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Aerobic Chromate Reducing *Bacillus cereus* Isolated from the Heavy Metal Contaminated Ennore Creek Sediment, North of Chennai, Tamil Nadu, South East India

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Abstract: Ennore creek sediments are often severely polluted with the heavy metal chromium. Several chromium-resistant bacteria were isolated and identified from the sediments and all the isolates exhibited multi-metal resistance. Chromate reduction assay showed that the isolated *Bacillus cereus* ECD strains were able to reduce chromate up to 76.1% at a concentration of 100 μg mL^{-1}. Growth of the *Bacillus* species in the presence of increasing concentration of chromate was also determined. Since the *Bacillus cereus* ECD strain has high resistance to chromate and other metals and also reduces chromate under aerobic conditions, this bacterium might be potentially applicable for the treatment of metal-based industrial effluents.

Key words: *Bacillus cereus*, chromate reduction, Ennore creek, heavy metals, metal tolerance

INTRODUCTION

Chromium is the seventh most abundant element on the earth's crust and exists in several oxidation states like most stable trivalent Cr (III) and hexavalent Cr (VI) species, with different chemical characteristics and biological effects (Mcgrath and Smith, 1990; Cervantes *et al*., 2001). Chromate is most toxic than trivalent chromium because of its high solubility, rapid permeability, ready interaction with proteins and nucleic acids, mutagenicity and carcinogenic properties. Due its many unique properties, the use and dispersion of chromate have increased vastly, resulting in extensive pollution in the soil, sediment and ground waters (Srinath *et al*., 2002).

Conventional methods for reduction/removal of chromate from industrial wastes include chemical reduction followed by precipitation, ion exchange and adsorption on activated coal, alum, kaolin and ash. However, most of these methods require high energy or large quantities of chemical reagents and produce secondary waste streams that require remediation (Komori *et al*., 1990; Viamajala *et al*., 2002).

Bioremediation is one of the promising technologies that are expected to play an important role in removal of chromate from industrial wastes/contaminated sites. The bioremediation strategy is to detoxify chromate in the wastes to reduce it to trivalent chromium. Microorganisms can play an

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important role in the detoxification and removal of chromate from the polluted sites. It has been reported that chromate is reduced to trivalent chromium by a number of bacterial species like *Enterobacter cloacae*, *Bacillus circulans* strain EB1 *Pseudomonas* strains (Wang *et al*. 1989; Silver and Walderhaug, 1992; DeLeo and Ehrlich, 1994; Shen and Wang, 1995; Rosen, 1996; Silver and Phung, 1996; Ganguli and Tripathi, 2002; Jeyasingh and Philip, 2005). The physiological mechanisms involved in chromate reduction vary widely among species. In some cases, the enzyme-catalyzed reaction is membrane associated, whereas in others the enzyme is in the soluble form. However, the availability of effective chromate reducing bacterial strain is an essential prerequisite for the bioreduction-based remediation of chromium contaminated water or soil. Hence, the present study was aimed to isolate potential aerobic chromate reducing bacterial flora from the sediments of Ennore Creek.

STUDY AREA-ENNORE CREEK

The Ennore Creek is a brackish water body located in the northeastern part of Chennai; a part of this water body also extends southwest. The total water spread of the creek is about 800 m wide and 1 to 4 m deep, being shallow near the mouth. The direct release of effluents from industries, urban wastes, depletion of the fresh water flow and regular siltation of Ennore Creek mouth enhance the accumulation of heavy metals in the sediments of the creek and pose a threat to the ecosystem. Earlier studies have reported the presence of more than the threshold level of chromium with an average of 409 ppm and maximum of 700 ppm in the sediments of Ennore Creek (Jayaprakash *et al*., 2005). Since the study area is being considered as a major source for variety of fishes, crustaceans, mollusks and oysters, the presence of chromium compounds in the sediments would be a primary source for the biomagnification of chromium in aquatic flora and fauna and cause ill effects in those who consume the contaminated fish.

MATERIALS AND METHODS

Sediment Sampling

Surface sediment samples at four fixed stations were collected during March 2004 using Petersen grab and transported on ice to the laboratory and processed within 18-24 h.

Isolation and Identification of Chromate Resistant Bacteria

Aerobic chromate resistant bacteria were isolated by serially diluting 1 g of the sediment in sterile distilled water and 0.1 mL of the appropriate dilution were plated by spread plate technique on ZoBell Marine Agar plates (Hi-media, India) amended with 50 mg L^{-1} of chromate as K_2CrO_4. Later, the plates were incubated at 26±1°C for 24 h and observed for bacterial growth. Morphologically distinct colonies were picked, purified and made glycerol stocks.

Later the isolates were identified using fatty acid fingerprinting method. Briefly, isolates were grown in Soya bean-Casein digest agar; fatty acids were extracted, methylated and analyzed by gas chromatography (Descheemaker and Swings 1995). Finally, the fatty acid profiles of unknown isolates were compared with the library entries of the Microbial Identification System (6890N GC).

Determination of Minimal Inhibitory Concentration

Minimal Inhibitory Concentration (MIC) of chromate and other heavy metals like zinc, nickel, lead, copper, cobalt and cadmium were determined by agar dilution method (Luli *et al*. 1983). The mid-

log phase cultures of the isolates grown in LB broth were inoculated into the ZoBell Marine Agar plates (Hi-Media, India) supplemented with increasing concentrations of the aforesaid heavy metals individually (5 μg mL^{-1} to 1.62 mg mL^{-1} with the interval of 5 μg mL^{-1} (Tanaka *et al*., 1977).

Study of Antibiotic Resistance

Chromate resistant strains were picked and transferred to series of LB agar amended with increasing concentration of Ampicillin (Am), Chloramphenicol (Cm), Tetracycline (Tc), Streptomycin (Sm) and Kanamycin (Kc) individually (Am, Tc and Sm was 3.1, 6.25, 12.5, 25, 50 and 100 μg mL^{-1}, Kc 2.15, 4.35, 8.75, 17.5, 35 and 70 μg mL^{-1} and Cm 1.85, 3.75. 7.5, 15, 30 and 60 μg mL^{-1}) (Cruickshank, 1968). Later, the plates were incubated at 26±2°C for 24 h and observed for the bacterial growth.

Isolation and Curing of Plasmid DNA

All the *Bacillus* species were screened for the presence of plasmids. Mini preps were made according to standard procedure Maniatis *et al*. (1989) with minor modification. Curing of the plasmids was performed by incubating the isolates overnight at 30°C in a LB broth containing 25 μg mL^{-1} of acridine orange. Samples of 0.1 mL from each culture after appropriate dilution were separately plated on LB agar plates by spread plate technique and the plates were incubated at 30°C for 24 h. To select the strains that lost antibiotic resistance, the colonies were transferred to LB agar plates amended with the respective antibiotics in appropriate concentration. The strains that had no antibiotic resistance were then analyzed for the metal tolerance.

Chromate Reduction

All the Gram-positive isolates were employed for chromate reduction by measuring the optical density at 380 nm using a spectrophotometer (Bopp and Ehrlich, 1988; Viti *et al*., 2003; Badar *et al*., 2004). Mid-log phase cultures of the isolates were aseptically transferred into the flasks containing 50 mL of LB broth supplemented with a final concentration of 100 μg mL^{-1} of chromate as K_2CrO_4. The flasks were incubated at 28°C and the aliquots were taken at different time intervals and cell-free filtrates (0.45 micron pore size) were prepared and later the optical density of the cell-free filtrates was measured at 380 nm using a spectrophotometer. Optical densities of the chromate in the cell-free solutions were used as a control.

Growth Kinetics of Gram-Positive Isolates

Growth kinetic studies were carried out by inoculating overnight cultures of *Bacillus* species in 50 mL of LB broth supplemented with final concentration of 100 and 200 μg mL^{-1} of chromate (Sangeeta and Tripathi, 2001). The flasks were incubated at 26±1°C and the growth were measured at different time intervals in terms of increase in optical density at 480 nm using a spectrophotometer (Model: Systronics 1304). The cultures grown in the absence of metal served as a control in this experiment.

RESULTS

Isolation and Identification of Chromate-resistant Bacteria

Eleven morphologically distinct chromate resistant, Gram-positive and Gram-negative bacilli were isolated from the sediment samples. Based on the fatty acid profile the isolates were identified as

Bacillus pumilus three strains designated as ECA, ECB and ECC, two *Bacillus cereus* strains designated ECD, ECE, *Bacillus sphaericus*, *Eschericha coli*, *Vibrio mimicus, Vibrio alginolyticus*, *Vibrio anguillarum* and *Vibrio parahemolyticus.*

MIC to Metals

MIC of chromate and other heavy metals for the isolates was determined and the results are presented in Table 1. *B. cereus* ECD showed higher tolerance to chromate, zinc, nickel, lead and copper. The strains *B. pumilus* ECC and *B. cereus* ECE exhibit higher tolerance to cobalt and cadmium. Nickel and lead showed less toxic to almost all the Gram positive isolates when compared to other heavy metals. In general Gram negative isolates were sensitive to all the heavy metals tested. Gram positive isolates were selected for further studies.

Growth Kinetics

Growth response of the *Bacillus* species in the presence of chromate was presented in the Fig. 1a-f. All the strains were able to grow and exhibit difference in growth phase according to the increasing concentration, indicating that the growth of the isolates were slightly changed in the presence of chromate. Marked difference were observed in the lag phase of *Bacillus pumilus* ECC, where as, in *Bacillus sphaericus* differences were observed in all the phases. *Bacillus pumilus* ECA strain exhibit longer lag phase at increasing concentration. Whereas, in *Bacillus pumilus* ECB and *Bacillus cereus* ECE difference were observed only after the lag phase according to the increasing concentration.

Antibiotic Resistance

In antibiotic resistance assay *Bacillus cereus* ECD showed resistance to Ampicllin (100 μg mL^{-1}) and Kanamycin (50 μg mL^{-1}) only. *Bacillus pumilus* strains exhibit resistance to Chloramphenicol (3.75 μg mL^{-1}) and *Bacillus sphaericus* was resistant to Ampicillin (12.5 μg mL^{-1}) and Chloramphenicol (3.75 μg mL^{-1}).

Chromate Reduction

Kinetics of the chromate reduction was carried out under aerobic condition with an initial concentration of 100 μg mL^{-1} for all the isolates. Among the isolates, *B. cereus* ECD strain reduce 76.1% of chromate followed by *B. pumilus* ECA (65%) and *B. sphaericus* (57.14%), after 72 h of incubation (Fig. 2) with reference to the original concentration in the medium under aerobic condition. These strains were used for further studies. No change in chromate concentrations was observed in the control indicating that the medium did not contain any substance to undergo abiotic chromate reduction.

Table 1: Minimal inhibitory concentration of the isolates to various heavy metals in μg mL^{-1}

Metal/Bacteria	Chromium	Cadmium	Nickel	Copper	Lead	Zinc	Cobalt
Bacillus pumilus ECA	600	10	495	355	1115	95	355
Bacillus pumilus ECB	545	10	510	335	990	105	345
Bacillus pumilus ECC	595	15	505	350	1010	95	340
Bacillus cereus ECD	610	95	815	535	1085	420	320
Bacillus cereus ECE	585	105	635	480	965	385	255
Escherichia coli	300	5	45	155	85	125	230
Vibrio mimicus	130	55	120	45	80	55	40
Vibrio alginolyticus	435	40	310	95	40	90	110
Vibrio anguillarum	330	10	325	110	60	25	255
Vibrio parahemolyticus	445	55	145	105	50	120	160
Bacillus sphaericus	605	20	750	435	855	35	255

Plasmid DNA Profile of Isolate

Plasmid profile of chromate resistant Gram positive isolates showed no visible bands, indicating the absence of lower molecular weight plasmid. After curing, there was no loss in Ampicillin and Chloramphenicol resistance in *B. sphaericus*, whereas 50.4% of *B. cereus* ECD strains become sensitive to Kanamycin and cobalt (50 μg mL^{-1}) to which it was previously resistant, indicating plasmid borne resistance.

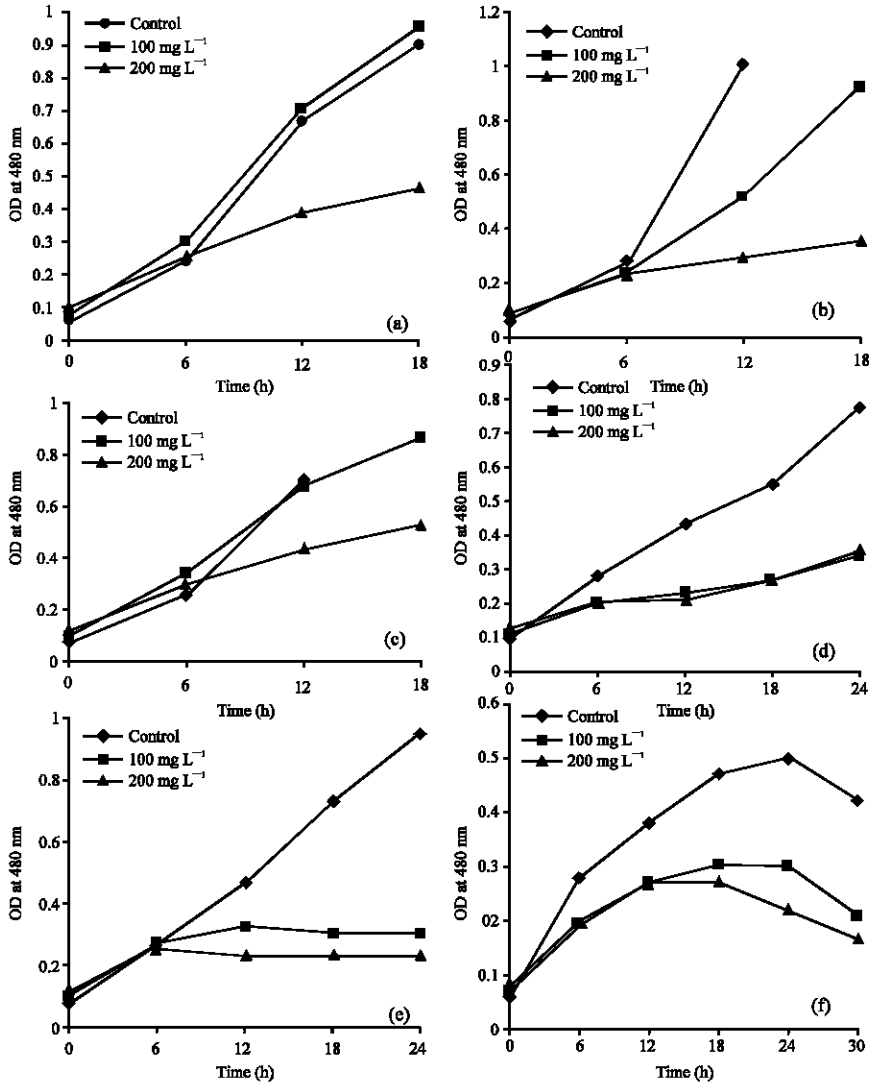

Fig. 1: a) Growth kinetics of *Bacillus pumilus* ECA strain in the presence of chromate. b) Growth kinetics of *Bacillus pumilus* ECB strain in the presence of chromate. c) Growth kinetics of *Bacillus pumilus* ECC strain in the presence of chromate. d) Growth kinetics of *Bacillus cereus* ECD strain in the presence of chromate e) Growth kinetics of *Bacillus cereus* ECE strain in the presence of chromate and f) Growth kinetics of *Bacillus* sphaericus strain in the presence of chromate

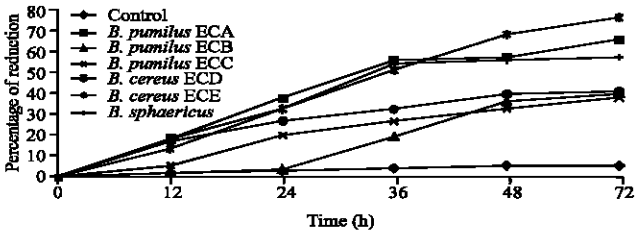

Fig. 2: Chromate reduction of *Bacillus* strains in LB broth supplemented with 100 μg mL^{-1}

DISCUSSION

Chromate resistant Gram positive and Gram negative bacilli have been isolated and identified from the Ennore Creek sediments. *Bacillus* species are the most abundant and also exhibit high resistance to almost all the heavy metals tested, which is similar to the results of earlier reports (Ross *et al*., 1981; Konopka and Zakarova, 1999) discussing the presence of *Bacillus* species in the heavy metal contaminated soils. All the isolated *Bacillus* strains were exhibited high resistance (~600 μg mL^{-1}) to chromate. However, differences in MIC of metal will also depend upon the type of the organic constituents and negatively charged ions present in the medium (Viti *et al*., 2003). Growth of the isolates in the liquid medium also depends upon the bioavailability and toxicity of the chromate. Due to its high diffusion rate, bioavailability and the inhibitory effect of chromate at high concentration, the bacterial cells show fewer differences in growth rate according to increasing concentrations of chromate in liquid media (Viti *et al*., 2003; Yilmaz, 2003; Jeyasingh and Philip, 2005).

In chromate reduction assay, *B. cereus* ECD strain showed a maximum efficacy when compared with other isolates. The decrease in chromate concentration during the log phase of the cultures may indicate the metabolic reduction of chromate. It has been noted that all the chromium resistant organisms do not have the ability to reduce chromate. Chromate reduction and resistance have been considered to be unrelated (Bopp and Ehrlich, 1988; Viti *et al*., 2003). There are many organisms in which chromate reduction is independent from chromium resistance and mediated by plasmids, but in this study it is in controversy, both chromium resistance and chromate reduction were chromosomally mediated.

Several authors have reported that the association between the heavy metal resistance and the antibiotic resistance was generally conferred by the transmissible plasmids (Hassen *et al*., 1990; Ramteke, 1997; Verma *et al*., 2001). Bhattacherjee *et al*. (1988) also reported that under metal stress, the bacteria adapt faster by the spread of R-factor than by natural selection and mutation. In our case, even though isolates shows high resistance to various heavy metals, all the isolates were mostly sensitive to the antibiotics tested, lack of plasmids in all these isolates may be the reason for high sensitivity. Tanaka *et al*. (1977) have reported the presence of large plasmids in *Bacillus* species, which can be observed only by pulse field agarose gel electrophoresis. In order to confirm whether the heavy metal resistance is mediated by larger plasmids, plasmid curing was performed for the Gram positive isolates *B. cereus* ECD, *B. sphaericus* and *B. pumilus*. Even after the curing, the isolates *B. sphaericus* and *B. pumilus* strains showed resistance to all the heavy metals tested, suggesting chromosomal encoded the resistance whereas *B. cereus* ECD showed susceptibility to cobalt, indicating plasmid borne resistance.

CONCLUSIONS

To conclude, the isolated *Bacillus cereus* ECD showed a good chromate reduction capacity. More than 76% chromate reduction was observed for 100 μg L^{-1} of chromate concentration within

72 h. MIC of the isolate clearly exhibited that the strain *Bacillus cereus* ECD have the ability to resist various heavy metals tested. This is an added advantage for isolates while performing the desired chromate reduction in the metal based industrial effluents. Other advantages of selecting bacterial based biological treatment of industrial wastes lie in the fact that it is inexpensive, does not require high energy and does not release any additional chemical to the environment.

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