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Hydrocarbon Degradation Potentials of Indigenous Fungal Isolates from Petroleum Contaminated Soils

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Abstract

Twelve fungal isolates recovered from oil-contaminated soils were screened for crude oil biodegradation activity in a shake-flask culture. Among the twelve fungal isolates, only eight showed potentials for biodegradation. Of these eight isolates, two of them identified as *Aspergillus versicolor* and *Aspergillus niger* which exhibited the fastest onset and highest extent of biodegradation were selected for further study on specific polycyclic aromatic hydrocarbon (PAH) biodegradation. Both isolates exhibited above 98% degradation efficiency for polycyclic aromatic hydrocarbon moieties when grown in a culture medium incorporated with 1% crude oil (hydrocarbon) and 0.1 %Tween 80 for 7 days.

Keywords: Fungi, biodegradation, hydrocarbon, petroleum contaminated soils.

Introduction

Although pesticides are hydrocarbon pollutants of the soils, the main sources of hydrocarbon pollution are the spills and leaks of petroleum products (Potter, 1993). The Exxon Valdez oil spill in South Central Alaska is an example (Pritchard *et al*., 1992). In Nigeria, the exploration and exploitation practices and the breaking of oil pipes lead to incessant pollution especially in the Niger Delta area and Southern part of Nigeria (Salu, 1999). These spills have the largest immediate and economic impact as they harm, to a large extent, the ecosystem more than just the isolated location. In many spills involving tankers or offshore oil wells, some of the spills catch fire and consequently their combusting results in emission of large quantities of toxic ash which is detrimental to human health.

In recent times, an increasing amount of microbiological research has been devoted to bioremediation of oil-contaminated sites using various microbial species (Atlas, 1981). Notable among them were the bacterial species of *Arthrobacter* (Edgehill and Finn, 1982), *Flavobacterium* (Saber and Crawford, 1985), *Sphingomonas* (a novel *Pseudomonas* sp) (Radehaus and Schimidt,1992), *Pseudomonas spp.* (Leung *et al*., 1997) and *Acinetobacter* (George-Okafor *et al*., 2005). Fungal species such as *Trichoderma* (Cserjesi and Johnson, 1972) and *Phanerochaete* (Andrea *et al*., 2001) have been implicated in hydrocarbon biodegradation.

Fungal bioremediation has been successful for clean-up of pentachlorophenol (PCP), a wood preservative and polycyclic aromatic hydrocarbon (Andrea *et al.*2001). The advantages associated with fungal bioremediation lay primarily in the versatility of the technology and its cost efficiency compared to other remediation technologies (such as incineration, thermal desorption, extraction) (Aust, 1990). The use of fungi is expected to be relatively economical as they can be grown on a number of inexpensive agricultural or forest wastes such as corncobs and sawdust. More so, their utilization is a gentle non-aggressive approach. The application of bioremediation capabilities of indigenous organisms to clean up pollutants is viable and has economic values. (Bijofp, 2003). The objectives of this study were therefore, to isolate and identity some of the indigenous fungal flora of oil contaminated soils and evaluate the biodegradation efficiencies of the potent isolates.

Materials and Methods

Sources of soil sample:

The three oil contaminated soil samples used for the isolation were from three different sites located at the environment of Nigerian National Petroleum Corporation (NNPC) Emene, Enugu-Nigeria. The three different sites were the Dual purpose kerosene (DPK) site, the Automotive gas oil (AGO) site and the Premium motor spirit (PMS) site. Samples from each site were collected randomly from different locations just 1cm below the soil surface and transported to the laboratory in white plastic bags and kept in a refrigerator (in order to keep the organisms viable and free from any contaminant) before analysis.

Media and Chemicals

Sabouraud Dextrose agar (SDA) and Czapek medium containing sucrose (30 g/1), $NaNO_3$ (3 g/1), K_2HPO_4 (1g/1), $MgSO_4.7H_2O$ (0.5 g/1), KCl (0.5 g/l), FeSO4.7H20 (0.01g/l) and agar (15 g/l) were utilized for the isolation of the fungi. Bacto Bushnell-Haas broth containing $MgSO_4$ (0.2 g/l), $CaCl_2$ (0.02 g/l), KH_2PO_4 (1 g/l), K_2HPO_4 (1 g/l), $FeCl_2$ (0.05 g/l) and NH_4NO_3 (1 g/l) was used for the screening test. Tween 80 (0.1%), redox reagent (2% 2, 6-dichlorophenol indophenols) and crude oil (1%) were all incorporated into the broth.

Isolation of indigenous fungi

Each oil-contaminated soil sample was homogeneously mixed and carefully sorted to remove stones and other unwanted soil debris using 2.5 mm sieve. One gram of each sorted soil sample was homogenously mixed with 1 drop (0.1ml) of Tween 80 and a loopful (3 mm) of it was collected and inoculated by sprinkling method onto SDA and Czapek agar plates, respectively. The plates were incubated at room temperature (28 – 30°C) for 3 to 5 days for SDA and 5 to 7 days for Czapek agar plates. The grown cultures were carefully and aseptically sub-cultured onto fresh SDA and Czapek agar plates to obtain pure cultures for biodegradation screening. The pure isolates were maintained on SDA slants.

Screening for biodegradation potentials

A modified method of Desai *et al*. (1993) was utilized for the screening test. Two agar plugs (1cm^{2}each) of a pure growth of each isolate were inoculated into Bacto Bushnell – Haas broth (50ml/250 Erlenmeyer flask) incorporated with sterile crude oil (1%$^v/_v$), redox indicator (2%$^v/_v$) and Tween 80 (0.1% $^v/_v$). The control flask had no organism. Incubation was at room temperature (28 – 30°C) with constant shaking at 180 rev/min for 7 days. The aliquots in the flasks were monitored daily for color change (from deep blue to colorless).

Identification of the potential isolates

Twenty four pure cultures of the potential strains maintained on SDA slant were identified at the Department of Mycology, University of Nigeria Teaching Hospital, Enugu, according to the methods of Campbell and Steward (1980) and Beneke and Rogers (1980), using colonial appearance and microscopic characteristics.

Biodegradation assay of the selected cultures

Three agar plugs (1 cm^2) of the 24 h pure cultures of each of the two best potential strains *(Aspergillus versicolor* and *Aspergillus nige*r) were inoculated into the Bacto Bushnell – Haas broth (100 ml/500 ml Erlenmeyer flask) containing 0.1%($^v/_v$)Tween 80 and 1% (v /v) crude oil (without redox indicator). Inoculation of each organism was carried out in triplicate prior to incubation as stated above. Control tube without the organism was prepared accordingly. On a daily basis, 5 ml of the aliquots were collected from each flask and centrifuged (5000 rpm) at room temperature for 5 minutes using MSE (Minor) centrifuge. The recovered supernatants were assayed spectrophotometrically for the residual hydrocarbon (both aliphatic and aromatic polymers) using the procedure of Leung *et al.* (1997). The mean results were then obtained.

After 7 days the extent of polycyclic aromatic hydrocarbon (PAH) degradation by *Aspergillus versicolor* and *Aspergillus niger* using undegraded crude oil as the control was determined by Gas Chromatography at Fugro Consultants Nigeria Limited, Port-Harcourt, Rivers State of Nigeria. The chromatographic equipment used was an Agilent Gas Chromatography, model 6890N. The analytic conditions of the Chromatograph were as follows: Carrier gas, helium, make-up nitrogen gas (flow – 30 ml/min), fuel-air flow rate, 450ml/min, fuel-H2 flow rate 45 ml/min, inlet temperature at 275°C and initial – final oven temperature at 65-290°C. The detector type was flame ionization with temperature at 300°C. The Chromatograph was then attached to an integrator.

The specific procedure for the assay was as follows: One mL of each sample (both undegraded and degraded crude oil) was diluted ten fold with dichloromethane and passed through a silica packed column for cleaning up as well as separating into aliphatics and polycyclic aromatic hydrocarbon using pentane and 40%/60% pentane/ dichloromethane mixture respectively. The aliquots for PAH only were concentrated to 1ml at 100°C using a Danish Kudana concentrator and then injected into a Gas Chromatography apparatus. The amount of PAH before and after the degradation were shown by the peak heights of the chromatographic runs. The amount of PAH (mg /L) was calculated by the integrator using a standard curve.

Results and Discussion

A total of 12 fungal isolates were initially obtained from SDA and Czapek medium. Of these, 8 isolates that showed potentials for hydrocarbon biodegradation were identified as *Aspergillus versicolor, Aspergillus niger, Aspergillus flavus, Syncephalastrum* spp., *Trichoderma* spp., *Neurospora sitophila, Rhizopus arrhizus* and *Mucor* spp. Some of these organisms have earlier been reported as hydrocarbon bio-degraders by April *et al.* (2000) and Oudot *et al.* (1993)

The ability of these isolates to produce a color change in the medium is presumably due to the reduction of the indicator by the oxidized products of hydrocarbon degradation. The total color change (blue to colorless) supports the fact that the isolates are potential hydrocarbon oxidizers. Among the 5 isolates that produced total color change, *Aspergillus versicolor* and *Aspergillus niger* displayed the fastest onset and highest extent of biodegradation (Figure1).

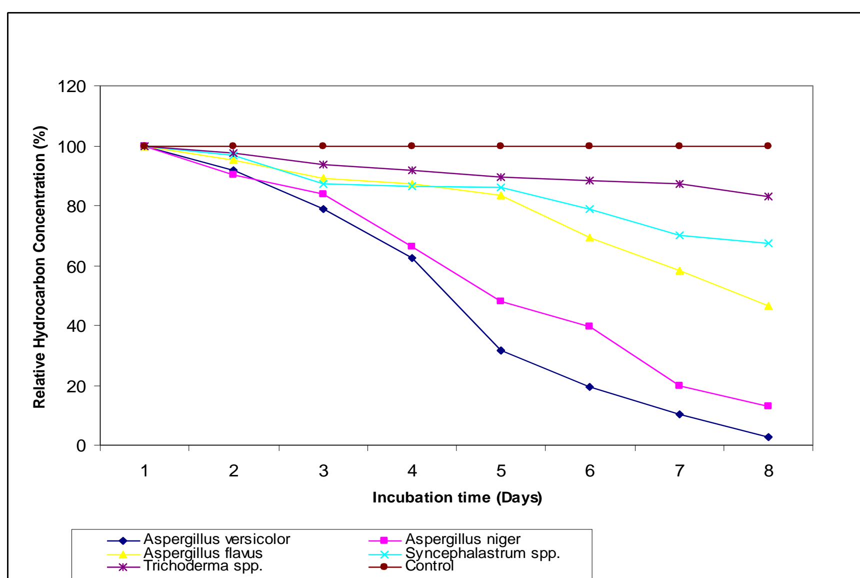

Fig. 1: Hydrocarbon degradation profile of the five isolates from 1-7days incubation in crude oil

Thus, they were selected for further study. 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 (1996), which showed that extracellular ligninolytic enzymes of white rot fungi are produced in response to their growth phases. The result of Gas Chromatography on PAHs degradation (Table 1) which showed that the two isolates exhibited biodegradation efficiency above 98% also confirmed their high degradation potentials.

Table 1: Degradation of polycyclic aromatic hydrocarbon (PAHs) by *Aspergillus* species after 7 days incubation in crude oil.

PAHs Parameters	Amount of PAHs (mg/l) in the undegraded crude oil	Percent depletion of PAHs of crude oil (%)	
		A. versicolor	*A. niger*
Acenapthylene	38.3	99.74	99.40
Benzo (K) fluoranthrene	117	99.95	99.95
Pyrene	118	99.95	99.92
Dibenzo (a,h) anthracene	127	99.92	99.93
Chrysene	145	99.97	99.88
Benzo (a) anthracene	156	99.96	99.94
Benzo (g,h,i) perylene	159	99.97	99.91
Benzo (a) pyrene	169	99.97	99.89
Fuoranthene	180	99.96	99.96
Phenanthrene	338	99.81	99.72

The abilities of these organisms in oxidizing the polycyclic aromatic hydrocarbons (PAHs) can be attributed to the non-specific nature of their enzymes especially the perioxidases on degrading chemicals. The fact that the isolates were able to degrade PAHs very effectively suggests that the degradation of the aliphatic moieties could be easier and faster than their polycyclic aromatic moieties (Englert *et al.* 1993). The implication of these two organisms in hydrocarbon degradation from our results is similar to the findings of April *et al.* (2000).

High biodegradation efficiency (> 98%) exhibited by *Aspergillus versicolor* and Aspergillus *niger* within 7 days of incubation showed that the cultural conditions were very appropriate for their growth and biodegradation. The utilization of 0.1% of Tween 80 during the assay was based on our earlier findings that it enhanced biodegradation activities (George-Okafor *et al.*, 2005). The hydrocarbon degradation abilities of *A.versicolor* and *A. niger* are similar to the findings of April *et al.*(2000) which showed that these two organisms were among the sixty-four species of hydrocarbon-degrading filamentous fungi isolated from flare pit soils in northern and western Canada. However, their chromatographic and radiorespirometry analyses only indicated the abilities of these fungi to degrade aliphatic fractions of crude oil [n-C_{12}-n-C_{26}] and not aromatic fractions as no observation was made on the degradation of the aromatic moieties.

Conclusion: Based on previous work, some bacteria such as *Pseudomonas, Bacillus* and *Acinetobacter* species were highly rated (70.6%-82%) in their abilities to degrade hydrocarbon. However, in this study we observed that a higher biodegradation efficiency (>98%) was exhibited by A. *versicolor* and A*. niger;* this proved these fungi to be better hydrocarbon degraders. Thus, they can be effectively utilized for the degradation of oil polluted farm lands especially those located within the vicinity of the isolation soil sites.

Field Application of the Organisms

After large scale production of the organism, the organism recovered should be ground and formulated with adequate carriers or extenders either in the powdered or liquid form using Tween 80 which enhances the degradation process. In order to prevent run-offs, 'inert' stickers or adhesives (such as molasses, corn syrup, skim milk, casein and latexes) may be incorporated into the formulation. A good sticker combined with charcoal can serve as a protectant. This will reduce the effects of ultra-violet light, desiccation and other detrimental environmental factors. During application, the oil contaminated plots to be treated should first be tilled to loosen the soil. Thereafter, the loosened soil should be enriched with adequate nutrients necessary for the growth of the organism before applying the formulated inoculants which must be properly mixed with the soil. Periodic moistening of the soil may be necessary to avoid excessive drying of the contaminated soil.

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