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Potential of engineered Pseudomonas putida for Bioremediation of Cr (VI) tannery polluted environment.

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Abstract -*Bioremediation of composite tannery wastewater appears to be incompatible for direct biological treatment. The present work aimed contaminated area can be treated with microbes that are genetically engineered for degradation specific toxic compounds. Tannery wastewater is heavily polluted containing appreciable biodegradable matters as well as inorganic substances like chromium, sulphide, chloride etc. Wastewater emanated from various sub-processes of tannery operation showed variable characteristics and also differential treatability.Pseudomonas putida isolated from tannery sludge, were examined for their tolerance to hexavalent chromium Cr (VI) and their ability to reduce Cr (VI) to Cr(III), a detoxification process in cell suspension and cell extracts.. Pseudomonas putida could reduce Cr (VI) up to 75% from the total content of different level of dilutions could confirm in terms of their Cr (VI) reducing ability and resistance to Cr (VI), and the Cr (VI) reduction was mainly associated with the soluble protein fraction of the cell.. P.putida used as a source of plasmid that carries the Chr R resistance gene inserted recombinant based device could be used for measurement pollutant in waste water and contaminated soil. This approach has a great potential for bioremediation of Cr (VI)-polluted environments. The bacterial isolates can be exploited for the bioremediation of hexavalent chromium containing wastes, since it seem to have a potential to reduce the toxic hexavalent form to its nontoxic trivalent form.*

Key Words: *Pseudomonas putida, Chromium, Plasmid, Pollution, Bioremediation.*

I.INTRODUCTION:

In the past few decades, environmental pollution has become one of the world's major concerns. A great number of toxic compounds, originating mostly from industrial and agricultural activities, are being released into environment continuously. In some cases harmful chemicals induce strong acute toxic effects to exposed organisms when released to the environment, but frequently the consequences are delayed due to the effects of bioaccumulation and bio-magnification[1]. In order to get more relevant information about environmental pollution risk, it is therefore inevitable to supplement the chemical analytical data with the results of methods providing information on biological impacts. The negative biological effects of pollutants present in all kinds of environmental samples can be assessed using different living organisms or cells as 'analytical devices'. The biological response following the exposure of living organisms or cells to environmental sample usually gives an information on toxicity, genotoxicity etc. of the whole mixture of chemical compounds present in that particular sample. The aquatic environment with its water quality is considered the main factor controlling the state of health and disease in both cultured and wild fishes. Pollution of the aquatic environment by inorganic and organic chemicals is a major factors posing serious threat to the survival of aquatic organisms including fish. The agricultural drainage water containing pesticides and fertilizers and effluents of industrial activities and runoffs in addition to sewage effluents supply the water bodies and sediment with huge quantities of inorganic anions and heavy metals [2]. The most anthropogenic sources of metals are industrial, petroleum contamination and sewage disposal [3].There are two types of tanning systems which are vegetable tanning, which does not contain chromium, and chrome tanning. However, due to the high pollution load and low treatability, conventional vegetable tanning can't be considered more environmentally friendly than chrome tanning. Moreover, vegetable tanned leathers have different physical properties and specific applications, but is biodegradable [4]. One of the major emerging environmental problems in the tanning industry is the disposal of chromium contaminated sludge produced as a by-product of wastewater treatment. Tannery effluents severely affect the mitotic process and reduce seed germination in extensively cultivated pulse crops [5].The main focus of the present day industrial toxicology is to effectively eliminate the industrial pollutants from their hazardous effect, which are a serious threat to the healthy environment and to the population. The effluent charged from tanning industry is highly complex and has a serious pollution potential. The present study is envisaged to investigate the effect of land disposal of tannery effluent and sludge of Dindigul in Tamil Nadu. India. In Dindigul about 80 million litres of untreated tannery effluent is discharged everyday into the barren land and occasionally to ponds and river basins. The effluent discharged from the tanneries pollutes the soil, water, plants, animals and workers of tannery industry of the surrounding area and hence the present study is undertaken to find means of reducing the hazardous influence of the tannery effluent near Dindigul area Tamil Nadu, India, by identifying the detoxification potentials of microbes that grow in the polluted area and effectively making use of them a biotechnological process of treatment. Hence the present study was aimed at the isolation of chromium reducing bacteria from a long term

tannery effluent contaminated soil. Metal specific coded sequence insert in recombinant bacteria have been constructed and used for the determination of chromium and detoxify hazardous contaminants, has long been considered a promising method to provide economical and ecologically sound clean up strategies. The paper has focused on the challenges impacts of tannery effluent and evaluates the alternative treatment options used to treat, recover or recycle chromium from the waste water.

II.MATERIALS AND METHODS:

Cultivation of Cr (VI) reducing bacterial strains:
Soil samples were collected from chromium contaminated site located at Dindigul**,** District of Tamil nadu, India. The soil was contaminated with the chromium sludge discharged from chromate manufacturing industry. Five grams of soil sample was added to 100mL of sterile growth media M1 with 10 mg/L of Cr (VI) and incubated in a shaking incubator for 24 h at 35 ◦C. After 1 day, when significant growth was observed, 1mL of the supernatant of the slurry was transferred to 100mL of fresh nutrient media (M1) and incubated at 35 ◦C. The consortia used for the Cr (VI) reduction was developed by a series of transfers at every 24 h by gradually increasing the Cr (VI) concentration. Once the enriched consortium was ready, bacterial isolates were prepared by repeated serial dilutions and streaking on agar plates. Identical colonies were separated based on their morphology and was streaked on agar slants using an inoculating needle, and incubated at 35 ◦C for 24 h and stored at 4 ◦C until needed for further experimentation. M9 minimal salts medium (Eisenstadt *et al* 1994) agar supplemented with 100µg Cr (VI)/ ml as $K_2Cr_2O_7$ and 0.5% (w/v) glucose as a carbon source. The biochemical tests were used to identify the bacterial isolates as per the guidelines of Bergy's manual of determination bacteriology [6].The strain used for present work (*Pseudomonas putida*) was isolated from tannery sludge and the strain was maintained in Luria- Bertini (LB) medium. The bacteria is gram negative, rod shaped and formed pale elevated colonies on LB plates. It also showed resistance to tetracycline up to 20µg per ml. *Pseudomonas putida* showed high potential to detoxify tannery effluents and hence was selected for the study.

Morphology studies
The morphology of the bacterium was studied under Transmission Electronic Microscope. Earlier cells were grown to an A_{660} of 2.5 and were harvested by centrifugation at 12,000 rpm for 3 minutes. The cells were washed, re-suspended in milliQ water. A drop of the re-suspended bacterium was layered on a glass slide and air dried. Thus prepared bacterial slide was used for imaging under transmission electron microscope.

Metal Screening
Pseudomonas putida strain was examined for its resistant against Hexavalent chromium and the selected form of hexavalent chromium is potassium chromate. Metal Screening was carried out with two different medium such as LB and M9 Medium containing antibiotic Tetracycline and also various concentration of Potassium chromate (10mM-1M). Plates were streaked quadrantly with the overnight grown culture of Pseudomonas putida on the surface of the agar plates. Then the plates were incubated at 28°C for growth.

Chromate reductase assay
The enzyme activity in the eluted fractions was assayed (Park *et al.*, 2000) at 30^0C in 0.5 ml reaction mixtures containing to a final concentration of 50mM Tris-Hcl (7.0), 0.05mM Potassium dichromate(K_2CrO_4), 0.1mM Nicotinamide Adenine Dinucleotide (reduced) [NADH] and 0.3 ml of enzyme preparations. The activity of the enzyme was measured spectrometrically at 540nm.

Growth and Cr (VI) Reduction
The bacterial strain *Pseudomonas putida* was pre cultured overnight in tryptic soy broth and the cells were harvested by centrifugation (6000g for 10 min at 10 ^{o} C) followed by, cells pellet washed in phosphate buffer (0.1M, PH 7.1). After two washes of the cells in same buffer , the cells were resuspended in the salts medium supplemented with Cr(VI) ranging from 10-100mgCr(VI)/l medium and 0.5% glucose were inoculated with the equal amounts of *P.putida* sp. Media without chromium, but inoculated with bacteria and uninoculated media containing chromium served as controls. All the cultures including controls were incubated for 72 hours at room temperature (37 ^{o} C) with shaking at 100rpm. Growth of the bacteria was monitored at definite time intervals, by measuring optical density of the cultures at 600nm. To measure the Cr (VI) reduction by growing cells, a 1-ml culture from each of the above flasks was centrifuged (6000 rpm for min at 10 ^{o} C) and the supernatant analyzed for Cr (VI).

Cell- Free preparation for analysis of Chromium reduction
To prepare the crude cell extract the bacteria were grown overnight in minimal salts medium supplemented with 0.5% glucose and harvested by centrifugation (6300 rpm for 10min, 10^{o}C) followed by washing twice in 10mM Tris-HCL buffer (pH7.2). The cells were placed on ice and disrupted (10 x 30s) with Sonifier. The resultant homogenate was centrifuged at 30,000g for 30min 4^{o}C and the supernatant was used as crude extract for Cr (VI) reduction activity. *Pseudomonas putida* was induced for Chromate Reductase and the rate of the method reduction of hexavalent chromium to trivalent chromium was measured by spectrophotometer. The amount of trivalent chromium formed by the action of

enzyme was assayed as stated by Greenberg *et al.*, 1981, To the reaction mixture H_2SO_4 and 1,5-diphenylcarbazide to final Concentration of 0.1M and 0.01%, respectively, and measuring the absorbance at 540nm (A_{540}) and the concentration was calculated using a calibration curve relating chromate concentration.

Bioremediation

The pH of the effluent/ sludge was altered to 7.0 with NaOH and then distributed in 250 ml conical flasks (100 ml in each). 1% sucrose was added and 1% of overnight grown culture was initiated in each conical flask inoculated with the organisms were used as single, and in immobilized forms and The flasks were kept on rotary shaker at 180 rpm at 37°C for a period of 10 days. Chromium was estimated at an interval of 12 h to calculate the chromium depletion from the effluent by the different test organisms. The results were calculated and tabulated and statistical analysis was performed to analyze and interpret the results.Recombinant construct of *Pseudomonous putida* was inoculated in different in dilution of tannery effluent and allow it to grown for 3 days. Simultaneously maintained effluent filtrate as control After 3 days the culture filtrate was used for germination studies of paddy seeds.Germination studies were carried out in petri dishes moist by a layer of cotton and a coarse filter paper. Thirty seeds per petri dish of *Oryza Sativa* variety IR -50 were used and five replicates were maintained for each concentration. A portion of 25ml of each concentration of the culture filtrate was added to the petri plate on alternate days and controls received the same amount of distilled water (cultrate filtrate ranging from 10%, 25%, 50% and 75%). The percentage was calculated 5 days after germination started. Germinated seeds were selected from different concentration also used for studied of root activity. Roots from 10 days old seedling washed in water and cut in to1- 2cm segment and pooled. The excess water removed by squeezing the roots with filter paper then used for α -naphthalene oxidizing activity of the roots. For that, 1-2cm of this sample was weighted and transferred to an Erlenmeyer flask containing 50.0 ml of 20-ppm alpha napthalamine solution. The flask were kept in a shaker for 2-3 hrs at 100-200rpm .20ml aliquot of α – napthalamine solution was pipetted out from after incubation and diluted to 100ml with distilled water. To this 1.0ml of sulphanilic acid (1g in 100ml 30%acetic acid) and 1.0 ml of 100ppm sodium nitrate solution was added, the contents were made up to 20 ml with distilled water. The tubes were incubated for the 30-40 min colour development and read 500nm in a colorimeter.

III.RESULTS AND DISCUSSION:

Bacteria of the genus *Pseudomonas* belong to the γ subclass of the Proteobacteria and are chemo-Organotrophic aerobic, Gram negative rods and a respiratory rather than a fermentative metabolism.The genus is a large taxonomic grouping of species that collectively exhibit a highly diverse range of activities: they are extremely versatile metabolically and engage in many activities such as element cycling, degradation, and recycling of biogenic and xenobiotic organic compounds, food spoilage, growth promotion and protection of plants from pathogens, parasitism of other bacteria.

Pseudomonas putida is a rapidly growing bacterium, frequently isolated from most temperate soils and water, particularly pollutants soils. It plays a key role in maintaining qualities of environment. *Pseudomonads* have considerable potential for biotechnological applications, particularly in the areas of bioremediation (Halt, J.G.1984).

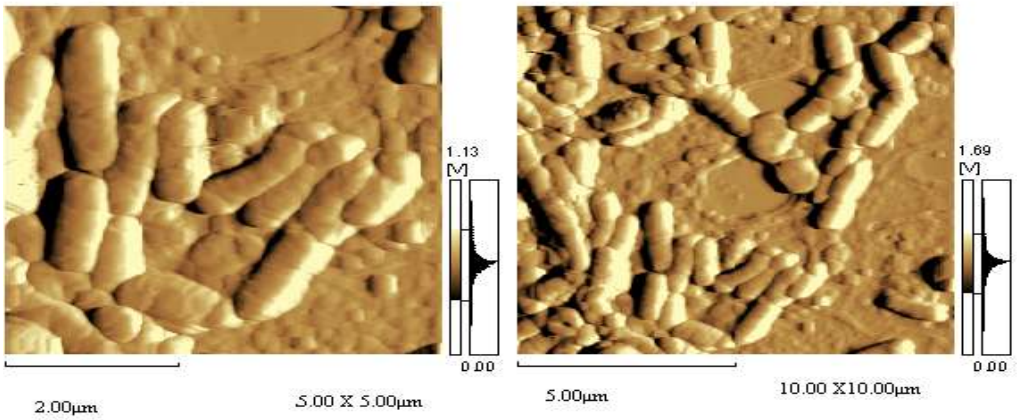

Figure 1: Transmission Electron Microscopic Images of *Pseudomonas putida*

Legends: image (Fig 1a) is an amplified portion of image 1b; due the higher concentration the cells are aggregated in the image.The leather industry being the chief contributor of pollution causes severe effects on plants, animals and human beings. Hexavalent chromium is the major main toxic constituents in tannery effluents. Chromate reductase enzyme is involved in reduction of hexavalent chromium to less toxic trivalent form. In this study, *Pseudomonas putida* isolated

from tannery sludge is exploited for its potency to detoxify tannery effluent. There are no strains of *Pseudomonas putida* that are animal or plant pathogens and this saprophytic species is considered to be environmentally innocuous. For this reason *Pseudomonas putida* was recognized to be a promising candidate from which to develop a safety strain for recombinant DNA experiments, requiring a host with a versatile catabolic physiology.

Microbial strains were examined its potential for metal screening to establish resistant against hexavalent chromium (Figure 2).

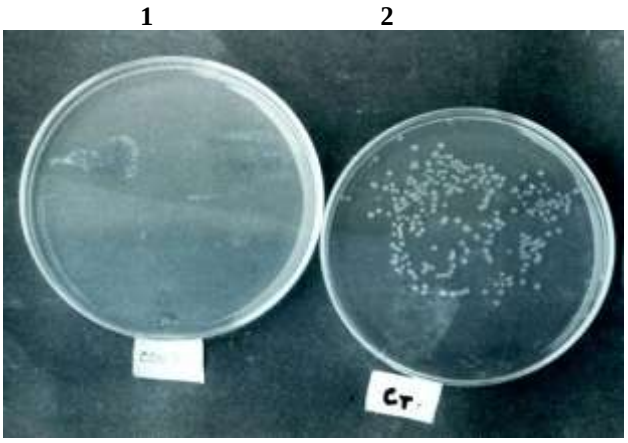

Figure 1: *Pseudomonas putida* strain showed Metal Screening

Legends: Figure1 indicated the results of ***Pseudomonas putida* strain** isolated from tannery sludge have the capacity to resist hexavalent chromium. Plate 1-control; plate 2-shows the growth of *Pseudomonas putida*.The chromate reduction ability of two strains was measured by the following method (Park et al., 2000). Growth of cells was followed by A_{660} and Chromate reduction rated was quantified in growing cultures, as well as cell suspension. These microorganisms have developed their capabilities to protect themselves from heavy metal toxicity by various mechanisms such as adsorption, uptake, methylation, oxidation and reduction. Residual chromate was measured by the diphenyl carbazide method (park *et al* 2002), after removing the cells by micro-centrifugation. *Pseudomonas putida* and *Escherichia coli* were selected for bioremediation of chromate (VI).

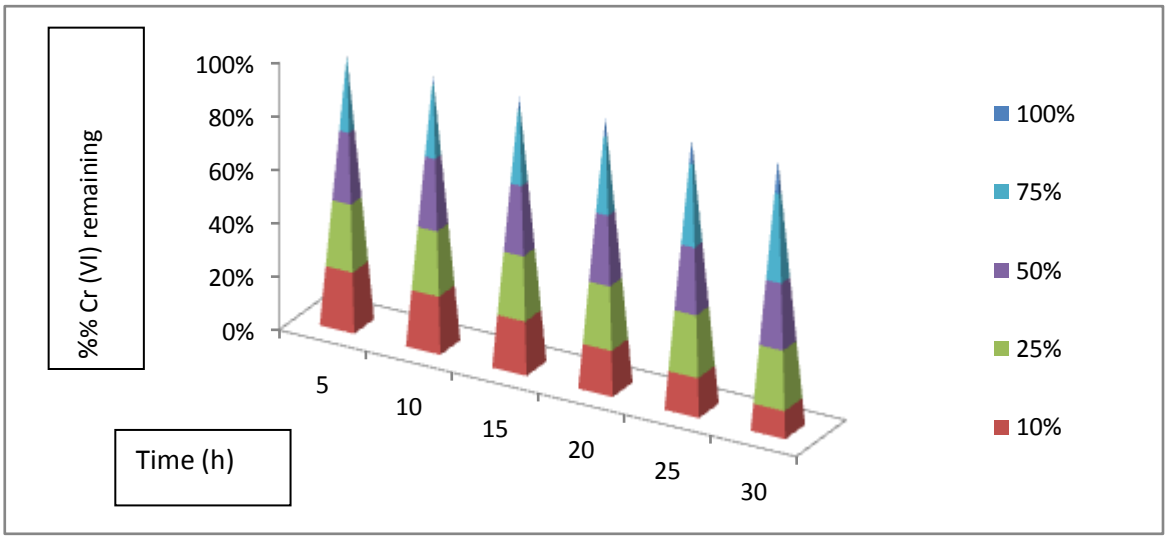

Figure: 3 Comparative analysis of Chromate reduction

Legends: Figure 3 indicates the rate of chromium reduction. In response to various dilution of culture filtrate such as 10%, 25%, 50%, 75%, 100%.The amount of chromium hexavalent reduction was increased with increasing with reduced dilution in the presence of chromium reductase of *Pseudomonas putida.*

Chromate reductase enzyme showed significant changes with varying substrate concentration. The rate of reduction was increased steadily with increasing dilution factor (Figure 3). Chromate was quantitatively converted to Cr (III) by chromate reductase (Chr R) enzyme had no activity with Cr (III).

1 2 3 4 5 6

Figure 4: Germination studies of *Oryza Sativa*

Legends: Fig: 4 showed the emerged stage of shoot from ***Oryza Sativa*** seeds and the length of shoot varied with respect to concentration of culture filtrate used in the experiments.

1- Control; 2-10%; 3- 25%; 4- 50% 5- 75% 6- 100%. In figure, maximum shoot length obtained in 10% of experimental setup.

1 2 3 4 5 6

Figure 5: Growth of *Oryza Sativa*

Legends: Fig: 5 indicated length of shoot & roots emerged from experimental samples a mention above.The culture filtrate had the reduction of chromate to Cr (III) to be considered as a remediation process there by results revealed remaining percentage of chromate. The uses of biological remediation technologous has bioremediation/phytoremediation, the cleanup of Cr-contaminated areas has received increasing interest from researchers from worldwide. To improve bacterial chromate remediation it is necessary to clone the gene encoded chromate reductase activity could be used for treating or bioremediation/phytoremediation.

IV.CONCLUSION:

Elimination of heavy metals from industrial wastewater is important for preserving the quality of aquatic systems. The hexavalent chromium compounds are comparatively more toxic than trivalent chromium compounds due to their higher solubility in water, rapid permeability through biological membranes and subsequent interaction with intracellular proteins and nucleic acids. A maximum acceptable concentration of 0.05 mg/l for hexavalent chromium in drinking water has been established on basis of health considerations. Studies showed that tannery waste disposals have led to severe contamination of productive agricultural land in India. Chromium level was found to increase with increase in the concentration of the tannery effluent. Chromium associated pollution is of increasing concern nowadays. Trivalent and hexavalent chromium differ widely in physicochemical properties and biological reactivity. While Cr (VI) species and dichromate's are extremely water-soluble and mobile in the environment, Cr (III) species are much less soluble and comparatively immobile. Moreover, Cr (VI) is recognized to be highly toxic, carcinogenic, mutagenic and teratogenic for mammals including humans, whereas Cr (III) is an essential trace element necessary for glucose, lipid and amino-acid metabolism as well as a popular dietary supplement [7]. Discovery of microorganisms capable of reducing Cr (VI) to Cr (III) have significant potential in development of *in situ* or on-site bioremediation strategies.

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