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Research Article

Hexavalent Chromium Bioreduction and its Functional Characterization Using *Vigna radiata*

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Abstract

Background and Objective: Microbial remediation of agriculture and natural water resources polluted by hexavalent chromium, a hazardous heavy metal pollutant by bioaugmentation with strains from extremophilic environments is an useful strategy for the xenobiotic remediation. This study reported the isolation of environmental friendly *Bacillus* and *Pseudomonas* species from a soil sample from the Mt Bromo, Indonesia volcano rim, capable of bioreducing up to $500 \mu\text{g mL}^{-1}$ Cr(VI). **Materials and Methods:** In this study, *Bacillus* RB1, RB2 and RB3 and *Pseudomonas* RB4 sp., isolated from volcanic rim sample of Mt Bromo, East Java, Indonesia were analyzed for Cr(VI) tolerance, bioreduction using the diphenylcarbazide assay, antibiotic sensitivity, biofilm formation and functional seed germination bioassay with *Vigna radiata*. **Results:** The isolates were characterized to be Cr(VI) tolerant (upto 2 mg mL^{-1}); halotolerant (upto 6% w/v), antibiotic sensitive and strong biofilm formers in the presence of Cr(VI). All the isolates were capable of reducing $500 \mu\text{g mL}^{-1}$ Cr(VI) by upto 66%. *Pseudomonas* RB4 also enhance seed germination of *Vigna radiata* by 70% in comparison to uninoculated Cr(VI) treated control. **Conclusion:** Microbial isolates from Mt Bromo, Indonesia volcanic rim sample were characterized to be hardy and capable of tolerating toxic Cr(VI) concentrations as well as possessing Cr(VI) reducing abilities. Such isolates from extreme environments are promising candidates to be developed as bioremediation strains for Cr(VI) polluting effluent treatments as they would be capable of surviving in harsh in field environments for successful and effective bioremediation strategies.

Key words: Hexavalent chromium, bioremediation, volcano, biofilm, bioreduction

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Microbial biodiversity provides immense opportunities for bioresource prospecting particularly for the bioremediation of environmental pollutants such as heavy metals¹. One of the major drawbacks of laboratory strains studied for their bioremediation properties is their lack of hardiness when applied in the environment. Bioaugmentation using microbial resources from hardy environments presents an effective means of overcoming these problems².

Chromium maybe present in nature due to erosion of soils containing chromium or via volcanic eruptions³. Investigations have reported that the heavy metal concentrations are highest near the source of volcanic emission which decreases with increasing distances. Cr and As were reported to at levels of 20.71 and 7.6 ppm near Masay volcano, Nicaragua⁴. The present investigation reports hexavalent chromate bioremedial activities of microbial isolates from the volcanic soil near the rim of Mt Bromo, East Java, Indonesia.

Hexavalent chromium [Cr(VI)] is a major polluting heavy metal of river Ganges along Kanpur city, which is famous for its leather and tanning industry⁵. In many instances, the industrial effluent is released directly or indirectly into the natural water resources. Chromium (Cr(VI)) is highly water soluble, carcinogenic and toxic to all biological forms. Its highly oxidative properties causes damage to various biomolecules like DNA and protein⁶.

Biological remediation of Cr(VI) to non toxic oxidative states of Cr(IV) and Cr(III) has been reported to be highly effective, non toxic as well as ecofriendly⁷. Several bacterial and fungal strains have been isolated that are capable of reducing Cr(VI) to non toxic oxidative states⁸. The major drawback with such laboratory isolates remains their poor viability in the face of adverse stresses in the field environments. Isolation of indigenous chromate reducing microorganisms or bioaugmentation of polluted sites using hardy Cr(VI) resistant and bioreducing strains are two potential strategies to ensure the survival of laboratory tested bioremediation microbial isolates in the field. Bioresource mining of microbial isolates from extreme environments exposed to heavy metals provides a means of utilizing such resources for the bioremediation of industrially heavy metal polluted sites.

Agricultural land contaminated with tannery effluents containing toxic levels of Cr(VI) adversely affect soil fertility and plant growth⁹. *Vigna radiata*, an important Indian pulse crop rich in protein has been shown to be susceptible to Cr(VI) toxicity and can be used in a functional bioassay for

determining the Cr(VI) remediating capacity of microorganisms^{10,11}. The use of *Vigna radiata* seed germination bioassay has been previously used for estimating the Cr(VI) bioremediation potential of microbial isolates¹¹.

In this study, the isolation of environmental friendly *Bacillus* and *Pseudomonas* species from a soil sample from the Mt Bromo, Indonesia volcano rim, capable of bioreducing up to 500 $\mu\text{g mL}^{-1}$ Cr(VI) is reported. The use of such antibiotic sensitive, halotolerant and hardy strains capable of tolerating upto 2 mg mL^{-1} Cr(VI) concentration with bioreduction ability provides an environmental friendly means of remediating the challenges of industrial pollution.

This study reports the isolation of resilient microbial isolates from extremophiles environments that can be beneficial for adaptation to Cr(VI) bioremediation of salt stressed environments, especially soils irrigated with Cr(VI) tannery effluents. This study will help the researcher to address the critical areas in bioremediation research wherein in field experiments fail due to the isolates being vulnerable to extreme conditions present in the field that many researchers are not able to overcome. Thus, the novel component of this study involved exploring use of extremophiles for bioaugmentation based bioremediation purposes.

MATERIALS AND METHODS

Microbial Isolation and characterization: The bacterial strains were isolated from sample near the rim of the volcanic crater of Mt. Bromo, East Java, Indonesia (7.9425°S; 112.9530°E) using Tryptone Soyapeptone Agar (TSA). The isolates were maintained on TSA plates at 37°C for 24 h. The isolates obtained were identified on the basis of microbiological and biochemical characterization as per Bergey's determinative manual¹².

Halo tolerance and Cr(VI) minimum inhibitory concentration: Halotolerance was determined for each isolate on TSA supplemented with increasing concentrations of NaCl and $\text{K}_2\text{Cr}_2\text{O}_7$. Plates were incubated at 37°C for upto 3 days to record growth.

Cr(VI) reduction assay: Reduction of Cr (VI) was determined by incubating media with Cr (VI) (0.5 and 1.0 mg mL^{-1}) for upto 120 h at 29°C. The chromate reduction was determined by 1,5 diphenyl carbazide method¹³. Briefly, the samples were acidified (pH 1.0) and 1,5 diphenyl carbazide was added and Cr (VI) concentration detected by spectrophotometer (Spectronics) at 540 nm. Growth curve was plotted by measuring absorbance at A610 nm.

Biofilm formation assay: Biofilm formation was assayed as per method of O'Toole¹⁴. The biofilm and associated planktonic cell absorbance at 620 nm was measured spectrophotometrically in a microplate reader (Thermo Fisher Scientific USA).

Antibiotic sensitivity: Antibiotic susceptibility tests for each of Cr (VI) resistant isolates were performed by disk diffusion method (Hi Media) as per CLSI nomenclature¹⁵. Plates were spread with cultures and antibiotic using Combi Octadisk and incubated at 30°C. The inhibition zones were measured after 18-24 h. Isolates were characterized as resistant, moderate and susceptible following the standard antibiotic disk sensitivity method. The antibiotic tested include Cephalothin (30 µg), Clindamycin (2 µg), Co-Trimoxazole (25 µg), Erythromycin (15 µg), Gentamicin (10 µg), Ofloxacin (1 µg), Ampicillin (10 µg), Vancomycin (30 µg).

Seed germination assay: Certified seed of *Vigna radiata* were surface sterilized with 0.1% aqueous HgCl₂ solution. Sterilized seeds were treated with log phase Cr reducing isolates for 30 min at room temperature, washed with saline and resuspended in sterile distilled water. For control experiments, seed were soaked in sterile water with out inoculum for the same time. Twenty pretreated seeds were uniformly spread in a sterile Petri dishes, lined with sterile filter paper and treated with distilled water or increasing concentrations of Cr (VI).

Statistical analysis: Statistical analysis was done using student's t test. All experiments were repeated at least thrice in triplicates. $p \leq 0.05$ was considered as biologically significant.

RESULTS AND DISCUSSION

Screening and identification of isolates: The soil (1 g) from Mt. Bromo volcanic rim was serially diluted and streaked on TSA as well as TSA amended with 100 µg mL⁻¹ Cr(VI). Four types of organisms were found to grow on both Cr(VI) amended as well as non-amended plates and were designated as RB1, RB2, RB3 and RB4 respectively. RB1, RB2 and RB3 were gram positive rods and RB4 was gram negative rod. Based on microscopic, microbiological and biochemical characterization, isolates RB1, RB2 and RB3 were characterized as *Bacillus* sp. and RB4 as *Pseudomonas* sp. Several studies have been reported wherein volcanic samples have been used to isolate microorganisms for diverse applications including thermophilic enzymes as well as bioplastics¹⁶. Reports of crude oil bioremediation using over 150 thermophilic microbial isolate from volcanic sample are an example of the vast diversity and potential of microbial isolates from extreme environments¹⁷.

Cr(VI) tolerance, halotolerance and antibiogram: Minimum inhibitory concentration of Cr(VI) as well as halotolerance for the four isolates was determined by plating them on increasing salt concentration on TSA (Table 1). Isolates RB1 and RB3 were found to be 3.0 mg mL⁻¹ Cr(VI) while RB2 and RB4 was 2.0 mg mL⁻¹ Cr(VI) respectively after 48 h of incubation at 37°C. Isolate RB3 was the fastest growing strain amongst the four isolates. Additionally, RB1, RB3 and RB4 could tolerate upto 6% w/v NaCl while RB2 could tolerate only 2% NaCl w/v after 72h of incubation at 37°C (Table 2). Since the isolates were isolated for their use in environmental bioremediation, their antibiogram was determined as per the Kirby Bauer disk diffusion assay as per CLSI nomenclatures¹⁵.

Table 1: Minimum inhibitory concentration of Cr(VI) and percentage Cr(VI) reduction in TSA incubated at 37°C

Strains	0.5 mg mL ⁻¹		1.0 mg mL ⁻¹		2.0 mg mL ⁻¹		3.0 mg mL ⁻¹		Cr(VI) reduction (%)	
	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h
RB1	++	+++	+	++	+	+	-	-	58.2	61.6
RB2	++	+++	+	+++	-	-	-	-	55.5	59.3
RB3	++	+++	++	+++	+	++	-	-	59.0	62.9
RB4	++	+++	++	+++	-	-	-	-	62.3	65.6

+: Poor growth, ++: Moderate growth, +++: Heavy growth, -: No growth

Table 2: Halotolerance of isolates in TSA incubated at 37°C

Isolates	0.5% NaCl				2% NaCl			4% NaCl			6% NaCl			8% NaCl		
	24 h	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h
RB1	++	++	++	++	++	+++	+++	++	+++	+++	++	+++	+++	-	-	-
RB2	++	-	++	++	-	-	-	-	-	-	-	-	-	-	-	-
RB3	++	++	++	++	++	++	+++	++	++	+++	++	++	+++	-	-	-
RB4	++	++	++	++	+	++	++	+	++	++	+	++	++	-	-	-

+: Poor growth, ++: Moderate growth, +++: Heavy growth, -: No growth

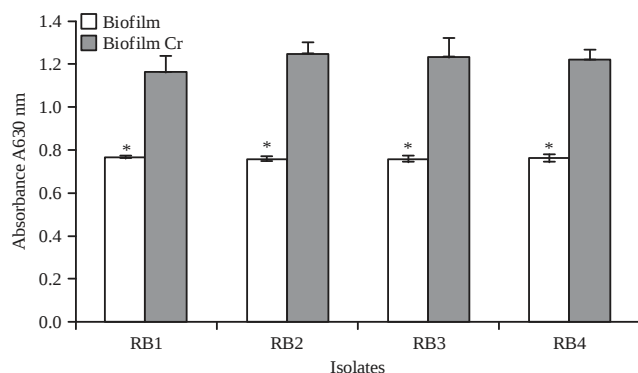


Fig. 1: Biofilm formation in TSB media in the absence and presence of 500 µg mL⁻¹ of Cr(VI).

*Indicates statistically significant differences within groups ($p \leq 0.5$)

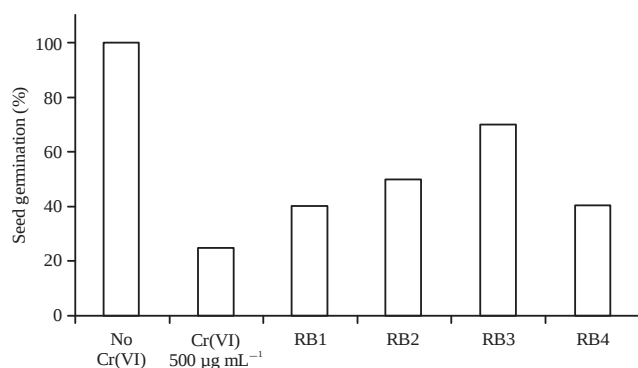


Fig. 2: Seed germination (%) of *Vigna radiata* in the presence of 500 µg mL⁻¹ of Cr(VI) following treatment with RB1, RB2, RB3 and RB4

All the isolates were found to be sensitive to the following antibiotics tested: Amoxycillin (10 µg), Cloxacillin (10 µg), Co-trimazole (25 µg), Cephalexin (30 µg), Erythromycin (15 µg) and Tetracycline (10 µg). Most environmental isolates show resistance to the second and third generation antibiotics used in this study. However, due to lack of exposure to antibiotics in volcanic rim region, these isolates have not developed any antibiotic resistance so far. Isolation of antibiotic sensitive but heavy metal resistant isolates ensures that such strains are useful for environmental free bioremediation.

Chromate reduction: The best isolates for the purpose of Cr(VI) bioremediation are those that are capable of reducing toxic Cr(VI) into less toxic Cr(IV) and Cr(III) derivatives. As the isolates were shown to be tolerant to high Cr(VI) concentrations, to bioreduce the Cr(VI) their ability were tested. The isolates were grown in TSB supplemented with 500 µg mL⁻¹ Cr(VI) and incubated at 37°C on shaker at

100 rpm. The ability to reduce Cr(VI) was tested using the diphenyl carbazide assay (DPC) for all the isolates at 500 µg mL⁻¹ Cr(VI) concentrations until 48 h post incubation. Table 1 showed that isolates *Bacillus* RB1 aerobically reduced Cr(VI) by 61.6%; RB2 by 59.3%; RB3 by 62.9% and RB4 by 65.6% respectively at 500 µg mL⁻¹ Cr(VI) concentrations after 48 h of incubation. There isn't a significant difference in bioreduction at 48 h in comparison to 24 h suggesting that Cr(VI) toxicity concentrations were reduced to levels that could be tolerated by *Bacillus* RB1 and RB2. The ability of the isolates to reduce Cr(VI) to its non toxic derivatives suggests that these are potential candidates which can be used in bioremediation strategies. There are several mechanisms at play for the aerobic reduction of Cr(VI) which primarily include intracellular or extracellular reduction of Cr(VI) via enzymatic means^{8,15}. Other mechanisms for chromate tolerance include metal sorption, uptake and accumulation, extracellular precipitation following reduction and presence of metal efflux pumps¹⁵.

Biofilm formation ability in presence of Cr(VI): One of the mechanisms by which microbial isolates tolerate high stress environments is by formation of biofilms¹⁸. Biofilms are microbial community organization that surround themselves with exopolymeric matrix comprising of polysaccharides, proteins and lipids which protect it from stressors in the external environment¹⁹. In order to determine if the isolates tolerated high Cr(VI) due to biofilm formation, the ability of isolates to form biofilm in presence of Cr(VI) was assayed by the crystal violet binding assay. All the isolates showed statistically significant increased biofilm formation in the presence of Cr(VI) ($p \leq 0.5$) (Fig. 1). The role of biofilm formation for protection against heavy metals has been previously reported¹⁹⁻²². Bioaccumulation as well as decreased cellular penetrations maybe other mechanisms that help protect microbial cells from the toxic effects of Cr(VI)⁸.

Seed germination assay: *Vigna radiata* seeds were used in a seed germination assay to study to bioremediation potential for the four isolates. Sterilized seeds (n = 20) were incubated with 10⁸ CFU mL⁻¹ of inoculums for 20 min for adsorption for biofilm formation. Seeds were then placed in sterile petri dishes with 500 µg mL⁻¹ of Cr(VI) solution. The ability of seeds to germinate was recorded and data shows that while only 25% of the seeds germinated in Cr(VI) treated samples, 40% for RB1 and RB4; 50% for RB2 and 70% for RB3 isolates was recorded for seed germination at day 2 of the assay (Fig. 2). Hence, the presence of isolates mitigates the toxic effects of Cr(VI) and helps in seed germination. The isolates were also tested for the production of plant growth promoting products

and were found to be negative (data not shown). Hence, the ability to promote seed germination is solely due to their ability to reduce toxic Cr(VI). The role of microbial remediation of Cr(VI) for improving plant growth has been reported previously¹⁰. The concurrent use of plant growth promoting and chromate reducing bacteria in microbial enhanced phytoremediation also is an effective means of improving sustainability of agricultural soils polluted with industrial effluents²¹.

CONCLUSION

All the four isolates found in the volcanic rim soils of Mt. Bromo, East Java, Indonesia showed high tolerance to hexavalent chromium as well as halotolerance. Of these, two isolates RB1 and RB2 showed chromium bioreduction activity at both 100 and 500 µg mL⁻¹ concentrations respectively. Interestingly, all the isolates were sensitive to second and third generation antibiotics and hence may be used for remediation without side effects. The isolates showed the ability to reduce Cr(VI) toxicity in a *Vigna radiata* seed germination assay. These strains may be further optimized for their ability to withstand high heavy metal stress as well as reduce Cr(VI) in environmentally polluted soils.

SIGNIFICANCE STATEMENT

In this study, we report the discovery of microbial isolates that can be beneficial for bioremediation of Cr(VI) contaminated sites and improve plant growth and soil fertility. This study will help the researcher to uncover the critical areas of bioremediation using extremophile that many researchers are not able to explore. Thus a new theory on use of bioaugmentation using isolates from extreme environments for bioremediation of environmental pollutants may be arrived at.

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