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Reduction of selenite to red elemental selenium by moderately halotolerant *Bacillus megaterium* strains isolated from Bhitarkanika mangrove soil and characterization of reduced product

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ABSTRACT

Two Gram (+) bacterial strains, BSB6 and BSB12, showing resistance and potential for Se(IV) reduction among 26 moderately halotolerant isolates from the Bhitarkanika mangrove soil were characterized by biochemical and 16S rDNA sequence analyses. Both of them were strictly aerobic and able to grow in a wide range of pH (4–11), temperature (4–40 °C) and salt concentration (4–12%) having an optimum growth at 37 °C, pH ~7.5 and 7% salt (NaCl). The biochemical characteristics and 16S rDNA sequence analysis of BSB6 and BSB12 showed the closest phylogenetic similarity with the species *Bacillus megaterium*. Both the strains effectively reduced Se(IV) and complete reduction of selenite (up to 0.25 mM) was achieved within 40 h. SEM with energy dispersive X-ray and TEM analyses revealed the formation of nano size spherical selenium particles in and around the bacterial cells which were also supported by the confocal micrograph study. The UV–Vis diffuse reflectance spectra and XRD of selenium precipitates revealed that the selenium particles are in the nanometric range and crystalline in nature. These bacterial strains may be exploited further for bioremediation process of Se(IV) at relatively high salt concentrations and green synthesis of selenium nanoparticles.

1. Introduction

Selenium is of considerable environmental importance as it is essential at low concentrations but toxic at high concentrations for animals and humans, with a relatively small difference between these values (Fordyce, 2005). Selenium occurs in different oxidation states as reduced form (selenide, Se^{2-}), least mobile elemental selenium (Se^0) and water soluble selenite (SeO_3^{2-})/selenate (SeO_4^{2-}) oxyanions. The seleniferous agriculture drainage and effluents from thermal power stations, oil refineries, smelting plants, glass production, pigments and semiconductor industries are the major sources of water soluble selenium species in aquatic environment (Kashiwa et al., 2000; Siddique et al., 2006). As such it is necessary to develop suitable methods to reduce the concentrations of these oxyanions in waste streams to permissible limits before their discharge into aquatic environment. In addition to physicochemical methods (Zhang et al., 2005; Rovira et al., 2008) like chemical precipitation, catalytic reduction and adsorption/ion exchange, there are several reports describing the bacterial reduction of selenite/selenate to less toxic elemental selenium and can form a viable and cost effective approach for abatement of excess selenium in contaminated water. A wide variety of bacteria occurring in diverse terrestrial and aquatic environments, both selenium-rich and selenium-free soils, have the ability to reduce aqueous selenite/selenate oxyions (Kessi et al., 1999; Ike et al., 2000; Kashiwa et al., 2000; Siddique et al., 2006; Narasingarao and Haggblom, 2007; Ghosh et al., 2008; Dhanjal and Cameotra, 2010). However, the potential of salt tolerant bacteria for reduction of selenite or selenate (Rathgeber et al., 2002; Borg et al., 2009) and other anions (Okeke et al., 2002) in high salt concentrations has been relatively less explored.

The mangrove is a typical costal biome located in the transition area in between land and sea. The periodic tidal flooding makes the salinity and nutrient content of mangrove highly variable and thus favours the growth of genetically diverse groups of aquatic and

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terrestrial microorganisms having potential for biotechnological applications (Kothari, 2002; Dias et al., 2009). Both halophilic and halotolerant microorganisms of the saline environments are suitable for bioremediation or biomineralization in saline soils because they are capable to grow in high salt concentration (Ventosa, 2004). Among the different groups of bacteria, the Gram (+) bacteria are well represented in the saline habitat and the *Bacillus* sp. and *Micrococcus* sp. are mimetically dominant (Zahran et al., 1992). It has also been reported that the halotolerant *Bacillus* species have greater biotechnological potential compared to other groups of non-tolerant bacteria (Oguntoyinbo, 2007).

Keeping the above in view, the present work was aimed to isolate some of the predominant halotolerant bacterial strains with high Se(IV) resistance potential from one of the biologically diversified and less explored saline habitat, the mangrove soils of Bhitarkanika, Orissa, India. The reduction potential of the two identified *Bacillus megaterium* strains with reference to reduction of Se(IV) to elemental selenium and the characterization of reduced product by various physicochemical methods were also reported in this study.

2. Materials and methods

2.1. Reagents

All the chemicals, reagents and microbiological media used in the present study were of analytical grade. Nutrient agar (NA) and nutrient broth (NB) (Hi-media) were used as supplied. Stock solution (250 mg L^{-1}) of selenite was prepared by dissolving $Na_2SeO_3 \cdot 4H_2O$ (E. Merck) in double distilled water, 2-mercaptoethanol (0.5 M) was prepared by diluting 3.5 mL (density, 1.14) of the original solution to 100 mL with double distilled water. Concentrated sulphuric and nitric acids (Merck, GR) were used as received.

2.2. Site description, sample collection and isolation of bacteria

Mangroves of Bhitarkanika (20°30′–20°50′N latitude and 86°30′–87°6′E longitude) are situated in the Kendrapara district which extends about 139.39 km^2 in the Brahamani and Baitarani deltaic regions of Orissa. Soil samples were collected from the Bhitarkanika mangrove forest and brought to the laboratory in sealed sterile polythene packets. The pH and conductivity of the soil samples were measured using a digital pH meter (Systronics, 361) and conductivity meter (Systronics, 308), respectively. The pH and conductance of soil samples were found in the ranges 5.9–7.5 and 13.0–17.3 mS cm^{-1}, respectively. The bacteria were isolated in NA media (g L^{-1}: peptone, 5.0; NaCl, 3.0; beef extract 3.0; agar, 1.8) and at pH ~7.0 containing 4–12% NaCl (w/v) following the dilution plate technique method.

2.3. Qualitative screening of bacterial isolates and bacterial growth

A qualitative screening of selenite resistance was done to identify the most effective isolate for further study. For this NA plates supplemented with 5 mM of sodium selenite at pH ~7.5 were inoculated with different isolates. The plates were incubated for 48 h at 37 °C. The intensity of orange–red/red colour around the bacterial colonies was taken as a qualitative efficiency for the reduction of selenite to elemental selenium. Two bacterial isolates viz. BSB6 and BSB12 which developed the most intense colour were selected for further characterization and assessment of selenite reduction. The growth of BSB6 and BSB12 in NA and NB media without selenite was studied in the presence and absence of common salt (4–12%) or sea salt (5–15%) at 37 °C and pH 4–9.

2.4. Characterization and 16S rDNA sequencing of the bacterial isolates

Cultural and morphological (Ghosh et al., 2008), physiological, biochemical and sensitivity to various antibiotics of the selected strains (BSB6 and BSB12) were performed following the standard methods. Partial 16S rDNA sequencing was done at ABI 3130xl analyzer based on Sanger's dideoxy termination method at the Institute of Microbial Technology, Chandigarh, India. The sequences obtained were analyzed using BLAST (ncbi.nlm.nih.gov). The best matching sequences were retrieved from the database, aligned and similarity analysis was performed using the Clustal_X program (Thompson et al., 1997). Phylogenetic tree was constructed using the maximum-parsimony methods implemented in the program MEGA5 (Kumar et al., 2001). The resultant tree topologies were evaluated by bootstrap analysis based on 500 resamplings.

2.5. Reduction of selenite (SeO_3^{2-})

The assay for selenium reduction was carried out in 100 mL NB as a static culture taken in 250 mL Erlenmeyer flasks and incubated at 37 °C in a BOD incubator. The BSB6 and BSB12 strains were cultured in the NB medium (pH ~7.5), at 37 °C for 8 h, centrifuged (8000*g*, 10 min at 4 °C) and the pellet was resuspended in 2 mL of Tris–HCl buffer (pH, 8.0). The cell suspension (100 μL) was inoculated into the test medium (100 mL) containing 0.05–2.0 mM of SeO_3^{2-}. At a fixed selenite concentration (2.0 mM), the pH and salt concentration were also varied in the ranges 4–10 and 4–12% w/v, respectively. Control experiments without selenite were performed simultaneously. At regular intervals, 2 mL culture was withdrawn from each flask and centrifuged at 10,000*g* for 10 min at 10 °C and the remaining selenite content in the supernatant was analyzed.

Selenium contents both in supernatant and pellet fractions were determined. The concentration of SeO_3^{2-} in the supernatant fraction was estimated spectrophotometrically as selenite equivalent following Afsar et al. (1989) using 2-mercapto ethanol (0.05 M) as the reducing/complexing agent and/or Kessi et al. (1999). For the estimation of total selenium reduced, the pellets associated with selenium particles were centrifuged, washed with deionized water and subjected to an acid digestion for 5 min with 10 mL of HNO_3 and 0.5 mL of H_2SO_4 followed by reduction of selenate with 6 M HCl at 100 °C for 30 min following Ike et al. (2000). The selenite content in the acid digested solution was estimated as described above.

2.6. Characterization of reduced elemental selenium

The bacterial cells associated with selenium particles, after 48 h incubation in presence of selenite (0.25 mM) at optimum pH (~7.5) and temperature (37 °C), were filtered through polycarbonate micropore filters (0.22 μm) and washed with Tris–HCl buffer (pH ~8.0) three times and fixed with 3% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 60 min. The suspension was centrifuged at 10,000*g* for 10 min at 4.0 ± 0.1 °C and the pellet was washed with Tris–HCl buffer followed by deionized water three times. The samples were dehydrated with 70% ethanol, mounted on an aluminium stub, coated with gold and examined under JEOL (840A, Japan) SEM at 200 kV. Analysis of elemental selenium was also carried out simultaneously by energy dispersive microanalysis (EDX).

For TEM, the 48 h culture grown in the presence of 0.25 mM selenite was centrifuged, washed three times with phosphate buffer saline (pH 7.4) followed by re-suspension in 1 mL of fixative solution (2.5% glutaraldehyde and 2% formaldehyde) and kept for 6 h at 4 °C. The post fixation and fixative solutions were removed by centrifugation. The cell pellet was then washed five times with phosphate buffer saline (pH 7.4) and finally re-suspended in 1 mL of