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ORIGINAL ARTICLE

Aerobic chromium(VI) reduction by chromium-resistant bacteria isolated from activated sludge

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Abstract A chromium-reducing strain isolated from activated sludge and subsequently identified as *Clostridium* sp. SS1 by a biomolecular method was studied for its resistance to toxic hexavalent chromium [Cr(VI)] and its ability to reduce Cr(IV). The strain was found to tolerate concentrations of Cr(VI) up to 50 mg/l; however, cell growth was severely affected by the presence of Cr(VI), especially at concentrations of more than 20 mg/l. *Clostridium* sp. SS1 also effectively reduced toxic Cr(VI) into the comparatively less toxic trivalent form. The optimum pH value for Cr(VI) reduction was 7.0, and the cells reduced Cr (VI) more efficiently at 30°C than at 20 and 40°C. Within the same incubation time, higher amounts of Cr(VI) were reduced at higher initial Cr(VI) concentrations (range 5–40 mg/l). *Clostridium* sp. SS1 had a higher volumetric [0.82 mg Cr(VI)/l/h] and greater capacity [72 mg Cr(VI)/g biomass] to reduce Cr(VI) at higher initial Cr(VI) concentrations (40 mg/l), suggesting that this strain could be potentially useful for the detoxification of Cr(VI) polluted sites.

Keywords *Clostridium* sp. SS1 · Chromium (VI) · Chromium (III) · Aerobic reduction · Activated sludge · Optimum pH

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Introduction

As countries become steadily more industrialized, increasing levels of chromium (Cr) are being discharged in industrial wastewater from industrial processes, such as plating, leather tanning, and wood preservation (Nkhalambayausi-Chirwa and Wang 2000). Chromium is an essential micronutrient required for the growth of many organisms; however, it is also toxic, carcinogenic, and teratogenic at higher concentrations and has been designated as a priority pollutant by the U.S. Environmental Protection Agency (EPA). Although chromium exists in nine valence states, trivalent chromium [Cr(III)] and hexavalent chromium [Cr(VI)] are of major environmental significance because of their stability in the natural environment (Thacker et al. 2006; Mishra and Doble 2008; Gupta and Rostagi 2008). Cr(VI) is approximately 100-fold more toxic (Yao et al. 2008) and 1000-fold more mutagenic than Cr(III) (Morales-Barrena et al. 2008).

The conventional remediation processes for Cr(VI)-contaminated water involve physical and chemical removal technologies. Physicochemical treatment strategies are, however, expensive and produce secondary waste streams that themselves require remediation (Viamajala et al. 2003; Barbooti et al. 2008). Bioremediation using microorganisms provides an attractive treatment option because the strategy is cost effective and environmentally compatible. Thus, there is a growing interest in the use of microorganisms to reduce Cr(VI) to Cr(III) (Carmago et al. 2005). It has been reported that Cr(VI) is reduced to Cr(III) by a number of bacterial species, including *Pseudomonas fluorescens* LB300, *Bacillus* sp., *Enterobacter cloacae* HO1, *Enterobacter aerogenes* (Thacker et al. 2006), *Escherichia coli* (Ackerley et al. 2004), *Shewanella* spp. (Myers C.R. et al. 2000; Viamajala et al. 2003), and *Pseudomonas aeruginosa*. In those studies, glucose or

organic acids were used as the carbon and energy source. The mechanisms by which these microorganisms reduce Cr (VI) are variable and species-dependent, with some species using Cr(VI) as the final electron acceptor in the respiratory chains (Bopp and Henry 1998; Faisal and Hasnain 2004), while in other strains certain soluble enzymes are responsible for the reduction of Cr(VI) to Cr(III) (Philip et al. 1998; Ackerley et al. 2004). Microbial chromate reduction becomes slightly more complex as a result of the effects of environmental conditions under which microbial Cr(VI) reduction proceeds. As such, it is therefore quite important to determine the optimum conditions for the maximum conversion of Cr(VI).

The aim of the study reported here was to isolate chromate-resistant and chromate-reducing bacteria from activated sludge, study the bacterial chromate reduction process, and determine the optimal conditions for bacterial chromate reduction, within the framework of obtaining knowledge useful for the bioremediation of chromate-polluted water.

Materials and methods

Bacterial strains and cultivation conditions

Bacterial strains were isolated from the Chinese Academy of Agricultural Sciences Sewage treatment plant in Wuhan, China. The sample suspension was prepared by adding about 1 g of sample obtained from activated sludge to 100 ml medium and then transferring 1 ml of the sample suspension to 9 ml medium. This dilution process was repeated to obtain serial 10-fold dilutions of the sample (10^{-1}–10^{-7}). Aliquots of 1 ml were taken from the 10^{-3} to 10^{-6} sample suspension dilutions and dropped directly onto culture plates; liquid agar medium was then poured into each plate and the agar plates incubated at 30°C for 48 h. Colonies of different morphologies were then selected and streaked on separate agar plates and incubated at 30°C for 24 h. The bacteria were then inoculated from the plates onto agar slants and stored at 4°C until needed for further experiments. The agar medium consisted of glucose (30 g), yeast extract (1 g), peptone (1 g), K_2HPO_4 (1 g), KH_2PO_4 (0.5 g), NH_4Cl (0.5 g), $MgSO_4{\cdot}7H_2O$ (0.5 g), agar (15 g) in 1 l distilled water. The pH value of the medium was adjusted to 7.0 with 10% (w/v) NaOH or 10% (w/v) HCl. All experiments were carried under aerobic conditions.

The ability of the isolates to reduce hexavalent chromium was determined by Cr(VI) reduction experiments. Those isolates with higher Cr(VI) reducing ability were identified using a biomolecular method.

Prior to each experiment, strains were enriched by transferring one loop of cells from the agar slants to 100 ml of previously sterilized liquid medium in 250-ml flasks and incubated at 30°C for 24 h with shaking at 150 rev/min in an orbital incubator (LRH-250-Z; Guangdong, China). The liquid medium contained the same components as those in the agar medium except for agar, and the pH value was also adjusted to 7.0 in the same way mentioned above. The cells grown in liquid medium was centrifuged at 6000 *g* for 10 min at 4°C, the supernatant was discarded, and the cell pellets were washed twice and then suspended in a sterile solution of 0.085% NaCl before being used in the experiments.

Cr(VI) reduction experiments

Chromium(VI) reduction experiments were carried out in batch reactors using 250-ml conical flasks which contained 100 ml liquid medium autoclaved at 121°C for 15 min inoculated with the Cr(VI) concentration to be tested (5, 10, 20, 40 mg/l) and 1 ml of cell suspension. The flasks were then incubated at 30°C with shaking at 150 rev/min in the orbital shaker. As described above, the cells had been previously centrifuged at 6000 *g* for 10 min and the cells pellet washed twice with a sterile solution of 0.085% NaCl (Pulane et al. 2008) prior to incubation. Cr(VI) was measured at the following time points: 0, 2, 6, 12, 24, 36, 48, 60, and 72 h. Control experiments were carried out under the same operating conditions, but the 250-ml conical flasks containing 100 ml liquid medium were inoculated with only the Cr(VI) concentration to be tested (no cell inoculant).

Analytical methods

Samples were withdrawn at regular time intervals and centrifuged under the same conditions described above. The concentration of Cr(VI) in the supernatant was determined by a colorimetric method involving a reaction with 1, 5-diphenylcarbazide and measurement on a UV-Vis spectrophotometer (UV 754N; Shanghai, China) at 540 nm (Camargo et al. 2005). The absorbance of the purple-colored solution was read after 5 min. The cell density of the liquid cultures was determined by measuring the absorbance of a 1-cm cuvette at 600 nm with a spectrophotometer. Samples for total chromium analysis were first digested with sulphuric–nitric acid (1:1,v:v) and then oxidized with 4% (w/v) potassium permanganate before reacting with diphenylcarbazide and determined calorimetrically (Liu et al. 2006). Cr(III) concentration was calculated from the difference between the concentration of total chromium and that of Cr(VI).

Biomass preparation Dry biomass pellets were obtained after centrifugation of 50 ml of cells at 6000 *g* for 10 min following by drying at 50ºC until a constant weight was

achieved. This step was repeated at regular time intervals for the different Cr(VI) concentrations tested. The experiments were performed under the same operating conditions, and all experiments were done in triplicate. At least three separate flasks were usually prepared for one concentration, and samples from each flask were read three times. The means and standard deviations of the readings were calculated using the computer software package OriginPro ver. 7.5 (OriginLab, Guangzhou, China).

The Cr(IV) removal performance of the *Clostridium* strain was evaluated according to three criteria, namely, removal efficiency (E, %), volumetric rate (RV), and capacity (RC) of Cr(VI) removal, which were calculated as follows:

$$\mathbf{E}(\%) = \left[\frac{Cr_0 - Cr_f}{Cr_0}\right] \times \mathbf{100}$$

$$\mathbf{R_C} = \left[\frac{Cr_0 - Cr_f}{X_f - X_0}\right]$$

$$R_v = \left[\frac{Cr_0 - Cr_f}{t_f - t_0}\right]$$

where Cr_0 is the initial Cr(VI) concentration at time t_0 =0 h, Cr_f is residual Cr(VI) concentration at time t_f, X_0 is initial biomass concentration at time t_0 =0 h, X_f is the biomass concentration at time t_f .

Results and discussion

Characterization and identification of the strains

Four colonies of different morphologies were selected to test their Cr(VI) reduction ability. Of these four strains, only SS1 was able to reduce Cr(VI) efficiently. All four strains were Gram-positive. SS1 was identified by a molecular method and eventually used in subsequent Cr(VI) reduction experiments.

The results of 16S rDNA sequencing indicated the predominance of *Clostridium* species at 99% certainty. A phylogenetic dendrogram was constructed with the neighbor-joining method using BLAST program ver. 2.2.3 to identify organisms with the highest similarity of 16S rDNA sequences. Phylogenetic analysis of the sequence was performed with the MEGA ver. 4.1 software program (Fig. 1). The phylogenetic tree was constructed using the neighbor-joining method and the Kimura2-parameter model of distance analysis, and 1000 boot-strap replications were assessed to support internal branches.

Bacteria used in the construction of the phylogenetic tree with their GenBank accession numbers included *Clostridium* sp. ArC5 AF443594, *Clostridium* sp. AB277866, *C. celerecrescens* X71848, *C. celerecrescens* AB294138, *C.* sp. CITR8 AY221993, *Desulfotomaculum* sp. CYP1 DQ 479411, *Desulfotomaculum* sp. CYP9 DQ 479419, *D. guttoideum* NR 026409, *D. guttoideum* AB294139, and Bacterium Te63R AY587830. Strain SS1 can be seen to be closely related to *Clostridium* sp. ArC5 AF443594. This is the first report of this strain being a chromium-reducing bacterium. However, *Clostridium* sp. GCAF1 has been reported to be a chromium-reducing bacteria (Sharma 2002) under anaerobic conditions. The 16S rDNA sequence of isolate SS1 has been deposited in GenBank under accession number HM004101.

Effect of Cr(VI) on the cell growth

The effect of Cr(VI) on the growth of *Clostridium* sp. cells was evaluated before the experiments of Cr(VI) reduction. The results of these experiments clearly showed that the growth of the cells was strongly affected at Cr(VI)

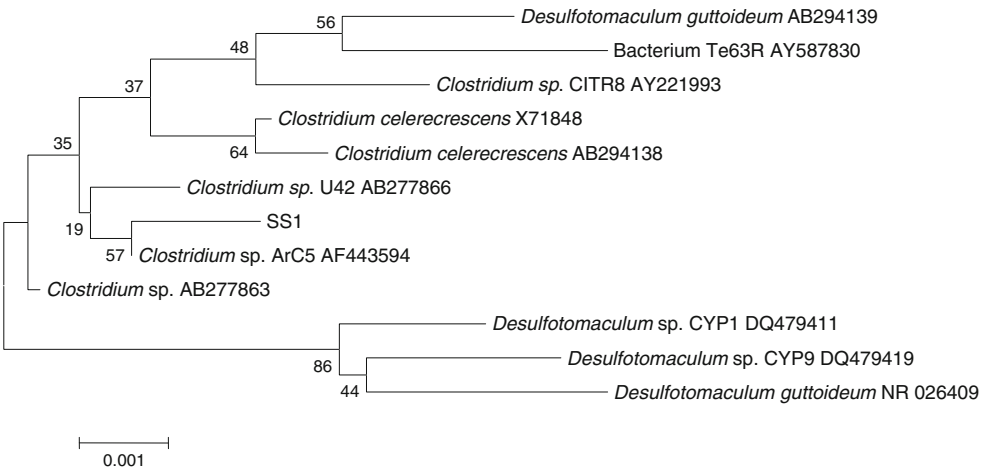

Fig. 1 Phylogenetic relationships by a neighbor-joining analysis of 16S rDNA sequences, showing the position of the *Clostridium* SS1 strain isolated in activated sludge

concentrations of 20 mg/l (data not shown), while Cr(VI) concentrations of 5 and 10 mg/l had only a slight effect, indicating the greater toxicity of Cr(VI) to the cells of *Clostridium* sp.SS1 at higher Cr(VI) concentrations. Liu et al. (2006) also reported that chromate at 80 mg/l significantly affected the cell growth of *Bacillus* sp. and that the cells failed to grow and reduce chromate in the presence of 100 mg/l chromate. Figure 2 shows a plot of the cell growth curves in the medium with or without different concentrations of Cr(VI). There was a difference in curve patterns between the cells grown in the control [no Cr(VI)] and those grown in the medium containing Cr(VI), indicating that the presence of Cr(VI) had a toxic effect on cell growth during the 24-h incubation time.

The maximum growth rates (μ) were calculated for different Cr(VI) concentrations according to th mathematical model: dx/xdt, with $\mu=\ln (x_2/x_1)/t_2 - t_1$ where x_1 and x_2 are biomass (g), t_1 and t_2 are different times (h). The results were summarized in Table 1, which shows that the maximum growth rate decreased as the concentrations of Cr(VI) increased. This was ascribed to the toxic effect of Cr(VI) on the cells.

Cr(VI) reduction

The results of the reduction experiments demonstrated that Cr(VI) reduction did occur in the culture and that about 5 mg/l Cr (VI) had almost been reduced in 24 h of incubation. The rate of reduction was faster during the initial stages (2 h) of incubation and slowed down gradually with time. Almost no abiotic Cr(VI) reduction occurred in the cell-free culture medium (control).

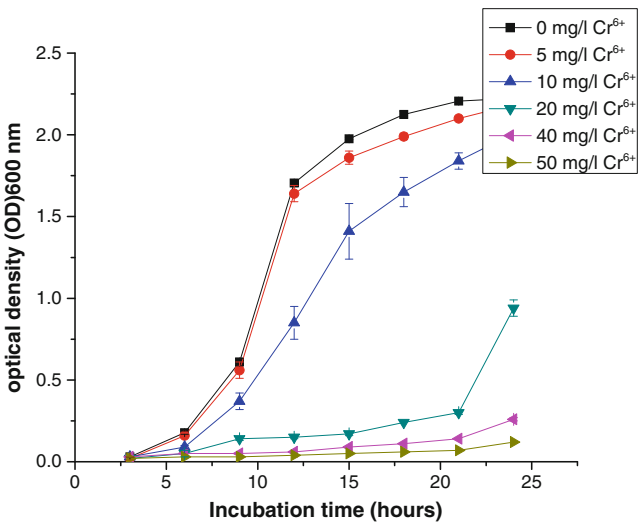

Fig. 2 *Clostridium* sp. (SS1) growth curves in medium containing different concentrations of hexavalent chromium (Cr^{6+}) and in the control (no Cr^{6+}). The optical density was tested every 3 h

Cr(VI) reduction with concomitant Cr(III) formation was observed in the reactor. According to the results, residual Cr(VI) concentration decreased as incubation progressed until no measurable concentration of Cr(VI) could be detected in the culture media. Cr(III) was the predominant product of Cr(VI) reduction in the cultures. In contrast, the total Cr concentration remained practically constant throughout the incubation time (more than 97% of the chromium initially added to the culture media was always present in solution). These results indicate that *Clostridium* sp. SS1 was capable of reducing Cr(IV) to the form of lower valence. As the stable forms of chromium are the trivalent and hexavalent forms, these results indicate that *Clostridium* sp. SS1 is most likely capable of transforming the highly toxic and soluble hexavalent chromium to the much less toxic and less mobile trivalent form (Pattanapipitpaisal et al. 2001; Stasinakis et al. 2004; Cheung and Gu 2005; Zakaria et al. 2007; Pulane et al. 2008; Liyuan et al. 2009).

Effect of pH on Cr(VI) reduction

Figure 3 shows the change in Cr(VI) concentration in the medium at different initial pH (range 4–8). The results suggest that the optimum initial pH value for Cr(IV) reduction was 7.0 (98% removal); complete Cr(VI) reduction had occurred after 24 h at an initial pH value of 7.0. Deviation from an initial pH of 7.0 resulted in decreased Cr(VI) reduction. Many other researchers have reported an optimum pH value for bacterial Cr(VI) reduction. Shakoori et al. (2000) reported that the optimum pH was 9.0 for Cr(VI) reduction by a Gram-positive bacterium, but Liu et al. (2006) found that the optimum pH was 7.0 in cases of *Pseudomonas aeruginosa* and *Bacillus coagulans*. The difference in optimum pH value suggests that pH modification is important for different cultures to achieve maximum Cr(VI) reduction in the bioremediation of chromate.

Effect of temperature on Cr(VI) reduction

Temperature is an important factor that has an effect on microbial Cr(VI) reduction. Cr(VI) reduction by the bacterium SS1 was evaluated at three different temperatures

Table 1 Maximum growth rate at different hexavalent chromium [Cr (VI)] concentrations

Cr(VI) concentrations (mg/l)	Maximum growth rates (μ/h)
0	0.53
5	0.48
10	0.42
20	0.22
40	0.08

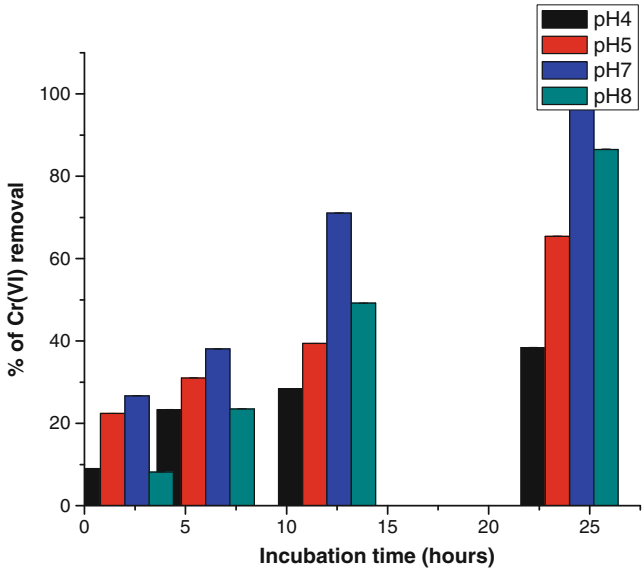

Fig. 3 Effect of initial pH of the medium (range 4–8) on Cr^{6+} reduction by *Clostridium* sp. (SS1). The initial Cr(VI) concentration was 10 mg/l

(20, 30, 40°C), and the results are presented in Fig. 4. Cr(VI) was reduced effectively at all three temperatures, with Cr(VI) removal being nearly 100% at 30°C after 24 h of incubation. This result indicates that the strain was able to reduce Cr(VI) better at 30°C than at 20 (47.7%) and 40°C (77.4%).

The 30% removal observed in the cell-free control experiment may be due to the presence of yeast extract in the medium. Enhanced Cr(VI) reduction has been observed in several cultures, including *Pseudomonas fluorescens* ATCC 27663, ATCC 31483, and ATCC 17573, *E. coli* ATCC 15489, and *Enterobacter cloacae* ATCC529, ATCC 29893, and ATCC 35930, in growth media containing yeast extract or nutrient broth. Yeast extract or nutrient broth alone may reduce Cr(VI), presumably by organic compounds with sulfhydryl groups, especially in the absence of oxygen (Wang and Xiao 1995).

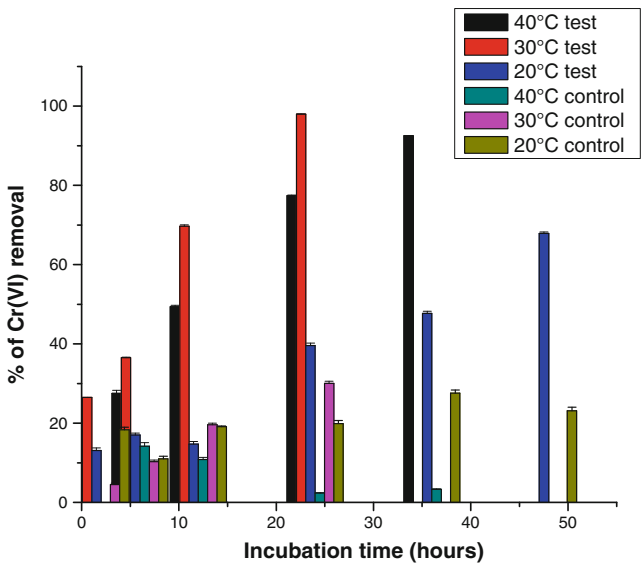

Fig. 4 Effect of temperature on Cr^{6+} reduction by *Clostridium* sp. SS1. Cr(VI) reduction was tested at 20, 30, and 40°C, respectively. The initial Cr(VI) concentration was 10 mg/l

Kinetics of Cr(VI) removal by *Clostridium* sp. SS1

Figure 5 shows the Cr(VI) concentration profiles for the different initial Cr(VI) concentrations tested. The residual Cr(VI) concentration in the culture medium decreased with increasing incubation time. Of the four initial concentrations of Cr(VI) tested, the removal efficiency in three batch cultures was 100% (i.e., no measurable Cr(IV) was detectable in the culture medium); only at an initial Cr (VI) concentration of 40 mg/l was a 100% removal efficiency attained, but only 42.3%, leading to the conclusion that this parameter was affected by Cr(VI) concentration. In contrast, total chromium concentration remained practically constant throughout the incubation time (more than 97% of the chromium initially added to the culture media was always present in the solution). These results indicate that *Clostridium* sp.SS1 was capable of reducing hexavalent chromium to the trivalent form. Similar findings have been previously reported with *Enterobacter cloacae*, *Escherichia coli*, *Pseudomonas fluorescens*, *Bacillus* sp., and *Hypocrea tawa* (Wang and Xiao 1995; Morales-Barrena et al. 2008).

Figure 6 shows that the average volumetric rate of Cr (VI) reduction exhibited by *Clostridium* sp. SS1 increased with increasing initial Cr(VI) concentration until it reached a maximum of approximately 0.82 mg/l/h, which occurred at an initial Cr(VI) concentration of 20 mg/l. This parameter

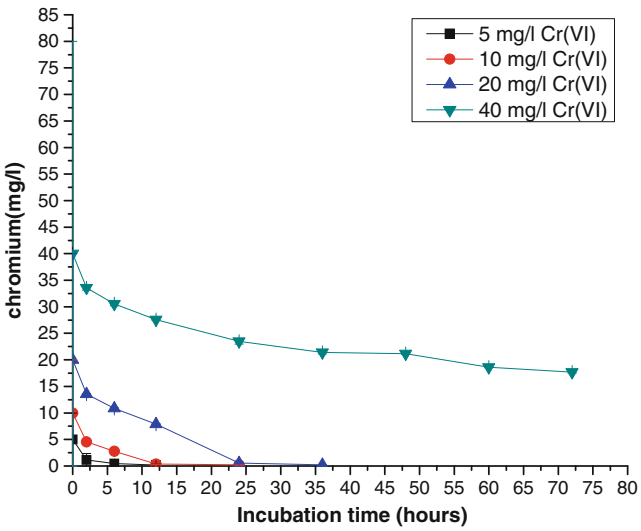

Fig. 5 Residual Cr(VI) concentration profiles for the batch cultures of *Clostridium* sp. (SS1)

was measured after 24 h of incubation because at this time no measurable concentration of Cr(VI) could be detected in the culture media for three of four concentrations tested. Nkhalambayausi-Chirwa and Wang (2000) reported that the average rate of Cr(VI) reduction exhibited by a consortium culture rose with increasing initial concentrations, reaching reached its maximum rate at 0.385 mM [0.95 mg Cr (VI)/l/h]. Above this concentration, the reduction rate decreased, and this was ascribed to toxic effects. In batch cultures of *Phanerochaete chrysosporium* conducted with initial Cr(VI) concentrations ranging from 0.01 to 0.45 mM, Pal (1997) found that the maximum volumetric rate of Cr(VI) reduction was approximately 0.11 mg Cr(VI)/l/h and was obtained at an initial Cr(VI) concentration of 0.01 mM. Stasinakis et al. (2003) reported that the average rate of Cr(VI) reduction observed 9 h after the start of Cr(VI) reduction experiments carried out with activated sludge increased from 0.052 to 0.71 mg Cr(VI)/l/h with increasing initial Cr(VI) concentrations from 0.0096 to 0.19 mM. Morales-Barrera et al. (2008) reported that the average Cr(VI) reduction exhibited by *Hypocrea tawa*, a fungus strain, rose as the initial concentration increased, reaching its maximum rate at 1.87 mM [1.75 mg Cr (VI)/l/h].

We estimated the Cr(VI) reduction capacity as the amount of Cr(VI) reduced by unit dry weight of biomass produced and evaluated the cultivation time at which Cr (VI) reduction was completed. Figure 7 shows that there is a linear correlation between this kinetic parameter and initial Cr(VI) concentration. This behavior confirms that the finite Cr(VI) reduction capacity of *Clostridium* sp. SS1 was not reached within the range of Cr(VI) concentrations tested. The highest Cr(VI) reduction capacity attained in our experiments was 72 mg Cr(VI)/g biomass. In comparison, the finite Cr(VI) reduction capacity of *Shewanella oneidensis* MR-1 (Middleton et al. 2003), *Bacillus* sp., *Escherichia coli* ATCC 33456, and *Pseudomonas fluorescens* LB300 (Deleo et al. 1994; Wang and Shen 1997) cultured under aerobic conditions was found to be 0.08 mg Cr(VI)/mg protein, and 2.08×10^{-12}, 7.18×10^{-12}, and 3.10×10^{-12} mg Cr(VI)/cell, respectively. Since the average dry weight of a typical bacterial cell is approximately 3×10^{-13} g (Atkinson et al. 1983), then the reduction capacities of *Bacillus* sp., *P. fluorescens* LB300 and *E. coli* ATCC 33456 would be 6.93, 10.33 and 23.93 mg Cr(VI)/g biomass, respectively.

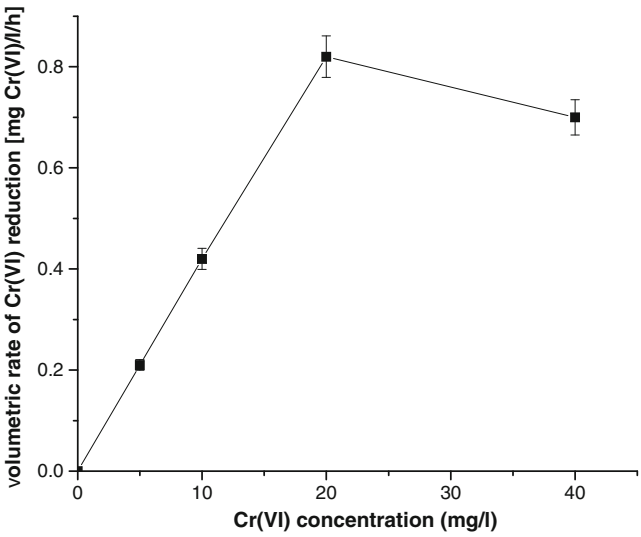

Fig. 6 Relationship between volumetric rate of Cr(VI) reduction and initial Cr(VI) concentration. *Error bars* Standard deviation of triplicate samples

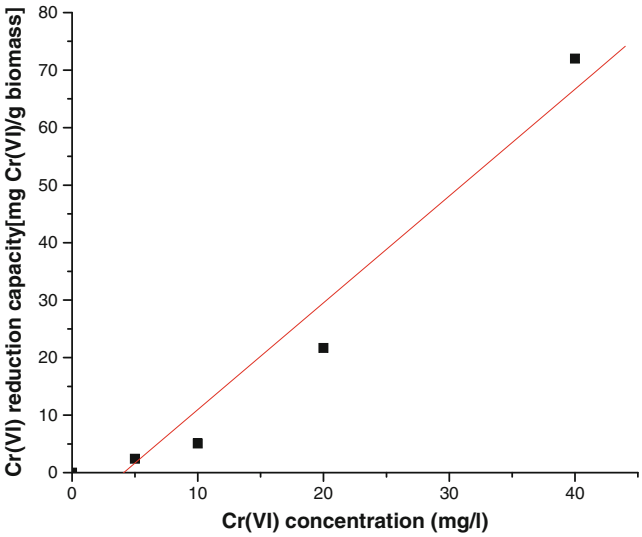

Fig. 7 Relationship between Cr(VI) reduction capacity and initial Cr (VI) concentration (R^2=0.9745), Y=1.859X − 7.655

The higher volumetric Cr(VI) reduction rate and greater Cr(VI) reduction capacity observed at higher Cr(VI) concentrations in this study indicate that the *Clostridium* strain tested has ability to reduce high concentrations of Cr(VI) and could, therefore, be a suitable candidate for the detoxification of Cr(VI)-polluted sites.

Conclusions

Four bacteria isolated from activated sludge were tested for their ability to reduce Cr(VI). Only strain SS1 was able to reduce toxic hexavalent chromium to the less toxic trivalent form under aerobic conditions. Complete reduction of 10 mg/l of Cr(IV) occurred after 2 days incubation. The SS1 strain was identified as *Clostridium* sp., by 16S rDNA sequence analysis and was subsequently used in further Cr (VI) reduction experiments.

The growth of SS1 cells was significantly affected by toxic Cr(VI) up to 20 mg/l. The optimum initial pH value for Cr(IV) reduction was 7.0, and Cr(VI) reduction was

more efficient at 30°C than at 20 and 40°C. The maximum growth rates decreased with increasing Cr(VI) concentrations in the culture media. A higher volumetric [0.82 mg Cr (VI)/l/h] rate and greater capacity [72 mg Cr(VI)/g biomass] to reduce Cr(VI) were obtained with higher initial Cr(VI) concentrations (40 mg/l). These results may provide valuable information for the bioremediation of chromate pollution. Further research is currently being conducted on the mechanisms by which this bacterium reduces Cr(VI).

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