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Characterization of Hydrocarbon-Degrading, Heavy Metal-Tolerant and Antibiotic Resistant Bacteria Isolated From Oil Contaminated Soil and Organic Wastes Within Katsina Metropolis

Mahuta, A. U.[1], Wagini, N. H.[2]*, Bello, A.[2], Kabir, K. [3], Riko, Y. Y.[3] & Mannir, K.[3]

[1]*Nigerian National Petroleum Corporation*
[2]*Department of Biology, Umaru Musa Yaradua University, Katsina.*
[3]*Department of Microbiology, Umaru Musa Yaradua University, Katsina.*
**Corresponding Author*

Abstract
The nexus between hydrocarbon degradation, heavy metal tolerance and antimicrobial resistance is critical in bioremediation and environmental health, and is being continually explored in these research spheres, of recent. As its contribution to this research subfield, this paper aimed at investigating the antimicrobial resistance and heavy metal tolerance profiles of petroleum hydrocarbons-degrading, heavy metal-tolerant and antibiotic resistant bacteria isolated from oil contaminated soil and organic waste, within Katsina metropolis. The bacteria were isolated using standard techniques of serial dilution and thereafter, the 38 isolates obtained were screened for hydrocarbons-degradation ability by culturing the isolates in mineral salts media supplemented with 2.5% petroleum hydrocarbons as sole carbon source, where growth indicates ability to survive at the sole expense of the petroleum hydrocarbons and hence, biodegradation ability. After the preliminary screening, 10 isolates were found to be hydrocarbon-degrading, and were subjected to heavy metals tolerance testing against three heavy metals: Cobalt, Iron and Zinc, which were incorporated at various concentrations (ppm) into the mineral salt media. The isolates selected from these screening procedures were identified using Vitek-2 Compact system. The identified isolates include: Routella ornithinolytica, Providencia stuartii, Citrobacter fruendii, Staphylococcus lentus, Routella ornithinolytica, Proteus mirabilis, Routella ornithinolytica, Klebsiella pneumoniae, Klebsiella oxytoca and Staphylococcus lentus. The hydrocarbon degrading, heavy metals-tolerant isolates (a total of 5 strains) were further subjected to antimicrobial susceptibility testing via Kirby/Bauer disk diffusion protocols, using UgoLabs, M13 and M14 antibiotic discs. Routella ornithinolytica, Citrobacter fruendii, Staphylococcus lentus and Klebsiella pneumoniae were found to be hydrocarbon-degrading, heavy metals-tolerant and multi-drug resistant. However, P. stuarti tested susceptible to most of the antibiotics, exhibiting resistance to just pefloxacine/reflacine, making it the potential best isolate in the research, as it is hydrocarbon-degrading, heavy metal tolerant, and susceptible to antibiotics. The heavy metal tolerance profile for these bacteria pointed out that the bacteria showed decreasing tolerance capacity with respect to increase in concentration in all the tested heavy metals. Statistical analyses confirmed that the survival of the isolates differs significantly with respect to concentration of heavy metals tested (p = 0.0004), the type of the heavy metals (p = 0.0007) and the bacterium involved (p = 0.0015). Significant differences were also obtained when the antibiotic resistance profile of the isolates was compared with respect to the isolates (0.015) and to the different antibiotics tested (0.044). The study recommends, amongst others, further studies seeking to establish the phenotypic characteristics and molecular basis of these and other isolates that manifest the phenomenon of hydrocarbon degradation, heavy metals tolerance and antimicrobial resistance.

Keywords: *Antimicrobial Resistance, Bacteria, Heavy Metal, Hydrocarbon Degradation, Tolerance.*

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I. Introduction

The highly electromagnetic metals with density greater than 5g/cm^3 are termed "Heavy Metals'' (Agarwal, 2009) [1]. Thus, these natural elements are characterized by their atomic mass and their high density, often used to differentiate them from other 'light metals'' (Koller, 2018) [9]. They persist in nature & tend to accumulate in food chains (de Silva *et al.,* 2012) [2].

Heavy metals include elements with atomic number greater than 20, excluding alkali metals, alkaline earth metal, and elements belonging to the lanthanide and actinides series. Although the natural background level of heavy metal fractions in air, soil and plants is highly variable, there are anomalous areas containing high levels of heavy metal pollution caused by man-made pollution, due to the mining of metal rich ores and other activities of metal smelting industries (Foy e*t al.*, 2003) [3]. The term heavy metal has particular application to cadmium (Cd), mercury (Hg), lead (Pb) and arsenic (As), all of which appear in the world health organization's list of 10 chemicals of major public concern. Other examples include manganese (Mn), chromium (Cr), Cobalt (Cu), Zinc (Zn), Selenium (Se), Silver (Ag), and Thallium (Ti). These heavy metals are non-biodegradable and must be reduced to acceptable limits before discharge into the environment, to avoid threats to living organisms (Alam *et al.*, 2012) [4].

Chisti (2004) [10] reported that several studies have been conducted to elaborate the effects of these heavy metals on living organisms including animals and plants. Heavy metals can damage the living organisms through different mechanisms such as by affecting cell membranes, by altering the enzymes specificity, by disrupting the cellular functions and by damaging the structure of DNA. Many countries have regulatory guidelines for heavy metal presence and exposure as well as remediation and treatment options. Screening of soil and water sources is conducted frequently to prevent overconsumption, but many of these programs and technologies are not readily available in developing nations (Li *et al.*, 2006) [5].

Bacteria that demonstrate the capacity of surviving in toxic heavy metal concentration have been isolated from different sources (Basu & Bhattacharya, 1997) [6]. Many bacteria have specific genetic mechanisms of resistance to toxic metals (Mindln *et al.*, 2001) [7]. In a previous study by Hassan *et al.* (2008) [13] it was reported that Cu and Cr were the best tolerated heavy metals by heavy-metals tolerant bacteria, and Hg was the most toxic component for all bacteria, followed by Co and Cd. *Pseudomonas aeruginosa* (strain S6), with a relatively high MIC for metals and a large spectrum of antibiotic resistance appears to be model for eco-toxicological studies.

It is relatively common to find association of metal and antimicrobial resistance, since both resistance genes are frequently located on the same mobile genetic elements (McIntosh *et al.*, 2008) [8]. Consequently, it can be assumed that the selective pressure exerted by heavy metals contribute to the indirect co-selection of antibiotic resistance, particularly in environments contaminated with both heavy metals and antibiotics.

The environmental pollution scenario in Nigeria is compounded by the frequency of oil spills. For example, in 2010 alone, about 3203 cases were reported and also about 8 million cubic feet of natural gas are flared every day in the Niger delta region (Opara, 2016) [11]. Again, about 50, 000 acres of mangrove vanished between 1986 and 2003 within Nigeria's Niger-Delta region (Opara, 2016) [11]. According to Opara, (2016) [11] over 600 cases of oil spills were reported in the Niger delta areas of Nigeria in 2014. The damages arising from heavy metal pollution in Nigerian auto mechanic workshops have induced large scale environmental degradation.

Mechanical workshop generates waste resulting from used engine oils and spills from other lubricants, which are discharged indiscriminately in open free land, sewers and gutters; and subsequently, this is channeled to inland/coastal water bodies. Such spent contaminated soil is not fit for plant proliferation, microbial survival and can be unaesthetically unsightly. When such contaminants seep into underground water, it renders it unfit for human usage. They also tamper with the survival and proliferation of soil macrofauna and loss in biodiversity; moreover, in coastal waters, they affect fish and other aquatic animals' growth and health, meaning a significant drop in fish population, bioaccumulation and biomagnification as well (Aboaba *et al.*, 2009) [14].

In Nigeria and Katsina state in particular, vehicular mechanical workshops often appear to be situated very close to residential and commercial areas and in some cases, near water bodies. This proximity poses a serious threat of heavy metal pollution to the inhabitants of the area. Moreover, Aboaba *et al.* (2009)[14] reported that mechanical workshop soil is relatively poor for agricultural purposes, thus many abandoned mechanical workshops are left redundant with black hardened soil.

Microbes deal with poisonous chemicals by applying enzymes to convert one chemical energy into another form and taking energy or utilizable matter therefrom, in a process termed biodegradation. This chemical transformation involves breaking of large molecules into simpler form. Therefore, research for more of such contaminants-degrading bacteria cannot be overemphasized (Darma *et al*., 2019) [18].

Increase in vehicular activities corresponds to increase in oil contamination. Therefore, deleterious effect of pollutants in the environment becomes an alarming event needing urgent attention (Darma *et al*., 2019) [18]. Moreover, day to day handling of such oil spent soil can render by the people in that area under threat of microbial infection by the indigenous microbial population of the area. Similarly, local people normally uses the organic wastes for agricultural and medicinal purposes which poses threat of microbial infection as well.

Therefore, there is a pressing need for a research endeavor of this kind, and to the best of our knowledge, no previous work of similar nature had been done involving the sampling locations in the study area (Kofar Durbi mechanical workshop and organic wastes from Mansho farmhouse).

II. Materials And Methods

Study Area

The study was conducted at selected locations within Katsina metropolis, as shown in figure 1 below.

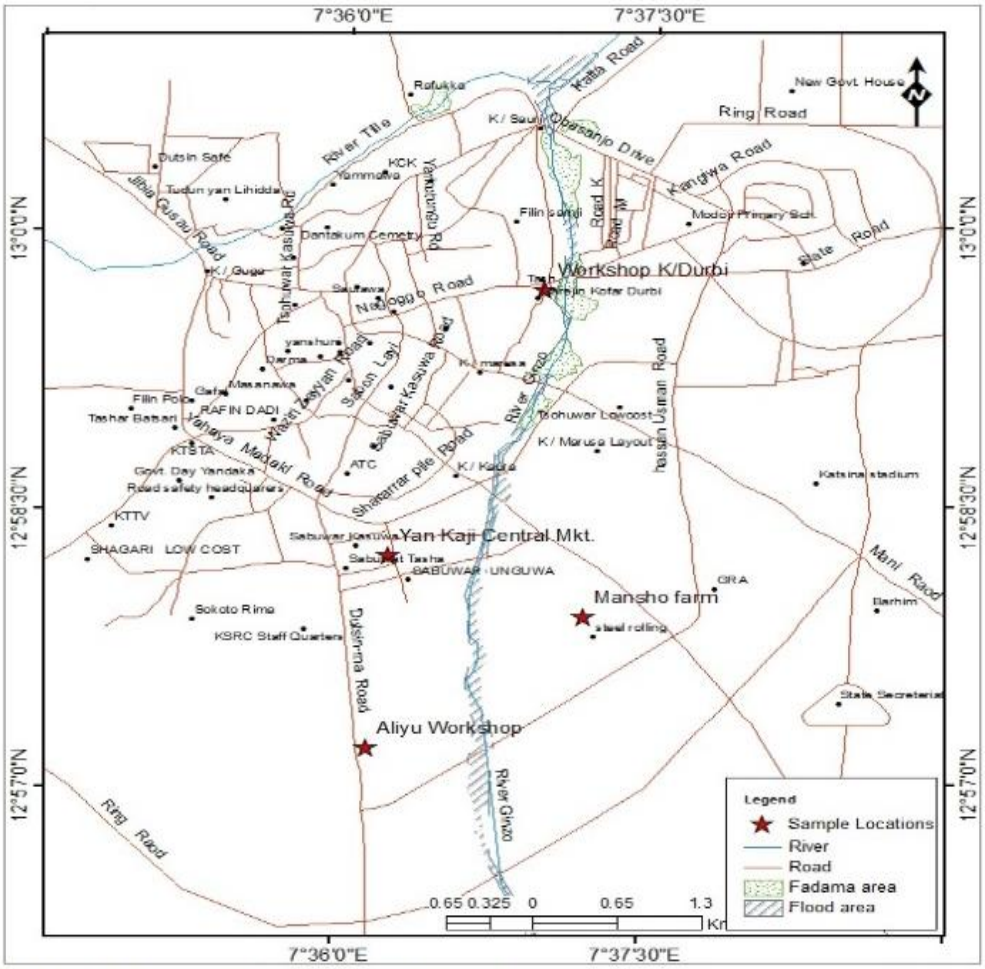

Fig.1:Map of Katsina Metropolis Showing Sample Locations
Source:-National Aeronoutic and Space Administration Spot Image 2020

Figure 1: Map of Katsina Metropolis showing sample locations

Source: NASA Spot Image (2020)

Sample-Collection

Soil-Samples Collection

Samples of oil contaminated soil was obtained from Kofar Durbi Mechanical Workshop, Katsina; following the procedure of (Olawale *et al.,* 2020) [15]. A hand trowel was used toexcavate 2kg of soil from depth of 0-30cm, which were subsequently transferred into a polythene bag.

Organic Matter Sample Collection

The Cow Dung was obtained from Mansho Farm House, Tudun Matawalle, Katsina and transferred into a polythene bag. The substrates were dried following the procedure of Adams *et al.* (2015)[16].

Isolation of Bacterial Isolates from the Samples Collected

The collected samples were aseptically transported to the Microbiology Laboratory, Umaru Musa 'Yar'adua University, Katsina, and subsequently processed. Isolation was carried out according to the protocols of American Public Health Association (APHA, 2017)[16].

Samples Preparation and Serial Dilution

From each of the collected samples, (i.e. oil contaminated soil sample and organic matter), 1g was measured and dispensed into a sterilized test tube containing 9 milliliters of distilled water, making the 10^{-1} dilution (stock). This allowed to soak for 10 minutes, with gentle agitation, using a rotary shaker. Subsequently, 1 milliliter was transferred into clean, sterilized test tubes containing 9 milliliters of distilled water, to obtain the 10^{-2} dilution. This was consistently continued in the next tubes, till the 10^{-3} to 10^{-6} dilutions were obtained (APHA, 2017)[16].

Media Preparation and Sample Inoculation

Nutrient agar was prepared according to manufacturer's instruction (Adams *et al*., 2015)[17]. Plating was done via the pour plate technique: 1ml from each of the10^{-4}, 10^{-5} and 10^{-6} dilutions was dispensed into a clean Petri dish. The medium was then added, the plate swirled and the medium allowed to solidify. Thereafter, incubation was carried out at 37^0C for 24 hours in an autoclave (APHA, 2017)[16].

Bacterial Enumeration

After 24 hours, colonies from the incubated plates were counted using Electronic Colony Counter SC6PLUS Model (Stuart Equipment, UK) Colony Forming Units = No of colonies counted x 1/dilution factor (APHA, 2017)[16].

Bacterial sub-culture

Pure colonies of the bacteria obtained were inoculated onto fresh nutrient agar plates to obtain pure cultures for each colony. The plates were incubated at 37°C in an autoclave for 24 hours (APHA, 2017)[16].

Isolation of Hydrocarbon-Degrading Bacteria

Preparation of Bushnell Hass Agar

The mineral salt medium prepared was Bushnell-Haas Agar (BHA), following the protocols of Darma *et al*. (2019)[18]; and it consists of Plain Agar Powder (15g); KH_2PO_4 (1g); K_2HPO_4 (1g); NH_4NO_3 (1g); $MgSO_4$ (0.2g); $FeCl_3$ (0.05g) and $CaCl_2$ (0.02g), which were dissolved in one liter of distilled water and sterilized by autoclaving at 121°C for 15 minutes. The medium was supplemented with 2.5% petroleum as sole source of carbon/energy.

Screening and Isolation of the Bacteria

Pure cultures of the bacterial isolates were streaked onto prepared Bushnell-Hass Agar plates and incubated at 37°C for 96 hours (Umar *et al*. (2020) [24]. The colonies from each plate were observed, enumerated and subcultured onto fresh nutrient agar slants, maintained at 4°C, for further analyses (Kabir *et al*., 2020)[29].

Bacterial Characterization

Gram staining

Gram staining was conducted according to the protocols of Cheesebrough (2006)[19]. Briefly, a smear of bacteria was made on a glass slide, which was subsequently air-dried and heat-fixed over a flame. A few drops of crystal violet were added and allowed to stand for 1 min. The slide was rinsed in water for five seconds, and then Gram's iodine was then applied on the smear and allowed to stand for one minute, allowing the formation of dye-iodine complex in the cytoplasm of the bacteria cell. The slide was then tilted and decolorized with solvent (acetone solution) for five seconds and then rinsed and shaken to remove excess. The slides were finally treated with safranin (counter-stain) and allowed to stand for 1 min. It was then washed briefly with water and shaken off to remove excess. It was then allowed to dry before examining under a microscope.

Bacterial Identification

The Bacteria were identified using VITEK-2 Compact system (Nisreen and Israa, 2020)[20]. Pure isolates from each bacterium were analyzed using VITTK-2 Compact Test (model number FUERR NO: 116), at the Federal Medical Center, Katsina, for exhaustive biochemical characterization and identification.

Heavy Metal Tolerance Studies

Media Preparation

The mineral salt medium prepared was Bushnell Haas Agar (BHA), as described by Darma *et al*. (2019)[18]; which consists of Plain Agar Powder (15g); KH_2PO_4 (1g); K_2HPO_4 (1g); NH_4NO_3 (1g); $MgSO_4$ (0.2g); $FeCl_3$ (0.05g) and $CaCl_2$ (0.02g), which were dissolved in one liter of distilled water and sterilized by autoclaving at 121^0C for 15 minutes. The prepared medium was enriched with 2.5% premium motor spirit (PMS) as a sole source of carbon and energy. Likewise, Nutrient Agar (BioLab, Budapest, Hungary) was prepared by dissolving 37 g of the commercially prepared powdered medium in 1L of distilled water, according to manufacturer's instructions.

Purity Testing of the Bacterial Isolates

Purity testing was carried out following the modified protocol reported by (Darma *et al*. 2019)[18]. The previously identified cultures of the bacteria were sub-cultured onto freshly prepared nutrient agar plates and streaked using four-way streaking. Distinct colonies were further sub-cultured before being used.

Standardization of Inocula

Inocula were standardized as described by Abdullahi (2019)[21]. Briefly, 24-hours-old cultures of the bacteria were emulsified in sterile normal saline till the turbidity matches 0.5 McFarland standard, which is equivalent to 1.5×10^8 cells/ml.

Heavy Metal Tolerance Testing

Heavy metal utilization was carried out following the protocol of Umar *et al.* (2020), with some modifications[22]. Briefly, three concentrations each of three selected heavy metals, comprising Iron (Fe), Cobalt (Co) and Zinc (Zn) were prepared, i.e. 0.1ppm, 1ppm and 10ppm for the primary screening, and then subsequently, 100ppm, 1000ppm and 10,000ppm were prepared secondary screening. These concentrations were then respectively incorporated into prepared Bushnell-Haas Agar Media to which 2.5% premium motor spirit (PMS) had been added as a sole source of carbon. These culture plates were then seeded with 100µl of the standardized inocula, and incubated at 37^0C for 72 hours. Thereafter, bacterial growths were enumerated using a colony counter, and tabulated as CFU/ml obtained after 72 hours for each concentration/treatment of heavy metal. Obtained counts were reported as $\times 10^3$, to ensure homogeneity and easy comparison of the results.

Antibiotic Susceptibility Testing

The protocol of Haruna *et al.* (2017)[23]was followed, with some modifications. Pure cultures of the identified isolates were used to prepare 0.5 McFarland Standard, as described before. A sterile swab stick was dipped into the culture of organisms and squeezed gently against the inside of the tube in order to remove excess fluid in the swab. The test organisms were streak using the swab on prepared nutrient agar plates, using lawn culture technique, and allowed to dry for 5 minutes. MastRing antibiotics discs (M13 and M14; Mast Group, Liverpool, UK) and UgoLabs antibiotic disks (UgoLabs, Kano, Nigeria) were placed on the surface of the agar using sterilized forceps and gently pressed onto the surface of the agar using flame-sterilized inoculation loop. Thereafter, the inoculated plates were carefully inverted and incubated for 24hours at 37°C. A centimeter rule was used to measure the diameter of the zone of inhibition for each antibiotic used. The measurements obtained from the individual antibiotics were compared to that of the interpretative chart, to interpret the obtained zone.

Statistical Analysis: Analysis of variance (ANOVA) was used to test the significant difference at ($p \leq 0.05$) in all treatments. All statistical analyses were carried out using SPSS, version 10.0. Similarly, Microsoft Excel (2019 version) was used to calculate the standard deviation for the replicate treatments, and to generate charts from the data.

III. Results

Isolation of Bacterial Isolates from the Samples Collected

A total of 38 colonies were identified from the isolation process, 27 are from oil contaminated sites, and 11 from organic matter.

Screening for Hydrocarbon-Degradation/ Heavy Metal Tolerance and Identification of the Bacteria

Upon isolation of the 38 isolates, they were subjected to screening for hydrocarbon-degradation ability, as described before. Only 10 isolates tolerated 2.5% petroleum as sole source of carbon and energy, and these were used for the heavy metal tolerance testing, at various concentrations, to find out the maximum tolerable limits. These isolates were also sent for identification via the VITTEK-2 Compact system.

Table 1: Identification of Hydrocarbon-Degrading, Heavy Metals Tolerant Bacteria Isolated from Petroleum Contaminated Soil and Organic Waste from Katsina Metropolis

S/No	Isolate ID	Isolate Name	Sample Source
1	A4	*Raoultella ornithinolytica*	Oil Contaminated Soil
2	A7	*Providencia stuartii*	Oil Contaminated Soil
3	A14	*Citrobacter fruendii*	Oil Contaminated Soil
4	A17	*Stylphylococcus lentus*	Oil Contaminated Soil
5	A22	*Raoultella ornithinolytica*	Oil Contaminated Soil
6	A23	*Proteus mirabilis*	Oil Contaminated Soil
7	A29	*Raoultella ornithinolytica*	Organic Matter
8	A33	*Klebsiella pneumoniae*	Organic Matter
9	A34	*Klebsiella oxytoca*	Organic Matter
10	A37	*Stylphylococcus lentus*	Organic Matter

Heavy Metal Tolerance Ability of the Isolates

Out of the 10 tested bacteria, five survived screening at the higher concentrations, the results of which were subsequently presented.

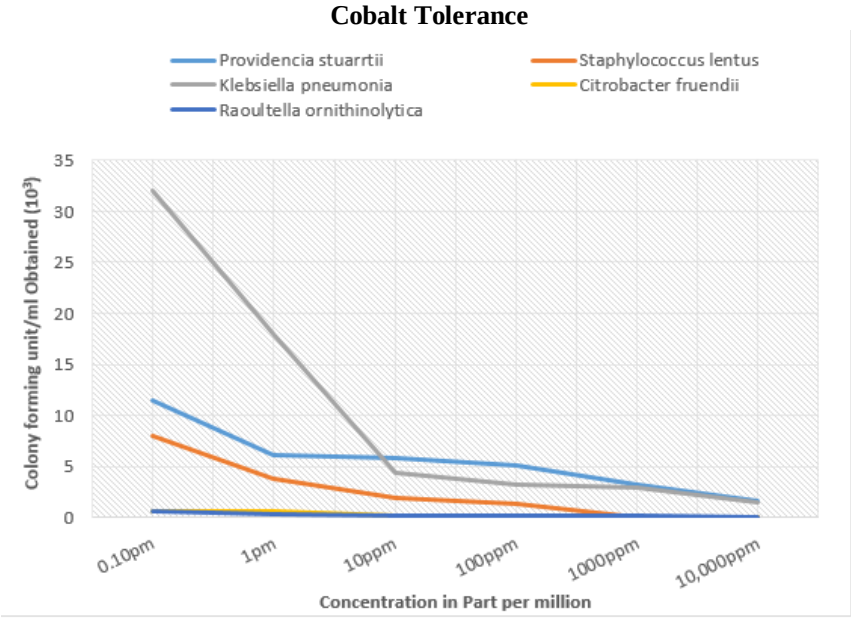

Figure 2: Cobalt Tolerance Ability of the Hydrocarbon-Degrading Bacteria

In the primary screening, *Providencia stuartii* showed a consistent decrease, with 11.52×10^3 CFU obtained in the lowest tested concentration of 0.1ppm, followed by 6.08×10^3 CFU at 1ppm and the lowest cell count was recorded at 10ppm(5.76×10^3). In the second batch of experiments, the maximum growth for cobalt was obtained inthe lowest concentration of 100ppm, (5.12×10^3) and thereafter, a significant drop in the CFU/ml was obtained at the highest tested concentration of 10,000ppm, with 1.55×10^3 CFU obtained. This indicates that at this concentration, the bacterium is very near to its tolerance threshold for cobalt.

In *K. pneumoniae*, the least prevalent isolate isolated from the organic waste sample showed diversification in terms of the tolerance ability of the isolates during the primary screening. There was a decrease in tendency of the bacterium to tolerate Cobalt with the highest colony count of 32.00×10^3 CFU/ml obtained at 10ppm concentration. Nevertheless, when the concentration is increased in the second batch of experiments, there was a uniform decrease in the number of cells capable of tolerating the heavy metals. For instance, at 100ppm, the colony count was 3.20×10^3 CFU, which decreased to 2.96×10^3 CFU at 1000ppm. However, at the highest concentration tested, i.e. 10,000ppm, only 1.44×10^3 CFU were obtained. Nonetheless, the result points out that these organisms may still survive beyond the 10,000ppm barrier.

C. fruendii, one of the least prevalent isolates from the oil contaminated soil sample, showed a near uniform tolerance ability in the results of the primary screening using Cobalt. There was a slight decrease in tendency of the bacterium to tolerate Cobalt with an initial colony count of 0.67×10^3 CFU/ml obtained at 0.1ppm concentration, followed by 0.57×10^3 CFU/ml at 1ppm and finally, a colony count of 0.16×10^3 CFU/ml was obtained at 10ppm. When the concentration is increased in the second batch of experiments, there is a uniform decrease in the number of cells capable of tolerating the heavy metals. For instance, at 100ppm, the colony count was 0.13×10^3 CFU, followed by 0.06×10^3 CFU at 1000ppm; however, at the highest concentration tested, i.e. 10,000ppm, only a minute amount of 0.02×10^3 CFU was obtained. Therefore, this is indicative that these organisms may cease to survive beyond the 10,000ppm barrier.

R. ornithinolytica, the most prevalent isolate from the oil contaminated soil sample, showed a slightly uniform tolerance ability in a decreasing manner, in the primary screening. There was a slight decrease in tendency of the bacterium to tolerate Cobalt with an initial colony count of 0.54×10^3 CFU/ml obtained at 0.1ppm concentration followed by 0.29×10^3 at 1ppm and final colony count of 0.16×10^3 CFU/ml, in the 10ppm concentration. However, upon acclimatization, in the second batch of the experiments, the bacterium tolerated all the concentrations (i.e 100ppm and 1000ppm and 10,000ppm), with growth of 0.14×10^3 CFU/ml, 0.11×10^3 CFU/ml and 0.02×10^3 CFU/ml respectively. However, this suggests that the bacterium is less likely to grow beyond a concentration of 10,000ppm.

Meanwhile, *S. lentus*, the isolate obtained from both organic waste and oil contaminated soil, exhibited varying tolerance levels to the three tested heavy metals. For Cobalt, in the first set of experiments, at 0.1ppm, the CFU/ml obtained was 8.00×10^3, which subsequently dropped to 3.84×10^3 CFU at 1ppm concentration and then 1.92×10^3 CFU/ml at 10ppm. However, upon acclimatization, in the second batch of experiments, the bacterium tolerated only 100ppm with 1.36×10^3 CFU/ml however, it failed to grow at 1000ppm and 10,000ppm.

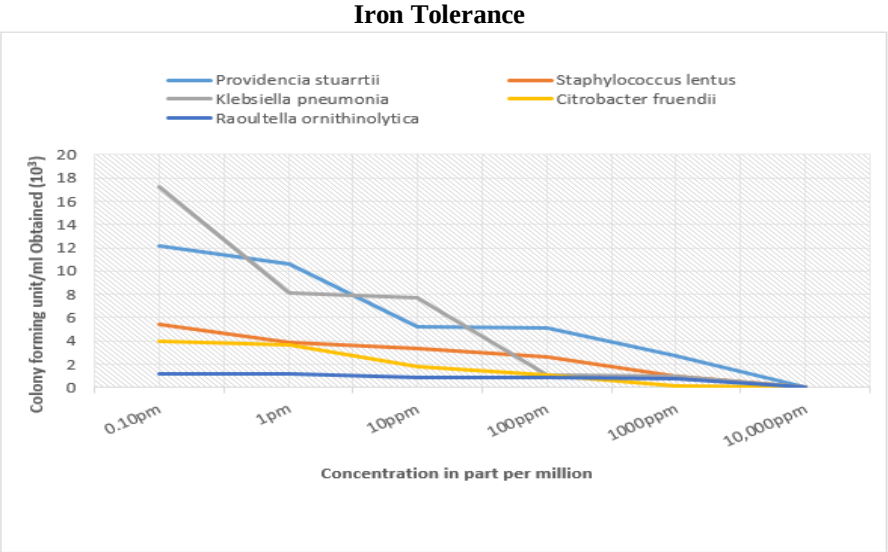

Figure 3: Iron Tolerance Ability of the Hydrocarbon-Degrading Bacteria

In respect to Iron, *P. stuartii*, showed similar pattern in the first batch of experiments: there was a significant decrease in growth with regards to an increase in concentration, such that at the minimum concentration of the first batch of experiments, i.e. 0.1ppm, 12.16×10^3 CFU was obtained, followed by 10.56×10^3 at 1ppm and the lowest growth of 5.20×10^3 CFU was obtained in the maximum concentration (10ppm). However, in the second batch of the experiments, a uniform drop in concentration from 5.12×10^3 CFU/ml at 100ppm to 2.72×10^3 CFU/ml at 1000ppm was obtained. At the highest tested concentration (10,000ppm), no growth at all was obtained.

For *K. pneumoniaee*, a uniform drop in concentration was also obtained in the first batch of experiments, with colony counts reducing from 17.28×10^3 CFU in the initial concentration of 0.1ppm, to 8.16×10^3 at 1ppm and finally 7.68×10^3 CFU in the final concentration of 10ppm. Similarly, in the second batch of experiments, the growth further dropped to 1.08×10^3 CFU/ml at 1000ppm. However, the maximum tolerance limit of the species was determined to be 10,000ppm, as the bacteria failed to grow at that concentration.

With regards to *C. fruendii*, a uniform drop in concentration was also obtained in the first batch of experiments, with colony counts reducing from 3.95×10^3 cells in the initial concentration of 0.1ppm, to 3.69×10^3 at 1ppmand then finally 1.83×10^3 CFU in the final concentration of 10ppm of the primary investigation. Similarly, in the second batch of experiments, the growth dropped to 1.08×10^3 CFU at 100ppm and then to 0.15×10^3 CFU at 1000ppm. At the maximum tested concentration of 10,000ppm, 0.02×10^3 CFU was obtained.

With regards to *R. ornithinolytica*, a uniform drop in concentration was also obtained in the first batch of experiments, with colony counts reducing from 1.20×10^3 CFU in the initial concentration of 0.1ppm, to 1.14×10^3 CFU at 1ppm and 0.91×10^3 CFU in the final concentration of 10ppm, in the preliminary screening. Upon acclimatization, in the second batch of experiments, the bacterium grows to 0.88×10^3 CFU (100ppm), reaching its maximum growth threshold (0.77×10^3 CFU) at 1000ppm. The bacterium failed to grow altogether at 10,000ppm.

For *S. lentus*, it exhibited the same pattern. There was a consistent decrease in CFU counts as the concentrations are increased, in the first batch of experiments, from 5.44×10^3 CFU at 0.1ppm followed by 3.84×10^3 at 1pm and then 3.36×10^3 CFU at 10ppm. However, upon acclimatization, in the second batch of experiments, the bacterium showed a decrease in growth from 2.64×10^3 CFU at 100ppm, to 0.96×10^3 CFU at 1000ppm. It failed to grow altogether at 10,000ppm. This result indicates that 1000ppm (0.96×10^3 CFU) is the maximum tolerable concentration for this bacterium with respect to iron.

Zinc Tolerance of the Isolates

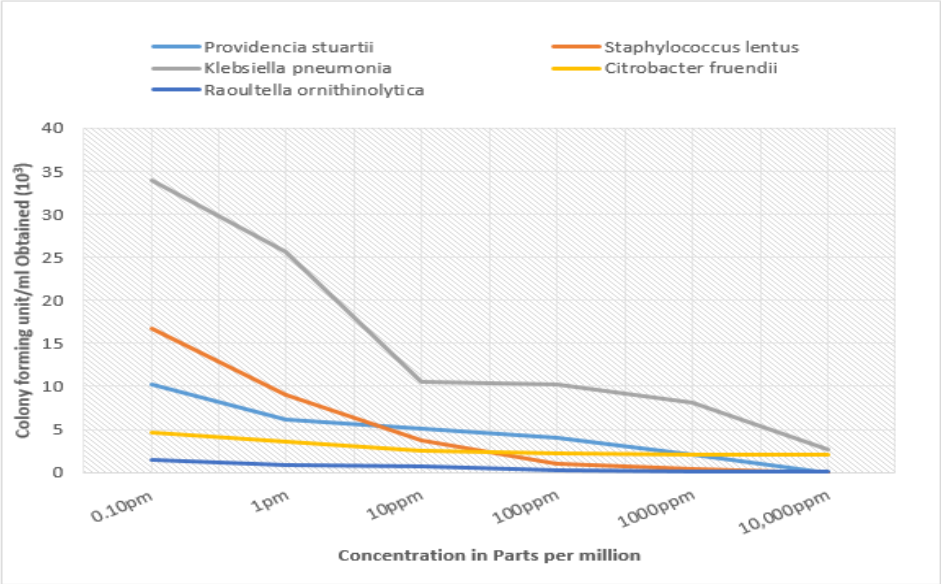

Figure 4: Zinc Tolerance Ability of the Hydrocarbon-Degrading Bacteria

With regards to Zinc, *P. stuartii* shows similar results: a slight decrease in CFU/ml with decrease in concentration from the minimum concentration (10.24×10^3CFU for 0.1ppm) followed by 6.08×10^3 for the 1ppmand then 5.12×10^3 CFU for the 10ppm concentration, in the maximum concentration. However, in the second batch of the investigation**,** the concentration of 10,000ppm was found to be intolerable for the bacterium, but it can grow at 100ppm (4.00×10^3 CFU) and at 1000ppm (2.08×10^3 CFU).

In the case of *K. pneumoniaee*, the growth of the bacterium showed a significant decrease with regards to increase in concentration for the first batch of experiments conducted. Thus, the highest growth was obtained (33.9×10^3 CFU) at the minimum concentration (0.1ppm) followed by 25.60×10^3 at the 1ppm; and the lowest growth was 10.56×10^3 CFU at the maximum concentration (10ppm). In the second batch of experiments, there was a uniform decrease in the growth measured as CFU/ml, with regards to increase in concentration, from 10.2×10^3 CFU at 100ppm to 8.12×10^3CFU at 1000ppm, until the highest tested concentration of 10,000ppm, where a growth of 2.72×10^3 CFU was obtained. Similarly, this indicates that the bacteria may still thrive beyond the maximal concentration of 10,000ppm.

In the case of *C. fruendii*, there was a significant drop in colony counts between the first and the second concentrations and a slight drop in the final concentration, with regards to increase in concentration in the first batch of experiments conducted. Thus, the highest growth was obtained at 4.58×10^3 CFU at 0.1ppm followed by 3.55×10^3 CFU at 1ppm which dropped significantly to 2.53×10^3CFU at 10ppm. However, in the second batch of experiments, there was a uniform decrease in the growth measured as CFU/ml, with regards to increase in concentration from 2.18×10^3 CFU at 100ppm to 2.12×10^3 CFU at 1000ppm, until the highest tested concentration of 10,000ppm, where a growth of 2.04×10^3 CFU was obtained. As before, this point out that the bacteria may still thrive beyond a concentration of 10,000ppm.

In the case of *R. ornithinolytica*, there was also a uniform drop in the concentration, with regards to increase in concentration in CFU/ml for the primary batch of experiments conducted. Thus, the highest growth was obtained at 1.52×10^3 CFU at 0.1ppm followed by 0.85×10^3 CFU at 1ppm which dropped significantly to 0.64×10^3CFU at 10ppm. However, upon acclimatization, in the second batch of the investigation, the bacterium showed a significant decrease in CFU from 0.28×10^3 CFU (at 100ppm) to 0.18×10^3 CFU (at 1000ppm) and finally 0.45×10^3 CFU (at 10,000ppm)

With regards to *S. lentus*, the bacterium exhibited a direct decrease in CFU from 0.1ppm to the final 10ppm in the first batch of the investigation, starting from 16.64×10^3 CFU followed by 8.96×10^3 CFU, and finally 3.68×10^3 CFU. In the same vein, upon acclimatization, the bacterium showed a significant decrease in CFU in the second batch of experiments from 0.98×10^3 CFU at 100ppm to 0.45×10^3 CFU at 1000ppm with no growth at all observed at 10,000ppm. Thus, we can say that the maximum tolerable concentration is 1,000ppm (0.45×10^3 CFU).

Antibiotic Susceptibility/Resistance Profiles of the Isolates

The isolates tested for heavy metal tolerance were further subjected to antibiotic susceptibility test in order to determine their antibiogram against antibiotics contained in MastRing and UgoLabs discs. The response of the bacteria in respect to the different antibiotics was classified as Susceptible, Intermediate and Resistant, in line with the guideline and provisions of the Food and Drugs Administration FDA, Clinical and Laboratory Standard Institute CSLI and European Committee for Antimicrobial Susceptibility Testing EUCAST (Riko*et al*., 2021) [31].

Table 2: Antibiogram of *Providencia stuartii* [evaluated using UgoLabs Disk]

S/No	Antibiotic (Abbreviation)	Average Zone of Inhibition Generated (mm±S.D.)	Interpretation*
1	Ampicillin (PN)	15.50±3.54	Intermediate
2	Augmentin (AU)	19.00±1.41	Susceptible
3	Ciprofloxacin (CPX)	28.00±1.41	Susceptible
4	Gentamicin (CN)	22.00±2.83	Susceptible
5	Nalidixic Acid (NA)	18.50±2.12	Intermediate
6	Ofloxacin/Tarivid (OFX)	17.00±1.41	Susceptible
7	Pefloxacin/Reflacine (PEF)	17.00±1.41	Resistant
8	Septrin/Cotrimoxazole (SXT)	14.00±1.41	Susceptible
9	Streptomycin (S)	17.50±0.71	Susceptible

Key: * Interpretation was based on EUCAST & CLSI (2018)[30] data

Table 3: Antibiogram of *Klebsiella pneumoniae* [evaluated using UgoLabs Disk]

S/No	Antibiotic (Abbreviation)	Average Zone of Inhibition Generated (mm±S.D.)	Interpretation *
1	Ampicillin (PN)	23.25±0.35	Susceptible
2	Augmentin (AU)	18.50±0.71	Susceptible
3	Ciprofloxacin (CPX)	17.00±0.00	Intermediate
4	Gentamicin (CN)	21.50±3.54	Susceptible
5	Nalidixic Acid (NA)	-	Resistant
6	Ofloxacin/Tarivid (OFX)	30.50±3.54	Susceptible
7	Pefloxacin/Reflacine (PEF)	20.00±1.41	Resistant
8	Septrin/Cotrimoxazole (SXT)	-	Resistant
9	Streptomycin (S)	20.75±0.35	Susceptible

Key: - No zone of inhibition obtained, * Interpretation was based on EUCAST & CLSI (2018)[30] data

Table 4: Antibiogram of *Citrobacter freundii* [evaluated using UgoLabs Disk]

S/No	Antibiotic (Abbreviation)	Average Zone of Inhibition Generated (mm±S.D.)	Interpretation*
1	Ampicillin (PN)	-	Resistant
2	Augmentin (AU)	12.50±0.71	Susceptible
3	Ciprofloxacin (CPX)	19.00±0.00	Intermediate
4	Gentamicin (CN)	14.50±3.54	Susceptible
5	Nalidixic Acid (NA)	-	Resistant
6	Ofloxacin/Tarivid (OFX)	15.50±3.54	Susceptible
7	Pefloxacin/Reflacine (PEF)	20.00±1.41	Susceptible
8	Septrin/Cotrimoxazole (SXT)	-	Resistant
9	Streptomycin (S)	-	Resistant

Key: - No zone of inhibition obtained, * Interpretation was based on EUCAST & CLSI (2018)[30] data

Table 5: Antibiogram of *Routella ornithinolytica* [evaluated using MastRing Disk]

S/No	Antibiotic (Abbreviation)	Average Zone of Inhibition Generated (mm±S.D.)	Interpretation*
1	Ampicillin (PN)	22.25±3.46	Susceptible
2	Cephalothin (KF)	11.00±2.83	Susceptible
3	ColistinSulphate (CO)	0.00±0.00	Resistant
4	Gentamicin (GM)	0.00±0.00	Resistant
5	Clotrimoxazole (TS)	24.50±3.69	Susceptible
6	Streptomycin (S)	0.00±0.00	Resistant
7	Sulphatriad (ST)	18.50±1.73	Susceptible
8	Tetracyclin (T)	13.25±0.96	Susceptible

Key:0.00±0.00 = No zone of inhibition obtained, * Interpretation was based on EUCAST & CLSI (2018)[30] data

Table 6: Antibiogram of *Staphylococcus lentus* [evaluated using UgoLabs Disk**]**

S/No	Antibiotic (Abbreviation)	Average Zone of Inhibition Generated (mm±S.D.)	Interpretation*
1	Amoxil/Amoxycilin (AML)	11.75±1.06	Intermediate
2	Ampicillin/Cloxacillin	12.75±0.35	Resistant
3	Chloramphenicol (CH)	14.00±1.41	Intermediate
4	Ciprofloxacin (CPX)	21.50±9.19	Susceptible
5	Erythromycin (E)	29.50±0.71	Susceptible
6	Gentamicin (CN)	16.50±0.71	Susceptible
7	Levofloxacin (LEV)	28.50±2.12	Susceptible
8	Norfloxacin (NB)	-	Resistant
9	Rifampicin (RD)	18.50±0.71	Resistant
10	Sparfloxacin (S)	21.00±2.83	Susceptible

Key: 0.00±0.00 = No zone of inhibition obtained, * Interpretation was based on EUCAST & CLSI (2018)[30] data

From the obtained results, some of the strains show resistance to multiple antibiotics. The results indicate that 4 of the isolates (*P. stuartii, K. pneumoniae, C. fruendii* and *S. lentus*) were susceptible to gentimicin, 3 (*P. stuartii, K. pneumoniae, C. fruendii*) to ofloxacin and augmentin, 2 (*P. stuartii* and *K. pneumoniae,*) to streptomycin, 2 (*K. pneumoniae* and *R. ornithinolytica*) to ampicilin, 2 (*K. pneumoniae and C. fruendii*) to nalixidic acid, and 2 (*P. stuartii* and *S. lentus*) to ciprofloxacin. One bacterium (*C. fruendii*) was susceptible to pefloxacin, one (*P. stuartii*) to septrin and similarly another one (*R. ornithinolytica*) shows susceptibility to cephalothin, clotrimoxazole, sulphatriad and tetracyclin; and one (*S. lentus*) was suceptible to erythromycin, levofloxacin, and sparfloxacin. Meanwhile, *P. stuarti* tested susceptible to most of the antibiotics, exhibiting resistance to just pefloxacine/reflacine, making it the potential best isolate in the research, as it is hydrocarbon-degrading, heavy metal tolerant, and susceptible to antibiotics.

With regards to resistance profile of the strains, *P. stuartii* and *K. pneumoniae* show resistance to Pefloxacin, while *S. lentus* and *C. fruendii* shows resistance to Ampicilin. *R. ornithinolytica* shows resistance to gentimicin. *K. pneumoniaee and C. fruendii* show resistance to nalixidic acid and septrin, *C. fruendii* and *R. ornithinolytica* show resistance to streptomycin; *R. ornithinolytica* shows resistance colistin sulphate; and *S. lentus* shows resistance to norfloxacin and rifampicin. Meanwhile, *C. fruendii* resisted four different antibiotics tested making it most antibiotic resistant strain in the research.

Based on the criteria outlined Riko*et al.* (2021) [31], it can be seen that *Citrobacterfreundii* (resistant to antibiotics from 4 different classes); and *Staphylococcus lentus*, *Routella ornithonilytica* and *Klebsiella pneumoniae* (resistant to antibiotics from 3 different classes each) were multidrug resistant, as they resist antibiotics from ≥3 classes, each.

Statistical Analyses

The two-way ANOVA results show that there is a significant difference in the growth response of the bacteria in the presence of varying concentrations of the heavy metal (0.1ppm, 10ppm, 100ppm, 1000ppm and 10000ppm), with a significant value of 0.0004 (less than the critical p-value of 0.05). There is also a significant difference in terms of the response to each heavy metal (zinc, iron and cobalt), with significant value of 0.0007 (also less than the p-value of 0.05). Furthermore, significant differences exist between the bacteria, concentration used * heavy metals; concentration used * bacterial isolate; type of heavy metal used * bacteria; and concentration used * bacteria; with p values of 0.0015, 0.0012, 0.0008, 0.0062 and 0.0075 respectively, (which are all less than the p-value of 0.05).

Statistical analyses also confirmed that significant differences were also obtained when the antibiotic resistance profile of the isolates was compared with respect to the isolates (0.015) and to the different antibiotics tested (0.044).

IV. Discussion

In the case of heavy metal tolerance, the organisms exhibited preference to certain metals over others. A critical look at the results obtained, shows that Iron is the least preferred metal. Only *C. fruendii* managed to survive it at 10,000ppm at a very low colony forming unit value of 0.02×10^3 CFU/ml. Conversely, Cobalt is the most tolerated metal: Most of the tested bacteria survived it even at 10,000ppm (with the exception being *S. lentus*, with a tolerance limit maximum of 1,000ppm). The capacity to tolerate heavy metals by the organisms utilized in this study can be attributed to two factors: the environment they live in prior to isolation, which is heavily polluted with heavy metals and the acclimatization they received in the first stage of the research. The research therefore shows that to some extent bacteria can get acclimatized to pollutants, and survive partially at higher concentrations.

The differences observed in terms of the ability of the bacteria to tolerate the heavy metals can be attributed to various factors, including the structure and molecular weight of the metal, its amenability or resistance to breakage/disintegration, the presence of specific genes within the bacteria that enable them to tolerate the respective heavy metal, or, in some cases, group of metals (Darma *et al*., 2019; Umar *et al*., 2020)[18, 22].

When compared to previous researches on heavy metal tolerance, the isolates used in this study have values exceeding some previously reported values. The lowest tolerance limit was 0.1ppm, corresponding to 0.00009g/L, while the highest tolerance limit was 10,000ppm, corresponding to 10g/L. However, in previous studies, Umar *et al*., (2020)[24] reported isolates from Katsina, Daura and Funtua that can survive at up to 3.5g/L, corresponding to 3500ppm. In another study, Darma *et al*., (2019)[18]. reported isolates from Katsina that can tolerate up to 1g/L, corresponding to 1000ppm.

Other studies obtained tolerance values similar to the present study, for instance, Hassen *et al.*, (2008)[13] recorded tolerance levels of 1.5mM for Zinc, corresponding to about 100ppm. However, they reported tolerance limits of 0.4mM for Cobalt, equivalent to about 25ppm.

The fact that at higher concentrations, heavy metals inhibit microbial activity can be attributed to many factors, majorly, the inhibition of key enzymes necessary for microbial life and growth, and causation of mutation in the DNA strands of the microbe (Umar *et al*., 2020; Nordberg *et al*., 2007)[24, 25]

It is established that heavy metals are essential as cofactors in influencing microbial metabolism. For instance, zinc is found in metalloenzymes and transcription factors which are involved in DNA-RNA transcription, DNA replication and signal transduction amongst microbial communities, amongst others; however, when present in high concentrations, zinc prevents the cell from absorbing other minerals and nutrients necessary for cell survival (Nordberg *et al*., 2007)[25].

Iron is also an important co-factor in enzymatic catalysis, and in the electron transport chain, nevertheless, iron is the least tolerated heavy metal in this research, and this can be attributed to the insoluble nature of iron and its tendency to react when in free form, thus reducing its bioavailability and increasing its potential toxicity (Darma *et al*., 2020)[24].

The results of susceptibility test in *P. stuartii* for aminoglycosides (i.e.gentimicine); streptomycins (i.e. streptomycin) and quinolones (i.e. ciproflaxicin and nalixidic acid) mimic those reported by da Silva *et al.* (2021)[26],where they turned out to be resistant.

In *K. pneumoniaee* the result of the susceptibility test for ampicillin disagreed with the work of Olawale *et al.* (2020)[15]where it turned to be resistant, whereas ciprofloxacin turned to be susceptible. In *C. fruendii* the result of its susceptibility test agrees with the work of Dennis *et., al* (1995)[27] where the bacterium turned out to be resistant to ampicilin, nalixidic acid and streptomycin. In *R. ornithinolytica,* the result of its susceptibility test in the case of gentimicin and streptomycin agrees with the work of Akinde & Obire (2008)[28] but differs with regards to susceptibility profiles against ampicilin and cephalothin.

The variation in terms of antibiogram profile between this work and some of the cited works and this study is not surprising, because of many factors, including mechanisms for innate and acquired resistance, and humans-associated factors, such as negligent antimicrobial stewardship techniques (Riko *et al*., 2021) [31]. Moreover, hotter temperatures are said to lead to increased bacterial growth and genetic mutation. Different genetic mutations can also confer different resistance abilities to bacteria. Bacteria can also exchange DNA with one another thus, spreading resistance through horizontal gene transfer whose rate increases at higher temperature. Increase in population and over prescription can led to development of resistance mechanism by the bacterium either by conformational changes or genetic mutation which enable the bacteria to survive and multiply in the presence of an antibiotic (MacFadden *et al*., 2018)[12].

V. Conclusion

Based on the findings of this research, the research concluded that diverse (namely: *Citrobacter freundii, Klesbisella pneumoniae, Providencia stuartii, Routella ornithinolytica* and *Staphylococcus lentus*) hydrocarbon-degrading and heavy metal-tolerant bacteria can be isolated from both oil contaminated soil and organic matter, which were also multidrug-resistant (with the exception of *Providencia stuartii,* which resisted just one antibiotic). *C. fruendii* was the most resistant, as it resisted antibiotics from four different classes. The bacteria showed a decreasing degree of tolerance with respect to increase in concentration of heavy metals; and showed variation in terms of their ability to tolerate the heavy metals. *K.pneumoniae,* despite being the least prevalent, was the most tolerant to the heavy metals, while the least tolerant was *S.lentus,* as it failed to survive 10,000ppm in any of the three tested heavy metals. Therefore, the bacterial strains identified in this study, especially *P. stuartii* (which was heavy-metals tolerant, hydrocarbon degrading and susceptible to antibiotics) could be potential agents for the bioremediation of heavy metal polluted environments.

Recommendations and future scope

The research work recommends the following based on the findings:

1. Molecular characterization of the bacterial isolates should be carried out to further study the diversity and genetic make-up of the isolates, which can highlight molecular underpinnings behind the hydrocarbon-degradation, heavy metals tolerance and antimicrobial resistance profiles of the bacteria.
2. Optimization studies involving laboratory (microcosm) and on-the-field trials of these isolates shall be done, to screen for those isolates with potential for use in bioremediation of petroleum contaminated environments, with co-contamination by various heavy metals, with a view to commercializing them, for bioremediation purposes.
3. Further studies should go beyond the highest concentration tested (10,000ppm) for the bacteria able to survive it, and shall incorporate other heavy metals which are more toxic than those involved in the current study, to ascertain the ability of the isolates to tolerate them.
4. The bacteria should be used to formulate consortia, and the performances of these consortia evaluated, to investigate their combined capacity to degrade hydrocarbons in the presence of heavy metal co-contamination.
5. The presence of multiple antibiotic resistant bacterial strains shows high vulnerability of drug resistance amongst persons living in oil contaminated areas or local people using organic waste for medical purposes, therefore it is recommended that the government should encourage proper hygiene by providing basic infrastructure and encouraging sanitation visits, etc. Mechanical workshops should also be cited away from water bodies, and shall have standard methods of disposal of waste.

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