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ISOLATION AND MOLECULAR CHARACTERIZATION OF CHROMIUM-REDUCING BACTERIAL STRAINS FROM CHROME-CONTAMINATED TANNERY WASTE SITES IN KARACHI, PAKISTAN

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ABSTRACT

The leather industry represents a key agricultural sub-sector in Pakistan with significant potential to contribute to economic growth, wealth creation, and employment. The Pakistani government actively encourages tannery establishment to boost leather processing, though this comes with unavoidable environmental challenges from Cr^{6+} pollution. Hexavalent chromium is non-biodegradable and classified as a Class A human carcinogen by the US Environmental Protection Agency (USEPA). While bioremediation offers promise for treating such hazardous waste, this technology remains underutilized in Pakistan and other developing nations. This study isolated Cr^{6+}-resistant bacteria from tannery waste in Karachi and evaluated parameters influencing chromium reduction. Analytical results revealed hexavalent chromium levels below Pakistan EPA's allowable limits, while total chromium exceeded regulatory thresholds. The tannery waste exhibited low fat content and acidic pH. Three bacterial isolates (CRB01, CRB02, CRB03) demonstrating Cr^{6+} reduction capability were obtained, showing varying reduction efficiencies across different chromium concentrations with minimum inhibitory concentrations of 60 mg/L, 80 mg/L, and 80 mg/L respectively. Through morphological, biochemical, and molecular characterization via 16S rRNA gene sequencing, the isolates were identified as Lysinibacillus pakistanensis NCCP-54 (CRB01), Bacillus pumilus SAFR-032 (CRB02), and Bacillus safensis NBRC 100820 (CRB03). This research confirms the capacity of native Pakistani microorganisms to bioremediate chromium-contaminated tannery waste, providing sustainable solutions to help tanneries meet environmental compliance standards through biodegradation of hazardous chrome wastes.

INTRODUCTION

Unchecked urbanization and push for industrialization has led to serious pollution problems due to the dumping of sewage and industrial effluents into water bodies. The leather industry is a major industry on an international scale and also of national economic importance. Four percent of Pakistan's agricultural Gross Domestic Product comes from hides, skins and the leather industry and this constitutes 1.5% of the overall GDP (Gathii, 2011). Value addition in the livestock sector has however been minimal and most of Pakistan's exports have been in the form of unprocessed, raw hides and skins. Recently, however, the government has laid out strategies to develop the leather industry. This springs from its Vision 2030 Programme which promotes industrialization and value addition in key sectors. This is because hides, skins and leather industry is one of Pakistan's main agricultural sub-sectors that can contribute to economic growth through expanding exports of both semi-processed and finished leather goods as well as job creation (Bekele et al., 2008).

There is however, a worldwide concern of the leather processing sector as a major polluter to the environment (Famielec and Wieczorek-Ciurowa,

2011). Traditionally, the public has always been concerned with odors associated with tanneries as well as water pollution from untreated discharges from tanneries. Important pollutants associated with tanneries include Chlorides, tannins, Chromium Sulphate and sulphides. In addition to these, trace organic chemicals and increasing use of synthetic chemicals such as pesticides, dyes and finishing agents, as well as from the use of newer processing chemical solvents are also associated with tanneries (Mwinyihija, 2010).

There are two main possible processes of tanning namely chrome tanning and vegetable tanning. Vegetable tanning is carried out in a series of vats that have increasing concentration of tanning liquor. Vegetable tannins are polyphenolic compounds which form hydrogen bonds with the peptide bonds of the protein chains (Riedl and Hagerman, 2001). Chrome tanning on the other hand uses chromium III as the active ingredient. The chromium tanning process is based on the cross-linkage of chromium ions with free carboxyl groups of the collagen which makes the hide resistant to bacteria and high temperature (Sharaf et al., 2013). Chrome tanning is preferred because the process is relatively cheap with respect to time since it only takes a day and produces a highly versatile leather suited to most industries while vegetable tanning can take as long as forty days (Krishnamoorthy et al., 2013). Chromium used in tanning is the more stable Cr^{3+} species which is considered nontoxic as compared to Cr^{6+}. Recently however, detection of significant levels of toxic Cr^{6+} in water bodies in various parts of the world have raised questions regarding disposal of chromium containing wastes (Förstner and Wittmann, 2012). Although Cr^{3+} is considered less toxic, the presence of certain naturally occurring minerals, especially MnO_2 oxides, and fats can enhance oxidation of Cr^{3+} to Cr^{6+} in the soil environment. Hexavalent chromium is bio available, and it is this form that is highly mobile and therefore poses the greatest risk of groundwater contamination (Avudainayagam et al., 2003).

Heavy metals such as Chromium, copper, lead, zinc, nickel, mercury, and cadmium are well-known to be powerful inhibitors of biodegradation activities (Deeb and Altalhi, 2009). These metals cannot be degraded. When Cr^{6+} accumulates in soils, the high levels become toxic to plants, animals, humans and aquatic life (Zayed and Terry, 2003).

Currently, chromium wastes are managed by recycling either directly or indirectly. In Pakistan, heavy metals including Chromium are removed from waste water by flocculation and precipitation (Ruhiu et al., 2009). Flocculation is a method of removing suspended particles from waste water by addition of alum. When alum is added to waste water, the suspended particles in water coagulate to form bigger heavier particles which then sink to the bottom to form sludge. Due to the large quantities of suspended solids, high quantities of sludge are produced which become a problem since the sludge has to be disposed somewhere. After flocculation waste water is then let into another stage of treatment called maturation ponds. Maturation ponds are designed such that ultra violet rays from sunlight can penetrate and kill harmful microorganisms. It is also in maturation ponds that the remaining non-biodegradable particles including heavy metals precipitate to the bottom of the pond as part of sludge (Cavallini, 1996). Indirect recycling involves the precipitation and separation of the chrome from the float containing residual chrome followed by re-dissolving it in acid for re-use. The direct form entails spent float being recycled directly to the chrome tanning processing for reuse. The big challenge to tanning industries is to adopt and practice technologies that are more efficient in recovering Chromium (Ludvík, 2000).

Bacteria have developed several types of mechanisms to tolerate the uptake of heavy metal ions to survive under metal stressed conditions. These mechanisms include the efflux of metal ions outside the cell, and reduction of the heavy metal ions to a less toxic state (Spain and Alm, 2003). Studies have found various species like Bacillus and Pseudomonas capable of remediating heavy metals. However, they need favorable environmental factors (Smith et al., 1998; Boopathy, 2000).

The main sources of river water pollution in Pakistan are industrial discharge, sewage, seepage from waste sites and illegal solid and liquid wastes disposals. The tanning industry in Pakistan releases large quantities of effluents and sludge rich in chromium (Cr) and salts into the environment (Wu et al., 2015).

Objectives

1. To determine physiochemical parameters such as percentage of fat, pH levels, total and Cr^{6+} concentration in chrome shavings from tannery waste sites in Karachi, Pakistan.
2. To isolate and characterize Cr^{6+} reducing bacterial strains from chrome shavings from tannery waste sites in Karachi, Pakistan.
3. To determine the bioremediation potential using native Cr^{6+} reducing bacteria isolated from

chrome contaminated tannery waste sites in Karachi, Pakistan.

4. To determine the phylogenetic relationship of bacterial strains isolated from chrome contaminated tannery waste site in Karachi, Pakistan.

MATERIALS AND METHODS

Sample Collection

Chromium contaminated tannery effluent and chrome shavings waste samples were collected from the disposal site around tannery located at Pakistan Industrial Research and Development Institute (PCSIR) and also from a tannery in UOK between November-December 2014. The samples were collected in triplicate. Solid wastes were collected from heaps of the waste from the sites. Three samples were collected from each heap from each site. In one heap, three samples were collected from the top part of the heap, three other samples from the middle part of the heap and three more samples from the bottom part of the heap. The sampled were then mixed based on the area of collection. That is, the three samples from the top part we mixed together and the same done for samples collected from the middle part and the bottom part. The result of these was that the total number of samples was 9. The samples were collected in sterile plastic containers and transported to the laboratory for bacteriological analysis.

Chemical Analysis of Tannery Waste

Samples were analyzed for a number of parameters such as pH, fat content, total Chromium and hexavalent Chromium. All reagents used were analytical grade.

Determination of Total Chromium

Total chromium was determined using flame atomic absorption spectrometry using the standard test ISO 5398-3:2007 (IULTCS/IUC 8-3). The main principle of this technique is that the chromium that is present in the leather is solubilized in the hexavalent state. The solution was then analyzed by flame atomic spectrometry. Samples, which were solid wastes were prepared by grinding using a Wiley mill. 100 mg of the ground sample was then weighed into a 125 mL conical flask containing 25 mL of 2N HCL. The contents were then filtered using Whatman #1 filter paper into a volumetric flask. The concentrations were then determined using prepared standards. The measurements were done in three triplicates and the mean taken as the final reading

Determination of Hexavalent Chromium (Cr^{6+})

Hexavalent chromium was determined using the standard 1.5–diphenyl carbazide method ISO 17075:2007 (IULTCS/IUC 18). The principle behind this method is that soluble Cr^{6+} is leached from the sample in phosphate buffer at pH 7.0 to 8.0. The Cr^{6+} in the solution oxidizes 1.5–diphenyl carbazide to 1, 5-diphenylcarbazone to give a red/violet complex with chromium which can be quantified photometrically at 540 nm.

The results were then read using a curve generated using prepared standards.

Determination of Fat content

The fat content was determined using the standard method ISO 4048:2008 (IULTCS/IUC 4).This method uses a soxhelet apparatus and a solvent that dissolves the fat in the leather. The solvent is then evaporated to leave the fat whose weight is then taken. In this method, samples were prepared by grinding the chrome shavings using a Wiley mill. 9 samples were prepared by grinding and mixing with 25 mL of analytical grade hexane.

Culture of Chrome Resistant Bacteria from Tannery Waste

The top, middle and bottom heap samples from the two selected locations PCSIR and UOK were mixed according to section 3.1. The presence of bacteria in the samples was determined by culturing the bacteria in basal peptone water as a diluent. 1 mL of the diluent containing bacteria was then aseptically inoculated on nutrient broth and incubating at 37 °C for 24 hours. This was followed by platting aseptically on nutrient agar amended with Cr^{6+} as $K_2Cr_2O_7$ to final concentration 40 mg/L using sterile filtered Cr^{6+} stock solutions using the standard plating method described by Robert Koch and incubating at 37 °C for 24 hours. Growth of colonies would confirm presence of bacteria. 3 colonies differing in morphological characteristics were selected and labeled for use in further studies.

Characterization of the Isolates

The bacterial isolates differing in morphological characteristics were grown on Eosin Methylene Blue (EMB) agar (Himedia, India). The shape and colors of the colonies were then examined under the microscope after Gram staining. This was followed by biochemical analysis for the activities of Oxidase, Catalase, MR-VP test, Citrate Utilization, Acid production from carbohydrates. These tests were used

to identify the isolates according to Bergey's Manual of Determinative Bacteriology (Holt et al., 1994).

Determination of Minimum Inhibitory Concentration

The minimum inhibitory concentration (MIC) of Cr^{6+} resistant isolates was determined by serial dilution method (Calomiris et al., 1984) in LB medium with Cr^{6+} concentrations ranging from 20 to 200 mg/L and the minimum concentration of metal in the medium inhibiting complete growth taken as the (MIC). Based on the evaluation MIC was determined at 37 °C for 24 hours. The minimum Concentration of the chromium ($K_2Cr_2O_7$) at which no growth was observed was considered the MIC.

Reduction of Chromium by the Isolates

Chromate-resistant bacteria isolates were inoculated into nutrient broth (pH 7.0) containing different concentration of Cr^{6+} (from 20 to 200 mg/L) and incubated for 72 hours at 30 °C under orbital shaking. The inoculum was 2% of the total volume of medium. Reduction of chromium was determined from extracted solution by using UVVis spectrophotometers at 540 nm with 1,5-diphenylcarbazide as a pink colored complex agent (APHA, 1992).

Molecular Identification of Isolates

Genomic DNA was isolated from the isolates using the CTAB protocol for molecular characterization and amplified by Polymerase Chain Reaction (PCR) using universal bacterial primers 1492R (5' - TACGGYTACCTTGTTACGACTT- 3') and Bac8f (5'AGAGTTTGATCCTGGCTCAG-3') for the rRNA gene (Weisburg, 1991). The amplified gene was then sequenced and the resulting 16S rRNA gene sequences were compared with sequences deposited in GenBank by performing a blast n search. (Thompson et al., 1994). Sequence data was then aligned and analyzed to find the closest homology for the microbes. Sequences were aligned with the ClustaLW algorithm using default parameters (Thompson et al., 1994). Phylogenetic trees were generated with a Neighbour-Joining (NJ) algorithm. Confidence values for NJ trees were generated by bootstrapping, based on 1000 replicates.

Statistical Data Analysis

The different Cr^{6+} reducing capabilities of the isolated Cr^{6+} resistant bacteria were then analysed using One-way ANOVA with post-hoc Tukey HSD Test. The statistical formula applies two steps. The first step ANOVA examines whether there is a difference in the gradient of the slope and the second step finds out which among the three is different if at all there is a difference.

RESULTS

Physicochemical properties of tannery waste

The results of the physicochemical properties of the effluent are shown in Table 1

TABLE 1: PHYSICOCHEMICAL PROPERTIES OF TANNERY WASTE

SAMPLE	Temperature (°C)	pH	Fat Content (%)	Total Cr(mg/L)	Cr^{6+}(mg/L)
PCSIR	28.1	3.55	0.441±0.03	35.077±0.07	0.0080±0.0002
UOK	27.9	3.23	0.628±0.07	37.565±0.5	0.0056±0.0002

± : Standard Deviation

The samples from both sites were found to have high concentrations of total chromium compared to the National Environmental Management Authority (NEMA) permissible limit of 1 mg/L. Concentrations of Cr^{6+} as well as those of fat were found to be lower than the limit set by the National Environmental Management Authority which is 0.05 mg/L for Cr^{6+}.

Isolation, Characterization and Identification of chrome resistant Bacteria

Three bacterial species were isolated from chrome shavings sampled from two sites in Karachi: the Institute of Environmental Studies, University of Karachi, and the Center for Environmental Studies, PCSIR Laboratories Complex. Two isolates from the University of Karachi site were labeled as CRB01 and CRB03, while the isolate from the PCSIR site was labeled as CRB02.

Morphological Characterization of Chrome Resistant Bacteria

The colonies of CRB01 were observed as being white with a rough dull surface. The colonies were also observed to be transparent with a butyrous (butter like) with flat elevation. The white colonies were observed to turn light yellow in older colonies. Fresh colonies exist as dots on the surface of nutrient media but on the second and third day they spread over the whole

surface of the media. Colonies of the second isolate CRB02 were observed to be slightly yellowish and mostly opaque. The texture of the colonies was smooth and were wrinkled and irregular. The colonies spread over the surface of the agar. The third isolate CRB03 was observed to have dull white colonies with irregular margins and were mostly opaque.

Biochemical Characterization of Chrome resistant Bacteria

Their morphological and biochemical characteristics are shown in Table 2.

TABLE 2: BIOCHEMICAL CHARACTERISTICS OF CHROME RESISTANT BACTERIA

Characteristic	CRB01 (UOK)	CRB02 (PCSIR)	CRB03 (UOK)
Shape	Rods	Short Rods	Rods
Gram Stain	Gram Positive	Gram positive	Gram Negative
EMB	+	+	-
Lactose	-	-	-
Methyl Red	-	+	+
VogesProskeur	-	+	-
Citrate Utilisation	+	+	-
Catalase	+	+	+
Oxidase	-	+	+

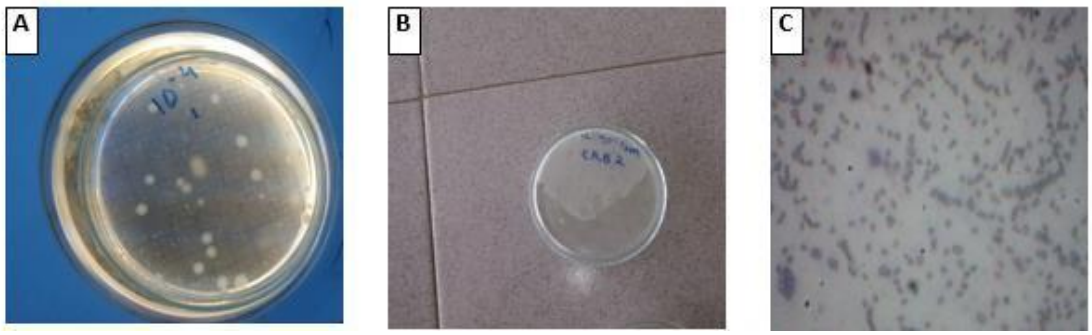

Figure 1: A: Colonies of CRB01, B: Spreading colonies of CRB02, C: Gram negative CRB03 on safranin counterstain

Minimum Inhibitory Concentration

A stock solution of potassium dichromate was prepared and different concentrations of the potassium dichromate amended to nutrient agar from 20 mg/L to 200 mg/L. The minimum concentration that inhibited growth of bacteria was found to be 60 mg/L, 80 mg/L and 80 mg/L for CRB01, CRB02 and CRB03, respectively.

Reduction of Cr^{6+} by the Isolates

The bacterial isolates were found to be able to reduce the Cr^{6+} to Cr^{3+} in different concentrations of potassium dichromate amended to nutrient broth. The results are as shown in Table 3 and demonstrated in figure 2

TABLE 3: TABLE SHOWING PERCENTAGE REDUCTION OF CR^{6+} BY THE ISOLATES

INITIAL CR^{6+} CONCENTRATION	PERCENTAGE CR^{6+} REDUCTION		
	CRB01	**CRB02**	**CRB03**
20	100	100	100
40	97.5	100	100
60	77.7	86.7	81.7
80	75	79	76.3
100	70	76.3	73.3

120	68.5	60.6	70.6
140	67.5	59.2	65.7
160	65	55	65
180	64	54.5	55
200	63.8	54.3	45

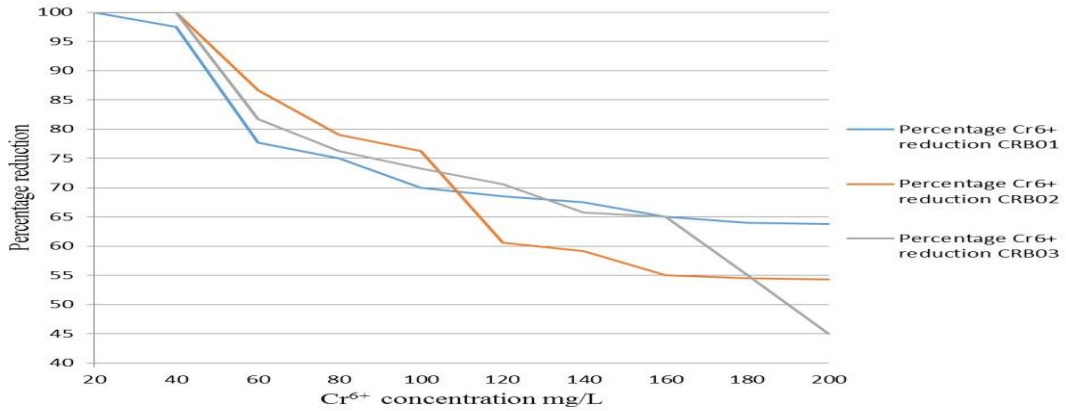

Figure 2: Graph showing different reduction capabilities of bacteria isolates

Molecular Identification

The DNA of the three bacteria species was extracted using CTAB protocol and amplified using PCR using two primers reverse and forward, 1492R and Bac_8F. The amplified 16S rDNA gene was then run on agarose gel electrophoresis using 1kbp ladder and the result observed under UV light. The amplicons were then sent to Macrogen for sequencing. The DNA sequences obtained were then used to identify the species that they closely matched based on the NCBI database. The Blast N results showed that the three chromium resistant bacteria belonged to the species Lysinibacillus pakistanensis NCCP 54, Bacillus pumilus SAFR-032and Bacillus safensis strain NBRC 100820.

TABLE 4: BLASTN RESULTS OF THE BACTERIAL ISOLATES

Isolate	Description	Max score	Total score	Query cover	E value	Ident	Accession
CRB01	Lysinibacillus pakistanensis NCCP 54	627	627	96%	6e-179	72%	NR 113166.1
CRB02	Bacillus pumilus SAFR-032	294	294	19%	9e-79	85%	NR 074977.1
CRB03	Bacillus safensis strain NBRC 100820	1659	1659	73%	0.0	94%	NR 113945.1

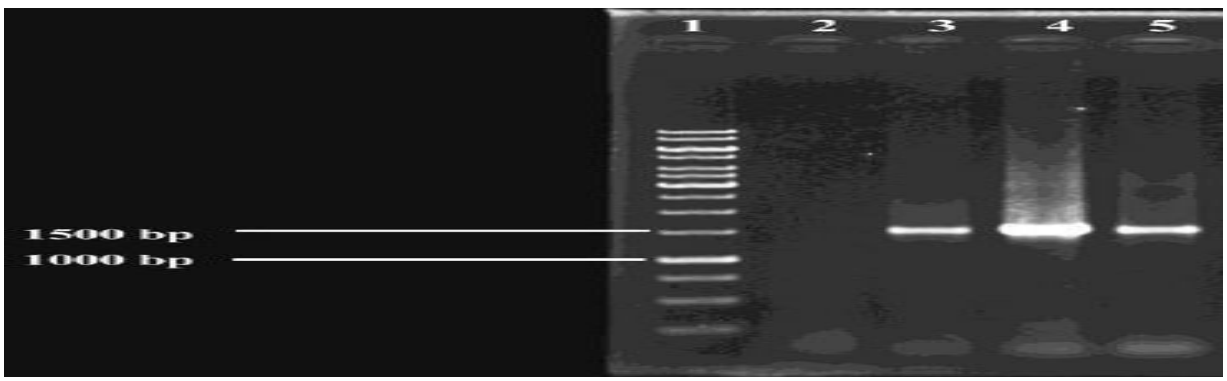

Figure 3 Agarose gel electrophoresis after 16S rDNA amplification. Lane 1: GeneRuler 1 kb DNA ladder Lanes 3 to 5: 16S PCR products of DNA isolated from chrome resistant bacteria CRB01 to CRB03 respectively.

Phylogenetic Analysis

The sequences of the isolates L. pakistanensis, B. pumilus and B. safensi, and that of a reference organism which a Picropilus species were taken through multiple sequence alignment and the result used to create a phylogenetic tree. The result is shown in figure 4.

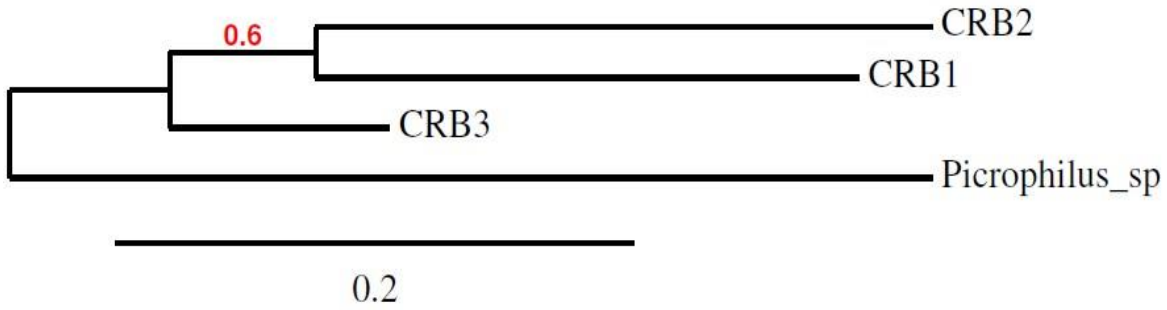

Figure 4: Neighbor-joining (NJ) tree of the three isolated bacteria based on the 16S rRNA gene sequences comparison, showing the relationship of Bacillus.

pumilus, Bacillus safensis and Lysinibacillus pakistanensis using Picropilus as an outgroup

DISCUSSION

5.1 Discussion

Chromium Sulphate is the tanning agent that is used for tanning in chemical tanning. The Chromium component is the trivalent form of Chromium. Total chromium in the tannery waste was found to be in high amounts compared to the National Environmental Management Authority (NEMA) permissible limit of 1 mg/L. This was expected because a very small percentage of the tanning agent is used during tanning while the rest is washed away with large quantities of water. In addition, after leather has been tanned, it has to be trimmed to the right shape and thickness through trimming and mechanical process called shaving. Through shaving, pieces of wet blue which contain high quantities of Cr^{3+} are produced to form part of the tannery waste and contribute to the levels of Cr^{3+} containing tannery waste. This finding is in line with a study undertaken by (Leghouchi et al., 2009) who found out that the concentrations of total chromium upstream of a tannery were lower compared with high concentration, up to 860 times downstream of the tannery.

Hexavalent chromium on the other hand was found to be in low amounts of 0.008 mg/L and 0.0056 mg/L, lower than the limit set by the National Environmental Management Authority which is 0.05 mg/L. In normal situations, Cr^{6+} is not expected to be detected in tannery waste but certain factors and environmental condition may lead to the oxidation of Cr^{3+} to Cr^{6+}. Some of these factors such as amount of fat and pH were determined in this study. The pH was found to be 3.55 and 3.23 and the fat content was found to be less than 1 percent. As mentioned in chapter 2, high pH or high fat content leads to the oxidation of Cr^{3+} to Cr^{6+} and since pH was found to be low then it was expected that there would be low concentrations of Cr^{6+} if any. These findings are similar to the findings of a study carried out by Palop et al., (2008) who found out that the type of fat-liquoring agent was vital in the formation of Cr^{6+} post tanning. Fatliquoring products with single or multiple unsaturated fatty acids either free or esterified are especially responsible for formation of Cr^{6+}.

The colonies of the isolated bacteria were observed to be white with rod shapes on nutrient agar amended with potassium dichromate. This is a characteristic that is common with bacteria of the genus Bacillus. B. subtilis, for instance, is a good example of bacteria that has white colonies with wrinkled form and with rod shapes. The most commonly known Bacilli are those that are pathogenic. However, it is important to note that most Bacilli are saprophytes that feed on dead decaying matter for instant decaying organic matter from industrial wastes. The colonies isolated were observed to spread throughout the media, which again is associated with members of the bacilli genus. Members of the genus Bacilli are often the major population found in sites with a high concentration of toxic substances such as heavy metals including chromium and because of their ability to produce spores they survive harsh environments.

The Minimum Inhibitory Concentration (MIC) of the three bacteria towards Cr^{6+} was determined to find out which among the three bacteria could tolerate the highest concentration of Cr^{6+}. This is because the best candidate for bioremediation is that microorganism that can tolerate the highest concentration of the pollutant (Spain and Alm, 2003). If those bacteria that

cannot tolerate high concentrations of the pollutant are used then they would die and bioremediation would not be achieved. Of the three bacterial species isolated, CRB02 and CRB03 had a higher MIC of 80 mg/L while the MIC of CRB01 was found to be 60 mg/L.

The three bacterial species each showed different reduction capabilities with different concentrations of initial Cr^{6+} concentration. However, upon statistical analysis using One-way ANOVA with post-hoc Tukey HSD Test, it was shown that the difference in the reducing potential among the three bacteria is not statistically significant. Therefore none of the bacterial species can be said to be better than the other as a potential candidate for application in bioremediation. Total reduction of Cr^{6+} was observed only at low concentrations of 20 mg/L and 40 mg/L.

The three Chromium resistant bacteria species, CRB01 and CRB03 from a tannery in UOK and CRB02 from a tannery at PCSIR were characterized in this study. Upon extraction of their DNA following the CTAB protocol and sequencing followed by comparison in the database at NCBI Gene bank, the bacterial isolates CRB01, CRB02 and CRB03 were identified as Lysinibacillus pakistanensis NCCP 54, Bacillus pumilus SAFR-032 and Bacillus safensis strain NBRC 100820 respectively. These three were the indigenous species of bacteria that were isolated from tanneries in UOK and PCSIR that were capable of reducing Cr^{6+} to Cr^{3+}.

This is the first time L. pakistanensis is being implicated in the biotransformation of Cr^{6+}. A different species, Lysinibacillus fusiformis ZC1 was found to contain quite a number of genes that confer metal resistance such as ChrA gene, yieF gene and several others that are known to encode for reductases (He et al., 2011). Lysinibacillus sphaericus has also been reported for its great larvicidal activity against mosquito larvae and toxic metal resistance. This implies that this specific genus should be studied more to identify the probability of having more species that are chrome resistant.

The revelation in this study that Bacillus pumilus can reduce Cr^{6+} is in agreement with a study carried out by Ejaz et al., (2013), who were able to isolate a different strain of B pumilus capable of reducing Cr^{6+}. They isolated Bacillus pumilus S-4 in Pakistan from a tannery effluent. In another study, Bacillus pumilus was found to be able to completely degrade protocatechuic and caffeic acids and reduce achieve a 50% reduction in the phenolic content of an oil mill waste water (McNamara et al., 2008). In our study, Bacillus pumilus SAFR-032 was isolated in Pakistan from tannery waste.

Bacillus safensis was originally isolated from a National Aeronautics and Space Administration (NASA) assembly plant (Satomi et al., 2006). Strains of this species have been reported to be resistant to Boron and Arsenic (Raja, 2014). It has also been isolated in Brazil from biodegraded petroleum (Laborda et al., 2014). Here we report isolation of Bacillus safensis strain NBRC 100820 from a Tannery site in UOK capable of reducing Cr^{6+}. There have been very few reports implicating B safensis in the reduction of Cr^{6+}.

Bacteria are organisms that can adapt to environmental stresses such as high concentration of heavy metals such as Cr^{6+}. In a study carried out by Megharaj et al., (2003), an Arthrobacter sp. and a Bacillus sp. were isolated from a long-term tannery waste contaminated soil. It is probable that in their study, Megharaj et al., (2003) isolated indigenous bacteria because the waste was long term meaning it had been in that for a period long enough to be colonized by native bacteria. The findings of this study that found native bacteria capable of biotransorming Cr^{6+} are also concur with findings of a study carried out by Alisi et al., (2009) where a formula of native bacteria were employed with success in bioremediation of wastes contaminated with heavy metals and diesel oil. It is important to note that the three species isolated in this study were Bacilli similar to the results obtained by a study carried out by Camargo et al., (2003) where it was observed that most of the isolates capable of bio transforming Cr^{6+} were those of the genus Bacillus.

From the phylogenetic tree, it was observed that there is a close relationship between L. pakistanensis, B. pumilus and B. safensis. Since all the three isolated species demonstrated ability to survive and biotransform Cr^{6+}, it could be that Mobile genetic elements (MGE) could be involved in the spreading of Cr^{6+} resistance determinants, facilitating the adaptation of bacterial communities to Cr^{6+}. Bacteria exposed to Cr^{6+} for a long period of time may acquire MGE such as plasmids carrying Cr^{6+} determinants and, therefore, they become Cr^{6+} resistant bacteria. In agreement with this hypothesis, is a study carried out by De et al., (2008) which found out that showed the presence of the copA gene in metagenomic DNA from the three Cu-polluted soils and the absence of copA gene in metagenomic DNA from the non-polluted soil.

Conclusions

Hexavalent chromium concentration in tannery waste was found below the NEMA threshold, yet total chromium levels remained elevated, contributing to heavy metal loads in sewage. From these waste samples, three bacterial strains—Lysinibacillus pakistanensis NCCP 54, Bacillus pumilus SAFR-032, and Bacillus safensis NBRC 100820—were isolated, each capable of reducing toxic Cr^{6+} to Cr^{3+}, indicating their potential for bioremediation applications. Phylogenetic analysis revealed close relationships among the strains, suggesting related species may harbor similar detoxification genes. Notably, this marks the first report of chromium-reducing bacteria isolated from tannery waste at PCSIR and the University of Karachi.

Recommendations

The three chromium-resistant bacterial strains identified—Lysinibacillus pakistanensis, Bacillus pumilus, and Bacillus safensis—hold promising potential for commercial bioremediation of Cr^{6+} in industrial settings, particularly in Pakistan's leather sector. However, to effectively harness their capabilities, further research is needed to explore their interactions with native microbial communities, which may enhance their natural waste-degrading efficiency. Additionally, the study did not investigate optimal conditions such as temperature or substrate type, both of which could significantly influence their bioremediation performance. It is also essential to elucidate the specific Cr^{6+} reduction mechanisms employed by each species to ensure strategic and effective deployment in future applications.

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