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Biodegradation of Oil and Diesel Compounds using Soil Isolates and Gravimetric Analysis for Utilization of the Individual Substrates

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ABSTRACT
Oil spills have become a global problem particularly in industrialized and developing countries. Microbial degradation process aids the elimination of spilled oil from the environment after critical removal of large amount of the oil by various physical and chemical methods. The parameters typically measured in laboratory tests of bioremediation efficacy include enumeration of microbial populations and determination of fate of hydrocarbon degradation. Microorganisms capable for degradation of oil and petrol were isolated by enrichment culture technique. Two isolates were found to be potent degraders which were identified from MTCC, Chandigarh and used for the present investigation. The degradation of diesel oil and groundnut oil was monitored over a twenty-seven day period using gravimetric method. These soil samples, together with the unpolluted control samples were seeded with the two isolates. The rates of degradation of oil by the isolates at the end of day one, day twelve to day twenty seven were determined. The weight of oil degraded by the isolates during the course of studies for all the samples showed a consistent decrease with respect to time. The present studies helped to investigate the role of gravimetric method in monitoring the degradation of the oil and petroleum samples using a simple cost effective protocol.
Keywords : Biodegradation, Groundnut oil, Diesel oil, Gravimetric Analysis.

INTRODUCTION

Oil spills have become a major world-wide problem more specifically in densely industrialized and developing countries. These oil spills occur during accidental leakages which may be during hydrocarbon fuel transportation and other fuel related activities which are quite inevitable [1, 2]. This makes the hydrocarbons the most common global environmental pollutants. The most concerned among these pollutants are the oil containing polycyclic aromatic hydrocarbons. The constituents of these contaminants are carcinogenic, mutagenic and potent intoxicants which cause serious threat to human and animal health [3]. It is practically very expensive to clean up such contaminants using invasive technologies. Besides, this may often damage the natural resources and properties of the soil, sediment or aquifer. The ability of microbes to degrade organic contaminants into harmless constituents has been used as a means to biologically treat contaminated environments. It is the subject of many research investigations and real-world applications and it is the basis for the emergent field of bioremediation [4].

Bioremediation makes use of the metabolic potential of microbes for the clean up of recalcitrant xenobiotic compounds. Bioremediation has thereby come up as a promising alternative to the invasive technologies. Successful application of the microorganisms for the bioremediation of PAH contaminated sites therefore requires a deeper understanding of the physiology, biochemistry and molecular genetics of potential catabolic pathways. Biological degradation represents one of the major routes through which hydrocarbons are removed from contaminated environments. Microbial degradation process aids the elimination of spilled oil from the environment after critical removal of large amounts of the oil by various physical and chemical methods [5]. This is possible because of microorganisms having enzyme systems to degrade and utilize oil and petrol as a source of carbon and energy [6, 7]. Biodegradation of diesel oil has been tried for contaminated cold-adapted and alpine soils earlier [8]. The present report deals with the isolation of groundnut oil and diesel-degrading bacteria and determination of the biodegradation potentials of the pure cultures of selected bacterial isolates.

MATERIAL AND METHODS

Isolation of Bacteria

Microorganisms capable of degrading groundnut oil were isolated from soil and petroleum containing soils. 1 gram of this soil sample was suspended in 9 mL of sterile distilled water. Dilutions (10^{-6}) were prepared from the soil samples using sterile distilled water. The soil samples were planted in and on solid feeding medium nutrient agar which was used here. After proper incubation period, typical colonies were picked up and inoculated in 500 mL

flasks containing 100 mL mineral salt medium. Enrichment was carried out in the flasks which were kept on rotary shakers at 120 rpm at room temperature for 7 days [9]. The medium comprised of 0.5 gL^{-1} NH_4Cl, 0.5 gL^{-1} NaH_2PO4. H_2O, 0.5 gL^{-1} KH_2PO_4, 0.5 gL^{-1} $MgSO_4.7H_2O$ and 0.4 gL^{-1} NaCl. The medium was autoclaved at 121^0C for 15 minutes [10]. The plates were incubated at 37^0C for 48 hours. Each bacterial colony type was sub cultured repeatedly over nutrient agar plates to obtain a pure culture.

The preliminary morphological attributes were studied in the form of colony characters and Gram character. The isolates were characterized based on cultural characteristics, by MTCC Chandigarh. Further screening was done for their utilization of oil and diesel using the methods of Abu and Ogiji [11].

Table-1: Identification of Isolates

Tests	Culture 1	Culture 2
Colony morphology		
Configuration	Irregular	Irregular
Margin	Undulate	Undulate
Elevation	Flat	Flat
Surface	Moist	Moist
Pigment	Cream	Greenish
Opacity	Opaque	Opaque
Gram's reaction	*-ve*	*-ve*
Cell shape	Short rods	Short rods
Size (µm)	1-2µ	1-2µ
Spore(s)	-	-
Motility	Motile	Motile
Anaerobic	-	-
Growth at temperatures		
4^0C	-	-
Between 15^0C-42^0C	+	+
55^0C	-	-
Growth at pH 5.0-11.0	+	+
Growth on NaCl (%)2 &4 %	+	+
6.0 and above pH	-	-
Methyl red test	-	-
Voges Proskauer test	-	-
Casein hydrolysis	-	-
Citrate	+	+
Arginine	+	+
Ornithine	+	-
Indole	-	-
Gelatin hydrolysis	+	+
Starch hydrolysis	-	-
Catalase test	-	+
Oxidase test	+	+
Growth on MacConkey's	NLF	NLF
Tween 20	+	-
Tween 40	+	-
Urea production	+	+
TSI results Butt	Yellow	Yellow
Slant	Pink	Pink
Acid production from		
Lactose	-	-
Melibiose	+	+

Xylose	+	-
Sucrose	+	-
Galactose	-	+
Fructose	-	+
Identified as	*Pseudomonas* sp. (Ps)	*Pseudomonas aeruginosa* (S)

+: Positive; -: Negative; NLF: Non-Lactose fermenting

Table-2: Weight of Oil Extracted from 2 gram Soil Samples polluted with 0.1ml (0.85g) of Groundnut Oil Using *Pseudomonas species*

Day	Sample Description	Weight of groundnut oil extracted (g)	Weight of groundnut oil extracted (g /h)
0	Sterilized Soil mixed with ground nut oil plus isolate (Ps)	0.085 ± 0.01	--
1	Sterilized Soil mixed with ground nut oil plus isolate (Ps)	0.085 ±0.01	--
	Sterile soil plus isolate (Ps)	0.00±0.00	--
	Sterilized Soil mixed with ground nut oil	0.085±0.01	--
3	Sterilized Soil mixed with ground nut oil plus isolate (Ps)	0.049 ±0.01	0.0005
6	Sterilized Soil mixed with ground nut oil plus isolate (Ps)	0.045 ±0.01	0.00028
9	Sterilized Soil mixed with ground nut oil plus isolate (Ps)	0.042 ±0.01	0.000199
12	Sterilized Soil mixed with ground nut oil plus isolate (Ps)	0.038 ±0.01	0.000163
15	Sterilized Soil mixed with ground nut oil plus isolate (Ps)	0.038±0.01	0.000163
18	Sterilized Soil mixed with ground nut oil plus isolate (Ps)	0.036 ±0.01	0.000113
21	Sterilized Soil mixed with ground nut oil plus isolate (Ps)	0.035 ±0.01	0.0000990
24	Sterilized Soil mixed with ground nut oil plus isolate (Ps)	0.032 ±0.01	0.0000920
27	Sterilized Soil mixed with ground nut oil plus isolate (Ps)	0.029 ±0.01	0.000086

Table-3: Weight of Groundnut Oil Extracted from 2 g Soil Samples Polluted with 0.1ml (0.85g) of Groundnut Oil using *Pseudomonas aeruginosa*.

Day	Sample Description	Weight of groundnut oil extracted (g)	Weight of groundnut oil extracted (g /h)
0	Sterilized Soil mixed with ground nut oil plus isolate (S)	0.085 ±0.01	--
1	Sterilized Soil mixed with ground nut oil plus isolate (S)	0.085 ±0.01	--
	Sterile soil plus isolate B1	0.00± 0.00	--
	Sterilized Soil mixed with ground nut oil plus isolate (S)	0.085 ±0.01	--
3	Sterilized Soil mixed with ground nut oil plus isolate (S)	0.066 ±0.01	0.00026
6	Sterilized Soil mixed with ground nut oil	0.064 ± 0.01	0.000135

	plus isolate (S)		
9	Sterilized Soil mixed with ground nut oil plus isolate (S)	0.060±0.01	0.000115
12	Sterilized Soil mixed with ground nut oil plus isolate (S)	0.052 ±0.01	0.000114
15	Sterilized Soil mixed with ground nut oil plus isolate (S)	0.049 ±0.01	0.00010
18	Sterilized Soil mixed with ground nut oil plus isolate (S)	0.042 ±0.01	0.000099
21	Sterilized Soil mixed with ground nut oil plus isolate (S)	0.035 ±0.01	0.0000992
24	Sterilized Soil mixed with ground nut oil plus isolate (S)	0.033 ±0.01	0.000090
27	Sterilized Soil mixed with ground nut oil plus isolate (S)	0.029 ±0.01	0.000086

Table-4: Weight of Diesel Oil Extracted from 2 g Soil Samples Polluted with 0.1ml (0.85g) of Diesel Oil Using *Pseudomonas species*

Day	Sample Description	Weight of diesel oil extracted (g)	Weight of diesel oil extracted (g /h)
0	Sterilized soil mixed with ground nut oil plus isolate (Ps)	0.085 ± 0.01	0.085 ±0.01
1	Sterilized soil mixed with ground nut oil plus isolate (Ps)	0.085 ±0.01	0.085 ±0.01
	Steriled soil plus isolate (Ps)	0.00±0.00	0.00±0.00
	Sterilized soil mixed with ground nut oil	0.085±0.01	0.085 ±0.01
3	Sterilized soil mixed with ground nut oil plus isolate (Ps)	0.067 ±0.01	0.00093
6	Sterilized soil mixed with ground nut oil plus isolate (Ps)	0.062 ±0.01	0.00043
9	Sterilized soil mixed with ground nut oil plus isolate (Ps)	0.057 ±0.01	0.00026
12	Sterilized soil mixed with ground nut oil plus isolate (Ps)	0.054 ±0.01	0.00018
15	Sterilized soil mixed with ground nut oil plus isolate (Ps)	0.051±0.01	0.00014
18	Sterilized soil mixed with ground nut oil plus isolate (Ps)	0.046±0.01	0.00010
21	Sterilized soil mixed with ground nut oil plus isolate (Ps)	0.043±0.01	0.000085
24	Sterilized soil mixed with ground nut oil plus isolate (Ps)	0.041±0.01	0.000071
27	Sterilized soil mixed with ground nut oil plus isolate (Ps)	0.037±0.01	0.000058

Table-5: Weight of Diesel Oil Extracted from 2 g Soil Samples Polluted with 0.1ml (0.85g) of Diesel Oil with *Pseudomonas aeruginosa*

Day	Sample Description	Weight of diesel oil extracted (g)	Weight of diesel oil extracted (g/h)
0	Sterilized Soil mixed with ground nut oil plus isolate (S)	0.085 ±0.01	0.085 ±0.01
1	Sterilized Soil mixed with ground nut oil plus isolate (S)	0.085 ±0.01	0.085 ±0.01
	Sterile soil plus isolate (S)	0.00± 0.00	0.00± 0.00
	Sterilized Soil mixed with ground nut oil plus isolate (S)	0.085 ±0.01	0.085 ±0.01
3	Sterilized Soil mixed with ground nut oil plus isolate (S)	0.069 ±0.01	0.00096
6	Sterilized Soil mixed with ground nut oil plus isolate (S)	0.064± 0.01	0.00044
9	Sterilized Soil mixed with ground nut oil plus isolate (S)	0.058±0.01	0.00027
12	Sterilized Soil mixed with ground nut oil plus isolate (S)	0.0556±0.01	0.00019
15	Sterilized Soil mixed with ground nut oil plus isolate (S)	0.052±0.01	0.00014
18	Sterilized Soil mixed with ground nut oil plus isolate (S)	0.048±0.01	0.000011
21	Sterilized Soil mixed with ground nut oil plus isolate (S)	0.044±0.01	0.000087
24	Sterilized Soil mixed with ground nut oil plus isolate (S)	0.042±0.01	0.000073
27	Sterilized Soil mixed with ground nut oil plus isolate (S)	0.040±0.01	0.000061

Sample collection and preparation

Top soil sample was collected from the premises of the PSGVPM's College, Shahada, district Nandurbar, in sterilized container and taken to the laboratory. Soil sample meant for degradation studies was sterilized by autoclaving at 121^0C for 15 minutes, after which it was allowed to cool for further treatments.

Treatment of samples

Treatment of the samples was carried out as per the method of Abu and Ogiji [11]. The soil samples in each group were treated. In group A two samples each of sterilized soil were taken and mixed with ground nut oil plus suspension. In group B two samples each of sterilized soil were taken and mixed with groundnut oil plus distilled water. In group C two samples each of sterilized soil were mixed with distilled water plus sample suspension. Group B and C served as controls. Similar protocols were followed for soil containing diesel.

Groundnut oil degradation studies

The ability of isolates to degrade ground nut oil was demonstrated in terms of reduction in the quantity of groundnut oil. The rate of utilization was monitored on the first day (day zero) of the study and subsequently at 3 day intervals for 27 days. Carbon tetrachloride was employed as the extractant. Treated sample at the interval of three days was analyzed for the quantity of residual oil using the methods of Udema and Antai [12]. Each of the 2 g soil treated samples were mixed with 8mL carbon tetrachloride, placed in separating flask, shaken vigorously for 3 minutes and allowed to settle for 5 minutes. The liquid phase was separated by allowing the mousse (oil- carbon tetrachloride) to pass gradually through a funnel fitted with filter paper (Whatman No.1). Anhydrous sodium sulphate was spread on the filter paper to remove the moisture in the mixture. Further liquid phase was collected in 50 mL pre–weighed Pyrex beaker containing the extract, and it was placed in an oven, allowed to cool to room temperature and weighed to determine the quantity of oil by difference [12].

The percentage of oil degraded at the interval of three days was determined from the equation: % oil degraded = (weight of oil degraded / original weight of oil degraded which was determined as original weight minus weight of residual oil obtained after evaporating the extractant. Rate of degradation = weight of oil degraded (g) per time taken [13].

Oil extraction and biodegradation measurements by GC-MS analysis

Inorder to confirm the biodegradation of oil by the isolate advanced analyses were accomplished by GC-MS. After the incubation period, 5 ml of the cultures were extracted with two 20ml volumes of n-hexane as a solvent by using separator funnels to remove cellular material. The residues were transferred to tarred vials and the volume of each extract was adjusted to 100 ml by adding further n-hexane. The vials were kept at 4^0C until the gas chromatographic analysis. Uninoculated control was incubated in parallel to monitor abiotic losses of the substrate. Biodegradation of diesel oil was monitored by quantitative gas chromatographic analysis by Perkin Elmer, GC autosystem X fitted with an electronic pressure control and mass selective detector using capillary column (30mX 250nm,0.25 film thickness). Helium was used as the carrier gas (30 ml min-1). The oven was programmed as follows: 80^0C (5 minutes) with injector temperature 250^{o}C. The degradation of diesel oil as a whole was in relation to the amount of the remaining fractions in the appropriate abiotic control samples (external standard technique). Combined areas under resolved peaks and the Unresolved Complex Mixture (UCM) were integrated to represent Total Petroleum Hydrocarbon (TPH). [14]

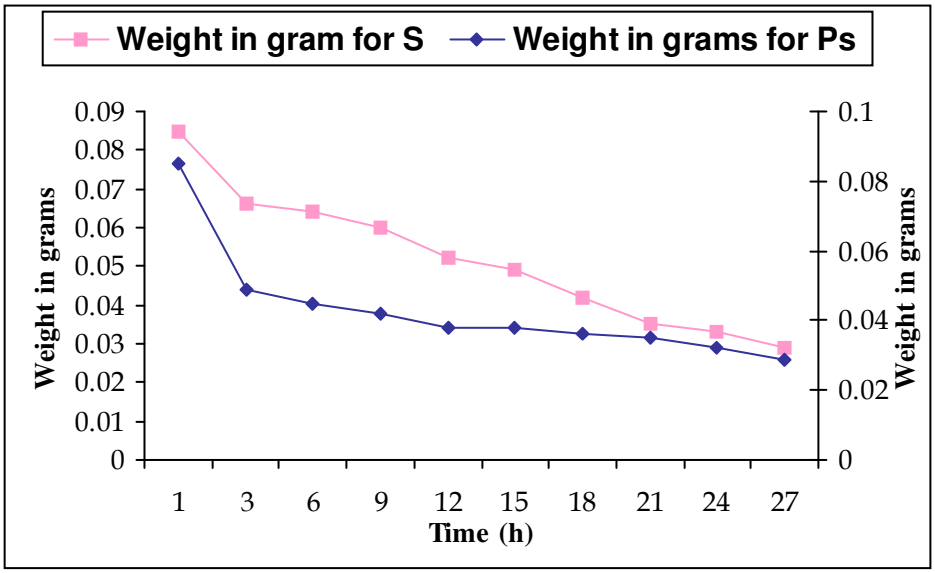

Fig.-1: Weight of groundnut oil extracted in time period of 1 to 27 days for Ps and S

RESULTS AND DISCUSSION

Isolation and Screening of organisms

On enrichment six isolates were isolated from petrol polluted soil. Out of these two isolates were finally screened on account of their potent ability of utilizing oil and diesel as carbon and energy source. The two isolates obtained and were identified from MTCC, Chandigarh on the basis of morphological, cultural and biochemical tests. The isolates were identified as *Pseudomonas* species and *Pseudomonas aeruginosa*. The results of the identification have been depicted in (Table 1).

Groundnut oil degradation studies

Sterilized soil was mixed with groundnut oil and isolates. Residual oil left was monitored over of period of 27 days. The oil and petrol utilizing bacterial strains were isolated first in mineral salt broth containing 1% oil and diesel oil. The isolated strains were examined further for substrate utilization for 27 days. The time course pattern of groundnut oil utilization and analysis of residual groundnut oil has been depicted in (Table 2) for *Pseudomonas species* and for *Pseudomonas aeruginosa* in (Table 3). 0.1 ml equivalent to 0.85 g of groundnut oil was used at zero time for the experimental studies and residual groundnut oil was found to be 0.049 and 0.066 grams for *Pseudomonas aeruginosa* and *Pseudomonas species* respectively in a period of three days and it subsequently

declined upto 0.029 for either organism in 27 days. Likewise the same quantity of diesel oil was used for the investigation and the quantity of diesel oil extracted was 0.069 and 0.067 grams in three days and 0.040 and 0.037 grams in 27 days for *Pseudomonas aeruginosa* and *Pseudomonas* species respectively (Table 4 & 5). The time course pattern of the utilization of oil and diesel has been parallely illustrated graphically in (Figure 1 & 2) respectively for the two isolates.

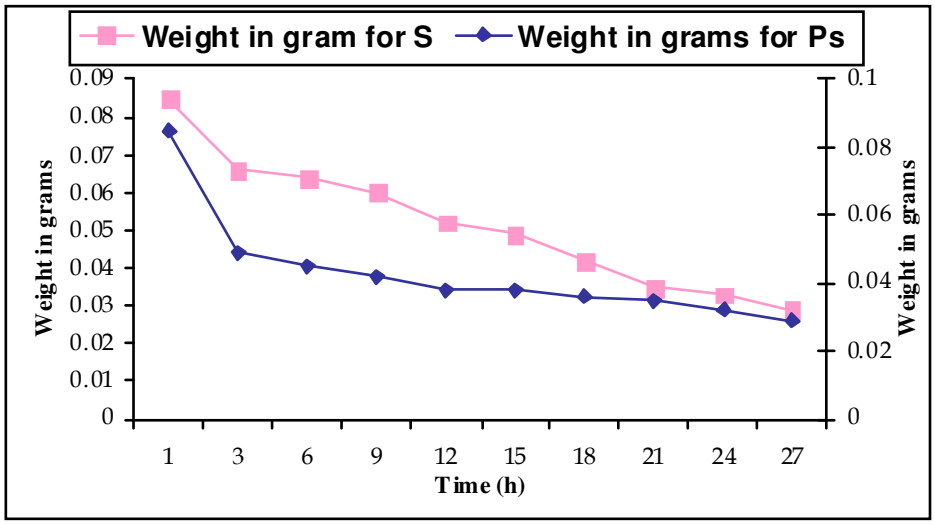

Fig.-2: Weight of diesel oil extracted in time period of 1 to 27 days for Ps and S

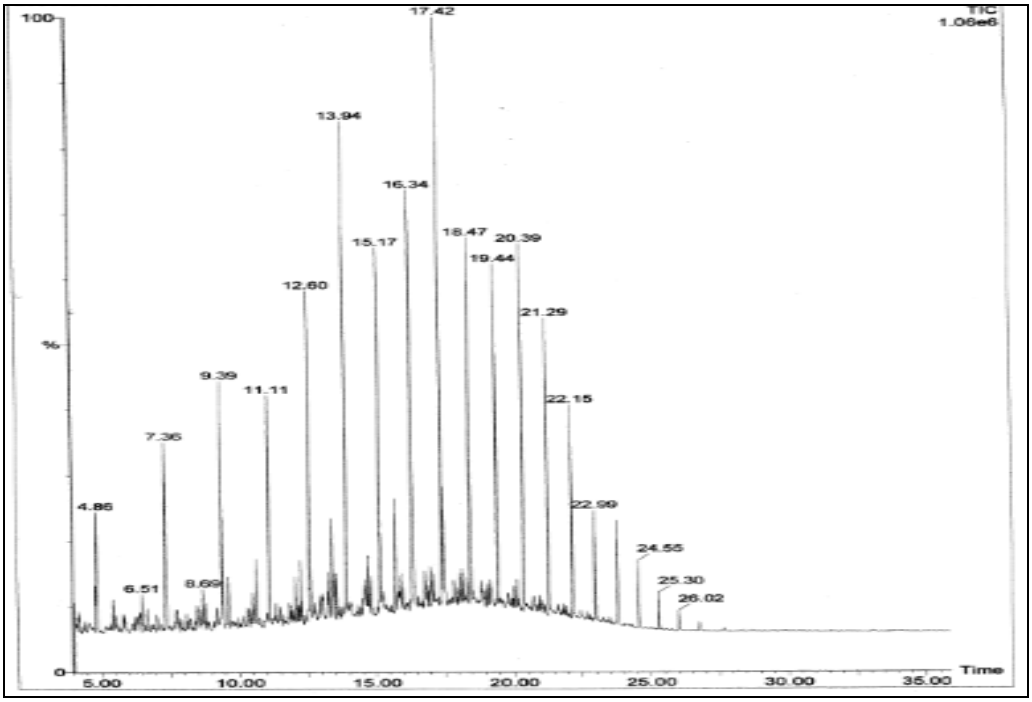

Fig.-3: GC-MS results of uninoculated control set of diesel oil after 15 days of incubation.

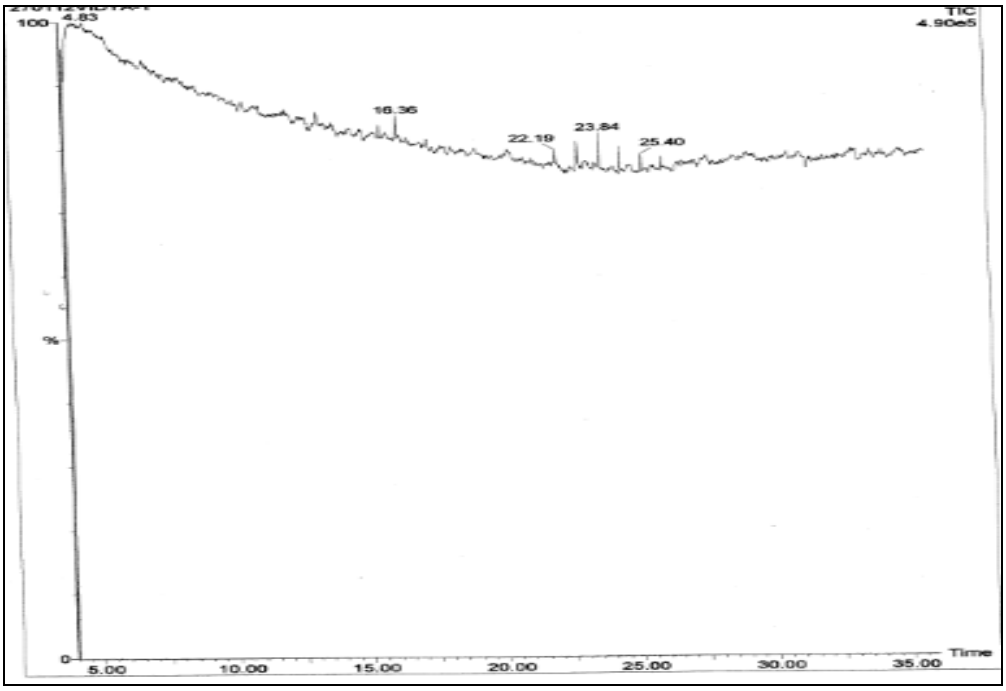

Fig.-4: GC-MS results of diesel oil inoculated with *Pseudomonas aeruginosa* after 15 days of incubation.

Oil extraction and biodegradation measurements by GC-MS

Figure 3 and 4 represent uninoculated and inoculated sets. They clearly reveal a reduction in diesel oil level contaminants with an increase in cellular number. The diesel carbon load was 1% and complete degradation of diesel represented by TPH could be achieved in 15 days of incubation at 37^0C. Figure 4 shows almost complete removal of diesel components as seen from the reduction in hydrocarbon peaks after 15 days of incubation.

CONCLUSION

The present experimentation clearly revealed that quantity of residual groundnut and diesel oil decreased consistently after day three and was found to be maximal on the third day and minimal on the 27^{th} day for both the isolates. This is indicative of the utilization of the two substrates by the organisms which has been further confirmed by GC-MS analysis. The gravimetric method performed was considered to be a cost-effective method very appropriate for preliminary investigations on monitoring of biodegradation of oil and diesel compounds. Results thus obtained after this investigation were comparable with those of GC-MS for drawing absolute conclusions on degradation potential.

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