Available online at www.ijpab.com

DOI: http://dx.doi.org/10.18782/2320-7051.6401

ISSN: 2320 – 7051

Int. J. Pure App. Biosci. **6 (2):** 782-792 (2018)

International Journal of Pure & Applied Bioscience

Research Article

OPEN ACCESS

Bacterial Approaches for Reclamination of Chromium (VI) Polluted Soil

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Received: 11.03.2018 | Revised: 16.04.2018 | Accepted: 20.04.2018

ABSTRACT

In recent day's industrialization is growing in very faster rate than any other activities. Due to industrialization, the heavy metals pollution load for soil, water and air has increasing day by day. The environmental pollution of toxic heavy metals is the major problem throughout the world since industrial evolution. Chromium is one of these heavy metals whose concentration in the environment is increasing due to many different industries, including metallurgical, electroplating, production of paints and pigments, tanning, wood preservation, Cr chemicals production, and pulp and paper production. There are two inorganic forms, Cr (VI) and Cr (III), in which chromium exists in nature. Cr (VI) is a serious environmental pollutant. A variety of microorganisms have been known for their ability to degrade these heavy metals. The current study aimed to isolate chromium reducing microorganism from industrial affected soil area of Mandideep, Madhya Pradesh. Among all the species diversity in soil, selected three bacterial isolated strains were named as (SS2, MR4, and MR5). These organisms were identified based on the cultural, morphology and biochemical characteristics and results of SS2 (Pseudomonas fluorescence), MR4 (Bacillus cereus) and MR5 (Bacillus decolorationis).The species were characterized according to criteria given in Bergey's Manual of Determinative Bacteriology[1]. The heavy metals degradation of isolated bacterial strains was analyzed based on the growth of Chromium containing medium. The efficiency of three isolates for chromium reduction was also determined, which shows that SS2 show 73% reduction, followed by MR4 65% reduction and MR5 58% reduction of chromium, degrading ability of isolated strains were analyzed at 48 hours after inoculation from the medium.

Key words: *Heavy metal, Biodegradation, Chromium reduction, Pseudomonas fluorescence, Bacillus cereus and Bacillus decolorationis.*

INTRODUCTION

Pollution of soils with heavy metals is a global problem. Among the heavy metals, Chromium (Cr) is an important metal pollutant that has been contaminating soil, sediment and ground water[2]. Contamination of Cr in soil mainly originates from its use in metallurgy, leather tanneries, dyes, textiles and wood preservation[3].

Cite this article: Ahirwar, N.K., Singh, R. and Gupta, P. K., Bacterial Approaches for Reclamination of Chromium (VI) Polluted Soil, *Int. J. Pure App. Biosci.* **6(2):** 782-792 (2018). doi: http://dx.doi.org/10.18782/2320-7051.6401

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In India alone about 2000 to 3200 tonnes of elemental Cr escape into the environment annually from the tanning industries with a Cr concentration ranging between 2000 and 5000 ppm in the effluent compared to the recommended permissible limit of 2 ppm. Remediation of contaminated soils is essential to mitigate the negative effects caused by heavy metals before they are incorporated into the ecosystems[4].

Heavy metals are often used as a group name for metals and semimetals (metalloids) that have been associated with contamination and potential toxicity or eco-toxicity. Contamination of environmental systems from heavy metals has been an immediate worldwide problem. Heavy metals are among the most persistent properties of pollutants in the soil land and water bodies' ecosystem because of their resistance to decomposition in natural condition[5]. Heavy metal residues in contaminated habitats may accumulate in microorganisms. In the fluvial environments, heavy metals are produced from a variety of natural and anthropogenic sources, such as atmospheric deposition, geological weathering, agricultural activities, and residential and industrial products[6].

Contamination of the environment by Cr, especially hexavalent Cr, has become a major area of concern. Chromium is used on a large scale in many different industries, including metallurgical, electroplating, production of paints and pigments, tanning, wood preservation, Cr chemicals production, and pulp and paper production[7]. The tanning industry is an especially large contributor of Cr pollution to water resources. The leather industry is the major cause for the high influx of Cr to the biosphere, accounting for 40% of the total industrial use[8]. Cr toxicity is of significant concern due to carcinogenic, mutagenic and teratogenic effects and tissue damage. The valency state of Cr ranges from −2 to +6, with the trivalent Cr (III) and hexa valent Cr (VI) states being stable and differ in certain properties and toxicity levels. Cr (VI) is classified as a priority pollutant and is listed as Class A human carcinogen by the US Environmental Protection Agency [9].

Toxic heavy metals cannot be recovered from the environment, and are remain ultimately indestructible. Chromium is one of the most toxic heavy metals whose concentration in the environment is still increasing and deteriorate the environment. Cr (VI) in water and soil environment poses pressure on micro flora. In contaminated soils, the availability of chromium is affected by several processes such as organic and inorganic complexes formation, precipitation ⁄ dissolution, oxidation ⁄ reduction or adsorption ⁄ desorption[10]. In soil these processes are affected by the microbial activities[11], however higher chromium levels in soil are toxic enough to decrease microbial biomass, diversity and there activities[12].

The effect of heavy metal toxicity depends on soil factors such as organic matter and clay content, pH and divalent cation concentrations (cation exchange capacity) [13]. These factors influence complex formation and the immobilization of heavy metals. However, the relative toxicities of different metals, namely Cd, Cu, Zn, and Pb, appear to be the same irrespective of soil type[14]. In soil contaminated for 40 years with high concentrations of Cr and Pb, the microbial biomass and activity were reduced and soil organic carbon had accumulated. These results indicated that Pb exerted a greater stress on soil microbes than Cr.

Soil microorganisms vary widely in their tolerance to heavy metal contamination, and the proportion of culturable resistant microorganisms can range from 10% to nearly 100%. The activities of enzymes in soil may serve as indicators of heavy metal contamination, as there are generally high correlations between reduced enzyme activities (of, e.g., dehydrogenases, acid phosphatases and ureases) and increased heavy metal contamination[14]. It has been reported that heavy metal contamination has different effects on soil bacteria and fungi [15].

The conventional processes are usually ineffective and highly expensive from economical view point[16]. It is therefore, necessary to introduce a low-cost, innovative and eco-friendly method for the removal of

toxic heavy metals from soil and water bodies[17]. Some bacteria producing plant growth activity like production of indole acetic acid, phosphate solublization, siderophores etc are capable of stimulating plant growth and phytoremediation of heavy metal contaminated soil [18-19]. Remediation of Cr through the plants and microorganisms was also advocated[20 -24]. Some indigenous bacterial species are found highly resistant to Cr (VI) in chromium contaminated sites[25] and also been identified to reduce toxic and soluble Cr (VI) to less toxic and less soluble form Cr (III), e.g. *Bacillus* sp, *Pseudomonas* sp, *Arthrobacter*, *and Ochrobactrum*, *Serratiamarcescens*, *Desulfovibrio vulgaris* and *Cellulomonas* sp. The potential use of these species for degradation purposes is much cheaper and ecofriendly in comparison with conventional degradation methods[26]. The present study was designed to explore the indigenous Cr resistant bacterial species from found in industrial affected area of Mandideep Madhya Pradesh.

MATERIAL AND METHODS

Study area

Mandideep is the largest industrial area and rapid growing city in Madhya Pradesh (India), situated about 27 km away from Bhopal. Mandideep is located between N 23°04' longitude and E 077°31' latitude, elevation 1496 meter above sea level (MASL). The average rainfall of Mandideep is 950mm. It has both large and small scale industries. Large number reputed companies are functioning in Mandideep **(Figure-1)**.

Fig. 1: Industrial area of mandideep (source-google earth map)

Soil Collection

In the present study the soil samples were first collected randomly from industrial contaminated soil areas nearby Pharmaceutical industries, Tractor manufacturing industry, Food & beverage industries, and Leather industry of Mandideep, district Raisen of Madhya Pradesh, India. Soil collected in sterilized polythene bags by proper marking and immediately bought to the laboratory for microbiological analysis.

Isolation and purification of bacteria: The microorganisms were isolated by serial dilution technique on nutrient agar media. In

this technique, a sample suspension was prepared by adding 1.0g sample to 9 ml distilled water and mixed well for 10 min and vortexed. Each suspension was serially diluted 10^{-1} to 10^{-6}. 0.1 ml was pipetted onto plates with nutrient agar media, spread with a glass spreader and incubated at 30°C for 24 hours for bacterial observation. The soil sample was streaked on the nutrient agar plates with further incubation at 30ºC for 24 hours. Each colony that appeared on the plate was considered as one colony forming unit (cfu) [27-28]. The pure culture was grown on nutrient agar medium and transferred to Mac-conkey agar medium, EMB agar medium and mannitol salt agar medium for differentiate and identified bacteria. The plates were incubated at 30°C in the incubator and readings were taken 24 h after inoculation. The bacterial isolates were indentified through microscopic analysis and biochemically characterized by oxidase test, catalase test, citrate test, urease test, indole production test and methyl red voges- proskauer test (IMVIC) [29,30,31].

Microscopic analysis of bacterial strains isolated from soil sample: The isolated bacteria were subjected to microscopic examination using Gram Staining[32], where the smear was prepared with a loop full of isolated bacterial culture via spreading over a clean slide in a drop of sterile normal saline and is allowed to dry in air. The smear is heat fixed by passing over the flame, brought to 25° ± 1°C and stained with crystal violet solution for one minute followed by rinsing with water and is allowed to air dry. The slide is then poured with Gram's iodine solution (Mordant) for another one minute, drained and decolorized with alcohol. Again rinsed with water and allowed to air dry. The smear thus obtained was stained with counter stain for two min., rinse with water, and allow to air dry and observed under the compound microscope. First with 40X then with 100X under oil immersion, gram-negative cell appears pink-red in colour and gram positive as violet.

Biochemical characterization of bacterial strains isolated from sludge sample: After Gram Staining Technique Biochemical tests were carried out for characterization purpose including, Motility test, Oxidase test, Catalase test, Urease test, Indole production test, H_2S production test Methyl red test and Voges-Proskauer.

Oxidase test- The test indicates the presence of cytochrome oxidase which catalyses the oxidation of reduced cytochrome by oxygen. It indicates the ability of microbes to oxidize amine. A freshly prepared 1% solution of oxidase reagent was soaked on a piece of filter paper and with a sterile loop the test organisms were smeared on the area impregnated with the oxidase reagent. Deep purple coloration after a few seconds shows a positive test. While other species remained colorless, indicated that they were Oxidase negative[3].

Motility test- This test was used to determine the presence or absence of Flagella. The motility medium in tubes were inoculated by making a fine stab of the isolates with an inoculating needle to the depth of about 1–2 cm short of the tubes bottom and were incubated for 24 hours at 30^0C. Motile organisms grow outside the line of stabbing, while the non-motile organisms grow on the line of stabbing.

Catalase test: Slide (drop) method was used. A drop of 30% H_2O_2 was placed on a glass slide using a wire loop, a little inoculums was removed and mixed with the H_2O_2 on the slide. A positive test was indicated by bubbling; the enzyme present on catalase positive organisms degrades hydrogen peroxide and releases O_2 which is detected as effervescence. The reason of bubble formation is that; Catalase enzyme present in bacteria causes the breakdown of hydrogen peroxide (H_2O_2) into water and oxygen[34].

$$2H_2O_2 + \text{Catalase} \longrightarrow 2H_2O + O_2$$

Citrate test: This test is used to study the ability of an organism to utilize Citrate present in Simmon's media as a sole source of carbon for growth. In order to identify an organism that is able to use citrate as carbon source, the test organisms were inoculated into Simmon citrate agar slant and incubated for 24–72 hours. The development of a blue colour

indicates a positive test. The reason of blue color production is that; citrate is a Krebs cycle intermediate generated by many bacteria[35] as described below.

Citrate = oxaloacetate + acetate

Oxaloacetate = pyruvate + CO_2

Urease test: The slants of Christian urea agar (0.1g peptone, 0.5g sodium chloride, 0.2g di-calcium hydrogen phosphate, and 600µl phenol red in 100mL distilled water, pH adjusted to 6.8-6.9 and then 2g agar was added and medium was autoclaved). After autoclaving, 1 mL filter sterilized 10% glucose and 10mL filter sterilized 20% urea added to the medium. It was readily poured into sterilized test tubes to make slants which were streaked with the bacterial isolates and incubated at 30^0C overnight (Benson, 2007). Appearance of pink color showed positive test, otherwise the test is negative. The pink color indicated the production of ammonia which has caused the pH of the solution to rise to about 8.2. This increase in pH is responsible for color change[36];

$(NH_2)2CO + H_2O \longrightarrow CO_2 + 2NH_3$

Pink color production in the solution confirmed that isolated species belong to genus *Pseudomonas*[37].

Indole production test: The test medium was prepared by dissolving 2g peptone and 0.5g NaCl in 100 mL distilled water, pH adjusted at 7.4. The medium was poured in test tubes and autoclaved at 121^0C, 15 psi for 20 minutes. The bacterial isolate was inoculated in the medium and incubated at 30^0C for overnight. Next day, 500µl of Kovac's reagent (isomyl alcohol 150 mL, p-dimethyl-aminobenzylaldehyde 10g, conc. HCl 50 mL) was added and shaken gently. The development of red color in the upper layer showed the positive test. The culture layer in the test tube became cloudy and appeared yellow in color indicated that cultures are indole negative and did not contained tryptophanase enzyme in them. So, it was confirmed that isolated species belong to genus *Pseudomonas*[38].

Methyl Red Test: The medium was prepared by dissolving 5g peptone and 5g K_2HPO_4 in 1L distilled water, pH adjusted at 7.6 and poured in test tubes. The medium was sterilized by autoclaving at 121^0C, 15 psi for 20 minutes. Then 0.25 mL glucose solution was added in 5 mL medium in each tube. The above media were inoculated with respective isolates and incubated at 30^0C for 48 hours. Then 5 drops of methyl red (0.1g methyl red and 300 mL ethanol in 100 mL distilled water) were added and mixed. The bright red color indicated the positive test, otherwise the test is negative[39]. The reason is that, after incubation when methyl red was added in the medium, change in pH was occurred. Methyl red is used as a pH indicator. The bacteria in test tube 2 showed the presence of the extreme acidity in acid fermentation[40].

Voges-Proskauer (V.P.) Test: The medium for V. P. test was prepared by dissolving 0.5g peptone, 0.5g dipotassium hydrogen phosphate in 100 mL distilled water, pH adjusted at 7.6 and then 0.5g of dextrose was added. The 5 mL medium was added to a test tube and autoclaved at 121^0C, 15 psi for 20 minutes. The medium was inoculated with respective bacterial isolate and incubated at 30^0C for overnight. Next day, 1 mL 40% KOH and 3 mL 5% -naphthol were added. The tubes were aerated for 30 minutes and the appearance of red colour indicates positive test. Voges proskauer test was performed to determine whether the selected bacterial cultures produce 2, 3-butanediol. No change in color was detected in all three of the test tubes indicated that both bacterial cultures were VP negative[41].

Hydrogen sulfide production test: The medium for H_2S production test was prepared by dissolving 3g lab lameco powder, 3g yeast extract, 20g peptone , 5g NaCl, 10g dextrose, 0.3g ferric citrate , 0.3g sodium thiosulphate, 0.05g phenol red in 1L distilled water, pH adjusted between 7.2-7.6, followed by addition of 12g agar. This medium (6ml) was taken in each test tube, autoclaved at 121^0C, 15 psi for 20 minutes and slanted. The bacterial isolates were streaked on these slants and incubated overnight at optimum temperature of the isolate. Blackening of medium indicated H_2S production by the isolate. After incubation no

black color was produced in each test tube indicated that bacteria did not have ability to cause reduction of sulfur containing compounds into sulfides i.e. they are H_2S negative and belong to genus *Pseudomonas*[42].

Screening of Cr (VI) degradation/ reducing activity of bacterial isolates-

Different concentrations of chromium prepared by dissolving required amount of potassium chromate $K_2Cr_2O_7$ (Qualigens), metal ion concentrations tested range from control, 20, 40, 60, 80 and100mg/L concentrations were selected for the experiment. Standard nutrient broth was prepared and autoclaved (jyoti scientific laboratory) at 121°C for 15 minutes and was cooled in a water bath. In 250 ml Erlenmeyer flasks, 100 ml nutrient broth was taken along with the above mentioned concentration of chromium (VI). A separate media with only Nutrient agar agar media was inoculated with bacteria served as control. Here a positive control media without bacterial inoculum and only Cr (VI) was also used. Under aseptic conditions, the three chosen bacterial isolates were inoculated individually into these flasks with 0.1ml cells. The flasks were incubated at shaking incubator 150rpm (Rotation/minute) at 30°C temperatures. Uninoculated control flasks were also maintained in the same manner. After 48 hours, samples were taken from each flask and centrifuged (sigma laboratory 3K30) at 10,000 rpm for 15 minutes. The supernatants were analyzed with Atomic Absorbance Spectrophotometer for chromium concentration adopting standard methods. Percentage reduction in chromium concentration was calculated for each chromium concentration based on the initial and final readings. The bacterial growth was determined after every 4 hours for the duration of 48 hours, through determination of the optical density at 600nm by UV visible spectrophotometer (U.V.-1601-SHIMADZU). Cr (VI) in the culture supernatant was measured by Atomic Absorption Spectrophotometer [43] (Perkin Elmer).

RESULTS

Isolation and purification of heavy metal resistant bacteria:

In the present study we identify and characterize heavy metals resistant bacteria isolated from industrial affected soil. 108 colonies were screened from initial level of heavy metal supplemented nutrient agar medium. 20 isolates were selected in the secondary screening from soil. Finally three strains were selected based on high degree of heavy metals resistances were used for further studies. The strains *Pseudomonas fluorescence* (SS2) was Gram-negative, rod shaped motile and *Bacillus cereus* (MR4), *Bacillus decolorationis* (MR5) was Gram-positive, rod shaped motile bacteria. The bacterial isolates showed optimum growth at 30°C and pH 7.0.

Biochemical characterization of isolated bacterial strains:

The isolated bacterial species were identified following the Bergey's Manual of Determinative Bacteriology. The identification criteria included the growth features (colony, shape and color), growth conditions (optimum temperature and suitable growth media), morphology of the cells (shape), physiological characteristics (motility, flagella and Gram reaction), production of enzymes (oxidase and catalase), and utilization of different carbon sources. Results of Morphological and Biochemical tests are listed in **Table-1 and 2**.

Table 1: Morphological characterization of isolated bacterial species

S.No.	Morphological characteristics of Isolated bacterial species			
		SS2 (*P. fluorescence*)	**MR4 (*B. cereus*)**	**MR5 (*B. decolorationis*)**
1.	Colour of the colony	White	White	White
2.	Shape of the cell	Rod	Rod	Rod
3.	Gram Staining	Negative	Positive	Positive
4.	Motility	Motile	Motile	Motile

Table 2: Biochemical characterization of isolated bacterial species

S.No.	Biochemical characteristics of Isolated bacterial species			
		SS2	MR4	MR5
1.	Oxidase test	Positive	Positive	Negative
2.	Citrate test	Negative	Positive	Positive
3.	Catalase test	Positive	Positive	Positive
4.	Urease test	Negative	Variable	Negative
5.	Indole production test	Negative	Negative	Negative
6.	H_2S production test	Negative	Negative	Negative
7.	Methyl red test	Negative	Negative	Negative
8.	Voges-Proskauer	Negative	Negative	Positive

SS2= (*Pseudomonas fluorescence*), MR4= (*Bacillus cereus*), MR5= (*Bacillus decolorationis*)

Determination of Cr (VI) reduction activity of selective bacterial isolates*:*

The reduction of Cr (VI) was measured after 48 hours in the supernatant solution obtained after centrifugation, by using Atomic absorption spectrophotometer. The bacterial reduction of Cr (VI) curve was plotted for each Cr (VI) concentration (20, 40, 60, 80 and 100mg/L). The rate of Cr (VI) reduction increased with decreasing concentrations of Cr (VI). It was found that the reduction of Cr (VI) activity of the bacteria was dependent on the growth. The bacteria growth was determined by the initial lag phase as well as second exponential phase and stationary phase with the final death phase. It was found from the results that bacterial lag phase increased with the increase in Cr (VI) concentration. The reason for initial rapid Cr (VI) degradation rate was that; in the beginning there was high concentration of Cr (VI) in the medium which increased the Cr (VI) degradation. *P. fluorescence, B. cereus* and *B. decolorationis* were inoculated into nutrient broth containing chromium at varying concentrations (control, 20, 40, 60, 80 and 100 ppm). The bacterial strain *P. fluorescence*, were found maximum removed 73%, 69%, 65%, 60%, 48% of chromium from medium in 48 hours starting with the initial concentration of 20 ppm, 40 ppm, 60 ppm, 80 ppm and 100 ppm respectively. Followed by *Bacillus cereus* were found maximum removed 65%, 61%, 54% 46%, and 40 % of chromium and *Bacillus decolorationis* were found maximum removed 58%, 51%, 44%, 38%, and 31 % of chromium. *Bacillus decolorationis* were showed low level of chromium degradation (**Table-3**).

Percentage removal of chromium was similar observation reported 97% removal of chromium *Bacillus subtilis* starting with an initial concentration of 2.5 mg/L. The strain was isolated from wetlands[44]. Results of the present study for chromium removal indicate that the isolates could tolerate to chromium. Percentage reduction in chromium concentration was calculated for each chromium concentration based on the initial and final readings. This is due to the fact that as the volume of inoculum was constant relatively less biomass was available for chromium removal from the media, in case of higher concentrations.

Table 3: Degradation of Chromium ability by isolated bacterial strains

S.No.	Selective Bacterial Isolates	Initial Cr VI Concentration in ppm	Final Cr VI Concentration in ppm	% of Cr degradation
1.	SS2 (*P. fluorescence*)	20	5.4	73
		40	14	69
		60	21	65
		80	32	60
		100	49	51
2.	MR4*(B. cereus)*	20	7	65
		40	15.6	61
		60	27.6	54
		80	43.2	46
		100	60	40
3.	MR5 (*B.decolorationis*)	20	8.4	58
		40	19.6	51
		60	33.6	44
		80	49.6	38
		100	69	31

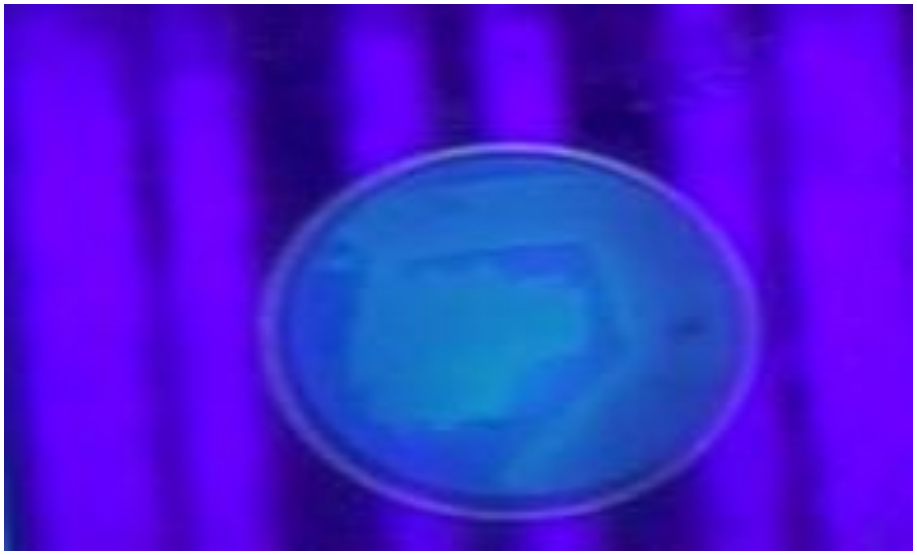

Fig. 2: *Pseudomonas fluorescence* strain (SS2) on Nutrient agar plates

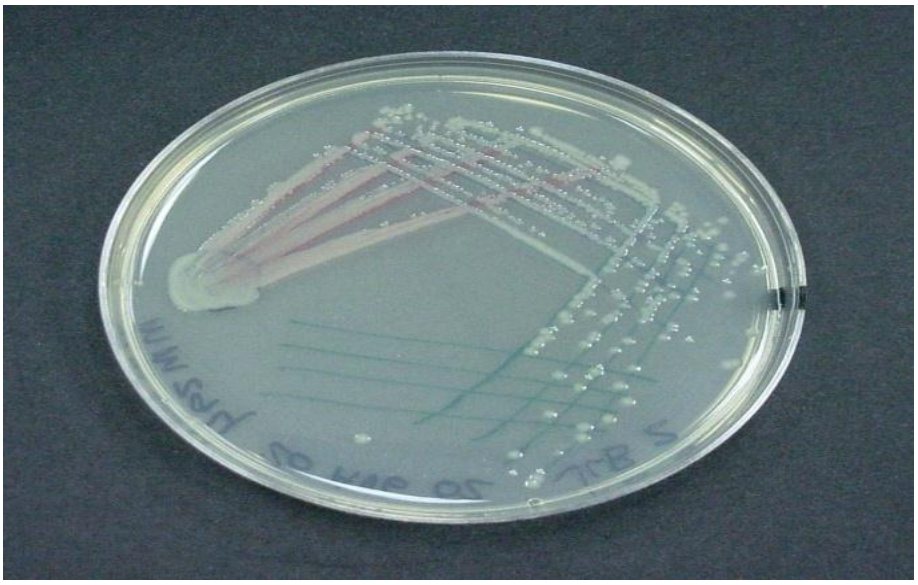

Fig. 3: *Bacillus Cereus* strain (MR4) on Nutrient agar plates

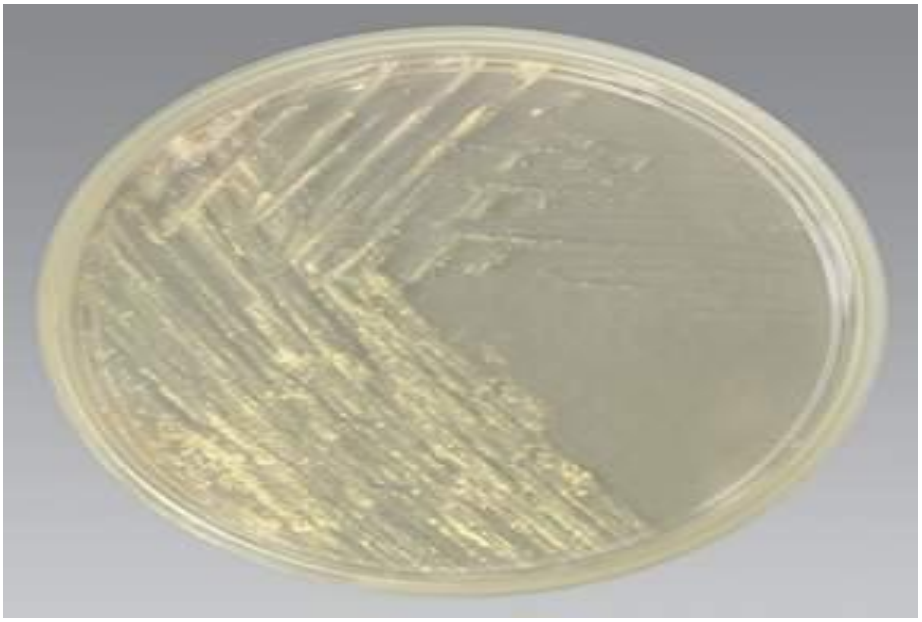

Fig. 4: *Bacillus decolorationis* strain (MR5) on Nutrient agar plates

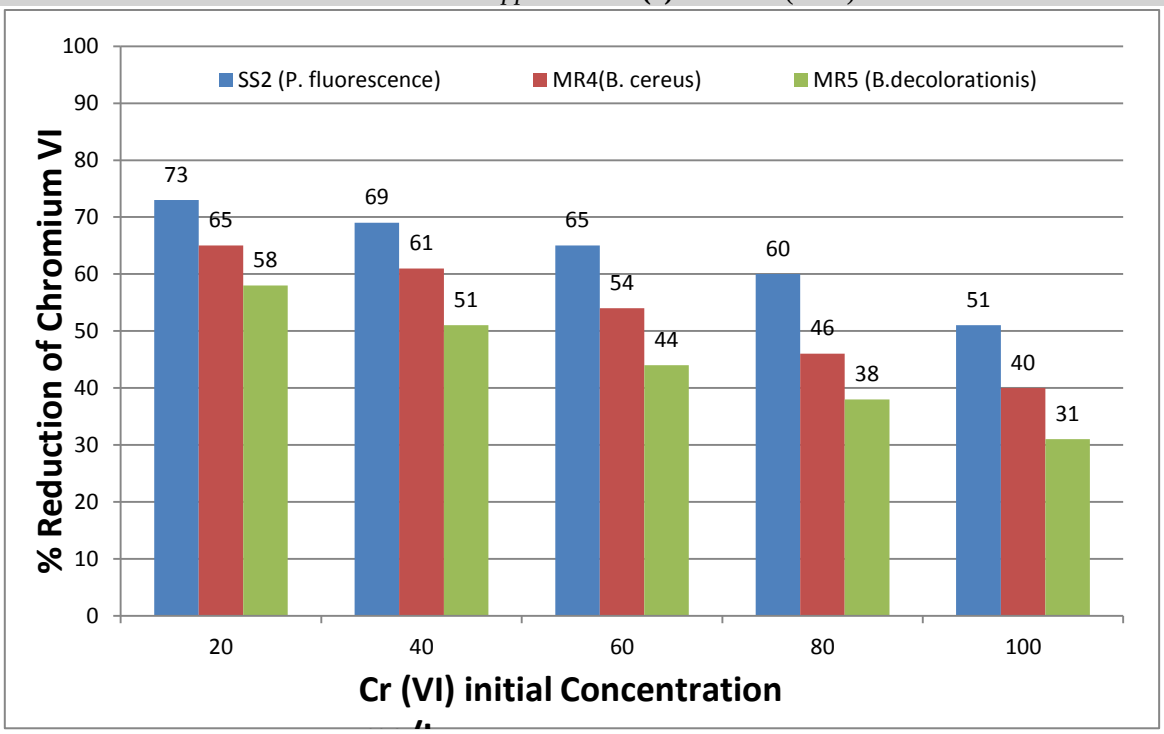

Fig. 5: Percentage reduction of Cr (VI) concentration by SS2, MR4 and MR5 isolated bacterial strain

CONCLUSION

Our result clearly demonstrated that Cr was highly toxic to microorganisms. The present study concluded that indigenous bacterial species from Mandideep industrial soil have their naturally existing machinery to degrade pollutants (chromium), which is cost effective as compared to conventional methods. Three different bacterial species were isolated from soil and identified that they have high degrading ability for Cr (VI) and have significant potential to degrade the toxic Hexavalent (VI) chromium into less toxic Trivalent (III) chromium. The species were biochemical characterized and results revealed close resemblance with *Pseudomonas fluorescence*, *Bacillus cereus* and *Bacillus decolorationis*. The degradation of chromium was calculated by the growth of these three bacterial isolates in the culture medium amended with chromium salt. It was observed that the identified bacterial species can efficiently degrade Cr (VI) up to 73%, 65% and 54% respectively in 48 hours at the temperature of 30ºC. Therefore, it was concluded that, selected bacterial isolates could be effectively recommended for the remediation of soils contaminated with Cr if the level of Cr is up to 100 ppm soil. These findings are potentially useful because the species can possibly be harnessed to detoxify chromium contamination sites.

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