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APPLICATION OF CHROMIUM RESISTANT ORGANISMS IN BIOREMEDIATION

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ABSTRACT

Water pollution resulting from the increased concentration of chromium is causing serious ecological problems necessitating its removal from the waste waters. In this investigation chromium resistant bacterial isolates viz. Escherichia coli, Enterobacter aerogenes and Bacillus globisporus were evaluated for their ability to abate pollution caused by chromium. Methods like bio-absorption using dead and live biomass and bio-reduction of chromium using live and resting cells were employed. All the isolates showed their potential as bio-cleansing agents in remediating chromium contaminated environments to varying degrees.

INTRODUCTION

Pollution of water resources, both surface and underground, by indiscriminate discharge of spent wastes of chromium-based industries has become a serious global concern. Hexavalent chromium is used extensively in various industrial applications such as leather tanning, chrome painting, metal cleaning and processing. Although there are laws to prevent the discharge of chromium-rich spent wastes into the land, it is estimated that in India alone about 2000-3000 tons of chromium escapes into the environment annually from the tannery industries, with chromium concentrations ranging between 2000 and 5000 mg/L in the aqueous effluent compared to the recommended permissible limits of 2 mg/L (Chandra and Kulshreshtha, 2004).

Hexavalent chromium produces harmful effects on organisms including humans, causing allergic and asthmatic reactions. Chromium poisoning among leather tanners has long been known. The workers have been found to suffer from ulcers, allergic dermatitis, lung cancer, and liver necrosis due to prolonged contact with chromium salts (Altaf *et al.* 2008). One of the highly catastrophic incidences of lung cancer as a result of inhaling dust containing Cr (VI) was reported in 1960 from the Kiryama factory of the Nippon-Denko concern on the island of Hokkaido, Japan (Chandra and Kulshreshtha, 2004). Study of the detoxification of chromium has hence gained importance due to the emphasis placed on the protection of the environment. This necessitates development of efficient, cost effective and environment friendly methods for the removal of chromium. The conventional physico-chemical technologies may not guarantee adequate treatment of the effluent, have high reagent and energy requirements, and generate toxic sludge that require disposal (Leung *et al*. 2001). Moreover, they are often laborious and expensive, unsuitable in case of voluminous effluents containing low metal contamination. Of all the technologies investigated in waste cleaning,

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bioremediation has emerged as the most desirable approach for cleaning up many environmental pollutants.

Bacteria, and other microorganisms, exhibit a number of metabolism-dependent and-independent processes or the uptake and accumulation of heavy metals. Recent studies show that the strains (bacteria, yeast and fungi) isolated from the contaminated sites possess excellent capability of metal scavenging. Resistance to toxic materials might prove useful in biotechnological processes by facilitating biomining of expensive/scarce metals or in the process of bio-remediation (Adarsh *et al*. 2007). Metal-resistant strains may also have applications in remediation of the metal-contaminated environments. Both living and dead cells as well as products derived from or produced by microorganisms can be effective metal accumulators and there is evidence that some biomass-based clean-up processes are economically viable (Gadd, 2005). Hence, in the present study, metal resistant microorganisms, isolated from the industrial wastewaters were evaluated for their remediating capability by employing bio-absorption using dead and live biomass and bio-reduction of chromium.

MATERIALS AND METHODS

All the media, chemicals (AR grade) and reagents were purchased from Hi Media Laboratories, Mumbai, India.

Sample Collection: Contaminated water samples from near the chrome plating industries were collected in the sterile glass bottles, transported on ice to the laboratory, and were processed in the laboratory within 2 h of their collection.

Isolation and identification of the chromium resistant organisms and determination of their MIC: Samples were initially plated on to the sterile Luria-Bertani (LB) (Tryptone 10g, NaCl 5g, Yeast extract 5g and Agar 25g in 1 L of distilled water) agar plates, supplemented with 50 µg/mL of (Cr). The plates were incubated at 28 ± 2°C for 48 h. In order to determine the minimal inhibitory concentration (MIC) of the isolates by the agar dilution method (Luli *et al*. 1983), LB agar plates supplemented with different concentrations of potassium dichromate (100-1000 µg/mL) were inoculated aseptically by the exponentially growing cultures of the isolates obtained in the earlier step. Minimum inhibitory concentration was taken as that highest concentration of the metal supporting the growth of the isolates. Single well-isolated colonies were picked, purified and identified using biochemical analysis using Bergey's Manual of Determinative Bacteriology (Holt *et al*. 2000) and Bergey's Manual of Systematic Bacteriology (Sneath *et al*. 1984).

Analysis of chromium (VI) ions : The concentration of the chromium (VI) ions was determined spectrophotometrically after complexation of the metal ion with 1, 5-diphenylcarbazide (DPC) to produce a red–violet coloured compound. The reaction is very sensitive, the molar absorptivity of the complex being ~40000 L/g/cm a 540 nm. This method determines only chromium (VI) and is applicable in the range of the 100-1000 µg/L of chromium (VI) concentration (American Public Health Association, 1986).

Biosorption of Cr (VI) using the dead and the live biomass: The bacterial cells of each isolate were grown in 250 mL conical flasks containing 100 mL of LB broth on an orbital shaker at 200 rpm at 28 ± 2°C. The 72-h cultivated cells were harvested by centrifugation (3000 rpm for 15 min). After two rinses with saline, the cells were dried in oven at 70-80°C for 6-8 h. 50 mg of dried and finely ground bacterial biomass per 100mL of 50 ppm of Cr (VI) in 250 mL beakers, was gently agitated at R.T. (28 ± 2°C). The pH of the metal solution was adjusted to 2.0 by adding 0.1 M HNO_3 just before the experiments. Samples (5 mL) were withdrawn from the solution after 1 h and were subsequently centrifuged at 3000 rpm for 10 min. The concentration of chromium in the supernatants was determined by diphenylcarbazide method. Biosorption experiments with the live biomass (harvested cells used without drying) were carried out exactly in the same manner as described for the dead biomass.

The removal efficiency the of metal ion was calculated by using the formula,

% Removal = (Ci-Cf)/Ci x 100

The amount of metal bound by the biosorbents was calculated as follows:

Q = V (Ci-Cf)/m

Where,

Q = Metal uptake (mg metal per g of the biosorbent),

V = Liquid sample volume (mL),

Ci = Initial concentration of the metal in the solution (mg/L),

Cf = Final concentration of the metal in the solution (mg/L) and

m = Amount of the added biosorbent on dry basis

(mg) (Parameswari *et al*. 2009).

Reduction of Cr (VI) by the isolates : The inocula of the bacterial isolates, were precultured overnight in LB broth and the cells were harvested by centrifugation (3000 rpm for 15 min) followed by washing the cell pellet in 0.1M phosphate buffer pH 7.0. After two washes, the cells were resuspended in the same buffer. 250mL Erlenmeyer flasks containing 80mL LB broth supplemented with different concentrations of Cr (VI) at pH 7.0 (50-300µg/mL at an interval of 50µg/mL), were inoculated with 20% of the bacterial suspensions. Media without Cr (VI), but inoculated with bacteria and uninoculated media containing Cr (VI) served as controls. All the cultures including controls (in duplicates) were incubated at room temperature with shaking at 100 rpm. To measure the Cr (VI) reduction by growing cells, 1mL culture from each of the above flasks was centrifuged (3000 rpm for 15 min) and the supernatant was analyzed for Cr (VI) concentration by diphenylcarbazide method at definite intervals of 6 h, 24 h and 48 h, at 600nm (Megharaj *et al*. 2003).

Resting cell assay for the reduction of Cr (VI) : The bacteria were grown overnight in LB broth and harvested by centrifugation (3000 rpm for 15 min), washed twice in 10mM tris-HCl buffer (pH 7.2) and resuspended in the same buffer. 20 mL portions (optical density at 600 nm of 1.6) from each of these bacterial suspensions were dispensed in separate sterile 100 mL Erlenmeyer flasks and spiked with potassium dichromate (pH 7.0) of varying concentrations (50-300µg/mL). Killed cells served as controls. All the flasks including controls were incubated with shaking at room temperature for 6 h. After incubation, cultures were centrifuged (3000 rpm for 15 min) and supernatant was analyzed for residual Cr (VI) (Megharaj *et al*. 2003).

RESULTS AND DISCUSSION

An enormous increase in the pollution due to the discharge of industrial effluents, domestic and agricultural wastes, sewage consisting of varying hazardous chemicals and heavy metals to the rivers and the lakes is a matter of great concern in India. Even the coastal marine environment of Mumbai and regions around, comprising the Arabian Sea to the west and a number of tidal inlets such as Thane Creek, Back Bay, Mahim Creek, Versova Creek, Ulhas estuary and Bassein Creek, receive mostly untreated domestic wastewater and industrial effluents containing toxic heavy metal contaminants. Hence, in the present study, highly polluted water bodies situated near the industrial zones and small units were selected for isolating the heavy metal resistant organisms.

In the present investigation, all the samples processed showed the presence of bacterial isolates. The isolates identified on the basis of biochemical tests showed resistance to chromium as follows- *Escherichia coli* (600 ppm), *Enterobacter aerogenes* (700 ppm) and *Bacillus globisporus* (600 ppm). It was observed by Mergeay, (1985) that generally heterotrophic bacterial adaptation to toxic metals is 2 to 4 orders of magnitude higher (millimolar) than the levels of resistance displayed in fungi (micromolar). Muneer (2005) isolated yeast and bacteria resistant to chromium (1000-2000 ppm) from the wastewater of tannery industries in Pakistan. Altaf *et al*. (2008) reported chromium resistance in *Rhizobium* spp. (1600 µg/mL) isolated from the Egyptian clover grown in soil contaminated with a high level of Cr, Ni, Zn, Cu & Cd.

The impact and long- term ecological ramifications of pollution on the biosphere have resulted in an increased interest in the evaluation of the interactions between the pollutants, the environment, and the biota. Among the various aspects of the interactions studied, the role of microorganisms in remediating contaminated water, soils, and sediments is gaining much appreciation. Microorganisms take up metals either actively (bioaccumulation) and/or passively (biosorption) (Hussein *et al*. 2003). However, feasibility studies for large-scale applications demonstrated that, biosorptive processes are more applicable than the bioaccumulative processes, because living systems (active uptake) are difficult to maintain in healthy condition due to the metal toxicity, often require the addition of nutrients. This results in increased biological oxygen demand (BOD) or chemical oxygen demand (COD) in the effluent. In

Table 1. Percent biosorption of 50 ppm of chromium (VI) by the dead and live biomass of the isolates

Isolates	% biosorption of 50 ppm of chromium (VI)	
	By dead s biomas	By live biomass
Escherichia coli	26.07	27.86
Enterobacter aerogenes	18.57	22.14
Bacillus globisporus	16.07	18.21

Table 2. Percent reduction of Cr (VI) ions at different time intervals by live cells.

Conc. of Cr (VI) ions in ppm	*Escherichia coli*			*Enterobacter aerogenes*			*Bacillus globisporus*		
	Time in hours (h)								
	6	24	48	6	24	48	6	24	48
50	58.54	95.61	100	52.2	94.15	100	50.73	89.76	100
100	34.84	68.56	86.69	30.59	64.31	84.14	25.78	59.21	78.75
200	17.93	43.43	66.93	16.14	41.04	64.34	13.94	39.64	59.96
300	14.07	37.16	53.98	12.69	34.56	50.61	11.16	32.57	47.25

Table 3. Percent reduction of Cr (VI) ions at different time intervals by resting cell assay

Conc.	Time	*E. coli*	*Enterobacter aerogenes*	*Bacillus globisporus*
50ppm	10 min	6.122	8.16	3.67
	6 h	58.78	54.29	50.61
100ppm	10 min	4.634	3.66	4.15
	6 h	35.37	32.2	28.05
200ppm	10 min	3.523	3.02	1.34
	6 h	25.34	22.48	17.95
300ppm	10 min	2.069	1.38	1.79
	6 h	17.52	16.97	15.17

addition, potential for the desorptive metal recovery is restricted since metals may be intracellulary bound. On the other hand, the alternative use of the microbial sorbents for the removal and the recovery of the toxic metals from effluents can be economical and competitive particularly for environmental applications (Tewari *et al*. 2005).

The phenomenon of biosorption is defined as a metabolism independent adsorption of the pollutants based on the partition process on a microbial biomass. It is a passive non-metabolically-mediated process of metal binding by the biosorbent, concentrating heavy metals from even very dilute aqueous solutions. The mechanism of biosorption is complex, mainly ion exchange, chelations, adsorption by physical forces, entrapment in inter and intra fibrilliar capillaries and spaces of the structural polysaccharide network as a result of the concentration gradient and diffusion through the cell walls and membranes. The major advantages of biosorption over conventional treatment methods include high efficiency; minimization of chemical and or biological sludge; no additional nutrient requirement; regeneration of the biosorbent; and possibility of metal recovery. These biosorbents can be highly selective and cheap competing with the commercial ion exchange resins and activated carbons (Tewari *et al*. 2005).

Biosorption is largely dependent on pH, metal ion, incubation time and biomass concentration. It is well known that both the cell surface metal binding sites and the availability of metal in solution are affected by pH. The interaction of the matrix with chromium ions is dependent on the extent of protonation of the cell wall functional groups, which in turn depends on the pH of the solution. As the pH of the aqueous phase is lowered, a large number of hydrogen ions can easily coordinate with the amino and carboxyl groups present on the biomaterial surface. Thus, a low pH makes the biomaterial surface more positive. The more positive the surface charge of the biomaterial, the faster the rate of chromium (VI) removal from the aqueous phase, since the binding of anionic chromium (VI) ion species with the positively-charged groups is enhanced (Park *et al*. 2004).

Hence, in the present investigation the potential of the dried as well as wet biomass of the isolates was evaluated in the detoxification of the dilute solution of chromium (50 ppm) using biosorption at pH 2.0. The drying and then grinding of the biomass increases the effective area; exposing more sites where metal ions could be sequestered, thereby increasing the probability of metal ions encountering such sites. Hence, in the present study the biomass of all the three isolates was dried at 80° C and ground with a mortar and pestle to obtain a fine biomass of uniform size. Both the dried as well as the wet biomass exhibited their property of biosorption efficiently. All the isolates though were effective in the removal of chromium, individual isolates differed in their capacities of biosorption as seen in Table 1. Highest biosorption was seen in case of *E. coli* followed by *Enterobacter aerogenes* and *Bacillus globisporus*. Hussein *et al*. (2004) observed that Cr (VI) biosorption ranged between 16 to 38% of metal influent by the dead biomass of Pseudomonas belonging to different species. Leung *et al.* (2001) carried out the batch biosorption experiments to investigate the metal-removing ability of *Pseudomonas, Bacillus* and *Aeromonas*

species. The biosorption capacity of these strains for three different heavy metals (copper, nickel, and lead) showed significant differences in the biosorption of metal ions.

Because metal biosorption from solution is predominantly due to physicochemical interactions between the biomass and the metal in the solution, morphologic differences existing within the biomass can greatly influence the biosorption process. The stereochemical differences in the structures of the cell envelope can make a significant difference in the acceptance of the metallic ions by these structures. Cell wall is the most important structure that may form the cell envelope, but capsules, S-layers, and sheaths are commonly found superimposed on the wall. The Gram-negative bacteria possess cell walls that are chemically and structurally more complex than the Gram-positive bacteria, resulting in different metal biosorption capacities. The lipopolysaccharides nature of the outer membrane is responsible for the efficient metal binding capacity (Mohanty *et al.* 2004). This difference was reflected in the present work with *Bacillus globisporus* proving inferior to both the Gram negative isolates.

The live biomass in all the cases was more effective showing more biosorption by *Escherichia coli, Bacillus globisporus* and *Enterobacter aerogenes* by 6.86%, 13.32% and 19.24% respectively. This can be attributed to the interaction of chromium ions with the microbial cells as well as the accumulation as a result of physico-chemical mechanisms and transport systems of varying specificity, independent on, or directly and indirectly dependent on the metabolism (Gadd, 1990). The increased uptake could also be attributed to the metabolic intracellular accumulation of the heavy metals by the living cells. Parameswari *et al.* (2009) evaluated the ability of the live biomass of *Azotobacter chroococcum*, *Bacillus* spp. and *Pseudomonas fluorescens* isolated from sewage effluent and sewage irrigated soils in the biosorption of chromium. The optimum time for maximum metal removal (82.58 %, 89.50 % and 95.54 % of Cr by *A. chroococcum*, *Bacillus* spp. and *P. fluorescens* respectively) was found to be 72 hours at an initial metal concentration of 25 ppm. The bioaccumulation of heavy metals by these bacterial isolates was observed to be in the order of *P. fluorescens* > *Bacillus* sp.> *A. chroococcum*. Srinath *et al*. (2001) evaluated the biosorption capabilities of living and dead cells. Biosorption of Cr (VI) was shown by *B. megaterium* and another strain, *B. coagulans*. Living and dead cells of *B. coagulans* biosorbed 23.8 and 39.9 mg Cr/g dry weight, respectively, whereas, 15.7 and 30.7 mg Cr/g dry weight was biosorbed by living and dead cells of *B. megaterium*, respectively. Biosorption by the dead cells was higher than the living cells. They attributed this to prior pH conditioning (pH 2.5 with deionized water acidified with H_2SO_4) of the dead cells. These results are contradictory to the results obtained in the present investigation.

Depending upon the environment, the microorganisms have adapted and evolved the ability to mediate various oxidation- reduction couples to conserve energy. Some Cr (VI) resistant bacteria are able to grow by reducing Cr (VI) to Cr (III). Bioreduction of Cr (VI) can occur directly as a result of microbial metabolism (enzymatic) or indirectly, mediated by a bacterial metabolite (such as H2S) (Poopal, 2008). In the last few decades, microbial reduction of soluble Cr (VI) to its insoluble Cr (III) form has been identified as an important process for the detoxification and remediation of soils contaminated with the toxic metals. It is a cost-effective way to prevent the mobility of Cr (VI) beyond the compliance boundaries and to eliminate the risk of health hazards to humans.

Microbial reduction of Cr (VI) is controlled by many factors, including cell density, initial concentration of Cr (VI), pH and redox potential. Rate of Cr (VI) reduction has been shown to be affected by cell density under both aerobic and anaerobic conditions. Wang *et al.* (1989) reported increase in the rate of Cr (VI) reduction with increase in cell density under both aerobic and anaerobic cultures of *Escherichia coli*. Hence, in the present investigation a high inoculum of 20% was employed. Sharma (2002) strongly suggested that the reduction process is related to the growth. Cr (VI) reduction was observed in cultures of *Enterobacter cloacae* at pH range of 6.0-8.5, and at pH range of 3.0-8.0 in cultures of *Escherichia coli* and *Bacillus coagulans*. However, the maximum initial specific rate of Cr (VI) reduction by all three bacteria was at pH 7.0, an optimal pH for most bacterial growth. Hence, in the present work the pH for reduction was maintained at 7.0.

All the isolates showed their efficiency in the reduction of chromium with *E. coli* again proving to be the most superior isolate followed by *Enterobacter aerogenes* and *Bacillus globisporus*. Depending upon the initial concentration of Cr (VI), its complete or incomplete reduction was observed. All the isolates at 50 ppm reduced more than 50% of chromium at the end of 6 h while, completely reduced it at the end

of 48 h (Table 2). The reduction of chromium at concentrations ranging from 50-300 ppm appeared to be a concentration and time dependent process. As the concentration of chromium increased, the rate of reduction by all the isolates examined decreased linearly. A doubling of concentration from 50 ppm to 100 ppm led to a decrease in the percent reduction obtained by the isolates at the end of six hours viz. 40.49% for *E. coli*, 49.18% for *Bacillus globisporus* and 41.40% for *Enterobacter aerogenes*. The difference was even more pronounced at 300 ppm with a decrease of more than 75% in the reduction of chromium by all the isolates (Table 2). Muneer, (2005) showed the ability of the chromium resistant bacterial and yeast isolates in removing 65-82% Cr+6 from the medium by reduction after 72 hours of incubation. Wani *et al.* (2007) reported the Cr (VI)-reducing bacterial strain Burkholderia cepacia MCMB-821, isolated from the alkaline crater lake of Lonar to be resistant to 1,000-ppm Cr (VI). This isolate reduced 98% of the 75 ppm Cr (VI) within 36 h at pH 9.0 in the presence of 2% salt and lactose as the electron donor.

The data on Cr (VI) reduction by the resting cells are shown in Table 3. Again the concentration of chromium seemed to affect the reduction capacity of the isolates as seen in case of the growing culture. Reduction by all the isolates though low was observed at the end of ten minutes. The reduction obtained at the end of six hours by the resting cells of all the isolates at 50 and 100 ppm was comparable with that by the growing cells. Interestingly, at higher concentrations the resting cells proved to be more efficient than the growing cells. Meghraj *et al*. (2004) also showed efficiency of resting cells of Arthrobacter spp. and *Bacillus* spp. (100% of 10mg/L in six hours) in reducing chromium. They attributed this to the bacterial reduction of Cr (VI) to Cr (III) to cell membrane bound or soluble proteins. Bridge *et al*. (1999) also confirmed that the microorganisms release a diverse range of specific and nonspecific metal binding compounds in response to high levels of toxic metals which can ameliorate the effect of toxic metals and mediate the uptake process (Meghraj *et al*. 2004).

The present investigation established the efficiency of the bacterial isolates in the removal of Cr (VI) from dilute solutions by sorption as well as reduction. Thus, these findings suggest the possibility of using the heavy metal resistant bacterial isolates for bioremediation of chromium from the heavy metal contaminated ecosystems.

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