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Exploring the Role of Selected Bacterial Strains in Chromium (Cr(V*i*) Reduction from Soil

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Abstract- World's rapidly evolving population and its progressive attraction towards luxurious life has unsurprisingly led to an increase in anthropogenic sources of pollution. Environmental contamination of hexavalent chromium Cr(VI) is of serious concern for its toxicity as well as mutagenic and carcinogenic effects. Bacterial chromate reduction is a cost-effective technology for detoxification and removal of Cr(VI) from polluted environment. In current study, a total of 15 Cr(VI) resistant bacterial isolates were isolated after enrichment at three different Cr(VI) concentrations- 25mg/L, 50 mg/L and 100 mg/L. Out of 15 only three- S1B, S10D and S3-A with a maximum tolerance of 100mg/L Cr(VI) concentration were selected for further study. On the basis of biochemical characterization isolates were identified, S1-B as *Enterococcus faecalis*, S10-D as *Bacillus megaterium* and S3-A as *Bacillus cereu*s. Efficiency of chromium reduction by selected isolates was evaluated at optimized growth conditions. All the three isolates have shown excellent chromium reduction with 100% by S10D, 78.6 % by S1B and 61.36% by S3-A at 25mg/L of Cr(VI) concentration. All three isolates had shown around 60% reduction within 48 h of incubation period. Multimetal resistance was also determined against six different heavy metals (Hg, Cd, Pb, Zn, Co, Mn). Cd and Hg were highly toxic and supressed the bacterial growth completely, only S10D was resistant to Cd. S2D, S3A and S10D were resistant against Co, Pb and Mn while growth of S3A was inhibited by Zn. These potential bacterial isolates could be used at industrial level for effluent treatment and also have great significance for bioremediation of chromium contaminated sites.

Keywords: *Anthropogenic, bioremediation, carcinogenic, degradation potential, multimetal resistance*

I. INTRODUCTION

Heavy metals are persistent and very rarely biodegradable. To combat heavy metal pollution, extensive research is going on advanced technologies for removal of heavy metals, other toxic contaminants from industrial wastewater. Their toxicity, especially in high concentrations, has become a global issue [1]. The term ''heavy metals'' refers to any metallic element that has a relatively high density >5g/cm^3 and is toxic or poisonous even at low concentration. Most common heavy metals present in industrial wastes are chromium, copper, cadmium, nickel and mercury. Chromium is the seventh most abundant element on earth and exists in several oxidation states. Main sources of Cr(VI) release in the effluent are electroplating industries, tannery industries, leather industries textile and ceramics. Chromium primarily exists in two stable oxidation states of Cr(III) and Cr(VI)[2], which have contrasting toxicities, mobility and bio-availabilities. In the last few decades, the amount of chromium in both aquatic and terrestrial ecosystems has increased as a consequence of anthropogenic activities [3,4]. It is now considered as one of the major environmental pollutants due to its toxicity for ecological, nutritional and environmental reasons. The discharge of waste water and other material from different industrial resources containing heavy metals have resulted in an increased population of the resistant bacteria. Therefore, these bacteria can be used as potential tools for bioremediation. The advantage of selecting indigenous bacteria from contaminated environments may be the minimization of inhibitory effects from other compounds that may result in present along with Cr(VI), as viable indigenous organisms will have developed some degree of resistance to these compounds [5]. Microorganisms and microbial products have been reported to efficiently remove soluble and particulate forms of metals, especially from dilute solutions. There are different mechanisms like

bioaccumulation, adsorption and reduction etc. therefore microbe-based technologies provide an alternative to the conventional techniques of metal removal or recovery [6]. Considering the metal threat to microbes, plants and consequently to human health and realising the biosorbing ability of microbes, the current study is aimed with isolation of efficient chromium resistant indigenous bacterial isolates that could be significant to be used in bioremediation effectively. For this screening of chromium resistant isolates for maximum tolerance level (MTL) was done and isolates having maximum resistance up to 100mg/L Cr(VI) concentration were selected for further study. Then biochemical characterization of selected bacterial isolates was done. Finally, chromium reduction study was performed by each isolate at optimized pH, temperature, inoculum level and initial chromium concentration values in Nutrient broth. Furthermore the multimetal resistance of the selected isolates was also evaluated as it is very important trait for survival of bacteria in contaminated site.

II. MATERIALS AND METHODS

Sample collection-
Soil samples were collected from different industrial sites in Gwalior, MP, India, by using standard methods [7]. Immediately soil samples were taken to the lab under 4^{o}C for further analysis.

Enrichment-
The enrichment of indigenous bacteria is carried out under aerobic conditions by modified method given by Joutey *et al.*, [8] 1.0 g soil samples were mixed with 50mL of sterile nutrient broth having 25mg/l Cr(VI) concentration and incubated at 37^{o}C at 120 rpm for 24 h using shaker incubator. After 24 h of incubation, 1mL of each suspension was transferred to 50mL of fresh medium amended with 50 mg/L Cr(VI) and finally with 100 mg/L Cr(VI). Residual chromium concentration was determined at regular time intervals from culture supernatant at 540nm spectrophotometrically (Shimadzu UV-1800) by diphenylcarbazide (DPC) method.

Isolation-
For isolation, enrichment culture was serially diluted, plated on nutrient agar medium (10 mg/L of Cr(VI) and incubated for 48 h at 37^{o}C [9]. Colonies were transferred thrice on similar medium and are subjected for further Cr(VI) reduction activity. Following three sequential transfers in increasing concentrations of chromium, the isolates were stocked in nutrient agar slants amended with 10 mg/L of Cr(VI), at 4^{o}C until use.

Screening of bacterial isolates for Maximum Tolerance Level (MTL)-
The ability of the bacterial strains to grow under increasing concentrations of chromium was tested by solid agar plate modified method by Malik and Jaiswal [10]. To determine maximum tolerance, bacterial isolates were aseptically streaked on nutrient agar plates supplemented with 25, 50, 75 and 100mg/L of Cr(VI) concentrations respectively. The highest concentration of metal supporting bacterial growth was defined as the maximum tolerance level (MTL).

Morphological and biochemical characterization of selected bacterial isolates-
For tentative identification bacterial isolates showing the highest MTL values were characterized morphologically and biochemically as per the Bergy's manual of Systematic Bacteriology [11]. Morphological study included- Grams staining, endospore staining and motility test. Colony characteristics were also observed. For biochemical characterization sugar fermentation test, IMVC (Indole, methyl red, voges proskauer and citrate), catalase, oxidase, amylase, urease, gelatinase, bile esculin, growth in 6.5% NaCl and nitrate reduction tests were performed.

Optimization of different parameters-

Bacterial growth is affected by several parameters like pH, temperature, initial Cr(VI) concentration and inoculum level. Chromium reduction is directly proportional to growth of bacteria. So, optimization of above parameters was done with reference of bacterial growth. For all experiments fresh overnight grown bacterial cultures were used as inoculum at 1% inoculum value. For pH, bacterial culture was inoculated into 50mL of nutrient broth (25 mg/L Cr(VI)) having different pH values from 2 to 10 by using dilute HCl or NaOH. The influence of temperature on growth was determined by incubating inoculated broth at different temperatures (30, 35, 40 and 45°C). For determination of optimum initial inoculum level, bacterial cultures at- 1%, 2% and 3% different inoculum levels were inoculated in 50 ml nutrient broth having 25 mg/L of Cr(VI) concentration. To evaluate the effect of the initial Cr(VI) concentration, bacterial culture was inoculated in 50 ml nutrient broth having different initial chromium concentrations of 50, 75, 100 and 125 mg/L. All sets were incubated in shaker incubator at 120rpm for 72h (REMI-CLS24 plus). Bacterial growth was measured in terms of optical density at regular time intervals at 600 nm spectrophotometrically (Shimadzu UV-1800).

Chromium reduction study by selected bacterial isolates-
Cr(VI) reduction study was carried out under aerobic conditions for efficient bacterial isolates at optimized values of pH, temperature, initial inoculum level and initial chromium concentration. Nutrient broth was inoculated with fresh overnight grown cultures of selected bacterial isolates. Cell growth was monitored by measuring the optical density at 600 nm at regular time intervals.

Determination of residual Cr(VI) in culture supernatant was done at 540nm following diphenylcarbazide method.

Determination of multi metal resistance-

Resistance of bacterial isolates against six different heavy metals (CO^{+2}, Hg^{+2}, Pb^{+2}, Cd^{+2}, Zn^{+2} and Mn^{+2}) was determined by inoculating bacterial isolates in nutrient broth having 50 mg/L concentration of different metals along with 25 mg/L concentration of chromium. The nutrient broth amended with Cr(VI) only was used as control. Resistance was determined as presence of bacterial growth and was measured in terms of optical density at regular time intervals at 600 nm spectrophotometrically.

III. RESULTS AND DISCUSSION

A total of 5 soil samples were collected from different metal contaminated sites at Gwalior and processed for pH and temperature determination. Soil sample enrichment was done at three Cr(VI) concentrations 25, 50 and 100 mg/L in nutrient broth at pH 6.8, 37°C and 120 rpm for 72 h in orbital shaker incubator. Uninoculated control flasks were also kept at the same conditions.

From 5 soil samples, 15 bacterial isolates were obtained after serial dilution of enriched samples. Maximum tolerance level (MTL) of bacterial isolates was evaluated by streaking bacterial cultures on nutrient agar plate supplemented with different Cr(VI) concentrations-25, 50, 75 and 100mg/L and visually compared for growth after 24 to 48 h incubation at 37°C. A comparative percentage of bacterial isolates having resistance at different concentrations is presented in the pie chart (Fig.1). Out of 15 bacterial isolates, three isolates S1-B, S10-D and S3-A (8%) had shown a good resistance at 100mg/L chromium concentration and were selected for further study.

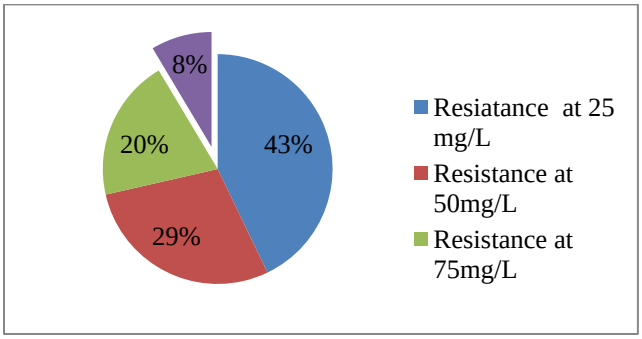


Fig. 1 Percentage of bacterial isolates having resistance at four different Cr(VI) concentrations.

On the basis of biochemical characterization bacterial isolates were identified as *Enterococcus faecalis* (S1-B), *Bacillus megaterium* (S10-D) and *Bacillus cereus* (S3-A). S1-B was positive for specific tests- Bile esculin and growth in 6.5% NaCl (Table.1 and 2).

Table 1 Morphological characteristics of selected bacterial isolates.

Name of Isolates	*Colony characteristics*	*Gram staining*	*Shape*	*Arrangement*	*Endospore*	*Motility*
S1-B	Cream, punctiform, entire margin, moist, opaque and flat,	+ve	Coccus	Cluster	Absent	Motile
S10-D	Cream, medium size, margin fringes, glossy, opaque and flat	+ve	Rod	Streptobacillus	Present	-
S3-A	Cream , small, entire margin, moist, transparent and raised	+ve	Rod	Streptobacillus	Central endospore	Motile

Table 2 Biochemical characterization of selected isolates

Biochemical tests	Bacterial isolates		
	S1-B	S10-D	S3-A
Indole	-	-	-
Methyl red	+	+	-
Voges proskauer	+	-	-
Citrate utilization	-	-	-
Catalase , Oxidase	-	+	+
Amylase, Urease	-	+	+

Gelatinase		-	-	-
Nitrate reduction		-	+	+
Sugar fermentation test-				
Lactose		-	-	-
Glucose		+	+	+
Maltose		+	+	+
Mannitol		+	+	-
Xylose		+	-	-
Arabinose		+	+	+
Triple Sugar Iron	Slant	R/R	R/R	R/R
	H2S	-	-	-
	Gas	-	-	-
Tentative identification		*Enterococcus faecalis*	*Bacillus megaterium*	*Bacillus cereus*

R/R- slant and butt both were red

For efficient chromium reduction optimization of different parameters- pH, temperature, initial inoculum level and initial chromium concentration was performed for selected three bacterial isolates in nutrient broth at 25 mg/L Cr(VI) concentration. Optimum pH values for isolates S1-B, S10-D and S3-A were pH 6.8, 10 and 8 respectively (Fig. 2). In the similar study by Joshi and Modi [12] has been reported that optimum pH for isolates SGPB1, PGNI2, PGCO3, KGCR4, PGMN6, SGZN7 was 7.5 while optimum pH for isolate PGCU5 was 6.5.

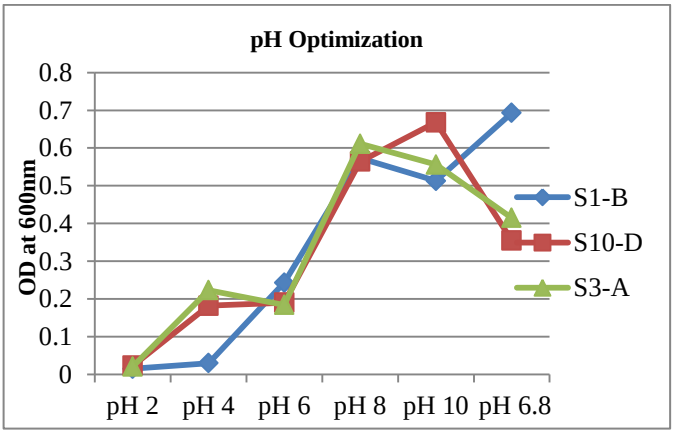


Fig. 2 *Effect of pH on the growth of selected bacterial isolates in nutrient broth with 25mg/L Cr (VI) at 37°C for 72 h*

Optimum temperature values for isolates S1-B and S3-A was 37°C while for S10D it was 45°C (Fig.4). Optimum temperature for bacterial growth is reported 37°C. So, two isolates are supporting this view. In another study by Liu *et al.*, Cr (VI) reduction by the bacterium, XW-4, was evaluated under three different temperatures: 20, 37 and 47°C. Similar result is reported that complete reduction was observed at 37°C after 72 h by *Bacillus sp*. (XW-4) isolated from chromium landfill site [13].

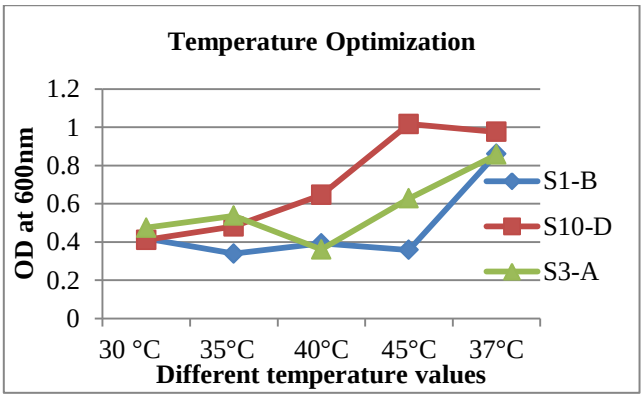


Fig. 3 Effect of temperature on growth of bacterial isolates in nutrient broth with 25 mg/L Cr(VI), pH-6.8 for 72 h

Optimum inoculum values for isolates S1-B, S3-A and S10-D was observed as 2% and 3% respectively (Fig.5). In another study, Biswas *et al.*, had reported that *Alcaligenes faecalis* strain P-2 showed the optimum growth when medium contained 5% of inoculum [14].

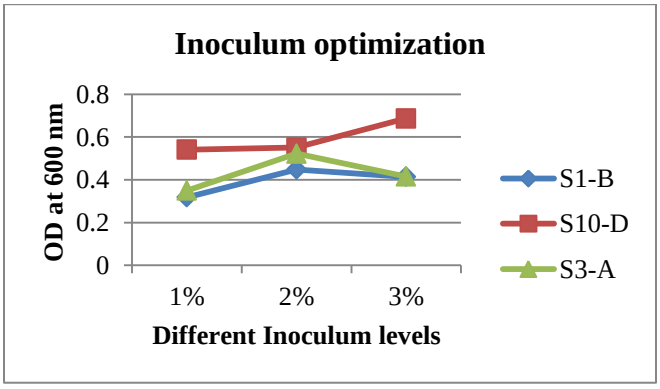
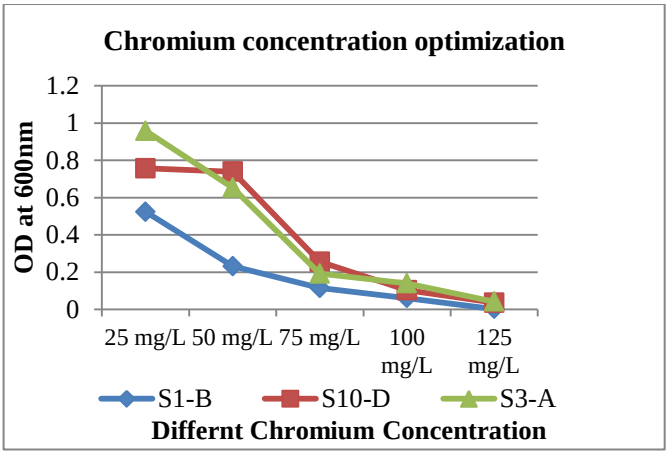


Fig. 4 Effect of inoculum levels on growth of bacterial isolates in nutrient broth with 25mg/L Cr(VI) at 37°C, pH 6.8 for 72 h

As represented in Fig.6, optimum initial chromium concentration value for isolates S1-B, S10-D and S3-A is 25 mg/L. Growth of isolates is decreasing with increase of Cr(VI) concentrations due to toxic effect of chromium but all the three isolates are showing resistance at 100mg/L Cr(VI) concentration. Similarly, Biswas *et al.*, has also reported that there was significant difference in the growth of the isolates at different concentration of chromium.



5 Effect of initial Cr(VI) concentrations on growth of selected bacterial isolates in nutrient broth at 37°C, pH 6.8 for 72 h

Final chromium reduction study was performed at above optimized values. Residual chromium concentration was measured at regular time periods for 120 h by DPC method at 540nm spectrophotometrically. Percentage of chromium reduction by bacterial isolates was determined. As represented in Fig.7, all the three isolates showed a gradual chromium reduction with increasing time intervals. S1-B showed highest chromium reduction (68%), while S10-D (60%) and S3-A showed minimum chromium reduction among three (53.36%) at 25 mg/L Cr (VI) concentration. Similarly, Bakiyaraj *et al.*, has reported that *Bacillus* sp. and *Pseudomonas* sp. removed 87%, 73%, 65% and 60% chromium from medium in 48 hours starting with the initial concentration of 5 ppm/L, 10 ppm/L, 25 ppm/L and 50 ppm/L respectively [15]. Similar work is reported by Basu *et al.*, that *Bacillus subtilis* had shown 97% removal of chromium with an initial concentration of 2.5 mg/L [16]. Dey and Paul in their study revealed that three bacterial isolates, SUK 1201(*Arthrobacter sp.*), SUK 1205 (*Arthrobacter* sp.) and SUK 1207(*Pseudomonas* sp.) were able to reduce more than 50 % and 80% of initial 2 mM Cr (VI) concentration [17].

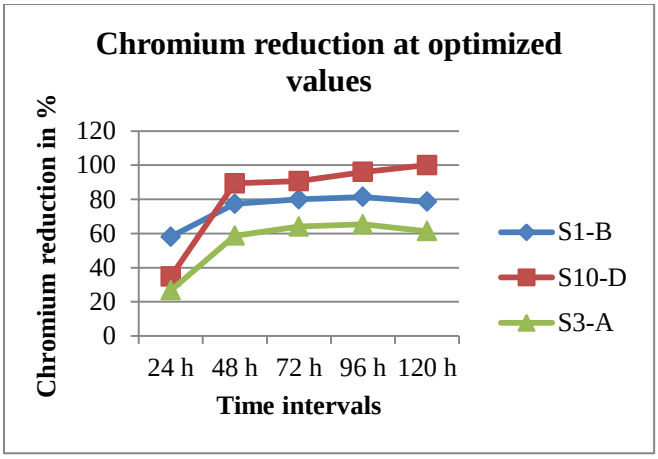


Fig. 6 Percentage reduction of Cr(VI) in nutrient broth at optimized values by bacterial isolates at optimized values for 120 h

Multimetal resistance was determined against six heavy metals and all three isolates showed a different resistance pattern for different heavy metals. S10 D showed the maximum resistance against five metals and S3A, showed minimum against three. Cd^{+2} and Hg^{+2} were highly toxic, all three isolates were inhibited by Cd^{+2} and only S10D was resistant against Hg^{+2}. Cadmium (Cd) is highly toxic, has a long biological half-life and causes the neurological disability in humans and animals by entering in brain parenchyma and neurons. That result in memory shortfalls, low concentration power etc. in humans [18]. Degree of sensitivity towards different heavy metals was as- Cd^{+2}> Hg^{+2}> Zn^{+2}> Co^{+2}> Pb^{+2}>Mn^{+2} (Fig.8). Similarly, U Thacker had also reported that *Brucella sp.* (DQ112027), showed multiple metal resistances to different levels. The degree of inhibition caused by tested metal cations was Hg^{+2} > Co^{+2} > Cr^{+6} > Ni^{+2} > Pb^{+2} > Zn^{+2} [19]. In another study Sultan and Hasnain, have shown that gram positive chromate resistant isolate exhibited tolerance against salts of Ba^{+2}, Cd^{+2}, Cu^{+2}, Fe^{+3}, Mn^{+2}, Ni^{+2} and Pb^{+2} but showed sensitivity to Co^{2+}, Hg^{+2} and Zn^{+2} [20]. It is very important to determine the multimetal resistance as the contaminated site also had other toxic metals and compounds. It is a valuable trait for microbes to withstand in remediation study of the natural contaminated sites. Genes responsible for metal resistance are also associated for antibiotic resistance and are transferred among phylogenetically distant bacterial groups [21].

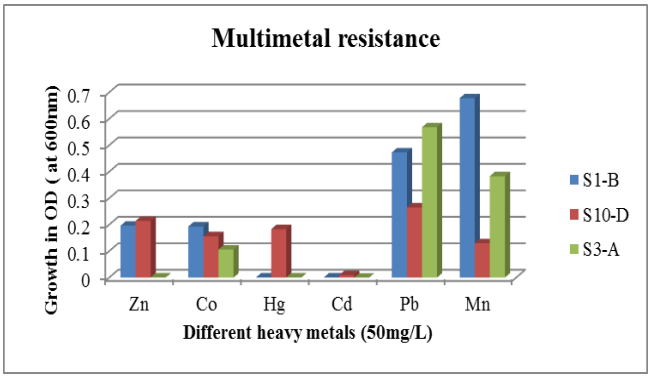


Fig.7 Determination of multimetal resistance by bacterial isolates for different heavy metals at 50mg/L of concentration

IV. CONCLUSION

Heavy metal toxicity is of great concern in the present scenario. To combat this toxicity attributed to these metals, there is an urgent need for an efficient industrial and domestic waste treatment strategy that eliminates priority pollutants at contaminated sites. These highly contaminated industrial sites have become the source of resistant microbes against different toxic heavy metals. The indigenous microorganisms can be used as an indicator of pollution and can be used to minimize the risk of public exposure to chromium contamination. In present research work three potent chromium reducing bacterial isolates with a significant chromium reduction efficiency, *Enterococcus faecalis*- (78.6%), *Bacillus megaterium*- (100%) and *Bacillus cerus*-(61.36%) were isolated at 25mg/L of Cr(VI) concentration and 37ºC. These isolates were found to exhibit multimetal resistance traits also for heavy metals such as Hg, Zn, Co, Pb and Mn, which opens a new path to use these isolates for bioremediation application. These isolates could be of great significance in finding a practical application in detoxification of chromium pollutants at industrial areas and is of great economic importance. Further research work to understand the mechanism of chromate reduction and evaluation of chromium reduction by preparing consortium is in progress.

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