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Potential of hexavalent chromium-resistant rhizosphere bacteria in promoting plant growth and hexavalent chromium reduction

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Abstract

Aim: This research aimed to evaluate three Cr(VI)-resistant rhizosphere bacteria (*Bacillus cereus*, *Bacillus aerius* and *Exiguobacterium profundum*) for their ability to produce plant growth-promoting (PGP) substances and to remove Cr(VI).

Methodology: Three rhizosphere bacteria were characterized for their ability to produce several PGP substances, including ammonia and indole-3-acetic acid (IAA) and solubilized phosphate. The Cr(VI) reduction ability of the rhizosphere bacteria was determined by diphenylcarbazide method with 60 mg l⁻¹ Cr(VI).

Results: Bioreduction of Cr(VI) in the reduction media by *B. cereus* was 13.7%, which was higher compared with those of *B. aerius* (4.4%) and *E. profundum* (3.6%). *B. aerius* adsorbed high Cr(VI) concentration of 56.1 mg g⁻¹. The best condition for these rhizobacteria to remove or adsorb Cr(VI) was at acidic pH (5.36–5.97). All rhizobacteria could not stand the toxic effect of Cr(VI) at 60 mg l⁻¹, which decreased almost 100% of rhizobacteria growth. *B. cereus* and *B. aerius* produced PGP substances, including ammonia and IAA and solubilized phosphate.

Interpretation: *B. cereus* and *B. aerius* with high PGP activities can be considered promising agents in microbe-assisted phytoremediation. All rhizosphere bacteria tolerated Cr(VI) and protected plants against the inhibitory effect of Cr(VI) by reducing Cr(VI) to Cr(III) and assisting the plant uptake of Cr(III).

Keywords : Bioreduction, Hexavalent chromium, Plant growth-promoting rhizobacteria, Rhizobacteria, *Scirpus grossus*

Ability of Cr(VI)-resistant rhizosphere bacteria

	<i>B. cereus</i>	<i>B. aerius</i>	<i>E. profundum</i>
NH ₃ production	✓	✓	✓
P solubilization	✓	✓	×
IAA Production	✓	✓	✓
Cr(VI) Reduction (%)	13.7	4.4	3.6
Cr(VI) biosorption (mg g ⁻¹)	4.3	56.1	8.1
CFU reduction (%)	99.9	99.8	99.9
Optimum pH to remove Cr(VI)	5.34-5.97	5.34-5.97	5.34-5.97

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Introduction

Environmental pollution of chromium has become a major concern nowadays. Chromium application in industrial processes, including metallurgy, tanning and textile industry, has led to serious groundwater and soil pollution (Augustynowicz *et al.*, 2010; Singh *et al.*, 2010; Razali and Said, 2017). Chromium at high concentrations is mutagenic, teratogenic and carcinogenic, and can pose toxic effects on both plants and animals. The effects of chromium are magnified when it is in the form of Cr(VI), which is more reactive, mobile and toxic than Cr(III), which is less soluble in water (Redondo-Gomez *et al.*, 2011; Paiva *et al.*, 2009; Halim and Phang, 2017).

Previous studies have treated Cr(VI) pollution using many conventional techniques, including chemical reduction, electrochemical reduction, membrane filtration and ion exchange. However, the applications and performance of these methods are often limited by their high operation costs, large required quantities of sludge or ineffectiveness (Ton *et al.*, 2015; Li *et al.*, 2018). Compared with conventional treatment, phytoremediation treatment yields better results, has cheaper operation costs, and is more environmental friendly.

Phytoremediation is a green technology where plants are utilized to remove and make pollutants harmless or decrease toxicity from the environment (Anh *et al.*, 2011; He *et al.*, 2013; Ali *et al.*, 2013; Lu *et al.*, 2018). This technology involves plant-microbe interactions to remediate inorganic pollutants, especially heavy metals (Monferran *et al.*, 2011). Plants play important role in phytoremediation through absorption, translocation, transformation and accumulation of heavy metals from the environment within their cells (Santana *et al.*, 2012; Grijalbo *et al.*, 2013). Prado *et al.* (2009) reported several plant species, such as *Eichhornia crassipes*, *Typha latifolia*, *Carex lurida*, *Prosopis* spp., *Allium sativum*, *Leptospermum scoparium* and *Polypogon monspeliensis*, have ability to reduce high percentage of Cr(VI) to Cr(III) and are excellent hyperaccumulators of Cr. Microbes also play a crucial role in soil ecosystems by maintaining biogeochemical cycles and plant productivity (Prado *et al.*, 2009; He *et al.*, 2016). Plant growth-promoting rhizobacteria (PGPR) have recently attracted considerable attention because of their ability to enhance plant growth, protection against pathogen infection and reduce the effects of toxic stresses, leading to increased biomass of plants (Son *et al.*, 2014; Rozainy *et al.*, 2017). PGPR produce PGP substances, such as indole-3-acetic acid (IAA) and ammonia and solubilize phosphate to promote plant growth and improve total metal uptake (Orhan, 2016).

Scirpus grossus is a native wetland plant that can survive in contaminated sites (Tangahu *et al.*, 2013; Al-Baldawi *et al.*, 2015; Ismail *et al.*, 2017; Yusoff *et al.*, 2019). *S. grossus* is a perennial tropical aquatic plant commonly known as *giant*

bulrush, *greater club-rush* and *rumpit menderong* (Malaysia), *mensiang* and *walingi* (Indonesia) (Tanghuet *et al.*, 2013; De'nanet *et al.*, 2017). Previous phytotoxicity studies reported that *S. grossus* can survive Cr(VI) concentrations up to 50 ppm within 90 days of exposure and remove 98% total Cr and 84% Cr(VI) (data not published). Therefore, the present study aimed to characterize and evaluate the potential of PGPR isolated from *S. grossus* to promote plant growth and remove Cr(VI).

Materials and Methods

Bioreduction of Cr(VI) by PGPR: Culture condition and inoculum preparation: Three Cr-resistance rhizosphere bacteria were isolated, screened, characterized and identified as *Bacillus cereus*, *Bacillus aerius* and *Exiguobacterium profundum* from a previous study (data not published). A fresh single culture was cultivated overnight at 37 °C in trypticase soy broth with agitation at 150 rpm (PROTECH S1-100D). After reaching the stationary phase, the cells were harvested and centrifuged at 4000 rpm for 15 min (Eppendorf Centrifuge 5810, Germany). The cell pellets were washed with aqueous solution to remove any residues adsorbed on the cell surfaces. The initial inoculum concentration was inoculated to an OD₅₅₀ of 0.5 (HACH DR6000, USA).

Cr(VI) stock solution: The reduction of Cr(VI) was studied in an aqueous solution. Cr(VI) stock solution was prepared by dissolving analytical-grade K₂Cr₂O₇ (1 g, SYSTERM, Malaysia) in 1 l of distilled water and mixing until complete dissolution. Cr(VI)-spiked medium was prepared by diluting this solution to the required concentration (60 mg l⁻¹) in the reduction media.

Bioreduction experiments: Bioreduction medium was prepared by using aqueous solution and spiked with an initial Cr(VI) concentration of 60 mg l⁻¹. A 20 ml aliquot of the PGPR inoculum with OD 0.5 was added into 400 ml of bioreduction media (10% v/v) and performed under sterile condition. The sample was incubated from Day 0 to Day 5 under shaking at 150 rpm and 37 °C (PROTECH S1-100D). Liquid samples of 10 ml were removed daily and centrifuged at 4000 rpm for 15 min (Eppendorf Centrifuge 5810, Germany). The pH of the supernatant was recorded (HACH sensION+, USA), and the remaining Cr(VI) was analyzed using the 1,5-diphenylcarbohydrazide method (Gnanamani *et al.*, 2010) at an absorbance of 540 nm with a UV spectrophotometer (HACH DR6000, USA). Pellet cell was naturally dried within 2 days and diluted to 1:1:3 (nitric acid: H₂O₂: DI water) within 24 hr at room temperature (Tangahu *et al.*, 2013). The digested Cr(VI) concentration was measured by 1,5-diphenylcarbohydrazide method.

Determination of rhizosphere bacteria population: The number of colony-forming units (CFUs) was performed using the standard plate count method on Day 0, Day 2 and final day of exposure (Day 5). Serial dilutions were prepared in duplicate, and

0.1 ml of each dilution was spread onto trypticase soy agar.

Plant growth-promoting activities: The following PGP abilities were determined as previously described by Orhan (2016) and Khan *et al.* (2017).

Ammonia production: For assessing the ability to produce ammonia, fresh culture was added to peptone water (Peptone 20 g l⁻¹ and NaCl 30 g l⁻¹) and incubated at 30 °C with constant shaking at 140 rpm for 5 days. After incubation, 0.2 ml of the culture supernatant was mixed with 1 ml of Nessler's reagent (Anees *et al.*, 2017). The OD of the mixture was measured at 450 nm using a UV spectrophotometer (HACH DR6000, USA), and development of yellow to brown color was considered as a positive result for ammonia production.

Phosphate solubilization ability: For phosphate solubilization assay, fresh culture was inoculated with Pikovskaya's modified medium and incubated at 30 °C for 7 days. Pikovskaya's modified medium contained the following per liter: glucose, 10 g; Ca₃(PO₄)₂, 5 g; (NH₄)₂SO₄, 0.5 g; MgSO₄·7H₂O, 0.1 g; KCl, 0.2 g; yeast extract, 0.5 g; MnSO₄·H₂O, 0.002 g; FeSO₄·7H₂O, 0.002 g; NaCl, 30.0 g; and agar, 15 g. Development of clearance zone on Pikovskaya's agar was considered positive for phosphate solubilization.

IAA production: The IAA concentration produced by each isolate was determined according to Salkowski's colorimetric method. Each bacterial culture was grown in a nutrient broth medium containing 0.1 mg ml⁻¹ l-tryptophan and 5% NaCl and then incubated at 30 °C for 2–4 days. After incubation, the broth was

centrifuged, the supernatant was retained, and 1 ml of the supernatant was mixed with 2 ml of Salkowski's reagent (2% 0.5 FeCl₃ in 35% HClO₄ solution) and stored in dark for a minimum of 30 min. After 30 min in dark, the absorbance was read at 530 nm (HACH DR6000, USA). The IAA concentration was determined using a calibration curve of pure IAA as standard.

Statistical analysis: All analyses were performed using SPSS version 16.0 (IBM, USA). The effects of Cr(VI) concentrations on bioreduction and CFU were analyzed using one-way ANOVA. DMRT was used to evaluate differences between all parameters at 95% confidence limit ($p < 0.05$), unless otherwise stated. Sampling and analyses were performed in triplicate.

Results and Discussion

Fig. 1 shows the result of Cr(VI) reduction abilities of three rhizobacteria (*B. cereus*, *B. aerius* and *E. profundum*) in reduction media spiked with 60 mg l⁻¹ Cr(VI). Bioreduction of Cr(VI) in the reduction media by *B. cereus* was 13.7%, which was higher compared with those of *B. aerius* (4.4%) and *E. profundum* (3.6%) (Table 1). The study on the biosorption of Cr(VI) within rhizobacteria cells showed that only *B. aerius* could adsorb high Cr(IV) (56.1 mg g⁻¹) after 5 days of exposure, whereas *B. cereus* and *E. profundum* adsorbed 4.3 and 8.1 mg g⁻¹, respectively (Fig. 2). A previous study reported that *B. cereus* could reduce Cr(VI) with initial concentrations of 60 and 70 mg l⁻¹ by up to 96.7% and 72.1%, respectively (Murugavelh *et al.*, 2013; Shaikh *et al.*, 2017).

The effect of bioreduction of Cr(VI) on the pH of the reduction media was also examined. The pH was acidic (pH

Fig. 1 : Cr(VI) in reduction media by *B. cereus*, *B. aerius* and *E. profundum* with an initial concentration of 60 mg l⁻¹ Cr(VI) (SW= saline water) [*represents statistically significant differences in Cr(VI) reduction for each rhizobacteria ($p < 0.05$)].

Fig. 2 : Biosorption of Cr(VI) by *B. cereus*, *B. aerius* and *E. profundum* cells [* represents statistically significant differences in the biosorption of Cr(VI) for each rhizobacteria ($p < 0.05$)].

Fig. 3 : Effect of Cr(VI) reduction on pH in saline water with an initial concentration of 60 mg l^{-1} Cr(VI) [* represents statistically significant differences in pH between control and presence of Cr(VI) for each rhizobacteria ($p < 0.05$)].

5.34–5.97) in all three rhizobacteria with an initial concentration of 60 mg l^{-1} Cr(VI) (Fig. 3). Compared with the pH of the reduction

media without Cr(VI), the media with Cr(VI) had neutral pH (6.88–7.93). Similarly, a previous study reported that pH 6 is the

Fig. 4 : Rhizobacteria population in Cr(VI) reduction medium [* represents statistically significant differences in CFU for each rhizobacteria ($p < 0.05$)].

Table 1 : Characterization of rhizosphere bacteria for plant growth-promoting activities and ability to reduce Cr(VI)

Heavy metal	Rhizobacteria	Plant Growth-Promoting Activities			Cr(VI) Reduction (%)	Cr(VI) Biosorption (mg g^{-1})	CFU Reduction (%)
		NH ₃	P Solubilization	IAA	Reduction media	Cell	
Cr(VI)	<i>B. cereus</i>	+	+	+	13.7	4.3	99.9
	<i>B. aerius</i>	+	+	+	4.4	56.1	99.8
	<i>E. profundum</i>	+	-	+	3.6	8.1	99.9

optimum for the reduction of Cr(VI) with *B. cereus* (Murugavelh and Mohanty, 2012). pH is an important parameter that affects Cr(VI) solubility and bacterial growth in wastewater treatment (Murugavelh and Mohanty, 2012). Rhizosphere microbes can reportedly improve metal solubility and availability by reducing the soil pH or by producing chelators and siderophores, which could enhance heavy metal extraction by plants (Rajkumar *et al.*, 2012; Islam *et al.*, 2014; Cho, 2017).

The effect of Cr(VI) reduction on rhizobacteria growth is shown in Fig. 4. The growth of each rhizobacteria reduced nearly to 100% CFU reduction at a Cr(VI) concentration of 60 mg l^{-1} (Table 1). In addition, the growth of rhizobacteria reduced drastically due to toxic effect of Cr(VI) compared with the control (Without Cr(VI)). A previous study reported that Cr(VI) may reduce microbial biomass completely, decrease specific population, or even cause shifts in microbial community structure even with a small amount of heavy metal (Zhuang *et al.*, 2007).

Table 1 summarizes the ability of each rhizobacteria to reduce Cr(VI) and the potential to produce PGP substances. *B. cereus* and *B. aerius* could produce more PGP substances, whereas *E. profundum* was not able to solubilize phosphate.

From the perspective of Cr(VI) reduction, *B. cereus* showed greater ability to produce PGP substances and reduce Cr(VI) than *B. aerius*. These results also prove that all rhizobacteria are not only resistant to high concentrations of Cr(VI) but also serve different functions within the rhizobacteria community. However, not all rhizobacteria can reduce toxicity and promote plant growth simultaneously. Each species of rhizobacteria has its inherent participation in plant-microbe interaction to survive toxicity. Hardoim *et al.* (2008) reported that the presence of associated bacteria in phytoremediation can benefit both parties, *i.e.*, plants can grow better, suffer less stress, solubilize macronutrients and establish in different ecosystems whereas microbes can benefit from nutrient availability from plants.

Bioreduction of Cr(VI) at an initial concentration of 60 mg l^{-1} in the reduction media by *B. cereus* was 13.7%, which was higher compared with that of *B. aerius* (4.4%) and *E. profundum* (3.6%). *B. aerius* could adsorb high Cr(VI) concentration of 56.1 mg g^{-1} . The best condition for these rhizobacteria to remove or adsorb Cr(VI) was at acidic pH (5.0–6.0). Cr(VI) was highly toxic to rhizobacteria and thus drastically reduced their growth. Both *B. cereus* and *B. aerius* could produce PGP substances, including ammonia and IAA, and solubilize phosphate. Although some

Cr(VI)-resistant rhizobacteria could not reduce Cr(VI) or produce PGP substances, all of them serve inherent functions to maintain the sustainability of plant growth and health while enhancing phytoremediation.

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