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REGULAR ARTICLE

Evaluating the diesel biodegradation potential of wild microfungi isolated from decaying wood in Nigeria

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ABSTRACT

We evaluated the potentials of pure wild microfungi isolated from decaying wood in utilizing diesel as source of carbon/energy and monitoring of their physiological responses via OD and pH gradient fluxes. The fungi diversity was obtained by conventional enrichment culture methods. Pure cultures of tentative fungal species namely: SC1, SC2, SC3, YI and Y2 were tested for their ability to utilize diesel oil as carbon and energy source. From the cultural, morphological and biochemical characterization and comparison with respect to the standard reference of fungi, the wild microfungi tentatively named as SC1, SC2, SC3, YI and Y2 were seemingly the members of Penicillum, Aspergillus, Mucor, Candida and Cryptococcus species. The diesel dependent growth of the pure microfungi was assessed by monitoring the fluxes in the pH and Optical density OD of the minimal media slurry for 288 hours. There were fluctuations in OD as well as pH values for the different microfungi. The mean pH data 5.90 ± 0.01-7.01± 0.01 and the OD 0.349± 0.001 -1.232 ± 0.002 were obtained. The ability of our fungal isolates to tolerate diesel and grow in it, suggests that the isolates can be employed as bioremediation agent.

1. Introduction

Petroleum like all fossil fuels consists primarily of complex mixtures of hydrocarbons. Diesel is a component of fossil fuel that contains aliphatic hydrocarbons with the carbon chain length between $C_{12}H_{30}$ – $C_{18}H_{38}$ (Endo and Schmidt, 2006; Cyplik et al., 2011). It is produced from fractional distillation of crude oil or petroleum. Over the last ten decades, there is huge demand for petroleum and its associated products. This has made petroleum spills inevitable consequences during fossil fuel production and refining. Despite the high cost and challenges emanating during its production, fossil fuels will remain a major source of energy in the next several decades because a reliable alternative has not yet been found.

The problems emanating from the spillage of diesel during production and transportation remain a top-

ical issue (Ojumu et al., 2004; Bola et al., 2006; Vieira, 2009; Sarang et al., 2013). When spillage of diesel occurs, especially in large concentrations, the hydrocarbon molecules that make-up the oil is highly toxic to many organisms, including humans (Alexander, 1994; Atlas and Philip, 2005). The accumulation of these pollutants in animal and plant tissues often causes death or mutations (Alvarez and Vogel, 1991). Diesel spills can inhibit photosynthesis, invariably threatens life and productivity. In some plants, they may die or survive but with possible sub-lethal responses.

Microbial degradation appears to be the most environmentally friendly method for the removal of oil spills especially for non-volatile components when other methods such as surfactant washing and incineration could generate more toxic compounds in the environment (Bola et al., 2006). According to Medina-Bellver and co-workers microbial degradation is an evolving method for the removal and degradation of many environmental pollutants (Medina-Bellver et al., 2005). Mycoremediation, an aspect of microbial degradation focuses on the degradation of organic compounds by fungi. Fungi can achieve the degradation of organic compounds by the production of extra-cellular and intracellular enzymes that catalyze various reactions in the degradation (Paszczynski and Crawford, 2000). Fungal degrading potentials differ among fungi. In white rot microfungi, they are capable of using their mycelia to bioremediate hydrocarbon products through their high production of organic acids, reduction of pH, chelators, oxidative enzymes and extracellular enzymes. According to Stamets, fungal enzymes and their peculiar physiological abilities enable it to utilize oil products rapidly and thus, serve as effective bioremediators (Stamets, 1999). For effective remediation of spilled oil sites, mixed cultures of bacteria and fungi have been proposed (Yuan et al., 2000). Conversely, single cultures of fungi have been found to be superior to mixed cultures (Okerentugba and Ezeronye, 2003). In the reports of Batelle, fungi have been found to better degraders of petroleum than bacteria (Batelle, 2000). It has also been documented that mycorrhizal fungi and wood rotting fungi are capable of degrading petroleum or oil (Adekunle and Adebambo, 2007; Bola et al., 2006; Boguslawska-Was and Dabrowski, 2001). Some of the fungi include: *Aspergillus, Trichodema, Penicillium., Rhizopus* and *Mucor* species. Some of the yeast species documented include: *Candida, Rhodotorula mucilaginosa, Geotrichum* and *Trichosporon mucoides*.

In spite of the progressive advances made in the use of mycoremediation, the majority of researches have focused on bacterial or mixed culture characterization. Thus, fungal biodegradation of diesel oil is still an open area of research. With the incessant occurrences of diesel spill in Nigeria and in the bid to provide solutions using a perceived waste (sawdust) product from wood, it would be desirous to nurture and develop bioremediation process using fungi from wood. This is because it has been noted that fungi has the capacity to incorporate rapidly into the soil matrix and improve the soil structure (Mollea et al., 2005). Fungi also have the advantage to grow in environments with low nutrient, low humidity and acidic pH (Thenmozhi et al., 2013; Sarang et al., 2013). In this study, pure wild microfungi diversity isolated from decaying wood were investigated for their potential in utilizing diesel as source of carbon and energy via monitoring of their physiological responses.

2. Material and Methods

2.1 Chemicals and Reagents

The $(NH_4)_2SO_4$, $MgSO_4.7H_2O$, $Ca(NO_3)_2.4H_2O$, K_2HPO_4, KH_2PO_4, Na_2HPO_4 of analytical grades were obtained from Merck, Germany. Potato Dextrose agar and Agar-agar were obtained from Biomark Laboratories, India. Petroleum diesel fuel (AGO), produced according to EN 590:2004, were purchased from a gas station in Canaanland, Ota,Ogun State Nigeria.

2.2 Stock solutions and Media

All the enrichment and degradation experiments were performed using chloride free minimal salts (MS) medium as described by Nwachukwu, (2000). The medium consists of 0.53g KH_2PO_4, 0.2g K_2HPO_4, 0.1g $MgSO_4.7H_2O$, 4.0g NH_4SO_4, 2.0g Na_2HPO_4 and 4.8g of agar-agar dissolved in 1000 mL of distilled water. The slurry MS medium was supplemented with the diesel oil.

2.3 Enrichment of Fungal Isolates

Decaying wood sample was selected from a construction site in Covenant University. The thickness of the wood was between 6-12 mm and occurred milky- brown in appearance. The wood had signs of fungal colonies but had dried off due to low or no moisture. The decaying wood was broken into smaller bits and exposed to moisture for about 8

days to enhance regeneration of the fungal diversity. At the end of the 8 days of incubation period, 5% (w/v) of the decaying wood sample bits were washed in distilled water.

The fungi diversity was obtained by conventional enrichment culture methods. For this, the wash distilled water containing the chipped particles of the wood was mixed with 0.005 ppm freshly prepared glucose in a 250 mL conical flask. The solution was stirred using a sterile glass rod and stoppered with sterile cotton wool. The solution was incubated at 25 $\pm$ 2°C for 5 days. Two milliliters aliquots of the solution were spread over freshly prepared sterile potato dextrose agar (PDA). The plates were incubated at 25 $\pm$ 2°C for 5 days.

2.4 Isolation, Purification and Characterization of Hydrocarbonoclastic Fungi Species

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 inoculated potato dextrose agar plates after incubation for 5 days at room temperature. These colonies were subcultured individually into different prepared PDA plates that were bored at the centre. The inocula were then transferred into minimal salt medium (Ms Medium) slurry supplemented with diesel (4% v/v) as carbon source. The enrichment slurry was prepared as described by Nwachukwu, (2000). The slurry media composition (per liter of distilled water) was 0.53g of KH_2PO_4, 0.2g of K_2HPO_4, 0.1g of $MgSO_4.7H_2O$, 4.0g of NH_4SO_4, 2.0g of Na_2HPO_4 and 4.8g of agar- agar.

Pure cultures from the diesel oil–enriched media were isolated by plating out 2.0 ml of the enriched cultures onto prepared PDA plates , sprayed with diesel oil on the surface. This was incubated at room temperature (25 $\pm$ 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). Cultural, biochemical and microscopic tests such as: germ tube test, urease activity, sugar fermentation test and lactophenol cotton blue stain was performed on the isolates.

2.5 Adaptation of Hydrocarbonoclastic Fungi in Minimal Salt Slurry

Pure fungal isolates were adapted again on freshly prepared sterile mineral salt slurry (30ml) in balch tubes. The tubes were stoppered with sterile cotton wool to enable aeration. The samples were incubated at room temperature for 5 days. The different fungal isolates were harvested by centrifugation at 40 x 100 rpm for 30 min. The different 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^{o}C for use during the growth / degradation studies (Verdin et al., 2004).

2.6 Growth on Diesel as Carbon and Energy Sources

Pure cultures of tentative fungal species namely: SC1, SC2, SC3, YI and Y2 were tested for their ability to utilize diesel oil as carbon and energy source. About 1000 µL 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, 500µL of diesel 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. The experiment was set up in triplicates. Abiotic controls were set up with the biotic controls (MS slurry medium and diesel 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 1, 72, 144, 216 and 288 hours intervals. Optical density was measured at 600 nm using Genesys 10 UVS Spectrophotometer.

2.7 Statistical analysis

Statistical tests (mean and standard deviation) were performed using the Graph pad prism 4.0 software programme.

3. Results and Discussion

The fungal isolates SC_1, SC_2, SC_3, Y_1 and Y_2 had different morphological and biochemical behaviour in the sugar test and urease activity as shown in (Table1). The morphology and biochemical characterization suggested the organisms SC_1, SC_2, SC_3, Y_1 and Y_2 similar to the members of the genus *Penicillum, Aspergillus, Mucor, Candida* and *Cryptococcus* species respectively.

	Isolates				
Tests	**SC_1**	**SC_2**	**SC_3**	**Y_1**	**Y_2**
Lactophenol stain	+	+	+	+	-
Colony morphology	Conidiophores with septate hyphae	Conidiospores are formed on a brush-like conidia head	Single sporangiophores with globular sporangium, spores are oval, Collumella present, non-septate hyphae and absence of rhizoids	Pasty and smooth colonies Blastoconidia produced singly and some in clusters	Mucoid in appearance Yellow after 48hours, pigmentation changes to pinkish orange Ovoid to elongated, single cells
Physical characteristics	Fluffy colonies with elevated mycelia that appeared black in coloration were observed. Reverse colour appeared pale yellow	Velvety texture, leaf-green in colouration. Reverse colour is creamish	Cottony colonies produced	Raised Cream to whitish	Slightly raised colonies
Urease activity				+	+
Glucose				†	‡
Sucrose				†	‡
Galactose				†	‡
Lactose				‡	‡
Germ tube				--	-

Table 1: Morphological and biochemical characterization of the pure fungal isolates capable of degrading spent oil.
Most probable organism— *Penicillium, Aspergillus, Mucor, Candida, Cryptococcus*
‡ = acid produced only, † = acid and gas produced, + = positive, - = negative

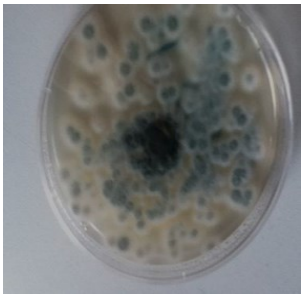

Figure 1: *Penicillum* specie had fluffy colonies with elevated mycelia that appeared black in colour were observed. Reverse colour appeared pale yellow.

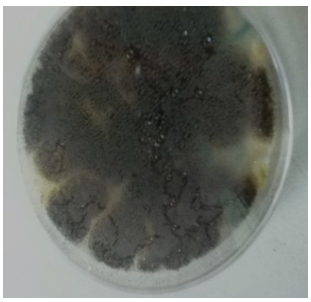

Figure 2: *Aspergillus* specie had velvety texture with leaf-green colouration. Reverse colour is creamy in appearance.

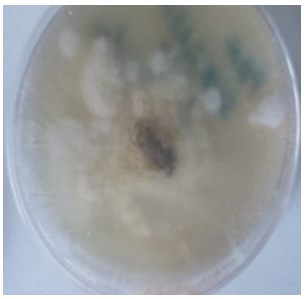

Figure 3: *Mucor* specie had cottony colonies when observed macroscopically.

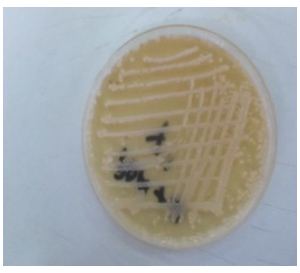

Figure 4: *Candida* specie had raised colony that are cream to white in appearance.

Fig.5. *Cryptococcus* specie had slightly raised colonies that were milky in appear-

In this study, increase in the turbidity measured by the optical density fluxes and the decrease in pH, of at least one order of magnitude when compared to the values of abiotic and biotic controls may be regarded as degradation/utilization of the diesel as carbon and energy sources by the fungal species. The initial and final values obtained for the abiotic and biotic controls had no significant difference with the values obtained at the initial stage for the test samples. In addition, the light colored diesel oil resulted in low optical density by the control and the test samples at the inception of this investigation. Conversely, the measure of degradation or utilization of diesel was indicated by turbidity in color which was equivalent to increase in optical density. Consequently, organisms that gave the highest optical density were adopted as the good degrader of the diesel oil in the slurry medium. Also, most hydrocarbon degradation often result in the production of acidic products that lower the pH of the medium, thus in this study, decreases in pH may therefore be regarded that degradation had occurred.

The (Figs. 6-10), demonstrate the mean changes in the pH and optical density of diesel- 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.

One of the major challenges facing environmental scientists worldwide is the remediation of sites contaminated with hydrocarbons, especially diesel oil. Most scientists have adopted the use of fungi mycelia mats that produce extra cellular enzymes and acids that can break down recalcitrant molecules, thus dismantling the long chains of hydrogen and carbon.

It is interesting to note that laboratory studies provides greater control and are essential in elucidating the basis to distinguish between biotic and abiotic processes, and possibly determine the optimized conditions for the biodegradation studies. In this study, the hydrocarbonoclastic fungal species isolated from the decaying wood were capable of growth in diesel. Okerentugba and Ezeronye, (2003) demonstrated aquatic *Penicillium, Aspergillus* and *Rhizopus* species capable of degrading hydrocarbons especially when single cultures were used. Furthermore, Batelle, (2000) showed that fungi were improved degraders much better than bacteria. In the present study, the utilization of diesel by our fungal isolates differed from one another. It showed a mean pH of 5.9 ± 0.02 - 7.01± 0.02 and the OD 0.349± 0.002 - 1.232 ± 0.002 for the 288 h incubation period. As shown in (Fig. 6), *Aspergillus* sp. exhibited a decreasing pH of 7.00± 0.02 – 5.89± 0.02 and OD of 0.733 ± 0.001- 1.232± 0.001. Initially, within the first three days of incubation, *Penicillium* sp. exhibited a decreasing pH of 7.20± 0.01 - 5.55± 0.02 and OD of 0.697± 0.001- 0.464± 0.001 (Fig.7). The strain identified as *Mucor* sp. showed a decreasing pH of 6.88 ± 0.01-5.90± 0.02 and increasing OD of 0.711± 0.001- 1.114± 0.001 (Fig.8). In (Fig. 9), the yeast species *Candida* sp. showed a pH of 6.91± 0.02 - 5.75± 0.02 and OD 0.656± 0.001- 0.369± 0.001. The *Cryptococcus* sp. exhibited a decreasing pH values of 6.78± 0.01- 5.55± 0.02 and the OD 0.740± 0.001- 0.496± 0.002 at the same incubation periods (Fig.10). These differences in the obtained readings were probably due to the difference in growth rates of each fungus. Furthermore, the ability of each fungi species to attain a maximum growth peak and decline after some days, can be linked directly to nutrient adaptation; and possibly release of toxic aromatic hydrocarbons from the diesel into the medium. This result is further corroborated by Chinedu et al., (2008) that experienced disparity among the activity of wild type *Aspergillus* niger ANL301 when exposed to different carbon sources. Similarly, Odjegba and Sadiq, (2002) reported of reduced growth behaviour of *P.tuberregium* due to presence of toxic aromatic hydrocarbons. In (Fig.10), *Cryptococcus* specie showed a decline in pH and OD. This may possibly be due to the aromatic components in the diesel that had potential to inhibit the growth of the organism. This was also reported by Li et al., (2008) of *Cladosporium* specie that had low degradative ability in aliphatic and aromatic hydrocarbons in diesel.

Prior to the slurry medium experiments, the various fungi species isolated grew better on solid medium than the slurry. The microfungi may have adapted better to a solid surface than a semi-solid state. Thus the extra cellular enzymes and acids required for breaking down the recalcitrant hydrocarbon molecules were not diluted out hence their ability to perform well on the solid surface. Generally, it has been documented that different microorganisms exhibit different abilities to degrade hydrocarbons. Some can suitably degrade aliphatic alkanes, whereas some possess strong ability to degrade both aliphatic alkanes and aromatic hydrocarbons (Odjegba and Sadiq, 2002). *Aspergillus*

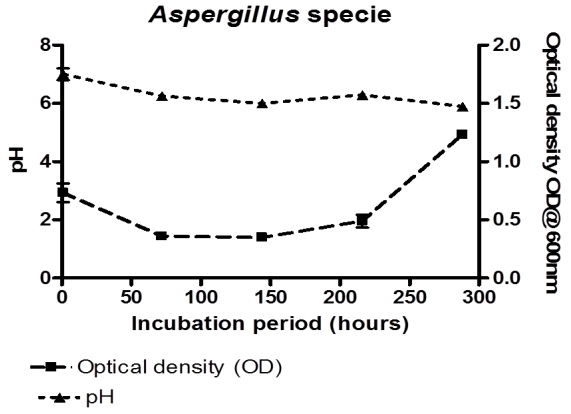

Figure 6: Diesel-dependent growth (OD) and pH fluxes of *Aspergillus* specie after 288 h-incubation. Data represent the mean of triplicate tubes for initial time represented as shown and the final time (288h). The error bars were due to differential responses of the cells in the triplicate tubes. The x-axis value range was chosen as such to allow for even spread of the pH and OD dynamics.

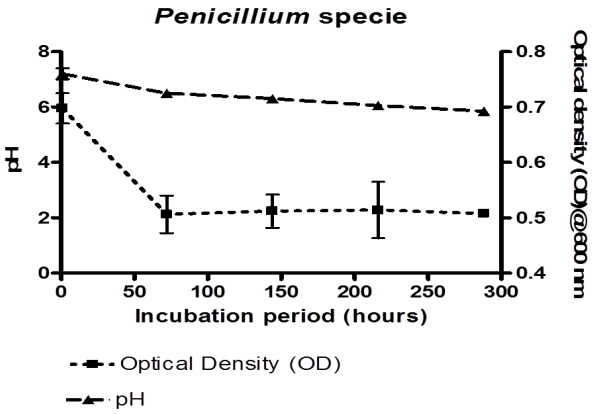

Figure 7: Diesel-dependent growth (OD) and pH fluxes of *Penicillium* specie after 288 h-incubation. Data represent the mean of triplicate tubes for initial time represented as shown and the final time (288h). The error bars were due to differential responses of the cells in the triplicate tubes. The x-axis value range was chosen as such to allow for even spread of the pH and OD dynamics.

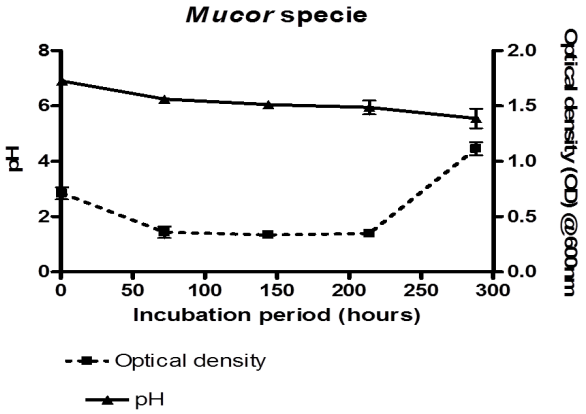

Figure 8: Diesel-dependent growth (OD) and pH fluxes of *Mucor* specie after 288 h-incubation. Data represent the mean of triplicate tubes for initial time represented as shown and the final time (288h). The error bars were due to differential responses of the cells in the triplicate tubes. The x-axis value range was chosen as such to allow for even spread of the pH and OD dynamics.

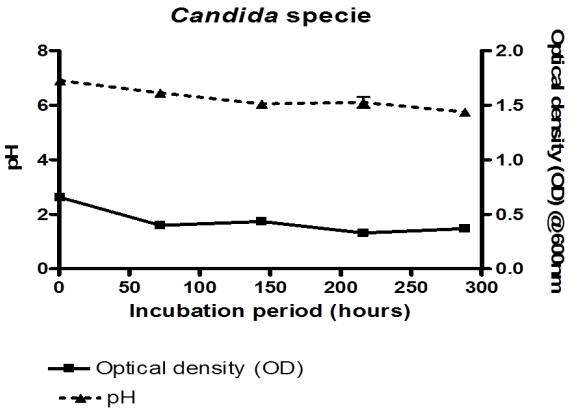

Figure 9: Diesel-dependent growth (OD) and pH fluxes of *Candida* specie after 288 h-incubation. Data represent the mean of triplicate tubes for initial time represented as shown and the final time (288h). The error bars were due to differential responses of the cells in the triplicate tubes. The x-axis value range was chosen as such to allow for even spread of the pH and OD dynamics.

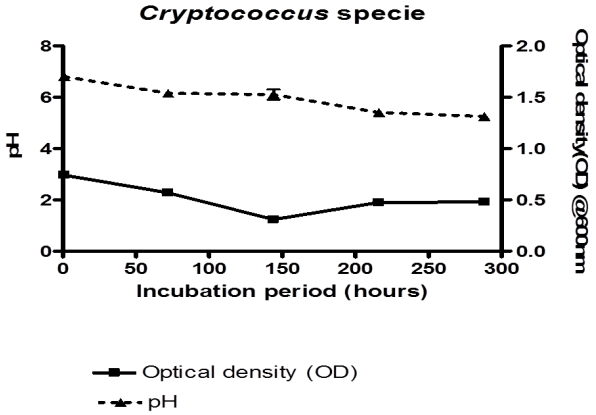

Figure 10: Diesel-dependent growth (OD) and pH fluxes of *Cryptococcus* specie after 288 h-incubation. Data represent the mean of triplicate tubes for initial time represented as shown and the final time (288h). The error bars were due to differential responses of the cells in the triplicate tubes. The x-axis value range was chosen as such to allow for even spread of the pH and OD dynamics.

niger and *A. fumigatus* have been documented to metabolize terpenes and PAHs. Potin and co-workers documented of *Penicillium* and *Aspergillus* species as having high ability to remove hydrocarbons (Li et al., 2008). In (Fig.1) It was evident that *Aspergillus* sp. recorded a gradual increase in turbidity that later became logarithmic towards the last 24 h. 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). The growth of our fungal isolates on diesel was significant. It has shown that our isolates might have the potentials to fuse relatively unspecific enzymes concerned in cellulose and lignin decay towards degradation of high molecular weight, complex and recalcitrant aromatic components in diesel.

In conclusion, the results of this study have shown that *Penicillum, Aspergillus, Mucor*, *Candida* and *Cryptococcus* species might have the potential of utilizing diesel 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 might have the potentials to access energy from the hydrocarbons. Also, the decrease in pH may be due to acidic products of the degradation of diesel. Conversely, it was observable that some of the fungi that were screened on solid surface grew better than when exposed to a slurry media. Future research work will be focused on intrinsic biodegradation of diesel using fungal species from decaying sawdust.

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