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Isolation and Molecular Characterization of Chrome Resistant Bacteria from Chrome Contaminated Tannery Waste from Disposal Sites in Kenya

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Abstract: *The leather industry is one of the key agricultural sub-sectors in Kenya with a high potential towards contributing to economic growth, creation of wealth and employment. Tanneries however, are known to pollute the environment with hexavalent chromium metal (Cr^{6+}). Cr^{6+} is non-biodegradable and is listed as a Class A human carcinogen by the US Environmental Protection Agency (USEPA). The treatment of environmental pollution by bioremediation is an evolving and promising technology although the application of this technology is uncommon in Kenya and other developing countries. Physicochemical characteristics of tannery waste such as pH and fat content were analyzed. Bacteria DNA was extracted using CTAB protocol from bacteria isolated from tannery waste. Isolates CRB01, CRB02 and CRB03 showed the ability to reduce different concentrations of Cr^{6+} by different percentages and exhibited MIC levels of 60mg/l, 80mg/l and 80mg/l respectively. Morphological, biochemical and 16S rRNA sequence analysis of these isolates identified CRB01, CRB02 and CRB03 to be Lysinibacillus pakistanensis NCCP 54, Bacillus pumilus SAFR-032 and Bacillus safensis strain NBRC 100820 respectively from the NCBI database. These three bacterial species can be exploited commercially for bioremediation of Cr^{6+}. This study therefore demonstrates that waste matter from tanneries in Kenya harbor microorganisms that can biodegrade Cr^{6+}. Different optimum conditions for the reduction of hexavalent Chromium and mechanisms of reduction should be investigated and the three isolates used to improvise bioremediation technology.*

Keywords: *Hexavalent Chromium, Remediation, Industrial waste.*

I. Introduction

Chromium exists in different oxidation states but Cr^{3+} and Cr^{6+} are the most stable forms (Güldal and Apak, 1980). One of the applications of Cr^{3+} is in chrome tanning of hide to leather and is usually applied in the form of chromium sulphate. Very little percentage of Cr^{3+} remains in the leather after tanning meaning that the rest is released together with waste from the tanneries (Chuan and Liu, 1996). More Cr^{3+} is also released as waste together with chrome shavings which are pieces of wet blue leather that arise after leather is trimmed to the required thickness and shape. In the environment, Cr^{3+} can be oxidized into Cr^{6+} by dissolved oxygen or free oxygen or by MnO_2 present in soil (Ling *et al.,* 2010). Cr^{6+} is more soluble than Cr^{3+} and can therefore permeate into biological membranes and interact negatively with proteins and nucleic acids. These interactions can lead to mutations and this is why Cr^{6+} has been listed as a Class A human carcinogen by the US Environmental Protection Agency (USEPA) (USEPA, 1998). Tanneries have different methods of removal of chromium in general from its effluent but in Kenya precipitation using alum is the method that is commonly used (Onyancha *et al.,* 2008). The major disadvantage of this is that it results in the generation of a huge amount of sludge and is expensive for small tanneries. Bioremediation is an evolving and promising method that can be employed because it is cheap compared to other methods (Ang *et al.,* 2005). First however, Chromium resistant bacteria native to the contaminated site need to be isolated and characterized. Indigenous bacteria have the advantage of being well adapted to the prevailing conditions of the contaminated site. The present study was carried out to determine concentration of total and Cr6+ in chrome shavings from waste sites, isolate and characterize native chrome resistant bacteria, determine the bioremediation potential using native Chromium reducing bacteria isolated and to determine the phylogenetic relationship of the bacterial strains isolated from Dandora and KIRDI in Nairobi.

II. Material and Methods

Sampling

Chromium contaminated chrome shavings waste samples were collected from the disposal site around tannery located at Kenya Industrial Research and Development Institute (KIRDI) and also from a tannery in Dandora between November-December 2014. The samples were collected in sterile plastic containers and transported to the laboratory for bacteriological analysis. The site map for the tannery at KIRDI is shown in figure 1 below.

Figure 1: Site map of KIRDI tannery

Chemical analysis of tannery waste

Samples were analyzed for a number of parameters such as pH, fat content, total chromium and hexavalent chromium. Total chromium was determined using atomic absorption spectroscopy using the standard test ISO 5398-3:2007 (IULTCS/IUC 8-3) while hexavalent chromium was determined using the standard 1,5–diphenyl carbazide method ISO 17075:2007 (IULTCS/IUC 18). The fat content was determined using the standard method ISO 4048:2008 (IULTCS/IUC 4). All chemicals used were of analytical grade.

Culture of bacteria from tannery waste

The presence of bacteria in the samples was determined by aseptically plating the bacteria on nutrient broth using the standard plating method described by Robert Koch and incubating at 37º C for 24 hours. Growth of colonies would confirm presence of bacteria. Colonies differing in morphological characteristics were selected and used for further studies.

Characterization of the isolates

The bacterial isolates were grown on MacConkey 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).

Isolation of chromium resistant bacteria

The bacterial isolates were serially diluted and plated on Nutrient agar for the isolation and enumeration of bacteria. The molten medium was amended with Cr^{6+} as $K_2Cr_2O_7$ to final concentration 40 mg/l using sterile filtered Cr^{6+} stock solutions. Plates were then incubated at 30º C in the dark which are the optimum conditions for growth of most bacteria and read after two days. Isolates were then be selected according to their morphological shapes according to Bergey's Manual of Determinative Bacteriology for further studies (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 will be determined at 37 ºC 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 bacterial 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 hrs at 30 ºC under orbital shaking. The inoculum was 2% of the total volume of medium. Reduction of chromium was determined from extracted solution by using UV spectrophotometer at 540 nm with 1, 5-diphenylcarbazide as a pink colored complex agent (APHA, 1992).

Molecular Characterization

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.

III. Results and Discussions

Table 1 gives data on the physicochemical properties of tannery waste

Table 1: physicochemical properties of tannery waste

SAMPLE	Temperature (ºC)	Odor	pH	% Fat Content	Total Chromium (ppm)	Cr^{6+} (ppm)
1	28.1	Non disagreeable	3.55	0.441±0.03	35.077±0.07	0.0080
2	27.9	Non disagreeable	3.23	0.628±0.07	37.565±0.5	0.0056
3	27.0	Non disagreeable	3.12	0.970±0.05	42.229±0.2	0.0220

± : Standard Deviation

All the samples from both sites were found to have high concentrations of total chromium compared to the Kenya Bureau of Standards (KEBS) limit of 2mg/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. Concentrations of the toxic 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 0.05mg/l for Cr^{6+}.

Isolation, Characterization and Identification of chrome resistant Bacteria

Three bacteria species were isolated from the chrome shavings sampled from the two sites in Dandora and KIRDI which were labelled as Dandora (CRB01 and CRB03) and KIRDI (CRB02). Their biochemical characteristics are shown in Table 2.

Table 2: Results of biochemical characteristics of chrome resistant bacteria

Characteristic	CRB01 (Dandora)	CRB02 (KIRDI)	CRB03 (Dandora)
Shape	Rods	Short Rods	Rods
Gram Stain	Gram Positive	Gram Negative	Gram Negative
EMB	-	-	-
Lactose	-	-	-
Methyl Red	-	+	+
Voges Proskeur	-	+	-
Citrate Utilisation	+	+	-
Catalase	+	+	+
Oxidase	-	+	+

The texture of the colonies of CRB01 was butyrous and the color is white in freshly grown colonies as shown in figure 2. As the colonies grow older they turn yellowish. 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 CRB02 were whitish and they spread on the surface of nutrient agar as shown in figure 3. CRB03 was isolated in this study from tannery waste and was found to be gram negative and rod shaped as shown in figure 4.

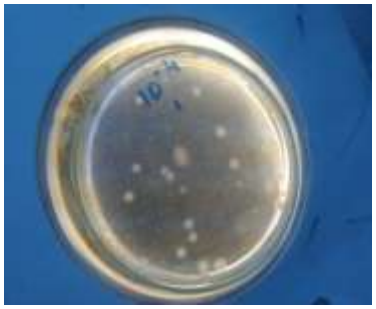
Figure 2


Figure 3

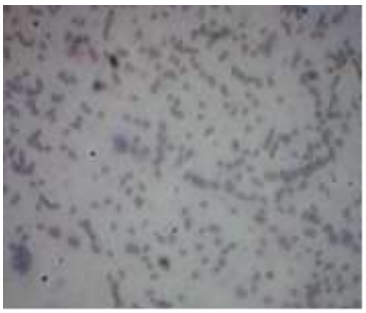
Figure 4

Figure 2: Colonies of CRB01
Figure 3: Colonies of CRB02 spreading out on nutrient agar
Figure 4: Gram negative colonies of CRB03

Minimum Inhibitory Concentration

This was carried out to identify the most resistant bacterial species. This is because the best candidate for bioremediation is that microorganism that can tolerate the highest concentration of the pollutant (Spain, 2003). If that bacteria that cannot tolerate high concentrations of the pollutant are used then they would die and bioremediation would not be achieved. The highest concentration that inhibited growth of bacteria was found to be 60mg/l, 80mg/l and 80mg/l for *L. pakistanensis, B. pumilus and B. safensis* 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 figure 5.

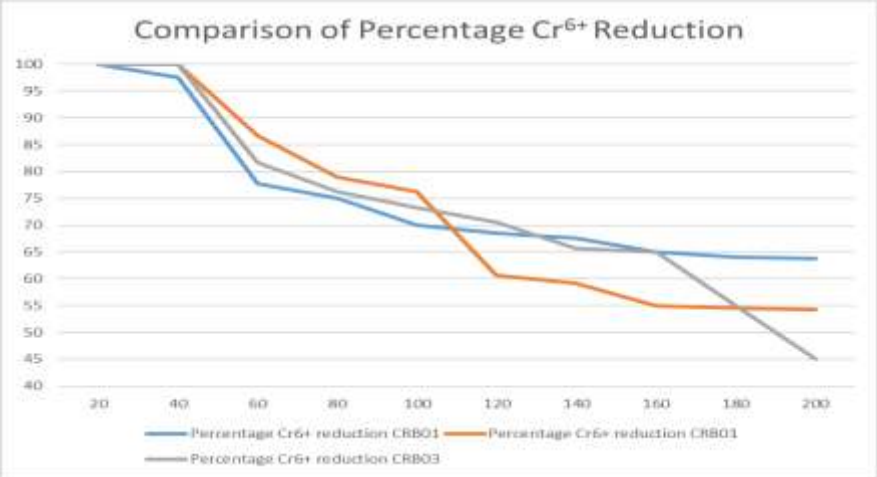

Figure 5: Comparison of the reduction percentages of the three bacterial species

The three bacterial species each showed different reduction capabilities with different concentrations of initial Cr^{6+} concentration as shown in figure 2. However, upon statistical analysis using One-way ANOVA with post-hoc Tukey HSD Test, it was shown that the differences 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. Complete reduction of Cr^{6+} was observed only at low concentrations of 20mg/l and 40mg/l

Molecular characterization

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 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. Blast N results showed that the three chromium resistant bacteria belonged to the species *Lysinibacillus pakistanensis* NCCP 54, *Bacillus pumilus* SAFR-032 *and Bacillus safensis* strain NBRC 100820. The blast N results are shown in Table 3

Table 3: Blast N results of the 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

This is the first time *L. pakistanensis* is being implicated in the reduction of Cr^{6+}. However, not long ago, a different species of *Lysinibacillus*, *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). 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 *B pumilus* can reduce Cr^{6+} is in agreement with a study carried out by Ejaz *et al.* (2013), who was 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 our study, *Bacillus pumilus* SAFR-032 was isolated in Kenya from tannery waste.

Bacillus safensis was isolated in this study from tannery waste and was found to be gram negative and rod shaped. This bacteria 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 and Omine 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 in Dandora capable of reducing Cr^{6+}. There have been very few reports implicating *B safensis* in the reduction of hexavalent Chromium.

IV. Conclusion

The three bacterial species isolated from tannery waste provide evidence that a community of microorganisms exist in the tannery waste that are tolerant to high concentrations of heavy metals. Each of the three bacterial species isolated had the capability of reducing Cr^{6+} to Cr^{3+} which suggests that they can be used to improvise bioremediation techniques for the cleaning of industrial wastes from industries associated with hexavalent Chromium. The leather industry in Kenya will particularly benefit from these findings since with the increase in the number of tanneries then the potential of formation of Cr^{6+} will increase too. Phylogenetic studies indicated that these three species *Lysinibacillus pakistanensis* NCCP 54, *Bacillus pumilus* SAFR-032 *and Bacillus safensis* strain NBRC 100820 are closely related which suggests that maybe their close relatives also possess the genes that enable these bacteria to bio transform Cr^{6+}.

References

[1] Ang, E. L., Zhao, H., & Obbard, J. P. (2005). Recent advances in the bioremediation of persistent organic pollutants via biomolecular engineering.*Enzyme and microbial technology*, *37*(5), 487-496.

[2] APHA(1992). Standard Methods for the Examination of Water and Waste water, 18 Edition. American Public Health Association. American water works Association. Water Environmental Federation, Washington, DC.

[3] Calomiris, J.J., Armstrong, J.L.andSeidler, R.J. (1984). Association of metal tolerance with multiple antibiotic resistances of bacteria isolated from drinking water. Applied and Environmental Microbiology 47:1238-1242.

[4] Chuan, M. C., & Liu, J. C. (1996). Release behavior of chromium from tannery sludge. *Water Research*, *30*(4), 932-938.

[5] Ejaz, S., Rizvi, F. Z., Anwar, S., & Faisal, M. (2013). Biotransformation potential of hexavalent chromium by Bacillus pumilus-S4, Pseudomonas doudoroffii-S5 and Exiguobacterium-S8 in association with hydrophytes.*International Journal of Environmental Science and Technology*, ***10***(4), 709-718.

[6] Güldal, Ü., & Apak, C. (1980). A study on Cr^{3+} Cr^{6+} equilibria in industrial emerald green glasses. *Journal of Non-Crystalline Solids*, *38*, 251-256.

[7] He, M., Li, X., Liu, H., Miller, S. J., Wang, G., & Rensing, C. (2011). Characterization and genomic analysis of a highly chromate resistant and reducing bacterial strain Lysinibacillus fusiformis ZC1. *Journal of Hazardous Materials*, *185*(2), 682-688.

[8] Holt, J. G; Krig. N. R; Sneath. P. H. A; Staley, J. T. and Williams, S. T. (1994), Bergey's manual of determinative bacteriology (9th edn). Baltimore, Maryland, Williams.

[9] Laborda, P. R., Fonseca, F. S., Angolini, C. F., Oliveira, V. M., Souza, A. P., & Marsaioli, A. J. (2014). Genome sequence of Bacillus safensis CFA06, isolated from biodegraded petroleum in Brazil. *Genome announcements*, ***2***(4), e00642-14.

[10] Ling, Z., Congzheng, Y., Hongmin, Y., & Qiang, H. (2010). Oxidation of Cr^{3+} to Cr^{6+} by hydroxyl radicals in leather. *Journal of the Society of Leather Technologists and Chemists*, *94*(4), 156-160.

[11] Onyancha, D., Mavura, W., Ngila, J. C., Ongoma, P., & Chacha, J. (2008). Studies of chromium removal from tannery wastewaters by algae biosorbents, Spirogyra condensata and Rhizoclonium hieroglyphicum. *Journal of Hazardous Materials*, *158*(2), 605-614.

[12] Raja, C. E., &Omine, K. (2014). Arsenic, boron and salt resistant Bacillus safensis MS11 isolated from Mongolia desert soil. African Journal of Biotechnology, 11(9), 2267-2275.

[13] Satomi, M., La Duc, M. T., & Venkateswaran, K. (2006). Bacillus safensis sp. nov., isolated from spacecraft and assembly-facility surfaces.*International Journal of Systematic and Evolutionary Microbiology*, ***56***(8), 1735-1740.

[14] Spain, A. (2003). Implications of microbial heavy metal tolerance in the environment, Reviews in Undergraduate Research 2: 1-6.

[15] Thompson.J.D., Higgins D.G., and Gibson.T.J. (1994). Clustal w: improving the sensitivity of progressive multiple sequence alignment through sequence weighing, position-specifc gaps penalties and weight matrix choice. Nucleic Acids Res 22:4673-4680.

[16] USEPA, A. (1998). *Guidelines for ecological risk assessment* (Vol. 2). EPA/630/R-95.

[17] Weisburg, W. G., Barns, S. M., Pelletier, D. A., & Lane, D. J. (1991). 16S ribosomal DNA amplification for phylogenetic study. Journal of bacteriology,173 (2), 697-703.