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Screening and Evaluation of Chromium (VI) Tolerant *Bacillus* sp. from Industrial Areas of Ranipet, Tamil Nadu for Bioremediation of Chromium Contaminated Soil

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ABSTRACT

The present study shows that bacterial species isolated from polluted sites show best results to resist and grow in high concentrations of hazardous pollutant like Chromium. A total of 10 chromium tolerance bacteria has been isolated and taxonomically identified to belong to genera *Bacillus, Pseudomonas, Enterococcus*. A strain isolated belong to *Bacillus* species show resistance to chromium at the concentration of 500 mg/l, which have been isolated from highly chromium polluted areas. They can be utilized as a best candidate for bioremediation studies due to their high tolerance to Cr(VI). These isolates can be further studied for their plant growth enhancing abilities for remediation and management of Cr contaminated soils.

Key words : *Chromium tolerance, Minimum Inhibitory Concentration (MIC), Maximum Tolerance Level (MTL), Chromium Bioremediation.*

Introduction

Heavy metal pollution is a matter of concern in recent years as it has had a direct effect on human and environmental health, exposure to heavy metal poses health risks to humans (Zaynab *et al*., 2022). Studies of Li *et al*., 2015; Wakeel *et al*., 2020 have shown that the bioavailability and reactivity of heavy metals are regulated by a variety of parameters, including oxidation-reduction potential (ORP), pH, temperature, ionic strength of the medium and the presence of chelating agents (e.g., EDTA, humic acids and amino acids), in addition to their quantities. Heavy metal accumulation in agricultural soils leading to environmental concerns due to high toxicity, non-biodegradability, and persistence for longer period (Cai *et al.*, 2019 Song *et al.*, 2021; Zhou *et al.*, 2022). Although heavy metals are hazardous to people, they are routinely used in industrial operations Chromium contamination of soil and water has become an issue of concern due to unregulated release of industrial (electroplating, alloying, tanning of animal hides, textile dyes and mordents, pigments, ceramic glazes, refractory

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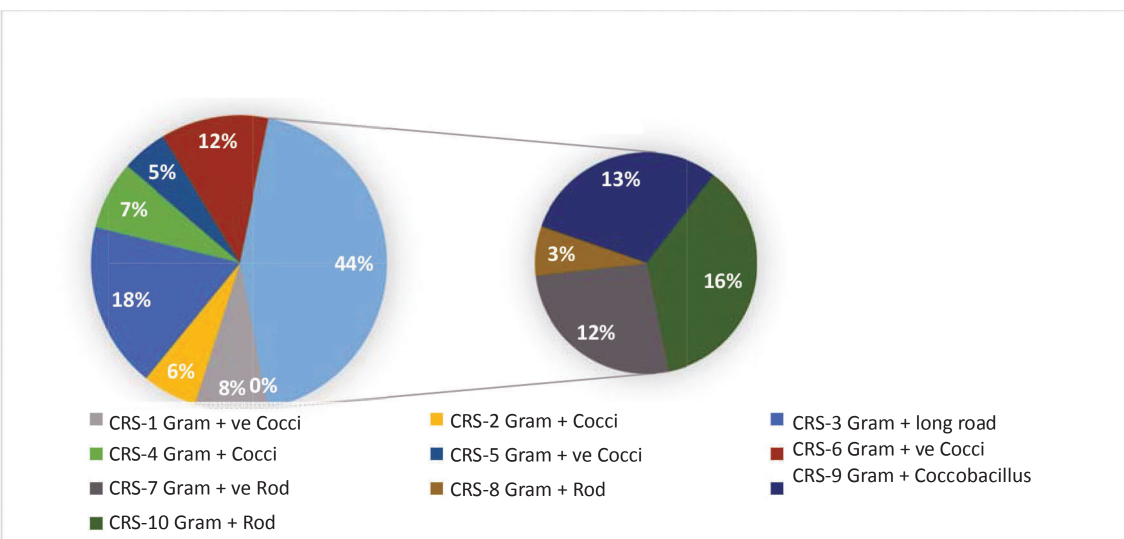
bricks, and pressure-related lumber) wastewaters into water bodies (Oluwatuyi *et al*., 2020; Sharma *et al*., 2021). As reported by Srivastava *et al*. (2021) total chromium toxicity in the environment is influenced by both natural and anthropogenic causes. The natural origin of chromium (VI) in groundwater is mainly through mineral leaching. However, anthropogenic sources account for more than 70 % of the total chromium in the environment. Tariq *et al*. (2019), asserts, that chromium in its hexavalent or trivalent state becomes highly mutagenic and carcinogenic after it enters cellular proteins and nucleic acids through sulfate uptake pathways.

As reported by Shukla *et al*. (2009), Ahamed and Kashif (2014), and Noorjahan (2014), Hasan *et al*. (2019), Sawalha *et al*. (2020), Tang *et al*. (2021) and Zaynab *et al*. (2022) more than 2500 tanneries in India, processing ~ 600 kilo tons of leather, releases 50–60 million liters of wastewater and 3 million tons of sludge into the environment and heavy metal toxicity has reached its peak in aquatic setting resulting in heavy metal speciation, bioaccumulation and toxicity. Venkatesan *et al*. (2020) claims, that as the tanneries, with long history in Southeast Asia, is rapidly expanding and driving the local economy in these places. As reported by Das *et al*. (2015), Tamil Nadu, West Bengal, Uttar Pradesh, Andhra Pradesh, Karnataka, Maharashtra, Rajasthan, and Punjab houses large numbers of mechanical tanneries in India. As reported by Song *et al*. (2004), Dogruel *et al*. (2006), Lofrano *et al*. (2013), Pavithra *et al*. (2019), Mohammed *et al*. (2020), and Hedberg (2020), chrome salts (trivalent) are used as principal tanning agents in the leather industry. However, under the right conditions, they get oxidized to chromium (VI), which is more toxic. Tanning industry generates a variety of pollutants (sludge, chrome-tanned leather shavings, and chrome leather trims), as chromium-based tanning is widely used due to its versatile nature. It is expected that 0.02 million tons of chromium shavings are produced each year (Zayed and Terry, 2003; Sangeetha *et al*., 2009). Chromium is classified under class A human carcinogen (Oluwatuyi *et al*., 2019; Mood *et al*., 2021, Chen *et al*., 2022), which requires effective preventive measures. As reported by Alvarez *et al*. (2021) this group includes chromium, which, despite being prevalent in our everyday food, can be detrimental to humans, causing skin allergies and raising the chance of lung cancer, among other health impacts.

Presently, the samples have been collected from Ranipet, an industrial area located near Chennai city in southern India witnessing dumping of chromium bearing waste, polluting groundwater in a radius of 30 km (Vijaykumar *et al.*, 2022). This is among the twelve sites identified across the country for hazardous waste contaminated dumpsites (The Hindu, 2015). These are the possible areas which may be naturally laden with microorganisms that tolerate and grow under extreme chromium concentrations. Hence, the purpose of the present study is to isolate, characterize and chromium tolerant bacteria from chromium contaminated sites of Ranipet, Vellore District, Tamil Nadu, India, to use it as efficient tool



Graph 1. Percentage of distribution of chromium tolerant Bacterial isolates.

for bioremediation.

Materials and Methods

Collection of soil samples and processing

Soil samples were collected from metal contaminated sites of chromate and chemicals industries in Ranipet of Vellore district, Tamil Nadu, India, (Latitude 12.953191 N, Longitude 79.310272 E). The samples were immediately transferred to the laboratory and preserved under 4°C until further studies.

Isolation and identification of Cr tolerant bacterial isolates

For bacterial isolation, 100 ml of serially diluted (10^{-1} to 10^{-10}) soil samples (Kalsoom *et al*., 2020) samples were aseptically inoculated on to LB agar medium amended with different concentrations of chromium. Bacteria were isolated based on distinct colony and morphological characteristics, pure cultures obtained and stored at 4 °C for further studies (Sanjay *et al*., 2018). All the isolates were biochemically characterized for production of catalase, oxidase, tryptophanase, urease, citrate, acetoin, and organic acids (Barrow and Feltham, 1993).

Primary screening of bacteria for chromium tolerance

The chromium tolerant bacterial strains were isolated by the spread plate method on Luria Bertani (LB) agar medium by spread plate method (Sarankumar *et al.*, 2020). The LB medium was autoclaved at 121°C for 15 min. Selective screening of chromium bacteria was demonstrated by incorporating sterilized Cr (VI) in the form of $K_2Cr_2O_7$ (Potassium dichromate) salt by a sterilized 0.22µ Whatman filter paper (Kafilzadeh and Saberifard, 2016) in varying concentrations of 100, 200, 300, 400 and 500 mg/l in LB agar medium plates (Peptone 10.00g/l, Yeast extract, 5.00g/l, NaCl 5.00g/l, dextrose anhydrous 10.00g/l, and agar 30.00g/l; pH - 7.00). Medium without chromium was used as control. Serially diluted samples were used as inoculum and spread evenly throughout the plate with the help of a spreader. Results were observed after 24 h of incubation at 37 °C for the development of any bacterial growth. After incubation, plates were observed for colonies with distinct colony morphology and sub-cultured regularly. After preliminary screening, isolates were streaked on LB agar without chromium and preserved at 4 °C for further studies.

Effect of chromium concentration on bacterial growth and Maximum Tolerance (Maximum Tolerance Level) Assay

Selected bacterial isolates grown on LB agar and broth supplemented with increasing concentrations of chromium (25, 50, 75, 100, and 125 mg/l). Plates and tubes without chromium were maintained as control.

Screening of bacterial isolates for Maximum Tolerance Level (MTL)

Modified LB agar plate method was employed to test the ability of bacterial isolates to tolerate and grow under increasing concentrations of chromium as per Malik and Jaiswal (2000). The efficiency of isolates was tested by spot inoculation of 0.5 µl of 24 h fresh inoculum at 10^8 cell/ml (Wani and Omozele, 2015), on LB agar medium supplemented with 0, 25, 50, 75, 100 and 125 mg/l of chromium concentrations (Singh and Gupta, 2019). The highest concentration of heavy metal supporting the growth of visible bacterial colony after 48 h of incubation at 37 °C is defined as MTL as per Singh and Gupta (2019).

Table 1. Morphological characterization of Cr (VI) tolerant bacterial isolates

S. No.	Isolates	Colony characteristics	Gram's nature
1	CRS-1	circular, cream, smooth, convex, opaque, slime	Gram + ve cocci
2	CRS-2	circular, white, smooth, raised, opaque, slime	Gram + cocci
3	CRS-3	irregular, cream, fringe, raised, opaque, glistening	Gram + long rod
4	CRS-4	irregular, white, smooth, flat, opaque, glistening	Gram + cocci
5	CRS-5	irregular, yellow, smooth, raised, opaque, glistening	Gram + ve cocci
6	CRS-6	circular, cream, lobate, flat, opaque, glistening	Gram + ve cocci
7	CRS-7	irregular, white, smooth, raised, opaque, glistening	Gram + rod
8	CRS-8	irregular, yellow, fringe, flat, transparent, dry	Gram + ve rod
9	CRS-9	circular, yellow, smooth, raised, opaque, glistening	Gram + coccobacillus
10	CRS-10	circular, orange, smooth, raised, opaque, glistening	Gram + ve rod

Determination of Minimum Inhibitory Concentration (MIC)

Minimum inhibitory concentration (MIC) is the lowest concentration of Cr(VI) inhibiting growth of bacteria (Cappuccino and Sherman, 2013). A modified agar disc diffusion method was employed to determine their Minimum Inhibitory Concentration MIC, ranging from 100 to 500 mg/l by preparing 24 h fresh culture discs with 0.5 µl of shortlisted isolates were placed on LB agar plates supplemented with increasing concentrations of chromium (100, 200, 300, 400 and 500 mg/l). Plate without Cr(VI) was used as a control. Plates were left to incubate at 37 °C for 48 h. in upright position. Chromium tolerance was recorded based on visual observation based on growth of colonies around the chromium diffused wells and diameter of the visible colonies were measured.

Acclimatization studies

Acclimatization studies were conducted in minimal salt medium (MSM) consisting of (sodium chloride - 0.5 g/l; potassium phosphate -3 g/l; magnesium sulphate anhydrous - 0.12 g/l; calcium chloride dehydrate - 0.015 g/l; sodium phosphate – 6 g/l and yeast extract - 3.0 in g/l). Sterilized potassium dichromate was incorporated in the above medium in the concentration of 100 mg/l. The pH was adjusted to 7.7 using 1N NaOH. The shortlisted isolates were inoculated separately into the MSM and incubated at 37±2 °C for 24 h in a rotary shaker at 150 rpm. The cultures were maintained for further studies (Sarankumar *et al.*, 2020).

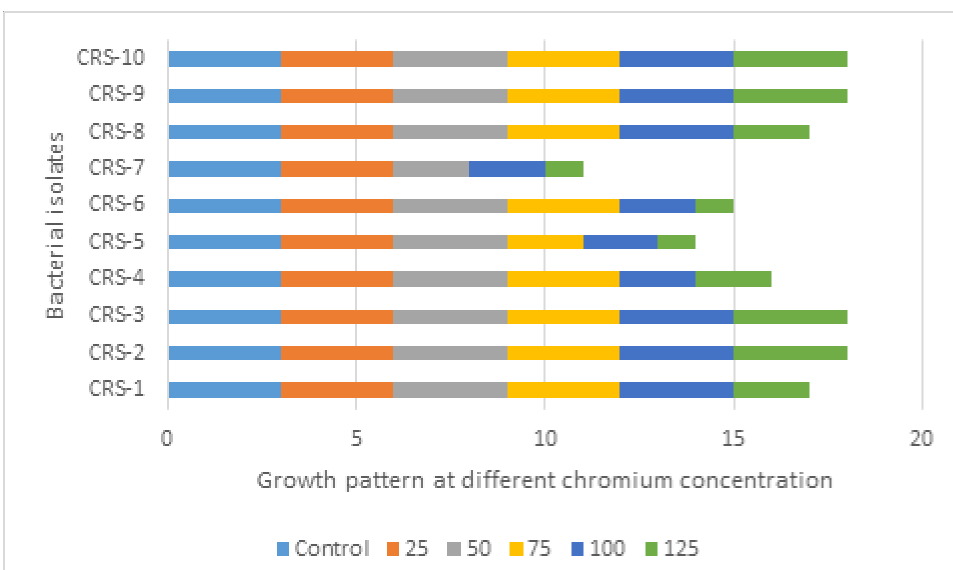
Results and Discussion

Statistical Analysis

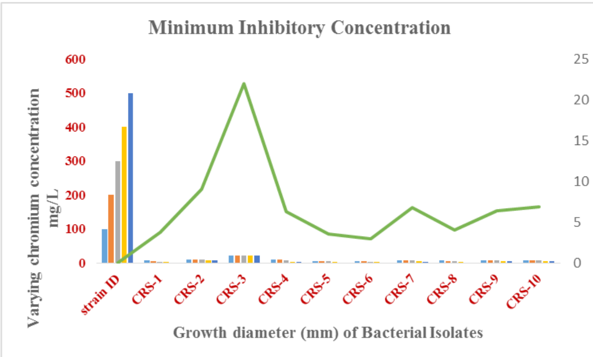
All experiments were conducted in triplicates and results obtained were compared using one-way analysis of variance.

Isolation and identification of chromium tolerant bacterial isolates

A total of forty-three chromium-resistant bacterial isolates were obtained from chromium rich soils. Ten isolates were shortlisted based their morphological characteristics, pigmentation varying from cream to orange, yellow, white with very small to large colony size, smooth margin to undulate, wavy, elevation from convex, raised, flat and consistency with slime to rough and glistening as presented in Table 1. Farag and Zaki (2010) reported the isolation of four strains capable of reducing hexavalent chromium from tannery effluents in the range of 20 to 200 mg/l. Strain *Morganella morganii* reported from tannery effluent collected from Vellore and Dindigul districts capable of reducing Cr (VI) at 4600 mg/l in minimal media (Princy *et al.*, 2020). Similar studies of Cr (VI) tolerance through reduction were observed at 3,700 mg/l at pH 7 as described by Princy *et al.* (2020). Mixed culture could reduce Cr (VI) at concentration up to 400 mg/l as reported by Kholisa *et al*. (2021).



Graph 2. Chromium tolerance determination of chromium tolerant bacterial isolates



Graph 3. Minimum Inhibitory concentration of chromium tolerant bacterial isolates at varying concentration (Colony diameter in mm)

Effect of chromium concentration on bacterial growth and Maximum Tolerance (Maximum Tolerance Level) Assay

In the present study, a consortium was developed with the organisms belonging to the genera *Bacillus*, *Pseudomonas*, *Enterococcus*, indicated unique combination of consortium to withstand and grow profuselyin chromium contaminated soils as presented in Fig. 1.

A significant reduction was observed in the growth of microbial population with increased chromium concentration (Ilamathi *et al*., 2008). Sarankumar *et al*. (2020) has reported two potent bacterial strains, *Bacillus cohnii* and *B. licheniformis* able to reduce 550mg/l of Cr (VI).

These isolates were further inoculated on LB agar medium supplemented with varying concentrations

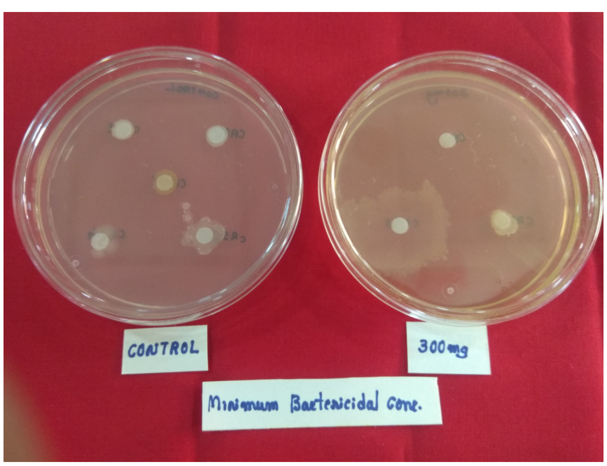


Fig. 1. Minimum inhibitory concentration (MIC)with isolate (CRS-3)showing luxuriate growth at 300 mg/l

of chromium, as potassium dichromate Cr (VI) tolerance from 25 to 125 mg/l and modified disc diffusion method to determine their MIC, ranging from 100 to 500 mg/l. Isolate CRS-3 showed highest tolerance level, growing profusely in the presence of 500 mg/l Cr(VI). Flavio *et al*. (2004), reported varying abilities of isolates to resist hexavalent chromium in the medium, which was reported to be directly co-related with the varying chromium concentrations.

Screening of isolates for maximum tolerance level (MTL)

On determination of Maximum Tolerance Level against varying chromium concentrations, four isolates showed tolerance to 125 mg/l of chromium concentration as indicated in Graph 2. Canizarez *et al*. (2017) has reported unaffected growth of *Chorella vulgaris* at 45-100ppm. Present study also shows similar results indicating a significant reduction in the growth of microbial population with the increased chromium concentration (Ilamathi *et al*., 2008) due to the toxic effect of chromium. whereas in *Scenesdesmus acutus* growth was completely arrested due to the presence of chromium at a concentration of 15 ppm. Wang *et al*., 2008 reported *E. cloacae* as a chromium resistant strain and was the most studied chromium resistant and reducing strain (Ohtake and Silver, 1994). Exposure to different concentrations of chromium caused significant reduction in the number of visible colonies with increasing Cr (VI) concentrations.

Determination of Minimum Inhibitory Concentration (MIC)

Ten isolates were shortlisted to check their chromium tolerance by subjecting them to Minimum inhibitory concentration assay was carried out by modified agar disc diffusion method. Strain CRS-3 found to be tolerant to Cr(VI) up to concentration of 500 mg/l, showing profuse growth with a colony diameter of 21.3 mm followed by CRS-2 with colony diameter of 8mm as indicated in the Table 2 and Graph 3. Similar results were indicated Camargo *et al*., 2003 showing 90 % Cr (VI) reduction as reported by *Bacillus* sp. Purwanti *et al*. (2017) reported total growth inhibition of strains, *Azotobacter* S8, *Bacillus subtilis* and *Pseudomonas putida* at 100 mg/l of $CrCl_3$. *Klebsiella* sp. reported to reduce 95 % of Cr (VI) as per Hossan *et al*. (2020). Works of Kalsoom *et al*. (2021) indicate the maximum tolerance of *Bacillus paranthacis* and *B. paramycoides* at 1600 ppm of Cr

Table 2. Study of Minimum Inhibitory Concentration of shortlisted isolates at varying concentration (Colony diameter in mm)

Isolate	100 mg/l	200 mg/l	300 mg/l	400 mg/l	500 mg/l
CRS-1	6.9±0.1	5.7±0.6	4.0±1.7	2.0±0.0	NG
CRS-2	10.3±0.5	9.3±0.6	9.3±0.6	8.3±0.6	8.0±0.0
CRS-3	22.4±0.5	22.3±0.6	22.3±0.6	21.6±0.6	21.3±0.6
CRS-4	10.7±0.6	9.7±0.6	7.0±0.0	3.0±1.0	1.3±0.6
CRS-5	6.0±1.0	5.3±0.6	4.3±1.1	2.3±0.6	NG
CRS-6	5.3±0.6	4.3±0.6	3.3±0.6	2.0±0.0	NG
CRS-7	8.7±0.5	8.7±0.6	7.6±0.6	5.0±1.0	4.0±1.7
CRS-8	7.0±0.0	6.0±0.0	5.0±0.0	2.3±0.6	NG
CRS-9	8.0±0.0	7.3±0.6	6.6±0.6	6.0±1.0	4.3±0.6
CRS-10	8.0±0.0	7.6±0.6	7.3±0.6	6.3±1.5	5.3±1.5

(VI). Bacteria isolated from the pearl millet fields, irrigated by wastewater from industrial area *Bacillus sp.* 1500 mg/l, *Brevibacillus agri*. 2500 mg/l, *Staphylococcus sp.* 2500 mg/l, *Exigobacterium sp*. 2500 mg/l and *Pseudomonas* sp. 1500 mg/l as reported by Hussain and Al-saadi (2021).

Isolate CRS-3 showed highest tolerance level, growing profusely in the presence of 500 mg/l Cr(VI). Flavio *et al*. (2004), reported varying abilities of isolates to resist hexavalent chromium in the medium, which was reported to be directly co-related with the varying chromium concentrations.

Conclusion

The present study concludes that bacterial species isolated from polluted sites show best results to resist and grow in high concentrations of hazardous pollutant like Chromium. A total of 10 chromium tolerance bacteria has been isolated and taxonomically identified to belong to genera *Bacillus*, *Pseudomonas*, *Enterococcus*. A strain isolated belong to *Bacillus* species show resistance to chromium at the concentration of 500 mg/l, which have been isolated from highly chromium polluted areas. They can be utilized as a best candidate for bioremediation studies due to their high tolerance to Cr(VI). These isolates can be further studied for their plant growth enhancing abilities for remediation and management of Cr contaminated soils.

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Conflict of Interest

Authors declare no competing interests.

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