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Cr(VI) removal from aqueous solution by thermophilic denitrifying bacterium *Chelatococcus daeguensis* TAD1 in the presence of single and multiple heavy metals

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Cr(VI) pollution is increasing continuously as a result of ongoing industrialization. In this study, we investigated the thermophilic denitrifying bacterium *Chelatococcus daeguensis* TAD1, isolated from the biofilm of a biotrickling filter used in nitrogen oxides (NO_X) removal, with respect to its ability to remove Cr(VI) from an aqueous solution. TAD1 was capable of reducing Cr(VI) from an initial concentration of 10 mg/L to non-detectable levels over a pH range of 7–9 and at a temperature range of 30–50°C. TAD1 simultaneously removed both Cr(VI) and NO_3^--N at 50°C, when the pH was 7 and the initial Cr(VI) concentration was 15 mg/L. The reduction of Cr(VI) to Cr(III) correlated with the growth metabolic activity of TAD1. The presence of other heavy metals (Cu, Zn, and Ni) inhibited the ability of TAD1 to remove Cr(VI). The metals each individually inhibited Cr(VI) removal, and the extent of inhibition increased in a cooperative manner in the presence of a combination of the metals. The addition of biodegradable cellulose acetate microspheres (an adsorption material) weakened the toxicity of the heavy metals; in their presence, the Cr(VI) removal efficiency returned to a high level. The feasibility and applicability of simultaneous nitrate removal and Cr(VI) reduction by strain TAD1 is promising, and may be an effective biological method for the clean-up of wastewater.

Keywords: thermophilic denitrifying bacterium, Cr(VI) removal, aqueous solution, heavy metals, cellulose acetate microspheres

Introduction

As contaminants in the environment, heavy metals are detrimental to human and ecosystem health, and are present in wastewater from various industrial sources. They pose a serious risk to the environment if they are not disposed of properly. Among heavy metals, Cr(VI) is particularly hazardous because of its oxidizing, mutagenic, and carcinogenic properties (Kimbrough *et al*., 1999; Cheung and Gu, 2007; Linos *et al*., 2011); almost every regulatory agency in the world has listed Cr(VI) as a priority toxic chemical to be controlled (Bryan and Langston, 1992; Cheung and Gu, 2007; Benazir *et al*., 2010; Dogan *et al*., 2011). Cr(VI) is widely used in electroplating, leather tanning processes, chromate ore processing, dyes and pigments, wood preservation, alloy making, and metal finishing industries. The extent of Cr(VI) pollution continues to increase as a result of continuing global industrialization (Saha *et al*., 2011; Zhang *et al*., 2013).

Traditionally, physicochemical processes such as chemical reduction followed by precipitation, ion exchange, adsorption, reverse osmosis, membrane separation, and solvent extraction are used to treat water contaminated with Cr(VI) and other heavy metals (Barrera-Diaz *et al*., 2003; Mohan and Pittman, 2006; Narayani and Shetty, 2013; Jiang *et al*., 2014). However, these processes can create secondary pollution and are characterized by high costs and low efficiency in terms of energy and chemical consumption, especially for wastewaters containing low metal concentrations (< 100 mg/L) (Kurniawan *et al*., 2006; Li *et al*., 2010). As a result, more economical and efficient alternatives, such as biological treatment methods, are being researched (Wang and Shen, 1995).

Since the discovery of the first microbe capable of reducing Cr(VI) in the 1970s (Romanenko and Koren'kov, 1977), the search for additional Cr(VI)-reducing microorganisms has been pursued extensively. Numerous strains, including *Shewanella putrefaciens* (Myers *et al*., 2000), *Pseudomonas aeruginosa* (Kilic *et al*., 2010), *Pseudomonas putida*, and *Vibrio desulfuricans* (Dogan *et al*., 2011) have been isolated. These chromium-resistant microbes combat Cr(VI) toxicity through various strategies, such as adsorption, intracellular accumulation of the metal, or the biotransformation of Cr(VI) to the less toxic Cr(III) through the activity of enzymes or metabolites (Narayani and Shetty, 2013). The mechanisms by which these microorganisms reduce Cr(VI) are varied and species-dependent (Wang *et al*., 1989; Viamajala *et al*., 2002; Cheung and Gu, 2007; Ahemad, 2014), and there is a need for further research to more fully understand the processes involved.

Most chromium-resistant strains are mesophilic, while a few studies have focused on the isolation and use of thermophilic (45–60°C) microorganisms (Fan *et al*., 2014; Ghalib *et al*., 2014). The bacterial reduction of Cr(VI) is limited to a narrow range of environmental conditions necessary to achieve a favorable reaction (Soni *et al*., 2014). Furthermore,

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wastewater that contains Cr(VI) often contains additional heavy metals (Zahoor and Rehman, 2009) that can severely inhibit the growth of the microbes, thereby decreasing the rate and extent of Cr(VI) removal. To lessen this inhibitory effect, some absorbent materials are often added to wastewater. Several studies have used cellulose for this purpose because of its abundance, low cost, and good performance as an adsorbent of heavy metal ions (Arthanareeswaran and Thanikaivelan, 2012; Yang *et al*., 2014).

Nitrate (NO_3^-) has become the most ubiquitous chemical contaminant in groundwater as a result of the excessive use of nitrogen fertilizers in agricultural activities and the inappropriate disposal of untreated industrial waste (Almasri and Kaluarachchi, 2004). As a result of the mixing of sewage from different sources, or the use of nitrate in the processes of electroplating, leather making, printing, and dyeing, which release chromium slag into water bodies, nitrate, and Cr(VI) commonly coexist as pollutants in aquatic environments (Peng *et al*., 2016). In a previous study, researchers observed that aerobic denitrification occurred during the biological treatment of tannery wastewater containing chromium (Farabegoli *et al*., 2004). This demonstrates that the denitrification process for simultaneous removal of nitrate and chromate is highly feasible.

Our research group has isolated a novel thermophilic aerobic denitrifying bacterium, *Chelatococcus daeguensis* TAD1, that has significant denitrification capacity and is tolerant to some heavy metals (Liang *et al*., 2012; Xiao and Huang, 2012). In this study, we investigated the performance of *Chelatococcus daeguensis* TAD1 with respect to Cr(VI) removal, the mechanisms involved, and the optimal environmental conditions under which this transformation takes place. We also investigated cellulose acetate microspheres as an absorbent material that can protect microbes from the inhibitory effects of multiple heavy metals.

Materials and Methods

Microbe

The thermophilic aerobic denitrifying bacterium *Chelatococcus daeguensis* TAD1 was isolated from the biofilm of a biotrickling filter for the removal of NOx (Liang *et al*., 2012; Yang *et al*., 2012). TAD1 is registered at the China General Microbiological Culture Collection Center (CGMCC no.5226). The 16S ribosomal DNA gene sequence of TAD1 has been submitted to the DNA Data Bank of Japan (DDBJ)/European Molecular Biology Laboratory (EMBL)/GenBank databases under accession no.HM000004. Its genetic composition is highly similar (99%) to *C. daeguensis* K106. TAD1 was stored in 30% glycerol at -20°C. The culture was maintained at 4°C on agar slants.

Media

We used the following growth medium for TAD1: Luria-Bertani (LB) medium (g/L): tryptone, 10.0; yeast extract, 5.0; NaCl, 5.0. The mineral salt medium (MSM) was composed of (g/L) (Ibrahim and Steinbuechel, 2010): KNO_3, 1.0; Na_2HPO_4, 7.9; KH_2PO_4, 1.5; $MgSO_4{\cdot}7H_2O$, 0.1; 1 ml of trace elements solution. The trace elements solution was composed of (g/L): EDTA, 50.0; $CaCl_2$, 5.5; $MnCl_2{\cdot}4H_2O$, 5.06; $FeSO_4{\cdot}7H_2O$, 5.0; $ZnSO_4$, 2.2; $CoCl_2{\cdot}6H_2O$, 1.61; $CuSO_4{\cdot}5H_2O$, 1.57; $(NH_4)_6Mo_7O_2{\cdot}4H_2O$, 1.1. The denitrification medium (DM) consists of 100 ml of the MSM medium with sodium succinate (9.4 g/L) as the sole carbon source.

Cr(VI) and nitrate reduction by strain TAD1

We investigated the ability of TAD1 to remove Cr(VI) in DM with an initial Cr(VI) concentration of 10 mg/L. First, we pre-cultured TAD1 in 50 ml LB medium for 12 h at 50°C and 150 rpm. We then inoculated 10 ml of this seed medium into 100 ml DM in 300-ml shaken flasks. During incubation, we removed 1.0-ml aliquots periodically (0, 4, 8, 12, 16, 20, and 24 h) from all the flasks for the estimation of residual Cr(VI). We centrifuged the samples at 5,000 × *g* for 15 min and we used the resulting supernatants for the analysis of residual Cr(VI).

We added Cr(VI) at a concentration of 10 mg/L to the MSM. We tested different carbon sources [glucose, sodium citrate, sodium succinate, and sodium acetate], initial pH levels [5, 6, 7, 8, and 9], and temperatures [30, 35, 40, 45, and 50°C] to determine optimal conditions for simultaneous nitrate removal and Cr(VI) reduction.

We investigated the influence of initial Cr(VI) concentration by performing the experiments at the following initial concentrations: 5, 10, 15, 20, 30, and 40 mg/L.

To determine the effect of individual heavy metals on the Cr(VI) removal efficiency of TAD1, we examined the culture media (pH 7), containing an initial Cr(VI) concentration of 15 mg/L, in three separate treatments: to the first we added Cu^{2+}, to the second, Ni^{2+}, and to the third, Zn^{2+}, at concentrations of 5 mg/L each. To examine the effect of a combination of multiple heavy metals, we investigated the mixture containing an initial Cr(VI) concentration of 15 mg/L, with 2 mg/L each of Cu^{2+}, Ni^{2+}, and Zn^{2+} in the same treatment. We also added 2 g of cellulose acetate microspheres, which have good mechanical properties, high surface porosity, and biocompatibility, to 100 ml of the culture media to investigate the inhibitory effect of the microspheres on Cr(VI) removal by TAD1.

Determination of different mechanisms of Cr(VI) removal by TAD1

Adsorption onto the carbon source and by inactivated bacteria may also be mechanisms causing a reduction in Cr(VI) concentrations. To determine the role that these factors might play in Cr(VI) removal, we ran five types of controls in triplicate, at an initial concentration of 10 mg/L Cr (VI): (1) MSM inoculated with inactivated microorganisms and containing a carbon source; (2) MSM without inoculation of microorganisms and with a carbon source; (3) MSM inoculated with activated microorganisms and without a carbon source; (4) MSM inoculated with inactivated microorganisms and without carbon source; and (5) MSM with no microorganisms and no carbon source.

To investigate mechanisms of Cr(VI) removal, first we pre-cultured the domesticated bacteria in 50 ml of LB medium

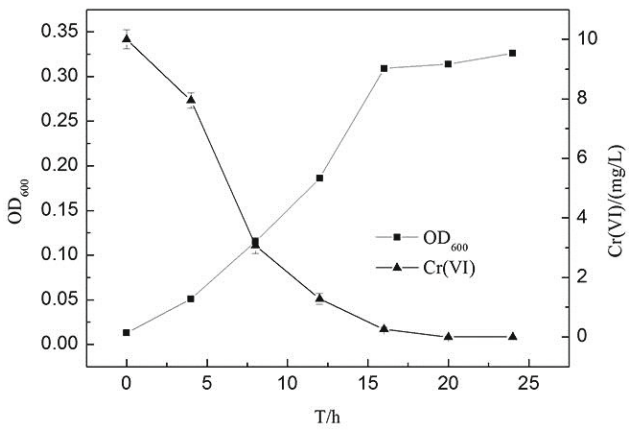

Fig. 1. **Growth and Cr(VI) removal of strain TAD1.**

for 12 h at 50°C and 150 rpm. We then inoculated 10 ml of this seed medium into 100 ml DM medium without Cr(VI) in a 300-ml flask and cultured it for 24 h. We centrifuged the bacterial liquid at 5,000 × *g* for 15 min to separate somatic cells and metabolites, then sieved the metabolites with a microporous membrane filter. We used the separated somatic cell and metabolites to treat a 15 mg/L Cr(VI) solution.

Analytical method

We determined the concentration of Cr(VI) colorimetrically at 540 nm using a diphenylcarbazide reagent in an acid solution (APHA, 1998). We determined the total chromium content by atomic absorption spectrophotometry (Murugavelh and Mohanty, 2013). We estimated the concentration of Cr(III) as the difference between total Cr and Cr(VI) concentrations. We determined bacterial contents by measuring OD600 (APHA, 1998). We measured nitrate using the standard methods (APHA, 1998).

We performed all experiments in triplicate and compared the mean values of the replicates as a quality control measure. We calculated standard deviations and performed one-way analysis of variance at $P \leq 0.05$ using SPSS 20.0, USA.

Results and Discussion

Performance of TAD1 in Cr(VI) removal Cr(VI)

The initial Cr(VI) concentration (10 mg/L) decreased by 97.5% after 15 h in the presence of TAD1, and by nearly 100% after 20 h; the average removal rate was 12 μM/h. The growth curve of the strain demonstrated that the adaptive period of the microorganism was initially 4 h, followed by a logarithmic phase lasting from 4 to 16 h, after which the concentration of the microorganism remained almost constant. In general, the initial pure aerobic denitrifier reaches its maximum concentration at 12 h (Yang *et al.*, 2012), so the presence of Cr(VI) appears to have led to a lag in microbial growth. The two curves show that the more vigorous the microbial growth, the more rapid the removal of Cr(VI). Specifically, the most effective reduction occurred between 4 h and 8 h, which is the first 4 h of the logarithmic phase; the maximum removal rate, 24 μM/h, occurred during this period. This behavior was related to the favorable environment for growth and metabolism of bacteria during this phase, resulting in increased enzyme activity, organic matter degradation, and products of denitrifying bacteria.

A moderately thermophilic bacterium, *Bacillus thermoamylovorans* SKC1, completely reduced 0.6, 1.8, and 3 mM Cr(VI) at 50°C at a rate ranging from 6 to 17.8 μM/h (Slobodkina *et al.*, 2007). The maximum Cr(VI) reduction rate (12 μM/h) we observed for TAD1 was quite high and fell within the range of those observed by various thermophilic bacteria. In comparison, Cr(VI) reduction rates and capacities of other mesophiles such as *Shewanella* sp. (Lall and Mitchell, 2007) and *Bacillus* sp. (Masood and Malik, 2011) were only 2 and 1 μM/h when the initial Cr(VI)concentrations were 10 mg/L and 30 mg/L, respectively. Although the exact rates of Cr(VI) reduction depend on various experimental conditions and on microbial physiology, thermophiles generally exhibit higher reduction rates than mesophiles.

Optimization of conditions for TAD1 for simultaneous Cr(VI) reduction and nitrate removal

As shown in Table 1, TAD1 utilized different carbon sources to remove Cr(VI). Removal efficiencies were very high in the presence of sodium succinate (100%) and sodium acetate (84.83%), followed by sodium citrate (77.54%). The lowest removal efficiency (19.44%) occurred when glucose was the only carbon source present. Removal efficiency was high in the presence of the sodium salts perhaps because sodium succinate and sodium acetate are structurally simple compounds that can be more easily utilized by the microorganisms. Alternatively, some researchers (Philip *et al.*, 1998) suggest that it is the degradation of organic matter via the tricarboxylic acid cycle that produces a reductive electron donor for the Cr(VI). Glucose must be first converted to pyruvate, which then participates in the tricarboxylic acid cycle. On the other hand, because succinic acid and other substances are the in-

Table 1. **Effect of different carbon sources on the removal of Cr(VI) and NO_3^--N by strain TAD1**

Carbon source	0 h		24 h		Removal efficiency (%/d)		OD_{600}
	NO_3^--N (mg/L)	Cr(VI) (mg/L)	NO_3^--N (mg/L)	Cr(VI) (mg/L)	NO_3^-N	Cr(VI)	
Sodium succinate	137.74±3.27	9.97±0.32	10.54±0.29^a	0^a	92.37±0.06^d	100^d	0.347
Sodium citrate	139.05±3.12	10.02±0.26	51.04±0.47^c	2.25±0.23^c	63.29±0.48^b	77.57±1.71^b	0.279
Sodium acetate	139.68±2.89	9.89±0.28	21.72±0.36^b	1.5±0.19^b	84.45±0.06^c	84.86±1.49^c	0.299
Glucose	138.93±3.07	9.93±0.17	128.23±1.67^d	8±0.27^d	7.74±0.76^a	19.45±1.34^a	0.094

One-way analysis of variance (ANOVA) was performed at $P \leq 0.05$. Values are means±SD of three replicates and means within the same column with different superscript small letters are significantly different.

Table 2. Effect of different initial pHs on the removal of Cr(VI) and NO_3^--N by strain TAD1

pH	0 h		24 h		Removal efficiency (%/d)		OD_{600}
	NO_3^--N (mg/L)	Cr(VI) (mg/L)	NO_3^--N (mg/L)	Cr(VI) (mg/L)	NO_3^--N	Cr(VI)	
5	140.21±2.34	10.01±0.18	136.44±1.24^e	7.82±0.21^c	2.69±0.78^a	21.89±0.69^a	0.032
6	139.63±3.25	9.98±0.24	92.35±1.17^d	4±0.16^b	33.85±0.71^b	59.93±0.64^b	0.077
7	140.79±3.12	9.88±0.27	7.72±0.36^a	0^a	94.52±0.13^e	100^c	0.315
8	139.68±2.78	9.91±0.22	9.48±0.35^b	0^a	93.21±0.12^d	100^c	0.271
9	138.71±2.65	9.94±0.27	13.66±0.42^c	0^a	90.15±0.11^c	100^c	0.207

One-way analysis of variance (ANOVA) was performed at $P \leq 0.05$. Values are means±SD of three replicates and means within the same column with different superscript small letters are significantly different.

termediate products, succinic acid is a more efficient carbon source for TAD1 to reduce Cr(VI). Additionally, 92% of NO_3^--N was removed when the carbon source was succinic acid. The mean difference is significant at the 0.05 level by one way ANOVA for Cr(VI) and nitrate, which presents that the carbon sources show great influence on Cr(VI) reduction and nitrate removal.

The pH is an important factor to consider in the Cr(VI) removal process because it determines the oxidation state of the chromium (Mohan and Pittman, 2006). The performance of TAD1 in simultaneously removing nitrate and reducing Cr(VI) was evaluated over a pH range of 5–9 (Table 2). The maximum reduction was observed at pH 7 both for nitrate and Cr(VI), with the removal efficiency exceeding 90%. At pH < 7, the removal of both nitrate and Cr(VI) was significantly inhibited. The mean difference is significant at the 0.05 level by one way ANOVA for nitrate at all pHs. However, for the Cr(VI), the mean difference is significant at the 0.05 level when the pH < 7 and has no significance when pH > 7.

The behavior of microorganisms with respect to Cr(VI) reduction is variable, and is species- and temperature-dependent, with an optimal temperature in the range of 20–40°C for most strains (Narayani and Shetty, 2013). For instance, the best temperature for *Bacillus* sp. and strain SDCr-5 are 35°C and 37°C, respectively (Sultan and Hasnain, 2007). Table 3 shows the removal of Cr(VI) at different temperatures (30–50°C) by strain TAD1. Essentially, we observed no differences in Cr(VI) removal. Temperature had negligible effect on Cr(VI) reduction ($P \leq 0.05$) since complete removal was achieved at all temperatures within 24 h. The mean difference is significant at the 0.05 level by one way ANOVA for nitrate. The maximum reduction of NO_3^--N occurred at 50°C, with an efficiency of 94.53%.

The optimum conditions for TAD1 for simultaneous nitrate removal and Cr(VI) reduction were the use of succinic acid as the carbon source at a pH of 7 and a temperature of 50°C; however, complete Cr(VI) reduction was achieved under a wide range of pHs (7–9) and temperatures (30–50°C). These findings are relevant to industrial applications, which can use this information to produce the ideal conditions to maximize Cr(VI) reduction and nitrate removal. Additionally, TAD1 appears to most effectively reduce Cr(VI) and simultaneously remove nitrate at higher temperatures.

Determination the role of active bacteria, inactivated bacteria, and medium on Cr(VI) reduction

Cr(VI) reduction may be due to active microbial growth and metabolism. Alternatively, Cr(VI) reduction may result from

Table 3. Effect of temperature on the removal of Cr(VI) and NO_3^--N by strain TAD1

T/°C	0 h		24 h		Removal efficiency (%/d)		OD_{600}
	NO_3^--N (mg/L)	Cr(VI) (mg/L)	NO_3^--N (mg/L)	Cr(VI) (mg/L)	NO_3^--N	Cr(VI)	
30	140.21±3.21	10.03±0.34	50.93±1.42^e	0	63.68±0.18^a	100	0.244
35	139.63±2.49	9.94±0.25	36.03±1.21^d	0	74.2±0.41^b	100	0.265
40	140.79±2.52	9.98±0.27	17.98±0.72^c	0	87.23±0.28^c	100	0.268
45	139.68±2.17	9.93±0.19	13.71±0.44^b	0	89.94±0.44^d	100	0.292
50	138.71±3.11	9.97±0.28	7.59±0.23^a	0	94.53±0.04^e	100	0.315

One-way analysis of variance (ANOVA) was performed at $P \leq 0.05$. Values are means±SD of three replicates and means within the same column with different superscript small letters are significantly different.

Table 4. Reductions in Cr(VI) concentrations in the presence of active bacteria, inactivated bacteria, and carbon source

T/h	Cr(VI)concentration / (mg/L)					
	Medium with carbon source			Medium without carbon source		
	Active bacteria	Inactive bacteria	Blank	Active bacteria	Inactive bacteria	Blank
	Experiment	Control 1	Control 2	Control 3	Control 4	Control 5
0	10.00 ± 0.31	10.00 ± 0.27	10.00 ± 0.14	10.00 ± 0.29	10.00 ± 0.27	10.00 ± 0.06
6	4.36 ± 0.22	9.88 ± 0.23	9.94 ± 0.16	9.76 ± 0.21	9.87 ± 0.25	9.96 ± 0.09
12	1.28 ± 0.18	9.72 ± 0.19	9.88 ± 0.08	9.41 ± 0.15	9.75 ± 0.11	9.91 ± 0.06
18	0.26 ± 0.04	9.63 ± 0.06	9.76 ± 0.08	9.30 ± 0.09	9.67 ± 0.04	9.89 ± 0.07
24	0.00 ± 0.01	9.50 ± 0.02	9.65 ± 0.04	9.18 ± 0.07	9.63 ± 0.04	9.88 ± 0.13

adsorption of Cr(VI) on inactivated bacteria or on ingredients of the medium, especially the carbon source, which accounts for a large proportion of the medium. To investigate the role of active bacteria, inactivated bacteria, and medium on Cr(VI) reduction, we ran five types of controls in triplicate, each with an initial concentration of 10 mg/L Cr (VI). The results are shown in Table 4.

The Cr(VI) (initial concentration, 10 mg/L) was removed completely within 24 h in the treatment with active bacteria. However, Cr(VI) concentrations decreased only slightly in the controls, most likely as a result of low-level physiochemical interaction between reducing substances in the medium and the Cr(VI). Overall, the results show that Cr(VI) reduction is most closely associated with the metabolic processes of the active bacteria, which utilize the carbon source.

Mechanism of Cr(VI) removal by TAD1

The mechanisms of Cr(VI) removal by microorganisms can be classified as direct or indirect (Sayel *et al.*, 2012). A direct mechanism refers to a situation in which the Cr(VI) reduction occurs mainly as a result of action by the somatic cell itself or the enzyme system; in contrast, an indirect mechanism is attributed to the bacteria's metabolites.

To explore the mechanism of Cr(VI) reduction by TAD1, we conducted three types of experiments using somatic cells of TAD1, metabolites of TAD1, and the total culture process of TAD1. Total chromium was measured at the end of Cr(VI) reduction experiments. There was only a small amount of total chromium removed in these three systems – 8.01%, 2.27%, and 12.90%, respectively (Fig. 2) – which demonstrates that the transformation of Cr(VI) into Cr(III) caused the removal of almost all of the Cr(VI). With the somatic cells, the Cr(VI) concentration decreased to 12.9 mg/L, with a removal efficiency of 14%; the reduction efficiency with the metabolites was 31.8%; and the Cr(VI) was completely removed with the culture process. These data indicate that the three different methods were all effective in reducing Cr(VI), so the mechanism of Cr(VI) reduction by TAD1 is complicated, being neither solely direct nor solely indirect, but a combination of the two. However, it was found that more than 85% of total chromium was present while no Cr(VI) in the supernatant of the liquid medium with the total culture process of TAD1. Thus, the decrease of Cr(VI) concentration (Fig. 2) was caused not by adsorption but mainly by microbial reduction.

The somatic cells were able to reduce the total chromium to a certain extent, which demonstrates that the TAD1 cells

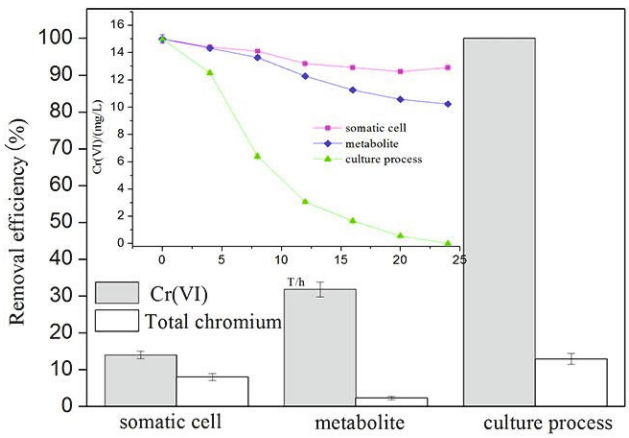

Fig. 2. Reduction of Cr(VI) and total chromium by somatic cells of TAD1, the metabolite of TAD1 and the total culture process of TAD1.

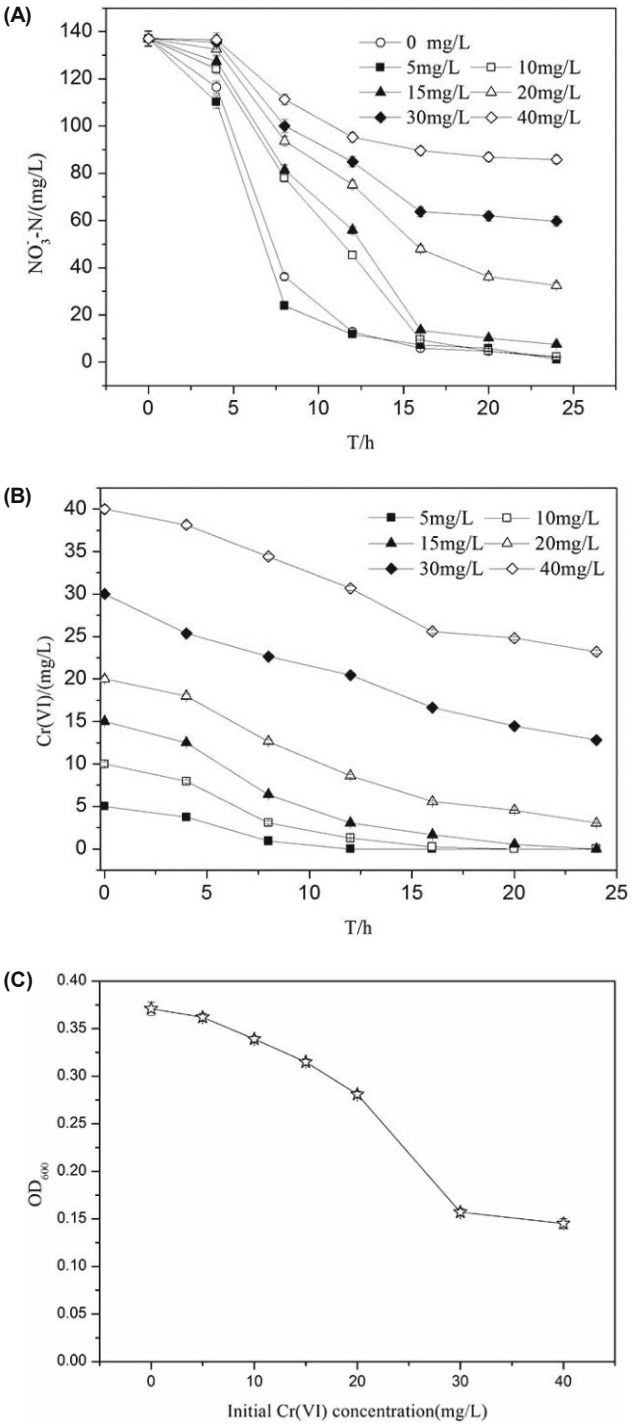

Fig. 3. Effect of initial Cr(VI) concentration on (A) nitrate removal (B) Cr(VI) reduction (C) strain TAD1 growth.

could absorb Cr(VI) and the adsorption ratio accounted for 57% of the Cr(VI) removal. That the Cr(VI) removal efficiency was higher than the total chromium showed the influence of both adsorption and chemical reduction. However, the removal efficiency by somatic cell and metabolite compared with the total culture process was quite lower, demonstrating that the culture process was more effective. This observation confirmed that the total culture process could supply sufficient electron donors to reduce Cr(VI) completely. The contribution of metabolites to total chromium removal was negligible in comparison with Cr(VI). As a whole, the culture process had greater Cr(VI) removal efficiency than either somatic cells or metabolites, confirming that Cr(VI) removal was mainly due to the reducing environment that was produced during the process of culturing TAD1.

Effect of varying initial Cr(VI) concentrations

Chromium is a trace element needed by microbes to survive, but that can also be toxic to bacteria at higher concentrations. Fig. 3 show the effect of rising Cr(VI) concentrations (5–40 mg/L) on simultaneous nitrate removal and Cr(VI) reduction and strain TAD1 growth.

At lower initial concentrations (5, 10, and 15 mg/L of Cr (VI)), NO_3^--N was reduced by 99.19%, 98.32%, and 94.52%, respectively, within 24 h. At Cr(VI) concentrations of 20, 30, and 40 mg/L, NO_3^--N was reduced by 76.31%, 56.39%, and 37.35%, respectively. The maximum reduction occurred at an initial concentration of 5 mg/L Cr(VI), rather than in the absence of Cr(VI), indicating that the presence of trace amounts of Cr(VI) could facilitate NO_3^--N removal. At concentrations of Cr(VI) < 15 mg/L, there was almost no negative effect on nitrogen removal, since almost 95% of NO_3^--N was removed.

At either higher or lower initial concentrations of Cr(VI), TAD1 can effectively remove Cr(VI). At concentrations < 15 mg/L, the removal efficiency reached 100% in 24 h, and at an initial concentration of 20 mg/L, Cr(VI) was reduced by 84.81% within 24 h. However, at higher initial Cr(VI) concentrations (30 and 40 mg/L), the extent of the reduction decreased significantly (57.27% and 42.01%, respectively). That is, the proportion of the initial Cr(VI) removed decreased as the initial concentration increased beyond 15 mg/L, especially during the first 12 h.

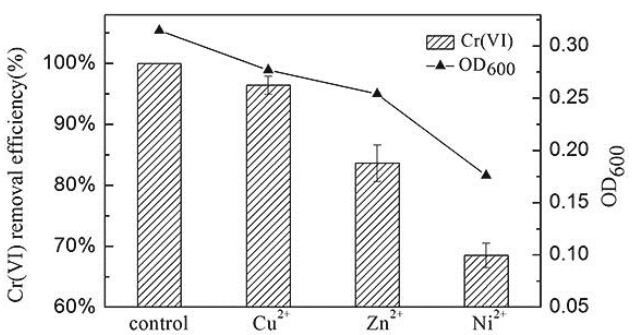

Fig. 4. Effect of different heavy metals (Cu, Zn, and Ni) on Cr(VI) reduction.

TAD1 was able to grow at different initial concentration of Cr (VI). The lower the concentration of Cr(VI), the higher the TAD1 growth rate and the higher the nitrate and Cr(VI) removal efficiencies. At higher concentrations of Cr(VI), bacterial growth was significantly lower, perhaps because of the acceleration of cell death caused by Cr(VI).

Clark (1994) found that *Enterobacter aerogenes* was resistant to chromate only under aerobic conditions and when chromate reductase was induced by nitrite. Additionally, the level of chromate reductase activity was similar to that of nitrite reductase. Other researchers (Zhao *et al.*, 2013) applied nitrite reductase to remove Cr(VI) directly, and concluded that the activity of nitrite reductase is the mechanism responsible for the removal of Cr(VI) in *Bacillus cereus* Cr2. The results of our study demonstrate that the simultaneous removal of nitrate and reduction of Cr(VI) by strain TAD1 is promising.

Effect of different heavy metals on Cr(VI) reduction

A variety of heavy metals were selected to explore their influence on the Cr(VI) removal ability of TAD1. The results are shown in Fig. 4.

Heavy metals at relatively high concentrations (5 mg/L) inhibited both the growth of microorganisms and the ability of TAD1 to remove Cr(VI). This finding could be attributed to the fact that heavy metals disrupt the synthesis of enzymes by microorganisms and the positive valence metal ions compete with Cr(VI) for the reducing substances. In the presence of Cu^{2+}, Zn^{2+}, and Ni^{2+}, the Cr(VI) removal efficiency decreased by 3.57%, 16.36%, and 31.48%, respectively, as compared with the control group, which did not contain additional heavy metals. Of the three heavy metals, Ni^{2+} had the strongest inhibitory effect on Cr(VI) reduction, followed by Zn^{2+}, and then Cu^{2+}. Other studies with different bacteria have produced different results, for example, Ni^{2+} was observed to stimulate the growth of isolate IFR-2 (Ilias *et al.*, 2011). Bhattacharya (Bhattacharya and Gupta, 2013) and Xu (Xu *et al.*, 2005) detected that Cu^{2+} promoted the Cr(VI)

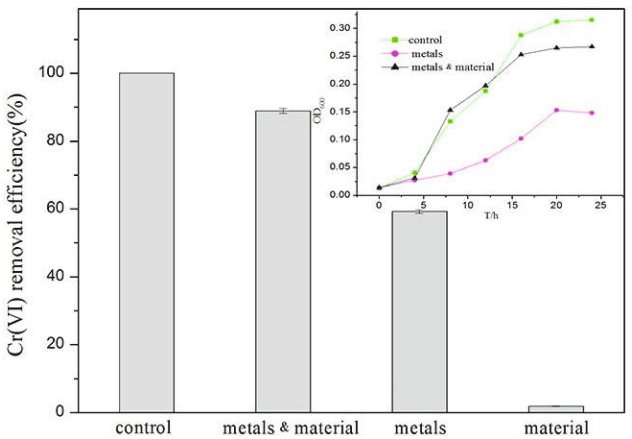

Fig. 5. Cr(VI) reduction in: the control group (without no heavy metals and no cellulose acetate microsphere material), a treatment containing multiple heavy metals, a treatment containing cellulose acetate microsphere material, and a treatment containing multiple heavy metals and cellulose acetate microsphere material.

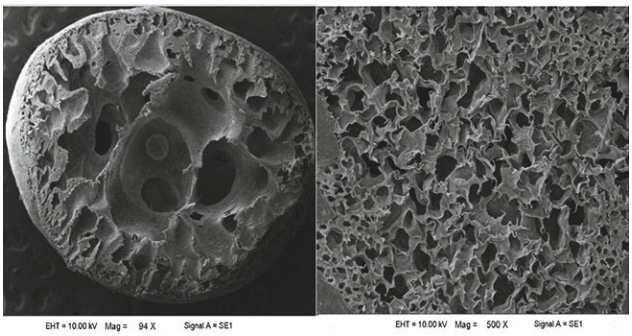

Fig. 6. Surface and fracture of cellulose acetate microsphere adsorbent, as captured by scanning electron microscopy (left was fracture, right was surface).

reduction ability of different strains. In summary, the effect of heavy metals on Cr(VI) reduction the ability of microorganisms depends on the type of metal and on the bacterial strain. The mean difference is significant at the 0.05 level by one way ANOVA.

Effect of multiple heavy metals on Cr(VI) reduction

The combined influence of multiple heavy metals on microorganisms can be divided into non-correlative, cooperative, and antagonistic effects (Chu and Chow, 2002; Ryan *et al.*, 2005; Sayel *et al.*, 2012). Some studies (Aston *et al.*, 2010) have shown that the toxicity of Cr(VI) was stronger when Cu^{2+} and Zn^{2+} are present together than when they are present separately, with the cooperative interaction of the two metals severely inhibiting bacterial growth. The effects of the complex system containing an initial concentration of 15 mg/L of Cr(VI), and 2 mg/L each of Cu^{2+}, Ni^{2+}, Zn^{2+} on Cr(VI) reduction are shown in Fig. 5.

Compared with the control group (containing no additional heavy metals) at an initial concentration of 15 mg/L Cr(VI), the presence of the heavy metals together inhibited not only the growth of TAD1 but also the bacteria's ability to remove Cr(VI). The OD_{600} of TAD1 decreased by 53.02% and the proportion of Cr(VI) that was reduced dropped to 59.26%.

The inhibitory effect of multiple heavy metals was stronger than the effect of the individual metals at almost the same concentrations, which indicates that the inhibitory effect of multiple heavy metals on TAD1 was cooperative, and this may aggravate the degeneration of the bacteria. Therefore, the kinds and concentrations of heavy metals need to be controlled in industrial applications of biological reduction of Cr(VI) in wastewater. Proper pretreatment with an absorbent material can address this problem, as described below.

Cr(VI) reduction in: the control group (without no heavy metals and no cellulose acetate microsphere material), a treatment containing multiple heavy metals, a treatment containing cellulose acetate microsphere material, and a treatment containing multiple heavy metals and cellulose acetate microsphere material.

The OD_{600} of TAD1 increased to 0.276 and the Cr(VI) reduction efficiency reached 88.89% following the addition of the cellulose acetate microsphere (shown in Fig. 6), which has high surface porosity and abundant active groups, such as hydroxyl and carboxy hydroxyl groups. The absorbent material protected the TAD1 from inhibitory effects of the heavy metals and provided a biological carrier and a carbon source for the denitrifying bacteria of TAD1. However, both the growth rate and Cr(VI) removal efficiency were lower than in the control because there were still trace amounts of heavy metals remaining after adsorption equilibrium was reached.

Conclusion

The Cr(VI) was removed by the thermophilic aerobic denitrifying bacterium *C. daeguensis* TAD1, initially in a highly effective manner, and the reducing environment resulting from the growth of TAD1 caused near complete removal of Cr(VI). The presence of heavy metals in addition to Cr(VI), both when additional metals were present individually or when a combination of metals was present, inhibited the reduction of Cr(VI). However, when cellulose acetate microspheres were introduced, the Cr(VI) removal efficiency returned to a high level. This study demonstrates the feasibility of harnessing bacteria as an effective tool in simultaneous nitrate removal and Cr(VI) reduction during the biological treatment of wastewater.

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