Journal of Advances in Biology & Biotechnology

19(4): 1-14, 2018; Article no.JABB.46012
ISSN: 2394-1081

Bioremediation Potential of *Aspergillus clavatus* and *Pichia* spp. on Oil Spill Dispersant Polluted Marshland

Janet Olufunmilayo Williams[1] and Barisi Samuel-Penu[2*]

[1]Rivers State University, Nkpolu-Oroworukwo, Port Harcourt, Nigeria.
[2]Kenule Beeson Saro-Wiwa Polytechnic P.M.B. 20 Bori, Rivers State, Nigeria.

Authors' contributions

This work was carried out in collaboration between both authors. Author WJO designed the study, author SPB collected the samples, did the analysis and wrote the first draft of the manuscript. Both read and jointly approved the final manuscript.

Article Information

DOI: 10.9734/JABB/2018/46012
Editor(s):
(1) Dr. Mohamed AliAbdel-Rahman, Associate professor, Department of Botany and Microbiology, Al-Azhar University, Cairo, Egypt.
Reviewers:
(1) Ibatsam Khokhar, Forman Christian College, Pakistan.
(2) Leera Solomon, School of Science and Technology, Captain Elechi Amadi Polytechnic, Nigeria.
Complete Peer review History: http://www.sciencedomain.org/review-history/27966

Original Research Article

Received 08 October 2018
Accepted 14 December 2018
Published 24 December 2018

ABSTRACT

Aims: The aim of the study is to identify the bioremediation potential of *Aspergillus clavatus* and *Pichia* spp. of oil spill dispersant on polluted marshland in Kegbara-Dere community in Gokana Local Government Area of Rivers State.
Study Design: The study employs experimental assay and statistical analysis of the data and interpretation.
Place and Duration of Study: Polluted marshland were collected from Kegbara-Dere community in Gokana Local Government Area of Rivers State with sterile spade from three different spot at the same location and put in black polythene bag and transported to the microbiological laboratory within 24 hours for physicochemical and microbiological analyses.
Methodology: Standard microbiological techniques were used to enumerate, isolate and identify the fungi. *Aspergillus clavatus* and *Pichia* spp. in contaminated soil samples with oil spill dispersants were observed for bioremediation potential for a period of 1day, 7days, 14days, 21days and 28days respectively.
Results: The results indicate that the total hydrocarbon (THC) content of soil samples at day one was 8006.58 mg/kg but reduces at day 28. Thus THC was: marshland polluted with Seacare (CTRL

**Corresponding author: E-mail: barisisam83@gmail.com;*

and CTRL 2b), 2988.49 mg/kg and 3453.95 mg/kg> polluted marshland and *Aspergillus clavatus,* 2942.11 mg/kg> polluted marshland and *Pichia,* 2973.68 mg/kg> polluted marshland and consortium, 1473.68 mg/kg. The bioremediation potential of the fungi expressed in percentage was: marshland and Seacare (control), *Aspergillus clavatus*, *Pichia* and consortium, 56.86%<64.50%>62.86%<81.59%.

Conclusion: These results show that bioremediation of dispersant pollutants by activation of naturally occurring microorganisms such as *Aspergillus clavatus* and *Pichia* spp. will be cost effective in cleaning up the environment.

Keywords: Bioremediation; dispersants; Aspergillus clavatus; Pichia species; Marshland

1. INTRODUCTION

Bioremediation is any process that uses microorganisms, decomposers and green plants, or their enzymes to improve the condition of contaminated environments. Bioremediation has been demonstrated to be effective against various types of shoreline [1-3]. Field studies have shown that bioremediation can be used successfully to clean polluted marshland and shorelines. Specific fungi can be used to bioremediate specific contaminants such as hydrocarbons which are present in oil and gasoline. Bioremediation is an efficient and environmentally safe technique for inexpensive decontamination of such environments [4]. Isolation of high numbers of certain oil degrading microorganisms from an environment is evident that those microorganisms are the active degraders in that environment. A number of well-known microorganisms are responsible for the biodegradation of oil spill dispersants [5].

In the aquatic ecosystems, fungi play an important role in removing hazardous compounds from the water, whereas sediment particles contaminated by crude oil spills is one of the desired ecological niche of fungi which inhabits such substrate and use carbon source of hydrocarbons in polluted sediment particles to biodegrade crude oil from the sediment in the beaches. Dispersants are chemical agents that reduce interfacial tension between oil and water or oil and solid, in order to enhance the natural process of dispersion by generating larger numbers of small droplets of oil that are contained in the water or soil [6]. Dispersant contains surfactants, which are surface-active agents with molecules that are composed of groups of opposing polarity and solubility, which mean surfactants have both oil-soluble hydrocarbon chains and a water soluble group.

Chemical dispersant biodegradability or the measure of oxygen needed to breakdown the chemical added to the oil contaminated marshland is a major interest when using dispersants because of the presence of water in a marshland environment. Dispersants exhibit a high demand for oxygen; hence, their use for spills in polluted water, coastal bays and marshland with limited circulation, could deplete or lower the dissolved oxygen, therefore causing harm to biological community such as water, marshland (Hamdan and Fulmer, 2011). Dispersants are most effective when applied immediately after a spill, before the evaporation of the lightest components in oil [7]. Fungi have been found to be better degraders of petroleum than traditional bioremediation techniques including bacteria, although hydrocarbon degraders may be expected to be readily isolated from a petroleum associated environment. The same degree of expectation may be anticipated for microorganisms isolated from a totally unrelated environment [8,9].

Several authors have made lists containing bacterial and fungal genera that are able to degrade a wide spectrum of pollutants, proceeding with marine atmosphere as well as the soil [10]. Recently, many researchers studied the role of fungi in biodegradation process of petroleum products and the most common fungi which have been recorded as biodegrading belong to the following genera: *Alternaria, Geotrichum, Candida, Cephalosporium, Cladosporium, Fusarium, Gliocladium, Mucor, Polyporus, Rhizopus Saccharomyces* [11-14]. Fungal genera are known fungi capable of utilizing hydrocarbon as carbon and energy sources and producing biosurfactants. Biosurfactants increased the oil surface area and the amount of the oil is actually available for bacteria to utilize it.

Oil spills dispersant is a combination of emulsifiers and solvents that lower the surface tension between two liquids or solids. Little oil droplets are easy to disperse throughout a water volume and these small droplets may be more readily biodegraded by microbes like fungi

(*Aspergillus, Pichia, Talarom Candida, Fusarium Mucor, Cephalosporium).* Oil spillage is the accidental discharge of crude oil into the environment. It involves the contamination of any part of the environment with any liquid hydrocarbon. These spills endanger public health, imperil drinking water, devastate natural resources, and disrupt the economy (Gensinde et al. 2008). Crude oil is a naturally occurring complex mixture hydrocarbon and non-hydrocarbon compounds which at appropriate concentration, possesses a measurable toxicity towards living systems. The toxicity of crude oil or petroleum product varies widely, depending on their composition, concentration, environmental factors, and on the biological state of the organisms at the time of the contamination [15]. Although oil spill from tankers and pipelines release crude oil particles to the marshland, water surface and move it to the beaches and contaminate living and non-living organisms. Micro-organisms especially fungi have a higher tolerance to the toxicity of hydrocarbons and dispersants due to their physiology and adaption to such variations in the environment and have the mechanism for the elimination of oil spill from the environment [16]. Soil or marshland is highly exposed to changes as a result of oil spill. The pollution can be very high and have a negative effect on soil. Oil spills can take place as a result of its release into the surrounding from a lot of sources which in turn causes pollution. The main source of crude oil and refined petroleum products such as kerosene, petrol, diesel and motor engine oil released on land come from the disposal of waste oil, leakage of oil stored in containers (Rina et al. 2009). The effect of oil on microbial population depends on the chemical composition of the oil and on the species of microorganisms present. The population of some micro-organisms increase and such microorganisms like fungi and bacteria are those that use the petroleum hydrocarbons as nutrients. Recently, many researchers studied the role of fungi in biodegradation process of petroleum products and the most common fungi have been noted as biodegraders. Some of the genera include *Geotrichum, Candida Cephalosporium, Fusarium Mucor, Talaromyces, Rhizopus, Saccharomyces* Species. Furthermore, effects of dispersants on the fate of dispersed oil have often showed conflicting results. Dispersanst may have little effect on oil biodegradation whereas some suggest a positive effect and other concluded inversely [2]. Practically, it has been proven that dispersion, whether chemically or mechanically in the water shortens the time required for microbial degradation. To assist oil removal in general views, the ultimate fate of oil spill is dependent primarily on the ability of microorganisms to use hydrocarbons as sources of carbon and energy [17]. The most vital role of soil microorganisms is their participation in nutrient remineralisation (Totora et al. 1995). The degradation of hydrocarbon, fungal mycelia can penetrate oil which brings to higher level of surface are available for biodegradation. Fungi are able to survive in a stressed environment, for example, low pH or low nutrients availability and in such environments, bacteria may not survive. It has also been studied and reported that though bacteria begins the degradation of a synthetic petroleum mixture, double as that of bacteria is degraded when fungi and bacteria were present (Obire and Ramesh, 2009). Fungi is a group of eukaryotic, non-phototrophic organisms with rigid cell wall, that include mushrooms, moulds, and yeasts. Many researchers investigate the role of fungi in biodegradation process of petroleum products and the most common fungi that have been recorded as degraders belong to the genera below, *Candida, Cephalosporium, Cladosporium, Fusarium, Geotrichum, Gliocladium, Mucor, Talaromyces, Polyporus, Rhizopus,* and *Saccharomyces* [18]. Fungi can be used to clean up oil spills in the water through biodegradation. Owing to the problems associated with physical, mechanical and chemical methods, there is need for a safer and less expensive approach to remediation of polluted environments (Obire and Putheti, 2009). Microbial degradation of petroleum hydrocarbons in a polluted water body in Lagos, Nigeria was reported by Adebusoye et al. [19]. *Pseudomonas* genera are the best known bacteria capable of utilizing hydrocarbons as carbon and energy source and producing biosurfactants. Some bacterial strains which are *Fluorescens, Pseudomonas aeruginosa, Bacillus subtilis,* Alcaligenes species, Acinetobater Iwoffi, Flavobacterium species, Micrococcus roseus and Corynebacterium species were isolated from the polluted stream which would degrade crude oil [19]. Several bacterial known to feed exclusively on hydrocarbons among *Pseudomonas, P. aeruginosa* is widely studied for the production of glycolipid type biosurfactants [20]. However, glycolipid type biosurfactants are also reported from some other species like P. *putida* and P. *chlororaphis*. The application of biodegradable dispersants namely Teepol, sodium Dodecyl sulphate (SDS) and corexit 9527 in brackish water were checked closely by the adoption of

ratio of dissolved organic carbon to total organic carbon which is primary biodegradation process, the ratio of organic carbon to total organic carbon known as mineralisation as well as total microbial population comprising of bacteria and fungi [7]. In the study dispersant utilizing bacteria were active and massive. Furthermore, teepol showed to be the highest and most degradable among the dispersants tested. This was followed by SDS and corexit 9527. Uzoigwe and Opokwasili [7] therefore recommended the analysis of the toxicity of dispersants before application in the ecosystem to safeguard the environment.

2. MATERIALS AND METHODS

2.1 Description of Study Area

Kegbara-Dere (K-Dere) in Gokana Local Government Area of Rivers State, Nigeria is situated in the Niger Delta Area of Nigeria. K-Dere is bounded by B-Dere and Biara communities in the north; Kpor and Bomu in the south: Bera in the east while it is bounded in the west by Onne in Eleme Local Government Area. It is situated between longitudes 7.01^0 and 7.07^0 E; and latitudes 4.08 and 4.2^0N. The area experiences two distinct seasons; the rainy and dry seasons and it is characterized by high temperature, rainfall (2000-2500 mm/yr), and high relative humidity. It is also characterized by poorly drained soil, low in nutrient due to the leaching of nutrient down the soil profile as a result of high rainfall.

2.2 Sample Collection

Soil sample was taken from Kegbara-Dere in Gokana Local Government Area of Rivers State with the aid of a sterile spade from three different spots of the same location and put in black plastic (polythene bag) and was taken to the laboratory within 24hours for analysis.

2.3 Analyses of Physicochemical Parameters

2.3.1 Temperature

The temperature was checked using the mercury in glass thermometer and this was done on day 1, 7, 14, 21 and 28 respectively. The thermometer was dipped in the soil sample and mercury rose to a certain level and the reading was taken.

2.3.2 Hydrogen ion concentration (pH)

The pH of soil samples was taken using a pH meter. After calibration, the electrode was rinsed in distilled water and dipped in the soil samples.

2.3.3 Moisture content analysis

This was performed by collecting and weighing 6g of marshland sample and using a top loading balance and was placed in a crucible and was heated. After heating, it was reweighed and the weight was taken. The final weight was removed from the initial weight to get the moisture content of the sample.

2.3.4 Nitrate

About 50g of dry soil sample was treated with 30-40cc of caustic lime (50% suspension) and sufficient quantity of water (H_2O) to bring up to a homogenous suspension. The later was well stirred and then treated with 2-4cc of copper sulphate (CUSO4) (10% solution) and basic lead acetate (10% solution) respectively. More H_20 was added and suspension again well stirred and filtered through Buchner funnel. The filtrate which is clear and colourless was then treated with standard permanganate in excess, followed by dilute Sulphuric acid in excess. The mixture was then shaken and treated with 10% KI. The iodine liberated was titrated against standard 0.05 normal thiosulphate in the usual way.

2.3.5 Phosphate

Phosphate was analysed by dissolving 10ml of the aliquot in a 50 ml measuring flask containing distilled water. 10 ml of the colour developing reagent was added, stirred, and allowed to stand for 15 minutes, then measured at 880 nm by using Spectrophotometer and glass cells.

2.3.6 Sulphate

The analysis of sulphate was done by measuring 5 ml of sample in a volumetric flask, and diluting to 100 ml distilled water, transferring into 250 ml conical flask. It was followed by the addition of 5.0 ml conditional reagents, mixing in stirring apparatus, during stirring, add spoon full $BaCl_2$ crystals 0.3 g. Begin timing immediately, stirring for 1 minute at constant speed. Immediately after stirring period has ended (at 30 sec intervals 4 min) pour solution in 4 cm sample silica cell. Measure the absorbance at 420 nm by using Spectrophotometer [21].

2.4 Microbiological Analyses

2.4.1 Preparation of media and diluents

About 65 g of Sabouroud Dextrose Agar (SDA) was weighed and dissolved in 1000 ml of distilled water and shaken thoroughly for proper mixing. The conical flask was then plugged with cotton wool and covered with aluminium foil to prevent moisture accumulation on the cotton wool which may lead to contamination. It was then sterilized in an autoclave at 121°C and 15 pound per square inches (Psi) for 15 minutes. The medium was cooled at 45-50°C and then poured aseptically in sterile petri dishes and allowed to solidify, ensuring there were no air bubbles [21].

Dispersant Agar was prepared for the isolation of hydrocarbon utilizing fungi (dispersant utilizing fungi). This media was prepared using the following: $ZnCl_2$ 0.03 g, MgSO4 0.03 g, K_2HPO_4 0.5 g NaCl 0.3 g, $NaNO_3$ 0.03 g $FeSO_4$ H_2O 0.02 g, Agar 1.6 g, dispersant (Seacare 0.1) and 1000 ml of distilled water [5]. The mixture media was stirred using a sterile rod and autoclaved at 121°C for 15 minutes.

2.4.2 Tenfold serial dilution

Normal saline was used as the diluent and was prepared by dissolving 8.5 g of NaCl (Sodium Chloride) in a conical flask and 1000 ml of distilled water was measured using a measuring cylinder and poured into the flask containing the NaCl with the aid of a sterile 10 ml. 1 ml of the diluent was pipette and put in a sterile test tube, the diluent was sterilized and autoclaved at 121°C for 15 minutes and allowed to cool before carrying out the serial dilution of the sample that had been contaminated with dispersant. Using a sterile spatula, sample contaminated with dispersant was taken. One gram was weighed and put in a sterile test tube containing the diluents, 1 ml was taken using a sterile pipette and put into another sterile 9ml diluent and shaked evenly.

2.4.3 Inoculation and incubation of cultures

Inoculation of total heterotrophic bacteria and fungi was done by aseptically transferring an aliquot (0.1 ml) of the dilution of 10^{-6} and 10^{-4} into properly dried nutrient agar plates and sabouraud dextrose agar plates containing antibiotic (Tetracycline and Penicillin) to inhibit bacterial growth in duplicate, spread evenly using bent glass rod and incubated at 35-37°C for 24 to 48 hr and 25-27°C for 72 hrs respectively [22].

Inoculation of total hydrocarbon degrading bacteria and fungi for each soil samples was done using spread plate method. An aliquot (0.1 ml) of the dilution of 10^{-3} was aseptically transferred into properly dried dispersant agar plates containing antifungi (Fluconazole) to inhibit fungi growth, and another, containing antibiotic (Tetracycline and Penicillin) to inhibit bacterial growth, in duplicate, spread evenly using bent glass rod and incubate at 35-37ºC and 25-27°C for 7days.

2.4.4 Enumeration and isolation of pure cultures

Enumeration was done as described by Prescott et al. [22].

2.4.4.1 Enumeration and isolation of Total Heterotrophic Fungi (THF)

After incubation, discrete bacterial colonies and fungal spores that grew on the plates were enumerated by sub-culturing into fresh nutrient agar plates and sabouroud dextrose agar plates. Counts were taken and fungal isolates were sub-cultured onto Potato Dextrose Agar slant in conical flasks for preservation and identification.

2.4.5 Maintenance of stock culture

Pure cultures of fungal isolates were sub-cultured into Sabouroud Dextrose Agar broth in conical flasks for preservation and identification.

2.5 Identification and Characterization of Microbial Isolates

2.5.1 Identification of fungal isolates

Macroscopic: Examination of growth was done by observing the colonial morphology, colour of colony, texture, shape and surface appearance [23]. The macroscopic pictures of pure culture of organisms are as shown in Fig. 1 a - b:

Microscopy: This was done by using the wet prep (needle mount) and slide culture characteristics like sexual and asexual reproduction structures e.g., the conidial head, sporangia; the vegetative mycellia, septate or non septate hyphae [24].

Wet prep (needle mount) small portion of the growth was picked with a sterile inoculation needle and suspended in a drop of water on a sterile slide, then leased out using two sterile needles. Using lactophenol, these slides were covered with clean cover slips; care was taken to

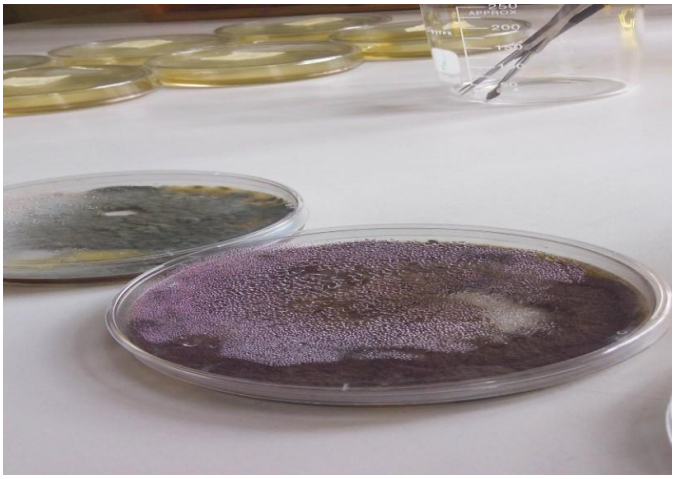
exclude air bubbles. The prepared slides were examined under the microscope. The microscope examination involved observing asexual and sexual reproductive structures [24].

2.5.2 Internal Transcribed Spacer (ITS) amplification

The ITS region of the rRNA genes of the isolates (*A. clavatus* and *Pichia* Spp.) respectively were amplified using the ITSIF: 5'-CTTGGTCATTTAGAGG AAGTAA-3' and ITS4: 5'-TCCTCCGCTTATTGATATGC-3, primers on a ABI9700 Applied Biosystems thermal cycler at a final volume of 30 µl for 35 cycles. The PCR mix comprise X2 Dream tag master mix supplied by Inquaba, south Africa (taq polymerase, DNTPs, MgCl), the primers at a concentration of 0.4 m and the extracted DNA as template. The PCR conditions were as follows: initial denaturation 95°C for 5 minutes, denaturation 95°C for 30 seconds, annealing 53°C for 30seconds, extension 72°C for 30 seconds for 35 cycles and final extension, 72°C for 5 minutes. The product was resolved on a 1% agarose gel at 120V for 15 minutes and visualized on a UV transilluminator.

2.5.3Sequencing

Sequencing was done with the aid of a Big Dye terminator kit on a 3510 ABI sequencer by Inqaba Biotechnological, Pretoria, South African. The sequencing was done at a final volume of 10 µl, the component are 0.25 µl BigDye Terminator v1.1/v3.1, 2.25 µl of 5x BigDye sequencing buffer, 10µM primer PCR primer, and 2-10 ng PCR template per 100 bp. The sequencing conditions were 32 cycles of 96°C for 10 seconds, 55°C for 5 seconds and 60°C for 4 m.



a: Pure culture of organisms

b

Fig. 1. Culture of organisms

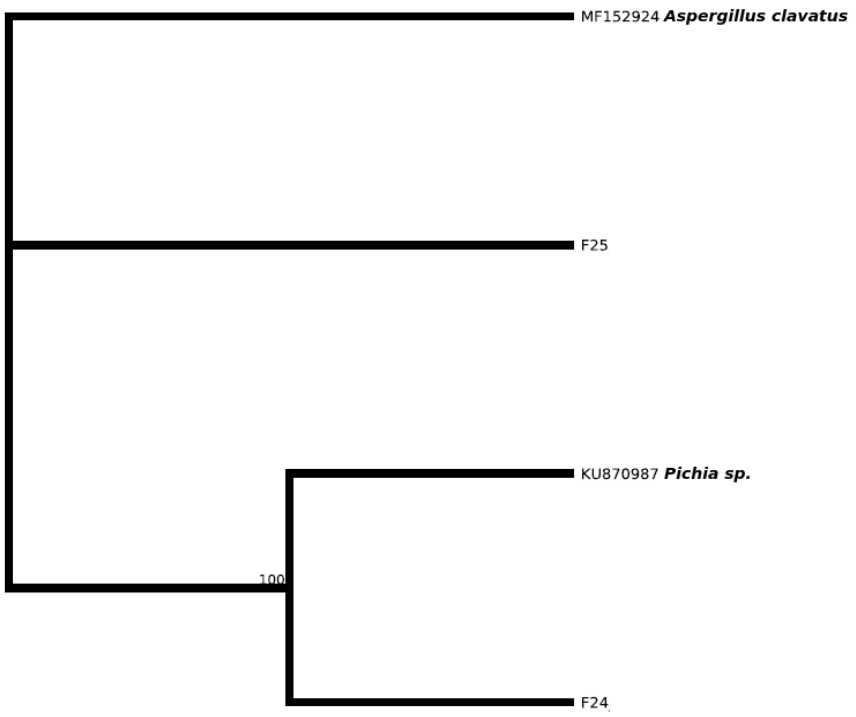


Fig. 2. Phylogenic Tree of *A. clavatus* and *Pichia* spp.

2.5.4 Phylogenetic analysis

The obtained sequence were edited using the bioinformatics algorithm Trace edit, similar sequences were downloaded from the National Centre for Biotechnology Information (NCBI) database using BLASTN. These sequences were aligned using ClustalX. The evolutionary history was inferred using the neighbour-joining method in MEGA 6.0 [25]. The bootstrap consensus tree inferred from 500 replicates [26] is taken to represent the evolutionary history of the taxa analyzed. The evolutionary distances were computed using the Jukes-Cantor method [27]; [28].

The Fig. 2 shows the phylogenic Tree of the organisms.

2.6 Bioremediation Set up

Soil samples marsh land which were taken from sites and weighed using top load balance 250 grams each and put in a plastic container. Eight (8) containers were used for dispersant Seacare for the sampled marsh land. Organisms were measured using a graduated measuring cylinder one was used as control 20 ml of dispersant (Seacare) and 100 ml of organism in broth was added to the set (*Aspergillus clavatus* and *Pichia* spp. and a container without organism which is the control).

Ten (10) grams of soil samples were weighed and put in a sterile beaker, 20 ml of n-Hexane was added and was shaken thoroughly to extract the dispersant after which it was sieved through a filter paper that was folded into funnel shape and was put in a test tube, 5 ml of the extract was poured into a spectrophotometer cuvette and placed in the open chamber to determine the concentration of the sample.

3. RESULTS

3.1 Physicochemical Parameters

Table 1 shows marshland contaminated with Seacare with a constant decrease in temperature 29°C to 28°C from day 1 to day 28. Consortium consumed the least amount of Nitrogen 24 mg/kg on day 28 while *Pichia* spp. and *A. clavatus* separately consumed the highest amount of Nitrogen 46 mg/kg respectively. Phosphorus content changed from 149 mg/kg in the control set up to 119 mg/kg in consortium set up. Sulphate changed from 334 mg/kg in the control set up to 235 mg/kg in consortium set up.

Table 1. Marshland + Seacare

Set up	Ctrl		*Pichia* spp.		*A. clavatus*		PIC+ASP	
	Day 1	Day 28	Day 1	Day 28	Day 1	Day 28	Day 1	Day 28
Temperature (^{0}C)	27	28	29	28	29	28	29	28
Nitrate(mg/kg	44	38	46	32	46	35	45	24
Phosphorous (mg/kg)	149	108	151	131	150	127	149	119
Sulphate (mg/kg)	334	323	335	317	336	287	335	235

Key: PIC= Pichia, ASP=Aspergillus clavatus

Table 2. Log counts (cfu/g) of *A. clavatus* and *Pichia* spp. on Seacare in Marshland soil during 28 days monitoring

Days	Logc	logA	logP	logPA
1	1.59±0.12^a	1.59±0.10^a	1.53±0.04^a	1.79±0.01^a
7	1.68±0.04^a	1.61±0.15^a	1.65±0.03^a	1.79±0.01^a
14	1.73±0.11^a	1.69±0.11^a	1.77±0.11^b	1.87±0.05^a
21	1.76±0.14^a	1.82±0.13^a	1.80±0.07^{ab}	1.89±0.04^a
28	1.81±0.04^a	1.77±0.02^a	1.84±0.08^{ab}	1.89±0.07^a

Means with the same alphabet across columns shows no significant difference (p>0.05)
Key: C-Control, P-Pichia, A-Aspergillus clavatus.

3.2 Microbiological Analyses

Table 2 shows the logarithm counts (Cfu/g) of *A. clavatus* and *Pichia* species on Seacare in marshland soil during 28 days monitoring. Day 1 revealed that the population of *A. clavatus* was higher (1.59±0.10) than that of *Pichia* spp (1.53±0.04) which was lesser than that in the control set up (1.59±0.12). During day 7 and day 14, the population of *A. clavatus* and *Pichia* rose from 1.69±0.11 to 1.82±0.13 and 1.77±0.11 to 1.80±0.07. The consortium however, maintained the highest population of 1.89±0.04 and 1.89±0.07 at day 21 and 28 respectively.

3.3 Bioremediation Results

Fig. 3 shows the total hydrocarbon content (THC-mg/kg) of bioremediated oil polluted (Seacare) on marshland soil using *A. clavatus* and *Pichia* spp. The THC for day 1 was 8006.56 mg/kg for all the samples and remediation occured over 28 days period.

Fig. 4 shows the bioremediation rate at day 28 of oil polluted (Seacare) on marshland using *A. clavatus* and *Pichia* spp. and the results indicated that *A. clavatus* had the least THC consumption (2942.11 mg/kg) and the consortium had the highest THC consumption rate (1473.68 mg/kg).

Fig. 5 shows the percentage bioremediation potential of oil polluted (Seacare) on marshland using *A. clavatus* and *Pichia* spp. Results indicated that *Pichia* spp had the least potential (62.86%) followed by *A. clavatus* (64.50%) and consortium with the highest potential (81.59%).

4. DISCUSSIONS

Results of the physicochemical analyses in Table 1 indicated that hydrogen ion concentration (pH) of the soil samples before contamination ranged from 6.90-7.79 showing marshland basic. The near neutral pH provides buffering property which may have contributed to the survival of the test organisms. Monod et al. (2009) confirmed that *Aspergillus clavatus* and most fungi grow very well at neutral and acidic pH. The moisture content of the soil samples before and after drying indicated uncontaminated marshland with the highest moisture content of 2.9/6 g. Moreso, *Aspergillus clavatus* have been reported severally to have certain ability to alter the temperature of its environment to favor its growth (Monod et al. 2009). *Aspergillus clavatus* can modify the temperature and pH of their environment by secreting acids such as butyrate, oxalate, malate, citrate, gluconate, and succinate. This however agrees with the work of Shehu and Bello [29] who reported that *Aspergillus clavatus* thrive in higher temperature even at 40°C.

Although microorganisms are ubiquitous in nature, they however thrive better under favorable environmental conditions [22]. The presence of Nitrate, Phosphate and sulphate were high in the soil samples analyzed. This could be as result of fertilizer application. It is important to say that the inhabitants of Kegbara-Dere in Gokana Local Government Area are

renowned farmers and most times, fertilizers are added to soil to enhance crop yield. Khan et al. [30]; Fan et al. (2010) reported that fertilizers in soil contribute immensely to nitrate, phosphorus and sulphate contamination of soil. However, the reduction in the nitrogen, phosphorus and sulphate at some certain points during the experimental monitoring was an indication that these nutrients were being utilized by the organisms [31].

Comparing the log counts of *Aspergillus clavatus* and *Pichia* spp. in OSD/Seacare (Table 2), consortium thrived more in Seacare implying that Seacare dispersant was more easily utilized. Obire and Ramesh (2009) suggested that microbial consortium degrades synthetic petroleum mixture faster than single organisms. Similar results were obtained by Nrior and Chinyere [5]. Another reason why *Aspergillus* and *Pichia* tend to sporulate better in OSD/Seacare than in OSD/LT may be as a result of the toxicity of the dispersants. OSD/LT has been reported by Nrior and Odokuma (2017), during their research to be more toxic than OSD/Seacare and microorganisms especially fungi have a higher tolerance to the toxicity of hydrocarbons & dispersant due to their physiology and adaption to such variations in the environment and have the mechanism for the elimination of oil spill from the environment [16]. Evaluating logarithm counts of *A. clavatus* and *Pichia* spp. in marshland environments (Table 2), one can infer that fungi population was less in the marshland samples and the major reason for this may not necessarily be tied to the toxicity of the dispersant alone but also the moisture content of the marshland soil samples. Although fungi grow very well in acidic environments, the amount of moisture present in soil can also retard their growth. Marshland soil sample had more moisture and this may have led to oxygen depletion which however may not favor fungal growth (Monod et al. 2009).

The results of the total hydrocarbon content (THC-mg/kg) of the bioremediation set up (Fig. 3), suggested that there was a reduction in the (THC-mg/kg) from 8006.58 mg/kg to 1276.32 mg/kg on the 28th day of monitoring (Fig. 2). The (THC-mg/kg) consumed by the different set ups on day 28, were: 2697.37 mg/kg (*A. clavatus*), 3197.37 mg/kg (*Pichia* species), and 1276.32 mg/kg (consortium). This experiment suggests that both *A. clavatus* and *Pichia* spp. have certain bioremediation potential as they could utilize petroleum products as their sole energy source. They have been often times implicated in the bioremediations of dispersants in brackish water samples [7]. Their ability to degrade

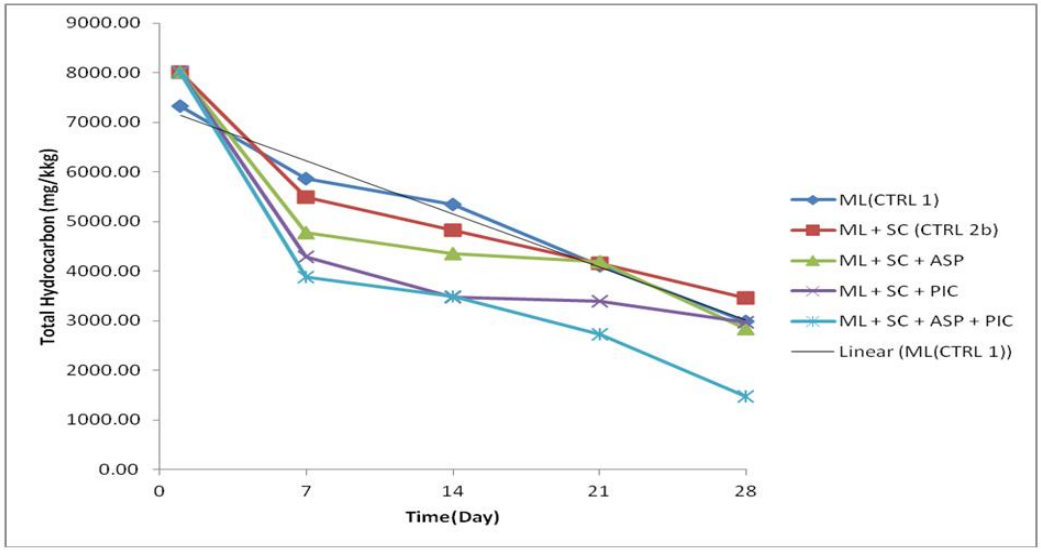


Fig. 3. Total Hydrocarbon Content (THC-mg/kg) of bioremediated oil spill dispersant (Seacare) polluted Marshland soil using *Aspergillus clavatus* and *Pichia* species over a 28-day period.
Key: ML= Marshland, CTRL 2b= dispersant (Seacare) without organism, SC=Seacare, ASP=Aspergillus clavatus, PIC=Pichia

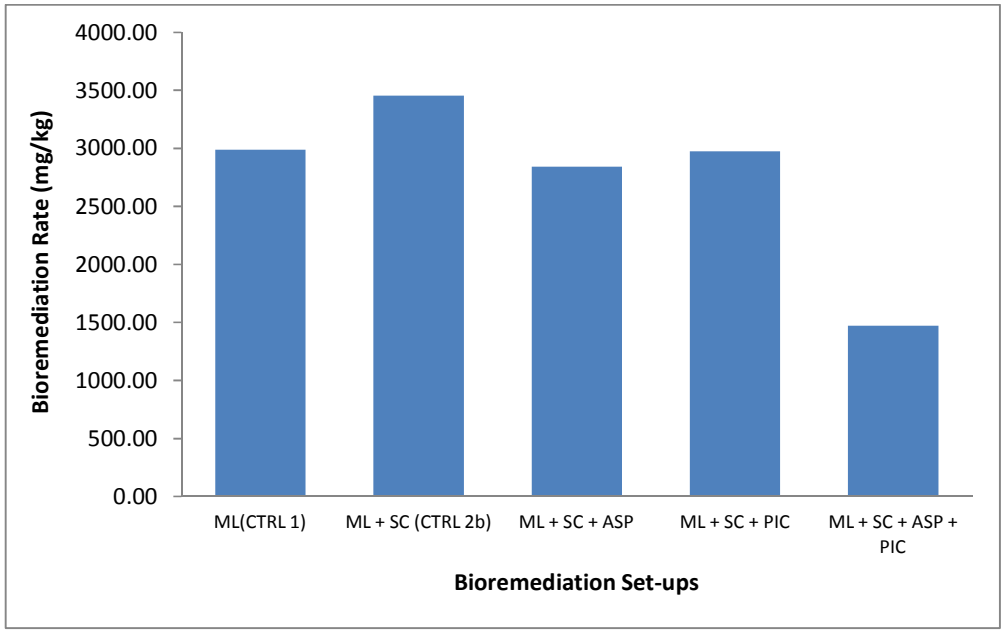


Fig. 4. Bioremediation rate of oil spill dispersant (Seacare) polluted Marshland soil using *Aspergillus clavatus* and *Pichia* spp. on day 28.

(key: ML= Marshland, CTRL 2b= dispersant (seacare) without organism, SC=Seacare, ASP=Aspergillus clavatus, PIC = Pichia)

dispersants and crude oil may be due to co-metabolism [32]. *A. clavatus* have been reported to possess 100% bioremediation potential [33]. The yeast (*Pichia* spp.*)* has also been reported to be a potent bioremediation organisms (Ortansa et al. 2010). The mould, *Aspergillus clavatus,* yeasts and *pichia* species have been reported severally to readily degrade hydrocarbon [16,34]; Ortansa et al. 2010; [35-37]. The mix culture (consortium) had higher bioremediation potential as reported in this study. A consortium of microorganisms known as the biomass can use the dispersant and the crude oil as food [38]. There are several reports proving that microbial mixed cultures have high bioremediation potential [39-41]. Oil spill dispersants (OSD/LT and OSD/Seacare) which contain chemicals of similar origin with hydrocarbons are regularly used to clean up oil spills. The chemical constituents of some oil spill dispersants such as the additives and base solvents have reportedly resulted in the inability of microbial cells to degrade dispersants and /or their mixtures with crude oil [42,43]. Bioremediation potentials of the fungi expressed in percentages on marshland and Seacare (Control), *A. clavatus*, *Pichia* species and consortium reveal 56.86% <64.50%>62.86%<81.59%. The results of the primary degradation of the dispersants or the bioremediation potential of the organisms according to OECD [44], is the alteration, structural change (transformation) in the chemical constituents of the substance brought about by biological actions of the microorganisms, resulting in the loss of a specific property. The percentage mineralization of the samples (OSD/Seacare in marshland) was the level of aerobic degradation obtained when the dispersants and/or crude oil (test compounds) are totally utilized by microbial cells resulting in the production of carbon dioxide, biomass, mineral salts and water [42]. The total hydrocarbon content (THC-mg/kg) consumed by *A. clavatus* and *Pichia* species is an indicator in bioremediation monitoring. Spectrophotometric absorption values of THC was 8006.58 mg/kg on the day 1 of monitoring on all the soil samples and significantly decreasing through day 28 as shown in Figs. 3 and 4. These results show that bioaugmentation of dispersant pollutants by activation of naturally occurring microorganisms such as *A. clavatus* and *Pichia* spp. will be cost effective in cleaning up the environment contaminated with petroleum products in this regard, oil spill dispersants.

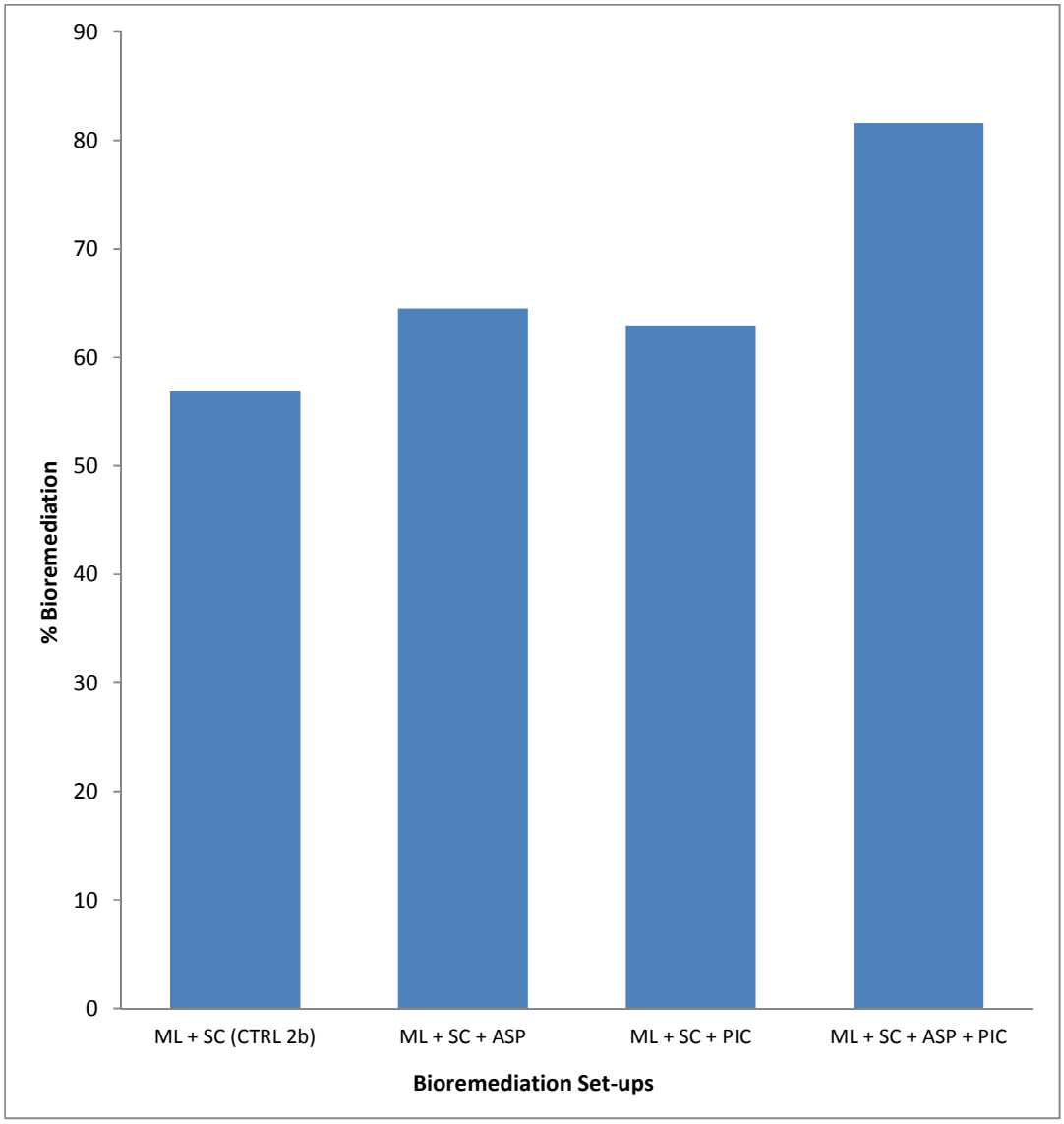


Fig. 5. Percentage bioremediation potential of 2 organisms on oil spill dispersant (Seacare) polluted Marshland soil using *Aspergillus clavatus* and *Pichia* spp.

Key: ML= Marshland, CTRL 2b= dispersant (Seacare) without organism, SC=Seacare, ASP=Aspergillus clavatus, PIC=Pichia

5. CONCLUSION

Selection of effective microbial strains is the most crucial in attainment to success in bioremediation. It is also noted that the effective removal and elimination of pollutants/ contaminants could be achieved through the application of microbial inoculants isolates in the immediate dispersant impacted site. The ability of the isolated innoculants to survive in the contaminated marshland determines the success of bioremediation in strong terms. This is sequel to the fact that the activities of microorganisms have great potentials to enhance renewal of the environment as well as maintenance of the ecosystem to support the flora and fauna. It is obvious, in most instances, that the potential degradation of contaminants is usually estimated at a typical laboratory by use of selected cultures and under favourable growth condition. Several factors ranging from composition of microorganisms, insufficient and inadequate supply of essential substrates as well as unfavourable external conditions (aeration, pH,

moisture, temperature etc) are implicated. To overcome the associated problems, the researcher deviced the process of supportive activation of indigenous micro-organisms in the contaminated marshland by bioaugmentation and/or bioremediation of their degradative potentials. Through this method, it will cause the reduction of waste, elimination of industrial pollution and make our environment safe from hydrocarbon contaminated. Based on the above assertions and evidences, study recommends the following:

i. The use of the consortium of fungi (*Aspergillus clavatus* and *Pichia* spp.) in the bioremediation and degradation in dispersant pollutants from the environment as they show high potential in remediation of hydrocarbon pollutions and elimination of dispersant pollutants.
ii. Natural bioremediation of dispersant pollutants by activation of naturally occurring microorganisms will be cost effective in cleaning up the environment to save the environment from hazardous effects of hydrocarbon on our agricultural and aquatic ecosystem.

ACKNOWLEDGEMENTS

The following people are acknowledged for their various contributions in ensuring the completion of this research: Dr. R. R. Nrior, Dr. (Mrs.) S.I. Douglas, Prof. D.N. Ogbonna, Prof. O. Obire and Dr. (Mrs.) N. A. Akani for the academic and experimental supports. Also, our appreciation goes to Mr. Penu L. Samuel, ACIB and Rev. Augustine Inordee for their financial support toward the completion of this work. Not forgeting Mr Dominic Girah for the assistance in laboratory erands. We most sincerely express our gratitude to you all.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Prince RC. Petroleum spill bioremediation in marine environment. Crit. Rev. Microbial. 1993;19(4):217-242.
2. Swannell RPJ, Lee K, McDonagh M. A direct respirometic method for the in situ determination of bioremediation efficacy. Microbial. Rev. 1996;60(2):342-365.
3. Okoh AI. Biodegradation of Bonny light crude oil in soil microcosm by some bacterial strains isolated from crude oil flow stations saver pits in Nigeria. African Journal of Biotechnology. 2003;2(5):104-108.
4. Williams JO, Youngtor TP. Remediaton of pesticide-polluted River using *Anabaena* and *Nostoc* spp. Journal of Environmental Science, Toxicology and Food Technology. 2017;11(7):71-76.
5. Nrior RR, Chinyere W. Biodegradation of oil spill dispersant in brackish water ecosystem of the Niger Delta Nigeria. Current studies in Comparative Education, Science and Technology. 2016;3(1):187-201.
6. Fingas M, Fieldhouse B, Mullin J. Water in oil emulsions. How they are formed and how they are broken in: Processings of The International oil spill Conference, Long Beach, California. 1995;829-830.
7. Uzoigwe CI, Okpokwasili GC. Biodegradation of oil spill dispersants in natural aquatic ecosystem. International Journal of Physical Sciences. 2012;7(38): 5477-5484.
8. Batelle CD. Mushrooms: Higher macrofungi to clean up the environment. Environmental Issues, Fall; 2000.
9. Ojo OA. Petroleum-hydrocarbon utilization by native bacterial population from a West water canal Southwest Nigeria. African Journal of Biotechnology. 2005;5:333-337.
10. Bouchez M, Blanchet D, Haeseler F, Vandeasteele JP. Les hydrocarbures aromaiques polycyclidques dans l'environment. *Revue de l'Institut francais du petrole*. 1996;51:797-828.
11. Gesinde AF, Agbo EB, Agho MO, Dike EFC. Bioremediation of some Nigerian and Arabian crude oils by fungal isolates. Int Jor P App Scs. 2008;2:37-44.
12. Hadibaratam T, Tachibana S. Microbial degradation of crude oil by fungi pre-grown on wood meal. Interdisciplinary Studies on Environmental Chemistry, Environmental Research in Asia; 2009.
13. Atagena HI, Haynes RJ, Wallis FW. Fungal bioremediation of creosote-contaminated soil: A laboratory scale Bioremediation study using indigenous soil fungi. Water Air Soil Pollution. 2006;172:201-219.
14. Husaini A, Roslan HA, Hii KSY, Ang CH. Biodegradation of aliphatic hydrocarbon by indigenous fungi isolated from used motor oil contaminated sites. Word Journal

Microbial Biotechnology. 2008;24:2789-2797.
15. Obire O, Anyanwu EC. Impact of various concentrations of crude oil on fungi populations of soil. International Journal of Environmental Science Tecchnology. 2009;6:21-218.
16. Atlas RM. Petroleum biodegradation and oil spill bioremediation. Mar Pollut Bull. 1995;31:178-182.
17. Varadaraj R, Robbins LM, Bock J, Pace S, MacDonald D. Net environmental benefit analysis of oil spill response. In: Proceedings of the 1995 International Oil Spill Conference, American Petroleum Institute, Washington, DC.; 1995.
18. Al-Nasrawi H. Biodegradation of crude oil by fungi isolated from Gulf of Mexico. Journal of Bioremediation and Biodegradation. 2012;3:14 7.
19. Adebusoye SA, Ilori MO, Amund OO, Teniola OD, Olutope SO. Microbal degradation of petroleum hydrocarbons in polluted tropical stem. World Journal of Microbiology and Biotechnology. 2007; (23)8:114-115.
20. Yakimov MM, Timmos KN, Golysin PN. Obligate oil degrading marine bacteria. Current Opinion in Biotechnology. 2007; (18)3:25-266.
21. Cheesbrough M. District laboratory practice in tropical countries. (Part two) Cambridge University Press; 2000.
22. Prescott LM, Harley JP, Klein DA. Microbiology. 6th ed. McGraw Hill, London. 2005;135-140.
23. Abbey DE, Lebowitz MD, Mills PK. Long-term ambient concentration of particulates and oxidants and development of chronic disease in a cohort of non-smoking California residents. Inhalation Toxicology. 1995;7:19-34.
24. Robert M. Burgess, Micheal J. Ahrens, Christopher Hickey W. Geochemistry of PAHs in aquatic environment; 2003.
25. Saitou N, Nei M. The neighbor-joining method: A new method for reconstructing phylogenetic trees. Molecular Biology and Evolution. 1987;4:406-425.
26. Felsenstein J. Confidence limits on phylogenies: An approach using the bootstrap. Evolution. 1985;39:783-791.
27. Jukes TH, Cantor CR. Evolution of protein molecules. In Munro HN, editor, Mammalian Protein Metabolism, Academic Press, New York. 1969;21-132.
28. Solomon L, Ogugbue CJ, Okpokwasili GC. Influence of biostimulation treatment using composted plant biomass on bacterial diversity of an aged petroleum contaminated soil as determined by culture-dependent and 16S rRNA gene PCR-DGGE based identification methods. South Asian Journal of Research in Microbiology. 2018;1(2):1-16.
29. Shehu KT, Bello MT. Effect of environmental factors on the growth of *Aspergillus* species associated with stored millet grains in Sokoto. Nigerian Journal of Basic and Applied Science. 2011; 19(2):218-223.
30. Khan MN, Mobin M, Abbas ZK, Alamri SA. Fertilizers and their contaminants in soils surface and Ground water. In: Dominick, A., Della, S., and Michaul Goldstein (eds). The encyclopedia of the Anthropocene. 2018;5(225-240).
31. Okpokwasili GC, Odokuma LO. Effect of salinity on biodegradation of oil spill dispersant. Waste Management. 1990;10: 141-146.
32. Chikere CB, Azubuike CC. Characterization of hydrocarbon utilizing fungi from hydrocarbon polluted sediments and water. Nigerian Journal of Biotechnology. 2014;27:49-54.
33. Mbachu AE, Chukwura EI, Mbachu NA. Isolation and characterization of hydrpcarbon degrading fungi from used (spent) engine oil polluted soil and their use for Polycyclic Aromatic Hydrocarbons (PAHs) Degradation. Universal Journal of Microbiology Research. 2016;4(1):31-37.
34. Diaz E. Microbial biodegradation, genomics and molecular biology. Ist Edn, Caister Academic Press, U.K. 2008;17. 402. Aaaaasss
35. Saadoun I. Isolation and characterization of bacteria from crude petroleum oil contaminated soil and their potential to degrade diesel fuel. Journal of Basic Microbiology. 2002;42:422–430.
36. Fernández-Luqueño F, Valenzuela-Encinas C, Marsch R, Martínez-Suárez C, Vázquez-Núñez E, Dendooven L. Microbial communities to mitigate contamination of PAHs in soil—possibilities and challenges: a review. Environmental Pollution Science Research. 2010;10:11-30.
37. Obire O, Anyanwu E, Okigbo R. Saprophytic and crude oil degrading fungi from cow dung and poultry droppings as

bioremediating agents. Journal of Agricultural Technology; 2008.
38. Ladhari N, Hayet G, Harrabi L. Study of the biodegradability of sodium salt from the condensation product of Naphthalene sulphonic acids and formaldehyde. Unit Textile Resources. 2000;1-10.
39. Mohan SK, Srivastava T. Microbial deterioration and degradation of polymeric materials. Journal of Biochemical Technology. 2010;2.210-215.
40. Zheng Xian J, Han-Qun Y. Inhibitory effects of butyrate on biological hydrogen production with mixed anaerobic cultures. Journal of Environmental Management. 2005;74. 65-70.
41. Silva DP, Calvalcant DP, De Melo EV. Bio-removal of diesel oil through a microbial Consortium isolated from a polluted environment. International Biodeterioration Biodegradation. 2015;97:85-89.
42. Nnadozie PC, Odokuma LO. Impact of rigwash oil spill dispersants' on the biodegradation of crude oil in Nigeria. Microbiol. Res. J. Int. 2017; 18(4):1-10.
43. Sogbanmu TO, Otitoloju AA. Efficacy and bioremediation enhancement potential of four dispersants approved for oil spill control in Nigeria. Journal of Bioremediation, Biodegradation. 2012; 3(2):1-5.
44. OECD. OECD Guideline for testing of chemicals. Draft Document, proposal for a new Guideline 302D. 2001;1-11.

Peer-review history:
The peer review history for this paper can be accessed here:
http://www.sciencedomain.org/review-history/27966