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BIODEGRADATION OF CRUDE OIL BY POTENTIAL HYDROCARBON-DEGRADING FUNGI IN SOIL SAMPLES OBTAINED FROM MESOGAR COMMUNITY IN DELTA STATE, NIGERIA.

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ABSTRACT

Soil samples were collected from petroleum exploring area of Mesogar Community in Warri South Local Government Area of Delta State to enumerate and identify hydrocarbon degrading fungi in the soil. Total indigenous fungi on potato dextrose agar were enumerated and those that were able to use crude oil as source of carbon and energy were enumerated using Mineral Salt Media supplemented with 1% and 2% crude oil. Fungal isolates were identified using macroscopic and microscopic examination. The fungi isolated from potato dextrose agar include: *Saccharomyces cerevisea, Doratomyces stemonitis, Fusarium poae, Paecilomyces variotii, Penicillium ferquentans, Penicillium chrysogenum, Aspergilus niger, Aspergilus flavus, Rhizoqus stotonifer*. Hydrocarbon-degraders isolated from mineral salt broth include: *Penicillium chrysogenum, Penicillium ferquentans, Aspergilus niger, Aspergilus flavus, Rhizopus stolonifer*. The biodegradative activities of hydrocarbon degrading fungi were determined by calculating the amount of CO_2 evolved using titrimetric method. The maximum amount of CO_2 evolved was observed with *Penicillium chrysogenum*, (4180mg/L) on 16th day of treatment and *Aspergillus flavus* (4180mg/L) on the 12th day with 2% crude oil concentration. *Aspergilus flavus* declined to 3520mg/L on the 16th day of treatment with 2% crude oil concentration. Increase in the amount of CO_2 evolved was observed with *Penicillium frequentans, Aspergilus niger, Aspergilus flavus and Rhizopus stolonifer* up to the 12th day of treatment, while decrease was observed on the 16th day of treatment with 2% crude oil concentration. The decrease in the amount of CO_2 evolved may be due to accumulation of toxic substances by microbes and exhaustion of nutrients to continue the degradation process with the exception of *Penicillium chrysogenum* in which the amount of CO_2 continues to increase throughout the period of treatment.

Keywords: Hydrocarbon-degrading, fungi, Crude oil, Carbon dioxide, incubation period.

INTRODUCTION

The major causes of hydrocarbon pollution are the spills and leaks of petroleum products (Potter, 1993). The 1989 Exxon *Valdez* oil spill in Prince William Sound, Alaska; the 2007 MT *Hebei Spirit* oil spill in South Korea; and the 2010 BP Deepwater Horizon oil spill, caused by an explosion on an offshore drilling platform are few examples (Chang et al., 2014). In Nigeria, the exploration and exploitation practices and the vandalization of oil pipes lead to continuous 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. Bioremediation is the process in which microorganisms metabolize contaminants either through oxidative or reductive processes. Under favorable conditions,

microorganisms can oxidatively degrade organic contaminants completely into non-toxic by-products such as carbon dioxide and water or organic acids and methane.

Fungal bioremediation has been successful for cleanup 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 forests wastes such as corn cobs and sawdust. The application of bioremediation capabilities of indigenous fungi to clean up pollutants is viable and has economic values, (Bijofp, 2003). The aim of this study is to isolate hydrocarbon degrading fungi from petroleum contaminated soil samples and determine the rate of biodegradation through CO_2 evolution.

METHODOLOGY

Sampling sites and collections of samples

Crude oil contaminated soil samples were collected from Mesogar Community, Warri South Local Government Area of Delta State, Nigeria and an uncontaminated soil sample that serves as control. The soil samples were collected aseptically into sterile polyethene bags and transported to the Microbiology Laboratory of the Federal University of Technology, Akure, Nigeria for analyses while bonny-light crude oil was obtained from Nigeria National Petroleum Commission (NNPC) Bayelsa State, Nigeria.

Enumeration of fungi from soil samples

One milliliter (1ml) aliquot of each serially diluted soil sample was aseptically dispensed for pour plate method using potato dextrose agar (PDA) which was incubated at 27^{o}C for 72 hours for the isolation of fungi. Streptomycin was added to prevent bacterial growth. After the incubation period, microbial loads were counted and the results were recorded in spore forming unit (Sfu/g) for the fungal load. Sub culturing was done until pure isolates were observed.

Characterization and identification of fungi.

The fungal isolates were characterized based on the colour of the colony, shape, size, vegetative parts, type of hyphae, and kind of spore. A small portion of the mycelium was stained with lactophenol blue on a slide using a compound microscope to observe the fungal features as described by Gaddeyya *et al*., 2012.

Enumeration of hydrocarbon degrading fungi

Mineral salt medium (MSM) containing K_2HPO_4 (0.36g), $MgSO_4.7H_20$ (0.04g), NaCl (0.02g), NH_4Cl (0.8g), $Na_2SO_4.7H_2O$ (0.02g) and agar (4.0g), dispensed in 100ml of distilled water was used for the isolation of the hydrocarbon degrading fungi. Mineral salt medium was prepared in conical flasks as modified by Okpokwasili and Oton (2006). The mineral salt medium was sterilized at 121^{o}C for 15 minutes in an autoclave and cooled to 45^{o}C under aseptic condition. Streptomycin was added to prevent bacterial growth and serially diluted soil samples (10^{-5} and 10^{-6}) were inoculated respectively. This was followed by the addition of membrane filter sterilized 1% and 2% crude oil each. The medium was incubated at 30^{o}C in an incubator with shaker and at an interval of 4 days. One milliliter (1ml) was withdrawn from the flask and inoculated into prepared PDA plate for isolation of organisms that can utilize the crude oil. The grown cultures were carefully and aseptically sub-cultured into fresh PDA plates to obtain pure cultures. The pure isolates were maintained on PDA slants.

Screening for biodegradative potentials

One hundred milliliters (100ml) of distilled water containing K_2HPO_4 (0.36g), $MgSO_4.7H_2O$ (0.04g), NaCl (0.02g), NH_4Cl (0.8g), $Na_2SO_4.7H_2O$ (0.02g) and agar (4.0g) with filter sterilized crude oil (1% and 2%) was prepared in 100ml conical flask and inoculated with 1ml broth of each isolate. Each of the flask was plugged with cotton wool wrapped with aluminum foil to prevent contamination and incubated at 30^0C on shaker at 100rpm for 16 days. One milliliter (1ml) of the incubated mineral salt broth was taken after 4, 8, 12, and 16 days of treatment and titrated against 0.05N of sodium hydroxide (NaOH) solution. Phenolphthalein was used as indicator and appearance of stable pink color was considered as the end point. The amount of CO_2 evolved by the fungal isolates was calculated using the following equation (APHA *et al*., 1995).

$$\text{Amount of free } CO_2 \text{ (mg/L)} = \frac{\text{Titre value X Normality of NaOH X 1000 X 44}}{\text{Volume of sample}}$$

Titre value = volume of base used (cm^3)
Normality of NaOH = 0.05N
Volume of sample =1ml

Statistical Analysis

The experiments were carried out in replicates of three. Data were statistically analyzed using SPSS version 20, the mean bacterial counts obtained were statistically analyzed using analysis of variance (ANOVA), and tests of significance carried out by New Duncan's multiple range test at $p < 0.05$.

RESULTS

Fungal population

The fungal loads of the soil samples collected from Mesogar Community, Warri South Local Government Area of Delta State, Nigeria and an uncontaminated soil sample are presented in Table 1. The soil sample showing the highest total fungal counts (TFC) was collected from S1 with 6.67 ± 1.5^b Sfu/g while the highest hydrocarbon degrading fungal count (HDFC), 5.33 ± 0.6^b Sfu/g and 5.33 ± 1.5^b Sfu/g were obtained from S1 and S2 respectively. The least HDFC was obtained from the control samples (1.00 ± 0.3^b) while the least TFC was 1.83 ± 1.5^a.

Table 1: Fungal counts from soil samples

Source	TFC (Sfu/g) x 10^4	HDFC (Sfu/g) x 10^4
S1	6.67 ± 1.5^b	5.33 ± 0.6^b
S2	1.33 ± 5.8^a	5.33 ± 1.5^b
S3	1.83 ± 1.5^a	1.00 ± 0.3^b

Each value is expressed as means ±standard error (n=3).
Mean difference is considered significant at the $P<0.05$

Key
S1 – Crude oil contaminated Site 1
S2 – Crude oil contaminated Site 2
S3 – Non- populated Site 3
TFC – Total Fungal count
HUF – Hydrocarbon Degrading Fungal count

Identification of fungal isolates

The identities of the fungal isolates obtained in this study are presented in Table 2. Nine (9) fungi were identified as *Aspergillus niger, Penicillium chrysogenum, Saccharomyces cerevisae, Fusarium poae, Penicillium frequentans, Doratomyces stemonitis, Aspergillus flavus, Paecilomyces varioti and Rhizopus stolonifer.*

Table 2: Microscopic and macroscopic observation of isolated fungi

Sources	Macroscopic description	Microscopic description	Probable organisms
S1, S2, S3	Black and powdery colonies which grows very fast	Conidiophores terminates in vesicles, smooth walled, colourless with brownish shades	*Aspergillus niger*
S1, S3	White fluffy coloured colonies tinted with green colour, growth rate is moderate	Conidiophore arising from the mycelium, hyphae cuts in many conidia.	*Penicillium chrysogenum*
S1, S2, S3	Creamy coloured smooth colonies, growth rate is slow.	Large, globose to ellipsoidal budding yeast like cell or blastoconidia. Hypha is absent.	*Saccharomyces cerevisae*
S1	Pink coloured colonies, growth rate is fast.	Produce both macro and micro conidia from slender phalide.	*Fusarium poae*
S2, S3	Grey-green coloured colonies, growth rate is fast.	Conidiophores are smooth walled, swollen at the apex.	*Penicillium frequentans*
S3	Pale-brown coloured colonies	Cylindrical conidia pointed at the apex.	*Doratomyces stemonitis*
S1, S2, S3	Green coloured colonies which grow very fast.	Simple upright conidiophores terminating in ovoid swelling, one celled conidia colour in mass and dry.	*Aspergillus flavus*
S2, S3	Brown coloured colonies which grow moderately.	Loosely branched irregular brush-like conidiophores with phalides at the tips. Conidia consist of hyphae and are borne in chains.	*Paecilomyces varioti*
S2, S3	White coloured colonies which grows very fast.	Non-septate, thin sporangiophore with sporangium in an umbrella-like form.	*Rhizopus stolonifer*

Key: S1 – Crude oil contaminated Site 1; S2 – Crude oil contaminated Site 2; S3 – Non- contaminated Site 3

Frequency of occurrence (%)

Table 3 shows the frequency of occurrence of the isolates obtained in this study. *Aspergillus niger* had the highest frequency of occurrence with 22% while *Fusarium poae, Paecilomyces varioti, and Doratomyces stemonitis* had the lowest with 4.87%.

Table 3: Frequency of occurrence of hydrocarbon degrading fungal isolates from soil samples.

Isolated organisms	S1	S2	S3	Total	Frequency of occurrence (%)
Aspergillus niger	4	3	2	9	22
Aspergillus flavus	3	3	1	7	17
Penicillium chrysogenum	5	0	1	6	15
Penicillium frequentans	2	2	1	5	12
Rhizopus stolonifer	3	2	0	5	12
Saccharoromyces cerevisae	1	1	1	3	7
Fusarium poae	1	1	0	2	5
Paecilomyces varioti	0	1	1	2	5
Doratomyces stemonitis	0	0	2	2	5
Total	19	13	9	41	100

Key: S1 – Crude oil contaminated Site 1; S2 – Crude oil contaminated Site 2; S3 – Non- contaminated Site 3

Biodegradation potentials of the fungal isolates

Amongst the nine (9) fungal isolates identified in this study, only five (5) had the ability to grow on crude oil (1% and 2%). They were *Penicillium chrysogenum, Penicillium frequentans, Aspergillus flavus, Aspergillus niger* and *Rhizopus stolonifer*.

Carbon dioxide (CO_2) evolution

Table 4 shows the amount of CO_2 released from five (5) fungal isolates (having the highest frequency of occurrence (%)) incubated with 1% concentration of crude oil. It was observed that *Penicillium chrysogenum* released the highest amount of CO_2 (3740 ± 2.3^{d}mg/L) on the 16^{th} day of incubation period while *Rhizopus stolonifera* released the least amount of CO_2 (2640 ± 1.1^{a}

mg/L) on the 16^{th} day of incubation period. The highest amounts of CO_2 evolved by *Aspergillus niger, A. flavus and Rhizopus stolonifera* were observed on the 12^{th} day which later decreased on 16^{th} day. The amount of CO_2 evolved by *Penicillium chrysogenum* was observed to increase from the 4^{th} day through 16^{th} day of incubation while that of *P. frequentans* increased up to 12^{th} day and remain constant till 16^{th} day of incubation period.

Table 4: Amount of Carbon dioxide (CO_2) in (mg/L) released from each of the isolates with 1% crude oil concentration.

Organisms	Incubation period			
	4th day	**8th day**	**12th day**	**16th day**
Aspergillus niger	2420±2.3[b]	2860±1.1[c]	3300±1.1[c]	2860±0.3[b]
Aspergillus flavus	2420±1.3[b]	2640±0.5[b]	3080±0.3[b]	2860±0.3[b]
Penicillium chrysogenum	2640±1.3[c]	3080±1.1[d]	3520±0.3[d]	3740±2.3[d]
Rhizopus stolonifera	2200±1.7[a]	2420±0.8[a]	2860±2.3[a]	2640±1.1[a]
Penicillium frequentans	2640±5.77[c]	2860±20.00[c]	3520±0.00 [d]	3520±0.00[c]

Each value is expressed as means ±standard error (n=3). Mean difference is considered significant at the P<0.05

Table 5 shows the amount of CO_2 released from five (5) fungal isolates (having the highest frequency of occurrence (%)) incubated with 2% concentration of crude oil. The maximum amount of CO_2 evolved was observed with *Penicillium chrysogenum*, (4180 ± 1.5[d]mg/L) on the 16th day incubation and *Aspergillus flavus* (4180 ± 0.3[d] mg/L) on the 12th day which decreased to 3530 ± 1.1[c] mg/L on the 16th day incubation period. The amount of CO_2 evolved by *Aspergillus niger*, *A. flavus*, *Rhizopus stolonifera* and *Penicillium frequentans* were observed to increase from the 4th day through 12th day and later decreased on the 16th day of incubation period while the CO_2 released by *Penicillium chrysogenum* increased throughout the incubation period.

Table 5: Amount of Carbon dioxide (CO_2) in (mg/L) released from each of the isolates with 2% concentration of crude oil.

Organisms	Incubation period			
	4th day	**8th day**	**12th day**	**16th day**
Aspergillus niger	2640±0.3[b]	3080±1.5[b]	3740±2.3[b]	3300±0.3[b]
Aspergillus flavus	2860±2.3[c]	3300±0.3[c]	4180±0.3[d]	3520±1.1[c]
Penicillium chrysogenum	2860±1.1[c]	3520±2.3[d]	3960±2.3[c]	4180±1.5[d]
Rhizopus stolonifer	2200±0.3[a]	2640±1. 1[a]	3300±1.1[a]	2860±2.3[a]
Penicillium frequentans	2860±0.3[c]	3300±0.3[c]	3740±2.3[b]	3520±2.3[c]

Each value is expressed as means ±standard error (n=3). Mean difference is considered significant at the P<0.05

DISCUSSION

In this study, crude oil contaminated soil sample obtained from S1 in Mesogar Community, Delta State had the highest total fungal count (TFC) (6.67 ± 1.5^{b}) while the crude oil contaminated sample from S2 had the least TFC (1.33 ± 5.8^{a}). The highest hydrocarbon degrading fungal count (HDFC) was obtained from crude oil contaminated soils SI (5.33 ± 0.6^{b}) and S2 (5.33 ± 1.5^{b}) while the least HDFC (1.00 ± 0.3^{b}) was obtained from the uncontaminated soil S3. The presence of fungi in both crude oil contaminated soils and uncontaminated soil proves the ubiquity of fungi. The lower HDFC obtained in this study implies that continuous discharge of crude oil into the ecosystem may result in selective decrease in fungal population due to toxicity of crude oil which is inhibitory to most microorganisms as reported by Ojumu *et al.,* (2004). This is however, in contrast to the findings of Boboye *et al*., (2010) and Olukunle *et al*., (2012) who reported higher number of oil-degrading microorganisms from oil polluted soils.

Aspergillus sp. (22% and 17%), *Penicillium* sp. (15% and 12%) and *Rhizopus stolonifera* (12%) were found to have the highest percentage of occurrence while species of *Saccharomyces* (7%), *Fusarium* (5%), *Paecilomyces* (5%) and *Doratomyces* (5%) had the least percentage of occurrence in hydrocarbon polluted sites from Mesogar Community, Delta State, Nigeria. In the same vein, Chikere and Azubike (2014) observed that *Aspergillus* sp. and *Penicillum* sp. also dominated with 31.23% and 22.91% in hydrocarbon polluted sites in Ogala- Bonny, Rivers State, Nigeria. *Fusarium* sp. had the least percentage of occurrence (4.26%) in the study.

A total of nine (9) fungal isolates were initially obtained from both the crude oil polluted and unpolluted soils. They were identified as *Aspergillus niger, Aspergillus flavus, Penicillium chrysogenum, Penicillium frequentans, Rhizopus stolonifer, Saccharoromyces cerevisae, Fusarium poae, Paecilomyces varioti and Doratomyces stemonitis*. Five (5) of the nine (9) isolates showed potential for hydrocarbon biodegradation on MSM. They were identified as *Penicillium chrysogenum, Penicillium frequentans, Aspergillus flavus, Aspergillus niger* and *Rhizopus stolonifer*. The results demonstrated that it is possible to select for potential hydrocarbon degrading fungi by using appropriate enrichment medium under favorable environmental condition. Some of these organisms have earlier been reported as hydrocarbon biodegraders by April *et al*., (2000) and Olukunle *et al*., 2012.

There is an increase in the amount of carbon dioxide (CO_2) evolved with increase in crude oil concentration (2%) as shown in table 5. Increase in CO_2 evolved during the incubation period indicates fungal growth due to utilization of crude oil as carbon source releasing CO_2 as a by- product. During degradation, most of the CO_2 is being released into the environment while some will be used for the synthesis of tissue and could be released when the mycelia are consumed (Hiscox *et al*., 2015). Del'arco and De (2001) observed that oil concentration greater than 5% leads to decrease in the microbial activity and that this high concentration may disturb C: N: P ratio and create oxygen limitations. *Penicillium* sp. and *Aspergillus* sp. isolated from soil samples in Mesogar community of Delta State were capable of degrading crude oil when used as single culture as indicated by an increase in the amount of CO_2 evolved. Similarly, Okerentugba and Ezeronye (2003) demonstrated that *Penicillium* sp., *Aspergillus* sp. and *Rhizopus* sp. were capable of degrading hydrocarbons especially when single cultures were used. These fungi have been isolated also from aquatic environment in Niger Delta area of Nigeria.

Fungi are capable of performing the mineralization of organic compounds, transforming crude oil ultimately to CO_2, water and biomass (Cunha and

Leite, 2000). Therefore, measurement of CO_2 released during the biodegradation process may be used as an index of hydrocarbon degradation. Table 5 summarizes the CO_2 released during biodegradation of crude oil by potential hydrocarbon degrading fungal isolated from soil sample. *Aspergillus* sp. and *Penicillum* sp. evolved the highest amount of CO_2 and the amount of CO_2 evolved increased throughout the period of treatment for *Penicillumm chrysogenum*. Therefore, *Penicillum* sp. was capable of growing at faster rate to decompose crude oil and this leads to an increase in the amount of CO_2 observed throughout the period of treatment. The variation in the capacity of the isolates to utilize hydrocarbon could be due to differences in the competence of crude oil degrading enzyme contained in each isolate. Similar results have been reported for *Penicillum* sp by Okoh (2006). The degradation of crude oil by fungi produces ultimately fungal cells and CO_2. Liberation of CO_2 during the degradation of crude oil can therefore be used as an indicator to study biodegradative activity of fungi in the growth medium.

Moreover, higher amount of CO_2 was released with 2% concentration of crude oil indicating that isolated fungi degrade crude oil more efficiently at higher concentration. Decrease in the amount of CO_2 evolved may be due to accumulation of toxic substances by microbes and exhaustion of nutrients to continue the degradation process with the exception of *Penicillium chrysogenum* in which the amount of CO_2 continued to increase throughout the period of treatment. The decline in CO_2 evolution would then indicate that those resources had been depleted (Hiscox *et al*., 2015).

CONCLUSION

This study reveals that potential hydrocarbon fungi are abundant and widely distributed in Mesogar community of Warri South Local Government Area of Delta State, Nigeria where crude oil exploration is taking place. Among the species of fungi isolated, it was observed that *Penicillium chrysogenum* and *Aspergillus flavus* were capable of producing enzymes at a faster rate to decompose the hydrocarbon and release more CO_2. Hence, these potential hydrocarbon degrading fungi can be utilized effectively as agents of biodegradation for oil contaminated lands especially those located within the vicinity of the isolation soil sites. Further study could be conducted on the enzymes produced by *Penicillium chrysogenum* and *Aspergillus flavus* for the degradation of crude oil polluted soils.

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