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SHORT CONTRIBUTION

A. Ganguli · A.K. Tripathi

Bioremediation of toxic chromium from electroplating effluent by chromate-reducing *Pseudomonas aeruginosa* A2Chr in two bioreactors

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Abstract The chromate-reducing ability of *Pseudomonas aeruginosa* A2Chr was compared in batch culture, with cells entrapped in a dialysis sac, and with cells immobilized in an agarose-alginate film in conjunction with a rotating biological contactor. In all three systems, the maximum Cr(VI) reduction occurred at 10 mg Cr(VI)/l. Whereas at 50 mg Cr(VI)/l concentration, only 16% of the total Cr(VI) was reduced, five spikings with 10 mg chromate/l at 2-h intervals led to 96% reduction of the total input of 50 mg Cr(VI) /l. Thus maximum Cr(VI) reduction was achieved by avoiding Cr(VI) toxicity to the cells by respiking with lower Cr(VI) concentrations. At 10 mg Cr(VI)/l, the pattern of chromate reduction in dialysis-entrapped cells was almost similar to that of batch culture and 86% of the bacterially reduced chromium was retained inside the dialysis sac. In electroplating effluent containing 100 mg Cr(VI)/l, however, the amount of Cr(VI) reduced by the cells immobilized in agarose-alginate biofilm was twice and thrice the amount reduced by batch culture and cells entrapped in a dialysis sac, respectively.

Introduction

Hexavalent chromium (chromate: $Cr0_4^{2-}$) is toxic and mutagenic to most organisms (Wong and Trevors 1988) and is known to cause irritation, corrosion of the skin and respiratory tract, and lung carcinoma in humans (Ajmal et al. 1984; Bidsrup and Case 1956). Hexavalent chromium is generated in wastewaters by several industrial processes, for example, leather tanning, electroplating, metal cleaning and processing, alloy preparation and wood preservation. Conventional methods for treatment of toxic chromate (Ohtake and Silver 1994) require large amounts of chemicals and energy and are unsuitable for small-scale leather, dye, and electroplating units. Biotransformation of hexavalent chromate to the non-toxic trivalent form by biological agents therefore offers a viable alternative. The potential of several bacterial strains to detoxify chromate has been described with a view to developing processes for microbiological detoxification of polluted waters (Ohtake and Silver 1994; Shakuri et al. 2000), and has been considered economical, safe and sustainable (Eccles 1995).

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 wastewaters; and developing an efficient and economical treatment process using these bacterial isolates. Although the potential of chromate-reducing bacteria to detoxify hexavalent chromium has been suggested by many investigators (Shakoori et al. 2000), only the exploitation of *Pseudomonas fluorescens* (Bopp and Ehrlich 1988), *Pseudomonas mendocina* (Bhide et al. 1996) and *Pseudomonas* sp. (Gopalan and Veeramani 1994) for bioremediation of toxic chromium from the industrial effluents has so far been described. Moreover, very little work has been done on aspects related to process development, which is required to demonstrate and recommend the method of application of potential strains for the bioremediation of chromate from industrial effluents.

The purpose of this study was to compare the chromate-reducing ability of *Pseudomonas aeruginosa* A2Chr (Ganguli and Tripathi 2001) in chrome-plating effluent in batch culture with cells entrapped in a dialysis sac or immobilized in an artificial biofilm. The possibility of using a rotating biological contactor (Mathys et al. 1997) for chromate bioremediation is also reported.

Materials and methods

Bacterial strain and culture conditions

Pseudomonas aeruginosa A2Chr is a Cr(VI)-reducing strain isolated from the effluent of a leather-tanning unit located in Kanpur,

A. Ganguli · A.K. Tripathi (✉)
School of Biotechnology, Faculty of Science,
Banaras Hindu University, Varanasi – 221005, India
e-mail: tripathianil@rediffmail.com
Fax: +91-542-368693/368174

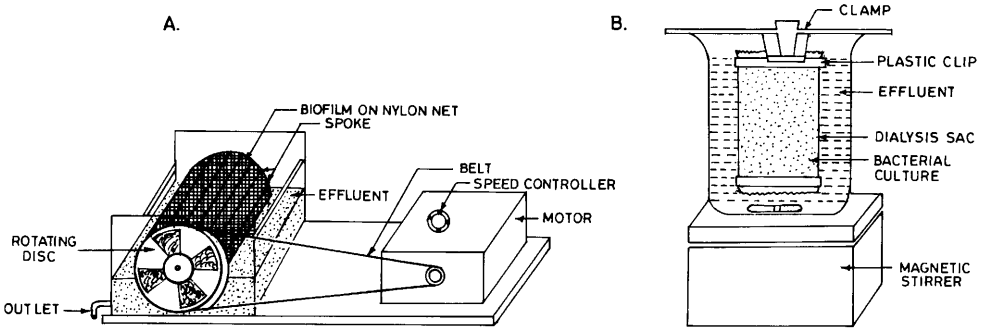

Fig. 1A, B Rotating biological contactor (**A**) and dialysis bioreactor (**B**)

India (Khare et al. 1997). This strain can tolerate and reduce Cr(VI) in tannery and electroplating effluents (Ganguli and Tripathi 1999). The culture of this strain has been sent to the Institute of Microbial Technology (IMTECH), Chandigarh, India, which is a national facility for type cultures: an identification number is awaited. This strain was grown in minimal medium with succinate as sole carbon source with or without potassium dichromate (Ganguli and Tripathi 1999). Cultures were incubated at 37 °C with shaking (150 rpm).

Effect of dichromate on growth of *P. aeruginosa* A2Chr

The effect of dichromate on the growth of *P. aeruginosa* A2Chr was studied in succinate minimal medium spiked with different concentrations of potassium dichromate. Cultures were withdrawn at intervals of 2 h and centrifuged. The pellet was washed with 0.85% NaCl, resuspended in 1.5 ml of 1 N NaOH and placed in a boiling water bath for 8 min. Appropriate amounts of such extracts were then used for the determination of total proteins (Lowry et al. 1951).

Collection of electroplating effluent

Effluent was collected from the main discharge tank of a chrome electroplating unit (pH 3.0, total chromium 5,000 mg/l, Cr(VI) 3,000 mg/l), and coarsely filtered with Whatman filter (no. 1) to remove the particulate matter. The pH of the electroplating effluent was adjusted to 7.2 by adding 1 N NaOH and the effluent was filtered again. The effluents prepared in this manner were used for various studies within 24 h of collection.

Estimation of Cr(VI) and total chromium

Hexavalent chromium, Cr(VI), was determined by its ability to specifically react with *s*-diphenyl carbazide (DPC) reagent and measuring the resulting absorbance at 540 nm (Clesceri et al. 1989). A standard curve was prepared by dissolving known amounts of $K_2Cr_2O_7$ in water. In solutions, Cr(VI) exists as chromate (CrO_4^{2-}) or dichromate ($Cr_2O_7^{2-}$) depending on pH and concentration (Losi et al. 1994). In the present study, Cr(VI) was added as $K_2Cr_2O_7$ in succinate medium or in effluent at neutral pH. Since dichromate ions above pH 6.5 and in the concentration range (10–50 mg/l) used in this study are transformed into chromate the actual ions available to the cells were those of chromate. In view of this, although potassium dichromate was used as source of Cr(VI), the term "chromate reduction" has been used to describe the enzymatic reduction of Cr(VI). Total chromium was estimated in an atomic absorption spectrophotometer (Perkin-Elmer model 2380, USA) using suitable standards (Merck, Darmstadt, Germany).

Estimation of Cr(VI) reduction in batch culture and dialysis bioreactor

For studying chromate reduction by free cells in the electroplating effluent, the effluent was diluted so as to achieve chromate concentrations of 10 mg/l, 25 mg/l, 50 mg/l and 100 mg/l, and supplemented with carbon, nitrogen and phosphorus (Ganguli and Tripathi 1999). Similar concentrations of chromate were also prepared in succinate medium, inoculated with log-phase cultures of a constant cell density of 5.5×10^8 CFU/ml and incubated with shaking at 37 °C for 12 h. Aliquots (1.5 ml) were withdrawn after intervals of 2 h, pelleted by centrifugation and the supernatant used to estimate residual Cr(VI). The pellet was washed and resuspended in 0.85% saline before recording absorbance at 600 nm in a spectrophotometer (Spectronic 1201, Milton and Roy) so as to obtain the desired cell density.

Dialysis tubing (Sigma, USA) was thoroughly washed in sterile triple-distilled water and clipped at one end to produce a sac. Log-phase cells were then suspended in succinate medium to a final cell density of 5.5×10^8 CFU/ml. After filling with bacterial culture, each sac was tied at the upper end with string and submerged completely in a beaker containing either succinate medium or electroplating effluent (Fig. 1b). Solutions in each beaker were supplemented with different concentrations of Cr(VI) and the dialysis sacs containing the cultures were incubated with stirring at 37 °C for 12 h. Aliquots (5 ml) of medium or effluent were withdrawn every 2 h and used to estimate residual Cr(VI). After 12 h, the cells from the dialysis sac were harvested by centrifugation and the supernatant tested for Cr(VI) before and after oxidation. The sacs were washed thrice with 0.1 N HCl and triple-distilled water to solubilize the precipitated chromium hydroxide formed inside the sac. The washings were combined, oxidized and estimated for Cr(VI) content. Dialysis sacs used for effluents were washed additionally with 0.1 M EDTA to remove the divalent cations present in the effluent.

Preparation of artificial biofilm and construction of a laboratory-scale rotating biological contactor

A laboratory-scale rotating biological contactor of standard design (Mathys et al. 1997) was fabricated (Fig. 1a). The trough was 33.5 ×23.8 ×15 cm; two flat discs connected by spokes were mounted on a spindle and suspended into the trough. The spindle was attached to a ridged disc on its outer side. The latter was rotated with a belt that was driven by a 0.25 HP motor equipped with a speed controller.

Cells of *P. aeruginosa* A2Chr were entrapped in biofilm by mixing 5 g cells, obtained from a log-phase culture in succinate medium, with 5 ml solution containing 4% (w/v) sodium alginate (Sigma, USA) and 4% agarose at 50 °C (Xu et al. 1996). The gel mixture was then uniformly spread to a thickness not exceeding 0.5 mm on nylon nets. The artificial biofilm so formed was wrapped around the spokes attached to two discs that were mounted on the spindle of the rotating biological contactor.

The trough was rinsed with 70% ethanol thoroughly before use. Minimal medium (250 ml) or electroplating effluent spiked

with desired concentrations of Cr(VI) was dispensed into the trough. The electroplating effluent was always amended with carbon, nitrogen and phosphorus. The drum was rotated at 10 rpm. The whole unit was kept at 37 °C and run for 12 h. Aliquots were withdrawn at 2-h intervals and used to determine residual Cr(VI).

Effect of respiking dichromate on Cr(VI) reduction

For elucidating the effect of respiking with low doses of dichromate on chromate reduction, cultures were inoculated in succinate medium containing 10 mg Cr(VI)/l and incubated with shaking at 37 °C. At 2-h intervals, 5 ml medium was removed and potassium dichromate added to the medium to a final concentration of 10 mg Cr(VI)/l. Ten mg Cr(VI)/l was supplemented every 2 h up to five times and the experiment terminated after 12 h. The 5-ml aliquots removed were centrifuged; the pellets were processed for estimating total proteins (Lowry et al. 1951) and the supernatants were analyzed for residual Cr(VI) by the DPC method (Clesceri et al. 1989).

Results

Effect of Cr(VI) on growth

The normal generation time of *P. aeruginosa* A2Chr in succinate minimal medium was 42 min (data not shown). Supplementation of 100 mg Cr(VI)/ml increased the generation time to 57 min; 10 mg Cr(VI)/l, however, did not have any significant effect on the generation time. Up to a concentration of 25 mg Cr(VI)/l, growth was very rapid during the initial 2 h but susbsequently tapered off. With 50 and 100 mg Cr(VI)/l, initial growth was slow and without any notable increase after 4 h. A concentration of 10 mg Cr(VI)/l was, therefore, determined to be the least toxic to the organism.

Cr(VI) reduction in batch culture

In batch cultures, maximum Cr(VI) reduction occurred at the lowest Cr(VI) concentration, i.e. 10 mg Cr(VI)/l). At 10–50 mg Cr(VI)/l, Cr(VI) reduction in minimal medium reached its maximum within 2 h (Table 1). At 100 mg Cr(VI)/l, however, reduction persisted for up to 4 h. The total amount of Cr(VI) reduced at 10 and 50 mg Cr(VI)/l was almost 2.5 and 2 times, respectively, more than that at 100 mg Cr(VI)/l. The amount of Cr(VI) reduced in electroplating effluent supplemented with carbon, nitrogen and phosphorus was approximately 25% lower than that in the minimal medium. Cr(VI) reduction >25 mg Cr(VI)/l was lower in electroplating effluent than in minimal medium.

Cr(VI) reduction in dialysis bioreactor

In dialysis sac cultures, maximum chromate reduction occurred at 10 mg Cr(VI)/l in minimal medium (Table 1). At 25 and 50 mg Cr(VI)/l, chromate reduction was inhibited by 15–20%. Cr(VI) reduction observed in the dialysis sac culture supplemented with 10 mg Cr(VI)/l effluent was 15% lower than that observed in the minimal medium. At lower dilutions of the effluent containing 25–100 mg Cr(VI)/l the Cr(VI)-reducing ability of the dialysis sac culture was adversely affected. The performance of the dialysis sac culture in the minimal medium, however, was much better since the Cr(VI)-reducing activity even at 100 mg Cr(VI)/l was very high. Estimation of trivalent chromium inside the dialysis sac showed that 86% of the total trivalent chromium formed after Cr(VI) reduction was retained within the dialysis sac. The dialysis sac method thus proved to be effective for chromium bioremediation only at higher dilutions of the effluent.

Cr(VI) reduction by biofilm in a rotating biological contactor

In view of the promise of biofilms (Palmer and White 1997) and rotating biological contactors (Mathys et al. 1997) in current wastewater treatment technologies, we evaluated the utility of this technique in the treatment of electroplating effluent. The artificial biofilm, mounted

Table 1 Comparison of Cr(VI) reduction by *Pseudomonas aeruginosa* A2Chr at different Cr(VI) concentrations in batch culture, dialysis bioreactor and rotating biological contactor (described in Materials and methods) in electroplating effluent and in succinate minimal medium. Each value represents average of three replicates

Test medium	Cr(VI) (mg/l)	Cr(VI) reduced (mg/l)											
		Batch culture				Dialysis bioreactor				Rotating biological contactor			
		2 h	4 h	6 h	8 h	2 h	4 h	6 h	8 h	2 h	4 h	6 h	8 h
Succinate minimal medium	10	9.4	9.9	9.9	9.9	8.6	8.7	9.0	9.1	8.5	9.0	9.5	9.5
	25	8.4	8.7	8.7	8.8	5.4	7.4	7.5	7.6	6.4	8.5	8.7	8.8
	50	7.6	7.8	7.9	7.9	5.4	6.4	7.0	7.4	4.9	7.1	7.2	7.3
	100	2.1	3.9	3.9	3.9	1.2	2.3	4.0	4.8	3.9	5.2	6.6	7.0
Electroplating effluent	10	7.5	9.6	9.9	9.9	7.2	7.6	7.6	7.6	6.5	7.9	8.5	9.3
	25	6.9	7.8	7.7	7.6	2.5	3.0	3.0	3.0	5.0	7.0	7.9	8.2
	50	6.1	6.9	7.0	7.1	2.0	2.4	2.39	2.38	4.3	5.9	6.9	6.9
	100	1.0	2.6	2.8	3.0	1.0	1.99	1.98	1.97	2.9	4.9	5.9	6.1

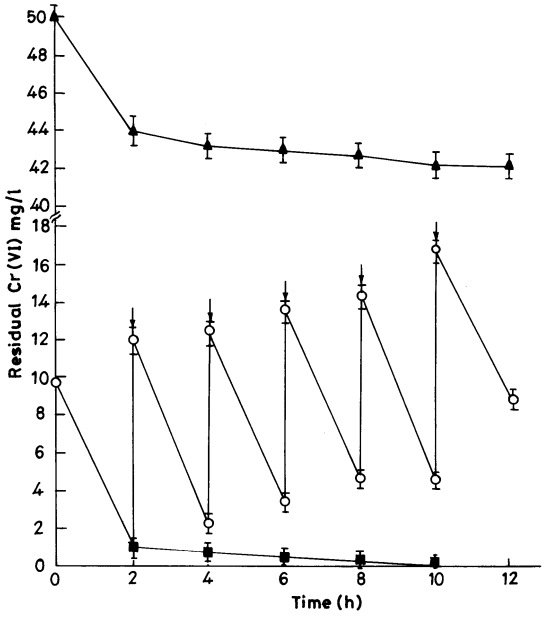

Fig. 2 Effect of respiking dichromate on Cr(VI) reduction by *Pseudomonas aeruginosa* A2Chr. ▲ Cells grown with –50 mg Cr(VI)/l, ■ cells grown with10 mg Cr(VI)/l, ○ cells grown in medium spiked with 10 mg Cr(VI)/l every 2 h. *Arrows* (↓) indicate the time of spikings).

on a rotating biological contactor, in succinate medium containing different concentrations of chromate, or chrome-electroplating effluent diluted to known concentrations of Cr(VI) showed that chromate reduction occurred in both cases with almost equal efficiency (Table 1). The chromate-reducing activity even at 100 mg Cr(VI)/l was much higher than that observed either in batch cultures or in dialysis sac cultures in which the cells were exposed directly to high Cr(VI) concentrations. The durability of the biofilms, however, was limited to 10 h.

Effect of periodic spiking of low doses of Cr(VI) on chromate reduction

In order to prevent the inhibitory effect of Cr(VI) on chromate reducting activity, cultures were grown with an initial low concentration of 10 mg Cr(VI)/ml and then spiked four times with 10 mg Cr(VI)/l at 2-h intervals (Fig. 2). This led to the reduction of 50 mg Cr(VI)/l within 12 h. However, when the initial concentration of Cr(VI) was 50 mg/ml, only 6 mg Cr(VI)/l was reduced in the same duration. This could be due to the fact that Cr(VI) at low concentrations (10 mg/l) was less toxic to the cells and reduced completely within 2 h. In the cultures spiked with higher initial concentrations of Cr(VI) (50 mg/l), the inhibitory effect of Cr(VI) on cellular metabolism may be responsible for the lower levels of chromate reduction.

Discussion

Growth of *P. aeruginosa* A2Chr was pronouncedly inhibited above 50 mg Cr(VI)/l. Therefore the electroplating effluent should be diluted to <50 mg Cr(VI)/l level before subjecting to treatment with *P. aeruginosa* A2Chr. The lower rate of Cr(VI) reduction at higher concentrations of Cr(VI) is likely due to an adverse effect of Cr(VI) and other ingredients of the electroplating effluent on bacterial metabolism. In order to prevent inhibition of growth and Cr(VI) reduction due to an higher initial concentration of Cr(VI), we respiked the cultures/effluent with lower, growth-permitting concentrations of Cr(VI) at regular intervals without addition of extra nutrients. Although this practice showed substantially improved Cr(VI)-reducing efficiency, it requires huge amount of water for dilution to achieve lower concentrations of Cr(VI) in the effluent. In order to achieve higher levels of chromate reduction even at higher concentrations of chromate in the effluent, two bioreactors were evaluated for their suitability. In one bioreactor a high density of *P. aeruginosa* A2Chr cells was entrapped in a dialysis sac, which was immersed in effluent containing different concentrations of Cr(VI); in the second bioreactor, the *P. aeruginosa* A2Chr cells were entrapped in an agarose-alginate biofilm and mounted on a rotating biological contactor.

The cells inside the dialysis sac are better protected from Cr(VI) toxicity than free cells of batch culture. The effective Cr(VI) concentration inside the dialysis sac is less than that outside. This might result from constraints in the diffusion of chromate across the dialysis membrane and also to faster Cr(VI) reduction by a dense culture inside the dialysis sac. Lower rates of chromate reduction observed while treating electroplating effluent are probably largely due to constraints in the diffusion of chromate. These constraints might be an effect of some components of the effluent, which might clog the pores and complicate free diffusion across the membrane. Most of the reduced Cr(VI) was found inside the dialysis sac as Cr(III), implying that the medium/effluent outside the dialysis sac can be freed from both hexavalent and trivalent chromium if either a low concentration of chromate in the effluent or a high cell density inside the dialysis sac is used.

Pseudomonads produce exopolysaccharides, which help in the formation of biofilms on a variety of substrata (Palumbo et al. 1987). However, the polysaccharides produced by the test strain of *P. aeruginosa* did not mediate its adhesion to the substratum to form a stable biofilm. Artificial biofilms could, however, be prepared by immobilizing *P. aeruginosa* cells in alginate-agarose films. We observed that the artificial biofilm mounted on the rotating biological contactor was better able to reduce Cr(VI) even at high concentrations. It appears that entrapment in the alginate-agarose film provided some protection against the toxicity of high concentrations of Cr(VI). The advantage of cells entrapped in biofilms in tolerating higher concentrations of toxic substances was suggested by Xu et al. (1996).

Bioremediation of Cr(VI) from industrial effluents was previously attempted using a dialysis sac bioreactor, an anion-exchange membrane reactor, and a continuous stirred tank bioreactor (Komori et al. 1990; Gopalan and Veeramani 1994; Bhide et al. 1996). Cr(VI) bioremediation using artificial biofilms or a rotating biological contactor, either separately or in conjunction, has not been tested so far. Our observations suggest that the rotating biological contactor could be a very effective approach to chromium bioremediation from industrial effluents provided that methods are developed for making stable biofilms containing the chromate-reducing strain. Free cells can also be made to reduce higher amounts of Cr(VI) provided that they are respiked with 10 mg Cr(VI)/l at regular intervals. Since *P. aeruginosa* A2Chr can reduce Cr(VI) only in an effluent containing C, N and P, the effluent should also be supplemented with appropriate sources of C, N and P. Chromium detoxification in industrial effluents can be economically achieved if mixed with a nutrient-rich wastewater (e.g. sewage), which may provide necessary energy and other nutrients to the bacteria in order to carry out Cr(VI) reduction in the mixed effluent.

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