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The effectiveness of Single and mixed Culture of Fungi Isolated from Oil-Polluted Soil in crude oil Degradation

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Abstract

This study aimed to explore the potentials of indigenous soil Fungi with the best degrading ability for effective and perpetual remediation. Soil sample was collected from a polluted site in Gbokoda, Warri, Delta state. *Aspergillus niger*, *Aspergillus flavus*, *Penicillium chrysogenum* and *Rhizopus stolonifer* were isolated using standard microbiological procedures. The degrading ability of each isolates was examined for forty days at $28^0C \pm 2^0C$ in an enriched medium containing 2% crude oil. From the degradation test results, the total mean dry weight of the mycelia increases as the residual oil and pH value decreased; proven mixed culture of the four fungal isolates having better degradative results, followed by *A. niger*. From this study, the pollution by crude oil should be avoided as much as possible. However, when it occurs, mixed culture of the four fungal isolates is recommended to remediate the spilled area. However, *Aspergillus niger* can as well be singly used.

Key Words: Fungi, Spilled area, Indigenous, Crude oil and Degrader

1. Introduction

The problem of oil contamination with petroleum and petroleum-based hydrocarbons has become compounded in recent years by increasing sabotage and vandalization of pipelines by restive oil communities particularly in the Niger Delta area of the country. The serious environmental and health defects of the pollutant; creates an increasing attention for developing and implementing innovative technology for its clean up (Li Q. *et al*., 2020).

Various techniques including mechanical and chemical methods have been employed for the bioremediation and degradation of hydrocarbons pollutants from the environments; however, some of these methods are generally expensive and may have detrimental effects on the environment. Hence, bioremediation is the alternative solution to hydrocarbon pollutants. Among microorganisms used in bioremediation technology, fungi are efficient, reliable, cost-effective, and environmentally friendly thus, can be used to clean-up and detoxify hydrocarbons contaminants from the environment: soil, water, and sediments (Tomer *et al*., (2021). Bioremediation using fungi ensures the complete degradation and mineralization of petroleum hydrocarbon contaminants into carbon dioxide, water, inorganic compounds, and cell biomass without causing any harm to the environments (Tasiu *et al*. 2022 and Rudakiya *et al* 2019).

The mechanisms of fungal biodegradation of petroleum hydrocarbon depend mainly on the kind, nature, and quantity of the hydrocarbon mixtures present. The activated hydrocarbon compounds are then converted to intermediate metabolites which are finally converted to excreted derivatives. Similarly, the mechanisms of fungal bioremediation of petroleum hydrocarbon under aerobic conditions were reviewed recently (Abdel-shafy and Mansour 2019).

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In recent times, an increasing number of microbiological researches have been devoted to bioremediation of oil-contaminated sites using various microbial species especially those indigenous to the contaminated environments. An ability to isolate high numbers of certain oil degrading microorganisms from an environment is commonly taken as evidence that those microorganisms are the active degraders of that environment (Grazyna *et al*., 2001).

This study aimed to highlight the isolated Fungi that could be more effective as a single and mixed culture in the remediation of crude oil spilled site.

2. Materials and Methods

2.1 Sampling site and Sample collection

The sampling site is an area that covered about 200 x 300m^2 located at Gbokoda, Warri North Local Government Area, Delta State (within 5% 50^0 North, 5% 70^0 East). All glasswares and culture media for sampling and analyses were sterilized and microbiological analyses were done based on the method of Fawole and Oso, 2007. The soil samples were taken with sterile tools at the depths of 0 -20cm following the method of Obayori *et al*. 2012. Bonny light crude oil was obtained from National Petroleum Development Centre (NPDC) in Warri, Delta State in a sterilized reagent bottle.

2.2 Isolation and Purification of oil-degrading fungi

Oil-degrading fungi were isolated from the soil samples by using enriched culture technique. One gram of the soil sample inoculated into 99ml of sterile mineral salt medium (2.0g Na_2HPO_4, 0.17g K_2SO_4, 4.0g NH_4NO_3, 0.53g KH_2PO_4, 0.10g $MgSO_4.7H_2O$ and 1000ml of distilled water supplemented with 2% crude oil as the sole carbon and energy source and incubated at room temperature (28±2°C) for 5 days (Obayori *et al*., 2012). Fungal isolates obtained from the resultant culture were plated on potato dextrose agar (Goddey and Dami, 2013). Pure culture of the fungal isolates obtained, characterized and identified based on the microscopic, macroscopic features; their morphology and cultural characteristics are then compared with known fungal species in the literature (Boddireddy and Singara, 2011; Shehu and Muhammad, 2011).

2.3 Fungal Biodegradation Tests

All the oil-degrading fungal isolates stored as stock cultures were used as test organisms for biodegradation tests. The enrichment procedure as described by Li Q. *et al* (2020) was used to determine the oil-degrading ability of the single and mixed culture isolates. Two hundred millimeter of the broth medium containing 2.0g of Na_2HPO_4, 0.17g of K_2SO_4, 4.0g of NH_4NO_3, 0.53g of KH_2PO_4, 0.10g of $MgSO_4.7H_2O$ dissolved in 1000ml of distilled water was dispensed into six flasks. Each of the flasks was supplemented with 2% crude oil as the sole carbon and energy source while their pH was adjusted to 5.6 with dilute HNO_3. The mineralized salt medium in all the flasks were then sterilized by autoclaving at 121^{0}C for 15mins (Panda *et al*., 2013).

The broth in four of the flasks was inoculated with each of the isolates containing 5 x 10^4 spore suspension/ml while the fifth contained all the four fungal isolates and the sixth, inoculum free; served as the control. The set-ups were incubated at ambient temperature (28±2 ºC) on shaker for continuous aeration at 3000rpm and monitored for 40 days. During incubation, samples were withdrawn from each of the flasks on day zero and at 5 day intervals for analysis. The degrading potentials of the fungal isolates were determined by changes in the pH of the culture medium, residual crude oil and mycelia dry weight of each fungus over a period of time.

2.4 Dry weight measurement of fungal mycelium during degradation

The mineral salt broth obtained from determination of residual crude oil was mixed with equal volume of n-hexane and thoroughly shaken. The mixture was again separated using separating funnel. The uppermost layer containing hexane and some crude oil were discarded while the aqueous part contained the hyphae. The aqueous part was filtered through a pre-weighed filter and dried at 80^{0}C for 1 hour. The difference in weight represents the weight of the mycelium (Sebiotimo *et al*., 2010).

2.5 pH measurement during degradation

The pH value of each of the culture medium was measured with a pH meter (Clida) at specified time interval starting from day zero till the 40th day.

2.6 Extraction of the residual crude oil during fungal biodegradation

Oil-weight loss due to fungal degradation was assessed gravimetrically after extraction with n-hexane dichloromethane (1:1). Values obtained were expressed as the difference between the amount of oil in broth on day zero and specified time interval (Li Q. *et al*., 2020). About 5mls of n-hexane was added to the equal volume of the broth; shaken vigorously for 2 minutes and was then allowed to settle for 5 minutes. The organic layer constitutes the residual crude oil and the hexane while the aqueous is the mineral salt broth. The two layers were separated using separating funnel. Extraction was carried out twice to ensure complete recovery of oil. The organic layer was collected in 50-ml pre-weighed Pyrex beaker (W_1). This was placed in an oven and the extractant allowed evaporating at 50ºC. The beaker with the residual oil was allowed to cool in a desiccator and weighed. This was repeated until a constant weight was obtained (W_2). The residual oil (W_3) was obtained as the difference in the weight (Lathal and Kalaivani, 2012). Evidence of degradation was then taken as the continuous decrease in the extracted residual crude oil.

Residual oil (W_3) = $W_2 - W_1$; Where W_1 = weight of Pyrex beaker;

W_2 = weight of Pyrex beaker + residual oil

2.7 Statistical Analysis

All the data collected were analyzed with a one-way Analysis of Variance (ANOVA) using, Statistical Package for Social Sciences (SPSS), version 19.0 (Goddey and Dami, 2013).

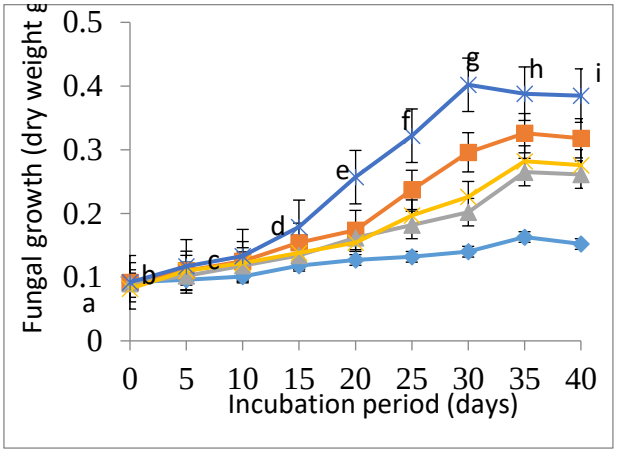

Fig.1: Growth of oil-degrading fungal isolates in the mineral salt medium containing 2% crude oil. Data are means of three replicates ± SEM.

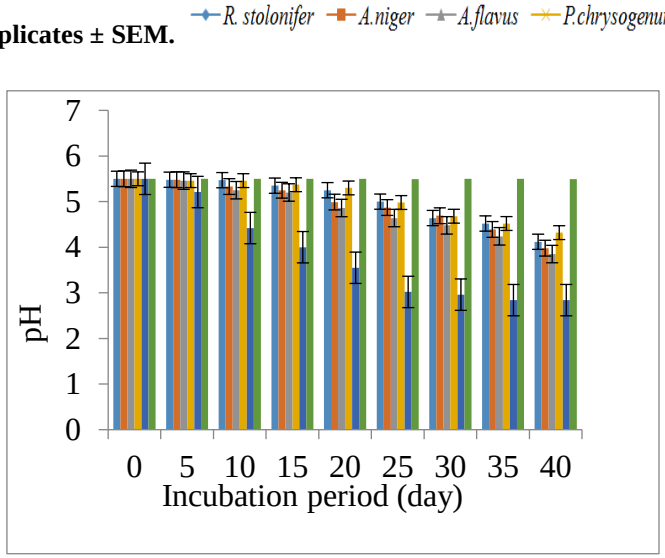

Fig 2: Changes in pH of the fungal culture medium during crude oil degradation. Data are means of three replicates ± SEM.

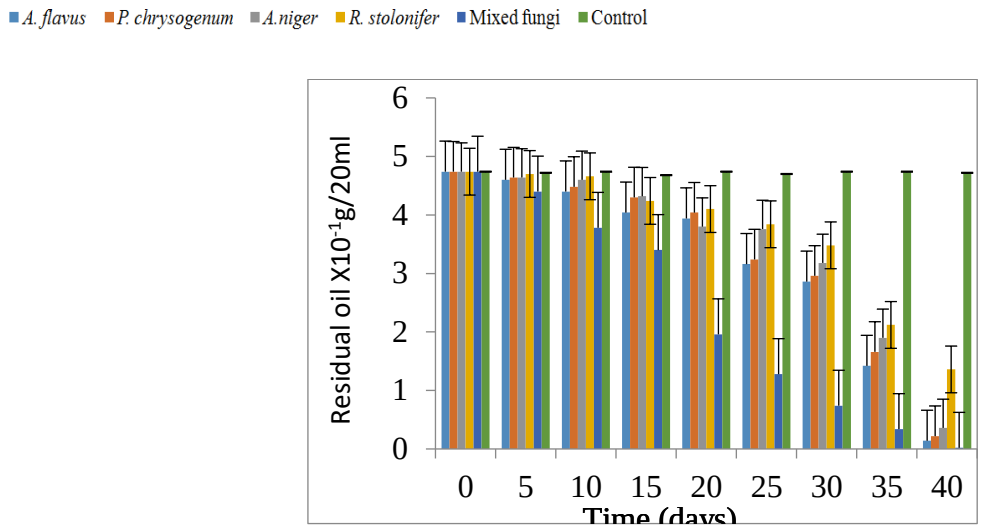

Fig.3: Residual crude oil in the fungal culture medium incorporated with 2% crude oil (0.477g/20ml) during degradation. Data are means of three replicates ± SEM.

3. Results and Discussion

Based on the findings in this study on screening test; the fungi isolates: *Aspergillus niger*, *A. flavus*, *Penicillium chrysogenum* and *Rhizopus stolonifer* identified from the oil polluted soils could utilize crude oil as energy and carbon source to grow. Some of the isolates are similar to those identified in a previous study that was conducted to assay for degrading activity of fungi on crude oil, kerosene, diesel and petrol using classical selective enrichment method. Ujowundu *et al*., (2011) isolated *Aspergillus* sp., *Rhizopus* sp. and *Fusarium* sp. from diesel contaminated soil. Olukunle *et al*. (2011) isolated *A. niger* in all the soil samples of oil-polluted sites in Ondo States. However, in the findings of Akpoveta *et al*. (2011) five hydrocarbon degrading fungi were isolated and identified (*Trichodema* sp., *Penicillium* sp., *Rhizopus* sp., *Fusarium* sp. and *Aspergillus* sp.) from crude oil polluted soil while Goddey and Dami (2013) also isolated *Penicillium chrysogenum*, *Aspergillus* sp. and *Candida* sp. from spent engine oil and Mohammed *et al*., (2023) isolated *Aspergillus* sp. as the most common genus among the 10 isolates recorded in his work on crude oil polluted soil.

Thus, the degrading activities of these indigenous isolated fungi on crude oil in this report could probably be employed for the bioremediation of the polluted sites. Similar reports were published by Okerentugba and Ezenronye (2003); Onifade and Abubakar (2007) and Mohammed *et al*. (2023). These aforementioned researchers reported high numbers of certain oil-degrading microorganisms from an oil-polluted environment and their implication as the active degraders of that oil.

However, the fungal isolates in this work showed different abilities in their breaking down and utilization of the crude oil. The resultant utilization of crude oil is proved by the progressive and significant increased dry weights, concomitant production of acid through the progressive decreased in pH value, continuous reduction in the residual crude oil contents in the culture medium during degradation. This pattern of result is similar to the findings of Oboh *et al*. (2006) and Sebiotimo *et al*. (2010). The presence of crude oil in each of the Mineral salt medium might be responsible for the significant increase in dry weight of the hydrocarbon degrading fungi. This may be because crude oil served as a good source of carbon and energy for cell growth thus enhance biodegradation rate. The mineralization of crude oil could also have possibly resulted into the production of toxic metabolites which might lead to the reduction in the proliferation of the mycelia cells by the organisms. This work revealed *Aspergillus niger;* the best degrader among the predominant degrading fungal isolates, showing the fastest growth on crude oil; thus recorded to have the highest potential to degrade crude oil as revealed by the degrading activity measured through the dry weight of the mycelium, residual oil content of the fungal culture medium with concomitant decreased in pH during degradation. This degree of degradative

potentiality is followed by *Penicillium chrysogenum, Aspergillus flavus* and *Rhizopus stolonifer* respectively in a decreasing order. This is in the support of the findings of Sebiotimo *et al*. (2010) who reported *A. niger* and *A. terreus* to be the best degrader that showed fastest growth on lubricating oil. The ability of the fungal isolates which include *A. fumigatus, A. oryzae, A. wenti, A. flavus, A. niger, Trichoderma sp., P. notatum, R. stolonifer*, and *Rhodotorula* sp. to utilize hydrocarbon substrate as carbon and energy source had also been reported by Oboh *et al*. (2006). Nevertheless, *Penicillium chrysogenum* was reported to be the best engine oil degrader in which about 43.33% of engine oil was consumed within 30 days (Goddey and Dami, 2013). Mohammed *et al*., (2023) recorded ten fungal species that could decompose crude oil; however, *Paecilomyces variotti* had the highest ability to decompose both medium and light crude oil while *Aspergilus flavus* had the lowest growth.

The slight reduction of the mycelia dry weight observed in most fungi from day thirty till day forty could possibly be attributed to decayed dead cells in soluble forms that might have been filtered off during the drying weight process (Mohammed *et al*., 2023). Therefore, a microbial consortium containing a number of microorganisms which produce the degradative enzymes for different parts of the decomposition pathway is considered to be well suitable for the degradation of aromatic hydrocarbons. Microorganisms not directly involved in the degradation process also probably play a role by producing micronutrients or surface-active agents for the solubilization of aromatic hydrocarbons (Ozaki *et al*., 2007). However, considering the degradation efficiency of single cultures of fungi on the crude oil; it is evident in this study that they both exhibit a definite degrading activity pattern respectively. The trend observed in this work is in support of the work of Okoro (2008) who observed that fungi have a great capacity and enzymatic capacity to degrade polycyclic aromatic hydrocarbons (PAHs). A mixed culture can therefore, be regarded as a better inoculum for oil spill clean-up.

4. Conclusion

It is interesting to know from this result that these oil degrading fungi could be isolated from soils in abundance; being able to grow under environmentally stressed conditions such as low pH and poor nutrient status, thus enhance the feasibility of their exploitation in oil spills clean-up. Furthermore, the use of fungi in bioremediation is of advantage; fungal mycelia having ability to penetrate oil, thus increasing the surface area available for biodegradation. And finally, the findings in this work showed that single culture of degrading fungi; *A. niger* may be useful in bioremediation of sites extremely polluted with crude oil hydrocarbons. However, based on the higher degradative potential; the mixed culture of *Aspergillus niger*, *Penicillium chrysogenum*, *Aspergillus flavus* and *Rhizopus stolonifer* could be employed for effective and efficient bioremediation of the spilled site.

Recommendation

It is recommended from this study that pollution of crude oil should be avoided as much as possible. However, when it occurs, *Aspergillus niger* is recommended for biodegradation among the fungal isolates, if single culture is to be considered. However, mixed cultures of the fungal isolates proved to produce better degradative results, when compared with the single cultures. The mixed cultures of *Aspergillus niger*, *Penicillium chrysogenum, Rhizopus stolonifer* and *Aspergillus flavus* is thus recommended for fungal degradation.

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