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Identification and hexavalent chromium reduction characteristics of *Pannonibacter phragmitetus*

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Abstract A hexavalent chromium [Cr(VI)] reducing bacterial strain was isolated from chromium-containing slag. It was identified as *Pannonibacter phragmitetus* based on physiological, biochemical characteristics and 16S rRNA gene sequence analysis. This bacterium displayed great Cr(VI) reduction capability. The Cr(VI) could be completely removed in 24 h under anaerobic condition when the initial concentration was 1,917 mg L^{-1}, with the maximum reduction rate of 562.8 mg L^{-1} h^{-1}. The Cr(VI) reduction rate increased with the increase of Cr(VI) concentration. *P. phragmitetus* was able to use many carbon sources such as lactose, fructose, glucose, pyruvate, citrate, formate, lactate, NADPH and NADH as electron donors, among which the lactate had the greatest power to promote the reduction process. Zn^{2+}, Cd^{2+} and Ni^{2+} inhibited, while Cu^{2+}, Pb^{2+}, Mn^{2+} and Co^{2+} stimulated the reduction. The optimum pH and temperature for reduction were 9.0 and 30 °C, respectively. The results indicated that this strain had great potential for application in the bioremediation of chromate-polluted soil and water systems.

Keywords Identification · Cr(VI) reduction · Cr(III) · *P. phragmitetus*

Introduction

Chromium is an important heavy metal that is widely used in industrial processes such as ore refining, electroplating, production of steel and alloys, metal plating, tannery, wood preservation, pigmentation, etc. [1]. Untreated Cr(VI)-containing waste generated from the above processes was directly released into the environment and caused serious pollution. There are nine valency states of chromium ranging from −2 to +6 in nature, but only Cr(III) and Cr(VI) are of major environmental significance [2]. Cr(VI) is relatively more water soluble, bioavailable, reactive and toxic than Cr(III). Compounds containing Cr(VI) were reported as mutagenic, carcinogenic and teratogenic [3–5]. Cr(III) is thermodynamically stable and less toxic. It is also an essential micronutrient for proper glucose metabolism that stimulates the enzyme system and stabilizes nucleic acids [6]. Consequently, the reduction of toxic Cr(VI) to stable Cr(III) is considered as an efficient way to recover chromate pollution from soil and water systems. In addition, Cr(III) is easily formed as precipitate $Cr(OH)_3$ or Cr_2O_3 [7].

Detoxification and removal of Cr(VI) through reduction of Cr(VI) to Cr(III) can be carried out with physicochemical or biological methods. The conventional physicochemical treatment technologies include ion exchange, chemical reduction, adsorption, precipitation and electrodialysis [8]. However, these methods consume high amounts of energy and large quantities of chemical reagents and therefore are not economically feasible. Furthermore, the resultant metal-containing chemical sludge is a potential source of metal pollution [9]. Alternatively, biological processes for treating chromium-contaminated sites are becoming very promising because of the high efficiency, low operating cost, short operation time and eco-friendliness [10].

Bioremediation of Cr(VI) by microorganisms has emerged as a potential alternative for detoxification and recovery of toxic and valuable metals from polluted environments. Three microbial Cr(VI) reduction mechanisms

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have been described: Chromate reduction under aerobic conditions is commonly associated with soluble reductases that use NADH or NADPH as cofactors; Cr(VI) was used as an electron acceptor in the electron transport chain under anaerobic conditions; Cr(VI) may also be reduced by unspecific reactions associated with organic compounds such as amino acids, nucleotides, sugars, etc. [11]. Various bacteria capable of converting Cr(VI) into less toxic Cr(III) have been identified under both aerobic and anaerobic conditions, including *Achromobacter* sp. [12], *Bacillus* sp. [13, 14], *Providencia* sp. [15], *Pseudomonas aeruginosa* [16], *Burkholderia cepacia* [17], *Escherichia coli* [18, 19], etc. However, the Cr(VI) reduction efficiency of these strains were not high enough.

In a previous study, we have reported a strain having the ability for Cr(VI) reduction. It was identified as *P. phragmitetus* [1]. *P. phragmitetus* strain had not been characterized as being able to reduce Cr(VI) previously (to our knowledge). In the present study, another strain stored in our laboratory was carefully investigated. It was also identified as *P. phragmitetus* and had much stronger Cr(VI) reducing power.

Materials and methods

Bacterial strain and cultural conditions

The Cr(VI) reducing strain was isolated from the soil collected from the slag sites of chromate ore processing in Changsha, China and stored in the School of Metallurgical Science and Engineering, Central South University, China. Cells were grown in the nutrient medium containing 5 g tryptone, 2 g NaCl, 5 g yeast extract and 5 g sodium lactate in 1 L distilled water (the contents of $K_2Cr_2O_7$ was properly adjusted according to experimental requirement) at pH 9 with shaking speed of 150 rpm. All media were autoclaved at 121 °C for 25 min before use.

Identification of the strain reducing Cr(VI)

Genomic DNA was extracted and purified using the TIANamp Bacteria DNA Kit (TIANGEN China). The 16S rRNA was amplified from chromosomal DNA of Cr(VI) reduced strain by PCR. The primers were 27F and 1492R [20]. The PCR amplification was performed as follows: each reaction was performed in a final volume of 50 μL, containing 25 μL 2× mix, 1 μL each primer, 1 μL DNA sample and 22 μL deionized water. The reaction mixture was subjected to 30 cycles of amplification, denaturation at 94 °C for 30, annealing at 55 °C for 60 s and extension at 72 °C for 60 s. PCR products were purified using the TIAquick Midi Purification Kit (TIANGEN China) and sent to Shanghai Biological Company for sequencing. The sequence was aligned with that of other bacterial species obtained from the GenBank database and corresponding sequences were downloaded. CLUSTALX program was used to align bacteria nucleotide sequences and construct phylogenetic tree. Physiological and biochemical characteristics of the strain reducing Cr(VI) were performed using the methods as described previously [21].

Preparation of cell suspension

The *P. phragmitetus* strain that grew for about 12 h in 200 mL of liquid nutrient medium was harvested by centrifugation at 10,000 rpm for 10 min at 4 °C, washed twice with Tris–HCl (100 mmoL L^{-1}, pH 9.0) and resuspended in 200 mL with the same buffer. Flasks containing cell suspensions were sealed with butyl rubber stoppers and purged with N_2 for 20 min to free O_2 and gain an anaerobic condition. Cell concentration expressed as OD_{600} was retained to 1.27. Then, the cell suspension was placed in flasks and stored at 4 °C in a refrigerator before use.

Cr(VI) reduction experiments

The reaction mixtures were set up in 40-mL sealed serum bottles, and the final volume was 20 mL. Cr(VI) reduction studies were started by the addition of Cr(VI) (200 mg L^{-1}) under anaerobic condition. Samples were withdrawn at intervals by a sterile syringe, and supernatants were analyzed for residual Cr(VI).

To characterize the Cr(VI) reduction efficiency of *P. phragmitetus*, the effect of temperature (20, 25, 30, 35, 40, 45 and 50 °C), pH (6, 7, 8, 9, 10, 11 and 12), initial cell concentration (0.059×10^9 to 3.41×10^9 cells mL^{-1}) and initial Cr(VI) concentration (104–2,031 mg L^{-1}) were investigated via resting cells or growing cells. Cr(VI) reduction was studied under anaerobic condition in 40-mL sealed serum bottles with a 20-mL mixture. The mixture was obtained from the suspension (2% sodium lactate was added) prepared above or from log bacterial culture with the desired concentration of cells and supplemented with appropriate amount of Cr(VI). It was incubated at the optimal temperature and pH with shaking condition (150 rpm). Samples were drawn at definite time intervals, centrifuged at 10,000 rpm for 10 min and the supernatant fluid was analyzed for residual Cr(VI). All the experiments were done in triplicate.

The effect of different carbon sources (2%) including lactose, fructose, glucose, pyruvate, citrate, formate, lactate, NADPH and NADH on Cr(VI) reduction was investigated in cell suspension containing 200 mg L^{-1} Cr(VI). Cell suspension with 200 mg L^{-1} Cr(VI) and no carbon

source was set as control. The effect of electron donors on Cr(VI) reduction was also investigated in cell suspension containing 200 mg L^{-1} Cr(VI), 2% sodium lactate and one of the following heavy metals: 20 mg L^{-1} (Co^{2+}, Cd^{2+}) and 100 mg L^{-1} (Cu^{2+}, Mn^{2+}, Ni^{2+}, Pb^{2+}, Zn^{2+}). The cell suspension with 200 mg L^{-1} Cr(VI), 2% sodium lactate and no heavy metal was set as control. All cell suspensions were incubated at the optimal temperature and pH with shaking (150 rpm). All the experiments were done in triplicate.

Analysis methods

Samples were withdrawn periodically, via a syringe, and the decrease in chromate concentration in supernatant with time was estimated using the Cr(VI)-specific colorimetric reagent *S*-diphenylcarbazide (DPC), which was prepared in acetone/H_2SO_4 to minimize deterioration as described previously [22]. Cell density was determined following previous work [12]. Reduction rates were designated as the amount of Cr(VI) reduced per hour (mg L^{-1} h^{-1}). Cell suspensions used in the above experiments were of original concentration, approximately 3.41×10^9 cells mL^{-1} ($OD_{600} = 1.27$), and the ratio of cell to Cr(VI) was 1.1×10^{10} cells mg^{-1}.

Droplets of a small amount of liquid mixture from different stages of Cr(VI) reduction were placed on glass slides. After 15 min adsorption, glutaraldehyde (2.5%) was added for immobilization for 1 h. Then the sample was dehydrated in gradient using 30–70% ethanol and replaced by isoamyl acetate for 30 min. Through critical point drying (HITACHI HCP-2 Critical PointDryer) and ion sputtering (Eiko IB-3 ion plating machine), the sample was observed via scanning electron microscopy (SEM) (JEOL JSM-6360LV).

Results and discussion

Identification of Cr(VI) reducing strain

The bacterial strain with high ability of reducing Cr(VI) was selected for identification. Its physiological and biochemical characteristics were basically similar to *P. phragmitetus* (Table 1). The 16S rRNA sequence size was 1,388 bp and displayed over 99% similarity with that of *P. phragmitetus*. The partial 16S rRNA gene sequence of the microorganism has been deposited in GenBank with the accession number JN626199. The phylogenetic tree (Fig. 1) showed that the stain tightly clustered with *P. phragmitetus*. Accordingly, the strain was indentified as *P. phragmitetus*.

Effects of temperature and pH on Cr(VI) reduction

Temperature is an important factor affecting biological Cr(VI) reduction. Cr(VI) reduction by *P. phragmitetus* was evaluated under seven different temperatures, namely, 15, 20, 25, 30, 35, 40 and 45 °C. The Cr(VI) was reduced fairly well (31.7–99.1%) from 15 to 45 °C with the optimum at 30 °C (Fig. 2a). However, the optimum temperature for growth was 35 °C (Fig. 2b), where the Cr(VI) removal was 96.9%. It is indicated that Cr(VI) reduction would be inhibited if the temperature is too high or low. Therefore, if the strain was used for chromium pollution recovery, the temperature should be controlled at 30–35 °C. As reported, a wide range of temperature from 10 to 40 °C was recorded for Cr(VI) reduction by *Enterobacter cloacae* strain HO1 with the optimum of 30 °C [25]. Bacterial growth and microbial Cr(VI) reduction of strain DM1 were investigated under 30–45 °C. The result showed that both the optimal growth temperature and Cr(VI) reduction temperature were 35 °C [26].

The variation of Cr(VI) concentration under different pH values of 6–12 is shown in Fig. 2c. The Cr(VI) was reduced when the pH ranged from 7 to 11. There was no obvious difference in Cr(VI) reduction at pH 9 and 10, but Cr(VI) reduction almost ceased at pH 6 and 12. The optimum value was 10. However, the optimum pH for growth was 8 (Fig. 2d). In addition, with the change of Cr(VI) concentration, the pH value of all media had a trend of change to about 8 (data not shown). The result indicated that the operation of *P. phragmitetus* reducing Cr(VI) mechanism may be priority to other physiological mechanisms to ensure the bacterial growth in the environment with high chromium content. In a previous report, the optimal pH was also 9 for Cr(VI) reduction by Gram-positive bacterium [27] and *Ochrobactrum* sp. strain CSCr-3 [28].

Effects of different electron donors on Cr(VI) reduction

During the process of reduction, the Cr(VI) was converted into Cr(III) via accepting three electrons. Consequently, there must be electron donor(s) to provide electrons. A variety of organic compounds were utilized by *P. phragmitetus* as electron donors for Cr(VI) reduction. In the resting cell suspension, when lactose, fructose, glucose, pyruvate, citrate, formate, lactate, NADPH and NADH were used as carbon source, the Cr(VI) reduction activity was 30, 20, 7, 78, 5, 17, 123, 70 and 90%, respectively. Those values were more than that of the control (Fig. 3). The result was consistent with previous report that *Streptomyces griseus* NCIM2020 was capable of using many substrates including glucose, sucrose, acetate, citrate, tartrate, glycerol and ethanol as electron donors, and the Cr(VI) reduction activity was also increased in varying

Table 1 Differential physiological and biochemical properties of the strain reducing Cr(VI) and its closest phylogenetic strains

Characteristic	Cr(VI) reduced strain	*Pannonibacter phragmitetus* [23]	Genus *Achromobacter* [23]
Micromorphology	Cells rod shaped, single, slightly curved, 0.3 × 2–3 μm	Cells rod shaped, single, slightly curved, 2–4 × 0.3–0.6 μm	Cells straight rod shaped, single, 0.8–1.2 × 2.5–3.0 μm
Colony morphology	White, round with smooth surface and margin, center raised	White, round with smooth surface and margin, center raised	ND
Colony size	Small, 2–4 mm	Small, 2–4 mm	ND
Motility	+	−	+
Oxygen concentration	Facultative anaerobe	Facultative anaerobe	Aerobe
Gram reaction	−	−	−
Citrate utilization	+	+	−
Urease activity	−	+	−
Phosphatase	+	+	ND
Oxidase	+	+	+
Catalase	+	+	+
Hydrolysis of	−	−	−
Methyl red test	+	–	ND
Voges-Proskauer test	−	−	ND
Acid production from			
D-Xylose	+	+	+
D-Fructose	+	+	−
D-Glucose	+	+	+
L-Arabinose	+	+	+
Lactose	+	+	−
Sucrose	+	+	−
Double hydrolysis of	+	+	−
Indole production	−	−	−
Salt (10%) tolerance	+	+	−
Nitrate reduction	+	+	+
H_2S production	+	−	ND
Optimum pH	7–10	7–10	ND

Characters are scored as: +, positive; −, negative

ND No data available

degrees [29]. Electron donors provide electrons for Cr(VI) reduction. The varying degrees of chromium reduction activity under various electron donors might be probably because the electron-accepting ability of reductase enzymes for different electron donors was different.

Effects of initial cell concentrations and chromate concentrations on Cr(VI) reduction

Effects of initial bacterial cell concentrations (0.059×10^9 to 3.41×10^9 cells mL^{-1}) on Cr(VI) (250 mg L^{-1}) reduction are normally distributed (Fig. 4a). Accordingly, Cr(VI) reduction rates were equal to the slope of the line. From the lowest cell concentration (0.059×10^9 cells mL^{-1}) to the highest cell concentration (3.41×10^9 cells mL^{-1}), the Cr(VI) was completely reduced within 9.5, 6, 4, 2.5 and 2 h, respectively, and the reduction rates increased from 26.5 to 125.0 mg L^{-1} h^{-1}. Cr(VI) reduction by *P. phragmitetus* increased with the increase in initial cell concentrations from 0.059×10^9 to 3.41×10^9 cells mL^{-1} as observed by other researchers [30, 31].

Effects of initial chromate concentrations on Cr(VI) reduction by growing and resting cell (3.41×10^9 cells mL^{-1}) of *P. phragmitetus* are shown in Fig. 4b, c. The initial rate of Cr(VI) reduction by growing cell increased with the increase of chromate concentration from 100 to 700 mg L^{-1}. When the chromate concentration was up to 900 mg L^{-1}, the initial rate was decreased. In addition, the higher the chromate concentration, the lower was the initial rate. This may be because the high concentration of Cr(VI) would adversely affect the growth of cells. But through a period of adaptation, the reduction rate increased to a relatively high value and then decreased. This may be attributed to the pH change ("Effects of temperature and

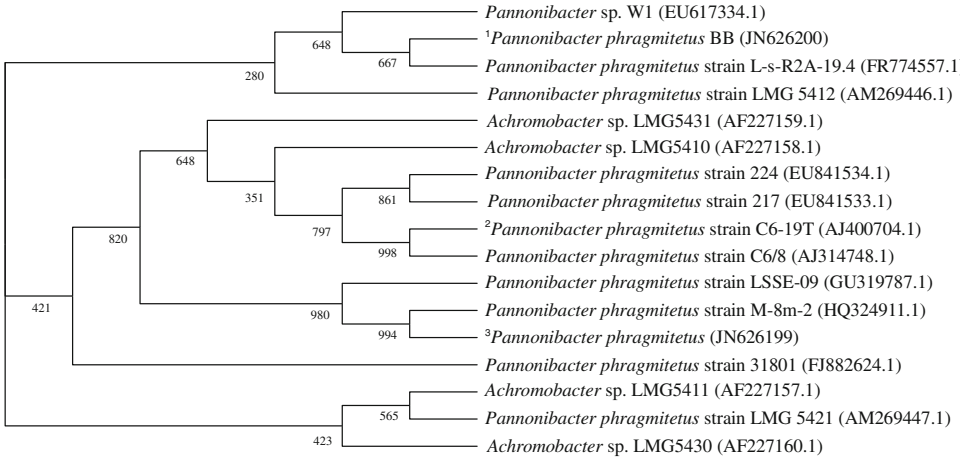


Fig. 1 Phylogenetic tree obtained from 16 SrRNA sequence comparisons 1,355 bp bases showing the relationship between members of the family *Pannonibacter* and the strain stored in our laboratory. The bootstrap neighbor-joining tree (random number generator seed = 57, trails = 1,000) was constructed with Clustal X version 2.0. GeneBank accession numbers are in brackets. *Scale bar* 0.01 base differences per position. *1* Previously reported strain; *2* the first reported *Pannonibacter phragmitetus* strain [24]; *3* the Cr(VI) reducing strain in the present study

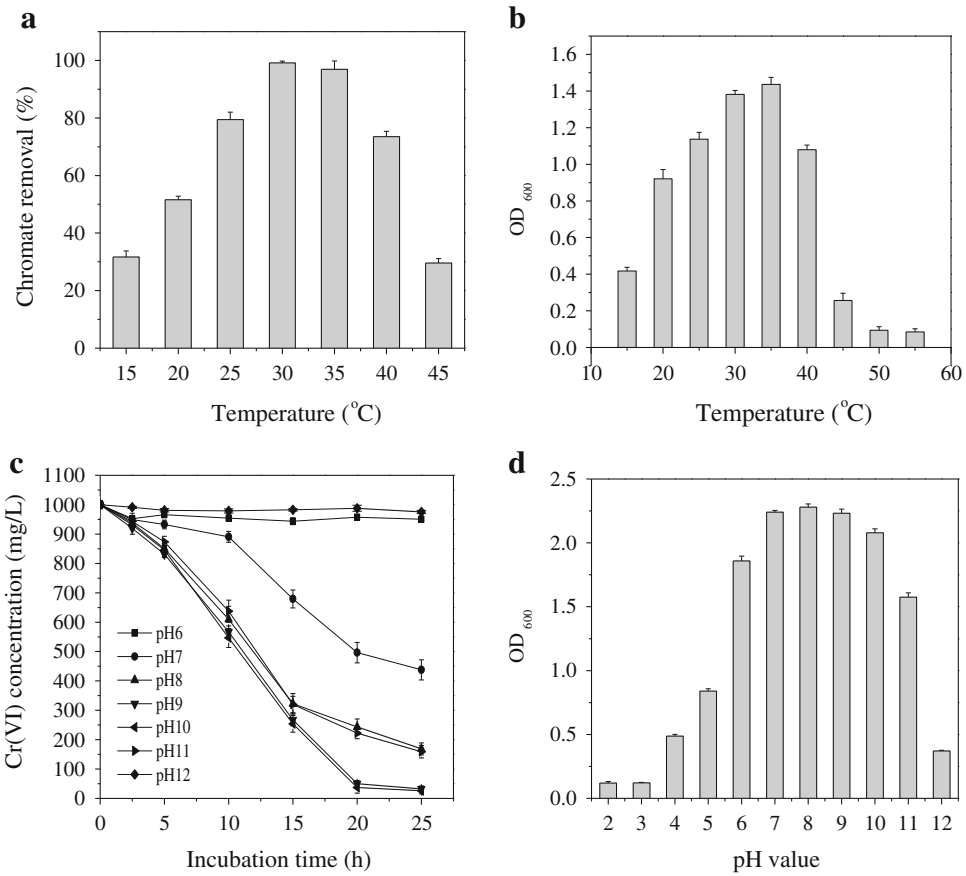


Fig. 2 Effects of temperature and pH on *P. phragmitetus* growth and Cr(VI) reduction

pH on Cr(VI) reduction") or cell degradation at the later stage of the reaction. The maximum Cr(VI) reduction rate and the largest reduction capacity by growing cell were up to 332.0 and 1,791 mg L^{-1}, respectively. Cr(VI) reduction by resting cell was different. The initial rate of Cr(VI) reduction increased with the increase of chromate concentrations from 300 to 2,031 mg L^{-1}, but decreased with the change of Cr(VI) concentrations for the same reason of pH change ("Effects of temperature and pH on Cr(VI) reduction"). The maximum Cr(VI) reduction rate and the largest reduction capacity were up to 562.8 mg L^{-1} h^{-1} and 1,917 mg L^{-1}, respectively. The maximum

Cr(VI) reduction rate and the largest reduction capacity of resting cells are all higher than those of growing cells; Cr(VI) concentration has little or no adverse effect on resting cells because these are not growing.

The Cr(VI) reduction efficiency by *P. phragmitetus* is very high. The maximum Cr(VI) reaction rate and the largest reduction capacity were both higher than those reported by other researchers [5, 30–32].

Effects of heavy metals on Cr(VI) reduction

As other metals also existed in industrial effluents, the effects of other heavy metal cations on Cr(VI) reduction by *P. phragmitetus* were also investigated (Fig. 5). The presence of Cu^{2+}, Pb^{2+}, Mn^{2+} and Co^{2+} significantly enhanced Cr(VI) reduction by 21, 12, 16 and 7%, respectively, while Zn^{2+}, Cd^{2+} and Ni^{2+} inhibited Cr(VI) reduction by 46, 28

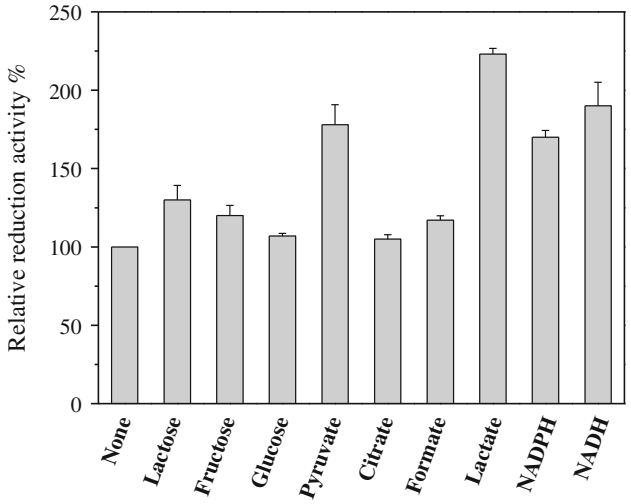


Fig. 3 Cr(VI) reduction by *P. phragmitetus* with different electron donors at pH 9 and 30 °C

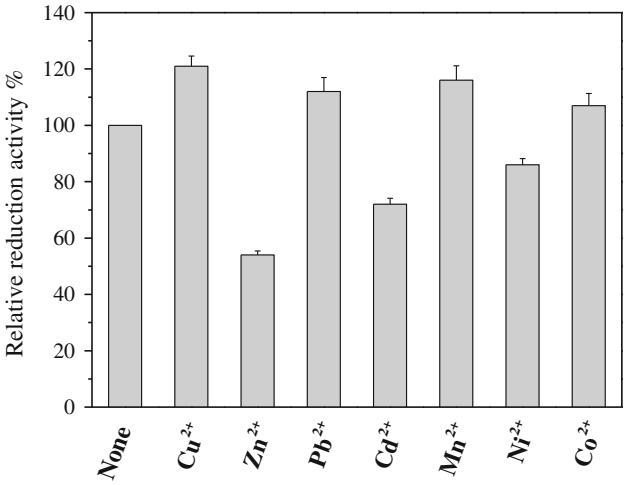
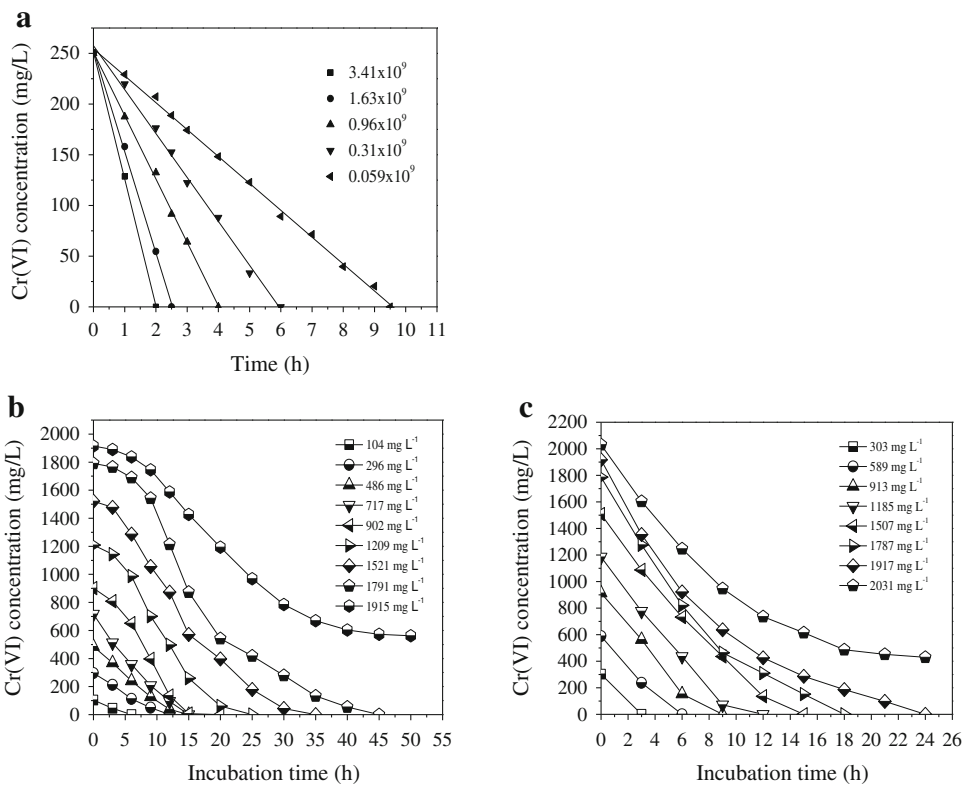


Fig. 5 Cr(VI) reduction by *P. phragmitetus* with different heavy metals at pH 9 and 30 °C

Fig. 4 Cr(VI) reduction by *P. phragmitetus* with different initial cell and Cr(VI) concentration at pH 9 and 30 °C



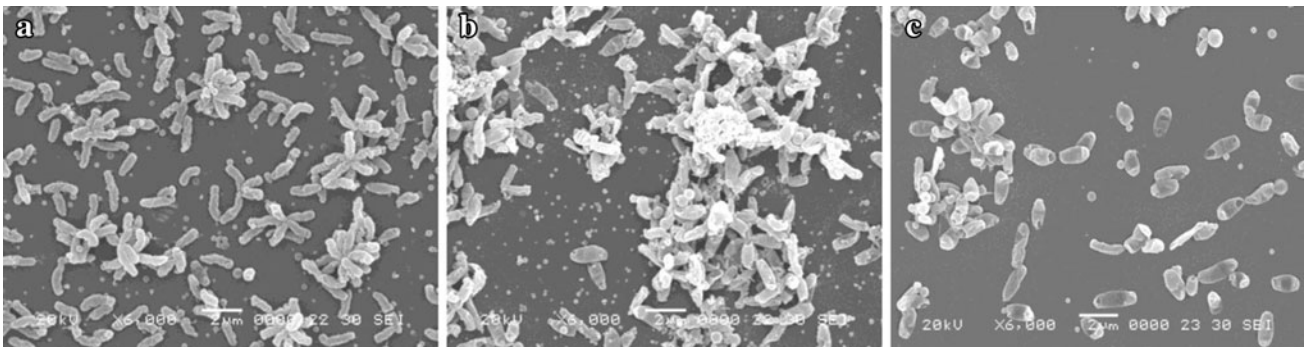


Fig. 6 Electron microscope photos of CRB1 cells that reduced 100 mg L^{-1} of Cr(VI): **a** initial stage of reduction; **b** middle stage of reduction; **c** last stage of reduction

and 14%, respectively. Thus, Cu^{2+} and Zn^{2+} showed the highest stimulatory and inhibitory effect on Cr(VI) reduction, respectively. Stimulatory effects of Cu^{2+}, Mn^{2+} and Co^{2+} on Cr(VI) reduction were also reported with *Ochrobactrum* sp. strain CSCr-3 [28] and *Ochrobactrum intermedium* strain SDCr-5 [30]. However, the inhibition of Cu^{2+} on Cr(VI) reduction by many other microorganism such as *Escherichia coli* [19, 31], etc., and the inhibitions of Pb^{2+} and Co^{2+} on Cr(VI) reduction by *Bacillus sphaericus* were also reported [33]. The mechanism of the effect of the heavy metals on Cr(VI) reduction is still unclear. As known, Cu^{2+} is the prosthetic group for many reductase enzymes. The main function of Cu^{2+} has been reported to be related to electron transport protection or acting as electron redox center and, in some cases, as a shuttle for electrons between protein subunits [34]. It was presumed that other stimulatory effect metals may have the same mechanism. Inhibitory effect metals may change pH or combine with functional groups of reductase enzymes during Cr(VI) reduction. Further studies of a reasonable mechanism are needed.

Bacterial cell morphology during the process of Cr(VI) reduction and reduction products

To further understand Cr(VI) reduction by *P. phragmitetus* at the macroscopic level, the resting cells of *P. phragmitetus* in three different Cr(VI) reduction periods were collected for SEM observation. As shown in Fig. 6, the distribution of resting cells was relatively uniform in buffer in the initial stage (Fig. 6a); in the middle stage, it was obviously observed that the resting cells agglomerated and reduced Cr(VI) synergistically (Fig. 6b); and in the last stage, resting cells became transparent, and the distribution was scattered again (Fig. 6c). The results indicated that Cr(VI) reduction by *P. phragmitetus* was a process of synergistic effect and that the reaction might be carried out more efficiently when the resting cells agglomerated. This gave an explanation for the above conclusion that Cr(VI) reduction was increased with an increase in the initial cell concentration (3.4).

During the process of Cr(VI) reduction by *P. phragmitetus*, the color of the reaction mixture changed from yellow to blue, and a large quantity of dark blue precipitate was formed. In our previous work [35], we have proved that the main components of the precipitate from Cr(VI) reduction by *P. phragmitetus* were chromium compounds. In addition, other microorganisms such as *Achromobacter* sp. [12] *Cellulomonas* spp. [36], etc. were reported to be able to produce chromium compounds during the process of Cr(VI) reduction.

Conclusions

A bacterial strain identified as *P. phragmitetus* was proved to be able to effectively reduce Cr(VI). Higher initial cell and Cr(VI) concentration and addition of lactate as carbon sources increased the Cr(VI) reduction ability of *P. phragmitetus*. The process of the reduction was enzymatic, so purification and characterization of chromate reductase is under way. Biological reduction of Cr(VI) by this strain can be used as an efficient and eco-friendly technique for Cr(VI) pollution control. Consequently, further understanding of the bacterial Cr(VI) reduction mechanism is necessary.

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