Characterization of Rhizosphere Bacteria and Their Potency as Phytoremediation Promoting Agents for Cr (VI) Contaminated Soil

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Abstract. Rhizosphere bacteria have an adaptation mechanism in the chromium contaminated environment and produce compounds that promote the heavy metal resistance of plants. Bacteria were isolated from tannery waste contaminated rhizospheric soil of Ficus septica Burm. F Screening was done based on the level of Cr (VI) tolerance. High tolerant isolates were tested for their potential in phosphate-solubilizing, Indole Acetic Acid (IAA) production, exopolysaccharide (EPS) production, and presence of chromium reductase (ChR) genes. Based on the results of screening, there were four rhizosphere bacterial isolates (R7, R9, R10, and R12) which were tolerant to 800 mg/L of Cr (VI). All isolates were able to produce IAA, EPS, and dissolve phosphate in medium containing 150 mg/L Cr (VI). The highest production capacity of IAA (25.08 μg/mL) and phosphate-solubilizing (150.98 μg/mL) was shown by R9 isolate, while the highest EPS production was found in R12 isolate (0.02 mg/L). ChR gene was found in R10 and R12 isolates. Bacterial isolates obtained from this study were potential to be used as promoting agents for phytoremediation of Cr (VI) contaminated soil.

Keywords: Rhizosphere bacteria, phytoremediation, heavy metal, chromium

1. Introduction
Chromium (Cr) is one of the heavy metal class that has the potential to be an environmental pollutant. Chromium can exist in different oxidation states, but only the trivalent chromium Cr (III) and the hexavalent chromium Cr (VI) are the most stable in the natural environment. Trivalent Cr (III) is a common form in nature, much less water-soluble and comparatively immobile, while hexavalent chromium is produced from various anthropogenic activities and has toxic effects on humans and other organisms. It can damage the environment because of its high water solubility and mobility than Cr (III) [1,2,3].

Chromium pollution can be sourced from various industries, including tanning industry [4,5]. Chromium containing tanning waste is the chromate form CrO₄²⁻[6]. Chromates are toxic, carcinogens, teratogens, mutagens and chronic that result in the death of organisms [7,8]. Based on the hazard of Cr (VI) pollution, a safe and effective technology is needed to handle it, one of them is by utilizing bacteria.

Some Cr (VI) tolerant bacteria can accumulate or reduce to Cr (III) [9,10]. If the concentration of these metals in the environment is too high, the heavy metals that enter the bacterial cell are no longer accumulated, but will be removed from the cell so as not to interfere with metabolism [11]. The
limited ability of accumulation by bacteria underlies the need for a combination of bioremediation with plants [12]. Bacteria can provide a variety of important compounds such as IAA, solubilize phosphate and EPS, so that bacteria are often used as bioinoculants to improve the metabolic function and permeability of root cell membranes and increase plant resistance and ability to remediate heavy metals contaminated soils [13,14,15].

Some bacteria are reported to be tolerant of Cr (VI) and have the ability to support phytoremediation in various types of plants. Previously, rhizosphere bacteria associated with Prosopis juliflora such as Pseudomonas aeruginosa sp. strain PJRS20, Bacillus licheniformis, and Arthrobacter sp. have been reported tolerant to Cr (VI) and potential as phytoremediation enhancing agents for Cr (VI) contaminated soil [16]. However, there have not been many studies on Cr (VI) tolerant bacteria associated with Ficus septica Burm. F. Therefore, this study aims to obtain rhizosphere bacterial isolates that have the potential to provide supporting compounds for phytoremediation ability of Cr (VI) contaminated soil.

2. Materials and Methods

2.1. Isolation of Bacteria

Rhizosphere soil samples are suspended in sterile physiological saline (0.85%) and serial dilutions are carried out until 10⁻⁷. A sample suspension of 0.1 mL of each dilution series was inoculated on sterile Nutrient Agar (NA) medium containing Cr (VI) in the form of K₂Cr₂O₇ 150 mg/L that had been sterilized by filtration through a 0.22 nm membrane filter (Millipore Corp., Bedford, MA). The sample was incubated at 30°C for ± 24 hours and the colonies were observed and purified.

2.2. Screening of Cr (VI) Tolerant Bacteria

The bacteria obtained were grown on NA medium containing Cr (VI) with multilevel concentrations from 150 mg/L to 1000 mg/L. Bacteria were incubated at 30°C for about three days and their growth was observed. Bacteria are categorized as the most tolerant if they can grow at the highest concentration of Cr (VI).

2.3. Bioassay of IAA Production, Phosphate Solubilizing, and EPS Production

Five milliliters cell suspension of Cr (VI)-tolerant bacteria were tested for their ability to produce IAA by growing at 50 mL Tryptic Soy Broth (TSB) with 2% (v/v) L-Tryptophan (1 μg/mL) and incubated at 28°C. Total cell of suspension was 10⁷-10⁸ or OD 0.5 at wavelength of 535 nm (measured by haemocytometer or spectrophotometer). Medium which was not inoculated with the bacteria served as the control. Three milliliters of bacterial suspension is harvested every day for four days to determine the IAA production of each isolate. The bacterial suspension was centrifuged at 10,000 rpm for 10 minutes, then 2 mL of Salkowski reagent was added to 1 mL of the supernatant and incubated for 30 minutes in a dark room until a pink color is observed which is an indicator of IAA production. The absorbance of the sample was measured at a wavelength of 535 nm. IAA concentrations is calculated based on IAA standard curves [18,19].

The activity of phosphate solubilizing bacteria were analyzed using a modified method [21]. Five milliliters of bacterial (OD 1 at wavelength of 540 nm) culture was grown on Pikovskaya Broth media added with 0.5% Tricalcium phosphate (TCP) and incubated at 28°C. Two milliliters of bacterial suspension were centrifuged at 10,000 rpm for 10 minutes, then 10 mL of chloromolybdate reagent and 0.1 mL of chlorostannous acid were added to 1 mL of the supernatant. The suspension was diluted to 25 mL using sterile aquades (ELGA LabWater), homogenized and incubated for 10 minutes. The absorbance of the sample is measured at a wavelength of 690 nm. Calculation of solubilized phosphate concentration is calculated based on a standard phosphate curve [31].
EPS extraction was performed by modified ethanol precipitation method [20]. Five milliliters of bacterial isolate (OD 0.5 at wavelength of 600 nm) was grown on NB medium containing 150 mg/L Cr (VI) enriched with 2% (w/v) sucrose and incubated for two days at 37°C. A total of 10 mL samples were centrifuged at 10,000 rpm for 30 minutes at 20°C. The resulting supernatants were boiled at 100°C for 15 minutes, after cooling, trichloroacetic acid was added (4% w/v) to protein precipitation followed by centrifugation at 10,000 rpm for 30 minutes at 4°C. The supernatants were removed and precipitated overnight at 4°C with two volumes of absolute ethanol. After centrifugation at 10,000 rpm for 30 minutes at 4°C, the pellets obtained were dried at 100°C for 15 minutes and then the EPS weight was calculated [20]. Data were analyzed using Two-way ANOVA using univariate models to evaluate interactions between treatments at a 95% confidence level and then Tukey tested using SPSS 16.0 for windows software program to evaluate differences in the ability of each rhizosphere bacterial isolate.

2.4. Detection of Chromate Reductase (ChR) Gene
Detection of the chromate reductase gene in the bacterial genome DNA was carried out by PCR amplification method using a specific primer Chromium Reductase Gene (ChR) Forward 5'TCACGCCGGAATATAACTAC- 'and Reverse 5'CGTACCCTGATCAATCACTCCTT3'. The PCR program used for ChR gene amplification was initial denaturation at 94°C for 5 minutes, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 50°C for 30 s and extension at 72°C for 1 min and a final extension of 72°C for 10 min. The PCR product obtained was qualitatively confirmed using DNA electrophoresis in 1.5% agarose gel and visualized using a UV transilluminator [22].

2.5. Identification of Selected Rhizosphere Bacteria Based on 16S rDNA
Bacterial chromosomal DNA was extracted using a modified heat treatment method. The sequence of 16S rDNA was amplified using universal primers 27f (5'-GAG AGT TTG CTG GCT ATC CAG- 3') and 1492r (5'-CTA CGG CTA TGT CCT TAC GA- 3'). PCR amplification of 16S rDNA gene was carried out in an icycferm cycle (Bio-Rad) subjected to denaturation at 94°C for 5 min, followed by 35 cycles consisting of denaturation at 94°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 1.5 min and a final extension of 72°C for 5 min. Amplicons of 16S rDNA were purified and sequenced in First BASE, Malaysia. The 16S rDNA sequences of isolates were aligned with reference strains using the MEGA V.6 program. Phylogeny tree was constructed and inferred with Neighbor-joining algorithm with Tamura-Nei model using 1000 replicates bootstraps [22,32].

3. Result and Discussion
3.1. Cr (VI) Tolerant Rhizosphere Bacteria
The density of the rhizosphere bacteria on NA medium containing 150 mg/L Cr (VI) was 4.8x10^5 CFU/g whereas in the control (NA media without Cr (VI)) 20.6 x10^5 CFU/g. The total isolates of rhizosphere bacteria obtained were 12 isolates. The isolates obtained have varying degrees of tolerance of Cr (VI). The most tolerant Cr (VI) to 800 mg/L are R7, R9, R10, and R12 (Figure 1). Previous studies [24] reported that Cellulosimicrobium sp. isolated from tannery waste was tolerant to Cr (VI) more than 100 mg/L. Some Cr (VI) tolerance mechanisms of bacteria include (1) balancing oxidative stress-induced Cr (VI) by activating ROS scavenging enzymes (eg. catalase and superoxide dismutase); (2) DNA repair with SOS response enzymes (RecA, RecA, RecG, RuvAB) to fight DNA damage; and (3) regulation of iron absorption to prevent the production of hydroxyl radicals through the Fenton reaction [24].
Table 1. Cr (VI) Tolerance of rhizosphere bacteria

| Isolate | Concentration Cr (VI) (mg/L) |
|---------|-----------------------------|
|         | 150 | 200 | 300 | 400 | 500 | 600 | 700 | 800 | 900 | 1000 |
| R1      | +   | +   | +   | -   | -   | -   | -   | -   | -   | -    |
| R2      | +   | +   | +   | -   | -   | -   | -   | -   | -   | -    |
| R3      | +   | +   | +   | +   | -   | -   | -   | -   | -   | -    |
| R4      | +   | +   | +   | +   | -   | -   | -   | -   | -   | -    |
| R5      | +   | +   | +   | +   | +   | -   | -   | -   | -   | -    |
| R6      | +   | +   | +   | +   | +   | +   | -   | -   | -   | -    |
| R7      | +   | +   | +   | +   | +   | +   | +   | -   | -   | -    |
| R8      | +   | +   | +   | +   | +   | +   | +   | +   | -   | -    |
| R9      | +   | +   | +   | +   | +   | +   | +   | +   | -   | -    |
| R10     | +   | +   | +   | +   | +   | +   | +   | +   | -   | -    |
| R12     | +   | +   | +   | +   | +   | +   | +   | +   | -   | -    |

Figure 1. Cr (VI) tolerant rhizosphere bacteria in nutrient agar containing 800 mg/L Cr (VI)

3.2. IAA Production, Phosphate Solubilizing, and EPS Production by Selected Rhizosphere Bacteria

Selected isolates that have the highest tolerance level of Cr (VI) show IAA production activities with varying concentrations (Figure 2a). Isolates R7, R9, and R10 produced an optimal IAA production at 72 hours where isolate R9 as the highest IAA producer (25.08 μg/mL) and significantly different from other isolates and controls, this can be seen from the difference in notation. R7, R9, and R10 were significant decrease in IAA concentration at 96 hours. The optimum IAA production by R12 isolates was achieved at 24 hours by 16.78 μg/mL.

The R9 isolate has similar capability with IAA-producing *Pseudomonas aeruginosa* OSG41 with 24.2 μg/mL IAA in medium containing Cr (VI) 200 μg/mL and L-Tryptophan 100 μg/mL. The difference in IAA concentration is due to differences in isolates tested and L-Tryptophan concentrations added to the medium as IAA precursors. IAA concentrations decrease with the addition of Cr (VI) [17].

The results showed that the R9 isolate can dissolve phosphate (P) 150.98 μg/mL in 48 hours incubation time in which performed the highest and fastest and significantly different to dissolve the phosphate rather than other isolates, this can be seen from the difference in notation, while R7 and R10 isolates dissolved P 146.69 μg/mL and 111.68 μg/mL, respectively. The three isolates (R9, R7, and R10) experienced a significant decrease in P concentration at 72 hours incubation to 121.92 μg/mL, 108.10 μg/mL, and 111.68 μg/mL, respectively. R12 can dissolve phosphate 116.28 μg/mL at 72 hours which the higher concentration than other incubation time (Figure 2b). The results of this study are similar to the study of Oves et al. (2013) Cr (VI) *P. aeruginosa* OSG41 reducing bacteria can dissolve phosphate to a concentration of 185.6 μg/mL on Pikoivskaya Broth media containing 200 mg/L Cr.
The highest IAA production and phosphate solubilizing of several isolates can indicate that at that time the isolate was in the final logarithmic phase towards the stationary phase. As for the decrease in IAA production and phosphate solubilizing indicate a phase of death.

The highest total EPS among the four bacterial isolates at 48 hours incubation time was at R12 isolates, 0.02 mg/L, while isolates R7, R9 and R10 were not significantly different, ie 0.017, 0.014 and 0.011 mg/L (Figure 2c). It was reported that *Pseudomonas aeruginosa* Rb-1 and *Ochrobactrum intermedium* Rb-2 intermediates grown for 48 hours showed a total EPS of 13.63 mg/g with the addition of Cr (VI) 1000 μg/mL [20]. Based on these results, it can be seen that the total EPS in this study is relatively low, this may be due to differences in the concentration of Cr (VI) as well as the characteristics of bacteria in their cell protection to Cr (VI) in the environment.

Figure 2. The ability of Cr (VI) tolerant bacteria for a) IAA production b) Phosphate solubilizing c) EPS production

Data were expressed as the mean ± standard deviation of three replicates experiment using Two-way ANOVA test at a = 0.05. Different notations indicate significant difference among treatments (p < 0.05).
3.3. Chromium Reductase (ChR) Gene of Bacteria
Four isolates tolerant of Cr (VI) in a concentration of 800 mg/L were detected for the presence of the chromium reductase (ChR) gene. Chromium reductase was found in isolates R10 and R12 (Figure 3). The gene is known to be able to express enzymes that catalyze the reduction reaction of Cr (VI) to Cr (III) [25]. The PCR amplification product of the ChR amplitude was 268 bp when confirmed using DNA ladder (Figure 1) as reported [22] that the partial amplification of the chromate reductase gene showed 268 bp fragment in three Gram positive bacteria isolated from the Cr contaminated area.

Previous studies also obtained DNA fragments of 268 bp in size from the bacterial chromate reductase gene isolated from tannery effluents. This confirms that the presence of the chromate reductase gene in both bacteria strengthens its ability to reduce Cr (VI) to Cr (III) [26].

Figure 3. Product of PCR amplification of ChR gene fragments.

3.4. Potency of Selected Rhizosphere Bacteria as Cr(VI) Phytoremediation Promoting Agent
The character of each Cr (VI) tolerant bacterial isolate showed that the isolate which had the highest IAA production ability and phosphate dissolution was R9 isolate, while the highest EPS production was R12 isolate. Between the two isolates (R9 and R12), isolate R12 was detected for the presence of the ChR gene. This shows that R12 is the most potential isolate, each isolate has a different ability in its role in supporting phytoremediation of heavy metal polluted soils. IAA can support the absorption of nutrients and metals by multiplying plant roots and facilitate adaptation and tolerance to heavy metals in plants which are gripped by heavy metals by inducing physiological changes [3]. Phosphate solvent bacteria can conduct metal sulfide precipitation in highly insoluble soils, causing the removal of heavy metals from solution. Some bacteria can free P by forming metal phosphate biomineral formation through the accumulation of high P concentrations that are broken down from glycerol-2-phosphate on the surface of bacterial cells, or bacterial P cycle processes [27,28].

EPS excretion by plant-bacteria is a protective barrier against the danger of heavy metals through metal biosorption. Mechanisms that include metal biosorption into EPS include metal ion exchange, complexation with negatively charged functional groups, adsorption, and bioprecipitation [29,30]. In addition to using the ability to produce phytoremediation supporting compounds (IAA, EPS, and phosphate dissolution), bacteria can also reduce the toxic effects of heavy metals Cr (VI) rhizosphere soils on plants by reducing them to Cr (III), so that translocation of Cr (VI) into plant tissue can be reduced and plant biomass increased. Previous studies [16] reported that the culture of Cr-resistant
rhizosphere bacteria could increase growth and resistance of *Prosopis juliflora* plants to heavy metal toxicity.

### 3.5. Phylogenetic Tree of Selected Rhizosphere Isolate Based on 16S rDNA Sequences

Phylogeny tree of selected rhizosphere bacterial isolate (R12) was constructed based on 16S rDNA sequences and compared with reference strains. As shown in the Figure 4, R12 isolate was identified as *Bacillus subtilis* NRS 744\(^T\) with similarity value 100%. Previous study has identified some rhizosphere bacteria from *Prosopis juliflora* that were Cr (VI) tolerant and have potential as Cr (VI) phytoremediation promoting agents. Those isolates were *Bacillus* sp., *Bacillus licheniformis*, *Pseudomonas aeruginosa*, *Staphylococcus* sp., and *Pseudomonas stutzeri*.

![Figure 4. Phylogenetic tree based on the 16S rRNA gene sequence of R12 isolate using Neighbor-joining algorithm](image)

### 4. Conclusion

Four Cr (VI) tolerant isolates (R7, R9, R10, and R12) from this study had the ability to produce IAA, EPS, and phosphate dissolution. Of the four isolates, those detected to have the ChR gene were isolates R10 and R12. All isolates can be a good choice for the application of bacterial association with plants in remediating a heavy metal contaminated environment, but R12 is the most potential isolate. Further research is needed for the application of bacterial inoculation in *Ficus septica* Burm.F plants in Cr (VI) contaminated soil, so that it can be known amount of Cr (VI) uptake to plant tissue.

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