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ISOLATION AND CHARACTERIZATION OF CHROMATE RESISTANT AND REDUCING BACTERIA FROM TANNERY EFFLUENT OF CHITTAGONG, BANGLADESH

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Abstract

Context: Waste water containing Chromium (Cr^{6+}) is by far the most important environmental challenge being faced.

Objectives: The present study was planned on the isolation and characterization of chromate resistant and reducing bacterial strains in order to use them for detoxification of chromate.

Materials and Methods: Water samples were collected to isolate microorganisms from tannery effluent of Baluchara, Chittagong and inoculated into Luria-Bertani medium with added Cr^{6+} as $K_2Cr_2O_7$. The organisms have been identified and studied for Cr^{6+} reduction-ability in growth dependent manner.

Results: A total of 35 isolates have been selected as potential organism belonging to the species of *Moraxella* (14.3%), *Bacillus* (11.43%), *Streptococcus* (25.72%), *Staphylococcus* (5.7%), *Salmonella* (12.3%), *E. coli* (13.3%), *Enterobacter* (11.3%), *Hafnia alvei* (2.45%) and *Alcaligenes* (3.5%). The selected isolates were able to tolerate at least 500 mg/l of Cr^{6+}. The total Cr^{6+} concentration of the effluent sample analysed was found to be about 23.73 mg/l as determined by Atomic Absorption Spectrophotometry. Two of the isolates reduced 38% and 32% of Cr^{6+} added to the medium. Another 7 isolates showed Cr^{6+} reducing capability ranging from 18 to 22%.

Conclusion: As the isolates have turned out to successfully reduce Cr^{6+} in this study, these can be used for the development of bioremediation process.

Key words: Enzymatic reduction, Bioremediation, Chromium, Ecotoxicity, Tannery.

Introduction

In the wake of industrialization, consequent urbanization and ever increasing population, the basic amenities of life viz. air, water and land are being polluted continuously (Chhikara *et al.* 2008) . The bio-magnification of heavy metals in ecosystem is a major threat to human life (Yigit and Altindag 2006, Hooda 2007). Chromium VI (Cr^{6+}) is one of the highly toxic heavy metals. It enters into the natural water bodies through the industrial effluents creating water pollution (Singh 1994). Chromium and its compounds are widely used in electroplating, leather tanning, cement, dyeing, metal processing, wood preservatives, paint and pigments, textile, steel fabrication and canning industries. These industries produce large quantities of toxic wastewater effluents (Raji and Anirudhan 1997). The industrial effluents containing chromium compounds in hexavalent form are released directly or indirectly into natural water sources, mostly without proper effluent treatment (Shakoori *et al.* 2000). Two stable oxidation states of Chromium persist in the environment, Cr^{3+} and Cr^{6+} (Raji and Anirudhan 1997).

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Leather tanning is one of the main sectors in Bangladesh's leather industry. Large amounts of chrome powder and chrome liquor are used during tanning process. More than 1,70,000 tons of chromium wastes are discharged to the environment annually as a consequence of industrial manufacturing activities (Kamaludeen *et al*. 2003). Due to its high oxidation potential, it can easily penetrate biological membranes and cause health hazards (Chaudhary *et al*. 2003). Feeds and fertilizer production from tanned skin-cut wastes (SCW) is the most direct phenomenon of chromium ecotoxicity leading to food chain contamination in Bangladesh (Rafiqullah *et al.* 2008). The SCW is protein-rich and indiscriminately used to produce poultry and fish feeds, and organic fertilizer. It is reported that feed ingredients produced from SCW contained chromium at levels as high as 2.49% (Hossain *et al.* 2007).

Reduction of Cr^{6+} is one of the important mechanisms for its detoxification from waste water. Hexavalent Cr is known to be 100−1000 times more toxic than the trivalent form (Gauglhofer and Bianchi 1991). Bacterial biomass can be used as an economical option for removing chromium from the effluent by reduction and bioaccumulation. The organics present in effluents are utilized as nutrients for the microorganisms which in turn reduce Cr^{6+} to Cr^{3+} leading to the removal of Cr^{6+} from the environment (Singh 1994). Microorganisms are advantageous for metal detoxification as they are easy to grow, resulting in a rapid production of biomass, and are part of natural environment (Faryal *et al.* 2007). It is therefore advantageous to develop a bioprocess utilizing selected indigenous microbes that are both Cr^{6+} - resistant and Cr^{6+} -reducing. Therefore, the present study was planned on the isolation and characterization of chromate resistant and reducing bacterial strains and to compare their growth behavior in different levels of Cr concentration in order to use them for detoxification of chromate in an integrated bioremediation system.

Materials and Methods

Study area and sample collection: Samples of tannery effluent releasing from two tanneries situated at Baluchara, Chittagong were collected once a month between March to May 2010. During sampling precautions were taken to minimize cross-contamination of samples. Samples were aseptically collected in sterile bottles and transported to the laboratory of Industrial Microbiology Research Division, BCSIR Laboratories, Chittagong in an insulated box with ice to maintain a temperature ranging from 4 ºC to 6 ºC (APHA 1998). Samples were stored in ice for up to 6 h from the time of collection for transport and subsequent analysis in the laboratory.

Enrichment culture of Cr(VI) tolerant bacteria: Five milliliters of the tannery effluent collected were added to 50 ml LB broth containing chromium as Potassium dichromate ($K_2Cr_2O_7$) at different concentration (100 mg /l, 200 mg /l, 300 mg/l, 400 mg/l and 500 mg/l) for enrichment and selection and incubated for 48-72 h at 37 ºC with shaking. A volume of 100 µl from enriched culture of the samples was inoculated on Luria-Berteni (LB) agar plate with addition of 500 mg/l Cr^{6+} (as $K_2Cr_2O_7$) and incubated at 37 ºC for 4 days. From all samples and agar plates, a total of 42 colonies have been selected and identified. Those colonies were isolated by sub-culturing onto fresh LB plate with same concentration of added Cr^{6+}. The isolates were purified by restreaking further on Nutrient agar (NA) and incubated for 18-24 h at 37 ºC. Following overnight incubation in nutrient agar at 37ºC, the isolates were preserved in 30% glycerol at −20 ºC.

Cultural and biochemical tests: Cultural and biochemical tests were conducted in order to avoid the involvement of similar type of microorganism in Cr(VI) reduction studies. The shape and type of Gram reaction were microscopically studied using 18 h culture from agar plate. Other cultural tests performed included selective and differential media such as MacConkey (MAC) Agar, Sorbitol MacConkey (SMAC)

Agar, Xylose Lysine Deoxycholate (XLD) Agar, Salmonella-Shigella (SS) agar, Mannitol Salt Agar(MSA), Bismuth Sulfide agar (BSA), Streptococcus Selection agar, Cetrimide agar and Eosine Methylene Blue (EMB) Agar. Biochemical tests were performed using Kliger's Iron Agar (KIA), Simmon's Citrate agar, Motility Indole Urea (MIU), Lysine Iron agar (LIA), Urea broth, Peptone water, Methyl Red-Voges Proskauer (MR-VP) broth, Nutrient Nitrate Broth (NB), carbohydrate fermentation broth with added lactose, sucrose, glucose as sole carbon source, Starch uitilization agar, Oxidase and Catalase tests. Identification of isolates obtained from pure culture was based on Gram staining, biochemical characterisitcs and growth pattern on selective and differential media according to the procedures recommended in the Bergey's Manual of Determinative Bacteriology (Holt 1984, Ewing 1986).

Estimation of total chromium content of the effluent: Tannery sample were digested and prepared for determination of total Cr load according to Fraga *et al*. (2002). The determination of Cr was performed on Atomic Absorption Spectrophotometer using an air- acetylene flame. The flow of gases was adjusted to obtain an oxidant flame. All measurements were made at the same wavelength and using the same hollow-cathode lamp.

Cr^{6+} reduction by the strains in growth dependent manner: Nine selected isolates were studied for Cr^{6+} reduction in growth dependent manner. The strains were inoculated from pure cultures into broth medium with added Cr^{6+} to obtain resultant Cr^{6+} concentration of 10 mg /l. Before addition of Cr^{6+} into inoculums, they were incubated for one hour in shake flask culture at 150 rpm and 37ºC. Then the optical density of the inoculums is adjusted around 0.60 at 600 nm as a measure for equal enzyme activity. After adding Cr^{6+}, the cultures were again incubated for 24 h in shake flask culture at 150 rpm and 37 ºC. After that the broth cultures were centrifuged at 5000 rpm for 15 minutes. The supernatants were separated. The residual Cr^{6+} concentrations in the supernatants were measured in comparison to the initial 10 mg/l.

Assay of hexavalent chromium by DPC method: DPC solution (125 µl) was added to one ml of the supernatant collected and mixed gently. Samples then kept at room temperature for 20 min. Then the absorbance of the color produced was measured at 540 nm by spectrophotometer. A standard curve was prepared to estimate the chromium concentration using different chromium concentrations ranging from 1 to 10 mg/L. The absorbance was measured for each concentration by using the Di-Phenyl Carbazide (DPC) method (Bartlet and James 1996). Using the concentration versus absorbance, a standard curve was plotted. From the standard curve Cr concentration was determined.

Results

Isolates: Identification of isolates obtained from pure culture based on Gram staining indicated that the isolates belong to the species of *Moraxella* (14.3%), *Bacillus* (11.43%), *Streptococcus* (25.72%) , *Staphylococcus* (5.7%), *Salmonella* (14.3%), *E. coli* (14.3%), *Enterobacter* (14.3%) , *Hafnia alvei* (2.45%) and *Alcaligenes* sp. (3.5%) respectively. The percentage of different genera isolated is shown in Fig. 1.

Cr^{6+} concentration and assay: It is clearly evident that the isolates selected were tolerant to Cr^{6+} concentration up to 500 mg /l. Though the growth expression by the isolates are differential, but a general trend of growth decrease is observed with increase in Cr^{6+} concentration. The total chromium load of the tannery effluents analyzed was found to vary from 22 to 24 mg/l with a mean value of 23.73 mg/l. Hexavalent chromium solution of different known concentration ranging from 1 to 10 ppm was prepared and assayed by DPC method. A standard curve was prepared with these data which was used to determine the reducing ability of the selected strains. The standard curve constructed was shown in Fig 2.

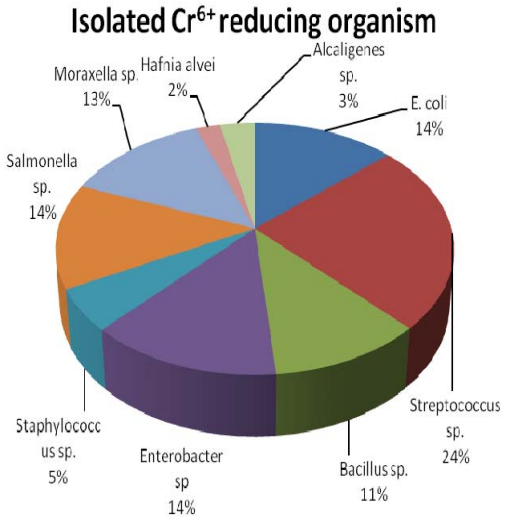

Fig. 1. Percentage of Cr^{6+} reducing genera isolated

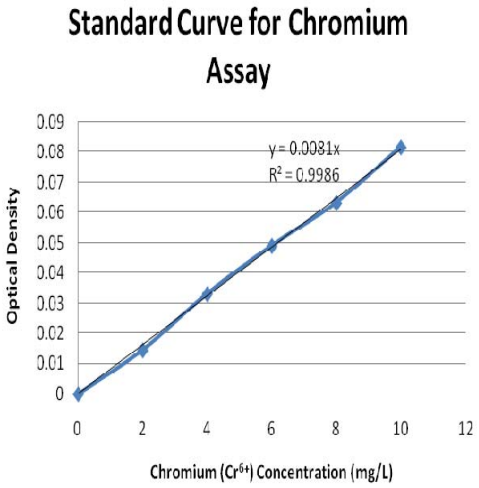

Fig. 2. Standard curve for determination of Cr^{6+} reduction

Table 1. Chromium reducing ability of the isolates (initial concentration 10 ppm)

Isolate	Identity	Percent reduction
4	*Alcaligenes* sp	20.0
8	*Hafnia alvei*	22.0
50	*Salmonella* sp.	17.0
58	*Moraxella* sp.	38.0
60	*Staphylococcus* sp.	32.0
63	*Bacillus* sp.	9.0
26	*Staphylococcus* sp.	16.0
25	*Staphylococcus* sp.	18.9
59	*E. coli*	8.0

Cr^{6+} reduction by the strains: The reducing ability varies among strains as shown in Table 1. It is clear that the selected isolates are able to reduce Cr^{6+} in laboratory environment. Of them Isolate 58 and 60 are most promising as they can reduce about 38% and 32% of Cr^{6+} added initially. They belong to the genus *Moraxella* and *Staphylococcus* respectively. Other isolates such as isolate4 (*Alcaligenes* sp.), 8 (*Hafnia alvei*), 50 (*Salmonella* sp.) and 25 (*Staphylococcus* sp.) have moderate reducing ability ranging from 18% to 22%. The rest of the isolates showed poor reducing ability, less than 10%. All of them can tolerate at least 500 mg/l Cr^{6+} in culture media. Those strains can be subjected to genetic manipulation to increase their chromium resistance capability as well as to increase their chromium reducing capability. As these organisms are indigenous of the tannery effluent, they hope to be better adapted to the environment of the effluent and will require less time for acclimation.

Discussion

Hexavalent chromium mainly causes both acute and chronic toxicity. The chromium load found in the present investigation is very high considering the fact that the natural total chromium content of surface waters is approximately 0.5–2 µg /l (WHO 1996) which in turn indicates heavy chromium pollution in tannery effluents of Bangladesh. The higher toxicity and mobility of Cr^{6+} compared to Cr^{3+} makes it to be of serious concern (Ross *et al.* 1981). The most important toxic effects, after contact, inhalation, or ingestion of hexavalent chromium compounds include dermatitis, allergic and eczematous skin reactions, skin and mucous ulcerations, perforation of the nasal septum, allergic asthmatic reactions, bronchial carcinomas, gastro-enteritis, hepatocellular deficiency, and renal oligo anuric deficiency (Baruthio 1992). In terms of carcinogenic behavior of chromium, chromate (CrO_4^{2-}) (which is a strong oxidizing agent) is reduced intracellularly to Cr^{5+} inside biological system and reacts with nucleic acids and other cell components to produce mutagenic and carcinogenic effects on biological systems (Clark 1994, McLean and Beveridge 2001).

To check chromium discharge into the environment, there are various treatment options. However, they are energy consuming and not very successful for being costly (Ohtake and Silver 1994). Besides, conventional methods for treatment of toxic chromate require a large amount of chemicals, energy and are unsuitable for small-scale leather, dye and electroplating units. In this context, biotransformation of Cr^{6+} into less toxic Cr^{3+} by certain bacteria offers a viable, economically safe and sustainable alternative (Eccles 1995). Nonetheless, development of a feasible chromate bioremediation process requires isolation of efficient chromate reducing bacterial strains, evaluation of their ability to survive, multiply and simultaneously reduce chromate in industrial waste water.

Conclusion

Reduction of very toxic Cr^{6+} to less toxic Cr^{3+} form is one of the promising approaches for combating tannery based chromium ecotoxicity in Bangladesh. Further means of bioremediation may also include biosorption and bioaccumulation of chromium by microorganisms. Development of processes coupling biological methods with chemical ones would concomitantly decrease the level of Cr^{6+} by reduction as well as precipitate Cr^{3+} through chemical reaction. This effort could make the effluents chromium free to a greater extent. As the isolates of *Moraxella sp., Staphylococcus aureus,* and *Salmonella sp.* have turned out to successfully reduce Cr^{6+} in this study, these can be used for the development of bioremediation process. But, for industrial adoption of remediation process, studies on feasible production of reductase enzyme or direct reduction of Cr^{6+} by the organisms are required for economically effective and rapid application

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