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Isolation of potential bacteria from tannery effluent capable to simultaneously tolerate hexavalent chromium and pentachlorophenol and its possible use in effluent bioremediation

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----------ABSTRACT----------
Environmental contamination of hexavalent chromium [Cr(VI)] and pentachlorophenol (PCP) has become a serious concern for tannery effluent. This study was conducted to isolate few efficient bacterial strains from treated tannery effluent which is tolerant to both Cr (VI) and PCP and could be employed for simultaneous bioremediation of both the pollutants. Fifteen bacteria resistant to high Cr(VI) and PCP concentrations simultaneously were isolated from tannery effluent by enrichment culture technique. Among them, five bacteria having high MIC values for Cr(VI) (850-950 mg/ L) and PCP (1100-1350 mg/ L) were selected for further studies. They exhibited remarkable ability to tolerate the presence of different heavy metals in addition to Cr(VI) and PCP. These bacteria appears to have great potential for simultaneous detoxification of Cr(VI) and PCP from tannery and other industrial effluents.

KEYWORDS: *Tannery effluent, Hexavalent chromium, Pentachlorophenol, Bacteria and Simultaneous bioremediation.*

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I. INTRODUCTION

In India, tanneries are one of the well developed industrial sectors. Unfortunately, they release huge amount of chromium and pentachlorophenol (PCP) through their effluent into the environment beyond the permissible limits due to the inefficient effluent treatment technology (Srivastava *et al*., 2007). In addition to tanneries, chromium is used together with chlorophenols including PCP in various industries such as petroleum refining, wood preservation, steel plants, coal conversion process, etc. (Shen and Wang, 1995). Thus, chromium and chlorophenols coexistence are introduced in water bodies and agricultural lands from the discharge of these industries. The predominant forms of chromium present in environment are the trivalent [Cr(III)] and hexavalent chromium [Cr(VI), chromate], in which Cr(VI) is dominant, highly soluble, rapidly permeable through sulfate transport system followed by interaction with proteins and nucleic acids, and also toxic, mutagenic and carcinogenic to all forms of life (Ackerley *et al*., 2004). During tanning, chromium salt is used to convert hide to leather and the wastewater generated is discharged into the environment which contains excess amount of Cr(VI).Pentachlorophenol (PCP) is used as a biocide in the leather manufacturing process. Pentachlorophenol being a polychlorinated aromatic compound is recalcitrant to biodegradation. PCP is carcinogenic and highly toxic to living beings as it causes inhibition of oxidative phosphorylation, inactivation of respiratory enzymes and damage to mitochondrial structure (Bevenue and Beckman, 1967). This has become a matter of prime concern since they are listed as priority pollutants by Environmental Protection Agency (EPA) and World Health Organization (WHO) (EPA, 1999; 2000). Removal of such toxic and persistent contaminants is highly imperative as they get biomagnified along the trophic level. Water supplies are also contaminated through leaching.

The presence of high levels of chromate and PCP in the environment also has an inhibitory effect on most microorganisms but some of them have evolved resistance mechanisms that lead to the selection of resistant variants that can tolerate their toxicity (Tziotzios *et al*., 2008; Huaxiao *et al*., 2009; Tripathi and Garg, 2010; Verma and Singh, 2012). Microbial reduction of Cr(VI) to Cr(III) is an efficient means of chromate detoxification (Verma *et al*., 2009) and reductive dechlorination has been suggested as the primary PCP biodegradation mechanism (Thakur *et al*., 2001) resulting in partially or fully dechlorinated product which is then more susceptible to ring cleavage (Kao *et al*., 2005). Several reports have been published on microbial detoxification of either Cr(VI) (Camargo *et al*., 2003; Rehman *et al*., 2008; Verma *et al*., 2009; Singh *et al*., 2013) or PCP (Premlatha and Rajkumar, 1994; Chen and Yang, 2008; Karn *et al*., 2011). However, there is very scanty information on bacteria,

particularly indigenous strains that could simultaneously bioremediate both Cr(VI) and PCP of industrial effluents (Shen and Wang, 1995; Liu *et al*., 2008; Tziotzios *et al*., 2008; Huaxiao *et al*., 2009; Tripathi and Garg, 2010; Verma and Singh, 2012). Also, majority of the strains reported were not able to survive in higher Cr(VI) and PCP concentration as they were not isolated from native environment, rather procured as a pure culture. Besides this, the removal of chromate and PCP took ~ 12-15 days. But such a long time for removal of Cr(VI) and PCP may lead to generation and accumulation of metal and toxic compounds in the environment. Efficient removal of these toxic contaminants in short time is the basic necessity of present time. These factors also limit the success of microbial bioremediation of Cr(VI) and PCP from tanneries at large scale. However, there are still many unknown bacteria in the industrial waste dumping sites that may have the exceptional ability to adapt and colonize such noxious polluted environments. These indigenous bacteria may contain tremendous bioremediation capacity for both the Cr(VI) and PCP simultaneously and can be utilized in the development of technologies for the simultaneous biological treatment of Cr(VI) and PCP contaminated waste prior to its release in lands and water bodies. Keeping it in view, the present study was aimed to isolate potential bacteria from the treated tannery effluent (natural environment) that could resist high concentration of Cr(VI) and PCP simultaneously for its possible use in bioremediation of dual pollutants of tannery effluent.

II. MATERIALS AND METHODS

2.1 Culture Media, Reagents and Chemicals

Bacteria were grown in liquid minimal salt medium (MSM, pH 7.5) containing (g/ L) K_2HPO_4 (1.73), KH_2PO_4 (0.68), $(NH_4)_2SO_4$ (1.0), $MgSO_4.7H_2O$ (0.1), $FeSO_4.7H_2O$ (0.02), $CaCl_2.7H_2O$ (0.03), $MnSO_4.7H_2O$ (0.03) and glucose 0.1% as a co-metabolite. Appropriate volumes of PCP (Sigma Aldrich chemicals, USA) from stock solution of 8,000 mg/ L as a carbon source and of Cr(VI) as $K_2Cr_2O_7$ (E-Merck, Mumbai, India) from 10,000 mg/ L stock solution were added to the sterilized medium before inoculation to get the desired effective Cr(VI) and PCP concentration. Millipore membrane filters of 0.22 μm were used for filter sterilization of stock solutions prior to their use and were stored in brown glass bottles to avoid photo-oxidation. The solid medium contained 16.0 g/ L agar (Hi-Media, India Ltd.) in addition to the components described above. The media components and all reagents used in the study were of analytical grade and purchased from Hi-Media, Merck, Qualigens, India Ltd. and Sigma Aldrich chemicals, USA.

2.2 Sampling of Tannery Effluent

The treated tannery effluent was collected from the release point of common effluent treatment plant (CETP) of tanneries located at Jazmau, Kanpur, India 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 ± 1^oC for physico-chemical and heavy metal analyses in the laboratory.

2.3 Physico-Chemical and Heavy Metal Analyses

Salinity, pH and conductivity were measured *in situ* employing portable water quality detection kit (Systronics, India). Total solids (TS), total dissolved solids (TDS) and total suspended solids (TSS) were determined gravimetrically (APHA, 1998). Total alkalinity, DO, BOD, COD, sulphate, chloride, phosphate, nitrate, total nitrogen, fluoride, phenol, PCP, oil and grease 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.

2.4 Enrichment and Isolation of Bacteria Resistant To Cr(VI) and PCP Simultaneously

The bacteria capable to tolerate Cr(VI) and PCP simultaneously were enriched from the treated effluent. Two milliliter of the effluent was added to 100 ml MSM broth, which was amended with 150 mg/ L Cr(VI) and 450 mg/ L of PCP in 250 mL conical flask. Only at this stage glucose (0.1%) was added to support the growth of the bacteria. The flask was then incubated for 7 days at 30 ± 1 °C and 120 rpm in an orbital shaking incubator (New Brunswick Scientific Excella E24, USA). Thereafter, 1 mL of the suspension was transferred after 7 days into a fresh MSM broth (without glucose) and cultured as above. At each culturing step, the concentration of Cr(VI) and PCP was gradually increased up to 600 mg/ L and 700 mg/ L, respectively. After five transfers, 1 ml of the grown culture was serially diluted and 0.1 ml of appropriate dilution (10^{-3}) was spread plated on MSM agar plates supplemented with 600 mg/ L of Cr(VI) and 700 mg/ L of PCP which were incubated for 7 days at 30 ± 1 °C. Ability of the isolates to tolerate Cr(VI) and PCP simultaneously was recorded by visible growth on MSM agar plates amended with Cr(VI) and PCP. Morphologically distinct bacterial colonies were selected and purified by repeated streaking on the same medium and refrigerated at 4 °C for further use.

2.5 Determination of Minimum Inhibitory Concentration (MIC)

The MIC of Cr(VI) and PCP for these strains was determined by the agar dilution method. The MSM agar plates supplemented with different concentrations of Cr(VI) (650-1000 mg/ L) and PCP (750-1400 mg/ L) were inoculated aseptically with about 3.2 X 10^7 colony forming units (CFU)/ ml bacterial cells of exponential phase. Plates were incubated for 7 days at 30 ± 1 °C and observed for growth. The minimum concentration of Cr(VI) and PCP, inhibiting complete bacterial growth, was considered the MIC of that isolate. Five bacterial strains showing higher MIC values for both Cr(VI) and PCP were selected for further studies.

2.6 Evaluation of Heavy Metal Tolerance

Tolerance of selected bacterial strains to various heavy metals was determined by agar dilution method (Cervantes *et al*., 1986). Freshly grown broth cultures of these isolates were inoculated aseptically on MSM agar plates supplemented individually with other heavy metals. The metal salts used were $MnCl_2.4H_2O$, As_2O_3 (E. Merck), $ZnSO_4.7H_2O$, $Co(NO_3)_2.6H_2O$, $CdCl_2$, $NiCl_2$ and $HgCl_2$ (Qualigens). The metal ion concentration tested ranged from 25 to 150 mg/ L. The MSM agar was also supplemented with respective concentration of Cr(VI) and PCP. Plates were incubated aerobically at 30 ± 1 °C for 6-7 days and observed for growth.

III. RESULTS AND DISCUSSION

3.1 Physico-Chemical Analyses of Effluent

The various physico-chemical parameters of treated tannery effluent was analyzed and it was found that the effluent was yellowish-brown in colour and its pH was 8.7 having conductivity of 11,050 moles/ cm. 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 yellowish-brown colour might be hindering the penetration of sun light causing depletion in the rate of oxidation process. It finally contributes to anaerobic oxidation which can be sensed from the putrefying odour of the receiving water bodies (Maiti, 2002). 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 and PCP are also discharged in significant amounts. Pentachlorophenol being a polychlorinated aromatic compound is toxic and recalcitrant to biodegradation hence they are not easily utilized by microorganisms as source of energy and carbon, thereby entering the food chain and adding toxicity. The exposure to chromium and PCP increases the risk of dermatitis, ulcer, lung cancer, immunodeficiency and neurological disorders (Bevenue and Beckman, 1967; Costa and Klein, 2006).

The low DO of treated effluent suggested an increase in the organic matter. This was also indicated by high BOD value. As the number of aerobic organisms increases, the demand for oxygen increases proportionately (Balasubramanian *et al*., 1999). The value of COD observed was higher when compared to the BOD value. Similar results were also observed by More *et al*. (2002). This may be attributed to a large amount of inorganic compounds present, which are not affected by bacteria, thereby resulting in higher COD. In a case study, Khwaja *et al*. (2001) reported that the tanneries of Kanpur (India) are polluting the holy river Ganges. Till date, the tanneries of Kanpur and adjacent Unnao regions claim that the treated effluent released by them is within or around the permissible limits. However, we infer from our study that their treatment technology is not adequate and stress for its revaluation prior to disposal.

Table 1: Physico-chemical characteristics of treated tannery effluent

Parameter	Effluent*	Permissible limit
pH	8.7	6.0-8.0
Conductivity (moles/ cm)	11,050	850
Alkalinity (mg/ L)	720	500
Total solids (TS) (mg/ L)	2,500	2,200
Total dissolved solids (TDS) (mg/ L)	2,219	2,100
Total suspended solids (TSS) (mg/ L)	281	100
DO (mg/ L)	2.5	4.0-6.0
BOD (mg/ L)	250	30
COD (mg/ L)	449	250
Sulfate (mg/ L)	2,490	1,000
Chloride (mg/ L)	360	600
Magnesium (mg/ L)	247	200
Phosphate (mg/ L)	5.4	5.0
Nitrate (mg/ L)	12.60	10
Total nitrogen (mg/ L)	234.50	780
Fluoride (mg/ L)	3.9	2.0
Phenol (mg/ L)	10.0	1.0
PCP (mg/ L)	14.9	0.1
Oil and grease	15.9	10

*Average of triplicate samples

3.2 Heavy Metals in the Treated Tannery Effluent

The heavy metal content of the treated effluent is presented in Table 2. The total chromium and Cr(VI) were found to be 19.50 and 3.0 mg/ L, respectively, which were above the statutory limit of Indian Standards IS:2296 and IS:2490. Nickel, iron and arsenic levels in the treated effluent also exceeded the permissible limit, whereas, other metals such as Cu^{2+}, Mn^{2+}, Zn^{2+}, Pb^{2+}, Cd^{2+} and Co^{2+} were present in significant quantities. Heavy metals may disperse both horizontally and vertically. Thus, there is an urgent need for removal of chromium and other metals from the treated wastewater before being discharged into the environment.

Table 2: Heavy metals content of the treated tannery effluent

Heavy metals	Concentration* (mg/ L)	Permissible limit
Total Cr	19.50	2.0
Cr^{6+}	3.00	0.1
Cu^{2+}	1.75	3.0
Mn^{2+}	1.62	2.0
Zn^{2+}	3.6	5.0
As^{3+}	0.6	0.2
Pb^{2+}	0.1	0.1
Cd^{2+}	0.5	2.0
Ni^{2+}	3.4	2.5
Co^{2+}	0.32	1.5
Fe^{2+}	3.6	3.0

*Average of triplicate samples

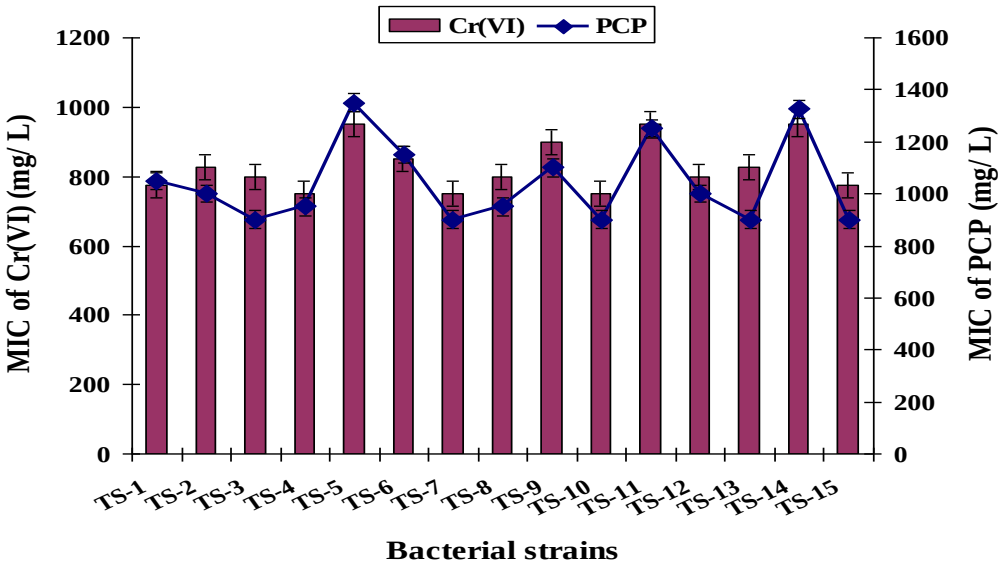
Environmental impact of tannery effluents on humans, animals and plants has been extensively studied (Upreti *et al*., 2004; Shanker *et al*., 2005). The phytotoxic impact of these heavy metals was observed on crops such as cabbage, tomatoes, rice, etc. Numerous diseases caused due to metal pollution, especially chromium toxicity, are very common among tannery workers in India (Gianello *et al*., 1999). Since these workers remain in constant touch with tannery wastes, they are prone to infection also by microbes, particularly pathogens present in the effluents and wastes.

3.3 Isolation of Bacteria Simultaneously Resistant to Cr(VI) and PCP and MIC Determination

Effective bioremediation of tannery contaminated sites requires detoxification of both the contaminants Cr(VI) and PCP simultaneously at higher rate by indigenous bacterial community. In the light of above facts, in the present investigation, fifteen bacteria simultaneously resistant to 600 mg/ L Cr(VI) and 700 mg/ L PCP was isolated from the treated tannery effluent after five rounds of enrichment and were capable to utilize PCP as a sole carbon and energy source. This reveals that chronic exposure to high levels of chromate and PCP may result in bacterial communities that have an exceptional ability to adapt in chromate and PCP polluted environments and have developed some detoxification mechanism as an effective tool for survival in the stress environments. Further, in order to achieve high removal efficiencies, bacteria were acclimatized by growing in successive increasing concentration of Cr(VI) and PCP as the sole carbon and energy source. This could be probably due to a marked increase in resistance among bacterial cells when continuously exposed to toxic chemicals (Srivastava *et al*., 2007). Such an increase in resistance may translate into enhancement of bacterial metabolic rate, which in our case may be detected as a remarkable increment in the simultaneous detoxification of chromate and PCP. Bacterial acclimatization to higher concentration of toxicants is one of the strategies to overcome substrate inhibition (Karn *et al*., 2011). Results of our study suggest that the tolerance efficiency of bacteria to high PCP concentration was not supported by glucose, whereas, other researchers have reported that the bacterial strains were able to grow and degrade PCP only when glucose was added as a co-substrate in the medium (Premlatha and Rajkumar, 1994; Singh *et al*., 2009).

The MIC of bacterial strains for Cr(VI) and PCP was evaluated in order to determine the maximum tolerance limit for both the pollutants simultaneously. The MIC of these strains for Cr(VI) ranged between 750-950 mg/ L and that for PCP between 900-1350 mg/ L (Fig. 1). Among them, based on higher MIC values for Cr(VI) and PCP, five bacterial strains (TS-5, TS-6, TS-9, TS-11 and TS-14) were selected for further studies. Their MIC values for Cr(VI) ranged between 850-950 mg/ L and for PCP between 1100-1350 mg/ L. Srivastava *et al*. (2007) have reported the isolation of *Acinetobacter* sp. from pulp-paper industry which was simultaneously tolerant to only 50 mg/ L PCP and 500 mg/ L chromate. Tripathi and Garg (2010) have also isolated bacteria from tannery effluent which was simultaneously tolerant to 500 mg/ L PCP and 200 mg/ L Cr(VI) concentrations. Contrary to the earlier research, the native bacteria of the tannery effluent reported in the present study were simultaneously tolerant to high PCP and chromate concentrations. The advantage of isolating indigenous potent isolate from polluted sites for bioremediation may be the minimization of inhibitory effects of other pollutants that may be present along with Cr(VI) and PCP in the treated tannery effluent.

Fig. 1. Minimum inhibitory concentration of Cr(VI) and PCP by bacterial strains simultaneously tolerant to chromate and pentachlorophenol. Error bars represent mean ± standard deviation. Each point is the mean of three independent experiments.



3.4 Tolerance to other Heavy Metals

The tannery isolates simultaneously tolerant to chromate and PCP was also tested for tolerance to different heavy metals. All the five selected isolates showed tolerance to different heavy metals at 100-140 mg/ L, except for mercury (25 mg/ L). A significantly higher number of strains exhibited resistance for Ni^{2+} (140 mg/ L), Mn^{2+} (120 mg/ L), Cd^{2+} (110 mg/ L) and As^{3+} (110 mg/ L). All isolates were found to be sensitive to as low as 25 mg/ L of Hg^{2+}. The bacterial viability is very important for simultaneous chromate and PCP detoxification (Tripathi and Garg, 2010). However, besides chromium and PCP, other metals are also released in tannery wastes which may possibly influence the performance of these bacteria (Verma and Singh, 2012). Thus, the resistance in bacteria to other metals in addition to Cr(VI) and PCP in industrial wastewaters must be considered for developing a biological treatment plant. Fortunately, the bacteria of this study appear to be significantly resistant to the presence of other metal cations indicating that these cells are still metabolically active under stressful conditions. Further, the industrial effluents are an enriched medium to propagate and spread microbial population which are resistant to multiple metallic ions and PCP. Thus, microorganisms having combined abilities of simultaneous resistance to chromate and PCP and their biotransformation to non-toxic form will be potentially useful for detoxification of chromate and PCP polluted waste waters.

IV. CONCLUSION

The various physico-chemical parameters and heavy metals content of the treated tannery effluent discharged into the environment were analyzed and the values reveals that it will enhance the pollution load and pose severe threats to human health and environment by ultimately entering the food chain thus, the discharged effluent requires further remediation. Five bacteria simultaneously tolerant to 850-950 mg/ L of Cr(VI) and 1100-1350 mg/ L PCP were isolated from the treated effluent. These bacteria were also significantly resistant to several heavy metals tested. They could be potentially useful for simultaneous bioremediation of Cr(VI) and PCP containing wastes at promising rate from industrial effluent and the environment.

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