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Isolation and characterization of hydrocarbon–utilizing fungi from fresh water swampy soil

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ABSTRACT

Enumeration, isolation and characterization of total fungi and hydrocarbon-utilizing fungi (HUF) were carried out on fresh water swamp forest soil collected at three depths. The ability of HUF to grow on mineral salt medium spiked with Bonny light crude oil was investigated as well as changes in pH of test media for three weeks. Fungal growth was measured by visual estimation of mycelial tissue, while pH was measured with a pH meter. Result indicates that total fungi and HUF increased from surface to sub-surface soil levels. HUF count ranged from 2. 27 ± 0.30 × 10^3 to 4.2 ± 1.08 × 10^3 cfu/g. Growth of *Mucor* species was profuse while *Penicilium* had sparse growth in the culture medium. pH of growth medium ranged from 5.5 to 5.8 and 5.4 to 6.0, thus lessening of acidity and toxicity due to petroleum hydrocarbon. Analysis of variance however indicated lack of significant difference between both fungal count and pH changes following hydrocarbon utilization ($p > 0.05$). Therefore, using petroleum hydrocarbon as carbon and energy sources by both fungi genera, which resulted in slight reduction of acidity implies that both species are suitable candidate genus for preparation of probiotics for stimulating the biodegradation of oil in vulnerable terrestrial environments and hence lessening of toxicity due to oil spill on arable land.

Keywords: Hydrocarbon-utilizing fungi, biodegradation, penicilium, mucor, hydrocarbon, pH.

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INTRODUCTION

The release of hydrocarbon into soil and water environment promotes the growth and proliferation of hydrocarbon utilizing microorganisms (HUM), which includes both bacteria and fungi. Incidentally, these hydrocarbon utilizing bacteria (HUB) and hydrocarbon utilizing fungi (HUF) are also the organisms that are responsible for the biodegradation and eventual clean up of oil spills in our environment (Atlas, 1981; Okpokwasili and Amanchukwu, 1988; Van Hamme et al., 2003; Dollah, 2004; Hamamura et al., 2006).

According to Leahy and Colwell (1990) and Atlas and Bartha (1992), microbial communities exposed to hydrocarbons become adapted, exhibiting selective enrichment and genetic changes. Ability of fungi and yeasts to utilize petroleum hydrocarbon as sole carbon and energy sources has been documented (Okpokwasili and Okorie, 1988; Adekunle and Adebambo, 2007; Al-Nasrawi, 2012). These independent reports confirm that moulds and yeast capable of growth on petroleum play active role in oil degradation in aquatic and terrestrial spills.

Although, fungi have been demonstrated to have petroleum hydrocarbon degradation potentials, the question of effectiveness or suitability of the genera and species for enhanced oil degradation for cleaning polluted environment has not been addressed. If seeding to clean oil spill in an environment is to be effective, then the use of organism with proven degradation capability is a sine qua non. In this paper, hydrocarbon utilizing fungi (HUF) from swamp forest soils were isolated and characterized and their ability to utilize crude oil was measured by the growth pattern in mineral salt agar (MSA) enriched with crude oil. pH changes accompanying fungal growth in crude oil medium was also determined in order to determine the extent to which toxicity of hydrocarbon due to high acidity can be

minimized.

MATERIALS AND METHODS

Collection of soil sample

Sandy loam soil sample was collected from a fresh water swamp area in Obrikom, Ogba Egbema Ndoni Local Government Area of Rivers State, Nigeria at three depths, 0 to 15 cm, 15 to 30 cm and 30 to 45 cm respectively using a hand held auger borer. Soil was placed in a sterile Ziploc bag and labeled accordingly and immediately transported to the laboratory.

Enumeration of HUF

Enumeration of fungi that are capable of utilizing hydrocarbons as their sole source of carbon and energy was carried out using standard method (APHA 9215C/9610C and ASTM 5465-93 spread plate method). Mineral salt agar (MSA) was weighed and prepared according to manufacturer's specification by dissolving the specified quantities of the listed mineral salts for fungal growth in deionized water. MSA was sterilized at 121°C for 15 min at a pressure of 15 psi and allowed to cool to about 43 to 47°C. 1.0 g of chloramphenicol antibiotics solution was added into each plate. MSA was aseptically poured into plates and swirled gently, allowed to solidify. 0.1 ml of each sample dilution was inoculated into each plate and spread using a sterile glass rod. Filter paper moistened with bonny light crude oil was placed in the lid of each plate. Plates were inverted and the edges sealed with masking tape to increase vapour pressure of the hydrocarbon which provides carbon source. Plates were incubated at 35°C for 3 to 7 days. Colonies that developed was counted and expressed as cfu/100 g soil.

Isolation and characterization of HUF

Distinct colonies which grew on MSA plates were isolated and purified on sterile plates of potato dextrose agar (PDA) enriched with bonny light crude oil and incubated for another 3 to 7 days. Two purified colonies were then transferred onto PDA slants and characterized to generic level.

Characterization of isolates

Characterization of the two isolates was done by determining the colonial morphology, cell micro-morphology and ability to ferment carbohydrate with or without gas production Gerhardt et al. (1981). Colony colour, texture, shape and surface appearance was determined by examining developed colonies on PDA plates, while cell micro-morphology was done by the methylene-blue method (Olds, 1983). Biochemical test was performed to determine ability to ferment carbohydrates such as glucose, fructose, lactose, sucrose, galactose, maltose and mannose (Odu, 1978; Holt, 1994). Identification to the generic level was performed using the key provided by Olds (1983).

Hydrocarbon utilizing ability of isolates was determined by visualizing the growth pattern and measurement of pH changes of medium with time (in weeks).

Statistical analysis

One way analysis of variance (ANOVA) was used to ascertain whether there was significant difference in fungal counts in both surface and sub-surface soil samples as well as changes in pH of test medium following hydrocarbon utilization by *Penicilium* and *Mucor* species.

RESULTS AND DISCUSSION

Table 1 shows the fungal load of surface and sub-surface soils under consideration.

Total fungi in soil sample ranged from $1.7 \pm 0.31 \times 10^5$ to $2.6 \pm 0.9 \times 10^5$ from topsoil to soil at 30 to 45 cm depth. On the other hand, total hydrocarbon-utilizing fungi ranged from $2.7 \pm 0.31 \times 10^5$ to $4.2 \pm 1.1 \times 10^5$ from topsoil to soil at 30 to 45 cm (Table 1). ANOVA statistics did not indicate any significant difference in fungal counts at both surface and sub-surface levels. In a related experiment, Onifade and Abubakar (2007) reported higher HUF in crude oil polluted soil than crude oil free soil. Increase of microbial load with depth of soil have been confirmed and attributed to migration of the oil downward (Leahy and Colwell, 1990). Adaptation of these HUF to hydrocarbon increases with exposure (Margesin et al., 2003; Head et al., 2006; Hamamura et al., 2006).

Table 2 summarizes the result of tests leading to the characterization and identification of fungal isolates. Isolates I and IV have similar morphology and biochemical characteristics and belong to the *Penicilium* genus while isolates II and V have characteristics typical of *Mucor* species. There was no growth observed by plates incubated with Isolate III. *Penicilium* species (isolates I and IV) are differentiated by colonial morphology, and gas production from glucose and maltose. While *Penicilium* fermented glucose with gas production, *Mucor* species weakly fermented glucose without gas production. Isolate III could not be identified because of lack of visible growth on culture medium.

Using mildly agitated liquid culture, hydrocarbon utilization by isolated fungi was accompanied by growth of the mycelial mat on the surface of the medium. Under the conditions provided, *Mucor* species grew more than the *Penicilium* species and utilization of base oil resulted in changes in pH of medium as shown in Figure 1. The pH of test medium increased following utilization of the hydrocarbon. Mean pH following utilization and subsequent degradation of crude oil for *Penicilium* and *Mucor* are 5.47 ± 0.003 and 5.53 ± 0.02 respectively while control had pH of 5.77 ± 0.6 (Figure 1). There was no significant difference in the pH of medium utilized by both fungal genera ($p > 0.05$).

The observed profuse growth of *Mucor* species within the given conditions of this experiment and its presence in most organic food wastes in the environment would suggest a wide versatility in organic matter utilization and genetic flexibility which concomitantly translates to its ubiquity. An increase in mycelial growth following utilization of petroleum hydrocarbon was reported by Al-Nasrawi (2012). Ability of these two fungal genera to

Table 1. Fungal load of crude oil impacted soil.

Soil depth (cm)	Total fungi (cfu/g × 10^5)	Hydrocarbon utilizing fungi (cfu/g × 10^5)
0 - 15	1.47 ± 0.31	2.7 ± 0.30
15 - 30	2.27 ± 0.09	3.2 ± 0.066
30 - 45	2.57 ± 0.95	4.2 ± 1.08

Table 2. Characterization and identification of isolates.

Isolate	Colony morphology	Micro-morphology	Fru	Gal	Glu	Lac	Mal	Man	Suc	Identity
I	Flat concentric colonies with radial furrows and a greyish green powdery surfaces	Short branches of conidiophores bearing sterigmata and produce chains of conidia, general appearance like brush	++	++,G	++,G	±	++	+	±	*Penicillum* sp.
II	Conidia are loose cotton-wool-like aerial mycelia, grey at first, dark later	No rhizoids, non septate mycelia, dark globose sporangia scattered over the myclelia	++,G	++,G	++	++	++,G	++	++	*Mucor* sp.
III	NG	-	-	-	-	-	-	-	-	NA
IV	Smooth powdery colonies with greenish grey surface	Has short filaments with conidiophores	++	+,G	++,G	±	+	+	+	*Penicillum* sp.
V	Loose conidia with dark grey colour	Mycelia is aseptate and has no rhizoid	+	++,G	+	+	+,G	+	++	Mucor sp.

Key: ++: acid; ++,G: acid gas; +: weak fermentation; ±: very weak fermentation; NG: no growth.

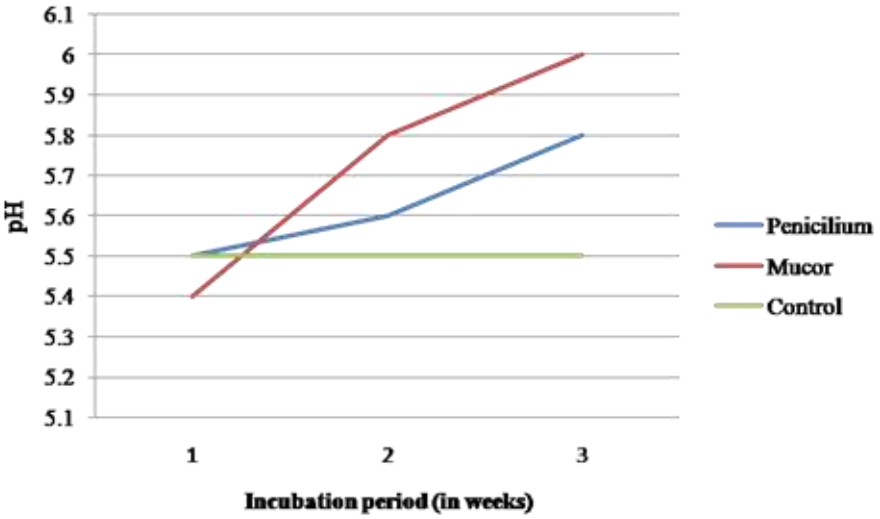

Figure 1. Growth of the hydrocarbon-utilizing fungi and change in pH of test media.

degrade petroleum has been previously reported (Okpokwasili and Okorie, 1988; *Wemedo* et al., 2002; Aj-Jahwari, 2014).

Hydrocarbon utilization is usually accompanied with pH changes. Oboh et al. (2006) reported an increase in total viable count with a decrease in pH of culture media. In a

related study, Al-Jawhari (2014) noted that the pH of all four fungal cultures including *Peniciluim* culture decreased after 28 days incubation, ranging from 7.0 to 5.1. The increase in pH of growth medium in this study (Figure 1) can be attributed to production of lesser acidic metabolites by secondary invaders-bacteria, made possible by initial fungal attack of the toxic petroleum hydrocarbon. This is because mycelial organisms can penetrate soluble fractions of petroleum hydrocarbon thus increasing the surface area available for bacterial attack. Besides, fungi can grow in environmentally stressed conditions such as low pH and poor nutrient status. The potential of fungi in the utilization and degradation of petroleum hydrocarbon and eventual oil spill cleanup has been established and polluted soils are vast reservoirs for these groups of beneficial microorganisms.

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