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Impact of Some Stimulants on Heavy Metals and Molecular Identification of Microorganisms in the Bioremediation of Crude oil Contaminated Soil

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ABSTRACT

This study was aimed at identifying the microorganisms involved in the remediation of hydrocarbon contaminated soil and the impact of some stimulants on some metals. Unpolluted soil was obtained from Rivers State University farm was contaminated with crude oil amended with various concentrations of stimulants. Setups of (20g and 40g) of NPK, Rhamnolipids and cow dung were used to supplement 1kg each of the contaminated soil while, another 1kg of the contaminated soil without amendment was used as control sample. The experimental setups were monitored on days 1, 56 and 70 for the concentrations of heavy metals. Standard microbiological procedures were used for the isolation and identification of organisms. The microorganisms were identified by conventional biochemical and molecular methods. The organisms identified includes *Serratia fonticola* AJ233429, *Enterobacter bugandesis* LR861527, *Alcaligenes faecalis* MT421929.1, *Pseudomonas fluorescens* CP03848.1., *Geotrichum Candidium* OP153873.1, *Candida viswanathii* NG0634191, *Penicillium griseofulvum* KJ881374.1, *Aspergillus niger* MF078659.1 were identified by 16S rRNA gene sequence. The percentage bioremediation of the heavy metals showed the highest reduction in concentrations in the contaminated soil stimulated with 40g NPK> 20gNPK> 40ml RM >40g CD >20ml RM>20g CD > Control sample for iron, chromium and Lead as shown in this study. The microorganisms isolated during the remediation study may be utilized for remediation of heavy metal contaminated soil; furthermore, NPK was observed as a better stimulant in the microbial degradation of heavy metals hence could be used in effective decrease in metals contaminated soil.

Keywords: Crude oil, soil, inorganic fertilizers, bioremediation, petroleum hydrocarbon, DNA sequencing, heavy metals

Introduction

Crude oil also known as petroleum is a naturally-occurring, unrefined petroleum product, composed of hydrocarbon deposits and other organic materials. Petroleum hydrocarbons are major environmental pollutants generated by wide-scale production, transport, coastal oil refining, shipping activities, offshore oil production, artisan or illegal crude oil refining activities and accidental spilling (Arulazhagan *et al*., 2010; Douglas *et al*., 2020). Human activities such as municipal run-offs and liquid release from industries cause oil pollution which impacts the environment and poses direct or indirect health hazard to lives (Sajna *et al*., 2015). Petroleum hydrocarbon leakage due to frequent accidental, illegal refinery and illegal disposal of oil waste at sea severely harms various ecosystems.

Crude oil drilling in Nigeria especially in the Niger Delta area of Nigeria has been the country's main source of income and foreign exchange (Chijioke-Osuji *et al*., 2014).

The Niger Delta region of Nigeria is the primary recipient of crude oil spills, which can occasionally cause significant environmental damage in these areas (Chijioke-Osuji et al., 2014). According to Costa *et al*. (2012), petroleum hydrocarbons are hazardous substances categorized as priority pollutants.

Studies have shown that Crude oil pollution and the introduction into the soil and water, interferes with the structure and texture of the soil, and also result in increase in the concentration and accumulation of heavy metals such as zinc, chromium, nickel, mercury, iron, Lead and copper (Igoni *et al*., 2023)

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Bioremediation is a scientific means of using microbial life and their products in the modification or removal of any pollutant or treatment of an environmental problem using biological processes(Agbaji *et al.,* 2020).Bioremediation has been applied in petroleum and oil field chemical units due to its ability to convert pollutants into less harmful forms (Agbaji *et al.,* 2020). Bioremediation process relies upon microbial enzymatic activities to transform or degrade the contaminants from the environment. Hydrocarbon-degrading bacteria and fungi are widely distributed in marine, freshwater and soil habitats environment (Adams *et al.,* 2015). The two major approaches to enhance bioremediation of a polluted site are biostimulation and bioaugmentation provided that environmental factors, which determine the success of bioremediation, are maintained at optimal range (Adams *et al.,* 2015).

Biostimulation is the addition of oxygen, electron, donor, electron acceptors or nutrients to coordinates sites in order to increase the population and activities of naturally occurring organisms for them to remediate sites of pollutants (Osu, *et al.,* 2021). Bio-stimulation utilizes the indigenous microbial populations to remediate contaminated soils. The added nutrients and other substances in soil catalyze natural attenuation processes (Awari *et al*., 2020).

Microbial remediation of heavy metal has a far-reaching progressive prospect among the decontamination methods. Microorganisms especially soil microbes can tolerate high levels of heavy metals, some microorganisms need certain types of metals as a micronutrient (i.e., Fe^{3+} is essentially utilized by all bacteria while Fe^{2+} is significant for anaerobic bacteria) to perform their metabolic activities (Ahemad 2019). The bioremediation process could be conducted *Ex-situ* by transferring the contaminated area to be treated or even *in -situ* by delivering the biological source to the polluted land (Abo-Alkasem *et al.,* 2023)

Microorganisms in these ecosystems may lack the ability or may not be in sufficient number to degrade the oil, hence there is need to increase the growth and degradation ability by enhancing their growth through the addition of amendments such as biosurfactants (Rhamnolipids),organic amendments (cow dung) and inorganic stimulant (NPK fertiliser) and which are readily available in the restoration of polluted soil containing metals.

Materials and Methods

Collection of Samples

Agricultural soil (unpolluted soil) samples and Cow Dung were collected from the Rivers State University farm. The soil was collected at 0-15cm depth using a sterile bag. This was transported to the lab for use.Crude oil was obtained from Total E & P, Obagi Town, Rivers State, Nigeria. The nitrogen phosphate potassium (NPK 10:15:15) fertilizer was obtained from the Agricultural Development Programme (ADP) farm at Rumuodumanya, Port Harcourt. Rhamnolipid was sourced and bought from Jochemicals Nigeria Limited, Mile 1, Port Harcourt, Nigeria.

Bioremediation Setups

The collected unpolluted soil was contaminated with 5% crude oil and mixed properly(Ogbonna *et al*., 2019). One kilogram (1kg) of the soil was measured into 8 clay pots. One of the contaminated soil set up served as control sample, 20g NPK was added to one of the contaminated setups, 40g NPK was added to another of the contaminated setups, 20g cow dung (CD) was added to other setups, 40g of CD was added with one of the setups, 20g and 40ml of Rhamnolipids (RM) were added each to 2 different contaminated soil. All the samples were monitored for a period of 70 days for heavy metals, bacteria and fungi and percentage bioremediation was determined. The microorganisms involved in the bioremediation were determined using conventional microbial procedures. The organisms were identified by biochemical and molecular methods (Nwadiokwu *et al.,* 2023).

Isolation of Hydrocarbon Utilizing Bacteria (HUB) and Fungi (HUF)

The hydrocarbon-utilizing microorganisms were isolated using a mineral salt medium adopting the method of Douglas and Cornelius, (2019). The vapour phase transfer method was employed in this study, wherein 1 ml of crude oil was poured onto sterile Whatman filter paper that was placed on the Petri dish lid. An aliquot from the $10^{-2}$to 10^{-3}dilutions were transferred into prepared mineral salt agar plates and tetracycline supplemented mineral salt agar for the enumeration and isolation of HUB and HUF, respectively. The inoculated plates were evenly spread using a sterile hockey stick, and the HUF was incubated at 25°C for 7 days and HUB plates were incubated at 30°C. Isolates obtained were purified.

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Characterization and Identification of Bacterial Isolates

The isolates obtained were purified by subculturing the bacterial and fungal isolates on nutrient agar and Potato Dextrose agar respectively. The bacterial isolates were characterized by observing them microscopically and subjecting them to series of biochemical tests such as Gram stain, catalase, citrate, oxidase, coagulase, Methyl Red, Motility, indole, starch hydrolysis, Voges Proskauer and sugar fermentation tests (Talaikhozani *et al.* 2015). They were identifed by comparing their characteristics with those of known taxa as outlined in Bergey's Manual of Determinative Bacteriology (Holt, 1994).

Molecular Characterization of the Isolates

DNA extraction (Boiling method)

Five millilitres of an overnight broth culture of the bacterial isolate in Luria Bertani (LB) was spun at 14000 rpm for 3 min. The cells were re-suspended in 500 µL of normal saline and heated at 95°C for 20 min. The heated bacterial suspension was cooled on ice and spun for 3 min at 14000 rpm. The supernatant containing the DNA was transferred to a 1.5mL microcentrifuge tube and stored at -20°C for other downstream reactions (Jukes and Cantor, 1969).

DNA quantification

The extracted genomic DNA was quantified using the Nanodrop 1000 spectrophotometer. The software of the equipment was launched by double clicking on the Nanodrop icon. The equipment was initialized with 2 µL of sterile distilled water and blanked using normal saline. Two microlitre of the extracted DNA was counted onto the lower pedestal; the upper pedestal was brought down to contact the extracted DNA on the lower pedestal. The DNA concentration was measured by clicking on the "measure" button (Jukes and Cantor, 1969).

16S rRNA Amplification

The 16s rRNA region of the rRNA gene of the isolates were amplified using the 27F: 5'-AGAGTTTGATC MTGGCTCAG-3' and 1492R: 5'-CGGTTACCTTG TTACGACTT-3' primers on ABI 9700 Applied Biosystems thermal cycler at a final volume of 40µl for 35 cycles. The PCR mix included: the X2 Dream taq Master mix supplied by Inqaba, South Africa (taq polymerase, DNTPs, MgCl); The primers at a concentration of 0.5µM and the extracted DNA as template. The PCR conditions were as follows: Initial denaturation, 95ºC for 5 minutes; denaturation, 95ºC for 30 seconds; annealing, 52ºC for 30 seconds; extension, 72ºC for 30 seconds for 35 cycles and final extention, 72ºC for 5 minutes. The product was resolved on a 1% agarose gel at 130V for 30 minutes and visualized on a blue light transilluminator (Jukes and Cantor, 1969).

Sequencing

Sequencing was done using the BigDye Terminator kit on a 3510 ABI sequencer by Inqaba Biotechnological, Pretoria South Africa. The sequencing was done at a final volume of 10ul, the components included 0.25 µLBigDye® terminator v1.1/v3.1, 2.25 µL of 5 x BigDye sequencing buffer, 10 µM primer PCR primer and 2-10ng PCR template per 100bp. The sequencing condition were as follows 32 cycles of 96°C for 10s, 55°C for 5s and 60°C for 4minutes (Jukes and Cantor, 1969).

Phylogenetic Analysis

Obtained sequences were edited using the bioinformatics algorithm Trace edit, similar sequences were downloaded from the National Center for Biotechnology Information (NCBI) data base using BLASTN. These sequences were aligned using MAFFT. The evolutionary history was inferred using the Neighbour-Joining method in MEGA 6.0 (Saitou and Nei, 1987). The bootstrap consensus tree inferred from 500 replicates (Felsenstein, 1985) is taken to represent the evolutionary history of the taxa analyzed. The evolutionary distances were computed using the Jukes-Cantor method (Jukes and Cantor, 1969).

Heavy Metal Analysis of the Samples

The detection of trace metals in the environment are accomplished by various methods but in this study, the atomic absorption spectroscopy (AAS) technique was used, which is relatively simple, versatile, accurate and free from interferences. Heavy metals readily form complexes with organic constituents; therefore, it is necessary to destroy them by digestion with strong acids. Digestion destroys the organic matter, removes interfering ions and brings metallic compounds in suspension to solution (Sriadibhatla, 2013).Analysis of Heavy metals: (Lead (Pb), Iron (Fe), and Chromium (Cr) of the samples were done by using atomic absorption spectroscopy (Sriadibhatla, 2013).

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Digestion of Sample

The EPA vigorous digestion method was adopted. One hundred grams (100g) of each of the representative samples were transferred into pyrex beakers containing 10ml of conc. HNO_3. The samples were boiled slowly and then evaporated on a hot plate to the lowest possible volume (about 20 ml). The beakers were allowed to cool and another 5ml of conc. Nitric acid was added. Heating was continued with addition of conc. Nitric acid as necessary until digestion was complete.

The samples were evaporated again to dryness (but not baked) and the beakers were cooled, followed by addition of 5ml of HCl solution (1:1 v/v). The solutions were warmed and 5ml of 5M NaOH was added, then filtered. The filtrates were transferred to 100ml volumetric flasks and diluted to the mark with distilled water. These solutions were then used for the elemental analysis. The concentrations of the heavy metals were determined using a Buck Scientific model 200A Atomic Absorption Spectrophotometer (AAS) equipped with air-acetylene (APHA, 2012).

Determination of Lead

Lead, stock solution corresponding to 1000mg/l of Lead (Pb) was prepared by weighing to the nearest + 0.0002gm, apx 1.0000gm Pb metal (minimum purity 99.5%) and diluted in a covered 250ml glass beaker with 10ml HNO_3. Then add 100ml of water. Boil to expel nitrous fumes, cool, transfer to 1000ml volumetric flask and fill to the mark with water. Lead, Standard Solution Corresponding to 10mg/ l of Pb was prepared by pipetting 10.00ml of Pb stock solution into a 1000ml volumetric flask. Add 20ml of nitric acid fill the mark with water and mix well. Lead, standard solution corresponding to 0.4mg/ l of Pb was prepared by pipetting 20.00ml of Pb standard solution into a 500ml volumetric flask. Add 10ml of nitric acid fill the mark with water and mix well. This was inserted and read in the AAS (APHA, 2008).

Determination of Chromium

Chromium, stock solution corresponding to 1000mg/l of Cr was prepared by Weighing to the nearest + 0.0002gm, apex 1.0000gm Cr metal (minimum purity 99.5%) and diluted in a covered 250ml glass beaker with 40ml HNO_3. Then add 100ml of water. Boil to expel nitrous fumes, cool, transfer to 1000ml volumetric flask and fill to the mark with water. Chromium (Cr), standard solution corresponding to 10mg/l of Cr was prepared by pipetting 10.00ml of Cr stock solution into a 1000ml volumetric flask. Add 20ml of nitric acid, fill to the mark with water and mix well.

Chromium (Cr) standard solution corresponding to 0.4mg/l of Cd was prepared by pipetting 20.00ml of Cr standard solution into a 500ml volumetric flask. Add 10ml of nitric acid, fill to the mark with water and mix well. Cr, standard solution corresponding to 0.02mg/l of Cr was prepared by pipetting 5.00ml of Cr standard solution into a 100ml volumetric flask. Add 2ml of nitric acid fill to the mark with water and mix well. Insert into AAS and take readings (APHA, 2008).

Determination of Iron (Fe)

The metal, iron, stock solution corresponding to 1000mg/l of Fe was prepared by Weighing to the nearest + 0.0002gm, apex 1.0000gm Fe metal (minimum purity 99.5%) and diluted in a covered 250ml glass beaker with 40ml HNO_3. Then add 100ml of water. Boil to expel nitrous fumes, cool, transfer to 1000ml volumetric flask and fill to the mark with water. Fe, standard solution corresponding to 10mg/l of Fe was prepared by pipetting 10.00ml of Fe stock solution into a 1000ml volumetric flask.

Add 20ml of nitric acid fill to the mark with water and mix well. Fe, standard solution corresponding to 0.4mg/l of Cd was prepared by pipeting 20.00ml of Fe standard solution into a 500ml volumetric flask. Add 10ml of nitric acid fill to the mark with water and mix well.

Fe, standard solution corresponding to 0.02mg/l of Fe was prepared by pipeting 5.00ml of Fe standard solution into a 100ml volumetric flask. Add 2ml of nitric acid fill to the mark with water and mix well. Insert into AAS and take readings (APHA, 2008).

Statistical Analysis

The mean and standard deviation of the microbial counts, the physicochemical parameters and TPH of the soil samples as well as the percentage occurrence of bacterial and fungal isolates were determined using the statistical package for social science (SPSS version 27).

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Results

The results of the microbial counts of unpolluted and polluted soil are presented in Table 1.The macroscopic and microscopic characteristics of the fungal isolates are presented in Table 2. *Rhizopus* sp, *Aspergillus* sp, *Rhodotorula* sp, *Gliocladium* sp, *Cunninghamella* sp, *Candida* sp, *Paecilomyces* sp, *Blastomyces* sp, *Penicillium* sp, *Geotrichum* sp and *Mucor* sp were isolated. *Rhizopus* sp, *Aspergillus* sp, *Rhodotorula* sp, *Penicillium* sp, *Geotrichum* sp and *Mucor* sp were hydrocarbon utilizing fungi.

The phenotypic and physiological characteristics of the bacterial isolates are presented in Table 3. Results revealed that these bacteria belonged to fourteen genera: *Staphylococcus*, *Klebsiella*, *Serratia*, *Escherichia*, *Pseudomonas*, *Shigella*, *Bacillus*, *Micrococcus*, *Alcaligenes*, *Cronobacter*, *Tatumella*, *Cedecea* sp. *Proteus* and *Providencia* sp. The hydrocarbon utilizing bacteria identified includes *Bacillus* sp, *Pseudomonas* sp, *Serratia* sp, *Cronobacter* sp, *Micrococcus* sp and *Alcaligenes* sp.

Table 1: Microbial Counts of Unpolluted and Polluted Soil

Sampling	Microbial counts (CFU/g)							
	Total heterotrophic bacteria		Total fungi		Hydrocarbon utilizing bacteria		Hydrocarbon utilizing fungi	
	Unpolluted	Polluted	Unpolluted	Polluted	Unpolluted	Polluted	Unpolluted	Polluted
Day 1	1.70×10^6	1.2×10^6	3.50×10^3	3.60×10^3	1.60×10^4	1.40×10^4	1.50×10^3	1.70×10^3
Day 14	6.50×10^6	4.10×10^6	4.00×10^3	3.20×10^3	3.60×10^4	1.10×10^5	3.50×10^3	4.80×10^3
Day 28	5.20×10^6	3.30×10^6	8.00×10^3	7.00×10^3	1.00×10^4	4.60×10^4	1.00×10^3	2.00×10^3
Day 42	4.10×10^6	3.40×10^6	3.40×10^3	2.50×10^3	7.00×10^3	4.60×10^4	8.00×10^3	3.50×10^3
Day 56	3.50×10^6	2.60×10^6	2.10×10^4	1.60×10^4	5.00×10^3	3.70×10^4	4.00x102	2.50×10^3
Day 70	2.80×10^6	2.70×10^6	1.80×10^4	3.10×10^4	2.00×10^3	3.70×10^4	2.00×10^3	1.90×10^3
P value	<0.001	<0.001	<0.001	<0.001	0.05	0.05	0.013	0.013

Table 2: Macroscopic and Microscopic Characteristics of Fungal Isolates

Isolate code	Macroscopy	Microscopy	Probable identity
A	White cottony, brownish grey to black-grey, brown reverse	Smooth walled and non-septate branched sporangiophores, presence of rhizoids	*Rhizopus* sp
B	White periphery, dense black spores, dark brown reverse	Hyaline conidiophore phialides borne on vesicles, chains of conidia with septate hyphae	*Aspergillus* sp
C	Pink-red smooth colonies	Ovoid, elongate budding cells	*Rhodotorula* sp
D	Dark green slimy suede-like conidia	Erect conidiophores with phialides bearing one-celled hyaline conidia	*Gliocladium* sp
E	White cottony colonies, pale white reverse	Aseptate hyphae with round head sporangiophores bearing spine-like structures	*Cunninghamella* sp
F	Cream to shiny round colonies	Large oval cells with budding cells	*Candida* sp
G	Suede-like, yellow-brown colony	Swollen phialides, long, dry chains of single-celled oval conidia	*Paecilomyces sp*
H	Glabrous, tan, non-sporulating	Hyaline, one-celled, smooth-walled conidia	*Blastomyces sp*
I	Green powdery surface bounded by white lawn, brown reverse	Septate hyphae with septate conidiophores bearing conidia	*Penicillium sp*
J	Flat white dry sued-like colonies, white on reverse	Cylindrical arthroconidia	*Geotrichum* sp
K	Fluffy white cottony, white on reverse	Aseptate hyphae bearing round sporangiospores	*Mucor* sp

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Table 3: Phenotypic and Physiological Characteristics of Bacteria Isolates

Isolates	Macroscopy	Microscopy	Motility	Catalase	Glucose	Mannitol	Lactose	Sucrose	Oxidase	Methyl red	Voges Proskauer	Indole	Citrate	Probable Organism
A.	Golden-yellow round smooth	+ve cocci	-	+	+	+	+	+	-	+	+	+	+	*Staphylococcus* sp
B.	Pink small round	-ve rod	-	+	+	-	+	+	-	-	+	-	+	*Klebsiella* sp
C.	Red round smooth	-ve short rods	+	+	+	+	-	+	-	-	+	-	+	*Serratia* sp
D.	Metallic-silver round flat	-ve short rods	+	+	+	+	+	+	-	+	-	+	-	*Escherichia coli*
E.	Green small round moist	-ve rods	+	+	-	+	-	-	+	-	-	-	+	*Pseudomonas* sp
F.	Pale round smooth	-ve rods	-	+	+	-	-	-	-	-	+	-	+	*Shigella* sp
G.	Milky flat dry	+ve rods	+	+	+	+	-	+	-	-	+	-	+	*Bacillus* sp
H.	Yellow small round	+ve cocci	-	+	-	+	+	+	-	-	-	-	+	*Micrococcus* sp
I.	Green small round moist	-ve rods	+	+	-	-	-	-	+	-	-	-	+	*Alcaligenes* sp
J.	Pink large round smooth	-ve short rod	+	+	+	+	+	+	-	-	+	-	+	*Cronobacter* sp
K.	Brown round raised	-ve short rods	-	+	+	-	-	+	-	-	-	-	-	*Tatumella* sp
L.	Cream round flat dry	-ve rods	+	+	+	+	-	+	-	+	+	-	+	*Cedecea* sp
M.	Pale small round	-ve rods	+	+	+	-	-	+	-	-	+	-	+	*Proteus* sp
N.	Pale round smooth	-ve short rod	+	+	+	-	-	-	-	+	-	+	+	*Providencia* sp

Genomic DNA was isolated from some of the dorminant isolates, a PCR product of 1500bp of the four isolates were analyzed on 1% agarose gel electrophoresis (Plates 1 and 2). Based on the partial 16S rRNA gene sequencing;

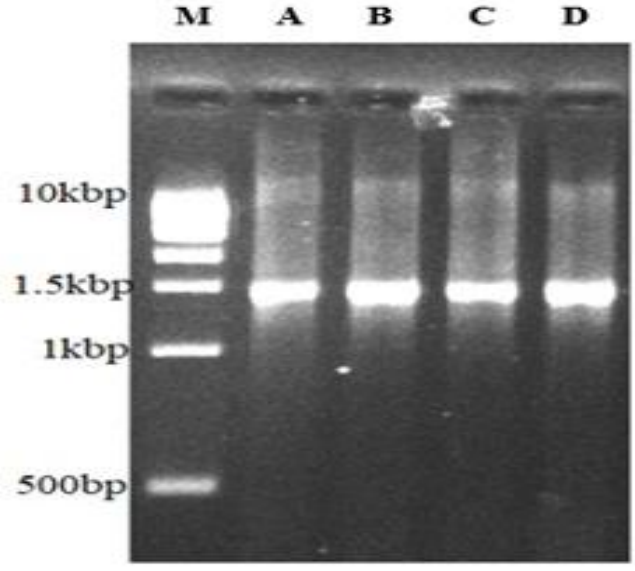

Plate 1: Agarose Gel Electrophoresis of the 16S rRNA of the Bacterial Isolates

The phylogenetic analysis of bacteria and fungi are presented in Figure 1 and Figure 2 respectively and their identities were compared to their closest relative species in the GenBank database and presented in Table 4.

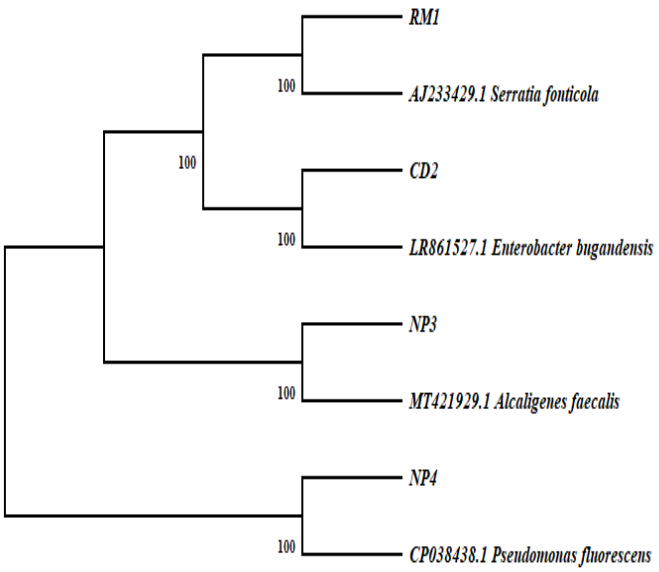

Fig. 1: Evolutionary Relationship of taxa

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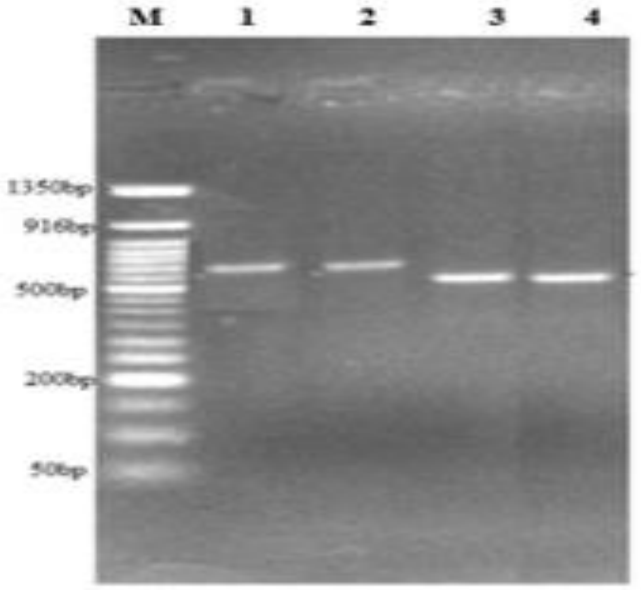

Plate 2: Gel Electrophoresis Image showing the Amplification of the ITS of the Fungi Isolates. M is a 50bp DNA ladder used to Estimate the Band Sizes

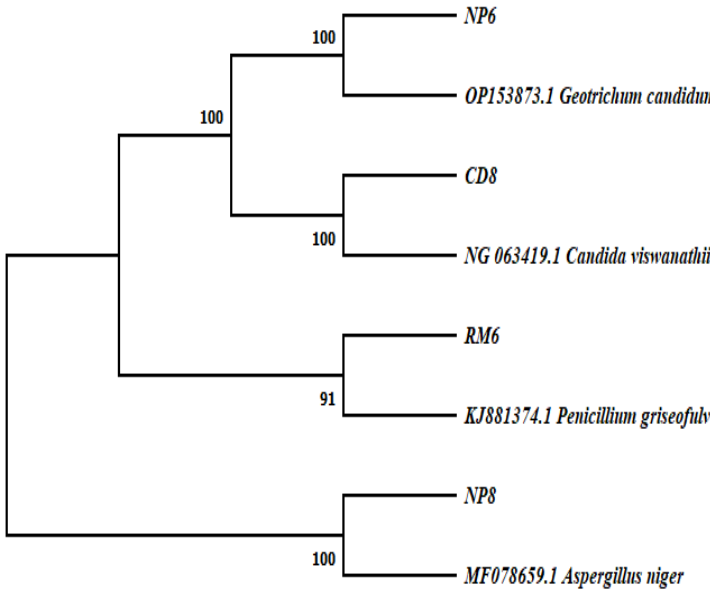

Fig. 2: Evolutionary relationships of fungi taxa

Table 4: Molecular Characterization of Bacterial and Fungal Isolates

Phenotypic identity	Molecular identity	Accession number	% Similarity
Serratia sp	*Serratia fonticola*	AJ233429.1	100
Cronobacter sp	*Enterobacter bugandensis*	LR861527.1	100
Alcaligenes sp	*Alcaligenes faecalis*	MT421929.1	99
Pseudomonas sp	*Pseudomonas fluorescens*	CP038438.1	98.9
Geotrichum sp	*Geotrichum candidum*	OP15873.1	100
Candida sp	*Candida viswanathii*	NG_063419.1	100
Penicillium sp	*Penicillium griseofulvum*	KJ881374.1	91
Aspergillus sp	*Aspergillus niger*	MF078659.1	99.8

The 16S rRNA gene sequence revealed that the strain RM1 was highly similar (100%) with *Serratia fonticola* AJ233429, the strain, CD2 show 100% relatedness to *Enterobacter bugandesis* LR861527, isolate, NP3 showed 99% similarity to the *Alcaligenes faecalis* MT421929.1 while the isolate NP4 also shown 98.9% similarity to *Pseudomonas fluorescens* CP03848.1. The NP6 showed 100% identity with *Geotrichum Candidium* OP153873.1, and the isolate, CD8 showed 100% relatedness to *Candida viswanathii* NG0634191. Isolate, RM6 showed 91% relatedness to *Penicillium griseofulvum* KJ881374.1 while the isolate, NP8 showed 99.8% similarity to *Aspergillus niger* MF078659.1.

The changes in the concentrations of the heavy metals are shown in Figures 3 to 5. The highest reduction in the concentrations of chromium was observed in the setup stimulated with 40g NPK from 0.112±0.0mg/kg on day 1 to 0.065±0.0mg/kg on day 56 to 0.038±0.0 mg/kg on day 70.

This was followed by set up soil amended with 20g NPK recording reduction from 0.112±0.0 mg/kg on day 1 to 0.076±0.0 mg/kg on day 56 and 0.042±0.0 mg/kg on day 70; followed by the setup with 40ml RM which reduced from 0.112±0.0 mg.kg on day 1 to 0.079±0.0mg/kg on day 56 and 0.044±0.0mg/kg on day 70.

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This was followed by the setup stimulated with 40g CD which recorded reduction in chromium concentrations (Figure 3) from 0.111±0.0 mg/kg on day 1 to 0.081±0.0mg/kg on day 56 to 0.048±0.0 mg/kg on day 70 followed by the contaminated soil with 20ml RM which recorded concentrations of 0.112±0.0 mg/kg, 0.083 mg/kg and 0.050±0.0mg/kg. on days 1, 56 and 70, respectively followed by 20g CD stimulated soil with concentration of 1.11mg/kg, 0.085±0.0 mg/kg and 0.050±0.0 mg/kg respectively on days 1, 56 and 70 while the control sample showed little or no difference in the concentrations with a mean range of 0.112±0.0 mg/kg, 0.10 ±0.0mg/kg and 0.1±0.0 mg.kg on days 1, 56 and 70, respectively.

The highest decrease in the concentrations of iron (Figure 4) was observed in the setup stimulated with 40g NPK with mean range from 1.481±0.0 mg/kg on day 1 to 1.083±0.0 mg/kg on day 56 to 0.57±0.0 mg/kg on day 70.

This is followed by set up with 20g NPK recording reduction from 1.443±0.0 mg/kg on day 1 to 1.102±0.0 mg/kg on day 56 and 0.68±0.0 mg/kg on day 70. Thereafter, the setup with 40ml RM reduced from 1.459±0.0mg.kg on day 1 to 1.168±0.0 mg/kg on day 56 and 0.762±0.0 mg/kg on day 70. The setup stimulated with 40g CD recorded reduction in chromium concentrations from 1.557±0.0 mg/kg on day 1 to 1.151±0.0 mg/kg on day 56 to 0.871±0.0 mg on day 70 followed by the contaminated soil with 20ml RM which recorded concentrations of 1.557±0.0 mg/kg, 1.237±0.0 mg/kg and 0.892±0.0 on days 1, 56 and 70, respectively followed by 20g CD stimulated soil with concentration of 1.515±0.0 mg/kg, 1.248±0.0 mg/kg and 0.898±0.0 mg/kg respectively on days 1, 56 and 70 while the control sample showed little difference in the concentration the iron with a mean range of 1.503±0.0 mg/kg, 1.422 ±0.0mg/kg and 1.373±0.0 mg.kg on days 1, 56 and 70, respectively.

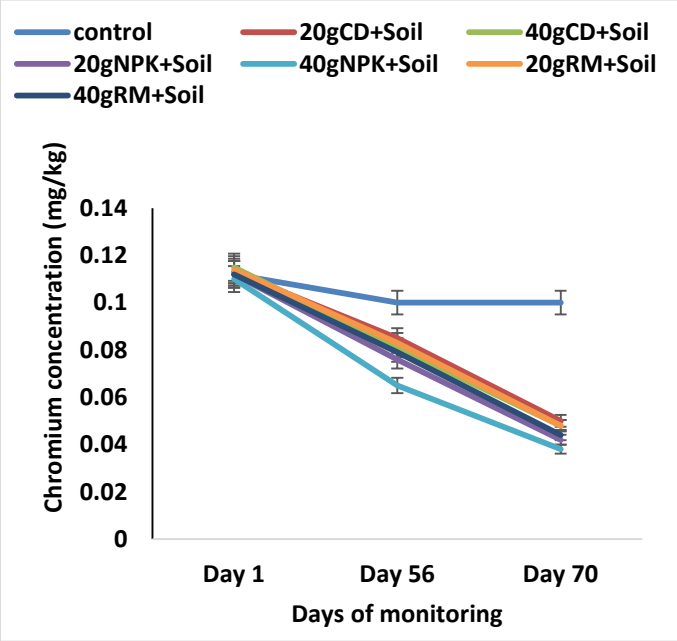

Fig. 3: Change in Concentration of Chromium during Remediation

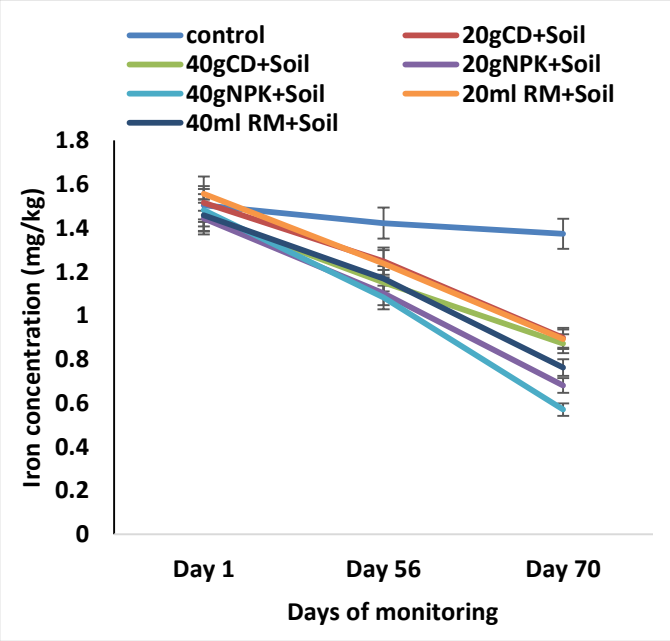

Fig. 4: Change in Concentration of Iron during Remediation

Keys: control (contaminated soil), CD: cow dung, NPK: nitrogen phosphate potassium fertilizer, RM: rhamnolipids

In the case of Lead (Pb) the highest decrease in the concentration (Figure 5) was also observed in the setup stimulated with 40g NPK with mean range from 0.112±0.0 mg/kg on day 1 to 0.073±0.0 mg/kg on day 56 to 0.051±0.0 mg/kg on day 70.

This was followed by contaminated soil stimulated with 20g NPK recording reduction from 0.122±0.0 mg/kg on day 1 to 0.085±0.0 mg/kg on day 56 and 0.063±0.0 mg/kg on day 70 followed by the setup with 40ml RM which reduced from 0.122±0.0mg.kg on day

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1 to 0.093±0.0 mg/kg on day 56 and 0.072±0.0 mg/kg on day 70.The next is the setup stimulated with 40g CD, which recorded reduction in chromium concentration from 0.112±0.0 mg/kg on day 1 to 0.094±0.0 mg/kg on day 56 to 0.077±0.0 mg on day 70 followed by the contaminated soil with 20ml RM which recorded concentration of 0.112±0.0 mg/kg, 0.095±0.0 mg/kg and 0.081±0.0 on days 1, 56 and 70 respectively followed by 20g CD stimulated soil with concentration of 0.112±0.0 mg/kg, 0.095±0.0mg/kg and 0.088±0.0mg/kg, respectively on days 1, 56 and 70

The control sample showed mean value of concentrations of Pb with mean values of 0.112±0.0 mg/kg, 0.10±0.0mg/kg and 0.10±0.0 mg/kg on days 1, 56 and 70, respectively.

Bioremediation of the heavy metals (Figure 6) revealed that the highest reduction in concentrations was in the contaminated soil stimulated with 40g NPK> 20gNPK> 40ml RM > 40g CD > 20ml RM >20g CD > Control sample for iron, chromium and Lead, as shown by the percentage removal of heavy.

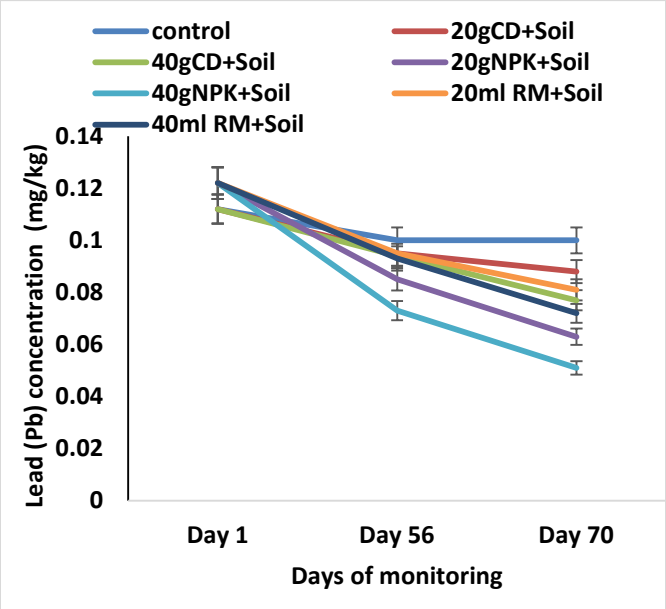

Fig. 5: Change in the Concentration of Lead during the Remediation

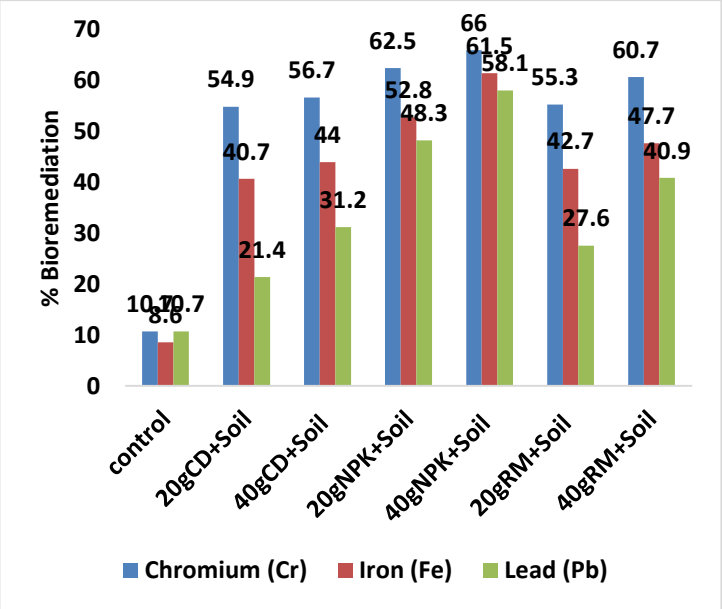

Fig. 6: Percentage Bioremediation of the Heavy Metals

Keys: control (contaminated soil), CD: cow dung, NPK: nitrogen phosphate potassium fertilizer, RM: rhamnolipids

Discussion

The genomic identification of the isolates in this study is similar to those reported by the study of Dilmi *et al.*, (2017). Organisms identified in this study include *Serratia fonticola* AJ233429, *Enterobacter bugandesis* LR861527, *Alcaligenes faecalis* MT421929.1, *Pseudomonas fluorescens* CP03848.1., *Geotrichum Candidium* OP153873.1, *Candida viswanathii* NG0634191, *Penicillium griseofulvum* KJ881374.1, and *Aspergillus niger* MF078659.1 The study of Nwankwo and Okpokwasili, (2019) among other organisms, reported *Pseudomonas* sp, *Alcaligenes* sp, *Serratia* sp, *Bacillus* sp and *Aspergillus* sp from crude oil contaminated soil.

Previous report found *Pseudomonas*, *Bacillus*, *Aspergillus* as the dominant microorganisms in crude polluted soil (Boboye *et al.*, 2010; Douglas *et al.*, 2020).

The effect of NPK, rhamonolipids and cow dung on the reduction of chromium, iron and lead showed significant reduction from initial values. Lai *et al.* (2014) investigated the biostimulation of Cr (VI) reduction in groundwater and demonstrated enhanced reduction rates through the addition of organic carbon sources, promoting the growth of indigenous Cr (VI)-reducing bacteria. Kapoor and Viraraghavan, (1995) investigated the biostimulation of lead removal found that the addition of organic amendments increased lead immobilization through microbial activity.

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Coates *et al.* (1996) investigated the biostimulation of Fe (III) reduction in subsurface sediments and demonstrated that the addition of electron donors (e.g., organic carbon) stimulated the activity of iron-reducing bacteria, influencing the reduction of Fe (III). Bioremediation of heavy metals and hydrocarbons in the environment is a complex process where quantitative and qualitative aspects depend on the nature and amount of heavy metal present. It was observed that the concentration of the different heavy metals (iron, chromium and Lead) decreases with time for the various applied nutrients (bio-stimulant) (Nwadiokwu *et al.*, 2023).

Microorganisms especially soil microbes can tolerate high levels of heavy metals, some microorganisms need certain types of metals as a micronutrient (i.e., Fe^{3+} is essentially utilized by all bacteria while Fe^{2+} is significant for anaerobic bacteria) to perform their metabolic activities (Ahemad, 2019).

The impact of the different treatments revealed that those with higher concentrations of the supplement (nutrient) had higher reduction. Hence, an increase in the amount of stimulant resulted in a corresponding increase in the loss of heavy metals by the indigenous microorganisms. For instance, the 40gNPK set-up had the highest percentage of bioremediation of heavy metals compared to the 20gNPK.

The treatments with NPK fertilizer showed higher metal bioremediation efficiency than the cow dung (organic nutrient) and rhamnolipids-treated soil when compared with the control and this shows that the amendment was effective in degradation (Nwadiokwu *et al*., 2023). Inorganic fertilizers have been used in Agriculture to restore nutrients in the soil. These inorganic supplements when applied more than necessary have been known to lead to some detrimental issues such as increasing soil pH levels and gas emissions (Gupta *et al*., 2016). Soil hardening, degradation, and eutrophication, are among the reasons why the use of inorganic compounds are discouraged. This is as mentioned previously by (Ekwuabu *et al.*, 2016). Inorganic fertilizers can readily leach out of soil when applied, run off into neighboring streams, and have negative effects on both humans and animals, according to Macaulay and Rees, (2014). However, crop residues and animal wastes made into organic fertilizers are more environmentally friendly and sustainable as they also possess the potential to enhance microbial degradation (Nduka *et al.*, 2012; Adams *et al.*, 2015; Chikere *et al.*, 2016). Hence, the rhamnolipid or cow dung could be used as alternative stimulants for metal bioremediation.

The study showed that NPK remediated more heavy metals in comparison to rhamnolipid and cow dung which are organic stimulants. Studies have shown that most microorganisms follow two common mechanisms in the bioremediation process; metal sequestering or immobilization and enhancement of solubility properties of the metal, other organisms oxidize or reduce the heavy metals to a less toxic form (Abo-Alkasem *et al.*, 2023).

In conclusion, this study has identified the following microorganisms: *Serratia fonticola* AJ233429, *Enterobacter bugandesis* LR861527, *Alcaligenes faecalis* MT421929.1, *Pseudomonas fluorescens* CP03848.1., *Geotrichum Candidium* OP153873.1, *Candida viswanathii* NG0634191, *Penicillium griseofulvum* KJ881374.1, and *Aspergillus niger* MF078659.1 from the crude oil contaminated soil. All these organisms identified have been shown to have potential for effective bioremediation. In this current study, the setup with higher amendment resulted in higher loss of heavy metals compared to the lower quantities. NPK resulted in a higher loss of metals than the biosurfactant, than the cow dung. Therefore, could be considered in the remediation of heavy metals and hydrocarbon-polluted soil as a result of the efficacy of biostimulation as shown in this study. It was also observed that the loss of the metals was also concentration-dependent.

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