Isolation and Characterization of Hydrocarbon Degrading Fungi from Used (Spent) Engine Oil Polluted Soil and Their Use for Polycyclic Aromatic Hydrocarbons (PAHs) Degradation

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Abstract Fungi capable of effectively degrading and cleaning up hydrocarbons was isolated from soil samples contaminated with used engine oil at auto-mechanic workshops (at Mgbuka-Nkpor, Nigeria) using vapour phase transfer method. The ability of the potential isolates to utilize used engine oil, diesel and petrol were assessed using gravimetric method. The ability of both the pure and consortium culture of the best potential strains to degrade the polycyclic aromatic hydrocarbons (PAHs) component of used engine oil, diesel and petrol was assessed using Gas Chromatography. A total of 8 fungal isolates were identified in this study based on their cultural and microscopic characteristics. Of these, 4 that showed high promise for hydrocarbon bioremediation potentials in screen flasks were confirmed as Candida tropicalis, Rhodosporidium toruloids, Fusarium oxysporium and Aspergillus clavatus based on their 18S rRNA gene sequencing. High biodegradation efficiency (> 70%) was recorded in the PAHs component in used engine oil, diesel and petrol with both the pure and consortium culture of the best potential strains; Candida tropicalis and Aspergillus clavatus, within 16 days of incubation at 28°C. However, there was complete (100%) depletion of some PAHs such as anthracene, naphthalene, acenaphthalene, acenaphthylene, phenanthrene and benzo (k) fluoranthene) in the hydrocarbon substrates with the pure and consortium culture of the isolates within 16 days of incubation at 28°C. Both the pure and consortium culture of the isolates (Candida tropicalis and Aspergillus clavatus) could therefore be utilized in the bioremediation of used engine oil, diesel and petroleum, as well as PAHs contaminated soil.

Keywords: Bioremediation, Used Engine Oil, Diesel, Petrol, PAHS, Consortium Culture

1. Introduction

The development of petroleum industry into new frontiers, the apparent inevitable spillages that occur during routine operations and records of acute accidents during transportation has called for more studies into oil pollution problems [1]. Oil pollution has been recognized as the most significant contamination problem [2]. The most notable oil spills at sea involve large tankers, such as Exxon Valdez, which spilled thousands of tones oil [3,4]. These oil spills can cause severe damage to sea and shoreline organisms [5]. Most responsible for the contamination are service stations, garages, scrap yards, waste treatment plants, sawmills and wood impregnation plants.

Engine oil is a complex mixture of hydrocarbons and other organic compounds, including some organometallic constituents [6]. It contains hundreds or thousands of aliphatic, branched and aromatic hydrocarbons [7,8], most of which are toxic to living organisms [9]. Used engine oil renders the environment unsightly and constitutes a potential threat to humans, animals and vegetation [10,11]. Fat soluble components may accumulate in the organs of animals and may be enriched in the food chain, even up to humans [12]. Prolonged exposure and high oil concentration may cause the development of liver or kidney disease, possible damage to the bone marrow and an increased risk of cancer [13-15]. In the long term, toxic and carcinogenic compounds can cause intoxication, diseases, cell damage, developmental disorders and reproduction problems [9]. In addition to toxic effects, oil products can affect plant and animals physically. A thick layer of oil inhibits the metabolism of plants and suffocates them. Destruction of plants affects the whole food web and decreases the natural habitat of numerous species [16].

Microbial remediation of a hydrocarbon-contaminated site
is accomplished with the help of a diverse group of microorganisms, particularly the indigenous bacteria present in soil. These microorganisms can degrade a wide range of target constituents present in oily sludge [17,18,15]. Hydrocarbon degrading bacteria and fungi are widely distributed in marine, freshwater and soil habitats. Similarly, hydrocarbon degrading cyanobacteria have been reported [19,20], although, contrasting reports indicated that growth of mats built by cyanobacteria in the Saudi coast led to preservation of oil residues [21]. Typical bacterial groups already known for their capacity to degrade hydrocarbons include *Pseudomonas* sp., *Marinobacter* sp., *Alcanivorax* sp., *Microbulbifer* sp., *Sphingomonas* sp., *Micrococcus* sp., *Cellulomonas* sp., *Dietzia* sp. and *Gordonia* sp. [22]. Molds belonging to the genera *Aspergillus* sp., *Penicillium* sp., *Fusarium* sp., *Amorphophoca* sp., *Neosartorya* sp., *Paecilomyces* sp., *Talaromyces* sp., *Graphium* sp. and the yeasts *Candida* sp., *Yarrowia* sp. and *Pichia* sp. have been implicated in hydrocarbon degradation [20].

Fungal bioremediation has been successful for clean-up of pentachlorophenol (PCP), a wood preservative and polycyclic aromatic hydrocarbon [23]. The advantages associated with fungal bioremediation lay primarily in the versatility of the technology and its cost efficiency compared to other remediation technologies (such as incineration, thermal desorption, extraction) [24]. The application of bioremediation capabilities of indigenous organisms to clean up pollutants is viable and has economic values [25]. This study was therefore undertaken with a view to isolate and characterize fungi from used engine oil-polluted soil and to access the polynuclear aromatic hydrocarbons (PAHs) degrading potentials of the isolates.

2. Materials and Methods

2.1. Collection of Soil Samples

Soil samples were collected randomly using a pre-cleaned hand scoop at a depth of 2–3cm from 3 auto-mechanic composite samples and placed into a sterile container. The samples were pooled together, homogenously mixed to obtain a composite sample.

2.2. Isolation of Fungi with Used Engine Oil Utilizing Abilities

Used engine oil utilizing fungi were isolated from soil samples obtained from auto-mechanic workshops on Mineral Salt agar Medium with composition as listed in Ekpenyong and Antai [26]. Fifty micrograms per millilitre (50μg mL⁻¹) of each of Penicillin G and Streptomycin was incorporated into the medium to inhibit interfering bacteria. The medium pH was adjusted to 5.5. The vapour phase transfer method was used with used engine oil as carbon and energy source supplied from the lid of the plates [27,28].

Each distinct colony on oil degrading enumeration plates were purified by repeated sub culturing onto Sabouraud Dextrose Agar (SDA) (Merck, Germany). The isolates were characterised and identified using colonial appearance and microscopic characteristics based on the schemes of Barnett and Hunter [29] and Efivuvwevwere [30].

2.3. Screening Tests for Used Engine Oil Utilization by Fungal Isolates

The isolates were screened for engine oil utilization capabilities in mineral salt broth medium. Screen tubes were incubated at 28°C for 16 days. Growth in tubes was scored as high (+++), moderate (++), low (+) and no growth (-). Viable count was taken at the end of 16 days incubation period by plating out onto Sabouraud Dextrose Agar and incubating at 28°C for 48 hours. Fungal biomass was also quantified at the end of 16 days incubation period at 540nm [31].

2.4. Determination of Used Engine Oil Biodegradation of the Potential Isolates

The rate and extent of biodegradation of used engine oil by four potential isolates; *Candida tropicalis*, *Rhodospiridium toruloids*, *Fusarium oxysporium*, and *Aspergillus clavatus* (confirmed using 18S rRNA gene sequencing) were assessed using the gravimetric method of Odu and Isinguzo, [32]. Degradation study flasks as well as control were incubated in triplicate at 28°C and 120 revolutions per minute (rpm) for 16 days. The amounts of hydrocarbon left after 16 days incubation was determined by extracting the residual oil with n-hexane (BDH Chemicals, England) in a separating funnel and noting their absorbance reading at 450nm, and the concentrations read off from the standard curve obtained from n-hexane extracts of used engine oil at different concentrations. Mean results were obtained and expressed as percentage weight loss of used engine oil. The whole process was repeated for diesel and petrol.

2.5. Determination of the PAHs Degradation by the Isolates

A 24 hour pure cultures as well as the consortium of each of the two best potential strains (*Candida tropicalis* and *Aspergillus clavatus*) were inoculated into Mineral Salt broth (100ml in 250ml Erlenmeyer flask) containing 1% (v/v) used engine oil, and incubated at ambient temperature of 28°C at 120 rpm for 16 days. Control flask without the organism was prepared accordingly. After 16 days the extent of polycyclic aromatic hydrocarbons (PAHs) degradation using undegraded engine oil as the control was determined by Gas Chromatography at Springboard Research Laboratory,
Awka, Anambra State, Nigeria. The whole process was repeated for petroleum and diesel oil.

The Buck 530 Gas Chromatography was equipped with a column oven, automatic injector, Mass spectrometer (Quadrupole Mass spectrometer, m/z 50 to m/z 400), HP 88 capillary column (30.0 m x 0.32 mm, film thickness 0.25µm) CA, USA. The analytic conditions of the chromatography were as follows: detector temperature, 250°C, injector temperature, 220°C, integrator chart speed, 2cm min⁻¹. Initial – final oven temperature was 70 – 280°C/min, and a holding time of 2 – 5 minutes. Carrier gas was helium (99.999% or 5.0 grade purity) at 5 psi, and injection volume was 1µL. The chromatograph was then attached to an integrator.

3. Result

A total of eight hydrocarbon utilizing fungi were isolated from the soil sample contaminated with engine oil. The fungal genera identified were Candida tropicalis, Rhodosporidium toruloids, Fusarium oxysporium, Aspergillus clavatus, Saccharomyces cerevisiae, Candida albicans, Microsporum gypseum and Trichophyton mentagrophytes, based on their cultural and microscopic characteristics (Table 1).

Candida tropicalis, Rhodosporidium toruloids, Fusarium oxysporium and Aspergillus clavatus showed the highest turbidity (+++) and viable count, with an absorbance of 1.597, 1.486, 1.422, 1.585 and viable count of 4.3x10⁵, 4.1x10⁵, 3.0x10⁵ and 4.8x10⁵ cfu/ml, respectively (Table 2). Based on these, they were confirmed using 18S rRNA gene sequencing and selected for further studies.

The hydrocarbonoclastic potentials of the selected isolates revealed that Candida tropicalis caused 86.2% weight loss of UEO in 16 days. This was closely followed by the weight loss of 85.0% caused by Aspergillus clavatus, Rhodosporidium toruloids and Fusarium oxysporium recorded 79.3% and 80.5% weight losses, respectively (Fig. 1). Candida tropicalis and Aspergillus clavatus also caused higher weight losses of 89.5% and 80.5% respectively, in diesel oil, while a weight loss of 66.8% and 64.2% was observed with Rhodosporidium and Fusarium species respectively (Fig. 1). Aspergillus clavatus caused a weight loss of 87.0% in petroleum oil, followed by the weight loss of 81.8%, caused by Candida tropicalis. Fusarium oxysporium and Rhodosporidium toruloids recorded weight losses of 74.4% and 68.8% respectively, in petroleum oil (Fig. 1).

The result of the Gas Chromatographic analysis for the removal of the PAHs in used engine oil by the two best potential strains: Candida tropicalis and Aspergillus clavatus, as well as their mixed culture (consortium) of the organisms are presented in table 3. Most of the PAH components of the used engine oil were completely removed by the single and mixed culture of the isolates. Aspergillus clavatus and the mixed culture achieved 100% depletion of the PAH components: phenanthrene, fluoranthene, pyrene and benzo (k) fluoranthene, while Candida tropicalis recorded 100% depletion of phenanthrene and benzo (k) fluoranthene, 96.76% depletion of fluoranthene and 99.27% depletion of pyrene. However, Candida tropicalis, Aspergillus clavatus and the consortium culture achieved 100%, 90.28% and 96.38% removal of dibenzo (a,h) anthracene respectively, as well as 94.09%, 71.82% and 87.09% removal of benzo (a) pyrene, respectively (Table 3).

Table 4 shows the result of the Gas Chromatographic analysis for the removal of PAHs in diesel by Candida tropicalis and Aspergillus clavatus as well as their mixed (consortium) culture. Candida tropicalis achieved 100% removal of the components:acenaphthene, acenaphthylene, phenanthrene, 1,2-benzanthracene, chrysene, benzo (k) fluoranthene, anthracene, naphthalene and fluorine, while benzo (a) pyrene and fluoranthene recorded 99.96% and 97.88% removal, respectively by Candida tropicalis in 16 days. However, Aspergillus clavatus and the consortium culture achieved 100% removal of acenaphthene, acenaphthylene, phenanthrene, chrysene, benzo (k) fluoranthene, anthracene and naphthalene. Also, Aspergillus clavatus and the consortium, recorded 98.71% and 80.75% respectively for the removal of 1,2-benzanthracene, 100% and 92.74% removal of chrysene, 78.0% and 75.02% removal of benzo (a) pyrene, 95.12% and 89.94% removal of fluoranthene, 78.07% and 100% depletion of fluorene in 16 days (Table 4).

Table 5 indicates that most of the PAH components found in petroleum are also completely removed by both single and mixed culture of the two best potential strains: Candida tropicalis and Aspergillus clavatus. Candida tropicalis, Aspergillus clavatus and the mixed culture achieved 100% depletion of benzo (k) fluoranthene, anthracene and naphthalene in 16 days. Candida tropicalis and Aspergillus clavatus achieved 100% depletion of fluoranthene, while the consortium recorded 87.89% removal of fluoranthene. However, Candida tropicalis and the consortia achieved 100% removal of fluorine, while Aspergillus clavatus recorded 76.88% removal of fluorine over a 16 day period. Moreover, Candida tropicalis and Aspergillus clavatus recorded only 80.22% and 79.76% removal of benzo (a) pyrene respectively, while the consortium achieved 99.88% removal of benzo (a) pyrene in 16 days (Table 5).
Table 1. Cultural and Microscopic Characteristics of isolates

| Isolates | Colonial Morphology | Microscopic Observation | Suspected Organism          |
|----------|---------------------|-------------------------|----------------------------|
| G        | White/creamy, smooth, soft and glabrous | Spherical to sub-spherical budding yeast-like cells | Candida tropicalis          |
| H        | Distinctive orange/red colony | Spherical to elongate budding yeast-like cells | Rhodosporidium toruloides    |
| I        | Purple/white and cone shaped colony | Branched conidiophores, smooth and rough conidia in pairs and chains | Fusarium oxysporium         |
| J        | Colonies are fast growing, flat and yellow-green to dark green in colour | Distinctive conidial heads with flask-shaped phialides arranged in whorls on a vesicle | Aspergillus clavatus        |
| K        | White/creamy smooth colonies | Large globose to ellipsoidal budding yeast-like cells. | Saccharomyces cerevisiae     |
| L        | Milky, waxy and butyrous texture | Small circular budding yeast cells | Candida albicans             |
| M        | Pale yellow-brown mycelium | Multiseptate, rough-walled, broadly spindle shaped macroconidia | Microsporum gypseum         |
| N        | Colonies are yellowish-brown and powdery | Smooth-walled, spherical and numerous microconidia produced in clusters | Trichophyton mentagrophytes  |

Table 2. Screen tests for utilization of used engine oil by fungal isolates

| Isolates         | Turbidity | Absorbance (540 nm) | Viable Count (x 10^5 cfu/ml) |
|------------------|-----------|---------------------|-------------------------------|
| Candida tropicalis | +++       | 1.597               | 4.3                           |
| Rhodosporidium toruloides | +++   | 1.486               | 4.1                           |
| Fusarium oxysporium | +++   | 1.422               | 3.0                           |
| Aspergillus clavatus | +++  | 1.585               | 4.8                           |
| Saccharomyces cerevisiae | +     | 1.274               | 2.6                           |
| Candida albicans  | ++        | 1.289               | 2.8                           |
| Microsporum gypseum | +      | 0.964               | 1.3                           |
| Trichophyton mentagrophytes | +    | 0.928               | 1.5                           |

+; little growth, ++; Moderate growth, +++; Heavy growth

Figure 1. Hydrocarbonoclastic potentials of some fungal isolates. G; Candida tropicalis, H; Rhodosporidium toruloides, I; Fusarium oxysporium, J; Aspergillus clavatus. Bars indicate the average of triplicate samples while the error bars show the standard deviation.
### Table 3. Degradation of PAHs by the isolates after 16 days incubation in used engine oil

| PAHs Component          | Percentage (%) depletion of PAHs in used engine oil | Candida tropicalis | Aspergillus clavatus | Consortium |
|------------------------|-----------------------------------------------------|--------------------|----------------------|------------|
| Phenanthrene           | 100                                                 | 100                | 100                  |
| Fluoranthene           | 96.76                                               | 100                | 100                  |
| Pyrene                 | 99.27                                               | 100                | 100                  |
| Benzo (K) fluoranthene | 100                                                 | 100                | 100                  |
| Benzo (a) pyrene       | 94.09                                               | 71.82              | 87.09                |
| Dibenzyl (a,h) anthracene | 100                                           | 90.28              | 96.38                |

### Table 4. Degradation of PAHs by the isolates after 16 days incubation in diesel oil

| PAHs Component          | Percentage (%) depletion of PAHs in diesel | Candida tropicalis | Aspergillus clavatus | Consortium |
|------------------------|------------------------------------------|--------------------|----------------------|------------|
| Acenaphthene           | 100                                     | 100                | 100                  |
| Acenaphthylene         | 100                                     | 100                | 100                  |
| Phenanthrene           | 100                                     | 100                | 100                  |
| Fluoranthene           | 97.88                                   | 95.12              | 89.94                |
| 1,2 Benzenanthracene  | 100                                     | 98.71              | 80.75                |
| Chrysene               | 100                                     | 100                | 92.74                |
| Benzo (K) fluoranthene | 100                                     | 100                | 100                  |
| Benzo (a) pyrene       | 99.96                                   | 78.0               | 75.02                |
| Anthracene             | 100                                     | 100                | 100                  |
| Naphthalene            | 100                                     | 100                | 100                  |
| Fluorene               | 100                                     | 78.07              | 100                  |

### Table 5. Degradation of PAHs by the isolates after 16 days incubation in petroleum

| PAHs Component          | Percentage (%) depletion of PAHs in petroleum | Candida tropicalis | Aspergillus clavatus | Consortium |
|------------------------|---------------------------------------------|--------------------|----------------------|------------|
| Anthracene             | 100                                         | 100                | 100                  |
| Fluoranthene           | 100                                         | 100                | 87.89                |
| Naphthalene            | 100                                         | 100                | 100                  |
| Benzo (K) fluoranthene | 100                                         | 100                | 100                  |
| Benzo (a) pyrene       | 80.22                                       | 79.76              | 99.88                |
| Fluorene               | 100                                         | 76.88              | 100                  |

### 4. Discussion

A total of 8 fungal isolate namely Candida tropicalis, Rhodosporidium toruloides, Fusarium oxysporum, Aspergillus clavatus, Saccharomyces cerevisiae, Candida albicans, Microsporum gypseum and Trichophyton mentagrophytes, were identified in this study based on their cultural and microscopic characteristics. Some of these organisms have earlier been reported as hydrocarbon bio-degraders [33,34]. Akpoveta et al. [35] reported the isolation of Trichoderma sp., Penicillium sp., Rhizopus sp., Fusarium sp., and Aspergillus sp. from crude oil polluted soil.

Among the 4 isolates that showed high promise for hydrocarbon bioremediation potentials (Table 2), Candida tropicalis and Aspergillus clavatus displayed the fastest onset and highest extent of biodegradation of used engine oil (Fig. 1). Thus they were selected for PAHs degradation studies. The high rate of hydrocarbon degradation by the two fungi could emanate from their massive growth and enzyme production responses during their growth phases. This could be supported by the reports of Bogan and Lamar [36], which showed that extracellular ligninolytic enzymes of white rot fungi are produced in response to their growth phases.

In this study, it was observed that the isolates utilized comparatively less amount of diesel than petroleum and used engine oil from the media. The high rate of degradation observed in petroleum and used engine oil compared to diesel may be attributed to the effect of compositional and structural complexity on biodegradability of petroleum derivatives. Octane fuel has the simplest atomic structure and has the least amount of double bonds as compared to diesel and kerosene, thus does not resist microbial attack [37,38].

The results of gas chromatographic analysis on PAHs degradation in used engine oil, diesel and petroleum (Tables 3-5) showed that the two isolates Candida tropicalis and Aspergillus clavatus as well as their consortium culture exhibited biodegradation efficiency above 70% after 16 days incubation also confirmed their high degradation potentials. The abilities of these organisms in oxidizing the polycyclic aromatic hydrocarbons (PAHs) can be attributed to the non-specific nature of their enzymes especially the peroxidases on degrading chemicals. The fact that both the pure and consortium culture of the isolates were able to degrade PAHs very effectively suggests that the degradation
of the aliphatic moieties could be easier and faster than the polycyclic aromatic moieties. The implication of these two organisms in hydrocarbon degradation from our results is similar to the findings of April et al. [33]. The inability of either the pure or consortium culture of the isolates to achieve a 100% depletion of Benzo(a)pyrene (BaP) in used engine oil, diesel and petrol in this study (Tables 3-5) could be attributed to the physical and chemical characteristics of the PAH. Numerous genera of microorganisms have been observed to oxidize PAHs [39]. While there is a great diversity of organisms capable of degrading the low molecular weight PAHs such as naphthalene, acenaphthene and phenanthrene, relatively few genera have been observed to degrade the high molecular weight PAHs, such as BaP [40]. Bishnoi et al. [41] reported that PAH adapted fungal strain Phanaerochaete chrysosporium, isolated from the soil of petroleum refinery, have the ability to degrade phenanthrene, anthracene, acenaphthene, fluoranthene and pyrene in sterilized as well as unsterilized soil in optimum conditions.

5. Conclusions

This study revealed that the isolates Candida tropicalis and Aspergillus clavatus as well as their consortium culture has promising potential in bioremediation of polynuclear aromatic hydrocarbon polluted soil. They could also be utilized in bioremediation of used engine oil, diesel and petroleum contaminated soil.

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