Novelty Journals

ISSN 2394-966X

International Journal of Novel Research in Life Sciences
Vol. 3, Issue 4, pp: (7-17), Month: July - August 2016, Available at: www.noveltyjournals.com

Biodegradation of N-Alkanes by Fungi Isolated from Waste Engine Oil Polluted Soil and Their Extracellular Enzyme Activities

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Abstract: **Fungi capable of effectively degrading and cleaning up alkanes was isolated from soil samples contaminated with waste engine oil (WEO) at auto-mechanic workshops (at Mgbuka-Nkpor), Nigeria. The ability of the potential isolates to utilize WEO, 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 n-alkanes component of WEO, diesel and petrol was assessed using Gas Chromatography. Enzymatic activity of the extracellular enzymes; catalase, lipase and peroxidase were also investigated. A total of 8 fungal isolates were obtained from soil samples contaminated with WEO. Of these isolates, 4 that showed high hydrocarbonoclastic potentials in the screen flask were confirmed as *Candida tropicalis*, *Rhodosporidium toruloides*, *Fusarium oxysporium* and *Aspergillus clavatus* using 18S r RNA gene sequence. *C. tropicalis* and *A. clavatus* exhibited the fastest onset and highest extent of biodegradation of WEO, diesel and petrol, and were therefore selected for further studies. High biodegradation efficiency (> 70%) was recorded in short-chain (C_8) and long-chain (C_{24} – C_{39}) alkanes in WEO, diesel and petroleum with both the pure and consortium culture of the isolates; *C. tropicalis* and *A. clavatus*, within 16 days of incubation at 28°C. However, there was a complete (100%) depletion of most of the medium-chain (C_{10} – C_{23}) alkanes in the hydrocarbon substrates with the pure and consortium culture of the isolates within 16 days of incubation at 28°C. Highest catalase and lipase production of 18.9±0.5UmL^{-1} and 11.66±1.04UmL^{-1} respectively was obtained with *A. clavatus* in the presence of petroleum oil. However, both organisms produced a high yield (> 30UmL^{-1}) of peroxidase enzyme in diesel and petrol, while a peroxidase production of 17.59±1.12UmL^{-1} and 15.83±1.17UmL^{-1} was obtained with *C. tropicalis* and *A. clavatus* respectively in the presence of WEO.**

Keywords: **biodegradation, bioremediation, catalase, diesel, extracellular enzymes, fungal isolates, lipase, n-alkanes, peroxidase, petrol, waste engine oil.**

1. INTRODUCTION

Waste oil is any petroleum or synthetic oil that has been used, and as a result of such use is contaminated by physical or chemical properties [1]. It contains hundreds or thousands of aliphatic, branched and aromatic hydrocarbons [2]; [3], most of which are toxic to living organisms [4]. Most motor oils are made from a heavy, thick petroleum hydrocarbon base stock derived from crude oil, with additives to improve certain properties. The bulk of typical motor oil consists of hydrocarbons with between 18 and 34 carbon atoms per molecule [5]. Indiscriminate disposal of used engine oil into gutters, water drains, open vacant plots and farms is a common practice in Nigeria especially by motor mechanics [6].

Alkanes are a major fraction (>50%) of the crude oil depending upon the oil source. Alkanes are saturated hydrocarbons and chemically very inert as apolar molecules [7]. They may be classified as linear (n-alkanes), cyclic (cyclo-alkanes) or branched (iso-alkanes) and found in three states: gaseous (C1–C4), liquid (C5–C16) and solid (>C17). Although highly

inflammable, alkanes are less reactive as organic compounds. They are highly essential for modern life, but their inertness poses serious ecological problems when released to the environment. However, microbes have developed effective strategies involving specific enzymes and metabolic pathways to use n-alkanes as a carbon source. Thus, microbes have the capability to degrade alkanes and convert them to easily metabolizable substrates.

A number of microbes including bacteria, fungi and yeasts have been reported to degrade alkanes using them as the source of carbon and energy [8]; [9]. Bacteria with alkane degradation ability have also versatile metabolism to use other compounds in addition to alkanes as source of carbon [10]; [11]. Use of bacteria in the degradation of alkane compounds has been extensively studied by Haryama *et al*. [11], who reported organisms such as *Arthrobacter* sp., *Acinetobacter* sp., *Candida* sp., *Pseudomonas* sp., *Rhodococcus* sp., *Streptomyces* sp., *Bacillus* sp., *Aspergillus* sp., *Arthrobacter* sp., etc., for the degradation of aliphatic compounds.

Whenever bioremediation is discussed, bacteria agents come into focus and fungi are much less studied [12]. It is now becoming apparent that fungi also play an important role in degrading organic materials in the ecosystem and that they have potential for remediating contaminated soils and water. Alexander [13] reported that the ability of fungi to transform a wide variety of hazardous chemicals has aroused interest in using them in bioremediation. Fungi are among nature's most powerful decomposers, secreting strong enzymes. The great potential of fungi in bioremediation is by virtue of their aggressive growth, great biomass production and extensive hyphae reach in the environment [14].

Susceptibility of a hydrocarbon to microbial degradation varies with type and size of the hydrocarbon molecule. Alkanes of intermediate chain length (C_{10}-C_{24}) are often degraded rapidly, while very long chain alkanes are increasingly resistant to microbial degradation [15]. Fungi isolated from oil spill environment can reduce oil pollution [16]; [17]. Nevertheless, interest on fungi receives a considerable attention for bioremediation of hydrocarbon contaminated sites associated fungi for enzyme secretion, to remove hydrocarbons from the environment.

The present study reports on the isolation of fungi from waste engine oil polluted soil and to access their n-alkane degradation potential and extrcellular enzyme activity

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 workshops that had a heavy spillage of waste engine oil (WEO) at Mgbuka-Nkpor ($6^{o}9$'N $6^{o}50$'E), Nigeria. The samples were pooled together, homogenously mixed to obtain a composite sample and placed into a sterile container. The hydrocarbon (waste engine oil) used in this work was subsequently collected direct from the engine of 911 Lorry (at Mgbuka-Nkpor) in a sterile container. Samples were transported in cold storage container to the laboratory for analysis. Other hydrocarbons (diesel and petrol) used in this work were purchased from Nigerian National Petroleum Corporation (NNPC) Mega Station, Awka.

2.2 Isolation of Fungi with Waste Engine Oil Utilizing Abilities

Waste engine oil utilizing fungi were isolated from soil samples obtained from auto-mechanic workshops (at Mgbuka-Nkpor, Nigeria) on Mineral Salt agar Medium of Zajic and Supplission, with composition as listed in Ekpenyong and Antai [18]. 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 waste engine oil as carbon and energy source supplied from the lid of the plates [19]; [20].

Each distinct colony on oil degrading enumeration plates were purified by repeated sub culturing onto fresh Sabouraud Dextrose Agar (SDA) (Merck, Germany) plates to obtain pure cultures of the isolates. The pure cultures were maintained on SDA slants and used for further studies.

2.3 Screening Test for WEO Biodegradation Potentials of the Fungal Isolates

The isolates were tested for waste engine oil utilization capabilities in mineral salt broth medium. 100ml of mineral salt broth supplemented with 1% (v/v) waste engine oil was measured into 250ml Erlenmeyer flasks. The whole preparation was autoclaved at 15psi (pounds/square inch) and $121^{o}C$ for 15 minutes and allowed to cool. A 24 hour pure culture of the isolates were aseptically inoculated into the 250ml Erlenmeyer flasks containing mineral salt medium in which 1% of the

waste engine oil had been introduced. Uninoculated control flasks were also set up. The flasks were incubated in an Orbital Shaker (VWR DS2 – 500 – 2 orbital shaker) at 120 revolutions per minute (rpm) and temperature of 28^{o}C for 16 days. During the period of incubation, the viable counts of each fungal isolate was determined (at 0, 4, 8, 12 and 16 days) by aseptically pipetting 1 ml sample from the Erlenmeyer flasks, serially diluted and plated onto the surface of freshly prepared Sabouraud Dextrose Agar (Merck, Germany) plates using the spread plate technique. Incubation followed immediately at 28^{o}C for 48 hours. Colonies formed were counted at the end of 48 hour incubation and expressed as colony forming unit per milliliter (Cfu/ml). The fungal biomass was also quantified at 540nm [21] (at 0, 4, 8, 12 and 16 days) using Spectrophotometer (WPA Lightwave S 2000 Diode Array Spectrophotometer).

2.4 Determination of WEO Biodegradation of the Potential Isolates

The rate and extent of biodegradation of waste engine oil by four potential isolates namely *Candida tropicalis*, *Rhodosporidium toruloides*, *Fusarium oxysporum*, and *Aspergillus clavatus* were assessed using the modified gravimetric method [22]; [23]. Degradation study flasks as well as controls were incubated in triplicate at 28^{o}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 waste engine oil at different concentrations. Mean results were obtained and expressed as percentage weight loss of engine oil. The whole process was repeated for diesel and petrol.

2.5 Determination of the n alkane 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) waste engine oil, and incubated at ambient temperature of 28^{o}C at 120 rpm for 16 days. Control flask without the organism was prepared accordingly. After 16 days the extent of n-alkane degradation using undegraded engine oil as the control was determined by Gas Chromatography. 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^{o}C, injector temperature, 220^{o}C, integrator chart speed, 2cm min^{-1}. Initial – final oven temperature was 70 – 280^{o}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.

2.6 Assay for Enzyme Activity

The test organisms were grown separately on Mineral Salt broth supplemented with three different oils namely waste engine oil, diesel and petrol as carbon sources and incubated in an Orbital Shaker for 7 days at 120 rpm and temperature of 28^{o}C. After 7 days incubation, the aliquots were centrifuged at 3000 rpm for 30 minutes at 4^{o}C and the supernatants (enzyme extract) were used for assay of extracellular enzymes such as catalase, lipase and peroxidase activities.

2.6.1 Determination of Catalase Activity

The activity of catalase (CAT) was determined according to Luck [24] by measuring the decomposition of hydrogen peroxide (H_2O_2), and the decline in absorbance at 240nm was followed every 60 seconds for 5minutes. The reaction mixture (3.0ml) contained 0.1ml of the enzyme extract in phosphate buffer (50mM phosphate buffer, pH 7.0) and 2.9ml of 30.0mM H_2O_2 in phosphate buffer. The reference reagent contained 0.1ml of phosphate buffer and 2.9ml of 30.0mM H_2O_2 in buffer. An extinction coefficient for H_2O_2 at 240nm of 40 $M^{-1}Cm^{-1}$ was used for the calculation. One unit of catalase was defined as the amount of enzyme that decomposes 1µmol of H_2O_2 per minute under standard assay conditions.

2.6.2 Determination of Lipase Activity

In the present investigation, one unit of lipase activity was defined as the amount of enzyme solution liberating 1µmole of p – nitrophenol per minute under standard assay conditions. The spectrophotometric method [25], using P – nitrophenylPalmitate (P^{NPP}) as substrate was applied for rapid and routine measurement of the lipase activity. Enzyme or

blank solution (480µl) was added to the reaction buffer (500µl) which has 50mM Tris-HCl and 30mM Triton x – 100. The content was incubated at 25°C for 5 minutes and 10mM P^{NPP} in 2 – propanol (200µl) was added to the enzyme buffer solution and shaken well. The progress of the reaction was followed by monitoring the change in absorbance at 400nm every 1 minute over a period of 5 minutes using Spectrophotometer (Genesys 10-S, USA). The molar extinction coefficient of p – nitrophenol (E = 16.900 M^{-1} Cm^{-1}) was estimated from the absorbance measured at 400nm of standard solution of P^{NP}.

2.6.3 Determination of Peroxidase (POD) Activity

The method proposed by Reddy *et al*. [26] was adopted for assaying the activity of peroxidase. This method is based on the principle that in the presence of the hydrogen donor, pyrogallol or dianisidine, peroxidase converts H_2O_2 to H_2O and O_2. The oxidation of pyrogallol or dianisidine to a coloured product called purpurogallin can be followed spectrophotometrically at 430nm. To 3.0ml of pyrogallol solution, 0.1ml of the enzyme extract was added and the spectrophotometer was adjusted to read zero at 430nm. To the test cuvette, 0.5ml of H_2O_2 was added and mixed. The change in absorbance was recorded every 60 seconds up to 5 minutes in a spectrophotometer (Genesys 10-S, USA). One unit of peroxidase was defined as the change in absorbance per minute at 430nm.

3. RESULTS

A total of eight hydrocarbon utilizing fungi were isolated from soil samples obtained from waste engine oil contaminated soil. The fungal genera identified were *Candida tropicalis*, *Rhodosporidium toruloides*, *Fusarium oxysporium*, *Aspergillus clavatus*, *Saccharomyces cerevisiae*, *Candida albicans*, *Microsporium gypseum* and *Trichophyton mentagrophytes*.

Candida tropicalis, *Rhodosporidium toruloides*, *Fusarium oxysporium* and *Aspergillus clavatus* showed higher counts during the screening test, which increased from 0.9 x 10^5 to 4.9 x 10^5, 0.8 x 10^5 to 4.3 x 10^5, 0.7 x 10^5 to 3.5 x 10^5 and 1.2 x 10^5 to 5.1 x 10^5 Cfu/ml, respectively from 0 to 12 days, and decreased to 4.3 x 10^5, 4.1 x 10^5, 3.0 x 10^5 and 4.8 x 10^5 Cfu/ml respectively, on the 16^{th} day. *Saccharomyces cerevisiae*, *Candida albicans*, *Microsporium gypseum* and *Trichophyton mentagrophytes* showed lower viable counts which increased from 0.5 x 10^5 to 3.0 x 10^5, 0.6 x 10^5 to 2.8 x 10^5, 0.3 x 10^5 to 1.5 x 10^5 and 0.4 x 10^5 to 2.0 x 10^5 Cfu/ml, respectively from 0 to 12 days, and decreased to 2.6 x 10^5, 2.7 x 10^5, 1.3 x 10^5 and 1.5 x 10^5 Cfu/ml respectively on the 16^{th} day.

However, measurement of the fungal biomass showed that *Candida tropicalis*, *Rhodosporidium toruloides*, *Fusarium oxysporium* and *Aspergillus clavatus* have higher turbidity with an optical density which increased from 0.088 to 1.617, 0.079 to 1.526, 0.075 to 1.470 and 0.085 to 1.605nm, respectively from 0 to 12 days and decreased to 1.597, 1.486, 1.422, and 1.585nm respectively on the 16^{th} day at 540nm. Moreover, *Saccharomyces cerevisiae*, *Candida albicans*, *Microsporium gypseum* and *Trichophyton mentagrophytes* showed lower turbidity which increased from 0.071 to 1.305, 0.064 to 1.313, 0.079 to 1.273 and 0.070 to 1.256nm, respectively from 0 to 12 days and decreased to 1.274, 1.289, 0.964 and 0.928nm respectively at 540nm. Based on these observations, *Candida tropicalis*, *Rhodosporidium toruloides*, *Fusarium oxysporium* and *Aspergillus clavatus* were selected for hydrocarbonoclastic potential studies. They were also confirmed using 18S rRNA gene sequences.

The hydrocarbonoclastic potentials of the selected isolates revealed that *Candida tropicalis* caused 86.2% weight loss of waste engine oil in 16 days. This was closely followed by the weight loss of 85.0% caused by *Aspergillus clavatus*. *Rhodosporidium toruloides* 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 80.5% and 82.1% respectively, in diesel oil, while a weight loss of 66.8% and 64.2% was observed with *Rhodosporidium toruloides* and *Fusarium oxysporium* 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 toruloides* recorded weight losses of 74.4% and 68.8% respectively, in petroleum oil (Fig. 1). Based on these observations, *Candida tropicalis* and *Aspergillus clavatus* were selected for further studies.

Novelty Journals

ISSN 2394-966X

International Journal of Novel Research in Life Sciences
Vol. 3, Issue 4, pp: (7-17), Month: July - August 2016, Available at: www.noveltyjournals.com

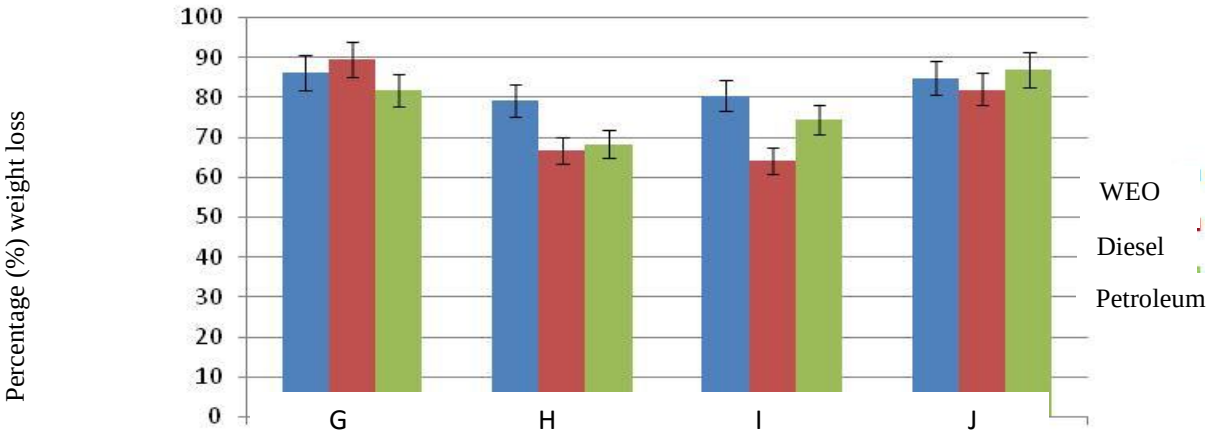

Fig.1: Hydrocarbonoclastic potentials of some fungal isolates. G; *Candida tropicalis*, H; *Rhodosporidium toruloids*, I; *Fusarium oxysporium*, J; *Aspergillus clavatus*. Bars indicate the average of triplicate samples while the error bars show the standard deviation.

The result of the Gas Chromatographic analysis for the removal of the n-alkane components of the hydrocarbons in waste engine oil by the two best potential strains (*Candida tropicalis* and *Aspergillus clavatus*, as well as their consortia) were presented in table 1. *Candida tropicalis* achieved 100% removal of the alkanes of chain length C_{15}, C_{16}, and C_{23}, while 76.58%, 99.77%, 98.26%, 91.26%, 85.61%, 94.08% 75.00%, 98.00%, 94.00% and 88.90% removal was recorded in C_8, C_{17}, C_{18}, C_{21}, C_{24}, C_{26}, C_{28}, C_{29}, C_{32} and C_{39} alkanes, respectively over a 16 day period. However, *Aspergillus clavatus* recorded 100% depletion of $C_{16} - C_{18}$, and C_{23} alkanes, while 87.65%, 97.99%, 96.31%, 76.36%, 97.74%, 74.04%, 98.76%, 90.46% and 85.08% removal was recorded in C_8, C_{15}, C_{21}, C_{24}, C_{26}, C_{28}, C_{29}, C_{32} and C_{39} alkanes, respectively. Moreover, the mixed culture (consortium) of the isolates (*Candida tropicalis* and *Aspergillus clavatus*) achieved 100% depletion of $C_{15} - C_{17}$, C_{21} and C_{23} alkanes, while 88.43%, 99.92%, 92.62%, 99.20%, 91.60%, 99.81%, 90.97% and 93.00% removal was recorded in C_8, C_{18}, C_{24}, C_{26}, C_{28}, C_{29}, C_{32} and C_{39} alkanes, respectively over a 16 day period (Table 1).

In diesel oil, *Candida tropicalis* achieved 100% depletion of C_{13}, C_{17}, C_{18}, and C_{23} alkanes, while 73.12%, 98.73%, 83.69%, 94.85%, 99.08%, 95.40%, 88.53%, 86.86% and 96.00% removal was recorded in alkanes of chain length C_8, C_{15}, C_{19}, C_{21}, C_{26}, C_{27}, C_{28}, C_{32} and C_{39} respectively (Table 2). *Aspergillus clavatus* recorded 100% depletion of $C_{13} - C_{17}$, $C_{19} - C_{23}$ alkanes, while 99.89%, 92.02%, 99.00%, 96.74%, 77.05%, 98.88% and 96.82% removal was observed in alkanes of chain length C_8, C_{18}, C_{26}, C_{27}, C_{28}, C_{32} and C_{39} respectively. Moreover, the mixed culture achieved 100% depletion of the alkanes of chain length $C_{15} - C_{18}$, and C_{21} while 89.77%, 98.01%, 86.31%, 90.65%, 99.56%, 98.42%, 88.97%, 79.36% and 93.37% removal was recorded in alkanes of chain length C_8, C_{13}, C_{19}, C_{23}, C_{26}, C_{27}, C_{28}, C_{32} and C_{39}, respectively over a 16 day period (Table 2).

The removal of the alkane component of the hydrocarbons in petrol by the isolates after 16 days incubation in petroleum oil revealed that *Candida tropicalis* achieved 100% removal of C_{17}, and C_{21} alkanes, while 99.75%, 99.99%, 99.97%, 87.95%, 82.51%, 98.74% and 90.89% removal was recorded in alkanes of chain length C_8, C_{15}, C_{18}, C_{24}, C_{28}, C_{31}, and C_{38} respectively (Table 3). *Aspergillus clavatus* achieved 100% depletion of C_{15}, C_{18}, and C_{21} alkanes, while 99.76%, 99.64%, 71.26%, 77.42%, 97.99% and 86.81% depletion was observed in C_8, C_{17}, C_{24}, C_{28} C_{31} and C_{38} alkanes, respectively. However, the consortium culture recorded 100% depletion of C_{15}, and C_{17} alkanes, while 99.70%, 99.54%, 98.77%, 99.99%, 99.00%, 98.45% and 98.32% removal was recorded in alkanes of chain length C_8, C_{18}, C_{21}, C_{24}, C_{28}, C_{31} and C_{38} respectively (Table 3).

Novelty Journals

ISSN 2394-966X

International Journal of Novel Research in Life Sciences
Vol. 3, Issue 4, pp: (7-17), Month: July - August 2016, Available at: www.noveltyjournals.com

Table 1. Degradation of the alkane components of the hydrocarbon after 16 days incubation in waste engine oil.

	Percentage (%) depletion of alkanes in waste engine oil		
Alkane components	*Candida tropicalis*	*Aspergillus clavatus*	Consortium culture
C_8; n – Octane	76.58	87.65	88.43
C_{15}; n – Pentadecane	100	97.99	100
C_{16}; n – Hexadecane	100	100	100
C_{17}; n – Heptadecane	99.77	100	100
C_{18}; n – Octadecane	98.26	100	99.92
C_{21}; n – Heneicosane	91.26	96.31	100
C_{23}; n – Tricosane	100	100	100
C_{24}; n – Tetracosane	85.61	76.36	92.62
C_{26}; n – Hexacosane	94.08	97.74	99.20
C_{28}; n – Octacosane	75.00	74.04	91.60
C_{29}; n – Nonacosane	98.00	98.76	99.81
C_{32}; n – Dotriacontane	94.00	90.46	90.97
C_{39}; n – Nonatriacontane	88.90	85.08	93.00

Table 2. Degradation of the alkane components of the hydrocarbon after 16 days incubation in diesel.

	Percentage (%) depletion of alkanes in diesel		
Alkane components	*Candida tropicalis*	*Aspergillus clavatus*	Consortium culture
C_8; n – Octane	73.12	99.89	89.77
C_{13}; n – Tridecane	100	100	98.01
C_{15}; n – Pentadecane	98.73	100	100
C_{17}; n – Heptadecane	100	100	100
C_{18}; n – Octadecane	100	92.02	100
C_{19}; n - Nonadecane	83.69	100	86.31
C_{21}; n – Heneicosane	94.85	100	100
C_{23}; n – Tricosane	100	100	90.65
C_{26}; n – Hexacosane	99.08	99.00	99.56
C_{27}; n – Heptacosane	95.40	96.74	98.42
C_{28}; n – Octacosane	88.53	77.05	88.97
C_{32}; n – Dotriacontane	86.86	98.88	79.36
C_{39}; n – Nonatriacontane	96.00	96.82	93.37

Table 3. Degradation of the alkane components of the hydrocarbon after 16 days incubation in petrol.

	Percentage (%) depletion of alkanes in petrol		
Alkane components	*andida tropicalis*	*Aspergillus clavatus*	Consortium culture
C_8; n – Octane	99.75	99.76	99.70
C_{15}; n – Pentadecane	99.99	100	100
C_{17}; n – Heptadecane	100	99.64	100
C_{18}; n – Octadecane	99.97	100	99.54
C_{21}; n – Heneicosane	100	100	98.77
C_{24}; n – Tetracosane	87.95	71.26	99.99
C_{28}; n – Octacosane	82.51	77.42	99.00
C_{31}; n – Hentriacontane	98.74	97.99	98.45
C_{38}; n – Octatriacontane	90.89	86.81	98.32

The result for the assay of catalase activity in *Candida tropicalis* and *Aspergillus clavatus* was presented in table 4. A low catalase production of 1.05±0.03, 0.89±0.02 and 2.50±0.04 Uml^{-1} was observed in *Candida tropicalis* in the presence of waste engine oil, diesel and petroleum respectively. In *Aspergillus clavatus*, the highest catalase production (18.9±0.54

Uml^{-1}) was observed in the presence of petroleum, while in the presence of waste engine oil, 9.68±1.21 Uml^{-1} catalase activity was observed. However, the least catalase production of 4.5±0.30 Uml^{-1} was observed in *Aspergillus clavatus* in the presence of diesel oil (Table 4).

For the assay of lipase activity, highest lipase production of 11.66±1.04 Uml^{-1} and 3.17±0.73 Uml^{-1} was observed in *Aspergillus clavatus* and *Candida tropicalis* respectively, in the presence of petroleum (Table 5). However, *Candida tropicalis* recorded lipase production of 2.96±0.53 Uml^{-1} and 1.18±0.30 Uml^{-1} in the presence of waste engine oil and diesel respectively. Similarly, *Aspergillus clavatus* recorded lipase production of 1.88±0.71 Uml^{-1} and 0.81±0.30 Uml^{-1} in the presence of waste engine oil and diesel respectively (Table 5).

The result for the assay of peroxidase activity was presented in table 6. Highest peroxidase production (37.35±0.57 Uml^{-1}) was observed in *Candida tropicalis* in the presence of diesel oil. This was followed by the peroxidase production of 36.81±1.02 Uml^{-1} and 36.27±1.45 Uml^{-1} observed in *Candida tropicalis* and *Aspergillus clavatus* respectively in the presence of petroleum oil. However, *Candida tropicalis* and *Aspergillus clavatus* respectively recorded peroxidase production of 17.59±1.12 Uml^{-1} and 15.83±1.17 Uml^{-1} in the presence of waste engine oil. Moreover, *Aspergillus clavatus* recorded peroxidase production of 30.86±0.87 Uml^{-1} in the presence of diesel oil (Table 6).

Table 4. Catalase activity (Uml^{-1}) of the isolates in waste engine oil, diesel and petrol

Isolates	Waste engine oil	Diesel	Petrol
Candida tropicalis	1.05±0.03	0.89±0.02	2.50±0.04
Aspergillusclavatus	9.68±1.21	4.5±0.30	18.9±0.54

Values are mean of three replicates ± Standard Deviation (SD)

Table 5. Lipase activity (Uml^{-1}) of the isolates in waste engine oil, diesel and petrol

Isolates	Used engine oil	Diesel	Petrol
Candida tropicalis	2.96±0.53	1.18±0.30	3.17±0.73
Aspergillusclavatus	1.88±0.71	0.81±0.30	11.66±1.04

Values are mean of three replicates ±SD

Table 6. Peroxidase activity (Uml^{-1}) of the isolates in waste engine oil, diesel and petrol

Isolates	Used engine oil	Diesel	Petrol
Candida tropicalis	17.59±1.12	37.35±0.57	36.81±1.02
Aspergillus clavatus	15.83±1.17	30.86±0.87	36.27±1.45

Values are mean of three replicates ±SD

4. DISCUSSION

A total of 8 fungal isolates were identified in this study. Of these, 4 isolates that showed high promise for hydrocarbon bioremediation potentials were confirmed as *Candida tropicalis*, *Rhodosporidium toruloides*, *Fusarium oxysporium* and *Aspergillus clavatus*, using 18S rRNA gene sequencing. Some of these organisms have earlier been reported as hydrocarbon bio-degraders [27] and [28]. Akpoveta *et al*. [29] reported the isolation of *Trichoderma* sp., *Penicillium* sp., *Rhizopus* sp., *Fusarium* sp., and *Aspergillus* sp. from crude oil polluted soil.

The viable count and optical density of the isolates during screening test indicates that the population of the organisms increased gradually from zero to 12 days during utilization of waste engine oil, and decreased slightly on the 16 day of incubation. The reduction in population of the hydrocarbon degraders could be due to the fact that the organisms have exhausted the available nutrient supplies present in the system. It could also be that mineralization of hydrocarbons could have possibly resulted in the production of toxic metabolites which on introduction into the system reduces the growth phase of the fungi. The findings of Amadi and Odu [30] who reported an initial gradual increase in the bacterial population following the application of petroleum hydrocarbon but a decline as the biodegradation progressed supports this explanation. Similarly, Akpoveta *et al*. [29] reports that hydrocarbon degrading fungi increased within the first seven

days from 2.16 x 10^4 cfu/g to 11.1 x 10^4 cfu/g and decreased progressively to 1.5 x 10^4 cfu/g within the next four weeks. A decrease in substrate will therefore result in a drop in the population of oil-degraders.

Among the 4 isolates that showed high promise for hydrocarbon bioremediation potentials, *Candida tropicalis* and *Aspergillus clavatus* displayed the fastest onset and highest extent of biodegradation of waste engine oil, diesel and petroleum (Fig. 1). 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 [31], 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 waste engine oil from the media. The high rate of degradation observed in petroleum and waste 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 – C = C – bonds as compared to diesel and kerosene, thus does not resist microbial attack [32]; [33].

The high biodegradation efficiency (> 70%) observed during gas chromatographic analysis for the removal of the n-alkanes (C_8 – C_{39}) in waste engine oil, diesel and petroleum (Tables 1-3), by the isolates: *Candida tropicalis*, and *Aspergillus clavatus* as well as their consortium culture showed that the cultural conditions were very appropriate for their growth and biodegradation. The incomplete (< 100%) removal of the short chain (C_8) and long-chain (> C_{23}) compared to the medium chain (C_{10} – C_{23}) n-alkane component observed in both waste engine oil, diesel and petrol (Tables 1-3) may be attributed to their high toxicity on the organisms used in this study. This result is in agreement with the findings of the National Academy of Sciences [34], who reported that petroleum components including the n-alkanes, the n-alkyl aromatics and the aromatic compounds in the C_{10} – C_{22} range are the least toxic and most biodegradable. The n-alkanes, alkyl aromatics and aromatic hydrocarbons in the C5 – C9 range are degradable at low concentrations, but are highly toxic and are removed primarily by volatilization. Shagufta [35] reported that short-chain alkanes are toxic to many microorganisms and are difficult to degrade, while intermediate chain length (C_{10} – C_{24}) are degraded most rapidly and very long chain alkanes becomes increasingly resistant to biodegradation. On a structural basis, the hydrocarbons in crude oil are classified as alkanes (normal or iso), cycloalkanes and aromatics. Alkenes, which are the unsaturated analogs of alkanes, are rare in crude oil but occur in many refined petroleum products as a consequence of cracking process [36]. The inherent biodegradability of these individual components is a reflection of their chemical structure, but is also strongly influenced by the physical state and toxicity of the compounds. As an example, while n-alkanes as a structural group are the most degradable petroleum hydrocarbons, the C_5 – C_{10} homologues have been shown to be inhibitory to the majority of hydrocarbon degraders. As solvents, these homologues tend to disrupt lipid membrane structures of microorganisms [36]. Similarly, alkanes in the C_{20} – C_{40} range, often referred to as "waxes", are hydrophobic solids at physiological temperatures. Apparently, it is this physical state that strongly influences their biodegradation [37].

Mukred *et al*. [38] reported that short-chain and medium-chain alkanes are generally easily degraded due to their lower hydrophobicity. Rahman *et al*. [39] reported that the maximum degradation was achieved of treatment n-alkanes in the range of C_8 – C_{11}, which were degraded completely followed by C_{12} – C_{21}, C_{22} – C_{31} and C_{32} – C_{40} with degradations percentage of 100, 83 – 98, 80 – 85 and 57 – 73% respectively using bacterial consortium. In another report, the biodegradation of n-alkanes C_{12} – C_{30} and of various aromatic compounds in crude oil using *Acinetobacter* sp. T4 culture showed that the n-alkanes were almost completely degraded, while the aromatic compounds were not [40]. In the culture of *Pseudomonas putida* PB4, neither the n-alkanes nor the aromatic compounds were degraded to any significant degree. Nikolopoulou *et al*. [41] reported that the capillary gas chromatography analysis of the degraded crude oil revealed that crude oil components of chain length C_{12} – C_{32} were extensively degraded by *Serratia marcescens* OCS-21 after 16 days of incubation, while *Acinetobacter calcoaceticus* COU-27 was able to degrade only C_{22} – C_{30} components of the crude oil after 16 days of incubation.

Enzymatic assay indicated a low yield of catalase and lipase enzymes in the hydrocarbon substrates by the isolates, except *Aspergillus clavatus* that had a moderate yield of catalase and lipase (18.9±0.54Uml^{-1} and 11.66±1.04Uml^{-1}) enzymes in petroleum oil (Table 4 and 5). However, *Candida tropicalis* and *Aspergillus clavatus* recorded moderate peroxidase activity (17.59±1.12Uml^{-1} and 15.83±1.17Uml^{-1}) in waste engine oil and high peroxidase activity (> 30Uml^{-1}) in diesel

and petroleum (Table 6). This wide disparity in enzyme activity may be attributed to the chemical nature of the hydrocarbon substrates. Chemical analyses have shown that crude oil is a complex mixture of polar and non polar compounds [42]. Carbon atoms of these polar and non polar constituents in oil serve as growth substrates and non growth substrates for microbial enzymes during degradation of hydrocarbon compounds. The low yield of the enzymes may also be attributed to the relative affinity of each of the substrate for the microbial enzymes. Measurement of catalase activity is a new approach for evaluating fungal ability to degrade hydrocarbons [43].

Since the same enzyme catalyze the initial degradation of both the growth and non growth substrates, competition for the enzyme can occur, reducing the rate of growth substrate degradation [44]. In addition to competitive inhibition, unproductive binding of a non growth substrate conceivably could cause uncompetitive inhibition or noncompetitive inhibition of growth substrate degradation as shown in the findings of Dixon and Webb [45], thus explaining the very low production of catalase and lipase obtained in this study. Previous studies showed highest lipase production ($108Uml^{-1}$) by *L. theobromae* and peroxidase enzyme production ($516Uml^{-1}$) by *Coprinus* sp. [25]; [46]. Kotic *et al*. [47] reported that enzymatic activity of microorganisms increased in the petroleum polluted soils. High activity of catalase and peroxidase was also reported in the soil microorganisms in petroleum-polluted soils [48]; [49].

5. CONCLUSION

The study showed that both the pure and consortium culture of the isolates; *Candida tropicalis* and *Aspergillus clavatus* have the potential for bioremediation of the alkane components in waste engine oil, diesel and petroleum. The isolates were able to produce extracellular enzymes; catalase, lipase and peroxidase, which indicates their potential to degrade hydrocarbons. Further research is needed to access the ability of the isolates to degrade polynuclear aromatic hydrocarbons (PAHs).

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