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Characterization of hydrocarbon utilizing fungi from hydrocarbon polluted sediments and water

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Abstract

Sediments from four different hydrocarbon polluted sites in Ogala-Bonny, Rivers State Nigeria and water samples from effluent discharge points of four different flow stations in Delta State were sampled. They were analyzed for presence of indigenous fungi. This was to establish possible fungal involvement in bioremediation of hydrocarbon polluted environments. Bushnell-Hass (mineral salt) medium supplemented with 0.05% (v/v) of streptomycin was used for the isolation and Okono medium crude oil severed as the only carbon source in the vapour phase transfer technique. The genera of fungi isolated from both samples were: *Aspergillus, Candida, Penicillium, Rhizopus, Saccharomyces*, *Cladosporium, Fusarium* and *Mucor. Cladosporium, Fusarium*, and *Mucor* were isolated only from the sediment samples. Among the genera of fungi isolated, *Aspergillus* had the highest frequency of occurrence 36.84% and 27.59% while *Rhizopus* had the least frequency of occurrence 5.26% and 3.45% for water and sediment samples respectively. Total heterotrophic count for water and sediment samples ranged from $1.9x10^3$ to $2.3x10^4$cfu/ml and $3.4x10^4$ to $3.8x10^4$cfu/g while hydrocarbon utilizing fungal count ranged from $1.0x10^2$ to $3.4x10^3$cfu/ml and $2.6x10^2$ to $5.7x10^3$cfu/g respectively. Similarly, it was observed that in both samples the total heterotrophic fungal counts were higher than hydrocarbon utilizing fungal counts which indicated chronic pollution of the sites sampled and availability of other sources of nutrient other than hydrocarbon. Species of these fungal genera isolated are known to secrete extracellular enzymes which aid in bioremediation. Under optimal environmental and nutritional conditions, the isolated fungi could be useful in the bioremediation of hydrocarbon polluted sites.

Keywords: Bioremediation, extracellular enzymes, fungi, hydrocarbon.

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Introduction

Microorganisms play diverse roles in biotechnology; one of such roles is bioremediation (the use of living things in the cleanup of polluted environments). Bacteria and fungi are among the major groups of microorganisms widely used in biotechnological applications, the former are easily manipulated genetically while the latter exhibit diverse growth pattern such as secretion of extracellular enzymes and invasive mode of growth. Artificial and natural release of petroleum and related products into the environments endanger aquatic and terrestrial life forms. Implications of such release include: devegatation, contamination of potable water sources, fall in reproduction of both plants and animals due to disruption in food chain, and death of plants and animal inhabiting the polluted environments (Okpokwasili and Nnorom, 1990; Chikere and Azubuike, 2013).

Technologies such as mechanical force, burying, evaporation, dispersant application, and washing have been applied in remediation of polluted environments. Nevertheless, bioremediation technologies offer greater advantages owing to its cost effectiveness and environmental friendliness (Pothuluri and Cerniglia, 1994; April et al., 2000; Jacques et al., 2008; Hesham et al., 2009; Das and Chandran, 2010). The success of bioremediation (science based on the knowledge of biodegradation) depends on complex sets of environmental and nutritional factors and some of these factors include: temperature, pH, oxygen, climatological conditions, nutrient availability, presence of alternative carbon sources, physical state of the oil, and the presence of microorganisms with appropriate

metabolic capabilities (Sabate et al., 2004; Claudia et al*.,* 2005; Okpokwasili and Oton, 2006; Das and Chandran, 2011; Chikere et al., 2011; Chikere and Ughala, 2011; Chikere and Azubuike, 2013).

Fungi have proven useful in bioremediation of polluted environments and among their features which enable them to play great role in bioremediation are: secretion of extracellular enzymes, ability to grow under stressed environmental conditions (low nutrient, pH, and water activity), extension in biomass location through hyphal growth, easy and rapid growth on agricultural or forest waste, and other enzyme systems (Obire and Putheti, 2008; George-Okafor et al., 2009). Fungi are known to secrete extracellular enzymes during biodegradation, such inherent capability make fungi to initiate primary attack of more complex and recalcitrant pollutant thereby facilitating secondary attack by bacteria. Futhermore, some fungal mycelia penetrate oil, by such means surface areas are increased for biodegradation and bacterial attack. Fungal genera (*Amorphoteca, Neosartorya, Talomyces*, and *Graphium*), yeast (*Candida, Yarrowia*, and *Pichia*) and terrestrial fungi (*Aspergillus*, *Cephalosporium,* and *Penicillium*) have been implicated in hydrocarbon degradation (Chaillan et al., 2004; Singh, 2006; Das and Chandran, 2011).

The aim of this study was to isolate and characterize hydrocarbon utilizing fungi from hydrocarbon polluted sites using vapour phase transfer technique with Okono medium crude oil as carbon source.

Materials and Methods

***Sampling*:** Sediment and water samples polluted with hydrocarbon were used for the analyses. Four different sediment samples were aseptically collected with Eckman grap from hydrocarbon polluted sites in Ogala-Bonny, Rivers State and were labelled SS_1, SS_2, SS_3, and SS_4, while the water samples were collected in sterile test tubes from discharge points of four different flow stations in Delta State and were also labelled WS_1, WS_2, WS_3, and WS_4. The samples collected were transported to laboratory and held at 4ºC until analyses were carried out.

Isolation of hydrocarbon utilizing fungi: Each of 1.0g of sediment and 1.0ml water samples were aseptically diluted in nutrient broth, following this, 0.1ml aliquots of each 10-fold serially diluted sample was transferred into triplicate plates of Rose-Bengal Chloramphenicol (RBC) agar and Bushnell-Hass (mineral salt) agar supplemented with 0.05% (v/v) streptomycin. In order to screen for hydrocarbon utilizing fungi, sterile Whatman filter papers soaked in Okono medium crude oil were aseptically placed into the lids of each inoculated Bushnell-Haas agar plates; this technique is called the vapour phase transfer (Chikere and Azubuike, 2013). After the inoculation procedures, RBC agar plates and Bushnell-Haas agar were incubated at 30ºC for 7days and 14 days respectively.

Total heterotrophic fungal counts were obtained from the inoculated RBC agar after incubation while colonies on Bushnell-Haas agar plates were further purified by subsequent subculture on RBC agar and final subculture on nutrient agar plates. The purified fungal isolates were identified based on their morphological characteristics (Barnett and Hunter, 1982; Malloch, 1997).

Results

In this work, it was observed that culture-dependent preliminary screening of hydrocarbon polluted sites revealed different genera of fungi which utilized the hydrocarbon as substrate for cellular activities. The two samples (sediment and water) used in this study were analyzed for yeast and filamentous fungi capable of utilizing hydrocarbon as their sole carbon source by plating on Bushnell-Haas (mineral salt) medium with Okono medium crude oil as the only carbon source.

A Total of forty-eight hydrocarbon utilizing fungal isolates were obtained, twenty-nine from sediment samples and nineteen from water samples respectively. The isolates covered eight fungal genera namely: *Aspergillus, Candida, Cladosporium, Fusarium, Mucor, Penicillium, Rhizopus,* and *Saccharomyces* (Tables 1).

It was observed that *Aspergillus* and *Penicillum* dominated with 31.25% and 22.91% frequency of occurrence respectively (Fig. 1). The total heterotrophic fungal (THF) counts ranged from $1.9x10^3$ to $2.3x10^4$cfu/ml and $3.4x10^3$ to $3.8x10^4$cfu/g, while hydrocarbon utilizing fungal (HUF) counts ranged from $1.0x10^2$ to $3.4x10^3$cfu/ml and $2.6x10^2$ to $5.7x10^3$cfu/g for water (cfu/ml) and sediment (cfu/g) samples respectively (Tables 2 and 3).

The water sampled sites were found to be richer with *Aspergillus* sp. than any other fungal genera whereas *Rhizopus* sp. was isolated only from WS_4 (Tables 4). Similarly, *Aspergillus* sp. was isolated from all the sediment sampled sites while *Rhizopus* sp. and *Saccharomyces* sp. were isolated from only one sediment sampled sites SS_1 and SS_2 respectively (Table 5).

Discussion

It has been reported by several researchers that continuous discharge of crude oil into the ecosystem may result in selective increase or decrease in microbial population (Okpokwasili and Nnubia, 1995; Okpokwasili and Odokuma, 1996). The fungal genera isolated from this study have been implicated in degradation of hydrocarbons such as crude oil, polyaromatic hydrocarbons and refined petroleum (Prince, 2005). The co-existence of different fungal isolates belonging to different genera was attributed to the concept of co-metabolism, a form of microbial interaction involving simultaneous degradation of two compounds. Heshman et al., (2006), in their study observed that *Penicillium anomala* could only degrade five ring benzo(a)pyrene through co-metabolism.

Table 1. Colonial and microscopic morphologies of the eight fungal isolates from both sediment and water samples.

S/N	Colonial morphology	Microscopic observation	Tentative identity
1	Greenish-yellow mycelium	Unbranched conidiophores, swollen apex with aseptate hyphae	*Aspergillus* sp.
2	Whitish felt mycelium	Branched conidiophores, smooth and rough conidia in pairs and chain	*Fusarium* sp.
3	Yellowish-green mycelium	Branched cell, smooth conidia in long chain	*Penicillium* sp.
4	Grayish velvety mycelium	Septate hyphae with lateral and terminal conidiophores bearing long branches of smooth walled and pointed conidia	*Cladosporium* sp.
5	Dark-brownish mycelium	Swollen rhizoid, sporangiophores arose without rhizoids	*Rhizopus* sp.
6	White/creamy smooth colonies	Budding yeast with no hyphae	*Saccharomyces* sp.
7	Milky, circular, and mucoid	Circular spores, no hyphae and no caedidial	*Candida* sp.
8	Whitish-gray mycelium	Branched sporangiophore, beards round terminal spore-filled sporangia	*Mucor* sp.

Table 2. Fungal counts from water samples

Sample	THF (cfu/ml)	Log10 (cfu/ml) THF	HUF (cfu/ml)	Log10 (cfu/ml) HUF
WS_1	$1.9x10^3$	3.28	$1.0x10^2$	2.00
WS_2	$1.6x10^4$	4.20	$1.1x10^3$	3.04
WS_3	$2.3x10^4$	4.36	$3.4x10^3$	3.53
WS_4	$2.8x10^3$	3.45	$2.6x10^2$	2.41

Prenafeta-Boldú et al., (2002), also reported in their study: “substrate interactions during the biodegradation of benzene, toluene, ethylbenzene, and xylene (BTEX) hydrocarbon by fungus *Cladophialophora* sp. strain T1” that neither benzene nor xylene was able to supported growth as single substrate, however the latter were successfully co-metabolized in the presence of toluene. Such observation and several others alike are in accordance with the standing hypothesis in microbial ecology that “effective bioremediation rely on the action of microbial consortia rather than on the action of single microorganism (Wackett and Hershberger, 2001). Synergistic form of microbial interaction exists between bacteria and fungi for mineralization of aromatic hydrocarbons in an acidic soil (Stapleton et al., 1998).

The predominance of filamentous fungi such as *Aspergillus* spp. and *Penicillium* spp. in crude oil polluted environments have been reported by several researchers (April et al., 2000; D’ Annibale et al., 2006; George-Okafor et al., 2009; Das and Chandran, 2011). In addition, *Candida* spp. has been reported to posses Cytochrome P450 monooxygenase systems which incorporate molecular oxygen into aliphatic hydrocarbons thus facilitate in effective bioremediation of hydrocarbon polluted environments (Das and Chandran, 2011). The higher HUF counts observed in SS_2 ($5.7x10^3$cfu/g) was attributed to the closeness of the site to petroleum industry; such closeness resulted in an increase in

total hydrocarbon content which in turn increased microbial activities. This observation was in line with (Eze and Eze, 2010) that: "the presence of excess hydrocarbon is considered a positive factor in biodegradation process. In all the microbial counts, it was observed that THF counts were greater than HUF counts which indicated that both samples sites were chronically polluted with hydrocarbon and the possible presence of other carbon sources other than hydrocarbon.

Table 3. Fungal counts from sediment samples

Samples	THF (cfu/g)	Log10 (cfu/g) THF	HUF (cfu/g)	Log (cfu/g) HUF
SS_1	$4.6x10^3$	3.66	$3.8x10^2$	2.58
SS_2	$3.8x10^4$	4.58	$5.7x10^3$	3.76
SS_3	$3.4x10^3$	3.53	$2.6x10^2$	2.41
SS_4	$2.9x10^4$	4.46	$1.4x10^3$	3.15

Table 4. Generic richness of fungal isolates from water samples

Fungal genera	WS_1	WS_2	WS_3	WS_4	Total
Aspergillus	++	+	+++	+	7
Penicillium	+	+	++	+	5
Saccharomyces	-	++	++	-	4
Candida	-	+	-	+	2
Rhizopus	-	-	-	+	1
Total	3	5	7	4	19

+: number of fungal genera isolated from each sample site.

Table 5. Generic richness of fungal isolates from sediment samples

Fungal genera	SS_1	SS_2	SS_3	SS_4	Total
Aspergillus	++	+++	+	++	8
Fusarium	-	++	-	+	3
Penicillium	-	+++	+	++	6
Cladosporium	+	-	+	-	2
Rhizopus	+	-	-	-	1
Saccharomyces	-	++	-	-	2
Candida	++	-	++	-	4
Mucor	-	+	-	+	2
Total	6	11	5	7	29

+: number of fungal genera isolated from each sample site.

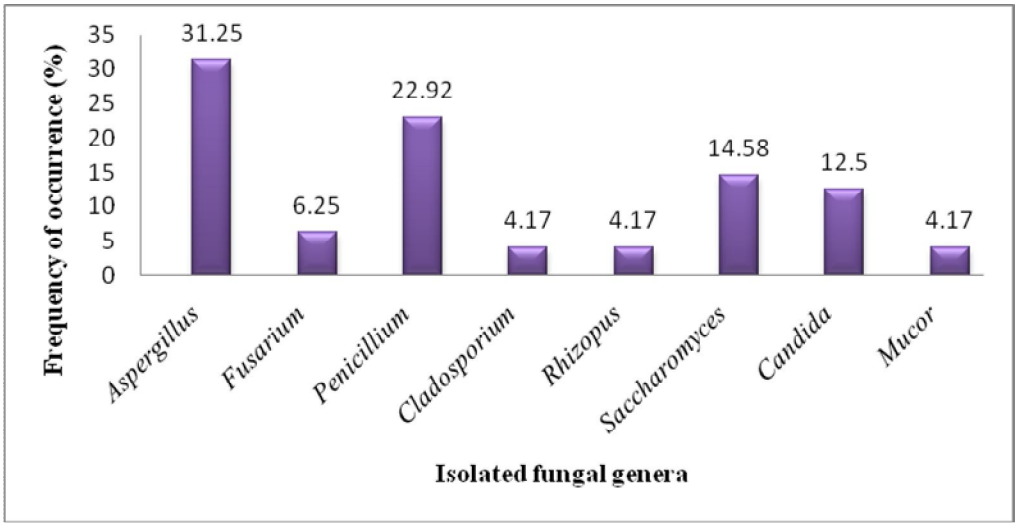

Fig. 1. Frequency of occurrence of fungal genera isolated from sediment and water samples.

Conclusion

The study revealed several genera of fungi which utilized hydrocarbon as carbon source in Ogala-Bonny, Rivers State and discharge points of four different flow stations in Delta State. Having been isolated from hydrocarbon polluted sites, under optimal environmental and nutrition conditions these fungi could be useful in the bioremediation of hydrocarbon polluted sites.

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