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Research article

Harnessing the bio-mineralization ability of urease producing *Serratia marcescens* and *Enterobacter cloacae* EMB19 for remediation of heavy metal cadmium (II)

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ABSTRACT

In the present study, urease positive *Serratia marcescens* (NCIM2919) and *Enterobacter cloacae* EMB19 (MTCC10649) were individually evaluated for remediation of cadmium (II) using ureolysis-induced calcium carbonate precipitation. Both the cultures were observed to efficiently remove cadmium from the media through co-precipitation of Cd (II) and Ca (II). *S. marcescens* and *E. cloacae* EMB19, respectively showed 96 and 98% removal of initial 5.0 mg L^{-1} soluble Cd (II) from the urea and $CaCl_2$ laden media at 96 h of incubation period. At higher Cd (II) concentrations of 10 and 15 mg L^{-1}, cadmium removal efficiency was much higher in case of *E. cloacae* EMB19 compared to *S. marcescens*. *In-vitro* cadmium (II) remediation study using urease containing cell-free culture supernatant of *S. marcescens* and *E. cloacae* EMB19 showed respective 98 and 53% removal of initial 50 mg L^{-1} Cd (II) from the reaction mixtures in co-presence of Ca (II). While in sole presence of Cd (II), only 16 and 8% removal of Cd (II) were detected for *S. marcescens* and *E. cloacae* EMB19, respectively. The elemental analysis of the co-precipitated mineral products using Energy Dispersive X-ray spectroscopy (EDX) clearly showed the prevalence of Ca and Cd ions. The morphology Cd-Ca composites formed with respect to both the cultures were observed to be of different shape and size as revealed through Scanning Electron Microscopy (SEM). Entire study hence comes out with a sustainable bioremediation option which could be effectively used to tackle Cd (II) or other heavy metal pollution.

1. Introduction

Heavy metal pollution is one of the serious environmental problems today. Though heavy metals are naturally found in environment and some are essential as trace elements for normal metabolism and survival of living beings (Colin et al., 2012; Yang et al., 2016). However, with rapid industrialization and urbanization there is tremendous increase in their concentrations and thus are of great concern.

At high concentrations, heavy metals alter the normal function of ecosystem through their toxic effects on living beings (Colin et al., 2012; Zhu et al., 2016a). The other major problems associated with heavy metals are their high persistence, unlike organic pollutants they cannot be degraded or completely removed from the environment and hence are continuously accumulated (Li et al., 2014). These and other different adverse effects of heavy metals, necessitates the development of suitable technologies for their safe remediation or disposal (Colin et al., 2012; Li et al., 2014).

Conventional physico-chemical methods used for remediation of heavy metals includes electro-chemical precipitation, membrane separation, adsorption, ion-exchange, electroflotation, electro-coagulation, evaporation, and reverse osmosis (Bazrafshan et al., 2015; Azimi et al., 2017). But high operational costs, high chemical and energy consumption along with generation of secondary pollutants has shifted the focus to better alternatives i.e. bioremediation based methods (Bhattacharya and Gupta, 2013; Azimi et al., 2017).

Bioremediation or microorganisms-mediated remediation alleviates pollutants in a sustainable way and is thus considered better over physico-chemical and other disposal methods (Bhattacharya

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and Gupta, 2013; Hou et al., 2014; Khan et al., 2017; Megharaj and Naidu, 2017). Bioremediation methods such as biocoagulation, bioaccumulation, bioleaching, biosorbents, and immobilization results in remediation of heavy metals without much alteration in natural properties of environment and are comparatively much cheaper and efficient (Bhattacharya and Gupta, 2013; Bhattacharya et al., 2014; Anbu et al., 2016). Realizing the wider implications of bioremediation, tremendous research is going in this area. Natural diversity of microorganisms and its metabolic potential is increasingly exploited to develop novel bioremediation techniques with greater efficiency and sustainability. Nevertheless, these methods too suffer from some limitations like re-release of immobilized or adsorbed metals back to the environment and change in valence state of metals to form species with varying toxicity and mobility due to alterations in pH or redox potential of the media/system (Achal et al., 2012; Anbu et al., 2016). Phytoremediation is another eco-friendly method for removal of heavy metals using growing plants. Major constrains associated with phytoremediation includes limitations of growing conditions for plants and long repair cycle time (Zhao et al., 2017).

In the midst of different bioremediation methods, microbial induced calcite precipitation (MICP) based removal of free metal ions is a newer technique of metal remediation and is capturing interest among researchers (Zhao et al., 2017). It results in mineralization of toxic metals from mobile species into stable minerals form and thus reduces metals mobility and toxicity (Zhu et al., 2016a; Zhao et al., 2017). It is one of the efficient, economic, and eco-friendly methods of heavy metal remediation (Anbu et al., 2016). The process is active and observed in almost all the environmental conditions exist on an earth (Achal et al., 2011).

The basic mechanism associated with MICP is based on enzyme urease (EC 3.5.1.5) produced by microbes, the enzymes act on its substrate (urea) to ultimately form carbonic acid (H_2CO_3), which further dissociates to form bicarbonate (HCO_3^-) and carbonate (CO_3^{2-}) ions. The carbonate then reacts with free metals to form respective metal carbonates (Achal et al., 2011; Li et al., 2013) or metal may co-precipitated with calcium carbonate in presence of calcium ions (Achal and Pan, 2011; Kumari et al., 2014).

$$CO(NH_2)_2 + H_2O \xrightarrow{U} NH_2COOH + NH_3 \qquad \text{U:Urease}$$

$$NH_2COOH + H_2O \rightarrow NH_2 + H_2CO_3$$

$$H_2CO_3 \leftrightarrow H^+ + HCO_3^- \text{(Rapid)}$$

$$HCO_3^- \leftrightarrow H^+ + CO_3^{2-}$$

$$CO_3^{2-} + M^{2+} \rightarrow MCO_3 \downarrow \qquad \text{M:Metals/Ca}$$

$$NH_3 + H_2O \leftrightarrow NH_4^+ + OH^- \text{(Increase the pH)}$$

Increased pH shifts the HCO_3^- equilibrium to formation of CO_3^{2-} ions.

Microbial based calcite precipitation is explored by number of researchers for formation of diverse calcium carbonate forms (Dhami et al., 2014; Achal and Mukherjee, 2015; Wong, L.S, 2015; Dapurkar and Telang, 2017). However, use of this process in remediation of toxic metals is quite recent and there are limited numbers of reports on this aspect. For instance, the ureolysis capabilities of different fungal strains (*Aspergillus* sp. UF3 and *Fusarium oxysporum* UF8) were tested for mineralization of heavy metal Pb and radionuclide Sr through carbonate precipitations by Dhami et al. (2017). Similarly, Mugwar & Harbottle (2016) illustrated remediation of Zn, Cd, Pb, and Cu by *Sporosarcina pasteurii*, through urease mediated calcite ($CaCO_3$) precipitation. Yang et al. (2016) tested Cu, Pb, and Cd remediation from mine tailing soil using indigenous ureolytic and calcifying bacterium *Bacillus firmus*. The effectiveness of *Lysinibacillus sphaericus* CH-5 for MICP based removal and precipitation of Cd is tested by Kang et al. (2014). Li et al. (2015) further examined urease positive fungi *Pestalotiopsis* sp. and *Myrothecium gramineum* isolated from calcareous soil for their properties of $CaCO_3$ and $SrCO_3$ biomineralization.

To the best of our knowledge there is no report on use of ureolytic *Serratia* species for remediation of metals using MICP based approach. Similarly, there are limited reports on use of ureolytic *Enterobacter* species for remediation of metals (Kang et al., 2015, 2016). The present study thus was attempted to study the potential of urease producing cultures of *Serratia marcescens* and *Enterobacter cloacae* EMB19 for remediation of toxic heavy metal cadmium (II). The comparison of metal removal efficiencies of the two microbes and characterization of their mineral products using SEM and EDX were accomplished. Finally an *in-vitro* study using urease containing cell-free supernatant of both the cultures was carried out to confirm the role of urease in metal precipitation and remediation.

2. Materials and methods

2.1. Materials

The media components were obtained from Hi- Media Laboratories (Mumbai, India). Analytical grade cadmium chloride ($CdCl_2$) was used as source of Cd (II) and procured from SISCO research laboratories (Mumbai, India). All other chemicals used were also of analytical grade.

2.2. Microorganisms

Extracellular urease producer and heavy metal tolerant *Serratia marcescens* (NCIM 2919)-NCBI GenBank accession No. KR185843 (16S rRNA) and *Enterobacter cloacae* EMB19 (MTCC 10649)-NCBI GenBank accession No. JF281095 (16S rRNA) were used throughout the study. Both the cultures were maintained on nutrient agar slants at 4 °C. They were repeatedly sub-cultured at an interval of 20 days.

2.3. Mother culture preparation

Nutrient media (pH 7.5) containing (g L^{-1}): Peptone, 5; NaCl, 5; Yeast extract, 1.50; Beef extract, 1.50 was used for preparation of mother cultures. Aseptically loop-full stock cultures of *S. marcescens* and *E. cloacae* EMB19 were individually transferred to above nutrient media and were kept for incubation at 30 °C and 150 rpm in an orbital shaker (Kuhner, Switzerland). The overnight grown cultures of individual strains were used as inoculums for further studies.

2.4. Media and culture conditions

Sterile nutrient media (50 mL) containing 25 mM (2774.5 mg L^{-1}) $CaCl_2$, 2% *(v/v)* (333.0 mM or 20,000 mg L^{-1}) urea, and 5.0 mg L^{-1} (0.0445 mM) of Cd (II) were separately seeded with two percent inoculum of *S. marcescens* ($A_{600\ nm}$—1.2) and *E. cloacae* EMB19 (A_{600nm}—1.0). The resultant inoculated media contained in 250 mL Erlenmeyer flasks were then kept for incubation at 30 °C and 150 rpm in an incubator for 96/120 h. Three types of control setups (C1, C2, and C3) were also run in parallel to experimental samples.