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Treatment of Chromium Pollution by the Reductase Enzyme Generated by a Chromium-Resistant Bacterium Isolated in Dewatering Sludge

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Abstract

An efficient Cr (VI)-resistant and reducing bacterial strain was successfully isolated in dewatering sludge collected from a sewage treatment plant, and characterized in vitro Cr (VI) reduction through a reductase enzyme. Phylogenetic analysis using 16S rDNA gene sequencing revealed that the newly isolated strain namely Pf-1 was closely related to *Bacillus cereus*. The strain almost reduced 0.17 mM Cr (VI) within 24h incubation and the presence of different substrates such as glucose, sucrose, or acetate significantly enhanced the reduction rate of Cr (VI) to Cr (III). However, addition of the same substrate at the stationary phase of the microbial growth increased the reduction rate as well as bacterial growth. Additionally, raising the concentration of thiosulfate in the medium doubled the reduction rate under similar conditions. Assay with different fractions of the cells demonstrated that the reductase enzyme activity was mainly associated with the cytoplasmic fraction. The maximum activity was 23.3 $\mu M\ h^{-1}\ mg^{-1}$ protein and was obtained at the Cr (VI) concentration of 2 mM. The promising strain can be efficiently employed for bioremediation of Cr (VI) polluted sites.

Keywords: *Bacillus cereus* Pf-1; reductase enzyme; Cr (VI); Cr (III); dewatering sludge

1. Introduction

Chromate compounds containing Cr (VI) are widely used in a variety of industries such as metal finishing industry, petroleum refinery, leather tanning, iron & steel industry, inorganic chemical production, pulp production processes, production of chromium metal and explosive (ATSDR, 2012). Cr (VI) is produced as a by-product of these industrial processes and wastewater containing Cr (VI) have become a well recognized hazard in water pollution control due to extremely toxic, mutagenic and carcinogenic effects on the biological system (Xu et al., 2012). Though chromium exists in several valence states, trivalent chromium (Cr (III)) and hexavalent chromium (Cr (VI)) are of major environmental significance because of their stability in natural environment (Kwak et al., 2003). Due to leakage, poor storage and improper disposal by these industries, hexavalent chromium has become one of the most frequently detected contaminant at waste sites. Removal of Cr (VI) in contaminated effluents is a challenge for the whole scientific community.

Conventional physicochemical methods for removing toxic Cr (VI) from industrial effluents include chemical precipitation, oxidation or reduction, reverse osmosis, ion exchange, membrane technologies and filtration (Cheung et al., 2006; Ozturk et al., 2009; Sau et al., 2010). However, there are several shortcomings of these methods including consumption of excessive energy, poor removal efficiency, production of large amounts of chemical sludge, in addition to the high cost of chemicals used for Cr (VI) removal, particularly for the removal of relatively low concentrations of Cr (VI) (He et al., 2011).

The maximum achievable Cr (VI) removal efficiency by conventional methods is not sufficient to attain the desired treated effluent quality standard for disposal by the industries. Due to its efficient, affordable, and environmental friendly advantages, biotransformation of Cr (VI) to Cr (III) by microorganisms is considered as an alternative viable option for the remediation of Cr (VI) pollution (Sarangi et al., 2008).

Cr (VI) biotransformation also produces Cr (III) – organic complexes with insoluble Cr (III) compounds. Chartterjee et al. (2009) reported that once Cr (III) – organic complexes are formed; they are relatively stable and recalcitrant in short-term biodegradation. In this way, Cr (III) – organic compounds are known as stable chromium species in the environment and taking part in natural biogeochemical cycle of the chromium.

Several bacterial strains including *Bacillus* have been shown to reduce toxic and soluble Cr (VI) to the less toxic and less soluble Cr (III) through enzymatic reaction (Puzon et al., 2002; Puzon et al., 2005; Ibrahim et al., 2012). However, a few of them can effectively produce the enzyme reductase responsible of Cr (VI) reduction. In this manuscript we investigated the potentials of the current strain to reduce Cr (VI) through reductase enzyme as well as the effect of different substrates in the reduction rate. Our results were compared to the previous isolated *Bacillus* strains. The current study could be helpful to the scientific community since bacteria are widely distributed in nature; screening of reductase enzyme producing strains from the contaminated source is not a difficult task.

2. Materials and methods

2.1 Chemicals

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

2.2 Bacterial Strain, Identification and Growth Conditions

The bacterial strain, Pf-1 tolerant to Cr (VI) was isolated in dewatering sludge collected from a sewage treatment plant, Wuhan, China by enrichment culture technique (Nguema et al., 2014). The culture was grown in Nutrient Broth (NB) agar plates previously sterilized amended with 1 mM Cr (VI) as $K_2Cr_2O_7$. Pf-1 was identified using biomolecular methods. Additionally, the analysis of the nucleotide sequence of 16S rDNA gene from Pf-1 was done using Blast-n tools at NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The phylogenetic tree was constructed by the Neighbor-joining method using MEGA version 5.05 (Tamura et al., 2011).

2.3 Chromium (VI) Reduction by Isolated Bacillus cereus Pf-1

The 250 ml conical flasks containing 50 ml NB (pH 7.0 ± 0.3) previously sterilized amended with different Cr (VI) concentrations (0.3-3.4 mM) were inoculated with overnight grown cells of *Bacillus cereus*. Uninoculated controls (sterilized NB medium + Cr (VI)) were used to compare abiotic Cr (VI) reduction during experiments. The inoculated cultures along with uninoculated controls were incubated at 30º C under orbital shaking (100 rpm) and 5 ml samples were withdrawn at different time intervals to monitor growth and Cr (VI) reduction.

The total chromium concentration in the supernatant was measured after 24h incubation to check whether chromium was absorbed by the bacterial cell.

2.4 Chromium (VI) Reduction Mediated by Different Substrates

Minimal Salt Medium (MSM) as previously used by Zacharia et al., 2007 was amended with different Cr (VI) concentrations (0.1-2 mM) and was supplemented with glucose, sucrose, acetate ($C_2H_2NaO_2$), sulfate (Na_2SO_4) and thiosulfate ($Na_2S_2O_3$) at final concentration of 0.2 mM. The Cr (VI) reduction was initiated after inoculation of culture flaks with Pf-1 strain and the reduction as well as growth was monitored from samples withdrawn at different time intervals. Control experiments (sterilized MSM medium + Cr (VI) were run in parallel without any substrate added.

2.5 Extraction of Reductase Enzyme

Bacillus cereus was cultured in sterilized MSM medium (pH 7.0) amended with glucose (0.2 mM) and 1 mM Cr (VI); the reaction was incubated at 30º C under orbital shaking (100 rpm) for 24h. At the end of incubation, the cells were centrifuged at 10,000×g for 5 min and the pellets obtained were washed three times with 50 mM phosphate buffer (pH 7.2) and re-suspended in 5 ml of buffer. These cell suspensions were placed in an ice bath and disrupted using ultrasonic probe for 2h. The power was supplied 5 times in 1-min pulse at 50 W. The cell suspensions were centrifuged at 10,000×g for 5 min and the cell pellets were discarded. The supernatant was centrifuged at 160,000×g for 1h at 4º C, followed by filtration through 0.22 µm filters and the supernatant or the cytoplasmic fraction or cell-free extract (CFE) thus obtained was used as source of soluble enzyme. The pellet or

cell membrane fraction (CM) was re-suspended in buffer. Aliquots (1 ml) of CFE and CM were used for the enzyme assay in order to localize the reductase enzyme.

2.6 Analytical Methods

Pf-1 growth was monitored by standard, or viable, plate count method.

Diphenylcarbazide method (APHA, 1998) using UV visible spectrophotometer with a detection limit of 1.7×10^{-4}mM was used to estimate the Cr (VI) concentration in the supernatants.

Protein concentrations of the CFE and CM were measured using Bradford reagent (Bradford, 1976) and Bovine serum albumin (BSA) was used as standard protein.

The enzyme assays were performed as previously described Pal et al. (2005). The reaction mixture containing Cr (VI) at the final concentration of 0.1 mM and 2 mM, glucose (0.2 μM) in 50 mM phosphate buffer and 1 ml of enzyme reductase.

The total chromium in the samples was analyzed by Atomic Absorption Spectrometry (AAS- Pgeneral TAS-990, Beijing Purkinjie General Instrument Co. Ltd). Chromium standard solutions were prepared by using appropriate dilution of stock Cr (VI) standard solution for AAS analysis. Limit of detection calculated on a 3s basis (a value three times the standard deviation of the blank) was 1×10^{-6}mM Cr

Each experiment was done in triplicate and the difference in their individual results in each set was less than 5%. The error bars shown in figures represent the standard deviation, calculated using Origin pro software 8.5.

The range of the concentrations chooses in this manuscript was according to the different literature of Cr (VI) reduction to Cr (III).

3. Results

3.1 Isolation and Identification of Bacterial Strain

A Cr (VI)-reducing bacterial strain namely, Pf-1 was isolated in dewatering sludge collected from a sewage treatment plant in Wuhan, China. Pf-1 was found to be Gram positive rod-shaped, motile, possesses oxidase and catalase activities and facultative anaerobe. Based on the 16S rDNA gene sequence results, Pf-1 has been identified as *Bacillus cereus* with 99% similarity to the corresponding sequences of *Bacillus* sp. in the GenBank databases. A phylogenetic tree was constructed using Pf-1 16S rDNA partial sequences from other *Bacillus sp*. The tree showed that the isolate Pf-1 clustered with high confidence to *Bacillus cereus* (GU321330) and which further confirm the identity of the 16S rDNA sequence with *Bacillus* sp. (Figure 1). The isolate was submitted to the GenBank with accession number (KC152883).

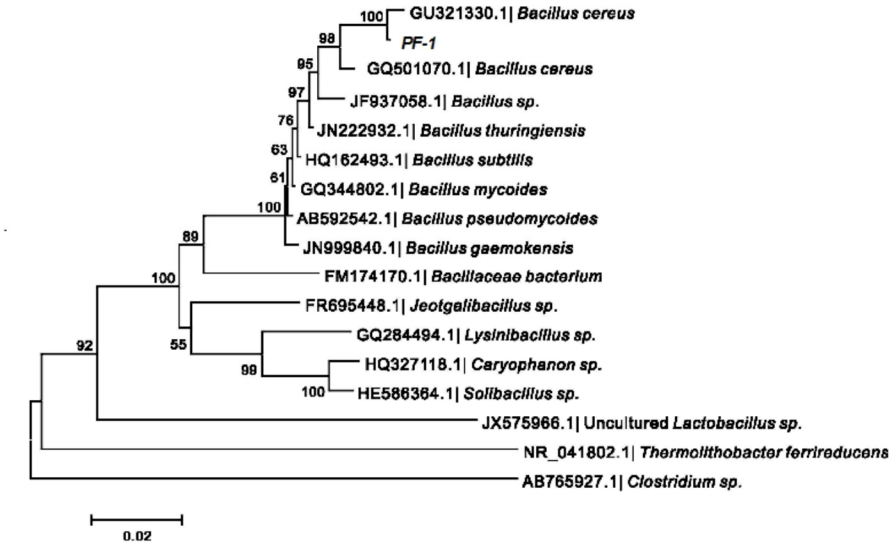

Figure 1. Phylogenetic tree constructed from the 16S rDNA gene of the strain Pf-1 using Neighbor-joining algorithm. Accession numbers are given before the strains name, and scale bar represents 1 base substitution per 20 nucleotide positions. The bootstrap probabilities calculated from 1,000 replications.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

3.2 Effect of Initial Cr (VI) Concentration on Growth and Cr (VI) Reduction

We investigated whether by raising the initial Cr (VI) concentration in the medium has a significance effect on the cell growth and reduction rate. The results show that the cell growth was affected by the concentration of Cr (VI) at different levels. The effect is higher at high Cr (VI) concentration. The Colony-forming Units (CFU) dropped from 5.5×10^5 to 1.1×10^5 when Cr (VI) concentrations increased from 0.3 to 3.4 mM (Figure 2a). Moreover, the relative reduction rates decreased as the concentrations of Cr (VI) increased (Figure 2b). The total chromium in the samples remained almost the same at different Cr (VI) concentrations (Table 1).

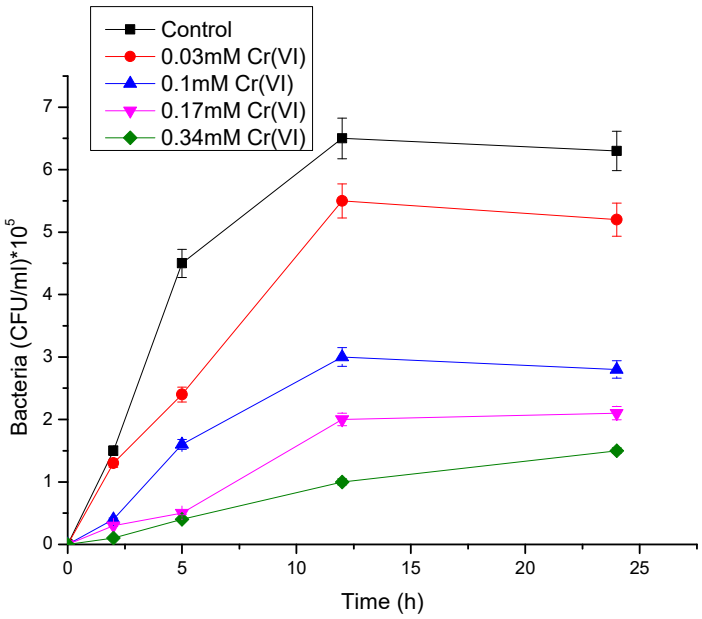

Figure 2a. growth curve of *Bacillus cereus* in NB medium (pH 7.0) amended with different Cr(VI) concentrations. NB medium (pH 7.0) amended with 0.1 mM Cr(VI) without bacterial inoculation as a control. Error bars represent standard deviation of triplicate samples

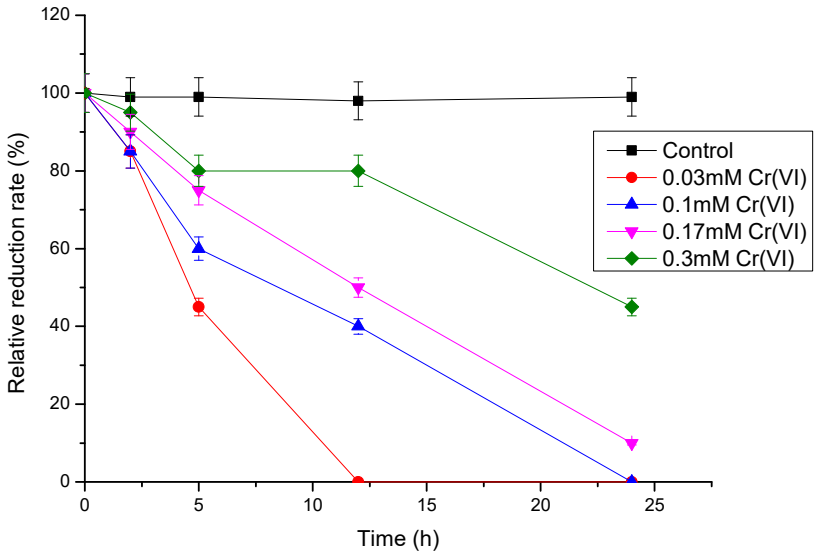

Figure 2b. chromium (VI) reduction by *Bacillus cereus* Pf-1 in NB medium amended with different Cr(VI) concentrations. NB medium (pH 7.0) amended with 0.1 Mm Cr(VI) without bacterial inoculation as a control. Error bars represent standard deviation of triplicate samples

Table 1. Cr(VI) reduction in MSM (pH 7.0) amended with different substrates at concentration of 0.2 mM. No substrate was added in the control experiments

Cr(VI) concentrations (mM)	Relative rate of reduction (μM/h)±standard error			
	Glucose	Sucrose	Acetate	Control
0.01	0.75±0.12	0.7±0.11	0.65±0.12	0.15±0.02
0.02	1.32±0.41	0.98±0.2	0.88±0.15	0.23±0.12
0.05	1.98±0.35	1.53±0.53	1.1±0.14	0.29±0.16
0.075	2.5±0.13	1.96±0.3	0.96±0.16	0.3±0.13
0.1	2.01±0.11	1.33±0.36	0.75±0.17	0.14±0.11
0.2	1.88±0.23	0.99±0.2	0.45±0.33	0.22±0.17

3.3 Cr (VI) Reduction in Presence of Different Substrates

The relative rates for time (h) versus percentage reduction of Cr (VI) showed maximum reduction with glucose, followed by sucrose and acetate (Table 1). However, the addition of the same substrate at the stationary phase of the microbial growth increased the rate of bacterial growth as well as Cr (VI) reduction (Figure 3A and B). Additionally, experiments conducted at 1mM Cr (VI) concentration in presence of different concentrations of sulfate and thiosulfate (0.5-1 mM) showed that, in contrast to sulfate, raising the concentration of thiosulfate in the medium doubled the reduction rate under similar conditions (Figure 4A, B, C, and D).

Table 2. Cr(VI) reduction with different fractions of the cells amended with 0.2 μM of glucose

Fractions	0.1mM Cr(VI)		0.2mM Cr(VI)	
	Enzyme activity (μM h^{-1} mg^{-1} protein) 1h	Enzyme activity (μM h^{-1} mg^{-1} protein) 12h	Enzyme activity (μM h^{-1} mg^{-1} protein) 1h	Enzyme activity (μM h^{-1} mg^{-1} protein) 12h
Membrane	7.3±0.5	11.5±1.1	4.2±0.1	10.6±0.9
Cytoplasm	9.6±0.7	18.6±1.6	15.6±1.4	23.3±1.9

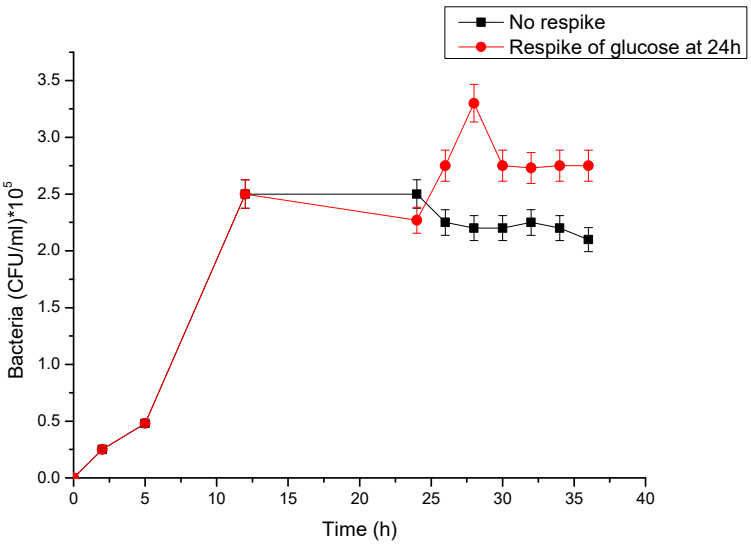

Figure 3a. growth curve of *Bacillus cereus* Pf-1 in MSM medium (pH 7.0) amended with 0.1 mM Cr(VI) after glucose was re-spiked during the stationary phase

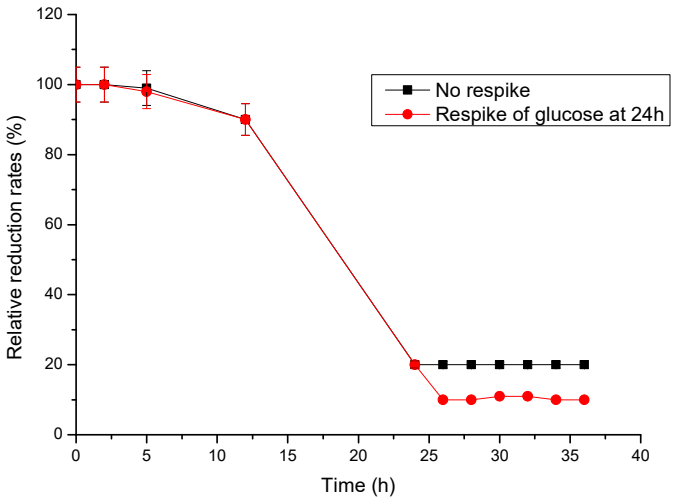

Figure 3b. chromium (VI) reduction of *Bacillus cereus* Pf-1 in MSM amended with 0.1mM Cr(VI) after glucose was re-spiked during the stationary phase

3.4 Cr (VI) Reduction by Cytoplasmic and Cell Membrane Fraction

By investigating which fraction of the cell has a higher enzyme activity, we found that the activities were higher with the cytoplasmic fraction at different time intervals and at different Cr (VI) concentrations (Table 2). Moreover, the enzyme activity also increased as the initial Cr (VI) concentration increased. Higher activity (23.3 µM h^{-1} mg^{-1} protein) could be obtained at higher Cr (VI) concentration (2mM), at 30ºC and pH 7. Previous experiments conducted at different temperature (10-65ºC) and pH (5-9) showed the maximum activity at 30ºC and pH 7, respectively (Data not shown). Considering the reductase enzyme activity at 30ºC as 100%, the reductase enzyme activity of Pf-1 at lower temperature of 10ºC and higher temperature of 65ºC decreased by 44% (13.1 µM h^{-1} mg^{-1} protein) and 57% (10 µM h^{-1} mg^{-1} protein), respectively. Considering also the activity at pH 7 as 100%, the reductase enzyme activity of Pf-1 at different pH was determined. At pH 5 and 9, the specific activities were decreased by 32% (15.9 µM h^{-1} mg^{-1} protein) and 14% (20.1 µM h^{-1} mg^{-1} protein), respectively.

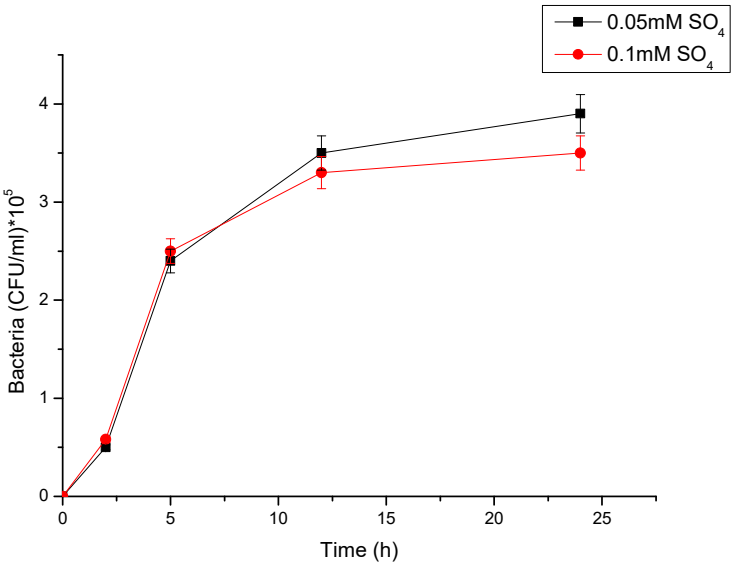

Figure 4a. growth curve of *Bacillus cereus* Pf-1 in MSM medium (pH 7.0) amended with different sulfate concentrations and 0.1 mM Cr(VI)

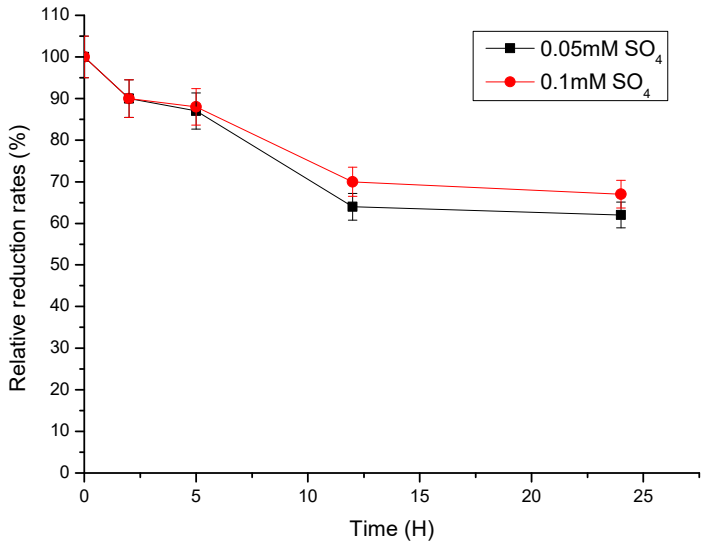

Figure 4b. chromate reduction of *Bacillus cereus* Pf-1 in MSM medium (pH 7.0) amended with different sulfate concentrations and 0.1 mM Cr(VI)

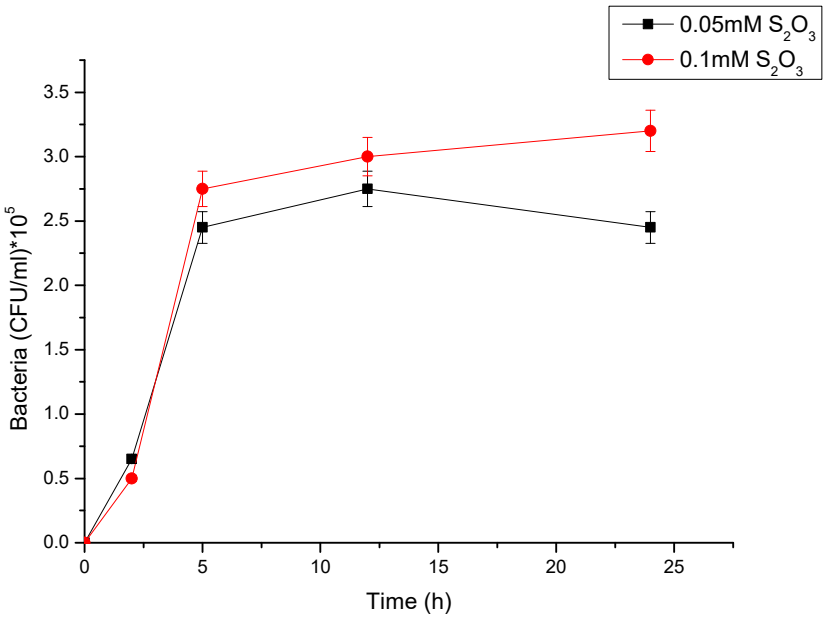

Figure 4c. growth curve of *Bacillus cereus* Pf-1 in MSM medium (pH 7.0) amended with different thiosulfate concentrations and 0.1mM Cr(VI)

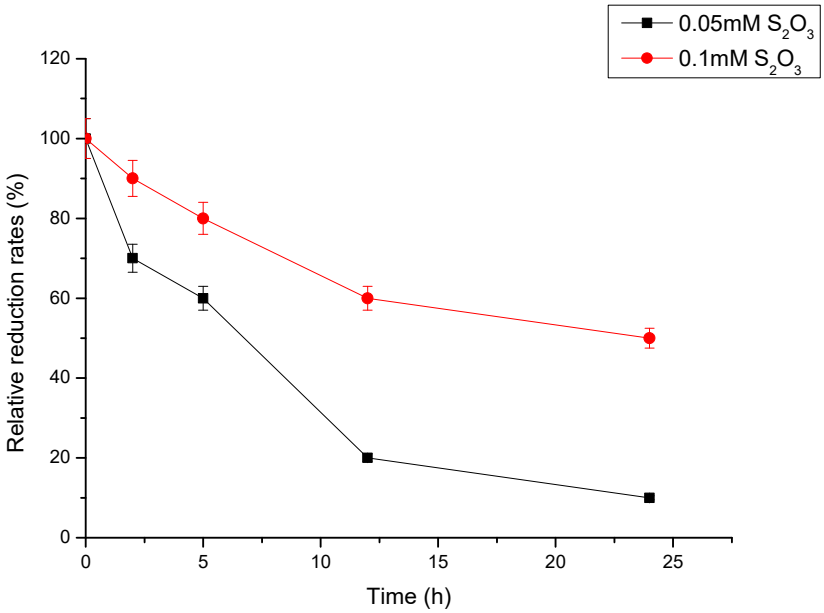

Figure 4d. chromium (VI) reduction of *Bacillus cereus* Pf-1 in MSM medium (pH 7.0) amended with different thiosulfate concentration. Cr(VI) concentration in the medium was 0.1 mM

4. Discussion

We have isolated a chromium-resistant and reducing bacterium from dewatering sludge and identified as *Bacillus cereus*. Therefore, the ability of the isolate to reduce Cr (VI) through reductase enzyme was investigated.

The investigation showed that *Bacillus cereus* grew well in the presence of different Cr (VI) concentrations and could almost reduce these concentrations within 24h incubation, suggesting that Pf-1 strain has a strong reducing capability. Strains of genus *Bacillus* are known to tolerate and reduce Cr (VI) (Campos et al., 1995; Liu et al., 2006; Soni et al., 2013). The tolerance level of Cr (VI) for our newly isolated strain of *Bacillus cereus* was 3.4 mM; which is more than other strain of *Bacillus cereus* those have been known to tolerate 2 mM Cr (VI) (Sarangi and Khrishan, 2008). However, a hyper-resistant *Bacillus sp*. ES29 has been reported who is resistant to very high level of Cr (VI) at > 9.6 mM (Camargo et al., 2003b). In our experiments, higher Cr (VI) concentration (3.4 mM) caused decrease in growth rate when compared to growth at lower concentrations; this was ascribed to the toxic effect of Cr (VI). Toxic effect of Cr (VI) on the cell growth at higher concentration has been mentioned in the literature (Pal et al., 2005; Cheng and Li, 2009). Further, the total chromium remained almost the same at different Cr (VI) concentrations and this suggests that the decrease of Cr (VI) concentration in the supernatant was the effect of reduction of Cr (VI) to Cr (III) and not adsorption (Pal et al., 2005).

It is also evident from our results (Figure 2b) that increase in chromate reduction was growth dependent; higher reductions were noticed during the first 12h corresponding to the exponential phase of the microbial growth. Most likely, bacteria growth and Cr (VI) reduction induced-damage are competing processes, and bacteria can cope with Cr (VI) exposure only as long as carbon sources are available. However, the addition of carbon sources such as glucose, sucrose or acetate in the media significantly enhances the reduction rate, suggesting the utilization of these carbon sources by *Bacillus cereus*.

Addition of the same substrate at the stationary phase (24h) of *Bacillus cereus* significantly enhances Cr (VI) reduction to Cr (III) as well as cell growth. In general, the stationary phase corresponding to the depletion of carbon sources and the necessary energy for the microbial growth, the addition of energy source such as glucose will reactivate the metabolism of the microorganism and this will have a positive effect on the bacterial growth as well as Cr (VI) reduction.

Meanwhile, in contrast with sulfate, raising the concentration of thiosulfate in the medium doubled the reduction rate of Cr (VI) to Cr (III) under similar conditions (Figure 5). However, a similar effect was not observed with the cell growth. In this regard, thiosulfate uptake was through bacterial cell wall or membrane which enhances the

biosynthesis of the reductase enzyme and enables the reduction of Cr (VI) to Cr (III). Moreover, all living organisms require sulfur for the synthesis of proteins and essential cofactors. Sulfur could be assimilated either from inorganic sources, such as sulfate and thiosulfate, or from organic sources, such as sulfate esters for example. It has been shown in the literature that sulfur is transported into the cell via an ATP-binding cassette-type sulfate-thiosulfate system (Guilouard et al., 2002). Our results can also corroborate with their finding.

The results on the assay with different fractions of the cell showed that the reductase enzyme was more active and more efficient in the cytoplasmic fraction. Therefore, it could be concluded that the reductase enzyme generated by *Bacillus cereus* may be primarily intracellular. Reports associate the reductase enzyme with cytoplasmic fraction of *Providencia* sp., *Bacillus* sp. and *Microbacterium* sp. were recently investigated (Pal et al., 2005; Thacker et al., 2006; Soni et al., 2013). The enzyme activity reported by Desai and his coworkers (2008) were lower compared to the values obtained in our experiments at the same Cr (VI) concentration, suggesting that our strain is a higher reductase producing bacterium among *Bacillus* strains reported up to date (Table 2). Moreover, the enzyme activity was affected by the temperature and pH of the medium. The temperature below and above 30ºC decreased the enzyme activity, suggesting that the enzyme was affected by the temperature. The observation was made with the pH, having the maximum activity at pH 7. Similar observations of the influence of temperature and pH in the enzyme activity have been made in the literature (Pal et al., 2005; Desai et al., 2008). Suziki et al. (1992) reported that some bacteria were known to use chromate as a terminal electron acceptor employing membrane bound enzymes, while others use soluble enzymes. Negligible reduction was noticed in abiotic control indicating the direct interaction of the cell in Cr (VI) reduction. Similar results indicating negligible reductions in abiotic control have been earlier reported by (Massod & Malik, 2011; He et al., 2010).

5. Conclusion

The results of the current study clearly demonstrate that *Bacillus cereus* Pf-1 has strong potentialities to reduce toxic Cr (VI) to the less toxic Cr (III) through enzyme reductase and hence can be considered as a reductase producing strain. Additionally, *Bacillus cereus* Pf-1 can be employed as a bio-agent for chromium detoxification from the contaminated effluents. Since bacterial growth is rapid, we could produce the reductase enzyme in 24h by providing nutrient and trace amount of chromate. Further, industrial symbiosis approach would be a feasible option; wastewater from food industries could be used as energy source for bacterial growth and reductase production.

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