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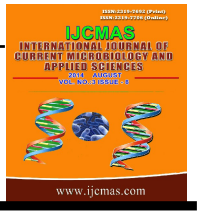

Original Research Article

Biosorption and Detoxification of Cr(VI) by Tannery Effluent Acclimatized Halotolerant Bacterial Strain pv_{26}

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Keywords

Biosorption, Chromium, Phytotoxicity, Pollution and Tannery Effluents.

A B S T R A C T

Tannery industries consume a considerable amount of water in their manufacturing processes and it has been rated as the most polluting among the industrial sectors. Tanneries serve as a major source of Chromium pollution, releasing about 40 – 25,000 mg/l of Cr in their effluents. Cr(VI) has been designated as a priority pollutant by the United States Environmental Protection Agency (USEPA) due to its ability to cause mutations and cancer in humans. Therefore treatment of tannery effluent containing hazardous compounds becomes necessary prior to their final discharge into the environment. In the present study, Five morphologically distinct Cr(VI) resistant bacterial strains designated as TVU-K1 to TVU-K5 were isolated from the treated tannery effluents were subjected for MIC. TVU-K1 (*Bacillus* Sp. Strain PV_{26}) isolate showed remarkable tolerance (upto 400 mg l^{-1}) towards Cr(VI). Atomic Adsorption Spectroscopy revealed that TVU-K1 showed 81% of Cr(VI) reduction and phytotoxicity assay using *Vigna cating* and *Phaseolus mungo* revealed the complete detoxification of Cr(VI).

Introduction

Leather industry in India, is one of the greatest contributors towards the economy of the nation. In India, thousands of industrial tannery units are spread mostly across Tamil Nadu, West Bengal, Uttar Pradesh, Andhra Pradesh, Karnataka, Maharashtra, Rajasthan and Punjab. Kanpur, which is otherwise known as the "Leather City of the World has over 1600 functional leather manufacturing units producing semi-finished, finished and value-added products. As per an investigation by the Inter Ministerial Group, India's leather export should approach the US $ 7 billion mark present year. Tannery industries consume a considerable amount of water in their manufacturing processes and it has been rated as the most polluting among the industrial sectors. Tanneries serve as a major source of Chromium pollution, releasing about 40 – 25,000 mg/l of Cr in their effluents. In addition, leakage due to improper handling and faulty storage containers also adds to the accumulation of chromium in the environment (Singh *et al*., 2011; Achal *et al*., 2005). The major users

of chromium are the metallurgical, chemical, and refractory brick industries. Other industries that employ chromium include pigment manufacture, metal finishing, corrosion inhibition, organic synthesis, leather tanning, and wood preservation (Sharma and Adholeya, 2012). According to WHO standards, the maximum permissible limit for total Chromium and Cr(VI) in drinking water is 2 mg/l and 0.05 mg/l respectively (Gupta and Rastogi, 2009).

Chromium, the 24th element on the periodic table, was first discovered in Siberian red lead ore (crocoite) in 1798 by the French chemist Nicholas-Louis Vauquelin. He named this new mineral chrom from the Greek word χρωμα, owing to the brilliant hues of the compound. It is a transition element located in the group VI-B of the periodic table with a ground-state electronic configuration of Ar 3d5 4s (Shanker *et al*., 2005). Chromium is a naturally found in many foods and drinking water, thus it makes its way into the body mainly from dietary intake. In addition, intake of chromium results from airborne dusts and mists, and cigarette smoke as well as from industrial and occupational exposures (Katz and Salem, 1994). In trace amounts, chromium is an essential component of human and animal nutrition (Mertz, 1993). It is associated with glucose metabolism and has been shown to be an integral component of glucose tolerance factor (GTF). Chromium functions by regulating and potentiating insulin action by increasing insulin binding to cell. Chromium is also known to be of importance in fat metabolism in animals (Anderson, 1989). The deficiency of chromium has been implicated in impaired insulin action, which can cause glucose intolerance, elevated glucose blood levels, diabetes, elevated cholesterol levels, obesity and heart diseases. Chromium is an essential micronutrient required for the growth of many microorganisms for the maintenance of normal glucose, cholesterol and fatty acid metabolism (Srivastava and Thakur, 2006).

In nature, Cr exists in 2 stable oxidation states: Hexavalent Chromium and Trivalent Chromium species with different chemical and biological characteristics (Cervantes *et al*., 2001). Characteristics like higher solubility, rapid permeability through biological membranes and subsequent interaction with nucleic acid and intercellular proteins makes Cr(VI) comparatively more toxic than Cr(III) (Sharma and Adholeya, 2012). At elevated levels, Cr(VI) compounds damage cell membranes, alter enzyme specificity, distrupt cellular functions and damage the DNA structure (Bruins *et al*., 2000) posing serious health hazards to all forms of life including humans, plants, animals and fishes (Srinath *et al*., 2002). Due to its carcinogenicity and mutagenicity, United States Environment Protection Agency (USEPA) has designated as a "Priority Pollutant" or Class "A" pollutant. The most commonly used conventional processes to remove Cr(VI) are: (a) reduction to Cr(III) followed by precipitation as chromium hydroxide, (b) removal by ion exchange and (c) removal by adsorption. These methods are costly due to operational, treatment and sludge disposal costs (Fiol *et al*., 2008). Recently, research for new and innovative technologies has centered on the biological treatment methods (Morales-Barrera *et al*., 2008). The two main biological treatment processes under investigation are: the adsorption of Cr(VI) onto microbial cells (i.e. biosorption), and the reduction of Cr(VI) to Cr(III) by enzymatic reaction or indirectly by reducing compounds produced by micro-organisms (i.e. biotransformation) (Cheung and Gu, 2003; Desjardin *et al*., 2003).

The biological reduction of hexavalent chromium has attracted increased interest, since this process may not only relieve the toxicity of chromium that affect living organisms, but may also aid in the precipitation of chromium at near-neutral pH (mainly as $Cr(OH)_3$) for further physical removal (Cheung and Gu, 2003). Microbial tolerance to Cr(VI) and reduction of Cr(VI) have been reported to be independent phenomena (Megharaj *et al*., 2003; Thacker *et al*., 2007). However, for reduction of Cr(VI) the cells should be able to tolerate Cr(VI) otherwise cell growth is inhibited. Chromium(VI)-reducing bacteria have also been isolated and characterized from chromium-contaminated soil, wastewater and industrial effluents (Pal and Paul, 2004). Bacteria can reduce Cr(VI) under aerobic or anaerobic conditions through electron-transport systems containing cytochromes. The process involved in Cr6+ reduction can be under aerobic or anaerobic conditions (Cheung and Gu, 2007). Bioreduction of Cr(VI) can occur directly as a result of microbial metabolism (enzymatic) or indirectly mediated by a bacterial metabolite, such as H_2S (Sultan and Hasnain,2005).

In view of the potential applications of Cr(VI) reduction, the present study was aimed to isolate and enrich the Cr(VI) resistant strains from the tannery effluents and to mediate biosorption and detoxification of hexavalent Chromium into non-toxic compound.

Materials and Methods

Sampling of Tannery Effluent

The tannery effluent was collected from the release point of common effluent treatment plant (CETP) of tanneries located at Ranipet in sterile plastic containers, transported in an ice box to the laboratory and processed for bacterial analyses within 6-8 h of collection. The sample was further stored at 4°C for physico-chemical and heavy metal analyses in the laboratory.

Physico-Chemical and Heavy Metal Analyses

Salinity, pH and conductivity, total solids (TS), total dissolved solids (TDS) and total suspended solids (TSS) were determined gravimetrically. Total alkalinity, DO, BOD, COD, sulphate, chloride, phosphate, nitrate, total nitrogen, fluoride and phenol were measured as per the standard methods of APHA (1998). The heavy metal content in the treated effluent was estimated by digestion of samples with concentrated nitric and perchloric acid (6:1) mixture till a clear solution was obtained (APHA, 1998). The heavy metals in the digest were then determined using atomic absorption spectrophotometer (Perkin Elmer model 5000). AAS grade metal solutions (Sigma Aldrich Chemicals, USA) were used as standards.

Enrichment of Cr(VI) Resistant Bacterial strains

For the isolation of Cr resistant bacteria, 10 ml of tannery effluent sample was inoculated into 250 ml Erlenmeyer's flask containing 100 ml sterilized Nutrient broth enriched with 1 ml of $K_2Cr_2O_7$ solution to make the chromium concentration of 200 mg l^{-1}. The flasks were incubated at 37°C for 48 h. Following incubation, 10ml of the broth culture was transferred to the freshly prepared Nutrient medium having same composition. The same procedure was successively repeated for 4 times to achieve the complete enrichment of chromium resistant bacterial culture (Tambekar and Gayakwad, 2013).

Isolation of Cr(VI) Resistant Bacterial isolates from Tannery Effluent

1ml of the enriched sample was serially diluted and plated onto Nutrient agar plates containing Cr(VI) to make the chromium concentration of 200 mg l^{-1} and incubated at 37°C, pH 7.0 for 48 h (Verma and Maurya, 2013). Following incubation, the well isolated and morphologically distinct colonies were selected and subcultured repeatedly to obtain pure cultures of Cr(VI) resistant bacteria. For frequent use, the culture was maintained by transfer to a fresh medium at 24 h intervals. When required for prolonged periods, it was maintained by sub-culturing once every 7 days on slants, prepared by solidifying the above mentioned medium with 2.0 (w/v) agar.

Characterization of the Cr(VI) Resistant isolates

The Cr(VI) resistant isolates (TVU-KI - TVU-K5) were examined by the following methods and compared with Bergey's manual of determinative Bacteriology (9th edition).

(i) Colony morphology
(ii) Microscopic examination

(i) Colony morphology

Cr(VI) resistant strains isolated from the tannery effluents were streaked aseptically in Cr(VI) supplemented Nutrient agar plates and incubated at 37°C for 48 h. After incubation, the colony morphology was investigated by using standard microbiological criteria, with special emphasis on pigmentation, diameter, colonial elevation, margin, consistency and opacity (Vijayanand *et al*., 2012).

(ii) Microscopic examination

Gram's staining was performed and the Cell morphology (Gram reaction, Cell shape & arrangement) was examined by light microscopy of the exponentially growing liquid cultures.

Determination of Minimum Inhibitory Concentration (MIC)

The Minimum Inhibitory Concentration (MIC) of five Cr(VI) resistant bacterial isolates (TVU-K1, TVU-K2, TVU-K3, TVU-K4, TVU-K5) were determined by broth dilution method (Calomoris *et al*., 1984) in LB medium with gradually increasing Cr(VI) concentrations ranging from 100 – 600 mg l^{-1}. The minimum concentration of Cr(VI) in the medium inhibiting the complete growth of the isolates were taken as the MIC. Two extremely tolerant isolates (TVU-K1, TVU-K2) were selected for further analysis. Simultaneously, MIC of the isolates were determined by Agar Dilution method.

Cr(VI) Reduction Analysis by DPC Method

The Cr(VI) reduction ability of bacterial isolates (TVU-K1, TVU-K2) were examined by inoculating 1ml of bacterial culture in 100 ml of LB broth amended with 400 mg l^{-1}. After every 24 h of incubation, aliquots were withdrawn, centrifuged at 10,000 rpm for 10 min at room temperature. Hexavalent chromium in the supernatant was determined calorimetrically with a spectrophotometer using the S-diphenylcarbazide (DPC) method (Camargo, *et al*., 2003). The absorbance of the color reduced was measured at 540 nm using a spectrophotometer.

Cr(VI) Reduction analysis by Atomic Adsorption Spectroscopy

50ml of the LB broth enriched with chromium (200 ppm) was incubated with the

bacteria isolates. Media without any bacterial inoculation was considered as control. After 72 h of incubation bacterial biomass were separated by centrifuging at 10,000 rpm for 10 min. Supernatant was subjected for AAS (Farag and Zaki, 2010). The percentage of reduction was determined by the following equation.

% of Reduction = (A-B) / A × 100

A = Cr(VI)Concentration in control
B = Cr(VI)Concentration in Test sample

Phytotoxicity Analysis

Phytotoxicity tests were performed inorder to assess the toxicity of the untreated and treated Cr(VI) sample. The ethyl acetate extracted products of Cr(VI) reduction were dried and dissolved in 5 ml sterile distilled water to make a final concentration of 100 ppm for phytotoxicity studies. The phytotoxicity tests were carried out on two kinds of seeds, *Phaseolus mungo* L. and *Vigna Cating* commonly practiced in Indian agriculture (Parshetti *et al*., 2006).

The study was carried out at normal room temperature. 10 healthy plant seeds of each variety were treated separately with 5 ml of control, Cr(VI) and its reduced products (100 ppm) per day. Control set was carried out using distilled water at the same time. Germination percentage as well as the length of plumule and radical was recorded after 7 days (Saratale *et al*., 2009).

Results and Discussion

Physico-Chemical Analyses of Effluent

The average temperature at the sampling site was around 35°C at day time. The physico-chemical characteristics of the treated tannery effluent sample were shown in the Table 1. The effluent was yellowish-cream in colour and its pH was 8.8 having conductivity of 11,230 moles/ cm. The coloured effluent might hinder the penetration of sunlight causing the depletion in the rate of oxidation process and thus contributing to the anaerobic oxidation which can be sensed from the putrefying odour of the receiving water bodies (Hemapriya *et al*., 2013). The level of BOD, COD, TDS, TSS, phenol, fluoride, phosphate, sulphate and nitrate were well above the permissible limits (Table 1).

The total nitrogen and chloride concentration were within the permissible limits. The slightly alkaline pH of treated effluent could affect biological property of the receiving water body. Increasing alkalinity results in increased conductivity which alters the chelating property of water bodies and creates an imbalance of free metal availability for flora and fauna. Phenols are also discharged in significant amounts.

The exposure to chromium increases the risk of dermatitis, ulcer, lung cancer, immunodeficiency and neurological disorders (Verma and Maurya, 2013). The low DO of treated effluent suggested an increase in the organic matter. As the number of aerobic organisms increases, the demand for oxygen increases proportionately (Sharma and Adholeya, 2012). The value of COD observed was higher when compared to the BOD value. Similar results were also observed by Verma and Maurya, 2013.

Enrichment and Isolation of Cr(VI) resistant bacteria

The effluent sample was acclimatized with 200 mg l^{-1} of Cr(VI) as the sole carbon and energy source to isolate potent bacterial strains to reduce toxic Cr(VI) from the

polluted environment. Acclimatization to toxic substances is considered as the best statergy to overcome substrate inhibition (Srivatsava *et al*., 2007). Five morphologically distinct Cr(VI) resistant bacterial strains designated as TVU-K1 to TVU-K5 were isolated from the treated tannery effluents. The morphological and cultural characteristics of the isolates were listed in Table 2. Cr(VI) degrading bacterial isolate *Bacillus subtilis*. was isolated from tannery effluent contaminated soil in Kanpur, India (Sharma and Adholeya, 2012). Many other strains have been reported to reduce Cr(VI) including *Nesterenkonia* sp. strain MF2 (Amoozegar *et al*., 2007) and *Sphaerotilus natans* (Caravelli *et al*., 2008). Tambekar and Gayakwad. (2013) reported the isolation and characterization of Cr(VI) resistant bacteria isolated from alkaline Lonar lake (MS), India.

Maximum Tolerance Limit (MTL)

MIC of the bacterial isolates towards Cr(VI) was evaluated to determine their maximum tolerance limit by both broth dilution and agar dilution methods. The MIC of the isolates (TVU-K1 to TVU-K5) ranged between 200 mg l^{-1} to 400 mg l^{-1} (Fig.1). Microbial load decreased with increase in Cr(VI) concentration. This result was in complete accordance with *Actinetobacter* sp. isolated from pulp-paper industry that showed tolerance up to 500 mg l^{-1} of Cr(VI) (Srivatsava *et al*., 2007). In contrast, the rest of the isolates were found to be susceptible to the increase in Cr(VI) concentration. TVU-K1 isolate showed remarkable tolerance (up to 400 mg l^{-1}) towards Cr(VI) and was selected and subjected for further studies. Based on 16 S r DNA analysis, TVU-KI was identified as *Bacillus* Sp. Strain $PV_{26.}$ The advantage of textile effluent adapted bacterial strains for bioremediation enhances dues to the minimization of toxic effect of other pollutants co-existing with Cr(VI) (Verma and Maurya, 2013).

Cr(VI) Reduction Analysis

Cr(VI) reduction by acclimatized bacterial isolate *Bacillus* Sp. Strain PV_{26} started gradually after 12 h of incubation and steadily increased with increase in incubation time. Maximum Cr(VI) reduction by the bacterial isolate was found to be achieved after 24 h (Fig .2). The result obtained was in contrast with the previous findings of Tambekar and Gayakwad. (2013). Further the Cr(VI) reduction efficacy of the isolates were investigated by Atomic Adsorption Spectroscopy. TVU-K1 showed 81% of Cr(VI). Similar results were reported by many researchers (Farag and Zaki, 2010, Camargo, *et al*., 2003).

Phytotoxocity Assay

Phytotoxicity tests were performed inorder to assess the toxicity of the untreated and treated Cr(VI) sample. *Vigna cating* seeds treated with tap water showed 100% germination, the mean plumule length of 15 cm and the mean radical length of 5 cm. In contrast, the seeds treated with untreated Cr(VI) sample showed only 90% germination, the mean plumule length of 10 cm and the radical length of 3 cm. Whereas, the seeds treated with treated Cr(VI) sample showed 100% germination, the mean plumule length of 13 cm and the radical length of 4 cm. *Phaseolus mungo* seeds treated with tap water showed 100% germination, the mean plumule length of 17 cm and the mean radical length of 3 cm. In contrast, seeds treated with untreated Cr(VI) sample showed only 40% germination, the mean plumule length of 3 cm, the mean radical length 1 cm, whereas, the seeds

treated with treated Cr(VI) sample showed 100% germination, the mean plumule length of 16 cm, the radical length of 2 cm. The result indicated that the extracted metabolites contains non-toxic metabolites, resulting in good germination rate as well as significant root and shoot length of *Vigna cating* and *P.mungo* when compared to Cr(VI) sample, where inhibition in all these parameters was observed (Table 3 and 4).

Table.1 Physico-chemical characteristics of Tannery effluent

Parameter	Effluent*	Permissible limit
pH	8.8	6.0-8.0
Conductivity (moles/ cm)	11,230	850
Alkalinity (mg/ L)	645	500
Total solids (TS) (mg/ L)	2,450	2,200
Total dissolved solids (TDS) (mg/ L)	2,300	2,100
Total suspended solids (TSS) (mg/ L)	282	100
DO (mg/ L)	2.8	4.0-6.0
BOD (mg/ L)	240	30
COD (mg/ L)	460	250
Sulfate (mg/ L)	2,290	1,000
Chloride (mg/ L)	350	600
Magnesium (mg/ L)	250	200
Phosphate (mg/ L)	5.5	5.0
Nitrate (mg/ L)	11.60	10
Total nitrogen (mg/ L)	235	780
Fluoride (mg/ L)	4.0	2.0
Phenol (mg/ L)	10.0	1.0
Oil and grease	15.9	10

Table.2 Cell and Colony Morphology of Cr(VI) Resistant Bacterial Strains

Strain	Cell Shape	Gram Staining	Cell Arrangement	Colony Morphology	Colony Size	Colony Elevation	Colony Density	Pigmentation
TVU-K1	Bacilli	G+ve	Single and paired cells	Circular	2.5mm	Convex	Mucoidal and glistening	Cream
TVU-K2	Bacilli	G-ve	Single, paired and regularly clustered cells	Circular	1-3mm	Slightly raised	Transparent and matt	Cream
TVU-K3	Cocci	G+ve	Single, paired and long chains	Circular	2.0mm	Convex	Translucent and matt	Yellowish cream
TVU-K4	Cocci	G+ve	Single, paired and irregularly clustered cells	Circular	0.5 - 1.5mm	Slightly convex	Transparent and glistening	Dark cream
TVU-K5	Bacilli	G-ve	Single, paired and short chains	Irregular	1.5-2.5mm	Slightly raised	Transparent and glistening	White

Table.3 Phytotoxicity study of Cr(VI) and its reduced products on *Vigna cating*

Parameters	Tap Water	Cr(VI)	Treated sample
Germination (%)	100	90	100
Plumule (cm)	15.0	10.0	13.0
Radical (cm)	5.0	3.0	4.0

Table.4 Phytotoxicity study of Cr(VI) and its reduced products on *Phaseolus mungo*

Parameters	Tap Water	Cr(VI)	Treated sample
Germination (%)	100	40	100
Plumule (cm)	17.0	3.0	16.0
Radical (cm)	3.0	1.0	2.0

Fig.1 MIC of the isolates (TVU-K1 to TVU-K5)

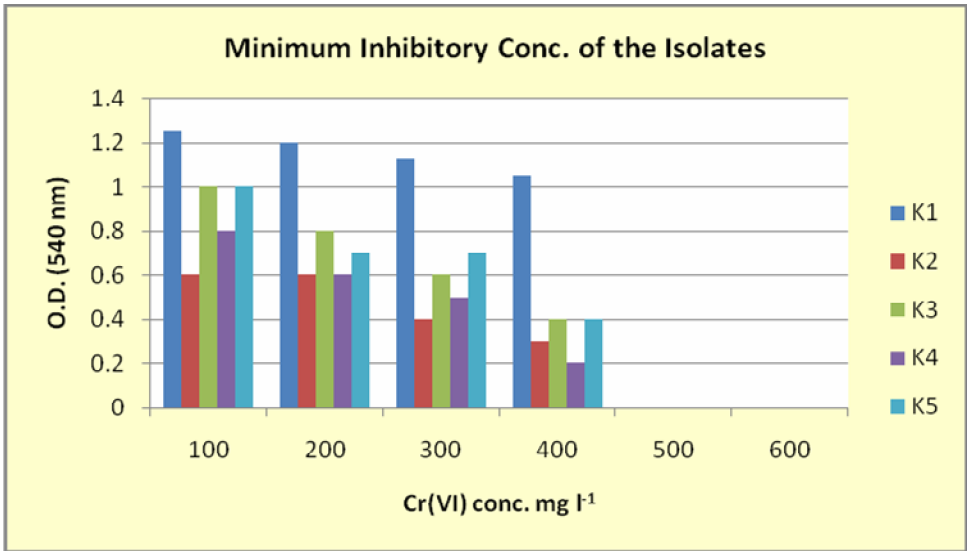
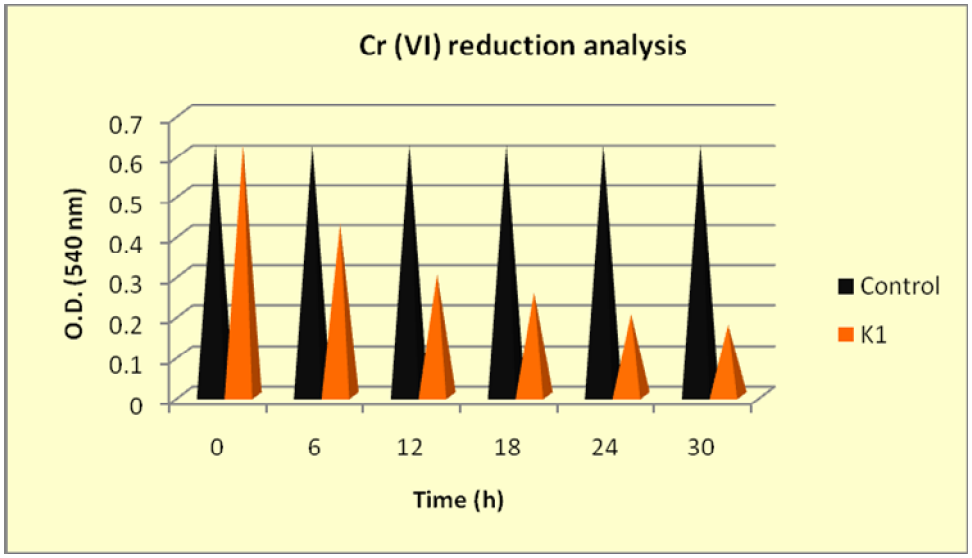


Fig.2 Cr(VI) Reduction Analysis by DPC Method



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