Detoxification hexavalent chromium by potential chromate reducing bacteria isolated from turnery effluent

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Abstract

Hexavalent chromium $Cr6^{+}$ is a non-essential well known carcinogen and the actual mechanisms of chromium toxicity is unknown. Approaches were made for the isolation of $Cr6^{+}$ resistant bacteria from tannery effluent in a common effluent treatment plant, located at Chrompet, Chennai. Five hexavalent chromium resistant bacterial strains such as V1, V2, V3, V4 and V5 were isolated. All the five strains exhibiting high level of resistant to hexavalent Chromium salts and could bear more than 50mg/L of K_2CrO_4 in Nutrient agar medium. Isolated bacterial strains were identified by various biochemical tests. Then the effectiveness of the bioremediation was evaluated with all the five strains. Based on the bioremediation efficiency, the strain (V3) *Psedomonas* sp. was selected for the detoxification experiment which exhibits maximum hexavalent chromium reduction. Hence we conclude that *Psedomonas* sp. isolated from tannery effluent have the potential for the bioremediation of heavy metal contamination.

Key-words: Effluent, Hexavalent chromium, *Pseudmonas* sp., and Reduction.

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Introduction

Contamination of the surface water and groundwater with hexavalent chromium [$Cr6^+$] is an issue of potential concern due to its toxicity [1]. It is well known for its toxic, mutagenic, carcinogenic on human beings and other living organisms and is classified under priority pollutants in many countries [2, 3]. Hexavalent chromium is a transition metal which is highly toxic and carcinogenic compound and is one of the major sources of pollutant. Chromate is generated as a byproduct of a large number and is discharged into the environment through the disposal of the wastes from the industries those engaged in welding, paper, pigment & wood production, leather tanning, chrome plating, metallurgical metal finishing, textiles & ceramics and thermonuclear weapons manufacturing etc.[4]. The wide spread use of chromium indiscriminate the disposal of byproducts and wastes from industrial processes have created serious problems of the environmental pollution in the urban areas and the other ecosystems associated with industrial discharge [5, 6]. Incidence of respiratory diseases among workers exposed to occupational and environmental risks of tannery industry. In many areas of Tamilnadu, groundwater is not suitable for domestic use, which forcing villagers to travel 4-5 km for the availability of water. The most of the groundwater is unsuitable for irrigation and maximum of wells in the region can no longer be used because of contamination. The tanning industry which commonly utilizes “Chroma liquor” for tanning process discharges the effluents into the environment containing chroma salts and mostly the concentration exceeds the maximum permissible limits which may be the cause of environmental risks [7, 8]. The hexavalent $Cr6^+$ compounds are comparatively much more toxic than those of trivalent $Cr3^+$ [9]. The reason for such toxicity appears due to its rapid permeability through the biological membranes and subsequently interaction with the intracellular proteins and nucleic acids [10] and to avoid the toxic effect of $Cr6^+$, it is necessary to convert it to lower toxic form $Cr3^+$. Reduction of $Cr6^+$ has been reported by many researchers in various different bacterial species such as *Desulfovibrio* sp. [11], *Microbacterium* sp. [12] and *Shewanella* sp. [13]. However, the availability of an effective $Cr6^+$ removing bacterial strain is an essential pre requisite for the developing bioremediation process aimed for detoxification of $Cr6^+$ contaminated waste waters. So, the present study was carried out to elucidate the isolation and characterization of potential chromate tolerant bacteria from tannery effluent for its detoxification.

Materials and Methods

Sampling, sample analysis and Enumeration of Tannery effluent microbial count

All the glass and plastic wares used were kept in 1.0 N HNO_3 solution overnight and then thoroughly rinsed with deionized water. The raw wastewater of a common effluent treatment plant, Chennai, Tamilnadu (India) receiving an amalgamated effluent of 25 tanning industries, was collected in sterile glass bottles, transported to laboratory on ice and processed within 6 h of collection. The total number of culturable, aerobic bacteria per milliliter of sample was determined by using nutrient agar plates [14]. About 1 ml of sample was transferred into 99 ml of water and further dilutions were made with 9 ml of water up to 7 dilutions. Hundred µl of the 3-7 dilution samples were used in the spread plate technique. The number of colony forming unit (CFU) were counted after 24 hours of incubation at 30º C.

Isolation of Cr-resistant bacteria

Sample was serially diluted in sterile phosphate buffer (pH 7.2) and spread inoculated on to nutrient agar amended with 50 mg/l of Cr (VI). A filter-sterilized solution of $K_2Cr_2O_7$ was used as the source of Cr (VI), which was added to the sterile molten nutrient agar to prevent problems associated with autoclaving chromate-containing solutions [15]. The inoculated plates were incubated at 30 ºC for 24 h.

Determination of minimal inhibitory concentration (MIC) of Cr (VI)

The minimal inhibitory concentration (MIC) of chromium at which no colony growth occurred was determined by broth agar dilution method. The isolates were inoculated individually into 25 ml peptone water (HiMedia, India) consisting of 1.0% (w/v) peptone and 5.0% (w/v) NaCl in a 150 ml conical flasks and incubated at 28 º C at 150 rpm to achieve log phase cultures. Nutrient agar plates containing different concentrations of Cr (VI) (50–200 mg/l) were inoculated aseptically from the exponential growing cultures of each bacterial strain. These plates were incubated at 37 ºC for 48 h. The MIC was considered to be the lowest concentration of Cr (VI) at which no growth occurred.

Growth kinetics

Growth of Cr(VI) resistance potential bacterial isolate V3 was studied in 1000 ml flasks containing 500 ml of nutrient broth supplemented with potassium dichromate concentrations ranging 20, 50, 100 and 200 mg/l. Flasks were inoculated with 0.2 ml of freshly prepared inoculum and incubated at 30°C with 150 rpm shaking for 48 hrs. Samples were drawn at regular 1h time intervals. The Optical density changes of the culture during growth were recorded at 540 nm using a SHIMADZU spectrophotometer UV-1601model [16].

Reduction of hexavalent chromium by bacteria

Nutrient broth amended with various initial concentrations of chromate ranging from 50 to 200 mg/l was inoculated with selected bacterial isolate V3 culture so as to get an OD of 0.05 from overnight grown culture; it was then incubated at 30°C in a shaker at 150 rpm. 10 ml aliquots were withdrawn at regular 5-h intervals and analyzed for chromium reduction [17].

Cr(VI) analysis

Samples (10 ml) were withdrawn at regular time intervals and centrifuged at 10,000 rpm for 20 min at 30°C. The supernatant was used to measure chromium concentration. Chromium reducing activity was estimated as the decrease in chromium concentration in supernatant with time using hexavalent chromium-specific colorimetric reagent S-diphenyl carbazide (DPC) at 0.25% (w/v) prepared in acetone (AR) to minimize deterioration (Monteiro *et al.* 2002). The reaction mixture was set up in 10 ml volumetric flask containing 200 or 400 µl sample and the same volume of standard $K_2Cr_2O_7$ (10 mg/l) volume was made up to 1 ml using glass distilled water. A further 330 µl of 6 M H_2SO_4 and 400 µl of DPC were added and the final volume was made up to 10 ml using glass distilled water. Optical density was measured immediately at 540 nm using a SHIMADZU spectrophotometer UV-1601 model [17].

Identification and characterization of the best Cr resistant isolates

Five best Cr resistant isolates determined by quantitative studies were identified up to genus level by studying phenotypic characters like gram staining, motility and biochemical characteristics like oxidase, catalase, IMVIC and selective medium. The methods described by

Microbiology: A laboratory Manual by [14] was followed for all the procedures. All these results were compared with Bergey's manual of determinative bacteriology to determine the genus [18].

Results

Enumeration of Tannery effluent microbial count

The total number of culturable, aerobic bacteria per ml of sample was determined by using nutrient agar plates [14]. The results are shown in Table-1.

Table. 1. Enumeration of microbial count from turnery effluent

Sr. No.	Dilution	Colony forming unit per ml
1	10^{-4}	140 CFU/ml
2	10^{-5}	73 CFU/ml

Isolation and screening of Cr-resistant bacteria and determination of minimal inhibitory concentration of Cr (VI)

Effluent was screened for Cr resistant isolates. After incubation the plates were enumerated and all the isolated colonies were sub cultured in nutrient agar medium followed by Cr resistant screening by qualitative minimal inhibitory concentration method. The minimal inhibitory concentrations of the isolated bacterial strains are shown in table-2.

Growth kinetics of V3 bacterial isolate

Growth of $Cr6^{+}$ resistance potential bacterial isolate V3 was studied in 1000 ml flasks containing 500 ml of nutrient broth supplemented with potassium dichromate concentrations ranging 20-200 mg/l. Flasks were inoculated with 0.2 ml of freshly prepared inoculum and incubated at 30°C with 150 rpm shaking for 48 hrs. Samples were drawn at regular interval of

1hr. The Optical density changes in the culture during growth were recorded at 540 nm using a SHIMADZU spectrophotometer UV-1601model [16]. Irrespective of the isolates the lag phase of growth was prolonged with increasing concentration of chromium and it was maximum with isolate V3 presented in figure 1. The extended lag period could be due to acclimatization of bacterial cells with high concentration of Cr^{6+} in the medium. The isolates were grown in Acetate minimal medium supplemented with 20 mg Cr^{6+}/l (■), 50 mg Cr^{6+}/l (▲),100 mg Cr^{6+}/l (○) and 200 mg Cr^{6+}/l (∗) under 150 rpm shake condition at 30°C.

Table. 2. Determination of minimal inhibitory concentration of Cr6+ by bacterial strains

Sr. No	Bacterial Isolates	Minimum inhibitory concentration (MIC)
1.	V1	50 mg/L
2.	V2	100 mg/L
3.	V2	200 mg/L
4.	V4	50 mg/L
5.	V5	50 mg/L

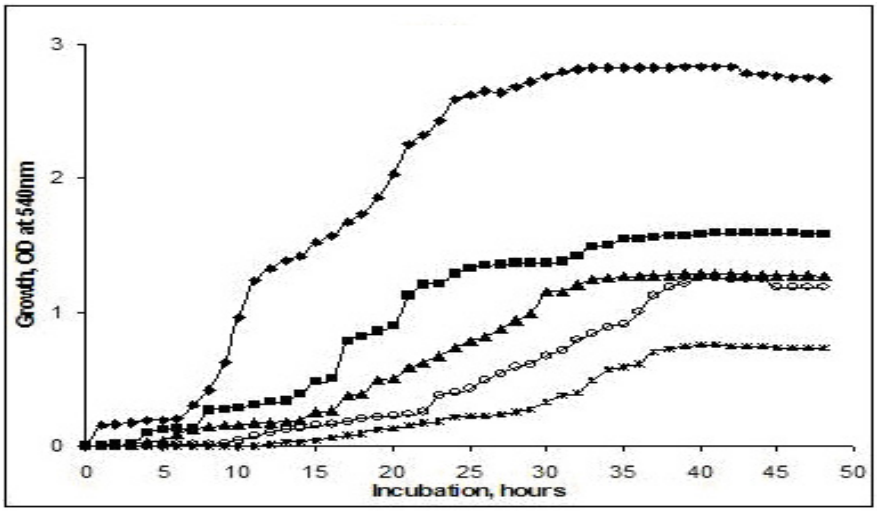

Figure 1. Growth kinetics of selected chromium resistant bacterial isolates.

$Cr6^+$ reducing capacity of V3 bacterial isolates

Based on the result obtained by MIC analysis V3 isolate was selected for further study. Nutrient broth amended with various initial concentrations of chromate ranging from 50 to 200 mg/l was inoculated with selected bacterial isolate V3 culture so as to get an OD of 0.05 from overnight grown culture. After inoculating the culture it was incubated at 30°C, under aerobic condition at pH 7 in a shaker at 150 rpm. 10 ml aliquots were withdrawn at regular 5hr intervals and analyzed for chromium reduction. V3 (*Pseudomonas* sp.) strain could reduce 50, 100, 150 and 200 mg/l of chromate maximally to zero at 30, 55, 60 and 80 hours, respectively shown in the figure 2. V3 strain showed very high level resistant against Potassium Chromate both in Nutrient Broth and Nutrient Agar.

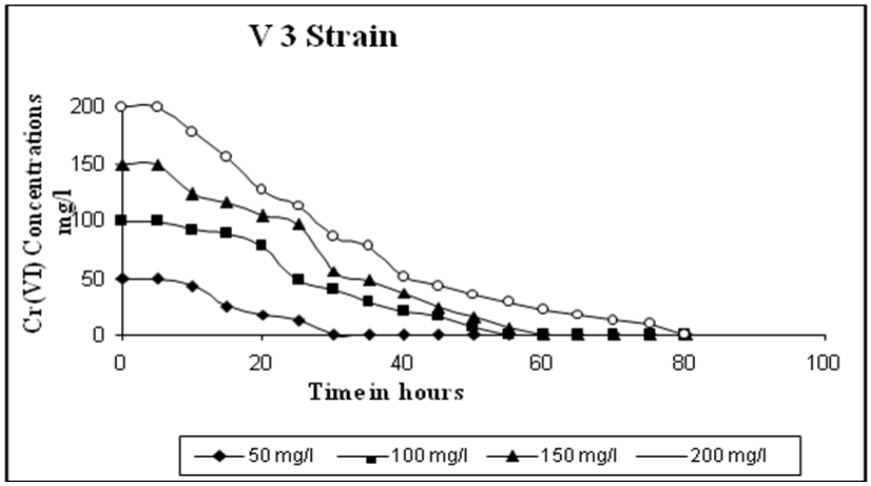

Figure 2. Chromate reduction by potential V3 bacterial isolate.

Identification and characterization of the best Cr resistant isolates

All the five chromium resistant isolates were identified up to genus level by studying phenotypic characters like Gram staining, motility, endospore staining and biochemical characteristics like oxidase, Indole, Methyl Red, VP, Citrate and selective medium by using the standard method described by Cappuccino [14]. All these results were compared with Bergey's manual of determinative bacteriology to determine the genus [18]. According to the biochemical

tests the bacterial strains V1, V2, V3, V4 and V5 were identified as *Bacillus* sp., *Vibrio* sp., *Pseudomonas* sp., *Salmonella* sp. and *E. coli* respectively by comparing the observed results with Bergey's manual of determinative bacteriology.

Discussion

Five morphologically distinct bacterial colonies V1, V2, V3, V4 and V5 was screened and identified up to genus level by studying phenotypic characters using various biochemical tests and identified as *Bacillus* sp., *Vibrio* sp., *Pseudomonas* sp., *Salmonella* sp. and *E. coli* respectively by comparing the observed results with Bergey's manual of determinative bacteriology which shows very high level of resistant against Potassium chromate both in Nutrient broth and Nutrient agar similarly high level of resistant was also reported [19] from the industrial effluent. Srinath *et al.*, [20] obtained seventy one morphologically distinct $Cr6^+$ resistant bacterial strains were screened for their $Cr6^+$ tolerance limit from common effluent treatment plant at Unnao. The growth kinetics V3 strain was also checked in Acetate minimal medium supplemented with different concentration of $Cr6^+$ under continuous shaking condition at 30^oC and OD was measured at regular interval which shows the chromium tolerance. Elangovan *et al.*,[21] isolated *Bacillus* sp. from chromate contaminated soil, which reduces $Cr6^+$ from 80 to 40mg/l after 42 hours in a nutrient medium. Megharaj and Avudainayagam[22] isolated *Arthrobacter* sp. from a long term tannery waste contaminated soil, which was examined for reduction of hexavalent chromium and found to be able to reduce 30mg/l of $Cr6^+$ during 46 hrs incubation and did not show any $Cr6^+$ reduction at 100 mg/l $Cr6^+$ concentration during this incubation period. According to Ganguli and Tripathi [23] the normal growth of *Pseudomonas aeroginose* was 42 minutes but with the addition of 0.1mg/ml of $Cr6^+$ increased time to 57 minutes. All the strains were able to reduce $Cr6^+$ after different incubation period. The reduction of $Cr6^+$ has also been reported by other investigators. Bacterial strains such as *Desulfomicrobium norvegicum* [24], *Bacillus*[25], *Shewanella oneidensis*[26,27,28], *Desulfovibrio* sp.[29], *Escherichia coli* [30], *Pseudomonas* sp.[31] and *Alcaligenes* [32] have been reported successfully reduce $Cr6^+$ to

$Cr3^+$. In the present investigation the V3 strain (*Pseudomonas* sp.) shows the maximum MIC value of 200mg/L of chromium growth tolerance and also found to the reduce different initial concentration of chromium up to zero after different incubation time period. This V3 strain could reduce maximum of the $Cr6^+$ after 40hrs of incubation period. Hardoyo and Ohtake [32] have also observed $Cr6^+$ reduction in the industrial effluent collected from metal fishing plant. They found that in *Enterobacter cloaca* HO1, significant inhibition in $Cr6^+$ reduction was observed when the effluent contain 220µg/ml of $Cr6^+$, 25 µg/ml of Cu, 26 µg/ml of Mn and 0.02 µg/ml of Zn. In another study Ganguly and Tripathi, [23] also observe reduction of $Cr6^+$ present in electroplating effluent with the help of *Pseudomonas aeruginosa* A2Chr in two bioreactor system. Thus, microorganisms having combined abilities of resistant to Chromate and its biotransformation to a non-toxic form of Chromium are potentially useful for detoxification of Chromate polluted waste waters. Industrial workplaces provide novel enrichment environments for natural selection of potent strains of metal resistant bacteria since there is little regulatory control over the industrial emissions and thus the environmental burden is severe. The present study highlights the prevalent occurrence of a chromate tolerant microbial population, which can reduce considerable amounts of chromate in tannery effluents. All the selected strains but especially *Pseudomonas* sp.V3 shows the best result with respect to others which suggest that hexavalent chromium reduction of the nature can be economical.

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