CHAPTER III

MATERIALS AND METHODS

3.1 HISTORY AND LOCATION OF THE STUDY AREA

Chennai Petroleum Corporation Limited (CPCL), formerly known as Madras Refineries Limited (MRL) was formed as a joint venture in 1965 by the Government of India (GoI), National Iranian Oil Company (NIOC) and the American Oil Company (AMOCO) having a share holding in the ratio 74%; 13%; and 13%; respectively.

In 1969 it had an initial refining capacity of 2.5 million tones per annum of imported lube bearing crude. Current capacity of the refinery is 10.5 Million Tones (MT) per annum. CPCL is located at 16 Kms North of the city of Chennai and at a 5 km distance west of the Bay of Bengal in the small township of Manali and is at an altitude of 32 m from Mean Sea Level. CPCL has a site area of about 750 acres.

The main products of the company are LPG, Motor Spirit, Superior Kerosene, Aviation Turbine Fuel, High Speed Diesel, Naphtha, Bitumen, Lube Base Stocks, Paraffin Wax, Fuel Oil, Hexane and Petrochemical feed stocks. CPCL has been contributing progressively greater measures to the progress of Indian economy besides meeting the total regional needs of the several petroleum products such as LPG, ATF, paraffin wax, petrol, naphtha, diesel, etc.

3.2 POLLUTED SOIL SAMPLE COLLECTION FOR MICROBIAL ASSESSMENT

Soil samples from a depth of 15 cm were collected from five places around oily sludge storage pit in CPCL. Soil samples were collected in polythene bags and kept in ice box during transport to the lab. Soil samples were homogenized and divided into aliquots for subsequent manipulations (Ashok and Musarrat, 1999 and Margesin, 2003).

3.2.1 Physico-Chemical Analysis of Soil Sample

The collected soil samples were air-dried, passed through a 2 mm sieve and then stored in a cold place. Soil pH was measured by the glass electrode method (McKeague, 1978) in a 1/1 (w/v) soil/water suspension. Moisture content of the soil was determined gravimetrically by drying at 105°C and cooled in a desiccator. Repeated this procedure until the constant weight attained and recorded the final weight of the sample (Saxena, 1994).

Soil temperature was recorded at site by using thermometer. Cations present in the exchange complex of the soil can be removed by leaching the soil with ammonium acetate solution. Calcium and magnesium were determined from the ammonium acetate leachate. Sodium and potassium were determined by flame photometric method. Available phosphorous

was determined colorimetrically by using spectrophotometer (Trivedy *et al.*, 1998). Methods of analysis of selected properties of soil samples were summarized in Table 3.1. The microorganisms were isolated and identified by using collected soil sample.

Table 3.1 – Parameters analyzed in oil polluted soil sample

S.No.	Parameter	Reference
1.	Temperature	Trivedy *et al.*, 1998
2.	Moisture content	Saxena, 1994
3	pH	McKeague, 1978
4	Calcium	Trivedy *et al.*, 1998
5	Magnesium	Trivedy *et al.*, 1998
6	Available Phosphorous	Trivedy *et al.*, 1998
7	Sodium	Trivedy *et al.*, 1998
8	Potassium	Trivedy *et al.*, 1998

3.2.2 Microbiological Analysis of Soil Sample

Initial laboratory studies were employed to isolate and enrich for a population of microorganisms capable of biodegrading oil wastes. Mineral salt medium plates were prepared with 1% crude oil for bacterial growth and Czapek Dox Agar with 1% crude oil was used for fungal growth

(Phillips *et al.,* 1974 and Mishra *et al.,* 2001). The antibiotic streptomycin (50 µg / ml) was added to the sterilized Czapek Dox Agar to suppress the bacterial population. Ten gram of polluted soil sample was serially diluted with 90 ml saline water (0.85 % NaCl). Appropriate dilutions were made (10^{-2}, 10^{-3}, 10^{-4}, $10^{-5}$and 10^{-6}) for enumeration of hydrocarbon utilizing microorganisms. The serially diluted samples were inoculated onto the medium by standard spread plate technique. Inoculated plates were incubated at 37°C and room temperature for bacterial and fungal species respectively for one week.

After incubation, the bacterial colonies, which developed on plates were randomly picked and purified by subculturing onto fresh agar plates, using the streak plate technique. Isolated colonies which appeared on the plates were then transferred onto basal salt medium slants properly labeled and stored as stock cultures (Okerentugba and Ezeronye, 2003). The bacterial isolates were identified based on their morphology, Gram reaction and their biochemical tests (Breed *et al.,* 1957).

Pure cultures of the fungi isolates were made and transferred onto Czapek Dox agar slants as stock culture. The microscopic and macroscopic features of the hyphal mass, morphology of cells, spores and nature of the

fruiting bodies were used for identification (Okerentugba and Ezeronye, 2003).

3.2.2.1 Bacterial Identification

Identification of isolates was carried out by using standard technique with reference to Bergey's Manual of Determinative bacteriology (Breed *et al.*, 1957).

3.2.2..1.1 Gram Stain

The colonies from the media were screened on a clean wax free glass slide, fixed and Gram stained. The smears were then examined under the microscope.

3.2.2..1.2 Motility Test

The hanging drop technique and soft agar tests were followed to observe the motility of the organism.

3.2.2..1.3 Biochemical Tests

3.2.2..1.3.1 Catalase test

The plate containing organisms to be identified was mixed with 0.5 ml of 3% of hydrogen peroxide solution. Immediate liberation of air bubble indicated that the organism was Catalase positive and no liberation indicated that the organism was Catalase negative.

3.2.2..1.3.2 Oxidase Test

The test reagent, tetra-methyl-p-phenylenediamine dihydrochloride was added to the slants containing bacterial colonies. Change in the colour of the colony to purple indicated positive results.

3.2.2..1.3.3 Triple Sugar Iron Agar Test

A loopful of colony was streaked on triple sugar iron agar slope surface and stabbed into the butt and incubated at 37°C for 24 hrs. Change of colour in the slant or butt or both indicated the sugar utilization.

3.2.2..1.3.4 Urea Utilization Test

The Christenson's urea agar plates were prepared and streaked with the colonies. The plates were then incubated at 37° C for 24-48 hrs. Change of the colour from yellow to pink indicated positive reaction. No colour change indicated negative reaction.

3.2.2..1.3.5 Starch Hydrolysis Test

The selected colonies were inoculated on starch agar medium and incubated at 37°C for 24 hrs. After the incubation period was over, few drops of Grams iodine solution was poured on the media.

3.2.2..1.3.6 Citrate Utilization Test

The test organisms were inoculated in Simmon's citrate agar slants. The tubes were incubated at 37°C for 24-48 hrs. Following incubation, bacteria that grow on the medium turn the bromothymol blue indicator from green to blue. Negative tubes remain green colour.

3.2.2..1.3.7 Voges - Proskauer Test

The test cultures were inoculated with separate MR-VP broth tubes and kept for incubation at 37°C for 24 hrs. After incubation 10 drops of Baritt's reagent A was added, with mild shaking. Immediately a few drops of Baritt's reagent B was added, mixed thoroughly. Positive result shows the colour change from yellow to pink.

3.2.2..1.3.8 Methyl Red Test

The selected microorganisms were inoculated in MR-VP broth and incubated at 37°C for 24 hrs. After incubation five drops of methyl red was added to each tube and observed. Positive result shows the colour change from yellow to red and negative result remains yellow.

3.2.2..1.3.9 Indole Production Test

The test organisms were inoculated in each separate tube with tryptophan broth. The tubes were incubated at 37°C for 24 hrs. After

incubation 10 drops of Kovac's reagent was added to that broth culture and slightly agitated for the development of cherry red colour ring, which is due to the action of p-dimethyl amino benzaldehyde with indole. The absence of ring shows negative result of the indole test.

3.2.2..1.3.10 Sugar Fermentation Test

To find out the fermentation capacity of the organisms in different sugars, it was inoculated independently in different media containing sugars like glucose, lactose, xylulose, sucrose, fructose and mannitol. The tubes were incubated at 37°C for 24-48 hrs. Positive tubes show acid and gas formation in Durham's tubes at the end of the incubation period.

The isolates were identified with the help of standard biochemical tests which were given in the Bergey's Manual of determinative bacteriology.

3.2.2.2 Fungal Species Identification

Fungal species were identified by wet mount preparation using lacto phenol cotton blue stain. This preparation was seen under microscope (Prakash, 2004 and Salle, 2006).

3.3 SOURCES OF OILY SLUDGE

The accumulation of sludge at the bottom of crude oil tanks and from ETP process is a major problem experienced by all refineries. The settling out of sand, rust and heavy fractions in the crude oil results in a loss of Storage capacity of crude storage tanks (Neelakandan, 2005).

3.3.1 Tank Bottom Sludge

Sludge accumulates at the bottom of crude oil and product storage tanks and has to be periodically removed. Contaminants in sludge vary with characteristics of crude oil and the shipping and handling methods prior to receipt at the refinery. The waste sludge, in general, consists of a mixture of water, sediments, oil and inorganic matter (Neelakandan, 2005).

3.3.2 Other Sources of Sludge

Other forms of sludge are generated from sources such as API separator, Dissolved Air Flocculation (DAF) units, bio-treatment wastes from refinery aqueous effluents and desalter units. This sludge has oily substances, fine and heavy solids and oil droplets, biological sludge with oil substances, and sludge interface emulsions from desalters which have high composition of oil and water (Neelakandan, 2005).

3.3 Collection and characterization of sludge

After oil recovered sludge samples were collected randomly from sludge storage pit and homogenized. The homogenized sample was analyzed for its composition (Oil fractions, sediments, water) by using standard method (ASTM, designation 473, 1998). Heavy metals were also identified using Atomic Absorption Spectrophotometry Spectra A5 with standard method. This analyzed sludge sample was used for the bioremediation study.

3.4 SELECTION OF CULTURE FOR BIOREMEDIATION

3.4.1 Feasibility Study

Each isolates were undertaken for ability study. Mineral salt medium were dispensed in 99 ml quantities into ten 250 ml Erlenmeyer flasks. Each flask was added with 1 ml of the crude oil. After sterilization, the identified bacterial isolates were inoculated separately. Inoculated flasks were incubated at 37 ^{o}C for 48 hrs.

After incubation, the cultures were transferred to the prepared plates with oily sludge. Nutrients were added to each plot to increase the microbial growth. This set up was maintained for one month period. Oil

percentage was calculated twice in a week by extraction method (ASTM, 1998).

3.4.2 Scaling up of hydrocarbon utilizing microorganism for inoculation into treatment plots

Isolated microorganisms were grown at 37°C in Mineral Salt Medium (MSM) containing crude oil at pH 6.8 to 7.0 to achieve enough bacterial cell biomass required for oil degradation, (Abd-El-Haleem, 2003).

In the process, basal salt medium slants of all the hydrocarbon utilizing bacterial genera isolated from the site were prepared. About 18 ml of sterile normal saline (0.85% NaCl) was transferred into a sterile test tube. Sterilized inoculation loop was used to scrap organisms from the slants into the normal saline. The suspension was then transferred into a conical flask containing 380 ml of sterile mineral salt medium (Mills *et al.*, 1978) containing 2 ml of crude oil. This mixture was placed on a shaker and incubated at room temperature of 34 ± 2°C for seven days. The pH was maintained in all stages at 7.2 by adjusting with standard phosphate buffer (APHA, 1985, Odokuma and Dickson, 2003).

The selected bacterial species by feasibility study were inoculated and incubated separately. The bacterial consortium was prepared by mixing equal volumes (400 ml) of each isolates after incubation (Okoh, 2003).

Each fungal species were inoculated separately in 18 ml sterile saline solution. This suspension was then transferred into 380 ml of mineral salt medium containing 2 ml of crude oil. It was kept for incubation at room temperature for seven days. The fungal consortium was also prepared by mixing equal volumes (400ml) of each isolates after incubation (Boonchan, 2000 and Okoh, 2003).

3.5 BIOREMEDIATION PROCESS

3.5.1 Work Design

Characterized oily sludge was spread in a pit where the bioremediation process held. From the results of ability test five bacterial species and five fungal species were selected for this present study. Based on selected microorganisms the following five models were prepared for bioremediation for three month study.

Plot 1 - Control

Plot 2 - BN (Bacteria with Nutrients)

Plot 3 - FN (Fungi with Nutrients)

Plot 4 - BFN (Bacteria and Fungi with Nutrients) (Boonchan, 2000)

Plot 5 - BF (Bacteria and Fungi without Nutrient)

The selected bacterial consortium was compared with commercially available bacterial consortium to determine the efficiency of selected

microorganisms. The following plots were prepared for the comparison study for one month.

Plot 6 - Com Control (Commercial Control)

Plot 7 - Com Mic (Commercial with Selected Microbes)

Nutrients such as magnesium sulphate 0.2 gm, calcium chloride 0.02gm, monopottasium phosphate 1.0 gm, diammonium hydrogen phosphate 1.0 gm, potassium nitrate 1.0 gm and ferric chloride 0.05 m were mixed in 1000 ml of distilled water. From above mixture 200 ml was added to the plot 2, 3 and 4 once in a two days and this process was conducted for three month period (Bushnell and Hass, 1941; Brown and Braddock, 1990). The samples were collected and analyzed for oil content once in a fifteen days. The total bacterial count was performed on basal salt medium using the spread plate method (APHA, 1985). Heavy metals were also identified in oil degraded samples before and after treatment.

Microbial cultures were added once in a twenty days. Plot 1 was maintained as control without addition of microbes and nutrients. In Plot 2 bacterial consortium with nutrients (BN) was maintained. In Plot 3 fungal consortium with nutrients (FN) were added. In Plot 4 mixed bacterial and fungal consortium with nutrients (BFN) were added. In Plot 5 consortium of bacteria and fungi without nutrients (BF) was maintained throughout the

study. In Plot 6 commercially available bacterial species were added. In Plot 7 commercial bacterial consortium with identified bacterial consortium were added for comparison study. This set up was maintained and monitored for 1 month.

3.5.2 Viable Cell Count

The total microbial count was compared to an estimate of the population present that would degrade the contaminants of concern. One gram of sludge sample was aseptically added into 10 ml of sterile normal saline solution and shaken vigorously to discharge the cells from the soil particles and allowed to stand for about ten minutes, after the supernatant was serially diluted. The cell densities of the appropriate dilution were determined by standard spread plate technique using 0.1 ml of the dilution from 10^{-1} to 10^{-6} onto agar (Nweke and Okpokwasili, 2003). All cultures were incubated at 37°C for 24 to 48 hrs.

3.6. ANALYSIS OF PERCENTAGE OF WATER CONTENT

Sludge samples were collected in polythene bags and transported to the laboratory for analysis. Water content was estimated in sludge sample by heating (ASTM D95). Total 100 gm of oily sludge from each sample were taken in Dean and Stark apparatus and toluene was added as solvent. This set up was heated upto 80°C. Due to heating water and solvent were

getting evaporated and collected in measuring tube (Plate 1). Water settled at the bottom of the measuring tube and thus water quantity in oily sludge was measured by following method (TERI, 2004).

$$\text{Percentage of water} = \frac{\text{ml collected in measuring tube}}{\text{Volume of sample}} \times 100$$

3.7 HYDROCARBON DETERMINATION

3.7.1 Analysis of Oil by Sedimentation

Total petroleum hydrocarbon (TPH) in oily sludge was extracted using solvent (toluene) by sedimentation process mentioned in ASTM 473. One gram of sample was kept in a pre weighed thimble and allowed for extraction by solvent. After extraction the thimble was kept in a hot air oven to evaporate the solvent. It was kept in desiccator until constant weight was attained. Finally, total petroleum hydrocarbon in sludge samples were quantified (TERI, 2007). The set up was given in plate 1. TPH was calculated by the following method

$$\text{Sediment (gm)} = \text{Final wt. of thimble–Initial wt. of thimble}$$

$$\text{Percentage of sediment} = \frac{\text{Sediment}}{\text{Volume of sample}} \times 100$$

Percentage of oil = 100 – (percentage of sediment + percentage of water)

3.7.2 Preparing Samples for TPH Analysis by Sim Dis, GC-FID and Gravimetric methods

Solvent extractable TPH which was collected by sedimentation process was kept in a round bottom flask. Excess solvent was evaporated in a fume hood by inert distillation with nitrogen gas. In this process the excess amount of toluene was evaporated. After solvent evaporation, the concentrated sample was used to quantify the TPH (TERI, 2007) by Sim Dis, GC-FID and Gravimetric methods.

3.7.3 Analysis of TPH by Simulated Distillation (Sim Dis) Method

Concentrated samples were subjected to Sim Dis Test. From this analysis the IBP (Initial Boiling Point) and FBP (Final Boiling Point) were determined and compared (ASTM, 1998).

3.7.4 Analysis of TPH by Gravimetric analysis

TPH analysis was carried out using gravimetric method, measure anything extractable by a solvent, not removed during solvent (toluene) evaporation and capable of being weighed. Known amount of concentrated sample was kept in pre weighed crucible and kept it in a hot air oven at 50°C. After evaporation the crucible was kept in Desiccator for cooling.

Then the constant weight was taken. The total TPH was calculated by using the following formula (Raghavan, 1998 and Weisman, 1998)

$$\text{Percentage of TPH} = \frac{W2 - W1}{\text{ml of sample}} \times 100$$

Where,

W1 – Weight of crucible before adding extract

W2 – Weight of crucible after experiment

3.7.5 Analysis of TPH by GC-FID

Screening by gas chromatography with flame ionizing detection has been carried out on extracted sludge sample (Douglas *et al.*, 1992 & Hamme *et al.*, 2000). After 90 days treatment, highly oil degraded plot which was confirmed by sedimentation process was analyzed on a Gas Chromatograph (GC) equipped with a flame ionization detector (FID) 9A (Arvin *et al.*, 1989). The OV1 capillary column had an inside diameter 0.53 mm, a length of 30 m and a 0.2 μm film thickness. The initial oven temperature was maintained at 40°C for 1 min, then increased at 10°C / min to 280°C and maintained at 280°C for 10 min for petroleum sludge samples. The temperature of the injection port was 250°C and the detector temperature was 300°C. The injection volume was 1 μl. The peaks formed were compared between before and after treatment.

3.7.6 Analysis of Selected Heavy Metals in Oily Sludge

Selected heavy metals were analyzed in composite samples of oily sludge undertaken for bioremediation process. Sludge samples (1 gm) were taken in microwave digestion teflon tube. Concentrated nitric acid (15 ml) was added to the teflon tube and the teflon tube was covered with a watch glass and heated at 140° C on a hot plate, in a fume hood, until most of acid was evaporated, the step was repeated for five times in order to soluble the metallic components. The solution was then filtered in another clean teflon tube through 0.45μ whatman filter paper number 42 and the insoluble residues on the filter paper was rinsed with 10% nitric acid. The residue was then discarded. The teflon tube containing filtered solution was again heated till complete evaporation of acid. After evaporation of acid teflon tube was heated at 400°C for six hours and cooled. The residue was dissolved in 1ml of concentrated nitric acid. The clear solution was then quantitatively transferred into 50ml volumetric flask and volume was made by using 10% nitric acid. The selected heavy metals from solution were analyzed using AAS Spectra A 55 (TERI, 2007).

3.8 STATISTICAL TEST

3.8.1 Analysis of Variance (ANOVA)

Statistical calculations were done using Smith's statistical package (SSP). The data obtained were analyzed using analysis of variance (ANOVA) at 5% level significance (Gupta 2005; Zar, 2006). This was done to determine statistical differences among the treatment groups.

3.9 GERMINATION STUDY

3.9.1 Vigna unguiculata (L.) Walp], *the Experimental Plant*

Vigna unuiculata (L.)Walp, (Sambamurthy and Subramaniyam, 1989) has been selected as the experimental crop since it is an economical and common grain used in most of the household. It is called as "Thattai Payaru" in Tamil and Cowpea in English.

3.9.2 Pretreatment of Seeds

Viable seeds were taken and prepared for germination study. Before, experimental treatment, the seeds were surface sterilized with 0.1% Mercuric Chloride solution for 5 minutes. The seeds were washed many times thoroughly with distilled water (Saraswathy, 2002; Salunke *et al.*, 2007).

3.9.3 Seed Viability Test

The seed viability can be defined as "the capacity of the seed to germinate under favourable conditions in the absence of dormancy" (Copeland and McDonald, 1985). Around 120 healthy seeds were soaked for overnight (18 hours) in 100 ml of distilled water to prepare them for the test. The seeds were decanted and placed in 0.5% aqueous solution of 2, 3, 5 - triphenyl tetrazolium chloride at 30°C in the dark. After 5 hours of treatment, the solution was drained (McDonald, 1985) and washed with distilled water 3 or 4 times. The numbers of stained (red) seeds were counted as viable **(Association of official seed analysts, 1970).**

$$\text{Percentage of viability} = \frac{\text{Number of stained seeds}}{\text{Total number of seeds tested}} \times 100$$

The experimental sludge samples were set up in plastic covers. Five *V. unguiculata* seeds were placed into each cover at 1 cm depth and watered daily. Three replicates were maintained. The treated seeds were allowed to germinate and germination percentage was assessed on the 30th day of seedling. The percentage of germination was calculated as follows;

$$\text{Percentage of germination} = \frac{\text{No. of germinated seeds}}{\text{Total number of seeds used}} \times 100$$

$$\text{Speed of germination (Maguire, 1962)} = \frac{X_1}{n_1} + \frac{X_2}{n_2} + \ldots\ldots\ldots\ldots + \frac{X_7}{n_7}$$

Where,

X_1, X_2 = Number of seeds germinated on 1st, 2nd, days

n_1, n_2 = Day of germination

After one month of growth, the root length and shoot length of the seedlings were measured using a scale and the following were calculated from this data (Vasanthy *et al.*, 2006).

1. Speed of germination
2. Root length
3. Shoot length
4. Vigour Index = Length of primary axis X Germination percentage (Abdul – Baki and Anderson, 1973)

The fresh weight of the seedling and the dry weight of the seedling (after complete desiccation in a hot air oven at 60°C until constancy in weight) were determined (Peach, 1954 and Vasanthy *et al.*, 2006). Electronic balance "Anamed" was used to weigh the fresh weight and dry weight of the seedlings.

3.9.4 Experimental Setup

Agricultural land soil was mixed thoroughly with treated sludge sample in controlled percentage such as 10%, 25%, 50%, 75% and 100% sludge separately. Then the mixed samples were filled in covers and then seedlings were done. Water was added and regularly monitored.

Model 1 – Control (100 % Soil)

Model 2 – 10 % Sludge with soil

Model 3 – 25 % Sludge with soil

Model 4 – 50 % Sludge with soil

Model 5 – 75 % Sludge with soil

Model 6 – 100 % Sludge