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Molecular Identification of Potent Chromium Reducing Bacteria Isolated from Hydrocarbon-Contaminated Soil within Sokoto Metropolis

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Abstract

Hydrocarbon-contaminated soils are recognised as reservoirs for heavy metal-utilizing bacteria due to the phenomenon of co-selection. These bacteria can have a potential in the biosorption of chromium heavy metal. This research aimed to screen the chromium reduction potential of bacteria isolated from hydrocarbon-contaminated soils. The soil samples used in this study were collected from hydrocarbon-affected sites in the Sokoto metropolis; additionally, a control sample was collected from non-polluted soil. Bacteria were isolated using standard protocols. Variable amounts of chromium were prepared using potassium monochromate (K_2CrO_4) and then incorporated into a nutrient broth medium. The most potent, molecularly-identified hydrocarbonoclastic bacteria were screened for chromium tolerance, and the percentage reduction in chromium content was also measured. Mean colony counts from the hydrocarbon-contaminated soil ranged from1.00×10^6 to 1.30×10^6 CFU/g while the control soil had 2.30×10^5 CFU/g. From the 14 strains, two, molecularly identified using NCBI BLAST as Brucellaintermedia and Bacillus sp., were shown to be the most potent chromium tolerant isolates. B. intermedia reduced Cr from an initial value of 350 mg/L to 198 mg/L within 72 hours (44 % removal efficiency). At the lowest concentration used in this study (50 mg/L), a removal efficacy of 96% was achieved. Bacillus sp. recorded the highest chromium reduction compared to Brucellaintermedia at the tested concentrations (50, 150, 250, and 350 mg/L). A 100% reduction in Cr was obtained at the 50 mg/L concentration. This study demonstrated that Bacillus sp. and Brucellaintermedia are particularly effective at reducing chromium from chromium metal solutions of different concentrations. These isolates can be used for bioremediation of chromium-polluted soils or water bodies.

Keywords: Bacteria, Chromium, Hydrocarbon, Reduction, Soil

INTRODUCTION

Heavy metals have detrimental effects (toxicity) on human health and the natural biota; they pose a serious environmental danger worldwide (Subhanullah *et al.*, 2024). These effects can be seen in the form of the formation of reactive oxygen species, DNA damage, lipid peroxidation, and binding to critical protein and enzyme –SH groups (Zaynab *et al.*, 2022). Heavy metals in soil pose serious threats to human and animal health. They are neither neutral to plants nor microorganisms (Li and Imran. 2024). They can also exert have inhibitory effect on the development of bacteria, fungi, and actinomycetes (Eshboev *et al.*, 2024).

Chromium as the element is found to have two stable oxidation states that are chromium (III) and chromium (VI) (Liu *et al.*, 2024). Chromium (VI) reduction by some microbes to chromium (III) is due to their physiological abilities (GracePavithra*et al.*, 2019). These forms of chromium can switch in a large-scale industrial plant operation (Mansor *et al.*, 2024). However, chromium (III) is less harmful to the environment because it is less toxic (Dubey *et al.*, 2024). Chromium is used in many industries, such as local tannery industries, and poses a threat to environmental contamination with chromium (Coetzee *et al.*, 2020).

In recent years, heavy metal pollution has now a global concern, and soil contamination with these metals is a significant contributor to the pollution (Gaur *et al.*, 2024; Giechaskie *et al.*, 2024). Hydrocarbon contamination of soils,

resulting from industrial activities and improper waste disposal, often threatens ecosystems (Frazer-Williams and Sankoh, 2024; Sajjad *et al.*, 2024). Chromium, a toxic heavy metal commonly found in such contaminated sites, further exacerbates environmental hazards (Budj *et al.*, 2024).

Bacteria adapted to hydrocarbon-contaminated environments often possess enzymes involved in hydrocarbon degradation, such as hydroxylases and dehydrogenases (Liu *et al.*, 2024; Riko and Darma, 2024). Additionally, some of these bacteria exhibit a remarkable ability to reduce chromium ions, transforming toxic Cr (VI) into less harmful Cr (III) (Ahmad, 2014; Ma *et al.*, 2024; Min *et al.*, 2024). This dual functionality suggests a potential synergistic role in the bioremediation of contaminated soils, making these bacteria promising candidates for eco-friendly clean-up strategies (Ma *et al.*, 2024).

Several factors influence the efficiency of chromium reduction by bacteria in hydrocarbon-contaminated soils (Wei *et al.*, 2024). Environmental parameters such as temperature, pH, and other co-contaminant presence can impact bacterial activity (Sharma *et al.*, 2024). Additionally, the availability of electron donors, such as organic compounds from hydrocarbon degradation, is vital in aiding hydrocarbon compounds (Yuan *et al.*, 2024). Understanding these factors is vital for optimizing bioremediation processes and developing sustainable strategies for soil clean-up in environments contaminated by tannery effluents (Min *et al.*, 2024). The study aimed to isolate and screen and isolate bacteria within soil contaminated with hydrocarbon in Sokoto Metropolis, Sokoto State, Nigeria, for their chromium reduction potential.

METHODOLOGY

Sample Collection and Processing

The samples were obtained from 3 different hydrocarbon-contaminated soils, namely: Usmanu Danfodiyo University Sokoto (UDUS) Mini Market, Kantin Daji (KD), and Works School (WK) in Sokoto metropolis, Sokoto State, Nigeria. Two hundred grams (200 g) of the samples were collected from a depth of 10 cm in the hydrocarbon-contaminated soils, in triplicates, and in sterile polythene bags. The fourth samples from non-hydrocarbon contaminated soil were collected within the UDUS main campus to serve as a control. The samples were properly labelled and transported immediately for analysis to the Postgraduate Microbiology Laboratory, UsmanuDanfodiyo University Sokoto. Samples were serially diluted and 1 mL of each suspension was plated based on spread plating onto Nutrient Agar (NA) plates. Plates were then incubated at 37˚C for 24 hours in accordance with the procedure of Ogodo (2022). Obtained pure cultures were subjected to Gram staining, as described before (Fardami *et al.*, 2022).

Spore Staining

A smear was formed and heat-fixed on a slide to check metallotolerant bacteria that indicates resilience in the face of adverse environmental stress such as heavy metalsA. After applying and heating with 5% Malachite green solution until steam rose, the mixture was allowed to cool. After that, the slides were carefully rinsed with water. After 30 seconds of counterstaining the smear with 0.5% safranin, water was used to wash it. After the slide was blot-dried, it was checked for spores using an oil immersion objective lens, and vegetative cells were dyed red, and spores were tinted green. The remaining slides underwent the same process (Fardami *et al.*, 2022).

Diphenylcarbazide (DPC) Method of Chromium Reduction Assay

Using the diphenylcarbazide (DPC) method, the ability of the bacterial isolates to convert Cr (VI) into a less hazardous form was assessed by measuring the decrease in hexavalent chromium concentration. To determine the chromium reduction activity, the 24-hour bacterial cultures were inoculated in an LB broth medium containing (50, 150, 250, and 350 mg/L) of Cr (VI) as K_2CrO_4 at 30 ˚C. Centrifugation was used to collect the isolates for 10 minutes at 13,000 rpm. Using a UV-visible spectrophotometer to assess the absorbance of the Cr (VI)-DPC complex against a reagent blank, the concentration of Cr (VI) in the supernatant was ascertained. Chromate and DPC interact to form a purple complex that absorbs light with a wavelength of 540 nm (Upadhyay *et al.*, 2017). The percentage was calculated as follows:

Percentage (%) of Cr (VI) = (Absorbance of control − Absorbance of sample /Absorbance of control) × 100

DNA Extraction

The isolated bacteria were cultured overnight on Luria-Bertani (LB) broth at 28°C. After

centrifuging two (2) milliliters of the culture for five minutes at 5000 rpm, the pellet was suspended in two hundred milliliters of pH 8 TE buffer that included 50 ng/ml of RNase. 400 mL of lysis buffer was added, thoroughly mixed, and incubated at 37°C for 15 minutes, shaking every 5 minutes. Chloroform and isoamyl alcohol (24:1) were added immediately, and the mixture was inverted. After five minutes of centrifuging the tubes at 10,000 rpm, the supernatant was carefully moved to another micro-centrifuge tube. 0.1 vol 3 M sodium acetate (pH = 5.2) and 0.6 vol isopropanol were added to the supernatant, well mixed, and inverted, and then the mixture was held at a very low temperature of 40°C for 10 minutes. This was followed by centrifugation at 1000 rpm for 10 minutes. After gently shaking the particle in 70% ethanol, it was centrifuged for three minutes at 10,000 rpm. After removing the supernatant, the pellet was allowed to air dry. The isolated DNA was seen using 0.8% agarose gel electrophoresis, and the pictures were recorded (Balakrishnan *et al.*, 2022).

PCR Amplification of 16S rRNA Gene

The universal primers 27F 50-AGA GTT TGA TCC TGG CTC AG-30 and 1492R 50-GGT TAC CTT GTT ACG ACT T-30 (Sigma) were used to amplify the 16S ribosomal RNA gene. 0.5 lM of each primer, 0.05 U Taq DNA polymerase enzyme (Sigma, USA), 200 µLdNTPs, 1× reaction buffer (10 mMTris [pH 8.3], 50 mMKCl, and 1.5 mM MgCl2), 50 µl reaction mixture, and one ng of template DNA were utilized to amplify the 16S rRNA gene. The temperature cycling parameters were as follows: 5 minutes at 94°C for the initial denaturation; 31 cycles of 30 s at 95.0C; 1 minute at 54°C for the annealing phase; 2 minutes at 72°C; and 5 minutes at 72°C for the final extension. The amplification reaction was conducted using a heat cycler (MyCycler, Bio-Rad, USA), and the PCR amplicons (about 1500 bp) was analyzed by electrophoresis on 1% (w/v) Agarose gel to confirm the expected product size (Balakrishnan *et al.*, 2022).

Purification of PCR Product for Sequencing

Two methods were used to purify the PCR products include the $ZnCl_2$ precipitation method of ammonium sulphate. The $ZnCl_2$ precipitation method involved concentrating 10 mL of the culture supernatants using $ZnCl_2$ to a final concentration of 75 mM. The Ammonium Sulfate precipitation method involved four steps: ammonium Sulfate fractionation, chilled acetone, hexane treatment, and silica gel column chromatography. The precipitated material was extracted twice using equal volumes of diethyl ether, dissolved in 10 mL of sodium phosphate buffer (pH 6.5), and the pellets were dissolved in 100 µl of methanol. Preparative TLC was used to achieve further purification (Esa *et al.*, 2023).

DNA Sequencing of the 16S rRNA Gene Fragment

An Applied Biosystems sequencer was utilized to sequence the 16S rRNA purified PCR product. The bacterial isolates' 16S rRNA gene was sequenced forward and reverse. BLAST was run identify the bacterial species based on the similarities with achieved gene bank sequences. After BLASTing, the sequences were uploaded to the NCBI GenBank to request the accession number (Balakrishnan *et al.*, 2022).

Phylogenetic Tree Analysis

Sequences of the 16rRNA genes that were sourced from the gene bank in relation to each molecularly identified isolate in this study. The sourced sequences were compared to those listed in the NCBI data base. The aligned sequences were modified using MEGA X software and the phylogenetic tree was built using the Maximum Likelihood Method with a bootstrap of 500, and the relationships between the strains were displayed (Balakrishnan *et al.*, 2022).

Analyzation of Data

Data analyzed were taken in triplicates, and almost all results were expressed in the form of mean ± standard deviation. Microsoft Excel Data Analysis Tool-Pak was the statistical tool used at the level of significance ($p<0.05$). Moreover, bioinformatics tools such as NCBI BLAST and Mega X were also used in analyzing the data obtained.

RESULTS

Bacterial Colony Counts from Hydrocarbon-Contaminated Soils Samples

The bacterial colony count result is presented in Table 1. The highest colony counts were recorded in the MN sample as 13.00 x 105 CFU/g and the lowest in the the KD sample as 10.00 x 10^5 CFU/g. The control soil sample (agricultural soil) was recorded as 23.00 x 10^5 CFU/g.

Table 1: Bacterial Colony Counts from Hydrocarbon Contaminated Soils Samples

Sampling Site	Mean of Bacterial Colony Count (CFU/g)
UDUS Minimart (MN)	$1.30 \times 10^{-5} \pm 04.14$
KantinDaji (KD)	$1.00 \times 10^{-5} \pm 14.41$
Works School (WK)	$1.07 \times 10^{-5} \pm 02.89$
Control (C)	$2.30 \times 10^{-5} \pm 0.01$

Morphological and Biochemical Identification

Table 2 presents the morphological characteristics of pure cultures of the fourteen bacterial isolates after subculturing, based on shape, availability or absence of spores, and Gram reaction based on positive or negative. They were given code as M-N-1, M-N-2, M-N-3, M-N-4, M-N-5, W-K-1, W-K-2, W-K-3, W-K-4, K-D-1, K-D-2, K-D-3, K-D-4 and K-D-5.

Table 2: Morphological Characteristics of Bacterial Isolates

Code	Shape	Spo	Gra
MN1	Rod	+	+
MN2	Rod	-	-
MN3	Rod	+	+
MN4	Rod	-	-
MN5	Rod	-	-
WK1	Rod	+	+
WK2	Rod	+	+
WK3	Cocci	-	+
WK4	Rod	+	+
KD1	Rod	-	-
KD2	Rod	-	-
KD3	Cocci	-	-
KD4	Rod	-	-
KD5	Rod	+	+

KEY:M N= UDUS Minimart, WK=Work School, KD= KantinDaji, Spo=Spore Staining, Gra= Gram Reaction

Screening for the Most Potent Bacterial Isolates Based on Chromium Tolerance

Results of the screening showed that the two most potent bacterial isolates were based on chromium tolerance after 48 hours at 250, 150, and 50 mg/L pottasiummonochromate (K_2CrO_4) concentration. Isolates W-K-1 and K-D-1 were found to be the most potent based on their ability to tolerate chromium.

Results of Molecular Identification and Phylogenetic of Two Most Potent Bacterial Isolates (W-K-1 and K-D-1)

After partial sequencing of the 16S rRNA gene, the sequencing results of two isolates, W-K-1 and K-D-1, were deposited in NCBI and were identified as *Brucellaintermedia* and *Bacillus* sp., respectively based on their high percentage similarity according to BLAST search results. Figure 1 and 2 shows the evolutionary relationship of the isolates to other species as indicated in the phylogenetic analysis based on similarities and disparities in their evolutionary genetic characteristics compared to related species from the GenBank of NCBI data base. Using closely related members from the NCBI GenBank, the phylogenic tree was created using the neighbor joining method.

Chromium Reduction Assay by *Brucellaintermedia* and *Bacillus* sp.

Table 3 displays the outcome of *Brucellaintermedia* chromium reduction. Within 24, 48, and 72 hours, *Brucellaintermedia* decreased the chromium concentration from 350 mg/L to 301, 249, and 198 mg/L. Furthermore, *Brucellaintermedia* lowered the chromium concentration from 250 mg/L to 198, 147, and 99 mg/L in 24, 48, and 72 hours, respectively. *Brucellaintermedia* lowered a chromium concentration from 150 mg/L to 110, 59, and 8 mg/L in 24, 48, and 72 hours, respectively. Finally, a chromium concentration of 50 mg/L was reduced to 1.5, 0, and 0 mg/L in 24, 48, and 72 hours, respectively.

Table 3 also displays the outcome of *Bacillus* sp. reduction of chromium. *Bacillus* sp. decreased the chromium concentration from 350 mg/L to 299, 248, and 196 mg/L in 24, 48, and 72 hours, respectively. *Bacillus* sp. decreased a chromium concentration from 250 mg/L to 197, 145, and 95 mg/L in 24, 48, and 72 hours, respectively. *Bacillus* sp. first decreased a 150 mg/L concentration of chromium concentrate to 108, 57, and 7 mg/L in 24, 48, and 72 hours, respectively. Next, 50 mg/L of chromium concentrate was reduced to 1.0, 0, and 0 mg/L in 24, 48, and 72 hours, respectively.

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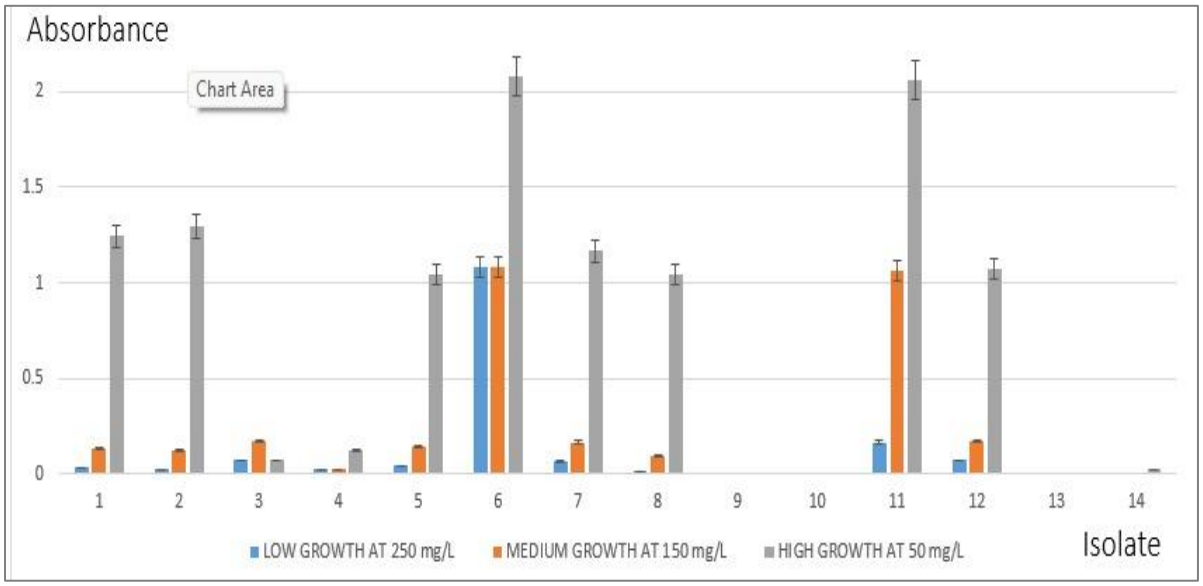

Figure 1: Absorbance Results of Fourteen Isolates Based on Chromium Tolerance after 48 hours at 250 mg/L of Potassium Monochromate (K_2CrO_4)
Key: Isolates were numbered 1 to 14 that respectively represented isolates M-N-1, M-N-2, M-N-3, M-N-4, M-N-5, W-K-1, W-K-2, W-K-3, W-K-4, K-D-1, K-D-2, K-D-3, K-D-4 and K-D-5.Where MN= UDUS Minimart, WK=Work School, KD= Kantin Daji.

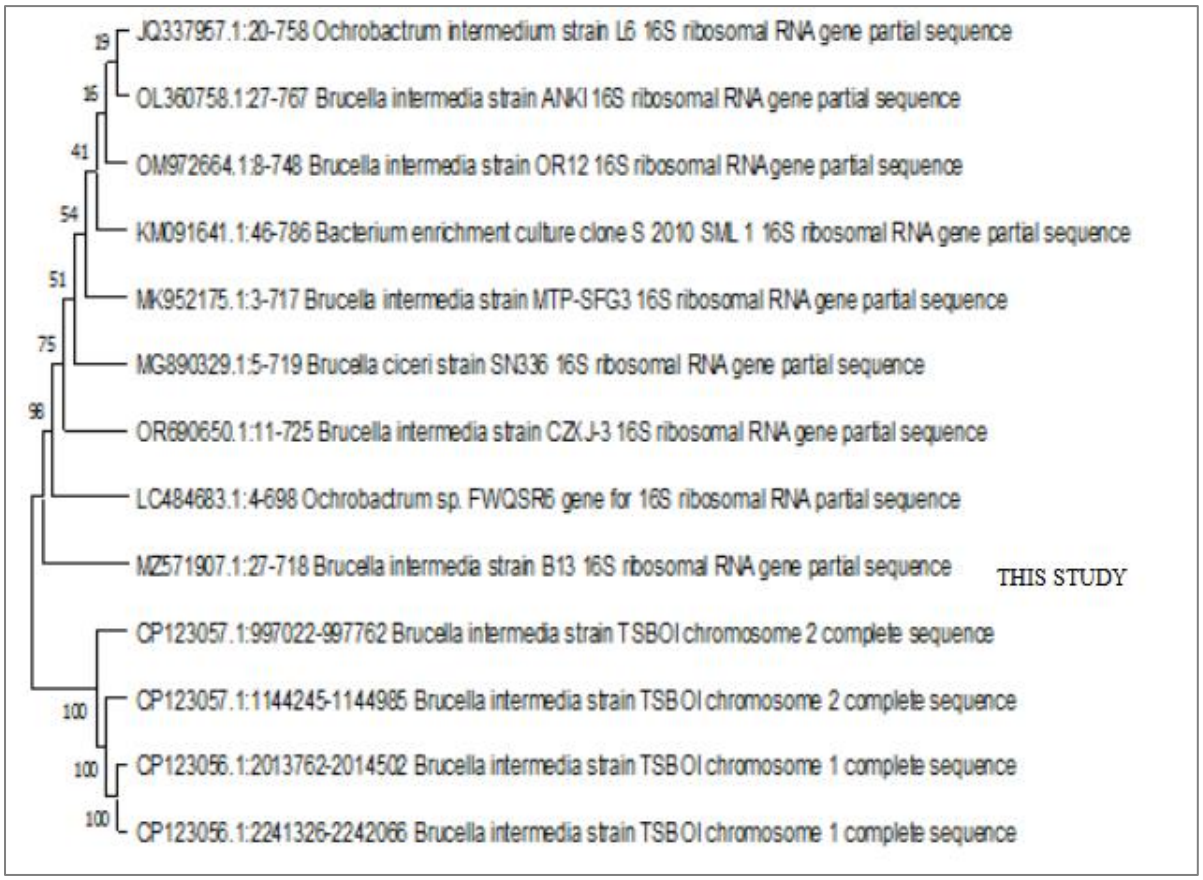

Figure 2: Phylogenetic Tree of Brucellaintermedia based on 16SrRNA Sequence using the Neighbor-Joining Method

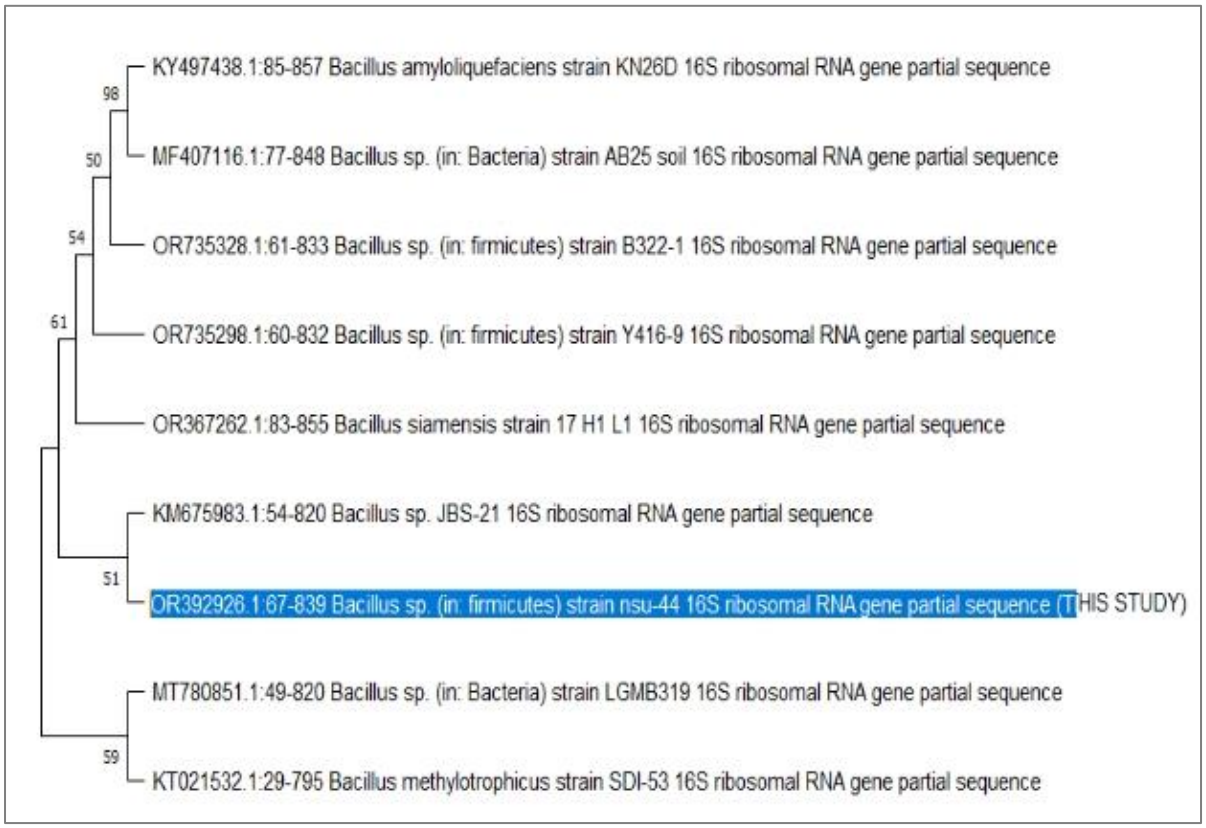

Figure 3: Phylogenetic Tree of *Bacillus* sp based on 16SrRNA Sequence using Neighbor-Joining Method

Table 3: Chromium Reduction by *Brucellaintermedia* and *Bacillus* sp

Brucellaintermedia	**(Cr) Concentration (mg/L)**			
Time (hours)	**350**	**250**	**150**	**50**
24	301±1.410	198±1.410	110±14.00	1.5±0.420
48	249±2.12	147±2.83	59±14.95	0±0
72	198±0.141	99±0.57	8±0.28	0±0
Bacillussp				
Time (hours)	**350**	**250**	**150**	**50**
24	299±0.42	197±0.14	108±0.57	1±0.01
48	248±0.28	145±0.56	57±0.42	0±0
72	196±0.28	95±1.27	7±0.28	0±0

DISCUSSION

The total heterotrophic bacterial count of the soil samples ranged from 1.0 to 3.0 x10^{-5}cfu/g (Table 1). This could be attributed to the fact that bacteria are abundant in the soil environment among which some are capable of chromium reduction. This result does not conform to the findings of Ogru and Olannye (2021), whose counts ranged between 1.0-1.2 x 106 cfu/g. It also disagreed with the results of Fardami *et al.* (2022), who reported bacterial counts that ranged between 1.10 to 8.4x 106 cfu/g. However, this might be due to differences in sample location and hydrocarbon-contaminated soil's physicochemical characteristics.

From the result of the morphological characterization, fourteen (14) bacterial isolates were isolated: s M-N-1, M-N-2, M-N-3, M-N-4, M-N-5, W-K-1, W-K-2, W-K-3, W-K-4, K-D-1, K-D-2, K-D-3, K-D-4 and K-D-5. The occurrence of all these isolates in hydrocarbon-contaminated soil could be attributed to the abundance of bacteria in the soil and their ability to utilize or degrade the hydrocarbons present in the soil due to the possession of so many enzymes such as hydroxylases and dehydrogenases, as reported by Li *et al.* (2024), Liu *et al.* (2024) and Riko and Darma (2024). The occurrence of *Brucellaintermedia*from hydrocarbon contaminated soil was confirmed by the 16S rRNA sequencing, which is in conformity with findings by Chen *et al.* (2022)

and Kumar *et al.* (2023), who both identified *Brucellaintermedia* from hydrocarbon contaminated soil. *Bacillus*sp was also confirmed from molecular partial sequencing of the 16S rRNA gene. The occurrence of *Bacillus* sp. might be due to its ability to survive in a wide range of pH and temperature because of its capability to form endospores. Abdelnasser *et al.* (2011) and Upadhyay *et al.* (2017) identified *Bacillus* sp from tannery-contaminated soils.

The present study employed *Bacillus* sp. and *Brucellaintermedia* for the chromium reduction assay. This might be due to the fact that these microorganisms can survive in chromium-contaminated water and soil because they can render heavy metals into less toxic forms through biosorption. The potential of *Brucellaintermedia* to reduce chromium was reported by several authors, including Chen *et al.* (2022) and Kumar *et al.* (2023), among others. Moreover, the potential of *Bacillus* sp to reduce chromium was reported by several authors, including Dhal *et al.* (2010), Wani *et al.* (2007), and Wróbel et al., (2023). Because they could withstand chromium, *Bacillus* sp. and *Brucellaintermedia* were the two most potent bacterial isolates among the fourteen isolates in this study. They were found to excel in chromium reduction during both the tolerance screening and assay of Cr reduction. BLAST results confirmed the sequences of the bacterial isolates that they were closely related to *Brucellaintermedia* strain B13 (89.52% similarity, with MZ571907.1 as accession number and *Bacillus* sp. strain nsu-44 with OR392983.1 as accession number. Thacker *et al.* (2007) and Kumar *et al.* (2023) had previously identified Brucella sp. strain DM1 and *Brucellaintermedia* strain BHO as chromium-reducing bacteria. Furthermore, Abdelnasser *et al.* (2011) identified molecularly *Bacillus* sp. strain KSUCr5 as a chromium-reducing bacterium.

CONCLUSION

From the fourteen bacterial isolates in this study, *Brucellaintermedia* and *Bacillus* sp. were the most potent chromium reducers . *Brucellaintermedia* was found to decrease the concentration of chromium from 350 to 198 mg/L in 72 hours (a 44% removal efficiency). Additionally, *Bacillus* sp. caused the chromium concentration to drop from 250 mg/L to 99 mg/L in 72 hours (a 60% removal efficiency). Considering that the *Bacillus* sp. identified in this study exhibited higher chromium reduction potential than *Brucellaintermedia* at concentrations of 50, 150, 250, and 350 mg/L, it is hereby recommended for subsequent research and applications in chromium biosorption.

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