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Reduction of hexavalent chromium by *Pannonibacter phragmitetus* LSSE-09 stimulated with external electron donors under alkaline conditions

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ABSTRACT

A novel Cr (VI) resistant bacterial strain LSSE-09, identified as *Pannonibacter phragmitetus*, was isolated from industrial sludge. It has strong aerobic and anaerobic Cr (VI)-reduction potential under alkaline conditions. At 37 °C and pH 9.0, growing cells of strain LSSE-09 could completely reduce 100 and 1000 mg L^{-1} Cr (VI)–Cr (III) within 9 and 24 h, respectively under aerobic condition. Resting cells showed higher anaerobic reduction potential with the rate of 1.46 mg $g^{-1}_{(dry\ weight)}$ min^{-1}, comparing with their aerobic reduction rate, 0.21 mg g^{-1} min^{-1}. External electron donors, such as lactate, acetate, formate, pyruvate, citrate and glucose could highly increase the reduction rate, especially for aerobic reduction. The presence of 3000 mg L^{-1} acetate enhanced anaerobic and aerobic Cr (VI)-reduction rates up to 9.47 mg g^{-1} min^{-1} and 4.42 mg g^{-1} min^{-1}, respectively, which were 5 and 20 times faster than those without it. Strain LSSE-09 retained high activities over six batch cycles and NO_3^- and SO_4^{2-} had slightly negative effects on Cr (VI)-reduction rates. The results suggest that strain LSSE-09 has potential application for Cr (VI) detoxification in alkaline wastewater.

1. Introduction

Chromium and its compounds are widely used in industry, such as electroplating, leather tanning, water cooling, metal finishing and wood preservation [1,2]. Chromium occurs in the environment primarily in Cr (VI) and Cr (III) states [3]. Cr (VI) is known to be toxic to both plants and animals, as a strong oxidizing agent and potential carcinogen whereas Cr (III) is less toxic than Cr (VI) compounds [4]. Hence, the removal of Cr (VI) is more important than Cr (III) in water pollution control. The discharging limit for Cr (VI) has been instituted by most industrialized countries. Environmental Protection Agency (EPA) in US recommends that the amount of Cr (VI) in drinking water should be less than 50 μg L^{-1} [5]. Therefore, it is essential to remove Cr (VI) from wastewater before disposal.

Conventional methods for removing metals from aqueous solutions include chemical precipitation, oxidation/reduction, ion exchange, filtration, membranes and evaporation [6,7]. The major shortcomings of conventional treatments include costly safe disposal of toxic sludge, incomplete reduction of Cr (VI) and high cost for Cr (VI) reduction, especially for the removal of relatively low concentrations of Cr (VI) [8]. Biotechnological approaches that are designed to cover such niches have attracted worldwide attention recently [9]. Among biotechnological approaches, microbial reduction which is cost-effective and eco-friendly can offer a viable alternative [10,11].

The application of microbial reduction seems very promising, which presents continuous metabolic uptake of specific metals, self-replenishment and avoidance of separating biomass production [9]. Microbial reduction of the toxic Cr (VI) to the less toxic Cr (III) represents a useful and cost-effective detoxification process [12]. Development of a feasible microbial reduction process requires isolation of efficient chromate-reducing bacterial strains [10]. To date, several bacteria which can reduce Cr (VI) under both aerobic and anaerobic conditions have been reported, such as *Bacillus* QC 1–2 [13], *Pseudomonas fluorescens* LB300 [14], *Pseudomonad* CRB5 [15], *Escherichia coli* ATCC 33456 [16], *Agrobacterium radiobacter* EPS-916 [17] and *Achromobacter* Ch1 [18,19]. However, previous researches on bacterial reduction of Cr (VI) were mainly conducted under neutral conditions. For the detoxification of Cr (VI)-contaminated alkaline wastewater from chromate factories, an effective Cr (VI)-removing strain under alkaline conditions is an essential pre-requisite. Until recently the information about it was quite insufficient.

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Herein, an effective Cr (VI)-reduction strain, identified as *Pannonibacter phragmitetus*, was isolated from industrial sludge. Its Cr (VI)-reduction potential was characterized with the presence of growing and resting cells. The effect of external electron donors on Cr (VI)-reduction potential under both aerobic and anaerobic conditions was also investigated. In addition, the Cr (VI)-reduction mechanisms were investigated by applying electron paramagnetic resonance (EPR), transmission electron microscopy (TEM), and energy-dispersive spectrometry (EDS).

2. Materials and methods

2.1. Isolation and identification of the bacterium

Cr (VI)-reducing strain was isolated from the industrial sludge of a chromate factory in Henan Province, China. Samples were enriched in Luria-Bertani (LB) medium which contained 10 g tryptone, 10 g NaCl and 5 g yeast extract in 1 L distilled water, modified by supplementing 300 mg L^{-1} Cr (VI) in the form of $K_2Cr_2O_7$. The pH of medium was adjusted to 9.0 with 1.0 M NaOH. Cultures were performed at 37 °C with 150 rpm shaking. After enrichment, the cultures were purified by isolating single colonies on LB solid medium which contained 1.5% (w/v) agar. All media were autoclaved at 121 °C for 20 min prior to use, and all chemicals were of analytical reagent grade.

The Cr (VI)-reducing strain was characterized by phylogenetic and phenotypic studies. 16S rRNA gene sequence was amplified as described by Gerhardt et al. [20]. The amplified products were sequenced on an automated DNA sequencer (TECHNE Techgene, Britain). DNA BLAST searched at NCBI GenBank database. The 16S rRNA gene sequences were aligned with the CLUSTAL_X program, version 1.64b [21], and positions with insertions or deletions were excluded during calculations. A neighbour-joining phylogenetic tree was constructed using MEGA 4.0, based on evolutionary distances that were calculated with the Kimura two-parameter model [22].

2.2. Cr (VI) resistance and reduction capacity by growing cells

P. phragmitetus LSSE-09 cells were inoculated into 100 mL LB medium, cultured aerobically at 37 °C and 150 rpm for 12 h. Thereafter, 1.0 mL of the bacterial cultures was transferred into 100 mL of LB medium which contained 300 mg L^{-1} Cr (VI). The effect of initial pH (6.0, 7.0, 8.0, 9.0, and 10.0) was investigated, respectively. Aliquots of solution were withdrawn using a syringe at designed intervals and centrifuged at 12,000×*g* for 5 min. The supernatants were analyzed for residual Cr (VI) concentrations.

To characterize Cr (VI) resistance and reduction capacity by growing cells at initial pH 9.0, 100 mL of LB medium containing 100, 300, 500 and 1000 mg L^{-1} Cr (VI), respectively, was added 1.0 mL of the bacterial cultures and then incubated aerobically at 37 °C and 150 rpm. Aliquots of solution were withdrawn to analyze OD_{600} and residual Cr (VI) concentrations as described above.

2.3. Cr (VI) reduction capacity by resting cells

P. phragmitetus LSSE-09 cells were harvested by centrifugation (4000×*g*) for 15 min at 4 °C, washed twice with de-ionized water and then suspended in different buffers. 50 mM phosphate buffer was used to suspend resting cells at pH 6.0. For other pH values ranging from 7.0 to 10.0, 50 mM Tris–HCl buffers were used. The dry weight of cells was approximate 1.95 g L^{-1} (2.04×10^9 cells mL^{-1}). Cr (VI)-reduction experiments were performed at 37 °C and 150 rpm under both aerobic and anaerobic conditions, as a function of pH values, initial Cr (VI) concentrations, electron donors and different co-ions.

For aerobic experiments, 20 mL of the reaction mixtures were incubated in 50 mL flasks. Anaerobic experiments were performed using 50 mL anaerobic glass bottles. Each bottle was sparged with nitrogen for 3 min, introduced by a syringe needle through the self-sealing rubber septum. Air displacement was achieved by inserting another syringe needle as the outlet. The composition of the reaction mixture depended on the parameters which were investigated as follows. Samples were taken periodically with a syringe to analyze residual Cr (VI) and total Cr concentrations.

To characterize the Cr (VI)-reduction efficiency of strain LSSE-09, effects of initial pH (6.0, 7.0, 8.0, 9.0 and 10.0), and Cr (VI) concentration (150–450 mg L^{-1}), and recycling stability were investigated, respectively. Experiments were conducted under both aerobic and anaerobic conditions as described above. Controls for all experiments were supplemented with all components except LSSE-09 cells.

To evaluate the effect of external electron donors on Cr (VI) reduction efficiency, 20 mL 50 mM Tris–HCl (pH 9.0) buffers were respectively added 3000 mg L^{-1} lactate, acetate, formate, citrate and glucose. The concentration of Cr (VI) in Tris–HCl buffer is 350 mg L^{-1}. Samples were drawn at regular intervals to analyze remaining Cr (VI). The Cr (VI)-reduction rate (TCR) is given by

$$\mathrm{TCR} = \frac{C_0 - C_\mathrm{f}}{1.95t} \quad (1)$$

$$\text{or} \quad \mathrm{TCR} = \frac{C_0 - C_\mathrm{f}}{C_\mathrm{d} t} \quad (2)$$

where

TCR the Cr (VI)-reduction rate (mg $g^{-1}_{(\text{dry weight})}$ min^{-1}, Eq. (1); mg $cell^{-1}$ min^{-1}, Eq. (2))
C_0 the initial Cr (VI) concentration (mg L^{-1})
C_f the finial Cr (VI) concentration (mg L^{-1})
1.95 the dry weight of cells (g L^{-1})
C_d the cell density (cells mL^{-1})
t the Cr (VI)-reduction time (min)

In addition, the influence of other anions on Cr (VI) reduction was also investigated by conducting 500 and 1000 mg L^{-1} chloride (NaCl), nitrate ($NaNO_3$) and sulfate (Na_2SO_4). All the experiments were set up in a completely randomized design with at least three replicate.

2.4. Analytical methods

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 [23]. Total concentrations of chromium in the samples were measured by an inductively coupled plasma atomic emission spectrometer (ICP-OES, Perkin Elmer Optima 7000DV, USA).

To investigate the valence states of chromium in the whole cell after chromium reduction, solid-state EPR analysis was conducted. EPR samples were prepared following the methods of Li et al. [24]. The EPR measurements were conducted with an ER 200D-SRC spectrometer at 9.53 GHz with 100 kHz modulation. Experiments were performed in a 1-mm-diameter glass tube at room temperature (300 K).

TEM samples were prepared following the methods of Li et al. [24]. All TEM measurements were carried out by JEM-2010 transmission electron microscopy coupled with an EDS (Oxford) system.

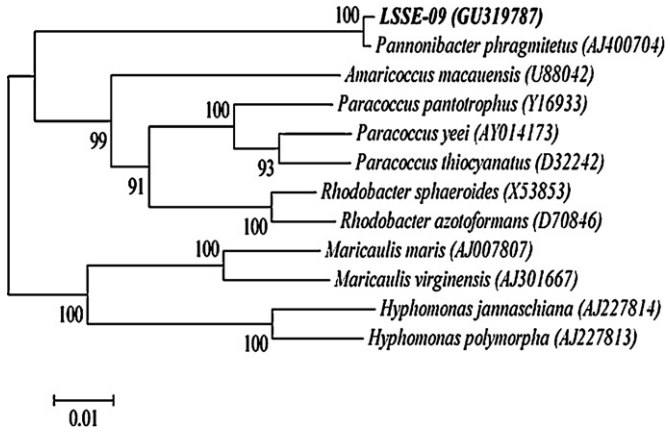

Fig. 1. Phylogenetic tree derived from 16S rRNA sequence data of *P. phragmitetus* LSSE-09.

3. Results and discussion

3.1. Identification of the bacterium

Cr (VI) resistant bacterial strain LSSE-09 with high Cr (VI)-reduction ability was isolated from industrial sludge. The 16S rRNA gene sequence analysis revealed that strain LSSE-09 was closely related to *P. phragmitetus* with the similarity of 99% (about 1370 bp, Genebank number: FJ882624.1). The neighbour-joining phylogenetic tree (Fig. 1) also showed that strain LSSE-09 tightly clustered with *P. phragmitetus,* together componented *Pannonibacte* genus. The phylogenetic tree was shown in Fig. 1. Strain *P. phragmitetus* LSSE-09 had been conserved in China General Microbiological Culture Collection Center (CGMCC No. 3512).

3.2. Cr (VI) resistance and reduction capacity by growing cells

3.2.1. Cr (VI) resistance capacity

Cr (VI) resistance and reduction capacity by growing cells was evaluated under aerobic condition. The cell growth under different concentrations of Cr (VI) at initial pH 9.0 was investigated to evaluate the Cr (VI) resistance of *P. phragmitetus* LSSE-09. As shown in Fig. 2, there was no remarkable difference in curve patterns among the tested Cr (VI) concentrations, and the maximal OD_{600} value was obtained nearly at 40 h. Strain LSSE-09 was tolerant to as high as 1000 mg L^{-1} Cr (VI) at initial pH 9.0. However, 1000 mg L^{-1} Cr (VI) caused a markedly slower growth and a significantly lower growth yield than lower Cr (VI) concentrations did. As the cells in Cr (VI)-containing mediums grew, the color of the medium became turbid and gray-green, while the control cultures (grown in the absence of Cr (VI)) showed no obvious coloration.

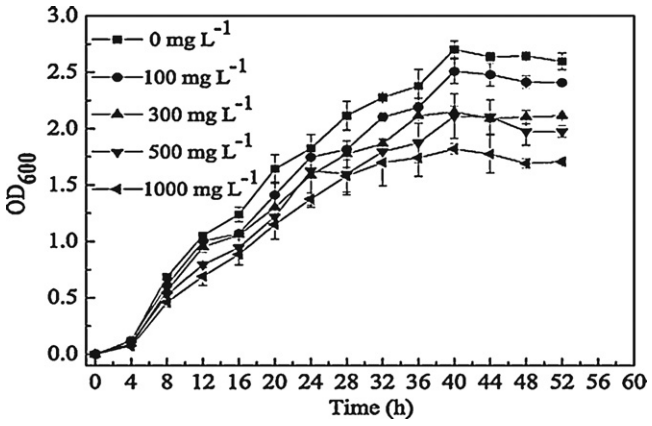

Fig. 2. Growth curves of *P. phragmitetus* LSSE-09 at different Cr (VI) concentrations at initial pH 9.0.

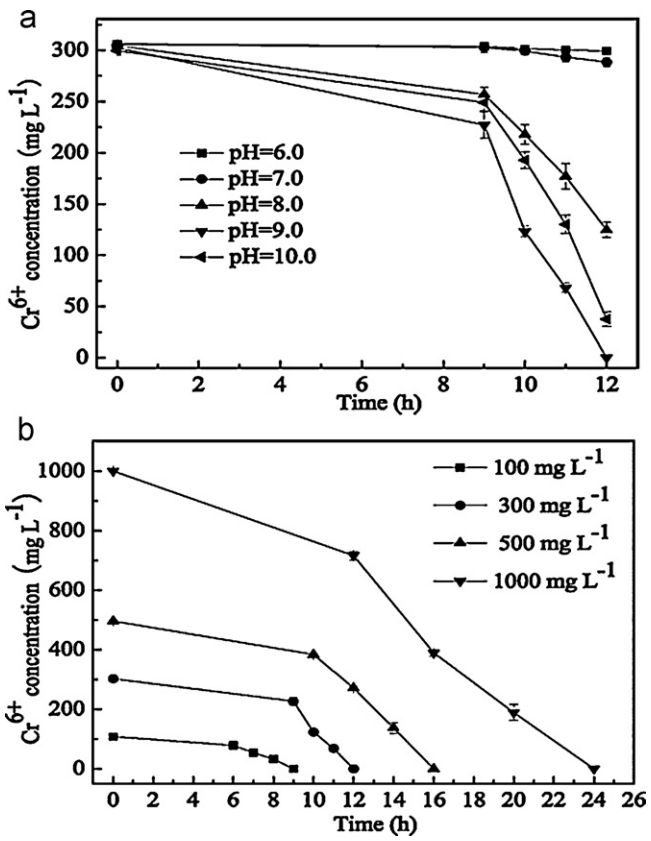

Fig. 3. Cr (VI) reduction capacity by growing cells: (a) effect of pH; (b) effect of different Cr (VI) concentrations at initial pH 9.0.

3.2.2. 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]. Fig. 3(a) shows the effect of pH on Cr (VI) reduction by growing cells of strain LSSE-09. It could reduce 300 mg L^{-1} Cr (VI) in 12 h at initial pH 9.0. There was no obvious difference in Cr (VI)-reduction yield when initial pH ranged from 9 to 10, but Cr (VI)-reduction rate highly decreased at pH 8.0. At initial pH 8.0 and 10.0, strain LSSE-09 reduced 180 and 262 mg L^{-1} Cr (VI), respectively. In addition, no obvious Cr (VI)-reduction occurred at initial pH 6.0 and 7.0. Apart from strain LSSE-09, some bacteria such as growing cells of *Ochrobactrum* sp. strain CSCr-3 [26] and a gram-positive bacterium ATCC700729 [27] were found to reduce Cr (VI) at pH 9.0.

3.2.3. Effect of Cr (VI) concentrations

Fig. 3(b) reveals that growing cells of strain LSSE-09 can completely reduce Cr (VI) at concentrations ranging from 100 to 1000 mg L^{-1}. No obvious abiotic reduction of Cr (VI) by LB medium occurred. Mclean and Beveridge [15] have reported that *Pseudomonad* strain CRB5 could completely reduce 20 mg L^{-1} Cr (VI) after 120 h. Complete Cr (VI)-reduction by *Ochrobactrum* sp. strain CSCr-3 was observed for 100 and 200 mg L^{-1} at 48 h [26]. Another *Pannonibacte phragmitetus*, strain BB could completely reduce 500 mg L^{-1} Cr (VI) in 24 h at 30 °C [28]. Comparatively, strain LSSE-09 could completely reduce 100 and 1000 mg L^{-1} Cr (VI) after 9 and 24 h of cultivation, respectively, with relatively higher reduction rate.

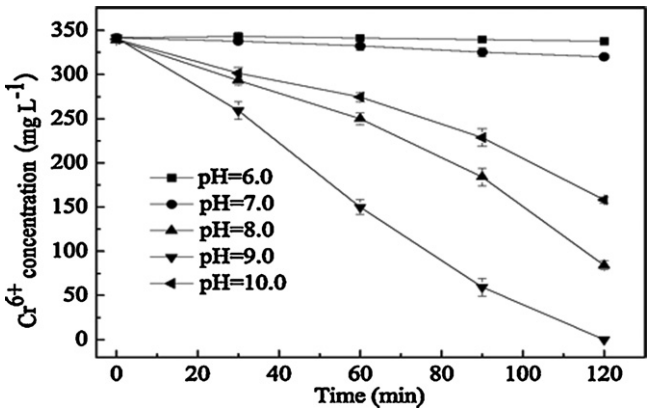

Fig. 4. Effect of pH on Cr (VI)-reduction capacity of resting cells under anaerobic condition without electron donors.

3.3. Cr (VI) reduction capacity by resting cells

In order to separate metal reduction from growth-related process, Cr (VI) reduction capacity by resting cells was evaluated. Truex et al. [29] suggested that metal reduction under resting conditions might simulate competition for limited substrates in natural environments. Cr (VI) reduction capacity by resting cells was evaluated under both aerobic and anaerobic conditions. Among various parameters, pH and temperature affected the Cr (VI)-reduction rate significantly.

3.3.1. Effect of pH

To find a suitable pH for effective chromium reduction by resting cells of strain LSSE-09, experiments were performed at different initial pH values (pH 6.0, 7.0, 8.0, 9.0 and 10.0). There was no increase in cell number during the Cr (VI) reduction process. Fig. 4 shows the effect of pH on Cr (VI)-reduction capacity under anaerobic condition without external electron donors. Strain LSSE-09 completely reduced 350 mg L^{-1} Cr (VI) at pH 9.0 after incubating 120 min. At pH 8.0 and 10.0, strain LSSE-09 reduced 255 and 182 mg L^{-1} Cr (VI), respectively. It is similar to growing cells that no obvious Cr (VI)-reduction occurred at initial pH 6.0 and 7.0. Most bacteria reported on Cr (VI)-reduction were mainly conducted under neutral conditions. Rare bacteria such as resting cells of *Achromobacter* Ch1 [18,19] was reported to reduce Cr (VI) at pH 9.0.

However, no obvious aerobic-reduction occurred for strain LSSE-09 at pH 9.0 (Fig. 5(a)). Similar result was also reported for *Enterobacter cloacae* HO1 [30]. Strain LSSE-09 probably utilized toxic chromate as a terminal electron acceptor anaerobically, but Cr (VI)-reducing activities were rapidly inhibited by oxygen which acted as the competing electron acceptor [31].

3.3.2. Effect of external electron donors

In order to increase the reduction rate, the effect of external electron donors on dichromate reduction by *P. phragmitetus* LSSE-09 was evaluated. No abiotic reduction of Cr (VI) by these electron donors occurred. External electron donors were essential to improve the reduction rate. As shown in Fig. 5(a), at pH 9.0 and 37 °C, when acetate was added to the medium, Cr (VI)-reduction rates were significantly improved under both aerobic and anaerobic conditions. Moreover, the anaerobic reduction rate was much faster than that of the aerobic one. When supplemented with 3000 mg L^{-1} acetate, strain LSSE-09 completely reduced 350 mg L^{-1} Cr (VI) within 20 min anaerobically, compared with 40 min aerobically.

Fig. 5(b) shows the effect of different electron donors on Cr (VI)-reduction rate at initial pH 9.0. The reduction rate was calculated by Eq. (1). For blank samples, which were conducted without external electron donors, anaerobic and aerobic reduction rates

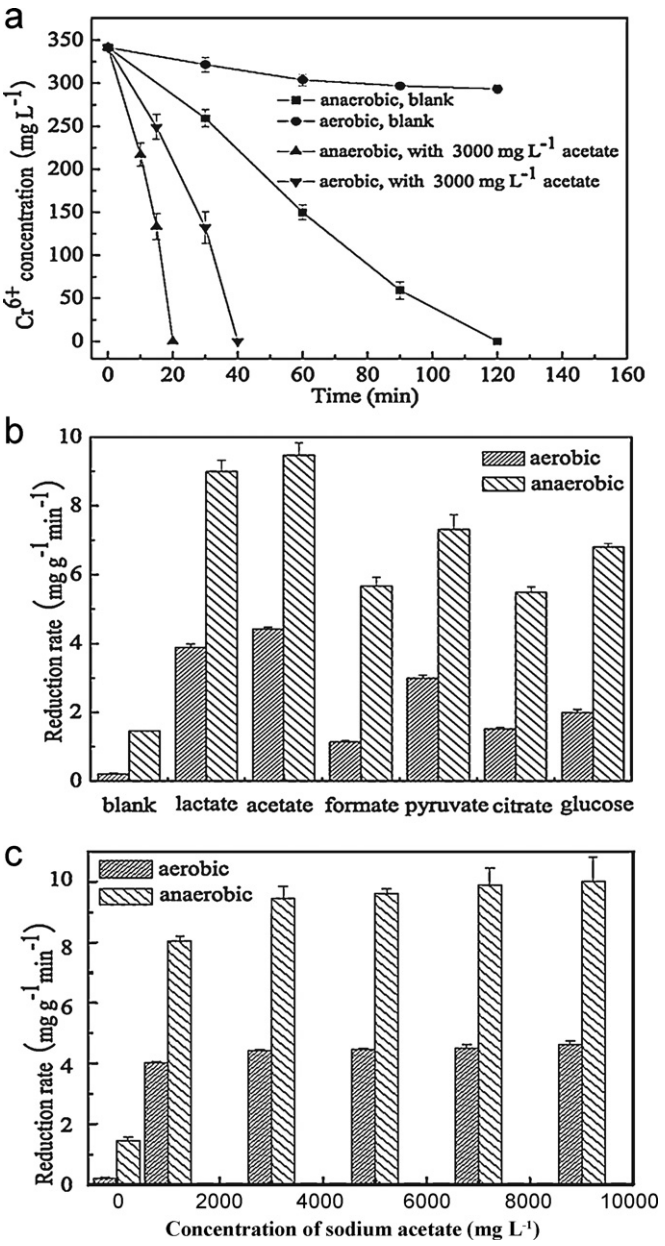

Fig. 5. Effect of electron donors on Cr (VI)-reduction rate at initial pH 9.0 under aerobic and anaerobic conditions: (a) with or without acetate; (b) effect of different electron donors; (c) effect of different acetate concentrations.

were 1.46 mg $g^{-1}_{(dry\ weight)}$ min^{-1} and 0.21 mg g^{-1} min^{-1}, respectively. The anaerobic reduction rate was much higher than the aerobic one, because this bacterium could anaerobically reduce Cr (VI) by using internally stored reserves as electron donors, whereas external electron donors such as lactate, acetate, formate, pyruvate, citrate and glucose were essential to stimulate the aerobic reduction. Among all tested electron donors, washed cells incubated with acetate showed the most significant enhanced effect on dichromate reduction activity. The presence of 3000 mg L^{-1} acetate enhanced anaerobic and aerobic Cr (VI)-reduction rates up to 9.47 and 4.42 mg g^{-1} min^{-1}, respectively, which were 5 and 20 times faster than those without it. For electron donors-containing cultures, the increased reduction rate indicated that it was an active process [15], because electron donors were also carbon and energy sources for resting cells. Some bacteria reported can utilize glucose [13,14], acetate or glycerol [30], pyruvate [18] and lactate [15,18,32] as external electron donors to mediate Cr (VI) reduction.

Table 1
Comparison of Cr (VI)-reduction rates between different reported bacteria.

Bacteria		Temperature (°C)	Cr (VI) concentration ($mg\,L^{-1}$)	Cell density ($cells\,mL^{-1}$)	Time (min)	Reduction rate ($mg\,cell^{-1}\,min^{-1}$)	References
Bacillus QC 1–2	Aerobic	30	15.6	1.0×10^9	1200	1.3×10^{-14}	Campos et al. [13]
Agrobacterium radiobacter EPS-916	Aerobic	30	26	2.0×10^9	360	3.6×10^{-14}	Llovera et al. [17]
Pseudomonad CRB5	Aerobic	30	20	1.5×10^7	7200	1.9×10^{-13}	J. McLean et al. [15]
Achromobacter sp. Ch-1	Aerobic	30	250	2.0×10^9	45	2.8×10^{-12}	Ma et al. [18]
	Anaerobic		104	3.64×10^9	35	8.2×10^{-13}	Zhu et al. [19]
Enterobacter cloacae HO1	Anaerobic	30	26	1.0×10^7	600	4.3×10^{-12}	Wang et al. [30]
Pannonibacter phragmitetus LSSE-09	Aerobic	37	350	2.04×10^9	40	4.3×10^{-12}	The present
	Anaerobic		350	2.04×10^9	20	8.6×10^{-12}	

Reduction rate was calculated by Eq. (2).

However, in our study, Cr (VI) could not be entirely reduced aerobically with the presence of 3000 $mg\,L^{-1}$ formate and citrate even though their initial reduction rates were highly stimulated. In order to compare the effect of different electron donors, reduction rates of blank, formate and citrate samples were calculated at 120 min by Eq. (1). To compare the Cr (VI)-reduction rates with other bacteria reported, the reduction rate of strain LSSE-09 was calculated by Eq. (2) with cell density of 2.04×10^9 $cells\,mL^{-1}$ (Table 1). The results obtained suggested that strain LSSE-09 has high-level aerobic and anaerobic reduction rates.

In addition, the effect of concentration of sodium acetate over a range of 0–9000 $mg\,L^{-1}$ on anaerobic and aerobic Cr (VI)-reduction rates was evaluated. As shown in Fig. 5(c), both aerobic and anaerobic reduction activities increased with increasing concentration. Nevertheless, when the concentration of sodium acetate was higher than 3000 $mg\,L^{-1}$, the reduction rate reached a plateau with minor increase, which could be attributed to the saturation of electron donor relative to the reductase in whole cells.

3.3.3. Effect of Cr (VI) concentrations

The effect of initial Cr (VI) concentrations on Cr (VI)-reduction efficiency was investigated (Fig. 6). Washed cells were added to 50 mM pH 9.0 Tris–HCl buffers containing various concentrations of Cr (VI) (150–450 $mg\,L^{-1}$) individually. Substantial Cr (VI) reduction occurred over the entire range of Cr (VI) tested. Resting cells of strain LSSE-09 reduced 450 $mg\,L^{-1}$ Cr (VI) within 25 min anaerobically (Fig. 6(b)), compared with 50 min aerobically (Fig. 6(a)).

Aerobic and anaerobic K_m and V_{max} values for intact cells were determined. The kinetic constants were calculated by fitting the initial rate data to a double-reciprocal Lineweaver–Burk plot of 1/V [mg of Cr (VI) min^{-1} g of $cells^{-1}$] vs. 1/[Cr (VI)] ($mg\,L^{-1}$) derived from a linear transformation of the Michaelis–Menten equation. This allowed the estimation of the specific K_m and V_{max} for the non-specific rate constants for whole cell reduction [15]. Initial reduction rates were estimated from the data in Fig. 6. For aerobic reduction, the nonspecific half-saturation or Michaelis-Menten constant, K_m, was estimated to be 140.00 $mg\,L^{-1}$ and the maximum nonspecific reduction rate or maximum velocity, V_{max}, was estimated to be 6.03 mg of Cr min^{-1} g of $cells^{-1}$ (Fig. 6(a)). For anaerobic reduction, K_m and V_{max} were estimated to be 50.91 $mg\,L^{-1}$ and 10.53 mg of Cr min^{-1} g of $cells^{-1}$, respectively (Fig. 6(b)). The anaerobic K_m value is less than the aerobic one, which means that the crude reductase in whole cells have higher activity under anaerobic condition.

3.3.4. Effect of different co-ions

Table 2 shows the Cr (VI)-reduction rate in presence of Cl^-, NO_3^- and SO_4^{2-}. No significant difference was observed when Cl^- was added in the resting buffer but NO_3^- or SO_4^{2-} had slightly negative effects on Cr (VI)-reduction rates. The higher concentrations of NO_3^- and SO_4^{2-} were added, the lower reduction rates were obtained. SO_4^{2-} at a concentration of 1000 $mg\,L^{-1}$ showed the most inhibitory effect on Cr (VI) reduction and decreased reduction rates to 3.70 $mg\,g^{-1}\,min^{-1}$ aerobically and 7.46 $mg\,g^{-1}\,min^{-1}$ anaerobically, which were 83.7% and 78.8% compared with the blank samples.

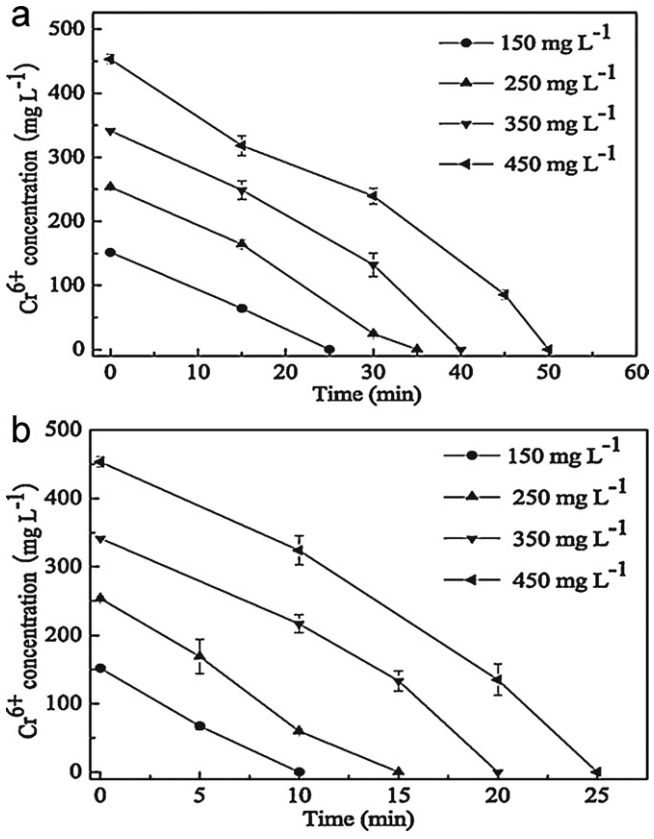

Fig. 6. Effect of Cr (VI) concentrations on Cr (VI)-reduction rate with 3000 $mg\,L^{-1}$ acetate at initial pH 9.0: (a) aerobic; (b) anaerobic.

Table 2
Effect of different co-ions on Cr (VI) reduction rates.

Treatment	Concentration ($mg\,L^{-1}$)	Reduction rate ($mg\,g^{-1}_{dry\,weight}\,min^{-1}$)	
		Aerobic	Anaerobic
Blank	0	4.42 ± 0.06	9.47 ± 0.36
Cl^-	500	4.55 ± 0.08	9.36 ± 0.53
	1000	4.32 ± 0.08	8.68 ± 0.76
NO_3^-	500	4.12 ± 0.05	8.55 ± 0.29
	1000	3.98 ± 0.15	8.15 ± 0.27
SO_4^{2-}	500	3.89 ± 0.03	8.06 ± 0.39
	1000	3.70 ± 0.08	7.46 ± 0.22

All samples including blank were added 3000 $mg\,L^{-1}$ acetate as an electron donor.

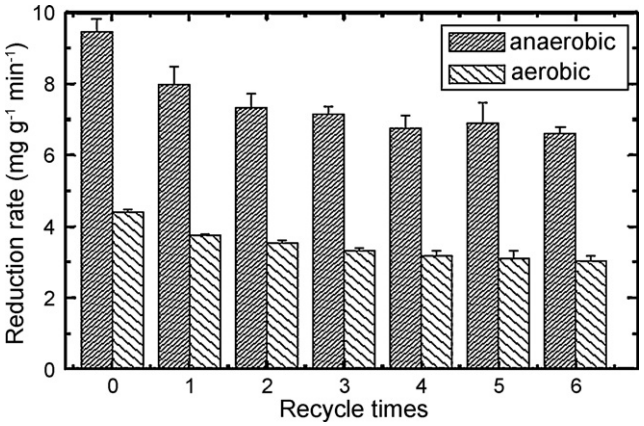

Fig. 7. Recycling stability of strain LSSE-09 over six batch cycles.

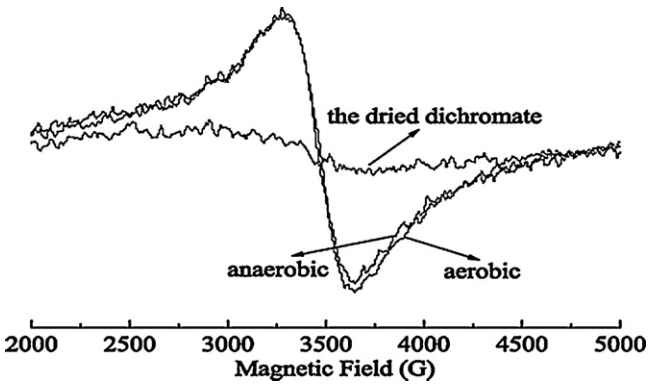

Fig. 8. EPR spectrum from a solid-state of strain LSSE-09 incubated with 350 mg L^{-1} Cr (VI) and 3000 mg L^{-1} acetate in Tris–HCl buffer after Cr (VI) was completely reduced.

As we know, Cr (VI) can be adsorbed directly by biomass and reduced to less toxic Cr (III) and then immobilized [24]. Cr (VI) mainly present in aqueous solution as chromate (CrO_4^{2-}) when pH is larger than 6.0 [33]. Due to the structural similarity of CrO_4^{2-} and SO_4^{2-}, the chromate anion can overcome the cellular permeability barrier, entering intracellularly via the SO_4^{2-} active transport system [32,34]. In this experiment, SO_4^{2-} may be a competitive ion for the uptake of CrO_4^{2-}, because the Cr (VI)-reduction rate decreased correspondingly. Das and Guha [35] also reported that the uptake of chromium by live biomass was reduced in presence of sulfate ion. The existence of NO_3^- also decreased the Cr (VI)-reduction rate but the mechanism may be different from SO_4^{2-}, because NO_3^- could act as another competing electron acceptor, similar to oxygen [31].

3.3.5. Recycling stability

The cells of strain LSSE-09 were tested repeatedly in a reaction mixture containing 350 mg L^{-1} Cr (VI) and 3000 mg L^{-1} acetate. At the end of each batch, the coated cells were collected by centrifugation and then reused in another test. Cr (VI) was completely reduced by strain LSSE-09 in each batch. As shown in Fig. 7, the activity of strain LSSE-09 decreased during the Cr (VI) reduction process. The reduction rate was originally 9.47 mg g^{-1} min^{-1}, and it decreased to 7.98 mg g^{-1} min^{-1} after first batch cycle. After that, the decreasing rate became slower. Six batch cycles later, the reduction rate was still as high as 6.61 mg g^{-1} min^{-1}, which was only 30% less than the original rate. For aerobic tests, the varying tendency was similar and the reduction rate was 3.04 mg g^{-1} min^{-1} after six batch cycles, which was 31% less than the original rate. The results suggested that strain LSSE-09 could be repeatedly used and retained high specific Cr (VI)-reduction activity.

3.3.6. Valence states of chromium binding to bacteria and the state of chromium inside the bacteria

During the reduction process, the original orange color became gray-green in both aerobic and anaerobic conditions as described previously. Whole amounts of bacteria for EPR were prepared to determine if this turbidity was in part due to the precipitation of reduced Cr (III).

To detect the possible valence states of chromium in the whole cell after reduction, EPR analysis of solid-state cells after centrifugation was conducted. Fig. 8 shows the EPR spectrums from solid samples of strain LSSE-09 incubated with 350 mg L^{-1} Cr (VI) and 3000 mg L^{-1} acetate after Cr (VI) was completely reduced. A abroad signal (about 500G) centered at a g factor of 1.97 was observed in both aerobic and anaerobic samples, which could be attributed to Cr (III) paramagnetic signal [24,36,37]. In comparison with solid samples, no change was observed in the dried dichromate powder because there is no paramagnetism for Cr (VI). Meanwhile, no other paramagnetic Cr species such as Cr (V) were found.

To investigate the distribution of chromium inside the bacteria, conventional thin-section TEM of bacteria together with EDS was applied. If Cr (VI) was not introduced, there was no Cr in EDS spectrum. U peaks were TEM specimen preparation artifacts from uranyl acetate used to stain the cells. After introducing 350 mg L^{-1} Cr (VI), no obvious changes were found on the cell morphology. EDS analysis showed that the inner parts of bacteria contained chromium after bioaccumulation (Fig. 9(b)) and the amount of

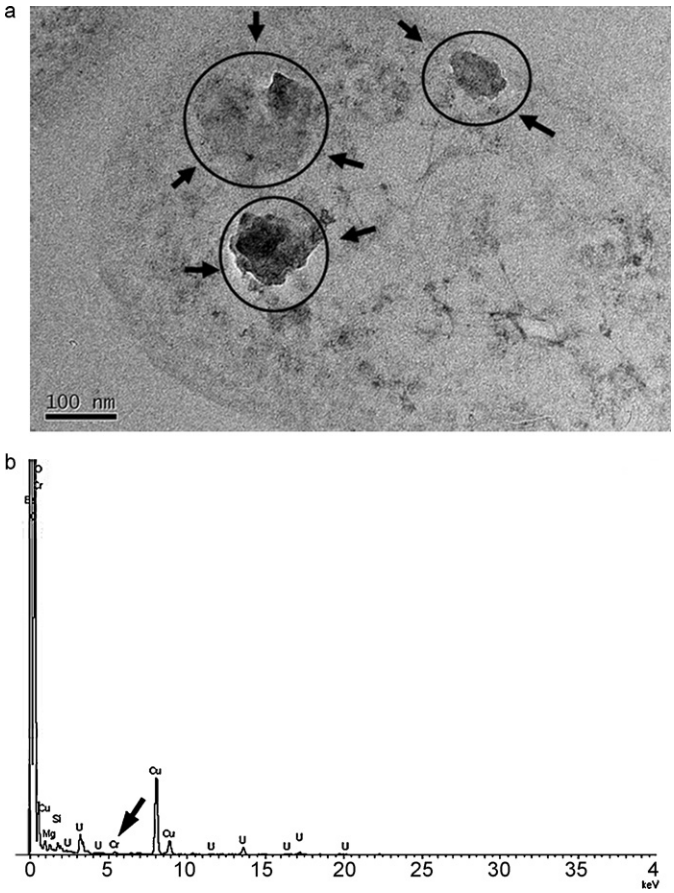

Fig. 9. Conventional TEM images of bacterial thin sections of bacteria after anaerobically incubated in Tris–HCl buffer with 350 mg L^{-1} Cr (VI) for 120 min: (a) TEM results of precipitates generated in bacteria after reduction; and (b) EDS spectrum of these precipitates (black circular portion).

Table 3
Total concentration of Cr in the supernatant after reduction.

Electron donors	Total Cr (mg L^{-1})	
	Aerobic	Anaerobic
Blank	305.70 ± 4.10[a]	300.55 ± 4.88
Acetate	280.42 ± 5.89	309.00 ± 9.76
Lactate	284.70 ± 0.96	293.15 ± 3.89
Formate	324.40 ± 4.10[a]	316.60 ± 4.95
Pyruvate	311.05 ± 3.75	297.40 ± 6.93
Citrate	321.55 ± 2.62[a]	315.75 ± 3.04
Glucose	314.15 ± 6.72	322.10 ± 8.20

[a] Means that Cr (VI) was not entirely reduced after 120 min. Total Cr was tested at 120 min for blank, formate and citrate samples.

chromium inside the bacteria was so small that few obvious precipitates were formed (Fig. 9(a)).

Considerable soluble Cr (III) was found outside the bacteria cells as described in Table 3. The final pH was between 9.0 and 10.0 when the initial pH of Tris–HCl buffer was 9.0 (results not shown), and the produced Cr (III) is thought to be insoluble $Cr(OH)_3$ [3]. However, most Cr (III) was not accumulated inside the cells and released into the supernatant. In this study, the minimum final concentration of total soluble chromium in the supernatant was 280.42 mg L^{-1} (Table 3). More than 82% of the Cr (III) retained in a soluble form in the culture supernatant.

As such, Cr (VI) reduction in the presence of cellular organic metabolites could form both soluble and insoluble organo-Cr (III) end-products [38]. Several research groups have reported that significant amounts of Cr (III) remained in the supernatant of bacterial cultures and did not precipitate after reduction [13,16,31,39]. It can be deduced that some soluble organo-Cr (III) end-products were probably formed after the reduction in this experiment. Further investigation is being undertaken to remove these soluble organo-Cr (III) end-products from waste water.

4. Conclusions

P. phragmitetus LSSE-09 showed a strong potential to reduce Cr (VI) to Cr (III) intracellularly by growing and resting cells aerobically and anaerobically under alkaline conditions. Resting cells of strain LSSE-09 have high anaerobic-reduction potential whereas no obvious aerobic-reduction occurred. External electron donors could highly increase the reduction rate, especially for aerobic reduction. Most reduced Cr (III) was extruded extracellularly and formed some soluble organo-Cr (III) end-products. In addition, NO_3^- and SO_4^{2-} had slightly negative effects on reduction rates. Strain LSSE-09 could be repeatedly used and retained high specific Cr (VI)-reduction activity. This bacterium shows great promise for detoxification of Cr (VI) at low or high concentrations in alkaline wastewaters.

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