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Isolation and Identification of BTEX-utilizing Fungi from Soil Polluted with Petroleum hydrocarbons

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ABSTRACT: Global consumption of petroleum and petroleum products draws the public's attention to the fate of petroleum hydrocarbons in the environment, as they can permeate the soil environment in an uncontrollable manner. This study examined the physicochemical properties of soil heavily polluted with petroleum hydrocarbons using standard methods as described by the International Soil Reference and Information Center (ISRIC). Isolation and identification of fungi was carried out using enrichment technique, cultural morphology and microscopic examination. The isolates obtained were tested for ability to utilize BTEX using mineral salts medium (MSM) supplemented with 1% v/v BTEX as the sole carbon source for 7 days. The hydrocarbon-utilizing fungal (HUF) spore counts were determined by direct counting using Neubauer Haemocytometer. The petroleum contaminated soil has a characteristic gasoline odour and black in color. The texture of the soil was observed to be sandy, with slightly alkaline pH (7.8) and a temperature of 22°C. Moisture content (2.16%) and water holding capacity (2.07%) were found to be very low. The soil had a high content of oil and grease (1870 Mg/l), and a very high organic carbon (6.54%) and organic matter (11.28%) contents. Nitrogen (0.93%), Phosphorus (3.84 mg/kg), Potassium (2.05 mEq/100g) and Sodium (53.94 mEq/100g) were all found to be in high quantities, while Calcium (8.0 mEq/100g) and Magnesium (2.40 mEq/100g) were observed to be moderate. On the other hand, the mean heterotrophic fungal count (HFC) was found to be 3.9×10^4 CFU/g and eight fungi were isolated from the soil. All fungal isolates were observed to be molds and identified as follows; *Gliocladium* sp., *Aspergillus flavus*, *Aspergillus terreus* DMW-3, *Penicillium* sp., *Aspergillus terreus* DMW-5, *Trichoderma* sp., *Aspergillus terreus* DMW-7 and *Aspergillus niger*. The highest percentage increase in hydrocarbon-utilizing fungal (HUF) spore counts was observed with *Aspergillus terreus* DMW-5 (98.4%) and *Gliocladium* sp. (98.1%) while the lowest percentage increase in hydrocarbon utilizing fungi was observed with *Aspergillus flavus* (94%). From the findings in this study, it appears that petroleum pollution did not inhibit the growth and variation of fungi in petroleum-contaminated soil. It seems that the fungi used petroleum hydrocarbons as nutrient.

Keywords: Petroleum hydrocarbons, BTEX, Soil, Biodegradation, Fungi.

Running title: Isolation and identification of BTEX-utilizing fungi.

Introduction

Benzene, toluene, ethylbenzene, and xylene isomers (BTEX) are mono-aromatic hydrocarbons found frequently in crude oil and its derivatives (Nagarajan and Loh, 2015). They are important industrial chemicals and are well-known volatile organic compounds (VOCs) of environmental and health concern

(Eldon *et al.*, 2012). The widespread use of crude oil and petroleum products in the world leads to an increasing contamination of the natural environment (Souza *et al.*, 2014; Nwaichi *et al.*, 2015). Petroleum products are a mixture of hydrocarbons which composed of carcinogenic and mutagenic compounds (Agata *et al.*, 2017), they are considered to be among the most toxic and dangerous pollutants in particular compartments of nature, especially in soil, which is the major site of their accumulation (Sutton *et al.*, 2013; Covino *et al.*, 2016; Marchand *et al.*, 2017). They can cause changes in fungal biodiversity in soil which can have an unfavorable impact on the soil health and fertility (Alrumman *et al.*, 2015).

BTEX account for up to 59% (w/w) of the gasoline pollutants and represent about 80% of the volatile organic compounds (VOCs) emissions in petrochemical plants (El-Naas *et al.*, 2014). In addition, they have higher mobility in the environment, either in gaseous, liquid or solid phase, and higher water solubility than other hydrocarbons (Mazzeo *et al.*, 2013). Prolonged exposure to BTEX compounds has adverse effects on human health (e.g., damage the central nervous system) and ecosystem functions (Picone, 2012). Hence, developing or improving current remediation methods that minimize the environmental damages caused by BTEX compounds has drawn the attention of environmental protection agencies (Kamal *et al.*, 2017). Much importance is attached to fungi in bioremediation of soils contaminated with petroleum products, one reason being their adaptability to extreme conditions (Rousk *et al.*, 2010; Agata *et al.*, 2017). In general, fungi demonstrate quite a large range of pH in which they can obtain an optimal growth (Rousk *et al.*, 2010). The biomass of fungi is positively correlated with their diversity (Agata *et al.*, 2017). They respond positively to the input of organic matter to soil (Islam *et al.*, 2011; Mohsenzadeh *et al.*, 2012). They can be effective in the removal of petroleum products from soil with some species of the genera *Aspergillus* (Díaz-Ramírez *et al.*, 2013; El-Hanafy *et al.*, 2017) and *Candida* (Fan *et al.*, 2014; Silva *et al.*, 2015) already exploited. High capacity to tolerate BTEX has also been ascribed to fungal genera such as *Cladophialophora*, *Exophiala*, *Leptodontium*, *Pseudeurotium*, *Cladosporium* (Prenafeta-Boldú, 2001). The ability of fungi to survive in environments contaminated with petroleum products and petroleum fuels alone suggests a reciprocal relationship between these fungi and the products mentioned (Alrumman *et al.*, 2015). Next to bacteria, fungi affect transformations of these products, whereas petroleum products produce some influence on the growth of fungi (Alrumman *et al.*, 2015; Agata *et al.*, 2017).

In view of the above, this study was aimed at isolation and identification of BTEX-utilizing fungi from soil polluted with petroleum hydrocarbons and the objectives' are to determine the physicochemical properties of the soil, enrich and isolate BTEX-tolerating fungi from the soil, identify the fungal isolates and to test for their abilities to utilize BTEX.

Materials and Methods

BTEX Compounds

The BTEX hydrocarbons used in this work comprised a mixture of benzene (99.9% purity, M & B, England), toluene (99.5% purity, BDH, England), ethylbenzene and xylene isomers (99% purity, JHD, China).

Mineral Salt Medium (MSM)

The mineral salt medium (MSM) used consisted of Na₂HPO₄ (2.0 g), K₂SO₄ (0.17 g), NH₄NO₃ (4.0 g), KH₂PO₄ (0.53 g), MgSO₄·7H₂O (0.5 g) and 1.0 mL of a trace salt solution per liter of distilled water (Fatuyi *et al.*, 2012). A stock solution of trace salt containing CoCl₂·6H₂O (30 mgL⁻¹), CuCl₂ (0.15 mgL⁻¹), H₃BO₃ (5.7 mgL⁻¹), MnCl₂·4H₂O (20 mgL⁻¹), Na₂MoO₄·2H₂O (2.5 mgL⁻¹), NiCl₂·2H₂O (1.5 mgL⁻¹), ZnCl₂ (2.1 mgL⁻¹) was prepared (Jian-zhong *et al.*, 2009).

Soil Sample

Soil samples with long history of petroleum contamination were collected randomly from five (5) different points at 0-15 cm depth and 15 m intervals at a mechanic workshop in Dan-Magaji, Zaria,

Kaduna State, Nigeria. The samples were collected using auger into sterile glass bottles and transported to the Environmental Microbiology Laboratory, Department of Microbiology, Ahmadu Bello University, Zaria for further analyses. The soil samples were mixed to make a composite sample, air dried, sieved through 2mm mesh to remove large unwanted soil debris. The samples were then stored in a cool dry place until further use (Prenafeta-Boldu *et al.*, 2004).

Determination of Physicochemical Properties of the soil

The physicochemical property of the soil sample was determined using the standards methods as described by The International Soil Reference and Information Centre (2002). The pH and temperature were determined using method described by (Uyovbisere *et al.*, 2013). Particle size analysis and textural class by the ISRIC (2002) method; moisture content by the evaporation method (ISRIC, 2002); organic carbon and organic matter contents by the Walkley-black procedure (ISRIC, 2002); Nitrogen by the micro-Kjeldahl procedure (Uyovbisere *et al.*, 2013); Phosphorus by the Olsen method (ISRIC, 2002); Potassium (K), Sodium (Na), Magnesium, (Mg), and Calcium (Ca) by total digestion method, using photometer (Sherwood – flame photometer 410) for sodium and potassium and atomic absorption spectroscopy (PG 500) for calcium and magnesium (ISRIC, 2002); oil and grease by the gravimetric method; and water holding capacity using method described by (Machido, 2008). With the exception of Particle size analysis and textural class, all other analysis were carried out in triplicates.

Enrichment and Isolation of BTEX-tolerant Fungi

Ten grams (10g) of the soil sample was suspended in 100mL of prepared mineral salt medium (MSM) and supplemented with chloramphenicol (500 mg/L) to inhibit bacterial growth. The pH was adjusted to 6.5 using 1.0 NH₄Cl, after which it was sterilized with an autoclave. BTEX (1% v/v) was added as sole source of carbon (Jin *et al.*, 2013). The enrichment was performed on a rotary shaker (180rpm) at room temperature for 14 days (Kamal *et al.*, 2017). After that, 10 mL of the enrichment culture was added into 90 mL of freshly-prepared MSM. It was further incubated for 7 days at room temperature on a rotary shaker (180rpm). After incubation period, 1mL of the culture was used to make ten-fold dilutions up to 10⁻⁵ (Prenafeta-Bold'u *et al.*, 2001).

The medium used for the isolation was sabouraud dextrose agar (SDA), prepared according to manufacturer's instructions and supplemented with chloramphenicol (500mg/L) to prevent bacterial growth. Spread-plate method was used to inoculate 0.1mL aliquot of dilutions 10⁻² to 10⁻⁵ onto SDA plates. The plates were incubated at an ambient temperature for 7days. The resulting fungal colonies were enumerated and recorded as colony forming units (CFU) per gram of soil (Prescott and Harley, 2002). Colonies obtained were aseptically sub-cultured into fresh SDA plates and incubated at room temperature for 7 days until pure isolates were obtained. The pure isolates were subcultured on SDA slant in McCartney bottles, incubated at room temperature for 5 days and stored at 4°C in the refrigerator until required (Fatuyi *et al.*, 2012).

Identification of fungal isolates

The fungal isolates were identified based on macroscopic and microscopic examination. Features of 7days old fungal isolates were examined based on colony morphology with regards to shape, color, presence or absence of aerial mycelium, nature of mycelia, texture, growth pattern, presence of wrinkles and furrows as well as pigment production (Nwankwegu *et al.*, 2016). Microscopic examination was carried out by lactophenol cotton blue staining and slide culture techniques as described by Barnett and Hunter (1972). The observations were then compared with a mycological atlas (Larone, 2002).

Preparation and Standardization of Fungal Spore Suspension

This was carried out following the method of Machido *et al.* (2014). The fungal isolates were grown on SDA slants for 5 days to obtain heavily sporulated cultures. The spores were scraped gently using a

sterile inoculating needle under sterile aseptic conditions. Spores suspensions of the isolates were obtained by dispensing 15 mL of sterile distilled water containing 0.005% Tween 80 into an agar slant and shaken properly for 15 minutes to wash off the spores. The spore suspensions were diluted with sterile distilled water to obtain concentration of spores/mL. The spores were standardized by direct counting, using Neubauer Hemocytometer and store in the refrigerator at 4°C until further used.

Assessment of BTEX-utilizing ability of the Isolates

The method of George- Okafor *et al.* (2009) was used to assess the BTEX-utilizing ability of the fungal isolates. An aliquot (1 mL) of standardized spore suspension (1.0×10^6 spores/mL) of the isolates were inoculated into an Erlenmeyer flask (250mL) containing MSM supplemented with BTEX (1%v/v) as sole source of carbon. The control was without inoculum. The set up was incubated at room temperature on a rotary shaker (Griffin Mechanical Shaker- Gallenkamp, England) (180rpm) for 7days. The hydrocarbon-utilizing fungi (HUF) were determined by direct counting using Neubauer Haemocytometer (Bekada *et al.*, 2008; Bekker *et al.*, 2009). This experiment was also carried out in triplicates.

Results

Textural classification and physico-chemical properties of the petroleum-contaminated Soil

The textural classification and physico-chemical characteristics of the soil sample obtained are shown in Table 1. The petroleum contaminated soil has a characteristic gasoline odour and black in color. The texture of the soil was observed to be sandy, with slightly alkaline pH (7.8) and a temperature of 22°C. Moisture content (2.16%) and water holding capacity (2.07%) were found to be very low. The soil had a high content of oil and grease (1870 mg/10g), and a very high organic carbon (6.54%) and organic matter (11.28%) contents. Nitrogen (0.93%), phosphorus (3.84 mg/kg), potassium (2.05 mEq/100g) and sodium (53.94 mEq/100g) were all found to be in high quantities while calcium (8.0 mEq/100g) and magnesium (2.40 mEq/100g) were observed to be moderate (USDA, 1993; DPR, 2002).

Table 1: Textural Classification and Physico-chemical Properties of the Petroleum-contaminated Soil

Properties	*Mean \pm S.E	Level (USDA, Standard)
Particle size analysis corrected to 20 ⁰ C (%)		
Clay	3	
Silt	7	
Sand	90	
Textural Class (USDA, Standard)	Sandy	
pH 1:2.5 (soil/H ₂ O)	7.80 \pm 0.09	Slightly alkaline
Temperature (°C)	22 \pm 2.67	
Moisture content (%)	2.16 \pm 0.56	Low
Water holding capacity (%)	2.07 \pm 0.14	Low
Oil and Grease (mg/10g) (DPR Standard)	1870 \pm 86.3	High (DPR, 2002)
Organic carbon (%)	6.54 \pm 0.13	Very High

Properties	*Mean \pm S.E	Level Standard)	(USDA,
Organic matter (%)	11.28 \pm 0.68	Very High	
Total nitrogen (%)	0.93 \pm 0.02	High	
Phosphorus (mg/kg)	3.84 \pm 0.46	High	
Exchangeable cations (mEq/100g)			
Potassium	2.05 \pm 0.16	High	
Sodium	53.94 \pm 2.87	High	
Calcium	8.00 \pm 0.89	Moderate	
Magnesium	2.40 \pm 0.52	Moderate	

*Sample was analysed in triplicate

Isolation and Identification of BTEX-tolerating fungi from the BTEX-treated soil

The mean heterotrophic fungal count (HFC) was found to be 3.9×10^4 CFU/g and eight (8) fungi were isolated from the soil. All fungal isolates were observed to be molds and identified as follows: *Gliocladium* sp., *Aspergillus flavus*, *Aspergillus terreus* DMW-3, *Penicillium* sp., *Aspergillus terreus* DMW-5, *Trichoderma* sp., *Aspergillus terreus* DMW-7 and *Aspergillus niger* as shown in Table 2.

Assessment of BTEX-utilizing Ability of the Fungal Isolates

All the isolates were able to utilize the BTEX (1% v/v) compounds. This was confirmed by the rate of growth observed among the isolates (Table 3). The highest percentage increase in hydrocarbon-utilizing fungal (HUF) spore counts was observed with *Aspergillus terreus* DMW-5 (98.4%) and *Gliocladium* sp. (98.1%) while the lowest percentage increase in hydrocarbon-utilizing fungal (HUF) spore counts was observed with *Aspergillus flavus* (94%).

Table 4.5: Average Hydrocarbon-utilizing Fungal (HUF) Spore Counts of the Isolates (N=3)

Isolate code	Isolate	Initial spore count (spores/mL)	Final spore count (spores/mL)	Percentage increase in spore count (%)
DMW-1	<i>Gliocladium</i> sp.	1.2 x 10 ⁶	6.3 x 10 ⁷	98.1
DMW-2	<i>Aspergillus flavus</i>	1.2 x 10 ⁶	2.0 x 10 ⁷	94
DMW-3	<i>Aspergillus terreus</i>	1.3 x 10 ⁶	4.2 x 10 ⁷	96.9
DMW-4	<i>Penicillium</i> sp.	1.1 x 10 ⁶	2.4 x 10 ⁷	95.4
DMW-5	<i>Aspergillus terreus</i>	1.3 x 10 ⁶	8.2 x 10 ⁷	98.4
DMW-6	<i>Trichoderma</i> sp.	1.2 x 10 ⁶	5.8 x 10 ⁷	97.9
DMW-7	<i>Aspergillus terreus</i>	1.3 x 10 ⁶	6.1 x 10 ⁷	97.9
DMW-8	<i>Aspergillus niger</i>	1.4 x 10 ⁶	5.2 x 10 ⁷	97.3

Discussion

The soil sample was of sandy texture, with little percentages of silt and clay particles (Table 1). This feature might decrease its capacity to retain nutrients for microbial survival. The particle size of a soil influences the diversity of fungi in the soil (Kakirde *et al.*, 2010) as well as the uptake of such hydrocarbons by indigenous fungi. The moisture content and water holding capacity, which determine the extent of moisture and water retention in the soil, were low in the petroleum contaminated soil. These two properties are important for the growth of biotic components in the soil. A study by Ding *et al.* (2010) found phenanthrene, a polycyclic aromatic hydrocarbon associated with petroleum contamination, as well as other fossil fuels to affect the sorption capacity of the soil. Presence of petroleum contaminants in the soil increases the soil hydrophobicity, reducing the water holding capacity of the soil (Osuji and Nwoye, 2007). The soil had a slightly alkaline pH, which could also play a role in the diversity of fungal population in the soil.

The soil had relatively higher quantity of oil and grease (DPR, 2002), which might be due to long history of petroleum contamination and was similar to the work of Raji *et al.* (2015) who reported a high content (1340 mg/g) of oil and grease in a mechanic workshop soil. Soils get exposed to gasoline, engine oil and diesel through activities such as repair of automobiles, changing of engine oil, etc. The presence of these nutrients (N, P, Na, K, Ca and Mg) in the soil sample might be due to other anthropogenic activities such as animal grazing, animal and human faeces, decayed plants and refuse disposal etc. The organic carbon content in the soil was significantly high (USDA, 1993), which might be due to hydrocarbons from the petroleum contamination. The nitrogen and phosphorous contents of the soil were also high (USDA, 1993). In the presence of high concentration of nitrogen and phosphorus, some microorganisms might be able to grow even with the contaminants present in the environment. Bundy *et al.* (2002)

reported that nutrient balance (C and N), pH and moisture content of soil were usually affected as a result of contamination by hydrocarbons. Petroleum contamination exerts adverse effects on soil conditions, microorganisms and plants (Uche *et al.*, 2011). This leads to deterioration of soil structure, loss of organic matter contents and loss of soil mineral nutrients such as potassium, sodium, calcium, magnesium, nitrogen, sulphate, phosphate and nitrate (Akubugwo *et al.*, 2009). Therefore, altered physico-chemical properties of a soil by petroleum contamination might make it unfit for the growth of normal soil flora.

The heterotrophic fungal counts (HFC) obtained (3.9×10^4 CFU/g) (Table 2) from the BTEX-treated soil was relatively high. This could be due to the reflective adaptive abilities of these fungi to thrive even in the event of deliberate anthropogenic intermittent discharges of petroleum products on the soil over long period of time. Another reason might be the presence of relatively higher quantities of macronutrients, which played a role in the multiplication of these fungi. Nwankwegu *et al.* (2016) in their study on auto-mechanic workshop soils reported a total heterotrophic fungal (THF) count range of 2.0×10^3 CFU/g to 2.7×10^3 CFU/g. In another study on petroleum contaminated sediment by Chikere and Azubuiké (2014), THF count of 3.4×10^3 CFU/g to 3.8×10^4 CFU/g was observed.

A total of eight fungi were isolated and identified as *Gliocladium* sp., *Aspergillus flavus*, *Aspergillus terreus* DMW-3, *Penicillium* sp., *Aspergillus terreus* DMW-5, *Trichoderma* sp., *Aspergillus terreus* DMW-7 and *Aspergillus niger* (Table 2). These fungal isolates belongs to the phylum Ascomycota and were all molds (a group of fungi called hyphomycetes, characterize by having a filamentous hyphae and producing spores as the asexual propagules). The fact that these fungi were isolated from the soil sample was a testimony that they were able to survive in the petroleum contaminated environment and able to tolerate the BTEX enrichments while those that could not survive are eliminated by the unfavorable conditions caused by the oil and the BTEX compounds. It has been reported by several researchers that continuous discharge of crude oil into the environment may result in selective increase or decrease in microbial population (Okpokwasili and Nnubia, 1995; Okpokwasili and Odokuma, 1996; Chikere and Azubuiké, 2014). All these fungal isolates obtained in the present study have earlier been reported as hydrocarbon degraders (April *et al.*, 2000). Cisneros-de *et al.* (2016) and Praveen *et al.* (2016) in their separate works, isolated different species of *Aspergillus* including *Aspergillus terreus*, *Aspergillus flavus*, and *Aspergillus niger* from petroleum contaminated soils. Fatuyi *et al.* (2012) isolated *Penicillium* sp., *Gliocladium* sp. and *Aspergillus* spp. from an oil contaminated site in Akure, Ondo State, Nigeria. Nwankwegu *et al.* (2016) identified *Aspergillus niger*, *Aspergillus fumigatus*, *Penicillium xingjiangense*, *Mucor racemosus* and *Rhodotorula* sp. as engine oil degraders using morphological and microscopic characteristics.

Even though the highest spore counts was observed in the strain of *Aspergillus terreus* DMW-5 (8.2×10^7 spores/mL) and *Gliocladium* sp. (6.3×10^7 spores/mL), the percentage increase in the spore count varied slightly from 94% to 98.4% (Table 3). This showed that the number of fungal spores are relatively similar with functional proficiencies. This points that the comparable spore counts of the fungi are evidence that they all thrived in the presence of the hydrocarbons, therefore studies on the abundance of functional genes for the hydrocarbon utilization will provide more comprehensive information on the metabolic abilities of the isolates (Higashioka *et al.*, 2009; Ding *et al.*, 2010; Korotkevych *et al.*, 2011). Though three different *Aspergillus terreus* were tested and showed varying capabilities of BTEX utilization. This might be of the difference in the strains tested even though they are from the same source. Further studies on molecular characterization of *Aspergillus terreus* will provide more information on the specific strain with the highest ability to utilize BTEX. There are several reports that *Gliocladium* spp., *Aspergillus flavus*, *Aspergillus terreus*, *Penicillium* spp., *Trichoderma* spp. and *Aspergillus niger* isolated from petroleum-contaminated soils were capable of utilizing crude oil components as carbon source (Sakineh *et al.*, 2012; Al-Jawhari, 2014; Chikere and Azubuiké, 2014; Nwankwegu *et al.*, 2016).

Conclusion

The soil sample was of sandy texture; with slightly alkaline pH (7.8). Moisture content (2.16%) and water holding capacity (2.07%) were found to be very low, with high content of oil and grease (1870 mg/10g) while nutrients were relatively high.

Gliocladium sp., *Aspergillus flavus*, *Aspergillus terreus* DMW-3, *Penicillium* sp., *Aspergillus terreus* DMW-5, *Trichoderma* sp., *Aspergillus terreus* DMW-7 and *Aspergillus niger* were isolated from the BTEX-treated soil.

The highest increase in hydrocarbon-utilizing fungal (HUF) spore counts was observed with *Aspergillus terreus* DMW-5 (98.4%) and *Gliocladium* sp. (98.1%) while the lowest HUF spore counts was observed with *Aspergillus flavus* (94%).

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