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Mycorestoration of crude oil polluted soil using *Pleurotus tuberregium*

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Abstract. Crude oil contamination is known to cause unwholesome damage to man, his environment comprising of soil, air and water bodies as well as other forms of life. This study determined the effect of crude oil polluted soils on the composition of different microorganisms and plants and the growth of *Pleurotus tuberregium*. Oil polluted soils in bowls were amended with sawdust from *Brachystegia nigerica* as substrate. Fruiting bodies and the diameter of the mushroom cap were found to increase with increasing weeks of exposure to oil as against the control which had no fruiting bodies throughout the experiment. *Pepperomia pellucida* was found to be the predominant weed (n = 20), followed by *Asystasia gangetica* (n = 11). The bacterial and fungal counts were higher at the oil polluted soil attached to sclerotia than the control. The growth of *Pleurotus tuberregium* in the polluted soil samples showed its ability to degrade and utilize hydrocarbon as the source of carbon and energy, thereby remediating the contaminated soil environment. This work has shown that the fungus has bioremediation and pollution control capacity.

Keywords: crude oil, environmental sustainability, *Pleurotus tuberregium,* sawdust

Introduction

The rapid and progressive development of the oil industry and allied companies have led to the ever increasing release of different environmental pollutants, such as halogenated and polycyclic aromatic hydrocarbons (Prakash,

2017). This has posed a lot of challenges to the environment owing to the high level of contaminants in the environment (Deshmukh *et al*., 2016). This has brought untold hardships to individuals in the affected regions including health issues plus breathing problems and skin lesions; many have been deprived of basic human amenities of life such as food and potable water (Enerijiofi and Ikhajiagbe, 2021).

Bioremediation, which is a cheap and effective process uses several agents such as microorganisms and higher plants as main tools in treating oil spills currently in the environment (Enerijiofi, 2020). However, it is still regarded as an evolving technology because despite the extensive variety of mechanisms by microorganisms, some are still not known (Adenipekun *et al.,* 2013). Mycoremediation which involves the use of fungi mycelia to decontaminate toxic wastes from contaminated areas is gradually gaining ground. Fungi thrive in soils of diverse climatic circumstances as well as the life-threatening ones and proliferate through spores' dispersal in the air as well as assist in sustaining ecosystem equilibrium (Khatoon *et al*., 2021). The variety of habitats and the capability to release lots of enzymes avail fungi as viable nominees for bioremediation at different sites (Zhang *et al*., 2013). The central issue in mycoremediation is to establish the correct species of fungi to aim at the precise pollutant. Fungi such as *Phanaerochaete chrysosporium* and *Polyporus* sp. are capable applicants for bioremediation, since they possess the ability to metabolise greatly different types of recalcitrant environmental pollutants. One main benefit that fungi have over bacterial is that they do not need to get acclimatize to the pollutant of interest (Adenipekun and Lawal, 2012).

The possibility of debasing pollutants that do not require a specific method just as the potential for in situ and ex situ are thoroughly examined as the reward of mycoremediation innovation which utilizes mushroom. The genus *Pleurotus* which belongs to the order *Agaricales* is considered as one of the commercially vital edible mushrooms throughout the world. *Pleurotus* species popularly known as Oyster mushrooms have been generally utilized in the degradation of different organic pollutants due to their applications in biotechnology and environment (Thapa *et al.*, 2012; Mohammadi-Sichani *et al.,* 2017). Crude oil / its derivatives contaminated soils are generally of no value to the farmer due to the attendants' negative effects on soil, soil microorganisms and plants. The study was aimed at degrading crude oil polluted soils with the aid of a very economical and environmentally responsive substrate, *Pleurotus tuberregium* so as to guarantee environmental sustainability.

Materials and methods

Collection of materials

Twenty seven (27) buckets were purchased and washed properly. Sclerotia of *P. tuber-regium* were also bought from Ikpoba Hill market in Benin City. Garden soil was collected from an area beside the Botanical garden, University of Benin, Ugbowo Campus. Waste engine oil was collected from Total Filling Station at Mission Road in Benin City. Sawdust, used as substrate was collected from Ugbowo sawmills in Benin City and the sawdust was wood shavings of *Brachystegia nigerica*. Ten (10kg) of the garden soil was polluted with 1kg of the waste engine oil at 10% w/w. The soils were divided into two batches; Experiment 1 and Experiment 2.

Experiment 1:

Oil polluted soil (at 10%w/w of soil) that is substrate amended with sclerotia (at 6%w/w of soil) and divided into sub Experiments A1-D1.

Experiment 1:

A1: Oil polluted soil + Cubed Sclerotia (all mixed)
B1: Oil polluted soil + Powdered Sclerotia (all mixed)
C1: Oil polluted soil + Powdered Sclerotia as mulch
D1: Oil polluted soil + no Sclerotia.

Experiment 2:

Only oil polluted soil 10%w/w and has no sawdust at all.

A2: Oil polluted soil + Cubed Sclerotia (all mixed)
B2: Oil polluted soil + Powdered Sclerotia (all mixed)
C2: Oil polluted soil + Powdered Sclerotia as mulch
In all there were 7 expts and 2 controls totaling 9x3rep =27bowls.

A total of 10kg of oil polluted soil were obtained and each combination was inoculated with 6% w/w of sclerotia of *P. tuberregium* (0.6kg) both cubed and powdered and then divided into 2 Expts (Expt1 and Expt2).

The Expts 1 were subsequently amended with 2kg of sawdust (i.e. 20% w/w); whereas the Expt 2 was not amended with 2kg of sawdust. The entire set up was left in a screen house for observation.

Determination of mushroom emergence

The emergence of fruiting bodies was carefully observed 3 times a week. After emergence parameters were taken weekly (once a week). The different parameters were checked.

Height of stipe

This was done for the buckets treated with sclerotia. It was carefully observed for increase in height. This was done by using a metre rule to measure from the base of the shoot to the terminated bud and records of the length taken.

Diameter of cap

This was done using a tape to carefully measure the cap of the fruiting bodies for all the buckets respectively.

Number of fruiting bodies

The number of newly emerging fruiting bodies was carefully observed and recorded. Each treatment (in 3 replicates) was counted individually and added together to obtain a mean. The same procedure was also used to determine the number of fruiting bodies with caps.

Number of observable weeds

The number of weeds was determined by counting each weed that appeared in the buckets (> 3cm).

Bacteria and fungi analyses

Soil samples from each replicate were collected and mixed for each concentration. Thereafter the soil was air-dried, sieved and 1g was weighed from each of the different concentrations into the test tubes. Then 9ml of normal saline was added using a syringe and stirred for 30 seconds with the Vortex Genie mixer after which it was covered with foil paper and then allowed to stay for 24 hours (that was the aliquot). Three serial fold dilution was carried out in test tubes containing 9ml of normal saline. Thereafter, aliquot 0.1ml was inoculated into Petri dishes containing already prepared Nutrient agar and 1 tablet of dissolved ketoconazole to inhibit the growth of fungi. This was incubated at 37^{o}C for 24hrs for bacterial growth. Also, 0.1ml aliquot was inoculated into already prepared potato dextrose agar containing

chloramphenicol, which inhibits bacterial growth. The plates were incubated at room temperature of 25^{o}C for 72hrs for fungal growth. The colonies observed for bacterial and fungal were counted thereafter and recorded in CFU/g (Enerijiofi *et al*., 2020).

Identification of bacteria and fungi species

The bacteria were identified according to the method of Holt *et al*. (1994) while the fungi were identified following the protocol of Fawole and Oso, 2001.

Determination of total hydrocarbon content

The total hydrocarbon content of the soil and fruiting bodies were analysed by adding 5ml of n-hexane to 1gram of the different treatments of the soil sample and the fruiting bodies respectively after two months of planting. These samples were placed in a cuvette and passed through a visible spectrophotometer with the wavelength of 460nm where the absorbance was recorded and the total hydrocarbon content was calculated (Mohammadi-Sichani *et al.,* 2017).

Statistical analysis

Data obtained from the analysis were subjected to statistical analysis under descriptive statistics, in a Mean of 3 replicates. Single factor analyses of variance was used to evaluate the data obtained since the soil used in the experiment was homogenized and homogeneity of the entire plot was also assumed to evaluate the data obtained. Means of 3 determiantions were presented in Tables. Mean separation was acheived by using Least significant difference (LSD) where neccessary.

Results

In Table 1, oil polluted with Sclerotia and sawdust, A1 had the highest fruiting bodies with a cap at week 6 of 11.3 while B1, C1, and D1 had no fruiting bodies at weeks 3, 4, 5 and 6. In contaminated soils with Sclerotia, A2 had the highest number of fruiting bodies with a cap at weeks 4 and 6 of 8.3 while B2 had the lowest, 1.3 at week 6. However, the control had no fruiting bodies with caps throughout the experiment. All experimental soil samples did not support mushroom cap at weeks 3 and 4. In week 6, A2 had the highest of 4.5cm while C2 had the least of 2.2cm. It was also noted that no record was

observed for the control throughout the period. For the height of the stipe A1 and A2 had the highest of 8cm at week 6 while C2 had the least of 4.5cm. The weeds that grew in the setup are recorded in Table 2. *Pepperomia pellucida* was the most predominant weed with a count of 16 at control F. *Pepperomia pellucida* was found to be the predominant weed in all the treatments having a count of 20. This is followed by *Asystasia gangetica* with a count of 11. No weed was found in control E as well as treatments A1 and B1.

Table 3 recorded the bacterial and fungal count in contaminated soil after two months of planting *P. tuberregium.* B1 had the highest bacteria count of 3.58 $x10^6$ cfu/g while A1 had the highest fungi count of $7.4x10^5$cfu/g in the sawdust amended soil. However, in soils without sawdust, B2 had the highest bacteria count of $7.0x10^5$ cfu/g while A2 had the highest fungal count of $7.3 x10^5$ cfu/g.

Table 4 above shows the bacterial and fungal identified in different treatments after two months of planting. It was observed that *Bacillus* sp., *Micrococcus* sp. and *Staphylococci* sp. were among the bacterial isolates that were identified in all soil samples while *Aspergillus niger, Penicillium* sp., *Rhizopus* sp. were the predominant fungal isolates. However, *Proteus* sp. was identified in only B1, *Trichoderma* sp. only in C2, *Proteus* sp. in only B1 while *Microsporium* sp. was present in A1 and C2. Table 5 shows the total hydrocarbon present in different soil treatment and fruiting bodies after two months of planting. It was observed that C1 had the highest THC of 1.625ppm while control, F had the least of 0.29ppm. In the fruiting bodies, A2 had the highest THC of 0.955ppm while A1 had the least of 0.395ppm.

Table 1. Growth parameters of mushroom growing in oil polluted soil

Treatments	**Week 3**			**Week 4**			**Week 5**			**Week 6**		
	A	**B**	**C**	**A**	**B**	**C**	**A**	**B**	**C**	**A**	**B**	**C**
Control E&F	0	0	0	0	0	0	0	0	0	0	0	0
A1 (O+X+S)	3.3	0	0	7.3	0	0	9.7	0	3	11.3	4.5	8
B1 (O+X+S)	0	0	0	0	0	0	0	0	0	0	0	0
C1 (O+X+S)	0	0	0	0	0	0	0	0	0	0	0	0
D1 (O+S)	0	0	0	0	0	0	0	0	0	0	0	0
A2 (O+X)	4	0	0	8.3	0	0	8	1.5	2.5	8.3	4.5	8
B2 (O+X)	0	0	0	1	0	0	1	0	0	1.3	2.7	6
C2 (O+X)	4.7	0	0	6.7	0	0	8.3	1.9	2.0	7.3	2.2	4.5

Legend: A= Mean number of mushrooms fruiting bodies with cap, growing in Oil polluted soil
B= Diameter of Cap of the mushrooms growing in Oil polluted soil (cm)
C= Height of mushrooms stipe growing in Oil polluted soil.
O= Oil-polluted soil, X= sclerotia, S sawdust, PS= pure soil

Table 2. Weeds grown from soil seed bank in the Oil polluted soil, with number per species of weed number

Treatments	Weeds Observed	Total
Control E (PS)	No weed	0
Control F (O)	*Pepperomia pellucida* (16) , *Asystasia gangética* (3)	19
A1 (O+X+S)	No weed	0
B1 (O+X+S)	No weed	0
C1 (O+X+S)	*Commelina erecta* (1)	1
D1 (O+S)	*Pepperomia pellucida* (1), *Cyperusspecies* (1)	2
A2 (O+X)	*Asystasia gangetica* (3)	3
B2 (O+X)	*Pepperomia pellucida* (3), *Asystasia gangetica* (2) *Eleucine indica* (2)	7
C2 (O+X)	*Asystasia gangetica* (3), *Cyperusspecies* (3)	6

Legend: O oil-polluted soil, X sclerotia, S sawdust, PS pure soil.
Values presented are means of 3 determinations and rounded off to the nearest integer

Table 3. Microbial load for bacterial and fungi count in oil polluted soil after two months of planting

Treatments	Oil polluted soil		Oil polluted soil attached to sclerotia	
	Bacterial counts ($x10^6$ cfu/g)	Fungal counts ($x10^5$ cfu/g)	Bacterial counts ($x10^7$ cfu/g)	Fungi counts ($x10^6$ cfu/g)
Control E (PS)	1.2	3.6	-	-
Control F (O)	0.77	4.9*	-	-
A1 (O+X+S)	2.34*	7.4*	2.19	4.45
B1 (O+X+S)	3.58*	5.3*	1.79	2.97
C1 (O+X+S)	2.34*	2.8	1.26	3.92
D1 (O+S)	0.66	3.8	-	-
A2 (O+X)	1.51	7.3*	2.46	2.77
B2 (O+X)	0.7	4.5	1.44	5.16
C2 (O+X)	1.15	2.7	1.4	3.57
LSD (0.05)	0.97	1.2	NA	NA
p-value	<0.001	0.004	NA	NA

Legend: O oil-polluted soil, X sclerotia, S sawdust, PS pure soil
*Means are significantly different from the control (PS), $p<0.05$

Table 4. Microorganisms present in the different oil polluted treated soil after two months of planting

Treatments	Oil polluted soil		Oil polluted soil attached to sclerotia	
	Bacterial isolates	**Fungal isolates**	**Bacterial isolates**	**Fungal isolates**
Control E (PS)	*Bacillus sp., Micrococcus letus,S. aureus*	*Penicillium* sp., *A. flavus., Rhizopus oryzea, A. niger, Mucor sp.*	-	-
Control F (O)	*Bacillus sp., Micrococcus sp, S. epidymis*	*Trichoderma* sp., *A. niger, Mucor* sp., *A. flavus, Penicillium* sp., *Rhizopus oryzea.*	-	-
A1 (O+X+S)	*Bacillus sp., Micrococcus sp.*	*Penicillium* sp., *Rhizopus oryzea, Trichoderma* sp., *A. niger.*	*Bacillus sp. Micrococcus* sp	*Rhizopus* sp., *A. niger, Microsporum* sp., *Mucor* sp., *Penicillium* sp.
B1 (O+X+S)	*Bacillus sp., Proteus sp., S. aureus, Micrococcus sp.*	*Candida sp., Penicillium sp., A. niger, Rhizopus* sp., *A. flavus.*	*Bacillus* sp, *Proteus* sp, *S. aureus, Micrococcus* sp.	*A. niger, Rhizopus oryzea, A. flavus, Penicillium* sp.,
C1 (O+X+S)	*Bacillus sp, Staph sp, Micrococcus*	*Mucor* sp., *Rhizopus* sp., *Penicillium* sp., *Tricodermal* sp., *A. niger.*	*Bacillus* sp, *Staph* sp, *Micrococcus*	*Fusarium sp., A. niger, Rhizopus* sp., *Mucor* sp., *Trichoderma* sp., *A. flavus.*
D1 (O+S)	*S. epidymis, Micrococcus*	*A. flavus, Fusarium* sp., *Rhizopus oryzea, Mucor* sp., *Penicillium* sp., *Aspergillus*	-	-
A2 (O+X)	*Bacillus sp, S. aureus, S. epidermidis*	*Rhizopus* sp., *Penicillium* sp., *A. flavis.*	*Bacillus* sp, *S. aureus, S. epidermidis*	*Rhizopus oryzea, A. niger, Fusarium* sp., *Penicillium* sp., *A. flavus*
B2 (O+X)	*Bacillus sp, S. epidermidis*	*A. niger, Trichoderma* sp., *Rhizopus oryzea, A. flavus, Penicillium* sp.	*Bacillus* sp, *S. epidermidis*	*A. flavus, Penicillium* sp., *Rhizopus* sp., *Fusarium* sp., *Mucor* sp., *A. niger.*
C2 (O+X)	*Bacillus sp, Micrococcus sp, S. aureus,*	*Penicillium* sp., *Aspergillus flavus, Trichoderma sp., Rhizopus oryzea, Mucor* sp.	*Bacillus* sp, *Micrococcus* sp, *S. aureus,*	*A. niger, A. flavus, Rhizopus* sp., *Microsporium* sp., *Mucor* sp., *Trichoderma* sp., *Penicillium* sp.

Legend: O oil-polluted soil, X sclerotia, S sawdust, PS pure soil

Table 5. Total Hydrocarbon Content of the soil sample and fruiting bodies after two months of planting

Treatments	Total Hydrocarbon Content (ppm)	
	Soil	Fruiting bodies
1st control (E) PS	1.375	-
2nd control (F) O	0.29*	-
A1 O+X+S	1.55	0.395
B1 O+X+S	1.42	-
C1 O+X+S	1.63	-
D1 O+S	0.65*	-
A2 O+S	0.77*	0.955
B2 O+S	2.53*	0.640
C2 O+S	1.42	0.590
LSD (0.05)	0.59	NA
p-value	0.024	NA

Legend: O oil-polluted soil, X sclerotia, S sawdust, PS pure soil
*Means different from the control (O), $p<0.05$

Discussion

The research was carried out to demonstrate the ability to utilize fungi for remediation purposes despite its importance as food to man. Three parameters: fruiting bodies, cap diameter and height of stipe were used to monitor the ability of the mushroom to grow in crude oil amended soil environment. The result as stated in Table 1 revealed that the mushroom was able to utilize crude oil in the presence of sawdust as a substrate over time for growth and proliferation. This could be responsible for the higher fruiting bodies recorded (Thenmozhi *et al.,* 2013). The increasing concern of the human population on the indiscriminate deposition of agricultural wastes into the environment has made focus on assessing the biodegradation ability of the white-rot fungi (mushrooms) on these waste (Isikhuemhen *et al*., 2010). Bioremediation of polluted soils by white-rot fungi is another area, using both local and exotic species to remediate different types of soils polluted with materials such as crude oil and its products of fractional distillation (Jonathan *et al.,* 2010). The usefulness of the treated soil was attested to by (Isikhuemhen *et al.,* 2011) where they reported its application on the cultivation of vegetables.

The ability of fungi to utilize crude oil profusely in the presence of sawdust is as also shown in Table 1. The isolates were found to proliferate with an increasing period of exposure. Similar work by Lawal *et al.* (2011), reported the cultivation of white-rot fungi which assessed the ability of white-rot fungi to degrade

agricultural wastes which are a nuisance to the environment. Also, Isikhuemhen *et al.* (2011) reported that white-rot fungi, *Pleurotus* species which are know to break down Polyaromatics (PAHs) and Polychlorinated biphenyls (PCB) into different fractions due to their ability to secrete lignocellulolytic enzymes are important in oxidizing persistent pollutants. Oluwafemi *et al*. (2011) reported that the use of Gas Chromatography-Mass Spetrophotometry and cation-exchange on sample analysis would enable one to characterize the genes that are regulated during growth and give a better understanding of the gene fractions involved by white-rot fungi during bioremediation.

The height of the stipe as recorded same Table 1 shows increased growth with prolonged exposure to the contaminated soil, which means that the mushrooms are able to utilize crude oil. The weeds that grew in the setup are recorded in Table 2. *Pepperomia pellucida* was the most predominant weed with the count at the oil polluted soil. This shows that the soil supported the growth of *Pepperomia pellucida* more than other experimental soils particularly the pure soil and oil polluted soil, and soil with soils with sclerotia and sawdust. This shows that the crude oil amended soil contained toxic substances that do not support the growth of weeds, hence no growth was recorded. Table 3 recorded the bacterial and fungal count in contaminated soil after two months of planting. The results of the bacterial and fungal counts revealed the ability of the isolates to grow profusely in the oil polluted soil particularly in the presence of sclerotia and sawdust. However, in general, the fungal isolates were observed to be more in numbers. This point to the obvious that fungi are better able to utilize and crude oil than bacterial species.

Table 4 above shows the bacterial and fungal identified in different treatments after two months of planting. It was observed that *Bacillus* sp., *Micrococcus* sp. and *Staphylococci* sp. were among the bacterial isolates that were identified in all soil samples while *Aspergillus niger, Penicillium* sp., *Rhizopus* sp. were the predominant fungal isolates. However, *Proteus* sp. was identified in only B1, *Trichoderma* sp. only in C2, *Proteus* sp. in only B1 while *Microsporium* sp. was present in A1 and C2. The bacterial and fungal isolates reported were not surprising as previous reports have documented them to be indigenous isolates in soil especially *Bacillus*, *Micrococcus, Staphylococci Aspergillus*, *Penicillium* and *Rhizopus* species. (Prakash, 2017; Thenmozhi *et al*., 2013). The fungal species reported were more than the bacterial isolates which also point to their robust morphology and diverse metabolic capacity. This makes fungal better agents of bioremediation and pollution control. Table 5 shows the total hydrocarbon present in different soil treatment and fruiting bodies after two months of planting. It was observed that C1 had the highest THC of 1.625ppm while control, F had the least of 0.29ppm. In the fruiting bodies, A2 had the highest THC of 0.955ppm while A1 had the least of 0.395ppm. It was observed that the crude oil polluted soil containing sclerotia and amended

with sawdust contained more hydrocarbons than the oil polluted soil with sawdust. This shows that fungi (sclerotia) are better degraders of hydrocarbons. In corroboration, Davis and Wilson (2005) reported that for efficient bioremediation, soil amendments are added to increase microbial activities. Also, *Pleurotus tuberregium* has been used to improve bioremediation of soils contaminated with crude oil (Adedokun and Ataga, 2007). The technology of bioremediation employed was simple, cheap, effective and environmentally friendly, whose biostimulant is pleasant and mainly of organic origin, i.e., sawdust, which is mostly referred to as waste and is of no economic value to the ordinary man.

Conclusions

This work has showed the potential of *Pleurotus tuberregium* in the degradation of crude oil polluted sites and confirmed the significant ecological role of fungi in petroleum-polluted environments.

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