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Isolation and characterization of chromate resistant bacteria from tannery effluent

O. P. Shukla, U. N. Rai*, N. K. Singh, Smita Dubey and V. S. Baghel

Ecotoxicology and Bioremediation Group, National Botanical Research Institute,
Rana Pratap Marg, Lucknow-226 001, India

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Abstract: *The tannery effluent emanating from Common Effluent Treatment Plant (CETP), Unnao (U.P., India) was found toxic in nature, having high BOD, COD, TDS and Cr content (5.88 mg l^{-1}), which supported growth of chromate tolerant bacteria. Several chromate tolerant bacteria have been isolated from these effluent and maximum tolerant four strains (NBRIP-1, NBRIP-2, NBRIP-3 and NBRIP-4) were characterized in this study. These strains showed multiple metal and antibiotic resistances. Growth of these strains was reduced at higher Cr concentration with extention of lag phase. Chromium accumlulation by these isolates may have a great potential in recovery and detoxification of Cr from tannery effluent.*

Key words: *Growth behaviour, Chromium, Bacterial isolates, Tannery effluent, Antibiotic, Bioremediation*

Introduction

The application of chromium in various manufacturing processes such as wood preservation, leather tanning, electroplating, metal cleaning and processing, textile, ceramics and photographic sensitizer manufacturing has led to release of this metal into the environment. About 80% of the tanneries in India are engaged in chrome tanning processes and they use nearly 40,000 tons of basic chromium sulphate every year. The conventional chrome tanning practices lead only to an uptake of 50-70% leaving behind 12,000-20,000 tons of Cr^{3+} in effluent annually. At high concentration, Cr^{3+} salts used in tanning process exhibit toxic and carcinogenic behaviour to the flora and fauna of the aquatic system and carcinogenic (Bartlett and James, 1979; Dessi *et al.,* 1989). Chromate (CrO_4^{2-}) is a strong oxidizing agent that is reduced intracellularly to Cr^{6+} and reacts with nucleic acids and other cell components to produce mutagenic and carcinogenic effects on biological systems (McLean and Beveridge, 2001). However, Cr^{3+} is readily being converted into Cr^{6+} under natural conditions through various oxidation processes. Cr^{6+} is much more toxic than Cr^{3+} and mutagenic to most organism and humans (Ajmal *et al.,* 1984; Tsou *et al.,* 1997). This has been mainly due to its rapid permeability through biological membranes and subsequent interaction with intracellular protein and nucleic acids (Horitsu *et al.,* 1978).

To check the chromium coming into environment there are various treatment options, however, they are energy expensive and not successful due to their high running cost. Besides, conventional methods for treatment of toxic chromate (Ohtake and Silver, 1994) required large amount of chemicals, energy and are unsuitable for small scale leather, dye and electroplating units. In this context, biotransformation of Cr^{6+} to non toxic Cr^{3+} by bacteria offers a viable, economically safe and sustainable alternative (Eccles, 1995). However, 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 waters. Despite the potential of microbial Cr^{6+} reduction envisaged by several authors for a long time, Cr bioaccumulation has gained attention during the recent past (Gadd and White, 1993; Sharma and Forester, 1993; Fude *et al.,* 1994). Although the potential of several chromate reducing bacteria to detoxify Cr^{6+} has been suggested (Shakoori *et al.,* 2000), only the exploitation of *Pseudomonas fluorescens* (Bopp and Ehrlich, 1988) and *P. mendocina* (Bhide *et al.,* 1996) for detoxification of chromium from the industrial effluent has so far been described. However, since Cr^{6+} reduction is enzyme mediated, changes in pH will affect the degree of ionization of the enzyme, proteins and ultimately affecting the enzyme activity (Farrrell and Randallo, 2000). Therefore, the present study was planned on the isolation and characterization of chromate resistant bacterial strains and to compare their growth behaviour in different levels of Cr concentration in order to use them for detoxification of chromate in an integrated bioremediation system.

Materials and Methods

The effluent sample from the aeration tank of common effluent treatment plant (CETP) at Unnao, UP, India, was collected in sterile glass bottles, transported on ice to the laboratory for physicochemical and microbiological analysis. Physicochemical characteristics 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). Toxic metals in the sample were determined using Perkin - Elmer 2380 atomic absorption spectrophotometer after acid digestion of sample with a mixture of concentrated HNO_3:$HClO_4$ (3:1, v/v).

Enumeration for bacteria started within 5-6 hr of collection using a serial dilution technique. The chromate-resistant bacteria were isolated and enumerated on nutrient agar supplemented

***Corresponding author:** E-Mail: rai_un@rediffmail.com, Tel.: +91-522-2205831-35, Extn. 229 (O), +91-522-2788182 (R), Fax: +91-522-2205836/39

with different concentrations of Cr^{6+} as $K_2Cr_2O_7$ following standard pour plate technique (APHA, 1992; Basu *et al.*, 1997). Plates were incubated at 37±2°C for 24-48 hr and the total number of bacteria were determined as CFU ml^{-1}. Such effluent was diluted 1-1000 times with sterile double distilled water and used for isolation of chromate resistant bacterial strains under selective pressure of chromium. Sixty eight morphologically distinct colonies were selected as chromate-resistant isolates. These isolates were further purified and stored onto nutrient agar slants at 4°C.

The minimum inhibitory concentration (MIC) of chromium at which no colony growth occurred was determined by agar dilution method (Luli *et al.,* 1983). Nutrient broth test tubes supplemented with different concentrations of Cr^{6+} were inoculated aseptically with a culture of selected bacterial isolates (NBRIP-1, NBRIP-2, NBRIP-3 and NBRIP-4) in exponential growth phase. The minimum concentration of Cr^{6+} allowing growth of the isolate was an indication of positive tolerance. Peptone water tubes amended with different concentrations of Cr^{6+} was used for the broth dilution. The minimum concentration of chromate at which no turbidity was observed was considered the MIC. Broth dilution method (Calomiris *et al.,* 1984) was used for the assessment of growth behaviour of selected bacterial isolates in the different levels of Cr^{6+} concentration and optical density was recorded at 540 nm of wavelength at spectrophotometer.

Chromate tolerant isolates were also studied for tolerance to other toxic metals. The fresh overnight peptone water broth culture of the isolates was inoculated aseptically on nutrient agar plates supplemented individually with other toxic metals. The metal salts used were; $CdCl_2$, $CuSO_4$, $ZnSO_4$ and Na_2HAsO_4. The metal ion concentrations tested ranged from 500 to 1500 μg ml^{-1}. The isolates exhibiting growth after overnight incubation were considered tolerant to the metal.

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 inhibition zones. The isolates were classified as resistant, intermediate and susceptible following the standard antibiotic disc sensitivity testing method. Disc containing the following antibiotics (mcg) were tested; ampicillin (10), chloramphenicol (30), gentamycin (10), kanamycin (30), tetracycline (30) and streptomycin (10).

Results and Discussion

The effluent collected from the aeration tank of CETP, Unnao was analyzed for physicochemical properties and microbiological characteristics (Table 1). The effluent was slightly alkaline (pH 8.1) having high biochemical oxygen demand (850 mg l^{-1}), chemical oxygen demand (1984 mg l^{-1}), total dissolved solids (11850 mg l^{-1}) and Cr content (5.88 mg l^{-1}). The effluent has sufficient number of bacteria with its count being $1.5x10^3$ CFU ml^{-1}. Besides, the effluent is also fortified with high amount of several ions like chloride, sulphate, sulphite and ammonia *etc.* It is worth mentioning that the Cr concentration in the treated effluent was much higher than the prescribed limits in Indian standards BIS: 2490 (BIS, 1974).

Sixty eight morphologically distinct Cr^{6+} resistant bacterial strains were isolated and their tolerance limit was determined. Out of these strains only four isolates namely; NBRIP-1, NBRIP-2, NBRIP-3 and NBRIP-4 have been found potential tolerant to elevated chromium concentration (Table 2). Amongst the four strain highest Cr-tolerance have been shown by NBRIP-4 (2100 μg ml^{-1}) followed by NBRIP-3 (1800 μg ml^{-1}) and NBRIP-1 (1400 μg ml^{-1}), while NBRIP-2 (1200 μg ml^{-1}) showed least tolerance to Cr concentration. Such a high tolerance of these bacterial strains to elevated Cr concentration is conferred under natural condition due to horizontal dispersion of genetic information, which depends upon the physicochemical characteristics of the site of isolation. These Cr tolerant bacterial strains were tested for their tolerance to other toxic metals, which showed high degree of tolerance (Table 3). Among other toxic metal tested, NBRIP-4 exhibited maximum tolerance to different metals; Cu, As, Zn and Cd. The other strain NBRIP-3 showed tolerance to As and Zn up to 1500 μg ml^{-1}, while it was found sensitive to 1000 μg ml^{-1} Cd and Cu. NBRIP-2 also exhibited tolerance to As and Zn up to 1500 μg ml^{-1}, while it showed the tolerance of Cd and Cu only up to 500 μg ml^{-1} of metal concentration. In contrast, strain NBRIP-1 showed least tolerance to these toxic metals and exhibited the tolerance of Zn up to 500 μg ml^{-1} only. Such resistance to other toxic metals may be possible either due to exclusion of ions, biotransformation, accumulation and production of low molecular weight binding proteins (Summers, 1978; Silver and Misra, 1988).

As toxic metal resistance is linked with antibiotic resistance in microorganism, the isolated Cr resistant strains were tested for their sensitivity which showed a differential response (Table 4). Isolate NBRIP-1 was found most resistant to streptomycin and kanamycin followed by gentamycin, while it was sensitive to tetracycline and showed intermediate properties with ampicillin and chloramphenicol. Isolate NBRIP-2 was found resistant to ampicillin, while it showed susceptibility to all other antibiotics with intermediate properties for streptomycin. The other isolate NBRIP-3 was found resistant to ampicillin, tetracycline and streptomycin showing susceptibility to other antibiotics. NBRIP-4 was found resistant to streptomycin, gentamycin and kanamycin, while it was sensitive to other antibiotics. The resistance to antibiotics is much of clinical concern, however, it may provide significant information on the mechanism of antibiotic resistant, plasmid biology and ecotechnological exploitation of microbes in polluted water environment (Cervantes and Silver, 1992; Ganguli and Tripathi, 2002).

Table - 1: Physicochemical and microbiological properties of the tannery effluent of aeration tank, CETP, Unnao, UP, India

Parameters	Concentration	Standards (BIS : 2490)
Colour	Dark brown	absent
Odour	Fowl smell	absent
Temperature (°C)	18.90 ± 0.06	40.0
pH	8.17 ± 0.61	5.5-9.0
Conductivity (μ Ω)	36.50 ± 0.71	-
Total dissolved solids	11885 ± 30.0	-
Biochemical oxygen demand	850.00 ± 56.9	30.0
Chemical oxygen demand	1984.00 ± 85.9	250.0
Chloride	6700.00 ± 10.0	1000.0
Sulphite	22.00 ± 1.5	-
Sulphate	300.00 ± 5.0	-
Ammonia nitrogen	213.00 ± 8.0	50.0
Total nitrogen	789.00 ± 1.0	-
Total hardness	1490.00 ± 32.0	100.0
Sodium	84.70 ± 5.2	-
Toxic metals		
Cr	5.88 ± 0.21	0.5
Cd	<0.001	2.0
Cu	<0.001	3.0
Fe	1.25 ± 0.11	-
Mn	0.05 ± 0.005	-
Ni	0.29 ± 0.01	3.0
Pb	<0.001	0.1
Bacterial count (CFU ml^{-1})	$1.5x10^3$	-

All values are expressed in mg l^{-1}, otherwise stated; Values are mean ± SD (n=3)

Table - 2: Minimum inhibitory concentration for Cr^{6+} of the selected chromate resistant isolates

Isolates	MIC of Cr^{6+} (mg ml^{-1})
NBRIP-1	1400
NBRIP-2	1200
NBRIP-3	1800
NBRIP-4	2100

The growth curve of chromate resistant bacterial isolates NBRIP-1, NBRIP-2, NBRIP-3 and NBRIP-4 at various concentration of chromium has been shown in Fig. 1a-d. Growth of all these isolates in the medium containing lowest concentration of chromate (200 μg ml^{-1}) was comparable to the control at this concentration. However, approximately 15 to 20% decline in growth was recorded as compared to control. Further at higher Cr concentrations the lag phase was extended as well as optical density attended by the strains was reduced depending upon the concentration of Cr in the medium. Although the general inhibitory response of all the strains was similar, it varied with respect to their growth pattern. Strain NBRIP-1 showed decrease of about 36.0% of the control and extended lag phase at 1000 μg ml^{-1} Cr, while strain NBRIP-2 showed an inhibition of approximately 60% with extension of lag phase up to 20 hr. In contrast, the strain NBRIP-3 showed 47.0% inhibition in growth at 1000 μg ml^{-1} Cr with an extended lag phase up to 24 hr. The extension of lag phase is dependent upon the Cr concentration in the medium. It is interesting to note that isolate NBRIP-4 although showed lower growth rate by increasing Cr concentration in the medium, it has no effect on the lag phase of the strain. Such a high growth values shown by all these strains at elevated Cr concentration further supported our contention about high Cr tolerance potential of these strains. Since the yellow colour of

Table - 3: Growth response of selected chromate-resistant bacterial isolates to different toxic metals

Isolates	Metal concentrations (μg ml^{-1})											
	Cu			As			Zn			Cd		
	500	1000	1500	500	1000	1500	500	1000	1500	500	1000	1500
NBRIP-1	-	-	-	-	-	-	+	-	-	-	-	-
NBRIP-2	+	-	-	+	++	+++	++	+	+++	+++	-	+
NBRIP-3	+	-	-	-	++	+++	+	-	++	++	-	-
NBRIP-4	++	++	++	+++	+++	+++	+++	+	+++	+++	+	++

+ : positive growth; ++, +++ : growth status; - : no growth

Table - 4 : Antibiotic sensitivity profile of chromate resistant bacterial isolates

Antibiotic	Disc content (mcg)	Diameter of inhibition zone (mm)			
		Isolates			
		NBRIP-1	NBRIP-2	NBRIP-3	NBRIP-4
Ampicillin	10	16.0 (I)	10.0 (R)	NI (R)	20.0 (S)
Tetracycline	30	40.0 (S)	36.0 (S)	NI (R)	30.0 (S)
Streptomycin	10	NI (R)	14.0 (I)	NI (R)	NI (R)
Gentamycin	10	10.0 (R)	26.0 (S)	20.0 (S)	6.0 (R)
Chloramphenicol	30	14.0 (I)	28.0 (S)	18.0 (S)	20.0 (S)
Kanamycin	30	NI (R)	24.0 (S)	20.0 (S)	12.0 (R)

NI – no inhibition, letters in parenthesis indicate sensitivity; R = resistance, I = intermediate, S = susceptible

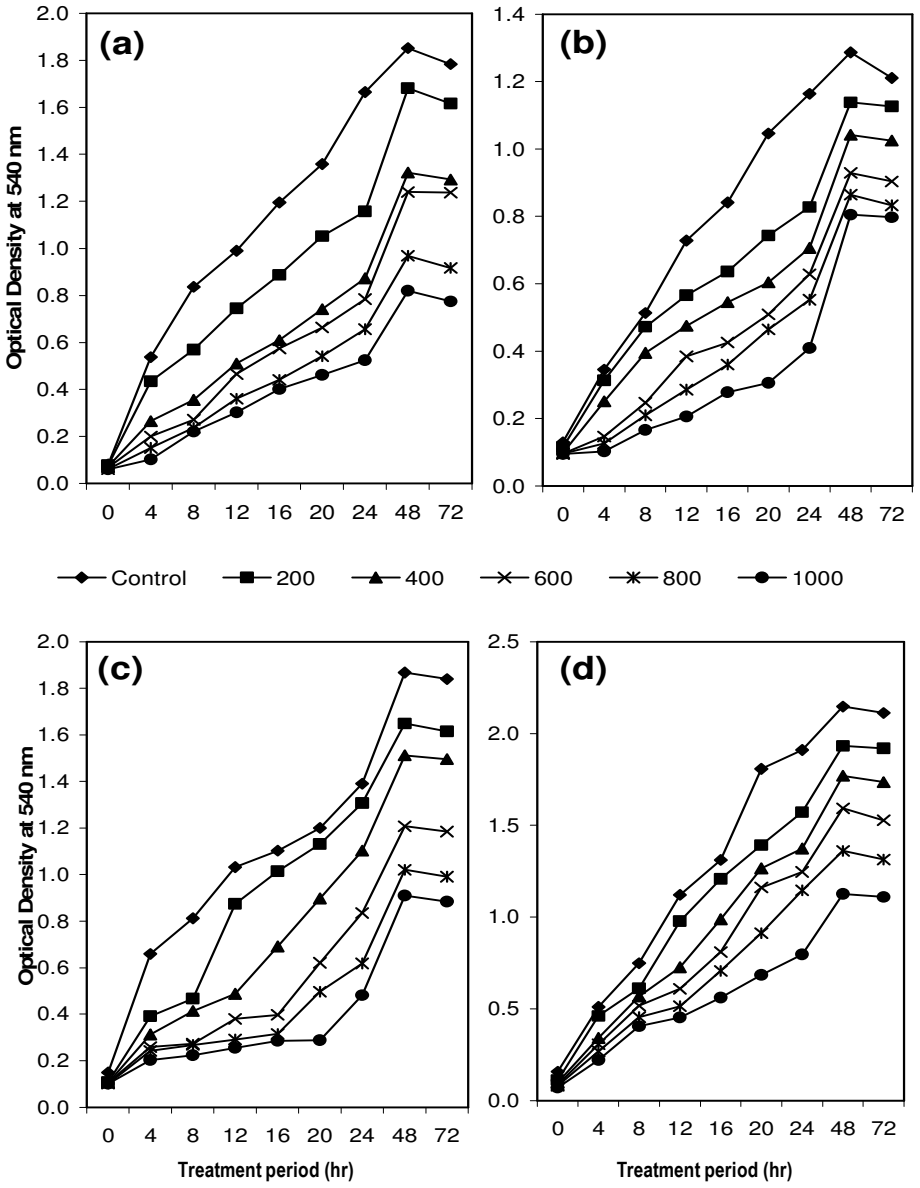

Fig. 1(a-d): Growth behaviour of chromate resitant bacterial strains NBRIP-1 (a) NBRIP-2, (b) NBRIP-3, (c) and NBRIP-4 (d) in the growth media containing different concentration of chromium

the medium turned white after incubation with all these bacterial strains for 48 hr, the reduction of Cr^{6+} to Cr^{3+} may be considered as reported using chromate tolerant parent and mutant *Pseudomonas* and *Bacillus* strains (Badar *et al.,* 2001). However, further experiments are required to prove this hypothesis. Results of this study indicated possible inheritance of plasmid determined Cr resistant factor in these strains, however, the mechanisms for chromate resistance along with their chromate reducing potential and accumulation needs to be worked out before the exploitation for detoxification of Cr from tannery effluent. Experiments are under way to develop an integrated bioremediation system utilizing some of these potential strains.

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