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Study on Hexavalent Chromium Reduction by Chromium Resistant Bacterial Isolates of Sukinda Mining Area

V. Mishra, D.P. Samantaray, S.K. Dash, B.B. Mishra, R.K. Swain

Post Graduate Department of Microbiology, Department of Animal Nutrition,Orissa University of Agriculture and Technology, Bhubaneswar- 751003, Orissa, India
E-mail: *dpsamantaray@yahoo.com*

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Abstract
Rapid industrialization coupled with explosive development of chemical and mining industries has not only resulted in global deterioration of the environmental quality but also has drawn attention of scientists for an effective measure to control environmental pollution. Sukinda in the district of Jajpur, Orissa has drawn worldwide attention as one of the most polluted area with chromium due to chromate rich. In the present experiment, attempt has made to detoxify Cr (VI) by chromium resistant bacterial isolates of Sukinda mining area. Random soil and water samples were collected aseptically from four different sites of the mining area and physiochemical parameters of the samples were estimated. Out of the twelve, four chromium resistant bacterial isolates *viz.*, *Micrococcus luteus*, *Pseudomonas putida*, *Serratia marcescens* and *Acinetobacter calcoaceticus* tolerated hexavalent chromium beyond 500 ppm and selected for reduction. Among all, *Acinetobacter calcoaceticus* shown highest amount of hexavalent chromium reduction of 67.14% incubated at 30°C for 24 hr at pH 7. Then *Acinetobacter calcoaceticus* was selected for parametric studies and observed to exhibit highest reduction i.e., 70.53% potential at pH 8.0, temperature 30°C/24 hr. Therefore, *Acinetobacter calcoaceticus* may be use in the bioremediation of hexavalent chromium toxicity.

***Key words*:** Bioremediation, hexavalent chromium, deterioration

Introduction

Sukinda valley of Jajpur district, the fourth most polluted place in the world (Blacksmith institute report, 2007) accounts 97% of India's chromites ore deposits. Intensive open cast mining for the last more than 70 years generated 30 million tones of hexavalent chromium wastes polluting soil, water, ruined agricultural fields and slowly poisoning the local flora and fauna including 2.6 million human population of the state. The environment is sensitive to metals due to their longevity and toxicity (Aravindhan *et al.*, 2007). Chromium, an essential micronutrient in animal physiology required for normal carbohydrate and lipid metabolism (Anderson, 1989) and also a priority pollutant is well known for the mutagenicity (Petrilli and Flora, 1977), carcinogenicity (Gruber and Jennette, 1978) and teratogenicity (Gale, 1978) of its hexavalent form in humans, experimental animals (IARC, 1990) and plants (Flora *et*

al., 1990). Cr (VI) is water-soluble, bioleachable form that can intracellularly reduced to Cr (V) and reacts with nucleic acids and other cell components to produce mutagenic and carcinogenic effects on biological systems (McLean and Beveridge, 2001). Conventional chemical treatment of Cr (VI) generated large volume of sludge, dangerous gases and expensive cost of the chemical. Reducing agents makes it imperative to look into safer and cheaper alternatives, where the metal resistant (Kasan and Baecker, 1989) microbial communities are of primary importance in bioremediation of metal contaminated sites and represent a substantial proportion of the *in situ* biomass and metabolic diversity. Bioreduction of Cr (VI) occurs directly due to microbial metabolism or indirectly by bacterial metabolites (Losi *et al*., 1994). Many scientists have investigated and demonstrated the feasibility of using biological processes for the treatment of Cr (VI) contaminated sites and industrial effluents by either pure culture or consortium of Cr (VI) reducing bacteria (Camargo *et al*., 2003; Aravindhan *et al*., 2007; Rahman *et al.*, 2007). Cr (VI) reduction by locally isolated strains and optimization of factors involved in Cr (VI) reduction carried out by monoculture of isolated bacteria to investigate its efficiency as an efficient tool for bioremediation of chromium-contaminated sites.

Methodology

Sample collection

Four different sites *viz*., Kalarangi mines, South Kaliapani mines, Kamardha mines and Dumsala canal were selected in the Sukinda mining area of district Jajpur, Orissa, located between 20°53′N-21°05′N and 85°40′E-85°53′E. Representative water samples were collected aseptically in sealed, sterile 500 ml containers from different sites. Soil and sediment samples were collected in sterile plastic bags from a depth of 5 inch below the surface and from the surface respectively during the month of February 2008 following the methods of (Zobell, 1946). Samples then transported aseptically and processed immediately in the laboratory for physico-chemical parameters analysis.

Physico-chemical parameters analysis

Physico-chemical parameter like temperature, pH, moisture content, total chromium and hexavalent chromium content were analyzed by using (Okatone) digital thermometer, (Systronics 361) pH meter, and oven drying method respectively. Hexavalent chromium content of the water samples was analyzed by 1, 5-Diphenylcarbazide (DPC) method (APHA, 1992) and the total chromium content was analyzed by acid digestion followed by (DPC) assay. The hexavalent chromium content of the soil and sediment samples were estimated by alkaline digestion followed by DPC assay and their total chromium content was analyzed by acid digestion followed by DPC assay (LATS, 2003).

Bacteriological analysis of sample

Luria Bertani broth, agar and phosphate buffer solution were used for microbial isolation and hexavalent chromium reduction studies procured from HiMedia Laboratories, Mumbai. Isolation of Chromium resistant bacteria was done by

enrichment culture technique, using Potassium dichromate as hexavalent chromium supplement in Luria Bertani broth ,samples were inoculated into the broth, incubated at 30°C overnight and were streaked onto Luria Bertani agar (LA) plates which were further incubated for 24 hr. The pure cultures of isolated strains were preserved in LA slants in vials under refrigerated (4°C) conditions and coded as CRB1 to CRB12 for further uses. The Gram-negative isolates were identified by standard biochemical tests (Collins and Lyne, 1970; Hansen and Sorheim, 1991) as per the requirements of bacterial identification software PIBWin (Byrant, 2004) and Bergey's manual of determinative bacteriology. Gram-positive isolates were identified by Api Staph strips (Biomeriux, France).

Estimation of heavy metal tolerance of bacterial isolates

For estimation of chromium tolerance molten LA medium was supplemented with Cr (VI) with final concentration ranging from 100-1100 mg/l by using filter (0.22 µm) sterilized $K_2Cr_2O_7$ solution. The isolates were streaked onto the LA plates and incubated at 30°C for 48 hr and the resistance pattern or minimum inhibitory concentration (MIC) was noted down. Of these, four isolates were selected for further reduction studies basing on their chromium tolerance. Similarly metal tolerance study was also carried by varying the concentration (100-1000 ppm) of each heavy metals like Fe^{2+},Cu^{2+}, Ni^{2+},$Hg^{2+}$and Co^{2+}.

Estimation pH tolerance of bacterial isolates

The pH tolerance test was conducted to study the cardinal pH of the chromium resistant bacteria. Five milliliter of the medium was taken in different test tubes and the pH was adjusted from 3-12 with help of 1N HCL, 1N NaOH. 100µl of the overnight culture (LB) was dispensed in to the test tubes and incubated at 30ºC for 24 hr. A loopful of overnight culture was sub-cultured on to LA plates and all the plates were incubated at 30ºC for 24 hr. Then the cardinal pH was determined from the observation.

Aerobic hexavalent chromium reduction by chromium resistant bacteria

For preliminary reduction study, selected bacterial isolates were inoculated into LB with 100 ppm of Cr (VI) and incubated at 30°C for 24 hr/100 rpm with incubator shaker (STM-225-IS). Sampling was done after 24 hr and cells were collected after centrifugation (Remi Compufuge) at 10,000 rpm for 10 minutes. Then supernatant was analyzed for residual chromium by 1, 5-Diphenyl carbazide method by measuring absorbance at 540 nm using a spectrophotometer (Systronics 104). Similarly in order to observe chromium reduction in a non-nutritive medium like Phosphate buffer solution the collected cells were washed twice with PBS and resuspended in 50 ml of PBS with 100 ppm of Cr (VI) and reduction was observed following DPC method at hourly intervals up to 6 hr and finally after 24 hr.

Optimization of pH and temperature on chromium reduction

The influences of pH and temperature on chromium reduction were assessed with LB medium and culture condition described earlier for chromium reduction. For the effect of pH, autoclaved culture medium was adjusted to pH 7 and 8 with HCL or NaOH and incubated at 30°C. Similarly keeping the optimum pH constant for reduction, temperature was varied *viz*., 20°C, 30°C and 37°C and optimum temperature was observed for chromium reduction.

Results

Physico-chemical parameters analysis

The results of physico-chemical parameter analysis of ten different samples are presented in table 1. The average hexavalent chromium content of the water, soil and sediments samples were 0.689 ppm, 39 mg/kg, 46.74 mg/kg respectively, but the chromium content of the water samples far exceeds the prescribed EPA standards i.e., 0.05 ppm. Presence of such high levels of hexavalent chromium in the water can be attributed to careless discharge of untreated wastewater from mines, rainwater running off the overburden and dumps collapsing and mixing with water in the river, which is co-related with the research report of Black Smith institute (2007).

Bacteriological analysis of sample

Twelve bacteria were isolated of which 7 were Gram positive and 5 were gram negative. The Gram-negative isolates were *Pseudomonas cepacia, Pseudomonas putida, Pseudomonas pseudomallei, Serratia marcescens, Acenetobacter calcoaciticus, Acinetobacter lwoffii,* and *Moraxella urethralis.* The Gram-Positive isolates were mostly cocci *viz*., *Staphylococcus xylosus, Staphylococcus cohniicohnii, Micrococcus luteus, Kocuria varians and Bacillus sp*.

Estimation of heavy metal tolerance of chromium resistant bacteria

Out of all the bacterial isolates, four isolates i.e., *M. luteus* (CRB6), *P. putida* (CRB8), *S. marcescens* (CRB9) and *A. calcoaceticus* (CRB10) could tolerate Cr (VI) chromium up to 500, 600, 900 and 1000 ppm respectively. Similar results have been found by most of the researcher (Srinath *et al*., 2001; Camargo *et al.*, 2003; Rahman *et al*., 2007) while working on Cr (VI) chromium reduction by different bacterial isolates. Natural habitats are generally characterized by the co-existence of a large number of toxic and non-toxic cations and therefore, it is necessary to study multiple metal effects on the physiology and biochemistry of microorganisms (Verma and Singh, 1995). *A. calcoaceticus* showed a broad range of tolerance to heavy metals like Fe^{2+}, Cu^{2+}, Ni^{2+}, Hg^{2+} and Co^{2+} up to concentration of 1000, 900, 1000, 100 and 300 ppm respectively. Apart from all the metals, the highest tolerance was observed towards Fe^{2+} and Ni^{2+}. These observations assume great significance because effluents from any metal related to industry have several metal ions or contaminants. Tolerance to other metals has an added advantage of withstanding the presence of other metal ions while performing the desired activity.

Estimation of pH tolerance of chromium resistant bacteria

The pH tolerance profile reveals that the optimum pH for most of the isolates ranges 7-9. All isolates except *P. putida* could tolerate a pH range of 4-12. Alkaline pH favours the growth of most of the isolates than acidic pH. This result corresponds to that of Camargo *et al*. (2003), who observed that isolates more tolerant to Cr (VI) grew better at pH 7-9. This might be a result of adaptation of the isolates to the natural habitat, which was mostly alkaline.

Aerobic hexavalent chromium reduction by chromium resistant bacteria

Hexavalent chromium reduction is dependent upon pH, temperature and Cr (VI) concentration. The twenty-four hours hexavalent chromium reduction result (Fig. 1) in a nutritive media (LB) reveals that out of all the four isolates, *A. calcoaceticus* reduced Cr (VI) by 67.14% at 30°C/24 hr/pH 7. Percentage of hexavalent chromium reduction by isolates *S. marcescens, M. luteus, P. putida* were 65.02, 53.14 and 50.72% respectively. Signs of chromate reduction like change in color of medium from yellow to greenish along with production of off-white residues were observed, the results were in accordance with Faisal and Shahida (2004) and Aravindhan *et al*. (2007). An interesting result was obtained by Zakaria *et al*. (2007) that *Acinetobacter haemolyticus* reduces Cr (VI) upto 100 mg/l but the rate of reduction was increased with Cr (VI) concentration i.e., upto30 mg/l. TEM studies showed that higher Cr (VI) concentration affects the shape and size of bacteria. The presence of electron dense particles in the cytoplasmic region of the organism suggested deposition of Cr (VI) in the cells. Optimum temperature and pH for Cr (VI) reduction has been reported to be 30°C and 7-7.8 by Losi *et al.* (1994) and Camargo *et al.* (2003), 30°C being the normal environmental temperature and pH 7 being the optimum pH for growth, these conditions were selected for the preliminary reduction studies.

However, the results of the hourly hexavalent chromium reduction by the selected bacterial isolates in PBS (Fig. 2) indicates that rate of reduction is directly proportional to time. *Acinetobacter calcoaceticus* reduced 20-28.5% of Cr (VI) within 3 hr, after that the rate was almost constant i.e., ranged 34.03 to 38%. This difference in trend of reduction in a non-nutritive medium (PBS) may be due to decrease in physiological and metabolic activities of the isolates (Losi *et al.*, 1994; Camargo *et al.*, 2003) and viability after some time and possible inhibition of biomass activity by prolonged chromate toxicity in a non-nutritive medium. This study indicates that the rate hexavalent chromium reduction is higher in nutritive medium (LB) than in non-nutritive medium. Therefore, optimization of pH and temperature for hexavalent chromium reduction has been conducted in a nutritive medium.

Optimization of pH and temperature on chromium reduction

It has been observed from the chromium reduction profile of all the four selected bacterial isolates, *A. calcoaceticus* reduced highest quantity of Cr (VI) in LB medium and selected for parametric study. It could

Table 1. Physico-chemical parameters of the mines sample.

SN	Sampling Sites	Type of sample	pH	Temp. (°C)	Moisture (%)	Total Cr content	Cr (VI) content
1.	Kalarangi mines	Water Soil Sediment	8.01 7.67 6.82	24 ND ND	- 2.59 27.2	0.277mg/l 5480mg/kg 5950mg/kg	0.192mg/l 66mg/kg 7.35mg/kg
2.	Dumsala canal	Water Sediment	8.00 7.62	24 ND	- 31.56	0.294mg/l 3900mg/l	0.238mg/l 66mg/kg
3.	South kaliapani mines	Water Soil Slurry	7.62 9.49 6.60	25 ND ND	- 12.5 34.22	3.515mg/l 3000mg/l 7800mg/l	2.036mg/l 12mg/kg 34mg/kg
4.	Kamardha Mines	Water Sediment	7.75 7.08	25 ND	- 36.6	0.387mg/l 4900mg/kg	0.292mg/l 119mg/kg

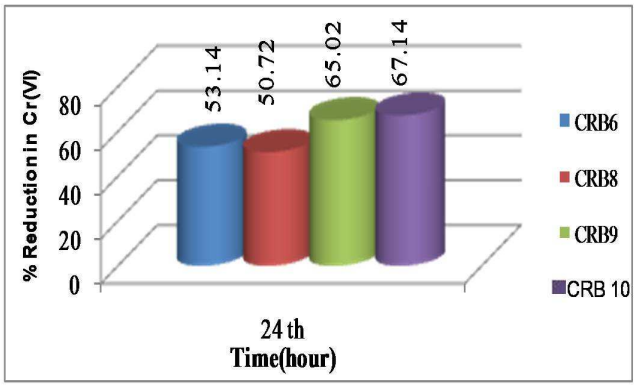

Figure 1. Hexavalent chromium reduction (24 hr) of bacterial isolates in LB.

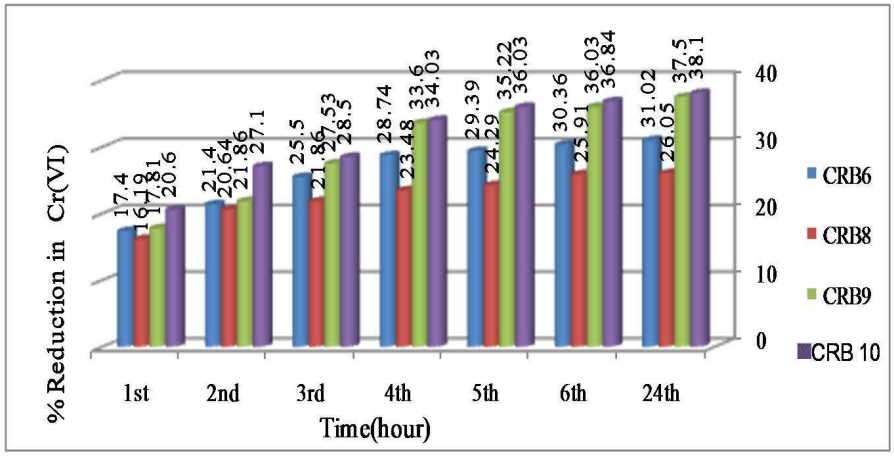

Figure 2. Hexavalent chromium reduction (24 hr) of bacterial isolates in PBS.

reduce 70.53% of hexavalent chromium optimally at pH 8, in LB within 24 hr (Fig. 3). For most of the isolates, optimum pH for growth correlates with highest rate of hexavalent chromium reduction (Camargo *et al.*, 2003). The relationship between pH and Cr (VI) reduction was not surprising because chromate is the dominant chromium species in aqueous environment at pH 6.5-9 (McLean and Beveridge, 2001). Optimum pH for growth of Cr (VI) resistant bacteria was reported at 7-7.8, but hexavalent chromium forms are soluble over a wide pH range and generally mobile in soil-water systems (Losi and Frankenberger, 1994).

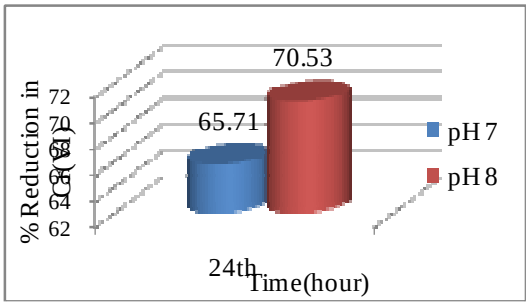

Figure 3. Hexavalent chromium reduction (24 hr) by isolate CRB 10 in LB at different pH.

The hexavalent chromium reduction profile was monitored at different temperatures ranging from 20-37°C in LB for 24 hr keeping constant the optimum pH at 8.0. Highest reduction (70.53%) was observed (Fig. 4) at optimum temperature of 30°C and percentage of reduction decreased with increase in temperature. This is possibly because of decreased enzyme activity with increase in temperature. This could be due to loss of viability or metabolic activity of the cells on prolonged incubation at higher temperature (Aravindhan *et al.*, 2007). Losi *et al*. (1994) and Camargo *et al.* (2003) who reported an optimum temperature of 30 to 37°C for chromate reduction also obtained similar results.

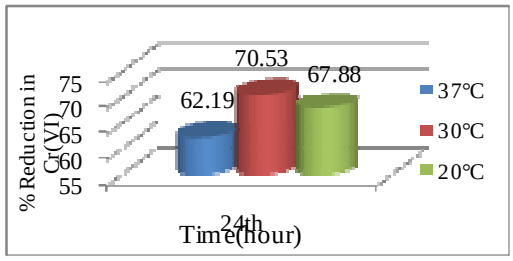

Figure 4. Hexavalent chromium reduction (24 hr) by isolate CRB 10 in LB at different temperatures.

Conclusion

The experimental observations indicate that the locally isolated strains show high Cr (VI) tolerance and demonstrate good metal removal capability. The advantages of selecting the indigenous bacteria from chromite mines of Sukinda for bioremedial purpose may be the minimization of inhibitory effects from other components that may be present along with Chromium, since viable indigenous bacterial isolates will have developed some degree of resistance to these components. It might be practical to use Cr (VI) reducing bacteria to reduce other waste metals simultaneously, which shows a positive sign for application of these locally isolated strains in the treatment or detoxification of hexavalent chromium from industrial effluents and Chromium contaminated sites. However, before exploiting the strain as an efficient biotechnological tool for chromium detoxification further investigation needs to be carried out in laboratory scale and in-situ

metal reduction potential of the Genus has to be assessed.

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