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To cite this article: Obinna C Nwinyi and P. J Ikhine 2019 IOP Conf. Ser.: Earth Environ. Sci. 331 012059

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Effect of Saw-dust on soils contaminated with waste lubricating oil

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Abstract. In this study, we evaluated the influence of sawdust as biostimulatory agent on waste-lubricating oil site. To achieve this, two sites were contaminated with waste lubricating oil, with one of the site amended with sawdust and the other left to serve as control. This study was conducted during the dry season. Decreases in pH and percentage moisture content were used to monitor the metabolic activities between the amended and control sites. Both sites (amended and control) showed decrease in the pH values however the control site showed just slight decrease. The mean pH value obtained ranged between 7.68± 0.15- 8.08± 0.57 for the amended and control while the mean moisture content (%) 38.3 – 48.6 respectively for the amended and control sites. We screened for fungal species that showed growth on sawdust amended site using waste lubricating oil as source of carbon/energy. This was done through conventional enrichment culture methods. The isolated fungal strains tentatively named as P1, P2, P3, P4 and P5 were identified by visual observation and micro-morphological technique. From the cultural, and morphological characterization and comparison with respect to the standard reference of fungi, the fungal species were identified as members Aspergillus niger, Rhizopus sp., Penicillium sp., Aspergillus flavus and Mucor sp. Pure cultures of these fungal species were tested for their ability to utilize waste lubricating oil as carbon and energy source. The ability of these fungal species to use the waste lubricating oil was done by monitoring their physiological responses via Optical Density (OD) and pH gradient readings. The mean pH obtained range from 5.90-7.33 and the (OD) 0.715-1.978. The fluctuations in OD readings as well as pH values for the different microfungi may be due to variation in growth patterns of the different fungal species.

1. Introduction
The quality of one’s environment affects the individuals’ life on earth. The releases and accumulation of persistent and toxic chemicals could have a harmful impact on human health and the environment [1]. Waste-lubricating oil is a common and toxic environmental contaminant not obviously found in the environment [2]. Waste-lubricating oil or mineral based crankcase oil occur, as brown-to-black liquid produced when lubricating oil is subjected to high temperature and pressure [3]. Waste-lubricating oil is a mixture of several different chemicals which include low and high molecular weight aliphatic hydrocarbons, aromatic hydrocarbons, lubricative additives heavy metals and decomposition products [3,4]. The waste-lubricating oil, is obtained following servicing and subsequent draining from automobile and generator engines. These are often released into the environment by motor and generator mechanics through unchecked releases into gutters, water drains, open vacant plots and farmlands [5-8]. When waste-lubricating oil is released into the environment it creates difficult condition within the soil matrix owing to poor aeration low pH and immobilization of soil nutrients[9-10]. In the environment, waste-lubricating oil spillages could spread horizontally into the ground waters [11], cause immense destruction to the mangrove and rainforest crops, farmland and aquaculture such as fish and periwinkle [12-13]. The toxicity of waste-lubricating oil or the refined products could be difficult to assess due to limited information on the effects of the additives, their synergistic or antagonistic effects of the mixtures [14-15]. Land is becoming scarce, polluted soil with waste-lubricating oil could be amended by adding materials to the soil that could improve its physical and chemical properties (Anon., 2012). Soil contamination with petroleum products have been treated with technologies such as thermal treatment, dig and dump method and stabilization/solidification technology [16]. Nonetheless, these technologies have their drawbacks [17]. These limitations include: the high cost, its operability at full scale and the destruction of soil texture and characteristics [18]. Bioremediation-mycoremediation presents as alternative technology to the pervious technologies currently in use.
Several soil improvement materials exist. One of such is sawdust. The economic disposal of sawdust is a serious challenge in the wood industries [19]. Enormous quantities of sawdust are produced yearly by sawmills. This could be diverted to soil conditioning/amendments efforts. Sawdust contain about 40% of lignin, 60% cellulose along with various waxes, resins and oils. [20]. The high lignin content makes sawdust potentially a good source of humus and thus good for soil amendments. Recently, fungi have received considerable attention for their bioremediation potential due to the range of enzymes they produce. These enzymes have capacity to degrade lignin and a range of recalcitrant pollutants. Due to the hyphae possessed by fungi, it has advantage over bacteria in the decontamination of soil polluted with hydrocarbons. Due to the overwhelming challenges that emanate from spent oil when released to the environment, authors aimed at using the sawdust to biostimulate the activities of soil fungi in the remediation of spent oil contaminated site.

2. Materials And Methods

Chemicals and Reagents
All chemicals and reagents were of analytical grade. Media used include: Potato Dextrose agar (manufactured by Biomark laboratories India), Chlorine-free MS medium (as described by [21], Agar-agar (manufactured by Biomark Laboratories India) to support the growth of fungi and Ethanol (manufactured by Sigma-Aldrich, Germany). Then (NH₄)₂SO₄, MgSO₄.7H₂O, Ca(NO₃)₂·4H₂O, KHPO₄, NaHPO₄ of analytical grades were obtained from LOBA Chemie lab, Mumbai, India, Qualikems lab, India, and Guangdong Guanghua Sci-tech Co., Ltd., China. Waste-lubricating oil, were obtained from a mechanic workshop in Ota, Ogun State Nigeria.

Instrumentation
Some of the instruments used in this study include: Genesys 10 UVS spectrophotometer, Incubator-shaker (Guangzhou healthy ling, Model HZQ-X 300), Centrifuge (Table centrifuge machine, model 800D), and pH meter (Jenway, 3505).

Stock solutions and Media preparation
Chloride free minimal salts (MS) medium as described by Nwinyi et al., (2014) with modification was used for enrichment and degradation experiments. The medium consists of 34.84g KH₂PO₄, 27.22g K₂HPO₄, 1.0g MgSO₄.7H₂O, 5.0g (NH₄)₂SO₄, 2.0g NaHPO₄, 0.76g Ca(NO₃)₂·4H₂O and 4.8g of agar-agar dissolved in 1000 mL of deionized water. The slurry MS medium was supplemented with the spent oil.

Study Location
The study was carried out as field study experiment in an open field within College of Science and Technology, Covenant University, Ota, Ogun State, Nigeria. A 1×1.5sqm of the test field in CST building, Covenant University was chosen. This test site was divided into two pieces. To both pieces, were added about 500ml of waste lubricating oil each and thoroughly mixed. One parts was amended with sawdust and the other without sawdust to serve as control. The experiment was left to stand for 40 days under the influences of natural factors. Moisture and pH were determined every 7days.

Determination of moisture content
The crucible were washed and dried. The weight of each crucible was measured and referred to as (W1). 5g of each soil sample were weighted into two sterile crucible dish (W2) and placed in the oven at 105°C for 3hours. This was allowed to cool before it was reweighed as (W31). The W31, were placed in the oven again for 30 minutes and allowed to cool and reweighed as (W3 ). This was repeated until similar weight value is obtained (W3*). The difference in weight between the initial weight and constant weight gained represents the moisture content. The mathematical calculation of moisture content is given below.

\[ \text{Moisture Content} \% = \frac{W3 - W1}{W2 - W1} \times 100 \]

Where W1 = initial weight of empty crucible, 
W2 = weight of crucible + soil before drying, 
W3= final weight of crucible + soil after drying

pH determination
Two (2g) each of the amended soil sample and control were dissolved in 10ml of deionized water and the pH value measured.

Enrichment of Fungal Isolates
Soil samples were collected from the study site and the enrichment slurry prepared as described by Nwinyi et al., (2014). The fungi diversity was obtained by conventional enrichment culture methods. For this, 10g each of soil samples were mixed with 50ml freshly prepared MS broth into two 250 mL conical flasks, 5ml of spent oil was added. The resulting mixture was stirred, stoppered with sterile cotton wool and incubated at 25 ± 2°C for 14 days. Thereafter, a loop full of the solution was streaked over freshly prepared sterile potato dextrose agar (PDA) and sprayed with waste lubricating oil and incubated at 25 ± 2°C for 5 days.

**Preliminary screening of hydrocarbon degrading fungal isolates**

For the estimation of hydrocarbon utilizers. A minimal salt broth (MSB) 34.84g KH₂PO₄, 27.22g K₂HPO₄, 1.0g MgSO₄.7H₂O, 5.0g (NH₄)₂SO₄, 2.0g NaH₂PO₄, 0.76g Ca(NO₃)₂.4H₂O and 5.0g of agar – agar dissolved in 1000ml of deionized water was prepared. Twenty 20ml of the minimal salt broth was measured into 6 test bottles (bioreactors) which was supplemented with 2ml of spent engine. The isolated fungal strains from the enrichment setup were seeded into 5 test tubes, while the 6th test tube with no fungi served as control. Each of the bioreactors were stoppered with sterile cotton wool to allow aeration and prevention of cross contamination. All the test tubes were then incubated at room temperature (25 °C - 27°C) for 12 days. The test tubes were slightly shaken periodically to facilitate oil-cell phase contract.

**Isolation and mass production of Hydrocarbonoclastic Fungi in Minimal Salt Slurry**

Pure cultures from the waste lubricating oil–enriched media were isolated by plating out 2.0 ml of the enriched cultures onto prepared PDA plates, sprayed with waste-lubricating oil on the surface. This was incubated at room temperature (25 ± 2°C) for 5 days. Colonies were periodically transferred to fresh PDA agar as they appeared to obtain pure culture. Fungi isolates were identified based on their cultural properties, colonial characteristics, and the microscopic features of their sporulating structures. The fungi isolates were compared with standard reference organisms (Bannet and Hunter, 1972).

**Harvesting of fungal isolates**

Pure cultures of fungal isolates were inoculated on freshly prepared sterile mineral salt slurry (30ml) in balch tubes stoppered with sterile cotton wool to enable aeration and incubated at room temperature for 5 days. The different fungal isolates were harvested by centrifugation at 400rpm for 60 min, fungal inocula were washed twice in phosphate buffer saline at pH of 7.25 and transferred into a sterile balch tubes and kept briefly in a refrigerator at 6°C for use during the growth / degradation studies (Verdin et al., 2004).

**Characterization of Hydrocarbonoclastic Fungi Species and determination of their degradation potentials**

Most fungi have been reported to be identified by visual observation and micro-morphological techniques (Thenmozhi et al., 2013). Based on this, the different fungal colonies were observed on the plated potato dextrose agar after incubation for 5 days at room temperature. The cultural and microscopic examination of the fungal isolates were carried out. These include: morphology of the sporulating structure and lactophenol cotton blue stain

Pure cultures of tentative fungal species namely: S1, S2, S3, S4 and S5 were tested for their ability to utilize spent oil as carbon and energy source using MS Medium slurry supplemented with spent engine oil (4% v/v) as carbon source for the determination of hydrocarbon degradation. The enrichment slurry was prepared as described by Nwinyi et al., (2014). For this, the slurry media composed of (per liter of distilled water) 34.84g KH₂PO₄, 27.22g K₂HPO₄, 1.0g MgSO₄.7H₂O, 5.0g (NH₄)₂SO₄, 2.0g NaH₂PO₄, 0.76g Ca(NO₃)₂.4H₂O and 5g of agar- agar.. About 1mL of the different fungal harvested inocula were transferred into 30ml of the different MS slurry tubes. In each of the aseptically prepared MS slurry tubes, 2mL of waste-lubricating oil were used to amend it. Incubation was carried out at room temperature for 12 days; however the tubes were intermittently shaken at 95 rpm using shaker (Model H2Q-X 300) to facilitate oil phase contact. Abiotic controls were set up with the biotic controls (MS slurry medium and spent oil devoid of organism) and incubated at the same conditions as the test samples. Determination of the extent of growth rate of the organism in MS slurry was measured by the turbidity (Optical density) and pH fluxes at 0, 3, 6, 9 and 12 days intervals. Optical density was measured at 600 nm using Genesys 10 UVS Spectrophotometer.

**3. Results And Discussions**

The Table 1.0 shows the cultural and microscopic characteristics of the obtained fungal isolates S1- S5. The most probable organisms include: *Aspergillus spp*, *Rhizopus*, *Penicillum* and *Mucor* species (See plate 1.0-4.0).

Table 1 Cultural and Microscopic characterization of the Soil Fungi
<table>
<thead>
<tr>
<th>Isolates</th>
<th>Cultural characteristics</th>
<th>Microscopic characteristics</th>
<th>Most probable organisms</th>
</tr>
</thead>
<tbody>
<tr>
<td>S1</td>
<td>Fluffy colonies with elevated mycelia that appeared black in coloration. Observing from underneath the plates showed a pale yellow color</td>
<td>Conidia in chains borne externally at the end of the vesicle. Long conidiophore present arising from septate hyphae</td>
<td><em>Aspergillus niger</em></td>
</tr>
<tr>
<td>S2</td>
<td>White cottony colonies</td>
<td>Sporangiospores within spherical shaped sporangium</td>
<td><em>Rhizopus sp.</em></td>
</tr>
<tr>
<td>S3</td>
<td>Mature colonies were observed, it showed velvety texture, leaf green in coloration. Reverse color is cream</td>
<td>Conidiosphores are formed on a brush –like conidia head. Microscopic appearance in blue green</td>
<td><em>Penicillium spp.</em></td>
</tr>
<tr>
<td>S4</td>
<td>Greenish and dusty colonies observed, reverse color appear pale yellow</td>
<td>Septate hyphae, non-septate conidiosphore with columnar conidial head bearing spherical spore</td>
<td><em>Aspergillus flavus</em></td>
</tr>
<tr>
<td>S5</td>
<td>White entire growth. Cottony with orange colored sporangium</td>
<td>Globular sporangium, spores are oval collumella present, non-septate hyphae and absence of rhizoids</td>
<td><em>Mucor sp.</em></td>
</tr>
</tbody>
</table>
Plate 1.0: Growth of *Aspergillus flavus* on PDA sprayed with spent engine oil.

Plate 2.0: Growth of *Mucor spp.* on PDA sprayed with spent engine oil.

Plate 3.0: Growth of *Rhizopus spp.* on PDA sprayed with spent engine oil.

Plate 4.0: Growth of *Aspergillus niger* on PDA sprayed with spent engine oil.
The Figures 1-5 shows the fluctuations in Optical densities and pH values of the different isolated fungal species growth on spent engine oil with an increasing OD and decreasing pH values.

Plate 5.0: Growth of Penicillium spp. on PDA sprayed with spent engine oil.

Figures 1.0: Data represent the mean value of pH and Optical Density (OD) against different time intervals with the same concentrate of spent engine oil (2ml) for 12 days by Aspergillus niger.

Degradation potential of Aspergillus niger

Figures 1.0: Data represent the mean value of pH and Optical Density (OD) against different time intervals with the same concentrate of spent engine oil (2ml) for 12 days by Aspergillus niger.
Figure 2.0: Data represent the Mean value of pH and Optical Density (OD) against different time intervals with the same concentration of spent engine oil (2ml) for 12 days by *Aspergillus flavus*.

Figure 3.0: Data represent the mean value of pH and Optical Density (OD) against different time intervals with the same concentration of spent engine oil (2ml) for 12 days by *Rhizopus spp.*
Fig 4.0: Data represent mean value of pH and Optical Density (OD) against different time intervals with the same concentration of spent engine oil (2ml) for days by *Mucor* spp.

Figure 5.0: Data represent the mean value of pH and Optical Density (OD) against different time intervals with the same concentration of spent engine oil (2ml) for 12 days by *Penicillium* spp.
Bar chart and Area chart showing the differences in moisture content and pH values respectively of the amended soil and the control.

Figures 6.1: Bar chart showing variation in % moisture content of amended soil and contaminated soil.
Fig 6.2: Bar chart showing the disparity in the degradation potential of amended soil and contaminated soil taking decrease in pH as an indicator of Degradation.

From the field study, it was observed from the moisture content and pH fluxes, that the amended soil showed evidence of the disappearance of the hydrocarbons. The decrease in pH observed for the contaminated soil could be due to the presence of microbial flora capable of utilizing waste-lubricating oil (Fig 6.1 and 6.2). This observation is in line with the reports of Ref [22] that opined that when hydrocarbon occur in the ecosystem, with the right micro-organisms capable of utilizing the hydrocarbons and the metabolites produced, there will be disappearance of the hydrocarbons. The moisture content recorded as shown in Fig 6.1 did not give clear evidence of degradation probably due to variations in the edaphic factors and the limitations on the indirect method of assessment. In this study, it was obvious that the fungal species *Penicillum, Mucor, Rhizopus, Aspergillus* species showed potential to utilize spent oil when the fluxes of pH and optical readings were measured (See plates 1-5). Okerentugba and Ezeronye (2003), reported that *Penicillum spp., Aspergillus spp., and Rhizopus spp.* isolated were capable of degrading hydrocarbons especially when single cultures were used. Furthermore, Batelle (2000) showed that fungi were better degraders than the traditional bioremediation techniques including bacteria. Different species of fungi respond differently to hydrocarbon sources as sole sources of carbon. The fungal species identified were *Aspergillus flavus, Aspergillus niger, Muscor sp., Rhizopus sp.* and *Penillicium sp.*

The (Figs 1-5), demonstrate the mean changes in the pH and optical density of spent engine oil degradation study by the fungi species. From the assessment of the fungal growth (increase in biomass) using the optical density at 600nm, it showed that the fungi species had different physiological dynamics. The results obtained from this work showed that the fungal species isolated from Sawdust (mostly considered as waste of the wood mill industries) were capable of degrading spent engine oil. *Apergillus flavus* showed a mean pH value of (7.42-6.16) and the mean values of OD (0.213-0.617) for the 12day incubation period. *Penicillium sp.* showed decreasing mean pH values of (7.40-6.40) and an increasing mean OD values of (0.175-1.457) within the 12 days incubation period. This result agrees with previous studies on *Aspergillus species* in utilizing hydrocarbons with the
resultant releases of CO$_2$, water and energy required to create cellular biomass (Thenmozhi et al., 2013). This explains the reason for the decrease in pH values as CO and H$_2$O forms weak acid.

From the onsite treatment, there was a slight reduction in soil pH which was insignificant compared to the control. Similar observations were made in the reports (Atuanya, 1987; Osuji and Nwoye, 2007). Petroleum hydrocarbon mediated decrease in soil pH has been attributed to the production of organic acids by microbial metabolism (Osuji and Nwoye, 2007). The strain identified as *Mucor sp.* showed a decreasing pH of 7.33-5.90 and increasing OD of 0.715- 1.978 (Fig.4.0). In (Fig.1.0), *Aspergillus niger* showed a pH of 7.43 – 6.50 and OD value of 0.292- 1.731. The *Rhizopus sp.* exhibited a decreasing pH values of 7.39- 6.41 and the OD 0.292- 1.728 at the same incubation periods (Fig. 3.0). The differences obtained in the readings were probably due to the difference in growth rates of each fungus and the continuous increase in the optical density means exponential growth of the fungal isolates and their ability to utilize spent as a sole carbon source.

4. Conclusion

This study have shown that *Penicillum sp.*, *Aspergillus niger*, *Mucor sp.*, and *Rhizopus* species have the potential of utilizing waste lubricating oil as carbon and energy sources. The observed significant increases in the turbidity may likely be explained by increase in cell growth (cell biomass) of the fungal organisms when compared with the controls. This showed that the fungal species have the potentials to access energy from the hydrocarbons. Also, the decrease in pH may be due to acidic products of the degradation of spent engine oil.

Acknowledgements

The authors would like to acknowledge Covenant University, Ota, Nigeria for defraying the cost of this publication.

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