Original Article

Bioremediation of Hexavalent Chromium Pollution by *Sporosarcina saromensis* M52 Isolated from Offshore Sediments in Xiamen, China*

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Abstract

Objective Cr(VI) removal from industrial effluents and sediments has attracted the attention of environmental researchers. In the present study, we aimed to isolate bacteria for Cr(VI) bioremediation from sediment samples and to optimize parameters of biodegradation.

Methods Strains with the ability to tolerate Cr(VI) were obtained by serial dilution and spread plate methods and characterized by morphology, 16S rDNA identification, and phylogenetic analysis. Cr(VI) was determined using the 1,5-diphenylcarbazide method, and the optimum pH and temperature for degradation were studied using a multiple-factor mixed experimental design. Statistical analysis methods were used to analyze the results.

Results Fifty-five strains were obtained, and one strain (*Sporosarcina saromensis* M52; patent application number: 201410819443.3) having the ability to tolerate 500 mg Cr(VI)/L was selected to optimize the degradation conditions. M52 was found be able to efficiently remove 50-200 mg Cr(VI)/L in 24 h, achieving the highest removal efficiency at pH 7.0-8.5 and 35 °C. Moreover, M52 could completely degrade 100 mg Cr(VI)/L at pH 8.0 and 35 °C in 24 h. The mechanism involved in the reduction of Cr(VI) was considered to be bioreduction rather than absorption.

Conclusion The strong degradation ability of *S. saromensis* M52 and its advantageous functional characteristics support the potential use of this organism for bioremediation of heavy metal pollution.

Key words: Hexavalent chromium; Sediment; *Sporosarcina saromensis*; Degradation

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INTRODUCTION

Chromium (Cr) is one of the most toxic heavy metals and is widely used in electroplating, steel production, wood preservation, tanning, and textile dyeing, resulting in the discharge of Cr-containing effluents[1-2]. Release of Cr without treatment causes serious anthropogenic contamination due to its nondegradable and persistent properties[3-5].

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Untreated industrial effluents are also a critical threat to public health because heavy metals have biomagnification properties and accumulate in the food chain, causing toxicity at a cellular level[6].

Cr has two stable forms in the environment: hexavalent chromium [Cr(VI)] and trivalent chromium [Cr(III)], whose toxicity depends on the metal redox state[7]. Cr(VI) has been reported to be 100 times more toxic and 1000 times more mutagenic than Cr(III)[8]. Due to its high solubility, availability, and mobility in soil as well as its ability to penetrate biological membranes, Cr(VI) in industrial effluent does great harm to organisms, including humans, through the food chain[9-10]. Moreover, its strong oxidizing properties give Cr(VI) carcinogenic, clastogenic, and teratogenic potential[11]. Thus, Cr(VI) has been classified as a class A contaminant by the US Environmental Protection Agency (USEPA)[12-13]. As a consequence, some agencies have established maximum allowed levels of Cr(VI) in water. For example, according to the Comprehensive Emissions Standard for Sewage, China (GB 20426-2006), the maximum permissible effluent concentration of Cr(VI) is 0.5 mg/L, and the total maximum allowable Cr is 1.5 mg/L.

Currently, the sheer number and diversity of contaminants in ground and drinking water are serious challenges[14]. Cr(VI) removal from the environment, particularly from industrial effluents and sediments, is an urgent goal for researchers and environmental organizations. Because Cr(III) has long been regarded as an essential human micronutrient[15], transformation of Cr(VI) into Cr(III) may be considered a simple, economical, and practical method to treat industrial effluents. Conventional chemical or physicochemical treatment processes, such as adding lime, ion exchange, membrane separation, and adsorption followed by chemical precipitation and coagulation as $Cr(OH)_3$, have been described in the last few years[16-17]. However, these methods suffer from a number of problems that restrict their application, including complex operational procedures, high cost, and low efficiency[18]. Moreover, some of these methods create secondary pollution that may be even worse for the environment.

The search for new and innovative technology has focused on bioremediation methods for heavy metal detoxification; such methods are thought to be economical and environmentally friendly[4,19]. Many native microorganisms have been identified and reported to have the ability to reduce Cr(VI) to Cr(III) under aerobic or anaerobic conditions[5,20-21]. Microorganisms belonging to polluted sites are usually preferred in the development of an efficient system for Cr(VI) bioremediation because they tolerate Cr(VI) and may evolve to develop some mechanisms to remove the pollutant[22]. Moreover, some native microorganisms may even have the ability to remove several pollutants simultaneously[23-24]. With the increase in offshore pollution, strains with high tolerance and Cr(VI) removal ability have been commonly isolated from offshore and intertidal zones[25-27]. Bioremediation of Cr(VI) involves different approaches, including biosorption, bioreduction, and bioaccumulation[28-29]. The degradation efficiency of Cr(VI) is influenced by many factors, including the presence of sufficient nutrients, the temperature and pH used during the bioremediation process, and the presence of other contaminants in the environment. Therefore, it is very important to identify bacteria with the capacity for efficient degradation, optimize degradation conditions, and clarify degradative mechanisms during the bioremediation process.

In the present study, bacteria used for bioremediation were isolated from sediment samples in intertidal zones and identified using 16S rRNA gene sequencing. A bacterial strain with high tolerance for Cr(VI) was then selected, identified, and characterized. Optimization of several parameters that affect practical treatment of industrial effluents was then performed. In addition, we obtained preliminary results to elucidate the main mechanisms involved in Cr(VI) bioremediation by this microorganism. Our present work will contribute to knowledge related to Cr(VI) bioremediation by a native microorganism and provide a potentially practical application to minimize heavy metal contamination by bioremediation.

MATERIALS AND METHODS

Sample Collection

Seven sediment samples were collected from the intertidal zones at low tide in Xiamen, Fujian Province, China. All sampling sites are shown in Figure 1. The samples were stored at 4 °C until use in microbiological analyses.

Nutrient Medium

All of the nutrient media in the study are

referred to as '216LB medium' and contained 1 g/L sodium acetate, 2 g/L yeast extract powder, 10 g/L peptone, 0.5 g/L ordinary gravy medium, 0.5 g/L sodium citrate, 0.2 g/L ammonium nitrate, and 0.5 g/L potassium dihydrogen phosphate in 1 L filtered seawater, pH 7.6. Agar powder (15 g/L) was added in solid medium before sterilization.

Isolation and Morphological Characterization of Cr(VI)-tolerant Strains

To select Cr(VI) tolerant strains, the samples were processed according to the following method. First, 2 g sediment from each sampling site was placed in an Erlenmeyer flask containing enrichment nutrient medium supplemented with 50 mg/L Cr(VI) as $K_2Cr_2O_7$. The flasks were shaken at 200 rpm and 28 °C for 7 days as a cycle. New bacterial solutions were obtained by serial dilution of the previous solution for each cycle, and the concentration of Cr(VI) remained the same during the entire process. Isolation of the bacteria was carried out by serial dilution and the spread plate method[30] after four cycles. Morphologically different colonies growing in the plates were isolated and selected for the following assays. All isolated bacteria were characterized according to their morphological features.

Evaluation of Cr(VI) Tolerance

To determine the maximum tolerance to Cr(VI), the isolated bacteria were spread on solid nutrient medium supplemented with different Cr(VI) concentrations (50-1000 mg/L). The maximum tolerated concentration (MTC) was established as the highest concentration of the contaminant at which bacterial growth could be observed after 7 days of incubation at 28 °C. One strain with the ability to tolerate high concentrations of Cr(VI), named M52, was selected, characterized, and identified for further Cr(VI) removal studies until identification.

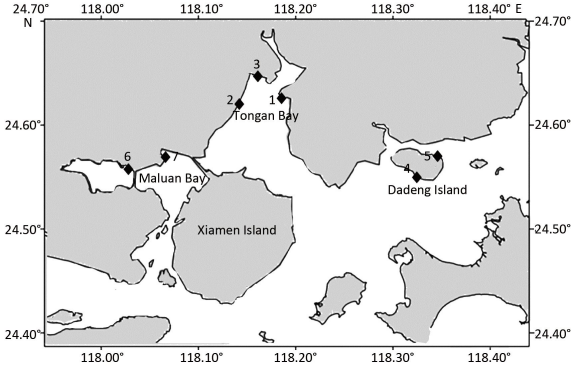

Figure 1. Distribution of sampling locations in Xiamen, China.

Molecular Identification and Phylogenetic Analysis

Molecular identification of the M52 strain was carried out using 16S rDNA analyses. DNA for polymerase chain reaction (PCR) amplification was extracted using a bacterial genome DNA rapid extraction kit. PCR was carried out using forward (5'-AGAGTTTGATCCTGGCTCAG-3') and reverse (5'-ACGGCTACCTTGTTACGACT-3') primers. The amplification program included an initial denaturing step of 94 °C for 4 min; 30 cycles of amplification for 60 s at 94 °C, 60 s at 55 °C, and 90 s at 72 °C; and a final extension at 72 °C for 10 min. The obtained sequence was initially analyzed on the National Center for Biotechnology Information (NCBI) website using the BLAST tool. The phylogenetic tree was constructed using aligned sequences by the neighbor joining (NJ) algorithm with more than 1000 replicates in Molecular Evolutionary Genetics Analysis (MEGA version 5.0) software[31].

Optimization of Parameters

Seed Liquid Preparation By inoculating a pure strain of M52 to the liquid nutrient medium, the seed liquid was obtained after 12-h incubation at 200 rpm and 37 °C.

pH and Temperature An experiment was conducted to optimize the growing conditions of M52 for practical applications. According to the Marine Environment Bulletin of Xiamen (2013), the optimum parameters were determined by evaluating the Cr(VI) degradation efficiency at 20, 25, 30, and 35 °C and pH 6.5, 7.0, 7.5, 8.0, and 8.5. At the beginning of the experiment, 50 mg/L Cr(VI) and 4% v/v seed liquid inoculum were added to the nutrient medium to determine the effects using a multifactor design. The degradation efficiency was calculated under aerobic conditions at 12 and 24 h from the beginning. The Cr(VI) concentration was determined according to the 1,5-diphenylcarbazide method of the national standard (GBT 5750.6-2006), as has also been reported by Pattanapipitpaisal et al.[32] Triplicates were carried out to assess reproducibility. An abiotic control was also analyzed with uninoculated medium under the same conditions.

Initial Inoculum Concentration The effects of the initial inoculum concentration (1%-10% v/v seed liquid) on the Cr(VI) degradation efficiency of M52 were determined under the optimal pH and

temperature. For other operations, the same experimental manipulation was applied as described above.

Cr(VI) Concentration The effects of different initial Cr(VI) concentrations (50, 100, 200, and 500 mg/L) on Cr(VI) degradation efficiency were determined under the optimal pH and temperature using 4% v/v seed liquid of M52 as the initial inoculum concentration. For other operations, the same experimental manipulation was applied as described above.

Preliminary Research on the Mechanisms Involved in Cr(VI) Degradation

This assay was performed in order to explore whether the mechanisms of degradation involved biological adsorption, reduction, or both. Erlenmeyer flasks containing 40 mL nutrient medium supplemented with 50 mg/L Cr(VI) were inoculated at 4% v/v seed liquid. Using the optimal pH and temperature, the flasks were incubated in an orbital shaker at 150 rpm for 24 h. Then, the cultures were centrifuged at 12,000 rpm at 4 °C for 20 min twice. The supernatants were filtered using 0.22-μm filters (Millipore, USA) to remove the remaining bacterial cells, and the pellets were washed twice with 0.9% NaCl. The Cr(VI) content was determined in the supernatants and the pellets after microwave digestion.

Statistical Analysis

The results of a minimum of three independent experiments are presented as the mean and standard error. Analysis of variance (ANOVA) for a two-way factorial design was used to evaluate the data obtained after 12 h. One-way ANOVA was performed to determine the significance of differences between pairs of means. Statistical analysis was performed using SPSS 19.0 software.

RESULTS AND DISCUSSION

Isolation, Characterization, and Identification of the M52 Strain

Fifty-five isolates resistant to Cr(VI) were obtained from the sediment samples; their morphological characteristics are shown in Tables 1-3. Among these isolates, one isolate, designated M52, which showed an MTC of 500 mg/L Cr(VI), was selected for further analysis. The results of 16S rRNA gene amplification and sequencing indicated that this

Table 1. Colony Morphology of the Isolates from Tong'an Bay

Latitude and Longitude	Genus and Species	Color	Geometry	Surface and Edge
E118.14° N24.62°	*Idiomarina* sp.	orange	circular, convex	smooth, regular
	Microbacterium esteraromaticum	beige	circular, convex	smooth, regular
	Leucobacter sp.	beige	circular, convex	smooth, regular
	Idiomarina sediminum	orange	circular, convex	smooth, regular
	Exiguobacterium profundum	beige	circular, convex	smooth, regular
	Leucobacter chromiireducens	beige	circular, convex	smooth, regular
	Falsochrobactrum sp.	beige	circular, convex	smooth, regular
	Pseudochrobactrum kiredjianiae	orange	circular, convex	smooth, regular
	Oceanobacillus polygoni	orange	circular, convex	smooth, irregular
	Leucobacter aridicollis	beige	circular, convex	smooth, regular
	Alcaligenes aquatilis	orange	circular, convex	smooth, irregular
E118.18° N24.59°	*Nitratireductor aquimarinus*	beige	circular, convex	smooth, regular
	Aliidiomarina taiwanensis	golden	circular, convex	smooth, regular
	Oceanobacillus profundus	beige	circular, convex	smooth, irregular
	Exiguobacterium mexicanum	beige	circular, convex	smooth, regular
	Exiguobacterium marinum	beige	circular, convex	smooth, regular
E118.19° N24.64°	*Exiguobacterium aestuarii*	beige	circular, convex	smooth, regular
	Halomonasaidingensis' sp.	beige	circular, convex	smooth, regular
	Alcaligenes aquatilis	orange	circular, convex	smooth, irregular
	Exiguobacterium profundum	beige	circular, convex	smooth, regular

Table 2. Colony Morphology of the Isolates from Maluan Bay

Latitude and Longitude	Genus and Species	Color	Geometry	Surface and Edge
E118.03° N24.55°	*Psychroflexus halocasei*	golden	circular, convex	smooth, regular
	Nitratireductor aquimarinus	beige	circular, convex	smooth, regular
	Arenibacter latericius	golden	circular, convex	smooth, regular
	Exiguobacterium aestuarii	beige	circular, convex	smooth, regular
	Sporosarcina saromensis	beige	circular, convex	smooth, regular
	Pseudochrobactrum sp.	golden	circular, convex	smooth, regular
	Microbacterium esteraromaticum	golden	circular, convex	smooth, regular
E118.06° N24.56°	*Exiguobacterium mexicanum*	beige	circular, convex	smooth, regular
	Bacillus sp.	ivory yellow	circular, convex	smooth, irregular
	Oceanimonas sp.	beige	circular, convex	smooth, irregular
	Marinobacter litoralis	orange	circular, convex	smooth, regular
	Sporosarcina saromensis	beige	circular, convex	smooth, regular
	Bacillus sp.	orange	circular, convex	smooth, irregular
	Halomonas shengliensis	tawny	circular, convex	smooth, regular
	Idiomarina sediminum	orange	circular, convex	smooth, regular

Table 3. Colony Morphology of the Isolates from Dadeng Island

Latitude and Longitude	Genus and Species	Color	Geometry	Surface and Edge
E118.32° N24.55°	*Nitratireductor aquimarinus*	beige	circular, convex	smooth, regular
	Dietzia aerolata	orange	circular, convex	smooth, regular
	Stappia indica	golden	circular, convex	smooth, regular
	Microbacterium sp.	golden	circular, convex	smooth, regular
	Halomonas sp.	orange	circular, convex	smooth, regular
	Idiomarina sediminum	golden	circular, convex	smooth, regular
	Mesonia sp.	golden	circular, convex	smooth, regular
	Aliidiomarina taiwanensis	golden	circular, convex	smooth, regular
	Exiguobacterium sp.	golden	circular, convex	smooth, regular
	Exiguobacterium sp.	beige	circular, convex	smooth, regular
	Marinobacter litoralis	orange	circular, convex	smooth, regular
	Microbacterium esteraromaticum	beige	circular, convex	smooth, regular
	Paenibacillus chungangensis	beige	circular, convex	smooth, regular
E118.33° N24.57°	*Dietzia aerolata*	orange	circular, convex	smooth, regular
	Nitratireductor aquimarinus	beige	circular, convex	smooth, regular
	Idiomarina maritima	golden	circular, convex	smooth, regular
	Idiomarina sediminum	golden	circular, convex	smooth, regular
	Idiomarina marina	white	circular, convex	smooth, regular
	Ornithinibacillus sp.	white	circular, convex	smooth, regular
	Pseudochrobactrum kiredjianiae	beige	circular, convex	smooth, regular

isolate had 100% similarity with species closely related to *Sporosarcina saromensis*; thus, this isolate was designated as *S. saromensis* M52. The M52 strain was a gram-positive bacterium and belonged to Firmicutes. Many reports[33-35] have suggested that Firmicutes exist in a wide variety of Cr(VI)-contaminated environments and may therefore be used as an effective bioindicator of Cr pollution in environmental monitoring.

The phylogenetic tree derived from 16S rRNA gene sequence of *S. saromensis* M52 AB243859 and sequences of the closest phylogenetic neighbors obtained by NCBI BLAST analysis demonstrated the relationships among selected isolates (Figure 2).

Evaluation of Optimum Conditions

Effects of pH and Temperature In the Cr(VI) removal process, pH and temperature are considered to be important factors. Chromium has been shown to exist as two main oxidation states in nature, i.e., trivalent chromium and hexavalent chromium[17]. Temperature has dramatic effects on enzyme activity and may play a predominant role during the degradation process[36]. The Cr(VI) degradation efficiencies by M52 under all conditions of pH and temperature at 12 and 24 h are shown in Figure 3. The best combination was obtained by analyzing the data at 12 h using ANOVA, and the data at 24 h were used for descriptive analysis.

ANOVA with a significance level of 0.05 for the data indicated that the main effects of both pH and temperature were statistically significant (P<0.05). Additionally, there was a significant interaction between the two factors (P<0.05). The optimum temperature was 35 °C after pairwise comparisons (P<0.05; Figure 3D), which suggested that M52 was a mesophilic bacterium. Extreme temperature restricted bacteria growth and Cr(VI) removal; therefore, the degradation was almost completely blocked at 20 °C. Similar results have been found in other studies[27,37-38]. For pH, there were no significant differences among the groups for pH values of 7.0 or higher (P>0.05); however, degradation was much higher at these pH values than that at pH 6.5. This was because M52 did not grow well under weakly acidic conditions, possible owing to inhibition of reductases at acidic pH. Earlier research has shown that the optimal pH for Cr(VI) bioreduction of *Bacillus* sp. and *Pseudomonas fluorescence* is 7.0, but that bioreduction is strongly inhibited at pH 6.5[39]. The optimum reduction of Cr(VI) by *Serratia proteamaculans* occurs at pH 7.0 and 30 °C under aerobic conditions[25]. This change in the optimal pH indicated that pH modification was important for different cultures to achieve the maximum Cr(VI) reduction in chromium detoxification. In the present study, the degradation efficiencies of M52 were almost 100% after 24 h at pH

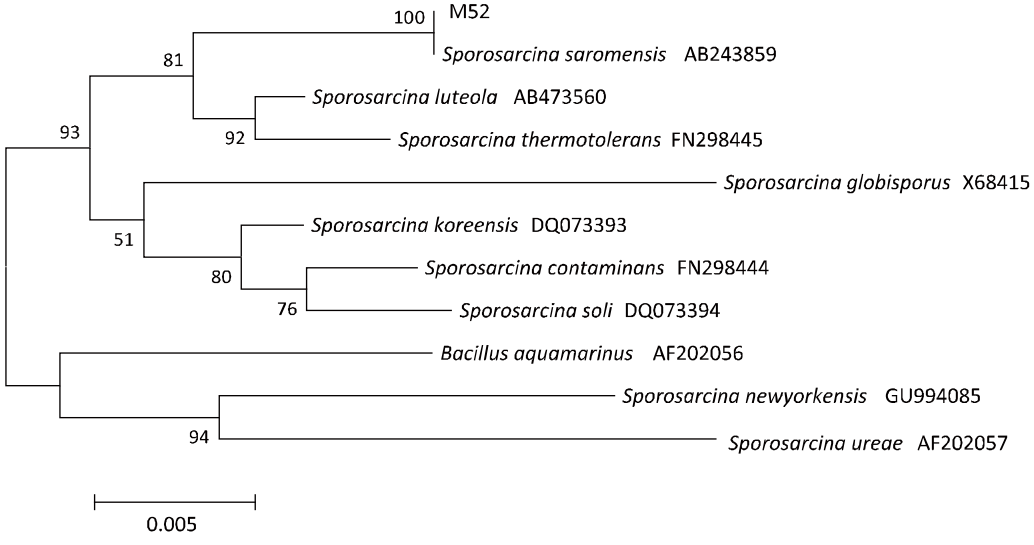

Figure 2. Neighbor-joining tree based on 16S rRNA gene sequences of *Sporosarcina saromensis* M52, as analyzed by MEGA 5.0 software. Numbers at nodes show the occurrence in bootstrap samples and provide an estimate of the confidence of the analysis. Accession numbers are indicated after the name of the isolate. Scale bars represent 0.005 substitutions per site.

values of 7.5 or more and temperatures of 30 °C or higher, but was strongly inhibited at pH 6.5. In comparison, the efficiencies were higher and the optimal pH and temperature obtained in this study were closer to the natural conditions, indicating that M52 was more useful for development of wastewater treatment technologies in the future.

Effects of Inoculum Concentration The experiment was conducted using 50 mg/L Cr(VI) and inoculum concentrations ranging from 1% to 10%. Obviously, the efficiency of Cr degradation increased as the inoculum concentration increased. Moreover, Cr(VI) degradation efficiencies for all groups reached 100% after 24 h, demonstrating that the bacteria had good performance in Cr(VI) degradation (Figure 4). However, the low inoculum concentration could lead to increased contaminant removal[22]. Therefore, our results indicated that a different mechanism was active during this process.

Effects of Initial Cr(VI) Concentration The degradation efficiencies of Cr(VI) in solution were calculated from the differences between the initial concentration and the residual concentration after 12 and 24 h of incubation at 35 °C in medium at pH 8.0 (Figure 5). After 12 h of incubation, the highest degradation efficiency (82.5%) was observed at 50 mg/L Cr(VI), whereas no degradation was observed at 500 mg/L, indicating a negative correlation between the initial concentration and the degradation efficiency. After 24 h of incubation, Cr(VI) was completely degraded when the initial concentration was no more than 100 mg/L, although almost no change was observed when the initial concentration reached 500 mg/L.

Previous studies have demonstrated that exposure to reasonable amounts of heavy metals triggers adaptive responses such as induction of metallothionein, which confers cells with resistance to heavy metal-induced toxicities[40]. The reduction in biomass caused by high Cr(VI) concentration-induced toxicity would affect the active metabolism of microorganisms, including bioaccumulation and enzymatic activities. Additionally, the lower growth rate observed at high Cr(VI) concentrations would result in a lower adsorption surface for metal binding; consequently, a low efficiency was observed in this condition. Cr-resistant bacteria isolated from Cr-polluted environments have been shown to be capable of reducing chromate[41-42]. However, another report showed that the tolerance level and the reduction efficiency were unrelated[32]. In our study, the concordance between tolerance level and degradation efficiency observed with this strain could be explained by the fact that *S. saromensis* M52 was isolated in an artificial Cr(VI)-contaminated environment. The results also revealed that the degradation mechanism may be related to biological reduction as a result of a bacterium-induced enzymatic reaction.

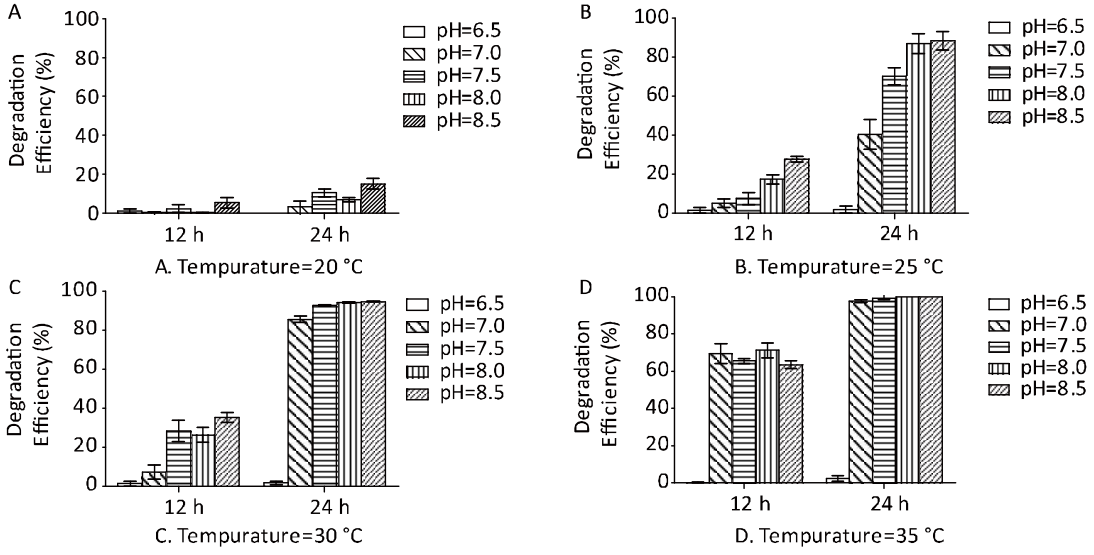

Figure 3. Cr(VI) degradation efficiencies at all conditions of pH and temperature at 12 and 24 h. A: 20 °C; B: 25 °C; C: 30 °C; D: 35 °C. Error bars indicate the standard error of the mean of degradation efficiencies from triplicate experiments.

Determination of the Bioremediation Mechanism

After 24 h of degradation at 35 °C and pH 8.0, the Cr(VI) content was determined in the supernatants and pellets using a photometric diphenylcarbazide method. Cr(VI) was not detected in the supernatants or pellets at the same time. Notably, Cr(VI) oxidized by potassium permanganate was identified again in the supernatants but not in the pellets, consistent with our previous results. Thus, we concluded that Cr(VI) was reduced to Cr(III) during the bioremediation process, and the mechanism of Cr(VI) degradation by M52 was considered to be bioreduction rather than adsorption.

Bioremediation of Cr involves biosorption, bioreduction, and bioaccumulation[28-29]. The oxidation/reduction state of toxic metals changes during bioreduction. Once it has entered bacterial cells, toxic hexavalent chromium Cr(VI) is reduced to the nontoxic trivalent form Cr(III), which has been frequently reported in recent years[22,43-45]. Priester et al. found that *Pseudomonas putida* cell lysis could release the reductases that catalyze the extracellular reduction of Cr(VI) to Cr(III)[46]. Puzon et al. demonstrated that Cr(VI) could be converted to a soluble and stable NAD^+-Cr(III) complex through an intracellularly located *Escherichia coli* enzyme system[47]. Furthermore, Cr(VI) reduction mostly involves a membrane-associated chromate reductase[26,38,48-51] or cytosolic chromate reductase[21,41-42]. However, the mechanism that governs Cr(VI) reduction by bacteria has not been fully elucidated. A part of mechanism involved in enzymatic Cr(VI) reduction under aerobic conditions was reported by Dermatas and Al-Tabbaa (Figure 6)[52]. Importantly, the reduction of Cr(VI) to Cr(III) is an important mechanism for Cr(VI) removal based on the low toxicity of Cr(III).

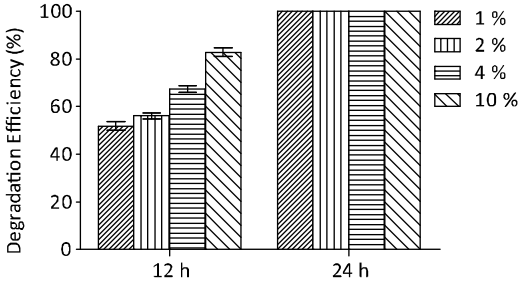

Figure 4. Cr(VI) degradation efficiencies at different inoculum concentrations. Error bars are the standard error of the mean of degradation efficiency from triplicate experiments.

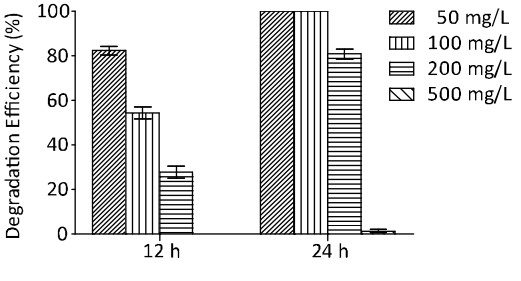

Figure 5. Cr(VI) removal efficiencies with different initial concentrations of Cr(VI). Error bars are the standard error of the mean of degradation efficiency from triplicate experiments.

CONCLUSION

Fifty-five strains with the ability to tolerate Cr(VI) were isolated from sediment samples. One bacterial strain designated as *S. saromensis* M52 was able to tolerate and degrade high concentrations of Cr(VI). Approximately 100% Cr(VI) bioremediation was reached under the optimal conditions of 35 °C and slightly alkaline conditions (7.0≤pH≤8.5) at 24 h; the mechanism most likely involved bioreduction. The strong degradation ability and suitable conditions for

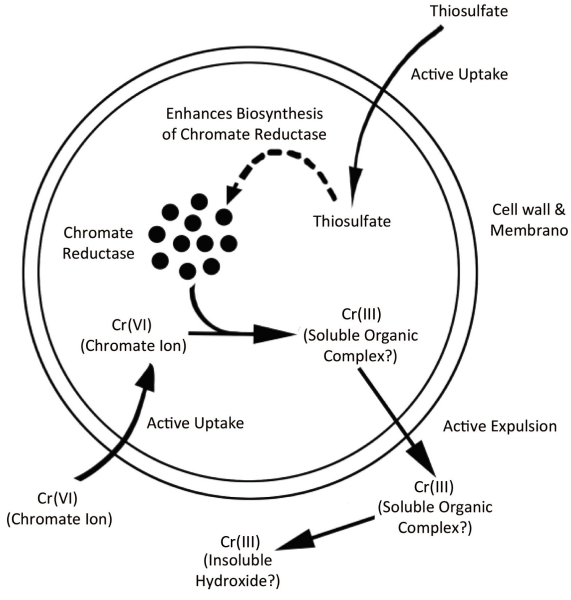

Figure 6. Mechanism of bacterial reduction of toxic Cr(VI) to nontoxic Cr(III) (from Dermatas and Al-Tabbaa)[52].

application showed that *Sporosarcina saromensis* M52 has significant potential for bioremediation of Cr(VI) contamination.

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