JOURNAL OF PURE AND APPLIED MICROBIOLOGY, June 2014. Vol. **8**(3), p. 1893-1900

Distributions of Soluble Hexavalent Chromate Reductase from *Leucobacter* sp. G161 with High Reducing Ability and Thermostability

Shimei Ge[1, 2], Weihong Zheng[1, 2], Xinjiao Dong[1,2], Maohong Zhou[1,2], Jiangmin Zhou[1, 2] and Shichao Ge[3]*

[1]College of Life and Environmental Science, Wenzhou University, Zhejiang Province, 325035, China.
[2]Key lab for water Environment and Marine Biological Resources Protection, Wenzhou, Zhejiang Province, 325035, China.
[3]State Key Laboratory of Molecular Biology, Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai, 200031, China.

(Received: 12 August 2013; accepted: 30 October 2013)

Chromate-reducing bacteria can convert soluble and toxic hexavalent chromate [Cr(VI)] to insoluble and less toxic trivalent chromate [Cr(III)]. Bioremediation can be more effective in removing chromate(VI) from the environments if chromate reductase is used in Cr(VI) reduction reaction. The bacteria strain *Leucobacter* sp. G161 isolated from chromate-contaminated soil has been demonstrated in its ability to reduce Cr(VI) to Cr(III) in the previous investigation. In this study, the cytoplasmic soluble fraction-associated chromate(VI) reductase was characterized and shown high reducing ability to catalyze reduction of chromate(VI). The enzyme is optimally active at a pH of 7.0 and 35 °C, and the specific Michaelis-Menten constant (K_m) and maximum reaction kinetic velocity (V_{max}) of the Cr(VI) reduction were 165.49 ìM and 1.44 ìM min^{-1} mg^{-1} protein respectively using NADH as an external electron donor. Interestingly, the enzyme was highly thermostable: 48% of its activity was remained when exposed at 80 °C for 30 min. Moreover, the reductase activity was enhanced by the addition of other external electron donors, with NADH being the most effective one. The Cr(VI) reductase tolerated several types of metal ions tested. These results suggested that the enzyme reductase could be used for bioremediation of Cr(VI)-polluted environments.

Key words: Chromate reductase, Cytoplasmic soluble fractions, Chromate(VI) reduction, Thermostability, *Leucobacter* sp. G161.

Chromium is one of the most widely used metals in several types of industries, including leather tanneries, pigment production, wood preservation and chemical plants[1-3]. Hexavalent chromate is a widespread environmental contaminant. Hexavalent chromate contaminated waste is often improperly treated and disposed of by industry, and this waste in turn pollutes the surrounding environment. Chromate(VI) is a strong oxidizing agent that reacts with nucleic acids and other cellular components causing mutagenic and carcinogenic effects on human and animals after absorption by the cells[4,5]. Chromium mainly exists in two oxidation states in the environment: trivalent (III) and hexavalent (VI). Of the two chromium oxidation states, Cr(VI) is much more toxic because it is highly soluble and can easily pass through cellular membranes; in contrast, trivalent chromium is far less soluble, less readily crosses cellular membranes than Cr(VI) and is therefore not as toxic[6]. Thus, the reduction of hexavalent chromium to trivalent chromium is a potential method of environmental decontamination. Typically, physicochemical treatments, such as chemical precipitation, ion exchange or adsorption are used to remove Cr(VI) waste. However, these practices

* To whom all correspondence should be addressed.
Tel.: +86 21 54921223; Fax: +86 21 54921011;
E-mail: gesc13@163.com

are not often utilized for full-scale bioremediation because they are expensive and require large amounts of energy[7]. Microbial reduction could provide a useful alternative economic method[3].

A variety of microorganisms have been reported to reduce Cr(VI) to Cr(III), such as *Pseudomonas fluorescens* LB300[8], *Enterobacter cloacae* HO1[9], *Escherichia coli* ATCC 3346[10], *Pseudomonad* CRB5[5] and *Arthrobacter rhombi-RE*[11], etc. However, the potential for biological treatment of Cr(VI) contaminated waste from intact cells is limited because some organisms lose viability in the presence of high concentration of chromate[12]. The use of cell-free Cr(VI) reductase enzyme has advantages over using the intact cells. Cell-free enzymes are not affected by the growth inhibitors, toxin or microbial competition in the environment. Moreover, cell-free enzymes do not require transport mechanisms that may impair microbial uptake of chromate. Furthermore, cell-free enzymes can be immobilized for pollutant removal in reactor[13].

The bacteria *Leucobacter* sp. G161 strain isolated from long-term chromium (VI) contaminated soil had a high Cr(VI)-reducing capacity with intact cells previously[14]. In this study the chromate(VI) reductase was purified from cytoplasmic soluble fraction of the cells. Its reducing ability was assessed by *in vitro* reduction of Cr(VI). The kinetic, pH and temperature parameters of Cr(VI) reduction, and thermostability of the enzyme were determined. The effect of external electron donors and metal ions on the reduction of chromate(VI) was assessed as well.

MATERIALS AND METHODS

Bacterial isolation and growth

The bacterial strain analyzed in this study was isolated from chromium contaminated soil waste from a tannery in Wenzhou, China[14]. The isolated strains were maintained on Luria broth (LB) agar plates supplemented with 100 ìM Cr(VI). The LB medium contained 10 g l^{-1} peptone, 5 g l^{-1} yeast extract and 10 g l^{-1} NaCl. The bacteria were grown at 35 °C with shaking at 150 rpm.

Preparation of sub-cellular fractionations

Cell-free extracts were prepared mainly following the previous reports[6, 15] with some modifications. Cells from overnight-grown aerobically in 100 ml LB medium were harvested, washed twice and sonicated in phosphate buffer (pH 7.0) at 10% of the original culture and disrupted in an ice bath by passage through a French pressure cell at 16,000 lb/in^2 (Thermo Electron FRENCH Press, FA-032)[16]. The sonicate was centrifuged at 6,000 × *g* for 10 min at 4 ºC, and the supernatant was filtered through 0.22 ìM filters (Millipore, Bedford, USA) to remove unbroken cells and debris. The supernatant was subsequently centrifuged at 12,000 × *g* for 20 min and 150,000 × *g* for 60 min at 4 ºC to yield cell soluble fractions S_{12} and S_{150}, and the corresponding pellets P_{12} and P_{150} (membrane fractions), respectively.

Periplasmic fractions were prepared by following the osmotic shock procedure[14, 17]. Briefly, harvested cells were washed twice with cold 10 mM Tris-HCl buffer (pH7.1) with 30 mM NaCl and resuspended in 33 mM Tris-HCl (pH 7.1). The re-suspension was rapidly mixed into 40% (w v^{-1}) sucrose and followed by the addition of EDTA at final concentration of 0.1 mM. The mixed suspention was placed on a rotary shaker for shaking 10 min and then spun to harvest whole cells. The harvested cells were rapidly dispersed in 10 mI cold 0.5 mM $MgCl_2$ in an ice bath for 10 min and spun again. Total proteins in the supernatant were measured using Coomasssie blue spectrophotometrically at 595 nm with bovine serum albumin as a standard[17] before and after treatment with EDTA to determine whether the outer membrane of the cells was permeabilized or not. After EDTA treatment to permeabilize the outer membrane of cells the periplasmic proteins should be released into the solution. The supernant was regarded as a periplasmic fraction with significantly increased in amount of proteins with EDTA permeabilization the outer membrane.

Chromate reductase assay

Chromate reductase activity of cell-free extracts from different components of the cells was assayed following the procedure of the previous report [13]. In brief, 1 ml reaction mixture was composed of 0.5 ml respective cellular fractions and 100 ìM Cr(VI) in 100 mM potassium phosphate buffer (pH 7.0). The amount of residual Cr(VI) in the reaction mixture was quantified spectrophotometrically at 540 nm using 1,5-diphenylcarbazide as the complexing reagent[12]. The Cr(VI) reductase activity was quantified by

measuring the decrease in Cr(VI) with or without NADH as an electron donor. One unit (1 U) of enzyme activity was defined as the amount of enzyme that reduced 1.0 ìM Cr(VI) per min at the optimum temperature.

Kinetic analysis of Cr(VI) reductase

The reaction kinetics of the Cr(VI) reductase in the extract S_{150} was evaluated with various concentrations of Cr(VI). Reaction mixtures (1 ml) containing 0.5 ml of cell-free extract S_{150}, 1 mM of NADH and various concentrations of Cr(VI) (0, 50, 100, 150, 200, 250, 300, 350 and 400 ìM) in 100 mM potassium phosphate buffer (pH 7.0) were incubated for 30 min at 35 °C. The amount of residual Cr(VI) in the reaction mixtures was quantified. The Michaelis–Menten equation was used to fit the data and calculate the Michaelis constant (K_m) and maximum velocity (V_{max}) of the enzyme.

Cr(VI) reduction and reductase activity over time

The reduction of Cr(VI) by chromate reductase in the bacterial extract S_{150} was monitored at 35°C at 0, 20, 30, 40, 60, 80, 100 and 120 min in the 100 mM potassium phosphate buffer (pH 7.0). The reaction was quenched, and the residual Cr(VI) in solution was quantified with the 1,5-diphenylcarbazide reagent described previously.

Effect of temperature, pH, electron donors and metal ions on Cr(VI) reductase

The Cr(VI) reduction reaction was incubated at 20, 30, 35, 40, 45, 50, 60, 70, 80, 90 and 100 °C with 100 ìM Cr(VI) to analyze the effect of temperature on enzyme activity. To further evaluate the thermostability of reductase activity, the cell-free extract was heated at 80 °C for 0, 10, 20, 30, 40 and 50 min, respectively, and then cooled in an ice bath. The *in vitro* reduction assay was subsequently performed at 35 °C for 30 min.

For testing the effect of pH, autoclaved culture medium was adjusted to 4.0, 5.0, 6.0, 7.0, 8.0, 9.0 and 10.0 with predetermined amount of filter-sterilized 1M HCl or NaOH. The initial Cr(VI) concentration was 100 ìM, and NADH was the electron donor. The reaction was incubated at 35 °C for 30 min and the residual enzyme activity was measured.

The effect of various electron donors on the Cr(VI) reductase activity of the extract S_{150} was also evaluated. The electron donors tested were NADH, sodium citrate, sodium acetate, sodium carbonate, sodium lactate and glucose; all of the electron donors were tested at a concentration of 1 mM. The reaction mixture (total volume of 1 mL) was comprised of 0.5 mL of cell-free extract S_{150}, 100 mM electron donor and 100 ìM of Cr(VI) in 100 mM potassium phosphate buffer (pH 7.0).

The mineral salts containing metal ions that were tested were $CuCl_2$, $NiCl_2$, $MgCl_2$, $MnCl_2$, $ZnCl_2$, $BaCl_2$, $CoCl_2$, $CaCl_2$, and $AgNO_3$. Each salt was tested at 1.0 mM in the 100 mM potassium phosphate buffer (pH 7.0).

Statistical analysis

All of the experiments were done in triplicate, and the results were statistically analyzed. Values are mean ± standard deviation from three independent experiments. $P < 0.05$ represented statistically significant differences. Data were compared using Student's paired 2-tailed *t*-test.

RESULTS AND DISCUSSION

Sub-cellular distribution of chromate(VI) reductase activity

Sub-cellular distribution of the Cr(VI) reductase from *Leucobacter* sp. G161 cells was investigated in cytoplasmic soluble fraction S_{12}, S_{150}, membrane fractions $P_{12,}$ P_{150} and periplasmic contents using ultracentrifugation and cell-EDTA permeabilized procedure described in Materials and methods. As shown in Table 1, the soluble fractions S_{12} and S_{150} exhibited the most potential reductase activity, and both showed similar enzymatic activity, while membrane or periplasmic fractions had low reductase activities, indicating that the G161 reductase was mainly associated with the soluble fraction of the cells. This finding was consistent with the previous reports that the Cr(VI) reductase was found to be mainly located in the soluble fraction of cells in *Rhodobacter sphaeroides* and *Pannonibacter phragmitetus* LSSE-09[15, 17]. In contrast, the chromate reductase was associated with the membrane fraction of the cells in *Enterobacter cloacae* HO1[18] and *Pseudomonas fluorescens* LB300[8]. In subsequent experiments, the examination of chromate reductase activity was monitored based on soluble fraction S_{150} of the cells.

Moreover, the Cr(VI)-reduction was enhanced approximately 3 times by addition of 1

mM external electron donor NADH compared with that without addition of NADH in cytoplasmic soluble fractions. There was a lower level of Cr(VI)-reduction without addition of NADH (Table 1). Previous studies had shown that Cr(VI) reduction depended on the presence of an electron donor [15], and that reductase activity was greatly enhanced by the addition of NADH in *Bacillus* sp.[19]. In contrast, *Bacillus sphaericus* AND 303 cell-free extract could reduce Cr(VI) without the presence of any additional electron donors[7]. Thus, the dependence on an electron donor for Cr(VI) reduction varies among different bacterial strains. Based on these data, an electron donor was helpful to Cr(VI) reduction *in vitro* by soluble reductase of strain G161.

Cr(VI) reductase kinetics

The kinetic data of Cr(VI) reduction provide an important parameters in assessing its potential remediation of Cr(VI) contaminants for quantitative analysis under realistic conditions[20]. Various concentrations of Cr(VI) substrate (0 - 400 ìM) were tested to assess the Michaelis-Menten kinetics of Cr (VI) reductase in the extract S_{150}. As shown in Figure 1a, the reductase activity increased with increasing Cr(VI) concentrations until 200 ìM, and there was a small increase in enzymatic activity from 200 ìM to 400 ìM. The K_m and V_{max} kinetic constants for Cr(VI) reductase activity were 165.49 ìM Cr(VI) and 1.44 ìM Cr(VI)/min/mg protein, respectively, which fit well with the linearized Lineweaver-Burk plot (Figure 1b). Sarangi ÿ Krishnan (2008)[21] previously reported that the *Leucobacter* sp. KCH4 strain had a K_m of 55 ìM , and that the reductase was inhibited by excessive substrate concentrations (75 ìM). These observations suggested that the reductase enzyme of strain G161 could tolerate the enhanced concentrations of the substrate.

Table 1. Distribution of chromate reductase activity in the cell fractions of strain G161 cells

Cellular fractions	Incubation without NADH Reductase activity Cr(VI) reduction$^{\alpha}$ (U/mg protein)	(%)	Incubation with 0.1M NADH Reductase activity Cr(VI) reduction$^{\alpha}$ (U/mg protein)	(%)
Soluble fraction S_{12}	0.59 + 0.06	18.3 + 0.71	1.89 + 0.48	48.7 + 3.56
Soluble fraction S_{150}	0.72 + 0.11	20.7 + 1.23	2.14 + 0.51	50.3 + 4.28
Membrane fraction P_{12}	0	0	0.12 + 0.02	1.43 + 0.22
Membrane fraction P_{150}	0.26 + 0.03	3.31 + 0.19	0.54 + 0.15	7.85 + 1.37
Periplasmic contents	0.34 + 0.21	4.83 + 0.33	0.78 + 0.71	9.73 + 2.23

$^{\alpha}$ Cr(VI) reductions were measured at incubation for 30 min.
Results represent mean + standard deviation of three independent experiments.

Table 2. Reductase activity of the cell-free extract from strain G161, which was heated to 80 °C for various periods of time and then incubated at 35 °C for 30 min

Incubation time at 80 °C (min)	Residual Cr(VI) concentration (µM)[a]	Reductase activity (U/min/mg protein)
0	22.16 ± 0.88	0.50 ± 0.02
10	40.41 ± 2.32	0.40 ± 0.05
20	52.89 ± 1.37	0.32 ± 0.07
30	61.94 ± 2.05	0.24 ± 0.04
40	79.95 ± 2.93	0.13 ± 0.03
50	97.32 ± 1.12	0.00 ± 0.01

[a]Initial concentration of Cr(VI) was 100 ìM. Results represent mean ± standard deviation of triplicate experiments.

Cr(VI) reduction and chromate reductase activity over time

The reduction of hexavalent chromium and reductase activity of the extract S_{150} was assessed by quantifying Cr(VI) reduction at various time points at 35 °C. Within the first 40 min of incubation, 75% of Cr(VI) was reduced by the S_{150}, and the reductase activity was 0.45 ± 0.02 units min^{-1} mg^{-1} protein (Figure 2). After 40 min, some additional Cr(VI) was reduced, although the reductase activity was decreased. After 120 min of incubation, 87% of the Cr(VI) was reduced and the reductase activity was 0.17 ± 0.01 units min^{-1} mg^{-1} protein. The results indicated that the highest reductase activity was at 40 min at 35 °C. In comparison, the *Bacillus* strains G1DM20,

G1DM22, G1DM64 and AND 303 could reduce approximately 50-65% of Cr(VI) within 20 min[6, 22].

Effect of temperature on Cr(VI) reductase

Cr(VI) reductase activity in cytoplasmic soluble fraction extract S_{150} of strain G161 was investigated at different temperature from a wide range of 20 to 100 °C. Maximum reductase activity was established at 35 °C (Figure 3). The activity of the S_{150} gradually increased from 20 to 35 °C and decreased above 35 °C for 30 min incubation. Similarly, the optimum temperature for *in vitro* Cr(VI) reduction by cell-free extracts of *Bacillus* sp. KCH3 and *Exiguobacterium* sp. KCH5 was 35 °C, whereas the optimal temperature for *Leucobacter* sp. KCH4 and *Bacillus* sp. RE was 30 °C[23,24].

Evaluation of reductase thermostability

To further investigate the thermostability of the chromate reductase, the cell-free extract S_{150} was incubated at 80 °C for various periods of time, and then *in vitro* Cr(VI) reduction was monitored at 35 °C for 30 min to evaluate the remaining enzyme activity with an initial concentration of 100 ìM Cr(VI). When the extract S_{150} was incubated at 80 °C for 20 min the remaining reductase activity was 64%; after 40 min incubation at 80 °C, 74% of the respective enzymatic activity was lost (Table 2). In a previous report, approximately 80% of reductase

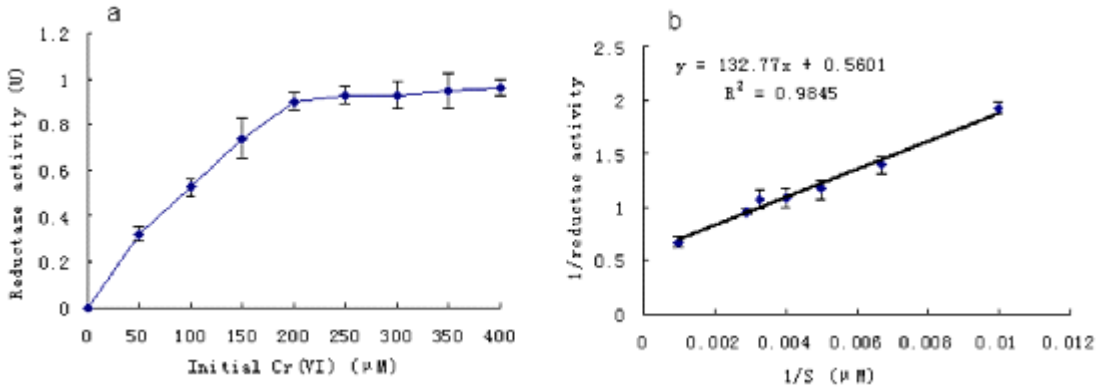

Fig. 1. Kinetic analyses of the Cr(VI) reductase in the cell-free extract S_{150} of strain G161. The Cr(VI) reductase activity was assessed with various initial concentrations of chromate (VI) (a), and the Lineweaver-Burk plot for the Cr(VI) reductase activity (b). The reaction was incubated at 35 °C for 30 min. Error bars represent standard deviation of triplicate samples

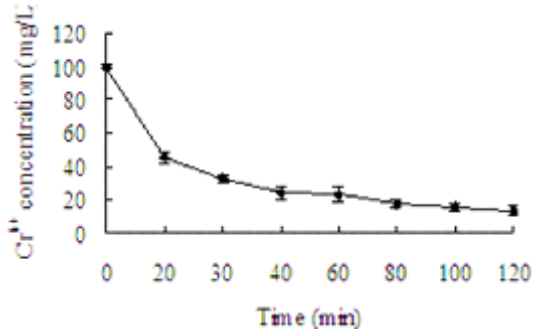

Fig. 2. Time course of Cr(VI) reduction by the S_{150} and effect of temperature. The reaction was incubated for various periods of time with the initial concentration of 100 ìM Cr(VI), and the residual Cr(VI) concentration was quantified. Error bars represent standard deviation of triplicate samples

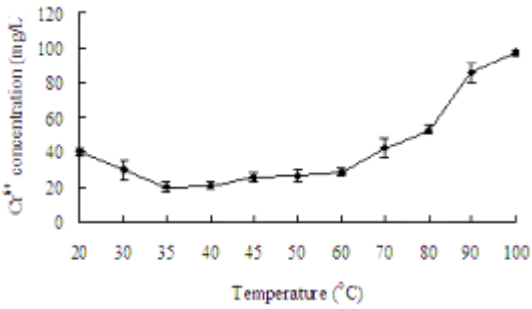

Fig. 3. Effects of external electron donors and metal ions on chromate(VI) reductase activity of the extract $S_{150.}$ The values at pH 7 (a) or blank representing only the S_{150} without any addition of electron donors (b) were set to 100%, the Cr(VI) reductase activity was quantified with various pH values (a) or 1 mM of each electron donor (b). The reaction was incubated at 35 °C for 30 min with 100 ìM Cr(VI). Error bars represent standard deviation of triplicate samples

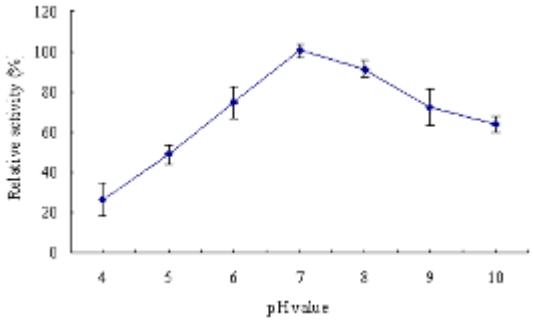

Fig. 4. Effects of pH value on chromate(VI) reductase activity of the extract $S_{150.}$ The reductase activity was quantified at the indicated pH values with 100 ìM Cr(VI) at 35 °C for 30 min

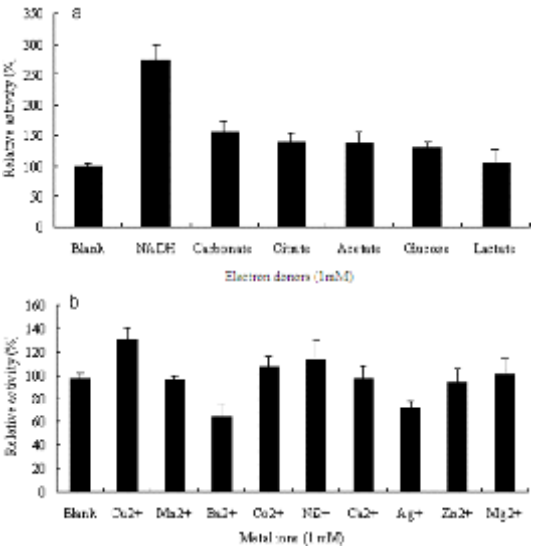

Fig. 5. Effects of external electron donors and metal ions on chromate(VI) reductase activity of the extract $S_{150.}$ The Cr(VI) reductase activity was quantified with 1 mM of each electron donor (a) and 1 mM of each metal ion (b). Blank represented only S_{150} without any addition of electron donors (a) or metal ions (b). The reaction was incubated at 35 °C for 30 min with 100 ìM Cr(VI)

activity was lost when cell-free extracts of *Bacillus sphaericus* AND 303 were incubated at 70 °C for 15 min[6]. A *Pseudomonas putida* chromate reductase was purified and the enzymatic activity drastically decreased to below 20% with incubation at 70 °C for 30 min although the activity remained virtually unaltered after 30 min of incubation at 50 °C[16]. Compared to these data, the strain G161 chromate reductase had a much higher thermostability. We proposed that the high thermostability was associated with long–term adaptation to the chromium polluted, high temperature industrial waste that was released from tanneries.

Effect of pH on Cr(VI) reductase

To test the effect of pH on Cr(VI) reductase activity, chromate reduction reactions were performed at a range of pH values between 4.0-10.0. Figure 4 demonstrated that the optimal reductase activity was at pH 7.0. Whereas acidic pH drastically decreased the enzymatic activity, alkaline pH only slightly decreased the enzymatic activity, and more than 85% of residual activity remained at pH 10.0. In *Bacillus* spp. strains the optimal pH for Cr(VI) reductase activity was 7.0, whereas the optimal pH was 5.5-6.0 for *Bacillus* sp. and *Leucobacter* sp. KCH4[6,23,24]. In all of these species there was rapid loss of enzymatic activity as the pH increased above the optimum values. Compared to these results from other bacterial strains, the reductase activity of strain G161 was more stable under alkaline pH conditions.

Effect of electron donors on chromate reductase activity

There was a threefold increase in Cr(VI) reductase activities of the soluble fractions S_{150} with the addition of an electron donor NADH compared to without a donor (Table 1). To investigate whether other electron donors could affect Cr(VI) reductase activity, we measured Cr(VI) reduction in the presence of glucose, sodium citrate, sodium acetate, sodium carbonate and sodium lactate. All of the electron donors tested could enhance Cr(VI) reduction, although the compounds had different chromate reduction efficiencies (Figure 5a). When the Cr(VI) reduction efficiency without an electron donor was set to 100%, the relative reduction activities of the electron donors in the order were as follows: NADH (273 %) > carbonate (155%) > citrate (140%) > acetate (137%) > glucose (130%) > lactate (105%). In previous reports, a momentous increase in reduction of Cr(VI) was observed with addition of NADH(P)[19,22,25]. Carbonate increased the reduction of Cr(VI) by 55% in the extract of strain G161 in the present study, while this electron donor had no effect on the reduction of Cr(VI) in *Bacillus* spp. [22]. Lactate expedited Cr(VI) reduction in cell-free extract of *Cellulomonas* sp.[26] while only 5% increase with the addition of this electron donor

was observed. The electron donor donated one electron to Cr(VI) to generate an intermediate form, Cr(V), which accepts another two electrons from two coenzyme molecules to produce stable Cr(III)[27, 28].

Effect of metal ions on chromate reductase activity

Various metal ions were added at a final concentration of 1 mM and the effect of each metal ion on Cr(VI) reduction by the cell-free extract was monitored. When the reaction was incubated for 30 min, the addition of Ba^{2+} and Ag^{+} significantly decreased the activity of chromate reductase by 36 and 28%, respectively (Figure 5b), which were similar to results previously seen with *Bacillus* sp. strains[22, 23] and *Arthrobacter rhombi-RE* spp.[11]. The addition of Ca^{2+}, Mg^{2+}, Mn^{2+} or Zn^{2+} had no effect on Cr(VI) reduction, whereas Co^{2+} or Ni^{2+} slightly increased the enzymatic activity 7 and 14% respectively, and Cu^{2+} significantly enhanced the activity of chromate reductase by 30% (Fig. 5b). Previous studies had shown that Cu^{2+} effectively stimulated Cr(VI) reduction by cell-free extracts of *Bacillus* spp. strains and *Leucobacter* sp. KCH4[13,23,24] . In contrast, the addition of Cu^{2+} inhibited hexavalent chromate reduction in *Pseudomonas putida*[16] and *Enterobacter cloacae*[29]. The effects of Ca^{2+} and Mg^{2+} on the activity of chromate reductase of strain G161 were consistent with the data from *Bacillus* spp. strains[22] The addition of Ni^{2+} had no effect on Cr(VI) reduction in *Bacillus sphaericus* AND 303[6] but it increased chromate reductase activity in *Ochrobactrum intermedium*[3]. Co^{2+} stimulation of Cr(VI) reduction was reported for *Bacillus* sp. G1DM22 , although no major effects were seen with *Bacillus* spp. G1DM20 and G1DM64[22] Zn^{2+} inhibition of Cr(VI) reduction was observed in *Bacillus* sp. RE[23] and *Ochrobactrum* sp. CSCr-3 strain[30]. These data indicated that the Cr(VI) reductase enzyme of strain G161 was tolerant to most of the metal ions tested, suggesting that this enzyme could be used for bio-treatment of Cr(VI) in contaminated wastes.

CONCLUSIONS

The enzyme Cr(VI) reductase activity of *Leucobacter* sp. strain G161 was mainly associated with the cytoplasmic soluble fractions of the cells. The maximum reaction velocity (V_{max}) was 1.44 ìmol min^{-1} mg^{-1} protein and the substrate binding affinity (K_m) was 165.49 ìM with the presence of NADH. The optimal conditions for Cr(VI) reductase activity are 35 °C and pH 7.0. In particular, the S_{150} was highly thermostable, 48% of enzyme activity was remained when exposed to 80 °C for 30 min which was the first report for Cr(VI) reductase, while 74% of the activity was lost when the incubation time was extended to 40 min. Addition of external electron donors enhanced the chromate reductase activity. Moreover, the enzyme activity of Cr(VI) reductase was tolerant to most of the metal ions. These data suggested that the chromate reductase from the strain G161 could be utilized for Cr(VI) bioremediation of contaminated environments.

ACKNOWLEDGMENTS

This work was supported by a grant (31300421) to Shimei Ge from the National Natural Science Foundation of China**.**

REFERENCES

1. Barnhart, J. Occurrences, uses, and properties of chromium. *Regul. Toxicol. Pharmacol*., 1997; **26** (1 Pt 2): S3-S7.
2. Viti, C.; Pace, A.; Giovanetti, L. Characterization of Cr(VI)-resistant bacteria isolated from chromium-contaminated soil by tannery activity. *Curr. Microbiol*., 2003; **46**(1): 1–5.
3. Sultan, S.; Hasnain, S. Reduction of toxic hexavalent chromium by *Ochrobactrum intermedium* strain SDCr-5 stimulated by heavy metals. *Bioresour. Technol,*. 2007; **98** (2): 340–344.
4. Losi, M.E.; Amrhein, C.; Frankenberger, W.T.J. Environmental biochemistry of chromium. *Rev. Environ. Contam. Toxicol.*, 1994; **136**: 91-121.
5. McLean, J.; Beveridge, T.J. Chromate reduction by a *pseudomonad* isolated from a site contaminated with chromated copper arsenate. *Appl. Environ. Microbiol.*, 2001; **67**(3): 1076–1084.
6. Pal. A.; Dutta, S.; Pau, A.K.; Reduction of hexavalent chromium by cell-free extract of *Bacillus sphaericus* AND 303 isolated from serpentine soil. *Curr. Microbiol.*, 2005; **51**(5): 327-330.
7. Krishna, K.R.; Philip, L. Bioremediation of Cr(VI) in contaminated soils. *J. Hazard. Mater.*, 2005; **121** (1-3): 109–117.
8. Bopp, L.H.; Ehrlich, H.L. Chromate resistance

and reduction in *Pseudomonas fluorescens* strain LB300. *Arch. Microbiol*., 1998; **150**(5): 426–431.

9. Wang, P.C.; Tsukasa, M.; Kohya, K.; Masanori, S.; Kiyoshi, T.; Hisao, O. Isolation and characterization of an *Enterobacter* cloacae strain that reduces hexavalent chromium under anaerobic conditions. *Appl. Environ. Microbiol.,* 1989; **55**(7): 1665–1669.
10. Shen, H.; Wang, Y.T. Characterization of enzymatic reduction of hexavalent chromium by *Escherichia coli* ATCC 33456. *Appl. Environ. Microbiol.*, 1993; **59**(11): 3771-3777.
11. Elangovan, R.; Philip, L.; Chandraraj, K. Hexavalent chromium reduction by free and immobilized cell-free extract of *Arthrobacter rhombi-RE. Appl. Biochem. Biotechnol.,* 2010; **160**(1): 81-97.
12. Pattanapipitpaisal, P.; Brown, N.L.; Macaskie, L.E. Chromate reduction and 16S rRNA identification of bacteria isolated from a Cr(VI)-contaminated site. *Appl. Environ. Microbiol.,* 2001; **57** (1-2): 257-261.
13. Camargo, F.A.; Okeke, B.C.; Bento, F.M.; Frankenberger, W.T. *In vitro* reduction of hexavalent chromium by a cell-free extract of *Bacillus* sp. ES 29 stimulated by Cu^{2+}. *Appl. Microbiol. Biotechnol.,* 2003; **62**(5-6): 569–573.
14. Ge, S.M.; Zhou, M.H.; Dong, X.J.; Lu, Y.; Ge, S.C. Distinct and effective biotransformation of hexavalent chromium by a newly isolated under aerobic growth followed by facultative anaerobic conditions. *Appl. Microbiol. Biotechnol.,* 2013; **97**(5): 2131-2137.
15. Nepple, B.B.; Kessi, J.; Bachofen, R. Chromate reduction by *Rhodobacter sphaeroides. J. Ind. Microbiol. Biot.,* 2000; **25**(4): 198-203.
16. Park, C.H.; Keyhan, B.; Wielinga, B.; Fendorf, S.; Matin, A. Purification to homogeneity and characterization of a novel *Pseudomonas putida* chromate reductase. *Appl. Environ. Microbiol.,* 2000; **66**(5): 1788–1795.
17. Xu, L.; Luo, M.F.; Jiang, C.Y.; Wei, X.T.; Kong, P.; Liang, X.F.; Zhao, J.M.; Yang, L.R.; Liu, H.Z. *In vitro* reduction of hexavalent chromium by cytoplasmic fractions of *Pannonibacter phragmitetus* LSSE-09 under aerobic and anaerobic conditions. *Appl. Environ. Microbiol.,* 2012; **166**(4): 933-941.
18. Wang, P.C.; Mori, T.; Toda, K.; Ohtake, H. Membrane-associated chromate reductase activity from *Enterobacter cloacae. J. Bacteriol.,* 1990; **172**: 1670-1672.
19. Garbisu, C.; Alkorta, I.; Marýa, J.L.; Serra, J.L. Aerobic chromate reduction by *Bacillus subtilis. Biodegradation,* 1998; **9**(2): 133–141.
20. Mabbett, A.N.; Macaskie, L.E. A novel isolate of *Desulfovibrio* sp. with enhanced ability to reduce Cr(VI). *Biotechnol. Lett.,* 2001; **23**(9): 683-687.
21. Sarangi, A.; Krishnan, C. Comparison of *in vitro* Cr(VI) reduction by CFEs of chromate resistant bacteria isolated from chromate contaminated soil. *Bioresour. Technol.,* 2008; **99**(10): 4130–4137.
22. Desai, C.; Jain, K.; Madamwar, D. Evaluation of *In vitro* Cr(VI) reduction potential in cytosolic extracts of three indigenous *Bacillus* sp. isolated from Cr(VI) polluted industrial landfill. *Bioresour. Technol.,* 2008; **99**(14): 6059–6069.
23. Elangovan, R.; Abhipsa, S.; Rohit, B.; Ligh, P.; Chandraraj, K. Reduction of Cr(VI) by a *Bacillus* sp. *Biotechnol. Let.,* 2006; **28**(4): 247–252.
24. Sarangi, A.; Krishnan, C. Comparison of *in vitro* Cr(VI) reduction by CFEs of chromate resistant bacteria isolated from chromate contaminated soil. *Bioresour. Technol.,* 2008; **99**(10): 4130–4137.
25. Puzon, G.J.; Peterson, J.M.; Roberts, A.G.; Kramer, D.M.; Xun, L. A bacterial flavin reductase system reduces chromate to a soluble chromium(III)–NAD+ complex. *Biochem. Biophys. Res. Commu.,* 2002; **294**(1): 76–81.
26. Viamajala, S.; Smith, W.A.; Sani, R.K.; Apel, W.A.; Petersen, J.N.; Andrew, L.N.; Roberto, F.F.; Newby, D.T.; Brent, M.P. Isolation and characterization of Cr(VI) reducing *Cellulomonas* spp. from subsurface soils: implications for long-term chromate reduction. *Bioresour. Technol.,* 2007; **98**(3): 612–622.
27. Suzuki, T.; Miyata, N.; Horitsu, H.; Kawai, K.; Takamizawa, K.; Tai, Y.; Okazaki, M. NAD(P)H-dependent chromium(VI) reductase of *P. ambigua* G-1: a Cr(V) intermediate is formed during the reduction of Cr(VI) to Cr(III). *J. Bacteriol*., 1992; **174**(16): 5340–5345.
28. Appenroth, K.J.; Bischoff, M.B.; Gabrys, H.; Stoeckel, J.; Swartz, H.M.; Walczak, T.; Winnefeld, K. Kinetics of chromium (V) formation and reduction in fronds of the duckweed *Spirodela polyrhiza*—a low frequency EPR study. *J. Inorga. Biochem*., 2000; **78**(3): 235–242.
29. Ohtake, H.; Fuji, E.; Toda, K. Reduction of toxic chromate in an industrial effluent by use of a chromate-reducing strain *Enterobacter cloacae. Environ. Technol.,* 1990; **11**(7): 663–668.
30. He, Z.; Gao, F.; Sha, T.; Hu, Y.; He, C. Isolation and characterization of Cr(VI)-reducing *Ochrobactrum* sp. strain CSCr-3 from chromium landfill. *J. Hazard. Mater.,* 2009; **163**(2-3): 869-873.