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Characteristics of a Novel *Acinetobacter* sp. and Its Kinetics in Hexavalent Chromium Bioreduction

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Cr-B2, a Gram-negative hexavalent chromium [Cr(VI)] reducing bacteria, was isolated from the aerator water of an activated sludge process in the wastewater treatment facility of a dye and pigment based chemical industry. Cr-B2 exhibited a resistance for 1,100 mg/l Cr(VI) and, similarly, resistance against other heavy metal ions such as Ni^{2+} (800 mg/l), Cu^{2+} (600 mg/l), Pb^{2+} (1,100 mg/l), Cd^{2+} (350 mg/l), Zn^{2+} (700 mg/l), and Fe^{3+} (1,000 mg/l), and against selected antibiotics. Cr-B2 was observed to efficiently reduce 200 mg/l Cr(VI) completely in both nutrient and LB media, and could convert Cr(VI) to Cr(III) aerobically. Cr(VI) reduction kinetics followed allosteric enzyme kinetics. The K_m values were found to be 43.11 mg/l for nutrient media and 38.05 mg/l for LB media. V_{max} values of 13.17 mg/l/h and 12.53 mg/l/h were obtained for nutrient media and LB media, respectively, and the cooperativity coefficients (n) were found to be 8.47 and 3.49, respectively, indicating positive cooperativity in both cases. SEM analysis showed the formation of wrinkles and depressions in the cells when exposed to 800 mg/l Cr(VI) concentration. The organism was seen to exhibit pleomorphic behavior. Cr-B2 was identified on the basis of morphological, biochemical, and partial 16S rRNA gene sequencing chracterizations and found to be *Acinetobacter* sp.

Keywords: *Acinetobacter*, bioreduction, kinetics, hexavalent chromium, isolation, pleomorphic behavior

Heavy metal pollution in wastewaters has become one of the major environmental concerns in the last decades. One of the most common polluting metals is chromium, arising from discharged effluents from leather tanning, chromium plating, cleaning and processing, wood preservation, and alloy preparation [27]. The trivalent [Cr(III)] and the hexavalent [Cr(VI)] forms of chromium are the two stable oxidation states [16]. Cr(VI), apart from being the predominant species involved in mutagenicity, carcinogenicity, and teratogenicity [23], is approximately 100 times more toxic than Cr(III), a form considered relatively innocuous [15]. Hexavalent chromium is known to cause irritation and corrosion of the skin and respiratory tract; it also causes lung carcinoma in humans [35]. Removal of heavy metals from wastewater is usually achieved by physical and chemical processes, which include precipitation, coagulation, reduction, membrane processes, ion exchange, and adsorption [11]. These methods can be ineffective or extremely expensive, especially when the metals in the solution are in a lower concentration range of 100–1,000 mg/l [18]. Hence, there is always a search for alternative techniques that are feasible in removing metals even at very low concentrations, and bioremediation using microorganisms has emerged to be a promising technology overcoming the demerits of conventional technologies. During the past three decades many researchers have isolated microbes which are highly resistant to Cr(VI), from various contaminated sources such as industrial sludges [10, 12], industrial wastewaters [6, 8, 29], activated sludge [9, 39], river sediment [5] and heavy metal contaminated soil [14, 21] and begun analyses of their role in removing this metal ion. Many researchers have reported bacteria that convert a more toxic form to a less toxic form of the metal [*i.e.*, conversion of Cr(VI) to Cr(III)]. However, the search for bacteria that are tolerant to high Cr(VI) concentrations and that possess a higher and faster Cr(VI) reduction capability needs to be continued with the objective of minimizing the size of industrial-scale bioreactors for treating Cr(VI)-contaminated wastewater.

The present article has been geared towards a study of the treatment of chromium-containing water using bacteria

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that have been isolated from chromium-contaminated water systems, and which feature the capability to convert the more toxic form of chromium to a relatively less toxic one. The kinetics of Cr(VI) reduction by the targeted organism has also been reported, with a view to facilitate the design of industrial scale wastewater treatment bioreactors which utilize these organisms.

Materials and Methods

Isolation of Bacterial Strain and Growth Conditions

Raw effluent from a nearby dye/pigment-based chemical industry was found to contain Cr(VI). Thus, it was anticipated that the aerator liquid of the activated sludge process in the effluent treatment facility of this plant would contain Cr(VI)-reducing bacteria. Hence, for the isolation of bacteria, aerator liquid samples were aseptically collected. The collected samples were serially diluted in sterile distilled water and plated in nutrient agar medium (Hi-Media Ltd, Mumbai, India) supplemented with 25 mg/l Cr(VI) [equivalent to 0.480 mM Cr(VI) or 70 mg/l of $K_2Cr_2O_7$] as potassium dichromate by the standard pour plate technique [2]. Plates were incubated at 30–35°C for 3–7 days. Isolated colonies that were morphologically distinct were selected and purified by repeated streaking on agar plates.

Evaluation of Chromium Resistance

The minimum inhibitory concentration (MIC) of Cr(VI) was determined by inoculating the bacteria into petriplates containing solid nutrient agar amended with potassium dichromate to provide Cr(VI) concentrations in the range of 50 to 1,200 mg/l in increments of 50 mg/l. The bacterial colonies, after full growth on plates, were subjected to higher concentrations of Cr(VI) successively. The Cr(VI) concentration above which the growth could not be observed in agar plate media was considered as the MIC of Cr(VI) for the bacteria.

Identification of the Isolated Strain

The isolated bacteria were identified by morphological, physiological, and biochemical characteristics and finally *via* 16S rRNA analyses.

16S rRNA Analysis

Total genomic DNA was isolated using GeneElute Genomic DNA isolation kits (Sigma-Aldrich, St. Louis, USA) and used as a template for PCR. FDD2 and RPP2 primers were used to amplify almost the entire 16S rRNA gene [26]. The PCR were performed and the product thus formed was precipitated and washed. An ABI Prism BigDye Terminator Cycle sequencing ready reaction Kit was used for the sequencing of the PCR product. A combination of universal primers was chosen to sequence the gene [17, 26]. After the preparation of the sequence reaction and template, the samples were run on an ABI Prism 310 Genetic Analyzer and the output sequence was analyzed. The sequence was compared with the National Center for Biotechnology Information GenBank entries by using the BLAST algorithm.

Chromium Reduction and Bacterial Growth Experiments

Luria–Bertani or nutrient broth (pH 7) with a 200 mg/l Cr(VI) concentration as potassium dichromate was inoculated with the selected bacterial isolate grown overnight and incubated at 37°C under aerobic conditions at 150 rpm. Aliquots were withdrawn at regular time intervals during the shake flask experiments and analyzed for bacterial growth and chromium reduction. Sterile inoculated broth without Cr(VI) served as the biotic control, and the uninoculated broth with Cr(VI) served as the abiotic control. The biotic control was used to compare the growth of bacteria with or without Cr(VI) and the abiotic control was used to test if any change in Cr(VI) was occurring as a result of the presence of the media components. All the experiments were performed in triplicate.

Analytical Method for Chromium Estimation

Samples were centrifuged at 10,000 rpm for 10 min at 4°C to remove any suspended biomass, and the concentration of Cr(VI) in the supernatant was determined spectrophotometrically at 540 nm, using diphenyl carbazide reagent under acidic conditions as the complexing agent for Cr(VI), as per APHA standard methods [2]. Absorbance measurements were conducted using a Hitachi U2000 model spectrophotometer. The total chromium concentration was determined by atomic absorption spectroscopy (AAS; GBC-932 PLUS). The concentration of Cr(III) was calculated from the difference between total Cr and Cr(VI) concentrations.

Biomass Estimation

For the measurement of microbial growth, the biomass concentration was determined by measuring the turbidity of the diluted sample at 600 nm and using a standard curve of absorbance against dry cell mass concentration. Dry cell mass for calibration was obtained by centrifuging the samples at 10,000 rpm for 10 min at 4°C, followed by drying of the pellets at 80°C till a constant weight was obtained. Triplicates of each calibration standard were taken to calculate the biomass.

Evaluation of Metal Tolerance

The resistance of the selected bacterial isolate to various heavy metals such as Ni, Cd, Zn, Fe, Cu, and Pb in the form of $NiCl_2$, $CdCl_2$, $ZnSO_4$, $FeCl_3$, $CuSO_4$, and $Pb(NO_3)_2$, respectively, at different concentrations, was examined in nutrient agar.

Antibiotic Resistance

Antibiotic susceptibility testing of the bacterial strain was carried out on Mueller–Hinton agar (Hi-Media Ltd, Mumbai, India) using the Kirby–Bauer disc diffusion method. The following discs were tested in this study: chlorampheicol (30 μg), nalidixic acid (30 μg), kanamycin (30 μg), streptomycin, (10 μg), novobiocin (5 μg), and gentamycin (10 μg).

Scanning Electron Microscopy (SEM)

Cell morphology was examined by SEM. Bacteria grown in liquid media with and without Cr(VI) were respectively harvested at their log phase by centrifugation at 8,000 rpm for 15 min. The cells were first fixed in 3% glutaraldehyde at 4°C for 24 h, and washed with phosphate buffer three times at 4°C. The fixed cells were then dehydrated through alcohol washing with a 10–100% ethanol series for 10 min at each stage and two washings in 100% ethanol. Thereafter, cells were washed with acetone, oven dried, and sputter coated with gold. The prepared samples were then examined with a JEOL JSM-6380LA scanning electron microscope.

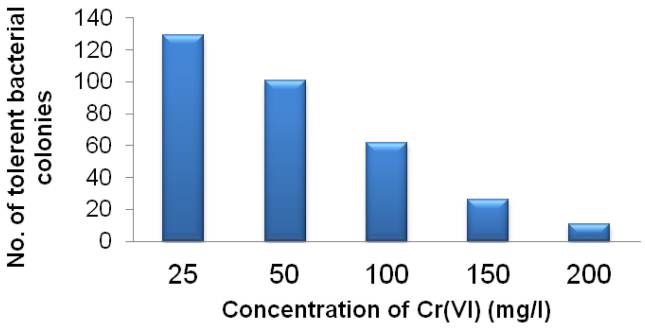

Fig. 1. Tolerant bacterial colonies number obtained during screening of Cr(VI)-resistant bacteria under each level of Cr(VI) concentration.

RESULTS AND DISCUSSION

Isolation and Identification of a Cr(VI)-Resistant Bacteria

Effluent obtained from a chemical industry showed the presence of 129 isolated colonies on media containing 25 mg/l Cr(VI). Isolated bacterial colonies differing in morphology were subjected to higher concentrations of Cr(VI). Fig. 1 shows the number of colonies screened at each level. Cr-B2 was one amongst those isolates, and was an aerobic, nonmotile, and Gram-negative rod-shaped bacteria. They could grow on solid media containing up to 1,100 mg/l Cr(VI). These strains exhibited pleomorphic behavior, often becoming coccoid. Their morphological, physiological, and biochemical characteristics are listed in Table 1. Results from 16S rRNA gene sequence analyses for Cr-B2 showed the highest homology (98%) with that of an uncultured *Acinetobacter* sp. clone TCCC 11087 (Accession No. EU567041.1), *Acinetobacter* sp. P11-B-4 (Accession No. EU016146.1), and *Acinetobacter haemolyticus* strain M116 (Accession No. HQ407291.1). *Acinetobacter haemolyticus* as a Cr(VI)-reducing strain was previously reported only once, at that time having been isolated from an effluent of a textile-related manufacturing premises, and demonstrated a resistance level of 90 mg/l Cr(VI) [39]. However, an *Acinetobacter* sp. that showed resistance towards Cr(VI) was also reported to have been isolated from pulp and paper mill effluent [33]. So far, *Acinetobacter* sp. with Cr(VI) tolerance levels as high as 1,100 mg/l has not been previously reported. Thus, the organism isolated in this study with a tolerance level of 1,100 mg/l differentiates itself from *Acinetobacter haemolyticus* and other *Acinetobacter* sp. with a homology of 98%, indicating its novelty. The sequence was submitted to the GenBank (NCBI) and an accession number (JF461086) was obtained.

Growth and Cr(VI) Reduction Batch Experiments

Fig. 2A shows the growth curve of Cr-B2 in the LB and nutrient media controls [absence of Cr(VI)], as well as in the LB and nutrient media containing 200 mg/l Cr(VI). It was observed that high growth was initially (up to 48 h) achieved in LB media containing Cr(VI), rather than in the absence of Cr(VI). After 48 h, the biomass growth with Cr(VI) was found to be less than the growth obtained without Cr(VI). In contrast with the LB media, the biomass was higher in the control as compared with that with Cr(VI), in the case of the nutrient media. Even though a reduction of Cr(VI) was observed in both the enrichments used in this study, it can be seen in Fig. 2B [Cr(VI) concentration vs. time] that during the 4 to 48 h of incubation period, the rate of Cr(VI) reduction was higher in the LB media than with the nutrient media during the batch runs. The higher rate of Cr(VI) in the LB media may be attributed to the higher biomass in the case of the LB media in comparison with the nutrient media, as can be seen from Fig. 2A. Pal and Paul [20] also observed that Cr(VI) reduction was higher when *Bacillus sphaericus* was grown in Vogel–Bonner (VB) as compared with a minimal salts broth. It was observed that >90% of Cr(VI) reduction was attained within 24 h when cells were grown in nutrient broth, whereas a similar reduction was observed within 12 h in the case of cells grown in LB media. However, the complete reduction was observed only after 78 h in both the media

Table 1. Morphological, physiological, and biochemical characteristics of Cr-B2.

Characters	Response
Morphological characteristics	
Colony morphology	Cream, smooth, circular
Cell morphology	rods
Gram reaction	-
Motility	+
Physiological characteristics	
Growth under aerobic condition	+
Growth under anaerobic condition	-
Biochemical characteristics	
Production of catalase	+
Oxidase	-
Indole production	-
Methyl red test	-
Voges–Proskauer	-
Hydrolysis of starch	-
Hydrolysis of urea	-
Hydrolysis of casein	-
Hydrolysis of gelatin	+
Nitrate reduction	-
H_2S production	-
Hemolysis on blood agar	+
Growth on MacConkey agar	+
Growth on eosin methylene blue agar	+
Growth on manitol salt agar	-

[a)]+: Positive response.
[b)]- : negative response.

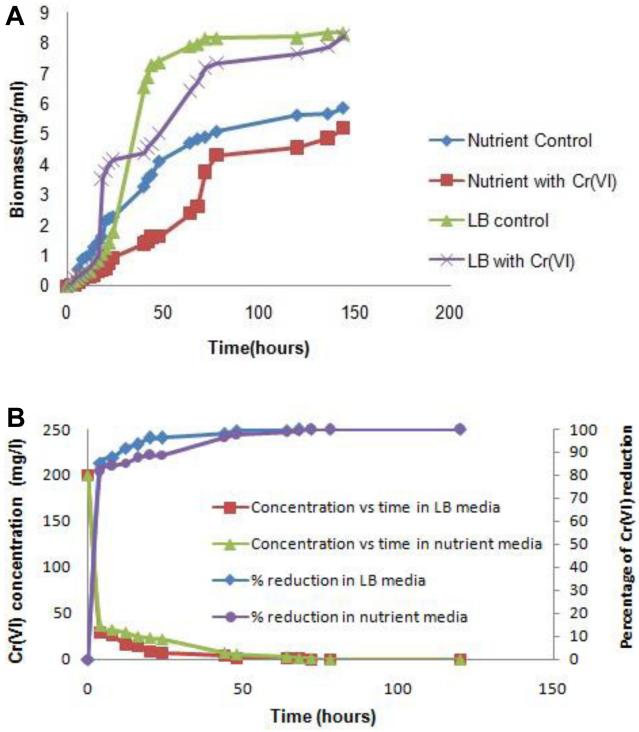

Fig. 2. Effect of media on Cr-B2 growth and Cr(VI) reduction. (**A**) Growth profile of Cr-B2 under nutrient media and LB media in the absence and presence of Cr(VI). (**B**) Time course variation of Cr(VI) concentration and the percentage Cr(VI) reduction in the presence of LB and nutrient media with Cr-B2.

used. In the abiotic controls, there was no change in the Cr(VI) concentration, indicating that bacterial cells are strictly responsible for the reduction. Strain Cr-B2 could completely reduce 200 mg/l Cr(VI), whereas *A. haemolyticus,* reported earlier, could reduce Cr(VI) only by up to 70 mg/l [39]. Zahoor and Rehman [40] reported that a *Bacillus* sp. and *S. capitis* could remove only 85% and 81% of Cr(VI), respectively, from water containing 100 mg/l of Cr(VI), although they had resistance of 4,500 and 2,800 mg/l, respectively, against Cr(VI). Another bacterial strain, *Bacillus sphaericus,* reported by Pal and Paul [20], could not remove Cr(VI) completely in the tested range of 10–100 mg/l, although it had an MIC of 800 mg/l. Similarly, *Ochrobactrum* sp. [35], *Brucella* sp. [36], and other strains have failed to reduce Cr(VI) completely at a concentration of 200 mg/l.

In the present study, the reduction of Cr(VI) was accompanied by a fading of the color (dark yellow) specific to Cr(VI) and an approach to a color similar to that of the biotic control, indicating the removal of Cr(VI). Thus, it was necessary to test whether the reduction in Cr(VI) concentrations during the biological process was due to sorption of Cr(VI), or due to a reduction of Cr(VI) to Cr(III). To accomplish this objective, total chromium concentration measurements of the samples during the shake flask experiments were performed using atomic absorption spectrometry (AAS), along with the Cr(VI) concentration measurements by the spectrophotometric method. The total soluble chromium measurement when deducted from the Cr(VI) measurement gives the soluble Cr(III) concentration. Although Cr(VI) may possibly be converted into pentavalent and tetravalent states, they are very short-lived and are unstable forms of Cr, and hence the difference in the total soluble Cr analyzed by AAS and the hexavalent chromium analyzed spectrophotometrically indicates the presence of the soluble form of Cr(III) being formed during the reduction process. Table 2 represents the various states of Cr formed during the time course of Cr(VI) reduction by different bacterial cultures. Although the initial Cr (as hexavalent) taken was 200 mg/l, the total Cr at all the times was lower

Table 2. Time course variation of concentrations of various forms of chromium in different media during biological reduction.

Media	Time (h)	pH	Total Cr[a] mg/l	Cr(VI)[b] mg/l	Soluble[c] Cr(III) mg/l	Insoluble[d] Cr(III) or cell associated Cr, mg/l
Nutrient	0	7	200	200	0	0
	24	8	166.45	22.21	144.23	33.55
	48	8.5	169.46	4.79	164.66	30.54
	78	9	173.57	0.1	173.46	26.43
	120	10	198.68	ND	198.67	1.32
LB	0	7	200	200	0	0
	24	8	164.44	7.49	156.94	43.05
	48	8.5	165.65	0.97	164.68	35.31
	78	9	167.59	0.16	167.43	32.56
	120	10	180.19	ND	180.19	19.80

[a]Total Cr (by AAS measurement), mg/l.
[b]Cr(VI) (by spectrophotometric analysis), mg/l.
[c]Soluble Cr(III) [difference of total Cr at any time and Cr(VI) at any time], mg/l.
[d]Insoluble Cr(III) adsorbed or accumulated in cell (difference of initial total Cr and total Cr at any time), mg/l.
[ND]Non-detectable.

than 200 mg/l. This differential quantity of Cr may be attributed to the insoluble form of Cr(III) precipitated after the reduction process, or may be related to the portion of Cr(VI) and/or Cr(III) adsorbed by the cells. It can be observed in Table 2 that the total chromium content decreased and then increased as the bioreaction proceeded and then approached the initial total Cr (200 mg/l) as Cr(VI) was taken for the batch study. The initial decrease may be due to sorption of the Cr(VI), or may be due to formation of insoluble organometallic complexes with the Cr(III) [which is formed after the reduction of Cr(VI) to Cr(III)]. The presence of the insoluble form of Cr(III) complexes in the media will not contribute to the AAS readings (total Cr) or spectrophotometric readings [Cr(VI)] as these measurement techniques involve analysis of supernatants that quantify only the soluble Cr.

There may be two possibilities for the increase in total chromium observed at the later phase: (i) If sorption of Cr by the cells had taken place in the initial phase, then a possibility for the increase in soluble Cr at the later phase may be desorption; (ii) if the insoluble form of Cr(III) had been formed in the initial phase, then this insoluble form would have been converted into the soluble form in the later phase. If the process of Cr(VI) removal is biosorption in the initial phase and desorption of Cr(VI) at a later time is the reason behind the increase in total chromium concentrations, then this should also be accompanied by an increase in Cr(VI) concentrations. However, in the present study, it was observed that the Cr(VI) concentration did not increase over time, indicating that the increase in total Cr is not attributable to the desorption of biosorbed Cr(VI). Therefore, the residual Cr, after deducting the total Cr at any time from the initial Cr, may be largely attributed to the insoluble form of Cr(III). During the reduction process, along with the formation of soluble Cr(III), the insoluble organo-Cr(III) complexes would have also been formed by the complexing of produced Cr(III) with certain organic constituents or metabolites present in the media. These results are supported by the work of Puzon *et al.* [24], who found that Cr(VI) reduction in the presence of cellular organic metabolites formed both the soluble and insoluble organo-Cr(III) end-products.

As the reaction proceeded, it was found that the pH increased from 7 to 10, and the increased pH led to an increase in the solubility of organo-Cr(III) compounds, which were insoluble at lower pH values, leading to an increase in the soluble Cr(III) concentration during the later phase. Such a dependency of Cr(III) solubility on pH has been reported by Remoundaki *et al.* [28]. Bolan *et al.* [4] also reported that Cr(VI) reduction is a proton consumption reaction, and the pH increases, as Cr(VI) is reduced. The latter study found that Cr(III) solubility increases with an increase in pH above 7 in the presence of yeast extract, nutrient media, and peptones with different weight ratios of organics to Cr(III). In the present study, it was observed that there was no change in pH in the case of the abiotic controls. Thus, the increase in total Cr concentrations during the later phase may be attributed largely to the conversion of the insoluble form of Cr(III) to the soluble form of Cr(III). Although the difference between the initial Cr and total Cr could have also been attributed to the biosorbed Cr(VI), the total Cr finally approached closly to the initial Cr concentration of 200 mg/l, showing that the contribution of biosorbed Cr, in the difference between the initial and final Cr concentrations, is very small. The biosorbed Cr component may only be of a magnitude equal to the difference between the initial Cr and the final total Cr (at the end of 120 h). This implies that the majority of the Cr(VI) initially present in the media had been reduced to Cr(III), and only a very small portion had been associated with the cell. These discussions are based on the qualitative and partial quantitative analyses of the experimental observations and results. Concrete quantitative analysis, like measurements of cells-associated Cr after the digestion of the cells, is needed for an understanding of the exact mechanism.

For the industrial treatment of wastewater, a high Cr(VI)-reducing capability is necessitated and Cr-B2 has the potential to provide it.

Cr(VI) Reduction Kinetics with Cr-B2

Cr(VI) reduction kinetics were studied in both LB and nutrient media. Rates of Cr(VI) reduction at different times were obtained by drawing tangents on plots of Cr(VI) concentrations vs. time data obtained by batch experiments with a 200 mg/l initial Cr(VI) concentration. Nonlinear regression analysis on the rate vs. concentration data were performed using Sigma Plot 12 software and it was found that the data fit well into the "Hill Equation" given by Eq. (1) in both cases.

$$V = \frac{V_{max} S^n}{K_m^n + S^n} \quad (1)$$

where V is the rate of removal of Cr(VI), mg/l/h
S is the concentration of Cr(VI), mg/l,
V_{max} is the maximum rate of Cr(VI) reduction, mg/l/h
K_m is related to the K_m of the Michalis–Menten equation
n is the Hill slope or the cooperativity coefficient

The values of kinetic parameters K_m, V_{max}, and n for Cr(VI) reduction kinetics with LB broth and nutrient media, along with the parameters representing the goodness of fit, are presented in Table 3. Fig. 3A and 3B show the plots of rate vs. Cr(VI) concentration obtained from the experimental data and from the kinetic model predicted data for nutrient media and LB media, respectively. Corresponding embedded plots indicate the goodness of the fit with R^2 values of 0.9943 and 0.9916, respectively.

Table 3. Kinetic parameters of allosteric model for Cr(VI) reduction in nutrient and LB media.

Media	V_{max} (mg/l/h)	K_m (mg/l)	n	R^2	Adj, R^2
Nutrient media	13.1715	43.1126	8.47791	0.9943	0.99271
LB media	12.5398	38.05446	3.49965	0.9911	0.98914

It was found from these plots that the rate of Cr(VI) reduction increased with an increase in the Cr(VI) concentration. The sigmoidal nature of the curves shows that this organism follows the trend of allosteric enzyme kinetics [31]. Allosteric enzymes have multiple active sites and their affinity for substrate [Cr(VI)] increases with increasing substrate [Cr(VI)] concentrations. The binding of one substrate to the enzyme facilitates the binding of the other substrate molecules. This behavior is known as allostery or cooperative binding [31]. It can be observed from Table 3 that the values of n are larger than 1. This indicates positive cooperativity. Suzuki *et al.* [34] reported a similar allosteric pattern for Cr(VI) reduction kinetics in the enzyme of *P. ambigua* G-1.

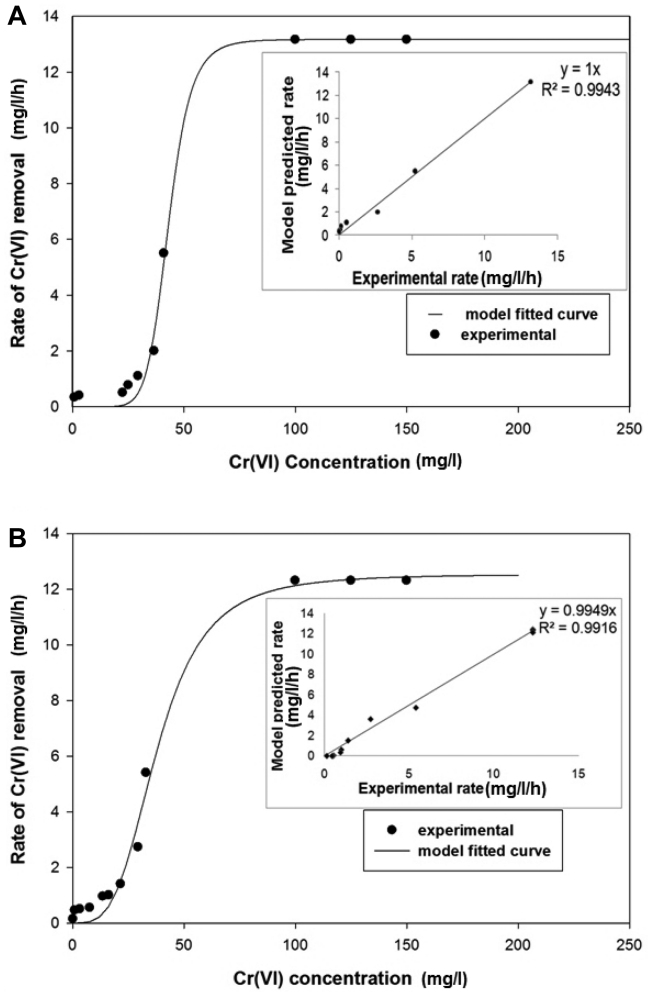

Fig. 3. Rate of Cr(VI) removal (mg/l/h) vs. Cr(VI) concentration (mg/l) (**A**) with Cr-B2 grown in nutrient media; and (**B**) with Cr-B2 grown in LB media.
Data were subjected to hyperbola nonlinear regression analysis to calculate K_m and V_{max}. The embedded plot shows the experimental rate vs. model predicted rate with the R^2 value.

Scanning Electron Microscopy (SEM) Analysis

Scanning electron microscopic images of these bacteria, when grown in the absence of Cr (VI) and in the presence

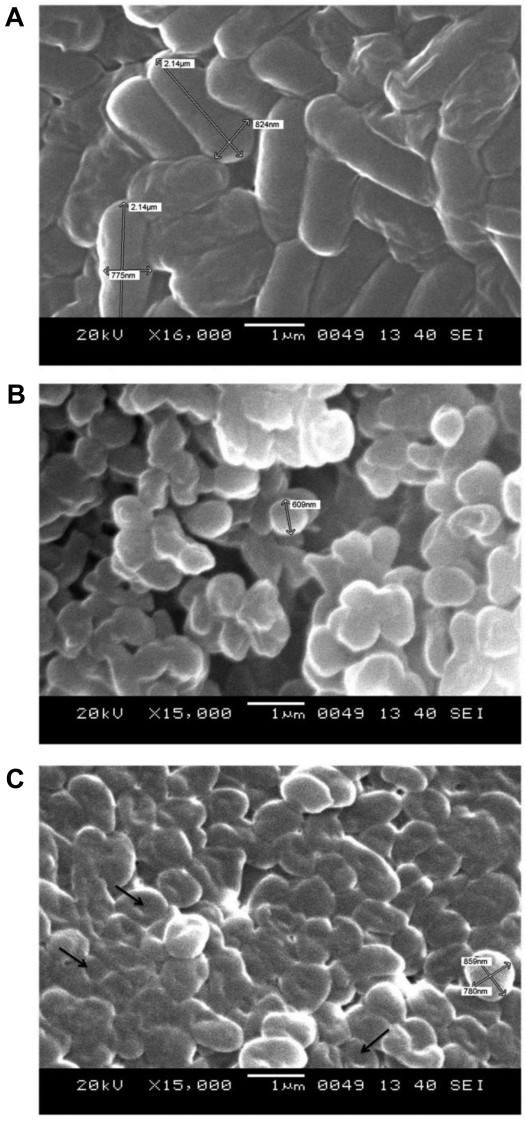

Fig. 4. Microscopy results.
SEM micrographs of Cr-B2 grown in the (**A**) absence of Cr(VI); (**B**) presence of 300 mg/l Cr(VI); and (**C**) presence of 800 mg/l Cr(VI). Arrows indicate the "depression" found on the bacterial cell surface.

Table 4. MIC of various heavy metals and antibiotic sensitivity of Cr-B2.

Heavy metal	MIC (mg/l)	Antibiotic	Resistant (R)/Sensitive (S)
Cu^{2+}	600	Chlorampheicol	S
Fe^{3+}	1,000	Nalidixic acid	S
Zn^{2+}	700	Kanamycin	S
Pb^{2+}	1,100	Streptomycin	S
Cd^{2+}	350	Novobiocin	R
Ni^{2+}	800	Gentamycin	S

of Cr(VI) at 300 and 800 mg/l, are presented in Fig. 4A, 4B, and 4C, respectively. SEM analysis revealed the morphological changes under Cr(VI) stress. Change in morphology with a reference to the size and surface characteristics, such as an increase/decrease in cell wall thickness [13, 39], irregular nodules formation [13], pilus-like structures [1, 39], small bodies over the cell surface, and ridges [33] by certain Cr(VI)-reducing species of bacteria, have been reported. However, in the present study, as can be observed from Fig. 4, the bacterial cells grown in the absence of Cr(VI) exhibited a rod-shaped morphology, whereas the cells grown in the presence of Cr(VI) exhibited a cocci shape. This indicates a pleomorphic pattern for the strain. Reports of such strains are very scarce, yet despite this, wrinkles were observed in the cells grown in the presence of 800 mg/l Cr(VI), indicating the toxicity affects of Cr(VI) on bacterial cells. A "wrinkled" cell surface in the presence of Cr(VI) has also been reported for *A. haemolyticus* by Pei *et al.* [22] and similar results have been observed in *Arthrobacter* sp. [33]. As can be seen in Fig. 4C, the Cr-B2 strain grown in 800 mg/l Cr(VI) exhibited single "depressions" or "indentations" (shown by an arrow mark in Fig. 4C) in the cocci-shaped cells, making them more concaved.

Effects of Other Heavy Metals on Cr-B2

Industrial effluents may be characterized by the presence of many metals. Resistance of a strain against a single metal ion may be altered by the presence of other metals. Hence, it was necessary to check the resistance of the isolated strain to other metals. Cr-B2 showed resistance not only against Cr(VI), but also to the other metals tested in this study. The strain Cr-B2 exhibited resistance in the following order: Pb> Fe> Ni> Zn> Cu> Cd. Table 4 lists the minimum inhibitory concentrations of all the heavy metals tested in this study.

Effects of Antibiotics on Cr-B2

The prevalence of metal-tolerant microorganisms is ecologically important, particularly if they are also antibiotic resistant. Several researchers [7, 19, 25, 30, 32] have studied the association between resistance to antibiotics and heavy metals and found that the genes encoding resistance to metals and antibiotics were located on the same transmissible plasmids. Hence, the isolates resistant to one should be analyzed for tolerance to the other. An attempt was made to check the antibiotic resistance of Cr-B2 in order to obtain a preliminary insight regarding the presence of plasmids. Cr-B2 exhibited activity against all the tested antibiotics, and the results are listed in Table 4. Since Cr-B2 was sensitive against all the antibiotics tested in this study, other than novobiocin, it is apparent that the plasmid-mediated resistance mechanisms are not well understood. Similar observations have been noted by other authors who have reported a specific strain resistant against heavy metals and a few antibiotics, and sensitivity to a few other antibiotics. However, an earlier report showed that most of the *Acinetobacter* strains carry multiple indigenous plasmids of various sizes [3, 37].

During the last two decades, much attention has been paid to the treatment of wastewater containing heavy metals, through biological techniques, and the search for highly potent strains continues. The *Acinetobacter* sp. Cr-B2 isolated in the current study has proved to be a promising bacterial strain that could be used in the bioremediation of wastewaters. This strain had a resistance of 1,100 mg/l Cr(VI) on agar media with the capability to convert Cr(VI) to Cr(III) efficiently in liquid media. The Cr(VI) reduction kinetics followed an allosteric model. This strain has a high resistance against most of the metals tested and hence its role may not be restricted to Cr(VI) removal alone. Since *Acinetobacter* strains exhibit metabolic versatility and robusticity, they can be easily employed. However, much research and further extensive study are needed in order to make its use practicable for industry application.

REFERENCES

1. Abboud, R., R. Popa, V. Souza-Egipsy, C. S. Giometti, S. Tollaksen, J. J. Mosher, *et al*. 2005. Low temperature growth of *Shewanella oneidensis* MR-1. *Appl. Environ. Microbiol.* **71:** 811–816.
2. American Public Health Association (APHA). 1998. *Standard Methods for Examination of Water and Wastewater*, 20th Ed. American Public Health Association, American Water Works

Association and Water Pollution Control Federation, Washington DC, USA.
3. Bergogne-Bérézin, E. and K. J. Towner. 1996. *Acinetobacter* spp. as nosocomial pathogens: Microbiological, clinical, and epidemiological features. *Clin. Microbiol. Rev.* **9:** 148–165.
4. Bolan, N. S., D. C Adriano, R. Natesan, and K. Bon-jun. 2003. Reduction and phytoavailability of Cr(VI) as influenced by organic manure compost. *J. Environ. Qual*. **32:** 120–128.
5. Bopp, L. H. and H. L. Ehrlich. 1988. Chromate resistance and reduction in *Pseudomonas fluorescens* strain LB300. *Arch. Microbiol.* **150:** 426–431.
6. Branco, R., M. C. Alpoim, and P. V. Morais. 2004. *Ochrobactrum tritici* strain 5bvI1 – characterization of a Cr(VI)-resistant and Cr(VI)-reducing strain. *Can. J. Microbiol.* **50:** 697–703.
7. Dhakephalker, P. K. and B. A. Chopade. 1994. High levels of multiple metal resistances and its correlation to antibiotics resistance in environmental isolates of *Acinetobacter. Biometals* **7:** 67–74.
8. Gopalan, R. and H. Veeramani. 1977. Development of a *Pseudomonas* sp. for aerobic chromate reduction. *Biotechnol. Tech.* **46:** 414–417.
9. Horitsu, H., H. Nishida, H. Kato, and M. Tomoyeda. 1978. Isolation of potassium chromate tolerant bacterium and chromate uptake by the bacterium. *Agric. Biol. Chem.* **42:** 2037–2043.
10. Kvasnikov, E. I., T. I. Klyushnikova, T. P. Kasatkinsa, V. V. Stepanyuk, and S. L. Kuberskaya. 1988. Bacteria reducing chromium in nature and in industrial sewage. *Mikrobiologiya* **57:** 680–685.
11. Lameiras, S., C. Quintelas, and T. Tavares. 2008. Biosorption of Cr(VI) using a bacterial biofilm supported on granular activated carbon and on zeolite. *Bioresour. Technol.* **99:** 801–806.
12. Lebedeva, E. V. and N. N. Lyalikova. 1979. Reduction of crocoite by *Pseudomonas chromatophila* species nova. *Mikrobiologiya* **48:** 517–522.
13. Lin, Z., Y. Zhu, T. L. Kalabegishvili, N. Y. Tsibakhashvili, and H. Y. Holman. 2006. Effect of chromate action on morphology of basalt-inhabiting bacteria. *Mater. Sci. Eng. C* **26:** 610–612.
14. Masood, F. and A. Malik. 2011. Hexavalent chromium reduction by *Bacillus* sp. strain FM1 isolated from heavy-metal contaminated soil. *Bull. Environ. Contam. Toxicol.* **86:** 114–119.
15. Luli, G. W., J. W. Talnagi, W. R. Strohl, and R. M. Pfister. 1983. Hexavalent chromium-resistant bacteria isolated from river sediments. *Appl. Environ. Microbiol.* **46:** 846–854.
16. McGrath, S. P. and S. Smith. 1990. Chromium and nickel, pp. 125. *In* B. J. Alloway (ed.). *Heavy Metals in Soils*. Wiley, New York.
17. Muyzer, G., E. C. De Waal, and A. G. Uitterlinden. 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Appl. Environ. Microbiol.* **59:** 695–700.
18. Nourbakhsh, M., Y. Sag, D. Ozer, Z. Aksu, T. Kutsal, and A. Calgar. 1994. A comparative study of various biosorbents for removal of chromium (e) ions from industrial wastewater. *Process Biochem.* **29:** 1–5.
19. Novick, R. P. and C. Roth. 1968. Plasmid linked resistance to inorganic salts in *Staphylococcus aureus*. *J. Bacteriol.* **95:** 1335–1342.
20. Pal, A. and A. K. Paul. 2004. Aerobic chromate reduction by chromium-resistant bacteria isolated from serpentine soil. *Microbiol. Res.* **159:** 347–354.
21. Pattanapipitpaisal, P., N. L. Brown, and L. E. Macaskie. 2001. Chromate reduction and 16S rRNA identification of bacteria isolated from a Cr(VI)-contaminated site. *Appl. Microbiol. Biotechnol.* **57:** 257–261.
22. Pei, Quek, Shahir Shafinaz, Santhana Raj, A. Zakaria Zainul, and Ahmad Wani. 2009. Chromium(VI) resistance and removal by *Acinetobacter haemolyticus*. *World J. Microbiol. Biotechnol.* **6:** 1085–1093.
23. Petrilli, F. L. and S. De Flora. 1977. Toxicity and mutagenicity of hexavalent chromium on *Salmonella* Typhimurium. *Appl. Environ. Microbiol.* **33:** 805–809.
24. Puzon, G. J., A. R. Roberts, D. M. Kramer, and L. Xun. 2005. Formation of soluble organo-chromium(III) complexes after chromate reduction in the presence of cellular organics. *Environ. Sci. Technol*. **39:** 2811–2817.
25. Ramteke, P. W. 1997. Plasmid mediated co-transfer of antibiotic resistance and heavy metal resistance in coliforms. *Indian J. Med. Microbiol*. **37:** 177–181.
26. Rawlings, D. E. 1995. Restriction enzyme analysis of 16S rRNA genes for the rapid identification of *Thiobacillus ferrooxidans, Thiobacillus thiooxidans* and *Leptospirillum ferrooxidans* strains in leaching environments, pp. 9–17. *In* T. Vargas, C. A. Jerez, J. V. Wiertz, and H. Toledo (eds.). *Biohydrometallurgical Processing.* 2nd Ed. Chile University.
27. Rehman, A., A. Zahoor, A. Munner, and A. Hasnain. 2008. Chromium tolerance and reduction potential of a *Bacillus* sp. env3 isolated from metal contaminated wastewater. *Bull. Environ. Contam. Toxicol*. **81:** 25–29.
28. Remoundaki, E., A. Hatzikioseyian, and M. Tsezos. 2007. A systematic study of chromium solubility in the presence of organic matter: Consequences for treatment of chromium-containing waste water. *J. Chem. Technol. Biotechnol.* **82:** 802–808.
29. Romanenko, V. I. and V. N. Korenkov. 1977. A pure culture of bacterial cells assimilating chromates and bichromates as hydrogen acceptors when grown under anaerobic conditions. *Mikrobiologiya* **46:** 414–417.
30. Schottel, L., A. Mandal, D. Clark, S. Silver, and R. W. Hedges. 1974. Volatilization of mercury and organomercurials determined by F factor system in enteric bacilli. *Nature* **251:** 335–337.
31. Shuler, M. L. and F. Kargi. 2005. *Bioprocess Engineering: Basic Concepts,* pp. 67–69. 2nd Ed. Prentice Hall of India, New Delhi.
32. Spain, A. 2003. Implications of microbial heavy metal resistance in the environment. *Rev. Undergrad. Res*. **2:** 1–6.
33. Srivastava, S and I. S. Thakur. 2007. Evaluation of biosorption potency of *Acinetobacter* sp. for removal of hexavalent chromium from tannery effluent. *Biodegradation* **18:** 637–646.
34. Suzuki, T., N. Miyata, H. Horitsu, K. Kawai, K. Takamizawa, Y. Tai, *et al.* 1992. NAD(P) H-dependent chromium(VI) reductase of *Pseudomonas ambigua* G-1: A Cr(V) intermediate is formed during the reduction of Cr(VI) to Cr(III). *J. Bacteriol.* **174:** 5340–5534.
35. Thacker, U. and D. Madamwar. 2005. Reduction of toxic chromium and partial localization of chromium reductase activity

in bacterial isolate DM1. *World J. Microbiol. Biotechnol.* **21:** 891–899.

36. Thacker, U., R. Parikh, Y. Shouche, and D. Madamwar. 2007. Reduction of chromate by cell-free extract of *Brucella* sp. isolated from Cr(VI) contaminated sites. *Bioresour. Technol.* **98:** 1541–1547.
37. Towner, K. J., E. Bergogne-Bérézin, and C. A. Fewson. 1991. *The Biology of Acinetobacter*: *Taxonomy, Clinical Importance, Molecular Biology, Physiology, Industrial Relevance*. Plenum Publishing Corp, New York.
38. Wang, P. C., T. Mori, K. Komori, M. Sasatsu, K. Toda, and H. Ohtake. 1989. Isolation and characterization of an *Enterobacter cloacae* strain that reduces hexavalent chromium under anaerobic conditions. *Appl. Environ. Microbiol.* **55:** 1665–1669.
39. Zakaria, A., Z. Zakaria, S. Surif, and W. A. Ahmad. 2007. Hexavalent chromium reduction by *Acinetobacter haemolyticus* isolated from heavy-metal contaminated wastewater. *J. Hazard. Mater.* **146:** 30–38.
40. Zahoor, A. and A. Rehman. 2009. Isolation of Cr(VI) reducing bacteria from industrial effluents and their potential use in bioremediation of chromium containing wastewater. *J. Environ. Sci.* **21:** 814–820.