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Detoxification of Cr (VI) by *Bacillus cereus* S-6*

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Abstract: A chromium resistant bacterial strain S-6 was isolated from chromium-contaminated soil. On the basis of different morphological, biochemical characteristics and 16S rRNA gene analysis the strain was identified as *Bacillus cereus* S-6. Hexavalent chromium resistance of the strain showed that it could tolerate very high concentration of K_2CrO_4 both in nutrient medium (25 mg mL^{-1} in nutrient broth and 50 mg mL^{-1} on nutrient agar) as well as in acetate-minimal medium (15 mg mL^{-1}). Chromate uptake and reduction studies revealed that the strain *Bacillus cereus* S-6 not only accumulate but can also reduce large amount of toxic Cr (VI) in to Cr (III). Heavy metals (Ni, Mn, Zn, Cu and Co) at low concentration did not affect the reduction capability of the strain. Strain could also reduce hexavalent chromium in industrial effluents.

Key words: *Bacillus cereus* S-6, heavy metal, chromium uptake, Cr (VI) reduction

INTRODUCTION

Different industries, including mining and electroplating, release aqueous effluents containing a variety of toxic heavy metals. Untreated effluents from these industries have an adverse impact on the environment (Chandra *et al*., 2004). Chromium occurs in oxidation states Cr (o) to Cr (VI), but only Cr (III) and Cr (VI) are biologically significant. Trivalent chromium, Cr (III) is an essential micronutrient for humans and is relatively less soluble (Bahijri and Mufti, 2002) than the hexavalent chromium whereas the high valence chromium is toxic (Lock and Janssen, 2002), mutagenic (Feng *et al*., 2004) and carcinogenic (Park *et al*., 2004). It is necessary to convert this highly toxic Cr (VI) to less toxic, immobile form Cr (III). Physico-chemical methods have been designed for the removal of toxic hexavalent chromium, but these methods are commercially impractical and expensive. The accumulation and reduction of hexavalent chromium has been observed in many bacterial genera such as *Pseudomonas* (Ackerley *et al*., 2004), *Bacillus* and *Escherichia* (Cheung and Ji-Dong, 2005) and *Desulfovibrio* (Mabbett *et al*., 2002). Present study was conducted in 2004-2005 at Microbiology and Molecular Genetics Research Lab, Department of Botany, University of the Punjab, Lahore, Pakistan where a chromium resistant/reducing bacterial strain S-6 was examined for its ability to accumulate and reduced toxic hexavalent chromium in industrial effluents.

MATERIALS AND METHODS

Microorganism Isolation and Characterization

Strain S-6 was isolated from chromium contaminated soil sample from Sheikhupura (Pakistan). The strain was initially maintained on nutrient agar plates supplemented with 1 mg K_2CrO_4 mL^{-1}.

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Chromate tolerance of this strain was determined in the nutrient broth (per liter of water: 5 g peptone; 3 g beef extract) supplemented with 0.5, 1, 2, 4, 8, 15 and 25 mg K_2CrO_4 mL^{-1} and in acetate-minimal medium (Pattanapipitpaisal *et al*., 2001). To determine the pH and temperature range of the isolate, nutrient broth was adjusted to different pH (5, 6, 7, 8, 9 and 10) and temperature (24, 28, 32, 37 and 42°C) conditions both in the presence and absence of 1 mg K_2CrO_4 mL^{-1}. Heavy metal resistance profile of the strain was also checked in presence of different heavy metals ($NiSO_4$, 500 μg mL^{-1}; $ZnSO_4$, 700 μg mL^{-1}; $MnSO_4$, 1500 μg mL^{-1}; $CuSO_4$, 1000 μg mL^{-1}; $CoCl_2$, 500 μg mL^{-1}; $HgCl_2$, 50 μg mL^{-1} and Pb $(NO_3)_2$, 1000 μg mL^{-1}).

16S rRNA Gene Sequencing

To confirm taxonomic identity, strain S-6 was selected for 16S rRNA gene sequencing. A part of the 16S rRNA gene (500 bp) was amplified and the amplicon sequenced using fluorescent di-deoxy terminator cycle sequencing chemistry. The extension product was then separated on an ABI PRISM® (automated DNA sequencer) and the data was compared to the MicroSeq® databases (ACCUGENIX™ Newark DE 19702).

Chromate Accumulation

For chromate accumulation experiments, strain S-6 was grown in nutrient broth. After 24 h, cells were harvested by centrifugation and washed thrice with autoclaved distilled water. All experiments were carried out with 100 mL of metal solution prepared in 250 mL flasks. The innocula were prepared as follow: i) One gram of fresh cell pellet was taken and dried at 60°C for 48 h (dried cells); ii) One gram of pellet was taken and heat-killed at 121°C for 20 min (Heat killed) and iii) same amount was used untreated (live cell mass). At regular time intervals samples were taken aseptically and were centrifuged at 10,000 rev min^{-1} for 10 min at 4°C. Pellets obtained were digested and the total amount of accumulated chromium was determined by first oxidizing any trivalent chromium into hexavalent with $KMnO_4$ and analyzed spectrophotometrically at 540 nm in a spectrophotometer using diphenylcarbazide as the complexing agent (Clesceri *et al*., 1998). All experiments were conducted in triplicate.

Cr (VI) Reduction

For Cr (VI) reduction, DeLeo and Ehrlich (1994) medium (grams per liter: tryptone 10, yeast extract 5, NaCl 5, citric acid 1, Na_2HPO_4 6.9) initially amended with different chromate concentrations (0.1, 0.5 and 1 mg K_2CrO_4 mL^{-1}) was used. Inoculation was given from freshly prepared overnight culture. The initial concentration of the inoculated strain were $2.4x10^7$ and $9.6x10^7$ cell mL^{-1}. Cultures were incubated at 37°C with shaking at 150 rev min^{-1}. Samples were withdrawn at regular time intervals (24, 48, 72, 96 h) in aseptic conditions and total chromate reduced was measured by using diphenylcarbazide as above.

Effects of Heavy Metals on Cr (VI) Reduction

To analyze the effect of different heavy metals on the reduction capability of the strain, some cultures were also amended with Zn, 200 μg mL^{-1}; Ni, 200 μg mL^{-1}; Mn, 200 μg mL^{-1}; Cu, 200 μg mL^{-1}; Co, 50 μg mL^{-1} and Ag, 50 μg mL^{-1} and amount of Cr (VI) reduced was determined as described above.

Cr (VI) Reduction in Industrial Effluent

To check the efficiency of strain S-6 to reduce hexavalent chromium present in the industrial effluents, sample from an electroplating industry was collected in sterilized bottles. Two different

dilutions of effluent sample were used: sample a* and sample b** that contained 0.15 and 0.3 mg mL^{-1} of Cr (VI), respectively. Cultures were harvested after 40 h and the amount of Cr (VI) reduced was measured as described above. All experiments were done in triplicate.

RESULTS

Microorganism Isolation and Identification

Strain S-6 that is highly resistant to chromate (up to 50 mg mL^{-1}) on nutrient agar and 15 mg mL^{-1} in acetate-minimal medium was used in this study. The pH optimum for growth of the strain was 7 but it prefers alkaline pH in the presence of 1 mg mL^{-1} of chromate. The temperature preferences remain same both in chromate supplemented and chromate free medium (37°C). Strain S-6 could grow up to 25 mg mL^{-1} of K_2CrO_4 in nutrient broth and it was not affected much up to 2 mg mL^{-1}, but at 4 mg mL^{-1} growth was reduced drastically (Table 1). Table 2 exhibits the heavy metal tolerance profile of this strain. On the basis of different morphological, biochemical and 16S rRNA gene sequencing results, strain S-6 was identified as *Bacillus cereus* S-6.

Chromium Uptake

The efficiency of strain S-6 to accumulate chromium from the medium at different contact times (15, 120 min) and chromate concentration (0.1, 0.5 and 1 mg mL^{-1}) is shown in Table 3. The living cells of strain S-6 accumulated 8.2 and 24.7 mg Cr g^{-1} dry weight from the medium containing an initial chromate concentration of 0.1 mg mL^{-1} after 15 and 120 min, respectively. Table 3 shows high affinity of chromate uptake by the strain in all the chromate concentrations applied and all cell treatments and the highest uptake value (64.6 mg Cr g^{-1} dry weight) was attained at high concentration of chromate (1 mg mL^{-1}).

Cr (VI) Reduction

The optimum pH and temperature for chromate reduction was 7 and 37°C, respectively. At an initial Cr (VI) concentration of 0.1 mg mL^{-1}, strain was able to reduce all chromate within 48 h when

Table 1: Growth of *Bacillus cereus* S6 in the presence of different concentrations of K_2CrO_4 mg mL^{-1} in nutrient broth and acetate-minimal medium after 24 h

	Growth of *Bacillus cereus* S6 in the presence of different concentrations of K_2CrO_4 mg mL^{-1}							
Media	0	0.5	1	2	4	8	15	25
Nutrient broth	1.859±0.09	1.234±0.07	1.027±0.08	0.862±0.02	0.259±0.04	0.241±0.009	0.205±0.006	0.129±0.004
Acetate- minimal	1.548±0.10	1.424±0.10	1.214±0.02	0.964±0.01	0.369±0.04	0.217±0.007	0.209±0.009	0.024±0.001

Table 2: MIC's of *Bacillus cereus* S-6 against different heavy metals

$NiSO_4$	$ZnSO_4$	$Pb(NO_3)_2$	$CuSO_4$	$CoCl_2$	$HgCl_2$	$MnSO_4$
400	500	1000	700	200	50	1500

Table 3: Uptake of potassium chromate at three initial K_2CrO_4 concentrations. Contact time (after 15 and 120 min)

		Chromium uptake at different initial chromate concentrations					
		100 μg mL^{-1}		500 μg mL^{-1}		1000 μg mL^{-1}	
Strains	Cell type	15 min	120 min	15 min	120 min	15 min	120 min
S6	Living	8.2±0.45	24.7±1.61	10.9±0.74	29.6±1.26	18.9±1.14	66.4±2.1
	Killed	7.3±0.24	12.5±0.41	10.7±0.65	21.4±1.51	16.8±1.05	41.2±1.5
	Dried	5.0±0.35	6.3±0.38	6.8±0.41	9.4±0.38	7.9±0.61	12.7±1.0

Table 4: Reduction of Cr (VI) in the presence of two inoculum sizes at different initial Cr (VI) concentrations. Reduction was monitored at various incubation time intervals

		% Cr (VI) reduction							
		100 μg mL^{-1}				500 μg mL^{-1}			
Strains	Inoculua	24 h	48 h	72 h	96 h	24 h	48 h	72 h	96 h
S6	2.4^{7} cells mL^{-1}	91.04±3.4	96.07±3.5	100±0.00	100±0.00	71.03±4.1	91.0±3.5	98.6±4.1	100±2.8
	9.6^{7} cells mL^{-1}	94.85±7.2	100±0.00	100±0.00	100±0.00	73.0±4.2	98.5±5.3	100±0.00	100±0.00

Table 4: Continue

		% Cr (VI) reduction			
		1000 μg mL^{-1}			
Strains	Inoculua	24 h	48 h	72 h	96 h
S6	2.4^{7} cells mL^{-1}	28.4±1.6	60.5±3.4	82.63±4.5	94.01±6.1
	9.6^{7} cells mL^{-1}	29.01±1.0	56.07±3.9	83.41±5.2	94.25±5.8

Table 5: Effects of different heavy metals on Cr (VI) reduction capability of the strain *Bacillus cereus* S-6 (Initial chromate concentration 0.5 mg mL^{-1}) and Chromate reduction in an industrial effluent

Source	Reduction (%)
Metals (μg mL^{-1})	
Control	87±2.16
Ni (200)	89±3.00
Mn (200)	87±2.50
Zn (200)	88±2.38
Cu (200)	83±2.78
Co (50)	84±2.30
Industrial effluents	
a*	94±3.60
b**	82±2.95

a* Initial Cr (VI) concentration 0.15 mg mL^{-1}, b** Initial Cr (IV) concentration 0.3 mg mL^{-1}, Cultures were harvested after 40 h

inoculated with 9.6x10^{7} cells mL^{-1}, but with an inoculum of 2.4x10^{7} cells mL^{-1}, complete reduction was accomplished after 72 h (Table 4). An increase in initial chromate concentration almost up to 5 times (0.5 mg mL^{-1}) strain reduced 71.03, 91, 98.6 and 100% after 24, 48, 72 and 96 h, respectively. At high initial Cr (VI) concentration (1 mg mL^{-1}), amount of Cr (VI) reduction was more but within 96 h complete reduction did not occur. The rate of reduction was much faster in first 24 h but with the passage of time it decelerated. Maximum Cr (VI) reduction was achieved after 96 h with 9.6x10^{7} cells mL^{-1} at an initial Cr (VI) concentration of 1 mg mL^{-1}. Controls showed no change in the chromate concentration from the initial to the final stage of the experiment (without any evidence of Cr (VI) reduction). No major difference in the reduction capability of the strain S-6 was observed with both cell concentrations used, although minute differences were observed at high initial Cr (VI) concentration (1 mg mL^{-1}).

Effects of Heavy Metals on Cr (VI) Reduction

Different heavy metals at low concentration in the medium seemed to have no effect on the reduction capability of the strain (Table 5). However, presence of Cu (200 μg mL^{-1}) and Co (50 μg mL^{-1}) caused slight decrease in reduction capability, whereas Ni (200 μg mL^{-1}), Mn (200 μg mL^{-1}) and Zn (200 μg mL^{-1}) enhanced the reduction rate to some extent.

Cr (VI) Reduction in Industrial Effluent

pH and temperature of the sample was recorded on-site (pH 5-6 and temperature 26°C). Other parameters of sample were also determined (Cr (VI) 800 μg mL^{-1}; Fe 121 μg mL^{-1}; Cu 65 μg mL^{-1}; Zn 4 μg mL^{-1}; Ni 109 μg mL^{-1}; Co 2 μg mL^{-1}; Pb <1 μg mL^{-1}; Mn <1 μg mL^{-1}). The rate of

reduction in industrial effluents was slightly less, as industrial effluents contain different pollutants. In sample I with an initial chromate concentration of 0.15 mg mL^{-1}, strain reduced 94% of the total Cr (VI) whereas in sample II (initial chromate concentration 0.3 mg mL^{-1}), it reduced almost 82% Cr (VI) within 40 h (Table 5).

DISCUSSION

The chromate resistance level of strain S-6 is very high both on nutrient agar (50 mg mL^{-1}) acetate-minimal medium (up to 15 mg mL^{-1}) which becomes the reason for the selection of this strain for further study. Such a high level resistance toward chromate was not found in the strains reported by other workers. The chromate tolerance level of strains CRB, 50 mg L^{-1} (McLean and Beveridge, 2001), CRRS, 2 $mmol^{-1}$ (Francisco *et al*., 2002) was not that high as the strain *Bacillus cereus* S-6 being discussed here. The initial chromate concentration used for reduction of Cr (VI) in the present study was also higher (0.1, 0.5 and 1 mg mL^{-1}) as used by other workers. Quintana *et al*. (2001) used an initial chromate concentration of 0.01 mg mL^{-1} in their experiment where as McLean and Beveridge (2001) applied a concentration of 0.02 mg mL^{-1}. The chromate resistance level of the strain was more on nutrient agar as compared to nutrient broth in the presence of chromate. This might be due to the fact that in aqueous conditions the cells are more exposed to metal toxicity as compared to solid surface. Metals are more toxic when soluble verses sorbed to humic or clay colloids. In the presence of Cr (VI) cells take more time to divide as compare to Cr (VI) free conditions. The presence of increasing concentration of Cr (VI) increased the time needed for bacterial growth (Francisco *et al*., 2002). According to Ganguli and Tripathi (2002), the normal growth time of *Pseudomonas aeroginosa* A2Chr was 42 min but with the addition of 0.1 mg mL^{-1} of Cr (VI) increase the generation time to 57 min. Besides chromium, strain *Bacillus cereus* S-6 exhibits multiple metal and antibiotic resistances. It might be attributed to the highly polluted source environment of this strain. Strains isolated from polluted environment with high metal concentration exhibits considerable tolerance to different metals and antibiotics. This tolerance may be due to biotic factors (pH, temperature, nutrient in the environment) or to the physiological and genetic adaptations of the microorganisms (Romero *et al*., 1999).

In the present study strain *Bacillus cereus* S-6 was found to be highly efficient in accumulating chromium at different concentration of chromate (0.1, 0.5 and 1 mg mL^{-1}). Uptake was more at pH 6 with a temperature of 37°C. The process of accumulation and uptake of metals by organisms is highly dependent on their bioavailability, which is greatly influenced by pH. At basic pH stable metal complexes, such as hydroxide, sulfide and carbonates are formed, making the metal less available for removal by biosorbents. The presented results are useful because the temperature and pH of the aqueous effluents also fall in this temperature range. Hence this strain can be easily opted for wastewater treatment. Because in the field condition it is difficult to maintain temperature.

In the present Cr (VI) reduction experiments, concentration of Cr (VI) at all the three initial Cr (VI) concentration decreased with increase in incubation time. Megharaj *et al*. (2003) observed that time needed for total Cr (VI) reduction increased with increasing initial Cr (VI) concentration. At low initial Cr (VI) concentration (0.1 mg mL^{-1}) strain *Bacillus cereus* S-6 was able to reduce all Cr (VI) present in the solution within 48 h. Wang and Xiao (1995) showed that under aerobic condition *Bacillus* sp. was unable to reduce all Cr (VI) within 96 h when initially supplied with 0.1 mM Cr (VI). It is generally believed that chromate reduction is inhibited in the presence of metals, presumably due to metal toxicity. High initial chromate concentration or the presence of other toxic compounds might slow down or prevent biological chromium reduction (McLean and Beveridge, 2001). But in the present study with the increase in initial hexavalent chromium concentration, more quantity of chromate was reduced and presence of different heavy metals did not show pronounced effects on the reduction capability of the strain.

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