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Enzymatic Chromium (VI) Reduction by Cytoplasmic and Cell Membrane Fractions of Chromate-Reducing Bacterium Isolated From Sewage Treatment Plant

Paul Fabrice Nguema[1], Zejiao Luo[1] & Jing Jing Lian[1,2]

[1] State Key Laboratory of Bio geology and Environmental Geology, Ministry of Education (BGEG), School of Environmental Studies, China University of Geosciences, Wuhan, China

[2] Schools of Earth Environment and Water Resources, Yangtze University, Wuhan, China

Correspondence: Zejiao Luo, School of Environmental Studies, China University of Geosciences, 388 Lumo Lu, Wuhan 430074, China. Tel: 86-134-7621-2088. E-mail: zjluo@cug.edu.cn

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Abstract

Hexavalent chromium is a toxic heavy metal in environment. To remove it from the system, reduction to the trivalent form using microorganisms is an alternative cost-effective method compared to the conventional physicochemical methods. *Bacillus cereus* Pf-1was isolated and tested for the reduction potential of Cr (VI) using different sub-cellular fractions. Diphenylcarbazide method was used to measure the remaining Cr (VI) concentration in supernatant after centrifugation, and the pellet was used for Scanning Electron Microscopy (SEM) and Energy Dispersive X-ray (EDX) analysis. Almost complete reduction of Cr (VI) was reported for initial Cr (VI) concentration from 10-50 mg/L. Assay with permeabilized cells (treated with Toluene and Triton X-100) and cells free assay demonstrated that Cr (VI) reduction activity was mainly associated with the cytoplasmic fraction of the cells. Considering the major amount of Cr (VI) being reduced within 24 h, the chromate reductase could have been released during growth. Additionally, SEM analysis showed that *Bacillus cereus* treated with Cr (VI) grew well and had uniform size. EDX analysis revealed certain amounts of chromium in cells treated with Cr (VI). Based on this, *Bacillus cereus* Pf-1 could be considered as a potential reducing agent for Cr (VI) in aqueous solutions.

Keywords: *Bacillus cereus* Pf-1, chromate-reductase, cytoplasmic fraction, membrane fraction

1. Introduction

Hexavalent chromium is a toxic heavy metal with corrosion resistance properties such as electroplating, textile dying, metal processing and wood treatment (Ibrahim et al., 2012) which is widely used in several industries. Chromium exists in several oxidation states but only trivalent (+3) and hexavalent (+6) forms are usually encountered in the environment, depending on the local redox conditions (Gheju & Balcu, 2010). Chromium compounds exhibit high mobility in the environment and exert toxic effects to most living organisms. The United States Environmental Protection Agency (2012) reported hexavalent chromium as one among the 17 chemicals posing as threat to humans. In contrast, the trivalent form is sparingly soluble, and has a stable oxidation property and thus less toxic (Ilias et al., 2011). It therefore implies that, removal of hexavalent chromium contaminant from polluted industrial aqueous effluents is an important step in pollution control of surface water and groundwater.

Several microorganisms have the exceptional ability to survive in noxious metal-polluted environments by developing mechanism like metal resistance plasmids, metal efflux channels, adsorption uptake, DNA methylation, and metal biotransformation either directly by enzymatic reduction to less mobile and non-toxic forms or indirectly through complexes formation with metabolites (Pei et al., 2009). Varieties of Cr-resistant with high potential of Cr (VI) reducing ability includes *Agrobacterium*, *E. coli*, *Shewanella*, *Enterobacter*, *Thermus*, *Pseudomonas*, *Bacillus* (Soni et al., 2013). However, Cr (VI) resistance and Cr (VI) reduction have been considered to be unrelated (Ohtake et al., 1987). The availability of selected strains able to resist and reduce chromate/dichromate increased the possibility of employing microorganisms for bioremediation of Cr (VI) contaminated sites.

There are different types of Cr (VI) reduction enzymes in bacteria with Cr (VI) reductase, aldehyde oxidase, cytochrome P450, DT-diaphorase (Patra et al., 2010) as prominent examples. Similarly, several oxidoreductases with different metabolic functions have also been reported to catalyze Cr (VI) reduction in bacteria, which include nitroreductase (Kwak et al., 2003), iron reductase, quinone reductases (Gonzalez et al., 2005) as well as flavin reductases (Ackerley et al., 2004).

Enzymatic reduction of chromium (VI) using bacteria has recently received much attention. However, a lot still needs to be done to fully understand the mechanisms that govern chromium (VI) reduction by bacteria.

The purpose of this study was to explore the localization and mechanisms associated with Cr (VI) reduction by *Bacillus cereus* strain Pf-1 isolated from dewatering sludge, with high chromate reduction activities. The optimum conditions as well as the efficiency of Cr (VI) reduction due to the presence of electron donors and proteins denaturants were also investigated.

2. Material and Methods

All the chemicals were of reagent grade and were used without further purification. Unless otherwise stated, all the chemicals were purchased from Sinopharm Group Chemical Reagents Co. Ltd., Shanghai, China, Fisher (Fair Lawn, Nanjing), and Tianjin Fu Chen Chemical Reagents Factory, Tianjin, China.

2.1 Isolation of Bacterial Strains

Dewatering sludge samples were obtained from the Chinese Academy of Agricultural Sciences Sewage treatment plant Wuhan, China. The samples were collected in sterile containers, while being maintained below 4 ºC, in our laboratory until use. Isolation of bacterial strains was done by the enrichment culture technique. Nutrient Broth was amended with 30 mg Cr (VI)/L as $K_2Cr_2O_7$ and 10% (v/v) soil suspension (10%, w/v) prepared in sterile distilled water, which was incubated at 30 ºC for 24 hours under orbital shaking (100 rpm). The Cr (VI) stock solutions were filtered and sterilized using a 0.22 µm membrane filter (Q/IEFJ01-1997, China). These isolated strains (6 in total) were evaluated for their tolerance to Cr (VI) at different concentrations (40-120 mg/L). On the basis of the chromium tolerance test, the higher Cr-tolerance strain was selected for further analysis.

2.2 Chromium (VI) Concentration Effect on Bacterial Growth and Chromium (VI) Reduction

Individual culture flasks (250 ml) containing 50 ml NB, pH 7.0 ± 0.3, supplemented concentrations of Cr (VI) at 10, 30, 50, and 100 mg/l, were inoculated with 1mL of bacterial cells cultured for 24h at 30 ºC with constant shaking at 100 rpm. The cultures including biotic [NB without Cr (VI)] and abiotic [NB with Cr (VI) but not inoculated with bacteria] were cultured as controls. The density of the bacteria was monitored at definite time intervals by the viable plate count method. To measure the Cr (VI) reduction, 10 mL culture from each of above flasks were centrifuged (10,000 × g for 10 min) and the supernatant was analyzed using Diphenylcarbazide (DPC) reagent as described by Ishibashi et al. (1990). Briefly, the Cr (VI) containing samples (in the range 1-10 microgram) were acidified by adding 330 µl of 6 N sulfuric acid. To this acidified solution of Cr (VI), 0.25% of DPC was added and the final volume was made up to 10mL. The mixture was incubated for 10 min at room temperature for the color development, thereafter; the complex was measured at 540 nm with spectrophotometer (WFZ800-D3B UV/VIS Spectrophotometer, Guangzhou, China).

2.3 Effect of Temperature and pH on Cr (VI) Reduction

Chromium (VI) reduction was studied at different temperatures (20, 30, 40, and 45 ºC) and pH (5, 6, 7, 8, and 9). The initial pH was adjusted using 10% (v/v) HCl or 10% (w/v) NaOH. Appropriate buffers (50mM), phosphate buffer (pH 7.2) and Tris-HCL buffer (pH 7.0) were added to avoid pH shift (Olajuyigbe & Ajele, 2005). Flasks containing 50 mL NB amended with $K_2Cr_2O_7$ to final concentration of 30 mg Cr (VI)/L and control cultures were incubated as mentioned above. All the cultures including biotic and abiotic controls, in triplicate, were incubated as mentioned earlier. Aliquots of the cultures (10 mL) were withdrawn at regular time intervals from each replicated tube and centrifuged as mentioned earlier. The concentration of Cr (VI) in the supernatant was analyzed as mentioned earlier. Experiments were conducted in triplicates and were repeated twice.

2.4 Resting Cell Assay

Bacterial culture in 50mL NB was grown overnight at 30 ºC with orbital shaking at 100 rpm. Cells were harvested from the culture by centrifugation at 10,000 × g for 10 min. The pellets were washed thrice with phosphate buffer (pH 7.2) and re-suspended in the same buffer. These cell suspensions were spiked with 30 mg Cr (VI)/L and adjusted the final volume to 10mL. The tubes were vortexed for 2 min and incubated for 12h. Samples were withdrawn at regular time intervals (2, 4, 6, 8, 10, and 12 h) and the remaining Cr (VI) concentrations were measured as mentioned above. Cr (VI) spiked in heat-treated (100 ºC for 30 min)

re-suspended cells served as controls.

2.5 Permeabilized Cell Essay

Bacterial cell permeabilization was carried according to previously reported protocols (Mangaiyarkarasi et al., 2011). Bacterial cell was grown for 24 h, harvested and washed thrice with phosphate buffer. The suspended cells were treated with Toluene and Triton X-100 at final concentrations of 0.1, 0.2, 0.5, 1 and 2%, and vortex for 20 min. Cr (VI) reduction was initiated by adding 2 mL of these permeabilized cells suspensions to 8 mL of 50 mM phosphate buffer containing Cr (VI) (30 mg/L) and glucose (1%), and incubated at 30 ºC in a water bath shaker for 12 h. Samples were withdrawn at regular time intervals (2, 4, 6, 8, 10, and 12 h), the reaction mixture was pelletized and the supernatant was analyzed for residual Cr (VI) concentration.

2.6 Cell-Free Assay and Localization of Chromate Reduction Activity

Cells were harvested at 10,000 × g after 24 h culturing, and washed thrice with phosphate buffer. These cells were re-suspended in phosphate buffer and disrupted using an ultrasonic probe according to Cheung et al. (2006). The cell lysates obtained were then centrifuged at 160,000 × g for 40 min at 4 ºC, and filtrated through 0.22 μm filters. The supernatant of the cytosolic obtained was used as source of soluble enzyme. The sonicated cell debris was washed thrice with phosphate buffer and accordingly re-suspended in 8ml of buffer. Aliquots (2 ml) of cell-free extract (CFE) or membrane fraction (CM) were used for enzymatic assay in order to localize the chromate reduction activity of the isolated either in extracellular, cytosolic or membrane-associated enzyme, respectively.

Chromate reduction was estimated using a standard calibration curve of Cr (VI) with reference to $K_2Cr_2O_7$. Protein concentrations of CFE were estimated using Bradford (1976) reagent and specific activity was defined as unit chromate reductase per milligram protein concentration. Known concentrations of bovine serum albumin (BSA) prepared in phosphate buffer were used for standard calibration curve.

2.7 Effect of Electron Donors and Proteins Denaturants on Cr (VI) Reduction by Cell-Free Extract

Hexavalent chromium reductase activity in the cell-free extract of bacterial isolate was determined in the presence of (NADH, glucose, succinate, citrate, urea, and thiourea) at the final concentration of 0.1 mM and incubated for 30 min at 30 ºC. Cr (VI) concentration in the medium was 30 mg/L.

2.8 Extraction of DNA From Bacterial Culture and Amplification of 16S rDNA

Bacterial genomic DNA was isolated from overnight grown cells using standard procedures (Chachaty & Saulnier, 2000). The extracted DNA was electrophoresed on 0.8% agarose gel in TAE buffer and visualized under UV in Uvitec to check for integrity. The quantity of extracted DNA was checked with a spectrophotometer.

Gene fragments specific for the highly variable V3 region of the bacterial 16S ribosomal DNA gene was amplified by PCR as described by Ovreas et al. (1997) using universal bacterial primers 7 f and 1540 r with their respective oligonucleotide (5'-CAGAAGTTTGATCCTGGCT-3') and (5'-AGGAGGTGATCCAGCCGCA-3'). The PCR products were subjected to 2% agarose gel electrophoresis stained with ethidium bromide and visualized on a UV transilluminator for the presence of about 200 bp PCR products. Amplified 16S ribosomal DNA gene PCR products were purified using strataprep® PCR purification kit (stratagene, USA) according to the manufacturer's protocol. Sequencing reactions were carried out using ABI-prism big dye terminator cycle sequencing ready reaction kit and the PCR products were purified by a standard protocol. The purified cycle sequenced products were analyzed with an ABI-prism 3730 genetic analyzer. The chromatogram sequencing files were edited using Chromas 2.32. Obtained 16S rDNA sequences were BLAST in NCBI using BLASTn program (http://www.ncbi.nih.gov/BLAST/). Sequences were aligned using CLUSTALW program (http://www.ebi.ac.uk/clustalw). Aligned sequences were used and phylogenetic tree constructed with the aid of Molecular Evolutionary Genetics Analysis (MEGA version 5.05) software based on Kimura 2-parameter evolutionary distances and evaluated by performing bootstrap analyses of 1000 replicates, to show the genetic relatedness of the isolate (Tamura et al., 2011).

2.9 SEM-EDX Analysis

The strain was cultured for 24h in the medium without and with Cr (VI) [30 mg/L]. After being centrifuged at 10,000 × g at 4 ºC, the cells were observed by SEM (model- Zeiss EVO40) and EDX (EDAX, USA).

2.10 Statistical Analysis

Experimental data, presented as mean ± standard error (SE) in table and with bars in figures, were analyzed using Student's *t* test and expressed at 0.05 probability level calculated by Origin version 8.5.

3. Results

3.1 Screening and Identification of Bacterial Isolate

Sequence similarity search was performed with obtained 16S rDNA sequences through BLAST-n program in NCBI. BLAST-n results revealed that our isolated strain namely Pf-1 belong to *Bacillus* and exhibited 99% similarities with *Bacillus cereus* (GenBank accession number GU321330). Phylogenetic analysis position in relation to other related organisms have been shown in Figure 1. Our sequence has been submitted to the NCBI GenBank under accession number (*KC152883*).

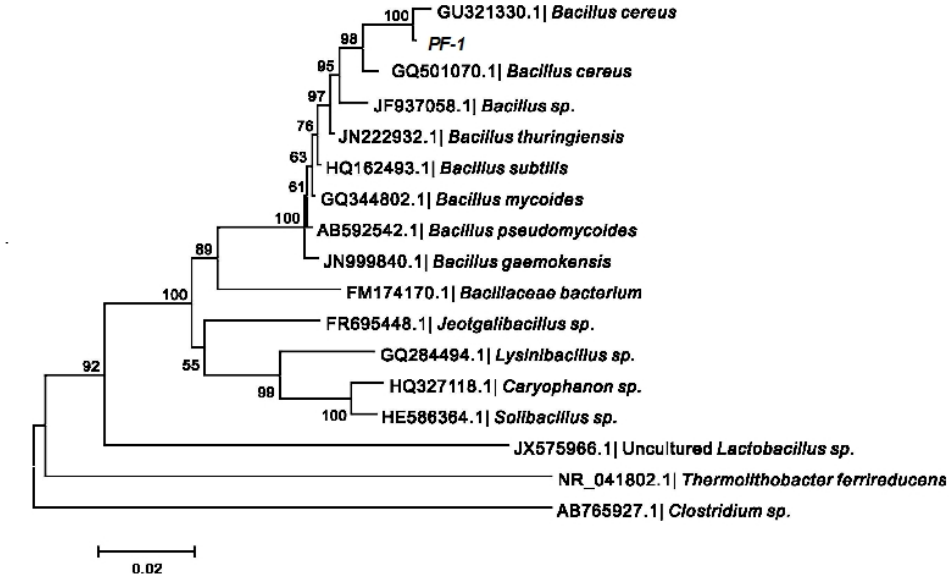

Figure 1. Phylogenetic tree constructed from the 16S rDNA gene of strain Pf-1 using neighbor-joining algorithm. Accession numbers are given before the strains name, and scale bar represents 1 base substitution per 20 nucleotide positions. The bootstrap probabilities calculated from 1,000 replications. *Clostridium sp*. (AB765927) was taken as an out-group

Table 1 shows different isolated strains in our dewatering sludge samples and their minimum inhibitory concentration (MIC).

Table 1. MIC values for different isolates

Strains	MIC (mg Cr (VI)/L)
Pf-1	60
SS1	30
Mp9	30
Zc5	20
Ng2	20
NN3	20

3.2 Cr (VI) Reduction and Growth of Bacterial Strain

The concentration of Cr (VI) affected bacterial growth and Cr (VI) reduction. As the concentration of Cr (VI) increased from 10-100 mg/L, the CFU decreased from 4.5×10^5 - 1.1×10^5 (Figure 2). At the same time the reduction efficiency decreased from 100% - 55% (Figure 3). There were negligible levels of chromate reduction in abiotic controls.

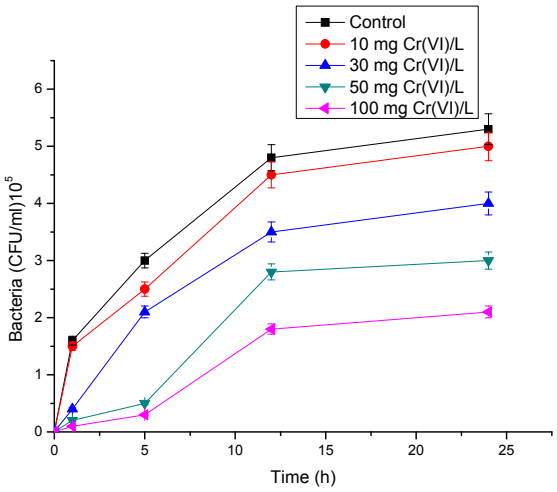

Figure 2. Growth of *Bacillus cereus* Pf-1 in NB medium amended with different Cr (VI) concentrations. NB medium with 10 mg Cr (VI)/L without bacterial inoculation as control

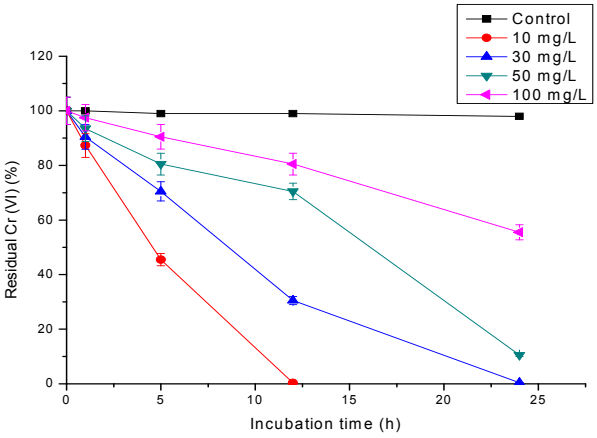

Figure 3. Kinetic of Cr (VI) reductions by *Bacillus cereus* Pf-1

3.3 Effect of pH and Temperature on Cr (VI) Reduction by Bacterial Strain

The strength of Cr (VI) reduction was found to be affected by strain identity, temperature, pH, and time. Chromate reduction was investigated at regular time intervals with variation of both temperature and pH (Figure 4). Maximum Cr (VI) reduction activity was established at 30 ºC and pH 7.0, this activity decreased at both lower (20 ºC) and higher (45 ºC) temperatures. Negligible Cr (VI) reduction was observed in abiotic control.

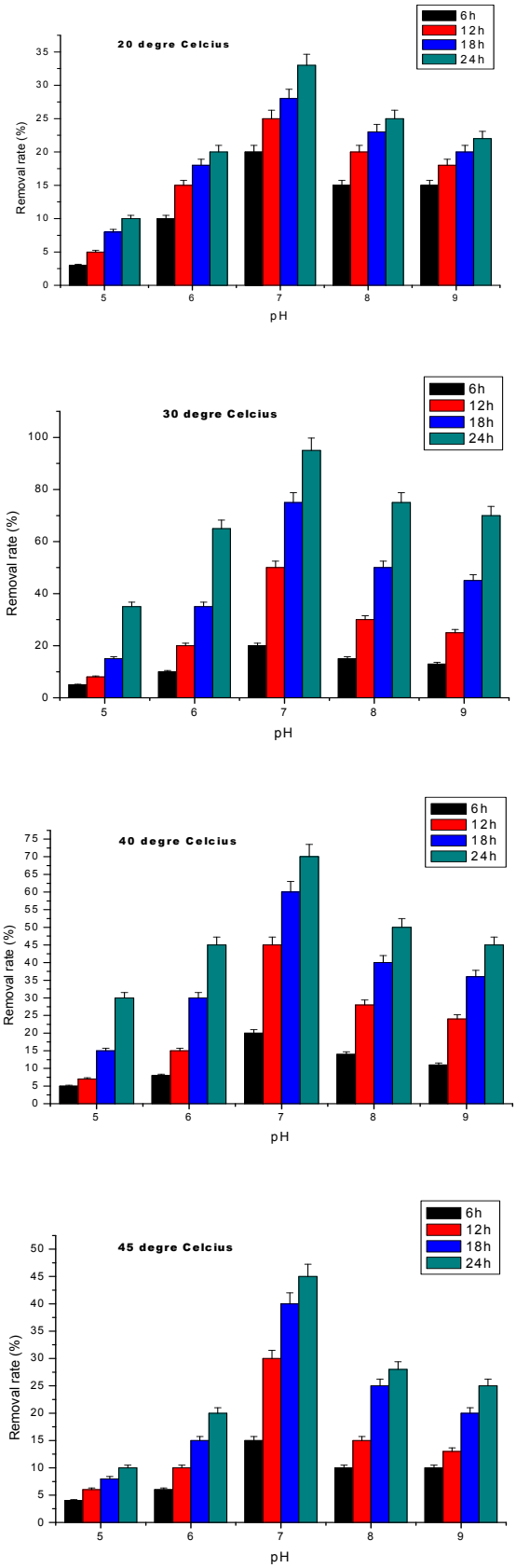

Figure 4. Effects of pH and temperature on Cr (VI) reduction by *Bacillus cereus* Pf-1 strain supplemented by 30 mg Cr (VI)/L

3.4 Localization of Chromium Reducing Activity

For detecting the localization, chromate reduction essays were carried out using resting and permeabilized cells by exposing the cells to 30 mg Cr (VI)/L at 30 ºC and the samples were withdrawn at regular time intervals (2-12 h) to measure the remaining Cr (VI) concentrations. Figure 5 shows the concentration of residual Cr (VI) upon exposure of resting and permeabilized cells of the strain. As observed from Figure 5, cell permeabilization significantly increased the Cr (VI) reduction. Among the two reagents used for permeabilization, Toluene increased the permeabilization of bacterial cells, which resulted in higher Cr (VI) reduction. Upon treatment with Toluene, complete reduction was observed at 0.5% of Toluene concentrations. Chromate reduction essays were followed using initial concentration of 30 mg Cr (VI)/L with cytosolic fraction (CFE) or cell-free extract and membrane fraction (CM). As observed from Table 2, reduction of Cr (VI) was mainly associated with soluble fraction (CFE), indicating the presence of chromate reductase in cytoplasmic fraction. No significant activity of chromate reduction was noticed in the membrane fraction derived from ultra-sonicated cells of the isolate. Heated kill cells, cell-free extracts acting as control failed to reduce Cr (VI). These results confirm the presence of soluble enzymatic mechanism in the cytoplasmic fraction (crude cell-free extract) of the strain. At the optimal temperature 30 ºC and pH 7.0, the specific activity of Cr (VI) reduction was determined to be 0.88 μmol min^{-1} mg^{-1} proteins.

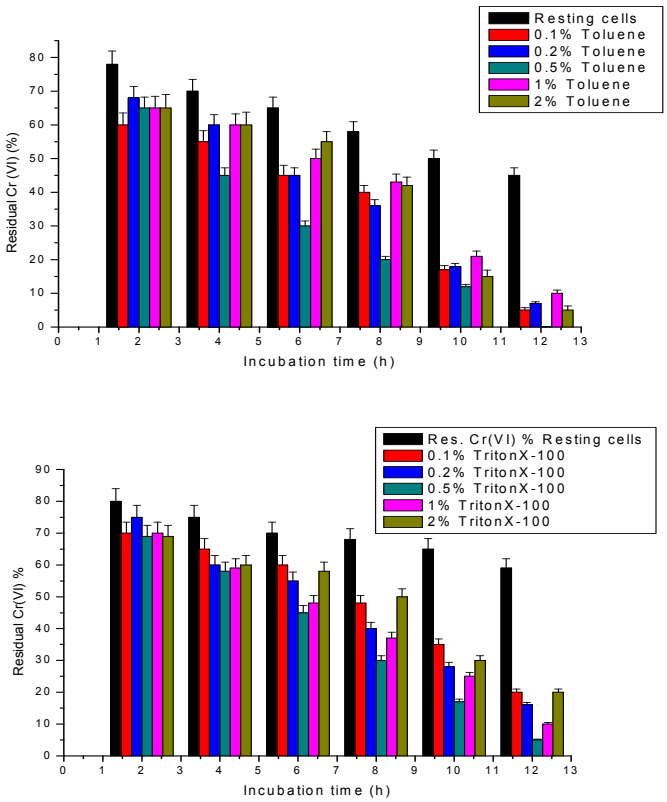

Figure 5. Hexavalent chromium reductions by resting and permeabilized cell of Pf-1 strain

Cr (VI) reduction activity of the soluble fraction was estimated in presence of 0.1 mM protein denaturants and electron donors at initial Cr (VI) concentration of 30 mg/L upon incubation at 30 ºC and pH 7.0 for 30 min in 50mM phosphate buffer. Urea, a protein denaturant, inhibited the Cr (VI) reduction by 85%, while another protein denaturant thiourea inhibited the Cr (VI) reduction by 95%, indicating the denaturation of proteins responsible for inhibition of Cr (VI) reduction. The specific activity of Cr (VI) reduction in the cell-free extract of the isolate showed an increase with the addition of 0.1 mM NADH; addition of 0.1mM NADH in the reaction mixture containing cell-free extract also stimulated the reduction of Cr (VI). Additionally, glucose, a possible electron donor, during the reduction of Cr (VI), increased Cr (VI) reduction as observed from data present in Table 3. Citrate increased Cr (VI) reduction to a significant level. However, succinate had no significant effect on

the reduction of Cr (VI) by cell-free extract.

3.5 SEM-EDX Analysis

The SEM images revealed that the strain has rod-shaped, elongated and uniform size in the control without Cr (VI) (Figure 6a). It could be clearly observed that the cells shape was considerably changed when Cr (VI) was added (Figure 6b). EDX analysis revealed an obvious peak at binding energy of 5.5 keV, which was corresponding to Cr for the intracellular sample of the strain that has been treated with Cr (VI) (Figure 6b).

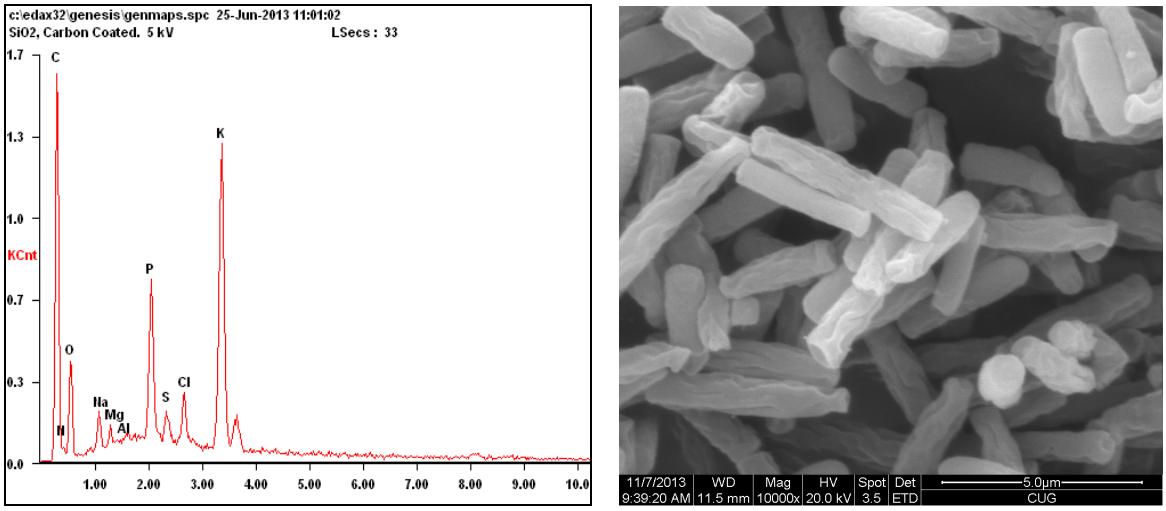

Figure 6a. SEM-EDX images without Cr (VI)

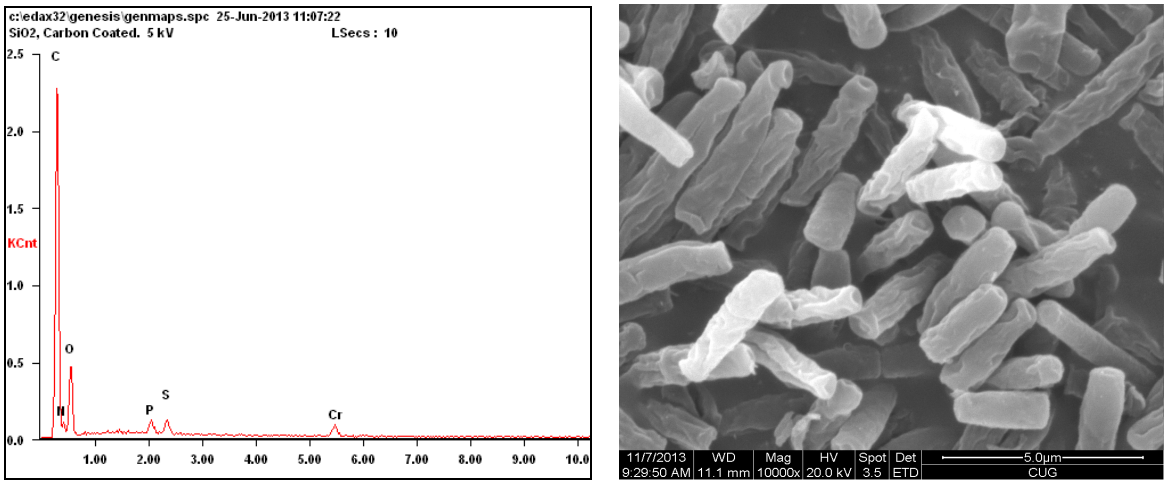

Figure 6b. SEM-EDX images treated with Cr (VI)

Table 2. Sub-cellular localization of chromate reductase activity in Pf-1 strain

Cellular fractions	Chromate reductase activity (µmol min^{-1} mg^{-1} protein)
Cytoplasmic fraction (CFE)	0.88 ± 0.15*
Membrane fraction (CM)	0.081 ± 0.032

*Standard error.

Table 3. Protein denaturants and electron donors on chromate reductase activity in the cell-free extract

Protein denaturants	Specific activity (μmol min^{-1} mg^{-1} protein)
CFE (control)	0.33 ± 0.16*
Urea	0.082 ± 0.025
Thiourea	0.045 ± 0.012
Electron donors	
NADH	0.79 ± 0.24
Glucose	0.67 ± 0.18
Succinate	0.32 ± 0.109
Citrate	0.65 ± 0.188

*Standard error.

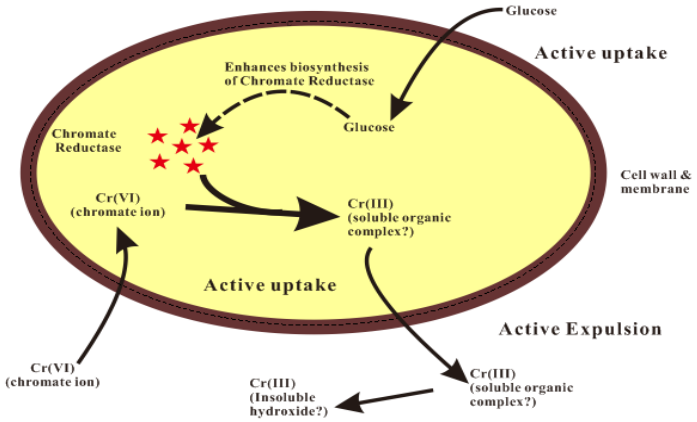

Figure 7. Mechanism of bacterial reduction of toxic Cr (VI) to non toxic Cr (III)

4. Discussion

Activated sludge/biosolid is a common environment for microbial communities with emergence of bacterial species having elevated metal tolerance. In the present investigation, bacterial strains tolerating and reducing Cr (VI) were isolated in dewatering sludge from a municipal treatment plant in Wuhan, China. The higher tolerant and reducing strain was selected for further analysis. The selected strain belongs to the genus *Bacillus*. Strains of the genus *Bacillus* are known to tolerate and reduce Cr (VI). However, the tolerance level and reduction ability varied with the strains identity.

In our experiments, higher Cr (VI) concentrations caused decrease in growth rate when compared to growth at lower Cr (VI) concentrations, suggesting that Cr (VI) was toxic to the cell. It is also clear from our results (Figure 3) that increase in chromate reduction was growth dependent; higher Cr (VI) reduction were noticed during the first 12 h corresponding to exponential phase of the microbial growth because Cr (VI) reduction is an active process; in the exponential phase of microbial growth, most of the cells are active and result to the increase of the reduction rate. Most likely, bacterial growth and Cr (VI)-induced damage are competing processes, and bacteria can cope with Cr (VI) exposure only as long as metabolizable carbon sources are available. Liu et al. (2006) noticed that this phenomenon might be explained as an increased time period for adaptation or repair during the exposure to high level of Cr (VI) in the growth medium.

The high level of Cr (VI) in the medium induces frame shift error and, to a greater extent, base pair substitution both in G-C and A-T base pairs (Deflora et al., 1990).

Hexavalent chromium reduction by *B. cereus* was investigated at different temperature (20-45 ºC), an important factor affecting microbial Cr (VI) reduction. Maximum Cr (VI) reduction was established at 30 ºC, which also corresponds to the maximum growth of the bacterial strain, confirming that Cr (VI) reduction was growth

dependent. Additionally, our strain can be considered as a mesophile bacterium with a moderate optimum temperature. Such growth dependent chromate reduction has also been earlier reported by Desai et al. (2008a). It has been reported that the optimal temperature of Cr (VI) reduction could be in the range of 25-37 ºC (Ibrahim et al., 2012). However, optimum temperature of Cr (VI) reduction of thermophilic *Thermus scotoductus* SA-01 (Opperman et al., 2008b) and *Bacillus firmus* KUCr1 (Sau et al., 2010) have been reported at 65 and 70 ºC, respectively.

Cr (VI) reduction was found to be also pH dependent. The growth and the reduction rate changed with the pH suggesting that pH is one of the key factors in microbial Cr (VI) reduction. Pf-1 has his optimum growth at pH 7.0, therefore it can be considered as a neutrophile strain. Wang et al. (1990) reported that reduction of Cr (VI) in bacterial strain occurred at neutral pH 6.0-8.0 and was strongly inhibited when the pH fall to acidic and alkaline conditions. Murugavelh and Mohanty reported the optimum pH 6.0 for the reduction of Cr (VI) with free cells of *Bacillus sp*. Our results clearly indicated that chromate reduction was dependent on pH, temperature, strain identity, suggesting that different strains perform differently under different temperature and pH.

Negligible reduction was noticed in abiotic control at all temperature and pH, confirming the direct interaction of microbes Cr (VI) reduction. Similar results indicating negligible reductions in abiotic control have been earlier reported by He et al. (2010), Masood and Malik (2011).

Further, Cr (III) readily precipitates as Cr $(OH)_3$ at pH 7.0 (Bopp et al., 1983). However, a number of recent studies suggest that Cr (VI) reduction by some bacteria strains such as *Pseudomonas* (Dogan et al., 2011) and *Bacillus sp*. (Desai et al., 2008a) led to the production of soluble Cr (III) end products instead of Cr $(OH)_3$. Dogan and his coworkers found that the release of exopolymeric substances (EPS) during microbial Cr (VI) reduction with *Pseudomonas* bacteria leads to enhance solubility of Cr (III) in solution. Puzon et al. (2002) suggested that an intracellular located *E. coli* enzyme system converts Cr (VI) to a soluble and stable NAD^+ - Cr (III) complex, and cytochrome c-mediated Cr (VI) reduction produces cytochrome c Cr (III) adducts. Their findings provide evidence that chromium exposure to cells produced elevated concentration of microbial EPS. Puzon et al. (2005) suggested that Cr (VI) intracellular reduced in the cytoplasm by a bacterial enzyme, using NADH as the reductant, and, after cells lysis, form soluble Cr (III) end products in solution. The metal reduction can also be mediated by the surfaces of bacterial spores (Junier et al., 2009) and such mechanism may be relevant for spore forming bacteria like *Bacillus*.

Resting and permeabilized cell assays provided the best evidence of the presence of a Cr (VI) reduction mechanism in cytosolic fraction as observed in literature. Permeabilization with Toluene and Triton X-100 at different concentrations resulted in increased Cr (VI) reduction, confirming that cytoplasmic proteins were released and the strain reduced Cr (VI) to Cr (III) through soluble cytosolic reductases and not through membrane associated reductases. Moreover, the reduction with Toluene was significantly higher compared to the reduction facilitated by Triton X-100, suggesting that phospholipids were more dissolved than proteins. Toluene and Triton X-100 are known to permeabilize the membrane phospholipids and solubilizing inner membrane proteins. The inability to reduce Cr (VI) by boiled cell-free extract, which served as control, showed that reduction process was enzymatic and not due to adsorption or chemical reaction. The cell lysis played an important role in Cr (VI) reduction. Elangovan and his coworkers observed that chromate reductase activity was associated with soluble proteins and not with the membrane fraction. Desai et al. (2008b) suggested that a soluble chromate reductase associated with the cytoplasmic membrane catalyzed Cr (VI) reduction by *Pseudomonas sp*. G1DM21 by transferring initial one electron to Cr (VI) to form an intermediate Cr (V), followed by two electron transfer to Cr (III) formation. Our results indicate that, although the chromate-reducing fraction was located in cytosol, this fraction may have been extracellular released considering major amount of chromium being reduced during 24 h incubation. Additionally, the presence of Cr peak in the EDX image for the sample treated with Cr (VI) indicated that there were certain amounts of Cr in the cells. These findings indicate that Cr (VI) was transferred to Cr (III) by *Bacillus cereus*. It is possible that Cr (VI) was first adsorbed to the cell walls, then absorbed into the cells and reduced to Cr (III) by the soluble cytosolic reductase as illustrated in Figure 7.

Electron donors have been known to affect chromate reduction activity. Glucose and citrate has significant stimulatory effect on Cr (VI) reduction and their activities were 2 fold higher than the control experiments. Glucose, by active uptake, enhances the biosynthesis of chromate reductase as mentioned in Figure 7. Studies by Mabbett et al. (2002) show the presence of low molecular organic molecules such as citrate protected the chromate reductase enzymes from inactivation by removing toxic products of microbial reduction and a close connection between the amount of Cr (VI) reduced and equilibrium constants of Cr-ligand complexes with more Cr (VI) being reduced with much stronger complexes. Succinate, on the other hand, did not show any

noteworthy effect in Cr (VI) reduction. The Cr (VI) reduction by this strain of *Bacillus cereus* belongs to the NADH and/or glucose dependent type. An addition of NADH or glucose improved Cr (VI) reduction to a significant level. There have been reports supporting NADH-dependent Cr (VI) reduction from *pseudomonas sp*., *E. coli*, and *Bacillus sp*.; probably, these can use NADH as electron donor (Bae et al., 2005).

Previous reports have demonstrated that intracellular Cr (VI) accepts a single electron from an NADH molecule forming a Cr (V) intermediate, which in turn accepts two electrons from two molecules of NADH to form stable Cr (III) (Suzuki et al., 1992). Urea and thiourea are well known protein denaturizing agents. In our study, both inhibited the Cr (VI) reduction by 85 and 95%, respectively.

5. Conclusion

The results of the current investigation clearly demonstrated that *Bacillus cereus* Pf-1 strain has strong potentials to reduce toxic and soluble Cr (VI) to the less toxic and less soluble Cr (III) and hence can be employed as a bio-agent for Cr (VI) detoxification from the contaminated effluents. Further, the findings of the current study would help the exploration of the reduction performance of the genus *Bacillus* and develop appropriate conditions for the treatment of contaminated effluents/soils.

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