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Biodegradation of Diesel Hydrocarbon in Soil by Bioaugmentation of *Pseudomonas aeruginosa*: A Laboratory Scale Study

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Abstract Bioremediation is an option that offers the possibility to destroy or render harmless contaminants using natural biological activity. This study examined the capacity of an indigenous isolate of *Pseudomonas aeruginosa* to remediate the diesel contaminated soil. Five different sets of cement bioreactors containing 2.5 kg soil; Bioreactor A (control, uncontaminated soil), B (sterilized soil), C (contaminated soil without any addition), D (contaminated soil with the addition of nutrients), E (contaminated soil with the addition of nutrients and bacterial inoculum) were prepared. Different soil enzyme activities like dehydrogenase, catalases, lipase and FDA hydrolase along with physicochemical parameters were investigated during the bioremediation process. The results obtained revealed that bioaugmentation of *P. aeruginosa* in diesel contaminated soil proved to be a better approach. 66 % diesel degradation was observed during the incubation period of 30 days. The soil enzyme activity increases as the hydrocarbon concentration decreased over time during bioremediation period. The FTIR and gas chromatographic analysis also confirmed the degradation of long chain alkanes of diesel hydrocarbons during bioremediation.

Keywords: *P. aeruginosa, bioremediation, diesel contaminated soil*

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1. Introduction

Soil contamination with oil spill is the major global concern today. This causes serious human health hazards and organic pollution of ground water, which limits its use, causes economic loss, environment problem and decreases the agricultural productivity of soil [1]. Environmental contamination with petroleum hydrocarbons is an inevitable that strikes many geographical regions to a variable extent depending on the local environmental law. Due to this fact, alternative biotechnological cleanup methods, such as bioremediation and phytoremediation, have raised much interest in the last two decades [2].

Diesel oil is a common product of crude oil distillation with a very complex composition. It consists mainly of low molecular weight alkanes and polycyclic aromatic hydrocarbons (PAHs) [3]. The fate of the latter compounds in nature may be of great human health importance, since PAHs have been considered toxic for plants and carcinogenic to people [3,4,5]. In case of an uncontrolled industrial leakage, diesel oil and its constituents might act as a persistent water and soil pollutant. According to Nogueira et al. [6], petroleum compounds can decrease the availability of water, oxygen and nutrients in the soil, which in consequence, may decline the rate of seed germination.

The world production of crude oil is increasing more than 3 billion/ year. Diesel oil is highly complex in nature, it is very difficult to understand the degradation mechanism. A diverse group of microorganisms has the ability to clean up the hydrocarbon contaminated sites [7]. Many researchers studied on petroleum degradation by using bacteria at various parameters to determine optimal degradation conditions. Microbes convert the chemical compounds into energy, cell mass and biological waste products [8]. Microbial decontamination of oils contaminated soils is claimed to be an efficient, economic, versatile and attractive in comparison to physicochemical treatments [9,10]. Various physical, chemical and biological techniques are used to reduce the negative effect of oil pollution on human health, flora and fauna [11]. Natural attenuation (NA), biostimulation (BS) and bioaugmentation (BA) techniques are used for bioremediation of diesel contaminated soils, since they are cost effective and environmentally friendly methods [12].

NA or BS occasionally show higher remediation efficiencies than BA treatment, according to environmental conditions, such as pollutants, soil types, pH and so on [13,14]. The need for a better understanding of the processes involved in the microbial remediation of

contaminated soils and of the physicochemical factors regulating the biotransformation of contaminants is particularly acute with respect to the use of biostimulation and bioaugmentation. Both technologies operate either through the addition of nutrients or enhanced aeration (biostimulation) or through the addition of microorganism (bioaugmentation). As such these approaches seek to ensure successful, remediation of the site by enhancing the activity of the right organism under the right environmental conditions [15].

In soil bioremediation, the health of the soil ecosystem has to be considered along with the remediation efficiency [11]. The degradation of hydrocarbons to simple molecules such as water and carbon dioxide involves many chemical reactions in which catalytic proteins are involved, which have a central role in hydrocarbon degradation and are attractive indicators for monitoring various impacts on soil [16].

The present study deals with the assessment of bioremediation efficiency of an indigenous strain of *P. aeruginosa* using soil biological methods as a monitoring tool and its validation through Fourier Transformed Infrared spectroscopy and Gas Chromatographic analysis.

2. Methodology

2.1. Bacterial Strain

P. aeruginosa used in experimental study was isolated from petroleum contaminated soil of oil depots of Madhya Pradesh (150 cm apart from oil depot from the depth of 15 cm) was deposited to Bacterial Germplasm culture collection centre, Bacteriology Lab, R. D. University, Jabalpur (M.P) and designated as BGCC # 2280.

2.2. Soil Preparation

25 kg soil (uncontaminated) was sieved through 0.2 mm sieve size mechanically. The water holding capacity of the soil was determined following the method of [17]. 2.5 1 diesel (10%v/v) was sprinkled over the sieved soil and allowed to get adsorbed for 30 min for further bioremediation studies following the method of Sharma and Rehman [18].

2.3. Inoculum Preparation

Single colony of bacterial isolate was inoculated into nutrient broth at 37°C for 24 hr in an orbital shaker at 120 rpm. The cells ($1x10^7$) were harvested by centrifugation at 10,000 rpm for 10 min. at 4°C, rinsed with sterile distilled water and resuspended in sterile Bushnell Hass broth for further use.

2.4. Bioremediation Experiment

The ability of bacterial isolate to remediate the diesel oil contaminated soil, was analyzed by carrying out the biodegradation experiment in bioreactors (12cm x 17 cm), made up of cemented walls, open from the top by following the method of Sharma and Rehman [18] with some modifications. Five bioreactors (A,B,C,D,E) were prepared in duplicates and 2.5 kg of soil was distributed in each experimental unit / bioreactor and covered by aluminum foil. The experiments were performed for 30 days at room temperatures (30±2°C) with following treatment combination:

Bioreactor A contained uncontaminated soil and was used to monitor the level of physiochemical and biological activities of soil.

Bioreactor B contained 356 ml of 6.5% sodium azide solution following the method of Riffaldi et al. [19] and to inhibit soil microorganisms, so as to monitor abiotic hydrocarbon degradation. In order to maintain the inhibiting effects, sodium azide was added at regular time interval.

Bioreactor C contained only contaminated soi1 without bacterial inoculum to evaluate hydrocarbon degradation by indigenous soil microorganisms.

Bioreactor D contained nutrients (yeast extract 0.28g/l; glucose 0.07g/l; $MnSO_4.2H_20$ 0.06g/l; $(NH_4)_2SO_4.2H_20$ 0.06g/1) in order to evaluate their effect on bioremediation

Bioreactor E contained contaminated soil seeded with nutrients (yeast extract 0.28g/1; glucose 0.07g/1; $MnSO_4.2H_20$ 0.06g/l; $(NH_4)_2SO_4.2H_20$ 0.06g/l) and bacterial inoculum [20].

For each experimental unit the soils were mixed thoroughly at every alternate day to provide adequate aeration. During the 30 days of incubation period the soil of each bioreactor was tilled and sterile distilled water was added at an interval of 5 days to maintain 60% of water holding capacity of soil [20]. Initial readings were taken immediately after the inoculation of the bacterial culture and further, the samples were drawn after every 5^{th} day from the respective experimental unit and analyzed up to 30 days by following the method of Riffaldi et al. [19] and Andreoni et al. [21].

2.5. Physicochemical Analysis of Soil

The soil pH and temperature of each bioreactor was determined following the method of Ashok and Mussarrat [22]. Distribution of soil particle size was determined by following the method of Ketler et al. [23]. Soil water content and total organic carbon content was determined by following the method of Craze [24] and Walkey and Black [25] respectively. Soil phosphate, nitrate, sulphate, chloride and carbonate content was determined quantitatively by following the method of APHA [26] and NEERI [27].

2.6. Soil Microbiological Analysis

Soil dehydrogenase, lipase, catalase and FDA hydrolase activity was determined by following the method of Casida et al. [28], Kuhnert-Finkernagel and Kandeler [29], Rodriguez-Kabana and True love [30] and Banadick and Dick [31] respectively. Total bacterial count was carried out using standard dilution plate method following the method of APHA [26].

2.7. Biodegradation of Diesel Hydrocarbon

Total diesel hydrocarbon was extracted by soxhlet apparatus using dichloromethane as a solvent following the method of Helalch et al. [32]. the degradation of diesel was determined by gravimetric analysis [13], FTIR analysis [33] and gas chromatographic analysis [34].

3. Results and Discussion

3.1. Soil Physicochemical Analysis

Although most of the constituents of petroleum oil are degradable [35], petroleum constituents are deficient in nitrogen and phosphorous [36,37] thus the overall rates of hydrocarbon degradation are govern by temperature, pH, water content, oxygen and inorganic nutrient. The physiochemical properties of soil undergoing bioremediation are shown in Table 1. In the present study the pH of the soil in all bioreactors ranged from 6.8-7.6 during the incubation period of 30 days. This indicated their neutral to slight alkaline nature. The soil pH is a limiting factor and is an important parameter to monitor. The neutral to slight alkaline nature of soil promote the biodegradation of diesel hydrocarbon in soil [38]. Maximum change in pH was observed in bioreactor E having biougmentation treatment of *P. aeruginosa*. The temperature of the soil ranged from 28-32°C during the incubation period of 30 days in all the bioreactor. All the treated soil showed 78.8% sand, 11.4%silt and 9.8% clay content. No significant change in particle size distribution was observed during the study period. Total organic carbon of the soil was found to be reduced in all biologically active treatment units during bioremediation process. Maximum reduction was observed in soil with bioaugmentation treatment (bioreactor E) i.e. from 1.63% - 1.12% at the end of the study period. This could be attributed to the removal of diesel hydrocarbons during decontamination process. Nutrients are very important ingredients for successful biodegradation of hydrocarbon pollutants especially nitrogen and phosphorus [39]. Nutrient deficiencies are often reported in soils contaminated with petroleum oil [40]. Therefore addition of the nutrients in these soils is necessary to enhance the degradation process [41,42]. Sabate et al. [43], reported addition of nutrients such as nitrogen and phosphorus which resulted in large decrease in total hydrocarbons by accelerating the growth of microbial population. In the present study 5 to 10 fold increase in biodegradation of diesel oil was observed in treatment units i.e. bioreactor D and E which may be due to the addition of nutrients (phosphate, nitrate, sulphate) during the bioremediation process.

Table 1. Physicochemical analysis of diesel oil (10%) contaminated soil during bioremediation period of 30 days

Incubation days	Bioreactor	Soil pH	Temperature (°C)	Soil texture			Phosphate (mg/kg)	Nitrate (mg/kg)	Sulphate (mg/kg)	Chloride (mg/kg)	Carbonate (mg/kg)	TOC (%)	TPH degraded (%)
				Sand%	Silt%	Clay%							
0th day	A	7.2	30	78.8	11.4	9.8	17.8	25.4	12.1	8.35	9.59	1.03	0.0
	B	7.3	31	78.9	11.5	9.6	17.5	25.6	12.9	8.40	9.44	1.54	0.0
	C	7.0	32	78.8	11.6	9.6	17.6	24.1	11.6	8.13	9.56	1.55	0.0
	D	7.1	30	78.5	11.7	9.8	118.4	87.6	36.5	26.7	9.39	1.67	0.0
	E	7.2	30	78.6	11.8	9.7	110.5	85.0	35.4	28.45	9.39	1.63	0.0
5th day	A	7.1	31	78.5	11.9	9.6	17.1	21.1	9.4	8.25	8.73	1.22	ND
	B	7.2	31	78.6	11.8	9.7	18.4	22.5	10.5	8.3	8.4	1.51	2.83%
	C	7.0	32	78.6	12	9.4	17.8	24.3	10.6	8.16	8.8	1.55	3.25
	D	7.1	32	78.6	11.5	9.9	114.5	87.5	35.9	21.5	9.4	1.64	5.56
	E	7.0	30	78.5	11.5	10	118.5	85.3	35.8	28.25	9.26	1.6	7.5
10th day	A	7.2	30	78.5	11.9	9.6	16.2	21.8	11.17	8.35	9.42	1.13	ND
	B	7.2	31	78.5	11.2	9.3	18.6	22.8	10.9	8.45	9.35	1.46	5.66%
	C	7.2	31	78.4	12.7	8.9	14.4	26.5	10.8	8.15	9.4	1.5	6.75
	D	7.3	32	78.9	11.1	10.0	112.3	81.4	31.8	21.45	8.48	1.49	9.15
	E	7.3	31	78.7	11.7	9.6	108.4	83.4	34.6	24.4	9.3	14.8	12
15TH day	A	7.2	29	78.7	11.5	9.8	15.61	20.6	10.5	8.4	9.21	1.10	ND
	B	7.1	30	78.8	11.9	9.3	15.8	19.8	9.4	8.3	9.34	1.43	8.52%
	C	7.1	31	78.7	11.7	9.6	14.8	16.5	10.3	8.31	9.42	1.44	10.5
	D	7.4	32	78.8	11.5	9.8	106.2	78.4	28.5	20.85	8.3	1.4	17.11
	E	7.5	32	78.9	11.9	9.2	97.1	82.4	31.6	24.15	9.3	1.3	21
20th day	A	7.2	28	78.6	11.6	9.8	12.41	20.6	10.2	8.65	9.6	1.13	ND
	B	7.1	30	78.5	12.1	9.4	12.5	21.5	10.5	8.2	9.55	1.4	11.35%
	C	7.1	31	78.4	11.9	9.7	12.25	17.2	9.1	8.3	9.3	1.42	15.5
	D	7.4	32	78.6	11.2	9.9	108.5	75.5	27.4	21.85	8.65	1.3	22.7
	E	7.6	32	78.8	11.9	9.3	95.4	79.4	31.5	26.25	9.5	1.3	48`
25th day	A	7.2	28	78.5	11.7	9.8	12.46	20.2	9.8	8.2	9.3	1.0	ND
	B	7.1	29	78.4	11.4	9.2	11.43	19.4	10.2	7.4	9.4	1.37	14.18
	C	7.1	30	78.5	11.2	9.9	10.41	15.9	8.8	7.15	10.2	1.38	17.4
	D	7.2	31	78.6	11.2	9.9	98.3	75.4	27.1	20.7	9.9	1.32	28.2
	E	6.8	31	78.4	11.5	10.2	92.4	75.4	28.5	26.75	9.6	1.2	54.1
30th Day	A	7.2	30	78.2	11.9	9.9	12.21	21.2	8.5	8.3	9.6	.97	ND
	B	7.3	30	78.3	11.7	9.0	11.26	18.2	9.4	7.8	8.9	1.35	17.01
	C	7.3	32	78.4	11.9	9.7	10.8	18.4	9.2	7.5	9.6	1.34	21.71
	D	7.0	31	78.5	11.2	9.9	98.5	71.6	25.6	22.6	8.8	1.28	34.5
	E	7.0	31	78.4	11.7	8-9	91.2	76.9	21.5	24.5	8.2	1.12	66

Bioreactor A: Uncontaminated soil; B: Sterilized soil; C: Diesel contaminated soil without nutrient; D: Contaminated soil with nutrients; E: Contaminated soil with nutrients and bacterial inoculum; ND: not detected.

3.2. Soil Microbiological Analysis

Enzymes are the catalysts of important metabolic functions, including the decomposition and detoxification of contaminants [44,45]. Enzyme activity therefore has been regarded as the indicator of microbial activity and are also useful for determining the intensity of microbial metabolism in soil. Dehydrogenase activity typically occurs in all intact, viable microbial cells. Thus, its measurement is usually related to the presence of viable microorganism and their oxidative capability [46]. In the present study soi1 treated with *P. aeruginosa* showed increase in the dehydrogenase activity (bioreactor E) up to 15 days which decreased upto 25th day and remained unchanged until the end of the incubation period (Figure 1). Dehydrogenases, catalases and FDA hydrolase have been found only to be useful for indicating the onset of the biodegradation process, as their activities decline rapidly after the rate of biodegradation has decreased [47,48]. However, in the present study lipase, catalases and FDA hydrolase activity was also found to increase with time (Table 2). Lipase activity, which degrade lipids in glycerol and fatty acid is produced by a large variety of microorganisms [49]. Margesin et al. [50] had shown a negative correlation between organic pollution and lipase activity in soil during the bioremediation period. In the present study all the soil enzyme activities increased after the initial stage of decontamination process and showed different pattern during the incubation period because not all enzymes are synthesized by a cell in the same amounts. Some enzymes are present in far greater number than others. Interpretation of the enzyme activities of soil is complex because both intracellular and extracellular enzyme activities contribute to the overall soil enzyme activities [21]. In soil the petroleum hydrocarbon compounds may likely exert different effects on diesel microbiological properties. This can be concluded from the present study that the presence of inorganic nutrients accelerated the lipase production significantly. The result obtained in the present study demonstrated the suitability of several enzyme activities and microbial properties to evaluate the early stage of remediation of freshly hydrocarbon contaminated soil. Total bacterial count observed in bioreactor A ranged from $2.8x10^4$-$16.4x10^5$ CFU/g soil, in bioreactor C $8.4x10^2$-$12.1x10^3$ CFU/g soil, bioreactor D $7.6x10^4$-$9.4x10^4$ CFU/g soil, in bioreactor E $4.8x10^8$-$12.3x10^5$ CFU/g soil (Table 2). The changes in bacterial population followed the pattern similar to soil enzyme activities during the experimental period and signified the role of these microbiological properties in the bioremediation of diesel contaminated soils.

Table 2. Microbiological and enzymatic analysis of soil contaminated with diesel oil (10%) during bioremediation period of 30 days

Incubation Days	Bioreactor	Dehydrogenase activity (µgTPF $g^{-1}h^{-1}$)	FDA hydrolase activity (µgfluorescein $g^{-1}min^{-1}$)	Lipase activity (Lipase unit)	Catalase activity (µmol O_2 $^{-1}min^{-1}$)	Total bacterial count (CFU g^{-1} soil)
0 day	A	2.4	3.31	0.489	1.63	$2.8x\ 10^4$
	B	ND	ND	ND	ND	ND
	C	1.03	3.96	1.62	1.15	$8.4x\ 10^2$
	D	1.53	5.9	1.95	1.33	$7.6x\ 10^4$
	E	1.9	6.1	2.43	1.47	$4.8x\ 10^8$
5th day	A	3.3	3.63	0.73	1.67	$16.1x\ 10^4$
	B	ND	ND	ND	ND	ND
	C	1.98	3.84	1.37	1.29	$12.4x\ 10^2$
	D	2.31	5.5	2.5	1.61	$6.8x\ 10^4$
	E	4.61	6.4	2.93	1.91	$4.2x\ 10^7$
10th day	A	3.1	2.5	1.28	1.87	$6.9x\ 10^4$
	B	ND	ND	ND	ND	ND
	C	1.5	3.8	1.65	1.35	$11.1x\ 10^2$
	D	2.1	5.5	2.84	1.72	$3.9x\ 10^5$
	E	4.33	7.8	3.51	2.12	$16.3x\ 10^7$
15th day	A	2.1	3.7	1.71	1.79	$4.8x\ 10^4$
	B	ND	ND	ND	ND	ND
	C	1.7	3.85	2.32	1.32	$2.9x\ 10^2$
	D	2.5	5.1	3.27	1.8	$4.7x\ 10^4$
	E	7.4	8.1	5.93	2.92	$1.9x\ 10^8$
20th day	A	1.4	3.5	1.68	1.72	$8.4x\ 10^3$
	B	ND	ND	ND	ND	ND
	C	1.6	3.8	2.63	1.57	$6.4x\ 10^3$
	D	1.8	5.5	4.71	1.7	$2.9\ x\ 10^5$
	E	7.1	8.5	7.06	3.83	$2.1\ x\ 10^8$
25th day	A	1.2	3.4	1.68	1.83	$46.1x\ 10^5$
	B	ND	ND	ND	ND	ND
	C	1.4	3.8	1.525	2.72	$21.9x\ 10^3$
	D	1.7	6.5	5.32	5.32	$3.8\ x\ 10^5$
	E	3.2	8.6	7.47	7.67	$14.1\ x\ 10^6$
30th Day	A	1.3	3.5	1.82	1.98	$16.4\ x\ 10^5$
	B	ND	ND	ND	ND	ND
	C	1.5	4.1	1.55	2.63	$12.1x\ 10^3$
	D	1.8	7.1	5.27	5.69	$9.4\ x\ 10^4$
	E	3.5	8.9	8.37	8.96	$12.3x\ 10^5$

Bioreactor A: Uncontaminated soil; B: Sterilized soil; C: Diesel contaminated soil without nutrient; D: Contaminated soil with nutrients; E: Contaminated soil with nutrients and bacterial inoculum; ND: not detected.

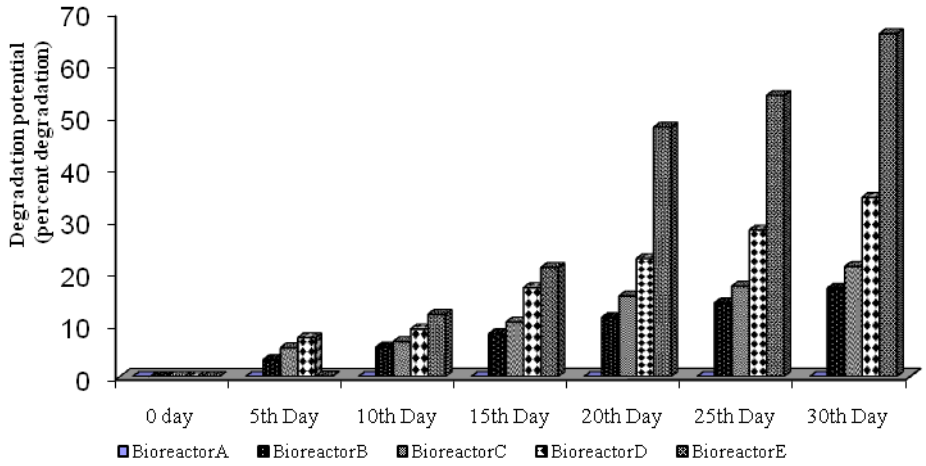


Figure 1. Percent degradation potential of diesel during 30 days of bioremediation process

3.3. Biodegradation of Diesel Hydrocarbon

The degradation of diesel hydrocarbon during experiment was investigated after every 5 days interval. Bioreactor B (abiotic control) showed 17.01% loss of diesel after 30 days of incubation. This petite loss of hydrocarbon during incubation period was observed probably due to their volatilization. Namkoong et al. [51] also observed a very small volatilization of total petroleum hydrocarbon (less then 3%) compared with biodegradation. Bioreactor C, D and E showed 21.71%, 34.5% and 66% degradation of diesel during experimental period (Figure 1). During the whole incubation period the sterilized soil showed a considerable decrease of contaminant level than that of biologically active soil. Highest degradation (66%) was observed in bioreactor E inoculated with *P. aeruginosa* after 30 days of incubation period showing significant bioremediation potential of *P. aeruginosa* within a period of 30 days.

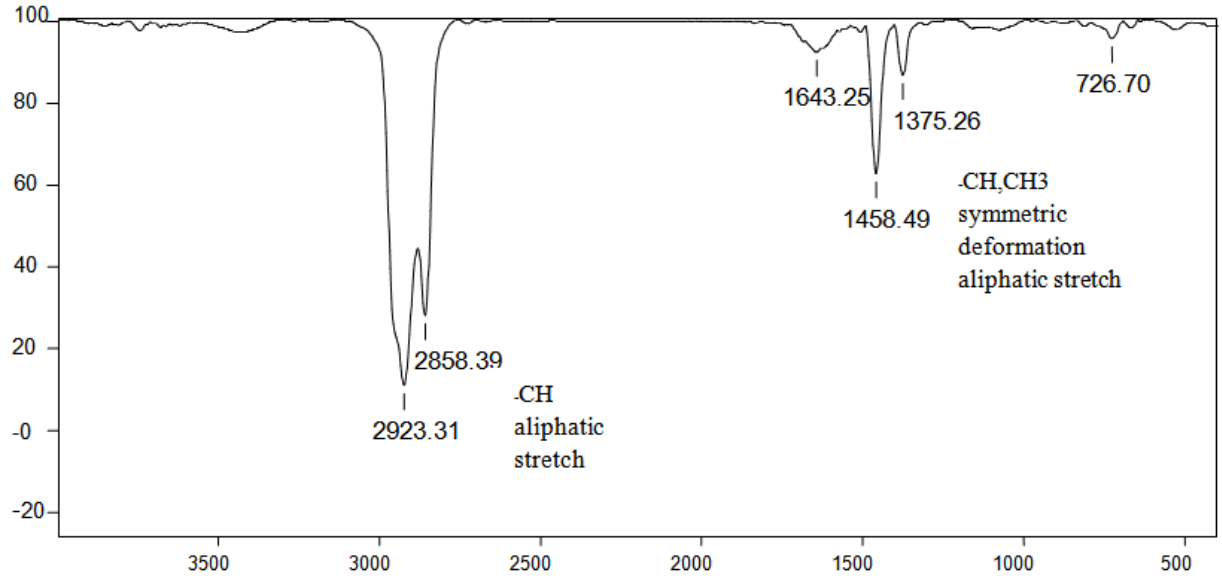
This study was found to be significant when compared to previous investigations. Karamalidis et al. [10] performed a laboratory scale bioremediation of petroleum contaminated soil by indigenous microorganism and added *P. aeruginosa* spet strain. They reported that total concentration of n alkane was reduced by 94% and total polycyclic aromatic hydrocarbon compounds by 79% after 191 days. A positive effect of microorganisms exposed to soil contaminated with petroleum derivatives has been described recently by Cameotra and Makkar [52,53]. Bioremediation of diesel-contaminated soil using laboratory scale bioreactors was investigated for 120 days which showed high total petroleum hydrogen (TPH) degradation (96%) and its removal at the end of bioremediation process [54]. Venketesh and Vedaraman [55] used waste frying rice bran oil to synthesize rhamnolipids- biosurfactants from *Pseudomonas aeruginosa* MTCC 2297.

A bioremediation study was performed in total petroleum hydrocarbon contaminated soil microorganism using bioaugmentation of *P. aeruginosa* N and NM isolated from petroleum contaminated soil sample of north east India (ONGC) oil field by Das and Mukherjee [56] and showed reduction of TPH level from 84-21 g/kg soil after treatment of 120 days. Although numerous diesel degrading bacteria have been isolated, however more strains with efficient or high diesel tolerance capacity need to be employed to cope with bioremediation or petroleum contaminated sites.

This study explored a new strain having a significant rate of diesel degradation capability showing 66% of degradation of diesel hydrocarbons in 30 days of bioremediation period. A high rate of degradation of diesel hydrocarbons in soil slurry system was reported by Hong et al. [57]. They isolated *Pseudomonas aeruginosa* IU5 from oil contaminated soil of gas station at Korea. This strain was capable of degrading 60% of applied diesel (8500 mg/kg) over 13 days therefore it is suggested that by using an engineered bioreactor in which optimum physical and nutritional conditions are provided, the rate of diesel degradation could be enhanced.

The FTIR spectrum was recorded for control soil (soil + diesel), uninoculated soil and treated soil on 0 day, 15th day and 30th day of bioremediation period (Figure 2). The FTIR spectrum of uninoculated/ untreated soil revealed bands at 2923.25 cm^{-1}, 2858.77 cm^{-1} indicating a CH stretch in aromatic and aliphatic compounds, and 1458.41, 1375 cm^{-1} peak showed –CH deformation and –CH_3 symmetrical deformation. The similar pattern of FTIR spectra of diesel extracted on 0 day was observed on the 15th day of incubation some short peaks at 666-1169.70 represents presence of substituted benzene, while a broad peak at 333.830 cm^{-1} showed presence of alcoholic OH group. Peak in the range of 1381.96-1689.06 cm^{-1} showed presence of C=O stretching and peaks in the range of 1400 cm^{-1}-1000 cm^{-1} showed C=O stretching for primary alcohol. Presence of some peaks in the range of 550-850 cm^{-1} showed presence of alkylhalide (CCI) group (Fig. 2d). The FTIR spectra obtained on 30th day revealed the presence of 4 new peaks in the range of 3418.64-3853.94 cm^{-1} was observed showing the presence of alcoholic and amine group and many small peaks in the range of 666.54-1121.12 cm^{-1} showed presence of ortho, meta and para di-substituted benzene derivative compounds (Figure 2e). FTIR analysis of diesel degradation by Patil et al. [58], showed characteristic bands at 3421.83, 3410.26, 2931.61-2673.43, 2281.87, 1753.35, 1606.76, 1456.30, 1373.36, 1292.32, 1184.33, 875.71, 740.84 indicating presence of phenol or amines alkanes or carboxylic acids or esters or aromatic rings. Extracted oil was analyzed by FTIR showed fingerprint pattern at 3422.98, 2922.25, 2828.60, 2727.44, 2675.36, 1042.68, 1610.68 and 1375.29. Absence of characteristic peak in the region of 1610 to

2042 in extracted sample indicates absence of ester linkage. In the present study the presence of alcoholic and ketonic group indicated the breakdown of aliphatic and aromatic alkanes during bioremediation process.



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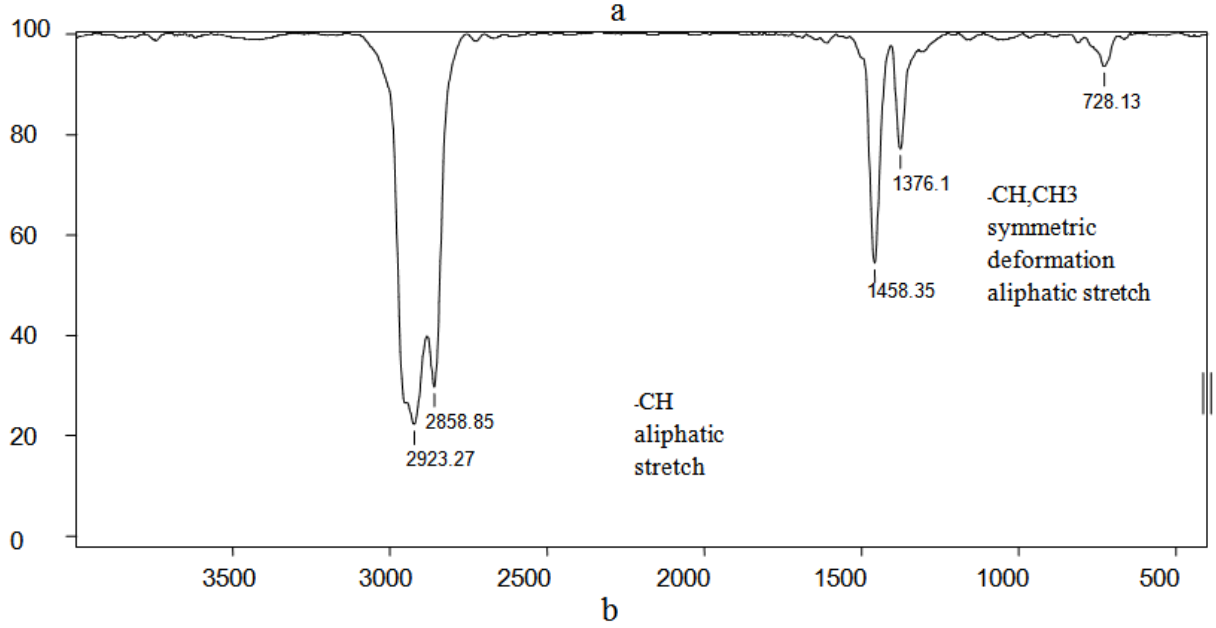

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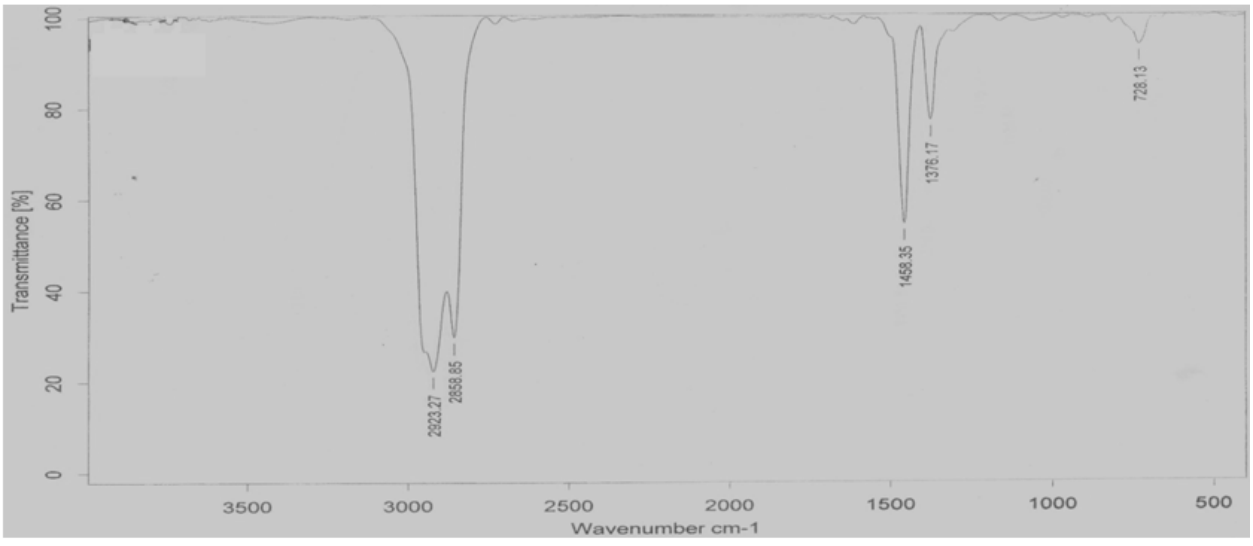

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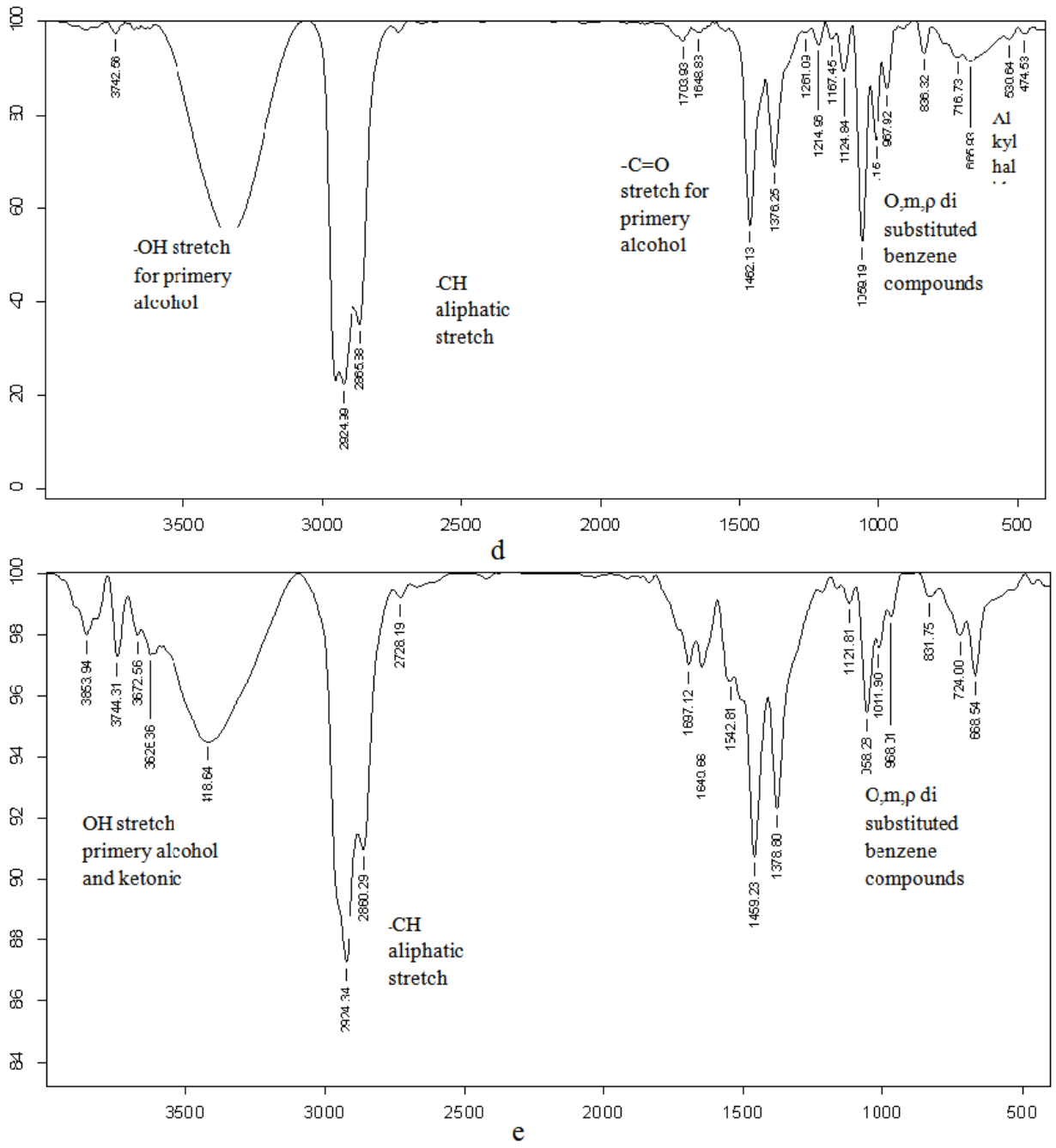
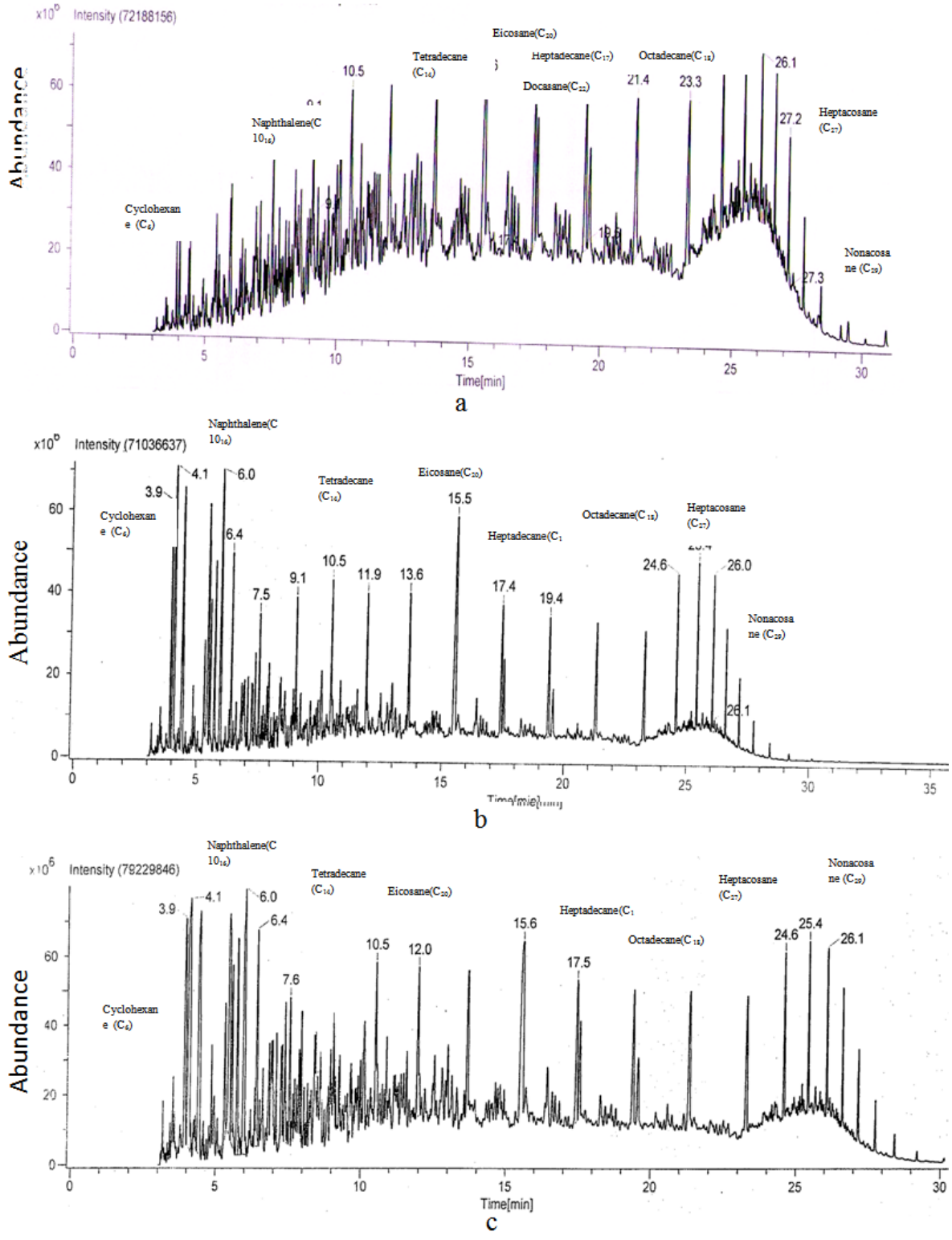


Figure 2. FTIR spectrum of (a) diesel oil (b) control: soil with diesel oil, (c) diesel oil extracted from soil bioaugmented with *Pseudomonas aeruginosa* at 0 day incubation at 10% diesel oil concentration (d) diesel oil extracted from soil bioaugmented with *Pseudomonas aeruginosa* (BGCC#2280) after 15th day incubation and (e) diesel oil extracted from soil bioaugmented with *Pseudomonas aeruginosa* (BGCC#2280) after 30th day incubation at 10% diesel oil concentration

The GC/MS analysis of diesel oil detected cyclohexane, benzene, ethyl benzene, decane, undecane, naphthalene ($C_{11}H_{10}$), dodecane, 1,7 isoprenoyl, 6 methoxybicyclo 2 ethanol ($C_{15}H_{24}0_2$), tetradecane ($C_{14}H30$), pentadecane ($C_{15}H_{32}$), hexadecane ($C_{27}H_{36}$) and nonacosane ($C_{29}H_{60}$) (Fig. 3a). While on 15th day of experimental period it was found that the long chain alkanes were degraded to some extent, while some of these compounds were completely degraded after 30 days i.e. benzene ($C_{10}H_{14}$), undecane ($C_{11}H_{24}$), naphthalene ($C_{11}H_{10}$), pentadecane ($C_{15}H_{32}$), hexadecane ($C_{16}H_{34}$), heptacosane ($C_{27}H_{56}$), heptadecane ($C_{17}H_{36}$), octadecane ($C_{18}H_{38}$), and docasane ($C_{22}H_{46}$), which were present on 0 day analysis (Fig. 3d, e). The gas chromatography analysis also confirmed the degradation of long chain alkane hydrocarbons by bioaugmentation of *P. aeruginosa* (BGCC#2280). The gas chromatogram obtained at different time interval clearly indicates the decrease of diesel hydrocarbons. Several bacterial strains can grow on C_2-C_4 gaseous alkanes, but not on methane [59]. Several bacterial strains can assimilate alkanes larger than C_{20}. These, strains usually contain several alkane hydroxylases those active on C_{10}-C_{20} alkanes are usually related to *P. putida* GP01 AlKB to *acinetobacter* sp. CB 104 cytochrome p450. However, the enzyme that oxidize alkanes larger than C_{20} seem to be totally different. For example *Acinetobacter sp*. M1 which can grow on C_{13}-C_{44} alkanes, contains a soluble, Cu^{2+} dependent alkane hydroxylase that is active on C_{10}-C_{30} alkanes has been isolated [60]. The results obtained revealed that different components of the hydrocarbons had different degrees of

degradability; for example, the aliphatic hydrocarbons and the aromatics may have been readily degraded but the resins and asphaltenes are inherently recalcitrant [61]. Some previous reports stated that *P. aeruginosa* can use a large variety of carbon sources [62]. Not all strains can grow on such broad range of hydrocarbons sps. *P. aeruginosa* RRI isolated from the oil contaminated soil could not utilize benzene, toluene and PAH's (Naphthalenes, Phenanthrene and Pyrene) but can utilize n 'alkanes as sole source of carbon as this strain contain gene encoding two alkanes hydroxylases (*alkBIand alkB2*) two rubredoxins (*alkG1 and alkG2*), and a rubredoxin reductase (*alk T*). *Pseudomonas aeruginosa* CM 323 obtained from hydrocarbon polluted soil could not grow on benzene and xylene but on toluene and ethlybenzene [63]. HPLC analysis showed presence of 36 peaks in control diesel sample while 35 peaks were observed in extracted sample. Many components in sample showed shift in retention time and peak area indicating changes in molecular weight and quantity of respective components present in control [58]. Studies of FTIR analysis of extracted oil by Patil et al.[58] showed absence of ester compound to pure diesel, GCMS analysis confirmed presence of naphthalene and its derivatives as hydrocarbon pollutant [12].



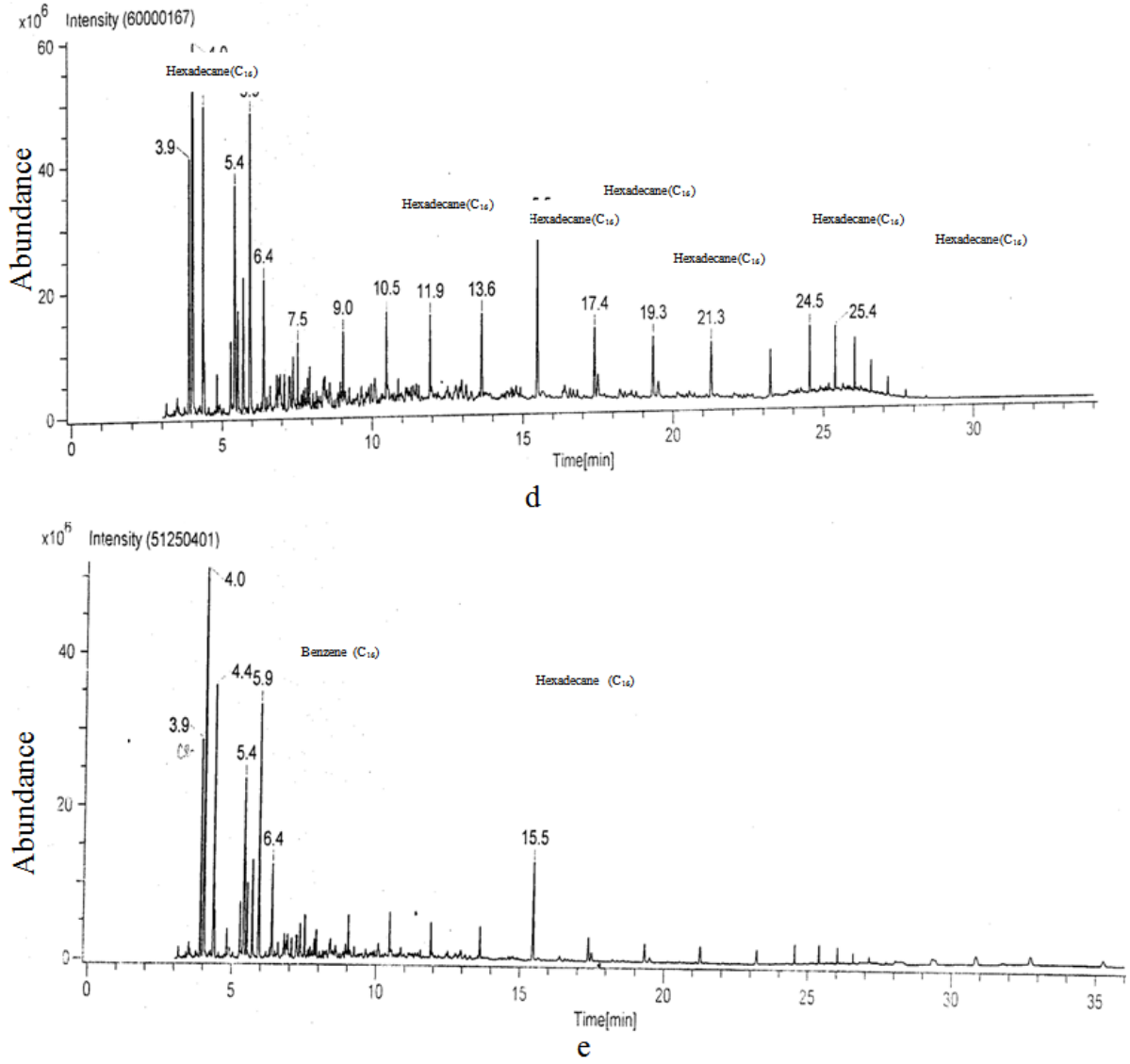


Figure 3. Gas chromatogram of (a) diesel oil, (b) soil with diesel oil (c) diesel oil extracted from soil bioaugmented with *Pseudomonas aeruginosa* (BGCC#2280) at 0 day incubation (d) diesel oil extracted from soil bioaugmented with *Pseudomonas aeruginosa* (BGCC#2280) on 15th day of incubation and (e) diesel oil extracted from soil bioaugmented with *Pseudomonas aeruginosa* (BGCC#2280) on 30th day of incubation at 10% diesel oil concentration

Nisha et al. [64] studied five bacterial strains including *Pseudomonas* which could be useful in bioremediation of sites which is highly contaminated with diesel oil. Streptomyces bacteria degraded long chains of carbon was proved by FTIR and gas chromatography studies performed by Ramadha et al. [11]. Therefore in the present study the ability to utilize a broad range of hydrocarbon (C_{10} -C_{23}) by this strain can differentiate this strain as a potent diesel degrader even within the same species. In the present study the bioaugmentation of *P. aeruginosa* resulted in complete removal of hydrocarbon compounds ranged from C_{10}- C_{29} this could be due to the presence of soluble Cu^{2+} dependent alkane hydroxylase enzymes system in this isolate. Further investigations are needed to reveal the mechanism employed during biodegradation of hydrocarbon compounds in the present study. It is meaningful to find new bacterial strains that can metabolize a broad range of the hydrocarbons contained in the diesel oil that are highly persistent. Lee et al. [12] also found indigenous *Rhodococcus* sp EH831 effective for remediation of diesel contaminated soil.

4. Conclusion

Environmental contamination by petroleum and its derivatives is a serious problem worldwide. Remediation of the contaminated areas with the use of microorganism offers a cost effective solution for restoring the ecosystem. In the present work, a laboratory scale bioremediation study was conducted to test the efficiency of *P. aeruginosa* as a potent candidate for the remediation of diesel contaminated soil. The study has successfully determined the baseline rate of hydrocarbon degradation (66%) within a bioreactor. It was also observed that addition of nutrients was mandatory for enhancing bacterial growth and degradation potential. Lipase activity was found to be the the best parameters for testing hydrocarbon degradation. FTIR and GC/MS analysis validated the biodegradation of diesel hydrocarbons by *P. aeruginosa*. Based on these observations, it was concluded that the degradation of diesel hydrocarbon was significantly achieved by indigenous strain of *P.*

aeruginosa and this study contributed a potent strain that can be further exploited in *in situ* treatment process.

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