Isolation and Identification of Chromium-Reducing Bacteria from Challawa Industrial Area Kano State, Nigeria

F. A. Ahmad a, F. Yusuf a, U. Shehu a, F. Muhammad a and H. M. Yakasai a*

a Department of Biochemistry, College of Health Sciences, Bayero University, Kano, PMB 3011, Kano Nigeria.

Authors’ contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Chromium (Cr) is toxic, mutagenic, carcinogenic, and harmful to most plants, animals, and bacteria inhabiting aquatic environments because it is mobile, highly toxic, soluble in water and it is a good oxidizing agent and it is detrimental to cell membranes. Chromium remediation with the help of microorganisms is believed to be the best and cheaper technology at hand to clean up Cr contamination, some chromium resistant bacteria can reduce chromium (VI) to (III) with the help of reductase enzymes. This research was aimed to isolate and identify bacteria capable of reducing chromium from tannery effluent. The level of chromium from different tannery industries was analyzed using Microwave Plasma Atomic Absorption Spectroscopy (MPAES) and compared to Environmental Protection Agency (EPA) standard which shows that all the samples were above the EPA standard except for one sample, a pour plating method was used to isolate the bacteria on Luria Bertani (LB) amended with K2Cr2O7 after serial dilution, the isolate was identified morphologically, biochemically and molecularly as Acetanaerobacter sp. based on 16s rRNA gene sequencing and molecular phylogenetic analysis, Atomic absorption spectrophotometry was carried out to determine the level of chromium from the bio-reduced sample. A reduction of 18.32% was observed in broth treated with the isolate within 48 hours of incubation at 37°C.

Keywords: Bioreduced; chromium; tannery industries; Acetanaerobacter sp.

*Corresponding author: E-mail: hmyakasai.bch@buk.edu.ng;
1. INTRODUCTION

Leather tanning and its related industrial pollution are one of the major industrial as well as environmental pollution problems in developing countries [1]. Kano has several tannery industries in Challawa, Sharada, and Bompa industrial estates. These areas are well known for their tannery industries. Hence, resolving environmental pollution from these industries have been a challenging issue because their operations could generate a large amount of organic and inorganic wastes known as Sludge [2] and the quantity is on the increase because of the high demand for leather materials and its position (as a second non-oil source of foreign exchange) in the nation’s economic development FME, 2012, [3] as this industry produces a wide range of goods such as leather bags, leather footwear, leather garments and so on. Leather tanning industries use chromium compounds intensively in the tanning process and discharge the crude effluents containing a higher level of chromium in the natural environments which leads to severe anthropogenic chromium pollution [4,5].

Chromium is the major pollutant in tannery waste. It is found in various oxidation states ranging from -2 to +6, among which chromium (VI) and chromium (III) are the most notable due to their persistence and stability. Chromium (VI) is listed in the Agency of Toxic Substances and Diseases Registry (ATSDR) priority list and also among the seventeen chemicals causing harm to human beings as stated by the United States Environmental Protection Agency (US EPA) (US Dept. of Energy) and the maximum concentrations of Cr(VI) into the inland surface water and drinking water have been set as 0.1 mg•kg•1 and 0.05 mg•kg•1 respectively [6] and the acceptable limit of 2 mg/L for wastewater discharge [7]. Currently, a maximum value of 10 µg/L for the general population and 20 µg/L for the occupationally exposed population is accepted [8].

Chromium is released into the environment through several industrial leachates, such as electroplating, leather tanning, pigment production, paints, steel manufacture. Other industrial processes using catalysts release huge amounts of chromium yearly and it has become a serious health issue [9].

Chromium compounds can cause cancer, mutation, and obstruct enzymes and nucleic acid synthesis [7]. At high accumulation, chromium is toxic, mutagenic, carcinogenic, and teratogenic. Chromium exists in oxidation states of +2, +3, and +6. The hexavalent chromium is much more poisonous to many animals, plants, and bacteria inhabiting aquatic environments. Most microorganisms are exposed to Cr (VI) toxicity but some groups possess resistance mechanisms to tolerate high levels.

One of the major environmental concerns in the tanning industry is getting rid of chromium-contaminated sludge produced as a by-product of wastewater treatment. There are various physicochemical methods (ozonation, electrocoagulation, ultrafiltration, and reverse osmosis,) for the treatment of tannery wastewater, but they have their limitation. For example, coagulation-flocculation by Al and Fe salts have shown acceptable outcomes in terms of chromium, total suspended solids (TSS), COD, and turbidity removals but at the expense of additional sludge formation. Similarly, electrochemical oxidation is expensive when applied to raw tannery wastewaters due to high energy demand and also due to the corrosive effects of effluent on the electrodes. Also, the main disadvantage of membrane application is significant fouling due to the clogging, adsorption, and cake layer formation by the pollutants onto the membrane [10]. Bioremediation, which includes the application of microorganisms or their enzymes is a potential technique for decontamination of industrial wastewaters [11]. Chromium remediation through microorganisms is accepted as the best and most cheaper technology at present to clean up Cr contamination. It was observed that some chromium-resistant bacteria have the ability to reduce chromium with the help of the enzyme reductase. The chromate reductases found in chromium-resistant bacteria are known to catalyze the reduction of chromium reduction [12]. This property may be due to the presence of chromate reductase gene or induced protein [11].

2. MATERIALS AND METHODS

2.1 Sample Collection

Effluent samples from the tannery industry were collected on August 28th, 2019 from Challawa industrial area Kano located on latitude 11°58-11°50 N and longitude range of 8°31- 8°40 E at an average elevation of 430m above the mean sea level in sterile bottles and transported to Microbiology laboratory, Bayero University Kano.
2.2 Detecting Chromium Level

The samples were digested and chromium level was quantified using Microwave Plasma Atomic Emission Spectroscopy (MP-AES).

2.3 Isolation of Chromium-Reducing Bacteria

The microorganisms were isolated from tannery effluent by the pour plating method. About 1 ml of the effluent was mixed with 9 ml of sterile physiological saline (0.85%) and serially diluted. Dilutions from $10^{-2}$ to $10^{-8}$ were pour plated on Luria Bertani (LB) medium amended with $K_2Cr_2O_7$ as hexavalent chromium compound and incubated for 24 h at 37°C. The selected colonies were then grown on tryptic soy broth throughout the study [13].

2.4 Pure Culture of Isolates

Out of the several colonies that were obtained from serial dilution by pour plating method, unique colonies were then selected based on the colony morphology and were purified by repeated streaking on Luria Bertani (LB) medium amended with $K_2Cr_2O_7$.

2.5 Screening for Hexavalent Chromium Tolerant Bacteria

The selected colonies labelled A, B, C, and D were then separately grown on tryptic soy broth (TSB) amended with different concentrations of $K_2Cr_2O_7$ as a source of hexavalent chromium 20, 40, 60, 80, and 100 mg/L along with control (without $K_2Cr_2O_7$) and incubated at 37°C for 24 h.

2.6 Identification of Cr (VI)-Reducing Bacteria

The isolate that tolerated the highest concentration of Cr(VI) was selected and further identified based on morphology, biochemical and molecular identification [14].

2.7 Gram Staining

A smear was prepared on a clean grease-free slide and gram staining was carried out according to [15].

After Gram Staining biochemical test was then carried out.

2.8 Catalase Test

A portion of the bacteria was transferred with a sterilized wire loop to a drop of $H_2O_2$ on a clean glass slide. The presence of catalase was observed by the effervescence of $O_2$ and no effervescence indicates a negative result.

2.9 Urea Utilization Test

A disinfected wire loop was used to transfer the inoculum onto a medium containing urea as a sole source of nitrogen and phenol red and was incubated for 24 h at 37°C. The development of pink-red indicates positive result and initial yellow indicate negative result.

2.10 Oxidase Test

p-phenylenediamide dihydrochloride solution and placed over a colony and then observed for colour change after 10-15 seconds. Dark purple indicates the presence of cytochrome oxidase in the test colony.

2.11 Methyl Red Test

A portion of the media was inoculated into a Glucose phosphate broth and incubated at 37°C for 24 – 48 h. After 24 hours few drops of methyl red reagent was added color change indicate positive result.

2.12 Voges-Prauskauer (VP) Test

Glucose phosphate broth was inoculated with culture and incubated at 37°C for 24 – 48 h. After 24 h few drops of 40% KOH, mix vigorously and alpha naphthol was added and results were observed immediately.

2.13 H₂S Production Test

Peptone broth was inoculated with test culture. A white filter paper strip was soaked in saturated lead acetate solution. Lead acetate paper strip was placed in the neck of the tube such that ¼ of the strip projects below the cotton plug and then broth was incubated at 37°C for 24 h. After 24 hours result was observed based on color change.

2.14 Nitrate Reduction Test

The test culture was inoculated in Peptone Nitrate Broth and incubated at 37°C for 24 h.
After 24 h, 0.5 ml of alpha naphthylamine was added, followed by the addition of 0.5 ml sulphanilic acid and observed for development of red colour.

### 2.15 Citrate Utilization Test

Simmon citrate agar slant was streaked with the test culture and incubated at 37°C for 24 – 48 h. Slant was observed for the color change, color change from green to blue indicates positive result.

### 2.16 Indole Production Test

Tryptone broth was inoculated with test culture and incubated at 37°C for 24 h. After 24 h, 1 ml of Kovac’s reagent was added on top of the medium and observed for the formation of pink ring or cherry red ring.

### 2.17 Starch Utilization Test

The nutrient agar plate having 2% starch was streaked at the center as a spot with test culture and incubated at 37°C for 24 – 48 h. After 24 h, the plate was flooded with iodine solution and checked for the zone of hydrolysis [16].

### 2.18 Molecular Identification

Genomic DNA was extracted from pure bacterial culture grown on tryptic soy broth according to (Livak, 1984). The PCR reaction was carried out using KAPAq DNA polymerase, and 16S rRNA gene fragment analysis was carried out by PCR amplification using forward primer Bact1442-F (AGAGTTGATCCTGGCTCAG) and reverse primer Bact1492-R (GGTTACCTTGTTACGACTT). PCR products were separated in 1.5% agarose gel stained with ethidium bromide, visualized under UV light, and photographed. The 16S rRNA gene sequences of the isolates obtained in this study were aligned and compared with the known 16S rRNA gene sequences in the Genbank database using the BLAST at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST/) to determine the closest available database sequences.

### 2.19 Assessment of Cr VI) Bio-Removal Efficiency

Atomic Absorption Spectroscopy (AAS) was carried out to analyze the level of chromium in the bioreduced (cultured) samples relative to control (uncultured). Both samples were digested and taken for AAS at the central laboratory complex Bayero University, Kano.

### 3. RESULTS

#### 3.1 Level of Chromium in Tannery Effluent

The level of chromium from discharged tannery effluent was assessed using MP-AES and compared with EPA standard as presented in Fig. 1 the result revealed that out of the six samples only one is below EPA standard for chromium discharged in industries.

Following serial dilution and a successful pour plating on LB media and incubating at 37 °C for 48 h, round milky colonies appeared, from which a distinct colony was isolated and streaked on a freshly prepared sterile media to obtain a pure culture.

#### 3.2 Screening for Cr (VI) Tolerant Bacteria

Four isolates were obtained from the tannery sludge, following serial dilution and pour plating they were labelled A, B, C, and D, the isolates were then screened for their tolerance to chromium salt by growing them on tryptic soy agar supplemented with various concentrations of Cr compound and incubated for 24 h at 37°C. Of the four isolates, only isolate B was able to tolerate the highest concentration tested, therefore was chosen for the study (Table 1).

<table>
<thead>
<tr>
<th>Isolate</th>
<th>20 (mg/L)</th>
<th>40 (mg/L)</th>
<th>60 (mg/L)</th>
<th>80 (mg/L)</th>
<th>100 (mg/L)</th>
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<tbody>
<tr>
<td>A</td>
<td>+</td>
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<td>B</td>
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<td>C</td>
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<td>D</td>
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**Key:** + = present, - = absent of growth
3.3 Identification Hexavalent Chromium-Reducing Bacteria

The morphology of the isolates from the tannery effluent grown on tryptic soy agar at 37°C for 24 h was visualized under the microscope following gram staining. The result shows that all the isolates (A, B, C, and D) were gram-positive with different morphology (Table 2).

3.4 Biochemical Identification of the Hexavalent Chromium-Reducing Bacterium

Biochemical test for the bacterial isolates from tannery effluent was presented in Table 3. It was observed that the candidate isolate was positive to Voges-prauskauer, catalase, oxidase, nitrate production, H₂S production, indole production, citrate utilization, starch utilization, and negative to methyl red, and urea utilization.

3.5 Molecular Identification of Hexavalent Chromium Reducing Bacterium

It was found that after subjecting the PCR product to sequencing using cycle sequencing kit genetic analyzer from both forward and reverse directions. The 16S rRNA gene sequences of the bacterium obtained was compared with the GenBank database using Blast Server at NCBI. The analysis shows that the DNA sequences obtained were closely related to the partial sequence of several *Acetanaerobacter* sp. Molecular phylogenetic tree studies using the neighbour-joining method is presented in Fig. 2.

3.6 Assessment of Cr (VI) Bio-Removal Efficiency

To determine the Cr bio-removal efficiency of the isolate, inoculated and non-inoculated Cr containing culture media was subjected to Atomic Absorption Spectrophotometry (AAS) to find the concentration of Cr, following 48 h incubation at 37°C. From the result (Fig. 3.) it was found about 18.32% of the Cr was removed from the cultured sample within 48 h of incubation.

4. DISCUSSION

Microbial reduction of Cr is an environmentally friendly technology that is cheaper compared to chemical processes [17]. To develop a competent process for Cr bioreduction, microbes that are indigenous to Cr-contaminated sites can be used because they have developed a potential detoxifying process that makes them survive with the toxic Cr. Indeed, a notable relationship was found between soil Cr content and the presence of Cr-tolerant and -resistant bacteria [18].

![Fig. 1. Level of Chromium from discharged tannery effluent](data:image/png;base64,iVBORw0KGgoAAAANSUhEUgAAAIgAAAAHCAIAAADo0JAAACXBIlKBOwAAAABJRU5ErkJggg==)  
*Data are mean ± SD of triplicate determinations*
Table 2. Morphological identification of the isolates following gram staining

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Gram staining</th>
<th>Shape</th>
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<tbody>
<tr>
<td>A</td>
<td>Positive</td>
<td>Rod-shaped</td>
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<tr>
<td>B</td>
<td>Positive</td>
<td>Rod-shaped</td>
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<tr>
<td>C</td>
<td>Positive</td>
<td>Rod-shaped</td>
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<tr>
<td>D</td>
<td>Positive</td>
<td>Cocci</td>
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Table 3. Biochemical identification for the candidate isolate

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<tr>
<th>Biochemical test</th>
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<tr>
<td>Methyl Red</td>
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<td>Catalase</td>
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<td>Voges-Prauskauer</td>
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<td>Nitrate Reduction</td>
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<td>H₂S Production</td>
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<td>Indole Production</td>
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<td>Citrate Utilization</td>
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<td>Starch Utilization</td>
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<td>Urea Utilization</td>
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Key: +Positive –Negative.

Fig. 2. Cladogram (neighbour-joining method) indicating the genetic relationship between unknown and referenced related microorganisms based on 16s rRNA gene sequence analysis. Accession numbers are accompanied by the specie names.
Since the first report of anaerobic Cr\textsuperscript{6+} reduction by Romanenko and Koren'Kov (1977) in uncharacterized Pseudomonas sp., globally aerobic and anaerobic Cr\textsuperscript{6+} reducing bacteria belonging to a range of genera from diverse environments have been isolated by researchers [19]. Up to now, several Cr-reducing bacteria have been isolated, including Bacillus [20], Desulfovibrio vulgaris, Enterobacter cloacae, Pseudomonas aeruginosa, Ralstonia metallidurans, P. putida, Cupriavidus metallidurans, Escherichia coli, Shewanella oneidensis, Caulobacter crescentus, Burkholderia cepacia and Bacillus firmus [21]. Enterobacter [22], Escherichia [23], Shewanella sp., Achromobacter sp., Pseudomonas sp. and others [24,25,26], Nesterenkonia sp. strain MF2, Sphaerotilus natans, Achromobacter sp. strain Ch1 [27], Escherichia [28], and Arthrobacter [29], Enterobacter cloacae HO1 [30], Bacillus sp. [31] and so on, indicating a vital approach in bioremediation of Cr contamination. The use of microorganisms to bioremediate metal-contaminated environment is an economical and environmentally friendly approach. Thus, chromate-reducing microbes with an inherent ability for chromium detoxification are considered to be suitable for on-site and in situ bioremediation [32].

5. CONCLUSION

The bacterium was isolated from tannery effluent, it was found to be gram-positive and identified as Acetanaerobacter sp. by 16S rRNA gene sequencing and molecular phylogeny and was capable of tolerating up to 100 mg/L of K\textsubscript{2}CrO\textsubscript{7}. The isolate was found to reduce Cr(VI) concentration by 18.32% within 48 hours. Thereby the isolate can be considered as a candidate for chromium bioreduction.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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Fig. 3. Chromium concentration in inoculated (Bioreduced) and uninoculated (control) samples incubated at 37°C for 48 h

Data are mean ± SD of triplicate determinations. Different letters over the bar indicate a significant difference (p<0.05)
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