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Physicochemical Characteristics and Mycoremediation of Ejamah-Ebubu Oil Spill Site located at Eleme Local Government Area in Rivers State, Nigeria

***[1]AKOMAH, ON; [2]ABU, GO**

*[1]Microbiology Technology option,School of Science laboratory Technology, [2]Department of Microbiology, University of Port Harcourt, PMB 5323 Choba Port Harcourt, Nigeria. *Email: onyinyechi.akomah@yahoo.com; onyinyechi.akomah@uniport.edu.ng*

ABSTRACT: Mycoremediation is the application of fungi isolate to contaminated sites. The mycological content of Ejamah-Ebubu oil polluted site was carried out. Using composite sampling technique, five sets of samples were collected; $_{At15}$, Bt_{15}, Ct_{15}, Dt_{15}, Et_{15} at depth (0 - 15cm) and Ab_{30}, Bb_{30}, Cb_{30}, Db_{30}, Eb_{30} at depth (15 - 30cm). The parameters analyzed include; pH, conductivity, nitrate, phosphate, sulphate, total heterotrophic fungal count (THF) and total hydrocarbon utilizing fungal count (HUF). The total heterotrophic fungi and hydrocarbon utilizing fungal count for A15 ranges between 5.0 x 10^{3}- 1.5x10^{4}cfu/g and 1.1 x10^{3} - 2.3x10^{3} cfu/g while A30 ranges between 4.0 x 10^{3}- 1.3x10^{4}cfu/g and 3.0 x 10^{3} - 1.3x10^{3}cfu/g. A total of nine fungal isolate were obtained and identified to belong to the genera: *Aspergillus* (44.44%), *Microsporum* (11.11%), *Fusarium* (11.11%), *Penicillium* (22, 22%) *Acremonium* (11.11%). The frequency of occurrence of the isolates have *Aspergillus*>*Penicillium* while *Microsporium,Fusarium* and *Acremonium* are the same. The unique ability of these isolates to adapt to such conditions of petroleum hydrocarbon content in soil can be effectively used in bioremediation of oil impacted areas in the Niger Delta.

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Mycoremediation is a form of bioremediation in which contaminated sites are converted into less contaminated ones by the use of fungal mycelium (Bennet *et al*., 2002). It is a complex and technical area of bioremediation. For the last two decades, Mycologists has employed fungal species in the degradation of organic compounds. Mycoremediation involved the mixing of the vegetative part of a fungus (mycelium) into contaminated soil; placing mycelial mats over toxic sites or/and combination of both. Pollution has significantly affected the ecosystem. Over the past few years the soil is getting more and more polluted due to advancement in technology. Remediation of these polluted soils is not an easy job. Mycoremediation technique has been applied to oil spill contaminated and polluted soil, industrial chemicals, contaminated water and even farm waste (Bennet *et al*., 2002).The cleanup of a contaminated site requires a consortium of microorganisms; bacteria as well as fungi. Fungi have some intrinsic feature that enable them carry out bioremediation. They secret extracellular enzymes, they also have the ability to grow under stress (low nutrient, pH and water capacity) (Obire and Anyanwu, 2009, George *et al*., 2009). Fungi secretion of extracellular substance during biodegradation initiate primary attack of more complex and recalcitrance pollutants thereby facilitating secondary attack by bacteria. Fungi mycelia penetrate oil, increasing surface area for biodegradation and bacteria attack (Chaillan*et al*., 2004).This presence study is aim at determining the physiochemical properties of soil sample and identifying the total heterotrophic and hydrocarbon utilizing fungi from Ejamah-Ebubu oil spill site located at Eleme Local Government Area in Rivers state Nigeria

MATERIAL AND METHOD

Sample Collection: Using composite sampling techniques soil samples were collected from Ejamah-Ebubu oil spill site located at Eleme Local Government Area in Rivers state Nigeria. The soil samples were collected by means of a manually driven clean auger at five (5) sampling points from (0 - 15) cm depth: At_{15}, Bt_{15}, Ct_{15}, Dt_{15}, Et_{15} and depth (15 - 30) cm Ab_{30}, Bb_{30}, Cb_{30}, Db_{30}, Eb_{30}. Samples were transferred aseptically into sterile flasks and transported to the laboratory for analysis.

Total Heterotrophic Fungal Count: This was done using spread plate technique. About 0.1 ml of the 10^{-3} and 10^{-4} dilution of each sample was spread on the surface of Potato dextrose agar (PDA) into which 0.1 ml of lactic acid was added and incubated at 28^{0}C for 5 days. Distinct fungi colonies were counted as cfu/g and sub-cultured into freshly prepared PDA for further identification.

Total Hydrocarbon Utilizing Fungal Count: About 0.1 of 10^{-3} and 10^{-4} dilution were spread onto the surface of freshly prepared acidified mineral salt medium which contain in g/l (0.4g of $MgSO_4$7H2O, 0.29g KCl, 1.25g

**Email: onyinyechi.akomah@yahoo.com; onyinyechi.akomah@uniport.edu.ng*

$KHPO_4$, 0.83 K_2HPO_4, 0.442g NH_4NO_3, 10g NaCl, 15g Agar). A filter paper dabbed with crude oil was inserted under the cover of the Petri plate and incubated at 28^0C for 5 days. Distinct fungi colonies were counted as cfu/g and sub-cultured into freshly prepared PDA for further identification.

Characterization, spore staining and identification of Hydrocarbon utilizing Fungi: The method of Bennet (2002) was adapted for characterization and identification. This include macroscopic examination and microscopic examination. Spore staining procedure was used to confirm the presence of spores. A loop of distinct colonies was emulsified in lacto-phenol cotton blue reagent in a clean glass slid and covered with a cover slip. It was then viewed under the microscope using the X40 objective lens.

Preparation of Fungi Innoculum and Biodegradation of crude oil by fungi isolate: Fungi isolates were innoculated into 100 ml of Potato dextrose broth into which 0.1 ml of lactic acid is added. The setup was incubated at 28^0C for 48 hours. About 5 ml of each fungi isolate were innoculated into 95 ml of potato dextrose broth into which 0.1 ml lactic acid was added. About 1% crude oil was added to the setup and allowed to form a thin layer over the medium surface. The stopper were placed on the flask and each was inverted several times allowing the microorganisms to mix with the oil. The flasks were incubated at 28^0C. Each flask was observed and inverted every 24hours for 5 days and the samples were then taken for estimation of total petroleum hydrocarbon degradation and each observation was recorded in the appropriate table.

pH and Conductivity Measurement: The pH of the sample was determined using the pH meter (Jenway model 015) while the conductivity was determined using conductivity meter (SC-300).

*Salts Content***:** Nutritive salts (nitrate, phosphate and sulphate) were determined by method outlined in APHA (1995). Nitrate was measured using brucine method, phosphate the ascorbic acid method while sulphate turbidometric method. The procedure had been described in earlier research work (Akomah and Abu, 2015).

*Total Petroleum Hydrocarbon and Polyaromatic Hydrocarbon***:** Residual total petroleum hydrocarbons (TPH) and polyaromatic hydrocarbons (PAHs) were extracted from soil sample and quantified using gas chromatograph-FID. Procedure had been described in previous research work (Akomah and Abu, 2015).

RESULTS AND DISCUSSION

pH and Conductivity: The result of the analysis of physico-chemical properties of the sediment sample are shown in Figures 1 and 2. The pH of the various sampling points is neutral, ranges from 7.21 - 7. 82. $(At - Et)_{15}$ recorded a higher pH, indicating decrease in pH as sampling depth increases. The conductivity of sampling points also increases as sampling depth increases indicating the present of ions.

Salts: The nitrate concentration of sampling points ranges from (10.5 - 12.6) mg/kg for $(At - Et)_{15}$ while $(Ab - Eb)_{30}$ ranges from (12.6 - 14.8) mg/kg. Phosphate concentration ranges from (3.7 - 4.4) mg/kg for $(At - Et)_{15}$ while (Ab - Eb)30 ranges from (2.56 - 2.92) mg/kg. The sulphate concentration ranges from (20 - 27) mg/kg for At15 - Et_{15}while $(Ab - Eb)_{30}$ ranges from (16 - 19) mg/kg.

TPH and PAHs Content: The TPH concentration of sampling points range between (15.65 - 31.65) mg/kg while PAHs ranges between (0.059 - 0.117) mg/kg. The concentration of TPH at most sampling point is below/close to the permissible limit (30 mg/kg) as indicated in table 1.

Table 1: Physicochemical properties, Salts, TPH and PAHs of various sampling points

Parameters	Depth	A	B	C	D	E
pH	0 - 15 cm	7.69	7.67	7.59	7.85	7.73
	15 -30 cm	7.47	7.51	7.58	7.59	7.78
Conductivity(µs/cm)	0 - 15 cm	161.3	160.3	158.7	160.7	164
	15 -30 cm	265.7	260	250.3	253.7	262.3
Nitrate(mg/kg)	0 - 15 cm	11.9	10.9	11.23	11.87	11.93
	15 -30 cm	13.53	13.8	13.5	13.37	13.33
Phosphate (mg/kg)	0 - 15 cm	4.1	4.07	4.13	4.0	4.03
	15 -30 cm	2.73	2.66	2.67	2.78	2.89
Sulphate (mg/kg)	0 - 15 cm	26.3	25.7	23.3	24.7	22
	15 -30 cm	17.3	18	17	18	18.3
TPH (mg/kg)	0 - 15 cm	31.49	31.2	31.57	31.65	30.42
	15 -30 cm	15.97	15.75	15.65	15.97	15.87
PAHs (mg/kg)	0 - 15 cm	0.097	0.098	0.098	0.117	0.098
	15 -30 cm	0.066	0.071	0.065	0.059	0.068

Total Heterotrophic Fungal Count: The result obtained for total fungal and hydrocarbon utilizing fungal were shown in table 2. The total heterotrophic fungal count for (At - Et)$_{15}$ ranges between 8.8 x 10^5 - 1.84 x 10^6cfu/g while (At - Et)$_{30}$ ranges between 4.4 x10^5- 1.04 x 10^6cfu/g.

The hydrocarbon utilizing fungal count for (At - Et)$_{15}$ ranges between 3.6 x 10^5 - 1.2 x 10^6cfu/g while (At - Et)$_{30}$ ranges between 5.6 x10^5- 8.8 x 10^5cfu/g.

Table 2: Total Heterotrophic Fungi count

Sample	Days	Dilution	Number of colonies	Fungal count (cfu/g)
(At-Et)$_{15}$	0	10^{-4}	184	1.84 x 10^6
	14	10^{-4}	152	1.52 x 10^6
	28	10^{-4}	136	1.36 x 10^6
	42	10^{-4}	88	8.8 x 10^5
(Ab - Eb)$_{30}$	0	10^{-4}	104	1.04 x 10^6
	14	10^{-4}	88	8.8 x 10^5
	28	10^{-4}	44	4.4 x 10^5
	42	10^{-4}	28	TFTC

Table 3: Total Hydrocarbon Utilizing Fungal Count

Sample	Days	Dilution	Number of colonies	Fungal count (cfu/g)
(At-Et)$_{15}$	0	10^{-4}	120	1.2 x 10^6
	14	10^{-4}	88	8.8 x 10^5
	28	10^{-4}	80	8 x 10^5
	42	10^{-4}	36	3.6 x 10^5
(Ab - Eb)$_{30}$	0	10^{-4}	88	8.8 x 10^5
	14	10^{-4}	56	5.6 x 10^5
	28	10^{-4}	56	5.6 x 10^5
	42	10^{-4}	20	TFTC

Cultural and Morphological Characteristic of fungal isolates: The number of hydrocarbon utilizing fungal isolates with their code were shown in table 4. A total of 9 fungal isolates were obtained. The probably organism are; *Aspergillus niger*, *Microsporumcanis*, *Aspergiluus fumigatus*, *Aspergillus flavus*, *Fusarium* sp., *Penicillium* sp., *Acremonium* sp. The percentage of occurrence has *Aspergillus* sp. (44.4 %), *Microsporum* sp. (11.11 %), *Fusarium* sp. (11.11 %), *Penicillium* sp. (22.22 %), *Acremonium* sp. (11.11 %).

Table 3: Cultural and Morphological Characteristics of Fungal Isolates

S/N	Cultural Characteristics	Microscopic Appearance using Lacto-phenol cotton Blue	Suggested Identification
F1	Black Sporing or Dotted Surface and Yellow crack reverse	The Presence of Septate Hyphae, Long Conidiophores	*Aspergillus niger*
F2	Greyish surface and Pink reserve	Large spindle-shaped muti-segmented Macroconidia with curved ends.	*Microsporum canis*
F3	Brownish surface and yellow reserve	Septate Hyphae with hemisherical vesicles	*Aspergillus fumigates*
F4	Light green, sporulating surface and yellow reserve	Vesicles are globose and phialides are produced directly from the vesicle surface	*Aspergillus flavus*
F5	White cottony surface and light or non-pigment reverse	Hyphae are small and septate and give rise to phialides	*Fusarium* sp.
F6	Greenish surface and light reverse	Hyphae are hyaline and septate and produce brush-like conidiophores	*Penicillium* sp.
F7	Lemon to green surface and light reverse	Vesicle are globose and phialides are produce directly from mutulae	*Aspergillus flavus*
F8	Green surface, velvety to powdery, conidia and light reverse	Hyphae are small and septate	*Penicillium* sp.
F9	Light grey surface and non-pigmented reverse	Small septate that produce single unbranched tube-like phialides	*Acremonium* sp.

*Estimation of Total Petroleum Hydrocarbon degradation (oil and grea*se)**:** The concentration of total petroleum hydrocarbon decreases as the experiment process, indicating loss of oil.

Ejamah-Ebubu oil spill site have been investigated for close to two decades (Amajor, 1984., Abu and Akomah, 2008., Giadom*et al.*, 2014., Zabbey, 2009). In 2007 the physicochemical analysis of the site revealed high concentration of total petroleum hydrocarbon, presence of polyaromatic hydrocarbon and low concentration of nutritive salts (Abu and Akomah, 2008).

The present study show that the nutritive salts are moderate when compared with the concentration of total petroleum hydrocarbon present. Nitrate concentration for top soil (At-Et)$_{15}$ ranges from 10.5 - 12.6 mg/kg while bottom soil (Ab-Eb)$_{30}$ ranges from 12.6- 14 mg/kg. Phosphate concentration for (At-Et)$_{15}$ ranges from 3.7 - 4.4 mg/kg while (Ab - Eb)$_{30}$ ranges from 2.56 - 2.90 mg/kg.

The total petroleum hydrocarbon concentration ranges from 15.57 - 32.21 mg/kg. The chromatogram shows carbon chain C9 - C40 including pristane and phytane. Most of the sample points have TPH concentration lower than the permissible limit for TPH (30 mg/kg).

Polyaromatic hydrocarbon concentration ranges from 0.059 - 0.1 mg/kg. Sixteen different PAHs were detected,

93.75% were among the 16 EPA PAHs; naphthalene, acenaphthylene, acenapthene, fluorene, phenanthrene, anthracene, fluoranthene, pyrene, benzo(a)anthracene, chrysene, benzo(b)fluoranthene, benzo(k)fluoranthene, benzo(a)pyrene, indeno (1, 2, 3-cd)pyrene and dibenzo(a, h)anthracene. About 37.5% benzo(a)anthracene, chrysene, benzo(b)fluoranthene, benzo(a)pyrene, indeno (1, 2, 3-cd)pyrene indeno (1, 2, 3-cd)pyrene and dibenzo(a, h)anthracene are suspected carcinogens and mutagens (IARC, 2007; Chaloupka *et al*., 1993).

Microbial analysis revealed nine isolate belonging to the genera: *Aspergillus niger*, *Microsporumcanis*, *Aspergillus fumigates, Aspergillus flavus, Fusarium* sp, *Penicillium* sp, *Acremonium* . The rate of degradation of crude oil by the isolate had *Fusarium* >*Acremonium*> *Aspergillus*>*Microsporum*>*Penicillium.*

Table 4: Concentration of Total Petroleum hydrocarbon (oil and grease)

Day	Treatment Setup	Oil and grease (ppm)	Percentage Different
0	Water with oil	75000.0	
14	*Aspergillus* sp innoculum with oil	36000.0	52%
	Microsporum sp innoculum with oil	31500.0	58%
	Acremonium sp innoculum with oil	36900.0	50.8%
	Fusarium sp innoculum with oil	17550.0	76.6%
	Penicillium sp innoculum with oil	53550.0	28.6%

Conclusion: The study revealed the presence of hydrocarbon utilizing fungi in the soil sample of Ejamah-Ebubu oil spill site. It is interesting that fungi species are presence at the site because they are considered as primary catalyses for correcting contaminated ecosystem and controlling the flow of nutrients.

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