Preliminary Study of Crude Oil Degradation by Microorganisms Isolated from Polluted Soil in Okarki, River State, Nigeria

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Abstract

Pollution of the environment by hydrocarbon compounds has become a significant management challenge in oil-producing countries. Presently, the use of biological means for the reclamation of polluted sites is the most acceptable technique owing to its eco-friendliness. This study was carried out to assess the crude oil degradation potential of indigenous microorganisms in polluted soil. The polluted soil was obtained from a crude oil-laden site in Okarki, River State, Nigeria. Bacterial and fungal organisms present in the polluted soil were isolated on Nutrient agar and Saboraud dextrose agar plates respectively. The isolates were identified based on their morphological, microscopic and biochemical characteristics. Gravimetric analysis of the crude oil degradation by the isolates was done in Bushnell Hass medium supplemented with 5% crude oil as the only carbon source. A total of 6 bacterial genera namely, Staphylococcus, Citrobacter, Micrococcus, Pseudomonas, Bacillus and Corynebacterium were identified while the fungal isolates were Aspergillus niger, Aspergillus flavus and Penicillium sp. Bacterial and fungal counts were 2.57±0.01 Log cfu/g and 2.08±0.07 Log cfu/g respectively. Bacillus sp. had the highest relative abundance (27.3%), while Micrococcus sp. and Corynebacterium sp. had the least occurrence (9.1%). Among the fungal group, A. niger showed the highest percentage occurrence in the polluted soil. All the indigenous organisms isolated from the polluted soil showed varying potentials for crude oil degradation. Bacillus sp. and Penicillium sp. were the highest crude oil-degrading bacterium and fungus respectively. The degradation potential of the bacterial consortium was significantly higher (P < 0.05) than the other consortia tested. This study has shown that indigenous organisms possess the potential for crude oil degradation.

Keywords: crude oil, degradation, bacteria consortium, reclamation, fungi consortium

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1. Introduction

The shift in economic base of coal to crude oil and petroleum products, significantly escalated the quantity of these commodities being shipped across the high seas. This, coupled with their storage underground, involve high environmental risks [1]. As a result of contamination from petrochemical products and industrial effluents, diverse components of crude oil and petroleum such as polycyclic aromatic hydrocarbons (PAHs) have been found in waterways [2].

Petroleum hydrocarbon pollution of the environment may also arise from oil well drilling production operations, transportation and storage in the upstream industry, marketing in the downstream industry, and intentional bunkering of pipeline [3]. Other sources of petroleum and its products in the environment also include accidental spills and ruptured oil pipelines, as pipelines are vulnerable to “tear and wear”, thus can fail with time [4].

The spilled petroleum hydrocarbons seeps into the soil due to gravity until an impervious horizon is met, for example bedrock, watertight clay or an aquifer. Poor miscibility of crude oil accounts for accumulation of free oil on the surface of ground water and this may drift over a wide distance to contaminate other zones very far away from the point of pollution [5].

Oil suffocates the soil particles, prevents ease of air diffusion in the soil pores, and creates an anaerobic environment which affects soil microbial communities [6]. Dense crude oil pollution can cause complete death of marsh vegetation [7]. In addition, crude oil-contaminated soils are hydrophobic compared with pristine sites [8]. Hydrocarbon contamination can also increase soil total organic carbon [9], and change soil pH values [10], and other soil chemical properties [11]. Crude oil affects soil fertility, germination and growth of some plants; however, the severity of the impact depends on the quantity and type of oil spilled [7]. Pollution of environment affects humans exposed to it. Some health conditions have been associated with exposure to crude oil pollution [12].
health problems may be through any or combinations of the following routes: contaminated food and/or water, emission and/or vapors [1]. The volatile organic components of crude oil have been implicated in the aggravation of asthma, bronchitis and accelerated aging of the lungs. They also affect the liver, kidney and spleen [12]. Epidemiological evidence suggests that oil spills affect neonates, contributes to infant mortality, and also increase the risk of abortion and stillbirth [13].

Oil spills are common occurrences in Niger Delta region of Nigeria, where over 40 million liters of crude oil spill have been recorded annually, resulting in human deaths and damage to the local ecosystem. It has been reported that more than 12,000 oil spill incidents have occurred in the oil-rich region between 1976 and 2014 [14].

Bioremediation recently has evolved as an emerging green technology of environmental conservation by removing, transforming and breaking down various contaminants, especially petroleum hydrocarbons, by applying living organisms [15]. Degradation can occur aerobically or anaerobically, however, greater percentage of hydrocarbon degradation occur under aerobic condition. This process uses microbial metabolism in the presence of optimum environmental conditions and sufficient nutrients to breakdown contaminants notably petroleum hydrocarbons [16]. Bioremediation requires the evaluation of both the intrinsic degradation capacities of the autochthonous microflora and the environmental parameters involved in the kinetics of the in-situ process [17].

Autochthonous microorganisms, which are the indigenous microorganisms living on polluted environment are usually well adapted to the presence of the pollutants, as a result of long-term exposure to site-specific stress factors [18]. The adaptation provides useful opportunities for bioremediation at such sites [19]. Therefore, several studies have focused on the community of microorganisms associated with oil-contaminated soil, and assessment of their bioremediation potentials [20,21,22]. Bacteria and fungi have been reported as the principal agents of hydrocarbon degradation [23]. Several genera have been reported as hydrocarbon degraders [24], and they include Achromobacter, Pseudomonas, Acinetobacter, Alkanindiges, Alteromonas, Arthrobacter, Burkholderia, Dietzia, Enterobacter, Kocuria, Marinobacter, Mycobacterium, Pandoraea, Bacillus, Staphylococcus, Streptobacillus, Streptococcus, Rhodococcus and Micrococcus [25].

The aim of the present study is to assess the crude oil degradation potential of autochthonous microorganisms in a polluted soil.

2. Materials and Methods

2.1. Description of Okarki Sampling Site

Okarki community in Ahoada East Local Government Area of River State, is a border town between Bayelsa and River state. It is located on latitude: 4°58′56″N and longitude 6°25′44″N. The sampling site is one of the sites of illegal refinery of crude oil [26].

2.2. Sample Collection

Soil samples contaminated with crude oil were aseptically collected by random sampling technique at 6cm depth in the sampling site. The soil samples were collected from 6 different points in the site, mixed and homogenized to obtain a composite sample.

2.3. Isolation of Indigenous Microorganisms

Indigenous microorganisms in the polluted soil samples were isolated and enumerated using standard pour plate method [26]. 1.0g of the composite soil was serially diluted tenfold and 1ml of 10⁻² dilution inoculated on Nutrient agar (Oxoid) plate. The plate was observed for bacterial growth after 24h incubation at 30°C. Triplicate plates were prepared. Developed colonies were counted and recorded as mean heterotrophic bacteria count ± standard deviation. The colonies were sub-cultured and pure cultures obtained were stored on Nutrient agar slants at 4°C for further studies.

The soil sample was similarly treated for the isolation of fungi. 1ml of 10⁻² dilution was inoculated on Saboraud dextrose agar (SDA) plate containing 0.05g/ml of chloramphenicol, and the plate incubated for 72h at 30°C. Fungal count was recorded and pure cultures obtained stored on SDA slant at 4°C for further studies.

2.4. Identification of Bacterial Isolates

Several biochemical tests were carried out to identify the bacterial isolates from the polluted soil sample following the method described by [27]. They include Gram reaction, catalase test, coagulase test, indole test, methyl red test, Voges-Proskauer test, citrate utilization test, sugar fermentation test.

2.5. Identification of Fungal Isolates

The fungal isolates were identified based on detailed studies of their colonial morphologies and microscopic features, and compared to standard description given by [28,29].

2.6. Shake Flask Experiment of Crude Oil Degradation by Indigenous Microorganisms

2.6.1. Preparation of Inoculum

A 24h old culture of the isolate was inoculated into 10ml of sterile distilled water in a test tube and standardized using 0.5 Mcfarland standard [30]. This served as the seed inoculum.

2.6.2. Biodegradation Assay

Shake flask experiment was carried out to assess the crude oil degrading potential of the isolated indigenous microorganisms [31]. 1ml of seed inoculum of the isolate was inoculated into 50ml of Bushnell-Haas broth supplemented with 5.0% w/v crude oil in 250ml Erlenmeyer flask. The flask was incubated at 30°C on a rotary shaker at 150rpm. An un-inoculated medium served
as control. After 7 days incubation, residual crude oil was extracted and crude oil degradation rate was estimated gravimetrically.

2.6.3. Extraction Process

To the culture broth was added 5ml of n-hexane and the flask contents mixed thoroughly before transferring to a separating funnel to extract the residual crude oil [31]. Extraction process was carried out twice to ensure complete recovery of oil. The extract was treated with 0.4g anhydrous sodium sulphate to remove the moisture, and the suspension decanted into a pre-weighed beaker, leaving behind sodium sulphate. The pre-treated extract was evaporated to dryness by heating in a water bath. The weight of the extracted crude oil was deducted from the previously weighed beaker.

The % degradation of the crude oil was calculated as:

\[ \text{Weight of residual crude oil} = \text{Weight of empty beaker} \]

Amount of crude oil degraded = Weight of crude oil added in the media - Weight of residual crude oil

\[ \% \text{ degradation} = \left( \frac{\text{Amount of crude oil degraded}}{\text{Amount of crude oil added in the media}} \right) \times 100 \]

2.7. Statistical Analysis

Data obtained was subjected to one way Analysis of Variance by Student-Newman-Keul (SNK) test at 95% confidence level using IBM SPSS statistics version 20 [32].

3. Results and Discussion

3.1. Isolation of Indigenous Microorganisms

The results obtained in this study show the presence of different genera of bacteria and fungi in the crude oil-contaminated soil (Table 1 - Table 2). The mean heterotrophic bacterial count in the polluted soil sample was 2.57±0.01 Log CFU/g. This is in line with the observation made by [33], in crude oil-contaminated soil in Port Harcourt, River State. However, our finding does not corroborate the report of [34], who recorded high bacterial count (5.903CFU/g - 9.69CFU/g) in oil-saturated desert soil samples. In another study, bacterial populations of crude petroleum-polluted soil counts ranged from 5.18 to 7.38CFU/g soil at a soil depth of 1-10 cm [23]. The reduced bacterial count observed in this study could be attributed to the long term impact of the crude oil pollution on the abundance of microbial communities in the site. The mean fungal count obtained in this study was 2.08±0.07 LogCFU/g. The range of heterotrophic fungal count obtained in this study supports that recorded by [35]. A significantly higher fungal count (p value < 0.05) was obtained in crude oil-polluted soil by [36]. The bacterial count in this study was significantly higher (p value < 0.05) than the fungal count. It is very likely that bacterial organisms were more adapted to the polluted soil than the fungi, hence were able to multiply and increase in population. In a study by [37], on the response of bacterial and fungal communities to high petroleum pollution in different soils, the bacterial communities were significantly higher than the fungal communities.

3.2. Identification of Bacterial Isolates

The characteristic features of the bacterial isolates from the polluted soil are shown in Table 1.

A total of six indigenous bacteria was identified from the crude oil-polluted soil and they belong to the genera Staphylococcus, Citrobacter, Micrococcus, Pseudomonas, Bacillus and Corynebacterium (Table 1). These bacterial isolates make the list of the commonly isolated microorganisms from hydrocarbon-polluted environment, and this observation supports the work of [35]. This finding is also in agreement with the work of [38], who isolated nine different genera of bacteria including Staphylococcus, Micrococcus, Bacillus, Pseudomonas, Acinetobacter, Enterobacter, Escherichia, Klebsiella and Proteus from hydrocarbon-polluted soil in Effurun, Delta State. Similarly, Bacillus spp., Pseudomonas spp., Micrococcus spp., Serratia spp., Arthrobacter spp., Proteus spp. and Shigella spp. were isolated from three different crude oil-polluted sites in Anambra state [39]. Bacillus and Pseudomonas spp. were recovered in a long-standing petroleum-contaminated sediments in Bohai Bay, China [40].

The relative abundance of the bacterial isolates as presented in Figure 1, shows that Bacillus subtilis was the predominant species (27.3%) while Micrococcus sp. and Corynebacterium sp. were the least occurring bacteria. Contrary to our findings, various researches showed that Pseudomonas spp. are predominantly detected in diverse hydrocarbon-polluted environment [34,41,42].

3.3. Identification of Fungal Isolates

The morphological and microscopic features of the fungal organisms are presented in Table 2.

The fungal isolates are Aspergillus niger, Aspergillus flavus and Penicillium (Table 2). This finding supports the work of [43] in which Penicillium spp., Aspergillus niger and Candida sp., Mucor sp., Rhodotorulla sp., Rhizopus sp, Trichoderma sp. and Cladosporium spp. were recovered from a crude oil-polluted soil in Bayelsa state, Nigeria. Our inability to isolate the other fungi may have been as a result of the medium used. In a study by [44] on the response of fungi to diesel contamination, Aspergillus niger, Aspergillus spp., Fusarium spp., Mucor spp., Rhizopus sp. and Saccharomyces spp. were isolated.

The dominant fungus recovered in this study was Aspergillus niger (Figure 1), having 60% relative abundance. This result corroborates the report of [45], who recorded Aspergillus niger, and Fusarium solani as the predominant fungi in a petroleum hydrocarbon polluted soil. However, [46] recorded Aspergillus oryzae and Mucor irregularis as the most abundant fungi in a crude oil-contaminated field with 56.67% and 66.70% abundances respectively.
Table 1. Characteristic features of the indigenous bacterial isolates from polluted soil

| Biochemical/morphological tests | S.aureus | Micrococcus | P.aeruginosa | Citrobacter | Bacillus | Corynebacterium |
|-------------------------------|----------|-------------|--------------|-------------|----------|----------------|
| Gram reaction                 | + cocci  | + cocci     | - rod        | - rod       | + rod    | + rod          |
| Motility                      | -        | -           | +            | +           | -        | +              |
| Citrate                       | +        | +           | +            | +           | -        | +              |
| Indole                        | -        | +           | -            | -           | -        | -              |
| Coagulase                     | +        | -           | -            | -           | +        | -              |
| Catalase                      | +        | +           | +            | +           | +        | +              |
| Oxidase                       | -        | +           | +            | -           | -        | -              |
| Methyl Red                    | +        | -           | -            | +           | +        | -              |
| Voges Proskauer               | -        | +           | -            | -           | -        | +              |
| Sugar fermentation            |          |             |              |             |          |                |
| Sucrose                       | +        | +           | -            | +           | -        | +              |
| Maltose                       | -        | +           | -            | +           | +        | +              |
| Glucose                       | -        | +           | -            | +           | +        | +              |
| Lactose                       | -        | -           | -            | +           | -        | +              |
| Rhamnose                      | -        | +           | -            | +           | +        | -              |

Key: - = negative, + = positive.

Table 2. Morphological and microscopic features of fungal isolates from polluted soil

| Isolate                  | Macroscopic features                                      | Microscopic features                                      |
|--------------------------|-----------------------------------------------------------|-----------------------------------------------------------|
| Penicillium sp.          | Greenish rough colonies with white edges on the front and milky colour on the reverse | Septate hyphae, branching conidiophore stipes with clublike phialides and conidia arranged in chains |
| Aspergillus niger        | Round brownish dark cottony colony with white edges on the front and milky on the reverse | Septate hyphae, variable length and smooth conidiophore, vesicles end in metulae which gives rise to the phialides with globular conidia |
| Aspergillus flavus       | Yellowish green cottony colonies on the surface and brown on the reverse | Septate hyphae, smooth conidiophore with round head. Conidia are clustered on the phialide head |

Figure 1. Percentage occurrence of bacterial and fungal isolates in the polluted soil

3.4. Shake Flask Experiment of Crude Oil Degradation by Indigenous Microorganisms

Results of crude oil degradation by the indigenous microorganisms isolated from the crude oil-polluted soil presented in Figure 2, show that all the bacterial and fungal isolates had the ability to degrade crude oil. Crude oil degradation rate ranged from 6.7± 2.3% to 98.8 ± 0.7%, with Bacillus subtilis achieving the highest degradation of crude oil, while Micrococcus sp. was the least. There was no significant difference (p-value > 0.05) in the degradation rates of S.aureus, P.aeruginosa, B. subtilis and bacteria consortium, indicating that they had the same level of performance. Some microorganisms have been reported to have inherent ability to degrade hydrocarbon, and probably their enzyme system aid them in the breakdown of different classes of hydrocarbons [47,48,49]. It has been reported that some organisms especially P.aeruginosa are capable of synthesizing different classes...
of biosurfactants in the presence of hydrophobic substrate, which makes the hydrocarbon bioavailable to them [50]. 
Pseudomonas sp. had high crude oil degradation rate (97.1%) in this study (Figure 2). Similar high crude oil degradation rate (93%) with Bacillus cereus was reported by [51], during their work on biodegradation of crude oil hydrocarbons by a newly isolated biosurfactant producing strain. Pseudomonas sp achieved 95% crude oil degradation and was reported as the most efficient crude oil degrader in Coastal area of Yanbu, Saudi Arabia by [52].

The optimal degradation of crude oil achieved by the bacteria consortium could be as a result of synergistic interaction among the bacterial organisms. This observation is in line with the report of [40], who recorded 80.4% crude oil degradation rate with mixed bacteria culture.

The crude oil degradation potential of the bacteria consortium was significantly higher (p-value > 0.05) than the consortium of the fungal isolates (Figure 2). [53], opined that bacteria, single species or consortium, are more efficient hydrocarbon degraders than other microorganisms. In a study carried out by [54], hydrocarbon degradation rates of 10.68 -15.67% was recorded by single bacterial strains, however, the synergistic effect of the bacterial strains in consortium produced higher total petroleum hydrocarbon degradation (19.59%).

The degradation rate (56%) of crude oil observed with Penicillium sp. (Figure 2) in this study is in line with the report of [55]. They noted a 57% and 55% crude oil degradation by Penicillium sp. RMA1 and Penicillium sp. RMA2 respectively from isolates recovered in Rumaila oil field, River State. In contrast to our findings, Aspergillus niger was reported as the highest crude oil-degrader against other isolates (Candida glabrata, Candida krusei and Saccharomyces cerevisiae) recovered in Basrah refinery field, Iraq [56].

The fungi consortium as presented in Figure 2, also achieved a degradation rate of 46.7±6.1% when compared to single fungal isolates. However, high degradation of petroleum hydrocarbon (92%) with fungi consortium comprising of Aspergillus terreus-SRF-15, Fusarium proliferatum-SRF-15, Fusarium sp-SRF-58 and Aspergillus sp-SRF-67 was reported by [57].

The poor degradation of crude oil (Figure 1) with bacteria and fungi consortium and Aspergillus spp. observed in this study is contrary to the report of [58]. They recorded a 42.24% crude oil degradation by bacteria and fungi consortium and 47.72% by Aspergillus fumigatus in their comparative study of petroleum crude oil degradation potential of microbes from petroleum-contaminated soil and non-contaminated soil.

Figure 2. Crude oil degradation by indigenous organisms (Key: B = bacteria; F = fungi)

4. Conclusion

The ability to remove crude oil from soil by indigenous microorganisms was observed in this study. Bacillus sp., Staphylococcus aureus, Pseudomonas sp. and the bacteria consortium achieved above 90% crude oil degradation. This shows that natural attenuation can be employed for reclamation of crude oil-polluted soil. Biodegradation is a complex process, which is influenced by several environmental factors, therefore, further research is necessary to improve the degradation potentials of the indigenous organisms.

Competing Interest

The authors have no competing interests.

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