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Biodegrading of Crude Oil by Micrococcus species

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ABSTRACT: Crude oil or petroleum hydrocarbons are major contaminants in the environment which can lead to ecological catastrophes when they destroy natural resources such as wild life habitats or farming land and might even cause ground water pollution if it penetrates fresh water aquifers.The present investigation was carried out to study the biodegradaion of crude oil by Micrococcus species isolated from crude oil contaminated soil. The nature of crude oil contaminated soil samples were analyzed for total nitrogen, phosphorus, potassium and pH. Rapid screening procedure was performed to determine the naphthalene degrading ability of Micrococcus on agar platesBioremediation of crude oil by Micrococcus sp. under shake flask condition was studied and its activity was monitored by gas chromatographic analysis. Twelve different hydrocarbons were exerted during GC analysis. Among them Hexadecane, Naphthalene and Phenantherene showed moderate degree of bioremediation and rest of the compounds showed considerable amount of mineralization.Studies can be made further by applying this Micrococcus species as a potential tool in field trials to clean up the crude oil spills.

Keywords;Biodegradation,crudeoil,rapidscreening, Hydrocarbon,Gas chromatography

I. INTRODUCTION;

Crude oil or petroleum hydrocarbons are major contaminants in the environment which can lead to ecological catastrophes when they destroy natural resources such as wild life habitats or farming land and might even cause ground water pollution if it penetrates fresh water aquifers. The present investigation was carried out to study the bioremediation of crude oil by Micrococcus species isolated from crude oil contaminated soil. The nature of crude oil contaminated soil samples were analyzed for total nitrogen, phosphorus, potassium and pH. Rapid screening procedure was performed to determine the naphthalene degrading ability of Micrococcus on agar plates. Bioremediation of crude oil by Micrococcus sp. under shake flask condition was studied and its activity was monitored by gas chromatographic analysis. Twelve different hydrocarbons were exerted during GC analysis. Among them Hexadecane, Naphthalene and Phenantherene showed moderate degree of bioremediation and rest of the compounds showed considerable amount of mineralization.Studies can be made further by applying this Micrococcus species as a potential tool in field trials to clean up the crude oil spills.Hydrocarbon is an organic compound consisting entirely of hydrogen and carbon. Petroleum derived hydrocarbons can be divided into four classes: the saturated, the aromatics, the asphaltenes (phenols, fatty acids, ketones, esters and porphyrins) and the resins (pyridines, quinolines, carbazoles, sulfoxides and amides) (Colwell and Walker, 1977). Hydrocarbons are unique in that microbial degradation especially of straight chained and branched form involves the initial addition of molecular oxygen.Many microorganisms have the ability to utilize hydrocarbon as the sole source of carbon and energy.Such microorganisms are widely distributed in nature. Microorganisms exhibit emulsifying activity by producing biosurfactant and utilize the hydrocarbons as substrate often mineralizing them or converting them into harmless products helping to clean up environment.Hydrocarbon is an organic compound consisting entirely of hydrogen and carbonPetroleum derived hydrocarbons can be divided into four classes: the saturated, the aromatics, the asphaltenes (phenols, fatty acids, ketones, esters and porphyrins) and the resins (pyridines, quinolines, carbazoles, sulfoxides and amides) (Colwell and Walker, 1977). Hydrocarbons are unique in that microbial degradation especially of straight chained and branched form involves the initial addition of molecular oxygen. Many microorganisms have the ability to utilize hydrocarbon as the sole source of carbon and energy. Such microorganisms are widely distributed in nature. Microorganisms exhibit emulsifying activity by producing biosurfactant and utilize the hydrocarbons as substrate often mineralizing them or converting them into harmless products helping to clean up

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environment.Micrococcus is a genus of bacteria in the Micrococcaceaefamily.Micrococcusoccurs in a wide range of environments including dust, water and soil. Some strains of Micrococcus can be used for hydrocarbon and wax degradation.Micrococcus has the ability to utilize a wide range of unusual substrates such as pyridine, herbicides, chlorinated biphenyls and oil. They are likely involved in detoxification or biodegradation of many other environmental pollutants.

II. MATERIALS AND METHODS;.

Sample collection

BiodegratedCrude oil and soil samples were collected from the oil spill near the drilling site of "Oil and Natural Gas Commission" Cauvery Project, Kalappal, Thiruthuraipoondi District, Tamil Nadu, India.

The characteristics of the crude oil contaminated soil were analysed. And Physiochemical parameters of soil was calculated.(Total Nitrogen,Potassium and Phosphorus and soil PH was analysed using soil analysis kit.

Nitrogen - 121.8 Kg/acre

- Phosphorus - 2.5 Kg/acre
- Potassium - 90 Kg/acre
- pH - 7.4

The crude oil was refrigerated at 4°C and used without preliminary treatment.

Isolation of bacterial isolates

Isolation and enumeration of bacteria were performed by standard plate count technique using Bushnell and Hass agar media. Colonies of Micrococcus sp. were sub cultured in Nutrient agar and incubated at 37°C for 24 hours. Pure cultures were stored at 4°C until use. The cell number of overnight broth culture of bacteria was counted using Haemocytometer

Rapid screening method

About 103 cells of Micrococcus sp. that had been grown overnight on Luria – Bertani agar were transferred with sterile tooth picks into mineral salt agar plate. Immediately thereafter an ethereal solution of naphthalene (about 10% wt/vol) was uniformly sprayed over the surface of the agar plate.

The ether immediately vaporized from the surface at ambient temperature, and a white, thin layer of Naphthalene remained on the entire surface. The plates were then incubated at 30°C for 48 hours (Kiyoharaet al., 1982).

Biodegradation of crude oil by Micrococcus sp.

About 50ml of Bushnell – Haas medium was dispensed into each of ten sterile 250ml Erlenmeyer conical flasks and sterilized. After sterilization, 0.5 ml of the crude oil sample was added to the medium and inoculated with 1ml of overnight grown Micrococcus sp. and labeled. Uninoculated flasks were kept as control. All the ten conical flasks were incubated on a mechanical rotary shaker (90 rpm, 28°C ± 2°C) at different time intervals of 24, 48, 72, 96 and 120 hours. After incubation, the residual crude oil present in the samples was well as in the controls were extracted with chloroform at the end of each time interval and the rate of bioremediation was estimated by gas chromatography analysis (Hanson et al., 1997).

Gas Chromatography analysis

Gas chromatograph, Shimadzu GC 15A was used for the analysis of crude oil bioremediation process. The packed column Gas chromatography was performed to evaluate the rate of bioremediation (Venosaet al., 1996). Operating conditions for GC was tabulated (Table 1). The chloroform extracted bioremediated crude oil sample was injected into the packed column Gas chromatography analysis after the above operating conditions were satisfied. The Gas Chromatography analysis data were observed in the form of peaks and folds determining each compound and the peak values of each compound were expressed in % (w/v) which determines the concentration of each compound (Van Hamme and Ward, 1999).

Peak value can be calculated as follows:

$$\text{Peak Value} = \frac{\text{Peak height + fold width}}{\text{Volume of sample}} \times 100$$

Operating conditions	Temperature
Injection port	290 °C
Transfer Line	310°C
Initial GC Oven Temperature	55°C held for 3 min
First Temperature ramp rate	5°C /min to 200°C held for 5 min
Second temperature ramp rate	5°C /min to 310 held for 10 min
Total run time	73 min. for each sample

Table 1. Operating conditions for gas chromatography;

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III. RESULTS

Biodegrated Crude oil and soil samples were collected from the oil spill near the drilling site of "Oil and Natural Gas Commission" Cauvery Project, Kalappal, Thiruthuraipoondi District, Tamil Nadu, India.

The characteristics of the crude oil contaminated soil were analysed. Physiochemical parameters of soil was calculated.(Total Nitrogen,Potassium and Phosphorus and soil PH)To determine the nature of the crude oil contaminated soil by analyzing its pH, N,P,K contents

Nitrogen - 121.8 Kg/acre
Phosphorus - 2.5 Kg/acre
Potassium - 90 Kg/acre
pH - 7.4

The crude oil was refrigerated at 4°C and used without preliminary treatment.

Isolation of bacterial isolates

Isolation and enumeration of bacteria were performed by standard plate count technique (Bushnell and Hass agar media.) The cell number of overnight broth culture of bacteria was counted using Haemocytometer before subjecting for experiment as the organism should possess a cell count,Total no of cepp count was 107 cells/ml.

Rapid screening method

A rapid screening procedure was done for detecting the hydrocarbon degrading ability of bacteria on solid media.It is evident that on minimal agar plate sprayed with an ethereal solution of naphthalene, Micrococcus sp. grew in the form of colonies which were surrounded with transparent areas (clear zones). The formation of clear zones indicated the utilization of naphthalene by Micrococcus sp. and the non utilized naphthalene remained as thin white layerThe detection procedure of Kiyoharaet al. (1982) found to be rapid, simple and useful for the isolation of PAH degrading bacteria and their mutants and it should contribute to genetic studies of bacterial degradation of poly aromatic hydrocarbons (PAH).Bioremediation of crude oil in shake flask experiment was conducted with Micrococcus sp. and its bioremediation activity was determined by Gas Chromatography analysisThe following twelve different hydrocarbons viz., Phenanthrene, Anthracene, Naphthalene, Pyrene, Isoprenoid, Hexadecane, Tetracosane, Pristane, Phytane, Methylbenzothiophene, Dibenzothiophenesulfone, Tetrahydrothiophenesufone were monitored for assessing the bioremediation activity.he peak values of these compounds present in the controls and in the samples were expressed in % w/v (Table 2).

The statistical representations of these values were shown in figure (1 – 12). The statistical data plotted by graphical method showed the comparison of bioremediation rate of exerted hydrocarbon compound present in the controls and in the samples during different time intervals of 24, 48, 72, 96 and 120 hoursIt is evident from the obtained data that hexadecane, naphthalene, phenanthrene showed moderate degree of bioremediation by Micrococcus sp. The percentage of bioremediation of these compounds in 120 h incubated sample was 50.8% (w/v), 28.5% (w/v) and 24.3% (w/v) respectively.The rest of the nine compounds also showed considerable amount of mineralization by Micrococcus sp. It is evident from the obtained data that hexadecane, naphthalene, phenanthrene showed moderate degree of bioremediation by Micrococcus sp. The percentage of bioremediation of these compounds in 120 h incubated sample was 50.8% (w/v), 28.5% (w/v) and 24.3% (w/v) respectively. The rest of the nine compounds also showed considerable amount of mineralization by Micrococcus sp.

CRUDE OIL SAMPLE

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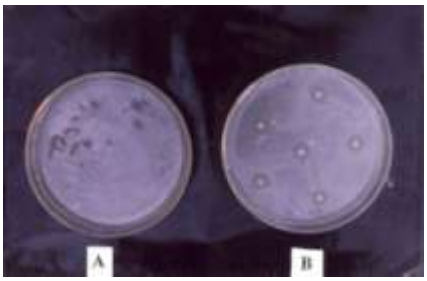
RAPID SCREENING METHOD



CONTROL
B – NAPHTHALENE SPRAYED MM2 AGAR PLATE INOCULATED WITH Micrococcus sp. SHOWING ZONE FORMATION
AERATION AND AGITATON USING MECHANICAL SHAKER DURING THE INCUBATION PERIOD OF THE BIOREMEDIATION PROCESS

BIOREMEDIATED

SAMPLE

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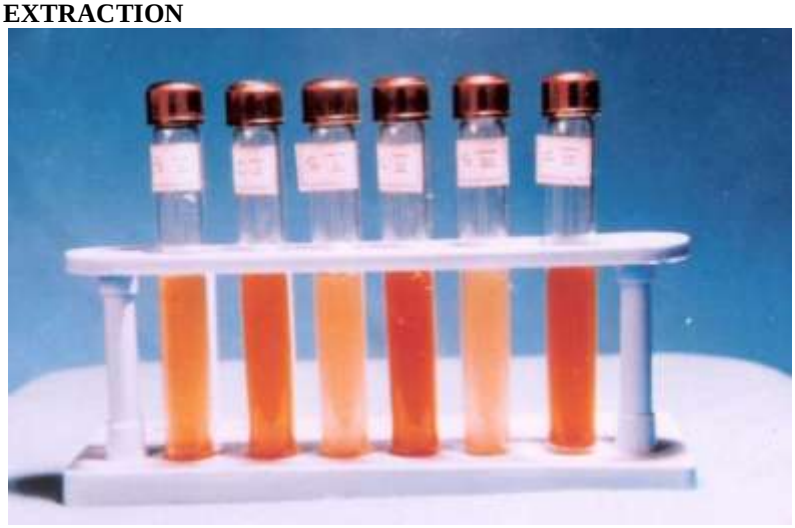
A – CONTROL
B – BIOREMEDIATED SAMPLE
CHLOROFORM EXTRACTION



SI – DAY ONE BIOREMEDIATED SAMPLE (24 HOURS)
CI – DAY ONE STERILE CONTROL
SII - DAY TWO BIOREMEDIATED SAMPLE (48 HOURS)
CII - DAY TWO STERILE CONTROL
SIII – DAY THREE BIOREMEDIATED SAMPLE (72 HOURS)
CIII – DAY THREE STERILE CONTROL

Table 2. Bioremediation of Crude oil by Micrococcus sp. in Shake flask experiment

Exerted Hydrocarbon Compounds	24 h		48 h		72 h		96 h		120 h	
	Control I	Sample I	Control II	Sample II	Control III	Sample III	Control IV	Sample IV	Control V	Sample V
Phenenthere ne	40.5	34.9	42.5	33.7	44.1	26.8	45.4	24.6	46.1	21.8
Anthracene	25.5	25.3	25.8	25.1	26.1	24.9	26.3	24.4	26.6	24.1
Naphthalene	44.8	35.8	67.3	52.2	47.3	21.4	48.1	20.8	48.7	20.2
Pyrene	40.1	37.4	41.8	35.3	43.1	30.6	43.6	28.4	44.4	27.3
Isoprenoid	21.6	37.4	41.8	35.3	22.8	19.7	23.1	19.6	23.8	19.3
Hexadecane	64.2	54.2	22.4	20.2	68.4	25.2	71.1	24.4	72.6	21.8

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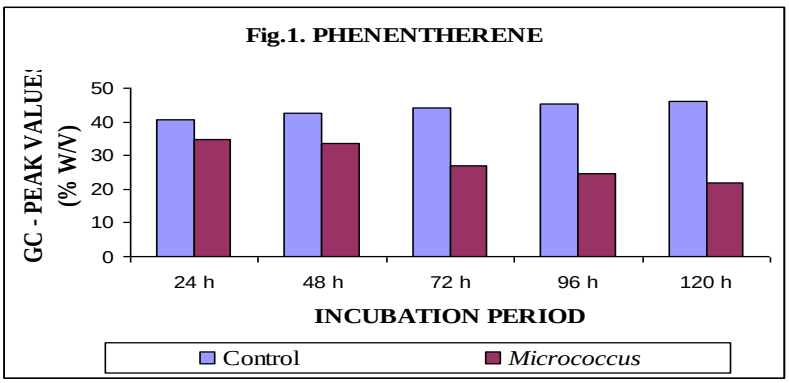
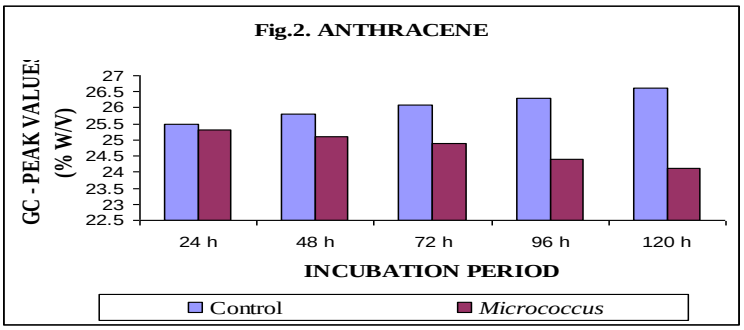
Tetracosane	40.9	37.9	41.1	35.7	43.4	33.3	44.6	31.1	45.8	29.2
Pristane	73.6	67.8	45.4	32.7	75.1	64.6	76.2	62.2	75.6	60.7
Phytane	44.1	43.7	74.8	66.2	45.4	42.8	46.4	42.1	47.3	41.3
Methylbenzo Thiophene	39.8	35.9	44.7	43.2	44.2	31.5	45.9	29.4	46.8	27.3
Dibenzo thiophene Sulfone	24.7	20.3	40.7	32.8	26.6	18.4	27.8	19.0	28.2	17.7
Tetrahydro Thiophene Sulfone	38.1	37.3	25.2	19.6	40.9	27.3	41.8	26.3	42.1	24.5

Gas Chromatography Analysis Peak Values (% w/v)
Bioremediation of Crude oil by Micrococcus sp. in Shake flask experiment
Gas Chromatography Analysis Peak Values (% w/v)





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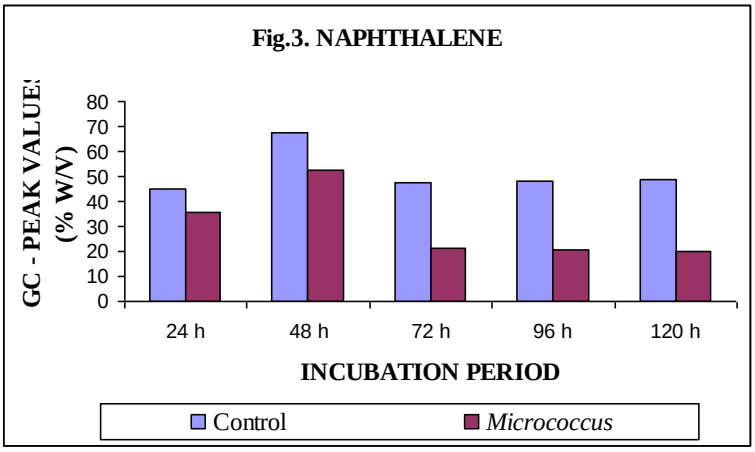

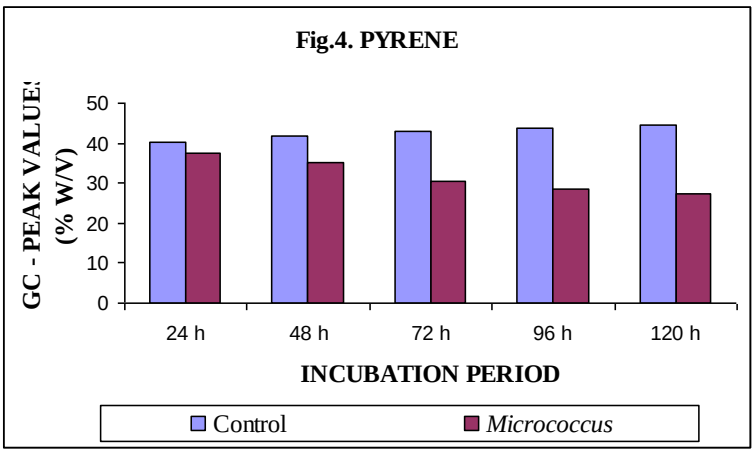

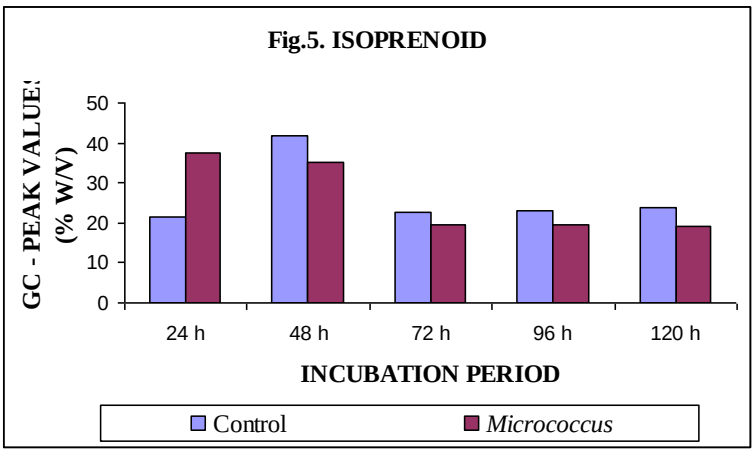

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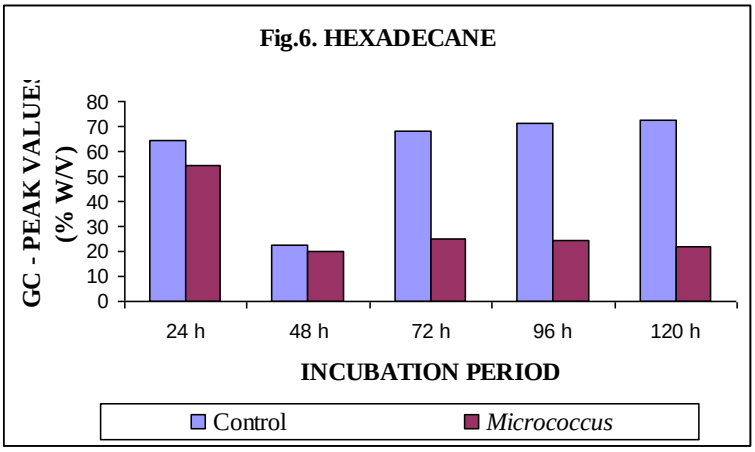

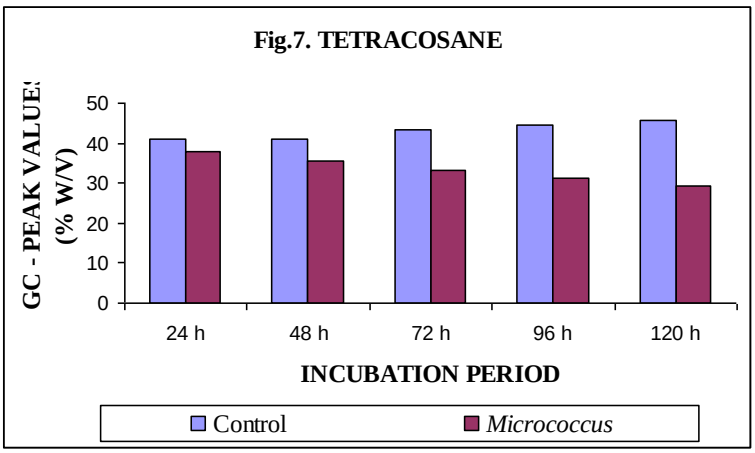

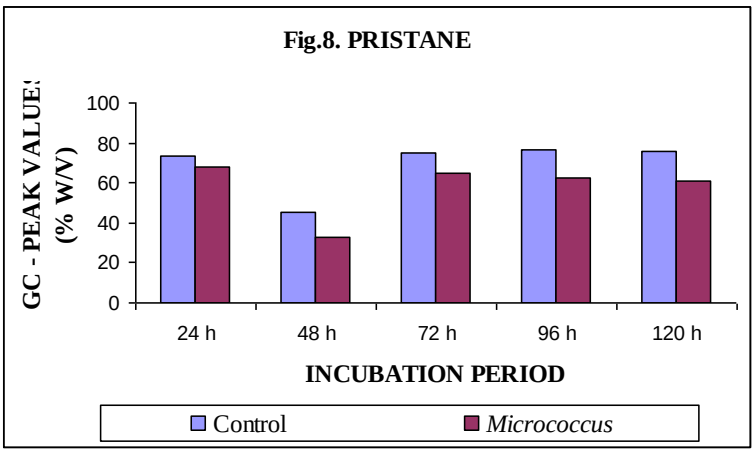

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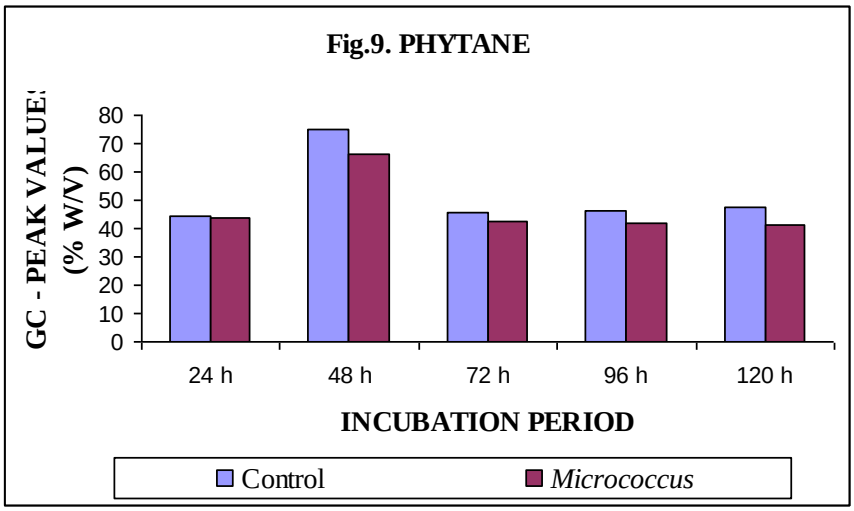

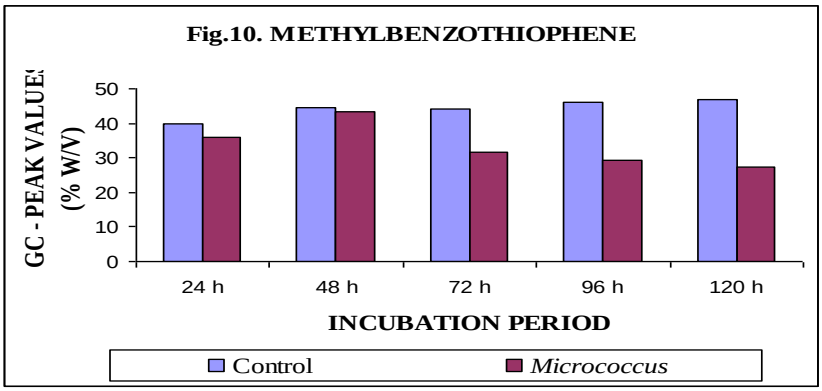

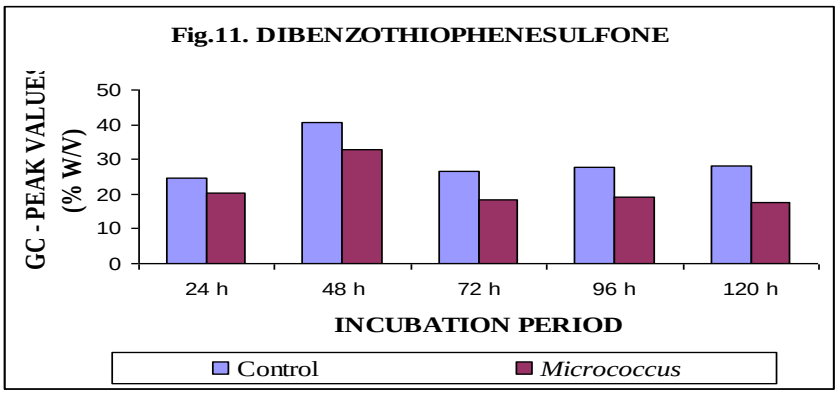

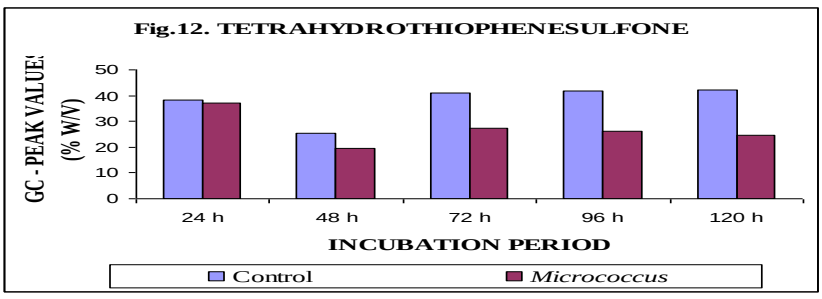

IV. COMCLUTIONS;

The nature of the crude oil contaminated soil collected from the drilling site of ONGC was assessed in terms of pH, N,P,K contents of soil. The pH of the soil was found to be alkaline and N,P,K contents found to be in a limited range

Naphthalene degrading ability of Micrococcus sp. was determined by rapid screening procedure .Bioremediation of crude oil by Micrococcus sp under shake flask condition was tudiedBioremediation activity was monitored by GC analysisAmong the twelve hydrocarbons exerted during GC analysis, Hexadecane, Naphthalene, Phenantherene showed moderate degree of bioremediation. Rest of the compounds also showed considerable amount of mineralization.The present study highlighted that Micrococcus sp. is a potential agent for use in bioremediation of crude oil contaminated sites.Studies can be made further by applying this Micrococcus sp. as a potential tool in field trials to clean up the crude oil spills.

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