

Isolation and identification of chromium-tolerant bacterial strains and their potential to promote plant growth

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Abstract. Remediation and management of Chromium (Cr) contaminated soils for safe usage has been a serious challenge. The aim of present work was to isolate and identify Cr tolerant bacterial strains and assessing their plant growth promoting potential under controlled conditions. Soil samples were collected from Gujrat District, Pakistan, and analyzed for Cr content and bacteria isolation. In-vitro screening was done for chromium tolerance and plant growth-promoting (PGP) abilities. The tolerant isolates which also exhibited PGP abilities were used as inoculants in germination and pot trial experiment. The isolates A5 and A6, identified as *Pseudomonas plecoglossicida* and *Staphylococci saprophyticus* through 16S rRNA gene sequencing, were found to be tolerant up to 700 mg L⁻¹ of Cr (VI). Both were effective in solubilizing phosphate but only A5 (*Pseudomonas plecoglossicida*) was able to produce indole acetic acid. A5 also increased the percentage of seed germination from 17 to 46% and spinach plant's biomass by 44% with respect to the control. Keeping in view the results obtained, A5 (*Pseudomonas plecoglossicida*) appeared as the best species that was able to tolerate Cr stress and promote plant growth. Further, it can be developed as a bio-inoculant for non-food agricultural applications for remediation of Cr contaminated soils.

1 Introduction

Industrialization, agricultural practices and various anthropogenic activities lead to the build-up of heavy metals in the environment. These heavy metals pose serious threats to terrestrial and aquatic ecosystems. Chromium (Cr) is one of the seventeen chemicals that pose the highest danger to human health according to the United States Environmental Protection Agency (US-EPA) [1]. It is a transition element which occurs naturally in rocks, plants, animals, soil, gases and in volcanic ash [2]. Chromium is one such toxic metal with regulatory limits set at 1.3 mg kg⁻¹ in plants (WHO), 0.1 mg L⁻¹ in water (WHO) and 100 mg kg⁻¹ in soil [3,4].

Chromium exists in the environment in several oxidation states of which the most stable and common are hexavalent and trivalent states which exhibit different properties. Cr(III) is considered to be a vital element, for glucose, lipid and protein metabolism in animals in small amounts whereas Cr(VI) is extremely toxic for biological systems [5]. It disseminates easily into soil and water as it is highly soluble, mobile and permeable through biological membranes [6].

Cr(VI) is usually present in the form of chromate (CrO₄⁻²) and dichromate (Cr₂O₇⁻²), strong oxidizing agents, which get reduced from Cr(VI) to Cr(V) inside the cells leading to the formation of reactive oxygen species (ROS)

[7]. The effects of chromium exposure in plants include reduction in seed germination, root length, plant biomass and plant height [8]. Toxic level of Cr(VI) in plants also shows drastic effect on plant's dry matter and yield [9].

In this scenario, management and remediation of Cr contaminated soils is a challenge for all the stakeholders. Remediation through microorganisms, like bacteria, is a cost effective method and may be beneficial for plant growth [10]. Many microorganisms can accumulate or reduce certain heavy metals easily [11]. Microbes have a high surface area to volume ratio hence they provide a large area of contact to interact and react with the metals in their surroundings [12].

These microorganisms can further benefit the environment if they work simultaneously to improve other aspects under concern like improving plant growth. Thus, heavy metal-resistant plant growth-promoting bacteria are a novel and promising approach to solve the soil toxicity and food crop issues [10]. In this context, the specific objectives of the present study were i) Isolation and identification of Cr tolerant bacterial strains from contaminated soil, ii) Assessment of plant growth promoting potential of Cr(VI) tolerant species under controlled conditions.

2 Materials and methods

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2.1. Soil sampling and characterization

Four soil samples were obtained from pearl millet fields, in Gujrat (32.57°N, 74.08°E), Pakistan, irrigated by wastewater from the industrial city. Soil was extracted in bulk amounts from 0-15 cm depth (upper surface) and from 15-30 cm depth (sub-surface). Physico-chemical parameters like electrical conductivity (EC) and pH were measured by adding soil to water in the ratio 1:5. The amount of hexavalent chromium (Cr(VI)) present was ascertained as per standard methods (APHA, 2012) using UV-Vis spectrophotometer (T-60U PG INSTRUMENTS UK) and soil sample with the highest chromium content was selected.

2.2 Isolation and identification of bacteria from chromium-rich soil

For the isolation and enumeration of bacteria, serial dilution technique was adopted [13]. One mL of each dilution was spread on nutrient agar plates followed by incubation at 37°C for 24 hours. After 24 hours, bacterial colonies were picked and cultures were purified by repeated streaking.

For their molecular characterization, the isolates were preserved in glycerol and sent to Genome Analysis Department Macrogen Inc., South Korea, for 16S rRNA gene sequencing. The sequences obtained were identified using BLAST search in the database of National Center for Biotechnology Information (NCBI). Phylogenetic analysis to check the relatedness of identified strains to existing ones was done using MEGA 7 software.

2.2. Minimum inhibitory concentration of chromium

The minimum inhibitory concentration (MIC) is the lowest concentration of Cr VI which inhibits growth of bacteria [14]. Nutrient agar plates, spiked with various concentrations of chromium (0, 100, 150, 200, 250, 300, 350, 500, 600, 700, 800 mg L⁻¹), were prepared and loops full of pure bacterial cultures were streaked on the prepared plates and were left to incubate at 37°C for 48 hours. Chromium tolerance was recorded based upon the growth of isolates in both types of media.

2.4. In vitro screening of plant growth-promoting (PGP) activity of Cr-tolerant isolates

2.4.1 Indole acetic acid (IAA) production

Indole acetic acid is a plant growth stimulating hormone. To check IAA production by the bacterial isolates, they were cultured on Luria Broth medium containing tryptophan. The inoculated media was incubated at 37°C for 24 hours. After the incubation period the culture broth tubes were centrifuged at 2,000 rpm for 20 min and 1 mL of the supernatants were transferred to empty sterile test tubes. One drop of Orthophosphoric acid and 2 mL of freshly prepared Salkowski's reagent (50 mL; 49 mL, 35% HClO₄ + 1 mL, 0.5M FeCl₃) was added to the tubes.

The tubes were left to incubate for 30 minutes [15]. The development of a pink color confirmed IAA production by the isolate.

2.4.2 Phosphate solubilizing activity

Chromium tolerant bacteria were tested for their phosphate solubilizing ability; a plant growth enhancing trait, by disc diffusion assay described by Gupta et al. [16]. Inorganic phosphate, calcium phosphate [Ca₃(PO₄)₂], was added to nutrient agar medium and each isolate was streaked in the center of the plates and left to incubate at 37°C for 5 days. The zone of clearing around the colony confirmed phosphate solubilizing ability. The larger the diameter of the halo, the better was the solubilizing activity.

2.4.3 Pot trial experiment for spinach seedlings

Clayey loam soil in bulk quantity was dried, crushed in a ball mill and sieved using a 2mm-mesh. The prepared soil was spiked with Cr (VI) salt (100, 200, 300, 400 mg kg⁻¹) and one set was fixed as control. Three week old spinach seedlings were obtained from a local plant nursery. These were carefully transplanted into the spiked soil. Organic compost and PGPB isolates, A5 and A6 (at 0.5 OD₆₀₀), were added to each of the five replicates of the four Cr(VI) treatments and the control. Spinach plants were provided the required cool temperature, constant watering and mulching for two weeks after which they were harvested and their physical parameters were recorded.

2.4. Statistical Analysis

Data of each treatment, was subjected to single factor and two-way analysis of variance (ANOVA). The means and standard deviations for all parameters were also calculated.

3 Results and Discussion

Equations should be centred and should be numbered with the number on the right-hand side.

3.1. Physico-chemical analyses of soil

The pH, electrical conductivity (EC) and the chromium composition of the four soil samples were tested. The pH of the soils fell in the same slightly acidic to neutral range i.e. 6.86, 6.85, 6.50 and 6.77 for the samples S1, S2, B1 and B2, respectively. EC, which is a measure of the amount of salts present, varied considerably due to differences in inherent factors like soil mineral composition and texture etc. The EC of S1, S2, B1 and B2 was recorded as 56.3, 163.1, 256 and 550 μS cm⁻¹ respectively. Analysis for hexavalent chromium via UV-Vis spectrophotometer revealed that the amount of Cr(VI) in B2 was the highest i.e. 177 mg kg⁻¹. This amount is higher than the permissible limit for Cr(VI) in soil, 100

mg kg⁻¹, according to Dutch Standards [4]. B2 sample, being chromium-rich, was appropriate for the isolation of chromium tolerant bacteria.

3.2. Identification and PGP activity of bacterial isolates

Bacterial colonies were isolated from soil sample B through the serial dilution method. Six isolates were able to solubilize inorganic phosphate to varying degrees however, only one tested positive for IAA production. Table 1 shows the results of all the tests conducted.

Table 2. Accession numbers and Plant Growth Promoting (PGP) characteristics of bacterial isolates.

Strain ID	Accession numbers	IAA production	P solubilization
A2	KX262672	-	+
A3	KX262673	-	-
A4	KX262674	-	+
A5	KX262675	+	+
A6	KX262676	-	+
A7	KX262677	-	-
A8	KX262678	-	-
A9	KX262679	-	-
A10	KX262680	-	+
A11	KX262681	-	-
A13	KX262682	-	+

The 11 isolates were characterized at molecular level and the predominant species were identified as *Bacillus* sp. (A2), *Staphylococcus haemolyticus* (A3), *Bacillus cereus* (A4), *Pseudomonas plecoglossicida* (A5), *Staphylococcus saprophyticus* (A6), *Bacillus stratosphericus* (A7), *Staphylococcus aureus* (A8, A9, A11), *Bacillus aryabhatai* (A10) and *Agrobacterium tumefaciens* (A13).

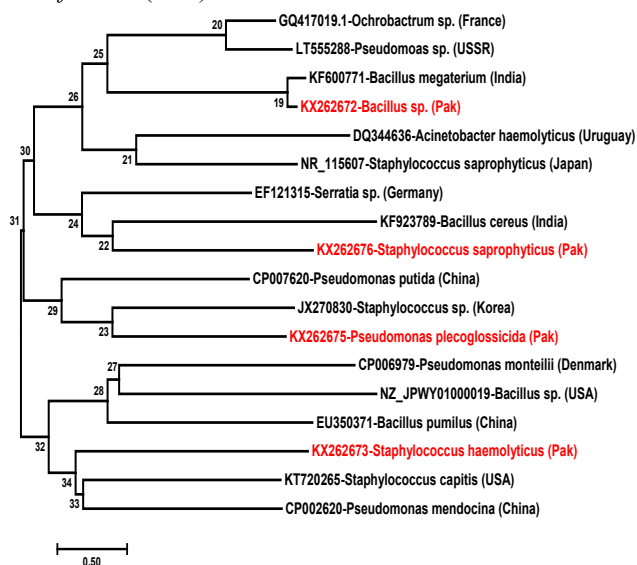


Fig. 1. Below the figure. Phylogenetic neighbor-joining tree of isolates created with MEGA 7 software.

The sequences obtained from 16S rRNA gene sequencing were submitted to GenBank under accession numbers listed in Table 1 and a phylogenetic tree was constructed.

The tree (Figure 1) lists the four most tolerant strains isolated in this study and other related chromium-tolerant strains that have been quoted in literature [5,10,17]. *Pseudomonas plecoglossicida* (A5) and *Staphylococcus haemolyticus* (A3) are the species being first reported and identified as chromium-tolerant bacteria from Pakistan.

2.4. Minimum inhibitory concentration (MIC) of chromium

Table 2. Chromium tolerance of bacterial isolates against various Cr (VI) concentrations in nutrient agar plate method.

Strain ID	Chromium Concentrations (mg L ⁻¹)							
	200	250	300	350	500	600	700	800
A2	+++	+++	+++	++	-	-	-	-
A3	+++	+++	+++	++	-	-	-	-
A4	+++	+++	++	-	-	-	-	-
A5	+++	+++	+++	+++	+++	++	+	-
A6	+++	+++	+++	+++	+++	++	+	-
A7	+++	+++	++	-	-	-	-	-
A8	+++*	++	-	-	-	-	-	-
A9	+	-	-	-	-	-	-	-
A10	+	-	-	-	-	-	-	-
A11	+++	+++	++	-	-	-	-	-
A13	+++	+++	++	-	-	-	-	-

+++ maximum growth, ++ moderate growth, + less growth, - no growth, *bacteria changes colour at specific concentration

The eleven isolates were screened to check their Cr(VI) tolerance and were subjected to MIC assays. Strains A5 and A6, from the genus *Pseudomonas* and *Staphylococcus* respectively, were found to be tolerant of Cr(VI) up to concentration as high as 700 mgL⁻¹ (Table 2). The next group of tolerant strains, for concentration up to 350 mg L⁻¹ Cr(VI), were A2 and A3 from genus *Bacillus* and *Staphylococcus* respectively. It is well reported that *Pseudomonas* sp. was exhibit high tolerance to Cr(VI) and is highly effective in its reduction [18] as were *Staphylococci* sp. [19] and *Bacillus* sp. [11].

2.4. IAA production and phosphate solubilization efficacy

The eleven isolates were tested in vitro to determine their ability for production of Indole acetic acid and solubilize inorganic phosphate. Six out of eleven isolates (A2, A4, A5, A6, A10, and A13) tested positive for phosphate solubilization (Table 1) which included the Cr (VI) tolerant A2, A5 and A6. According to Freitas et al. [20], good phosphate solubilizers are known to produce a clearing zone of diameter more than 15 mm around their colonies. This was true for the isolates A1, A2, A5, A6, A10, and A13 which can be regarded as efficient phosphate solubilizers. On the other hand, only A5 out of the eleven tested positive for IAA production. Bacteria

belonging to the genus *Pseudomonas* are known to produce IAA and that too higher than many other species [21]. The results showed that many heavy metal tolerant bacteria, like *Pseudomonas* sp. (A5), *Bacillus* sp. (A2) and *Staphylococci* sp. (A6) from this work, were also plant growth-promoting as reported earlier by Gadd [22].

2.4. Effect of PGPB and Cr (VI) on spinach

After 2 weeks growth in inoculated soil, the root and shoot lengths and wet and dry biomass of the spinach plants were recorded. The root and shoot length of the plants remained unaffected by the presence of PGP bacteria as shown by Figure 2. Root length increased by 1% with A5 and the shoot length decreased by 13% with respect to the control. In the presence of A6, the shoot length decreased by 1% and the root length increased by 20% with respect to the control but then showed inconsistent behaviour with increasing Cr(VI) concentrations. Sayel et al. [8] reported similar trend of decreasing root (30, 46 and 66% decrease, respectively) and shoot (17, 20 and 55% decrease, respectively) lengths of clover plants with the addition of 100, 200 and 300 mg L⁻¹ Cr(VI). It was also reported in the same study that inoculation with *Pseudomonas* sp. enhanced the root and shoot lengths by 15 to 20% with respect to the control which was not observed in our study.

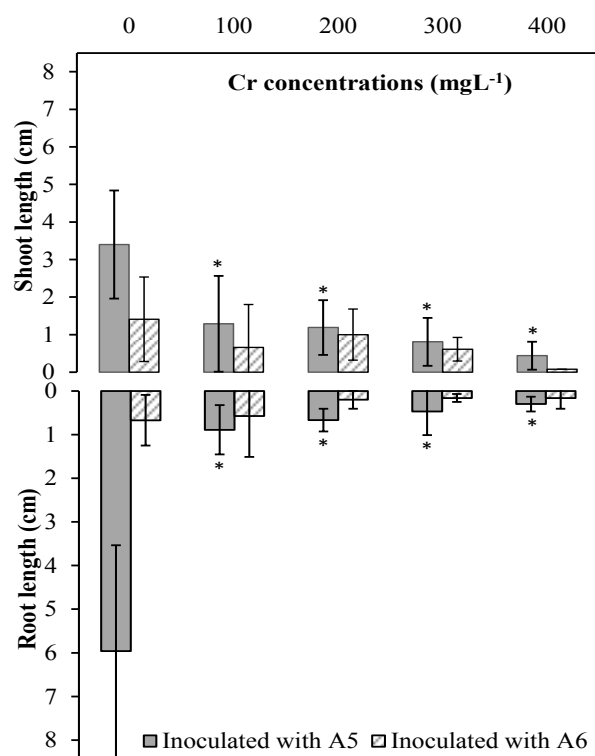


Fig. 2. Growth of spinach (root and shoot) with PGPB and various Cr treatments. Error bars represent standard deviations from using five replicates.

The addition of isolates A5 (*Pseudomonas* sp.) and A6 (*Staphylococci* sp.) brought about changes in the plant biomass. As shown in Figure 3, both the isolates were able to enhance biomass, A5 by 44% and A6 by 7% with respect to the control, in the presence of 100 mg kg⁻¹

Cr(VI). As the concentrations increased the biomass decreased significantly ($P < 0.05$) with application of inoculants of both the isolates.

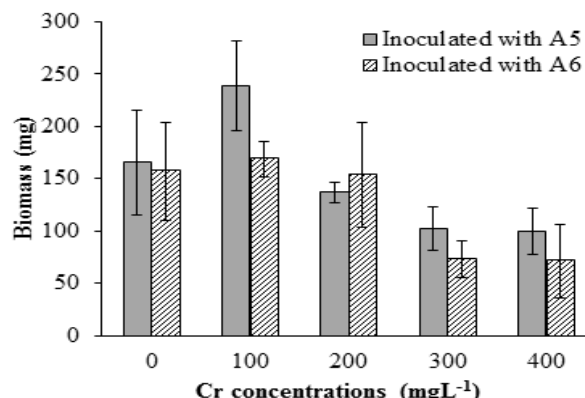


Fig. 2. Biomass (dry) of spinach seedlings with various Cr treatments. Error bars represent standard deviations from using five replicates (All at 5% level of significance).

Application of bacterial inoculants to plants has been reported to not only improve plant growth but also enhance the yield of crops [23]. Another study in which the addition of PGP bacteria have been known to significantly increase fresh and dry weights of plants; maize seedlings, after 20 days of growth in chromium stressed soil [15] also supported the results obtained with spinach. These encouraging results have very high significance in management of heavy metal contaminated soils, particularly with Cr. Beneficial traits can help to fight Cr toxicity to plants under stress conditions. Moreover, growth of non-food crops at Cr contaminated soils can help to produce biomass that can be further tested for biofuel production.

4 Conclusions

The study examined the capacity of 11 isolated bacterial species to tolerate and grow at various concentrations of chromium (VI). The screening process revealed that only *Pseudomonas plecoglossicida* and *Staphylococcus saprophyticus* were able to tolerate Cr (VI) presence up to 700 mg L⁻¹. *Staphylococcus saprophyticus* solubilized Phosphate but was unable to produce IAA. *Pseudomonas plecoglossicida* utilized both these plant growth promoting mechanisms to enhance spinach plant's growth in Cr spiked soil. Inoculation of *Pseudomonas plecoglossicida* increased spinach plant's biomass by 44% with respect to the control. This reported capability of *Pseudomonas plecoglossicida* can help to develop inoculum to be used for remediation and management of Cr contaminated soils.

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