Isolation and identification of Chromium-reducing bacteria from Challawa industrial area Kano State, Nigeria

Abstract

Chromium (Cr) is toxic, mutagenic, carcinogenic, teratogenic, and much more toxic to many plants, animals, and bacteria inhabiting aquatic environments because it is motile, highly toxic, soluble in water and it is a strong oxidizing agent that causes severe damage to cell membranes. Chromium remediation through microorganisms is accepted as the best and economically affordable technology at present to clean up Cr contamination, some chromium resistant bacteria possessed the property of reducing 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 $K_2Cr_2O_7$ 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.

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 Bompai industrial estates. These areas are well known for its tannery industry. 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 continually increases because of the high demand in leather materials and its status (as a second non-oil source of foreign exchange) in the nation's economic development FME, 2012,(3) as this industry produces a broad range of goods such as leather footwear, leather bags, leather garments and so on. Leather tanning industries use chromium compounds extensively in the tanning process and release the untreated effluents and solid wastes containing a higher level of chromium in the natural environments which results in severe anthropogenic chromium pollution (4,5).

Among heavy metals present in tannery waste, Chromium is one of the most common pollutants. Chromium is found in several oxidation states ranging from -2 to +6, among which chromium (VI) and chromium (III) are the most significant as of their persistence and stability. Chromium (VI) catches its place in the priority list arranged by the Agency of Toxic Substances and Diseases Registry (ATSDR) and also Cr(VI) has been listed as one of the seventeen chemicals causing harm to human beings by the United States Environmental Protection Agency (US EPA) (US Dept. of Energy) and the limited concentrations of Cr(VI) into the inland surface water and potable water have been set as 0.1 mg•kg-1 and 0.05 mg•kg-1 respectively (6) and the permissible limit of 2 mg/L for wastewater discharge (7).

Currently, a maximum value is accepted of 10 µg/L for the general population and 20 µg/L for the occupationally exposed population (8).

Chromium is discharged into the environment through various industrial wastewaters, such as leather tanning, electroplating, paints, pigment production, steel manufacture. Other industrial processes using catalysts discharge worldwide huge amounts of chromium every year and it has become a serious health problem (9)

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

One of the major emerging environmental problems in the tanning industry is the disposal of chromium contaminated sludge produced as a by-product of wastewater treatment. There are several physicochemical methods (electrocoagulation, ozonation, reverse osmosis, and ultrafiltration) available for the treatment of tannery wastewater, but they have one or more shortcomings. For instance, coagulation-flocculation by Fe and Al salts have shown reasonable results in terms of total suspended solids (TSS), chromium, COD, and turbidity removals, albeit at the expense of additional sludge formation. Likewise, electrochemical oxidation proved uneconomic when applied to raw tannery wastewaters due to the very high energy requirement and also due to the corrosive effects of effluent on the electrodes. Similarly, the main drawback 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 utilization of microorganisms or their enzymes is a potential technique for detoxification of industrial wastewaters (11). Chromium remediation through microorganisms is accepted as the best and most economically affordable technology at present to clean up Cr contamination. It was noted that some chromium resistant bacteria possessed the property of chromium reduction with the help of reductase enzymes. 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).

Materials and Methods

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.

Detecting Chromium level

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

Isolation of chromium-reducing bacteria

The microbes were isolated from tannery effluent (semi-solid) by 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)

Pure culture of isolates

Out of the various 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 $K_2Cr_2O_7$.

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.

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).

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.

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 indicate negative result.

Urea Utilization Test

A sterilized 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

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.

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.

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.

H_2S 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.

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 napthylamine was added, followed by the addition of 0.5 ml sulphanilic acid and observed for development of red colour.

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.

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.

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).

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 KAPATaq 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 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.

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.

Results and Discussion

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 **Figure** I, the result revealed that out of the six samples only one is below EPA standard for chromium discharged in industries

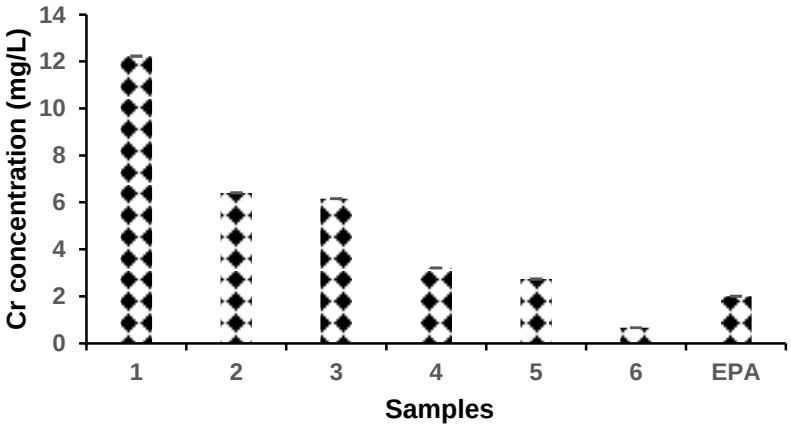

Figure I: Level of Chromium from discharged tannery effluent. Data are mean ± SD of triplicate determinations

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.

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** I).

Table I: Screening of various isolates from tannery effluent for tolerance to various Cr concentrations on tryptic soy broth (TSB) media at 37 °C for 24 h

Isolate	20	40	60	80	100
			(mg/L)		
A	+	+	+	–	–
B	+	+	+	+	+
C	+	+	–	–	–
D	+	+	+	–	–

Key: + = present, - = absent of growth

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** II).

Table II: Morphological identification of the isolates following gram staining

Isolate	Gram staining	Shape
A	Positive	Rod shaped
B	Positive	Rod shaped
C	Positive	Rod shaped
D	Positive	Cocci

Biochemical identification of the hexavalent chromium-reducing bacterium

Biochemical test for the bacterial isolates from tannery effluent was presented in **Table** III. It was observed that the candidate isolate was positive to Voges-prauskauer, catalase, oxidase, nitrate production, H_2S production, indole production, citrate utilization, starch utilization and negative to methyl red, and urea utilization.

Table III: Biochemical identification for the candidate isolate

Biochemical Test	Result
Methyl Red	–
Catalase	+
Oxidase	+
Voges-Prauskauer	+
Nitrate Reduction	+
H_2S Production	+
Indole Production	+
Citrate Utilization	+
Starch Utilization	+
Urea Utilization	–

Key: +Positive -Negative

Molecular identification of hexavalent chromium reducing bacteria

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 **Figure** II

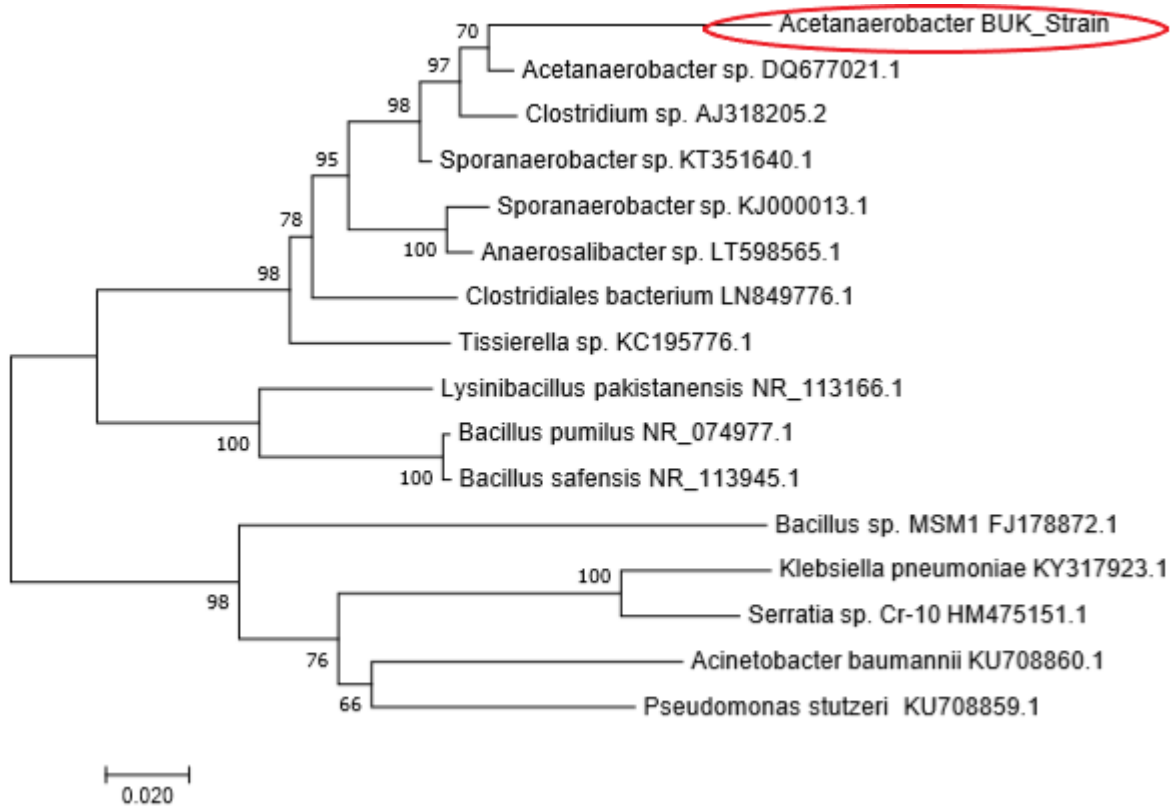

Figure II: Cladogram (neighbour-joining method) indicating the genetic relationship between unknown and referenced related microorganisms based on 16s rRNA gene sequence analysis. Accession numbers are accompanied by the specie names

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 determine the concentration of Cr, following 48 h incubation at 37 °C. From the result (**Figure** III) it was found about 18.32% of the Cr was removed from the cultured sample within 48 h of incubation.

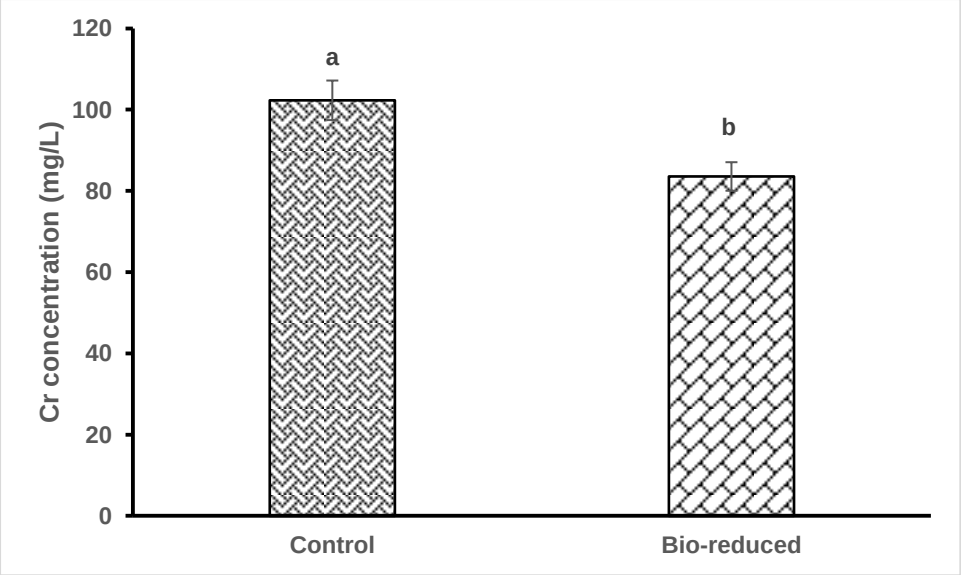

Figure III: Chromium concentration in inoculated (Bioreduced) and uninoculated (control) samples incubated at 37 °C for 48 h. Data are mean ± SD of triplicate determinations. Different letters over the bar indicate a significant difference ($p<0.05$).

Discussion

Microbial reduction of Cr is a green technology that is more cost-effective compared with chemical processes (17). To develop an efficient process for Cr bioreduction, microbes that are native to Cr-contaminated sites can be utilized. Since the site is their natural habitat, we hypothesize that they have developed a potential detoxifying process that allows them to cope with the otherwise toxic Cr. Indeed, a significant relationship was found between soil Cr content and the presence of Cr-tolerant and -resistant bacteria (18).

Since the first report of anaerobic Cr^{6+} reduction by Romanenko and Koren'Kov (1977) in uncharacterized *Pseudomonas* sp., worldwide researchers have isolated both aerobic and anaerobic Cr^{6+} reducing bacteria belonging to a range of genera from diverse environments (19) Up to now, several Cr-reducing bacteria have been reported, including *Bacillus* (20), *Enterobacter cloacae, Desulfovibrio vulgaris, Pseudomonas aeruginosa, Cupriavidus metallidurans, Ralstonia metallidurans, P. putida, Escherichia coli, Caulobacter crescentus, Shewanella oneidensis, Bacillus firmus* and *Burkholderia cepacian* (21). *Enterobacter* (22), *Escherichia* (23), *Achromobacter* sp., *Shewanella* sp., *Pseudomonas* sp. and others (24,25,26), *Nesterenkonia* sp. strain MF2, *Achromobacter* sp. strain Ch1, *Sphaerotilus natans* (27), *Escherichia* (28), and *Arthrobacter* (29), *Enterobacter cloacae* HO1 (30), *Bacillus* sp. (31) and so on, indicating an important approach in bioremediation of Cr contamination. The utilization of microbes 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).

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_2CrO_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 bio-reduction.

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