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Bioremediation of Hexavalent Chromium by Heavy Metal Acclimatized Bacterial Isolate *Cytobacillus solani*

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Abstract: Chromium resistant bacterial strains of *Cytobacillus solani* were isolated from the rhizosphere soil samples and selected based on its growth on enriched media. The strain showed resistance to high concentration of Cr (MTC 12.5 mg/ml). Chromium degradation assay revealed that *C. solani* showed 27% of chromium reduction. It was evident that maximum bacterial growth may be obtained by allowing the bacteria to grow at 40°C. Bacteria showed maximum growth in pH 10 and growth was absent in low pH that suggests that isolate prefer alkaline pH. Investigating the effect of chromium on *Pheretima posthuma* revealed that they showed death within 10 min in 12.5 mg/ml of chromium concentration. However, it was observed that they survived for almost 35 min in chromium degraded broth. Thus, effective processes are necessary to overcome the mutagenic and carcinogenic properties of chromium.

Keywords: Bioremediation, *Cytobacillus solani*, Rhizosphere, *Pheretima posthuma,* Chromium, Carcinogenic

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Introduction

Consumption of heavy metals has grown significantly with the commencement of rapid industrialization and urbanization. The environment's living ecosystem was destroyed as a result of the uncontrolled disposal of the heavy metals containing effluent. Heavy metals are metallic elements and metalloids with density higher than 5 gcm^{-3}. Heavy metals like Chromium have a significant impact on health due to their high levels of toxicity (Kalsoom *et al*., 2023). Among toxic heavy metals, Cr is designated amidst the top sixteen toxic contaminants that have detrimental impacts on human health. Cr is one of the most hazardous heavy metal and is being widely used in many industries such as electroplating, dyeing, textile, steel fabrication, and canning. The high level of Cr entering the biosphere is mostly caused by the leather industry. These anthropogenic

activities have increased the bioavailability and biomobility of chromium and contributed to the widespread contamination of it in the environment (Sundarraj *et al*., 2022).

The Cr atom can exist in seven different oxidation states (0–VI). Depending on the oxidation state, Cr can either be a non-hazardous material or a noxious and carcinogenic contaminant (Akkurt *et al*., 2022). The highest stable oxidation state of chromium that may be found in living things is the trivalent form. The most important biological characteristic separating Cr^{3+} from Cr^{6+} is its poor reactivity and inability to readily pass cell membranes. Trivalent form of Cr is less soluble under neutral pH and is less toxic due to its inability to cross cell membrane (Harboul *et al*., 2022). Chromium (III) can assist to regulate blood sugar levels by collaborating with insulin and promote the glucose uptake into cells. (Chaudhary *et al*., 2021). Cr (Vl) readily traverses biological membranes and interacts with nucleic acids and protein constituents inside of cells before being deoxygenated to Cr (III). Cr (VI) is mutagenic, carcinogenic and teratogenic to humans and animals and thus regarded as a group A human carcinogen (Akkurt *et al*., 2022). Cr (VI) when breathed in at high concentrations can irritate the nose and result in nosebleeds, ulcers, and holes in the nasal septum. The workers exposed to hexavalent chromium dust are most at risk for developing lung cancer (Barceloux and Barceloux, 1999). Chromium (VI) compounds exerts genotoxicity both *in vivo* and *in vitro*. Cr (VI) has also been reported to induce developmental toxicity in animals (Anusha *et al*., 2021).

There are several chemical, physical and biological methods for the removal of Cr (VI). Biological agents such as bacteria, fungi, algae and plants are used to restore chromium contaminated sources. Among them, bacteria and fungus have been proved to be the best remedial agents. It has been demonstrated that organisms that can live in contaminated environments may have the capacity to remediate those environments on their own, up to a point, by biosorption, bioaccumulation, and biotransformation and can convert hazardous chemicals into harmless ones (Mishra *et al*., 2020). Chromium Reduction Bacteria (CRB) are the common name for the bacterial strains that can reduce Cr (VI). In the bacterial cytoplasm, cell wall, or both, highly poisonous Cr (VI) is reduced into less hazardous Cr (III) during the bioreduction process (Kumar and Dwivedi, 2019). In comparison to physiochemical methods, biological methods are thought to be more advantageous from an economic and environmental perspective because of their low installation and operating costs, produce far fewer secondary pollutants, and convenient and easy to use (Mishra *et al*., 2020). Bioremediation leads to the full eradication of a wide range of pollutants instead of moving toxins from one environmental medium to another and generates little to no secondary wastes during the process (Espinoza- Sanchez *et al*., 2019).

In the present study chromium resistant bacterial strains of *Cytobacillus solani* were isolated from the rhizosphere soil samples and evaluated for its effect on *Pheretima posthuma.*

Materials and Methods

Enrichment and Isolation of Cr Resistant Bacterial strains

Soil was collected from St. Mary's College Garden area. The surface soil was scooped into a plastic bag with a plastic scooper.(Panda and Sarkar, 2012). For the isolation of Cr resistant bacteria, 1 g of soil sample was inoculated into 250 ml Erlenmeyer's flask containing 100 ml sterilized Nutrient broth enriched with 1 ml of Potassium dichromate solution to make the Cr concentration of 200 mg l^{-1}. The flasks were incubated at 37°C for 48 h. Following incubation, 10 ml of the broth culture was transferred to the freshly prepared Nutrient medium having same composition. The same procedure was successively repeated for 4 times to achieve the complete enrichment of Chromium resistant bacterial culture (Piñón-Castillo *et al*., 2010) 1ml of the enriched sample was serially diluted and plated onto Nutrient agar

plates containing Cr to make the concentration of 200 mg l^{-1} and incubated at 37°C, pH 7.0 for 48 h. Following incubation, the well isolated and morphologically distinct colonies were selected and subcultured repeatedly to obtain pure cultures of resistant bacteria (Chen *et al*., 2009)

Analysis of Maximum Tolerance Capacity (MTC) of isolates

To determine the Cr MTC, the isolates was cultured on Agar Agar Type 1 and Nutrient broth with different Cr concentrations and growth was recorded. The plates were incubated at 37°C, for 24 h. Following incubation, the well isolated and morphologically distinct colonies were selected and sub cultured repeatedly (Wang *et al*., 2010).

Identification of bacterial isolate by 16 S rRNA sequencing

The identification of bacterial strains was done by the employment of 16 S rRNA sequencing (Nakamura *et al*., 2002).

Optimization of cultural conditions for maximizing bacterial growth

Two loops full of organism inoculated on to 100 ml Nutrient Broth and incubated at 37°C for 24 h. The absorbance was read at 610 nm in colorimeter at every 1 h intervals. Bacteria were incubated at various temperatures ranging from 10 to 70° C to find out the most favourable temperature for bacterial growth. Bacterial growth media was maintained in different pH by administrating three buffers to it; acetate (3.6-5.6), phosphate (5.8 – 7.4) and bicarbonate (9.2-10.6). Three different pH (4,7,10) was taken as a parameter to find out the effect of pH on bacterial growth. Similarly, the growth curve of bacteria was estimated by analysing bacterial growth in calorimeter. Absorbance of bacterial culture was taken at 600 nm and found log, lag and stationary phase (Kristjánsson *et al*., 1994) and the bacterial concentration (cells/ml) was found by the following equation:

$$1\ OD = 8x10^8\ CFU$$

Analysis of degradation through Colorimetric analysis

50 ml of the L B broth enriched with chromium was incubated with the bacteria isolate. Media without any bacterial inoculation was considered as control. After 14 days of incubation bacterial biomass were separated by centrifuging at 10.000 rpm for 10 min. Supernatant was subjected for colorimetric analysis at 610 nm (Bankar *et al*., 2009). The percentage degradation was calculated as:

Percentage degradation= O.D. of control- O.D of test/ O.D of control x 100

Investigating the effect on Pheretima posthuma

P. posthuma were maintained in moisture water in the lab. The stock solution (Cr- 12.5 mg/ml) was used to perform mortality assay and to detect the time of paralysis. The time taken for paralysis and time taken for the death were noted (Chai *et al*., 2009).

Histological analysis

After the incubation period the treated samples were washed with distilled water. After a depuration of 6 h earthworms were fixed in 10% formalin and then sequentially dehydrated in graded ethanol concentrations and embedded in paraffin wax. Sections were cut at 6 µm, stained with hematoxylin-eosin method for light microscopic observation, and photographed with an Labomed microscope equipped with a digital color camera Micaps pro HDMI (Okeke, 2008).

Results and Discussion

Selection of Superior Strain

A sterilized solution of chromium was used as the source of Cr, which was added to the sterile molten agar. The inoculated plates were incubated at 37°C for 24 h. Four morphologically distinct Cr resistant bacterial colonies are found from soil samples after serial dilution. After incubation plates were subcultured in concentration method (Fig. 1).

Analysis of Maximum Tolerance Level

The ability to withstand Cr concentration of the isolated bacterial strain was determined by

Fig. 1: Diversity of Cr Resistant strains.

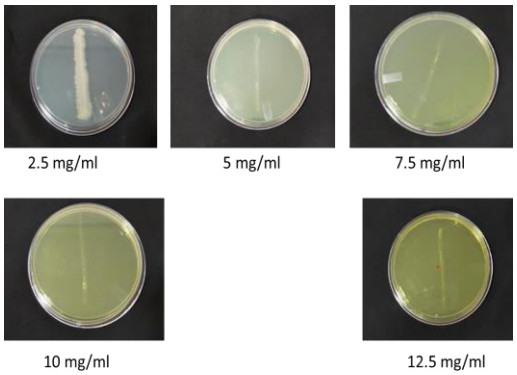

Fig. 2: Growth of isolate in increasing concentration of Cr.

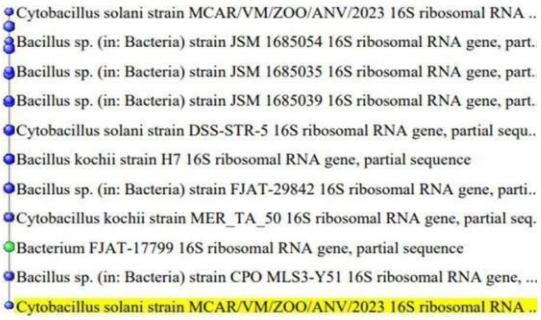

Fig. 3: Phylogenetic relation of bacterial isolate.

growing it in increasing order of concentration starting from 2.50 mg/ml to 12.5 mg/ml (Fig. 2). Strain was successfully grown up to 12.5 mg/ml concentration which suggests its ability to withstand high chromium presence

Identification of superior strain

The superior strain was chosen for its ability to withstand high chromium. To avoid further contamination, the strain was subcultured in pure plates and designated as MCAR/VM/ZOO/ANV/ 2023. Using 16s rRNA sequencing, a bacterial isolate was identified. According to the phylogenetic analysis, the bacteria is *Cytobacillus solani*. *Cytobacillus solani* MCAR/VM/ZOO/16s ANV/2023's rRNA sequence has been submitted to the National Centre for Biotechnology Information (NCBI) under the accession number OQ947116.1 (Fig. 3).

Optimization of cultural conditions for maximizing bacterial growth

Various factors such as temperature and pH were monitored for its effect on the growth and biomass production of bacteria. It was observed that all these factors have great importance upon the growth of bacteria and slight change in them could manipulate the growth. The optimum temperature for maximizing bacterial growth was found to be 40°C (Fig. 4) whereas the higher temperature and lower temperature inhibited the growth. However, the bacteria preferred basic pH for its optimum growth as the highest growth was seen in pH that of ten (Fig. 5) when compared with neutral and growth was inhibited at low pH (Table 1).

Bioremediation of Chromium by bacterial isolate

The analysis suggested that bacterial isolates collected from isolation process were potential as chromium degrader due to their capability to grow in chromium containing medium. 27%

Table 1: Optimum conditions for maximizing bacterial growth

Temperature (°C)/ Bacterial Concentration (CFU)	10/0	40/0.48 x10^8	70/0
Hydrogen Ion Concentration (pH)/ Bacterial Concentration (CFU)	4/0	7/0.64 x10^8	10/0.96 x10^8

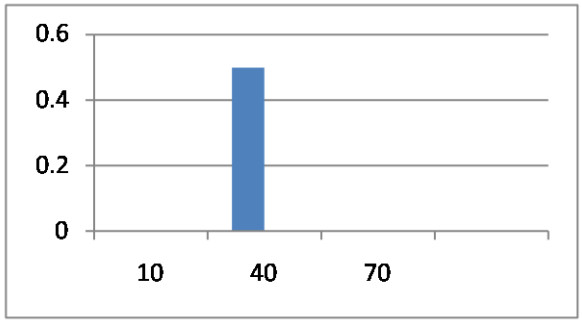

Fig. 4: Optimum temperature for bacterial growth (CFU).

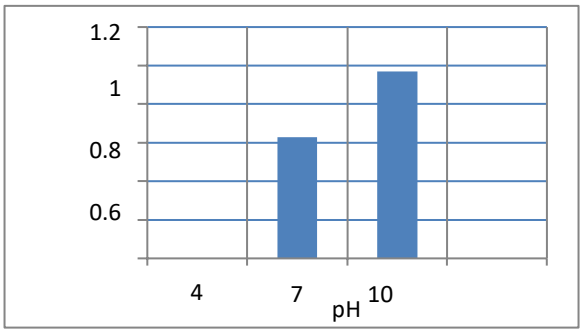

Fig. 5: Optimum pH for bacterial growth (CFU).

degradation activity was observed by the bacterial strain (Fig. 6).

Detoxification analysis in Pheretima posthuma

The earthworms were treated with test and control samples. After 15 min earthworm became paralysed and it takes about 35 min to die in test samples. In control sample it takes only 5 min to become paralyzed and about 10 min to die. The paralysis time and death time of earthworm is recorded. These results indicated that there is a clear reduction in toxicity due to microbial treatment. The histopathological analysis indicated thick and intact epithelial layer, compact circular and longitudinal muscle layers and intestine. Untreated sample indicated aberrations such as ectodermal spoilage with increased concentration. The treated sample showed lower distortions like vacuole formation with compactness in tissues indicating that the degree of damage was reduced by the bacteria (Fig. 7).

One of the most prevalent heavy metals that degrade soil quality is chromium, which enters the environment through the tanning of leather, electroplating, and the manufacture of inorganic pigments. The cleanup of Cr- contaminated areas is a really challenging task (Ahirwar *et al.*, 2015) There are numerous ways for bacteria, fungi, ciliates, algae, mosses, and higher plants to remove heavy metals from aqueous solutions (Holan and Volesky, 1995).

The reduction of Cr (VI) to Cr (III) by microbes is one such technique; this process was first reported by using *Pseudomonas* spp. (Romanenko and Koren'Ken, 1977). Another bacterial strain

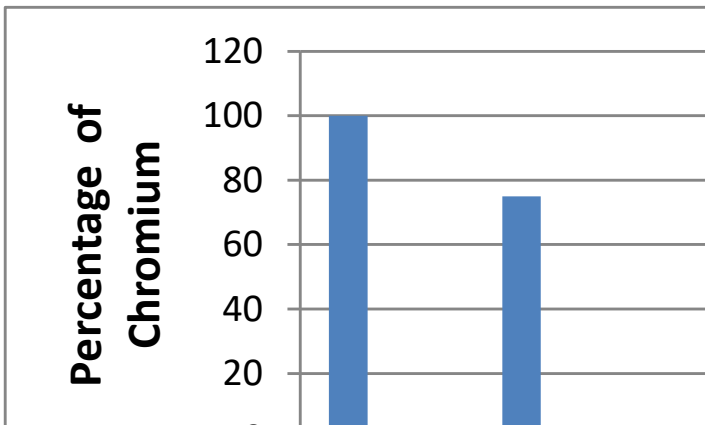

Fig. 6: Microbial Mediated remediation of Cr.

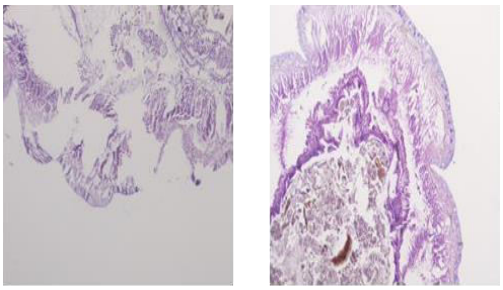

Fig. 7: Histopathological analysis.

Bacillus subtilis was able to reduce 11% of chromium at a concentration of 75 mg/l (Purwanti *et al.*, 2015). In another study, five chromium-removing bacterial strains were successfully isolated from Cr (VI) contaminated soils and identified by their 16S rRNA gene sequences. Among them *Pseudochrobactrum saccharolyticum* strain W1 could grow at Cr (VI) concentrations ranging from 0 to 1225 mg l^{-1}, and shown a high capacity to remove chromium. This is the first report of chromium removal by a *Pseudochrobactrum* genus member. At 0.79 mg h^{-1} mg^{-1} biomass, *Sporosarcina saromensis* W5 exhibited the best rate of chromium removal (He *et al.*, 2014).

In the present investigation the bacterial strain *C. solani* was found to be highly resistant to chromium at a concentration of 12.5 mg/ml. *C. solani* showed 27% of chromium reduction and showed optimum growth at a pH of 10 and temperature of 40°C. Investigating the effect of chromium on *P. posthuma* revealed that they showed death within 10 min in 12.5 mg/ml of chromium concentration. However, they survived for almost 35 min in chromium degraded broth. Thus, effective processes are necessary to overcome the mutagenic and carcinogenic properties of Chromium.

Conclusion

The growing use of heavy metals in most industrial activities has led to it being considered as the most important environmental pollutant that may cause harm and toxicity to animals and humans. Agricultural land contamination with heavy metals is a serious problem, which has gathered considerable public attention in recently. Hexavalent chromium is considered the most toxic form because of its high solubility in water. Chronic exposure and bioaccumulation of chromium, which is a carcinogen, can cause

toxicity and numerous pathophysiological defects. Conventional methods for removing metals from contaminated sites include chemical precipitation, ion exchange, evaporation and adsorption on activated coal, alum, and ash. However, most of these methods are costly and require large quantities of chemical reagents, with possible production of secondary pollution. But bioremediation offers the possibility to destroy or render harmless various contaminants using natural biological activity so that, bioremediation seems to be a good alternative to conventional clean-up technologies research in this field. It uses relatively low-cost, low-technology techniques, which generally have a high public acceptance and can often be carried out on site. But the application of bioremediation will not always be suitable, however, as the range of contaminants on which it is effective is limited, the time scales involved are relatively long, and the residual contaminant levels achievable may not always be appropriate.

The present study provides a greater understanding of Cr resistance and reduction by *C. solani* isolated from rhizosphere. Cr resistant bacterial isolate showed promising results for the bio reduction of toxic hexavalent chromium *in vitro*. The isolate should be potentially useful for the bioremediation of industrially contaminated soil ecosystem and as biomarkers in detecting environmental pollution.

Bioremediation has already proven itself to be a cost effective and beneficial addition to all physical methods of managing wastes and environmental pollutants.

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Conflict of Interest

The authors declare no conflicts of interest.

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