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Bioremediation of hexavalent chromium by bacterial strains isolated from chromium contaminated site

[1]Kamalambigeswari R, [1]Agathiya A, [2] Pooja Karni B, [2]Sowntharya P, [2]Haripriya J
[1]Department of Industrial Biotechnology, Bharath Institute of Higher Education and Research, Chennai, Tamil Nadu.

ABSTRACT

Chromium contamination due to increased industrial activities poses a serious threat to people's health and the surrounding environment. The bioreduction of highly toxic Cr(VI) to less toxic Cr(III) is a promising alternative to chemical chromium remediation methods. In this study, ten bacterial strains were isolated from the soil samples of leather tanning industries and subjected to analysis of their Cr(VI) resistance and reduction. The isolates C5, C8, C14, C16, and C20 were found to be resistant to 1000 mg/l Cr(VI). Of the five isolates, C5, C8, and C20 reduced the Cr (VI) of (1000 mg/l) by 80%, 89%, and 84%, respectively, when analysed using the diphenylcarbazide assay. The potentiality of C8 (highest Cr VI reduced strain) to remediate toxicity of Cr(VI) on plants was assessed on Vigna radiata (Green gram), which shown significant improvement in the seed's germination and plant growth of Vigna radiata in the presence of Cr(VI) treated with C8 than untreated, proving its capability to remediate Cr(VI) contamination by reducing the phytotoxicity of Cr(VI). By gram staining, the bacterial strain (C8) was identified as a gram-negative cocci, and biochemical analysis revealed that it was positive for the indole test. This study suggests that the bacteria found in Cr-contaminated sites could resist and reduce Cr(VI) at a very high level, making it a promising and feasible approach for the remediation of Cr contamination.

Keywords: Chromium contamination, Chromium reduction, bioremediation

INTRODUCTION :

Chromium is widely used in tanneries, pulp and paper, textile, mining, dying, and painting industries. According to estimates, more than 170 thousand tons per annum of chromium waste are being discharged globally into environments due to various anthropogenic activities, thus causing severe environmental pollution and human health problems (Kaur, Kumar & Kaur, 2014). Hexavalent chromium is highly toxic and found to be hemotoxic, genotoxic, and carcinogenic when inhaled or exposed. In both humans and animals, trivalent chromium is a necessary trace element. Chromium has no harmful effects when existing as a pure metal. When trivalent chromium is present in significant concentrations, it has little harmful effect. Hexavalent compounds are the principal source of chromium poisoning, both acute and chronic. Adverse health effects associated with Cr(VI) exposure include occupational asthma, eye irritation and damage, perforated eardrums, respiratory irritation, kidney damage, liver damage, pulmonary congestion and edema, upper abdominal pain, nose irritation and damage, respiratory cancer, skin irritation, and erosion and discoloration of the teeth. Expensive safe disposal of toxic

sludge, incomplete Cr(VI) reduction, and high costs for Cr(VI) reduction, particularly for the removal of relatively low Cr(VI) concentrations, are inconvenient from an economic standpoint (Nourbaksh et al. 1995). Biosorption is a quick, independent, and metabolically passive process responsible for the selective sequestration of heavy metal ions by dead/inactive biomaterials (Hansda et al., 2016). There is always a solid phase in the biosorption process that serves as the biosorbent (various biological materials). The sorbate is drawn and bonded by numerous mechanisms due to the sorbent's increased affinity for the sorbate species. The mechanisms behind their resistance include adsorption, uptake, methylation, oxidation, and reduction of toxic, highly soluble Cr (VI) to less soluble and less toxic Cr (III) (**Chandhuru et al., 2012**). The aim of the present study includes isolation of chromium resistant bacteria from the chromium contamined soil sample, to analyse the hexavalent Cr reducing ability of the bacterial isolates and to assess the bioremediation potential of the selected isolate, in order to use it for practical application of Cr bioreduction.

MATERIALS AND METHODS

Sample collection

The soil samples were collected in a sterile polythene cover from the effluent discharge sites of two different leather tanning industries situated at Pallavaram, Tamil Nadu. The collected soil samples were immediately brought to the laboratory and stored at 4°C in a refrigerator until further use.

Isolation of bacteria from soil samples

About 1g of soil samples were suspended in 9 ml of sterile saline solution and serially diluted. From which, 100 µl of aliquot from each dilution (10^{-2} to 10^{-4}) was allowed to spread on a nutrient agar plates and kept in incubator at 37°C for 24 hours. The obtained colonies were streaked & re-streaked on a fresh nutrient agar plates to get pure and isolated colonies. Based on the morphological characteristics, the distinct colonies were picked up and grown in nutrient broth and incubated at 37°C under shaking for 24 hours.

Isolation of chromium resistant bacteria

The isolated strains were screened for chromium resistance based on the capability to grow in presence of Cr(VI). About 5% of broth cultures were inoculated in to the nutrient broth amended with 1000 mg/l of potassium dichromate ($K_2Cr_2O_7$) as a source of Cr(VI) and incubated at 37°C under shaking conditions for 120 hours. The media with no inoculum remain as control. After incubation, the growth of the bacterial strains in the Cr amended medium was determined by measuring the absorbance at 600 nm. The isolates showing maximum growth were proceeded with further investigations.

Reduction of hexavalent chromium

The ability of the bacterial strains to reduce Cr(VI) to its less toxic form Cr(III) was analysed by DPC (Diphenyl carbazide) assay by estimating the residual amount of unreduced Cr(VI) in the

broth according to the procedure described by Alok Prasad Das and Akalabya bissoyi (2011). At the end of 120 hours of incubation in ($K_2Cr_2O_7$) amended medium, the medium was withdrawn and centrifuged at 7000 rpm to collect the supernatant. The DPC reagent was prepared by dissolving 0.025g of DPC in 9.67 ml of acetone and 330 µl of H_2SO_4. To 400 µl of culture supernatant, 800 µl of 20mM MOPS-NaOH buffer, 66 µl of 3M H_2SO_4, 80 µl of DPC reagent and 654 µl of distilled water were added and absorbance was read immediately at 540 nm and the percentage reduction of hexavalent chromium by each bacterial isolate was calculated using the following formula;

$$\textbf{Cr(VI) reduction (\%)} = \textbf{((A-B) ÷ B) × 100}$$

Where, A = Absorbance of control

B = Absorbance of sample

Morphological and biochemical characterization of the isolate :

The morphological characteristics of the colony of the isolate was examined. The bacterial isolate was subjected to gram staining according to the standard procedure. The bacterial strain was subjected to the following biochemical characterization tests such as Indole test, Methyl red test, Voger proskauer test, citrate utilization and catalase test and the results were interpreted in accordance with the standard protocol.

Antibiotic susceptibility test :

The antibiotic susceptibility of the bacterial isolate was determined for 4 different antibiotics, such as streptomycin, amoxicillin, gentamicin, and tetracycline by disc diffusion method. The wells were placed at equal distances on the agar plate swabbed with 100 µl of bacterial culture. Then, 100µl of each of the antibiotics was added into the wells and the plate was incubated at 37°C for 24 hours. After incubation, the bacterial isolate was classified as resistant or sensitive to antibiotics based on the diameter of zone of inhibition.

Phytotoxicity assay using Vigna radiata seed germination:

To assess the toxicity of Cr(VI) on the seed germination, the seeds of Vigna radiata were placed on a petri plate containing sterile filter paper moistened with sterile water. The plates were provided with the isolate treated Cr solution and untreated Cr solution respectively. The plate with no Cr solution remain as a control. The experiment was done in duplicates. All the plates were incubation at the room temperature for 72 hours allowing seed germination to occur. The root and shoot length of the germinated seeds were measured at the end of incubation.

Field studies :

The plant pots were filled with cleaned and sterilized soil. The soil in the pots were mixed with 24h old culture grown in 1000 g/l of Cr(VI) amended medium and only Cr(VI) solution at a concentration of 1000 g/l, followed by sowing with the seeds of *Vigna radiata*. The pot soil with no inoculum and no Cr(VI) solution served as a control. All the pots were kept under shade near

sunlight allowing the growth of plantlets. Periodically, the pot were sprinkled with water and were supplemented with 24 h old Cr amended broth and Cr(VI) solution respectively in every 3 days. This study was continued for a period of 10 days. At the end of study, the grown plants were carefully removed from the soil. Then, the root length and shoot length was measured using a graduated scale.

RESULTS AND DISCUSSION

Isolation of bacteria from soil samples:

The mixed bacterial colonies were obtained on agar plates spreaded with serially diluted samples. It was noticeable that variety of bacteria strains survive in the Cr contaminated site. A number of bacteria have the extraordinary capacity to adapt to and settle in environments that are contaminated with toxic metals which are inadaptable by higher organism, these microorganisms possess the ability to protect themselves from the harmful effects of heavy metals through a variety of processes, including adsorption, absorption, methylation, oxidation, and reduction (Rehman et al. 2008). The colonies with distinct morphological characteristics were picked up and purified by streaking. The well-established pure colonies were obtained after 24 hours of incubation in a streak plate, out of which, 10 single colonies (C1, C2, C3, C5, C8, C11, C14, C16, C19, C20) were selected and proceeded for the further investigations.

Isolation of chromium resistant bacteria :

The Chromium resistant bacteria were isolated by growing them in Cr(VI) amended NB medium. Following 120 hours of incubation, visible turbidity was observed in the bacterial cultures. Furthermore, the growth of the strains was quantified by reading OD at 600nm. Five isolates (C5, C8, C14, C16 and C20) were found to have higher OD values when compared to other isolates (Fig.1) which indicates that these five isolates grown by tolerating the toxicity of Cr(VI) in $K_2Cr_2O_7$ amended medium at a concentration of 1000mg/l. Moreover, C20 isolate shows highest OD at 600 nm of about 0.471 which was much closer to the OD of C8 isolate of about 0.451. Only few studies have reported about the tolerance of 1000 mg/l concentration of Cr(VI), therefore it may be concluded that C8 isolate was able to resist 1000mg/l of Cr(VI) which is considered to be the highest concentration of Cr that the bacteria could resist.

Reduction of hexavalent chromium:

Diphenyl carbazide (DPC) assay was performed to measure the residual amount of unreduced hexavalent chromium in 120 h grown culture which in turn provides the amount of reduced hexavalent Cr. The culture was centrifuged and supernatant was collected, addition of DPC reagent to the supernatant, resulted in color change of the sample from clear to purple with varying color intensities in accordance with varying concentrations of unreduced Cr(VI). After reading the absorbance, the percentage removal of hexavalent chromium by bacterial isolates was calculated using the formula reported by **Neha et al., (2017**). The results shows that the three bacterial isolates (C5, C8 and C20) have reduced Cr(VI) of 1000mg/l by 80%, 89% and 84% respectively, while the C14 and C16 shown reduction percentage of about 4% and 34%

respectively which were found to be resistant to Cr (VI). This is in accordance with the results of **Rahman et al. 2021** that not all Cr(VI)-resistant microbes could reduce Cr(VI).

Phytotoxicity assay using seed germination

After 3 days of incubation, the growth of seedlings was observed. The toxicity of hexavalent chromium on seed germination was analysed by measuring the growth of roots and shoots. Even though, the chromium treated seeds had growth on 1st days of incubation, but however the growth of the seedlings was completed inhibited in the following days while the C8 treated seeds were survived with significant amount of growth. Mung bean (Vigna radiate L.) tolerant and sensitive cultivars were used to assess the impact of Cr contamination on the germination medium. The results showed that while germination was unaffected in tolerant plants, it was affected in sensitive plants when exposed to 96 or 192 M Cr(VI) (Samantry et al. 2002).

Field studies:

At the end of 10 days of study, the plantlets were removed carefully from the pot and the total length of the grown plants was analysed in the treated and untreated pots (Fig.1 &2). As a result, the pot with C8 treated Cr solution shown significant growth in the plantlets while the pot with untreated chromium shows retarded plant growth.

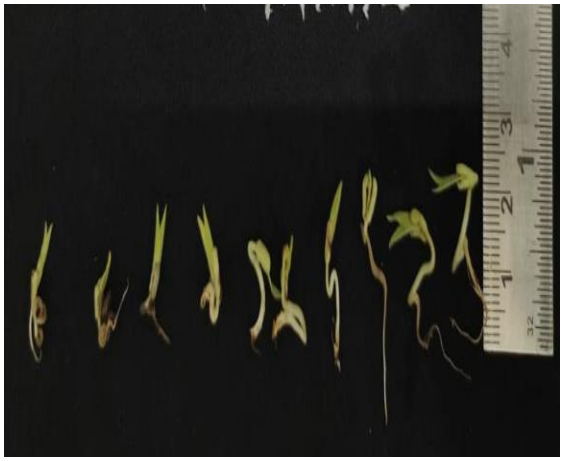


Fig.1 Growth of the seedlings (treated with cr)

Fig.2 Growth of plantlet (untreated Cr)

Morphological and biochemical characterization of the isolate:

The selected bacterial isolate C8 was characterized by morphological and biochemical tests using 24 hours old grown bacterial culture (Kamalambigeswari et al., 2018). The bacterial strain C8 was identified as gram-negative, cocci shaped bacterium (Fig.3). The colony of the bacterial isolate C8 was found to be mucoid, round in shape with smooth edges and off-white in colour.

The biochemical tests shown that the strain C8 was positive for indole test while negative for Methyl red test, Voger Proskauer test, citrate utilization test and catalase test.

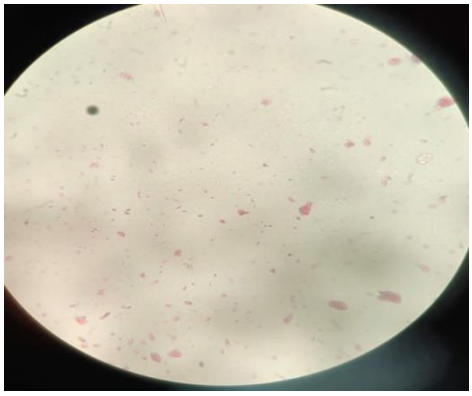


Fig.3 Gram staining of C8 isolate

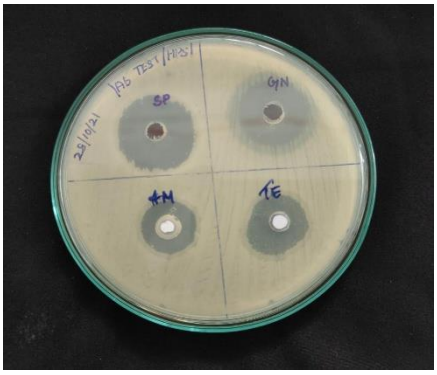
Antibiotic susceptibility test:

The sensitivity of the strain C8 against four antibiotics was identified by measuring the diameter of the zone of inhibition and it was found that the isolate C8 was sensitive to all the four antibiotics such as Streptomycin, Amoxicillin, Gentamicin and Tetracycline (Fig4). The diameter of the zone of inhibition (mm) of the C8 isolate by the antibiotics were presented in the table 1.

Table 1. Antibiotic susceptibility profile of the isolated strain C8.

S. No.	Antibiotics	Zone of inhibition (mm)
1.	Streptomycin	1
2.	Amoxicillin	1.05
3.	Gentamicin	0.65
4.	Tetracycline	0.75

Fig 4 Antibiotics susceptibility disc of C8 isolate



CONCLUSION

Heavy metal chromium is very toxic and carcinogenic to humans and animals. Hexavalent chromium pose a serious threat to aquatic life and the environment. Chromium remediation through microorganisms is accepted as the best and economically affordable technology at present to clean-up Cr contamination. From this study, it can be concluded that the indigenous bacteria isolated from contaminated site would be the potential option for bioremediation. However, further studies required to find out the nature of species of the bacterial isolate C8 and optimization studies could further enhance the bioreduction of Cr(VI) by the isolate.

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