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Full Length Research Paper

Hydrocarbon degradation potentials of indigeneous fungal isolates from a petroleum hydrocarbon contaminated soil in Sakpenwa community, Niger Delta

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In this study, hydrocarbon degradation potentials of indigenous fungal species were investigated employing colonial morphologies and molecular techniques. Soil samples were collected from Saakpenwa community, Tai L.G.A, Rivers State, an oil exploration zone of the Niger Delta, Nigeria which was later prepared for laboratory analyses. Nine (9) fungal isolates were screened for hydrocarbon biodegradation potentials in a shake-flask culture incorporated with 1% crude oil (hydrocarbon), redox reagent (2% 2,6-dichlorophenol indophenols) and 0.1 % Tween 80 for 14 days. Among the nine fungal isolates, eight showed potentials for hydrocarbon degradation employing colour change, optical density and THC for 14 days. Five of the isolates exhibited the fastest onset and highest extent of biodegradation and were selected for further identification following polymerase chain reaction using 5'TCCGTAGGTGAACCTGCGG 3' and 5'GCTGCGTTCTTCATCGATGC 3' and were identified as: *Aspergillus japonicus, Yarrowia lipolytica* YPY 01*, Yarrowia lipolytica* ATCC 9773*, Zygorrhinchus* sp.(1) *and Zygorrhinchus* sp.(2) respectively. In this study, it was observed that a higher THC biodegradation efficiency was exhibited by *Yarrowia lipolytica* ATCC 9773 and *Zygorrhinchus* sp.(2). This indicated that these fungal species are efficient hydrocarbon degraders. Thus, they can be considered in strain development programme for biodegradation of oil polluted farm lands (soil) especially those located within the sampled sites.

Keywords: Hydrocarbon, fungal species, Saakpenwa community

INTRODUCTION

There is an increased interest in promoting environmental methods in the process of cleaning oil-polluted sites. These methods are less expensive and do not introduce additional chemicals to the environment. Compared to physiochemical methods, bioremediation offers a very feasible alternative for an oil spill response. This technique is considered an effective technology for treatment of oil pollution. One reason is that the majority of the molecules in the crude oil and refined products are

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biodegradable.

Biodegradation is most often the primary mechanism for contaminant destruction including petroleum contaminants (Leahy and Colwell, 1990). Bioremediation, the enhancement of natural biological degradation processes, has been proposed for cleanup of oil-spills in soils (Kerry, 1993) as cost-effective technology of removing contaminants. There have been many studies on microbial oil degradation in soils in which hydrocarbon-degrading microbes have been detected (Kerry, 1990; 1993; Aislabie *et al*., 1998). The activity of microbial types naturally present can be enhanced by bioremediation techniques which include increased aeration of the polluted area and nutrient additions (Ivshina *et al*., 1998; Christofi and Ivshina, 2002).

Objectives of the study were to isolate the indigenous fungal flora of an oil contaminated soil in Saakpenwa Community, Tai Local Government Area, Ogoniland, Rivers State of the Niger Delta, identify indigenous fungal isolates capable of hydrocarbon degradation, identify indigenous fungal isolates capable of hydrocarbon degradation using molecular techniques, establish a base line analysis for on-field remediation of a hydrocarbon polluted soil using mould strains, create a bio-data on our indigenous fungal strains capable of hydrocarbon degradation.

MATERIALS AND METHODS

Source of soil sample

Two hundred grames of the oil-contaminated soil sample used for the isolation was collected from Saakpenwa community, Tai L.G.A Ogoniland, Rivers State of the Niger Delta using a soil sampler. Ogoniland region covers 1,000km^2 in Rivers State, southern Nigeria and it has been the site for oil industry operations since the late 1950s, Ogoniland also has a tragic history of pollution from oil spills. Samples from the site were collected from the polluted area, 500m and 1000m away the polluted area and transported in plastic bags to the laboratory for analysis.

Media and Chemicals

Sabouraud's dextrose agar and Czapek medium containing sucrose (30g/1), $NaNO_3$ (3g/1), K_2HPO_4 (1g/1), $MgSO_4.7H_2O$ (0.5g/1), KCl (0.5g/l), $FeSO_4.7H_20$ (0.01g/l) and agar (15g/l) were used for the isolation of the fungi. Bushnell-Haas broth containing $MgSO_4$ (0.2g/l), $CaCl_2$ (0.02g/l), KH_2PO_4 (1g/l), K_2HPO_4 (1g/l), $FeCl_2$ (0.05g/l) and NH_4NO_3 (1g/l) was used for the screening test. Redox reagent (2% 2, 6-dichlorophenol indophenols), Tween 80 (0.1 %) and crude oil (1%) were all incorporated into the broth. This method has been previously adopted by George-Okafor *et al*.,(2009).

Isolation of fungi

The oil-contaminated soil sample was homogeneously mixed and carefully sorted to remove stones and other unwanted soil debris using 2.5 mm sieve. Ten grammes of the sorted soil sample was introduced into a 250ml conical flask. After which 90ml of sterile water was added and the flask was vigorously shaken for 2 minutes to obtain soil solution representing 1:10. 10ml of the supernatant of the 1:10 solution was transferred into 90ml of sterile water representing 1:100. In the same way, 1:1000, 1:10000 and 1:100000 were made.

A ml of the last three dilutions was spread onto Sabouraud's dextrose agar and Czapek agar plates respectively. The Petri dishes were rotated by hand to disperse the medium and the soil suspension (Soil-dilution Plate Method). The plates were incubated at room temperature (28 – 30^{o}C) for 3 to 5 days for Sabouraud's dextrose agar and 5 to 7 days for Czapek agar plates. The colonies were carefully and aseptically sub-cultured onto fresh Sabouraud's dextrose agar plates to obtain pure cultures for biodegradation screening. The pure isolates were maintained on Sabouraud's Dextrose agar slants.

Screening for biodegradation potential

A modified method of Desai *et al*. (1993) was utilized for the screening test. Two agar plugs (1cm^2) of a pure growth of each isolate was inoculated into Bushnell – Haas broth (50ml/250ml Erlenmeyer flask) incorporated with sterile crude oil (1%v/v), Tween 80 (0.1%) and redox indicator (2%v/v). The control flask had no organism. Incubation was done at room temperature (28^{o}C – 30^{o}C) with constant shaking for 14 days. The aliquots in the flasks were monitored daily for color change, optical density and THC variations for 14 days.

IDENTIFICATION OF ISOLATES

Cultural Identification

Pure cultures of the potential strains maintained on Sabouraud's Dextrose agar slants were identified at the Environmental microbiology laboratory of the Department of Microbiology, University of Port Harcourt, Rivers State, using colonial morphologies, and microscopic characteristics.

Molecular identification

Pure cultures of the potential strains maintained on Sabouraud's Dextrose agar slants were identified at the Biotechnology Centre, Federal University of Agriculture Abeokuta-Nigeria employing DNA extraction, DNA sequencing and sequence blasting on NCBI (National Centre for Biotechnological Information).

Extraction of DNA using fungi Genomic DNA isolation kit

Five millilitre of collection solution made up of 0.9% (w/v) NaCl prepared with distilled water was added to the fungi growing on plates. One milliliter of washed spores and wet mycelium were aseptically transferred into a microcentrifuge tube ensuring not to collect agar debris and centrifuged at 14,000 x g for 1 minute to pellet the cells. The supernatant was carefully discarded so as not to disturb or dislodge the cell pellet. Five hundred µL of lysis solution was added to the cell pellet and the cells were re-suspended by gently vortexing. The mixture was transferred to a bead tube; the tube was secured horizontally on a flat-bed vortex pad with tape and was mixed by vortexing for 5 minutes at maximum speed. The bead tube with lysate was incubated at 65^{o}C for 10 minutes and the lysate was occasionally mixed (2 or 3 times) during incubation by inverting the tube. The tube was briefly spun to remove liquid from the cap and all the lysate including all debris were transferred to a DNase-free microcentrifuge tube by pipetting ensuring that the beads were not transferred during pipetting and the tube was centrifuged for 2 minutes at 14000 x g. The supernatant was carefully transferred to a DNase-free microcentrifuge tube without disturbing the pellet and an equal volume of ethanol was added to the lysate collected above and was mixed by vortexing. 300 µL of Binding solution was added and briefly mixed by vortexing. A spin column assembled with its collection tube was obtained, 650 µL of the lysate with ethanol was applied onto the column and centrifuged for 1 minute at 6,000 x g. The flow through was discarded and the spin column with the collection tube was reassembled and this step was repeated with the remaining lysate. Five hundred µL of wash solution made up of 42 ml of 96 – 100% ethanol + concentrated wash solution giving a final volume of 60 ml was applied to the column and centrifuged for 1 minute at 6,000 x g, the flow through was discarded and the column with its collection tube reassembled. The above step was repeated to wash column a second time and the flow through was also discarded and the spin column with its collection tube reassembled. The column was spun for 2 minutes at 14,000 x g in order to thoroughly dry the resin and the collection tube was discarded.

The column was placed into a fresh 1.7 ml elution tube, 100 µL of elution buffer was added to the column and centrifuged for 2 minutes at 6,000 x g. Finally the purified nucleic acids were stored at -20^{o}C.

PCR analysis (MJ Research Thermal cycler, PTC-200 model)

PCR analysis was run with 18S primer. The PCR mixture comprises of; 1µl of 10X buffer, 0.4µl of 50mM $MgCl_2$, 0.5µl of 2.5mm dNTPs, 0.5µl 5mM forward primer, 0.5µl of 5mM Reverse primer, 0.05µl of 5units/µlTaq with 2µl of template DNA and 5.05µl of distilled water to make-up 10µl reaction mixture. Initial denaturation temperature of 94^{o}C for 3minutes, 30 cycles of 94^{o}C for 60seconds, 56^{o}C for 60seconds , and 72^{o}C for 120seconds were employed.

Purification of PCR products

The amplicon were further purified before the sequencing using 2M sodium acetate wash technique. An equal volume of 2 ml sodium at pH 5.2 was added to the PCR product, followed by 20µl absolute ethanol, kept at -20^{0}C for 1hr, spun at 10,000rpm for 10 minutes, and then washed with 70% ethanol and air dried. It was resuspended in 5µl sterile distilled water and kept at 4^{0}C for sequencing.

Preparation of sample for Gene Sequencer (Applied biosystem, AB1 3130xl model)

The Cocktail mixture is a combination of 9µl of Hi di formamide with 1µl of purified sequence making a total of 10µl. The samples were loaded on the machine and the data in form A, C, T, and G were released.

Genotyping

Multilocus sequence typing (MLST) was adopted and 400-500 base pair fragments of DNA were sequenced at seven different conserved genes which allowed small variations within the species to be detected.

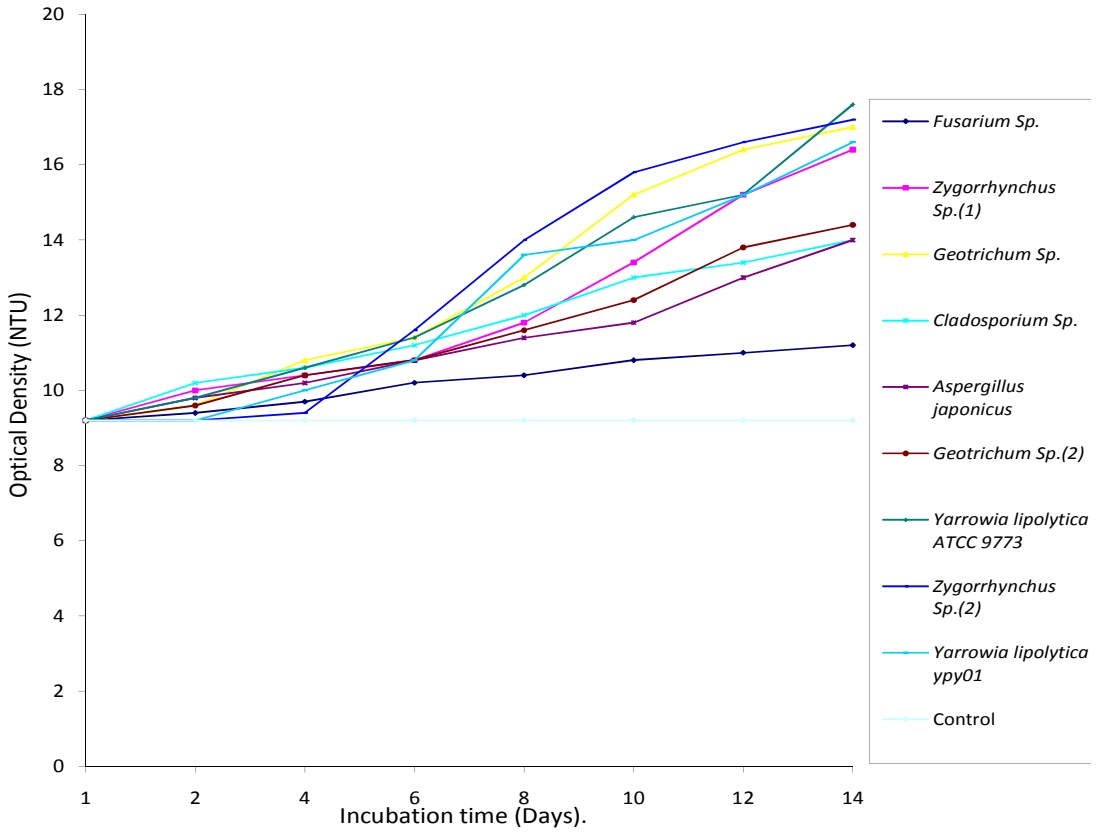

Figure 1. Graph showing optical density profile of fungal hydrocarbon degradation simulation from 1 – 14 days.

Table 1. Total Hydrocarbon Content (Mg/ml) concentration during the study period

ISOLATE	Day 1	Day 2	Day 4	Day 6	Day 8	Day 10	Day 12	Day 14	% Degra- dation
Fusarium sp.	8100	7850	7300	6905	6510	5900	5400	5265	35%
Zygorrhynchus sp.	8100	6130	5800	4800	2800	2100	1805	1665	79.4%
Geotrichum sp.	8100	7500	6580	5860	4200	2605	1680	1597	80.2%
Cladosporium sp.	8100	7080	6645	5775	4820	3900	3210	1755	78.3%
Aspergillus japonicus	8100	7525	7010	6400	5877	5215	3688	1620	80%
Geotrichum sp.(2)	8100	7680	6600	5540	4415	3533	2980	1620	80%
Yarrowia lipolytica ATCC9773	8100	7600	8515	5912	4799	2945	1740	1350	83%
Zygorrhynchus Sp.(2)	8100	8100	7635	5757	3589	2680	1887	1440	82.2%
Yarrowia *lipolytica* ypy 01	8100	8100	7335	6519	3911	2813	1990	1485	81.6%
Control	8100	8100	8100	8100	8100	8100	8100	8100	0%

RESULTS

Nine (9) fungal isolates were obtained from SDA and Czapek media, eight isolates showed potentials for hydrocarbon degradation. Five of these potential microbial degraders exhibit the fastest onset and the highest extent of biodegradation were identified with ITS 1 **5'**TCCGTAGGTGAACCTGCGG**3'** ITS 2 **5'**GCTGCGTTCTTCATCGATGC**3'** as *Aspergillus japonicus* (AJ279993.1), *Yarrowia lipolytica* ypy01 (HQ412612.1), *Yarrowia lipolytica* ATCC 9773 (GQ458037.1), *Zygorrhynchus* sp.(1) *and Zygorrhynchus* sp.(2) respectively employing DNA extraction, DNA sequencing and sequence blasting on NCBI. This result is supported by the findings of Chaillana *et al.*(2004).

There was progressive increament in the optical density of various experimental setup with the exception of the control which was not inoculated (Figure 1). It was

observed that within 6 days of microbial inoculation, the increase in biomass as shown by optical density was highest in the option containing *Yarrowia lipolytica* ATCC 9773, followed by *Zygorrhynchus* sp.(2), *Geotrichum* sp.(1), *Yarrowia lipolytica* ypy 01, *Zygorrhynchus* sp.(1), *Aspergillus japonicus*, *Geotrichum* sp.(2), *Cladosporium* sp., and control was the least(Figure 1).

The increase in biomass is an indication that the biodegradative processes were driven by the inoculated microorganisms and available nutrients in the bush-nell Hass medium (Nitrogen, phosphorus and petroleum hydrocarbon).

From biodegradation experiment, it was observed that at baseline the concentration of the total hydrocarbon in the medium was 8100mg/ml (Tab. 1). In addition, on the 14th day of incubation of microorganisms in the broth containing microorganisms, different percentages of biodegradation was observed. Highest degradation was observed in the option inoculated with *Yarrowia lipolytica* ATCC 9773, where as *Zygorrhynchus* option had 82% reduction in total hydrocarbon content (Tab. 1).

In addition, 35%, 79%, 89.4%, 80.2%, 78.3%, 80% and 80% reduction in total hydrocarbon content was observed in Fusarium sp., Zygorrhynchus sp., Geotrichum sp., Cladosporium sp., Aspergillus japonicas., Geotrichum sp.(2) respectively. It was observed from the first to the 14th day, the control set up which was not inoculated with microorganisms had no reduction in total hydrocarbon content.

DISCUSSION

Color change in the medium due to microbial activities during the biodegradation screening was as a result of the reduction of the indicator by the oxidized products of hydrocarbon degradation. The reduction of the indicator also indicates increase in optical density following increase in absorbance (Figure 1).

The high rate of hydrocarbon degradation by the five fungi was as a result of 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 Total Hydrocarbon contents which showed that the five isolates exhibited biodegradation efficiency above 98% also confirmed their high degradation potentials.

The utilization of the hydrocarbons resulted in increase in cell densities (Oboh *et al*., 2006) and optical densities with a concomitant visual gradual reduction in the oil layer and redox indicator (2,6 Dichlorophenol indophenols) and gradual disappearance of the oil with prolonged incubation. The colour changes in the culture fluids in the experimental flasks within the 14-day incubation period further confirmed chemical changes of the hydrocarbon substrates which must have been precipitated by microbial enzymes (Atlas and Bartha, 1973).

It is evident from this investigation that oil degrading microorganisms could readily be isolated from soil without the need for time consuming traditional enrichment protocols. Further understanding of the metabolic process of these organisms on the hydrocarbons will increase possibilities of developing models and strategies for removing hydrocarbon pollutants from oil impacted environment.

High biodegradation efficiency (>83%) exhibited by *Yarrowia lipolytica* ATCC 9773 and (>82%) by *Zygorrhynchus* sp.(2) within 14 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 findings that it enhanced biodegradation activities (George-Okafor *et al*., 2005). The hydrocarbon degradation ability of *Aspergillus japonicus* was similar to the findings of George-Okafor *et al*.,(2009) and April *et al*.(2000) which showed that this organism was among the sixty-four species of hydrocarbon-degrading filamentous fungi isolated from flare pit soils in northern and western Canada.

CONCLUSION

In this study, it was observed that a high biodegradation efficiency was exhibited by *Yarrowia lipolytica* strain ATCC 9773 and *Zygorrhinchus* sp.(2). This proved these fungal species to be efficient hydrocarbon degraders. Thus, they can be considered in the strain development programme for the degradation of oil polluted soil especially those located within the vicinity of the sampled sites.

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