

In Vitro Reduction of Hexavalent Chromium by Cytoplasmic Fractions of *Pannonibacter phragmitetus* LSSE-09 under Aerobic and Anaerobic Conditions

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Abstract Hexavalent chromate reductase was characterized and was found to be localized in the cytoplasmic fraction of a chromium-resistant bacterium *Pannonibacter phragmitetus* LSSE-09. The Cr(VI) reductase activity of cell-free extract (S_{12}) was significantly improved by external electron donors, such as NADH, glucose, acetate, formate, citrate, pyruvate, and lactate. The reductase activity was optimal at pH 7.0 with NADH as the electron donor. The aerobic and anaerobic Cr(VI)-reduction enhanced by 0.1 mM NADH were respectively 3.5 and 3.4 times as high as that without adding NADH. The Cr(VI) reductase activity was inhibited by Mn^{2+} , Cd^{2+} , Fe^{3+} , and Hg^{2+} , whereas Cu^{2+} enhanced the chromate reductase activity by 29% aerobically and 33% anaerobically. The aerobic and anaerobic specific Michaelis–Menten constant K_m of S_{12} fraction was estimated to be 64.95 and 47.65 $\mu\text{mol L}^{-1}$, respectively. The soluble S_{150} fractions showed similar activity to S_{12} and could reduce 39.7% and 53.4% of Cr(VI) after 1 h of incubation aerobically and anaerobically while the periplasmic contents showed no obvious reduction activity, suggesting an effective enzymatic mechanism of Cr(VI) reduction in the cytoplasmic fractions of the bacterium. Results suggest that the enzymatic reduction of Cr(VI) could be useful for Cr(VI) detoxification in wastewater.

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Introduction

Chromium and its compounds are widely used in industry, such as electroplating, leather tanning, water cooling, metal finishing, and wood preservation [1]. Hexavalent chromium [Cr(VI)] is generated as a by-product of these industrial processes [2]. Cr(VI)-containing wastewaters have become a well-recognized biohazard in water pollution control due to the extremely toxic, mutagenic, and carcinogenic effects of Cr(VI) on biological systems [3]. Cr(VI) is usually soluble under oxidizing conditions, and only limited removal can be achieved by conventional precipitation methods [4]. The major shortcomings of conventional treatments include poor removal efficiency, the production of large amounts of chemical sludge, and high cost of chemicals used for Cr(VI) reduction, especially for the removal of relatively low concentrations of Cr(VI) [5]. Several bacteria possess chromate reductase activity that can convert Cr(VI) to Cr(III), which is much less toxic and less soluble. Thus, biological reduction offers a viable alternative for Cr(VI) bioremediation [6–9].

Bioreduction of Cr(VI) can occur directly as a result of microbial metabolism [6, 10] or indirectly through a bacterial metabolite such as H₂S [11, 12]. The enzymatic reduction of Cr(VI) mostly involves a soluble cytosolic chromate reductase under aerobic conditions [6, 10, 13–16] or a membrane-associated chromate reductase under anaerobic conditions [17, 18]. On the other hand, *Pseudomonas* CRB5 [3] and *Escherichia coli* ATCC 33456 [19] possess a soluble reductase which can reduce Cr(VI) to Cr(III) both aerobically and anaerobically. It is suggested that the soluble chromate reductases found in chromate-reducing bacteria may have different primary roles [3]. Soluble chromate reductases are more amenable to protein engineering, which leads to increase the ability of the enzyme to function in the presence of other contaminants [6, 20]. In addition, other reductases such as hydrogenases [21] and nitroreductases [2] have been identified to function as chromate reductases.

Previous work has shown that a Cr(VI)-reducing strain, *Pannonibacter phragmitetus* LSSE-09 has the ability to reduce Cr(VI) under both aerobic and anaerobic conditions [22]. Strain LSSE-09 shows strong Cr(VI)-reduction potential under alkaline conditions, but the chromate reductase from *Pannonibacter* sp. has not been fully characterized to date. Elucidation of the enzymatic mechanisms involved in direct chromate reduction is crucial in designing an efficient Cr(VI) bioreduction process [16]. Hence, the objective of this study was to assess in vitro reduction of Cr(VI) by cytoplasmic fractions of this strain under both aerobic and anaerobic conditions. In addition, the present study evaluated the effects of electron donors and metal cations on Cr(VI)-reduction activity after 1 h of incubation.

Materials and Methods

Microorganisms and Culture Conditions

P. phragmitetus LSSE-09 was isolated from industrial sludge of a chromate factory in Henan province, China [22]. This strain was inoculated into 300 mL of Luria–Bertani medium (10 g tryptone, 10 g NaCl, 5 g yeast extract, and 1 L de-ionized water), cultured aerobically at 37 °C and 200 rpm for 18 h.

Preparation of Cell-Free Extracts and Periplasmic Components

Cells were harvested by centrifugation ($4,000\times g$) for 20 min at $4\text{ }^{\circ}\text{C}$, washed twice with de-ionized water, and then suspended in 20 mL Tris–HCl buffers (50 mM; pH 7.0). Suspensions of the cells were kept in an ice bath and disrupted with an ultrasonic probe (JY92-II, China) at 300 W with 6 s pulse and 12 s off mode for 99 times. The sonicate was centrifuged at $12,000\times g$ and $4\text{ }^{\circ}\text{C}$ for 20 min to pellet unbroken cells. The supernatant thus obtained was then filtered through $0.22\text{ }\mu\text{m}$ filters (Millipore, USA) and diluted to 60 mL to produce cell-free extract (S_{12}). Thirty milliliters of the S_{12} was then spun at $150,000\text{ g}$ for 1 h at $4\text{ }^{\circ}\text{C}$ to produce a soluble fraction (S_{150}) and a membrane pellet. The membrane pellet fraction was re-suspended in 10 mL Tris–HCl buffer (50 mM; pH 7.0).

Extraction of periplasmic components was accomplished using the osmotic shock procedure [3, 23]. Harvested cells were washed twice with cold 10 mM Tris–HCl buffer (pH 7.1) with 30 mM NaCl. Cells were rapidly mixed into 40% (w/v) sucrose in 20 mL Tris–HCl buffers (33 mM; pH 7.1), followed by the addition of 0.1 M EDTA to a concentration of 0.1 mM EDTA. The suspension was placed on a rotary shaker at $25\text{ }^{\circ}\text{C}$ for 10 min (180 rpm) and centrifuged at $12,000\times g$ and $4\text{ }^{\circ}\text{C}$ for 10 min to recover whole cells. After that, the obtained cell pellet was rapidly dispersed in 10 mL 0.5 mM MgCl_2 , gently stirred in an ice bath for 10 min and centrifuged. After centrifugation, the supernatant containing the osmotic shock proteins (periplasmic contents) was assayed for Cr(VI) reduction activity. Total protein in the supernatant fraction was measured before and after treatment with EDTA to ensure that the outer membrane was permeabilized.

Chromate Reductase Assay

Cr(VI)-reduction experiments by S_{12} were performed at $37\text{ }^{\circ}\text{C}$ under both aerobic and anaerobic conditions, as a function of electron donors and metal cations. The electron donors evaluated were NADH, glucose, acetate, formate, citrate, pyruvate, and lactate at 0.1 mM. The metal ions tested were NaCl, MgCl_2 , MnCl_2 , CaCl_2 , CdCl_2 , CuCl_2 , FeCl_3 , and HgCl_2 at 1.0 mM. The reaction system for the enzyme assay contained 5.6 mg L^{-1} of Cr(VI), 1.9 mL of S_{12} (2.0 mg mL^{-1}), and 0.1 mM of the electron donor or 1.0 mM of the metal cation to be tested in 50 mM Tris–HCl buffer (pH 7.0) with a total volume of 8.0 mL. Meanwhile, the Michaelis constant (K_m) and the maximum reaction rate (V_{max}) for S_{12} were determined by varying the substrate concentration from 3.5 to 9.3 mg L^{-1} at pH 7.0 under aerobic and anaerobic conditions. Controls for all experiments were supplemented with all components except S_{12} .

For aerobic experiments, the reaction mixtures were incubated in 20-mL glass bottles. Anaerobic experiments were performed using 20 mL anaerobic glass bottles. Each bottle was sparged with nitrogen for 3 min, and introduced by a syringe needle through the self-sealing rubber septum. Air displacement was achieved by inserting another syringe needle as the outlet. Samples were taken to analyze residual Cr(VI) concentrations after 1 h of incubation. Cr(VI)-reduction experiments by cell extracts (S_{12} , S_{150} , membrane fraction, and periplasmic contents) were performed under aerobic and anaerobic conditions, with 0.1 mM of NADH as the electron donor. Experiments were conducted in duplicate as described above. One unit of enzyme activity was derived as amount of enzyme that reduced 1.0 nmol of Cr(VI) per min at $37\text{ }^{\circ}\text{C}$. Protein was determined using Coomassie blue at 595 nm, with bovine serum albumin as the standard. The analysis of Cr(VI) in aqueous samples was performed using a UV–vis spectrophotometer (UNICO7200, USA) at 540 nm after complexation with 1,5-diphenylcarbazide [24].

Results and Discussion

Effect of pH

Since Cr (VI) reduction is enzyme-mediated, changes in pH will affect the degree of ionization of the enzyme, the protein's conformation, and the enzyme activity [25]. To find a suitable pH for effective chromium reduction by *S*₁₂, experiments were performed at different initial pH values (pH 6.0, 7.0, 7.5, 8.0, and 9.0) with NADH as the external electron donor. As shown in Fig. 1, chromate reductase activity was observed in a wide pH range with an optimum at 7.0, and the relative activity was much lower at pH 9.0 after 1 h of incubation. However, growing cells of the alkaline-tolerant strain LSSE-09 show a strong potential to intracellularly reduce Cr(VI) to Cr(III) at pH 9.0 [22]. It could be deduced that strain LSSE-09 maintains a cytoplasmic pH lower than the ambient pH under alkaline conditions. This could contribute to an alkaline pH homeostasis which plays an essential and dominant role in strain LSSE-09 to lower the cytoplasmic pH, because bacteria must maintain an optimal cytoplasmic pH that supports growth [26]. In addition, the reductase activity proceeded at a higher rate under anaerobic conditions, which was similar to *Pseudomonas* CRB5 [3]. The anaerobic reduction would be advantageous for bacterial removal of chromate due to the low energy requirement and small excess sludge production [27].

Effect of External Electron Donors

Figure 2a shows the effect of different electron donors on chromate reductase activity of *P. phragmitetus* LSSE-09 after 1 h of incubation. The blank in Fig. 2a is only *S*₁₂ without electron donors. The reductase activity increased on supplementation in the reaction mixtures with all tested electron donors, suggesting that external electron donors were essential to improve the reductase activity. Glucose has been reported to act as an electron donor to increase Cr(VI) reduction rate by *Bacillus sphaericus* AND 303 [13]; also, formate-dependent Cr(VI) reductases have been reported in *Shewanella putrefaciens* MR-1 [28]. Among all tested electron donors, *S*₁₂ incubated with NADH showed the most significant enhanced effect on dichromate reduction activity. The aerobic and anaerobic Cr(VI)-reduction enhanced by 0.1 mM NADH were respectively 3.5 and 3.4 times as high as that without

Fig. 1 Effect of pH on chromate reductase activity of *S*₁₂ under aerobic and anaerobic conditions with 0.1 mM NADH (100% relative activity—aerobic, 1.31 ± 0.12 units mg^{-1} protein; anaerobic, 1.79 ± 0.06 units mg^{-1} protein)

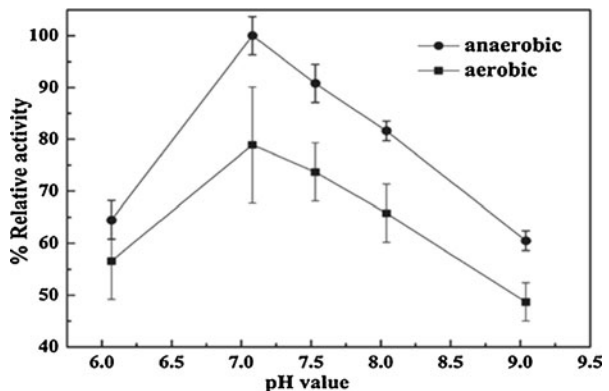
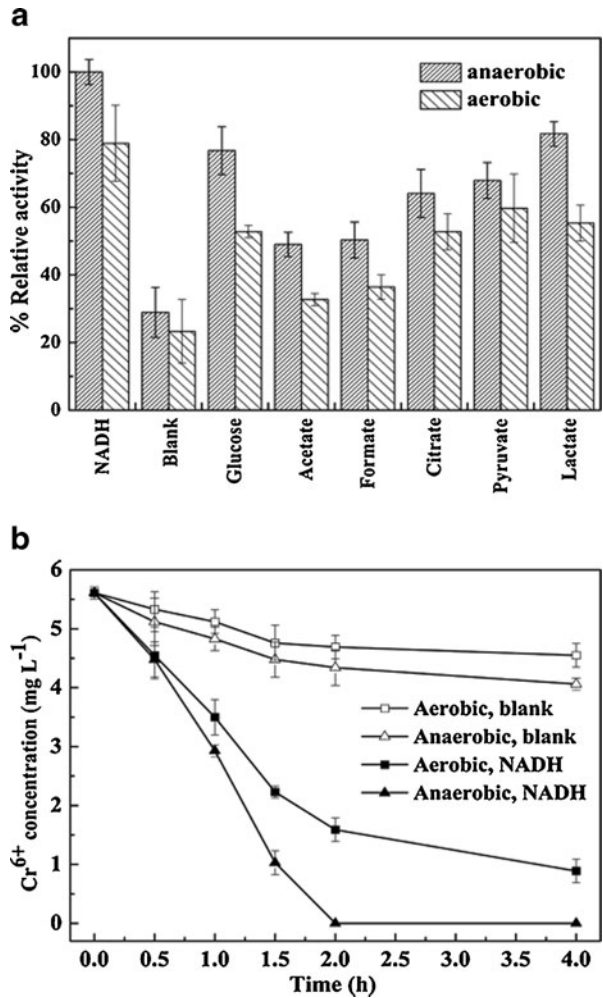


Fig. 2 Effect of electron donors on chromate reductase activity of S_{12} at initial pH 7.0 under aerobic and anaerobic conditions: **a** effect of different electron donors (100% relative activity—aerobic, 1.31 ± 0.12 units mg^{-1} protein; anaerobic, 1.79 ± 0.06 units mg^{-1} protein); **b** Cr(VI)-reduction by S_{12} with or without NADH



the addition of NADH after 1 h of incubation. Other researchers have also reported that Cr(VI)-reduction by the soluble fractions of *Bacillus* sp. [10, 29], *Pseudomonas putida* PRS2000 [14], and MK1 [6] required NADH as an electron donor for activity. In comparison, pseudomonad CRB5 [3], *B. sphaericus* AND 303 [13], and *E. coli* ATCC 33456 [19] can use endogenous electron donor reserves and do not require the presence of an external electron donor.

Time course of Cr(VI)-reduction by S_{12} at an initial Cr(VI) concentration of 5.6 mg L^{-1} Cr(VI) is shown in Fig. 2b. There was only a slight level of Cr(VI)-reduction without external electron donors under both aerobic and anaerobic conditions after 4 h of incubation, whereas the reductase activity was significantly improved by NADH. When supplemented with 0.1 mM NADH, the extent of reduction was rapid during the first 1 h of incubation, and S_{12} at a concentration of 2.0 mg mL^{-1} completely reduced 5.6 mg L^{-1} Cr(VI) within 2 h anaerobically, compared with 71.6% aerobically. Approximately 84.1% reduction was observed aerobically after 4 h of incubation with NADH.

Effect of Metal Cations

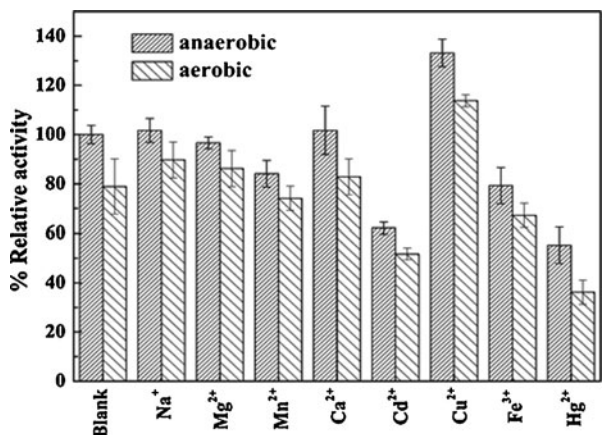
The effect of different metal cations on the chromate reductase activity of *P. phragmitetus* LSSE-09 after 1 h of incubation was determined as exhibited in Fig. 3. The blank in Fig. 3 is S_{12} added with 0.1 mM NADH without metal cations. Among the metal ions tested, Na^+ , Mg^{2+} , and Ca^{2+} did not exhibit any significant effect on the reductase activity, whereas the presence of Mn^{2+} , Cd^{2+} , Fe^{3+} , and Hg^{2+} inhibited Cr(VI) reduction at various levels. Hg^{2+} strongly inhibited the enzyme with 59% and 45% reduction in relative activity aerobically and anaerobically, presumably because mercury could bind to a variety of enzyme systems, with a particular affinity for ligands containing SH groups [10]. The negative effect of Hg^{2+} on Cr(VI) reduction by other chromate reductases was also reported [10, 29].

It is important to note that Cu^{2+} enhanced the chromate reductase activity by 29% aerobically and 33% anaerobically. Similarly, enhancement of chromate reductase activity by Cu^{2+} has been observed in case of *Bacillus* sp. ES 29 [10], *Bacillus* sp. RE [29], and *Pseudomonas* sp. G1DM21 [16] under aerobic conditions. It has been reported that Cu^{2+} is a transition metal that is a prosthetic group for many reductase enzymes. The role of Cu^{2+} in stimulation of Cr(VI) reductase could be attributed to its action as a protective agent for electron-transport, as a single electron redox center and as a shuttle for electrons between protein subunits [10, 30]. In addition, for the oxygen-sensitive chromate reductase of strain LSSE-09 which shows higher activity under anaerobic conditions, it is also possible that Cu^{2+} is indirectly involved in the protection of chromate reductase from O_2 [10]. On the other hand, Cu^{2+} has been known to inhibit the chromate reductase from *P. putida* [6] and *B. sphaericus* AND 303 [13].

Kinetic Analysis

Cr(VI)-reduction by chromate reductase showed strong dependence on substrate concentration, and the aerobic and anaerobic apparent K_m and the maximum specific reduction rate V_{max} values for S_{12} were determined. The kinetic constants were calculated by fitting the initial rate data to a double-reciprocal Lineweaver–Burk plot of $1/V$ [micromoles Cr(VI) per minute per gram protein] versus $1/[Cr(VI)]$ (micromoles per liter) derived from a linear transformation of the Michaelis–Menten equation. This allowed the estimation of the specific K_m and V_{max} for cell-free extract reduction. The total protein in the S_{12} fraction was measured and then added at a concentration of 2.0 mg mL^{-1} .

Fig. 3 Effect of different metal cations on chromate reductase activity of S_{12} (100% relative activity—aerobic, 1.31 ± 0.12 units mg^{-1} protein; anaerobic, 1.79 ± 0.06 units mg^{-1} protein)



As shown in Fig. 4a, aerobic reduction by S_{12} resulted in an apparent K_m of 64.95 and a maximum specific velocity, V_{max} , of 2.07 nmol Cr(VI) $\text{min}^{-1} \text{mg}^{-1}$ protein. Accordingly, the anaerobic K_m and V_{max} was 47.65 $\mu\text{mol L}^{-1}$ and 2.83 nmol Cr(VI) $\text{min}^{-1} \text{mg}^{-1}$ protein, as shown in Fig. 4b. The aerobic and anaerobic K_m values are similar to that reported for *Arthrobacter rhombi* RE without the presence of NADH under aerobic conditions [31]. In this study, the anaerobic K_m value is less than the aerobic one, suggesting that the crude reductase has higher activity under anaerobic condition.

Localization and Characterization of Chromate Reductase Activity

The cellular distribution of the soluble enzyme was investigated by monitoring the Cr(VI)-reducing activity in the cell-free extract fractionated by ultracentrifugation and the isolated periplasmic contents of *P. phragmitetus* LSSE-09. As shown in Table 1, the soluble S_{150} fractions showed similar activity to S_{12} and could reduce 39.7% and 53.4% of Cr(VI) after 1 h of incubation aerobically and anaerobically. No obvious reduction of Cr(VI) using the membrane fraction was observed under aerobic and anaerobic conditions. The results

Fig. 4 Effect of Cr (VI) concentrations on chromate reductase activity of S_{12} with 0.1 mM NADH at initial pH 7.0: **a** aerobic; **b** anaerobic

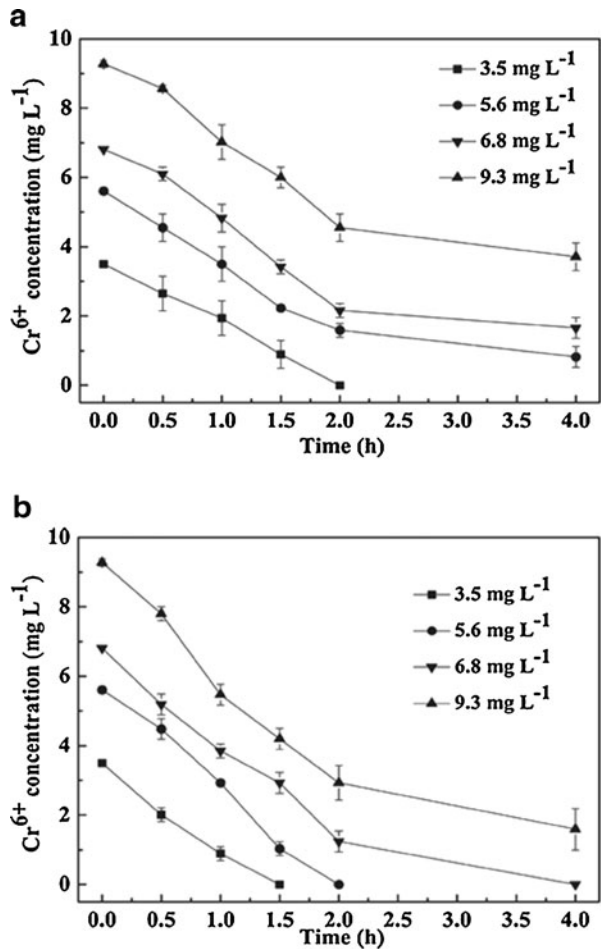


Table 1 Localization of chromate reductase activity in cell fractions of *P. phragmitetus* LSSE-09

Cell fractions	Initial protein concentration, mg mL ⁻¹	Chromate reductase activity, units mg ⁻¹ protein		Cr(VI) reduction, %	
		Aerobic	Anaerobic	Aerobic	Anaerobic
Cell-free extract (S ₁₂)	0.518	1.31±0.12	1.79±0.06	37.2±3.5	50.9±1.8
Soluble fraction (S ₁₅₀)	0.470	1.54±0.14	2.07±0.20	39.7±3.5	53.4±5.3
Membrane fraction	0.249	0	0.27±0.13	0	3.7±1.8
Periplasmic contents	0.457	0	0.34±0.09	0	6.2±1.8

Initial Cr(VI) concentration, 5.7 mg L⁻¹

Initial NADH concentration, 0.1 mM

Cr(VI) reduction was measured after 1 h of incubation

indicated that the enzyme activity was mainly associated with the soluble fraction (S₁₅₀), not with the re-suspended membrane fraction as reported for pseudomonad CRB5 [3]. For Cr(VI) reduction by *E. coli* ATCC 33456 [19], under aerobic and anaerobic conditions, Shen and Wang have indicated that there was minor Cr(VI) reductase activity associated with the membrane electron transport chain, although Cr(VI) reduction this strain was largely due to soluble reductase activity. However, whether Cr(VI) reduction in strain LSSE-09 is carried out by the respiratory-chain-associated electron transport activity remains unclear. On the other hand, most soluble cytosolic chromate reductases reported could only reduce Cr(VI) under aerobic conditions [6, 10, 13–16].

Since reduction is associated with the soluble fraction, the enzyme responsible could be either cytoplasmic or periplasmic in origin [3]. After EDTA treatment to permeabilize the outer membrane, the amount of protein in solution outside the cells increased significantly, indicating that periplasmic proteins were released. However, there was only slight Cr(VI)-reduction using the periplasmic fraction under aerobic and anaerobic conditions when the periplasmic protein concentration was approximately the same as the concentration in the S₁₅₀ fraction. It could be deduced that the soluble chromate reductase was present in cytoplasmic fractions of *P. phragmitetus* LSSE-09. Further investigation is being undertaken to purify and characterize the soluble chromate reductase from *P. phragmitetus* LSSE-09.

Conclusions

The present study presented the localization and characterization of the Cr(VI) reductase of *P. phragmitetus* LSSE-09. External electron donors were essential to improve the Cr(VI) reductase activity, and NADH showed the most significant increase in activity under both aerobic and anaerobic conditions. The Cr(VI) reductase activity which processed at a higher anaerobic rate was not stable in presence of Mn²⁺, Cd²⁺, Fe³⁺, and Hg²⁺, whereas the activity was obviously enhanced by Cu²⁺ under both aerobic and anaerobic conditions, presumably because of its electron-transport protection, acting as a single electron redox center and as a shuttle for electrons between protein subunits. Cu²⁺ could also be indirectly involved in the protection of chromate reductase from O₂ for oxygen-sensitive reductase of strain LSSE-09. Chromate reductase assays of the soluble fraction (S₁₅₀) have shown a high Cr(VI) reductase activity while the periplasmic contents showed no obvious reduction activity, implicating the localization of enzyme in the cytoplasmic fraction. Characterization

of the chromate reductase present in strain LSSE-09 indicates that it may be useful for Cr (VI) bioremediation.

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