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Isolation and Characterization of Cr^{6+} Reducing Bacteria and Their Potential Use in Bioremediation of Chromium Containing Wastewater

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Abstract.- In this paper the ability of *Bacillus pumilus*, *Alcaligenes faecalis* and *Staphylococcus* sp. to reduce Cr^{6+} into Cr^{3+} was evaluated. *B. pumilus* could tolerate Cr^{6+} up to 2 mg/ml. *A. faecalis* and *Staphylococcus* sp. resisted Cr^{6+} up to 1.4 and 1.6 mg/ml, respectively. *B. pumilus* and *Staphylococcus* sp. showed optimum growth at pH 8 while *A. faecalis* showed maximum growth at pH 7. All bacterial strains showed optimum growth at 37ºC. *B. pumilus* was found sensitive against amoxicillin and tetracycline while found least sensitive against polymyxin B. *A. faecalis* was found sensitive to tetracycline while showed least sensitivity against amoxicillin. *Staphylococcus* sp. showed high sensitivity against vancomycin while minimum against erythromycin. All bacterial strains showed different degrees of sensitivity against to kanamycin, erythromycin, streptomycin, oxytetracyclin and neomycin but all bacterial strains showed resistance against bacitracin. *B. pumilus* showed 95%, *A. faecalis* showed 97% and *Staphylococcus* sp. showed 91% ability to reduce Cr^{6+} into Cr^{3+} within 24 hours from the medium containing 100 µg Cr^{6+}/ml. Bacterial strains can be exploited for bioremediation of hexavalent chromium containing wastes, since they seem to have potential to reduce the toxic hexavalent form to its nontoxic trivalent form.

Keywords: Cr^{6+} reducing bacteria; bioremediation; *Bacillus pumilus*; *Alcaligenes faecalis*; *Staphylococcus* sp.

INTRODUCTION

Environmental pollution is an important consequence of industrial processes and human activity. Heavy metals have many industrial applications due to their technological importance. Wastewaters from these industries have permanent toxic effects to human and the environment. Heavy metal contamination and the problems that it poses to the biota have been well documented (Raskin and Ensley, 2000). Accumulation of toxic metals, *e.g*., Cd, Cr, Cu, Hg and Zn, in humans has several consequences such as growth and developmental abnormalities, carcinogenesis, neuromuscular control defects, mental retardation, renal malfunction and wide range of other illnesses (Thiele, 1995).

Chromium is the seventh most abundant element on earth and exists in several oxidation states from Cr^{2+} to Cr^{6+}. In nature, chromium can be found either as Cr^{6+} or as Cr^{3+}. Cr^{3+} is rather benign and easily adsorbed in soils and waters, whereas Cr^{6+}, which is the toxic form, is not readily adsorbed and is soluble (Kotas and Stasicka, 2000). Industrial wastewaters contain both chromium and salts ions which have toxic affects on the microbial consortia of wastewater treatment systems (Stasinakis *et al*., 2003). Removal of Cr^{6+} either by reduction (Kamaludeen *et al*., 2003) or by biosorption can significantly reduces the risks to human health.

Traditional metal removing methods from industrial effluents include chemical precipitation, chemical oxidation or reduction, ion exchange, filtration, electrochemical treatment, reverse osmosis, membrane technologies and evaporation recovery (Ahluwalia and Goyal, 2007). These processes may be ineffective or extremely expensive especially when the metals in solution are in the range of 1-100 mg/l (Nourbakhsh *et al*., 1994). Therefore, it is important to develop an innovative, low cost and eco-friendly method for removal of toxic heavy metal ions from the wastewater.

Microorganisms such as bacteria, yeast, algae, protozoa and fungi are frequently found in waters receiving industrial effluents. These microorganisms have developed the capabilities to

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protect themselves from heavy metal toxicity by various mechanisms such as adsorption, uptake, methylation, oxidation and reduction. Many microorganisms have been reported to reduce the highly soluble and toxic Cr^{6+} to the less soluble and less toxic Cr^{3+}, *e.g., Acinetobacter* and *Ochrobactrum* (Francisco *et al*., 2002), *Arthrobacter* (Megharaj *et al*., 2003), *Pseudomonas* sp. (Rajkumar *et al*., 2005), *Serratia marcescens* (Campos *et al*., 2005), *Ochrobactrum* sp. (Thacker and Madamwar, 2005), *Bacillus* sp. (Elangovan *et al*., 2006), *Desulfovibrio vulgaris* (Goulhen *et al*., 2006), *Cellulomonas* spp. (Viamajala *et al.,* 2007).

The present work deals with the isolation of chromium resistant bacteria from contaminated environment, its molecular characterization, the ability of the bacteria to reduce hexavalent chromium and optimization of temperature and pH for maximum chromium reduction.

MATERIALS AND METHODS

Sample collection

Wastewater samples were collected in screw capped sterilized bottles from Hadiara drain, Lahore. Some physicochemical parameters of wastewater *viz.,* temperature (ºC) and pH were measured (APHA, 1989).

Isolation of Cr resistant bacteria

For isolation of chromium resistant bacteria, 100 µl of the wastewater sample was spread on Luria-Bertani (LB) agar plates containing 100 µg of Cr^{6+} /ml of the medium. LB agar plates were prepared by dissolving 1 g NaCl, 1 g tryptone and 0.5 g yeast extract in 100 ml distilled water, pH adjusted at 7 to 7.2 and then 1.5 g agar was added in the 250 mL flasks. The medium was autoclaved at 121ºC and 15 lb pressure for 15 minutes. The growth of the bacterial colonies was observed after 24 hours of incubation at 37 ºC. Effect of Cr(VI) on the growth of bacterial isolates was determined in acetate minimal medium which contained (g/l): NH_4Cl, 1.0; $CaCl_2.H_2O$, 0.001; $MgSO_4.7H_2O$, 0.2; $FeSO_4$ $.7H_2O$, 0.001; sodium acetate, 5; yeast extract, 0.5; K_2HPO_4, 0.5 (pH 7) supplemented with $K_2Cr_2O_7$ (Pattanapipitpaisal *et al.,* 2001). It was again incubated at 37ºC for 24 hours. This process was repeated with successively higher concentrations of Cr^{6+} until the minimum inhibitory concentration (MIC) of bacterial isolate was obtained.

Identification of the bacterial isolates

For biochemical characterization the isolates were tested for catalase activity, motility, oxidase acivity, nitrate reduction, urease activity, hydrolysis of casein, blood agar test, MacConkey agar test, utilization of citrate and different sugars, Voges-Proskauer test, and hydrolysis of starch and casein. The procedures of these biochemical tests were taken from Cappuccino and Sherman (2001).

For molecular identification, genomic DNA was isolated as described by Carozzi *et al*. (1991) and the 16S rRNA gene was amplified by PCR using two general bacterial 16S rRNA primers (RS-1; 5′-AAACTC-AAATGAATTGACGG-3′, RS-3; 5′-ACGGGCGGTGTGTAC-3′). The PCR product of 0.5kb was removed from the gel and cloned in pTZ57R/T vector. The amplified 16S rRNA gene was purified with a Fermentas purification kit (#K0513) and the amplified products were electrophoresed on 1% agarose gel. Sequencing was carried out by Genetic analysis system model CEQ-800 (Beckman) Coulter Inc. Fullerton, CA, USA. The 16S rRNA gene sequences were compared with known sequences in the GenBank database to identify the most similar sequence alignment.

Determination of optimum growth conditions

For optimum growth of the bacterial isolates, two parameters *i.e.* temperature and pH were considered. For determination of optimum temperature, 5 ml LB broth was added in 4 sets, each of three test tubes, autoclaved and inoculated with 20 µl of freshly prepared culture of each bacterial isolate. The four sets of tubes were incubated at 25ºC, 30ºC, 37ºC and 45ºC. After an incubation period of 12 hours, their absorbance was measured at 600 nm using a Lambda 650 UV/Vis Spectrophotometer (PerkinElmer, USA). For determination of optimum pH, test tubes having 5 ml LB broth were prepared in 9 sets, each containing 3 test tubes and their pH was adjusted at

5, 6, 7, 8, 9 and 10 then autoclaved. These tubes were inoculated with 20 µl freshly prepared culture of each bacterial isolate. After an incubation period of 12 hours, their absorbance was measured at 600 nm.

Effect of Cr on bacterial growth

Growth curves of bacterial isolates were determined in acetate minimal medium with (100 µg Cr^{6+}/ml) and without chromium (Control). For each bacterial isolate 50 ml medium was taken in one set consisting of 3 flasks, autoclaved and then inoculated with 20 µl of the freshly prepared inoculum. The cultures were incubated at 37 ºC in a shaker at 80-100 rpm. An aliquot of culture was taken out in an oven sterilized tube, at regular intervals of 0, 4, 8, 12, 16, 20, 24 and 28 hours. Absorbance was measured at 600 nm. Growth was plotted graphically.

Determination of antibiotic resistance

Twelve different antibiotic discs were used to check the resistance or sensitivity of locally isolated Cr-resistant bacterial isolates. For this purpose antibiotic discs were placed on agar plates with bacterial cultures. The plates were incubated at 37^{o}C. After 15 hours of incubation diameter of the clear zone around the antibiotic discs was measured with the help of scale in millimeters and results were recorded in terms of sensitive (S) or resistant (R).

Reduction of hexavalent chromium by bacteria

In order to determine the ability of bacterial isolates to reduce Cr^{6+} (100 µg/ml) to Cr^{3+}, diphenylcarbazide method was used (Fulladosa *et al*., 2006). Samples (1 ml) from cultures were taken after 6, 12, 18 and 24 hours, spun down at 14000 rpm for 5 min and supernatants were used for estimation of Cr^{6+} left in the medium. Supernatant (100 µl) was added to 10 ml of glass-distilled water in a test tube, followed by addition of 1 ml of diphenylcarbazide solution (prepared by dissolving 0.25g diphenylcarbazide in 100 ml acetone) and 1 drop of H_3PO_4. The mixture was kept at room temperature for 10 minutes to allow for color development and then optical density was measured at 540 nm.

Statistical analysis

Observations were made and all the experiments run in triplicate. At least three separate flasks were usually maintained for one treatment. Each time three readings were taken, their mean, and standard error of the mean were calculated.

RESULTS

Physicochemical characteristics of wastewater

Some physicochemical characteristics of industrial wastewater were ascertained, from where chromium tolerant bacteria were isolated. The temperature of different samples ranged between 26ºC to 30ºC and pH ranged between 5.5 and 7.5.

Identification of the bacterial isolates

Biochemical characteristics of the Cr-resistant bacterial isolates are given in Table I. The partially amplified (500bp) and sequenced 16S rRNA gene from local isolates (Cr1, Cr2 and Cr3) was blast to check the maximum homology of this gene to confirm the species of these local isolates. The blast query revealed that this gene is 98% homologous to *Bacillus pumilus* (Cr1), 100% homologous to *Alcaligenes faecalis* (Cr2) and 98% homologous to *Staphylococcus* sp. (Cr3). The nucleotide sequences coding for 16S rRNA gene of *Bacillus pumilus*, *Alcaligenes faecalis* and *Staphylococcus* sp. have been submitted to the GenBank database under accession numbers FN691760, FN796460 and FN796461, respectively.

Optimum growth conditions

The optimum temperature for Cr-resistant bacterial isolates was found to be 37^{o}C. *B. pumilus* and *Staphylococcus* sp. showed maximum growth at pH 8 while the optimum growth of *A. faecalis* was observed at pH 7. The growth curve pattern was studied by growing the organisms in the presence of Cr^{6+} (100 µg/ml) and comparing with the control culture in which no metal ions were added. Although the growth pattern of the isolates was not significantly different from those of control but the growth rate of isolates was lower in the presence of Cr^{6+}. The growth pattern is shown in Figure 1.

Table I.- Biochemical characteristics of the Cr-resistant bacterial isolates.

Biochemical tests	*B. pumilus*	*A. faecalis*	*Staphylococcus* sp.
Gram staining	-ve	+ve	+ve
Endospore staining	+ve	+ve	+ve
Acid fast staining	+ve	+ve	+ve
Motility test	+ve	+ve	+ve
Catalase test	+ve	+ve	-ve
Urease test	+ve	+ve	-ve
Gelatin hydrolysis test	-ve	+ve	+ve
Starch hydrolysis test	+ve	+ve	+ve
Glucose fermentation test	+ve	+ve	+ve
Fructose fermentation test	+ve	+ve	+ve
Lactose fermentation test	-ve	-ve	-ve
MRVP test	+ve	+ve	+ve
Citrate test	+ve	+ve	-ve
H_2S production test	-ve	-ve	+ve
Blood agar test	+ve	+ve	+ve
MacConkey agar test	+ve	+ve	+ve
Oxidase test	-ve	+ve	-ve
Indole test	ve	-ve	-ve
Casein hydrolysis test	+ve	+ve	+ve
Nitrate reduction test	+ve	+ve	+ve

+ve = Positive; -ve = Negative

Bacterial antibiotic resistance

Bacterial strains were tested against twelve antibiotics and *B. pumilus* was found sensitive against amoxicillin and tetracycline while showed least sensitivity against polymyxin B. *A. faecalis* was also found sensitive to tetracycline while showed least sensitivity against amoxicillin. *Staphylococcus* sp. showed high sensitivity against vancomycin and minimum against erythromycin. All three bacterial strains showed different degrees of sensitivity against kanamycin, erythromycin, streptomycin, oxytetracyclin and neomycin but all strains were resistant against bacitracin (Table II).

Cr reduction ability of the bacterial isolates

Chromium reducing capability of the bacterial isolates was checked by adding Cr^{6+} at 100 µg/ml in the acetate minimal culture medium. *B. pumilus* could reduce Cr^{6+} (100 µg/ml) 35%, 60%,

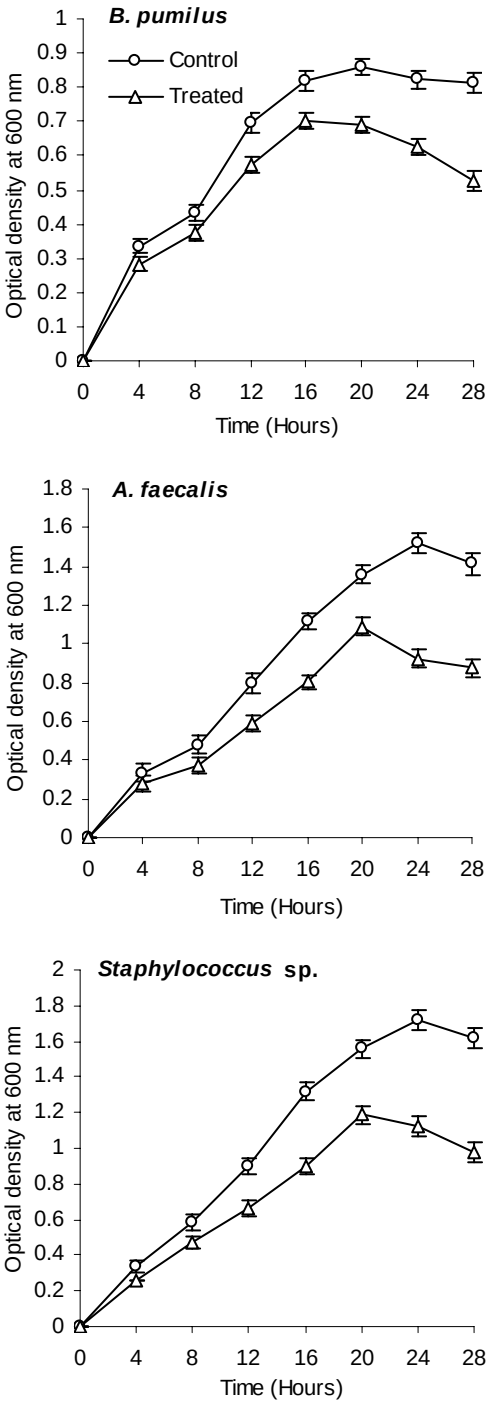

Fig. 1. Growth curves of Cr-resistant *B. pumilus, A. faecalis* and *Staphylococcus* sp. in acetate minimal medium LB medium containing chromium (100 µg/ml) and without chromium after incubation at 37°C.

80% and 95% from the medium after 6, 12, 18 and 24 hours, respectively. *A. faecalis* could reduce 97% of Cr^{6+} from the medium after 24 hours of incubation. The bacterium was also capable to reduce Cr^{6+} by 40%, 65% and 84% from the medium after 6, 12 and 18 hours, respectively. *Staphylococcus* sp. was also capable to reduce Cr^{6+} 30%, 53%, 73% and 91% from the medium after 6, 12, 18 and 24 hours, respectively (Fig. 2).

DISCUSSION

A wide variety of mechanisms exist for the removal of heavy metal from aqueous solution by bacteria, fungi, ciliates, algae, mosses, macrophytes and higher plants (Holan and Volesky, 1994; Pattanapipitpaisal *et al*., 2002; Rehman *et al*., 2007). The cellular response to the presence of metals includes various processes such as biosorption by cell biomass, active cell transport, binding by cytosolic molecules, entrapment into cellular capsules, precipitation and oxidation-reduction reactions (Gadd, 1990; Lovely and Coates, 1997) as well as protein-DNA adduct formation (Zhitkovitch and Costa, 1992) and induction of stress proteins (Ballatori, 1994).

Chromium resistant bacteria have been isolated from tannery effluents by several groups (Basu *et al*., 1997; Shakoori *et al*., 2000; Zahoor and Rehman, 2009). During the present investigation *B. pumilus* could tolerate Cr^{6+} up to 2 mg/ml. *A. faecalis* and *Staphylococcus* sp. were also able to resist Cr^{6+} up to 1.4 and 1.6 mg/ml, respectively. Shakoori *et al*. (2000) reported the isolation of a dichromate-resistant and reducing gram-positive bacterium from effluents of tanneries. The organism was able to tolerate Cr^{6+} up to 40 mg/ml.

Shakoori *et al*. (2010) reported that arsenic-resistant *Citrobacter freundii* and *Bacillus anthracis* were sensitive to erythromycin, kanamycine, nalidixic acid and tetracycline while *Klebsiella oxytoca* was found to be resistant to these antibiotics. All these isolates were sensitive to amoxicillin, chloramphenicol, neomycine, oxytetracycline, streptomycine, and polymixin B. Berg *et al.* (2005) described elevated antibiotic resistance in copper-amended field using culture-based assays. Copper resistant isolates had a higher incidence of antibiotic resistance as compared to copper sensitive isolates, indicating that these metal and antibiotic traits are associated . Present work also supports the hypothesis that metal exposure results in increased frequency of antibiotic tolerance in bacteria. In the present study *B. pumilus*

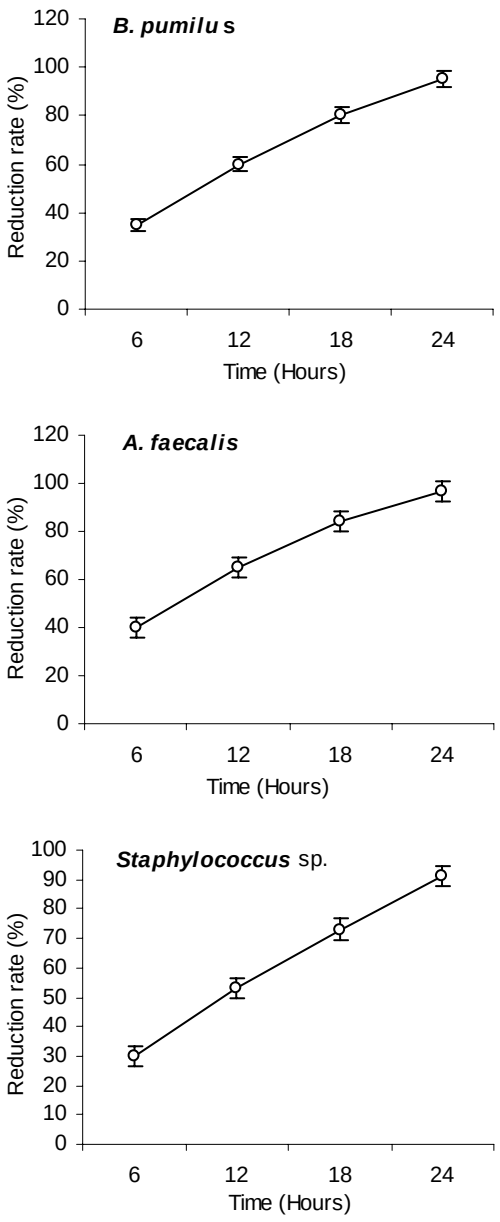

Fig. 2. Chromium reduction by *B. pumilus, A. faecalis* and *Staphylococcus* sp. from the medium containing (100 µg Cr^{6+}/ml). Estimations were done at different time periods.

was found highly sensitive against amoxicillin, *A. faecalis* against tetracycline and *Staphylococcus* sp. showed high sensitivity against vancomycin. All bacterial strains were resistant against bacitracin but showed different degrees of sensitivity against kanamycin, erythromycin, streptomycin, oxytetracyclin and neomycin (Table II).

Table II.- Resistance of antibiotics by Cr-resistant bacterial strains.

Antibiotic	*B. pumilus*	*A. faecalis*	*Staphylococcus* sp.
Ampicillin	S (0.233mm)	S (0.133mm)	S (0.04mm)
Amoxicilin	S (0.933mm)	S (0.2mm)	S (0.4mm)
Bacitracin	R	R	R
Chloramphenicol	S (0.633mm)	S (0.533mm)	S (0.566mm)
Eryhromycin	S (0.6mm)	S (0.533mm)	S (0.036mm)
Kanamycin	S (0.533mm)	S (0.466mm)	S (0.6mm)
Neomycine	S (0.4mm)	S (0.533mm)	S (0.4mm)
Polymyxin B	S (0.233mm)	S (0.233mm)	S (0.3mm)
Oxytetracycline	S (0.533mm)	S (0.8mm)	S (0.7mm)
Streptomycine	S (0.366mm)	S (0.666mm)	S (0.733mm)
Tetracycline	S (0.866mm)	S (0.866mm)	S (0.533mm)
Vancomycin	S (0.5mm)	S (0.233mm)	S (0.933mm)
Polymixin B	S (0.08mm)	S (0.07mm)	S (0.06mm)

S: Sensitive; R: Resistant

Hexavalent chromium is a common pollutant introduced into natural waters from a variety of industrial effluents and its removal by reduction has been well documented (Pattanapipitpaisal *et al*., 2002; Zahoor and Rehman, 2009). One potential method is microbially catalyzed reduction of Cr^{6+} to Cr^{3+}, which was first reported with *Pseudomonas* spp. (Romanenko and Koren'Ken, 1977). Since then, significant progress has been made towards understanding the processes controlling enzymatic reduction of Cr^{6+} in Gram-negative bacteria, especially those belonging to the genera *Pseudomonas, Desulfovibrio* and *Shewanella* (Chardin *et al*., 2003; Ackerley *et al.,* 2004). Several Gram-positive bacteria are also known to reduce Cr^{6+} including several members of the genus *Bacillus* (Campos *et al*., 1995; Camargo *et al*., 2003).

Ganguli and Tripathi (2002) reported that *Pseudomonas aeruginosa* cells reduced chromate 10 µg/ml completely within 2 hours. Hexavalent chromium was reduced to undetectable level from 10 µg/ml. Experiments with cell free extracts of *Bacillus* sp. indicated that soluble type of enzymes were responsible for Cr^{6+} reduction (Wang and Changsong, 1995). In the present study *B. pumilus* could reduce Cr^{6+} (100 µg/ml) 95% from the medium after 24 hours, *A. faecalis* could reduce 97% and *Staphylococcus* sp. could reduce 91% from the medium after 24 hours. Several researchers have also been reported the reduction of Cr^{6+} directly in contaminated effluents of metal finishing industry (Hardoyo and Ohtake, 1991; Ganguli and Tripathi, 2002; Zahoor and Rehman, 2009).

CONCLUSION

B. pumilus, *A. faecalis* and *Staphylococcus* sp. showed excellent ability to reduce hexavalent chromium to trivalent chromium *i.e.,* 95%, 97% and 91%. The bacterial isolates can be exploited for bioremediation of hexavalent chromium containing wastes, since they seem to have potential to reduce the toxic hexavalent form to its nontoxic trivalent form.

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