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Bioremediation of Hexavalent Chromium Contaminated Soil Using Chromium-Tolerant *Bacillus* Strains Isolated from Agricultural Sites

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ABSTRACT
This study investigates the potential of chromium-tolerant bacteria isolated from agricultural soil near the Manikgarh cement factory in Gadchandur for the bioremediation of hexavalent chromium (Cr (VI)). Three bacterial strains, *Bacillus flexus, B. subtilis*, and *B. stercoris*, were isolated and identified by 16S rRNA sequencing. These strains showed significant chromium tolerance and degradation capabilities. *B. flexus* showed optimum growth at 200 ppm Cr (VI), achieving 84% degradation, *B. subtilis* and *B. stercoris* demonstrated 76 and 24% degradation at 100 ppm and 50 ppm, respectively. Optimal pH and temperature conditions for chromium reduction were identified for all three species. Co-culturing the strains significantly enhanced the degradation efficiency, achieving a 91% reduction in Cr (VI) in 72 hrs, compared to 84% in 120 hrs by *B. flexus* alone. This synergistic effect highlights the potential of co-culturing for more efficient bioremediation.

Key words: Chromium degrading bacteria, Gadchandur, Co-culture, Hexavalent chromium, *B. flexus, B. subtilis*, *B. stercoris*

INTRODUCTION

Rapid growth in the global population and industrialization of habitable regions are the leading causes of the contamination of land, air, and water (Chhikara and Dhankhar 2008). This phenomenon has led to the bioaccumulation of hazardous chemicals in nature. Heavy metals' bioaccumulation in the biosystem seriously threatens human survival (Hooda 2007). The environment mostly accumulates heavy metals from a variety of sources. The cement industry has been identified by the Central Pollution Control Board (CPCB) as the most polluting industry, which has been assigned the red category. Cement kilns are the source of highly hazardous and cancer-causing pollution. Heavy metals like nickel (Ni), arsenic (As), cadmium (Cd), cobalt (Co), lead (Pb), manganese (Mn), chromium (Cr), and zinc (Zn) are found in cement dust (Pidurkar et al. 2014). The most hazardous form of this metal, Cr (VI), is known to be released into the environment by the cement industry, and this is one of the main ways that soils become contaminated with chromium. This leaching mainly impacts the quality of surface and groundwater. These heavy metals can pollute groundwater through various means, including leaching from surrounding heaps and landfills, storm water runoff, and leakage from storage ponds (Moncur et al. 2005). Water from surrounding water streams has also been contaminated by leaching from overburdened waste rock dumps. These poisonous metal ions are causing harmful effects on human health. It can quickly infiltrate cell membranes, posing a significant risk to human health (Chaudhary et al. 2003). According to De Filippis and Pallaghy (1994), chromium concentrations in clean waters range from 0.1 to 0.5 ppm in fresh waters and 0.0016 to 0.05 ppm in oceanic waters. However, wastewater from paper mills has been shown to release up to 80 ppm of chromium (Sudhakar et al. 1991). Heavy metal-containing dust can seriously endanger the health of humans, animals, and aquatic life when it lands on surface soil, agricultural soil, or natural water resources (Robin et al. 2022). Hazardous to humans, microorganisms, plants, and animals, Cr, Pb, and Cd can harm cell membranes, change an organism's genetic composition, and lessen an enzyme's specificity. This toxicity is caused by heavy

metals interacting with ligands or by displacing critical metals from their natural binding sites.

Chromium can be found in the environment as hexavalent Cr (VI) and trivalent Cr (III). In addition to being a possible pollutant of soil, surface water, and groundwater, Cr (VI) is carcinogenic. It is a hundred to a thousand times more dangerous than Cr (III). Even a tiny increase in Cr (VI) levels can harm the environment and human health, including cancer and mutagenicity (Venitt and Levy 1974). Cr (III) is necessary for humans and is less poisonous and insoluble than its reduced trivalent version. Owing to its toxicity, the United States Environment Protection Agency (U.S. EPA) has strict regulations limiting the discharge of Cr (VI) to surface water to less than 0.05 mg/l and total Cr to less than 2 mg/l (Baral and Engelken 2002). Anthropogenic chromium inputs have surged since the industrial revolution (Baral et al. 2006). According to Ryan et al. (2002), chromium is widely utilized in cement, leather tanneries, resistant alloys (such as stainless steel), and electroplating (chrome plating). The environment's primary Cr (VI) sources are mine tailings and effluents from the non-ferrous metals industry (Moore and Ramamoorthy 2012).

Gadchandur town and its surroundings are home to several cement factories, including Ambuja, Manikgarh, ACC, and Murli Agro Cement (Singh and Chauhan 2002). When cement dust settles on open land, it contains chromium in both forms. The neighboring water stream exhibits contamination from Cr (VI) well above allowable limits because of the water seepage from this contaminant. The risks associated with Cr (VI) pollution include stillbirths, gastrointestinal bleeding, asthma attacks, tuberculosis, infertility, and birth abnormalities. Regulating organizations have often warned the sectors to decrease Cr (VI) contamination, but the companies have not implemented suitable treatment facilities. One crucial process by which microorganisms can be employed for chromium detoxification is the conversion of Cr (VI). The health of those living in this area is at risk due to an overabundance of these chemicals in the water and soils (Pidurkar et al. 2014). Installing suitable measures and treating dust before it is released into the environment is the only step toward reducing this enormous problem. Water treatment plants should use simple, efficient, economical, and environmentally friendly methods when working with groundwater and already-contaminated bodies of water.

As a result of their ongoing exposure to toxic substances, several bacteria have evolved systems that aid in the detoxification of heavy metals and enable them to acquire resistance to them (Ezaka and Anyanwa 2011). Numerous scientists have looked at the potential of bacteria *Arthrobacter* sp., *Bacillus* sp., and *Aerococcus* sp. identified from tannery effluents in Kanpur (Saxena and Bharagava 2015). Every microbiological strain that has been reported has a lower minimum inhibitory concentration for Cr (VI). The use of microbial strains for mining wastewater treatment, specifically for Cr (VI) pollutants, has received very little research. Thus, the current study focuses on identifying the chromium-degrading bacterial strain from the nearby agricultural fields contaminated with chromium and optimizing growing conditions for the isolated microbial strain for Cr (VI) degradation.

MATERIAL AND METHODS

Sample collection

The sample was collected from the metal-contaminated agricultural site near the Manikgarh cement factory at Gadchandur town (19°42'35.3" N 79°10'15.7" E, 19°43'12.9" N 79°10'50.9" E, and 19°43'31.9" N 79°10'05.1" E). The sampling sites were very close to the Manikgarh cement factory (Fig. 1). The samples were collected in polythene bags, stored at 4 ºC, and used within 24 hrs of sample collection.

Enrichment cultures

Soil samples (10 g) were added to 100 ml of media containing (2 g/L K_2HPO_4, 0.5 g/L KH_2PO_4, 0.2 g/L $MgSO_4 \cdot 7H_2O$, 1 g/L $(NH_4)2SO_4$, 0.01 g/L $FeSO_4$ (mineral salts). This enrichment media is supplemented with the Cr (VI) 10 ppm. The flask was incubated at 37°C at 160 rpm for 5 days. After every 3 days, the cultures were sub-cultured (Marzan et al. 2017). The sample was diluted 5-fold by serial dilution technique and inoculated on the nutrient agar plates. The plates were incubated at 37ºC for 24 hrs. The control consisted of mineral salts lacking Cr

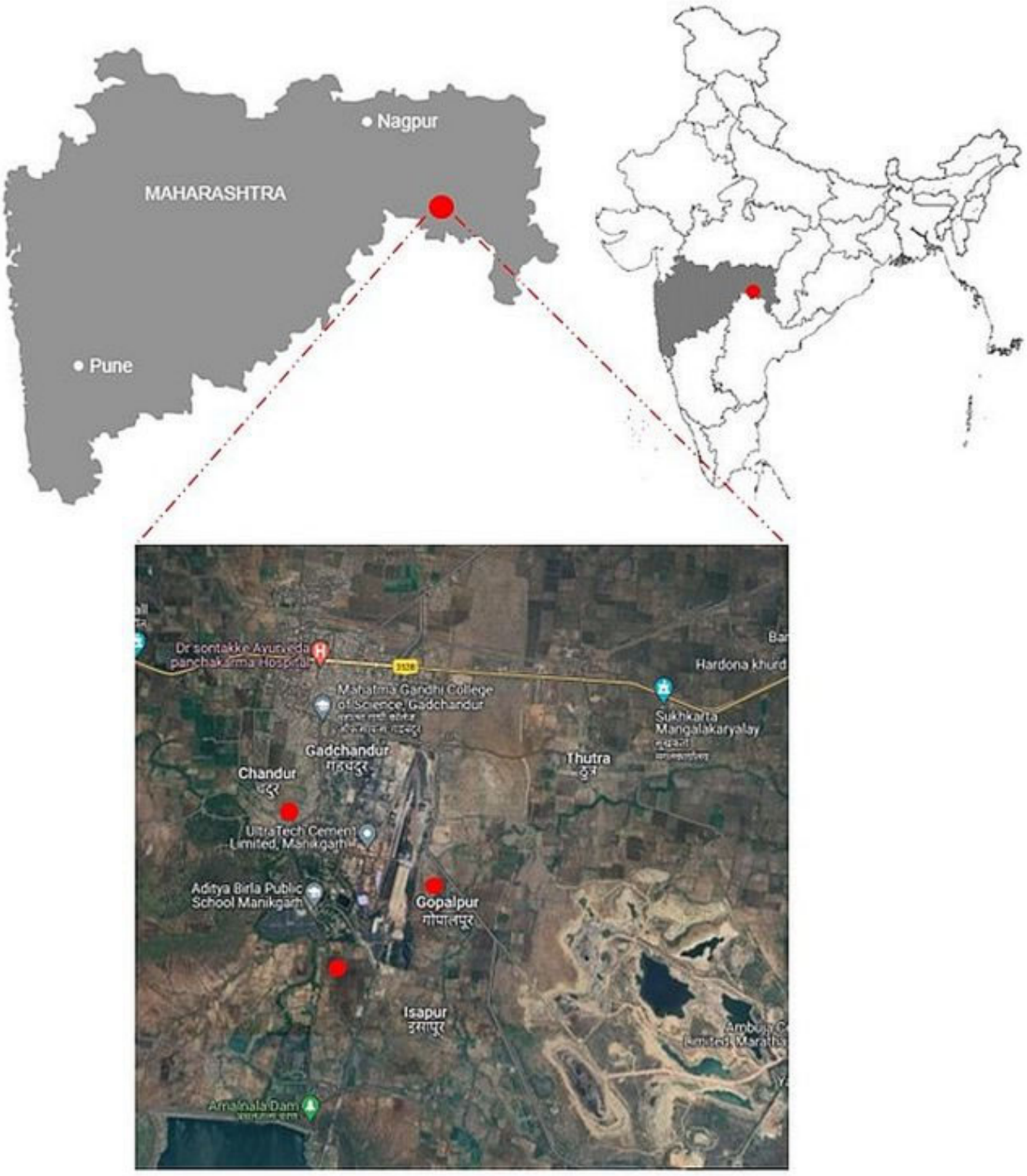

Figure 1. Sample collection sites (red dots) near Gadchandur town

(VI). The colonies with different morphological characteristics were selected, picked, and preserved for further studies in 30 % glycerol stocks.

Identification of microorganisms

Morphological characters were studied using Gram staining and microscopy, and the biochemical test (methyl red, Voges-Proskauer, catalase, oxidase) was performed according to standard protocols (Chauhan et al. 2020). The isolated bacterial colonies were identified by 16 S rRNA sequencing. The bacterial colonies were grown on nutrient broth for 24 hrs at

37°C. Centrifugation was done for 10 minutes at 8000 rpm to break down the cells. Genomic DNA Isolation was done using a modified CTAB procedure (Williams et al. 2017). The universal PCR primers of 5'-AGAGTTTGATCCTGGCTCAG-3' forward and 5'- CGGTTACCTTGTTACGACTT-3' reverse were used to amplify the 16S rRNA region of bacteria as demonstrated by Choi et al. (2015). The amplified PCR products and 500 bp DNA ladder (NEB, Beverly, Massachusetts) were then separated on a 1.2% agarose gel. For DNA sequencing, 50 ng of PCR product combined with 8 ìl of ready reaction mix (Applied Biosystems' BDT v3.0 in Foster City, California) and 5 picomoles of the forward primer. This sequencing was performed using an ABI Prism 3100 Genetic Analyzer (Applied Biosystems). Subsequently, the BLASTN search was done within microbial nucleotide databases to identify the closest matching bacterial species.

Study of pH and temperature variations on the growth of the bacteria

To investigate the influence of pH on bacterial growth, a series of controlled experiments using minimal salt solutions at different pH (4, 5, 6, 7, 8, 9, and 10) was employed. The pH of the solution was maintained using 1 N NaOH or 1 N HCl. For each experiment, 100 mL of pH-adjusted medium with 1 mL bacterial culture in the log phase (OD600, 0.8) was prepared separately for each bacterial strain. To achieve optimal growth conditions, the cultures were incubated at 37°C for chromium degrading strain with continuous shaking at 160 rpm. Optical density at 600 nm at regular intervals was taken after every 6 hrs. OD600 measurements were conducted using a spectrophotometer calibrated with minimal salt solution as the reference. Each experiment was carried out in triplicate. The influence of temperature growth and chromate reduction were investigated at range of temperatures (25, 28, 33, 35, 37 and 42°C) at the same conditions with pH 7.

Chromium degradation assay

The culture was grown in different concentrations of Cr (10-100 ppm). After 24 hrs the samples were centrifuged and supernatant will be tested for the Cr (VI) concentration. The reaction mixture consists of sample (before and after incubation), H_2SO_4 (1 N), $KMnO_4$ (0.1 N), sodium oxide (4%) and diphenyl carbazide (0.25%). After heating the reaction mixture at 60ºC for 3 min and cooling it, the optical density of the pink color complex was taken at 540 nm (Krishna and Philip 2005). The MSM with Cr (VI) without bacterial inoculation served as negative control. The MSM with Cr (VI) with bacterial inoculation is a positive control. The MSM without Cr (VI) and bacterial inoculation served as blank. The residual Cr (VI) concentration was calculated using the formula:

Cr (VI) degradation (%) = (Initial Cr (VI) concentration – Residual Cr (VI) concentration)/ Initial Cr (VI) concentration

Experimental set up of lab scale bioreactor

The lab scale bioreactor was constructed from acrylic measuring 60 cm in length, total volume of 3 L and a working volume of 2.5 L, was sealed securely on both ends. The lower terminus of an internal plate was connected to an aerator, which served to agitate the contents therein. Sampling ports were installed 16 cm from the lower and upper boundaries of the reactor. A mixture of mineral salt medium and chromium in a 4:1 ratio was introduced into the vessel. An aseptically administered co-culture (1 ml each at log phase, O.D 600, 0.8), functioning as a seed, was added. Optimal conditions within the entire assembled system were meticulously regulated and maintained. Monitoring of chromium degradation was monitored after every 6 hrs.

RESULTS AND DISCUSSION

Isolation of chromium tolerant bacteria

Three bacterial strains (20.1 b, 20.3 b and 30 b) were isolated from the contaminated soil of the Gadchandur region. All the strains could easily tolerate the concentration of 50 ppm Cr (VI). The highest tolerant species were selected after screening various colonies for the chromium tolerance range of 10 to 500 ppm. All the isolated strains showed Gram positive nature. The morphological and biochemical characters of the colonies were also studied and shown in Table 1. Previous studies indicate some bacteria have shown a remarkable capacity to reduce chromium, mainly its toxic

Table 1. Morphological and biochemical characters of bacterial strains

Characteristics	***Strain 20.1 b***	***Strain 20.3 b***	***Strain 30 b***
Colony shape	Circular	Circular	Irregular
Colony size	Medium	Medium	Medium
Colony colour	Off-white	Off-white	Creamish
Catalase	Positive	Positive	Positive
Citrate utilization	Positive	Positive	Positive
Methyl red test	Negative	Negative	Negative
Voges-Proskauer test	Positive	Positive	Positive
Indole production	Negative	Negative	Negative

hexavalent state Cr (VI). *Pseudomonas aeruginosa* strain M3, isolated from heavy metal-contaminated soil, could reduce Cr (VI) to 75% within 48 hrs (Desai et al. 2008). *Bacillus cereus* strain SJ1 reduced ~95 % of Cr (VI) within 72 hrs. The efficiency of this strain is demonstrated by the detoxification process in Cr-contaminated environments (He et al. 2009). *Shewanella oneidensis* MR-1, could reduce 100 µM Cr (VI) entirely in less than 24 hrs (Viamajala et al. 2003). Smrithi and Usha (2012) reported isolating 34 bacteria belonging to diverse categories, predominantly *Bacillus, Micrococcus*, and *Lactobacillus* types, from tannery waste disposal sites. Numerous studies have also noted *Bacillus* varieties as chromium tolerant bacteria.

Identification of microorganism

The isolated microorganisms were identified as *Bacillus flexus* (Strain 20.1 b, Accession no. PQ056843), *Bacillus subtilis* (Strain 20.3 b, Accession no. PQ056873) and *Bacillus stercoris* (Strain 30 b, Accession no. PQ056885) by 16 S rRNA gene sequencing. The sequences were compared with the available nucleotide sequences by BLASTN search algorithms to find the closest homologous bacterial species. Multiple sequence alignment was done by the CLUSTALW program with the obtained 16 S rRNA sequences. The phylogenetic map was built using MEGA5 software. The built phylogram was documented along with the close homology of the bacteria isolated. *Bacillus* spp. effectively reduced hexavalent chromium Cr (VI), indicating their potential for bioremediation applications (He et al. 2009). Under heavy-metal contaminated soil conditions, the strain SJ1 of *B. cereus* had 95% removal of Cr (VI) in 72 hrs (He et al. 2009). *B. subtilis* shows evidence of reducing chromium (Camargo et al. 2003), up to nearly an 84% reduction in less than a day under optimal conditions. *B. thuringiensis* studied for treating industrial effluents was able to reduce Cr (VI) by up to 70%, whereas *B. sphaeicus* reduced Cr (VI) by about a maximum of 55% in two days (Sultan and Hasnain 2005). These studies illustrate a variety of efficient mechanisms used by *Bacillus* species to reduce toxic Cr (VI) into a relatively less harmful trivalent state, which is an approach that reduces environmental pollution and leads to cleaner ecosystems and habitats. All the sequences were submitted to NCBI using GenBank, and accession numbers were acquired.

Study of different chromium concentrations on growth

The bacterium was grown under 10-500 ppm of chromium concentration to study its effect on the growth. *B. flexus* and *B. subtilis* could grow in 10, 50, 100, 200, and 500 ppm of chromium, but the optimum growth was achieved for *B. flexus* at 200 ppm and for *B. subtilis* at 100 ppm (Fig. 2). The bacterial growth was reduced at 500 ppm of the chromium. *B. stercoris* can only grow up to 50 ppm, and the growth reduced in 100-500 ppm chromium (Fig. 2). Strains of *Pseudomonas putida* reduce Cr (VI) concentrations of up to 100 ppm (Desai et al. 2008). Similarly, studies on chromium-resistant strains isolated from heavy-metal contaminated soil showed that *Bacillus cereus* can reduce Cr (VI) at concentrations as high as 100 ppm (He et al. 2009). *Escherichia coli* can reduce chromium at concentrations up to 10 ppm (Abskharon et al. 2009).

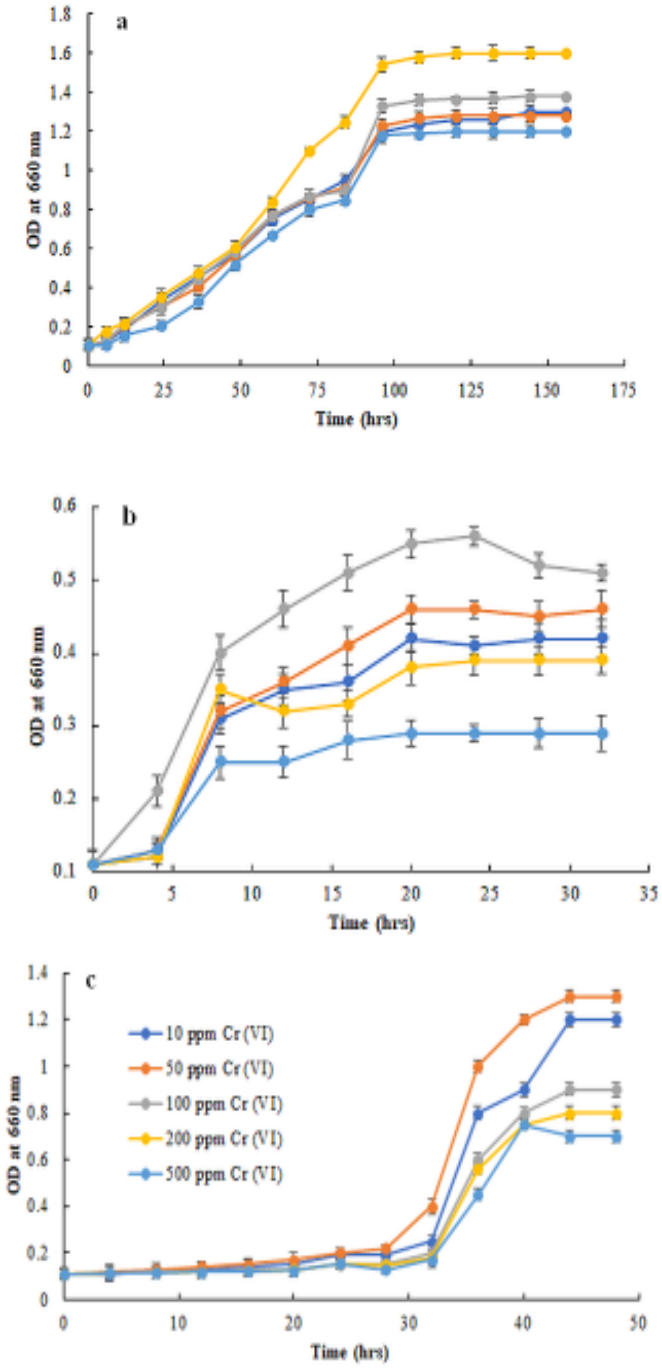

Figure 2. Effect of different chromium concentrations on the growth of of *Bacillus flexus* (a), *B. subtilis* (b), and *B. stercoris* (c) from agricultural fields of Gadchandur. Each value is the mean ± SD from three independent experiments. Mean values are significantly different at $P \leq 0.05$ level, n = 3

Effect of pH on growth

B. flexus showed the highest chromium reduction potential at pH 7 and 8, signifying its favourability towards neutral-slight alkalinity. *B. stercoris* showed the maximum degradation capability at pH 7. *B. subtilis* showed maximum chromium reduction activity at pH 7. The biodegradation capability of the bacterial strains increased from 4 to 6 pH values. It remained highest for *B. flexus* and *B. stercoris* at pH 7-8 and 7, respectively, and 7 for *B. subtilis*, followed by the decrease in the biodegradation capability up to pH 9 (Fig. 3). The degradation of Cr (VI) is usually enzyme associated. Although minor changes in pH are known to affect this, the data show that natural background levels not only alter but severely restrict Cr toxicity and impact the enzyme ionization ability, which can modify protein shape by inhibiting enzyme activity (Farrell and Ranallo 2000). Maximum degradation of Cr (VI) occurred at pH 7 Cr (VI) which reduced it by 98% from 50 to 0.78 ppm. The optimal pH for growth chromium-degrading bacteria is usually 7.0-7.8 (Losi et al. 1994). *Enterobacter cloacae* was able to grow between pH 6.5 and 8.5, but its Cr (VI) reduction rate was considerably reduced at low pH 5.0 and high pH 9.0 (Wang et al. 1991). The Cr (VI) reduction occurred at optimum pH 6-7 in *Streptomyces griseus* (Laxman et al. 2007). The highest Cr (VI) reduction activity was reported at pH 7.0 for many species of Gram-positive bacteria, including different *Bacillus* sp., along with *E. coli* and *P. fluorescence* (DeLeo and Ehrlich 1994, Philip et al. 1998). Maximal growth of *Bacillus* spp. was reported at pH 7 for most species tested (Smrithi and Usha 2012).

Effect of temperature on growth

The optimum growth for the *B. flexus* at 200 ppm Cr (VI) is at 35°C. The temperature range was studied from 25 to 42°C. The bacteria also showed growth at 42°C, which showed the capability of the strain to grow above room temperature, which may be helpful in the biodegradation of the metal above room temperature. *B. stercoris* also showed an optimum growing temperature of 37°C at 50 ppm Cr (VI). *B. subtilis* is similarly effective in reducing chromium under moderate warmth conditions. Its highest activity tends to occur within the range of

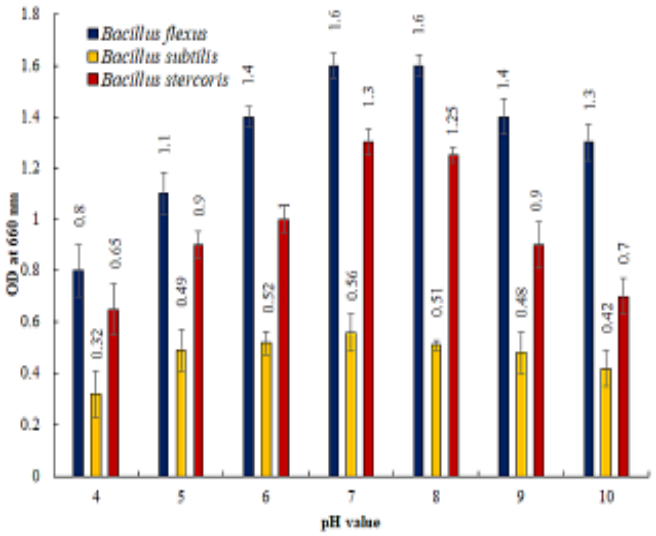

Figure 3. Effect of different pH values on growth of *Bacillus flexus*, *B. subtilis* and *B. stercoris*. Each value is the mean ± SD from three independent experiments. Mean values are significantly different at $P \leq 0.05$ level, n = 3

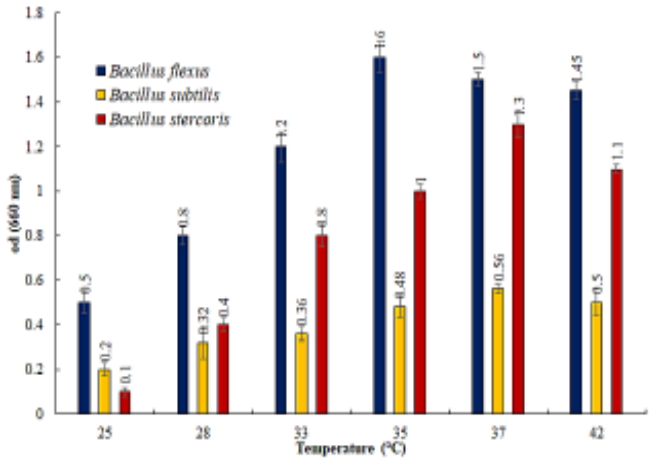

Figure 4. Effect of different temperatures on growth of *Bacillus flexus, B. subtilis,* and *B. stercoris*. Each value is the mean ± SD from three independent experiments. Mean values are significantly different at $P \leq 0.05$ level, n = 3

temperature 30-37ºC with optimum growth at 37°C at 100 ppm Cr (VI) (Fig. 4). Chromium reduction by *Pseudomonas putida* at 30°C indicates that the bacterium prefers moderate temperatures (Camargo et al. 2003). However, *Acinetobacter* spp. performs excellently at slightly lower temperatures, from about 25°C to around 30°C (Francisco et al. 2002). Reducing chromium by *D. vulgaris*, a sulfate-reducing bacterium, occurs at temperatures ranging from 30-35°C, which agrees with its adaptation to mesophilic thermal conditions (Lovley and Phillips 1994). The temperature of 30°C was optimum for the growth of *B. megaterium* strain TKW3 (Cheung and Gu 2005). *B. ciruclans* optimally grows at 30°C as compared to 25°C and 35°C (Chaturvedi 2011).

Chromium degradation

The degradation of the chromium was achieved either individually or in the lab-scale bioreactor. All the optimum conditions were used for individual chromium degradation with the isolated bacteria. It was found that *B. flexus* could reduce the 200 ppm Cr (VI) to 24 ppm Cr (VI), i.e., 84% chromium reduction after 120 hrs (5 days) of incubation (Fig. 5). *B. subtilis* could achieve 76% degradation of Cr (VI) after 32 hrs of incubation and *B. stercoris* could degrade Cr (VI) to 48% from 50 to 24 ppm after 48 hrs of incubation (Fig. 5). The degradation of the chromium was enhanced by 7% from highest degradation rate, i.e., from 84 to 91% after 3 days with the co-culture of all the three isolated strains (Fig. 6). It is to be noted that the time required by *B. flexus* to degrade Cr (VI) was 5 days but with co-culture, the degradation is achieved within 3 days. Co-culturing microbes can enhance degradation abilities by capitalizing on the strengths of distinct organisms and creating more efficient and effective bioremediation systems by synergistic effect, enhanced nutrient utilization, reduced toxicity, and improved environmental adaptation (Tong et al. 2023). In oil spill clean-ups, co-cultures containing hydrocarbon-eating bacteria *Alcanivorax* and *Pseudomonas* have proven to improve the pace and extent of oil breakdown compared to sole cultures (Radwan et al. 2019). The co-cultures of *Dechloromonas* and *Geobacter* microbes decreased the concentration of pollutants more effectively than either could individually (Ueki et al. 2018). The pair works synergistically, with one breaking contaminant down partly while the other completes the conversion, expediting the clean-up. Biodegradation of chromium in bioreactors is an advanced technique used for environmental clean-up. Bioaugmentation with *Shewanella oneidensis* in a continuous-flow bioreactor achieved 85% chromium removal efficiency over 30 days (Tang et al. 2006). Liu et al.

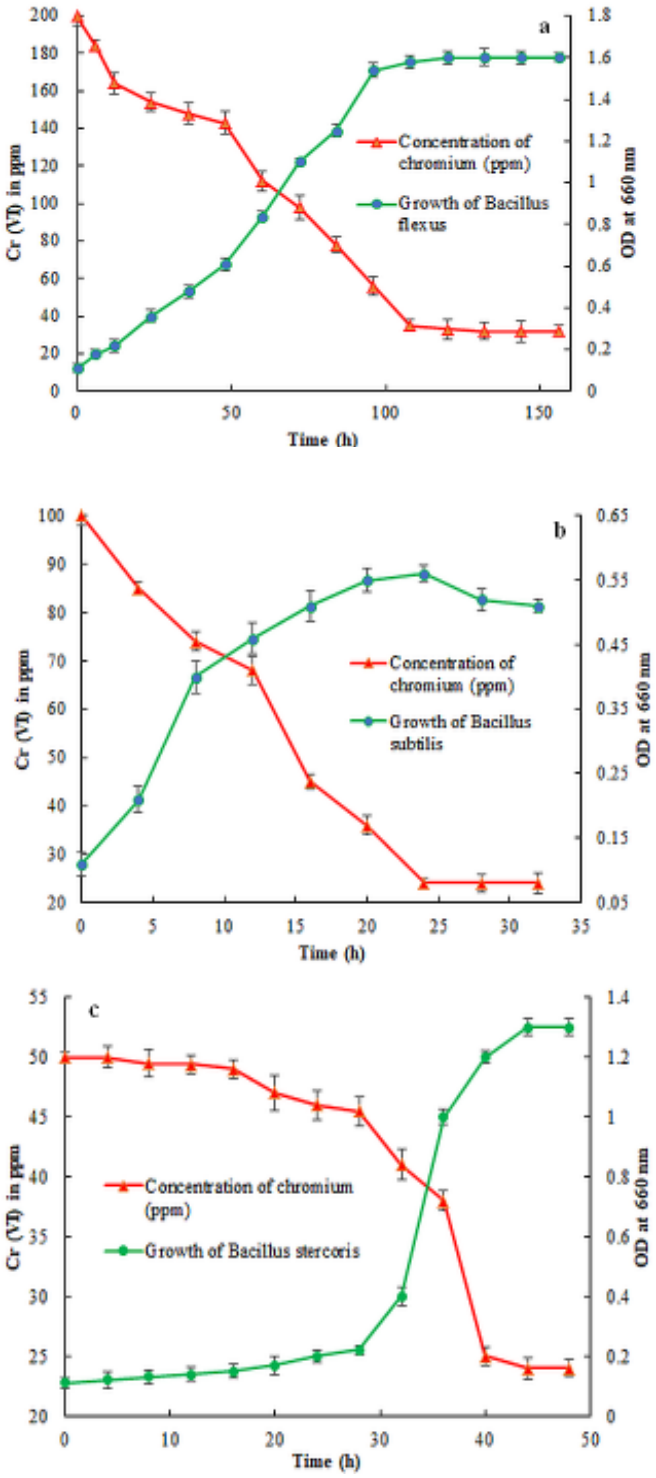

Figure 5. Chromium degradation and growth of *Bacillus flexus* (a), *B. subtilis* (b), and *B. stercoris* (c). Each value is the mean ± SD from three independent experiments. Mean values are significantly different at $P \leq 0.05$ level, n = 3

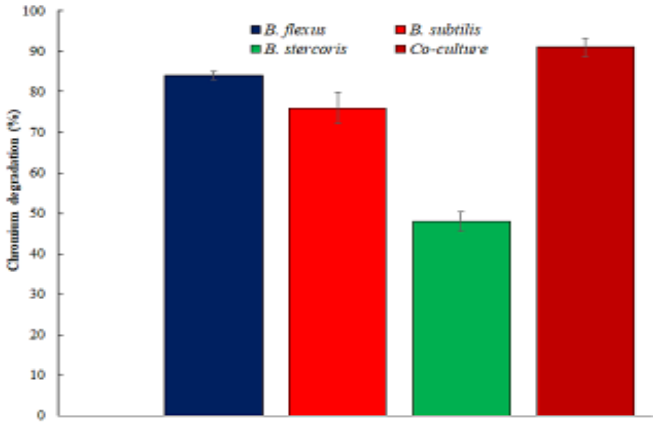

Figure. 6 Comparison of chromium degradation by individual isolated bacterium and by co-culture technique in lab-scale bioreactor. The values represented in this experiment are the mean of all the values ± SD. In total five independent experiments were performed (n = 5) and values of mean are notably different when $P \leq 0.05$ level

(2020) demonstrated that the use of nano-scale zero-valent iron along with microbial cultures in a bioreactor resulted in an 88% removal efficiency of Cr (VI) within 24 hours. Bioreactor level optimization of chromium reduction was demonstrated by John and Rajan (2022). Their studies indicate the enhancement of chromium degradation by 40% by *Pseudomonas putida* APRRJVITS11 after optimization of the parameters. The chromium mineralization was achieved by using lab scale bioreactor, which showed mineralization of Cr (VI) (Kamalasini et al. 2023).

CONCLUSIONS

The present investigation analyzed the possibility of chromium-tolerant microbes isolated from agricultural soil close to Gadchandur for bioremediation of hexavalent chromium (Cr (VI)). Three bacterial strains, *B. flexus*, *B. subtilis*, and *B. stercoris*, could degrade the chromium effectively. *B. flexus* showed the optimum tolerance at 200 ppm Cr (VI), which showed 84% degradation in 120 hrs by lowering 200 ppm Cr (VI). *B. subtilis* and *B. stercoris* indicated significant chromium degradation capacities, with optimal growth and reduction of 76%

in 32 hrs and 24% in 48 hrs.

The co-culture of all the strains enhanced the Cr (VI) degradation rate to 91% in 72 hrs, compared to the 84% reduction in 120 hrs by *B. flexus*. This proposes that co-culturing improves the bioremediation procedure, making it progressively productive. These findings underscore the potential of using *Bacillus* spp. in bioremediation strategies to mitigate chromium contamination in agricultural soils. The strains' ability to thrive and reduce chromium under various conditions suggests their adaptability and effectiveness in dealing with chromium degradation. Moreover, the success of co-culturing approaches opens new avenues for developing more robust bioremediation systems. Future studies could focus on optimizing these bacterial consortia in bioreactors and exploring their application on a larger scale to achieve sustainable environmental clean-up. In summary, the isolated Bacillus strains offer promising solutions for reducing Cr (VI) pollution, contributing to cleaner ecosystems, and demonstrating the potential of microbial bioremediation as a viable approach for managing heavy metal contamination.

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Author's contributions: Utkarsh Ravindra Moon performed and designed all the experiments. He was also responsible for final approval of the manuscript. Arpana Ashokrao Durge performed all the statistical analysis. Vijay S. Wadhai was responsible for manuscript preparation and overall responsibility of the manuscript.

Conflict of interest: Authors declare no conflict of interest.

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