peristaltic pump was used at the outlet of the reactor for recycling the broth and an AC/DC converter (power pack) along with fiber pipes was used for connecting the components. The peels of *Cucumis sativus* was used as the substrate in the bioreactor (Fig. 1).

2.7. Biofilm Formation on the Substrate

The overnight seed culture of the effective bacterium was prepared

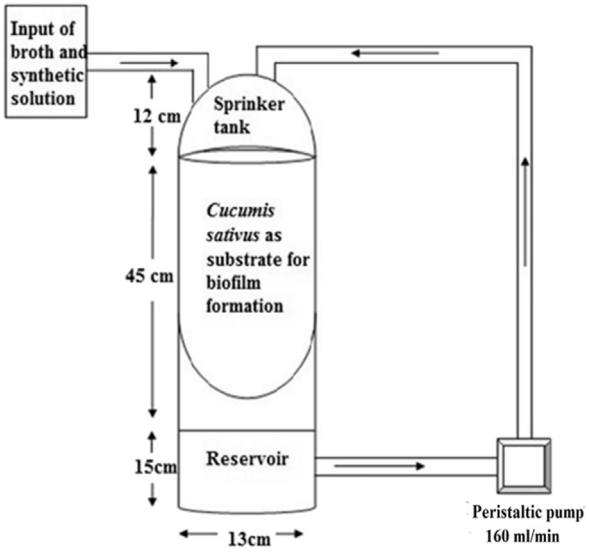

Fig. 1. Schematic representation of the packed bed bioreactor.