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RESEARCH ARTICLE

ISOLATION AND IDENTIFICATION OF BIOSURFACTANT PRODUCING CHROMATE RESISTANT BACTERIA ISOLATED FROM CHROMIUM CONTAMINATED SITE.

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Abstract

The occurrence of chromate tolerant and biosurfactant producing bacteria with an ability to bioremediate Cr(VI) through bioreduction was investigated in the soil of waste dumping site of battery manufacturing industry. Two bacterial strains TB9 and TB10 showed high Cr(VI) MIC values of 1350 and 1400 $\mu\text{g/ml}$, respectively, and also reduced 83% and 90.0% of chromate after 20 h of growth. Interestingly, both the strains were capable to produce biosurfactant in significant amount which was confirmed by methylene blue plate assay, blood hemolysis test, emulsification test and oil displacement activity. The TB9 strain showed highest emulsification index (E_{24}) after 24 hours of incubation for sunflower oil (74 ± 1.60) followed by kerosene (72 ± 0.5) and olive oil (65 ± 0.3), whereas, strain TB10 showed highest E_{24} value for olive oil (74 ± 1.2) followed by sunflower oil (70 ± 0.29) and kerosene (68 ± 0.19) after the same period of exposure. Results for oil spreading test of the kerosene oil revealed that TB9 and TB10 caused the spreading of the oil at the rate of 6 and 5 minutes with a diameter of 2.7 cm and 3.3 cm, respectively. Based on the results of concomitant biosurfactant production efficiency and chromate reduction potential, strains TB9 and TB10 seems to be most efficient strains that can be potentially be used for Cr(VI) bioremediation and were identified as *Pseudomonas aeruginosa* and *Bacillus cereus*, respectively.

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Introduction:-

The discharge of chromium in the environment through the industrial wastes has become a subject of great concern due to its higher toxicity and non-biodegradable nature. The waste discharged from the battery manufacturing industries contains various chemicals including chromium sulfate in large amount which is ultimately released into the cultivable lands and nearby water bodies beyond the permissible limit (Rehman *et al.*, 2008). The predominant forms of chromium present in environment are the trivalent [Cr(III)] and hexavalent chromium [Cr(VI), chromate]. However, Cr(III) is immobile in nature and acts as a micronutrient in trace amounts, whereas Cr(VI) is highly soluble thus present in large amount and increases the environmental pollution load (Kimbrough *et al.*, 1999). Chromate cause cellular toxicity due to its carcinogenic, mutagenic and teratogenic nature, thus listed as priority pollutant by Environmental Protection Agency (EPA) and World Health Organization (WHO) (Nariagu and Pacyna, 1989; USEPA, 1998). The recommended acceptable limit for Cr(VI) in potable water and household usage should be less than 50 $\mu\text{g/l}$ (EPA, 2000). Exposure to chromium may cause dermatitis, skin ulcer, mental disorders, lung cancer, kidney and liver damage in humans and animals (Cohen *et al.*, 1993). Presence of Cr(VI) and other metals in

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the soil also effects plant growth due to inhibition of photosynthesis, reduced shoot and root length, decrease in enzyme activity, seed germination rate and total protein content (Narain *et al.*, 2012; Verma and Maurya, 2014). Strategies aimed to detoxify chromate contaminated environment by appropriate methods are therefore urgent.

Several treatment methods such as chemical precipitation, ion exchange, adsorption, chemical oxidation, etc. have been earlier used for the treatment of metal contaminated waste. However, these treatments are very expensive, require large amount of chemical and energy, produce secondary waste into the environment and affect soil texture (Szpyrkowicz *et al.*, 2001; Verma and Singh, 2013). Bacteria show potential approach in this regard as they could adapt and inhabit such polluted environments and remediate the toxic metals by converting them into less toxic and easily disposable forms.

Several bacteria have been reported to bioremediate Cr(VI) by bioreduction, bioaccumulation, biosorption, enzyme catalysed transformations, use of immobilized cells and enzymes and application of biosurfactants (Srinath *et al.*, 2002; Verma *et al.*, 2009; Panda and Sarkar 2012; Singh *et al.*, 2013; Vijayanand and Divyashree, 2015). However, the simple metabolic activity alone is not efficient for Cr(VI) remediation especially at highly polluted sites. Thus, use of bacterial detoxification activity along with the bacterial biosurfactants is a promising strategy for Cr(VI) detoxification at higher rate from polluted soil/water. It would increase the efficiency of metal sequestration and thus economically viable and environmentally compatible (AmeerBasha, and Rajaganesh, 2014; Adamu *et al.*, 2015; Govarthanam *et al.*, 2017).

Biosurfactants are surface active compounds that are produced by bacteria, yeasts and fungi either extracellularly or as part of the cell membrane (Juwarkar *et al.*, 2008). They are non-toxic and possess high surface activity and emulsifying activity. Biosurfactants are able to reduce surface and interfacial tension at the interfaces between liquids, solids, and gases, thereby allowing them to mix or disperse readily as emulsions in water or other liquids, thus, can be safely used for heavy metal bioremediation. Hence, the present work was focused to isolate chromate resistant indigenous bacteria from chromium contaminated industrial waste and to investigate its biosurfactant production efficacy in view of Cr(VI) bioremediation.

Methodology:-

Media and Chemicals:-

The media components and reagents used in this study were of analytical grade and procured from Merck, Hi-Media, Qualigens, India Ltd. and Sigma Aldrich chemicals, USA. For the determination of hexavalent chromium, diphenylcarbazide (DPC) solution (0.25%, w/v) was prepared by dissolving 125 mg 1,5- diphenylcarbazide in to 25 ml of high performance liquid chromatography (HPLC) grade acetone and stored in brown glass bottle.

Sampling and isolation of chromate resistant biosurfactant producing bacteria:-

The soil sample was collected from the dumping site of battery manufacturing High Flow Industry Private Limited, Faizabad, India in sterile containers, transported on ice to the laboratory and was processed for bacterial isolation within 4-6 h of collection. One gram of soil was serially diluted into saline (0.6% NaCl) up to 10^{-7} dilution and bacteria were isolated by the standard pour plate technique on nutrient agar plates (pH 7.5) amended with varying chromate concentration (100-500 $\mu\text{g/ml}$) (APHA, 1998). The plates were incubated at $30 \pm 1^\circ\text{C}$ for 24-36 h. Morphologically different bacterial colonies were selected and purified by repeated streaking on selective agar plates to obtain pure isolates.

Analysis of heavy metals in soil sample:-

The heavy metal concentration present in the chromium contaminated soil was estimated using AAS, after acid digestion of samples with a mixture of concentrated nitric acid:perchloric acid (6:1, v/v) (APHA, 1998). AAS grade metal solutions (Sigma Aldrich Chemicals, USA) were used as standards.

Minimal inhibitory concentration of Cr(VI):-

The minimal inhibitory concentration (MIC) of chromate was determined by the agar dilution method (Verma *et al.*, 2001). The log phase cultures of the bacterial isolates were inoculated aseptically on nutrient agar plates supplemented with different concentrations of Cr(VI) (250–1450 $\mu\text{g/ml}$). Plates were incubated for 24–36 h at $30 \pm 1^\circ\text{C}$. Minimum concentration of Cr(VI) that completely inhibited growth of bacteria was considered the MIC of that isolate.

Cr(VI) reduction Assay:-

The five selected bacterial strains were evaluated for their chromate detoxification ability by reduction of Cr(VI) to less toxic Cr(III) at the respective maximum chromate tolerance concentrations. Chromate concentration was determined in the supernatant fraction of overnight grown cultures by diphenylcarbazide (DPC) method at 540 nm using UV-Vis spectrophotometer by measuring absorbance of the purple complex of Cr(VI) with 1,5-diphenylcarbazide after 6, 12, 18, 24, 28 and 32 h of growth (APHA 1998), and the Cr(VI) concentration was determined by the standard curve of $K_2Cr_2O_7$ (100–1500 mg/l) (Maurya and Verma, 2014). Uninoculated Cr(VI) amended broth served as control. The initial (0 h) and final (after incubation) Cr(VI) concentration was determined by the DPC method and the Cr(VI) reduction efficiency of bacteria is determined in terms of “% Cr(VI) reduction”. Total Cr i.e., Cr(VI) + Cr(III), in the supernatant was determined by atomic absorption spectrophotometer (AAS) at 357.9 nm, after digesting the supernatant with the mixture of nitric acid and perchloric acid (6:1, v/v).

Screening of bacteria for biosurfactant production:-

Five bacterial isolates exhibiting high MIC values for Cr(VI) and reducing it to less toxic Cr(III) were selected to evaluate their biosurfactant production efficiency by following methods:

Methylene Blue Plate Assay:-

The selected bacteria were screened for biosurfactant production by streaking on minimal salt medium plates amended with respective chromate concentrations. The medium was supplemented with 200 µg/ml cetyl-trimethyl-ammonium bromide (CTAB; Himedia), 5 µg/ml methylene blue, and 1.5% agar agar (Satpute *et al.*, 2008). The inoculated plates were incubated at $30 \pm 1^\circ C$ for 24-36 h. The ability of bacteria to form dark blue halo zone around the culture was considered positive for biosurfactant production.

Blood hemolysis test:-

Overnight grown bacteria were streaked on blood agar plates followed by incubation at $30 \pm 1^\circ C$ for 24 h and the haemolysis was measured (Satpute *et al.*, 2008). Presence of clear zone (hemolytic activity) around the bacterial colonies indicated the production of biosurfactant by the test strain. The zone appears due to the lyses of red blood cells by the biosurfactant produced.

Emulsification test:-

This test was performed to measure the stability of biosurfactant and its emulsification ability towards hydrocarbons (sunflower oil, olive oil and kerosene) (Paraszkievicz *et al.*, 1992). Bacterial colonies were grown in test tubes containing 2 ml of nutrient broth and incubated for 24 h. Thereafter, 2 ml hydrocarbon oil was added to each tube and it was then vortexed at high speed for 2 mins, left to stand for 24 h and the height of the stable emulsion layer (mm) is measured. The emulsification index (E_{24}) was calculated as the percentage of the height of the emulsion layer developed (mm) divided by the total height of liquid medium (mm).

$$E_{24} = \frac{H_{\text{emulsion}}}{H_{\text{total}}} \times 100$$

where,

E_{24} is emulsion index after 24 hours,

H_{emulsion} is the height of emulsion layer developed,

H_{total} is the total height of the liquid medium

Oil spreading technique:-

The oil spreading method was followed according to Morikawa *et al.* (1993). Twenty milliliters of distilled water was taken in a Petri dish and 0.5 ml of kerosene oil was added onto the distilled water surface. Then one drop of the culture supernatant was added on the centre of kerosene oil surface. The biosurfactant producing bacteria displaced the oil and spread in the water. The diameter of the clear zone visualized on the oil surface was measured after 25-40 seconds, which correlates to the surface tension reduction efficiency of the biosurfactant. It is also called as oil displacement activity.

Identification of the selected bacteria:-

Among the five bacterial isolates, TB9 and TB10 was the most promising isolate in terms of concomitant biosurfactant production and Cr(VI) reduction efficiency and was identified following the scheme of Cowan and

Steel (1993). The various biochemical tests were performed and results were interpreted according to Bergey's Manual of Determinative Bacteriology (Holt *et al.*, 1994).

Results and Discussion:-

Isolation of bacteria and Cr(VI) MIC Determination:-

A total of eleven morphologically different bacteria tolerant to varying Cr(VI) concentration between 250-500 µg/ml were isolated from chromium contaminated soil of battery manufacturing industry. The MIC of Cr(VI) for these strains was evaluated in order to determine the maximum tolerance limit for hexavalent chromium. The Cr(VI) MIC of these strains ranged between 500–1400 µg/ml (Fig. 1). Among them, on the basis of higher MIC values, five bacterial strains (TB1, TB5, TB9, TB10 and TB11) were selected for further studies. Their MIC for Cr(VI) was 1100, 1150, 1350, 1400 and 1200 µg/ml, respectively. This study highlights the prevalent occurrence of Cr(VI) resistant bacterial population in the soil of dumping site of battery industry waste. It also reveals that continuous exposure to high levels of Cr(VI) may result in bacterial community that have an exceptional ability to adapt in chromate polluted environments and might have developed some detoxification mechanism as an effective tool for survival in the stress environments (Rehman *et al.*, 2008).

Heavy metal Analysis:-

The Cr(VI) and total Cr in the soil (mg/kg) of battery industry waste dumping site was 1170 ± 0.2 and 2900 ± 0.09 , respectively, which is much above the permissible limit. Further, the concentration (mg/kg) of other heavy metals detected were manganese (540 ± 0.2), copper (790 ± 0.06), nickel (650 ± 0.4), lead (540 ± 0.07) and zinc (860 ± 0.5). Results revealed that in addition to Cr(VI), the waste discharged from battery industry contains other metals in significant quantities which are ultimately causing pollution of soil, water bodies and the agricultural lands close to it. Since, large amount of waste is generated everyday from industries; chromium and other metals discharged through their waste into the environment get biomagnified along the trophic level, thus leading to ecotoxicological risk (Armienta *et al.*, 2001). Further, ground water quality is getting deteriorated due to seepage of chromate ions from the waste dumping site (Verma *et al.*, 2004). Results proved that the industrial waste discharged is not properly treated and stresses for its revaluation prior to disposal. The phytotoxic impact of chromium was observed in tomatoes, cabbage, rice, waterchestnut, pulses, etc. as visible symptoms of yellowing and immature fall of leaves, chlorosis, poor growth and retarded flower and fruit yield. This was possibly attributed to imbalance of nutrients and nutritional disorders in the plants due to metal interactions with plant nutrients (Chunillal *et al.*, 2005). Also, entry of toxic Cr(VI) in the food chain through contaminated edibles may cause health hazards (Sharma *et al.*, 1996). Moreover, Cr(VI) is non-degradable in nature and are dispersed both horizontally and vertically. Therefore, there is an urgent need for the bioremediation of Cr(VI) from the waste dumping sites.

Cr(VI) reduction Assay:-

The Cr(VI) reduction potential of the selected five bacterial strains was determined and the results are depicted in Figure 2. All the five isolates in this study were capable of reducing Cr(VI) aerobically and the reduction values ranged between 59.0-90.0%. Significant reduction was observed in the supernatant fraction of TB10 strain (90.0%) after 20 h of growth followed by TB9 (83%), TB11 (72.0%) and TB1 (65.0%) strain. However, the TB5 isolate reduced Cr(VI) less efficiently. The Cr(VI) concentration in uninoculated broth (control) remained the same throughout the experiment, indicating that medium components were unable to reduce Cr(VI) to a lower valency state. Reduction of Cr(VI) to Cr(III) has been reported by many bacterial types taking 2–9 days (Ma *et al.*, 2007; Panda and Sarkar, 2012). This prolonged period for chromium removal may lead to accumulation of metal and toxic compounds in the environment. The effective removal of chromium in a short time is a basic necessity of the present time. Since, strain TB9, 10 and 11 reduced 72-90% Cr(VI) in ~20 h, it may definitely improve the efficiency of the treatment of chromate contaminated wastes.

Screening of bacteria for biosurfactant production:-

The five bacteria selected on the basis of high Cr(VI) MIC values, were screened for their ability to produce biosurfactant following the standard qualitative and quantitative tests and the results are presented in Table 1. Appearance of dark blue halo zone around the culture of all bacterial isolates except TB5 strain on CTAB-MB MSM agar indicated the production of anionic biosurfactant by bacteria. The ability of biosurfactant producing bacteria to form clear halos in methylene blue agar plate assay has also been reported by other researchers (Adamu *et al.*, 2015; Vijayanand and Divyashree, 2015; Okore *et al.*, 2017). Blood hemolysis test is used to screen the microbes for their ability to produce biosurfactants on hydrophilic media. Table 1 revealed that bacterial strains TB5, TB9 and TB10 showed positive results for beta haemolysis on blood agar medium, thereby, indicating production of surface active

molecules (biosurfactant) which causes lyses of red blood cells. Vijayanand and Divyashree (2015) and Okore *et al.* (2017) have demonstrated the hemolytic activity of *Bacillus*, *Citrobacter*, *Pseudomonas*, *Staphylococcus*, *Corynebacterim* and other bacterial species on blood agar. Hemolytic activity has been regarded as indicative of biosurfactant production and used as a rapid method for bacterial screening (Satpute *et al.*, 2008).

The quantitative tests for biosurfactant production comprises of emulsification capacity test and oil spreading method. Results for emulsification of hydrocarbon revealed that bacterial strains TB9, TB10 and TB11 emulsified sunflower oil, olive oil and kerosene at varying rates and were also hemolytic (Table 1). This proves that these isolates are capable to produce biosurfactant which has ability to degrade hydrocarbon. Further, strain TB1 could emulsify sunflower oil and kerosene at very low rate. Among the three isolates, interestingly TB9 showed highest emulsification index (E_{24}) after 24 hours of incubation for sunflower oil (74 ± 1.60) followed by kerosene (72 ± 0.5) and olive oil (65 ± 0.3), whereas, strain TB10 showed highest E_{24} value for olive oil (74 ± 1.2) followed by sunflower oil (70 ± 0.29) and kerosene (68 ± 0.19) after the same period of exposure. However, the E_{24} values of TB11 strain was significantly lower for all the three hydrocarbons tested. Further, all the isolates that were hemolytic were also able to emulsify the hydrocarbons tested except the strain TB5. The inability of TB5 to emulsify the oil indicated its failure to produce biosurfactant and hence, the hemolytic activity could be attributed to extracellular secretions. Similar findings were reported by Adamu *et al.*, (2015) and Govarthanam *et al.*, (2017). Results for oil spreading test (Table 1) of the kerosene oil revealed that strain TB9 and TB10 caused the spreading of the oil at the rate of 6 and 5 minutes with a diameter of 2.7 cm and 3.3 cm, respectively. Results also showed that the TB11 strain showed positive results for hemolysis and emulsification but was unable to spread the oil. This might be because some bacteria do not produce extracellular material as biosurfactant but the cells itself acts as biosurfactant (Adamu *et al.*, 2015).

Bacterial Identification:-

Based on the results of concomitant biosurfactant production efficiency and chromate reduction potential, bacterial strains TB9 and TB10 seems to be most efficient strains that can be potentially be used for Cr(VI) bioremediation and environmental restoration. Thus, both strains were chosen for detailed study on optimization of biosurfactant production and chromate removal applications. On the basis of morphological and biochemical characteristics both the strains were identified as *Pseudomonas aeruginosa* and *Bacillus cereus*, respectively (Table 2).

Table1:-Screening of bacteria for biosurfactant production

Bacterial isolates	Methylene Blue Plate Assay	Hemolysis test	Emulsification index E_{24} (%)			Oil displacement	
			Sunflower oil	Olive oil	Kerosene	Diameter (cm)	Time (min)
TB1	+	-	20 ± 0.72	-	23 ± 0.28	-	-
TB5	-	+	-	-	-	-	-
TB9	+	+	74 ± 1.60	65 ± 0.3	72 ± 0.5	2.7	6
TB10	+	+	70 ± 0.29	74 ± 1.2	68 ± 0.19	3.3	5
TB11	+	+	19 ± 1.0	36 ± 0.5	24 ± 1.2	-	-

(+) Positive, (-) negative

Table 2:-Morphological and biochemical characteristics of selected TB9 and TB10 isolate.

Characteristics	Bacterial strain	
	TB9	TB10
Colony Morphology	Bluish-green, raised, non-mucoid, circular colonies with smooth margin	Cream, raised, non-mucoid, circular colonies with serrated margin
Gram reaction	Gram negative, rod	Gram positive, rod
Endospore formation	-	+
Indole test	+	-
Methyl Red test	-	+
Voges-Proskauer	+	-
Citrate utilization	-	+
Casien hydrolysis	-	+
Starch hydrolysis	-	+

Urea hydrolysis	-	+
Gelatin hydrolysis	-	+
Catalase test	-	+
Oxidase test	+	+
Nitrate reduction	+	-
H ₂ S production	-	-
Cytochrome oxidase test	+	-
Acid production from carbohydrates		
Glucose	+	+
Lactose	-	-
Fructose	+	+
Sucrose	-	+
Arabinose	+	-
Possible bacterial strain	<i>Pseudomonas aeruginosa</i>	<i>Bacillus cereus</i>

(+) Positive, (-) negative

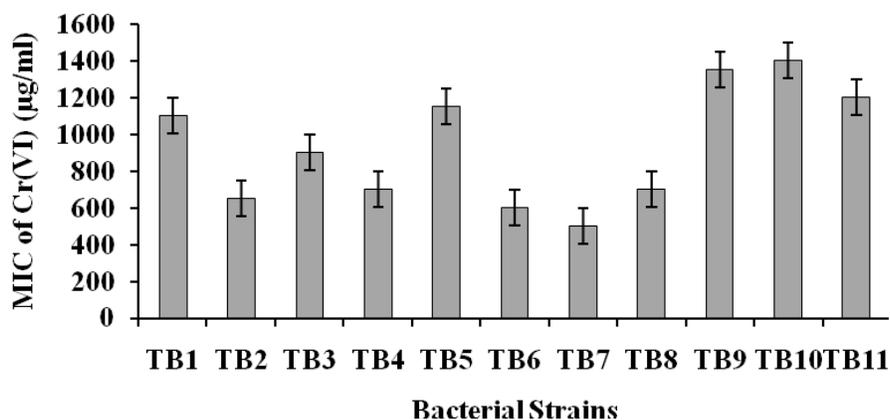


Figure 1:- Minimum inhibitory concentration for Cr(VI) by chromate tolerant bacterial strains. Error bars represent mean ± standard deviation.

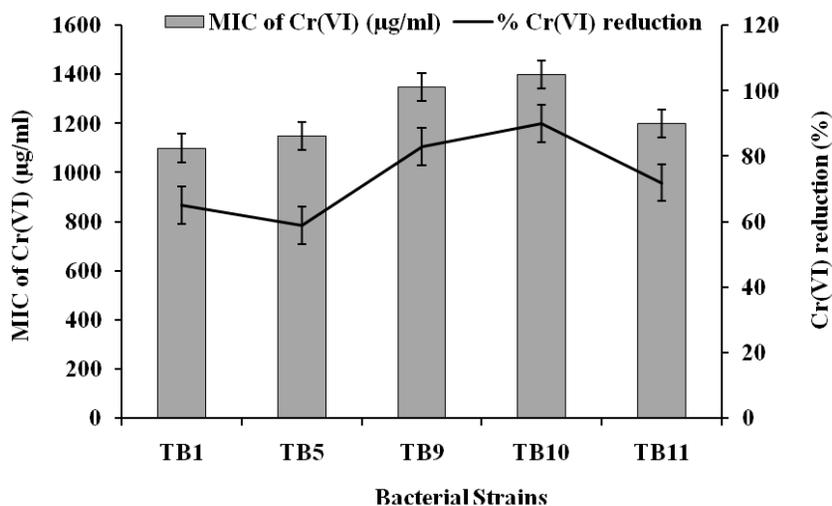


Figure 2:- Chromate reduction potential and the respective MIC for Cr(VI) among selected bacterial strains. Error bars represent mean ± standard deviation.

Conclusion:-

Metal contamination of soil due to disposal of industrial waste has increased considerably in recent years leading to contamination of the environmental reservoirs such as water bodies and soils. Soil usually acts as a sink for harboring metals. These metals being immobile in soil accumulate and influence the properties of soil adversely. A novel approach for enhanced bioremediation of metal contaminated site is the use of microbial surfactants. The present study focuses on the biosurfactant production by chromate tolerant *Pseudomonas aeruginosa* and *Bacillus cereus* and assessing their potential for bioremediation of chromate from contaminated site. The bacteria of this study offers an interesting model for further studies, as well as an alternative to be exploited for Cr(VI) bioremediation in a wide range of environments.

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