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Comparative Assessment of Crude Oil Degradation by *Monocillium* sp. and *Aspergillus niger*

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ABSTRACT: Fungi dwelling in soils contaminated by petroleum products can survive on these hydrocarbons due to the highly effective extracellular enzymes. Species belonging to the genus, *Aspergillus* are known to be efficient degraders of various classes of hydrocarbons as well as other organic contaminants. In this study, the biodegradation of crude oil by *Aspergillus niger* and *Monocillium* sp. were compared using laboratory microcosms. The moulds were isolated from a site receiving effluent from a petroleum refinery. They were identified using their macroscopic and microscopic characteristics and subsequently screened for their ability to utilize hydrocarbons for their metabolic requirements. Following the biodegradation studies, *Aspergillus niger* and *Monocillium* sp. recorded an increase in hydrocarbon utilizing fungal counts of 8.5 x 10^7 spores/ml and 6.1 x 10^7 spores/ml and crude oil weight loss of 80 % and 70 %. Both fungi were tested singly and in a consortium for their ability to degrade crude oil, it was observed that *Monocillium* sp. and *A.niger* performed better when tested individually (94.2 %; 92.8 %) than in consortium (76.3 %). This may suggest that their combined metabolism may have created some antagonistic effect on the degradation process as opposed to their enzymatic capabilities which appeared to be more favourable to the process. The biodegradation experiment analysis showed that contact time plays a significant role in biodegradation of crude oil (p<0.05), and *Monocillium* sp. and *Aspergillus niger* are excellent crude oil degraders and can be used in the bioremediation of petroleum-contaminated soil and water.

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The increase in the use of fossil fuels over the past several decades has led to an increase in petroleum-related pollution and contamination of the natural environment (McDonald, 2001; Nwachi *et al*., 2013). One of the major challenges in the last century is oil spillages into the environment leading to contamination of soil and water bodies, these spillages have been rising with the corresponding increase in petroleum production (Onifade *et al.,* 2007; Olukunle and Oyegoke, 2016; Barnes *et al.,* 2017). Accumulation of pollutants in plant tissues and animals caused by hydrocarbon contamination is detrimental and may cause death and mutation (Nilanjana and Preethy, 2011). Therefore, there is a need to remediate contaminated sites to protect human health and the ecosystem (McDonald, 2001). Physical and chemical treatment methods such as the use of absorbents (clay and straws), dispersants, the use of booms and skimmers, oil sinking agents, and oil coagulation have been investigated in recent times in order to effectively clean up polluted sites (Wilde, 2017), however, these treatment processes are expensive, and also leave byproducts which are either incinerated or buried which may lead to air pollution and ground water pollution as the case may be (Doeffer, 1992; Abdel Rahman, 2011; Nilanjana and Preethy, 2011). The biological treatment method utilizes microorganisms in treatment of oil-contaminated sites and it is preferred to physical and chemical treatments due to its reliability and capability to a high removal efficiency and low cost (Al-Hawash *et al.,* 2018). The challenge in oil spill remediation is to have the effective cleanup of oil spills with treatment processes that are sustainable, eco-friendly, non-toxic, biodegradable, and very cost-effective. This can be achieved with "oil-degrading microbes". These microbes degrade the oil in just days leaving the water clean and safe for marine life. Crude oil is a black, thick, and viscous liquid found in the earth. It is a complex mixture of hydrocarbon and non-hydrocarbon compounds which contains some metals and non-metals that are toxic to humans. (Orjiude, 2018; Chikwe and Ogbole, 2019). They are organic compounds made up of hydrogen and carbon atoms. The components of crude oil are mostly biodegradable. Biodegradation refers to the use of

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microbes to reduce complex organic pollutants to smaller chemical compounds (Joutey *et al.,* 2013). These organisms utilize organic pollutants as their sole carbon source and are able to break down these compounds due to the type of enzymes the produce, these enzymes oxidize a wide range of hydrocarbons (Raji, 2016).

Microorganisms such as bacteria, algae, yeast, and some fungi have been reported to be hydrocarbon degraders (Nilanjana and Preety, 2011; Joutey *et al.,* 2013; Al-Hawash *et al.,* 2018). Bacteria are known as the most dynamic agents in hydrocarbon degradation and they work as principal degraders in a contaminated environment. Some bacteria are known to utilize hydrocarbons as their sole carbon source (Nilanjana and Preethy, 2011). Among the most studied bacteria in this process are *Pseudomonas*, *Bacillus, Alcaligenes*, S*phingomonas*, *Rhodococcus*, and Mycobacterium, these degrade aliphatic and aromatic hydrocarbons, using them as the only source of carbon and energy. Fungi have been reported to be good hydrocarbon degraders due to the nature of extracellular enzymes they produce, this enzyme makes it easy for the fungi to assimilate complex carbohydrates thereby degrading a wide range of pollutants (Nilanjana and Preethy, 2011). Fungi can expand by means of their hyphae and are less sensitive to environmental conditions such as pH, temperature, nutrients, and aeration that affect its growth, unlike bacteria whose growth is dependent on environmental factors (Xenia and Refugio, 2016). Some fungi used as hydrocarbon degraders are, *Alternaria*, *Aspergillus, Candida, Cephalosporium, Cladosporium, Fusarium, Geotrichum, Gliocladium, Mucor, Paecilomyces, Penicillium, Pleurotus, Polyporus, Rhizopus,* and *Rhodoto* (Nilanjana and Preethy, 2011; Joutey *et al.,* 2013).

Fungi constitute more of the soil biomass than bacteria, depending on the soil depth and nutrient conditions, and are an important component of the soil microbiota. The role of fungi in the soil is extremely complex and is fundamental to the soil ecosystem. Indigenous microorganisms have played a significant role in the biodegradation of crude oil due to their adaptation to environments that require treatments (Al-Hawash *et al.,* 2018). Many studies have been conducted on isolation and characterization of hydrocarbon degraders from oil spill sites (Onifade *et al.,* 2007; Olabisi *et al.,* 2009; Abdel Rahman, 2011; Nilanjana and Preethy, 2011; Isaac, 2018; Orjiude, 2018), this study, therefore, aims at isolating indigenous fungi species capable of degrading crude oil and compare its biodegradation abilities singly and in a consortium.

MATERIALS AND METHODS

Sample Collection and Isolation: Soil samples contaminated with crude oil were collected randomly from five different points from the effluent point of the Kaduna Refining and Petrochemical Company (KRPC), depths of 0-15cm. The samples were collected using a sterile spatula and placed in clean Ziploc bags, they were then stored in an icebox to preserve the samples. In the laboratory, stones and debris were removed using a 2mm sieve (Prenafeta-Boldu *et al.,* 2001). Crude oil was collected from the oil movement Department of the Kaduna Refining and Petrochemical Company (KRPC) in a clean plastic bottle and transported to the Department of Microbiology, Ahmadu Bello University, Zaria.

Fungi were isolated from the hydrocarbon contaminated soil using the enrichment technique in a mineral salt medium with the following composition (1L of sterile distilled water): Na_2HPO_4 (0.2 g), K_2SO_4 (0.017 g), NH_4NO_3 (0.4 g), KH_2PO_4 (0.053 g), $MgSO_4.7H_2O$ (0.05 g) as described by Nwachukwu (2000). The salts were dissolved in 100 ml of distilled water and sterilized by autoclaving at 121°C for 15 mins at 15 psi (Fatuyi *et al*., 2012). In this method, 5g soil samples were suspended in 100 ml of already prepared sterile mineral salt medium and 0.025 g of chloramphenicol and supplemented with 1 % crude oil as the sole carbon source. The flask was incubated for 7 days at room temperature on a rotary shaker at 130 rpm (Prenafeta-Boldu *et al.,* 2001). After shaking, a ten-fold dilution was carried out and 1 ml of each dilution was poured into duplicate Potato Dextrose Agar (PDA) plates for the isolation of crude oil utilizing fungi. The plates were incubated at 28 ^{0}C for 7 days (Abdel Rahman, 2011).

Identification of Fungal Isolates: The microscopy technique was used to identify fungal isolates. The fungal isolates were characterized using cultural characteristics like mycelial type, aerial colour, and shape, and kind of spores, presence of foot cell, conidiophores as well as the microscopic and macroscopic characteristics of spore. Identification of fungal isolates was achieved by comparing their characteristics with those of known taxa as described by Larone (2002).

Biodegradation Studies: Biodegradation studies were carried out using a slightly modified method as described by Mittal and Singh (2009). The individual and mixed culture of the fungi 1 % (vol/vol) was added to the different conical flasks. The fungal consortium was formulated by mixing equal proportions of the pure culture of *Monocillium sp. and A.niger.* The liquid medium was inoculated with 1% of the fungal

suspension. The flasks were set up in duplicates and the control flasks contained only the crude oil and uninoculated MSM. All flasks were incubated at ambient temperature on a mechanical shaker (SHA-C, China) at 130 rpm (Prenafeta-Boldu *et al.,* 2001). The setup was monitored for 15 days, after 3 days time interval, the contents of the flasks were assessed for the amount of crude oil degraded by gravimetric analysis (AbdelRahman, 2011). The absorbance reading of each isolate and control was taken and recorded. Weight loss of crude oil was calculated after determining the amount of crude oil from a prepared standard using known amounts of crude oil in Equation 1 (AbdelRahman, 2011; Barnes *et al.,* 2017).

$$\% W_{CO} = \frac{C_{Cl} - C_F}{C_C} \times 100 \qquad 1$$

Where W_{CO} = weight loss of crude oil ; CC = control concentration; CF = final concentration

Extraction of Crude Oil from Medium: The extraction of crude oil was conducted according to the methods reported by Al-Jawahri (2014) with a slight modification. Extraction of crude oil was carried out by taking 5 mls of culture broth and mixing it with 5 mls of toluene (1:1 v/v) and placing it in a centrifuge to shake for 10 mins, the residual oil was monitored gravimetrically by toluene cold extraction and measuring the optical denstiy at 600nM using a spectrophotometer (Nwiyi and Olutubo, 2014).

Statistical Analysis: Analysis of Variance (ANOVA) was carried for this study on all treatments using Microsoft Excel (version 2016) package to determine the significant difference of crude oil degradation.

RESULTS AND DISCUSSION

Identification of Hydrocarbon Utilizing Fungi: A total of nine (9) fungi capable of growing on crude oil as the sole carbon source were isolated. The macroscopic and microscopic characteristics of the fungal isolates revealed that they are all moulds. They were identified as; *Madurella grisea, Monocillium* sp., *Aspergillus niger, Trichophyton megnini, Trichophyton tansurans, Aspergillus flavus, Fusarium* sp., *Actinomadura* sp., and *Chrysosporium* sp. All isolates were able to utilize crude oil as their sole carbon source and this could be as a result of their various enzymatic capabilities (Al-Dossary *et al.,* 2019), and have been reported to be hydrocarbon degraders (Ijah et al., 2013; Al-jawahri, 2014).

*Ability of the Selected Isolates in Degradation of Crude Oil***:** The isolate *Monocillium sp.* showed the best degradative capability with 84.8 % degradation from day 3 to 94.2 % at day 15, followed by *Aspergillus niger* with a degradative ability of 78.3 % at day 3 to 92.8 % at day 15. Crude oil degradation was monitored by both isolates singly and in consortium and showed a steady increase in the percentage degradation pattern from day 3 to day 15. The fungal consortium showed the least degradative ability with a degradative ability of 68.5 % to 76.3 % from day 3 to day 15 respectively. Figure 1 shows the percentage degradation of crude oil.

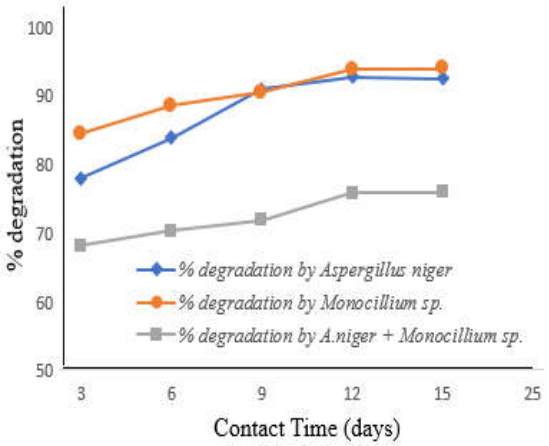

Fig 1. Percentage degradation of Crude Oil by *A.niger* and *Monocillium* sp.

From Figure 1, percentage degradation of *Monocillium* sp., *A.niger*, and the consortium in decreasing order. This shows that the degradation of crude oil was highest by the individual isolates than when they were used in combination. The percentage degradation of crude oil (Figure 1) shows that *Monocillium* sp. had the highest value at the beginning of the experiment at >80 % and it maintained the highest value to the end of the experiment; while the consortium markedly lower values than the fungi used individually. This means that as the fungi are in their log phase of growth they were actively producing enzymes that enable them to break down the hydrocarbons in crude oil. However, it will appear that the enzymes produced by *Monocillium* sp. and *A. niger* are more effective when the isolates are present as a single culture than in consortium. This might be due to the fact that competition for the same active site might slow down the degradative process or the metabolites resulting from the degradation are affecting the ability of the enzymes to perform effectively. This study does not agree with the study conducted by Adebusoye *et al*. (2007) who demonstrated that a consortium of microorganisms is required to complete biodegradation of oil pollutants because the necessary enzymes needed for biodegradation cannot be found in a single organism because of differences in volatility, solubility and

susceptibility of hydrocarbons. Also, according to Silva *et al.* (2015), the microbial consortium provides a greater spectrum of enzyme activity since it belongs to microorganisms of different taxa. A study conducted by Fatuyi *et al.,* (2012) and Al-Jahwahri (2014) demonstrated that single fungi isolates had a higher percentage of petroleum hydrocarbon degradation than consortium used which is in line with the work done in this study. Fungi have been reported to play an important role in the biodegradation of crude oil due to the extracellular enzymes they produce which helps in breaking down organic matter or recalcitrant hydrocarbon molecules into simpler nutrition of the fungi (Fatuyi *et al.,* 2012; Vanishree *et al.,* 2013; Olukunle and Oyegoke, 2016). This makes fungi very useful in cleaning up oil spills. Odu (1975) and Ijah (1998) stated that crude oil-degrading organisms are dominant in the Nigerian environment. Fungi isolated from crude oil-contaminated environments are believed to be more efficient than their counterparts from uncontaminated environments (Ijah, 1998, Fatuyi *et al.,* 2012). Microorganisms dwelling in soil likely contaminated with petroleum hydrocarbons have adapted to these compounds for their metabolic needs. The two isolates used in this study, *Monocillium* sp. and *A.niger* were the most efficient hydrocarbon degraders hence, their use in the final biodegradation experiments. Filamentous fungi such as *Aspergillus niger* and *Penicillium* sp. have been reported to grow on hydrocarbons and are capable of breaking down and utilizing hydrocarbons (April *et al.,* 2000; Mittal and Singh, 2009; Majekodunmi, and Adongbede, 2016). Davies and Westlake (1979) reported that although fungi are capable of adapting in a hydrocarbon-contaminated environment, their abilities to degrade a specific hydrocarbon as their sole carbon source differs, the chemical composition of crude oil may also be determining factor in the type of fungi that grows on it.

Hydrocarbon Utilizing Fungal Counts of Fungi during Biodegradation Experiment: Utilization of crude oil as the sole carbon source was observed in both *Monocillium sp.* and *Aspergillus niger* as this was evident in the increase in spore count from 1 x 10^6 spores/ml to 6.2 x 10^7 spores/ml after 9 days by *Aspergillus niger*, 1 x 10^6 spores/ml to 8.3 x 10^7 spores/ml after 9 days by *Monocillium* sp., and also from 1x 10^6 spores/ml to 8.1 x 10^7 spores/ml after 9 days by the consortium as shown in Figure 2. The highest growth of the isolates was observed on the 9^{th} day for all the isolates used in biodegradation studies. From the figure above, *Monocillium* sp. had the highest increase in spore count, followed by the consortium and *Aspergillus niger* showed the least increase in spore count. An increase in the optical density during biodegradation studies indicates the fungal growth which may be due to the utilization of crude oil as the sole carbon source by the isolates used in the study (Vanishree *et al.,* 2013).

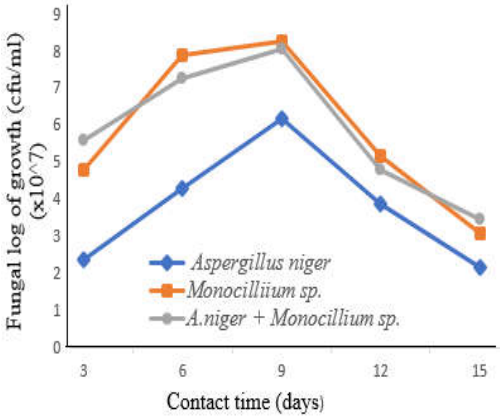

Fig 2. Utilization of Crude Oil by *A.niger* and *Monocillum* sp. using Hydrocarbon Utilizing Fungal (HUF) Count

The hydrocarbon utilizing fungal (HUF) count monitored during the experiment (Figure 2) is indicative of a steady increase in the number of fungal spores and a subsequent drop in the three treatments, by the end of the incubation period. The eventual drop in the number of spores could be as a result of depletion of the only source of carbon in the medium, which is the crude oil (Majekodunmi and Adongbede, 2016). On day 3 of the experiment, the most number of spores recorded for the three treatments in decreasing order are as follows: the consortium, *Monocillium* sp. and *Aspergillus niger*. And the same trend was observed at the end of the experiment, however, by day 6, *Monocillium* sp. had higher counts than the consortium and sustained high numbers until day 15 when the counts dropped slightly lower than the consortium. This implies that the high percentage degradation recorded by *Monocillium* sp., was actively proceeding between day 6 and day 12 as noticed in the growth curve in Figure 2. Even though *A. niger* consistently showed lower counts all through the experiment, it can be seen to record higher degradation percentage than the consortium (Figure 1), perhaps it could be that its enzymatic capabilities were very efficient even when the fungus is present in relatively low numbers. The fungus was also observed to have a comparatively steady rate of growth during the log phase unlike *Monocillium* sp., and the consortium. *A. niger* has been isolated from petroleum-contaminated environments and shown to be an efficient hydrocarbon degrader in many studies (Machido *et al*., 2005; Al-Jawhari, 2014; Chikere and Azubuike, 2014; Usman *et al*., 2019); and the genus, *Aspergillus* has been reported to have superior spore-forming

abilities compared to other filamentous fungi (Abdullahi, 2007). Amongst all isolates used in this study, *Monocillium* sp. degraded crude oil best and this may be due to differences in the range of activity of the degradative enzymes and pathways used by the organisms. This study revealed that crude oil degradation by the isolates both individually and in consortium increased with an increase in the length of incubation (Figure 1). Fatuyi *et al*. (2012) and Agarry and Jimoda (2013) all reported that the degradative ability of petroleum hydrocarbons increased with an increase in incubation time. This suggests that the rate of hydrocarbon degradation is directly proportional to incubation time. The highest percentage degradation of crude oil was observed with *Monocillium* sp. (94.2 %) followed by *A.niger* (93 %) and the least percentage was observed with the consortium (76.3 %) after 15 days of incubation. Statistical analysis of percentage degradation of crude oil and mean of HUF counts were carried out using analysis of variance (ANOVA), the results obtained showed that the *P* values for both percentage degradation of crude oil and HUF counts were highly significant having obtained a probability value less than 0.05 ($P<0.05$). This may be as a result of the differences in capabilities of the degradative enzymes and pathways of degradation used by the different organisms (Olukunle and Oyegoke, 2016), and may also indicate that *Monocillium* sp. had higher production of extracellular enzymes and organic acids than the other organisms which enables the organism to utilize hydrocarbon faster thus the differences in significance. Sakineh *et al.,* (2012) also reported significant differences in the degradation of hydrocarbons by the different organisms used in the study.

Conclusion: Degradation of crude oil was more efficient when *Monocillium* sp. and *Aspergillus niger* were used individually than when they were used as a consortium. *Monocillium* sp. recorded the highest hydrocarbon utilizing fungal counts during the biodegradation experiment. Statistical analysis of means of the HUF counts by ANOVA showed that they are statistically significant. The result also shows that all isolated fungi can be used in hydrocarbon degradation.

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