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**Full Paper**

# Isolation of hexavalent chromium-reducing Cr-tolerant facultative anaerobes from tannery effluent

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**Several facultative anaerobes tolerant to high levels of chromate (>400 μg/ml) were isolated from tannery effluents. These isolates displayed varying degrees of Cr(VI) reduction under aerobic and anaerobic conditions at room temperature (24±2°C). Interestingly, eight isolates were efficient in reducing 70% Cr(VI) anaerobically. This includes 5 isolates of genus *Aerococcus*, two isolates of *Micrococcus* and single isolate of genus *Aeromonas*. These isolates were subjected to further characterization for possible use in Cr(VI) detoxification of industrial wastes. This is the first report of *Aerococcus* sp. capable of Cr(VI) reduction >70% anaerobically. These bacteria were further checked for tolerance to a variety of other heavy metals. Our study indicates the possible use of these bacteria in environmental clean up.**

**Key Words**——aerobic; *Aerococcus*; *Aeromonas*; anaerobic; Cr(VI) reduction; facultative anaerobes; *Micrococcus*

## Introduction

Chromium, a priority pollutant is well known for its mutagenicity (Petrilli and Flora, 1977), carcinogenicity (Gruber and Jennette, 1978) and teratogenicity (Gale, 1978) in humans, experimental animals (IARC, 1990) and plants (Flora et al., 1990). Extensive use of chromium in industries such as leather tanning, stainless-steel production, electroplating and wood preservatives have resulted in chromium contaminated soil and ground water at production sites (Riley and Zachara, 1991; Turick et al., 1996) which pose a serious threat to human health. Chromium is present in industrial wastes primarily in the hexavalent form as chromate and dichromate, which is an oxyanion that is actively transported into cells by the sulfate transport system.

Conventionally, Cr(VI)-containing industrial effluents are treated by chemical means that are relatively chemical- and energy-intensive and that may be a source of potential metal pollution from the resultant metal-containing chemical sludge. Methods to remediate these sites include excavation, to pump and treat, in situ vitrification and chemical treatment with a reductant (Vermeul et al., 1995).

Several reports have indicated biological reduction of Cr(VI) by microorganisms, both by aerobes and anaerobes (Wang and Shen, 1995). Biological reduction of Cr(VI) usually occurs at a neutral pH-range and generates an insignificant quantity of chemical sludge as well as offers potential cost-effective remediation strategy (Mahesh et al., 1997). The objective of the present investigation was to screen the chromate-resistant microbial population for their ability to reduce

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Cr(VI) biologically, for environmental clean up and bioremediation of heavy metal-contaminated industrial wastes.

## Materials and Methods

*Sampling.* Effluent samples were collected from an Upflow Anaerobic Sludge Blanket (UASB) treatment plant, Kanpur (26.28°N, 80.24°E), Uttar Pradesh (India). The UASB plant receives a mixture of domestic sludge and chromium-contaminated tannery wastewater for treatment. Samples were collected in sterile glass bottles aseptically and transported on ice to the laboratory and were analyzed within 6 h of collection.

*Isolation of chromate-resistant microorganisms.* Chromate-resistant bacteria were isolated from raw and treated tannery effluents on anaerobic agar plates (Hi-Media Ind. Pvt. Ltd., Mumbai, India) that contained (/L): casein 20 g; dextrose 10 g; sodium chloride 5 g; sodium thioglycolate 2 g; sodium formaldehyde sulphoxylate 1 g; methylene blue 2 mg and agar 20 g/L with a final pH of 7.2±0.2. These plates were amended with Cr(VI) (200 and 400 μg/ml) by standard spread plate method (APHA, 1992; Baldi et al., 1990). The inoculated plates were incubated in anaerobic jars (E. Merck, Mumbai, India) at room temperature (24±2°C) up to 5 days. After incubation, clones representing different colony types were purified on anaerobic agar plates. All the cultures were stored at −20°C in agar stabs.

*Evaluation of metal tolerance.* The anaerobes were tested for their resistance to chromate both by agar dilution method (Cervantes et al., 1986) and broth dilution method (Calomoris et al., 1984). In agar dilution method, freshly prepared anaerobic agar plates amended with Cr(VI) as dichromate at various concentrations ranging from 50 to 800 μg/ml were inoculated with overnight grown cultures. Plates were then incubated at room temperature (24±2°C) for 48 h. The minimal concentration of metal in a plate inhibiting complete growth was taken as minimal inhibitory concentration (MIC). Tolerance to Cr(VI) was further confirmed by broth-dilution method. Tolerance to other heavy metals, viz. Co, Pb, As, Zn, Cu, Cd, Ni, and Hg was determined by agar dilution method. All the compounds were chloride salts, except for Pb (nitrate) and As (sodium arsenite).

*Cr(VI) reduction assay.* Reduction of Cr(VI) was determined by inoculation of test organisms in diluted peptone water flasks amended with Cr(VI) (20 μg/ml) and their incubation at room temperature (24±2°C) up to 3 days. For anaerobic reduction, flasks were incubated in anaerobic jars. The decrease in Cr(VI) concentration in experiment flasks after appropriate time intervals was determined by 1,5-diphenyl carbazide method (APHA, 1992). Briefly, the test-samples were acidified (pH 1.0–2.0) and 1,5-diphenyl carbazide (50 μg/ml) was added and Cr(VI) concentration was detected by spectrophotometer (Spectronic 1001, Bousch and Lomb, Rochester, USA) at 540 nm. For estimation of total chromium, samples were digested with hydrochloric and perchloric acid mixture (6 : 1). Metal level was estimated by atomic absorption spectrophotometer (Labtam 8440, Plasmalab, Braeside, Australia).

*Characterization of bacterial isolates.* Bacterial isolates were characterized according to Bergey's Manual of Systematic Bacteriology (Holt et al., 1989). The tests included Gram staining, spore formation, motility, growth in presence/absence of air, indole, nitrate reduction, oxidase, catalase, oxidation/fermentation, acid production after sugar utilization, $H_2S$ production and gelatin liquefaction.

## Results and Discussion

The microbial numbers of facultative anaerobes yielded from tannery effluent is given in Table 1. The bacterial counts of $4.2\times10^6$ and $5.0\times10^5$ colony forming units (cfu)/ml were obtained at a chromate concen-

Table 1. Isolation of Cr(VI)-tolerant facultative anaerobes from tannery effluents.

| Cr(VI) (μg/ml) | cfu/ml |
|---|---|
| 200 | $4.2\pm3.23\times10^6$ |
| 400 | $5.0\pm3.32\times10^5$ |

Table 2. MIC of Cr(VI) of 45 chromate-resistant facultative anaerobes.

| Cr(VI) (μg/ml) | No. of isolates |
|---|---|
| <400 | 10 |
| >400 | 19 |
| >800 | 16 |

Table 3. Anaerobic reduction of Cr(VI) by facultative anaerobes.

| MIC of Cr(VI) (μg/ml) | No. of isolates | % Reduction of Cr(VI) | | | | |
|---|---|---|---|---|---|---|
| | | 0 | <50 | 51–70 | 71–90 | >90 |
| <400 | 10 | 0 | 1 | 1 | 7 | 1 |
| >400 | 19 | 1 | 4 | 8 | 5 | 1 |
| >800 | 16 | 0 | 4 | 9 | 3 | 0 |
| Total | 45 | 1 | 9 | 18 | 15 | 2 |

Table 4. Aerobic reduction of Cr(VI) by facultative anaerobes.

| % Cr(VI) reduction in anaerobic conditions | Total no. of isolates | No. of isolates | % Cr(VI) reduction in aerobic conditions |
|---|---|---|---|
| >90 | 2 | 1 | 10–20 |
| | | 1 | 41–50 |
| 71–90 | 15 | 2 | <10 |
| | | 3 | 11–20 |
| | | 2 | 21–30 |
| | | 3 | 31–40 |
| | | 3 | 41–50 |
| | | 1 | 51–60 |
| | | 1 | 61–70 |
| 51–70 | 18 | 2 | <10 |
| | | 1 | 11–20 |
| | | 4 | 21–30 |
| | | 6 | 31–40 |
| | | 4 | 41–50 |
| | | 1 | 51–60 |
| <50 | 10 | 1 | 11–20 |
| | | 2 | 21–30 |
| | | 4 | 31–40 |
| | | 2 | 41–50 |
| | | 1 | 51–60 |

tration of 200 and 400 μg/ml, respectively.

MIC of chromate in isolates is represented in Table 2. A majority (>77%) of the isolates were observed tolerant to concentration of chromate >400 μg/ml. Around 35.5% isolates were observed tolerant to chromate level of >800 μg/ml.

Anaerobic reduction of Cr(VI) by isolates and their tolerance to chromate is depicted in Table 3. Out of 45 isolates studied, 35 (78%) isolates showed the ability to reduce Cr(VI) >50%. Two isolates were able to reduce >90% Cr(VI) and one of them was tolerant to concentrations of Cr(VI) >400 μg/ml.

Isolates capable of anaerobic Cr(VI) reduction >70% were also studied for aerobic reduction of Cr(VI) (Table 4). The results indicate overall reduced levels of Cr(VI) reduction when these facultative anaerobes were subjected to reducing Cr(VI) aerobically. The highest level of Cr(VI) reduction under aerobic conditions was 62.65% by an *Aeromonas* sp. Similar predominance of anaerobic Cr(VI) reduction over the aerobic Cr(VI) reduction have been observed earlier (Bopp and Ehrlich, 1988; Wang et al., 1989).

Selected isolates of facultative anaerobes capable of Cr(VI) reduction >70% were identified and their tolerance to heavy metals was also estimated (Table 5). Except for a single Gram negative rod, all isolates were found to be Gram positive cocci demonstrating physiological characteristics primarily indicative of the

Table 5. Reduction of Cr(VI) and heavy metal tolerance in selected isolates.

| Genus (Strain no.) | MIC of Cr(VI) (μg/ml) | % anaerobic reduction of Cr(VI) | % aerobic reduction of Cr(VI) | Metal tolerance (μg/ml) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Co | Pb | As | Zn | Cu | Cd | Ni | Hg |
| *Aerococcus* (S-31) | 400 | 93.50 | 17.65 | 100 | 100 | 200 | 100 | 200 | 100 | 50 | 25 |
| *Aerococcus* (S-26) | >400 | 90.4 | 40.41 | 100 | 200 | 200 | 100 | 200 | 50 | 50 | 100 |
| *Micrococcus* (S-39) | 400 | 87.2 | 40.04 | 50 | 100 | 200 | 50 | 200 | 100 | 200 | 25 |
| *Aerococcus* (S-24) | >400 | 84.2 | 8.92 | 50 | 100 | 100 | 200 | 200 | 200 | 200 | 25 |
| *Aerococcus* (S-21) | >400 | 82.1 | 52.83 | 50 | 50 | 200 | 100 | 200 | 100 | 50 | 25 |
| *Aeromonas* (S-33) | 400 | 81.4 | 62.65 | 50 | 200 | 200 | 100 | 200 | 200 | 100 | 25 |
| *Micrococcus* (S-37) | 400 | 81.4 | 44.58 | 50 | 200 | 200 | 50 | 200 | 100 | 50 | 25 |
| *Aerococcus* (S-23) | 400 | 72.5 | 48.98 | 50 | 200 | 100 | 100 | 200 | 200 | 50 | 100 |

Table 6. Physiological and biochemical tests of the isolates identified as *Aerococcus* sp.

| Morphological/physiological/ biochemical characteristics | Results/observations | | | | |
|---|---|---|---|---|---|
| | S-21 | S-23 | S-24 | S-26 | S-21 |
| Gram stain | + | + | + | + | + |
| Cell shape | cocci | cocci | cocci | cocci | cocci |
| Endospore formation | − | − | − | − | − |
| Motility | − | − | − | − | − |
| Growth in air | + | + | + | + | + |
| Anaerobic growth | + | + | + | + | + |
| Indole test | − | − | − | − | − |
| Nitrate reduction | − | − | − | − | − |
| Oxidase | − | − | − | − | − |
| Catalase test | − | − | + | − | + |
| Oxidation/fermentation (O/F) | F | F | F | F | F |
| Dextrose fermentation | + | + | + | + | + |
| Sucrose/lactose fermentation | + | − | + | − | − |
| Acid production from glucose | + | + | + | + | + |
| $H_2S$ production | − | − | − | − | − |
| Gelatin liquefaction | + | + | + | + | + |

genera *Micrococcus* and *Aerococcus*. The Cr(VI) reduction by *Micrococcus* and *Aeromonas* sp. has been reported earlier (Gvozdyak et al., 1986; Kvasnikov et al., 1985). However Cr(VI) reduction by *Aerococcus* sp. has yet not been reported. The morphological and biochemical character of the *Aerococcus* sp. is presented in Table 6. Besides their resistance to high levels of Cr(VI) (400 μg/ml or above), these isolates were able to tolerate different concentrations of various heavy metals. All the isolates were found tolerant to a

Cu concentration of 200 μg/ml. These isolates also showed tolerance to varying levels of metallic ions such as Hg, Co, Pb, As, Zn, Cd, and Ni.

Serious concern about the toxicity of chromium compounds necessitates recovery and reuse of chromium from tanneries and other industrial wastes or rendering it to a less toxic form (Yamamoto et al., 1993). All the eight bacterial isolates in this study were quite efficient in detoxification of Cr(VI). They were able to reduce 70–90% of Cr(VI) anaerobically, at room temperature (24±2°C) within 3 days. These isolates were also capable of the reduction of Cr(VI) aerobically although with lower efficiency. Thus these strains can be exploited in Cr(VI) detoxification operations.

Natural habitats are generally characterized by the coexistence of a large number of toxic and nontoxic cations and therefore it is necessary to study multiple metal effects on the physiology and biochemistry of microorganisms (Verma and Singh, 1995). All the eight bacterial isolates in this study were found tolerant to multiple metals. These observations assume great significance because effluent from any metal-related industry contains several metal ions/contaminants. Tolerances to other metals have an added advantage of withstanding the presence of these metallic ions while performing the desired activities. The advantage of selecting for indigenous bacteria from contaminated environments may be the minimization of inhibitory effects from other compounds that may be present along with Cr(VI), since viable indigenous organisms will have developed some degree of resistance to these compounds. Furthermore, when there are other metal contaminants, it might be practical to use Cr(VI) reducing microorganisms, to reduce other metals simultaneously (Lovely, 1995).

Recent interest in bacterial Cr(VI) reduction has been evoked by the potential use of this process with bioremediation purpose. Given the notable properties of these isolates to both tolerate and reduce Cr(VI), we are currently analyzing the ability of these isolates to detoxify chromate from chromium-containing industrial discharges.

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**References**

APHA (1992) Standard Methods for the Examination of Water and Wastewater, 18th American Public Health Association. American Water Works Association, Water Environmental Federation, Washington, D.C., 981 pp.

Baldi, F., Vaughan, A. M., and Olson, G. J. (1990) Chromium(VI)-resistant yeast isolated from a sewage treatment plant receiving tannery wastes. *Appl. Environ. Microbiol.*, **56**, 913–918.

Bopp, L. H. and Ehrlich, H. L. (1988) Chromate resistance and reduction in *Pseudomonas fluorescens* strain LB 300. *Arch. Microbiol.*, **150**, 426–431.

Calomoris, J. J., Armstrong, T. L., and Seidler, R. J. (1984) Association of metal-tolerance with multiple antibiotic resistance of bacteria isolates from drinking water. *Appl. Environ. Microbiol.*, **47**, 1238–1242.

Cervantes, C., Chavez, J., Cardova, N. A., de la Mora, P., and Velasco, J. A. (1986) Resistance to metal by *Pseudomonas aeruginosa* clinical isolates. *Microbios*, **48**, 159–163.

Flora, S. D., Bagnasco, M., Serra, D., and Zanacchi, P. (1990) Genotoxicity of chromium compounds, a review. *Mutat. Res.*, **238**, 99–172.

Gale, T. F. (1978) Embryonic effect of chromium trioxide in hamsters. *Environ. Res.*, **16**, 101–109.

Gruber, J. E. and Jennette, K. W. (1978) Metabolism of the carcinogen chromate by rat liver microsomes. *Biochem. Biophys. Res. Commun.*, **82**, 700–706.

Gvozdyak, P. I., Mogilevich, N. F., Ryl'skii, A. F., and Grishchenko, N. I. (1986) Reduction of the hexavalent chromium by collection strains of bacteria. *Microbiologiya*, **55**, 962–965.

Holt, J. G., Krieg, N. R., Sneath, P. H. A., Staley, J. T., and Williams, S. T. (1989) Bergey's Manual of Systematic Bacteriology (Vol. 1–4), Williams and Wilkins, Baltimore, 2648 pp.

IARC (1990) Chromium, Nickel and Welding, Monograph on the Evaluation of the Carcinogenic Risk to Humans, Vol. 49, International Agency for Research on Cancer, Lyon, France, 677 pp.

Kvasnikov, E. I., Stepanyuk, V. V., Klyushnikova, T. M., Serpokrylov, N. S., Simonova, G. A., Kasatkina, T. P., and Panchenko, L. P. (1985) New Gram variable bacterium which reduces chromium and has a mixed type of flagellation. *Mikrobiologiya*, **54**, 83–88.

Lovely, D. R. (1995) Bioremediation of organic and metal contaminants with dissimilatory metal reduction. *J. Ind. Microbiol.*, **14**, 85–93.

Mahesh, A., James, R., Peterson, N., Johnstone, L., Johnstone, C., Turick, E., David, R. Y., and William, A. A. (1997) Bacterial reduction of hexavalent chromium by *Enterobacter cloacae* strain HO1 grown on sucrose. *Biotechnol. Lett.*, **19**, 691–694.

Petrilli, F. L. and Flora, S. D. (1977) Toxicity and mutagenicity of hexavalent chromium in *Salmonella typhimurium. Appl. En-*

*viron. Microbiol.*, **33**, 805–809.

Riley, R. G. and Zachara, J. M. (1991) Nature of chemical contaminants on DOE lands and identification of reproductive contaminant mixtures for basic subsurface science research. OHER Subsurface Science Program, PNL, Richland, W.A.

Turick, C. E., Apel, W. A., and Carmiol, N. S. (1996) Isolation of hexavalent chromium reducing anaerobes from hexavalent chromium contaminated and non-contaminated environments. *Appl. Microbiol. Biotechnol.*, **44**, 683–688.

Verma, S. K. and Singh, S. P. (1995) Multiple chemical resistance in the cyanobacterium, *Nostoc muscorum. Bull. Environ. Contam. Toxicol.*, **54**, 614–619.

Vermeul, V. R., Gorky, Y. S., and Teel, S. S. (1995) Geochemical, microbiological and hydrologic characterization at the in situ redox manipulation test site PNL-10633, Pacific North-West-Laboratory, Richland, W.A.

Wang, P., Mori, T., Komori, K., Sasatsu, M., Toda, K., and Ohtake, H. (1989) Isolation and characterization of an *Enterobacter cloacae* strain that reduces hexavalent chromium under anaerobic conditions. *Appl. Environ. Microbiol.*, **55**, 1665–1669.

Wang, Y. T. and Shen, H. (1995) Bacterial reduction of hexavalent chromium. *J. Ind. Microbiol.*, **14**, 159–163.

Yamamoto, K., Kato, J., Yano, T., and Ohtake, J. (1993) Kinetics and modeling of hexavalent chromium reduction in *Enterobacter cloacae*. *Biotechnol. Bioeng.*, **41**, 129–133.