INTERNATIONAL JOURNAL OF NATURAL AND APPLIED SCIENCES (IJNAS) VOL. 1, No. 1: pp. 15 – 20, 2 TABLES, 2 FIGS.

The bioremediation potentials of fungal species isolated from soils polluted by petroleum products in Cross River University of Technology, Calabar, Nigeria.

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ABSTRACT

A comparative assessment of the biodegradative abilities of fungal species isolated from Nigerian soils contaminated with engine oil, diesel and petrol was carried out. Microbiological and physico-chemical properties such as moisture content, pH, particle size, total hydrocarbon (THC), organic carbon, nitrogen and phosphorus contents of the contaminated soils were also investigated, and the biodegradative potentials of the isolated fungal species were determined by measuring the optical densities (OD) of the fungal cultures spectrophotometrically. Incidence of fungal growth was highest in diesel-contaminated soil (41%) after an initial response to the diesel toxic effect. Among the fungal isolates, *Saccharomyces*, *Aspergillus*, *Cladosporium*, *Rhizopus*, *Mucor* and *Penicillium* species, *Cladosporium* species from diesel-contaminated soil had the highest growth turbidity of OD = 1.010 while *Penicillium* from petrol-contaminated soil showed the highest growth turbidity of OD = 0.848. All the fungal isolates showed low ability to degrade engine oil. It was concluded that *Cladosporium* and *Penicillium* were the best microorganisms for the degradation of diesel and petrol respectively.

Keywords: Bioremediation potential, isolated fungal species, petroleum products, environmental impact, contaminated soil.

INTRODUCTION

Increased oil prospecting, distribution and use have resulted in increased pollution of water and soil around the world. Several environmental impacts resulting from accidental spills of oil and its products are mainly attributed to the petroleum industry (Pala *et al.*, 2002). Inland oil spillages are almost a daily affair especially in developing countries like Nigeria, where tankers are prone to accidents because of poor maintenance of roads and vehicles. Besides, automobile workshops are not regulated in the disposal of waste engine oil and other lubricants (Eja *et al.*, 2003). The result is that water courses and agricultural lands are polluted mostly by petroleum products such as petrol (gasoline), engine oil and kerosene.

Because of the serious environmental impacts associated with oil pollution (GESAMP, 1993; Clark *et al*., 1997), increased awareness has led to a dramatic increase in research on various strategies that may be employed to clean up the environment (Pandey and Jain, 2002). In the past, contaminated soils had been recuperated by physico-chemical methods, but it is now realized that bioremediation could be more efficient and less expensive in treating soils and sediment contaminated by petroleum and its derivatives (Gazso, 2001; Pala *et al.*, 2002; Goksungur *et al.*, 2003). A number of microbial agents (bacteria and fungi) which are capable of biodegrading petroleum and its derivatives have been identified (Atlas and Bartha, 1992, 1993), but what is now necessary is a search for the most efficient genera and species. Some have already been identified, and they include *Lentinus lepideus*, *Trametes vesicolor*, *Phanerochaeta chrysoporium* and *Bjerkandera adustra*. Thus, there is at present a worldwide search for suitable fungi which are capable of degrading petroleum products. In addition, some research is known to have been carried out to investigate the possibilities for large-scale production of these fungi (Mahro *et al.*, 1994; Field *et al.* 1996; Anon, 1998). This appears to be particularly useful in combating the menace caused by inland spillages of petroleum products.

In the present study, a comparative assessment of the biodegradative abilities, and the bioremediation potentials of fungal species isolated from Cross River University of Technology Laboratory premises soil impacted by petroleum products was carried out. It was further aimed at examining the prospects of using fungal species isolated from petroleum product-contaminated soil for bioremediation in comparison with those isolated from uncontaminat-

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ed soil.

MATERIALS AND METHODS

Soil sampling

Four soil sampling sites (S_1, S_2, S_3 and S_4; $1m^2$ each) within the laboratory premises of Cross River University of Technology were identified. The sites were 5m apart from each other. S_1, S_2, and S_3 were each contaminated with 1 litre of engine oil, diesel and petrol respectively by pouring the petroleum products evenly on the sites. Site 4 (S_4) was uncontaminated and served as the control.

Sampling was carried out at 7 days interval. Two hours after contamination, two sets of soil samples (10g each) were collected from each of the sites. One set of the sample was analysed microbiologically for isolation of fungal species, while the other set was analysed for physico-chemical properties such as moisture content, pH, particle size, total hydrocarbon (THC), organic carbon, nitrogen and phosphorus contents. Soil samples were taken at about 4cm depth by the use of hand-driven auger. All samples were taken to the laboratory in labeled polyethylene bags stored in ice-cooled boxes at approximately $4^{O}C$. Microbiological analysis was carried out within three hours of sample collection while physico-chemical analysis was carried out within five days. The means of the triplicate samples were calculated from the results of the analysis.

Total fungal counts and isolation of fungal species from soil samples

Soil sample (1.0g) was suspended in 9ml sterile distilled water. Ten-fold serial dilutions in the range of 10^{-1} – 10^{-6} were prepared using sterile distilled water (Atlas and Bartha, 1992). Aliquots (1ml) of sample dilutions of 10^{-3} – 10^{-6} were each plated on Malt Extract Agar (Oxoid) acidified to a pH of 4.8 to suppress the growth of bacteria and incubated at room temperature (about $28.4^{0}C$) for three to four days. Total fungal counts were made on any plate showing discrete colonies. The number of visible colonies was multiplied by the reciprocal of the dilution factor and recorded as colony-forming units (cfu) per gram of soil (Fawole and Oso, 1988; APHA, 1998).

Discrete colonies were sub-cultured onto malt extract acidified to a pH of 4.8 until pure cultures were obtained through identification. The isolates were identified according to the methods described by others (Beneke and Rogers, 1970; Lodder, 1974; APHA 1998).

Screen test for the ability of fungal isolates to utilize engine oil, diesel and petrol

Engine oil, diesel and petrol were tested directly for the ability of fungal isolates to degrade them using the method earlier described by Okpokwasili and Okorie (1988), as their soil sources of carbon and energy by the determination of growth turbidity. This was carried out by dispensing 9ml amount of mineral salt medium (MSM) into test tubes (Zajic and Supplison,1972). Following sterilization by autoclaving and cooling, 0.1ml amounts of aliquots of the isolates from 10^{-4} to 10^{-6} dilutions were seeded into the medium, followed by 0.1ml filter-sterilized (0.45μm pore size filter, Milipore Corp. England) engine oil. The same treatment was given to diesel and petrol respectively. The cultures were then incubated at room temperature for seven days. For each isolate, a control was set up in petrol respectively. The cultures were then incubated at room temperature for seven days. For each isolate, a control was set up in which no organism was seeded. At the end of the incubation, the optical density (OD) of each culture was measured at 400nm (Eja *et al.,* 2003) using spectronic 20 Genesys spectrophotometer. In this case, the OD was an index of growth reflecting the potential for the biodegradation of the petroleum products by the respective fungal species.

Physico-chemical analysis of soil samples

In the laboratory, the moisture content of the soil samples was immediately determined by determining the difference in weight before and after drying in the oven at $120^{o}C$ to constant weight. Soil samples were then air-dried, ground with a wooden roller and sieved through a 2mm mesh. The pH of the soil samples was determined in 1:2 soil/water ratio. The method of Fawole and Oso (1988) was used in determining the organic carbon. This was done by igniting the dried sieved soil sample (2.5g) in a preweighed crucible, and calculating the loss in weight by difference, followed by the calculation of the percentage of organic carbon in the soil sample. Available phosphorus was determined by the methods of Bray and Kurtz (1945). Total nitrogen was determined by the micro-kjeldahl digestion method (Black *et al.,* 1965). Total hydrocarbon (THC) was determined following extraction with redistilled n-hexane before measuring the total hydrocarbon content colorimetrically at 430nm using a DR/3000 HACH spectrophotometer (England). Particle size measurement was determined by the Bouyoucos hydrometer method (Day, 1965) as modified by Gee and Bauder (1986). This involved weighing about 100g of air-dried soil samples into a container and adding 50ml of sodium hexametaphosphate solution followed by stirring for 30 minutes. The mixture was left overnight in a 250ml measuring cylinder followed by shaking and inverting the cylinder several times. After 40 seconds, the first reading which gave the percentages of clay and silt was recorded (Gee and Bauder, 1986), while second reading was taken after two hours as the percentage of sand (Gee and Bauder, 1986).

Statistical test

A two-way analysis of variance (Miller and Miller, 1986) was carried out to determined significant differences, if any, between fungal counts in the soils contaminated with petroleum products and days of sampling.

RESULTS

The effects of soil contamination with petroleum products on fungal counts are shown in Table 1. There was no significant difference ($P<0.05$) in fungal counts between the days of sampling. However, high significant differences ($P<0.01$) in fungal counts were observed between the soil samples. Least significant difference (LSD) test showed that there was a high significant difference between diesel-contaminated soil samples and control (uncontaminated soil samples) and any other soil samples at $P = 0.01$, but there was no significant difference between petrol and control ($P > 0.01$) (Table 1).

Table 1. Effect of contaminated and uncontaminated soil samples with petroleum products on total fungal counts ($x10^4$cfu/g) of soil samples.

Sampling intervals (days)	Sites contaminated with Petroleum Products			
	Engine oil (S_1)	Diesel (S_2)	Petrol (S_3)	Control site (S_4)
0	134 ± 0.3	400 ± 1.1	189 ± 0.5	264 ± 1.2
7	110 ± 0.3	2400 ± 0.3	181 ± 0.3	193 ± 0.3
14	118 ± 0.2	1800 ± 0.1	160 ± 0.1	185 ± 0.3
21	102 ± 0.4	1200 ± 0.5	170 ± 1.1	171. ± 0.4
Total	464 ± 1.2	5800 ± 2.0	700 ± 2.0	813 ± 2.2

Each value is the mean ± standard deviation of fungal counts of triplicate soil samples collected from each site on each day of sampling.

The mean levels of some physico-chemical characteristics of the contaminated soil samples with petroleum products are presented in Table 2. The contaminated samples showed very high levels of THC compared with the control. THC levels ranged from 8.0 mg/kg in the uncontaminated soil (control) to 3,158.1mg/kg in the engine oil-contaminated soil. The carbon-nitrogen ratio (C/N) calculated from Table 1 ranged from 12.0 in diesel-contaminated soil to 14.2 in engine oil-contaminated soil (Figure 1).

The incidence of fungal species in the soil samples during each sampling interval is shown in Figure 1. The contaminated and uncontaminated (control) soil samples supported the growth of fungal species. The first sampling interval (day 0), the uncontaminated soil (control) had the highest incidence (32%) of fungal species, while the least incidence (7%) occurred in the diesel – contaminated site. In the second sampling interval (day 7), diesel contaminated soil (S_2) showed the highest incidence of fungal species (41%) which declined to 31% during the third sampling interval (day 14). On day 21 of sampling, the incidence of fungal species appeared to be relatively the same with the contaminated soil and control except petrol-contaminated soil (24%). The carbon/Nitrogen ratios calculated from Table 2 for all sampling sites (S_1, S_2, S_3 and S_4) were 14.2, 12.0, 12.4 and 13.4, respectively as shown in Figure 1.

Figure 2 shows a multiple bar chart representing the results obtained from the screen test for the utilization of petroleum products (engine oil, diesel and petrol) by the isolated fungal species (*Saccharomyces* species, *Aspergillus* species, *Cladosporium* species, *Rhizopus* species, *Mucor* species and *Penicillium* species. *Cladosporium* species from diesel contaminated soil had the highest optical density (OD = 1.010), followed by *Saccharomyces species* (OD = 0.786) and *Penicillium* species (OD = 0.772). In the petrol – contaminated soil, *Penicillium species* showed the highest OD of 0.848 followed by *Saccharomyces* species of OD = 0.615 and *Mucor* species of OD = 0.560. (Figure 2).

DISCUSSION

Contaminated soil samples showed mean THC levels exceeding the Department of Petroleum Resources Standard (DPR, 1991). The mean THC level in the uncontaminated soil (control) was as low as 8.0 mg/kg indicating that hydrocarbons could be present in uncontaminated soils and sediments, as earlier reported elsewhere (Geiger and Blumer, 1974).

The uncontaminated site had the highest fungal count (813 cfu/g), indicating that the petroleum products in the contaminated sites could have initially adversely affected the growth of the organisms. However, the susceptibility of petroleum products to biodegradation varies with the types and size of the components, as is evident in the rapid microbial degradation of alkanes of intermediate chain length ($C_{10} - C_{24}$), compared with the resistance of alkanes with very long chains to degradation (Atlas and Bartha, 1993, Atlas, 1996). This accounts for the high significant differences ($P<0.01$) in fungal counts between soils contaminated with diesel ($C_{15} - C_{20}$), petrol ($C_4 - C_6$) and engine oil ($C_{25} - C_{40}$) as a result of differences in the ability of the petroleum products to support fungal growth (Clark *et al*., 1997). Moreover, petroleum is a mixture of many different classes of hydrocarbons, and a given microorganism has the potential to degrade only a part (Rosenberg *et al.*, 1992). This, besides physico-chemical factors, was observed to be responsible for the differences in the fungal degradation of the different petroleum products.

The low incidence of total fungi in diesel-contaminated soil in the first day (day 0) of sampling (Fig.1) was as a result of a lag period caused by toxic effect (Clark *et al*., 1997) on fungi, which was less evident in soils contaminated with engine oil and petrol. Petroleum products with higher molecular weight components such as engine oil are less toxic than products with middle weight constituents (Clark *et al.*, 1997). Also, low molecular weight compounds (e.g., petrol) are generally volatile and rapidly lost into the atmosphere, and thus have less toxic effect (Clark *et al.*, 1997). In the second day (day 7) of sampling, some fungal species having recovered from 'shock', became adapted and showed greater enzymatic activity for the degradation of susceptible diesel molecules for carbon and energy, resulting in the sudden rise in their incidence (Fig. 1). In the third and fourth sampling days (days 14 and 21), the microbial flora was

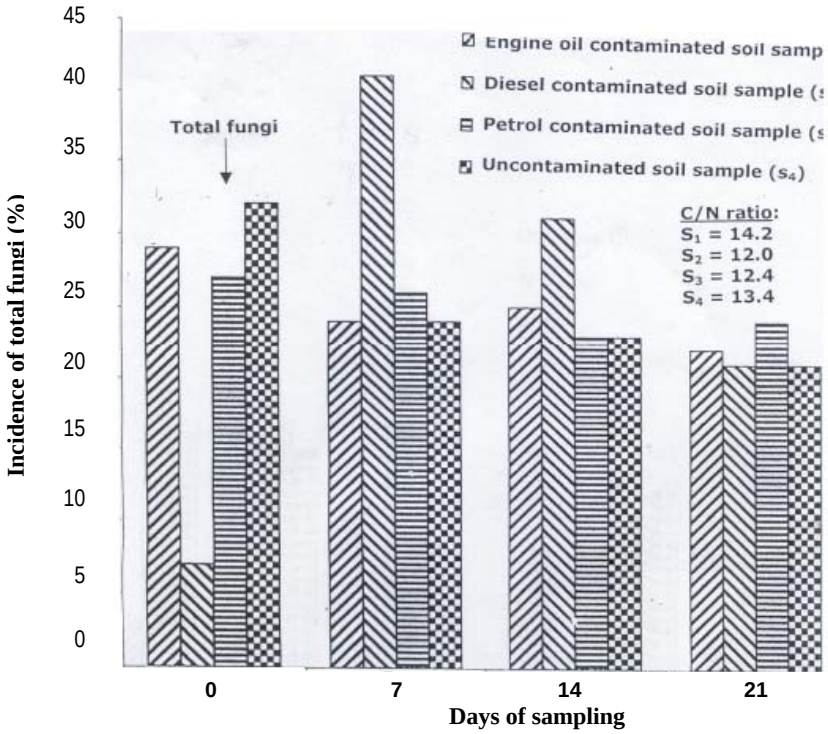

Fig. 1. Incidence of total fungi at the sampling sites at various intervals of sampling

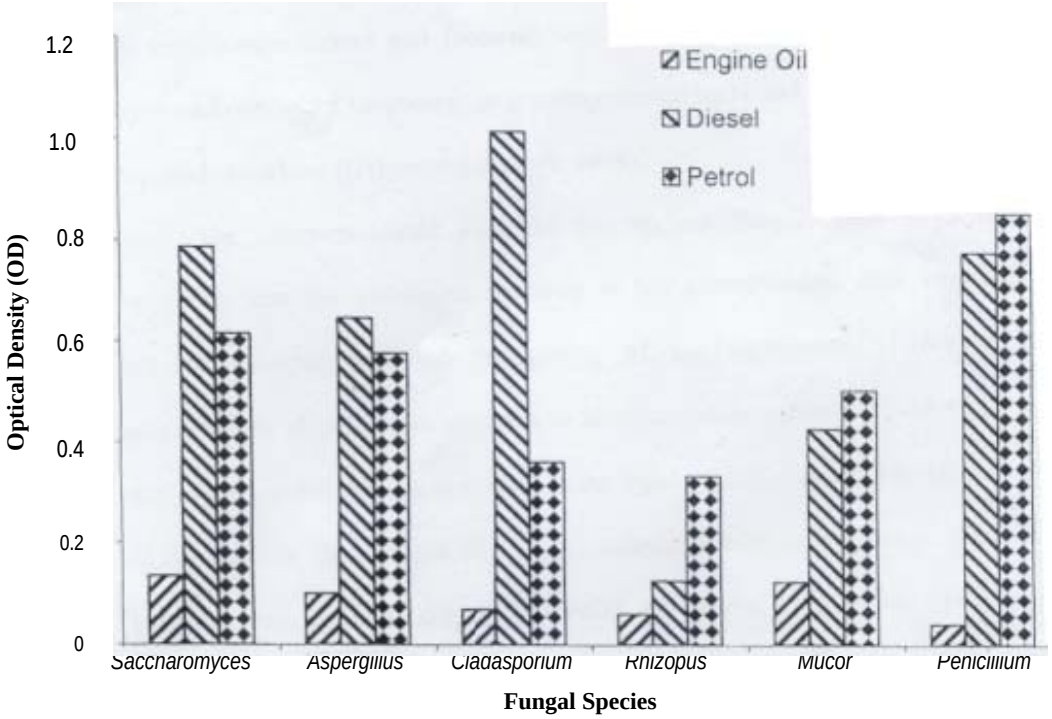

Fig. 2. Utilization of petroleum products (Engine oil, Diesel and Petrol) by fungal species

restored. It is apparent that the C:N ratio, an index of biomass (McEldowney *et al.*, 1994; Eja, *et al.*, 2003) (Fig. 1), and soil physico-chemical properties (Onwurah, 2000) (Table 2) were responsible for the general restoration of the microbial flora. However, organic materials have widely different carbon: nitrogen ratios, and generally, if the ratio is below 20, this is often adequate to satisfy the nitrogen requirements of the microflora that decompose the residues (Lynch and Poole, 1979; Eja *et al.*, 2003). Thus, the differences in the C:N ratios of the soil samples also reflected in the incidence of total fungi at the sampling sites.

Saccharomyces, Aspergillus, Cladosporium, Rhizopus, Mucor and *Penicillium* species isolated in this study showed evidence of relative ability to degrade petroleum in the contaminated soil samples. Diesel was most susceptible to degradation by fungal species isolated in this study whose degradative abilities were observed to be in the following order: *Cladosporium* > *Saccharomyces* > *Penicillium* > *Aspergillus* > *Mucor* > *Rhizopus* (Fig. 2).

Table 2. Effect of contaminated and uncontaminated soil samples with petroleum products on some physicochemical characteristics of soil samples from the petroleum-contaminated sites ($S_1 – S_3$) and the uncontaminated (control) site (S_4)

Soil sample	Total Moisture (%)	pH	THC (mg/kg)	Total Organic Carbon (C)	Total Nitrogen (%)	Available Phosphorus (mg/kg)	Particle size %			Kcmol per kg	Texture
							Clay	Silt	Sand		
S_1	12.04 ± 0.20	5.4 ± 0.03	3,158.1 ± 0.8	2.99 ± 0.01	0.21 ± 0.30	4.00 ± 0.10	7.0 ± 0.10	8.7 ± 0.20	84.3 ± 0.01	0.04 ± 0.01	LS
S_2	13.86 ± 0.40	5.9 ± 0.12	1,236.2 ± 0.1	1.92 ± 0.3	0.16 ± 0.20	7.00 ± 0.30	8.0 ± 0.20	12.7 ± 0.15	79.3 ± 0.20	0.22 ± 0.02	SL
S_3	10.69 ± 0.30	5.6 ± 0.10	1,128.6 ± 0.2	0.99 ± 0.03	0.08 ± 0.1	7.62 ± 0.40	6.0 ± 0.12	12.7 ± 0.20	81.3 ± 0.01	0.24 ± 0.03	LS
S_4	13.99 ± 0.10	5.7 ± 0.12	8.0 ± 0.10	1.89 ± 0.12	0.14 ± 0.03	3.37 ± 0.02	4.0 ± 0.20	16.7 ± 0.31	79.3 ± 0.02	0.17 ± 0.01	SL

S_1 = Engine oil contaminated soil, S_2 = Diesel contaminated soil, S_3 = Petrol contaminated soil,
S_4 = Control, LS = Loamy sand, SL = Sandy loam.
Each value is the mean ± standard deviation of physicochemical parameters of triplicate soil samples collected from each site.

On the other hand, the abilities of the fungi to degrade petrol was observed to be in the following order: *Penicillium* > *Mucor* > *Saccharomyuces* > *Aspergillus* > *Cladosporium* and *Rhizopus*. For engine oil, the order was *Saccharomyces* > *Mucor* > *Aspergillus* > *Cladosporium* > *Rhizopus* > *Penicillium.* The order of biodegradative abilities of various species differ remarkably. Thus, *Cladosporium* was observed to be the best fungus for a rapid degradation of diesel, while *Penicillium* was the best fungus for the degradation of petrol. All the fungal isolates showed no appreciable abilities to degrade engine oil. This could have resulted from the fact that engine oil has a high molecular weight aromatic component (>20 carbon atoms), a factor that favours resistance to biodegradation of petroleum (Atlas and Bartha, 1993).

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