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Biodegradation Studies of Benzene, Toluene, Ethylbenzene and Xylene (BTEX) Compounds by *Gliocladium* sp. and *Aspergillus terreus*

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ABSTRACT: Benzene, toluene, ethylbenzene and xylene (BTEX) are monoaromatic hydrocarbons found frequently in petroleum and its derivatives; and they are among the most important pollutants of soil and groundwater. This study focused on harnessing the enzymatic capabilities of filamentous fungi *Gliocladium* sp. and *Aspergillus terreus*, dwelling in a petroleum-contaminated soil to degrade benzene, toluene, ethylbenzene and xylene (BTEX) compounds. The biodegradation experiment was carried out using the fungi individually and in consortium in a batch culture containing mineral salts medium supplemented with 1% v/v BTEX. The experiments were carried out in triplicates at room temperature on a rotary shaker (180rpm) for twenty five days and aliquots were taken on a five day interval to determine the hydrocarbon utilizing fungal (HUF) count and residual BTEX in order to monitor the rate of biodegradation. The hydrocarbon utilizing fungal counts were determined by direct counting using a Neubauer Haemocytometer while, the residual BTEX was determined using absorbance values measured using a spectrophotometer and the corresponding concentrations determined from a standard curve. The highest percentage degradation of BTEX was observed with *Aspergillus terreus* (89.1%) while, the least was observed with *Gliocladium* sp. (84.4%). The growth peak was attained on the 15th day in all treatments after which the HUF counts declined. Statistical analysis showed no significant difference (P>0.05) in the mean amounts of BTEX degraded and hydrocarbon-utilizing fungal counts between the treatments. The strains of *Gliocladium* sp. and *Aspergillus terreus* used in this study showed high ability for BTEX degradation thus, they are potential candidates for bioremediation of soils contaminated with monoaromatic hydrocarbons.

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Mono-aromatic hydrocarbons such as benzene, toluene, ethylbenzene and xylene isomers (BTEX) are found frequently in crude oil and its derivatives; and are among the most important pollutants of soil and groundwater. BTEX compounds are released into the environment through accidental spills of diesel fuel or gasoline during transportation and leakages from underground storage tanks and pipelines (Tabani *et al.,* 2016). BTEX are volatile aromatic hydrocarbons responsible for health problems such as irritation, headaches, liver and kidney damage as well as cancer (Gallastegui, *et al.,* 2011). BTEX have been classified by United States Environmental Protection Agency as Group A known human carcinogens (Wang *et al.,* 2013). BTEX are widely used as chemical substances in various industrial processes and significant amount are found in fossil fuels (Mazzeo *et al.* 2010). High mobility of such hydrocarbons in soil–water systems favors their contamination of water resources. Conventional (physical and chemical) treatments remove these contaminants from the environment without destroying or transforming them, leading to an accumulation of toxic residues. Contrarily, biological processes such as bioremediation can remove these contaminants from the environment as well as transform them into harmless substances and are, therefore, regarded as a promising and clean technology, particularly because of their simplicity, low cost and efficacy when compared to other alternatives (Alexander, 1994; Bertin *et al.,* 2007; Massalha *et al.,* 2007). During the past several decades, a number of remediation techniques including biological (e.g., bioventing, phytoremediation etc.), chemical (e.g., chemical oxidation, soil flushing etc.) and physical (e.g., soil vapor extraction, thermal treatment etc.) methods have been developed for the subsurface remediation of BTEX contaminated soil and groundwater systems (Mazzeo *et al*., 2010; Guo *et al*., 2012; Jin *et al*., 2013; Zhang *et al*., 2013; Mahmoodlu, 2014; Firmino *et al*., 2015; Nagarajan and Loh, 2015; Stasik *et al*., 2015; Tabani *et al*., 2016). Among these methods, biological treatment of contaminated soil and groundwater is a well-established technique and is also known as being cost effective and environmentally friendly (Firmino *et al.,* 2015). During the biodegradation process,

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microorganisms are able to directly degrade toxic hydrocarbons such as BTEX into less toxic compounds by consuming the available carbons within the structure of the hydrocarbons (Farhadian *et al*., 2008).

Globally, biological cycles include fungi as ideal decomposers that can decompose almost everything found on earth. Animals, plants or even non-living objects are all readily broken down into simpler constituents by the diverse metabolic activities of fungi. Fungi are cosmopolitan and their spore spread through the air and water that makes possible ubiquitous biodegradation. Fungi generally withstand harsher environmental conditions than bacteria and could play an important role in the degradation of petroleum hydrocarbons in the soil (Prenafeta-Boldu *et al.,* 2002). Fungi have received considerable attention for their bioremediation potential due to nature of the enzymes they produce; the extracellular lignin-degrading enzymes are capable of oxidizing a wide range of aromatic hydrocarbons. In addition, fungi have advantages over bacteria such as fungal hyphae that can penetrate contaminated soil to reach the pollutants (Hussaini *et al.,* 2008). Chemically, aromatic hydrocarbon molecules have benzene-based structure. As compared to most other cyclic compounds, aromatic compounds are more stable because of sharing of delocalized electrons by pi (π) bonds. There are two major steps involved in the biodegradation of an aromatic molecule; activation of the ring and ring cleavage (Kothari *et al.,* 2004). Generally, the aerobic degradation of monoaromatic compounds follows different metabolic pathways based on the enzyme system present in the microorganism (Cao *et al.,* 2009). The biodegradation of phenol, for example, is initiated by the formation of catechol, which later undergoes ring cleavage either via *Meta* fission or *ortho* fission (Nilotpala and Ingle, 2003) to intermediates of the central metabolism. In view of the above, this study was aimed at using *Gliocladium* sp. and *Aspergillus terreus* isolated from soil polluted with petroleum hydrocarbons for BTEX-degradation and the objectives' are to determine the percentage of BTEX-degradation, Hydrocarbon-utilizing fungal (HUF) spores counts and statistical analysis of the mean values for amount of BTEX degraded and hydrocarbon-utilizing fungal counts using one-way analysis of variance (ANOVA).

MATERIAL AND METHODS

Chemicals: The BTEX hydrocarbons used in this work comprised of a mixture of benzene (99.9% purity, M & B, England), toluene (99.5% purity, BDH, England), ethylbenzene (99% purity, JHD, China) and xylene isomers (99% purity, JHD, China). The extracting solvent, dichloromethane (DCM) is a product of Merck and 98% purity. It was further distilled to obtain higher purity (99.9%) and also of analytical standard.

The mineral salt medium (MSM) used consisted of Na_2HPO_4 (2.0 g), K_2SO_4 (0.17 g), NH_4NO_3 (4.0 g), KH_2PO_4 (0.53 g), $MgSO_4 \cdot 7H_2O$ (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 $CoCl_2 \bullet 6H_2O$ (30 mgL^{-1}), $CuCl_2$ (0.15 mgL^{-1}), H_3BO_3 (5.7mgL^{-1}), $MnCl_2 \bullet 4H_2O$ (20mgL^{-1}), $Na_2MoO_4 \bullet 2H2O$ (2.5 mgL^{-1}), $NiCl_2 \bullet 2H_2O$ (1.5 mgL^{-1}), $ZnCl_2$ (2.1 mgL^{-1}) was prepared (Jian-zhong *et al.,* 2009).

Fungal Isolation: Gliocladium sp. and *Aspergillus terreus* were isolated from petroleum contaminated soil collected from a Mechanic Workshop located in Zaria, Kaduna State, Nigeria; using the standard methods described by Prenafeta-Bold´u *et al.* (2004). Ten grams of soil sample was transferred to 100 mL of Mineral Salt Medium (MSM) in 250ml of conical flask with 1% (v/v) BTEX as the sole carbon source. The flask was incubated at 25^oC on a rotatory shaker at 180 rpm for 14days (Kamal *et al.,* 2017). Then 10 mL of the culture was transferred to fresh MSM containing 1%v/v BTEX and incubated for another 7 days. After two successive cycles of such enrichment, 1mL of the culture was used to make ten-fold dilution, up to a dilution of 10^{-5} (Prenafeta-Boldu *et al.,* 2001). Spread plate method was used to inoculate 0.1mL aliquot of dilutions 10^{-2} to 10^{-5} onto the surface of saboraud dextrose agar (SDA). The plates were incubated at 25^oC for 7days (Prescott and Harley, 2002). The prominent fungal colonies were selected and pure culture obtained for further study. The cultural characteristics of the purified isolates were noted and the microscopic features of the isolates were observed using the wet mount technique (Sharma, 2009). Purified cultures were stored on SDA slants for further characterization.

Lactophenol cotton blue stain was used as mordant to examine the microscopic structures of the isolates, which are recorded and compared to those stated in previous studies (Aneja, 2005). The isolates were screened on both agar and broth medium, with BTEX as the sole source of carbon.

Preparation and standardization of fungal spore suspensions: This was carried out following the method of Machido *et al.* (2014). The fungal isolates were grown on SDA slants for 5days 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 15mL of sterile distilled water containing 0.005% Tween 80 into an agar slant and shaken properly for 15minutes to wash off the spores. The spore suspensions were diluted with sterile distilled water to obtain concentration of spores/mL. The spores were enumerated by direct counting, using Neubauer Hemocytometer.

The standardized spore suspensions (2mL) were inoculated into 5mL of mineral salt medium supplemented with 1%v/v BTEX and 0.1%v/v Tween 80 (George- Okafor *et al.,* 2009) and incubated at 180rpm on a rotary shaker (Griffin Mechanical Shaker- Gallenkamp, England) at room temperature for 96 hrs (Nwankwegu *et al.,* 2016).

Table 1: Experimental Design

*Treatment	Contents of Mineral Salt Medium (MSM)
1	1 % (v/v) BTEX + 1mL (1.0 x 10^6 spores/mL) of *Gliocladium* sp.
2	1 % (v/v) BTEX + 1mL (1.0 x 10^6 spores/mL) of *Aspergillus terreus*
Consortium	1 % (v/v) BTEX + 0.5mL (1.0 x 10^6 spores/mL) of *Gliocladium* sp. and *Aspergillus terreus*, respectively
Control	1 % (v/v) BTEX only

**In triplicates*

BTEX Degradation Experiment by *Gliocladium* sp. and *Aspergillus terreus:* Biodegradation of BTEX compounds by *Gliocladium* sp. and *Aspergillus terreus* was carried out as shown in Table 1. The treatment flasks contained 150mL of Mineral Salts Medium (MSM). The pH was adjusted to pH 6 and supplemented with 1% v/v BTEX as the sole carbon source. An aliquot (1.0mL) of 96hrs prepared spore suspensions (1.0 x 10^6 spore/mL) was inoculated into each treatment flask. The control was left uninoculated. Experiments were carried out in triplicates at room temperature on a rotary shaker (Griffin Mechanical Shaker- Gallenkamp, England) (180rpm) for 25 days (Kamal *et al.*, 2017). An aliquot (5mL) samples were taken aseptically at 5 day intervals (Ijah and Ukpe, 1992). The residual BTEX were extracted using 5mL dichloromethane (DCM) and centrifuged (Griffin-Gallenkamp, England) (5000rpm) for 5minutes. The absorbances were determined at 600nm wavelength using a UV-spectrophotometer (Bausch and Lumb Inc., A CE 303) and concentrations were determined from a prepared standard curve (Nwankwegu *et al.,* 2016). Percentage loss of BTEX by the isolates was analyzed using the formula below. The hydrocarbon-utilizing fungi (HUF) were determined by direct counting using Neubauer Haemocytometer (Bekada *et al.,* 2008; Bekker *et al.,* 2009). The percentage BTEX degradation was calculated as follows: (Manal, 2011).

$$\% \text{ BTEX degradation} = \left[\frac{\text{TBTEX control} - \text{TBTEX treatment}}{\text{TBTEX control}}\right] \times 100$$

Where: TBTEX control represents the total residual BTEX of the control; TBTEX treatment represents the total residual BTEX of each inoculated flask

Statistical Analysis: Means of values obtained for BTEX degradation and hydrocarbon-utilizing fungal (HUF) counts were used to determine whether or no significance difference using one-way analysis of variance (ANOVA) (Microsoft Excel, 2013).

RESULTS AND DISCUSSION

Macroscopic and Microscopic Characteristics of the Filamentous Fungi: Gliocladium sp. (plate I): Colonies grow faster; surface is creamy in colour and velvety in texture. It has a septate hyphae, with branched conidiophore bearing penicillate branches forming a characteristics compact "brush" structure as in *Penicillium* spp. *Aspergillus terreus* (plate II): Colonies are rapidly growing; surface is cinnamon brown and velvety in texture. It has a biseriate hyphae, with short conidiophores compactly in column.

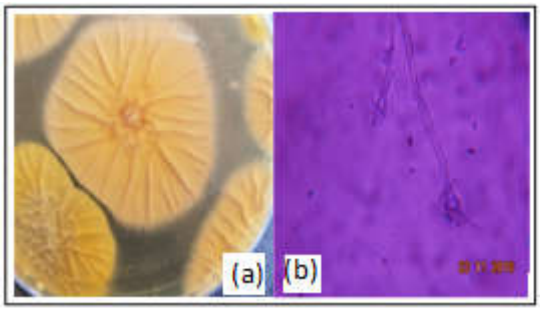

Plate I. Photograph of *Gliocladium* sp. (a) colonial morphology (b) Micrograph of its spores (X1000 magnification)

Plate II. Photographs of *Aspergillus terreus* (a) colonial morphology (b) Micrograph of its spores (X1000 magnification)

Gloicladium sp. and *Aspergillus terreus* belong to the phylum, Ascomycota, and are all moulds (a group of

fungi called hyphomycetes, characterize by having a filamentous hyphae and producing spores as the asexual propagules). The ability of these fungi to survive in the petroleum contaminated soil played a big role in their ability to tolerate BTEX enrichment. Both isolates 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 *Penicllium* sp., *Gliocladium* sp. and *Aspergillus* spp. from an oil contaminated site in Akure, Ondo State, Nigeria.

Biodegradation of BTEX by Gliocladium sp. *and Aspergillus terreus singly and in Consortium*: Reduction in amount of BTEX by the two fungi (singly and in consortium) showed progressive rise in the rate of BTEX degradation (Figure 1). The highest percentage BTEX degradation was observed in the treatment containing only *Aspergillus terreus* (89.1%), followed by the consortium (85.5%) and the least by *Gliocladium* sp. (84.4%). The observed level of significance (0.9197) was greater than 0.05 (p>0.05). This implies that there was no significant difference in the reduction of BTEX between the treatments.

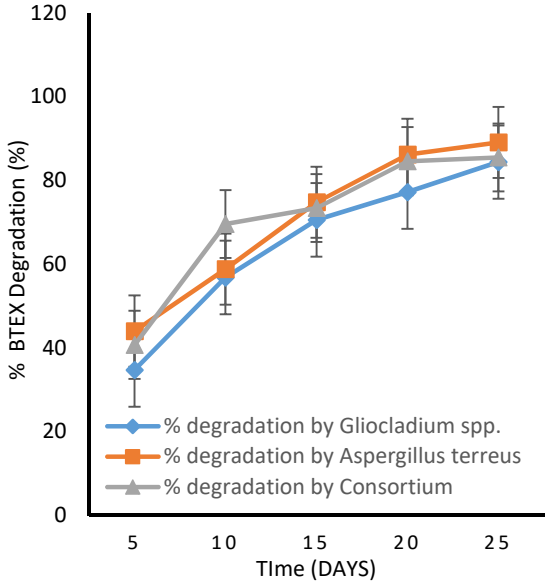

Figure 1: Mean Biodegradation rates of Benzene, Toluene, Ethylbenzene and Xylene (BTEX) by *Gliocladium* sp. and *Aspergillus terreus* (P>0.05; Not statistically significant)

The biodegradation of BTEX by the fungi (singly and in consortium) showed progressive increase in percentage BTEX degradation (Figure 1). The highest percentage degradation was observed with *Aspergillus terreus* (89.1%), followed by the consortium (85.5%) and least percentage reduction was observed with *Gliocladium* sp. (84.4%) after twenty five days. This showed that degradation increased as time progresses in all the treatments. It was also observed that total elimination of the BTEX (100%) was not achieved during the period of the experiment which is often the case in most studies. This is because in a batch culture, as the biodegradation progresses, the source of nutrients which is the hydrocarbon gets depleted consequently leading to a decline in the number of viable fungi. The ability of *Aspergillus terreus* to degrade BTEX better than *Gliocladium* sp. and the consortium might be due to its ability to express more degradative genes, or it may have utilized more than one mechanism to degrade the compounds (e.g. assimilation, emulsification, coagulation, production of biosurfactant). The individual fungus performed more efficiently than the consortium in degrading the hydrocarbons probably because of competition between the two fungal antagonistic effects (secretion of toxic substances by one organism that is detrimental to the other) thus, reducing their collective effectiveness. Fatuyi *et al.* (2012) reported that fungi degraded higher percentage of petroleum hydrocarbons individually than their consortium. This is similar to the work of Sakineh *et al.* (2012) which showed that *Aspergillus terreus* had the higher degradative ability than that of *Penicillium* sp, *Aspergillus niger* and *Aspergillus terreus* in consortium. AI-Jawhari (2014) in a related study on single and mixed culture of fungi reported similar findings; but it contradicts the findings of Nagarajan and Loh (2015), Nwankwegu *et al.* (2016) and Praveen *et al.* (2016), in which the degradation rate was observed to be greater in the consortium than the individual fungi. A similar study by Odili *et al*. (2020), it was observed that *Aspergillus niger* and *Monocillium* sp. degradaed crude oil better when they were used individually than in consortium. The hydrocarbon-utilizing fungal (HUF) counts in the treatments (Figure 2) showed that the growth peak was attained at the 15th day in all treatments (*Gliocladium* sp., 6.3 x 10^{7}spores/mL; *Aspergillus terreus,* 7.9 x 10^{7}spores/mL and in consortium, 8.3 x 10^{7}spores/mL) and then subsequently declined (*Gliocladium* sp., 1.7 x 10^{6}spores/mL; *Aspergillus terreus*, 2.8 x 10^{6}spores/mL and the consortium, 2.2 x 10^{6}spores/mL). The observed level of significance (0.5288) was greater than 0.05 (p>0.05), which showed that there was no significant difference in hydrocarbon-utilizing fungal (HUF) counts between the treatments. The dynamics of fungal growth in all the treatments (Figure 2) showed that the growth peak was attained at the 15th day in all the treatments after which the growth declined. This showed that there was

decrease in the number of spores after the 15th day, which might be due to depletion in the amount of nutrients in the medium as time elapsed. Most likely, there was shortage of nutrients subsequently leading to growth decline.

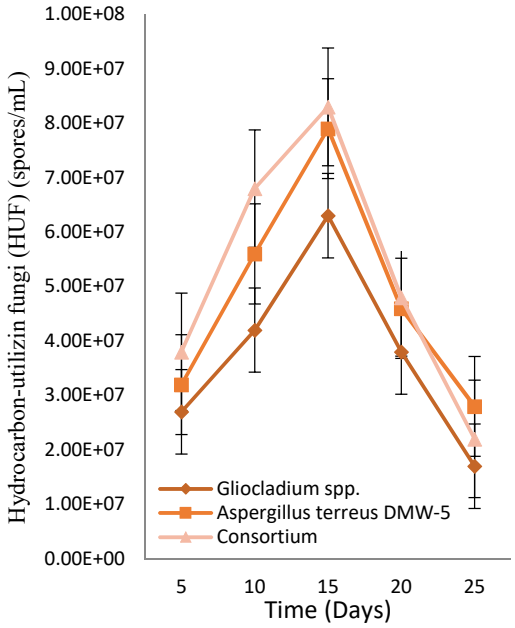

Figure 2: Hydrocarbon-Utilizing Fungal Spore Counts of *Gliocladium* sp. and *Aspergillus terreus* singly and in Consortium (P>0.05; Not statistically significant)

This is similar to the work of Prenafeta-Bold'u *et al.* (2002), who reported that, the fungus, *Cladophialophora* sp. Strain T1 attained its maximum growth on the day 15. Cisneros-de *et al.* (2016) in their work on aerobic degradation of diesel by a pure culture of *Aspergillus terreus* KP862582 also reported that the fungus attained its growth peak on the day 15, after which it entered stationary phase and then declined. AI-Jawhari (2014) also reported that fungi attained their maximum growth on the 14th day in his work on the ability of some soil fungi in biodegradation of petroleum hydrocarbons. This work contradicts with Ukpaka (2017) who reported that the fungi attained their growth peak on the 28th day. The reason for the difference from this work might be due to difference in the conditions of the experiment of the studies. One main reason is that Ukpaka (2017) used the BTEX compounds singly and not in combination (mixture), which is not the case in this study and several others (Prenafeta-Bold'u *et al.* 2002; Abuhamed *et al.* 2004; El-Naas *et al.,* 2014). Statistical analysis of the mean of percentage BTEX degradation and HUF spore counts revealed no significant differences ($P > 0.05$). This might be because the fungi were isolated from the same source and went through the same processes of enrichments, isolation and screening before been selected for this study. Another reason might be because they had been subjected to the same process conditions (nutrients, pH, temperature, agitation, duration etc.). These findings are similar with the findings of AI-Jawhari (2014), who reported that there was no significant difference in the degradative ability of different species of fungi singly and in consortium, but contradicts Sakineh *et al.* (2012), who reported that analysis of variance (ANOVA) showed that the concentrations of remediated hydrocarbons of each of the four fungi were significantly different from other fungi ($P < 0.05$).

Conclusion: The highest percentage BTEX degradation was observed with *Aspergillus terreus* (89.1%) and the least performance was observed with *Gliocladium* sp. (84.4%). Growth peak was attained at the 15th day in all the three treatments (i.e., 6.3 x 10^7, 7.9 x 10^7, 8.3 x 10^7) after which the hydrocarbon utilizing fungi declined (i.e., 1.7 x 10^6, 2.8 x 10^6, 2.2 x 10^6). Statistical analysis showed that there was no significant difference ($P>0.05$) in the mean values obtained for amount of BTEX degraded and hydrocarbon-utilizing fungal counts between the treatments.

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