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MOLECULAR DESIGN OF PSEUDOMONAS PUTIDA FOR REMEDIATION OF HEXAVALENT CHROMIUM IN TANNERY EFFLUENT

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Abstract: The leather industry, which is one of the major contributors to pollution, has serious impacts on plants, animals and humans. The present study revealed the isolation of *Pseudomonas putida* from tannery effluent for its potency to detoxify toxic pollutants. Thus, the efficiency of hexavalent chrome reduction by the bacteria *P. putida* isolate was investigated by looking behind the plasmid-coded gene responsible for the degradation potential. Plasmid DNA was extracted from *P. putida* and then transformed into an XL-blue strain in order to check the role of plasmids in metal tolerance on LB plates containing 60 mM Cr. The metal tolerance and toxicity reduction coded by the plasmid gene confirmed by the PCR-coding gene might be around 9 kb and 4 kb, respectively. The coding region of the chrome reductase gene was amplified using appropriate primers. Based on the efficient Cr (VI) reducing ability of *P. putida*, this bacterium has great potential for use in the detoxification of Cr (VI) in contaminated soil and water. Thus *P.putida* was recognized as a promising candidate to develop a safety strain for recombinant DNA. The present work focused on providing an attractive strategy for remediation of chromium from effluent, and then subsequently treated waste water could be considered for irrigation practices. Plant seed germination experiments were conducted to confirm its application for agricultural purposes. Results obtained by measuring shoot growth and root development, as well as the alpha-naphthalene oxidizing root activity of *Oryza sativa* seedlings, might explain the remediating efficiency of molecularly designed *Pseudomonas putida*, which plays a key role in the remediation of tannery effluent.

Keywords - Alpha naphthalene assay, Chromate reductase, PCR, Plasmid, *Oryza sativa*.

I. INTRODUCTION

The global economy greatly benefits from the leather industry, which produces a wide range of leather products using raw hide. Interestingly, 95% of these raw materials are actually by-products of the meat and dairy industries (Sivaram and Barik, 2018). India has emerged as a key player in this industry, thanks to its abundant livestock, water resources, skilled labor force, and advanced leather processing technology. Within the leather processing industry, the tannery sector plays a crucial role in converting raw leather into finished materials. However, it is important to note that this sector is also the most polluting, as it generates toxic pollutants at every stage of the process. Unfortunately, only 20% of the raw materials are transformed into usable leather products, while a staggering 60% is converted into solid and liquid waste (Saran et al., 2019). The pollution caused by the liquid waste depends on the type of hides and skins being processed, as well as the chemicals used. Disappointingly, the discharge of this untreated or partially treated effluent into freshwater bodies has resulted in severe environmental consequences.

Tannery effluents are considered the most significant pollutants among industrial wastes. They are classified into different types: vegetable tanning, which is chromium-free and utilized for heavy leather products such as shoe soles, handbags, straps, and belts; chrome tanning, which contains chromium and is suitable for light leather (Kanagaraj *et al.*, 2008). The chrome tanning process involves the utilization of chromium to form cross-linkages among free carboxyl groups found in collagen, making the hide resistant to bacterial degradation and high temperatures. The hexavalent chromium Cr (VI) produced in tanneries is highly toxic, as it directly interacts with intracellular proteins and nucleic acids, leading to mutagenic and carcinogenic effects in animals and humans (Ahmed and Chowdhury, 2016).

The primary objective of this research is to address the detrimental effects of industrial pollutants on the environment and the population by effectively eliminating them. The discharge of effluent from the tanning industry poses a significant pollution risk due to its complex nature. Bioremediation has recently emerged as a safe and cost-effective alternative to traditional physico-chemical methods (James 1996). However, the successful implementation of bioreduction-based remediation for Cr (VI)-contaminated water and soil requires the availability of effective Cr (VI)-reducing organisms (Campos *et al.*, 1990). Therefore, this study aims to explore the biodegradability of microbes in the vicinity of the effluent discharge area and develop bioprocesses to efficiently eliminate toxicity. The development of a methodology utilizing a specific strain to effectively remove toxicity is crucial. By creating new plasmids, we can construct a highly efficient strain that can be utilized for pollution control. The research aims to identify the detoxification capabilities of microbes thriving in polluted areas and utilize them in a biotechnological treatment process. In this study, we have selected a specific bacterial strain from our previous research that shows promise in eliminating metal toxicity and degrading tannins. Therefore, our objective is to understand the mechanism

behind the removal of metal toxicity, whether it is plasmid-encoded or not. In other words, we aim to screen for the presence of plasmid DNA and determine its role in the organism's metal resistance.

II. MATERIALS AND METHODS

Isolation of *Pseudomonas putida*

Heavy metal resistant bacterial strain was isolated from tannery effluent collected from contaminated soil or sludge of waste disposal site at Dindigul, Tamilnadu, India using M9 minimal salts medium (Eisenstadt *et al.,* 1994). The medium was enriched with 100µg Cr (VI)/ ml in the form of $K_2Cr_2O_7$ and 0.5% (w/v) glucose was added as the carbon source. The isolated strain was biochemically characterized and identified as *Pseudomonas putida*.

Isolation of plasmid DNA

The plasmid DNA isolation procedure followed the methodology outlined by Silhavy *et al*., (1984). Initially, 0.5 ml of overnight culture was combined with ampicillin (50 µg/ml) and inoculated into 100 ml of LB medium in a 500 ml flask. The mixture was then shaken at 37 °C until the OD reached 0.5–1.0. Subsequently, the cells were centrifuged at 5000 rpm for 10 minutes at 4 °C. Following this, 2.4 ml of TE (10 mM-Tris HCl, pH 8.0; 11 mM EDTA) and 30 µl of Lysosome (910 mg/ml) were added and thoroughly mixed. The solution was then incubated for 10 minutes on ice and centrifuged at 15000 rpm for 20 minutes. The supernatant was extracted with an equal volume of phenol chloroform at 4 °C, and the plasmid DNA was collected by centrifugation at 10000 rpm for 10 minutes at 4 °C. The resulting pellet was washed with 70% ethanol, air dried, and dissolved in a minimal volume of 100 ml of TE. Finally, the plasmid DNA was analyzed using agarose gel electrophoresis as described by Maniatis *et al*., (1982).

Plasmid curing:

The metal + bacterial strain was cured of its plasmid by using acridine orange as the curing agent. The bacterial strain was inoculated into LB medium and incubated overnight with shaking at 37ºC. Subsequently, the cultures were diluted 1:1000 in LB medium, and 2 ml samples were aseptically distributed into a series of tubes containing varying concentrations of acridine orange, ranging from 50 to 500 g/ml. All the tubes were then incubated at 37oC for 24 hours. To assess the loss of ability to grow in metal-containing media, the master plates were replica-plated on LB agar plates containing the minimal inhibitor concentration (MIC) of different metals.

Transformation of plasmid into E.Coli:

Competitive cells were prepared using the Chung and Miller method (1988). The recipient cells, DH5, were cultured until early log phase in 50 ml of LB (0.3 OD). After centrifugation at 1000 rpm for 10 minutes at 4°C, the pellet was resuspended in a 1/10th volume of transformation and storage buffer (TSB) containing 10% PEG (MW.3350 pH 6.1, 5% DMSO), and 20 mM Mg++. The suspension was then incubated on ice for 10 minutes. Following that, 100 mg of plasmid DNA was added to 100 µl of competent cells and incubated on ice for 30 minutes. The cells were diluted to 1 ml with TSB containing 29 mM glucose and incubated at 37 °C for 1 hour with shaking (225 rpm). Aliquots of 100 ml were plated on LB plates and incubated overnight at 37 °C. Transformants were selected for metal tolerance by plating on appropriate metal plates.

Primer designing and PCR amplification

Chromate reductase gene sequence: Accession no.AAK56853, Size 561 base pairs; Primers were designed for the Chromate reductase sequence using the software OLIGOTES. The software provides options for checking to determine the GC content, Tm values of primer, and size of primer. Size - 24 mer; Tm value - 57.90 °C; GC content - 45%. Synthetic oligonucleotides were utilized as primers in the polymerase chain reaction (PCR), which was conducted in a Perkin Elmer Cetus DNA thermal cycler.

For detection of gene,

One **24 mer Forward primer** -5’ TGG ATC CTA TGA GCC AGG TGT ATT 3’ and

One **24 mer Reverse primer** -5’ AAA ATC CTA TGA GCC AGG TGT ATT 3’ were used to amplify a chromate reductase gene. The reaction components were deoxynucleoside triphosphates, primers, Taq DNA polymerase, and $MgCl_2$. They were employed to facilitate the amplification of DNA. . The thermal profile consisted of a single cycle at 94°C for 3 minutes, followed by 30 cycles at 94°C for 1 minute, 60°C for 1 minute, and 72°C for 2 minutes, and concluded with a final cycle at 72°C for 15 minutes. The amplified product run on 1% gel was used to analyze the chromate reductase gene PCR product along with a hundred base pair marker to confirm the size of the products, and it was examined under UV illumination. The results were photographed in gel documentation.

Analysis of chromium reduction:

Pseudomonas putida was cultured overnight in tryptic soy broth. The cells were then collected through centrifugation at 6000rpm for 10 minutes at 10°C. Subsequently, the cell pellet was washed in phosphate buffer with a pH of 7.1. After two additional washes in the same buffer, the cells were suspended in a salts medium supplemented with Cr (VI) ranging from 10-100 mg Cr (VI)/l medium. A 0.5% glucose solution was inoculated with equal amounts of *P. putida* media without chromium but containing bacteria. Uninoculated media with chromium served as controls. All cultures, including the controls, were incubated for 72 hours at room temperature (37°C) with shaking at 100 rpm. The growth of the bacteria was monitored at specific time intervals by measuring the optical density of the cultures at 600nm. To determine the reduction of Cr (VI) by the growing cells, a 1-ml culture from each flask was centrifuged at 6000 rpm for 10 minutes at 10°C. The supernatant was then used to estimate the Cr (VI) concentration. *P. putida* culture was utilized to assess the rate of hexavalent chromium to trivalent chromium reduction, which is facilitated by chromate reductase. The amount of trivalent chromium formed by the action of the enzyme was determined following the method described by Greenberg *et al.,* 1981[14]. In the reaction mixture, H2SO4 and 1, 5-diphenylcarbazide was added to achieve final concentrations of 0.1M and 0.01%, respectively. The absorbance at 540nm (A540) was measured, and the concentration was calculated using a calibration curve correlating chromate concentration.

Germination studies:

A genetically engineered version of *Pseudomonas putida* was introduced into various dilutions of tannery effluent and allowed to cultivate for a period of 3 days. In parallel, the effluent filtrate was maintained as a control. Following the 3-day incubation period, the culture filtrate was utilized to determine the reduction of chromate, as well as to conduct germination experiments with paddy seeds. The germination studies were conducted in petri dishes that were moistened with a layer of cotton and coarse filter paper. Each petri dish contained thirty seeds of *Oryza sativa*-50, with five replicates maintained for each concentration. A volume of 25ml of the culture filtrate at varying concentrations was added to the petri dish every other day, while the control group received an equivalent amount of distilled water (with culture filtrate concentrations ranging from 10%, 25%, 50%, to 75%). The percentage of germination was calculated after 5 days, and the germinated seeds from different concentrations were also utilized to assess root activity. The root activity was evaluated using the α-naphthalene assay method standardized by Koptiyeva and Tanstsiurenko (1971), with the α-naphthalene oxidizing activity of the roots measured in colorimeter at 500nm.

III. RESULTS AND DISCUSSION

The bacterial strains isolated from the discharge area have been biochemically characterized. Among the isolates, *Pseudomonas putida* has shown high efficiency in converting hexavalent chromium to trivalent chromium. The selected bacterial strain exhibits tolerance to toxic metals which may be effectively used to eliminate the metal toxicity found in effluent. The five bacterial strains isolated from the effluent discharge area of the tannery industry have been biochemically characterized and published previously by Renuga and Rajamanickam (2003). In the present study, the response of one bacterial strain, *P. putida* was selected to find out its efficiency against hexavalent chromium reduction in tannery effluent. The work was devoted towards the efforts to characterize the plasmid based origin of metal tolerance property of the specific microbe identified.

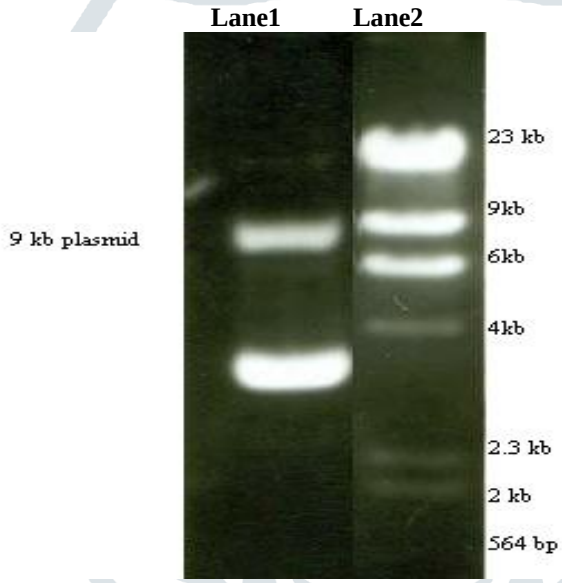

Figure 1: Agarose gel electrophoresis of Plasmid DNA

Legend: separation of plasmid DNA isolated from *Pseudomonas putida*

Lane1: Plasmid sample; Lane 2: Lambda Hind III digest DNA marker

The plasmid isolated from *P.putida* is shown in Figure 1. It revealed that the two bands were visualized under the illumination of UV. The results indicated that the size of the plasmids may be around 9 kb and 4 kb respectively. There could be a possibility of the strain processing two plasmids. The restriction analysis would reflect knowing the sequence homologies of the plasmid with known plasmids with information coding for metal tolerance. Deletion of specific regions of restriction digests of the plasmids, transformation of the deleted and undeleted native plasmids into *E. coli* strains, and subsequent metal tolerance contributed by the transformant strains confirmed the specific region of the plasmids that contribute to the metal tolerance. Further curing of the transformants (transformed with native plasmids) might confirm plasmid coded metal tolerance.

The isolated plasmids were then transformed into an XL-blue strain (Figure 2). To check the involvement of the plasmids in metal tolerance, the transformants were plated on LB plates containing 60 mM Cr, whereas the untransformed blue strain did not show growth at the above concentration of metal. The ability of transformants to grow at higher concentrations here suggests that the metal tolerance and its toxicity reduction were plasmid coded genes. The coding region of the chromate reductase gene was amplified using appropriate primers.

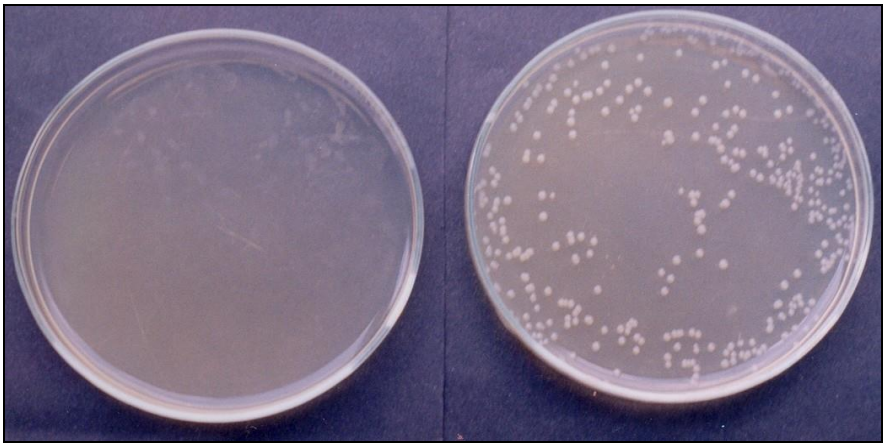

Figure **2:** Control and Transformed colonies on LB plates containing 60mM Cr

Legend: LB agar plates showed that there was no bacterial growth on control plate and transformed colonies grown at chromium at 60 mM concentration which expressed the capability of chromium resistant of *P.putida*.

PCR amplification of chromate reductase is represented in Figure 3. The amplified products run on 1% Agarose gel were examined for bands under UV illumination. The results were photographed in the gel documentation system. To assess the metal tolerance observed in *P. putida* due to the presence of plasmids, attempts were made to cure the plasmids using two methods, such as acridine orange, heat shock, and ethidium bromide treatment. After curing treatment, colonies were checked for their growth on LB plates containing a minimal inhibitor concentration of chromium. There was no loss of ability to grow on metal containing media with the above metal concentration. Since the acridine orange curing was not successful, heat shock (42^{o}C) and ethidium bromide treatment were used for curing but treatment also could not help in curing the plasmids.

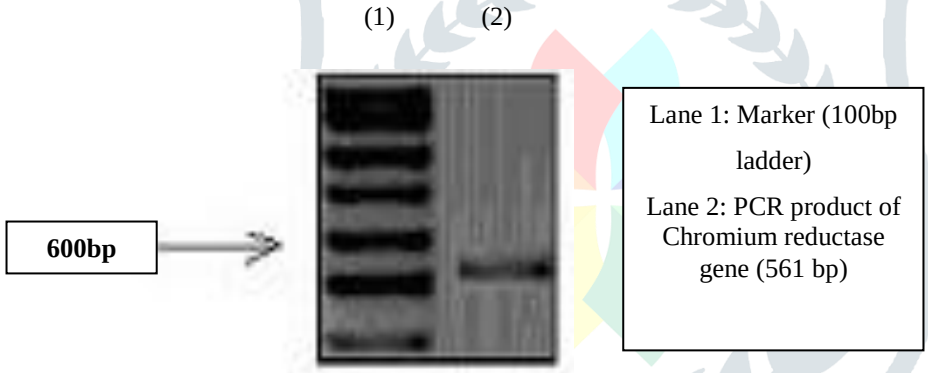

Figure 3: PCR Amplification products

The bacterium after curing showed the same resistance to all the tested concentrations of chromium as it had shown before curing. The curing may not have been very efficient due to several reasons. The curing agents were not strong enough to cure as was the presence of productive curing which might have prevented the entry of the curing agents and the organism might have effluxed the curing agents. One such mechanism might have prevented the curing. This was checked further with the isolation of plasmids from uncured and cured cells. Both the cells exhibited the presence of two plasmids with the same copy number and bands could establish that the plasmids found are responsible for the Cr (VI) resistance mechanisms and to ascertain the resistance to chromate of this isolate was due to plasmid DNA as reported by Cario *et al.,* (2003).

The growth response of *Pseudomonas putida at* different concentrations of tannery effluent represented in Figure 4 revealed the efficiency of chrome reduction in different concentrations of tannery effluent inoculated with *P. putida and P. putida* transformant. The rate of chromium reduction was higher in 25% of effluent dilutions in the presence of *P. putida* transformant *than* that of control (without bacterial culture) and *P. putida* culture inoculated in other dilutions of effluent. The estimation of chrome reductase explained the amount of hexavalent chromium and thereby calculated the toxicity of the effluent. As depicted in Figure 4, the time required for the complete reduction of Cr (VI) was extended with higher concentrations of Cr (VI). The bacteria's capability to reduce Cr (VI) was dependent on its growth. The data from Figure 4 indicates that the *P. putida* transformant was a more effective reducer of Cr.

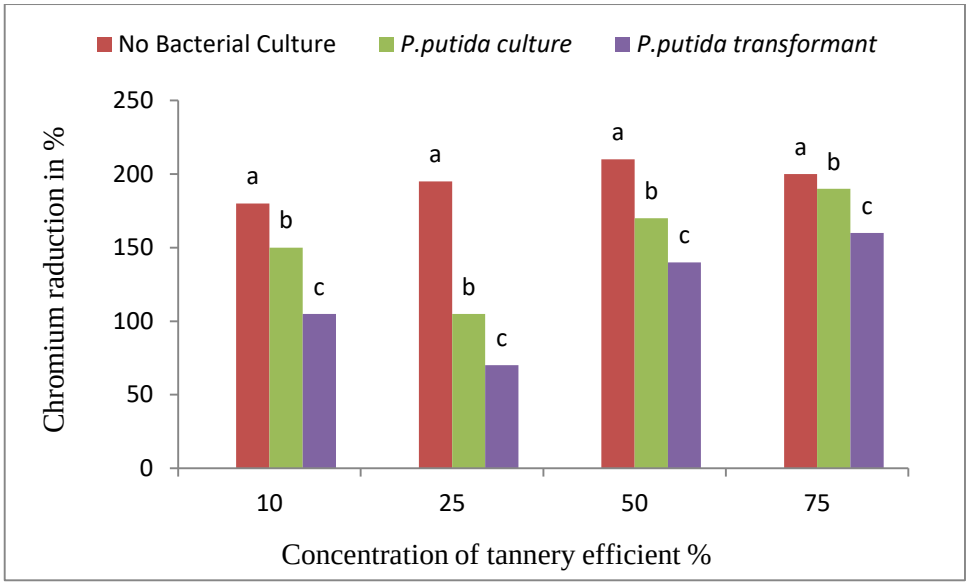

Figure 4: Analysis of Chromium reduction

Legend: X indicated dilution of tannery effluents as 10, 25, 50 and 75 %; Y elucidated the decrease in chromium levels observed in the effluent when exposed to the bacterial cultures of *Pseudomonas putida* and *Pseudomonas putida* transformant culture during the experiments.

The chromate reduction rate and heavy metal resistance of *P.putida* were found to be similar to those of other chromate-resistant bacterial strains typically associated with plasmids, as reported by Malik and Jaiswal (2000). Moreover, plasmids are commonly associated with chromate resistance in natural isolates. Specifically, the Pseudomonas putida Cr (VI) resistant isolate was found to contain plasmids, indicating the presence of a mechanism for both resistance and reduction of Cr (VI) on the Plasmid DNA. Additionally, the strains were subjected to screening to detect the presence of plasmid DNA, which proved to be successful and unveiled the existence of two plasmids.

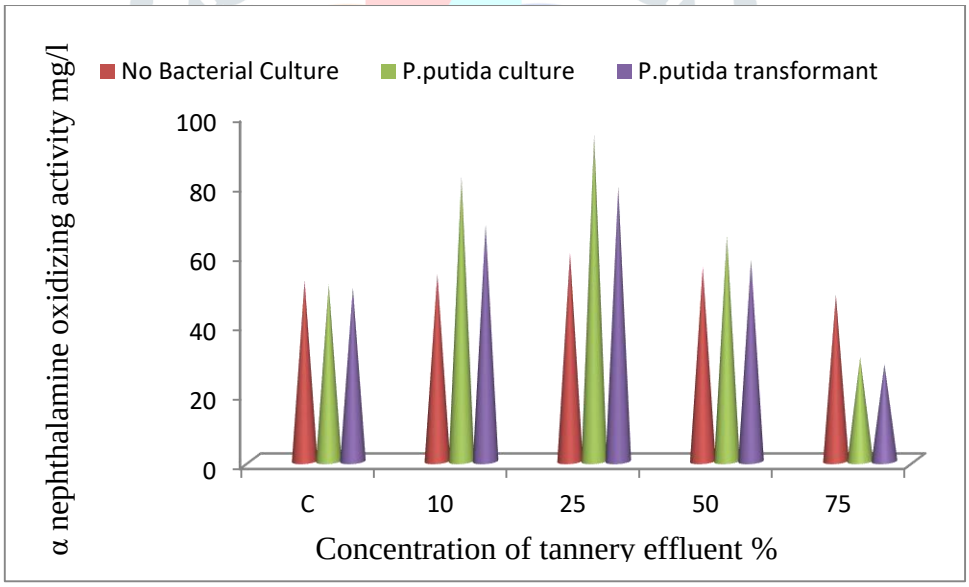

Figure 5: Alpha napthalamine oxidizing root activity of *Oryza sativa* seedling

Legend: X denoted tannery effluents dilution as 10, 25,50 and 75 % ; Y indicated Alpha napthalamine oxidizing root activity of *Oryza sativa* seedling mg/l after treatment of *Pseudomonas putida* and *Pseudomonas putida* transformant culture filtrate treated *Oryza sativa* seeds and its growth responses showed remediation of chromium in tannery effluents.

Alpha napthalamine oxidizing root activity of *Oryza sativa* seedlings was compounded and presented in Figure 5. Increased activities were observed in seeds treated with a 25% dilution of culture filtrate of recombinant strain ChrR gene-transformed strain grown in tannery effluent used as a control. An attempt was made to treat the tannery effluent biologically using chromate reductase coded gene transformations in *P.putida* inoculated at different concentrations of tannery effluent. Hence, the present investigation concluded that root activity showed growth in a 25% dilution. There was a decreased amount of heavy metal chromium in the effluent thereby utilizing nontoxic trivalent chromium for their growth; sufficient minerals were obtained by seedlings for their growth due to the detoxification process of the tannery effluent. However, it was interesting to note that culture filtrate treated seeds showed a higher shoot length and root length.

Pseudomonas putida is a species that is not known to cause any diseases in animals or plants. It is considered to be harmless to the environment as it is a saprophytic organism. This characteristic makes P. putida a promising candidate for the development of a safe strain for recombinant DNA. The efficient ability of Pseudomonas putida to reduce Cr (VI) suggests its potential in the detoxification of contaminated soil and water containing chromium (VI). By utilizing protein engineering techniques on P. putida chromate reductase, it is possible to enhance the efficiency of chromate reduction and minimize chromium toxicity in the presence of pollutants. The chromate reductase enzyme would convert soluble and toxic chromate into insoluble and less toxic Cr(III), thereby reducing the overall toxicity. The research focuses on identifying Pseudomonas putida and its ability to degrade heavy metals and exhibit resistance to metals. This research holds great significance in terms of efficiently treating tannery effluent and remediating chromate from the environment.

IV. CONCLUSION

The composite tannery effluent used for the present study might consist of a mixture of the discharges from all stages of processing**.** In the present work, *Pseudomonas putida* was isolated and characterized for its efficiency in the bioremediation of effluent. The chromate reductase activity of *Pseudomonas putida* enables the reduction of Cr (VI) to Cr (III), which is significantly less soluble and less harmful. Consequently, this enzymatic reduction provides a viable approach for chromate bioremediation. In addition, *P. putida* stains underwent a screening process to confirm the existence of plasmid DNA. This screening proved to be successful, revealing the presence of two plasmids with molecular masses of 9 kb and 4 kb, respectively. In order to enhance the efficacy of bacterial chromate remediation, it is essential to clone the genes that encode the activity of chromate reductase. For this reason, *Pseudomonas putida* has been identified as a promising candidate for the development of a safe strain for recombinant DNA due to its efficient Cr (VI) reducing ability. This bacterium shows great potential for detoxifying Cr (VI) in contaminated soil and waste. The detoxification mechanisms used for removing Cr (VI) through biotransformation and bioremediation can transform harmful pollutants into harmless forms. Thus the biological agents, such as pollutant degrading bacteria, are essential for detoxifying heavy metals and improving bioremediation efforts for tannery effluents.

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