



BIODEGRADATION POTENTIAL OF RHIZOSPHERIC MICROORGANISMS OF RHIZOPHORA RACEMOSA IN CRUDE OIL CONTAMINATED MANGROVE SWAMP IN THE NIGER DELTA

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ABSTRACT

Rhizophora racemosa (red mangrove tree) belongs to the family Rhizophoraceae; it is an important constituent of the mangrove swamp in Niger Delta, an oil producing region in Nigeria. The remediation of soils containing organic pollutants is possible with the use of microbial communities when the ecology is understood for potentials maximization. This study investigated the biodegradation potential of rhizospheric microorganisms of *Rhizophora racemosa* in crude oil- contaminated mangrove swamp in the Niger Delta. The total microbial count was determined by the serial dilution method. The hydrocarbon-utilizing bacteria and fungi were enumerated using Mineral Salts Agar containing crude oil as the sole carbon source. The biodegradation potential of these rhizomicrobes was determined using screen test, shake flask degradation tests, Total Organic Gas (TOG) and Total Petroleum Hydrocarbon (TPH) InfraCal Analyzer (HATR-T2 and CH). The turbidity, total organic gas (TOG-N) and total petroleum hydrocarbon were measured weekly for twenty-eight days. Hydrocarbon-degrading microbes isolated from the rhizosphere were identified as *Marinococcus* sp., *Azotobacter* sp., *Acinetobacter* sp., *Aspergillus niger*, *Aspergillus flavus* and *Candida albicans*. The highest rate of TPH reduction was recorded in *Acinetobacter* sp. (from 150 mg/L on day 1 to <0.0031 mg/L on day 14). This was followed by *Candida albicans* (148mg/L on day 1 to 2.68mg/L on day 28) and *Aspergillus flavus* (150mg/L on day 1 to 4.21mg/L on day 28) In conclusion, it can be inferred that the some rhizospheric microbes of *Rhizophora racemosa* can efficiently degrade hydrocarbon up to 100% rate over a period of 28 days.

Keywords: *Rhizophora racemosa*, Rhizospheric, Rhizomicrobes, Mangrove swamp, TPH and TOG analyzer, Shake flask degradation test, Biodegradation potential

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INTRODUCTION

Rhizophora racemosa is a special plant which can survive in marine environment. It is common along the coastal regions where there is constant flooding. It belongs to the family Rhizophoraceae and it is the most common of this family in West Africa. Other species in this family include *Rhizophora mangle* and *Rhizophora harrisoni*. *R. racemosa* has a forklike root which has breathing pores called pneumatophores (Adegbehin, 1993; Giri *et al.*, 2010). Mangrove swamp is an ecosystem which can be aquatic in nature during the raining seasons and a little bit dry in dry seasons. It is a waterlogged ecosystem and can also be salty. The ecosystem is made up of some plants that have the ability to survive and live conveniently in water; some of these plants are ferns, palms, trees, shrubs and vines (Adegbehin, 1993; Flowers and Colmer, 2015). This ecosystem is a major source of food and materials for people in the coastal region to which the Niger Delta belongs. Mangrove forests provide fruits, firewood, timber, charcoal, fish etc. for people living in the region. (Adegbehin, 1993; Eleanya *et al.*, 2015).

Environmental pollution is one of the woes brought upon humanity as a result of infrastructural and industrial development. In the past century our environments have been facing a lot of challenges as a result of constant contamination with persistent substances, some are organic, inorganic and xenobiotic in nature (Atlas and Hazen, 2011). The use of advanced technologies for production especially in the oil sector has a lot of adverse environmental health effects. Also the disposal of wastes generated from crude-oil production is a worldwide problem in that hydrocarbon wastes pollute and destabilize the air, aquatic, terrestrial and mangrove ecosystems (John *et al.*, 2010).

Different methods have been employed to eradicate or minimize these environmental challenges. Methods like excavation, incineration and land filling have been used in past times; these methods were found to be costly, vigorous and time consuming (John *et al.*, 2010). This led to the search for better, less expensive, easy and time saving technologies. The breakdown of toxic substances such as hydrocarbons can be done naturally by microbial degradation (Amanchukwu *et al.*, 1989). In most cases, microbial degradation is a detoxifying mechanism and in the cases of some composite compounds, it is the only mechanism, which leads to comprehensive mineralisation (Nnubia and Okpokwasili, 1992).

It was discovered that the association between the plant and the microorganisms found in the root region called rhizosphere can be effectively used to clean up polluted ecosystems (Kuiper *et al.*, 2004). This process is referred to as rhizoremediation and it is a widely accepted technology. Researches are still ongoing in order to improve on these methods or better still to discover new methods. Rhizosphere is a unique region between the root of a plant and its immediate soil environment. The rhizosphere is rich in organic substances produced from the root of the plant; these organic substances include exudates, mucilage and cellulose which are by products of processes like photosynthesis and shedding off old cells (Brime-Combe *et al.*, 2007). A lot of microbial activities take place in this region. The activities of these microbes bring about plant growth and root development. Microbes found in the rhizosphere can also help the plant to withstand the toxicity of contaminants including crude oil hydrocarbons and heavy metals. Most mangrove plants appear to tolerate toxicity of crude oil better than other plants (Atuanya *et al.*, 2013). Since *R. racemosa* is commonly found in mangrove swamps and no study has demonstrated the biodegradation potential of its rhizospheric microorganisms in crude oil contaminated mangrove swamps in the Niger Delta, hence this study. The objectives of the study were to isolate and identify bacteria and fungi found in the

rhizosphere of *R. Racemosa* and to determine their degradation potentials in a crude-oil contaminated mangrove swamp.

SIGNIFICANCE OF THE STUDY

Environmental pollution by petroleum products remain an unavoidable consequence of oil exploitation, transportation and distribution activities. In finding solutions to the problem of environmental pollution posed by activities of oil exploration such as oil pipe cleaning, oil spillages and inappropriate disposal of hydrocarbon contaminated wastes, hydrocarbon- degrading microorganisms can be identified and harnessed to formulate products that will boost the biodegradation of petroleum hydrocarbon in hydrocarbon contaminated environments. This in turn will benefit the United Nations, in that it will contribute to the achievement of the Sustainable Development Goal (SDG) 15. This SDG 15 is to protect, restore and promote sustainable use of terrestrial ecosystems, sustainably manage forests, combat desertification, halt and reverse land degradation and halt biodiversity loss.

METHODOLOGY

DESCRIPTION OF SAMPLING SITE

The mangrove swamp soil sample (sandy loam) used for this research work was collected from Ekpan community near (Edjeba-Warri) of Uvwie Local Government area of Delta state, Nigeria. Uvwie is located in the area roughly between longitudes 5.40' and 5.50' east of latitudes 5.30 and 5.50 north. (Wikipedia).

COLLECTION OF RHIZOSPHERIC SOIL FOR MICROBIAL ANALYSIS

A seedling of *Rhizophora racemosa* was uprooted and the soil surrounding the root of the mangrove seedling was collected together with some of the adhering roots. Five gram of the soil was weighed and air dried at normal room temperature

ENUMERATION OF HYDROCARBON UTILIZING BACTERIA AND FUNGI

A serial dilution up to 10^{-3} and triplicates of each dilution was prepared. Fungal plates were incubated aerobically at 25°C for 72 hours and bacterial plates were incubated at 30°C for 48 hours. The total counts were estimated manually after incubation period. This represents a viable count, that is, plate count (CFU/mL) = (No of colonies on plate) x (dilution factor) (Obire and Anyanwu, 2009).

After cooling two drops of 1mL sterilized flagyl (Metronidazole B.P.400 mg) were added to a proportion of mineral salt agar (MSA) to prevent the growth of fungi and two drops of 1mL sterilized tetracycline were added to prevent the growth of bacteria. A loopful of the various colonies was added to 9mL of distilled water and serial dilution was carried out, 0.1mL of 10^{-3} and 10^{-4} of the dilutions were aseptically taken and placed on the sterile Petri dishes. For isolation of hydrocarbon utilizing bacteria, cooled MSA containing sterilized flagyl was poured on to the aliquot and rotated properly to mix well. Also, MSA containing sterilized tetracycline was used for the isolation of hydrocarbon utilizing fungi. The control sample was set up by adding 0.1mL sterile distilled water into the Petri dishes in place of aliquot of dilute soil samples. Incubation was carried out at 30°C for 14 days (Atuanya and Ekanem, 2008). After 14 days, microbes that were able to grow on MSA were considered to be the hydrocarbon utilizing microorganisms in the soil sample.

CHARACTERIZATION OF HYDROCARBON UTILIZING BACTERIAL AND FUNGAL ISOLATES

Pure stock cultures of crude oil utilizing bacterial and fungal isolates were examined for colonial appearance and used to carry out the following test: Gram staining, motility test, catalase test, citrate utilization test, indole test, hydrogen sulphide production test, methyl red-Voges Proskauer test, oxidase test and sugar fermentation test. Confirmatory identities of the bacteria were made using the Bergey's manual of determinative bacteriology and mycology.

SCREEN TEST FOR CRUDE OIL UTILIZATION

Bacteria and fungi that are able to grow on Mineral Salts Agar (MSA) containing hydrocarbons as energy and carbon source are considered to be the hydrocarbon-utilizing rhizomicrobes of *Rhizophora racemosa*. Pure cultures of these hydrocarbon-utilizing bacterial and fungal isolates were made by picking each of the colonies and streaking them on Mineral Salts Agar, Nutrient Agar and Potato Dextrose Agar separately. They were incubated at $28 \pm 2^\circ\text{C}$ for three days in the case of bacteria and five days in the case of fungi. Each of the isolates was later sub-cultured on MSA. Whatman size No1 filter paper was soaked with sterile bonny light crude oil and was placed on the cultured MSA. The plates were incubated at $28 \pm 2^\circ\text{C}$ for three days in the case of bacteria and seven days in the case of fungi. The plates were incubated upside down to prevent contamination. The slant stock cultures were made for both bacterial and fungal isolates (Atuanya and Ekanem, 2008).

CRUDE OIL BIODEGRADATION USING SHAKE-FLASK TEST

Organisms which are positive in the screening test were selected for shake flask degradation test. The biodegradation of the crude oil by these isolates were carried out using the same Mineral Salts Medium (MSM). One hundred and fifty millilitres of sterilized Mineral Salts broth were dispensed into 250mL conical flask, autoclaved and mixed with isolates which were inoculated by carefully picking a loopful of each isolate. One of the conical flasks was not inoculated which served as control. Five millilitres of 1% v/v crude oil was added to each flask and all the flasks were incubated at room temperature ($28 \pm 2^\circ\text{C}$) on a rotary shaker at 120rpm for 15 days (Atuanya and Omonigho, 2007).

DETERMINATION OF TOTAL PETROLEUM HYDROCARBON (TPH) DEGRADATION

Concentrations of total petroleum hydrocarbon were determined by taking 5ml each of the well shaken samples of the filtrate into cuvette (cell) and ran for absorbance using Total Organic Gas (TOG) and Total Petroleum Hydrocarbon (TPH) InfraCal Analyzer (HATR-T2 and CH) in order to determine the level of hydrocarbon and organic gas-nitrogen. Calculation of total petroleum hydrocarbon was as follows:
 $DF \times MF \times ABS \times Vol. \text{ of solvent}$

KEY: *DF* is Dilution Factor, *MF* is Multiplication Factor, and *ABS* is Absorbance

RESULTS

ISOLATION OF RHIZOMICROBES

The following rhizomicrobes were isolated: ISO B1, ISO B2, ISO B3, ISO F1, ISO F2 and ISO F3

TOTAL HYDROCARBON UTILIZING MICROBIAL COUNT

Table 1 shows the total count for the hydrocarbon utilizing bacterial and fungal isolates; ISO B2 had the highest count among the bacterial isolates, followed by ISO B1 and ISO B3 had the least count. For fungal isolates *ISO F3* had the highest count, followed by ISO F2 and ISO F1 had the least count.

Table 1: Total Hydrocarbon-Utilizing Bacterial Count and Total Fungal Count

Organisms	ISO B1	ISO B2	ISO B3	ISO F1	ISO F2	ISO F3
Population (x 10 ³ cfu/mL)	1.16	1.38	1.11	1.38	1.59	1.66

CULTURAL AND MICROSCOPIC CHARACTERISTICS OF HYDROCARBON UTILIZING FUNGAL ISOLATES.

Table 2 presents the cultural and microscopic characteristics of the fungal colonies isolated from rhizosphere of *Rhizophora racemosa*. The fungi include the following, *Aspergillus niger*, *Aspergillus flavus* and *Candida albicans*. These fungi were found to degrade crude oil.

Table 2: Cultural and Microscopic characteristics of fungal colonies from the rhizosphere of *Rhizophora racemosa*.

Organism	Size	Stripes colour	Wall	Surface	Margin	Shape	Conidia surface
Iso.F1	400-3000	Slightly brown	Smooth	Biserate	Entirely	Glucose	Very rough irregular
Iso.F2	400-800	Palebrown	Roughened	VessicleBiserate	Metillacoverly	Glucose ellipsoid	Smooth finely
Iso.F3	16mb (haphoid)	Cream	Thick	Quieshy spherical	Short inflated	& Septate hyaline	Roughened Smooth

Iso.F1: *Aspergillus niger*, Iso. F2: *Aspergillus flavus*, Iso. F3: *Candida albicans*

Taxonomic characteristics of Hydrocarbon utilizing bacterial isolates from rhizosphere of *Rhizophora racemosa*.

Table 3 shows the taxonomic characteristics of bacterial isolates from rhizosphere of *Rhizophora racemosa*. The bacteria include the following, *Marinococcus* sp, *Azotobacter* sp and *Acinetobacter* sp.

Table 3: Taxonomic characteristics of bacterial isolates from rhizosphere of *Rhizophora racemosa*.

Cultural characteristics	Iso.B1	Iso.B2	Iso.B3
Cultural shape	Circular	Irregular	Rhizoid
Colour	Cream	Cream	Milky
Elevation	Flat	Raised	Raised
Margin	Entire	Lobate	Pointed
Wetness/dryness	Wet	Wet	Dry
Transparency	Translucent	Opaque	Opaque
Morphological characteristics	Iso.B1	Iso.B2	Iso.B3
Gram staining	+	-	-
Cell morphology	Cocci	Cocci	Rod
Arrangement	Cluster	Cluster	Single
Biochemical tests	Iso.B1	Iso.B2	Iso.B3
Catalase	+	+	+
Oxidase	+	-	-
Indole	Nil	+	+
Citrate	+	+	+
Motility	-	+	+
VP test	+	+	+
Acid test	-	-	-
Strict Anaerobic	Nil	+	+/+
Nitrate Reduction	Nil	Nil	
Coagulase	-	-	
Lactose	+	+	-
Glucose	+	-	-
Starch	+	+	+
Mannitol	+	+	+
Probable Isolate	<i>Marinococcus</i> sp	<i>Azotobacter</i> sp	<i>Acinetobacter</i> sp

- is negative and + is positive

SCREEN TEST FOR CRUDE OIL UTILIZATION

The result of the screen test for the utilization of crude oil (hydrocarbon) as the sole carbon source by the rhizomicrobes is presented in Figure 1; it showed that *Acinetobacter* sp, *Azotobacter* sp and *Marinococcus* sp produce turbidity in the broth culture after seven (7) days and the turbidity increased over the period of twenty-eight days. Among the rhizobacteria *Micrococcus* sp. showed the highest turbidity followed by *Acinetobacter* sp. and *Azotobacter* sp., showed the lowest turbidity after the 28 days. Among the fungi, *Aspergillus flavus* showed the highest turbidity after 28 days, followed by *Candida albicans* and *Aspergillus niger* showed the least turbidity. Among the hydrocarbon-degrading rhizomicrobes. *Aspergillus flavus* was found to have the highest turbidity and *Azotobacter* had the lowest turbidity.

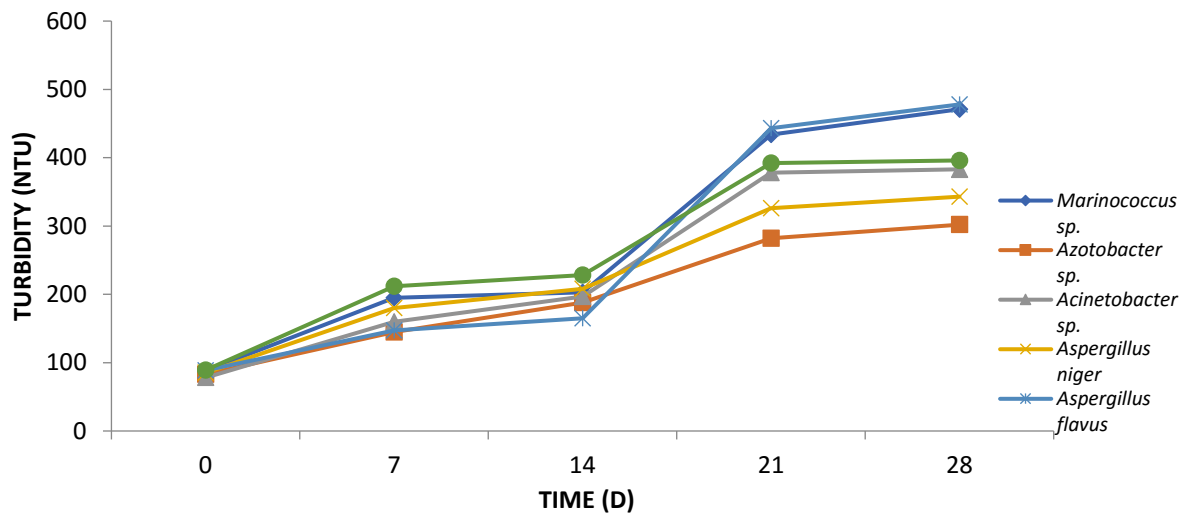


Figure 1: Turbidity of the rhizomicrobes during biodegradation of crude oil.

3.6. TOTAL ORGANIC GAS (NITROGEN) CONTENT REDUCTION IN CRUDE OIL CONTAMINATED SOIL BY THE ISOLATES.

Results in Figure 2 shows that the rate and level of nitrogen reduction in *Azotobacter* sp. was the highest from (0.92 to 0.06) mg/L, followed by *Acinetobacter* sp. (0.90 to 0.07) mg/L while *Marinococcus* sp. (0.92 to 0.08) mg/L demonstrated the least reduction. Also, for fungi, *Candida albicans* (0.90 to 0.078) mg/L had the highest nitrogen reduction followed by *Aspergillus niger* (0.92 to 0.08) mg/L and *Aspergillus flavus* (0.90 to 0.08) mg/L.

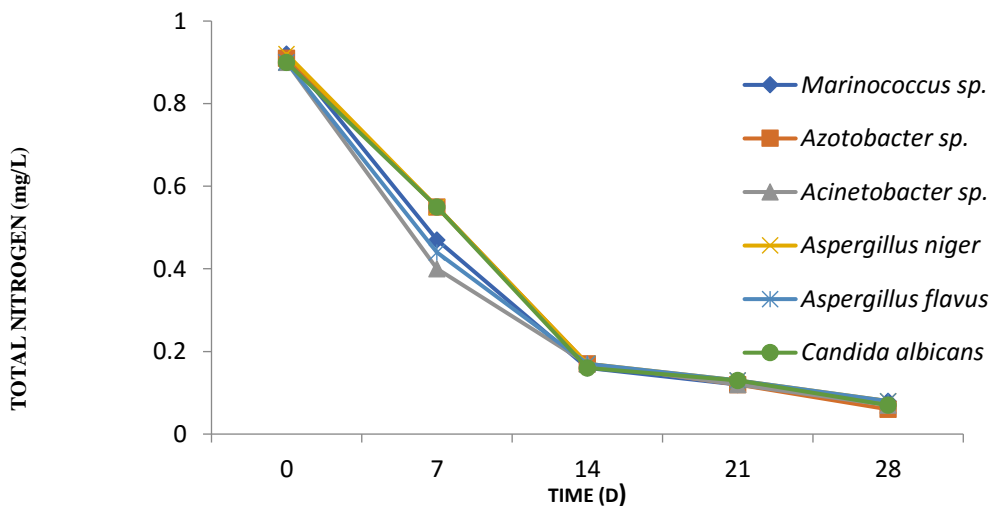


Figure 2: Total Nitrogen Reduction in Soils Contaminated with Crude Oil by The Rhizomicrobes

TOTAL PETROLEUM HYDROCARBON (TPH) REDUCTION IN CRUDE OIL CONTAMINATED SOIL BY THE ISOLATES

Figure 3 shows that the level of total petroleum hydrocarbon (TPH) decreased gradually during the 28 days. Results showed that the rate and level of TPH reduction was more in *Acinetobacter* sp. than in others from (150 to <0.0031) mg/L, followed by *Azotobacter* sp. (149 to 43.49) mg/L while *Marinococcus* sp. (150 to 61.69) mg/L had the least reduction. Figure 3.3 also showed that for fungi, *Candida albicans* (148 to 2.68) mg/L had the highest reduction followed by *Aspergillus niger* (150 to 4.21) mg/L and *Aspergillus flavus* demonstrated the least reduction.

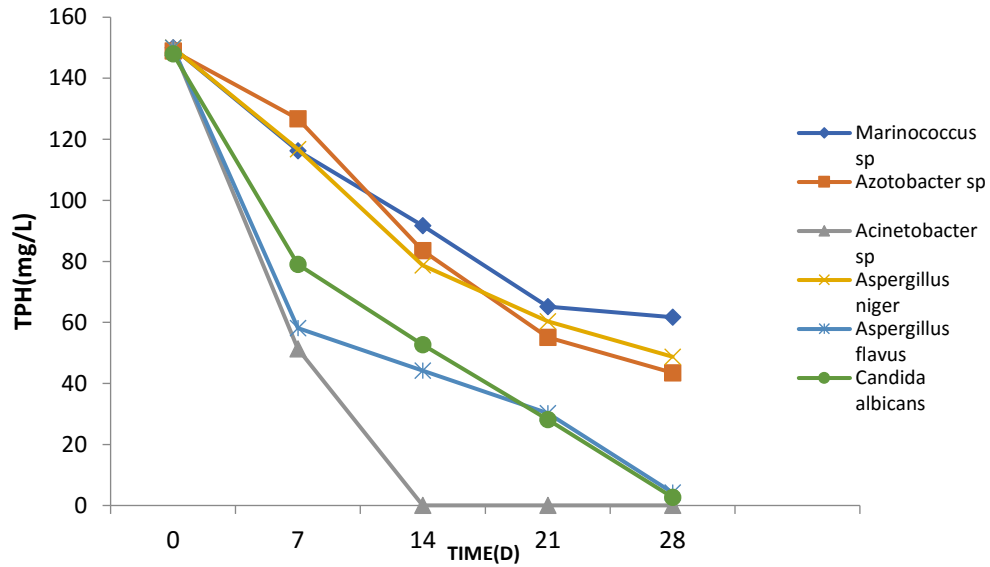


Figure 3: Total Petroleum Hydrocarbon (TPH) reduction in soil contaminated with crude oil by the isolates.

HYDROCARBON -UTILIZING MICROBIAL COUNTS DURING BIODEGRADATION OF CRUDE OIL

Figure 4 showed that HUM count increased gradually from day (7) and throughout the 28 days except for *Acinetobacter sp.* and *Candida albicans* which exhibited reduction after 21 days.

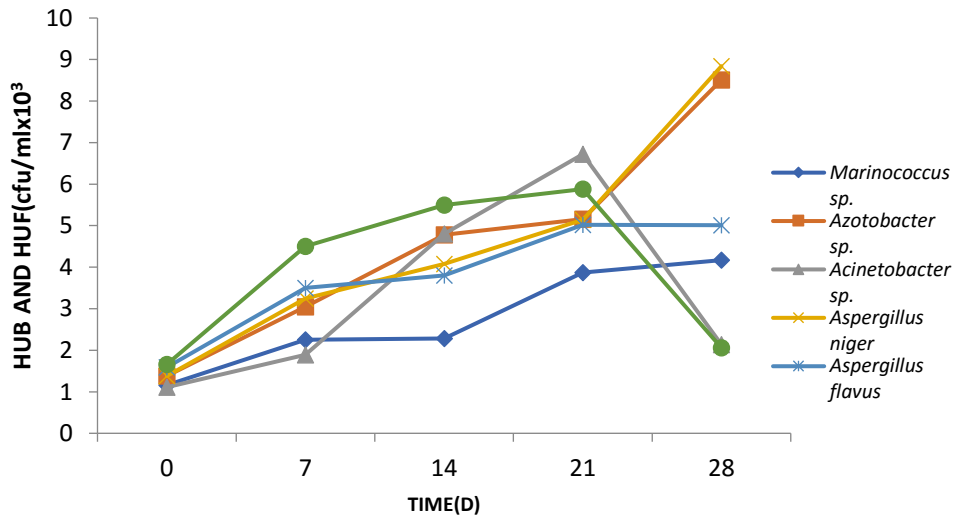


Figure 4: Total Microbial Count during biodegradation of crude oil.

DISCUSSION

Findings from this work on the biodegradation potential of the rhizospheric microorganisms of *R. racemosa* in crude oil contaminated mangrove swamp in the Niger Delta demonstrated that there were presence of fungi (mycorrhiza) and bacteria (rhizobacteria) in the rhizosphere of *R. racemosa*. This result supports a previous report that diverse groups of bacteria and fungi occur in the rhizosphere of plants (Compant *et al.*, 2010; Kumar *et al.*, 2012). The microorganisms found in the rhizosphere of *Rhizophora racemosa* have the capacity to degrade hydrocarbon in polluted soil. These finding agreed with the report of Atlas and Hazen (2011) that when there is hydrocarbon in an environment, there must be in existence hydrocarbon utilizing microorganisms.

In this research the bacteria and fungi found to be associated with effective degradation of hydrocarbon are *Marinococcus* sp., *Azotobacter* sp., *Acinetobacter* sp., *Aspergillus niger*, *Aspergillus flavus* and *Candida albicans*. This agreed with the report of Sarkar *et al.* (2018) that several bacteria such as *Acinetobacter* sp. have been found to play vital roles in petroleum hydrocarbon degradation. Also Compant reported that hydrocarbons can be degraded by some microorganisms examples of such include fungi belonging to the genera *Aspergillus* and yeasts which include *Candida* (Compant *et al.*, 2010).

The results in Figure 1 show that all the rhizomicroorganisms considerably utilized crude oil as the sole carbon source. The turbidity increased gradually from day (7) and throughout the 28 days of experiment for most of the isolates except for *Acinetobacter* sp and *Candida albicans* which started to retrogress after 21 days. This confirmed a previous report that the microbial load increases considerably over time (Atuanya *et al.*, 2013). *Acinetobacter* sp and *Candida albicans* were the only organisms that demonstrated three major phases of microbial population these are growth phase, the stationary phase and the decline phase.

The results in Figure 2 show that the level of total nitrogen (TN) reduced gradually during the 28 days. This indicates that these rhizomicrobes both bacteria and fungi found in the rhizosphere of *Rhizophora racemosa* effectively utilized nitrogen for growth. The results confirmed the fact that *Azotobacter* sp. and *Acinetobacter* sp. were involved in nitrogen fixation. The results indicated that *Azotobacter* sp. had the highest potentials for utilisation nitrogen in contaminated mangrove swamp soil. Also, for fungi, *Candida albicans* (0.90 to 0.078) mg/L had the highest potentials for utilization of nitrogen in crude oil contaminated mangrove swamp soil. This was followed by *Aspergillus niger* (0.92 to 0.08) mg/L and *Aspergillus flavus* (0.90 to 0.08) mg/L.

The results in Figure 3 indicate that the level of TPH reduced gradually during the 28 days. This indicates that the hydrocarbon utilizing rhizomicrobes found in the rhizosphere of the *Rhizophora racemosa* effectively utilized the crude oil for growth. According to the results *Acinetobacter* sp. (150 to <0.0031) mg/L had the highest potentials for utilisation and degradation of crude oil in contaminated mangrove swamp soil, followed by *Azotobacter* sp. (149 to 43.49) mg/L while *Marinococcus* sp. (150 to 61.69) mg/L demonstrated the least potentials. This agreed with the report of Kumar *et. al.* (2015) that formulated root endophytic fungus and plant growth promoting rhizobacteria enhances the biodegradation process. Also, for fungi, *Candida albicans* (148 to 2.68) mg/L had the highest potentials for utilization and degradation of hydrocarbon in crude oil contaminated mangrove swamp soil, this agreed with a former report that mycorrhizal associations possess some unique characteristics which enable them to enhance the degradation of organic pollutants (Meharg and Cairney, 2000; McNear 2013). *Aspergillus niger*

(150 to 4.21) mg/L had the second highest potentials for hydrocarbon utilization and degradation and *Aspergillus flavus* (150 to 48.74) mg/L demonstrated the least potential for utilization and degradation of crude oil.

The results in Figure 4 showed that total hydrocarbon – utilizing microbial count increased gradually throughout the 28 days of the research for most of the isolates except for *Acinetobacter* sp. and *Candida albicans* which started to regress after 21 days. This confirmed a previous report that the microbial load increases considerably over time (Atuanya *et al.*, 2013). For all the rhizomicrobes *Aspergillus niger* showed the highest increase, followed by *Aspergillus flavus* and *Candida albicans*, this also confirmed Lynch's report that fungi are the most abundant in the rhizosphere (Lynch, 1990).

CONCLUSION

In conclusion, the investigations showed that the rhizosphere of *Rhizophora racemosa* contains some indigenous microorganisms, both bacteria and fungi, which are capable of effectively degrading hydrocarbons in crude oil-contaminated mangrove swamp soil in the Niger Delta etc. Hydrocarbon removal efficiencies of these rhizomicrobes in a hydrocarbon impacted mangrove ecosystem can reach 100% using *Acinetobacter* sp and up to 98% using *Candida albicans* over a period of 28 days.

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