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Chromium bioremediation by *Alcaligenes sp.* strain newly isolated from chromite mine of Sabzevar

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ABSTRACT

In this work, CKCr-6A strain was found to be highly resistant to some toxic heavy metals such as Cr^{+6}, Cr^{+3}, Cu^{+2}, Co^{+2}, Cd^{+2}, Pb^{+2}, Hg^{+2}, U^{+6}, tellurium, and selenite. Herein, high chromate tolerance of an isolated strain is reported with a high minimum inhibitory concentration value of 80,000 mg/L and the effective parameters (pH, temperature, shaking, and glucose concentration) were selected for Cr(VI) removal by this isolated strain. Cr(VI) elimination by the target strain increased with glucose addition to the culture medium. We optimized the possible parameters and their interactions using design experimental software. After optimization, this strain showed high efficiency in detoxifying chromate; this could reduce up to 100 mg/L of Cr(VI) to Cr(III) over 3 h. The CKCr-6A strain exhibited ability to *in vitro* reduction after 3 h and repeated removing of Cr(VI) without any amendment of nutrients, suggesting its possible application in continuous bioremediation.

Keywords: Bioremediation; Carcinogenic Cr(VI); Chromium reduction; Reductase; Response surface methodology

Introduction

Heavy metals pollution is one of the main problems in the world and it is increasing day to day. The wide use of chromium in electroplating and leather-tanning methods, among others, has caused higher chromium concentrations in aquatic systems.[1-3] The high mobility, bioavailability, and toxicity of Cr(VI) make this ion an important environmental concern.[4,5] Cr(VI) enters the cell through non-specific anion channels and is metabolically reduced to Cr(V), Cr(IV), and Cr(III). Removal of chromium from mining and industrial effluents is important before discharging it into the aquatic environment. Cr(VI) reduction or chromium immobilization can be produced biotically by different substances.[6-8] However, recent reports have proved the consequences of using biological materials, including living and non-living microorganisms, for the treatment of Cr(VI) containing wastes. Microbial actions on heavy metals availability include absorption, bioleaching, mineralization, intracellular accumulation, and enzyme-catalyzed transformation by redox processes.[9-12] The classical method of chromium removal optimization involves varying one parameter at a time and keeping the others constant. But the method is inefficient as it fails to understand relationships between variable parameters and the chromium uptake percentage. As a solution, the statistical method of response surface methodology (RSM) has been proposed to include the influence of individual factors as well as their interactive influences. The process efficiency could be increased by optimizing these factors. RSM has been proposed to determine the influence of individual factors and their interactive influences.[13, 14]

Recently, in my research laboratory at Hakim Sabzevari University, a total of 11 Cr-resistant bacteria, following plating on media amended up to 1,000 mg/L Cr(VI), were isolated from contaminated soil of the Cheshmeh Khan mine near Sabzevar City in Khorasan Razavi province. We have reported that one strain isolated was *Bacillus Cereus*,

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which is capable of Cr(VI) reduction in aerobic conditions and has the highest minimum inhibitory concentration (MIC) value (75,000 ppm).[15] The *Aalcaligenes sp.* strain which is reported in this article, like *Bacillus Cereus*, is able to reduce greater concentrations of chromium. Furthermore, presence of other toxic contaminants does not inhibit this process. As well as, the CKCr-6A strain mentioned in this work, like *Entrobacter sp.* strain, can eliminate the selenium oxy anions from a culture medium and reduce them to elemental SeNPs in the range of ~100 nm being capable of producing selenate reductase enzyme.[16, 17] In order to evaluate the effect of different parameters on biomass growth and Cr(VI) removal and to determine the optimum conditions, central composite design (CCD) and RSM were employed in the present study.

Materials and Methods

Strain isolation, the MIC, and antibiotic resistance determination

The contaminated samples were collected from Cheshmeh Khan chromium mine, around Sabzevar City in the Khorasan Razavi Province of Iran. For isolation and enumeration of Cr(VI)-reducing bacterial strains, 10 g soil was added into 10 ml of normal saline and incubated at 37 ºC overnight. A 0.1 ml of 10^{-6}, 10^{-7}, and 10^{-8} dilution sequence was spread on LB agar plates containing 200 mg/L sterilized solution of K_2CrO_4 and was incubated at 37 ºC. A purified colony for strain was injected into 50 ml of LB medium at 37 ºC (180 rpm) for overnight growth. Three ml of the grown bacterial cultures was transferred into 50 ml of fresh liquid nutrient medium containing 0–1000 mg/L Cr(VI) and incubated in the same conditions. Furthermore, the effects of aerobic and anaerobic conditions on Cr(VI)-reduction by strain CKCr-6A in the presence and absence of aeration, by incubator shaker, was investigated. At time intervals, 5.0 ml aliquots were drawn from the medium and Cr(VI) was measured in the supernatant. All the experiments were done at least in triplicate.

MIC of the Cr(VI)-resistant isolate was determined by broth dilution methods in LB medium, with various Cr(VI) concentrations (200–100,000 mg/L). The minimum concentration of heavy metal in the medium which inhibited complete growth was taken as MIC.[11] An EC50 value was also calculated by Probit analysis using computer software (SPSS 16.0). Antibiotic sensitivity and resistance of the CKCr-6A strain were assayed according to the Kirby–Bauer disc diffusion method.[18, 19] Thickness of the clear zone around the antibiotic discs was determined after 18 h of incubation. The CkCr-6A Strain was considered susceptible when the inhibition zone was 12 mm or more in diameter.

PCR amplification and 16S rRNA sequencing

The genomic DNA of a single colony of CKCr-6A strain was extracted according to Sambrook and Russell.[20] Universal 16S rRNA PCR forward primer (5'-AGTTTGATCCTGGCTCAG-3') and reverse primer (5'-GGC/T TACCTTGTTACGACTT-3') were used in the amplification of 16S rRNA genes.[21] After DNA sequencing, the nucleotide sequence was initially analyzed using BLAST (Gen Bank database of the National Center for Biotechnology Information [Bethesda, MD], www.ncbi.nlm.nih.gov/GenBank/).

The sequence similarity analysis was performed using the ClustalW program. The CKCr-6A 16S rRNA sequence has been deposited in GenBank under accession number of KC993901. A phylogenetic tree was constructed using PhyloDraw software between different members of genus *Alcaligenes*.

Atomic Force Microscopy (AFM) and Scanning Electron Microscopy (SEM)

AFM was also performed to study of the structural changes of the bacterial surface exposed to heavy metals. AFM measurements of CKCr-6A (grown both in the absence and presence of 100 mg/L Cr[VI]) were taken. For this reason, 5 µl of the medium containing bacteria was centrifuged and the pellet was washed with sodium phosphate buffer (50 mM, pH 7). Bacterial cells grown in LB media with and without Cr(VI) were picked up by centrifugation at 8,000 rpm for 10 min at 4 ºC. Cells were fixed, dried, and then SEM was obtained on a VEGA TESCAN.

Analytical method for evaluation of Cr(VI) reducing ability

In culture medium

The culture medium was centrifuged at

8,000 rpm for 15 min, then the supernatant fractions were analyzed for the remaining Cr(VI). The decrease in Cr(VI) concentration with time was estimated via spectrophotometry using 1,5-Diphenylcarbazide (DPC) as the complexing agent at 540 nm. The 1 ml of sample containing free Cr(VI) ions was mixed with 3 ml of H_2SO_4 (200 mM) and of 1 ml of DPC. The pink-violet colored solution was examined for the Cr(VI) ions.[22] Furthermore, total chromium determination was analyzed using the inductively coupled plasma atomic emission spectrometry (ICP-AES).

In-vitro removal with resting and permeablilized cells

Culture suspension of CKCr-6A was grown overnight in 50 ml LB (pH 7.0) and harvested by centrifuge at 5,000 rpm for 6 min at 4 ºC. Resting cell pellets were washed with 1 ml of 20 mM potassium phosphate buffer (pH 7.0) and resuspended in the same buffer with 40 mg/L Cr(VI). The tubes were stirred and incubated for 2 h at 25 ºC. The remaining Cr(VI) concentration was estimated using DPC reagent. For permeabilized cell preparation, overnight grown cells of CKCr-6A strain were harvested and washed and the bacteria sedimentation suspension (1 ml) in the same buffer was prepared. Suspended cells were treated with 0.02 M $CaCl_2$. All of these cell suspensions were spiked with 40 mg/L K_2CrO_4 solution and incubated at 25 ºC for 2 h, and then the Cr(VI) concentration was measured from the supernatant. Experiments with each set of resting cells and permeabilization treatment were performed in triplicate. Heat-destroyed cells were used as controls.

Analysis of chromate reductase activity in cell-free extract

Isolated strain was cultured in LB amended with 50 mg/L of Cr(VI) at 37 ºC and pH 7 for 24 h to increase the chromium reductase enzyme production. The pellet was washed with buffer; the cells were disrupted by sonication and cell-free extract was applied to the pre-incubated reaction mixture (0.05 mM chromate, 0.1 mM NADH in 600 µL of 20 mM phosphate buffer pH 7). Samples were incubated at 37 ºC for 20 min and the residual chromium was measured. Cr(VI) was determined as defined above. Control mixtures included 20 mM buffer and respective Cr(VI) concentrations without any addition of cell-free extracts.

Effects of different parameters on chromate uptake/reduction

This step served as a screening test to identify which factors had a significant effect on the *in vivo* and *in vitro* chromate removal. In this set of experiments, the level of each factor was altered, while all other experimental factors remained constant. In this work, the resistance and reduction of Cr (VI) for the selected strain (CKCr-6A) were performed under varying incubation periods, initial Cr(VI) concentration (50–1,000 mgL^{-1}), pH (3-9), temperature (25–50 ºC), shaking speed (0–240 rpm), and commonly used carbon source with different structures and reducing abilities (glucose, fructose, sucrose, lactose, ammonium acetate, potassium carbonate, and glycine [1.0 %]) were used to optimize the parameters.

Experimental design for optimization of Cr(VI) removal

According to the outcomes obtained in tests using one factor at a time, Design Expert software (version 7.0, Stat-Ease, Inc., Minneapolis, MN) was applied to improve the chromium uptake efficiency.[13] The four factor-five coded considered level of experimental variables in this paper are presented in Table 1.

Table 1. Independent variables and their levels in the experimental design

Level	Coded Level	Uncoded Level			
	(x_i)	pH (i =1, X_1)	Temperature (°C) (i = 2, X_2)	Glucose con. (%) (i = 3, X_3)	Stirring speed (RPM) (i = 4, X_4)
Lowest	-2	4.75	33.0	0.00	0.00
Low	-1	5.50	37.0	0.50	60.0
Mid	0	6.25	41.0	1.00	120.0
High	+1	7.00	45.0	1.50	180.0
Highest	+2	7.75	49.0	2.00	240.0

The quality of the fit of the polynomial model equation was expressed by different criteria; furthermore, the correctness of the results, which were not included in the model estimation. Clearly, all empirical models were valid just within the variable ranges that were

model was verified through comparing the model predicted values with the experimental used to predict the model and any extrapolation led to considerable errors. The optimum values of the selected variables were found by resolving the regression equation and the response surface contour plots were also studied.[23]

Instrumental analysis of the reduced products Fourier transforms infrared (FTIR) spectroscopy

FTIR spectroscopy using Perkin Elmer FTIR spectrometer in the region of 400–4,000 cm^{-1} was performed to explain the modifications in the functionalities of the chromate reducing bacteria in the presence and absence of various concentrations of chromium. The cells grown overnight in the lack and presence of Cr(VI) were harvested by centrifugation. The sample cells and control cells pelleted were washed with 0.85% NaCl to remove the loosely bounded ions and then centrifuged for collecting the washed cells. The collected biomass was dried in an oven and then the sample/KBr ratio of 1/100 was prepared.

X-ray diffraction analysis (XRD)

The bacterial cells linked with reduced product were centrifuged and then the obtained pellets were washed with phosphate buffer (pH 7) before drying at 50 ºC. The analysis of samples was carried out by XRD methods.

Results and Disruption

Isolation, identification, and estimation of chromium tolerance of isolated strain

In this study, a total of 11 Cr-resistant bacteria were isolated from contaminated soil following plating on media amended up to 1,000 mg/L Cr(VI). The isolated CKCr-6A was selected due to its high Cr(VI) tolerance, which this paper showed 99% homology with *Alcaligenes sp.* Growth and chromate removal in the LB medium supplemented initially with different concentrations of Cr(VI) were measured at time intervals (3 h). This strain grew in the presence of high chromium strain was resistant to Cu^{+2}, Co^{+2}, Cd^{+2}, Pb^{+2} Hg^{+2}, uranium, tellurite, and selenite. The phylogenetic analysis of the isolated strain in concentration and reduction was initiated immediately in the presence and absence of electron donor such as glucose (Fig. 1A& Fig 1A, inset). The color of the medium changed from yellow to greenish with longer incubation. Alteration of Cr(VI) to Cr(III) due to the media color modification was detected. Furthermore, the results showed that various Cr(VI) concentrations up to 1000 mg/L does not particularly affect bacterial growth in this strain (Fig. 1B).

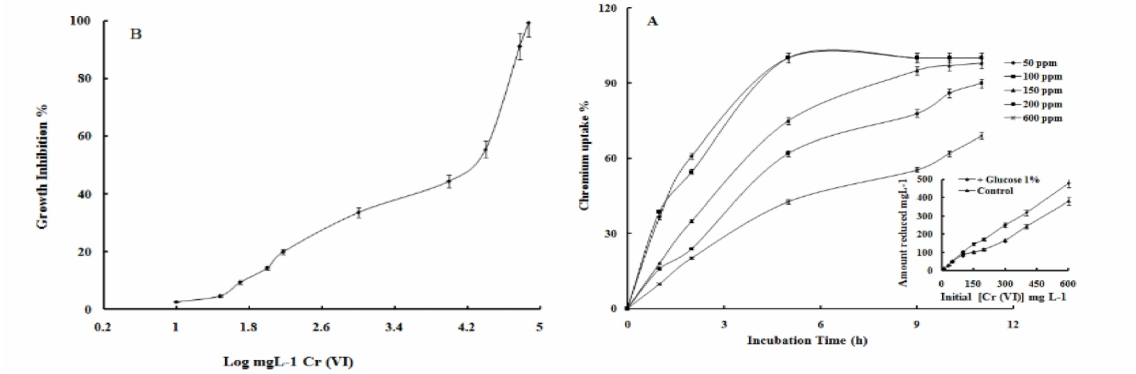

Fig. 1. (A) The effect of [chromium] on Cr(VI) reduction at 37 ºC, pH 7.0, and glucose (1%). Inset: amount of Cr(VI) reduced after 10 h in the absence (control) and presence of an electron donor, such as glucose (1%) with varying initial Cr(VI) concentration. (B) Inhibition of growth in CKCr-6A strain in the presence of different concentrations of Cr(VI) upto 100,000 mg/L

The slightly reduced growth at high chromium concentrations was mentioned to be most likely related to the modification of genetic material as well as to changed metabolic and physiological reactions of bacteria. For these reasons, pH of cultures amended with Cr(VI)

was changed from alkaline to acidic range during the bacterial growth. Moreover, Cr(VI) reduction was not affected by increase of different heavy metals (Cd^{+2}, Cu^{+2}, Co^{+2}, Pb^{+2}) under culture conditions (Fig. 2A). Metals like Hg^{+2} and Ag^{+} slightly reduced Cr(VI) uptake. These results may provide a useful organism for the bioremediation of chromate under a wide range of environmental pollution conditions. For more investigations, repeated reduction of chromium on continuous inputs investigation was performed to check the ability of the CKCr-6A isolate to continuously reduce the repeated additions of Cr(VI), as presented in Fig. 2B. This strain exhibited reduction of Cr(VI) up to five consecutive inputs. After optimization, the initial 100 mg/L of Cr(VI) got reduced near to zero within 3 h at 37 ºC under aeration (shaking at 180 rpm).

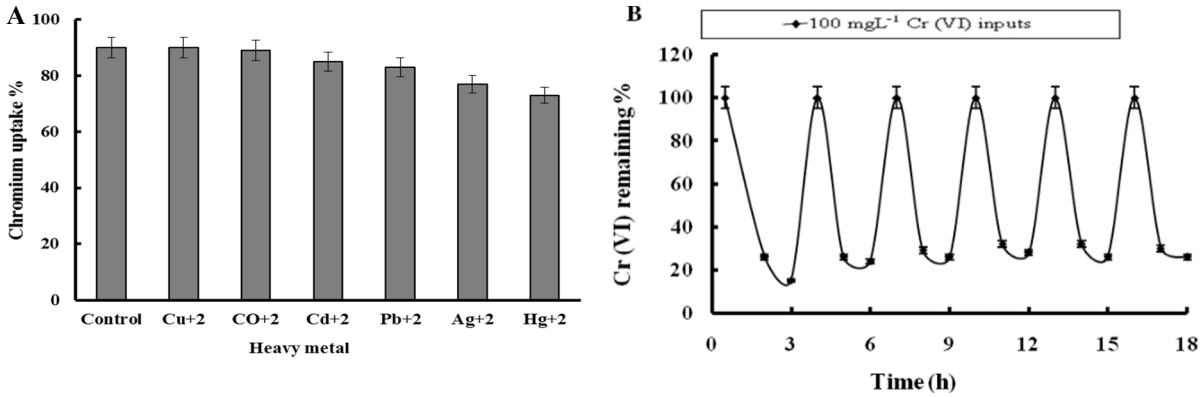

Fig. 2. (A) The effect of heavy metals on Cr(VI) reduction (%) by *Alcaligenes sp.* CKCr-6A, Error bars represent standard error. (B) Repeated detoxification of 100 mg/L Cr(VI) by CKCr-6A strain at 37°C in LB medium under 180 rpm of shaking without any amendment of nutrients

AFM could more directly and realistically image the characteristics of bacterial surfaces. Results showed that the intact bacteria in the absence of chromium as control exhibited long-rod shapes and smooth surfaces (Fig. 3A). After the bacterium was exposed to Cr (VI), the bacterial surfaces became rough (Fig. 3B). SEM analysis was performed, which exposed the surface changes after treatment with chromium 50 mg/L. The bacterial cell morphology of *Alcaligenes sp.* CkCr-6A was observed by SEM after cultivation of bacteria for 24 h without and with Cr (VI) (Figs. 3C and 3D).

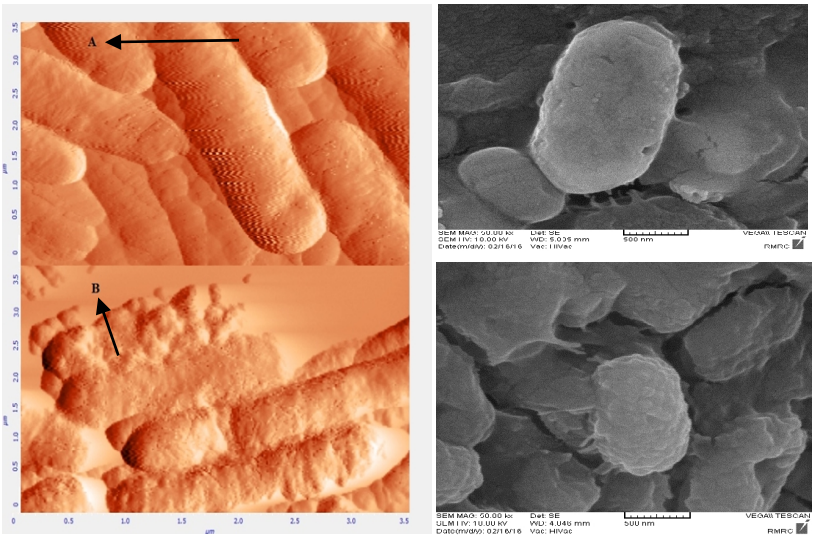

Fig. 3. AFM amplitude image of (A) CKCr-6A grown in the absence of Cr(VI) as a control and (B) grown in the presence of chromium. SEM micrographs of *Alcaligenes sp.* cells grown in: (C) LB medium without Cr(VI); (D) LB medium amended with 50 mg L^{-1} Cr(VI) for 24 h

MIC and antibiotic resistance

The MIC showed by isolated strain, *Alcaligenes sp.* was 80,000 mgL^{-1}. When percentage inhibition data was analyzed it was found that EC_{50} of Cr(VI) for this strain was 25,000 mgL^{-1}. The antibiotic sensitivity results are shown in Table 2. In the absence of Cr(VI), maximum resistance was shown for oxacillin and cefixime and minimum resistance for chloramphenicol. Results propose the hypothesis that heavy metal exposure causes an increased or modified frequency of antibiotic tolerance in bacteria. In other scientific articles, metal resistance has been described to hold a relationship with antibiotic resistance.[27-28]

Table 2. Result for Antibiotic resistance

Antibiotic	Cefixime (5 g/disc)	Chloramphenicol (30 µg/disc)	Cefoxitin (30 µg/disc)	Oxacillin (1 µg/disc)	Ampicillin (10 µg/disc)	Vancomycin (30 µg/disc)
Control (Without Cr(VI))	Resistance	Sensitive	Sensitive	Sensitive	Resistance	Sensitive
Cr(VI) 50 mg/L	Resistance	Sensitive	Resistance	Resistance	Resistance	Sensitive
Cr(VI) 100 mg/L	Resistance	Sensitive	Resistance	Resistance	Resistance	Sensitive
Cr(VI) 600 mg/L	Resistance	Sensitive	Resistance	Resistance	Resistance	Sensitive
Cr(VI) 800 mg/L	Resistance	Resistance	Resistance	Resistance	Resistance	Resistance

Chromate reductase activity in Alcaligenes sp.

The resting of the bacterium were studied in reducing 25–200 mg/L Cr(VI) concentrations in 3 h. The cell permeabilization considerably increased the Cr(VI) uptake by the resting cells, as the Tween 80 and Triton X100 permeabilized cells could reduce more than 80% of 100 mg/L of Cr(VI) in 3 h (Fig. 4A). Moreover, reduction of Cr(VI) was mainly associated with the soluble component of the cells. For more studies, the localization of chromate reductase activity was evaluated by carrying out the assays using the sub-cellular fractions. These results presented that the Cr(VI) reductase activity was related with the CFE (cytosolic fractions [S12]). As previously reported, bacterial chromate reductase was localized either in the membrane fraction[29] or in the cytosolic fractions.[30, 31] The minimal activity difference was observed for the cytoplasmic fraction of cells grown in the presence Cr(VI) and in absence of Cr(VI), proposing constitutive nature of the chromate reductase within this isolate bacterium. In the other works, constitutive chromate reductase has been reported.[32, 33] Reduction of Cr (VI) needs a source of an electron; cellular nicotine amine adenine dinucleotide (NADH) has been reported to serve as an electron donor for Cr (VI) reduction. Adding of NADH (1 mM) in the reaction with crude cell-free extract of this strain increased the reduction of Cr (VI) that established a dependence of bacterial Cr (VI) reductases on NADH.[25, 34]

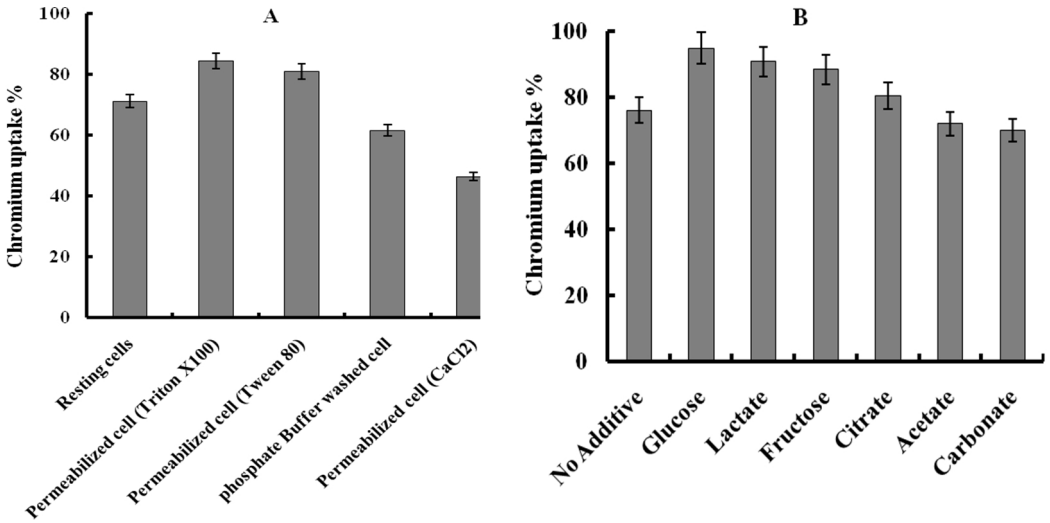

Fig. 4. (A) The influence of permeabilization of whole cell agent on the Cr(VI) reduction. (B) The effect of electron donor on the *in vitro* chromium reduction

In the *in vitro* Cr (VI) uptake, the effect of different electron donors on the chromate reductase activity of permeabilized cells of CKCr-6A was determined, as is exhibited in Fig. 4B. The reductase activity increased when the reaction mixtures were supplemented with electron donors like glucose, lactate, and fructose at 18%, 14%, and 12%, respectively. Whereas, acetate and carbonate showed negligible effects on chromium reduction.

Effect of different in vivo and in vitro conditions on the chromate reduction Effect of pH and shaking speed on Cr(VI) uptake

In a primary experiment, the Cr (VI) concentration was 50 mg/L and the uptake was analyzed after 9 h. It was well documented that pH is an important parameter affecting bioremoval of heavy metals.[35] The chromium removal was studied in the wide range of pH (3.5–9.0), the reduction was increasingly increased with enhancing pH up to 7.0. The optimal pH values of ~7.0 and 9.0 have been reported for Cr(VI) reduction by *Bacillus sp.* from chromite mine soil and *Bacillus sp.* from chromate landfills, respectively.[36-39] The maximum uptake was observed in the pH range of 5.5–7. Under the *in vivo* conditions, as the pH of the media was modified, the growth was affected, which might have a noticeable effect on the metabolism and Cr(VI) reduction by this strain. The results concerning the effect of solution pH on Cr(VI) removal are shown in Fig. 5A. Results showed that also the Cr(VI) reduction in this strain is enzyme-mediated and changes in pH will affect the induction of enzyme production, altering the protein's conformation or enzyme activity. Furthermore, the reduction of standard redox potential for Cr(VI)/Cr(III) couple at higher pH is one of the parameters which ultimately affects the total Cr(VI) reduction.[37]

Results for the *in vitro* removal of Cr(VI) showed that medium pH can affect the availability and solubility of metal ions and the ionization properties of the metal groups like carboxyl, hydroxyl, and phosphate as well as amino groups of the bacterial cell wall. This optimum pH range for *in vitro* chromium uptake was 3–6. In the acidic pH, the overall cell surface charge would become positive, and the surface would also be enclosed by the hydronium ions, which could increase some heavy metal connections (Fig. 5A, inset). The variation of shaking speed in the range of 0–300 rpm is counted as another factor with the highest *in vivo* and *in vitro* Cr(VI) reduction at 180 rpm and 100 rpm, respectively (Fig. 5B & Fig. 5B, inset).

Effect of chromium concentration and electron donors

It was obvious that the growth of cells was slightly influenced by Cr(VI) at concentrations above 1,000 mg/L, but these concentrations above 1,000 mg/L did not suppress the cells growth, and the overall efficiency of Cr(VI) reduction was not expressly affected by initial Cr(VI) concentrations up to 200 mg/L. For whole reduction of chromium, the required time was increased with enhancing the Cr(VI) concentration. The decrease in removal percentage can be attributed to the fact that enzymes responsible for Cr(VI) reduction were probably inactivated at high Cr(VI) concentrations. On the other hand, decrease in pH of the media, due to formation of $Cr(OH)_3$, may also contribute to the inactivation reductase enzyme.[37] The effects of various concentrations of glucose (w/v) as that of the electron donor (Fig. 5C & Fig. 5C, inset) were also investigated in the Invivo and Invitro condition.

Effect of temperature

Cr (VI) bioremoval at different temperatures (30–55 ºC) was also investigated. As is revealed in Fig. 5D, the optimum temperature for the *in vivo* chromium uptake was 37 ºC and high reductase enzyme production was observed. As shown in Fig. 5D, inset, the *in vitro* uptake percentage of Cr(VI) was increased up to 45 ºC. The increase in bioremoval with temperature could be attributed to the increase in the number of adsorption locations produced due to breaking of some initial bonds of the bacterial cell wall. On the other hand, the increase in temperature can also favor the adsorbed transport of the absorbent within the pores.

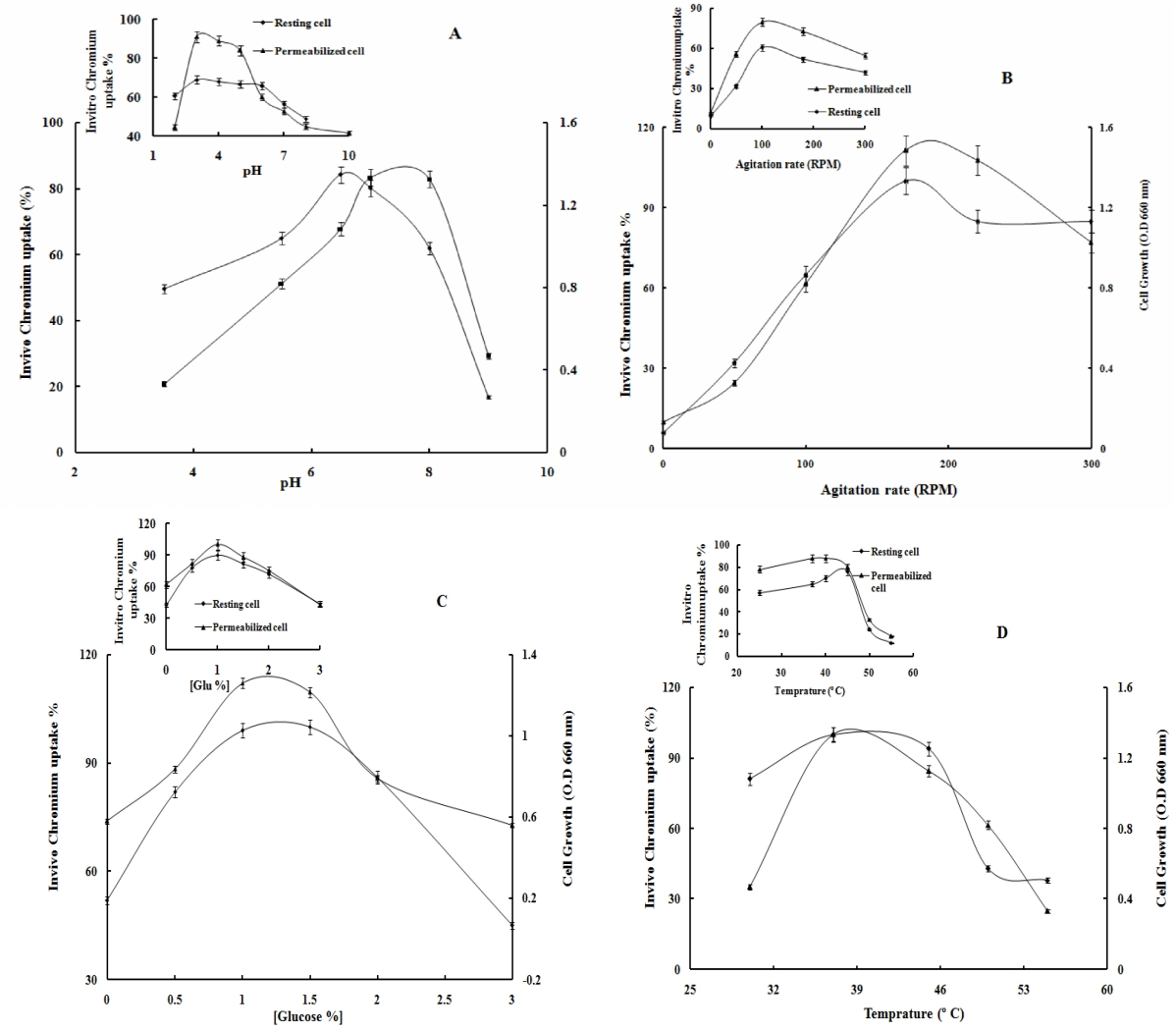

Fig. 5. Influence of pH (A), aeration level (B), glucose as an electron donor concentration (C) and incubation temperature (D) on Cr(VI) bio-reduction by *Alcaligenes sp*. strain CKCr-6A

Experimental design of in vivo Cr(VI) bio-uptake optimization

For chromium uptake optimization, 100 mg/L concentration was selected. The results for each test were performed and the experimental plan is given in Table 1S. Results obtained by one factor at a time method revealed the prominent effect of four parameters (pH, temperature, shaking speed, and glucose concentration) on the yield of chromium uptake that was expressed by the quadratic model. It can be proved that the main effects of X_1, X_2, X_3 and X_4 , pH, Temperature (ºC), Glucose con. (%) and Stirring speed (RPM) respectively, are the only ones which influence the Cr(VI) in the ranges investigated. In order to analyze and determine the effect of factors, the regression equation was calculated to check for all the polynomial models to fit the CCD data. As represented in Table 2S, the analysis of variance (ANOVA) was evaluated for the statistical significance of the model.

The details regarding the statistical analysis were mentioned in Lawson et al.[40] The value of R2; 0.9220 and adjusted R2; 0.8893 supported a high correlation between the observed and predicted values. The associated Prob>F value for the model is lower than 0.05, which indicates that the model is considered to be statistically significant. From ANOVA analysis, low value of the coefficient of variation (C.V = 10.16%) indicates a better precision and reliability of the trials performed. For reduction of Cr(VI) efficiency, pH was found to have maximum

effect on the response with the highest F value. Fig. 1S represents the "Normal Probability Plot" of experimental response versus the predicted ones (chromium uptake %).

Since the point's cluster is around the diagonal line, it can be concluded that there is no significant difference between the predicted and experimental values. Fig. 6A-D show the 3D response surfaces which were generated to show the effects on the percentage of Cr(VI) removal. These graphs characterize the effect of two variables at their studied range. Before optimization, initial Cr(VI) concentration of 100 mg l^{-1} completely reduced after 24 h. Interestingly, results showed that after optimization this strain could remove 100 mg/L of Cr(VI) to a non-detectable level over 3 h.

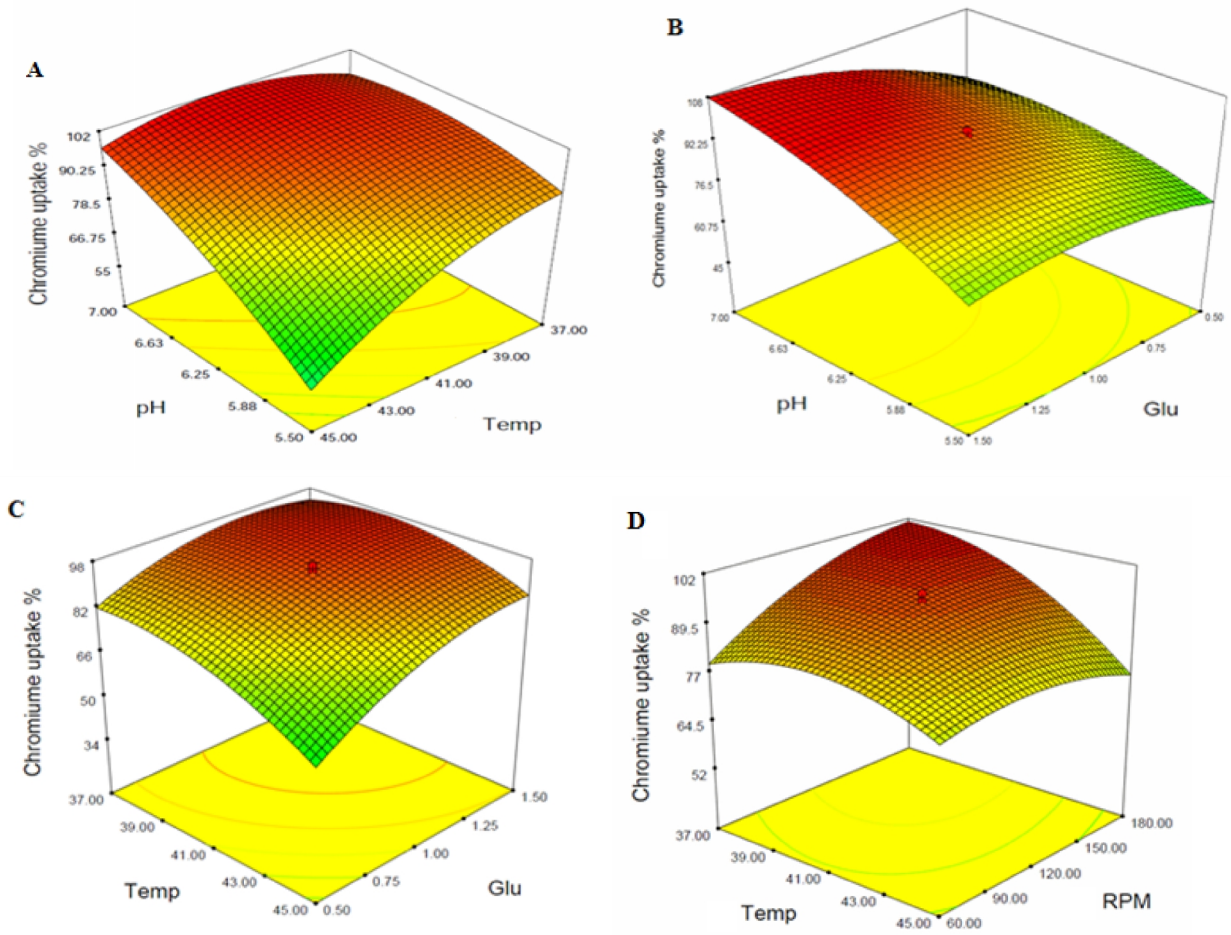

Fig. 6. Design-Expert plot. 3D surface plot of chromium removal efficiency showing (A) the interacting effect of pH and temperature at [glucose] and shaking rate of constant. (B) The interacting effect of pH and [glucose] at temperature and shaking rate of constant. (C) Interacting effect of temperature and [glucose] at pH and shaking rate of constant. (D) Interacting effect of temperature and RPM at pH and [glucose] of constant

Spectral analyses

The FTIR spectra of the CKCr-6A strain control and metal-loaded bacteria were taken to obtain information on the nature of the possible cell metal ion interactions.[41] The functional groups involved in the interaction with chromium were ionizable functional groups that included amino, carboxyl, and hydroxyl groups.[42-47] The Cr(VI) reduction in this strain was observed. To investigate the nature of

interactions, the FTIR spectra from 400 to 4,000 cm^{-1} wave number ranges were recorded. The existence of characteristic bacterial signatures showed in the FTIR spectrum (Fig. 7).

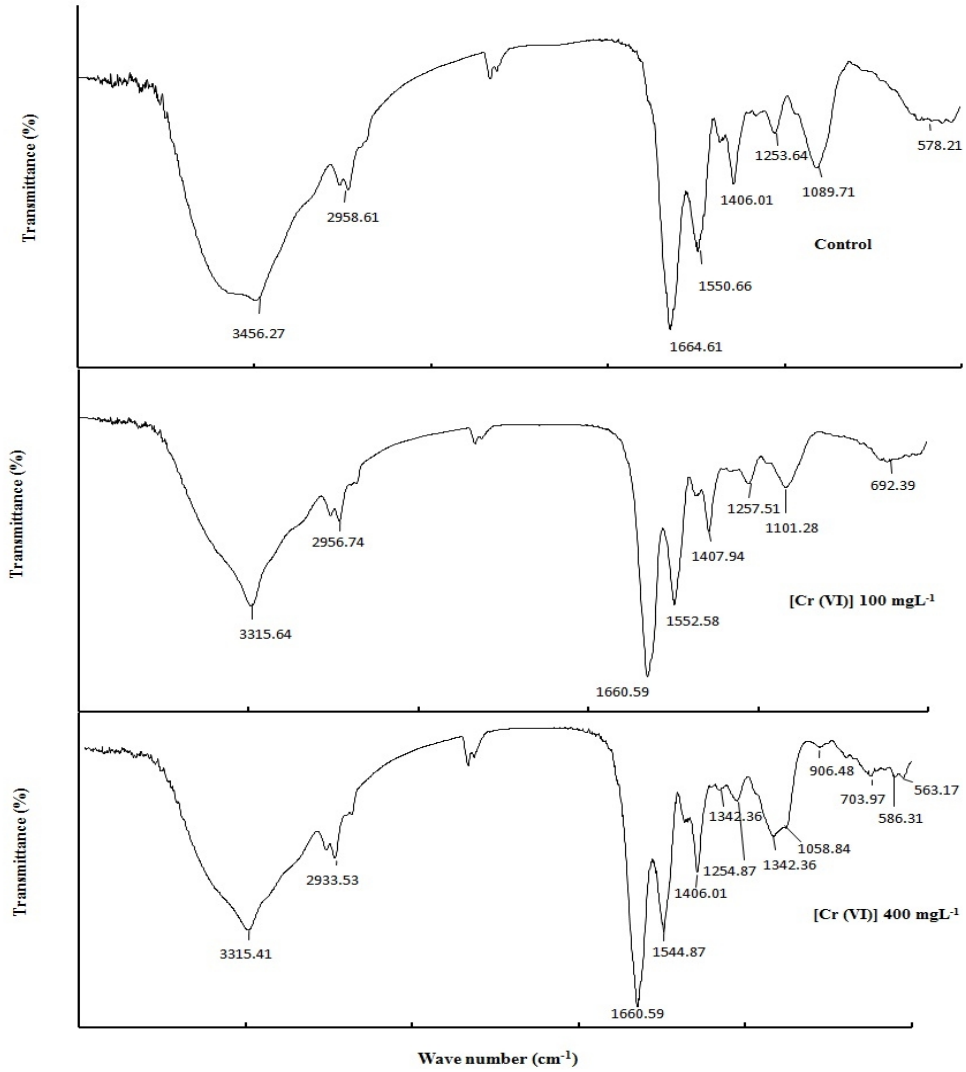

Fig. 7. FTIR spectra of the *Alcaligenes sp.* CKCr-6A strain with and without Cr(VI) in LB medium after 24 h

Results showed a broad stretching peak around 2,958 cm^{-1} which is representative of a weak C-H stretching band from the alkyl groups. A peak that was observed around 1,661 cm^{-1} suggested the presence of ester C, O groups. Furthermore, the spectrum showed the presence of prominent carboxyl (around 1,400 cm^{-1}) and amide groups (1,253, 1,550, and 1,660 cm^{-1}), which are specially expected for bacterial cultures. In the presence of metal, some delicate changes were observed. The FTIR spectra of metal-loaded bacteria exhibited a major shift in frequency to lower ranges from 3,456 to 3,315 cm^{-1}, echoing the strong interaction of the OH, and the NH stretching groups in the chromium binding by this strain. It indicates that the conversion of hydroxyl groups into acids caused the chromium reduction. The peak at 1,342 cm^{-1} became more noticeable on exposure to 400 mg/L of Cr(VI); hence suggesting the contribution of either the phosphate moiety or the C,O group in the interaction with chromium. The strong peak at 1,089 cm^{-1}, which corresponds to the C-O bond of polysaccharides, was shifted to 1,058 cm^{-1} as the chromium concentration was increased. This peak may also be recognized as the characteristic peak of orthophosphate (1.03-

1.100 cm^{-1}). The slight shift from 1,664 cm^{-1} to the lower frequency 1,660 cm^{-1} indicating the intervention of the C,O group of the amide (I) bond (CO-NH), where the peak position at 1,552 cm^{-1} remained unaltered, represents the non-involvement of the amide (II) bond in the chromium adsorption procedure. The appearance of a low intensity peak at 550-840 cm^{-1} represents Cr-O vibration. The areas of the carboxyl and ester increased with the chromium addition, while reduction of the areas of hydroxyl and alkyl groups occurred. Although, amide I and amide II bands were unaltered and their areas were almost the same in all the cases, the decrease in protein/lipid ratio gave an indirect signal for the contribution of proteins in the chromium reduction.

More insights into the nature of reduced product were obtained from XRD analysis. The powder XRD patterns of the CKCr-6A strain cells grown with or without Cr(VI) in LB media showed peaks at identical positions due to the presence of some polysaccharides in the cell wall (data not shown). The peak intensities were reduced in the case of bacterial cells grown with 100 mg/L Cr(VI). Also, no peaks corresponding to the crystalline end product were observed, which indicated its amorphous nature, or formation of a very small amount which did not produce any noticeable diffraction peak in the XRD pattern as also observed previously with the reduction of Cr(VI) by *Bacillus sp*. It seems that the culture media composition greatly affects the general distribution and nature of Cr(III) species. However, more studies are needed to gain further insights into the mechanism of chromium reduction.

Conclusion

In summary, this study highlights the enrichment and application of the bacterial strain CKCr-6A for detoxification Cr(VI). It is interesting to note the tolerance capacity of a new strain of *Alcaligenes sp.* (CKCr-6A) against heavy metal toxicity and its ability to detoxify a variety of toxic heavy metals, like Cr(VI). Optimization of potential to uptake chromium was tested by employing RSM and resulted in the high removal of carcinogenic Cr(VI) over 3 h.

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