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Bio-removal of carcinogenic Cr (VI) by whole cells and cell-free extracts of a new native and highly chromate-resistant *Enterobacter sp.*

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ABSTRACT

In this study, whole cells and cell-free extracts of a new reducing bacterium CKCr-8 are prepared and used for the removal of carcinogenic Cr (VI). This strain showed high resistivity towards toxic metal ions such as chromium, cobalt, cadmium, lead, and mercury to the concentrations up to several folds more than normal levels available in polluted areas. Cr(VI) removal efficiency in the in-vitro conditions was accomplished considering important parameters such as pH, temperature, stirring speed, and glucose concentration. It was also found that addition of glucose, as an electron donor agent, caused a dramatic enhancement in Cr(VI) uptake. The high potential capacity of CKCr-8 strain in the in-vitro uptake of Cr (VI) without any supplements, proposed this strain as an effective bio-adsorbant for the bioremediation of high-valent Cr(VI) in industry.

Keywords: Cr(VI); Bio-remediation; Chromium uptake; Reductase; In-vitro

1. Introduction

The extensive use of Cr (VI) in electroplating, leather-tanning and in numerous aquatic industrial processes caused environmental pollution with this carcinogenic pollutant [1–5]. Cr (VI) Simply diffuses into the cells through non-specific anion carriers and can be metabolically reduced to Cr^{5+}, Cr^{4+}, Cr^{3+} [4,5]. Therefore, the expulsion of Cr (VI) from mining and industrial effluents is vital before discharging it into the earth. Cr (VI) uptake can be accomplished biotically by various mechanisms; however, recent reports have demonstrated the feasibility of the cell-wall of bacteria, as biological adsorbants [6–8]. Microorganism functions on the evacuation of cancer-causing metals ions involve biosorption onto the surface groups anchored to the cell-wall, bio-reduction, bio-mineralization, and so on [9–12]. The established strategies for the removal of Cr (VI) involve different important parameters which strongly affect efficacy of the selected methodology [13–15].

This investigation is focused on the ability of the cell wall of a particular bacteria isolated from a local chromite mine for the uptake of Cr (VI) in the presence of other toxic metal ions. Besides, in order to evaluate the impact of various parameters on the Cr (VI) evacuation, central composite design and response surface methodology (RSM) were employed.

2. Experimental

2.1. Instrumental analysis of the reduced products

2.1.1. Fourier transforms infrared spectroscopy (FT-IR)

FT-IR spectroscopy was carried out to clarify the adjustments in the functionalities of the chromate reducing bacteria under various concentrations of Cr (VI) on a Perkin Elmer FT-IR spectrometer in the region of 400–4000 cm^{-1}. The cells were grown overnight in the absence and presence of Cr (VI); then, harvested by centrifugation. The sample and control cells pellets were washed with NaCl (0.85%) to remove the loosely bound ions and impurities and again centrifuged. At last, the collected biomass was dried at 50°C

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to complete dryness and was pressed into spectroscopic quality KBr pellets with a sample/KBr ratio of 1/100.

2.1.2. X-ray diffraction analysis (XRD)

To identify the nature of the reduced products, the bacterial cells were characterized by XRD. The bacterial cells associated with the reduced product were separated by centrifugation at 10000 rpm at 4°C for 10 min. The supernatant liquid was discarded while the pellets were washed with potassium phosphate buffer (pH 7) prior to air-drying at 50°C overnight.

2.1.3. Atomic force microscopy (AFM)

AFM analysis of CKCr-8, ripped in the absence and presence of Cr (VI), showed the ability of the cell wall of the isolated bacteria to absorb Cr (VI). This study also envisaged the probable changes in the extracellular polymeric substances. For this purpose, 5 µl suspension of the centrifuged pellet washed with potassium phosphate buffer (20 mM, pH 7) was utilized.

2.2. Strain isolation and growth conditions

The soil samples were gathered from Cheshmeh-khan chromite mine at Sabzevar, Iran. For isolation of Cr(VI)-reducing bacterial strains, 10 g soil was added to 10 ml of normal saline and incubated at 37°C for 24 h. The LB agar plates containing 200 mg l^{-1} of the sterilized solution of potassium chromate (K_2CrO_4) was prepared and incubated at 37°C. A purified colony for the strain was inoculated into 50 ml of LB medium and incubated at 37°C (180 rpm) overnight. 3 ml of the matured bacterial cultures were transferred into 50 ml of fresh liquid nutrient medium involving 0–1000 mg l^{-1} Cr (VI) and incubated at the same condition. At pre-determined time intervals, 5.0 ml aliquots were drawn from the medium and the bacterial growth was measured in the supernatant. All the experiments including controls were repeated for at least three times.

2.3. Determination of the antibiotic resistance and minimum inhibitory concentration (MIC)

Antibiotic sensitivity and resistance of the CKCr-8 strain were assayed according to the Kirby-Bauer disc diffusion method [16–20]. After 18 h of incubation, the diameter of the clear zone around the antibiotic discs was measured. The strain was viewed as susceptible when the inhibition zone reached 12 mm or more in diameter. MIC of the Cr (VI)-resistant bacteria was estimated by broth dilution method for LB medium containing different concentrations of Cr (VI) (200–100,000 mg l^{-1}). The minimum concentration of Cr (VI) in the medium which restrained complete growth was considered as MIC.

2.4. Analytical method for the in-vitro uptake of Cr (VI) with resting and permeabilized cell wall of CkCr-8

Cr (VI) uptake studies were carried out in the LB medium during the bacterial growth. Before analysis, samples were centrifuged at 10,000 rpm for 5 min; then, the supernatant fractions were separated and analyzed for the remaining Cr (VI). The decrease in Cr (VI) concentration with time was evaluated by means of spectrophotometry using 1,5-diphenylcarbazide (DPC) as the complexing agent at 540 nm. The sample containing 1 ml of Cr (VI) was mixed with 3 ml of H_2SO_4(0.2 M) and 1 ml of DPC. Finally, the obtained pink–violet solution was analyzed for Cr (VI) [21].

Bacteria deposition of CKCr-8 was harvested by centrifugation at 5000 rpm for 6 min at 4°C. Then, resting cell pellets were washed twice with 1 ml of potassium phosphate buffer, 20 mM, pH-7, and re-suspended in the same buffer. The tubes were vortexed briefly for 1–2 min and incubated at 25°C for 2 h. At the end of incubation, tubes were centrifuged and the remaining Cr (VI) was measured from the supernatant following the DPC method. For the preparation of the permeabilized cells, bacteria pellet of CKCr-8 strain was harvested and washed twice with 1 ml of potassium phosphate buffer (20 mM, pH 7) as described above and suspended to the final volume of 1 ml in the same buffer. Thereafter, the suspended cells were treated with $CaCl_2$, 0.02 M. All cell suspensions spiked with a K_2CrO_4 solution (40 mg l^{-1}) and incubated at 25°C for 2 h. The tubes were centrifuged and the remaining Cr (VI) was estimated from the supernatant as described above. Heat-killed cells were used as controls in these series of experiments.

2.5. Analysis of chromate reductase activity in the cell-free extracts of CkCr-8

The isolated strain of CkCr-8 was cultured in the LB amended with 50 mg l^{-1} of Cr (VI) at pH 7 at 37°C for 24 h. The pellet was washed twice with potassium phosphate buffer and the cells were disrupted by sonication and the supernatant (cell-free extract) was applied for the pre-incubation in 0.05 mM chromate solution involving NADH (0.1 mM) in 600 µL of 20 mM potassium phosphate buffer at pH 7. After 30 min incubation at 37°C, samples were taken; and the remaining Cr (VI) was measured. Control samples involving potassium phosphate buffer (20 mM) and the respective Cr (VI) concentrations were prepared without addition of cell-free extracts.

2.6. Effect of different parameters on the in-vitro Cr (VI) uptake

This activity was served as a screening test to identify which factors significantly affect the in vitro Cr (VI) removal. In these experiments, the level of each factor was changed, while all other experimental variables remained constant. Then, to optimize the factors, uptake of Cr (VI) with the selected strain (CKCr-8) was carried out under varying incubation period, initial Cr (VI) concentration (50 –1000 mg l^{-1}), pH (3–9), temperature (25–50°C), shaking speed (0–240 rpm) in the presence of commonly used carbon sources (1.0 wt.%) with different structures and reducing abilities.

3. Result and discussion

3.1. Isolation and estimation of the Cr (VI) tolerance of CkCr-8 strain

In this study, eleven Cr-resistant bacteria were isolated from the selected contaminated soil following plating on

media amended up to 1000 mgl^{-1} Cr (VI). The isolated CKCr-8 showed high Cr (VI) (~60000 mg l^{-1}) tolerance, which is normally higher than the reported values for other microorganisms [22,23]. Furthermore, this strain was resistant to Cd^{2+} (10 mM), Co^{2+} (10 mM), Hg^{2+} (10 mM), Pb^{2+} (10 mM), Se^{4+} (200 mM) and tellurite (50 mM). The phylogenetic analysis of this strain confirmed 99% homology with *Enterobacter sp.* which has been reported in GenBank under the accession number of KC993899.1. Cr (VI) removal in the LB medium supplemented initially with different concentrations of Cr (VI) and was measured at 3 h intervals. This strain actively grew in the presence of high Cr (VI) concentrations and Cr (VI) uptake began immediately. It seems that the real toxicity of Cr (VI) may be decreased due to the complexation of Cr (VI) with organic components such as tryptone and yeast extract and the microbial metabolism would probably be less affected [24]. Moreover, Cr(VI) uptake was not affected by adding different metal ions such as cadmium, cobalt, and lead, with 10 mM concentration, under the culture condition (Fig. 1A). These results may provide a useful pattern for the bioremediation of Cr(VI) under a wide range of environmental pollutions with repeated detoxification potential (Fig. 1B). A technique such as atomic force microscopy (AFM) could directly show the features of bacterial surfaces. AFM study proved that the intact bacteria in the absence of Cr(VI) as control, exhibited smooth surface; whereas, after treatment with Cr (VI) for 24 h, the bacterial surface became rough (Figs. 2A,B).

3.2. Antibiotic resistance and MIC determination

The antibiotic sensitivity tests were done and the outcomes appear in Table 1. Greatest resistance was found for oxacillin, cefixime and the least protection was achieved for chloramphenicol in the absence of Cr (VI). These findings bear in mind that the metal ion exposure caused significant changes in the antibiotic tolerance of the bacteria [25]. Under conditions of metal stress, metal and antibiotic resistance in microorganisms possibly help them to adopt faster by the spread of resistant factors than by mutation and natural selection [26]. In this study, the MIC of 60000 mg l^{-1} was obtained by the isolated strain.

3.3. Evaluation of chromate reductase enzyme in Enterobacter sp. CkCr-8 strain

The resting and permeabilized cells of CkCr-8 strain were desirable in reducing Cr(VI) (25–200 mg l^{-1}). One of the bacterial cells components can be the enzyme reductase content. The localization of chromate reductase activity was evaluated by achieving the assays by means of ultrasonicated sub-cellular fractions. Results demonstrated that Cr (VI) reductase activity was associated with the cell-free extract (CFE). For CFE preparation, bacterial cells were gathered by centrifugation at 10000 rpm and at 4°C for 10 min and washed with potassium phosphate buffer (20 mM, pH 7); then suspended in 25 ml of same buffer containing 1 mM PMSF. Bacterial cells were permeabilized and then disrupted by sonication. The broken-cell suspension was centrifuged (14000 rpm, 4°C for 20 min) and the supernatant was used as a CFE for enzyme assays. It has been shown that non inducible bacterial chromate reductase activity would be localized either in the membrane fraction or in the cytosolic fractions [27–29]. Similar constitutive chromate reductases have been deposited in other studies [30,31].

Permeabilization of the cells in the presence of agents such as EDTA, citric acid, and lactic acid significantly enhanced Cr (VI) removal and enzyme activity (Fig. 3A). Furthermore, the reduction of Cr (VI) needs an electron donor, cellular nicotine amine adenine dinucleotides (NADH) have been introduced to serve as electron donors for Cr (VI) reduction [23,32]. Effect of different electron donors on the chromate reductase activity and Cr (VI) removal by permeabilized and resting cells of CKCr-8 were examined under the in-vitro Cr (VI) removal conditions (Fig. 3B). The Cr (VI) removal activity was improved when the reaction mixtures were supported with a suitable electron donor such as glucose, fructose, and acetate in the permeabilized cells and with citrate, fructose, and carbonate in the resting cell.

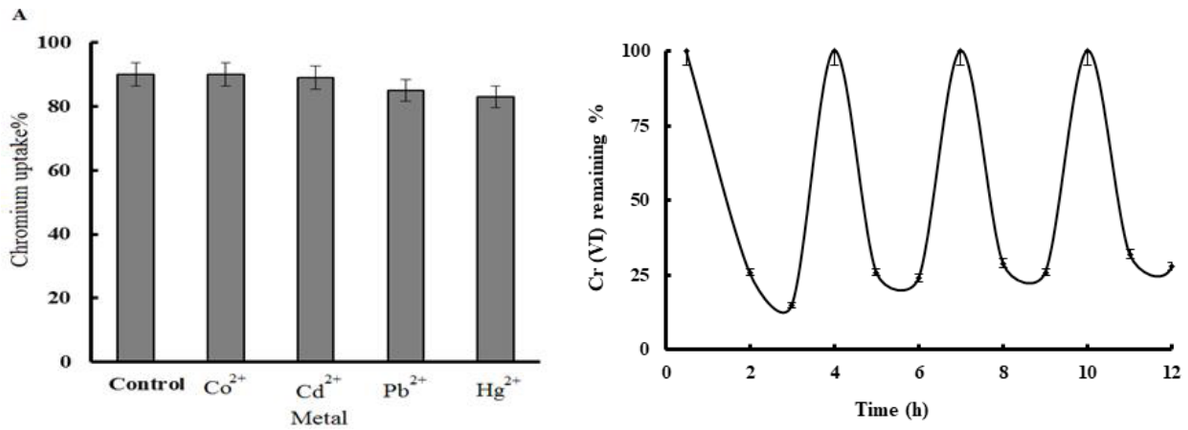

Fig. 1. Effect of different metal ions (10 mM) on Cr (VI) uptake by *Enterobacter sp.* CKCr-8; error bars represent standard error (A). Repeated detoxification of 100 mg l^{-1} Cr(VI) by CKCr-8 strain at 37°C without any amendment of nutrients (B).

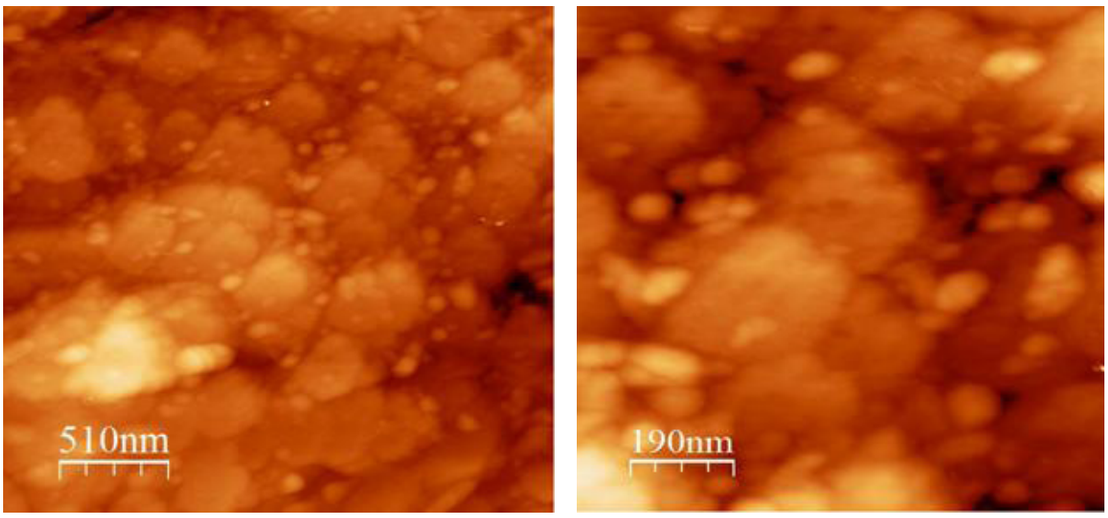

Fig. 2. AFM amplitude image of CKCr-8 strain grown in the presence of Cr(VI) (100 ppm).

Table 1
Results for the antibiotic resistance

Disc name	100 ppm Cr (VI)	No Cr (VI)
C30	<13	20mm
CFM5	17mm	17mm
FOX30	14mm	15mm
OX	<13	<13
V30	<13	<13

3.4. FT-IR analyses

FT-IR of control and metal ion loaded CKCr-8 strains was obtained to explore some information on the nature of the interactions between the cell wall and the metal ion [33]. The functional groups involved in this interaction would be ionizable functional groups including amino, carboxyl, and hydroxyl groups [34]. To investigate the nature of these interactions, FT-IR spectra from 400 to 4000 cm^{-1} were recorded. The results showed the most important assignments for the control and metal ion loaded bacteria (Fig. 4).

Results displayed a broad stretching vibration around 2958 cm^{-1} which is characteristic for weak C–H stretching bands of alkyl groups. An asymmetrical stretching band observed around 1643–1645 cm^{-1} suggested the presence of ester C=O groups. Furthermore, the presence of prominent carboxyl (around 1427 cm^{-1}) was preferentially expected for the bacterial cultures. As far as the FT-IR spectra of the metal ion loaded strain are concerned, they showed some subtle changes. The FT-IR spectra of the metal ion loaded bacteria showed a significant shift in the frequency to lower values (3242 cm^{-1}) echoing the strong interactions of OH and NH stretchings in the Cr (VI) bonded strain. This finding proved involvement of the hydroxyl groups in the Cr (VI) reduction and subsequent conversion of hydroxyl groups into acids. The peak at 1375 cm^{-1} became more prominent on exposure to 500 mg l^{-1} solution of Cr (VI); thus, suggesting the involvement of either the phosphate moiety or the C=O group in the interaction with Cr (VI) metal ion. The unchanged peak at 1517 cm^{-1} indicated the non-involvement of amide (II) bond in the Cr (VI) uptake process. The appearance of a low-intensity peak at 980–550 cm^{-1} was due

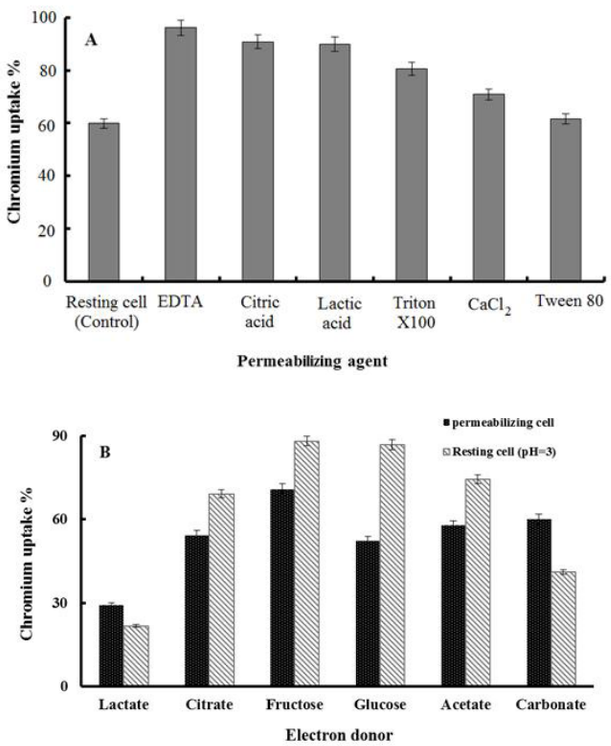

Fig. 3. (A) Influence of permeabilization whole cell agent on the Cr(VI) uptake. EDTA (5 mM), citric acid and lactic acid (1% W/V), $CaCl_2$ (0.2 M), Triton X100 and tween 80 (0.5 V/V) was used. (B) Effects of electron donors (1% W/V) on the in-vitro Cr (VI) uptake of permeabilized and resting cells.

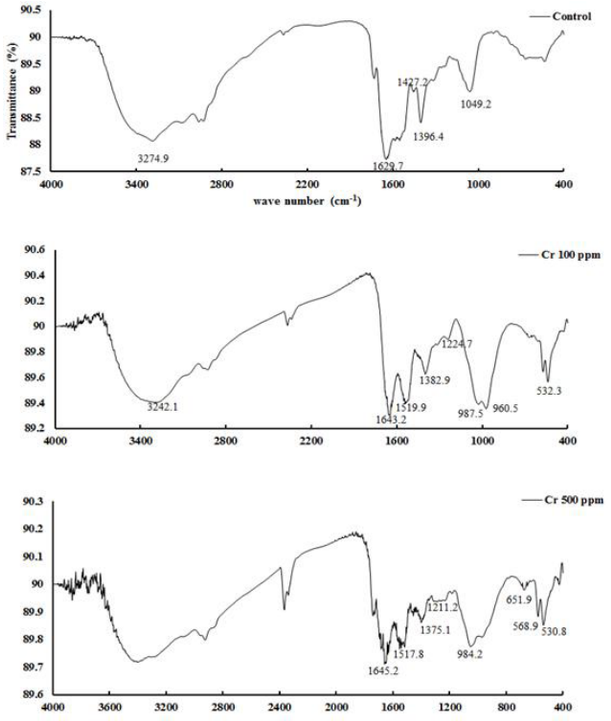

Fig. 4. FT-IR spectra of the *Enterobacter sp.* CKCr-8 strain with and without Cr(VI) in LB medium after 24 h.

to Cr–O vibration. The areas of the carboxyl and ester C=O increased after the metal ion addition; whereas, the areas of hydroxyl and alkyl groups were decreased. Although amide I and amide II bands were unaltered and their areas were almost the same in all cases, the reduction in protein/lipid ratio gave an indirect evidence for the participation of the proteins in the Cr (VI) uptake.

More information on the nature of the reduced products was obtained from XRD analysis. The powder XRD patterns of CKCr-8 strain cell grown in the presence and/or absence of Cr(VI) in LB media revealed similar peaks due to the presence of some polysaccharides in the cell wall at identical positions (data not shown). However, the peak intensities were reduced in the case of bacterial cells grown with 100 mg l^{-1} Cr(VI) [35].

3.5. Effect of different conditions on the Cr (VI) uptake

3.5.1. Effect of pH and shaking speed on Cr (VI) uptake

It is well understood that pH, as an important parameter, affects bio-removal of various metal ions [36]. The Cr(VI) uptake was studied in a wide range of pH's (2–10) and findings revealed that the uptake was significantly increased by enhancing pH up to 8.0 in the both permeabilized and resting cells. The optimum pH for the selected strain accords with other reported cases [37–40]. Clearly, pH can affect the accessibility of metal ions and ionization of the metal functional groups like carboxylate, hydroxyl, phosphate as well as amino groups of the bacterial cell wall. In the acidic pH, the overall surface charge of the cell surface would be positive, and the surface can be surrounded by the hydronium ions, which could enhance some metal interactions with bio-sorbent (Fig. 5A). Shaking speed in the scope of 0–300 rpm has been represented as another critical factor for the in-vitro Cr (VI) uptake of permeabilized and resting cells (Fig. 5B).

3.5.2. Effect of Cr (VI) concentration

Obviously, the growth of cells was somewhat influenced by Cr (VI) at concentrations over 1000 mg l^{-1}; however, the overall efficiency of Cr (VI) uptake was not significantly affected by the initial Cr (VI) concentration up to 200 mg l^{-1} (data not shown). Changing initial concentration of Cr(VI) showed that the time required for complete uptake of high Cr (VI) increased with enhancing the initial concentration of Cr (VI). Moreover, effects of different concentrations of glucose (w/v) as the electron donor were also investigated as shown in Fig. 5C.

3.5.3. Effect of temperature

In-vitro Cr(VI) uptake was performed at different temperatures from 25 to 55°C. As shown in Fig. 5D, the optimum temperature for the effective in-vitro uptake of Cr (VI) by permeabilized and resting cells of CkCr-8 strain was achieved at 45ºC. Increase of uptake with temperature could be attributed to the increase in the number of adsorption sites generated due to the breaking of some initial bonds close to the active surface sites of the bacterial cell wall.

3.6. Experimental design for optimization of Cr (VI) removal

Based on the results obtained at one factor at a time experiments, central composite design (CCD) was applied to optimize the chromium uptake efficiency by using Design Expert software (version 7.0, Stat-Ease, Inc., Minneapolis, MN) [41]. The investigated range and levels of experimental variables in this study are presented in Table 2. For statistical calculation, the variables X_i have been coded as x_i according to the following transformation [Eq. (1)] by assigning the lowest values listed in Table 2 as –2 and the highest values as +2:

$$x_i = \frac{X_i - \bar{x}_i}{\Delta x_j}, i = 1, 2, 3, \ldots, k \quad (1)$$

where x_i is the dimensionless value of an independent variable, X_i represents the real value of the independent variable, x_{ji} is the real values of an independent variable at the center point and Δx_j is the step alteration.

The CCD permits the response surface to be modeled by fitting a second-order polynomial with a number of experiments equal to $2f + 2^f + n$, where f and n are the number of factors and center runs, respectively (f = 4, n = 6). The repetition of the central runs was carried out to give information on the variation of the responses about the average, the residual variance, and eventually, approximate the pure experimental uncertainty. A four factor-five coded level,

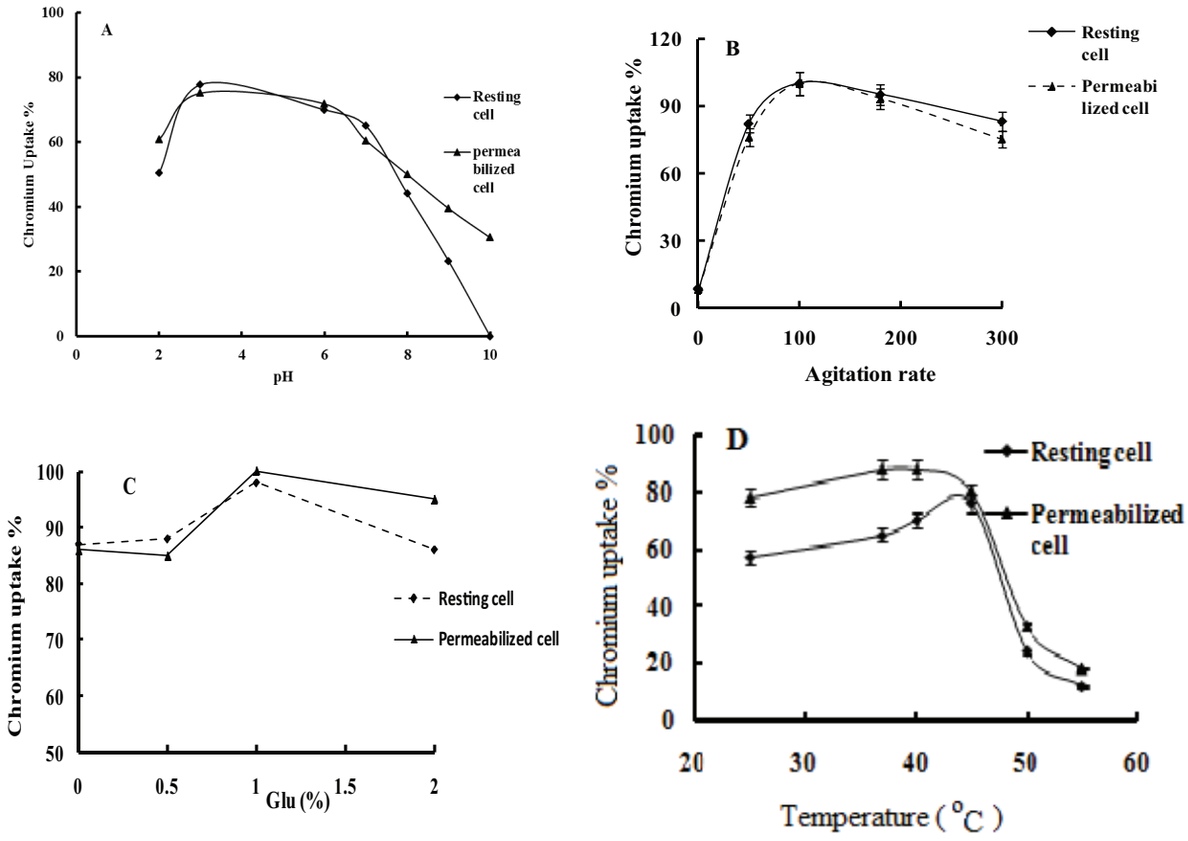

Fig. 5. Influence of pH (A), aeration level (B), glucose concentration (C) and incubation temperature (D) on the Cr(VI) uptake by *Enterobacter sp.* strain CKCr-8.

Table 2
Independent variables and their levels in the experimental design

Level	Coded Level	Uncoded level Coded level		
	(x_i)	pH (i = 1, X_1)	Strring speed(RPM) (i = 2, X_2)	Glucose (%) (i = 3, X_3)
Lowest	–2	1.64	0	0
Low	–1	3	51	1
Mid	0	5	125	2
High	+1	7	200	3
Highest	+2	8.36	250	3.68

CCD, 30 runs, was carried out to fit the following general model and to find the optimum conditions [Eq. (2)].

$$Y = \beta_{\circ} + \sum_{i=1}^{4} \beta_i X_i + \sum_{i=1}^{4} \beta_{ii} X_i^2 + \sum_{i=1}^{4} \sum_{j=i+1}^{4} \beta_{ij} X_i X_j \quad (2)$$

where Y (the response or dependent variable) is chromium removal% and β_is are model coefficients calculated from experimental data. The data represented in Table 3 are the means of three independent experiments and the average values of three repeated experiments were used as final values for developing the model. The quality of the fit of the polynomial model equation was expressed by different criteria; furthermore, the correctness of the model was verified through comparing the model predicted values with the experimental results, which were not included in the model estimation. Clearly, all empirical models were valid just within the variable ranges that were used to predict the model and any extrapolation led to considerable errors. The optimum values of the selected variables were obtained by solving the regression equation and also the response surface contour plots were analyzed [42].

In this work, the results of each test performed and the experimental plan, for chromium(VI) uptake optimization, 100 mg l^{-1} concentrations, are given in Table 3. The application of the RSM on the estimated factors indicated an empirical relationship between the response and input variables expressed by the quadratic model. Results obtained by one factor at a time method revealed the prominent effect of three parameters (pH, shaking speed and glucose concentration) on the yield of chromium uptake. It can be proved that the main effects of X_1, X_2, and X_3 are influencing the Cr (VI) in the ranges investi-

gated. These experiments were carried out to minimize the effects of uncontrolled variables on the response. The statistical significance of the model was then determined by the analysis of variance (ANOVA), represented in Table 4. The associated Prob > F value for the model is lower than 0.05 (i.e. $\alpha = 0.05$, or 95% confidence), which indicates that the model is considered to be statistically significant. For uptake of Cr(VI) efficiency, pH was found to have maximum effect on the response with the highest F value. In addition, the "Normal Probability Plot" of experimental response versus the predicted ones was made (Fig. 6). Fig. 7A-C 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 2 variables at their studied range. Interestingly, these results showed that this strain could significantly remove 100 mg l^{-1} of Cr (VI) to a non-detectable level.

Table 3
Results of CCD conditions and experimental/predicted values

				Permeabilized cell	
Run No.				Experimental	Predicted
	X_1	X_2	X_3		
1	+1	–1	–1	96.03	96
2	+2	0	0	91.13	92
3	+1	+1	–1	96.03	95
4	0	0	–2	98.22	95
5	+1	+1	+1	96.26	95
6	+1	–1	+1	96.96	96
7	0	0	0	91.37	90
8	+1	–1	+1	91.91	90
9	0	0	0	96.03	92
10	0	0	0	93.84	94
11	0	0	0	96.18	94
12	0	0	+2	96.03	93
13	–1	–1	+1	92.15	91
14	0	0	0	96.61	96
15	+1	+1	+1	91.48	90
16	–1	–1	–1	93.73	90
17	–1	–1	–1	97.38	90
18	0	0	0	96.03	95
19	–1	+1	–1	90.55	91
20	–1	+1	–1	96.03	95.1111

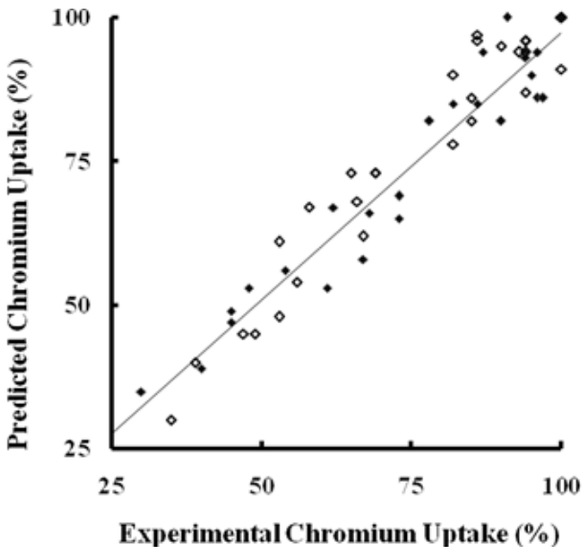

Fig. 6. Predicted *vs.* versus experimental chromium(VI) uptake. (◊) Validation conditions and (♦) model development conditions.

Table 4
ANOVA for Cr (VI) uptake with the permeabilized cell wall of CKCr-8 strain

Source	Sum of Squares	DF	Mean Square	F Value	Prob. > F	
Model	90.36	2	10.04	19.74	< 0.0002	Significant
A	5.96	9	5.96	11.71	< 0.0091	
B	1.04	1	1.04	2.04	0.1910	
C	2.44	1	2.44	4.79	0.0601	
AB	9.83	1	9.83	19.33	0.0023	
AC	15.15	1	15.15	29.79	0.0006	
BC	1.87	1	1.87	3.68	0.913 >	
A^2	37.52	1	37.52	37.52	0.0001	
B^2	3.6	1	3.6	3.6	0.0288	
C^2	12.55	1	12.55	12.55	0.0011	
Residual	4.07	8	0.51			
Lack of Fit	4.07	5	0.81	114.46	< 0.0001	Significant
Pure Error	0	3	0			
Cor. Total	100.29	19				

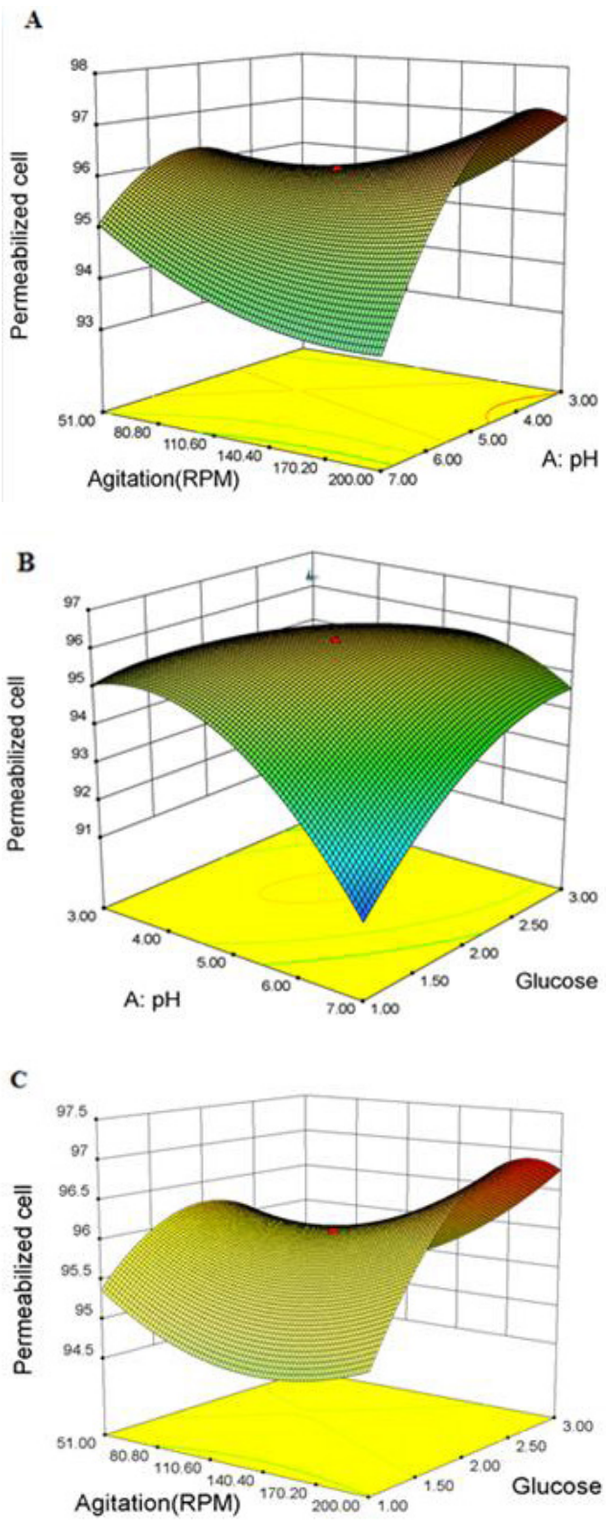

Fig. 7. Design-Expert plot. The 3D surface plot of chromium(VI) uptake efficiency showing (A) effect of pH and shaking rate at constant [Glucose]. (B) Effect of pH and [Glucose] at constant shaking rate. (C) Effect of shaking rate and [Glucose] at constant pH.

4. Conclusions

In summary, the present study introduced a new bacterial strain, *Enterobacter sp.* (CKCr-8), for the detoxification of Cr(VI) in the presence of other metal ions. Optimization studies employed by RSM proved the high potential of the strain for the uptake of Cr(VI). Finally, the CKCr-8 strain exhibited the ability to the in-vitro uptake by both permeabilized and resting cells. Repeated removing of Cr (VI) by this strain in the in-vitro condition without any amendment of nutrients, suggested its possible application in continuous bioremediation.

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