CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Collection of samples

The soil sample used was collected from the Nursery section of Asia-European Institute, University of Malaya, Kuala Lumpur in a sack and transported to the laboratory for analysis. Used lubricating oil was collected from Perodua car service centre, Petaling Jaya, while the organic wastes were collected from different locations; banana skin (BS) was collected from IPS canteen, University of Malaya. Brewery spent grains (BSG) was collected from Carlsberg brewery, Shah Alam, Selangor and spent mushroom compost (SMC) was collected from Gano mushroom farm, Tanjung Sepat, Selangor. All the organic wastes were dried at 60^0C and grinded. The *Jatropha curcas* seedlings (3 weeks old) used for phytoremediation were provided by Dr John of the Nilai University College, Nilai, Malaysia, while, the *Hibiscus cannabinus* seeds were purchased from National Tobacco Board of Malaysia, Kelantan, Malaysia. The two plants were selected based on three factors: hardiness, non edible and they are commercially viable plants. While organic wastes was selected based on their abundance and availability in Malaysia.

3.2 Organic wastes utilized in this study

Three organic wastes (banana skin, brewery spent grain and spent mushroom compost) were used in this study for amendment of soil contaminated with used lubricating oil. These wastes contain appreciable quantities of nutrients that soil microorganisms can use for multiplication in the contaminated soil. The use of such wastes, besides providing alternative substrates, helps to solve environmental problems, which are caused by their disposal (Osma et al., 2007).

3.2.1 Banana skin

Banana (*Musa sapientum*) fruit skin is an organic waste that is highly rich in carbohydrate content and other basic nutrients (nitrogen and phosphorus) that could support microbial growth (Essien, et al., 2005). Nutritional compositions of banana skin are presented in Table 3.1. Plate 3.1 shows the banana skin used for the studies.

Table 3.1 dry matter and chemical composition of banana skin

Compound (g per 100g)	
Dry matter	14.08
Crude protein	7.87
Crude fat	11.60
Crude fibre	7.68
Total ash	13.44
Carbohydrates	59.51
Moisture	78.4
Mineral content (mg per 100g)	
Calcium	7.0
Sodium	34
Phosphorus	40
Potassium	44
Iron	0.93
Magnesium	26
Sulphur	12

(Essien et al., 2005)

Plate 3.1 Dry banana skin used for bioremediation

3.2.2 Brewery Spent Grain

Brewery spent grain (BSG) is the residue left after separation of the wort during the brewing process. Composition of BSG may vary with barley variety, time of harvest, characteristics of hops and other adjuncts added, and brewery technology (Santos et al., 2003). Brewery spent grain (BSG) represent around 85% of the total by-products generated by brewery industry. It is available in large quantities throughout the year, but its main application has been limited to animal feeding. Nevertheless, due to its high content of protein and fibre (around 20 and 70% dry basis, respectively, Mussatto et al., 2006), it can also be used to enhance the activities of indigenous microorganisms in hydrocarbon contaminated soil. Santos et al., (2003) found, besides fibre, 24.2% protein, 3.9% lipid and 3.4% ash in oven-dried BSG.

Minerals, vitamins and amino acids are also found in BSG. The mineral elements include calcium, cobalt, copper, iron, magnesium, manganese, phosphorus, potassium, selenium, sodium and sulphur, all in concentrations lower than 0.5% (Huige, 1994). Plate 3.2 shows the picture of BSG used for the studies.

Plate 3.2 Brewery spent grain used for bioremediation

3.2.3 Spent Mushroom Compost

Spent mushroom compost (SMC) is the remains of the compost in which mushrooms are produced (Lau et al., 2003). Mushroom production is the biggest solid-state-fermentation industry in the world (Moore and Chiu, 2001). Correspondingly, 5 kg of SMC will be generated from the production of 1 kg of mushrooms (Semple et al., 2001). Spent mushroom compost (SMC) has proven to be an attractive material for improving soil structure in tilled soils and increasing dry matter production in grassland soils, owing to its high organic matter content and availability of essential plant nutrients (Jordan et al., 2008)

There are also many potential agricultural, horticultural and industrial uses of SMC including: An agricultural fertiliser (Gent et al., 1998; McCahey et al., 2003); a ruminant feed for sheep (Fazaeli and Masoodi, 2006); environmental enrichment in intensive pig farms (Beattie et al., 2001); treatment for coal mine drainage (Stark et al., 1994); bioremediation (Lau et al., 2003); enzyme extraction (Ball and Jackson, 1995); and a novel biosorbent (Chen et al., 2005). Picture of SMC used for the studies is shown in Plate 3.3.

Plate 3.3 Spent mushroom compost used for bioremediation

3.3 Methodology used for biostimulation studies under laboratory conditions

3.3.1 Microcosm Set-up Description

1.5kg of soil (sieved with 2mm mesh size) was placed in plastic vessels labeled A to E with a volume of about 3000 cm^3. The soil was polluted with 5% (w/w) (Ijah and Antai, 2003a) used lubricating oil (50,000 mg kg^{-1} soil) and left undisturbed for two days. After two days, 10% (Ijah and Antai, 2003a) of each organic waste (ground dry banana skin (BS), brewery spent grain (BSG) and spent mushroom compost (SMC)) were individually introduced into each oil polluted soil labeled A, B and C respectively and thoroughly mixed. The moisture content was adjusted to 60% water holding capacity and incubated at room temperature (28 ± 2^0C). Vessel D with only soil and used lubricating oil served as control. Additional control (comprising of autoclaved soil and 0.5% (w/w) NaN_3) was set up with the same quantity of used lubricating oil to monitor degradation of oil due to non-biological factors in plastic vessel labeled E. The content of each vessel was tilled twice a week for aeration and the moisture content maintained at 60% water holding capacity by the addition of sterile distilled water. The experiment was set up in triplicate. The same method was used for soil contaminated with 10% and 15% (w/w) used lubricating oil.

3.3.2 Sampling

Periodic sampling from each vessel was carried out at 14 days intervals up to 84 days. Composite samples (30 g) were obtained by mixing 5g of soil collected from four different areas of the microcosm for isolation and enumeration of bacteria and determination of total petroleum hydrocarbon.

3.3.3 Determination of physicochemical properties of soil and organic wastes

Nitrogen content of soil used for bioremediation and organic wastes was determined using the Kjeldahl method Ijah and Antai, (2003), while phosphorus, lead and zinc contents were determined using inductively coupled plasma – optical emission spectrometry (ICP-OES) (after acid digestion of the samples in a microwave digester). Furnace method was used to determine the total organic carbon by heating 5 g of the soil sample for 7 hours in a furnace at 500^0C (Ijah et al., (2008). The pH was determined with a pH meter (HANNA HI 8424) on 1:2.5 (w/v) soil/distilled water after 30 minutes equilibration. Triplicate determinations were made.

3.3.4 Total Petroleum Hydrocarbon Determination

Hydrocarbon content of the soil samples was determined by toluene cold extraction method (Adesodun and Mbagwu, 2008). Ten grams (10g) of soil sample was weighed into 50 ml flask and 20 ml of toluene (AnaLar grade) added. After shaking for 60 minutes on an orbital shaker (model N-Biotek-101M), at 200 RPM, the liquid phase of the extract was measured at 420nm using a DR/4000 Spectrophotometer. The total petroleum hydrocarbon (TPH) in soil was estimated with reference to standard curve derived from fresh used engine oil diluted with toluene. TPH data was fitted to first-order kinetics model of Yeung et al., (1997).

$$y = ae^{-kt}$$

Where y is the residual hydrocarbon content in soil (g kg^{-1}), a is the initial hydrocarbon content in soil (g kg^{-1}), k is the biodegradation rate constant (d^{-1}) and t is time (d). The

model estimated the biodegradation rate and half-life of hydrocarbons in soil relative to treatments applied. Half-life was then calculated from the model of Yeung et al., (1997) as

$$\text{Half life} = \ln(2)/k$$

This model was based on the assumption that the degradation rate of hydrocarbons positively correlated with the hydrocarbon pool size in soil (Yeung et al., 1997).

3.3.5 Gravimetric measurement of used lubricating oil loss in soil (Biodegradation)

The total extents of used lubricating oil biodegradation in soil were also determined gravimetrically by suspending 10 g of soil (dried with 10 g of anhydrous sodium sulphate) in 20 ml of diethyl ether in a 250 ml capacity Erlenmeyer flask. After shaking for 30 minutes on an orbital shaker (model N-Biotek-101), the solvent – oil mixture was filtered using Whatman No. 1 filter paper into 100 ml Florentine flask of known weight, the solvent was evaporated completely using rotary evaporator. The process was repeated in order to ensure complete extraction of the oil from the soil. The new weight of the flask (now containing residual oil) was recorded. Percentage biodegradation of used lubricating oil was calculated using the formula of Ijah and Ukpe (1992):

$$\% \text{ biodegradation} = \frac{\text{weight of oil (control)} - \text{weight of oil (degraded)}}{\text{Weight of oil (control)}} \times 100\ \%$$

3.3.6 Enumeration and identification of bacteria in soil

Three replicate samples from each oil polluted soil were withdrawn every 14 days for the enumeration of total aerobic heterotrophic bacteria (AHB). 0.1ml of serially diluted samples was plated on nutrient agar medium (Oxoid) supplemented with 50 µg/ml nystatin to suppress the growth of fungi. Triplicate plates were incubated at 30^0C for 24 hours before the colonies were counted. Hydrocarbon utilizing bacteria (HUB) in the soil samples

were enumerated using mineral salt medium of Zajic and Supplisson (1972) (1.8 g K_2HPO_4, 4.0 g NH_4Cl, 0.2 g $MgSO_4.7H_2O$, 1.2 g KH_2PO_4, 0.01 g $FeSO_4.7H_2O$, 0.1 g NaCl, 20 g agar, 1% used engine oil in 1000 ml distilled water, pH 7.4). The oil agar plates were incubated at 30^0C for 5 days and the colonies were counted and randomly picked based on the frequency of the colonies on the agar plate. Pure isolates were obtained by repeated sub-culturing on nutrient agar (Oxoid). The bacterial isolates were characterized using microscopic techniques (Gram staining) and biochemical tests, and further confirmed by using analytical profile index (API) 20NE for Gram negative bacteria and BBL Crystal rapid identification kit for Gram positive bacteria. For Gram positive bacterial identification, colonies of pure culture of bacteria was introduced into the BBL inoculums fluid with the aid of sterile wire loop and vortexed for 10 – 15 seconds. The turbidity was adjusted to the equivalent of McFarland No. 0.5 standard, the entire inoculums was poured into the BBL base that contains different wells. The inoculums was gently roll with both hands to ensure that all the wells are filled, the wells containing the inoculums were later covered with BBL lid that contained 29 dehydrated biochemical and enzymatic substrates and a fluorescence control on tips of plastic prongs. The inoculated panels were incubated for 18 – 24 hours at 35 – 37^0C, at the end of incubation period the wells were examined for colour changes or presence of fluorescence that resulted from metabolic activities of the microorganisms. The resulting pattern of the 29 reactions was converted into a ten digit profile number that was used as the basis for identification. The resulting profile number derived from different colour changes and cell morphology was entered into PC in which the BBL Crystal mind software has been installed to obtain the bacterial identification.

Gram negative bacterial isolates were identified by using API 20 NE. Pure culture colonies of bacterial sample was transferred into an ampoule of API NaCl 0.85% medium (2 ml)

with the aid of inoculating wire loop to prepare a suspension with a turbidity equivalent to 0.5 McFarland standard. Tests NO_3 to PNPG in the API panel were inoculated by distributing the saline suspension into the tubes using sterile pipette. 200 µl of the remaining suspension was added into an ampoule of API AUX medium and homogenized. The cupules tests GLU to PAC were filled with the suspension from API AUX medium this was followed by addition of mineral oil to the test cupules labeled GLU, ADH and URE until a convex meniscus was formed. The incubation box was closed and incubated at $29^0C \pm 2^0C$, at the end of the incubation period (24 hours), the results was recorded based on colour changes and converted into numerical profile. The identification was performed by using the database (V7.0) with the API which was earlier installed into the PC.

3.3.7 Measurement of CO_2 evolution from contaminated soil amended with organic wastes

The rate of microbial breakdown of oil was also assessed by the carbon dioxide (CO_2) evolution method of Cornfield (1961). One hundred gram (100 g) of soil contained in screw cap bottles in triplicates was treated with 10% (w/w) used lubricating oil. Ten grams (10g) of organic wastes (BS, BSG and SMC) was added individually to each bottle and the soil moisture was adjusted to 60% water holding capacity. To trap the CO_2 liberated during oil biodegradation, glass vials containing 0.5 g of barium peroxide and 4.5 ml of distilled water were placed in the screw cap bottles containing oil treated samples. Two control experiments were set up as follows: one control without added organic wastes, while the second had neither used lubricating oil or organic wastes. All treatments were incubated at room temperature (28 ± 2^0C) for 28 days. At 7 days intervals, a set of three vials per treatment were withdrawn and titrated with 1M HCl. The amount of CO_2 evolved during

oil degradation was calculated by the method of Cornfield (1961) and Stotzky (1965). The same method was used to determine CO_2 evolution for soil contaminated with 5% and 15% used lubricating oil.

3.3.8 Germination toxicity test of remediated soil

Toxicity of the remediated soil was assessed using germination test. Lettuce was used in this study owing to its sensitivity to hydrocarbon in soil (Vaajasaari et al., 2002; Plaza et al., 2005). The germination test was conducted over a 5 days test period. Seeds of lettuce were obtained commercially. For each soil samples 150 g of thoroughly mixed remediated soil was placed in 100 x 15 mm Petri dish bottoms. Ten viable seeds of lettuce (*Lactuca sativa* L.) were placed evenly throughout each Petri dish and covered with 10 g of dry sand. Three replicates of the samples were prepared. The moisture content of the soil was maintained at 80% water holding capacity. The Petri dishes were placed in a room with 16 hours light and 8 hours darkness for 5 days. At the end of 5 days, the number of seedlings that emerged from the surface of the sand were counted and recorded.

Germination index of lettuce seed on the remediated soil was calculated using the formula of Millioli et al., (2009).

$$\text{Germination index (\%)} = \frac{(\%\ SG) \times (\%GR)}{100}$$

% SG = (% EG/%CG) x 100

% GR = (GERm/GERCm) x 100

Where % SG = seed germination

% GR = growth of the root

% EG = germination on contaminated soil

% CG = germination on control soil

GERm = elongation of root on contaminated soil

GERCm = elongation of root on control soil

3.4 Methodology used for biostimulation studies under natural conditions

Top soil (20 cm) was air-dried and passed through a 2 mm sieve to remove stones, root materials and other debris. The air-dried, sieved soil samples were spiked with 5%, 10%, 15% (w/w) used lubricating oil and thoroughly mixed. The oil contaminated soil were amended with (10% w/w) different organic wastes: ground dry banana skin (BS), brewery spent grain (BSG), spent mushroom compost (SMC), after thorough mixing of the oil contaminated soil with the organic wastes; 1.5 kg each of the soil were packed into polythene plastic bags and set up at the experimentation site, exposed to sunlight and rainfall for the period of one year. Two different control treatments were set up; one control was oil contaminated soil without organic wastes amendment while the second control as well did not contain organic wastes but the soil was autoclaved in order to determine the oil loss due to abiotic factor. The biodegradation of the used lubricating oil was determined using GC/FID to analyzed the hydrocarbon fractions from the oil extracted from the soil as follows: 1 microlitre of the extracted oil sample was analyzed using gas chromatography with flame ionization detector (GC/FID). The GC was equipped with cross-linked 5% phenyl methyl siloxane capillary column; HP-5MS. Helium was used as carrier gas. The oven temperature program was started at 50 ^{0}C and raised by 25^{0}C/min until 325 ^{0}C, which was maintained for 11 minutes. The major hydrocarbon fractions were identified on the basis of their retention time and by comparing them to those of analytical standards.

3.4.1 Sampling and analysis

Replicate samples were withdrawn from each treatment every three month throughout the one year period of the experiment for the analysis of TPH loss, enumeration of total bacteria and HUB as described in the methods stated above.

3.5 Methodology used for phytoremediation studies

3.5.1 Determination of physicochemical properties of soil and organic wastes

Nitrogen content of soil used for phytoremediation and organic wastes was determined using the Kjeldahl method, while P, Fe, Zn and Pb contents were determined using inductively coupled plasma-optical emission spectroscopy (ICP-OES optima 4100 DV, Perkin Elmer, USA) after acid digestion in a microwave oven. The pH was determined with a pH meter (HANNA HI 8424) on 1:2.5 (w/v) soil/distilled water after 30-min equilibration. Triplicate determinations were made.

3.5.2 Phytoremediation with *Jatropha curcas* under laboratory condition and natural conditions

Four kilogramme (4 kg) of sieved (2mm) soil was contaminated with 2.5% and 1% (w/w) (these percentages were selected because in the initial test conducted the plants died at 3% oil concentration and above) of waste lubricating oil and thoroughly mixed. Five percent 5% (w/w) of different organic wastes (BS, BSG and SMC) were also mixed separately with the oil contaminated soil. Plastic bags were filled with the soil-oil-organic waste mixture

which was allowed to stabilize for four days before transplanting the seedlings of *Jatropha curcas* into the contaminated soil. Control treatment consisting of bags of the plant without waste lubricating oil or organic wastes was also set up. Additional control treatment comprising of autoclaved soil containing 0.5% (w/w) NaN_3 was also set up to determine non-biological loss of waste lubricating oil from the soil. All the treatments were set up in triplicate at room temperature (28 ± 2^0C) with 24 hours fluorescent lamps. The plants were moderately watered every 2 days with tap water to prevent leaching from the plastic bags. The same set of experiment was conducted with *Jatropha curcas* at a field (fifth floor of Institute of postgraduate studies, Universiti Malaya) exposed to sunlight and rainfall for the period of six months (April to October, 2009).

The appearance of the plants in response to the oil in soil was monitored to determine if there is phytotoxicity of the oil to the plants. The design of the experiment (randomized complete block design) is shown in Table 3.2.

Table 3.2 Experimental Design of phytoremediation

Treatment	Details of Treatment
A	4Kg soil + 2.5% oil + 5% BS + Jatropha/Hibiscus
B	4Kg soil + 2.5% oil + 5% BSG + Jatropha/Hibiscus
C	4Kg soil + 2.5% oil + 5% SMC + Jatropha/Hibiscus
D	4Kg soil + 2.5% oil + Jatropha/Hibiscus
E	4Kg soil + 2.5% oil only
F	4Kg autoclaved soil + 2.5% oil + 0.5% NaN_3
G	4Kg soil + 1% oil + 5% BS + Jatropha/Hibiscus
H	4Kg soil + 1% oil + 5% BSG + Jatropha/Hibiscus
I	4Kg soil + 1% oil + 5% SMC + Jatropha/Hibiscus
J	4Kg soil + 1% oil + Jatropha/Hibiscus
K	4Kg soil + 1% oil only
L	4Kg autoclaved soil + 1% oil + 0.5% NaN_3
M	4Kg soil without oil + Jatropha/Hibiscus

3.5.3 Phytoremediation with *Hibiscus cannabinus* under laboratory condition and natural conditions

Phytoremediation with *Hibiscus cannabinus* unlike that of *Jatropha curcas* was carried out by planting the seed of the plant directly into the oil-contaminated soil amended with organic wastes. A trial with *Hibiscus cannabinus* under laboratory condition did not survived because the plant requires lot of sunlight to survive, therefore phytoremediation with *Hibiscus cannabinus* was solely carried out at the experimental site exposed to sunlight and rainfall. The studies with *Hibiscus cannabinus* was carried out for the period of 3 months, unlike studies with *Jatropha curcas* that lasted for 6 months, the reason been that *Hibiscus cannabinus* grows fast and produce seed within 3 months period. The methodology used for soil preparation and sample analysis was the same as those used for phytoremediation with *J. curcas*, the only difference are the points stated above.

3.5.4 Sampling and analysis of samples

Soil samples were taken within the rhizosphere zone (20 cm) of Jatropha from each plastic bag every 30 days for analysis of TPH, pH, AHB and HUB counts. At the completion of the experiment (180 days), the plants were uprooted. The root was rinsed thoroughly with tap water and the plant dry matter was determined after drying at 50^0C for 48 hours (Palmroth et al., 2002). The root tissue was extracted with dichloromethane in a Soxhlet extractor for 12 hours to determine if the roots absorb the hydrocarbon from soil. The extracts were analyzed for hydrocarbons using gas chromatography with a mass-selective detector (GC/MSD) HP-6890 in scan mode. The GC was equipped with cross-linked 5% phenyl methyl siloxane capillary column; HP-5MS. Helium was used as carrier gas. The temperature program was started at 40^0C and raised by 10^0C/min until 300^0C, which was

maintained for 8 min. Heavy metals (Fe, Zn and Pb) in plant tissue were determined by drying at 40 ^{0}C and grinding them with laboratory blender (Waring model), followed by digestion with mixture of acids (HCl, HNO_3, and H_2SO_4) and analysis with ICP-OES. Bioconcentration factor (BCF) and Translocation factor (TF) of Zn and Fe by the two plants was calculated using the formula of Santosh et al., (2009).

$$\text{Bioconcentration factor (BCF)} = \frac{\text{Average metal conc. in the whole plant tissue (mg kg}^{-1})}{\text{Metal in the soil (mg kg}^{-1})}$$

$$\text{Translocation factor (TF)} = C_{aerial} \times 1/C_{root}$$

C_{aerial} = meal concentration in the aerial parts of plant

C_{root} = meal concentration in root of plant

3.5.5 Rate of metal uptake by *Jatropha curcas* and *Hibiscus cannabinus* under laboratory condition

The uptake rate of heavy metals (Fe and Zn) by *Jatropha curcas* and *Hibiscus cannabinus* were determined by using first order kinetic model to calculate the uptake rate of each metal per month by the plants (Kamath et al., 2004).

$k = -1/t \ (\ln M/M_o)$

Where:

k = first order rate constant for metal uptake per month

t = time in month

M = mass of residual metal in the soil (mg/kg)

M_o = initial mass of metal in the soil (mg/kg)

3.5.6 Isolation and identification of hydrocarbon utilizing bacteria

HUB counts in the soil was determined by plating a serially diluted 1g of the soil on oil agar (OA) [1.8 g K_2HPO_4, 4.0 g NH_4Cl, 0.2 g $MgSO_4.7H_2O$, 1.2 g KH_2PO_4, 0.01 g $FeSO_4.7H_2O$, 0.1 g NaCl, 20 g agar, 1% used engine oil in 1000 ml distilled water, pH 7.4], and incubated at 30^0C for 72 hours . The colonies on each plate were counted and recorded as colony forming unit per gram of soil (CFU/g). The pure culture of the bacterial isolates were identified by Gram staining technique and API 20NE for Gram negative bacteria and BBL Crystal rapid identification kit for Gram positive bacteria as described earlier.

3.5.7 Measurement of oil loss in phytoremediated soil

The total extents of waste lubricating oil biodegradation in soil were determined by suspending 10 g of soil in 20 ml of dichloromethane in a 250 ml capacity Erlenmeyer flask. After shaking for one hour on an orbital shaker (Model N-Biotek-101), the solvent-oil mixture was filtered using Whatman number 4 filter paper into a beaker of known weight and the solvent was completely evaporated. The new weight of the beaker (now containing residual oil) was recorded. Percentage biodegradation of used oil was calculated using the formula of Ijah and Ukpe (1992).

$$\% \text{ biodegradation} = \frac{\text{weight of oil (control)} - \text{weight of oil (degraded)}}{\text{Weight of oil (control)}} \times 100$$

3.6 Biodegradation studies with microorganisms isolated from oil contaminated and non-contaminated soil

Bacteria and Yeast were isolated from oil-contaminated and uncontaminated soils which were collected from mechanic workshop and the university environment respectively. The capabilities of the isolates to degrade used lubricating oil were tested in the laboratory.

3.6.1 Isolation and identification of microorganisms

Soil enrichment technique was used for the isolation of oil degrading microorganisms. In this method, 2 g of either oil polluted soil or unpolluted soil samples were added to 100 ml of sterile mineral salt medium (MSM) [1.8 g K_2HPO_4, 4.0 g NH_4Cl, 0.2 g $MgSO_4.7H_2O$, 1.2 g KH_2PO_4, 0.01 g $FeSO_4.7H_2O$, 0.1 g NaCl, pH 7.4] in 250 ml capacity Erlenmeyer flasks. Two milliliters (2 ml) of used lubricating oil were added to the medium and the flasks were incubated for 7 days at 30 ^{0}C on an incubator shaker (Thermo-line, Japan) operated at 200 rpm. After enrichment (2 times), suspensions of the enriched soil in physiological saline were inoculated into two sets of duplicate oil agar plates (Ijah, 1998). In one set of the plates meant for the isolation of crude oil utilizing bacteria, nystatin was added at a concentration of 50 mg/ml to suppress the growth of fungi. The plates were then incubated at 30 ^{0}C for 3 days. Colonies which appeared on the oil agar plates were randomly picked and pure isolates obtained by repeated sub-culturing on Nutrient agar (Oxoid). The bacterial isolates were characterized and identified by Gram staining technique and the use of API 20NE for Gram negative bacteria and BBL Crystal rapid identification kit for Gram positive bacteria.

The second sets of plates meant for the isolation of yeasts were added 100 mg of streptomycin to inhibit the growth of bacteria. The plates were then incubated at 30 ^{0}C for 5 days. Suspected yeast colonies which appeared on the oil agar plates were isolated in pure cultures by repeated sub-culturing on sabouraud dextrose agar (Oxoid). The yeast isolates were Gram stained and identified using API 20 C AUX identification kit as follows: 24 hours old pure culture of yeast colonies were inoculated into an ampoule suspension medium containing 2 ml of 0.85% NaCl to prepare a suspension with turbidity equivalent to 2 McFarland standard, this was followed by transfer of 100µl of the suspension into an ampoule of API C medium and gently homogenized with pipette. The API cupules were filled with the suspension covered with the lids and incubated at 29^0C ± 2^0C for 48 hours. At the end of the incubation period, the growths in each cupule were compared with the 0 cupule, which was used as a negative control. A cupule more turbid than the control indicates a positive reaction. Identification was obtained with the numerical profile entered into apiweb^{TM} identification software.

3.6.2 Biodegradation studies with the microbial isolates

A total of 10 hydrocarbon utilizing bacteria species were isolated from oil-contaminated and un-contaminated soil (7 from oil contaminated and 3 from un-contaminated soil) and 6 hydrocarbon utilizing yeast (4 from oil-contaminated and 2 from uncontaminated soil). Out of the 16 microbial isolates, 2 bacteria and 2 yeasts were selected for the biodegradation studies due to their rapid growth on oil agar and efficient utilization of oil in the preliminary test in test tubes. The rates and extent of used lubricating oil degradation by

these four selected microbial isolates were determined using gravimetric analysis and chromatographic technique (Ijah et al., 2008).

The biodegradation studies was carried out by inoculating 2 ml of 24 hour broth culture of each microbial isolates into 100 ml of sterile MSM (Zajic and Supplisson, 1972), that contained 0.5 g of used lubricating oil in an Erlenmeyer flask. The experiment was set up in triplicates with control flasks which contained 100 ml of sterile mineral salts medium plus 0.5 g of used lubricating oil but without added microorganisms. The flasks were incubated in an incubator shaker (Thermo-line, Japan) maintained at 30^0C at 150 rpm for 28 days. At seven days intervals, triplicates flasks per organisms plus control flasks were removed from the incubator shaker and the residual used lubricating oil extracted twice with 150 ml of n-hexane (Merck brand) and dried with anhydrous sodium sulphate. The solvent was removed by rotary evaporator and the weight of the residual oil was measured and recorded, and the percentage biodegradation of the used lubricating oil was calculated using the formula of Ijah and Ukpe, (1992). The residual oil was diluted with n-hexane (Merck brand) and cleaned up with silica gel (HyperSep SI column), 1 microlitre of the extracted oil sample was analyzed using gas chromatography with flame ionization detector (GC/FID). The GC was equipped with cross-linked 5% phenyl methyl siloxane capillary column; HP-5MS. Helium was used as carrier gas. The oven temperature program was started at 50^0C and raised by 25^0C/min until 325 ^{0}C, which was maintained for 11 minutes. The major hydrocarbon fractions were identified on the basis of their retention time and by comparing them to those of analytical standards.

3.7 Statistical analysis

Statistical analyses of all the data obtained from these studies were carried out using one – way ANOVA and LSD with SPSS Statistics version 17.0.