Postprint: Characteristics of Cr(VI) Removal by an Endophytic Bacterium from Leersia hexandra

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Abstract

Leersia hexandra is the first chromium hyperaccumulator plant discovered in China. This study investigated the endophytic bacteria of L. hexandra and their chromium removal performance. Using beef extract peptone solid plate culture supplemented with Cr(VI), an endophytic bacterial strain G04 with strong Cr(VI) resistance was isolated and screened from the roots of L. hexandra. Molecular biological identification revealed that this strain belongs to Enterobacter cloacae. Employing shake-flask culture, with Cr(VI) removal rate, total Cr removal rate, and bacterial biomass as evaluation indices, the effects of pH, temperature, substrate concentration, liquid volume, inoculum size, shaker rotational speed, and reaction time on Cr(VI) removal, total chromium removal, and strain growth were investigated. The results demonstrated that in beef extract peptone liquid medium, the optimal conditions for Cr(VI) removal by E. cloacae G04 were initial pH 5.0, temperature 37 °C, Cr(VI) concentration 100 $mg \cdot L^{1}$, liquid volume 80 mL (250 mL Erlenmeyer flask), inoculum size 15%, shaker speed 120 $r \cdot min^{1}$, and reaction time 48 h. Under these conditions, the removal rates of Cr(VI) and total chromium by E. cloacae G04 were 84% and 8%, respectively. Based on the Cr(VI) and total chromium removal rates, the mechanism of Cr(VI) removal by this strain is speculated to be primarily reduction with adsorption playing a secondary role. The findings indicate that the L. hexandra endophytic bacterium E. cloacae G04 exhibits promising application potential, including potential direct application in remediation of chromium-contaminated soil and aquatic environments, utility as a candidate strain for enhancing phytoremediation of chromium contamination, and value as a reference for in-depth investigation of the chromium accumulation mechanism in L. hexandra.

Full Text

Characteristics of Cr(VI) Removal by an Endophytic Bacterium Isolated from *Leersia hexandra* Swartz

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Abstract

Leersia hexandra Swartz is the first chromium hyperaccumulator plant discovered in China. This study investigated the endophytic bacteria of *L. hexandra* and their capacity for chromium removal. Using beef extract peptone solid plates supplemented with Cr(VI), a Cr(VI)-resistant endophytic bacterium G04 was isolated and screened from the roots of *L. hexandra*. Molecular biological identification revealed that this strain belongs to *Enterobacter cloacae*. The effects of pH, temperature, substrate concentration, liquid volume, inoculum size, shaking speed, and reaction time on Cr(VI) removal rate, total Cr removal rate, and bacterial biomass were examined using shake-flask cultivation. The results demonstrated that in beef extract peptone liquid medium, the optimal conditions for Cr(VI) removal by *E. cloacae* G04 were: initial pH 5.0, temperature 37 °C, Cr(VI) concentration 100 mg · L ^{1}, liquid volume 80 mL (in 250 mL flask), inoculum size 15%, shaking speed 120 r · min ^{1}, and reaction time 48 h. Under these conditions, the removal rates of Cr(VI) and total chromium by strain *E. cloacae* G04 were 84% and 8%, respectively. Based on the differential removal rates of Cr(VI) and total chromium, the mechanism of Cr(VI) removal by this strain appears to be primarily reduction with adsorption as a secondary process. These findings indicate that the *L. hexandra* endophytic bacterium *E. cloacae* G04 possesses promising application potential, both for direct remediation of chromium-contaminated soil and water environments and as a candidate strain for promoting phytoremediation of chromium pollution. Additionally, this work provides valuable insights for further investigation into the chromium accumulation mechanisms of *L. hexandra*.

Keywords: *Leersia hexandra*, endophytic bacteria, *Enterobacter cloacae*, hexavalent chromium, reduction

Introduction

With the development of modern technology, heavy metal chromium has been widely applied in pharmaceuticals, electroplating, and various other industries. Consequently, environmental contamination by chromium and its compounds has become increasingly severe. Long-term exposure to chromium and chromium compounds can affect the gastrointestinal system, immune system, liver, and kidneys, and may even induce respiratory cancers (Costa, 1997). Among various chromium species, Cr(VI), which primarily exists as chromate and dichromate ions, is considered the most toxic form (Shanker et al., 2005; Wang and Liu, 2010). Internationally, bioremediation approaches—leveraging microorganisms or plants for flocculation, absorption, accumulation, and enrichment of heavy metal ions—are advocated for chromium-contaminated water and soil (Chibuike and Obiora, 2014; Malik et al., 2010). Microorganisms isolated from chromium-contaminated environments, including *Leucobacter* sp. (Ge et al., 2013), pseudomonads (Zhang et al., 2016), *Streptomyces griseus* (Chen et al., 2014; Poopal and Laxman, 2008), *Bacillus* sp. (Alsalamah, 2011; Dhal et al., 2010), and *Thermus scotoductus* (Opperman and Van, 2007), have demonstrated effective Cr(VI) removal capabilities.

Hyperaccumulator plants offer significant advantages for environmental remediation, including high biomass, heavy metal absorption capacity 10–500 times greater than conventional plants, and robust growth in contaminated soils (Shi et al., 2015), leading to their increasing application in pollution cleanup (Brooks et al., 1998). Concurrently, research on endophytic bacteria associated with hyperaccumulator plants has gained attention, with studies reported on arsenic hyperaccumulator *Pteris vittata* (Dong, 2012), zinc hyperaccumulator *Sedum alfredii* (Long et al., 2013), cadmium hyperaccumulator *Solanum nigrum* (Cao et al., 2009), and manganese hyperaccumulator *Phytolacca acinosa* (Lu, 2015). *Leersia hexandra* Swartz, discovered in Guilin, Guangxi by Zhang et al. (2007), represents the first chromium hyperaccumulator found in China, with demonstrated strong accumulation capacity for both Cr(III) and Cr(VI) (Hu, 2008; Chen et al., 2008; Lu et al., 2013). However, no studies have yet reported on Cr(VI) reduction by endophytic bacteria from *L. hexandra*. This study isolates, screens, identifies, and characterizes the Cr(VI) removal properties of endophytic bacteria from this chromium hyperaccumulator, laying the foundation for their application in chromium pollution remediation.

Materials and Methods

1.1.1 Plant Material Chromium hyperaccumulator plant *Leersia hexandra* was collected from the laboratory of the College of Environmental Science and Engineering at Guilin University of Technology.

1.1.2 Culture Media Solid medium: beef extract 3 g · L ^{1}, peptone 10 g · L ^{1}, NaCl 5 g · L ^{1}, agar 20 g · L ^{1}, distilled water, pH 7.0.
Liquid medium: beef extract 3 g · L ^{1}, peptone 10 g · L ^{1}, NaCl 5 g · L ^{1}, distilled water, pH 7.0.
Cr(VI)-containing media: prepared by adding appropriate amounts of potassium dichromate to the above solid or liquid media.

1.2 Isolation and Screening of Cr(VI)-Resistant Endophytic Bacteria Healthy *L. hexandra* root tissues were washed clean, surface-sterilized by soaking in 70% ethanol for 40 s, followed by 2.5% sodium hypochlorite for 2 min, and rinsed six times with sterile water to remove residual disinfectant. To verify complete surface sterilization, the final rinse water was plated on solid medium and confirmed to be sterile. Sterilized root tissue was ground with 1 mL of 0.9% NaCl solution, and 1 mL of the homogenate was inoculated into 100 mL beef extract peptone liquid medium (in a 500 mL flask) and incubated at 37 °C with shaking at 120 r · min ^{1} for 2 days. The culture was serially diluted to 10 ^{2}–10 , and 20 L aliquots were spread on beef extract peptone plates containing 100–1000 mg · L ^{1} Cr(VI). After incubation at 37 °C for 24–48 h, colonies with good growth and strong chromium resistance were selected and purified by streak plating on chromium-containing plates. Pure cultures of Cr(VI)-resistant endophytic bacteria were obtained and stored on slant medium at 4 °C after incubation at 37 °C for 24 h.

1.3 Identification of Cr(VI)-Resistant Bacteria Genomic DNA of strain G04 was extracted using a bacterial genomic DNA extraction kit (Sangon Biotech, Shanghai). PCR amplification was performed in a 50 L reaction containing 2×Taq DNA polymerase 25 L, template DNA 2 L, primers 1492R and 27F (2 L each), and ddH O 19 L. The thermal cycling program was: 94 °C for 3 min; 30 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min; and final extension at 72 °C for 5 min. PCR products were analyzed by 1% agarose gel electrophoresis in 0.5×TBE buffer at 80 V. The PCR product of strain G04 was sequenced by Sangon Biotech (Shanghai) and the sequence was blasted against the NCBI database (http://blast.ncbi.nlm.nih.gov/) for strain identification.

1.4 Cr(VI) Removal Experiments with Endophytic Bacterium G04 Strain G04 preserved on slant medium was activated on beef extract peptone solid plates at 37 °C for 24 h. Two loops of cells were inoculated into 100 mL beef extract peptone liquid medium (in a 250 mL flask) and incubated at 37 °C with shaking at 120 r · min ^{1} for 24 h to prepare the seed culture. Uninoculated medium processed identically served as the blank control.

The seed culture was inoculated at 10% (v/v) into beef extract peptone liquid medium (pH adjusted to 7.0 with 2 M NaOH or HCl) containing 100 mg · L ^{1} Cr(VI) with a liquid volume of 100 mL/250 mL flask. Cultures were incubated at 37 °C with shaking at 120 r · min ^{1}. At appropriate intervals, samples were

aseptically collected and centrifuged at 10,000 r · min ^{1} for 10 min to remove cells and suspended impurities. The pellet was resuspended in distilled water for OD measurement, while the supernatant was used for Cr(VI) and total Cr determination. Uninoculated medium processed similarly served as the blank control.

1.5 Determination of Cr(VI) and Total Cr **Cr(VI) standard curve:** Aliquots of 0, 0.2, 0.4, 0.8, 1.2, 1.6, and 2.0 mL of 5 mg · L ^{1} Cr(VI) standard solution were transferred to 10 mL volumetric tubes, diluted to volume with distilled water, then mixed with 0.1 mL each of (1+1) sulfuric acid and phosphoric acid, followed by 0.4 mL diphenylcarbazide solution. After 5–10 min reaction, absorbance was measured at 540 nm. A standard curve was constructed with Cr(VI) concentration as the x-axis and absorbance at 540 nm as the y-axis.

Cr(VI) determination: 1.0 mL of sample was diluted to 10 mL with water. One mL of this diluted solution was transferred to a 10 mL colorimetric tube, diluted to mark, mixed with 0.1 mL (1+1) H SO and 0.1 mL (1+1) H PO , then 0.4 mL diphenylcarbazide solution was added. After 5–10 min, absorbance was measured at 540 nm. Cr(VI) concentration was determined from the standard curve, and removal rate was calculated using Equation (1).

Total Cr determination: 1.0 mL sample was diluted to 10 mL. One mL of diluted solution was mixed with 0.1 mL (1+1) H PO . Four percent KMnO solution was added dropwise until a persistent pink color remained. The solution was boiled down to ~4 mL. After cooling, 0.2 mL of 20% urea solution was added, followed by dropwise addition of 2% NaNO solution with thorough mixing until the pink color just disappeared. After bubbles dissipated, the solution was transferred to a 10 mL tube, diluted to mark, and analyzed as for Cr(VI). Total Cr removal rate was calculated using Equation (2). Final Cr(VI) and total Cr removal rates were corrected by subtracting blank control values to exclude medium effects.

1.6 Statistical Analysis Experimental data were analyzed using Microsoft Office Excel 2007 for significant differences (t-test, confidence level 0.05).

Results

2.1 Strain Identification Following the method described in Section 1.2, Cr(VI)-resistant endophytic bacteria from *L. hexandra* roots were isolated and purified, yielding strain G04 with strong Cr(VI) resistance. DNA sequence analysis (Section 1.3) and phylogenetic tree construction [Figure 1: see original paper] revealed that strain G04 showed 99% sequence homology with *Enterobacter cloacae* strain R2-5A (HQ154552.1) and *E. cloacae* strain R6-354 (JQ659813.1), identifying it as *Enterobacter cloacae*. This species is widely applied in biotechnology, exhibiting plant growth promotion (Xu et al., 2001), nitrogen fixation

(Yang et al., 2001), diesel degradation (Ramasamy et al., 2017), pesticide degradation (Lin et al., 2008), and Cd^2 adsorption (Xu et al., 2017). Cr(VI) removal by *E. cloacae* has also been reported (Rahman et al., 2016; Zhang, 2011; Wang et al., 1989).

2.2.1 Effect of Initial pH Using the method in Section 1.4 with other conditions fixed, the effect of pH on Cr(VI) removal by strain G04 is shown in [Figure 2: see original paper]. *E. cloacae* G04 exhibited a broad pH range for growth and Cr(VI) reduction, performing well between pH 5–8. Bacterial growth was significantly inhibited at pH 4, while growth slowed above pH 8. Statistical analysis indicated pH significantly affected Cr(VI) removal rate and bacterial growth ($P < 0.05$) but not total Cr removal rate. Maximum Cr(VI) removal (~84%) occurred at pH 5.0, which was selected as the optimal pH.

2.2.2 Effect of Temperature With other conditions fixed, temperature effects are shown in [Figure 3: see original paper]. Strain G04 grew and reduced Cr(VI) effectively between 25–40 °C. Statistical analysis showed temperature significantly influenced Cr(VI) removal rate and bacterial growth ($P < 0.05$) but not total Cr removal. Cr(VI) removal decreased significantly above 40 °C, likely due to thermal inactivation of intracellular reductases. Optimal growth and maximum Cr(VI) removal occurred at 37 °C, which was selected as the optimal temperature.

2.2.3 Effect of Initial Cr(VI) Concentration With other conditions fixed, substrate concentration effects are shown in [Figure 4: see original paper]. Cr(VI) removal rate and bacterial biomass decreased significantly with increasing Cr(VI) concentration, presumably due to chromium toxicity inhibiting growth. Conversely, total Cr removal rate increased after 100 $mg \cdot L^{1}$ Cr(VI), suggesting enhanced adsorption. Statistical analysis confirmed significant effects of initial Cr(VI) concentration on all parameters ($P < 0.05$). The maximum amount of Cr(VI) removed occurred at 100 $mg \cdot L^{1}$, which was selected as the optimal substrate concentration.

2.2.4 Effect of Liquid Volume With other conditions fixed, liquid volume effects are shown in [Figure 5: see original paper]. Cr(VI) removal rate decreased slightly with increasing liquid volume, though not significantly, while bacterial growth declined, likely due to insufficient dissolved oxygen. Statistical analysis showed no significant effects of liquid volume on any parameter ($P > 0.05$). A liquid volume of 80 mL (in 250 mL flask) provided optimal growth and Cr(VI) removal, and was selected as the optimal condition.

2.2.5 Effect of Inoculum Size With other conditions fixed, inoculum size effects are shown in [Figure 6: see original paper]. At inoculum sizes below 15%, bacterial growth and Cr(VI) removal were positively correlated. Statistical analysis showed inoculum size significantly affected Cr(VI) removal rate and

bacterial growth ($P < 0.05$) but not total Cr removal. Maximum Cr(VI) removal occurred at 15% inoculum, which was selected as optimal.

2.2.6 Effect of Shaking Speed With other conditions fixed, shaking speed effects are shown in [Figure 7: see original paper]. Both Cr(VI) removal

Note: Figure translations are in progress. See original paper for figures.