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Microbial Load and Enzyme activities of Microorganisms isolated from waste oil contaminated soil in Akwa Ibom State, Nigeria.

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Abstract

Soil enzyme activities, physicochemical parameters and microbiological analysis of automobile waste oil contaminated soil were investigated in this study using standard microbiological techniques. Total heterotrophic bacterial count, phosphate stabilizing bacterial count, lipolytic bacterial count and nitrifying bacterial count for waste oil contaminated soil were 1.7 x 10^3 cfu/1g, 1.4 x 10^1 cfu/g, 1.2 x 10^2 cfu/g and 0.7 x 10^1 cfu/g respectively. While that of the control soil were 3.7 x 10^6 cfu/g, 3.1 x 10^4 cfu/g, 2.7 x 10^3 cfu/g and 2.4 x 10^4 cfu/g respectively. Five bacterial isolates were obtained and their frequencies of occurrence were Bacillus sp. (37.5%), Proteus sp. (25.0%), Pseudomonas sp. (12.5%), Micrococcus sp. (12.5%) and Enterobacter sp. (12.5%). Fungi isolated were Rhizopus stolonifer, Aspergillus flavus, Aspergillus glacus, Monilla sp, Cladosporium sp, Aspergillus terreus and Verticillium sp. Determination of soil enzyme activities showed that dehydrogenase was 15.32 and 27.22 mg^{-1} 24h^{-1} in the impacted soil and control soil respectively, followed by alkaline phosphatase (0.97 and 3.87 µmol-p-nitro phenol), lipase (3.01 and 3.71 mg-g^{-1} 24h^{-1}) and acid phosphatase (1.71 and 3.67 µmol-p-nitro phenol). In all cases, control soil had higher activities than the waste oil contaminated soils. The enzymes activities correlated positively with the bacterial loads. The results showed that waste oil contamination cause decrease in bacterial loads and enzyme activities of the impacted soils and also affects soil physicochemical parameters.

Key Terms – *Aspergillus, Bacillus,* phosphatase, pollution, *Rhizopus* and waste oil.

1. Introduction

Environmental pollution with petroleum and petroleum products (complex mixture of hydrocarbons) has been recognised as one of the most serious current problems especially as when associated with accidental spills on large scale. Waste or used oil according to the US Environmental Protection Agency is defined as oil that has been refined from crude oil or any synthetic oil. Petroleum products such as engine oil, petrol, diesel and kerosene are used daily in various forms in mechanic workshops. These products tend to harden and change the colour of the soil, which may have untold health hazard on the technicians and artisans (Udeani *et al.*,

2009). Apart from altering the physiochemical state of the soil, oil pollution has an unprecedented effect on the soil microbial population (Bossert and Bartha, 1984). Contamination of environment by crude oil or its component could lead to a depression of microbial number and activities even in case of light contamination (Odu, 1972). Soil constitutes the major habitat of terrestrial microorganisms. A higher number of these organisms occur in the rhizosphere in association with plant roots. Thus, this study is aimed at determining the microbial load of the polluted soil, characterization and identification of microbial isolates, determination of physicochemical parameters of impacted soil and the determination of soil enzyme activities of the oil contaminated soil.

2. Materials and Methods

2.1 Media and Materials

The media used include Nutrient agar, Sabouraund dextrose agar and Peptone water. The media was prepared according to the manufacturer's direction on the media labels. The Materials employed in this work include; 100 ml conical flasks, test tubes, petri dishes, slides, inoculating loop, test tube rack, weighing balance, incubator, pressure cooker, measuring cylinder, auger, sample bags, aluminium foil and cotton wool. All the media and materials used in this work were purchased at the Science Laboratory equipment Store, Uyo, Akwa Ibom State, Nigeria.

2.2 Study Area

The study area was automobile mechanic village, Abak road, Uyo, Akwa Ibom State, Nigeria. This area has been constantly polluted with waste oil over a long time. There is no plant growth in this area.

2.3 Sample Collection

Soil samples were collected from the waste oil polluted site as well as control site which was an agricultural farm in same study area. The soil samples were collected with the aid of an auger into a sterile black polythene bags. The soil samples were transported to the Department of Microbiology, University of Uyo Laboratory for analysis.

2.4 Isolation of Microorganisms

10g of waste oil contaminated soil sample was weighed into 90ml sterile distilled water in 250ml conical flask. The soil suspension was agitated and allowed to stand for 10 minutes. Serial dilution of the supernatant (1.0ml) were carried out on 9ml of sterile distill water in test tubes. Desired dilutions were plated on nutrient agar and Sabouraund dextrose agar respectively. This process was also carried out for the control sample.

2.5 Enumeration of Bacteria

The enumeration of bacteria was carried out by pour plate technique. One milliliter of series of dilution 10^{-5}, 10^{-4}, 10^{-3} was plated in duplicates on nutrient agar medium and incubated at room temperature (28 ± 2^{0}C) for 24 – 28 hours. Counts were recorded from duplicates plates as colony forming units/ml.

this process was also carried out for the control sample.

2.6 Isolation of Fungi

The enumeration of fungi was carried out by using pour plate technique. 1ml of the dilution 10^{-5}, 10^{-4}, was pipetted into sterile petri dishes in duplicate using a sterile 1ml pipette. Also 1ml of aliquot from dilution 10^{-5}, 10^{-4} of the control sample was pipetted into sterile petri dishes in duplicate using a sterile pipette. Sabouraund dextrose agar supplemented with 50uglml of streptomycin (antibacterial agent) was poured into the plates. Swirled gently and allowed to set. The plates were incubated at room temperature for 4 – 5 days.

2.7 Purification and maintenance of bacterial isolate

Distinctive colonies of bacteria from the primary plates were sub cultured repeatedly onto nutrient agar plate. Stock cultures were prepared and stored in the refrigerator for further use.

2.8 Identification and characterization of bacterial isolate

Bacterial identities were determined based on motility, morphological feature, and biochemical characteristics using Bergey's manual of determinative bacteriology (Buchanan and Gibbons, 1974).

2.9 Physicochemical properties of the soil

The physiological properties of the soil were determined according to the recommended methods of Association of Official Analytical Chemists (AOAC, 2005).

2.10 Methods for different groups of Bacteria

The soil physiological bacteria groups were determined using various culture media. Tryptone soy agar was used for total heterotrophic bacterial count, modified mineral salt agar for the nitrifying bacterial count and Pikovskay's media for the phosphate solubilizing bacterial count. The bacteriological loads of the soil samples were determined after ten-fold serial dilutions with 0.2ml of the desired dilution being inoculated on the various media using the spread plate technique (Cheesbrough, 2002). The counting was done after 24 to 48 hours aerobic incubation. The bacteria species observed in the Pikovskay's media (PSB) were characterized and identified to the general level using morphological and microscopic features in addition to biochemical tests (Holt *et al.*, 1994) (Cheesbrough, 2002).

2.11 Soil Enzymes activities

The enzymes whose activities were assessed in this study include; dehydrogenase, lipase and the phosphatase (acid and alkaline). The soil was dried at room temperature for 24 hours and passed through 0.5mm sieve. The sieved soil used for the analysis. Dehydrogenase activities was determined by the method involving the reduction of Triphenyl tetrazolium chloride (TTC) to triphenyl formazon (TPF) after incubation of the TTC amended soil at 30^0C for 6 hours (Alef and Nannipieri, 1995). Activities of

both acid and alkaline phosphatases were determined as decribed by Tabatabai (1997), which involve the use of P-nitro phenyl phosphate and read at 110nm. White acid phosphatase activity was determined at pH 6.8 and alkaline phosphatase was determined at pH 11.5. Lipase was determined according to Saisuburamaniyan *et al.* (2004) using olive oil amended soil.

3. Results

Automobile waste oil contaminated soil samples were analyzed along with an agricultural soil which served as control. Table 1 shows the result of the bacteriological loads of the automobile waste oil contaminated soils which showed considerable variations in the values of bioloads of the different groups estimated. Total heterotrophic count, phosphate solubilizing bacterial count, lipolytic bacterial count and nitrifying bacterial count for the waste oil contaminated soil were 1.7 x 10^3 cfu/g, 1.4 x10^1 cfu/g, 1.2 x 10^2 cfu/g and 0.7 x 10^1 cfu/g respectively while that of the control soil was 3.7 x 10^6 cfu/g, 3.1 x 10^4 cfu/g, 2.7 x 10^3 cfu/g and 2.4 x 10^4 cfu/g respectively.

Table 1: Bacteriological loads of the automobile waste oil contaminated soil (cfu/g)

Microbial Group (cfu/g)	**Waste oil polluted soil**	**Control**
Total heterotrophic bacterial count	1.7 x 10^3	3.7 x 10^6
Phosphate solubilizing bacterial count	1.4 x10^1	3.1 x 10^4
Nitrifying bacterial count	0.7 x 10^1	2.4 x 10^4
Lipolytic bacterial count	1.2 x 10^2	2.7 x 10^3

The results of the characterization of bacterial isolates are presented on Table 2. The bacteria isolates obtained and their frequencies of occurrence were *Bacillus sp.* (35.5%), *Proteus sp.* (35.0%), *Pseudomonas sp.* (12.5%), *Micrococcus sp.* (12.5%) and *Enterobacter sp.* (12.5%).

Table 2: Morphological and Biochemical characterization of Bacterial Isolates

Cell sample	Gram	Motility	Spore stain	Indole	Catalase	Coagulase	Oxidase	Mp-test	Vp-test	Urease	Citrate	Carbohydrate Utilization Test					ORGANISM
												Glucose	Sucrose	Lactose	Galactose	Mannitol	
Rods	+	+	+	+	+	-	+	-	+	+	+	AG	AG	AG	AG	AG	*Bacillus sp*
Rods	+	-	+	-	-	-	+	-	+	-	-	AG	AG	AG	AG	AG	*Proteus sp*
Rods	-	+	-	-	+	-	+	-	-	+	+	A	AG	AG	A	A	*Pseudomonas sp*
Cocci	+	-	-	-	+	-	+	-	-	+	+	AG	A	A	AG	A	*Micrococcus sp*
Cocci	+	-	+	+	-	-	+	+	+	+	+	AG				AG	*Enterobacter sp*
																A	

KEY:

AG – Acid and Gas

A – Acid

- - No fermentation (Negative

Table 3 shows the morphological and cultural characterization of the fungal isolates. The fungi isolated were *Rhizopus stolonifer*, *Aspergillus flavus*, *Aspergillus glacus*, *Monilia sp.*, *Aspergillus ferreus*, *Clasdosporium sp.*, and *Verticilium sp.*

Table 3: Morphological and cultural characterization of fungal isolates

Isolate number	**Colony colour**	**Type of soma**	**Nature of hyphae**	**Special vegetative structure**	**Asexual spore**	**Special reproductive structure**	**Conidial head**	**Vesicle head**	**Probable organism**
1	White becoming grayish	Filamentous	Coenocytic	Rhizoids	Ovoid sporangiospores	Tall sporangiospores	-	-	*Rhizopus stolonifer*
2	Dense felt yellow green colour	Filamentous	Septate	Footcell	Glubose conidia	Phallides borne directly on the vesicle sclerotia	Radiate	Subglubose	*Aspergillus flavus*
3	Grayish green	Filamentous	Septate	Footcell	Subglubose conidia	Hyaline conidiophores	Radiate	Subglubose	*Aspergillus glacus*
4	Fast growing white colony with irregular tufits	Filamentous	Septate	Footcell	1-celled conidia in chains	Conidiogenous hyphae	-	-	*Monilia species*
5	Brownish colony becoming darker with age	Filamentous	Septate	Footcell	Glubose conidia	Short conidiophores	Long columnar	hemispherical	*Aspergillus ferreus*
6	Powd	Filame	Septat	-	Acropetal	Short	-	-	*Cladosp*

	ery olivaceaus	ntous	e		branched conidial chains	conidiophores			*orium species*
7	Cotton y white to pale yellow	Filamentous	Septate	-	1-celled conidia in heads (cylindrical in shape)	Solitary Phallides chlamydospores Absent	-	-	*Verticilium species*

Table 4 shows the value of enzyme activities analyzed. Determination of soil enzyme activities showed that dehydrogenase was 15.32 and 27.22mg^{-1} 24 h^{-1} in the impacted soil and control soil respectively, followed by alkaline phosphatase (0.97 and 3.87 µmol-p-nitro phenol). Lipase (3.01 and 3.71 mg-g^{-1} 24h^{-1}) and acid phosphatase (1.71 and 3.67 µmol-p-nitro phenol). In all cases, control soil had higher activities than waste contaminated soil. The enzyme activities correlated positively with the bacterial loads.

Table 4: Soil enzymes activities of automobile waste oil contaminated soil

Enzyme	Waste oil polluted soil	Control
acid phosphatase	1.71	3.67
Dehydrogenase	15.32	27.22
Lipase	3.01	3.71
alkaline phosphatase	0.97	3.87

Table 5 shows the results of physicochemical properties of the soil. Waste oil affected soil physicochemical properties adversely. Results show slight increase in temperature. Decrease in pH and total phosphate was observed. However, the total nitrate and total sulphate was observed to increase.

Table 5: Physicochemical properties of the contaminated soil

Parameters	Soil sample	control
pH	6.5	7.6
Temperature ^{o}C	31.5	30.7
Total phosphate (mg/c)	27.63	29.38
Total nitrate (mg/c)	14.69	8.783
Total sulphate (mg/c)	30.73	10.93
Ca (mg/l)	21.50	18.00
Mg (mg/l)	30.27	30.18
K (mg/l)	198.2	236.1
Na (mg/l)	81.93	96.53
Cu (mg/l)	17.93	5.99
Zn (mg/l)	50.17	20.57

4. Discussion

The results obtained from this study showed that automobile waste oil affected soil physicochemical parameters adversely at high concentrations. The slight increase in temperature is attributed to oxidation of the oil on soil and heat released from the automobile engines. The decreased in pH

observed could be attributed to the activity of microorganisms which may have produced organic acid. Tang *et al*. (2010) reported similar observation in bioremediation of petroleum polluted soil. In mechanic workshops there is constant change in the soil mechanism as a result of deliberate spillage of used engine oil, This alter the biomass and ecology of the soil such that both microbial communities and plants can no longer grow on the polluted soil. The colour and texture of the soil are affected; this leads to different microbial flora establishment in an attempt to remedy the petroleum product spillage (Bartha and Atlas, 1977). The total heterotrophic bacterial count (THB) was the most prevalent among the various bacterial groups observed, followed by phosphate solubilizing bacterial count, lipolytic bacterial count, and while nitrifying bacterial count was the least. Since the THB is the total bacterial group that could be seen on general purpose media, it is not a specialized group. Some bacteria of the other groups could be found among the THB. The specificity of the contents of the impacting agents influenced the prevalence of available organisms in the soil. The aromatic compounds have been shown to have more drastic effect than alkanes. The compounds inhibit photosynthesis and growth, reduced enzymes activities and microbial biomass (Megharaj *et al*., 2000). The enzyme analysis positively correlated with bacterial bioloads observed earlier. Dehydrogenase is produced by every organism, no matter the species, hence the high enzymatic actions observed. It could be said to be from organisms either heterotrophic or specialized. On the other hand, the activities of the other enzymes, alkaline phosphatase, lipase and acid phosphatase could be said to be substrate induced following the content of the wastes. Also acid and alkaline phosphatase was adversely affected by the impaction of automobile waste oil in high concentrations. Though acid was more affected than alkaline phosphatase, this could be due to decrease in pH and decrease in the number of bacterial species producing it. Nwaugo *et al*. (2008) stated that the activities of alkaline phosphatase increased with increase in pH in cattle market waste impacted soil.

5. Conclusion

Studies of microbial load and enzyme activities associated with petroleum degrading microbes have the potential to enhance our understanding of the roles played by microbes in natural genesis of long term effect of petroleum waster product pollution and to determine new remediation. From this study, the used engine oil was poorly disposed. Therefore, regulations should be placed on disposal of automobile waste oil and pit systems should be built, where this used oil will be collected and properly treated before disposing them to avoid environmental degradation.

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