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Bioremediation of Engine Oil Polluted Soil by the Tropical White Rot Fungus, *Lentinus squarrosulus* Mont. (Singer)

[1]Clementina O. Adenipekun and [2]Omoanghe S. Isikhuemhen
[1]Department of Botany and Microbiology, University of Ibadan, Ibadan, Nigeria
[2]North Carolina Agricultural and Technical State University, Greensboro, NC 27411, USA

Abstract: This study was conducted to test the efficacy of an indigenous white rot fungus *Lentinus squarrosulus* in degrading engine oil in soil. Flasks containing sterilized garden soil (100 g) moistened with 75% distilled water (w/v) were contaminated with engine oil 1, 2.5, 5, 10, 20 and 40% w/w concentrations, inoculated with *L. squarrosulus* and incubated at room temperature for 90 days. Levels of organic matter, pH, total hydrocarbon and elemental content (C, Cu, Fe, K, N, Ni, Zn and available P) were determined post-fungal treatment. Results indicate that contaminated soils inoculated with *L. squarrosulus* had increased organic matter, carbon and available phosphorus, while the nitrogen and available potassium was reduced. A relatively high percentage degradation of Total Petroleum Hydrocarbon (TPH) was observed at 1% engine oil concentration (94.46%), which decreased to 64.05% TPH degradation at 40% engine oil contaminated soil after 90 days of incubation. The concentrations of Fe, Cu, Zn and Ni recovered from straw/fungal biomass complex increased with the increase of engine-oil contamination and bio-accumulation by the white-rot fungus. The improvement of nutrient content values as well as the bioaccumulation of heavy metals at all levels of engine oil concentrations tested through inoculations with *L. squarrosulus* is of importance for the bioremediation of engine-oil polluted soils.

Key words: Bioremediation, engine oil, heavy metals, *Lentinus squarrosulus*, polyaromatic hydrocarbons (PAH)

INTRODUCTION

Many industrial activities release hazardous organopollutants into the environment. The pollutants are usually persistent and known to have carcinogenic or mutagenic effects (Reddy and Matthew, 2001). Bioremediation is the onsite enhancement of the live soil organisms such as fungi, bacteria and plants to breakdown hydrocarbon and organic contaminants (Atlas and Bartha, 1972).

Efforts to achieve biodegradation of oil products have involved bacteria and fungi, since they are the only biological species which have the metabolic capability of utilizing petroleum carbon for cell synthesis (Jobson, 1974). A number of bacteria such as *Pseudomonas flourescens*, *Acenobacter* spp. and *Micrococcus varians* (Obire, 1988) and fungi have been investigated for their potential for breaking down and or removing hazardous compounds associated with petroleum spills. The use of fungi for the treatment of heavy-metal containing effluents has been well established due to their ability to accumulate metals from their external environment (Siegel *et al*., 1990; Gadd, 1993; Kalac *et al*., 1999).

White-rot fungi are increasingly being investigated and used in bioremediation because of their ability to degrade an extremely diverse range of very persistent or toxic environmental pollutants (Isikhuemhen *et al*., 2003). Atagana *et al*. (2006) reported that *Pleurotus* sp. performed best as a biodegrader of creosote in soil compared to four other non white rot fungi. Stamets (1999) reported the successful use of a *Pleurotus* species to reduce more than 95% PAH inoculated into test soil to non-toxic components. Another white-rot fungus-*Pleurotus tuber-regium*, (Fr.) Singer, indigenous to Nigeria and distributed across sub-Saharan Africa (Isikhuemhen *et al*., 2000), has also been shown to have the capability to ameliorate crude oil polluted soil. Once remediated the soil was demonstrated to support seed germination and seedling growth of *Vigna unguiculata* at levels, which in some cases was better than control (Isikhuemhen *et al*., 2003). Similarly, *Lentinus squarrosulus* Mont. has been found to mineralize soil contaminated with various concentrations of crude oil resulting in increased nutrient contents in treated soils after 6 months of incubation (Adenipekun and Fasidi, 2005).

Corresponding Author: Clementina O. Adenipekun, Department of Botany and Microbiology, University of Ibadan, Ibadan, Nigeria Tel: 234-8055265186 Fax: 234-02-8103043

Although there is growing evidence for the potential of white rot fungi as an efficacious bioremediator of petroleum products, more investigations are needed to identify the remediation parameters such as oil content and heavy metal concentrations for which the fungi can be most effective. Towards this goal the present research investigated the ability of *L. squarrosulus* to bioremediate engine-oil polluted soils, bioaccumulate heavy metals and the improvement on nutrient conditions of treated soils.

MATERIALS AND METHODS

Garden soil used for this experiment was collected from the Nursery Unit of the Department of Botany and Microbiology, University of Ibadan, Nigeria from June, 2005 to June, 2007. The engine-oil was obtained from a fuel station in Ibadan. Rice straw used was collected from a rice farm, cut into 5 mm pieces and soaked in boiling water for 30 min before use. A tissue culture was made from fresh fruit bodies of *L. squarrosulus* obtained from a dead log of wood in the Department of Botany and Microbiology.

Fungal cultivation and incubation: A modified method of Baldrian *et al*. (2000) was employed. One hundred gram of sterilized soil moistened with 75% distilled water (w/v) was weighed into 350 cm^3 conical flasks. Varying concentrations (1, 2.5, 5, 10, 20 and 40% w/w) of engine oil was added and mixed thoroughly and 20 g (dry wt. equivalent) of rice straw, was laid on the contaminated soil, in each flask, covered with aluminum foil and autoclaved at 121°C for 15 min. Agar plugs (7 mm in diameter) from actively growing mycelia of test fungus were made with a sterile cork borer. Two agar plugs were inoculated onto the straw in each flask and cover with aluminum foil. All flasks were incubated at room temperature (28 ±2°C) for 90 days. Each experiment was replicated three times. The control treatment had soil contaminated with engine oil (0, 1, 2.5, 5, 10, 20 and 40% w/w), but not inoculated with fungus.

Nutrient content analyses: The soil pH measured at 0 and 90 days, was determined according to the procedure developed by Bates (1954). Twenty grams of soil sample was weighed into a 50 mL beaker followed by the addition of 20 mL of deionized distilled water. It was stirred manually for 5 min, allowed to stand for 30 min and pH measured using a pH meter. Organic matter, carbon, percentage nitrogen, phosphorus and potassium contents were determined according to Association of Agricultural Chemists (AOAC, 1980).

Total TPH and heavy metal analysis: The total petroleum hydrocarbon content of the soil was determined using a Perkin Elmer Spectrum GX FTIR (Fourier Transform Infrared) Spectrometer USA. The mycelial-ramified rice straw substrate was carefully separated from the soil layer in the flasks dried to constant weight in an oven at 80°C and ground into powder with a mortar and pestle. Three to five grams of samples were weighed into crucibles and ashed in a muffle furnace at 500°C. The ash content of each crucible was dissolved in 20 mL 1M nitric acid and analyzed for the heavy metals using Flame atomic absorption spectrophotometer (Crosby, 1977).

A simple pre-post design with 6 oil treatments and a control was used in the experimental set-up and carried out in three replicates. Statistical analysis was done using Duncan's multiple range test.

RESULTS AND DISCUSSION

After 90 days of incubation there was decrease in pH for all oil contaminated concentrations and the control showed significant differences ($p \leq 0.05$) in pH at 1 and 10% concentrations (Fig. 1). The lignin degrading enzymes (Laccase and other peroxidases) secreted by white-rot fungi are known to function best at low pH, sometimes as low as pH of 3.5 (Hossain and Anatharaman, 2006). Also, white rot fungi are known to secrete organic acids into their substrate which presumably can lower the pH to levels optimum for their enzymes to function best (Hofrichter *et al*., 1999). Dibble and Bartha (1979) reported that the pH range of 5.0 to 7.8 favored the degradation of oily sludge in the soil. The changes were more at 0 to 20% crude oil in soil. Coincidentally, these are the levels of contamination where TPH loss (degradation) was prominent. It could be interpreted to mean that those are the level of contamination where the fungus had more metabolic and physiological activities to produce substances that modified the pH of the substrate.

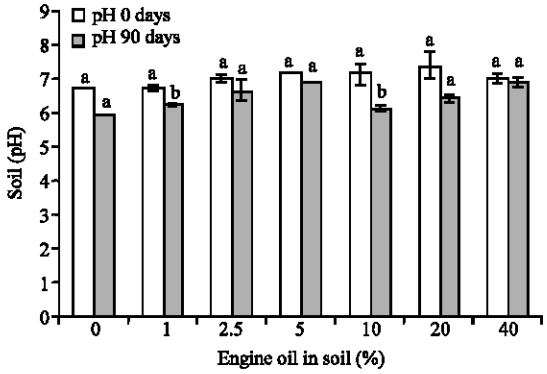

Fig. 1: Changes in soil pH contaminated with engine oil after 90 days of incubation with *L. squarrosulus*

Table 1: Changes in nutrient contents of engine-oil contaminated soils after 90 days of incubation

Treatments		Organic matter (%)	Carbon (%)	Nitrogen (%)	Phosphorus (μg mL^{-1})	Available potassium (meq/100 g)
Control	0 day	3.31^{a}	1.92^{a}	0.24^{a}	10.71^{b}	0.79^{a}
	90 day	4.47^{a}	2.59^{a}	0.14^{a}	29.28^{a}	0.67^{a}
1%	0 day	5.05^{a}	2.93^{a}	0.37^{a}	15.70^{b}	0.94^{a}
	90 day	8.85^{a}	5.13^{a}	0.15^{a}	30.40^{a}	0.53^{b}
2.5%	0 day	5.23^{b}	3.03^{a}	0.38^{a}	15.39^{b}	1.01^{a}
	90 day	10.05^{a}	5.83^{a}	0.14^{b}	29.43^{a}	0.52^{b}
5%	0 day	5.60^{b}	3.25^{a}	0.41^{a}	14.35^{a}	0.59^{a}
	90 day	10.85^{a}	6.29^{a}	0.14^{b}	20.25^{a}	0.24^{b}
10%	0 day	6.31^{b}	3.66^{a}	0.46^{a}	10.26^{b}	0.54^{a}
	90 day	12.54^{a}	7.27^{a}	0.14^{b}	16.87^{a}	0.22^{b}
20%	0 day	3.33^{b}	1.93^{a}	0.24^{a}	9.23^{a}	0.44^{a}
	90 day	11.89^{a}	6.90^{a}	0.14^{a}	13.25^{a}	0.18^{b}
40%	0 day	2.05^{b}	1.19^{b}	0.15^{a}	4.33^{b}	0.33^{a}
	90 day	11.71^{a}	6.79^{a}	0.13^{a}	12.32^{a}	0.16^{a}

Each value is the mean of 3 replicates, Values in column followed by the same letter(s) are not significantly different according to Duncan's multiple range test ($p \leq 0.05$)

At all levels of oil concentration in soil, there were significant differences ($p \leq 0.05$) in organic matter content at 0 and 90 days of incubation. Organic matter content increased with increasing oil concentration in soil to reach 12.54% at 10% oil in soil and thereafter declined to 11.71% at 40% oil concentration in treated soils (Table 1). A similar trend was observed in carbon content in soil with highest concentration of 7.27% at 10% oil in soil, followed by a decrease to 6.79% at 40% oil in soil. Similar observations were reported by Adenipekun and Fasidi (2005). Atlas and Bartha (1972) also reported that an addition of crude oil to an ecosystem enriches micro-organisms capable of utilizing hydrocarbons. *L. squarrosulus* seems to have improved the organic matter and carbon content of the soil compared to the control after 90 days of incubation through biodegradation of the applied engine oil.

After the 90 days incubation period, increase in oil concentration in soil resulted in increased nitrogen content, reaching 0.46% value at 10%, but N content decreased to 0.15% at 40% oil concentration (Table 1). For Phosphorus content there were significant increases at 1, 2.5 and 5% levels of oil compared to the control condition (0% oil in soil). The highest level observed was 30.40 μg mL^{-1} at 1% oil in soil. However, beyond 2.5% oil concentration, phosphorus content decreased to reach lowest levels (12.32 μg mL^{-1}) at 40% oil in soil. Potassium levels showed a significant decline after 90 days and a pattern of lower potassium levels for the higher oil concentrated soil samples (Table 1). The observed differences in N, P and K contents are consistent with reports in literature. Low values for nitrogen, potassium and phosphorus reserve in petroleum hydrocarbon contamination were reported (Lehtomaki and Niemela, 1975). Benka-Coker and Ekundayo (1995) reported low levels of Nitrogen and Phosphorus from a crude oil spill site in the Niger Delta of Nigeria. In general, oil products when added to the soil create a very high C:N ratio, where the essential elements of nitrogen, potassium and phosphorus become the limiting factor in oil degradation by bacteria and fungi (Ayotamuno *et al*., 2006). Isikhuemhen *et al*. (2003) reported that white rot fungi bioremediated crude oil polluted soil and resulted in improved percentage germination in *Vigna unguiculata*.

Table 2: TPH of engine oil contaminated soils incubated with *L. squarrosulus*

Treatment (conc. of engine oil)	TPH (mg kg^{-1}) 0 days (control)	90 days	TPH (% lost)
1%	8623±0.02	1599±0.01	94.46
2.5%	14106±0.05	1913±0.01	86.40
5%	15446±0.15	3484±0.03	77.44
10%	17132±0.20	4169±0.03	75.66
20%	23300±0.22	6196±0.05	73.40
40%	19302±0.30	6939±0.05	69.05

Each reading is a mean of 3 readings±standard error

Table 3: Heavy metal contents (mg kg^{-1}) after 90 days incubation of engine oil contaminated soil with *L. squarrosulus*

Oil in soil (%)	Fe	Zn	Cu	Ni
Control	0.002±0.50^{a}	0.0015±0.00	0.0006±0.05^{a}	0.000005±0.00^{a}
1	0.038±1.20^{b}	0.0020±0.05^{a}	0.0008±0.04^{b}	0.000050±0.02^{a}
2.5	0.054±1.20^{c}	0.0080+0.1^{b}	0.0011±0.22^{c}	0.000036±0.02^{b}
5	0.088±1.50^{d}	0.0260±0.08^{c}	0.0032±0.06^{d}	0.000920±0.01
10	0.190±0.80^{e}	0.0360±0.16^{d}	0.0046±0.20^{e}	0.003500±0.04^{d}
20	0.635±2.20^{f}	0.0580±0.08^{e}	0.0048±0.25^{f}	0.005900±0.05^{e}
40	0.354±3.50^{g}	0.0300±0.13^{f}	0.0032±0.05^{g}	0.001700±0.01^{f}

Each reading is a mean of 3 readings±standard error, Values in the same column followed by the same letter(s) are not significantly different according to Duncan's multiple rang test ($p \leq 0.05$)

Table 2 shows the Total Petroleum Hydrocarbon (TPH) in engine oil-contaminated soils and inoculated with *L. squarrosulus*. A decrease in TPH lost (%) was highest at 1% engine-oil contaminated soil recording 96.46% which decreased to 64.05% at 40% engine-oil contaminated soil after 90 days. It has been reported that spent sawdust cultures of *Lentinus edodes* removed 44-61% of the pentachlorophenol in fermented sterile contaminated soil, after 21 days of incubation (Okeke *et al*., 1993). Rosado and Pitchel (2004) reported that after 150 days in the clover treatment study, added oil was no longer detected in the clover treatment. A total of 67% oil was removed in sunflower/mustard treatment.

Table 3 shows that *L. squarrosulus* accumulated Fe and Zn onto straw/fungal biomass, from soil contaminated with engine oil after 90 days of incubation. The Fe content increased steadily to reach a peak (0.635 mg kg^{-1}) and decreased to (0.354 mg kg^{-1}) at 20 and 40% oil concentration in soil respectively. Zn contents followed similar pattern, although at levels of concentrations that are lower than Fe concentration

values. A consistent increase in Cu content was observed to 0.0048 dry weight to 0.0048 mg kg^{-1} followed by a decrease to 0.0032 mg kg^{-1} dry weight of straw applied. Ni content increased up to 20% (0.0059 mg kg^{-1}) and thereafter decreased to 0.0017 mg kg^{-1} at 40% oil concentration in soil. At 20% oil in soil, which appeared to be the level at which most metals were accumulated into the straw/fungal biomass complex, the trend of accumulation was Fe>Zn>Ni>Cu.

The experiment revealed the ability of the white-rot fungus, *L. squarrosulus* to improve the nutrient contents of the engine-oil contaminated soil and an accumulation of Fe, Zn and Ni to an appreciable extent. This could represent a process that could be exploited in remediation of engine oil contaminated soils.

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