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ISOLATION AND MOLECULAR IDENTIFICATION OF PHENOL TOLERATING BACTERIA FROM PETROLEUM CONTAMINATED SITES

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ABSTRACT

This research was conducted to isolate and identify phenol tolerating bacteria from petroleum contaminated sites. Two bacterial species were isolated from petroleum contaminated sites (NWPK and NWPKD). The isolation was based on morphological, microscopic and molecular identification. The total viable counts of phenol utilizing bacteria from NWPK and NWPKD are 2.71×10^7 and 4.0×10^6 cfu/g respectively. The isolates showed capacity of phenol tolerance at 500 mg/L, 1000 mg/L and 1500 mg/L when grown on phenol tolerant nutrient agar. The NWPK showed its capacity to tolerate phenol at 2.3×10^7 , 2.5×10^7 and 1.0×10^7 cfu/g of 500, 1000 and 1500 mg/L of phenol concentration respectively while NWPKD tolerate 1.5×10^7 , 3.8×10^7 and 1.0×10^7 cfu/g of 500, 1000 and 1500 mg/L of phenol respectively. The isolates were identified as *Citrobacter* and *Acinetobacter* species respectively based on 16S rRNA gene sequence analysis. These isolates showed their ability to withstand and survive high phenol concentrations in the environment.

Key words: Phenol tolerance, bacteria, petroleum contaminated sites, 16S rRNA

INTRODUCTION

Efforts have been made towards limiting the perceived threat of pollution around the world specifically by the United Nations Organs including the United Nations Environmental Programme. The environment is a very vital and necessary component for the existence of man and other biotic organisms (Dankaka *et al.*, 2018). Environmental pollution is considered as a side effect of modern industrial society. The presence of man-made (anthropogenic) organic compounds in the environment is a very serious public health problem. Soil and water of lakes, rivers and seas are highly contaminated with different toxic compounds such as phenol, ammonia, cyanides, thiocyanate, phenol formaldehyde, mercury, heavy metals. Monoaromatic hydrocarbons such as benzene, toluene and phenol are obvious choices for studies on biodegradation. Among these, phenols are considered to be pollutants (Sridevi *et al.*, 2012).

Phenol is a basic structural unit for a variety of synthetic organic compounds. It is a white crystalline solid with molecular weight of 94.14 g/mol and formula of C₆H₅OH (ATSDR, 1989; US Environmental Protection Agency, 1990). The United States Environmental Protection Agency (EPA) had published the current list of 126 Priority Pollutants (Ahmad *et al.*, 2014). Phenol is included in the list of priority pollutants by the

US Environmental protection Agency (Jena and Patil, 2016). Phenol is among the common toxic environmental pollutants that characterized as the first priority pollutants by European Union (Soudi and Kolahchi, 2011; Claudio *et al.*, 2009). Phenol is extremely toxic and carcinogenic; ingestion or exposure to phenol can burn the skin and destroy tissues, and it has been shown to cause liver damage, blurred vision, and diarrhea (El Gaidoumi *et al.*, 2019).

Phenolic compounds are some of the major hazardous compounds in industrial wastewater due to their poor biodegradability, high toxicity and high ecological aspects. Petroleum is also a source of phenol which are discharged into the water from petroleum refinery wastewater (El-Ashtoukhy *et al.*, 2013).

In spite of the toxicity of phenol, a number of microorganisms have been reported to tolerate and degrade it, including *Candida tropicalis*, *Bacillus brevis*, *Corynebacterium* sp., *Bacillus amyloliquefaciens*, *Gliomastix indicus* and *Pseudomonas putida* (Wang *et al.*, 2013).

To date, several number of phenol tolerating bacteria linked with *Enterobacter*, *Acinetobacter*, *Serratia* and *Stenotrophomonas*, *Bacillus circulans*, *Bacillus cereus*, *Pseudomonas aeruginosa* strain HSD38, *Bacillus thuringiensis* (Castillo *et al.*, 2012; Vieira *et al.*, 2018; Hank *et al.*, 2010 and Zheng *et al.*, 2010; Ereqat *et al.*, 2017) have been isolated. Hence this study

focused on the isolation and molecular identification of phenol tolerant bacteria from petroleum contaminated sites.

MATERIALS AND METHODS

Sample Collection and Isolation of Bacteria

Two soil samples were collected from refined petroleum products contaminated oil spill sites located in NNPC depot in Hotooro Kano and Kaduna refinery. All samples were transferred to sterile polyethylene bags, and transported to the Microbiology laboratory in Bayero University Kano for analyses. A total of 10 g of each sample was initially inoculated and homogenized in 250-mL Erlenmeyer flasks containing 90 mL of Bushnell-Hass (BH) mineral medium (g.L⁻¹:KH₂PO₄, 1.0 g; K₂HPO₄, 1.0g; NH₄NO₃, 1.0g; MgSO₄, 0.2g;CaCl₂, 0.2g; FeCl₃, 0.05g) (Bushnell and Hass, 1941) and was supplemented with phenol as sole carbon source at a final concentration of 400 mg.L⁻¹ and of synthetic marine salts (18 g.L⁻¹) (Dias *et al.*, 2009). The flasks were then incubated under orbital agitation (130 rpm) at 30°C. After 72 h of growth, a 10 mL aliquot of each sample was collected and transferred to new BH broth (90 mL) added with phenol and synthetic marine salts at the previously described concentrations. It was then succeeded by incubation under agitation as earlier specified. This procedure was repeated four more times. Subsequently, each sample was serially diluted from 10⁻¹ to 10⁻⁶ in sterile distilled water and proper dilutions (10⁻⁵ to 10⁻⁶) was plated on Nutrient agar, and BH Agar (BHA) supplemented with glucose (1.0 mg/L) and of synthetic marine salts (18 g/L). The substances were then incubated at 30°C for 24/48 h. Bacterial colonies grown were isolated according to their morphology and selected morphotypes was purified by repeated streaking on nutrient agar. After completing the purification, recovered isolates were grown on stock culture of nutrient agar. The stock culture of nutrient agar was incubated at 37°C for 24 h, bacterial strains was then stored at low temperature in the refrigerator for further studies.

Screening of Phenol Tolerance

Phenol tolerance was assessed by cultivation of the isolated strains on mineral salt medium (BH broth) containing phenol in crescent concentrations (500, 1000 and 1,500 mg/L). Microorganisms were previously grown on Mineral salt medium at 30°C for 48 h and seeded onto the surface of the phenol containing Nutrient Agar (NA) (Steers *et al.*, 1959). The development of a single bacterial colony within 24-48 h at 30°C was considered as

a tolerant strain for the evaluated phenol concentration (Vieira *et al.*, 2018).

Molecular Identification of the Bacterial Isolates

DNA Extraction

Genomic DNA was extracted using DNA extraction kit from pure bacterial culture; 24 h grown in fresh nutrient agar medium at 37^o C. Genomic DNA was extracted following the method described by Van Soolingen *et al.* (1994). Total genomic DNA (25-50 ng) was used as template for the amplification of the 16S ribosomal RNA (16S rRNA) coding region with the universal primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') (Weisburg *et al.*, 1991).

Polymerase Chain Reaction

The Polymerase Chain Reaction (PCR) was carried out using a reaction mixture of 50 µL containing 35.5 µL of water, 5.0 µL of PCR buffer 10 X, 1.5 µL of MgCl₂ (50 mM), 1.0 µL of each primer (10 mM), 4.0 µL of deoxynucleoside triphosphate solution (2.5 mM) and 1.0 µL of Taq DNA polymerase (5.0 U). Amplification conditions were adjusted on a thermocycler as follows: initial denaturation step at 94°C for 3 min; 30 cycles of 94°C for 1 min, 55°C for 30 s, 72°C for 30 s; and a final elongation step at 72°C for 10 min. PCR products were analyzed by electrophoresis on a 1.2% agarose gel electrophoresis (Ahmed *et al.*, 2015). The gel was stained with ethidium bromide, visualized under light, and photographed. The amplified products with the correct size were then purified and sequenced (Abdelhaleem *et al.*, 2019).

DNA Sequencing

The purified PCR product was sent to Malaysia for sequencing. DNA sequencing was performed with the universal primer (forward). The sequence obtained was assembled using BioEdit software to get the consensus sequence for each strain (Ahmed *et al.*, 2015), and was compared with bacterial 16S rRNA gene sequences deposited in the GenBank database from the National Center for Biotechnology Information (NCBI) using Basic Local Alignment Search Tool (BLAST) algorithm (Altschul *et al.*, 1990).

Determinations of Physicochemical Parameters and Some Heavy metals

Physicochemical parameters including pH and electrical conductivity were assessed following the method of Jackson, (1967), organic matter content (Addis and Abebaw, 2014). Potassium, Calcium, and Magnesium Content were determined according to Chaudhari, (2013), Nitrate and Phosphate (Ogofure *et al.*, 2016), while sodium was determined by the method described by Brupbacher, (1968). Heavy metals

(Iron, Nickel, Copper and Lead) were determined according to Kumar and Chopra, (2012) and Ogofure *et al.*, (2016).

RESULTS AND DISCUSSION

From the result of the physicochemical parameters, the pH from the two sites NWPK and NWPKD are 5.98 ± 0.021 and 5.75 ± 0.012 respectively and this plays a role in determining both the qualitative and quantitative abundance of the microorganisms present in petroleum contaminated soil. Even though the pH of Kano was slightly higher than that of Kaduna, there is no significant difference between the pH value recorded in Kano and Kaduna. Fakhrudeen *et al.* (2018) reported neutral pH in a study conducted on degradation of diesel and phenol using bacteria isolated from petroleum hydrocarbon contaminated soil. Similar pH (6.5-7.5) was reported by Upasani *et al.* (2013) in a research on isolation and screening for hydrocarbon utilizing bacteria (HUB) from Petroleum samples. Osazee *et al.* (2014) observed the rise in the pH from 6.107 to .60 after the crude oil contaminated soil was amended with cow dung. This is because the type of microorganisms that participate in hydrocarbon degradation is determined by the pH of the soil. Bacteria have limited tolerance for acid conditions and fungi are more tolerant (Osazee *et al.*, 2014). The above previous research was not in agreement with our finding. Since the pH in Kano and Kaduna soil was at low pH, it could be assumed that the fungi were more involved in the degradation of the oil in that soil than the bacteria or the acidic pH might be due to the origin of the petroleum during exploration.

Conductivity of the petroleum contaminated soil from Kano is 0.273 ± 0.012 dS/m while that of Kaduna is 1.527 ± 0.006 dS/m. The lower electrical conductivity of the petroleum contaminated soil affects soil structure and its physicochemical properties. According to Kumar *et al.* (2013), who observed that there was reduction in the electrical conductivity of soil contaminated with gasoline which affected soil structure and its physicochemical properties when compared with uncontaminated soil in a study conducted on physicochemical properties, heavy metal content and fungal characterization of an old gasoline-contaminated soil site in Anand, Gujarat, India. Increased concentrations of oil-derivative compounds in soil modify its physical and chemical properties.

The OM obtained from petroleum contaminated soil sites in Kano was $0.267\% \pm 0.025$ while that of Kaduna was $0.123\% \pm 0.006\%$. From the

result, O.M from Kano is slightly higher than that of Kaduna petroleum contaminated soil, though, the values are not significantly different. However, this may be as a result of decomposition of plants and animals remnants in that environment for a longer period of time. Ekhaise and Nkwelle (2011) reported the organic matter between 5.76 - 8.30% in a study conducted on microbiological and physicochemical analyses of oil contaminated soil from major motor mechanic workshops in Benin city metropolis, Edo State, Nigeria. Wang *et al.* (2015) recorded an O.M of 18.69% from soil contaminated with recent oil spill. The O.M recorded from the above previous researches differs from this research and this might be due to the abundance of the microbes in these sites (Kano and Kaduna) that utilized the O.M content in higher percentage than that of the sites recorded in those previous researches.

Nitrogen, phosphorus, potassium, sodium, calcium and magnesium are inorganic sources needed by the microbes for their activity. Inorganic sources such as nitrogen, phosphorus, potassium, hydrogen or oxygen are essential for microbial metabolism and affect the growth and the activity of microorganisms. The ratios of carbon/nitrogen or carbon/phosphorus are considered as a determining factor of biodegradation rates and are high in hydrocarbon-contaminated sites which limit and affect the degradation rate (Mahjoubi *et al.*, 2018). Phosphorus is not an abundant component of the ecosystem. It had been shown that its availability is further restricted by its tendency to precipitate in the presence of bivalent metals (Ca^{2+} , Mg^{2+}) and ferric (Fe^{3+}) ion at neutral to alkaline pH.

Heavy metal analysis of the petroleum contaminated soil sites of Kano and Kaduna which include Cu mg/kg (0.000, 0.503 ± 0.091) Ni mg/kg (0.560 ± 0.040 , 0.700 ± 0.095) Fe mg/kg (404.787 ± 4.145 , 389.400 ± 4.411) and Pb mg/kg (32.473 ± 0.244 , 29.840 ± 0.704), were determined. The finding showed the heavy metals were present in considerable amount in the soil. This is so because of the wide use of chemicals containing heavy metals being released into the environment as a result of petroleum activities. The result of the study revealed that Fe and Pb present in the soil sites are in higher concentrations than Cu and Ni that are in trace amount. Iron is present in concentration higher than other metals may be because of geographical origin of the soil. Lead was also high due to its wide usage being the combustion of leaded gasoline.

The high Fe and Pb concentrations showed that the sites are highly polluted with heavy metals where anthropogenic activities are heavy. While the lower concentration recorded for Cu and Ni showed that anthropogenic activities containing the metals are lower and the high Fe level could be as a result of wastes containing Fe dumped in the sites, or occurred naturally in the soil, or might be due to difference of iron salt.

The concentrations of Fe from the two sites have exceeded the limit prescribed by WHO (150 mg/kg) while that of Kaduna falls within the safe limit of FEPA (400 mg/kg). Pb concentration from Kano and Kaduna are within the permissible limit set by WHO (85 mg/kg). But with all, the two sites have exceeded the limits set by FEPA for Pb (1.60 mg/kg). Nickel fall within the limit prescribed by WHO (35 mg/kg). Cu is also within the limit of WHO (150 mg/kg). The concentration of Cu from Kano is not detected may be because of the complete mineralization of the Cu by the indigenous microbes.

Similar finding by Asia *et al.* (2007) on the effects of petroleum exploration and production

operations on the heavy metals contents on soil and groundwater in the Niger Delta reported the values of Fe, Pb, Cu and Ni ranges from 536 – 12872 mg/kg, 3.40 – 99.40 mg/kg, 5.10- 49.30 mg/kg and 1.60 – 13.80 mg/kg respectively.

Morphological characteristics of the bacterial isolates (Table 3) from this study showed that the color of the isolates NWPK and NWP KD on nutrient agar are white to cream and white to milky respectively. The gram staining reaction showed that the NWPK and NWP KD are pink in color after viewing under microscope at x100 objective lens which gives gram negative reaction base on the color.

Most of the bacteria involved in the biodegradation of phenol are predominantly gram negative. The gram negative bacteria are ubiquitous and have been recovered from various environments due to its ability to withstand harsh environmental conditions, and are capable of degrading phenol at various concentrations. There are many literatures that reported gram negative bacteria for phenol degradation.

Table 1: Physicochemical Analysis of the Petroleum Contaminated Soil Sites

Variance	SAMPLE ID		
	Kano	Kaduna	Control
pH	5.976 ± 0.021 ^a	5.753 ± 0.012 ^a	6.326 ± 0.025 ^c
EC (dS/m)	0.273 ± 0.012 ^a	1.527 ± 0.006 ^b	ND
O.M (%)	0.267 ± 0.025 ^a	0.123 ± 0.006 ^a	0.663 ± 0.091 ^b
N (%)	0.117 ± 0.020 ^a	0.105 ± 0.000 ^a	0.198 ± 0.040 ^b
P (mg/kg)	15.317 ± 0.099 ^a	16.680 ± 2.244 ^a	72.330 ± 4.689 ^b
K (mg/kg)	150.183 ± 0.021 ^b	116.070 ± 0.346 ^a	709.747 ± 3.218 ^c
Na (mg/kg)	915.000 ± 2.425 ^b	1199.153 ± 3.988 ^c	92.040 ± 0.010 ^a
Ca (mg/kg)	3362.157 ± 4.452 ^c	2777.273 ± 2.816 ^b	2317.813 ± 2.829 ^a
Mg (mg/kg)	485.250 ± 0.052 ^c	360.667 ± 0.065 ^a	455.263 ± 0.064 ^b

Control = soil not contaminated with petroleum

NB: The values with the same superscript across the rows are not significant (a, a).

The values with different superscript across the rows are significant (a, b, c).

Table 2: Heavy Metal Analysis of the Petroleum Contaminated Soil Sites

Variance	Sample ID			WHO/FEPA limit
	Kano	Kaduna	Control	
Ni mg/kg	0.560 ± 0.040 ^a	0.700 ± 0.095 ^a	1.070 ± 0.110 ^b	35/NA
Cu mg/kg	ND	0.500 ± 0.091 ^a	1.900 ± 0.542 ^b	150/ NA
Fe mg/kg	404.790 ± 4.145 ^c	389.400 ± 4.411 ^b	124.300 ± 2.141 ^a	150/400
Pb mg/kg	32.470 ± 0.244 ^c	29.840 ± 0.704 ^b	19.350 ± 0.325 ^a	85/1.60

Control = Soil not contaminated with petroleum

NB: The values with the same superscript across the rows are not significant (a, a).

The values with different superscript across the rows are significant (a, b, c).

Table 3: Morphological Characteristics of the Bacterial Isolate

S/N	Isolate code	Shape	Color	Gram reraction
1	NWPK	Short rod	White- cream	Negative (-ve)
2	NWP KD	Short rod	White-milky	Negative (-ve)

The total viable counts of heterotrophic/hydrocarbon utilizing bacteria (HUB) from two sites (Kano and Kaduna) are $27.0 \times 10^6 \pm 2.828$, 4.1×10^6 cfu/g ± 0.00 respectively. Surprisingly, the total viable counts of bacterial isolates from Kano is higher than that of Kaduna, which indicates that the petroleum contaminated soil site from Kano has higher microbial population compared with that of Kaduna. The presence of macronutrients and other favorable environmental conditions have also played a major role for the bacterial growth than the other sites. However, in this current research the toxicity caused by the hydrocarbons and environmental stress may be the reason for lower counts of bacteria in Kaduna sites compared to Kano. It may also be due to the fact that the indigenous bacteria are

acclimatizing to the new carbon sources in that environment, thus resulting in low counts.

Eze *et al.* (2014) reported the viable count of hydrocarbon utilizing bacteria from 3.8×10^6 - 7.8×10^6 cfu/g in a study conducted on microbiological and physicochemical characteristics of soil contaminated with used petroleum products in Umuhahia, Abia State, Nigeria.

Uzor *et al.* (2020) reported the total culturable heterotrophic bacteria (THC) counts from the polluted sites ranged from 7.2×10^7 to 9.5×10^7 cfu/g while the hydrocarbon utilizing bacterial (HUB) counts from the polluted sites ranged from 2.8×10^6 to 3.3×10^6 cfu/g. The hydrocarbon utilizing bacterial isolates PFP 1 has counts of 3×10^7 cfu/g. This isolates has been described as *Acinetobacter sp.*

Table 4: Total Viable Count (Mean) of Phenol Utilizing Bacteria from Petroleum Contaminated Soil Sites

S/N	Soil sites	CFU/g
1	NWPK	$2.70 \times 10^7 \pm 2.828$
2	NWPKD	$4.1 \times 10^6 \pm 0.000$

Previous study has reported on the phenol tolerating bacteria linked with *Enterobacter*, *Acinetobacter*, *Serratia* and *Stenotrophomonas*, *Bacillus circulans*, *Bacillus cereus*, *Pseudomonas aeruginosa* strain HSD38 have been isolated *Bacillus thuringiensis* (Castillo *et al.*, 2012; Vieira *et al.*, 2018; Hank *et al.*, 2010 and Zheng *et al.*, 2010; Ereqat *et al.*, 2017).

Two bacterial isolates were recovered from petroleum contaminated soil sites base on colony morphology and molecular analysis of 16S rRNA gene. The isolates were identified as *Citrobacter* and *Acinetobacter* species after blasting the gene sequence in NCBI database with 97.36% and 97.02% similarity with *Citrobacter sedlakii* strain NBRC 10 and *Acinetobacter baumannii* strain respectively. The present study aimed to assess the phenol tolerance of the bacteria isolated. Some factors could explain the isolation of only 2 bacterial strains. Firstly, the concentration of phenol applied (400 mg/L) in the screening medium as a sole source of carbon for the organisms which may turned out to be high and probably hindered bacteria with the ability to metabolize the phenol, which should be initially isolated in an environment with lesser concentration of phenol. The steps involved in the screening of

the isolates in the preparatory stage were vital in evaluating their capacity of tolerance. Applying different concentrations of phenol (500, 1000 and 1500 mg/L) in the mineral salt medium as in Table 5 below is a proof that the strains can tolerate a high concentration of phenol after plating on phenol tolerant nutrient agar. The isolates were able to tolerate phenol through the use of their metabolic pathways, thereby transforming the compound into a less toxic one preventing it from being toxic to their body.

This finding was in conformity with that of Cordova-Rosa *et al.* (2009) who reported that indigenous consortium of phenol-degrading bacteria was able to survive in high phenol concentrations (1200 mg/L) and Banerjee and Ghoshal (2011), reported that the *Acinetobacter lowffii* strain UW7 could grow best at 2.5 g/L of phenol but could not tolerate up to 4.0 g/L. Castillo *et al.* (2012) investigated the potential of bacterial communities in cock-processing wastewaters. He found that *Acinetobacter* was capable of tolerating up to 10 mM of phenol. Mohite *et al.* (2011) also reported the isolation of two bacterial strains among which are *Citrobacter freundii* from oil contaminated soil and are found to tolerate phenol (100 mg/L).

Table 5: Mean Value of Phenol Tolerance by the Bacterial Isolates

S/N	Isolate code	Concentration (mg/L)		
		500	1000	1500
1	NWPK (CFU/g)	2.3×10^7	2.5×10^7	1.0×10^7
2	NWPKD (CFU/g)	1.5×10^7	3.8×10^7	1.0×10^7

Deng *et al.* (2018) conducted the isolation of bacteria from saline wastes of petroleum industry. The strains HP-1 was identified based on 16S rRNA gene sequence and revealed that

the strain had the closest relation with genus *Citrobacter*. This strain HP-1 was having the ability of tolerating up to 1100 mg/L of phenol in an environment with different salinity

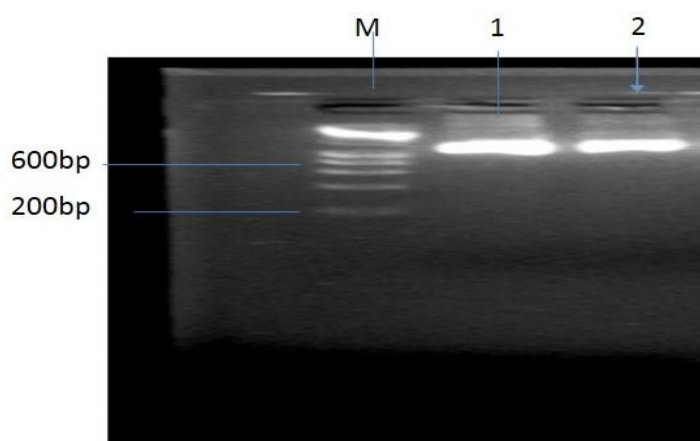


Figure 1 Agarose gel electrophoreses of the isolates, lane 1, 2 indicates extracted DNA from 2 isolates (NWPK and NWPKD). Lane M 200bp marker (Bioline).

CONCLUSION

From the petroleum contaminated sites, the physicochemical characteristics of the soil was analyzed. Two bacterial isolates NWPK, and NWPKD which belong to the genus of *Citrobacter* and *Acinetobacter* respectively were isolated and identified based on 16S rRNA gene sequence analysis. These isolated strains showed their capacity to tolerate higher phenol

concentration and hence can be useful for usage in the environmental cleanup.

RECOMMENDATION

It is remarkable that optimization of culture condition such as time of incubation, pH, medium salinity, temperature should be assessed to know the exact and best optimum condition required for the isolates to grow best in the medium.

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