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Bioremediation of Crude Oil Contaminated Soil Using *Pleurotuspulmonarius*, a White-rot Fungus

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Abstract: *This study was conducted to investigate the extent of degradation of crude oil in contaminated soil by harnessing the remedial potential of Pleurotuspulmonarius when grown in soil with saw dust and rice bran as amendment additives. Physico-chemical parameters such as pH, total organic carbon (%TOC), total organic matter (%TOM), total nitrogen (TN), available phosphorus (P), available potassium (K) and total petroleum hydrocarbon (TPH) of the contaminated soil and soil amended with saw dust and rice bran were monitored during the study period. Homogenized sterilized soil (700g) each was amended with 150 g of saw dust, rice bran and inoculated with P.pulmonariusspawn,to establish A (soil + saw dust + rice bran + P.pulmonarius), B (soil + rice bran + P.pulmonarius), C (soil + saw dust + P.pulmonarius) and D (soil + saw dust + rice bran) experimental set-up. The experiment was allowed to run for 60 days with periodic sampling every 15 days intervals for analysis. The results revealed a sharp decrease in TPH concentration after 15 days, which progressively decreased further over the subsequent sampling intervals; with a corresponding increase in the removal rate of nitrogen, phosphorus and potassium. Set-up A and D emerged as the maximum and minimum for the removal of TPH components at the end of the bioremediation process as illustrated in the order: A (90.12%) > B (77.42%) > C (72.20%) > D (9.98%). It was observed that the saw dust and rice bran blend had sufficient amounts of nitrogen, phosphorus and potassium (NPK) to sustain biodegradation of crude oil components in the contaminated soil. Biodegradation of the hydrocarbon components of the crude oil was confirmed using gas chromatography and reduction of crude fractions with carbon atoms ranging from C_{10}-C_{40} was observed. This study showed that P.pulmonarius is effective at decontaminating crude oil polluted soil.*
Keywords*: Pleurotuspulmonarius, soil,biodegradation saw dust, rice bran*

I. Introduction

Petroleum is the major sources of energy for industrial and domestic use (Axelsson, 2012). Exploration of Petroleum crude comes at a cost to the environment and its attendant pollution has been described as the most prevalent problem in the environment. Leaks and accidental spills occur regularly during the exploration, production, refining, transport and storage of petroleum and petroleum products (Milliolie*t al.*, 2009). The release of hydrocarbons in the environment whether accidentally or due to human activities is a main cause of water, air and soil pollution. Soil contamination with hydrocarbons causes extensive ecosystem system damage with attendant death or mutation of animals and plant (Nwilo and Badejo, 2001).

A biological process termed bioremediation, which involves the application of living organisms (bacteria, fungi and plants) and nutrients to detoxify or remove pollutants (Atlas and Bartha, 1992). Bioremediation transforms chemical compounds to other forms nonhazardous to the environment (Gradi, 1985). The ability of fungi to transform a wide variety of hazardous chemicals has aroused interest in using them in bioremediation (Alexander, 1994). Fungi are among nature's most powerful decomposers, secreting strong enzymes. The great potential of fungi in bioremediation is by virtue of their aggressive growth, great biomass production and extensive hyphae reach in the environment (Ashoka*et al*., 2002; Hamman, 2004). Fungi are unique among microorganisms in that they secrete a variety of extracellular enzymes involved in pollutant degradation. They use a variety of mechanisms to accomplish the degradation of lignin and a wide variety of other environmental pollutants (Asamudo*et al*., 2005).

Crude oil consists of many constituents; the most problematic in terms of bioremediation are those having ring structures like polycyclic aromatic hydrocarbons (PAHs). White-rot fungi are the most active lignin degraders. It has been demonstrated that a lot of species belonging to the group of white-rot fungi are able to degrade lignin, which is a naturally occurring polymer. They are able also to breakdown xenobiotic pollutants such as crude oil and compounds structurally related to lignin (Kirk *et. al*., 1992). *Pleurotus tuber-regium* has been reported to ameliorate crude oil polluted soil to support plant growth(Isikhuemhen*et al*., 2003). Many studies have reported the use of *Pleurotusostreatus*in bioremediation exercises (Bezalel*et al*., 1996a; Bezalel*et*

al., 1996b; Bezalel*et al*., 1997; Young, 2012; Baldrian*et al*., 2006). *Lentinussubnudus* has also been reported to decontaminate soil contaminated with crude oil (Adenipekun and Fasidi, 2005; Stanley and Immanuel, 2015).

White-rot fungi have been adopted in the field of bioremediation with quite a number of successes. There is however the need to find a better candidate from this group and suitable substrates that optimizes their potential for use in mycoremediation. This study aimed at examining the efficiency of *Pleurotuspulmonarius* as a candidate for mycoremediation using rice bran and saw dust nutrient amendment.

II. Materials And Methods

Sample collection

The white-rot fungus used for this study is *Pleurotuspulmonarius.* The spawn of the organism was obtained from diplomat mushroom farm located at the Rivers State University of Science and Technology, Port Harcourt, Rivers State. The crude oil contaminated soil sample was collected from Bomu, in Ogoni Land of Gokana Local Government Area of Rivers State 8cm deep from the soil surface. The contaminated site lies on the geographic coordinate of 7.4° E and 4.5° N.

Experimental Design

700g of the crude oil contaminated soil was weighed out into four different containers labelled A-D and sterilized by adding 0.2 wt% of mercury chloride into the contaminated soil samples. 150g of saw dust and 150g of were added to set-up A. Set-up B had 300g rice bran only. Set-up C had 300g of saw dust only. Set-up D was the control with equal amount of the substrates added. Contents were mixed thoroughly to get a composite mixture. Thereafter, they were inoculated with 100g of the spawn and kept at room temperature except for the control. Samples were taken every 15 days and analysed for reduction in total petroleum hydrocarbon (TPH).

Soil Physico-chemical Analysis

pH

A pH probe (Lonalyzer, model 407A, Orion research, USA) was used to measure pH.

Total Organic Carbon

Total Organic Carbon (TOC) was estimated using the approved method of Walkey-Black (Walkey and Black, 1934).

Total Nitrogen

Nitrogen in the sample was estimated following the micro Kjeldhal method.

Available Phosphorous

Phosphate content of the soil sample was estimated using Ascorbic acid method.

Available Potassium

Potassium content of the sample was measured using Atomic Absorption Spectrophotometer (AAS) after acid digestion.

Total Petroleum Hydrocarbon

The soil sample was extracted for Total Petroleum Hydrocarbon with Analar grade Hexane and Acetone (1:1, v/v) in an extraction bottle, equipped with Teflon cover. The sample was sonicated for 1hr and the two phases was separated by decanting. Extracted organic phase was concentrated or reduced to a volume of 1mls using vacuum rotary evaporator. 1.0 µl of the final extracting solution was injected and eluted in already calibrated Gas Chromatograph, HP 5890. The calibration was carried out by using commercially available TPH primary standards (Accustandards, USA). The peak areas were used in the quantifications of the TPH concentration.

Statistical Analysis

Results were statistically analyzed by one-way ANOVAusing SPSS 20.

III. Results

Physico-chemical Analysis of Contaminated soil and Nutrient additives

Physico-chemical properties of the contaminated soil as well as the nutrient supplements used in the baseline study are shown in Table 1. The test soil under investigation can be described as been heavily contaminated with hydrocarbon contaminants; with mean TPH concentration of 21410 mg/kg. The pH of the contaminated soil i.e. 5.62 falls within a suitable range (5 – 9) required for bioremediation process to occur. However, the nitrogen content of the contaminated soil was found to be 48.12 mg/kg which is low compared to the nitrogen content of rice bran (3410 mg/kg) and saw dust (2608 mg/kg). The pH of the nutrient additives were found to range from 6.11-6.97.

TPH degradation by *Pleurotuspulmonarius*

Figure 1 shows result for TPH degradation by *Pleurotuspulmonarius* under different nutrient additives, expressed as percentage loss. After 15 days of growth, the fungus effectively reduced the amount of TPH in the contaminated soil. This continued progressively till the 60th day of sampling. At the end of the 60 days study period it was observed that TPH reduction generally increased in all experiment sets in this order; A (90.12%) > B (77.42%) >C (72.20%) > D (9.98%). Chromatographic profiles of degradation are shown in Figures 2 – 6.

Nutrient Removal by*Pleurotuspulmonarius*

It was observed that the essential nutrients levels decreased after 15 days and remained fairly stable before decreasing further with time (Tables 2 - 6). Significant changes with time were observed in all the treatments as compared to the control.

Table 1: Physico-chemical parameters of the test soil with and without amendment before incubation with *P. pulmonarius*

Physico-chemical parameter	Soil	Soil + rice bran	Soil + saw dust	Soil + saw dust + rice bran
pH	5.62	6.11	6.97	6.85
Total Organic Carbon (%)	5.02	8.08	9.74	9.26
Total Organic Matter (%)	9.72	13.97	16.84	16.01
Total Nitrogen (mg/kg)	48.12	3410	2608	3247
Available Phosphorous (mg/kg)	5095	7411	9877	8850
Available Potassium (mg/kg)	102.52	1368	484.55	961.55
TPH, mg/kg	21410	-	-	-

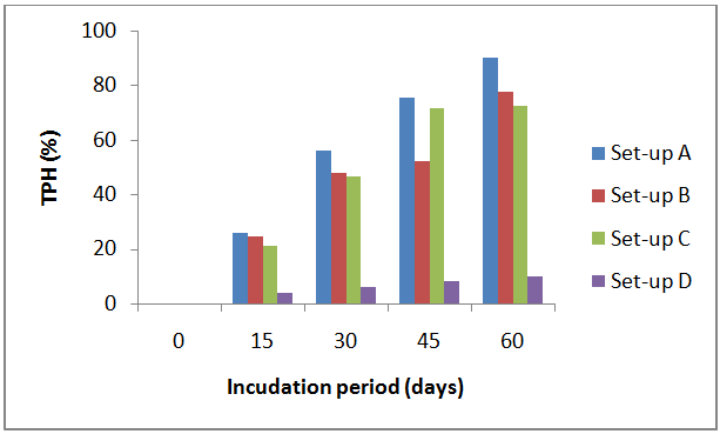

Figure 1: Percentage loss in TPH in soil incubated with *P. Pulmunarius* for 60 days

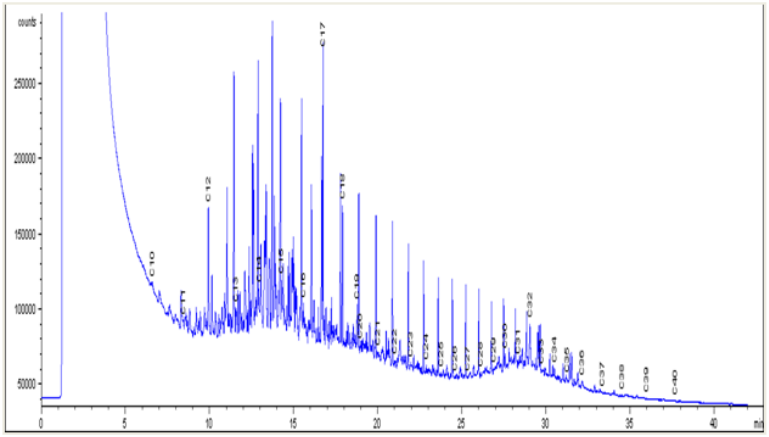

Figure 2: Chromatographic profile of untreated crude oil contaminated soil

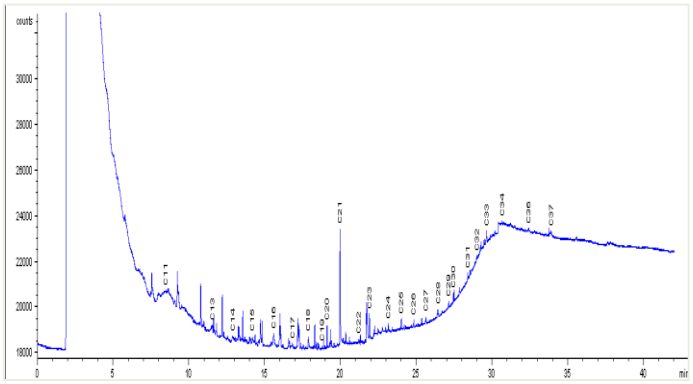

Figure 3: Chromatographic profile at day 60 for set-up A

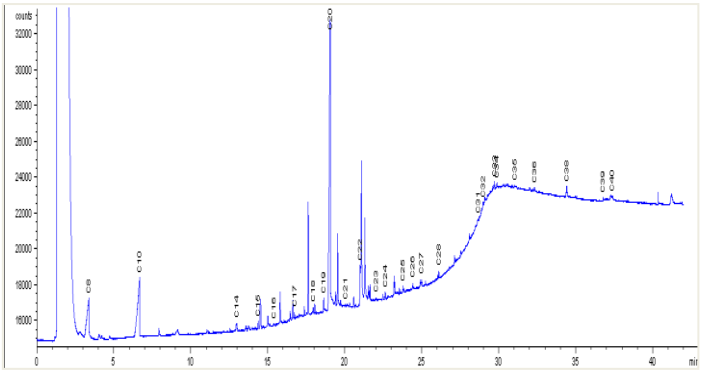

Figure 4: Chromatographic profile at day 60 for set-up B

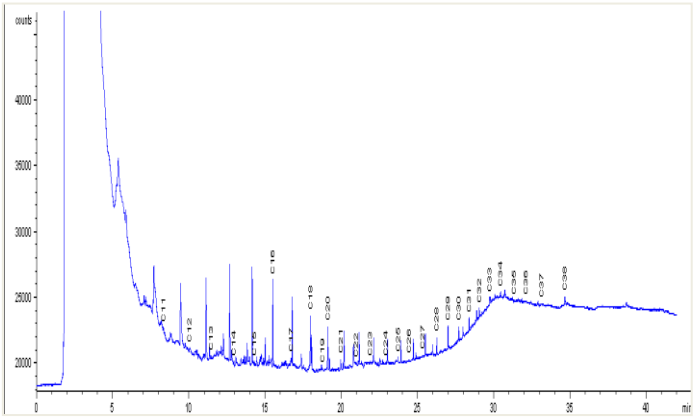

Figure 5: Chromatographic profile at day 60 for set-up C

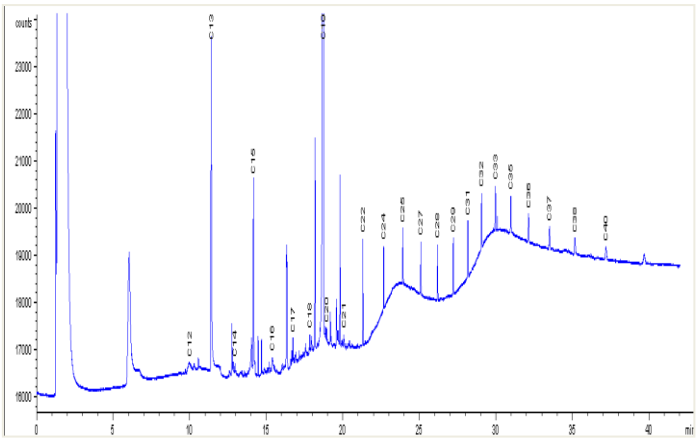

Figure 6: Chromatographic profile at day 60 for set-up D

Table 2: Nitrogen concentrations in soilincubated with *P. pulmonarius*for 60 days

Treatment	Nitrogen Concentration (mg/kg)				
	0	**15th day**	**30th day**	**45th day**	**60th day**
A	3247	2517	2498	2447	2221
B	3410	1577	1577	2001	2004
C	2608	1608	1520	1479	1006
D	3247	2145	2445	2520	2488

Table 3: Phosphorous concentrations in soilincubated with *P. pulmonarius*for 60 days

Treatment	Phosphorous Concentration (mg/kg)				
	0	**15th day**	**30th day**	**45th day**	**60th day**
A	8850	4114	4112	3005	2997
B	7411	4002	4002	3988	3942
C	9877	3147	3147	3210	3114
D	8850	4887	5087	4977	4887

Table 4: Potassium concentrationsin soilincubated with *P. pulmonarius*for 60 days

Treatment	Potassium Concentration (mg/kg)				
	0	**15th day**	**30th day**	**45th day**	**60th day**
A	961.55	345.55	340.29	321.88	304.11
B	1368	397.14	388.10	356.24	311.24
C	484.55	171.08	155.44	161.02	155.44
D	961.55	731.29	604.05	597.36	512.29

Table 5: Total organic carbon concentration in soil incubated with *P. pulmonarius* for 60 days

Treatment	TOC Concentration (%)				
	0	**15th day**	**30th day**	**45th day**	**60th day**
A	9.26	7.62	7.62	7.69	7.66
B	8.04	6.83	6.83	7.01	6.99
C	9.74	7.04	7.04	7.11	7.08
D	7.90	7.98	7.95	7.95	7.91

Table 6: Total organic matter concentrations in soil incubated with *P. pulmonarius* for 60 days

Treatment	TOM Concentration (%)				
	0	**15th day**	**30th day**	**45th day**	**60th day**
A	16.01	13.14	13.30	13.30	13.24
B	16.84	11.81	11.11	12.26	12.20
C	13.97	12.17	12.14	12.24	12.54
D	13.75	13.75	13.85	13.78	13.82

IV. Discussion

The baseline study crude oil contaminated soil revealed that the amount of limiting nutrients such as nitrogen, phosphorus and potassium present in thepolluted soil were very low. The nutrient levels (NPK) were moderate in the treatments with the amount of K>N>P. This necessitated the amendment with saw dust and rice bran to supply additional nutrients for microbial activities. Soil amendment with saw dust and rice bran had impact on the Physico-chemical properties of the soil. A decrease in the nutrient content of the soil with time was observed for soil incubated with *P. pulmonarius*. Ibiene*et al*. (2011) likewise reported in their study that concentrations of phosphate, nitrate, decreased significantly during bioremediation study. Nitrogen, phosphorous and potassium are essential nutrients for microbial growth and are required for the degradation of hydrocarbons as reported by Eziuzor and Okpokwasili(2009). Crude oil has been reported to have a fertilizer effect on *P. pulmonarius* grown on crude oil contaminated soil Ogbo*et al*, (2009).There were slight decreases in total organic matter (TOM) and total organic carbon (TOC) concentrations in the soil as corroborated by Ibiene*et al.* (2011).Adenipekun*et al*., (2013) however reported slight increases in TOC and TOM in their study of bioremediation of cement and battery polluted soil.

Microorganisms require nutrients to be able to breakdown complex compounds. Organic compounds serve as sources of carbon and energy for the microorganisms and can be measured or estimated based on the concentrations of TOC. Provided that carbon is present in sufficient amounts, the total amount of nitrogen suitable to microorganisms in the form of organic nitrogen, ammonia, and nitrate can significantly influence the rate of contaminants degradation.Loss in TOC has been correlated with biomass increase in microbial systems.

The pH values of the various treatments were slightly acidic. The average soil pH level was 5.62 and this is typical of the pH level of soils in Niger Delta. The pH levels of incubated enriched soilwere slightly higher than the control. This may be attributed to the enhancement or enrichment of the nutrient levels (by supplementing with sawdust and rice bran) of the soil. Atlas and Bartha (1992) reported that addition of nutrient to soil

increases the pH of the soil mixture. Wood fungi can change pH that is not conducive to their environment to suit pH that favours growth.

The levels of the petroleum hydrocarbon in the crude oil contaminated soil decrease over time and varied significantly with each treatment option. The percentage reduction in TPH over time from the native soil to that of the *P. pulmonarius* pre-grown on soil enriched with saw dust + rice bran, rice bran only and sawdust only at day 60 were 90.12%, 77.42% and 72.20% respectively. These values are far higher than those reported by Chiu *et al*. (2009); in which values ranging between 31-64% were reported. High percentage degradation (90.0%) of TPH at 10% contamination level was reported by Adenipekun*et al*. (2013) for both fresh and spent contaminated soil samples. The *P. pulmonarius* pre-grown on soil enriched with saw dust + rice bran was more effective in the bioremediation of the contaminated soil and soil pre-grown on sawdust only was the least effective. The reduction in the amount of the hydrocarbon was as a result of the effectiveness of the *P. pulmonarius* used for the remediation of the contaminated soil. The chromatographic profile of the TPH levels of treated soil shows reduction in hydrocarbons particularly within the C_{10}- C_{30} range.

Bioremediation of crude oil by *P. pulmonarius*has compared favourably with other candidates used for mycoremediation and bioremediation. Ibiene*et al*. (2011) reported up to 99.91% loss in TPH when spent mushroom substrate was used for bioremediation of hydrocarbon. Tsai *et al*. (2009) reported up to 95 % reduction in TPH when *Pseudomonas* sp. and *Shewanella*sp. Where used in microcosm study for 120 days. Consortia of bacteria isolates have been reported to degrade TPH by a number of authors but the advantage of using the white-rot fungi far outweighs this fit by bacteria. Use of white-rut fungi in soil remediation requires low maintenance of sites treated with the fungi with minimal handling. Mycoremediation requires very cheap substrates like saw dust and rice bran used in this study. The degradation potential of the white-rot fungi makes them utilize the most toxic components of crude oil which often inhibit bacterial growth.

V. Conclusion

Managing crude oil spills requires the expedient use of available technology to minimize impact within a short space of time and to save cost. Mycoremediation offers this advantage but this advantage could be lost if the right fungal candidates and suitable substrates are not found. The results obtained from this study revealed that the white-rot fungi *Pleurotuspulmonarius* was effective for bioremediation of crude oil contaminated soil. The results equally revealed that a cheap nutrient source in form of saw dust and rice bran can be used for the enhancement or enrichment of the soil nutrient. Waste utilization in bioremediation is currently receiving great research attention globally, and the findings of this research work identified the usefulness of the substrate blend in bioremediation of crude oil-impacted soils in the Niger Delta.

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