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Hexavalent Chromium Induced Changes in Growth and Biochemical Responses of Chromate-Resistant Bacterial Strains Isolated from Tannery Effluent

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Hexavalent chromium is generated in wastewater by several industrial processes, for example, leather tanning, electroplating, metal cleaning and processing, alloy preparation and wood preservation. The large scale use of chromium in various industrial processes makes these industries potential source of chromium pollution. Conventional methods for treatment of toxic chromate (Ohtake and Silver 1994) require large amount of chemicals and energy those are unapplicable for the small scale tanneries. Microbes can be used to remove toxic metals and metalloids from contaminated soil, water and waste streams (White et al. 1997; Kamaludeen et al. 2003). The potential of several bacterial strains to detoxify chromate has been described with a view to develop processes for microbial detoxification of toxic metal ions from polluted waters (Ohtake and Silver 1994; Shakoori et al. 2000). The cellular response to the presence of metals includes various processes such as biosorption by cell biomass, active 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).

Several species of bacteria are capable of accumulating metal ions up to concentrations several orders of magnitude higher than the ambient concentration (Krauter et al. 1996; Kratochvil et al. 1998; Khattar et al. 1999). However, development of a chromium bioremediation process requires isolation of efficient chromate – tolerant bacterial strains; evaluation of their ability to survive, multiply and simultaneously reduce chromate in industrial saline effluent (Dönmez and Koçberber 2005). Bioremediation of chromium is of a special interest since this metal has a variety of industrial applications. Although the potential chromate reducing bacteria to detoxify hexavalent chromium has been suggested by many investigators (Shakoori et al. 2000), the only exploitation of *Pseudomonas fluorescens* (Bopp and Ehrlich 1988), *P. mendocina* (Bhide et al. 1996) and *Pseudomonas* sp. (Gopalan and Veermani 1994) has been for bioremediation of toxic chromium from the industrial effluent, however the Cr accumulation values were less. The purpose of this study was to isolate chromate tolerant bacterial strains having high growth rate and chromium bioaccumulation potential for its use in bioremediation of tannery effluent under different background concentration of Cr^{+6}. These strains were characterized with respect to metal and antibiotic resistances and the growth behavior of these strains under different growth inhibitory concentration of Cr^{+6}.

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MATERIALS AND METHODS

The effluent sample from the aeration tank of Common Effluent Treatment Plant (CETP) at Unnao, Uttar Pradesh, India was collected in sterile glass bottles, transported on ice to the laboratory for physico-chemical and microbiological analysis. Physico-chemical characteristic of the effluent was determined following standard methods (APHA 1992), while a few parameters were recorded at spot using a portable water analysis kit (CMK 731). The concentration of Cr present in the effluent was estimated using a Perkin Elmer model atomic absorption spectrophotometer following digestion of samples with concentrated HNO_3 and $HClO_4$ (3:1, v/v) mixture. Enumeration for bacteria was started within 5-6 h of collection using a serial dilution technique. The chromate-resistant bacteria were isolated and enumerated on nutrient agar supplemented with different concentrations of Cr^{+6} as $K_2Cr_2O_7$ following standard pour plate technique (APHA 1992). Plates were incubated at 37 ± 2°C for 24-48 h and bacterial population were determined as CFU ml^{-1}. For isolation of Cr-resistant bacteria, samples were serially diluted in sterile phosphate buffer (pH, 7.2) and spread on to nutrient agar plates supplemented with 50 mg l^{-1} Cr^{+6}. A filter-sterilized solution of $K_2Cr_2O_7$ was used as the source of Cr^{+6}, which was added to the sterile molten nutrient agar to prevent problems associated with autoclaving chromate – containing solutions (Babich et al. 1982). The inoculated plates were incubated at 37±2°C for 48 h.

One hundred twenty morphologically distinct colonies were selected as Cr-resistant isolates. These isolates were purified and stored on to nutrient agar slants at 4°C. The maximum Cr^{+6} tolerant bacterial isolates (NBRIE-01, NBRIE-02, NBRIE-03, NBRIE-04) were selected for further study. The selected bacterial isolates were subjected to morphological as well as biochemical tests in order to evaluate their status as Cr resistant bacteria isolates from tannery effluent. The morphological as well as biochemical test exercised were gram reaction, motility, catalase-production, urease production, citrate utilization, nitrate reduction and NaCl tolerance etc.

Chromate tolerant isolates were also studied for tolerance to other toxic metals. The fresh overnight peptone water broth culture of the isolates was inoculated (1.0 ml) aseptically on nutrient agar plates supplemented individually with different concentrations of Ni, Hg, Cu, Cd and As. Susceptibility to different antibiotics for the chromate tolerant isolates was determined by the disc diffusion method (Bauer et al. 1966). The antibiotic impregnated discs were placed on freshly prepared lawns of each isolates on Muller Hinton agar plates and examined for the diameter of inhibition zones. The most promising bacterial isolate was reinoculated in nutrient broth in axenic condition and maintained on a shaker device for proper shaking for 12 h. After an optimum turbidity was maintained in the broth, the inoculum was subjected to the varying concentrations of Cr^{+6} supplemented nutrient broth and growth of isolates were recorded in spectrophotometer in terms of the optical density (O.D.) at 540 nm using the uninoculated broth as the blank at the different time periods of the incubation. Overnight cultures of strains were inoculated into 100 ml of peptone water containing 50 mg l^{-1} Cr^{+6}. The inoculated flasks (1000 ml) were incubated at 28°C for 24 h under continuous shaking at 150 rpm. After 12, 24 and 36 h, bioaccumulated chromium was estimated by harvesting and processing the cells as described above. The amount of chromium accumulated was calculated as μg Cr g^{-1} dw. The uninoculated peptone water containing the appropriate concentration of Cr^{+6} was used as blank. All the tests were performed in triplicate.

RESULTS AND DISCUSSION

Physico-chemical and microbiological characteristics of the raw tannery effluent which has been used as a seed material for the isolation of chromate-resistant bacterial strains showed high pH (8.2-8.4), electrical conductivity (19.90-20.84 μΩ), total dissolved solids (10428-10620 mg l^{-1}) and chlorides (4.103-5.021 mg l^{-1}). Besides the effluent is fortified with high amounts of chromium ranging from 10 to 15 μg ml^{-1} and bacteria ($3.0x10^{-5}$ to $4.4x10^{-6}$ CFU ml^{-1}) as evidenced by high B.O.D. (850-1647 mg l^{-1}) and C.O.D. (4225-6627 mg l^{-1}) contents. It showed high bacterial population which is in confirmation with earlier reports (Luli et al. 1983; Losi-Frankenberger 1993). The extensive chromate pollution in several other sites receiving tannery discharges has been described (Basu et al. 1997; Burman et al. 2000).

The morphological and biochemical characteristics of selected bacterial strains have been shown in Table 1. Based on the comparison of these characters strains were differentiated and designated as NBRIE-01, NBRIE-02, NBRIE-03 and NBRIE-04. However, a more detailed study is needed for confirmation.

The chromate tolerant strains were tested for antimicrobial susceptibility profile and the results have been depicted in Table 2. The strain NBRIE-01 represents sensitivity (S) to streptomycin, nalidixic acid, kanamycin, gentamycin and tetracycline, while vancamycin showed resistance (R) and cofactor showed intermediate (I) status until ciproflaxacin, norflaxacin, chloramphenicol, ampicillin and bacitracin showed no inhibition (NI). The strain NBRIE-02 showed I status for ampicillin; vancamycin and chloramphenicol showed NI status and another antibiotics showed S status. NBRIE-03 showed S status to nalidixic acid; ampicillin, kanamycin, gentamycin, chloramphenicol, vancamycin, narfloxacin and ciprofloxacin; cafeclor and tetracycline showed I and R status, respectively, while other antibiotics showed NI status. NBRIE-04 showed R status for ciprofloxacin and nalidixic acid, I status has been found for streptomycin and norfloxacin; ciprofloxacin and nalidixic acid showed R status and another antibiotics showed S status.

Table 1. Morphological and biochemical characteristics of bacterial strains.

Characters	**Strain and their responses**			
	NBRIE-01	**NBRIE-02**	**NBRIE-03**	**NBRIE-04**
Gram-reaction	+	+	+	+
Morphology	Cocci, single	Cocci, single	Rod, in pairs	Rod, in pairs
Color	Translucent white	Orange	Bright yellow	
Catalase production	+	+	+	+
Urease production	+	+	+	+
Critate utilization	-	-	-	-
Carbohydrate Utilizations; Mannitol	+	+	+	+
Glucose	+	+	+	+
Sucrose	+	+	+	+
Maltose	+	+	+	+
Nitrate reductase	-	+	+	+
Motility	+	+	+	+
NaCl-tolerance	>17%	>15%	>20%	>16%

(+) Positive, (-) negative

Table 2. Antimicrobial susceptibility test profile for selected bacterial isolates.

Antibiotic doses	Diameter(mm) of Inhibition Zones			
	NBRIE-01	NBRIE-02	NBRIE-03	NBRIE-04
Streptomycin (10 mcg)	16 (S)	28 (S)	NI	14 (I)
Bactracin (10 Units)	NI	26 (S)	NI	30 (S)
NalidixicAcid (30 mcg)	24 (S)	22 (S)	30 (S)	10 (S)
Ampicillin (10 mcg)	NI	16 (I)	30 (S)	32 (S)
Kanamycin (10 mcg)	22 (S)	26 (S)	26 (S)	18 (S)
Gentamycin (10 mcg)	30 (S)	34 (S)	30 (S)	24 (S)
Tetracycline (30 mcg)	26 (S)	28 (S)	16 (R)	28 (S)
Chloramphenicol (30 mcg)	NI	NI	20 (S)	30 (S)
Vancomycin (30 mcg)	8 (R)	NI	18 (S)	16 (S)
Norfloxacin (10 mcg)	NI	26 (S)	24 (S)	16 (I)
Cafeclor (05 mcg)	16 (I)	26 (S)	16 (I)	26 (S)
Ciprofloxacin (10 mcg)	NI	32 (S)	42 (S)	14 (R)

NI = No Inhibition; S = Sensitive; R = Resistant; I = Intermediate

Selected four chromate tolerant strains were further tested for their tolerance to other toxic metals like; nickel, mercury, copper, cadmium and arsenic by taking two concentrations (100 and 200 μg ml^{-1}). These strains were found susceptible to mercury and cadmium exposure, however, showed differential level of tolerance to other toxic metals. The strains NBRIE-01, NBRIE-02 and NBRIE-03 were found tolerant to nickel at the level of 100 μg ml^{-1} while the other strain NBRIE-04 was found sensitive to these concentrations. All the four strains were able to tolerate As concentration upto 200 μg ml^{-1} except strain NBRIE-01. Similarly strain NBRIE-01 and NBRIE-02 were found tolerant to 200 μg ml^{-1} Cu, while other two strains were able to grow at 100 μg ml^{-1} Cu only.

Growth behavior were studied by taking different Cr concentration ranging from 10-200 μg ml^{-1}. Based on their growth performance in different concentration of Cr^{+6}, all the strains were found to be promising showing 5-20% growth at highest Cr concentration tested during the study. The growth pattern have been shown in Figure 1. Growth of these isolates was comparable to that of control at lowest Cr concentration of 10 μg ml^{-1}. However, it declined at higher concentration irrespective of isolates. A comparative study of growth behavior of isolates showed NBRIE-01 and NBRIE-02 was most promising and showed optimum growth, which was delayed at higher Cr concentration. It is interesting to note that at higher concentration the lag phase of these isolates has been extended by increasing Cr concentration in the medium. Bioaccumulation of chromium was studied in these isolates by taking different Cr concentration and treatment

Table 3. Tolerance potential of selected strains to toxic metals.

Isolates	Concentration (μg ml^{-1})											
	Chromium		Nickel		Mercury		Copper		Cadmium		Arsenic	
	100	200	100	200	100	200	100	200	100	200	100	200
NBRIE-01	+++	++	++	-	-	-	+++	++	-	-	++	-
NBRIE-02	+++	+	+++	++	-	-	++	-	-	-	+	+
NBRIE-03	++	+	+++	++	-	-	++	-	-	-	+	+
NBRIE-04	+	+	-		-			-	-	-	++	++

+++ = Normal growth; ++ = Inhibitory growth; + = Minimum growth; - = No growth

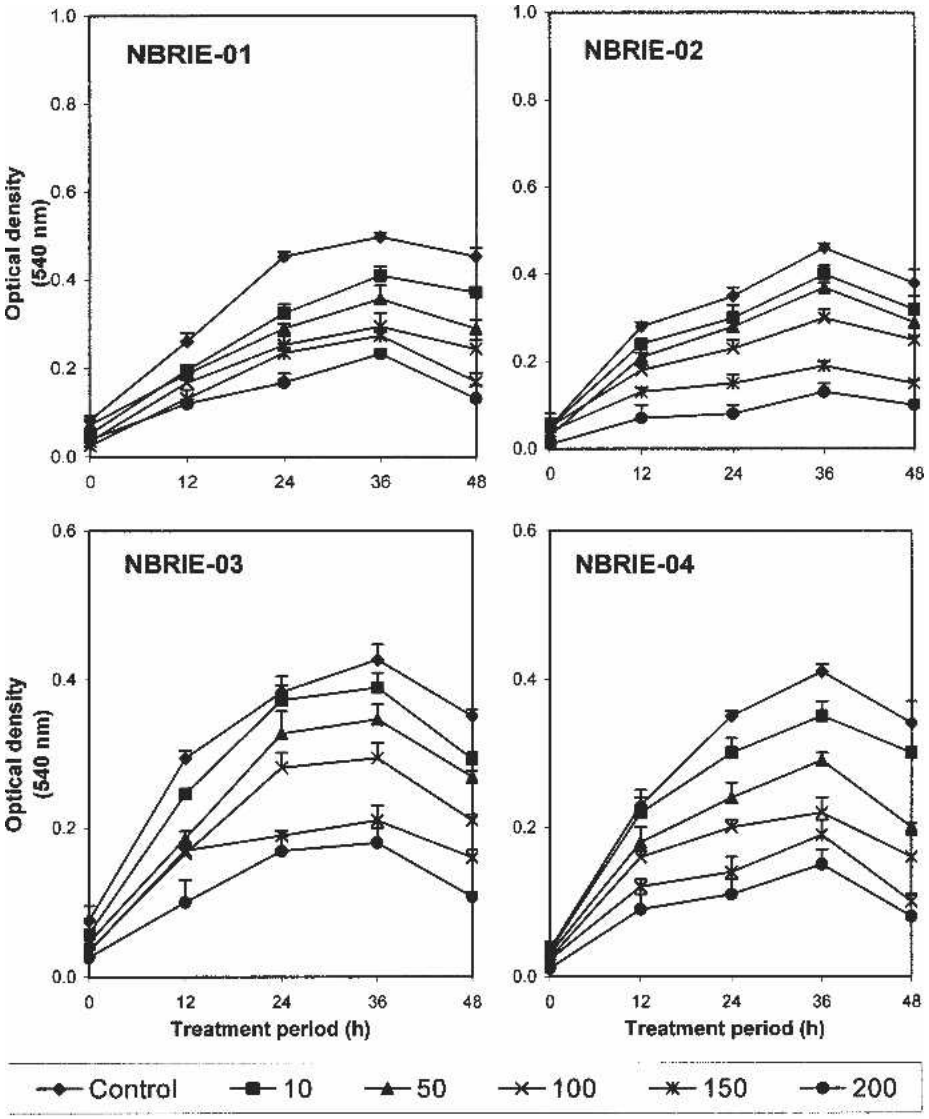

Figure 1. Growth behavior of chromate-resistant bacterial strains NBRIE-01, NBRIE-02, NBRIE-03 and NBRIE-04 in the growth media containing different concentration of Cr (μg ml^{-1}). Bars represent +SE (n=3)

duration. The data presented in Figure 2 showed a concentration and duration dependent accumulation of Cr inside cells. Maximum amounts of Cr (4994. 4 μg g^{-1} dw) was found accumulated at 200 μg ml^{-1} Cr after 36 h of treatment. It may be noted that there was not much increase in metal accumulation value at different treatment durations; however, it is apparent in case of different concentration.

The bacterial resistance to chromate may be either due to chromosomal mutation or it's plasmid born nature (Silver and Mishra, 1988). Although the bacterial resistance to metal

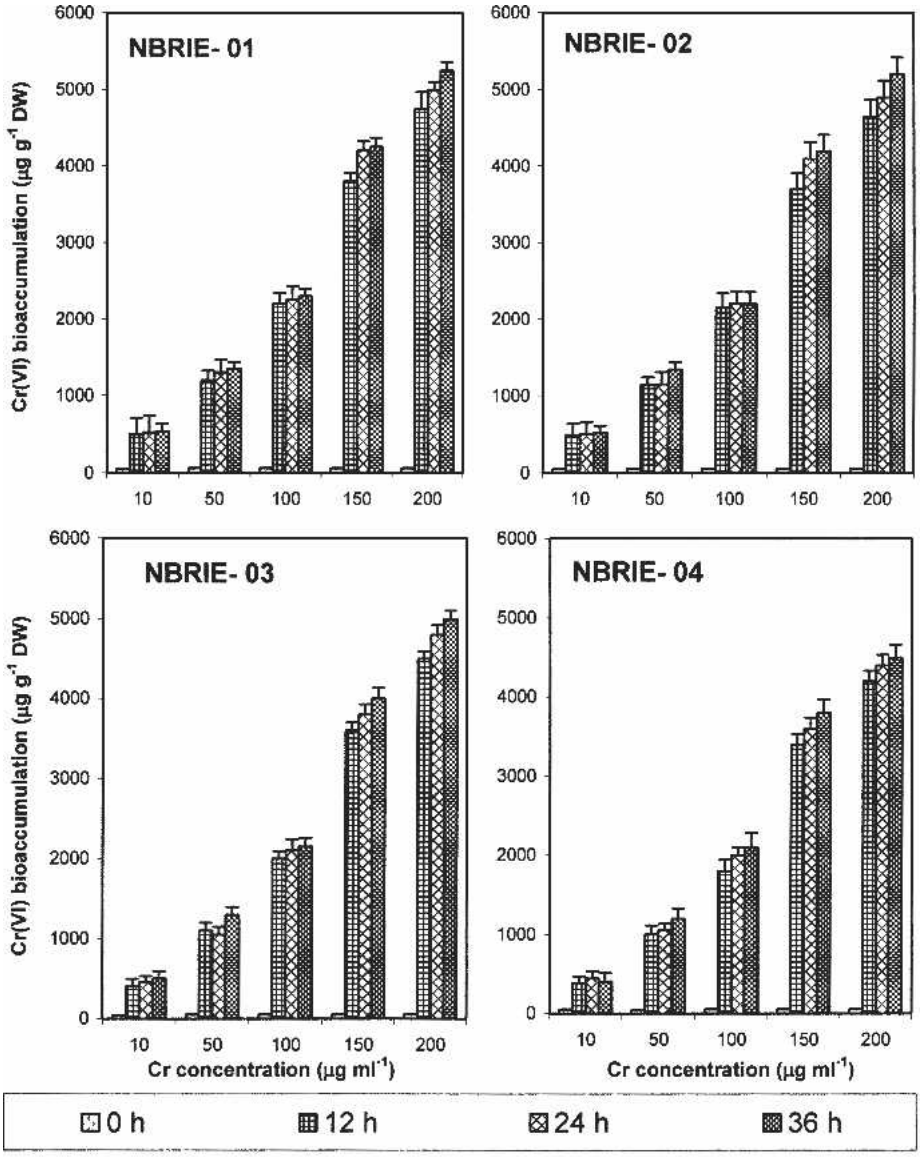

Figure 2. Cr^{+6} bioaccumulation by the isolates NBRIE-01, NBRIE-02, NBRIE-03, NBRIE-04 at different exposure concentration. Bars represent +SE (n=3)

ions is a potential health hazard, it is essential to study their tolerance level while using in bioremediation of metal pollutants. Under environmental conditions of metal stress such metal and antibiotic resistance bacterial population adapt faster by these spread of R-factor than by mutation and natural selection needed to a rapid increase in their numbers (Bhattacharjee et al. 1988).

The growth response curve of all these strains at various concentration of Cr^{+6} showed that the lag phase as well as optical density is dependent upon the Cr concentration in

the medium. A comparison of growth reduction by Cr^{+6} in different chromate tolerant strains revealed NBRIE-01 to be most tolerant strain followed by NBRIE-03, NBRIE-04 and NBRIE-03 showing approximately 53, 57, 63 and 71% reduction respectively at 200 μg ml^{-1} Cr^{+6} concentration after 36 h in comparison to the control. Such effects on growth pattern of chromate tolerant bacterial strains have been reported in earlier studies (Basu et al. 1997). The biosorption potential of these tolerant strains showed a concentration dependent accumulation of Cr^{+6}. Many microorganisms are known to remove Cr^{+6} from waste waters. These microorganisms have developed a variety of mechanisms to remove Cr^{+6}, such as adsorption to cell surfaces, transport into the cell, intra-cellular accumulation (Badar et al. 2000). It has been suggested that accumulated Cr^{+6} may act as a terminal electron acceptor and reduced to Cr^{+3} then binds to cell wall. Many bacteria belonging to the genera *Pseudomonas, Acromonas, Enterobactor, Escheritia, Bacillus* and *Streptomyces* have been reported to reduce Cr^{+6} to Cr^{+3} (Bhide et al. 1996, Bopp and Ehrlich 1988, Ganguli and Tripathi 2002).The chromate tolerant strains isolated during present study showed high level of chromate tolerance and accumulated substantial amount of Cr^{+6} from the medium. Thus these isolates can be exploited for bioremediation purposes. Further experiments are underway for studying the mechanism of Cr^{+6} bioaccumulation under various environmental conditions and the mechanism of its reduction to Cr^{+3}.

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