MATERIALS AND METHODS

II-1. Samples Collection and Locations

Three different locations were chosen for their high agriculture activities. The first location was represented by 11 samples from 4 different sites along Cairo- Alexandria agriculture Road. The second location was represented by 1 sample which was collected from Toushky area, South Egypt. The third location was represented by 5 samples from 5 different sites, these sites were at different distances from an oil field (Well West Dikirinis 3-MDT of Mansoura Petroleum Company in Dikirnis, El-Mansoura). All the samples were collected during August 2005. Samples collection and locations are illustrated in Table 5 and Fig.7.

The first location was studied to evaluate the effect of traffic activities on the agriculture soil around Cairo- Alexandria agriculture Road. The second location was studied due to the relatively its low traffic activity and absence of oil industrial activities in this area. The third location was chosen to evaluate the effect of the presence of oil field in an agriculture area.

The samples were collected with a grab and placed in clean plastic containers and then preserved in an ice box. Then in the laboratory the samples were grinded, care should be taken not to break the individual soil minerals during the grinding. The purpose of grinding is to reduce heterogeneity and to provide maximum surface area for physical and chemical reactions. The soil samples were then crushed mixed and sieved to < 2 mm. A sample of each sieved soil was air-dried, so soil aggregates should be broken carefully to accelerate the drying procedure. Then stored in desiccators and care was taken to avoid contamination and to prevent the occurrence of further chemical and biochemical reactions in order to determine soil physical–chemical properties. For the biotreatment, the used sample was stored at 4°C without drying or sieving.

Table 5: Samples locations and description.

Location	Site	Sample No.	Sample description
The first location	10km away from Cairo to Alex. (1st stage)	1	Road surface soil sample.
		2	Surface soil sample, 1km aside the road.
	20km away from Cairo to Alex. (2nd stage)	3	Road surface soil sample.
		4	Surface soil sample, 1km aside the road.
		5	Soil sample from depth of 30cm.
		6	Soil sample from depth of 30cm, 1km aside the road.
	30km away from Cairo to Alex. (3rd stage)	7	Road surface soil sample.
		8	Surface soil sample, 1km aside the road.
		9	Soil sample from depth of 30cm.
		10	Soil sample from depth of 30cm, 1km aside the road.
	32km away from Cairo to Alex.	11	Surface soil sample, from a protected area followed to the ministry of agriculture.
The second location	Toushky area, south Egypt.	12	Surface soil sample, from Toushky area, south Egypt.
The third location	Dikirnis, El-Mansoura	13	Surface soil sample.
	1km away from an oil field	14	Surface soil sample.
	10km away from an oil field	15	Surface soil sample.
	20km away from an oil field	16	Surface soil sample.
	50km away from an oil field	17	Surface soil sample.

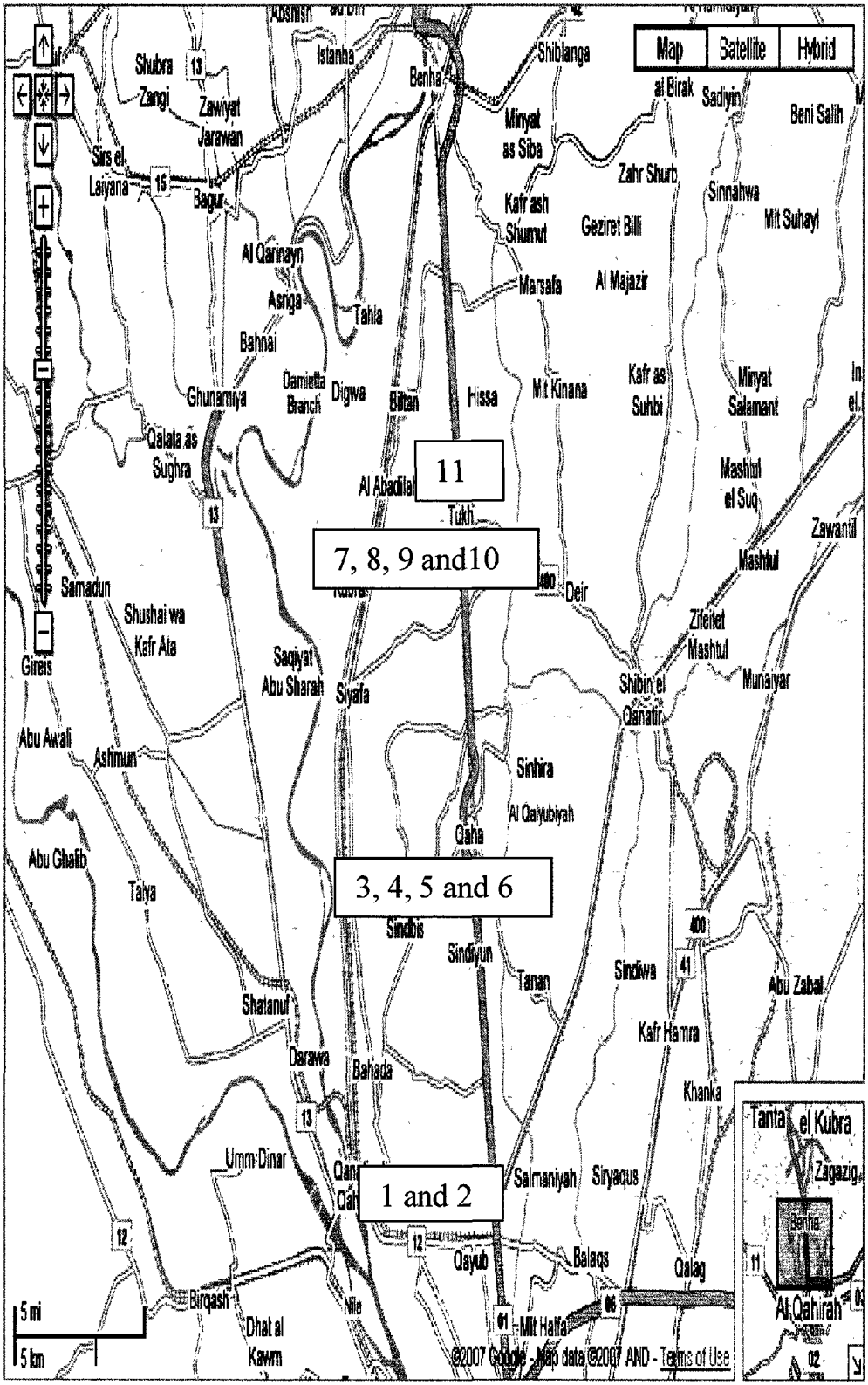
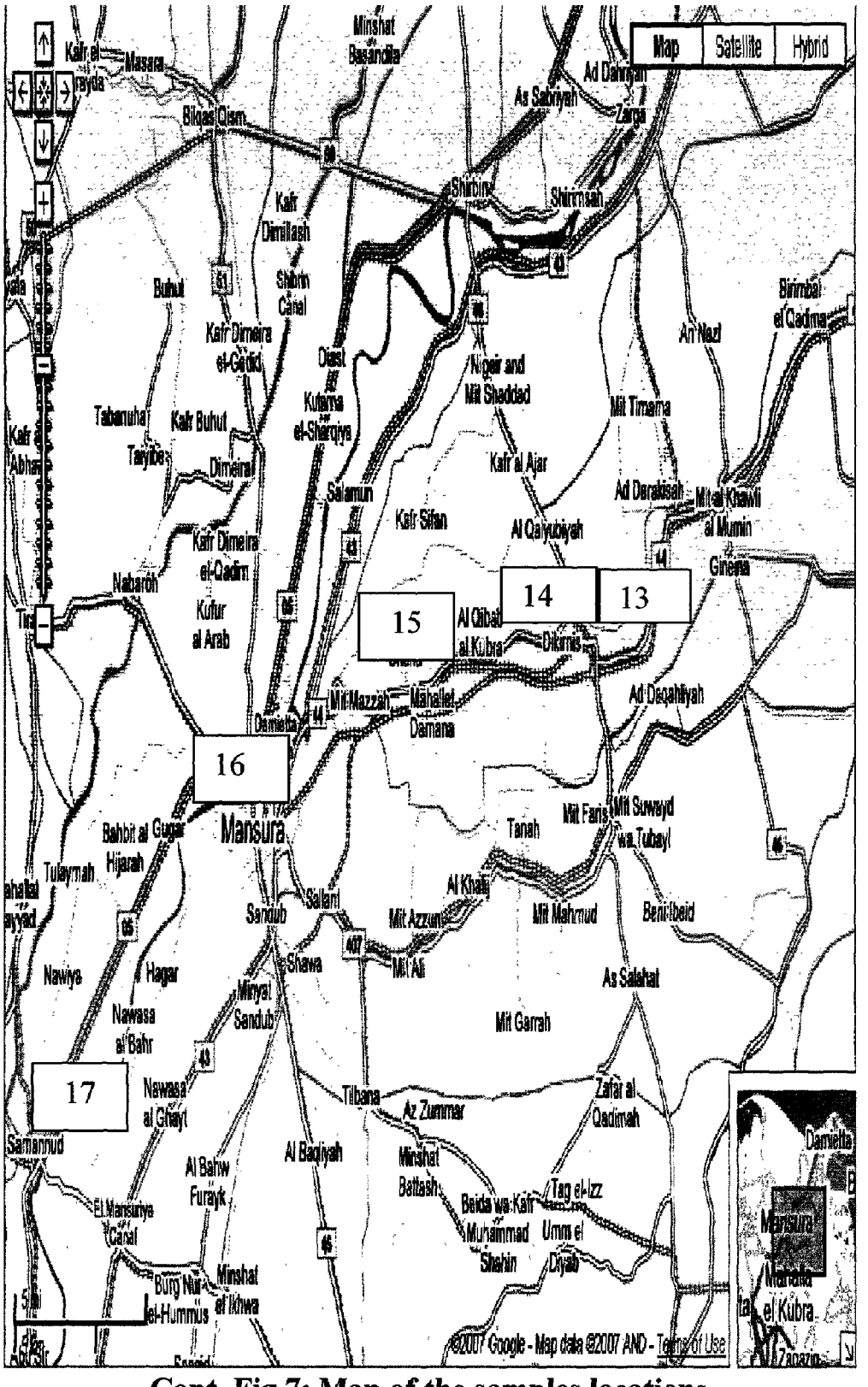


Fig.7: Map of the samples locations.



Cont. Fig.7: Map of the samples locations.

II-2. Experimental

All solutions were prepared using analytical-reagent grade chemicals and ultrapure quality deionised water.

II-2-1. Physicochemical analysis

II-2-1-1. Moisture content: is measured according to **(ASTM: D4643-93).**

II-2-1-2. Bulk density: was determined by the core method **(Black, 1965).**

II-2-1-3. Particle size: distribution of the soil samples was determined using the international pipette method as described by **(Gee and Bauder, 1986).**

II-2-1-4. Soil pH: was measured in (1 : 2.5) soil : water suspension by using a glass electrode microcomputer pH-meter (HNNA, model HI 8424) according to **(ASTM: D4972-95a).**

II-2-1-5. Electrical conductivity (EC): in the soil was measured in (1 : 2.5) soil : water suspension by **(Bohn et al., 1979).**

II-2-1-6. Soluble anions and cations: were estimated in the soil saturated paste extract according to the procedures described by **(Jackson, 1973).**

II-2-1-7. Organic matter content: was determined by oxidation with dichromate method according to Walkley and Black method, **(Black, 1982).**

II-2-1-8. Total heavy metals content: One gram of the air dried sample was digested with HNO_3, $HcLO_3$ and HF (1:1:1) in wide-mouth crucible using a microwave mixer. The mixture was heated to dryness and few drops of concentrated HCl were then added to dryness. 20ml HCl (5N) was added and heated to boiling and the transferred to 50ml measuring flask and filled by deionized water to 50ml and then the heavy metals were determined by UNICAM 969 Atomic Absorption Spectrometer **(Zhang and Shan, 1997).**

II-2-1-9. Loss upon ignition: was estimated by using a platinum crucible and cover according to **(Bariller-Boyer et al., 2003)**

II-2-1-10. Total Petroleum hydrocarbons content (TPH): The dried soil samples were extracted in a soxhelet extractor using a mixture of n-hexane and dichloromethane (1:1v/v). The total hydrocarbons concentrations were determined gravimetrically **(Viguri et al., 2002).**

II-2-2. GC-FID analysis:

All extracted oil from PCS samples before and after biotreatment were analyzed using Aglient model 6890 plus gas chromatograph. The conditions of analysis were as follow:

- Column: HP-1 (100% methyl silicon siloxane) 30m length and 0.25mm ID.
- Oven temperature: 80-300°C (3°C/min).
- Detector: flame Ionization Detector (FID) 325°C.
- Injector: splitter injector 300°C.
- Carrier gas: N_2 (2ml/min).

Procedure: The column was left over night with the carrier gas flowing through it, and at a maximum working temperature used, disconnected from the detector. The extracted hydrocarbons were dissolved in the least amount of methylene chloride, and about 1µl was injected into the apparatus. The temperature of the oven was programmed (before injection) from 100-300°C at fixed rate (5 degree/min). The column was then left at its maximum temperature until the sample has been completely eluted i.e. no more peaks appears. The identification of the n-paraffin peaks was established by the chromatographic a reference mixture of n-paraffin of known composition.

II-2-3. Impact of heavy metals on petroleum contaminated soil (PCS)

II-2-3-1. Basal salts medium (BSM) used in this study was prepared according to **Piddington et al., 1995** but with modifications and consists of 5.57g/l Na_2HPO_4, 2.44g/l KH_2PO_4, 2.0g/l NH4Cl, 0.2g/l $MgCl_2.6H_2O$, 0.001g/l $FeCl_3.6H_2O$, 0.001g/l $CaCl_2.2H_2O$ and 0.1g/l of yeast extract, dissolved in 1liter of deionized water. The pH was adjusted to 7 with 10% NaOH and it was sterilized by autoclaving at 121°C for 15min. Phenanthrene

(Phe) dissolved in ethyl ether was added as a sole source of carbon in a final concentration of 500ppm (BSM/Phe medium).

II-2-3-2. Luria-Bertani medium (LB) used for obtaining biomass was prepared according to **Kirimura et al., (2001)** and consists of 10g/l Tryptone, 5g/l Yeast extract and 10g/l NaCl, dissolved in 1liter deionized water and adjusted to pH 7 with 10% NaOH before sterilization.

II-2-3-3. Heavy metals tested were copper, manganese, nickel and zinc. They were incorporated as soluble salts: $CuCl_2.2H_2O$, $MnCl_2.4H_2O$, $NiCl_2.6H_2O$ and $ZnCl_2$. A sterilized concentrated solution of each of the salts was prepared by dissolving a calculated quantity containing a desired concentration of the metal in deionized water to produce separate metal solutions which were served as stock solutions. Desired lower concentrations were obtained by serial dilutions. To cover the whole concentration of the chosen four heavy metals in the PCS used in this study. Cu and Ni (5, 10, 25, 50 and 100mg/l) but for Mn and Zn the different concentrations prepared were (5, 10, 25, 50, 100, 250, 500, 750 and 1000mg/l). Aliquots of individual metal solutions were mixed to obtain a mixture containing concentration ranges of 1.25, 2.5, 6.25, 12.5, 25, 62.5, 125, 187.5 and 250 mg/l of each of the metals.

II-2-3-4. Soil sample

Hydrocarbon polluted soil sample was collected from the top layer (0-10cm) of an oil field; Well West Dikirinis 3-MDT of Mansoura Petroleum Company in Dikirnis, Mansoura, Egypt. Physico-chemical characterizations of soil sample were determined according to the methods listed in (II-2-1).

II-2-3-5. Enrichment and isolation of heavy metals tolerable bacteria

Ten grams of PCS were mixed with 100ml of sterilized LB medium of pH7 amended with mixture of equal concentration of the four heavy metals (Cu, Mn, Ni and Zn) in different 500ml Erlenmeyer conical flasks to obtain heavy metals/LB broth media containing different concentrations of heavy metals ranging between (0-1000mg/l). Flasks were incubated at 30°C for 48hours in a rotary shaking incubator (150rpm). Serial dilutions in 10ml

saline solutions (8.5g NaCl per one liter deionized water) of each of these suspensions were spreaded on LB/agar plates to enumerate total viable count (TCFU/g soil) able to grow and tolerate different concentrations of heavy metals. Plates were incubated at 30°C for 48hours and separate colonies were picked and purified on LB/agar plates.

II-2-3-6. Identification of isolates

Identification of isolated bacterial strains was done using Biolog system model; Biolog/Microlog 3420 program (Egyptian Plant Disease Research Institute).

II-2-3-7. Selection of phenanthrene degrading microorganisms

Each of the isolated heavy metal tolerable bacteria was inoculated separately in LB broth media of pH 7 at 30°C for 48hours in a rotary shaking incubator (150rpm) to obtain a biomass. Cells were harvested by centrifugation at 500rpm for 15min and then washed three times with sterilized BSM free from any C-source and heavy metals. Washed cells of each bacterial isolate were inoculated separately into 20ml BSM/Phe in 100ml Erlenmeyer conical flasks. The inocula were adjusted so that the beginning absorbance for each was (A_{600} 0.1). The cultures were incubated at 30°C for 7 days, in a shaking incubator (150rpm). The growth was monitored by optical density at 600nm using Vis spectrophotometer (JENWAY, model 6300), non-inoculated BSM was used as blank. pH of the cultures was also monitored using microcomputer pH-meter (HNNA, model HI 8424). To determine the biodegradation efficiencies of the bacterial isolates the cultures were acidified with 1mM HCl to pH 2.0 then extracted with equal volumes of ethylacetate. The extracted solutions were analyzed by HPLC. All the experiments were done in duplicates and the data listed are the average of the results obtained.

II-2-3-8. Biosurfactant production test

Biosurfactant production was examined by inoculating bacteria in 20ml BSM supplemented with n-hexadecane (1% v/v) as a carbon and energy source. The cultures were incubated at 30°C for 7 days, in a shaking incubator (150rpm). Growth was monitored with total viable count (TCFU/ml) on LB/agar plates. The medium was centrifuged and the surface tension of the supernatant was

measured using ring tensiometer model Kruss 8451 at 25°C and compared to the surface tension of sterilized uninoculated flask of BSM/hexadecane medium.

II-2-3-9. Emulsification index determination (E24)

E24 was the method used to quantify the emulsification caused by the produced biosurfactant. It was determined by the addition of 2ml of paraffin oil to 3ml of culture (bacteria/BSM/Phe, centrifuged at 500rpm for 5min and the supernatant was used) at the end of incubation period, mixing with a vortex for 2min, and leaving to stand for 24hours. BSM/Phe uninoculated flask was used as a negative control. The E24 index is given as percentage of height of emulsified layer (mm) divided by total height of the column (mm) **(Fleck et al., 2000).**

II-2-3-10. Impact of heavy metals on biodegradation of phenanthrene

Seven groups of 100ml conical flasks were used where each of them contained 20ml BSM/Phe medium.

- One group amended with mixture of equal concentrations of Cu, Mn, Ni and Zn to obtain HM/BSM/Phe broth media containing different concentrations of HM ranging between (5-1000mg/l).
- One group amended with $CuCl_2.2H_2O$ to obtain Cu/BSM/Phe broth media containing different concentrations of Cu ranging between (5-100mg/l).
- One group amended with $MnCl_2.4H_2O$ to obtain Mn/BSM/Phe broth media containing different concentrations of Mn ranging between (5-1000mg/l).
- One group amended with $NiCl_2.6H_2O$ to obtain Ni/BSM/Phe broth media containing different concentrations of Ni ranging between (5-100mg/l).
- One group amended with $ZnCl_2$ to obtain Zn/BSM/Phe broth media containing different concentrations of Zn ranging between (5-1000mg/l).
- Another set of flasks were the positive control group free from any HM.
- For each group another set of flasks were prepared as the negative control group without inoculation with bacteria.

Cellulomonas hominis N2 was inoculated in LB broth media of pH 7 and incubated at 30°C for 48hours in a rotary shaking incubator (150rpm) to obtain a biomass. Cells were harvested by centrifugation at 500rpm for 15min and then washed three times with sterilized BSM free from any C-source and heavy metals. Washed cells were inoculated into 20ml BSM/Phe in 100ml Erlenmeyer conical flasks. The inocula were adjusted to have TCFU of about 10^5cells/ml.

The cultures were incubated at 30°C for 7 days, in a shaking incubator (150rpm). Growth was monitored with total viable count on LB/agar plates. To determine the biodegradation efficiencies of the bacterial isolates, the cultures were acidified with 1mM HCl to pH2.0 then extracted with equal volumes of ethylacetate. The extracted solutions were analyzed by HPLC. All the experiments were done in duplicates and the data listed are the average of the results obtained.

II-2-3-11. Biotreatment of petroleum contaminated soil (PCS)

One kg of the petroleum contaminated soil (PCS) was placed in each of a set of aluminum pans with surface area of 780cm^2 and a volume of 6240cm^3. The soil was biostimulated by adding 250mg/kg of $(NH_4)_2SO_4$ and 100mg/kg of K_2HPO_4 every 14 days. Soil without addition of nutrients was used as negative control. The soil in each pan was mixed daily to provide sufficient air and oxygen. The microcosms were incubated at 30°C for five weeks. The soil was moistened by the addition of sterile deionized water twice a week until the end of the experiment to keep the moisture content about 40%. Total viable count (TCFU), pH and TPH were determined at time intervals of zero, 7, 10, 14, 21, 28 and 35 days. GC analysis was done to determine the effect of biotreatment on n- and iso- alkanes and unresolved complex mixture (UCM) of the extracted oil. All the experiments were done in duplicates and the data listed are the average of the results obtained.

II-2-3-12. Tools of Analysis

II-2-3-12- 1. HPLC analysis

Biodegradation efficiency of bacterial isolates for Phe was followed by measuring the concentration of Phe using HPLC

model Waters 600E equipped with auto sampler Waters 717 plus and dual wavelength absorbance detector Waters 2487 set at 254nm. Phe Standard compound was obtained from Supelco. The conditions of operation were as follow:

- Column: Supelcosil LC-PAH, 15 cm x 4.6mm ID, 5μm particles size.
- Mobile phase: Acetonitrile/water 60:40 of HPLC grades.
- Flow rate: 1.0 ml/min.
- Calibration curve for Phe (0-500mg/l) was done.

II-2-3-12- 2. GC-FID analysis

All extracted oil from PCS samples before and after biotreatment were analyzed using the method listed in (II-2-2).