ISSN 2349-7203

IJPPR
INTERNATIONAL JOURNAL OF PHARMACY & PHARMACEUTICAL RESEARCH
An official Publication of Human Journals

HUMAN

Human Journals
Research Article
May 2016 Vol.:6, Issue:2

Diesel Degradation by Microbes and Its Control

IJPPR
INTERNATIONAL JOURNAL OF PHARMACY & PHARMACEUTICAL RESEARCH
An official Publication of Human Journals
ISSN 2349-7203
HUMAN

Karuppasamy Murugan* and M.A. Mohideen

PG Research Scholars, PG Department of Microbiology, Sri Paramakalyani College, Alwarkurichi – 627 412, Tirunelveli – District, Tamilnadu, India.

Submission: 9 May 2016
Accepted: 14 May 2016
Published: 25 May 2016

www.ijppr.humanjournals.com

Keywords: Muck sample, Bushnell-Haas agar medium, Diesel degrading bacteria, Total viable count, Cetyl trimethyl ammonium bromide (CTAB), FTIR analysis and NMR analysis

ABSTRACT

The present study reveals that the effect of microbial contamination of diesel was examined through controlled experiments in a synthetic medium. For this, Bushnell-Haas broth was employed with diesel supplementation. Both in diesel supplemented and unsupplemented, bacterial cultures were inoculated and after 15 days, the population changes was examined in all the five test bacteria mainly *Moraxella* sp., *Thiobacillus* sp., *Sulfobacillus* sp., *Gallionella* sp. and *Brucella* sp. were found to utilize the hydrocarbon, diesel on the basis of an increase in their population. Simultaneously culture filtrate was subjected to FTIR and NMR analysis to understand the nature of diesel degradation in the Bushnell-Haas broth. In order to check the application of biocide such as CTAB, experiments conducted with CTAB at two different concentrations. Even though CTAB exhibited degradation on bacteria after 7 days, slow recovery of bacterial cells was recorded. NMR analysis indicates the effective degradation of diesel with the test bacterium. The probable mechanism of diesel degradation by these organisms could be oxidation as revealed by the respective peaks in the NMR spectrum. On the basis of this analysis, it could be concluded that *Gallionella* sp. was the most efficient organism in diesel degradation followed by *Moraxella* sp., *Thiobacillus* sp. and *Sulfobacillus* sp. similar pattern was recorded in FTIR analysis also.

INTRODUCTION

In 1946, Zobell[1] reviewed the action of microorganisms on hydrocarbons. He recognized that many microorganisms have the ability to utilize hydrocarbons as a sole source of energy and that such microorganism are widely distributed in nature. He further recognized that microbial utilization of hydrocarbons was highly dependent on the chemical nature of the compounds within the petroleum mixtures and on the environmental determinants.

Biodegradation of petroleum in a natural ecosystem is complex. The evolution of the hydrocarbon mixture depends on the nature of the oil, the nature of the microbial community and a variety of environmental factors, which influence microbial activities.

Attention has been focused on marine environments as they are the ultimate receptors of hydrocarbon pollutants (Gibson, 1968; Zobell, 1973; Vander Linden, 1978; Atlas, 1981)[2-5].

The ability to degrade petroleum hydrocarbons is not restricted to a few microbial genera. A diverse group of bacteria and fungi have been shown to have this ability. Zobell (1946)[1] in his review noted that more than 100 species representing 30 microbial genera had been shown to be capable of utilizing hydrocarbons. In a review, Bartha and Atlas (1977)[6] too listed 22 genera of bacteria, 1 algal genus and 14 genera of fungi, which had been demonstrated to contain members, which utilize petroleum hydrocarbons. All of these microorganisms had been isolated from aquatic environments. The most important (based on frequency of isolation) genera of hydrocarbon utilizers in aquatic environments were *Pseudomonas* sp., *Achromobacter* sp., *Micrococcus* sp., *Nocardia* sp., *Candida* sp., *Vibrio* sp., *Acinetobacter* sp., *Brevibacterium* ap., *Rhodotorula* sp., *Sporobolomyces* sp., *Corynebacterium* sp. and *Flavobacterium* sp. (Bartha and Atlas, 1977)[6].

Walker *et al*. (1975)[7] compared the abilities of bacteria and fungi to degrade hydrocarbons. The following genera were included in their study *Candida* sp., *Sporobolomyces* sp., *Hansenula* sp., *Rhodotorula* sp., *Cladosporium* sp., *Penicillium* sp., *Aspergillus* sp., *Pseudomonas* sp., *Vibrio* sp., *Leucothrix* sp., *Nocardia* sp. and *Rhodium* sp. Walker *et al*. (1976)[8] isolated *Vibrio* sp., *Pseudomonas* sp. and *Acinetobacter* sp. from oil contaminated sediments and *Pseudomonas* sp. and *Coryneform* sp. from oil free sediment. A large number of *Pseudomonas* sp. have been isolated that are capable of utilizing petroleum hydrocarbons. The genetics and enzymology of

hydrocarbon degradation by *Pseudomonas* sp. has been extensively studied (Chakrabarty, 1972; Chakrabarty *et al*., 1973; Dunn and Gunsalus, 1973)[9-11]. The genetic information for hydrocarbon degradation in these organisms generally has been found to occur on plasmids. Numerical taxonomy has also been used to examine petroleum degrading bacteria (Austin *et al*., 1977)[12]. Storage of fuel in refineries and by distributors allows microbial activities to cause the production of biomass at an oil / water interface.

Lepetit and Barthelemy (1968)[13] reported that the concentration of available nitrogen and phosphorus in seawater are severe limiting factors for microbial hydrocarbon degradation. Another investigator (Kinney, 1968)[14], however, are of the opinion that nitrogen and phosphorus are not the limiting in seawater since microorganisms require nitrogen and phosphorus for its incorporation into biomass and that the availability of these nutrients within the same area as that of hydrocarbon is critical. Colwell *et al*. (1978)[15] concluded that metula oil is degraded slowly in the marine environment most probably because of limitations imposed by the relatively low concentrations of nitrogen and phosphorus available in seawater.

As with nutrients, there has been controversy over whether oxygen is absolutely required for hydrocarbon degradation or whether hydrocarbons are subject to anaerobic degradation. The current evidence supports the view that anaerobic degradation by microorganisms at best proceeds at negligible rates in nature. The existence of microorganisms which are capable of anaerobic hydrocarbon metabolism has not, however, been excluded (Atlas, 1981)[5]. In the case of *Pseudomonas* strain studied by Senez and Azoulay (1961)[16], the organisms consumed oxygen when grown on heptane even though it had an n-heptane dehydrogenase enzyme. The importance of oxygen for hydrocarbon degradation is well indicated by the fact that the major degradative pathways for both saturated and aromatic hydrocarbons involve oxygenases and molecular oxygen.

Degradation of hydrocarbons has been reviewed (Atlas, 1981)[5] and n-alkanes are generally considered the most readily degraded bacteria (SRB) can use a wide range of fatty acids including acetate, lactate, propionate, butyrate and fatty acids up to C_{18} and some phenyl substituted acids (Widdel, 1980)[17]. The generally accepted view is that anaerobic degradation of hydrocarbons is a very slow process as compared to aerobic degradation.

Schwarz *et al*. (1975)[18] examined the growth and utilization of hydrocarbons at ambient and *insitu* pressure for deep-sea bacteria. The rate of hydrocarbon utilization under high pressure and ambient temperatures were found to be significantly low than the rates found under conditions of ambient temperatures and atmospheric pressure, whereas 94% hexadecane was utilized within 8 weeks at 1 bar while at 500 bars it took 40 weeks for similar degradation. It appears that oil, which enters deep-ocean environments, will be degraded very slowly and persist for long periods of time. Water is essential for biodegradation but less than 0.1% is enough for microbial activity (Degray and Killian, 1962)[19]. Most hydrocarbon degrading microorganisms prefer a near neutral pH while some organisms can tolerate extreme values.

For a fuel to be of marketable quality, it must be clear and bright (C+B). Microbial growth can often cause severe turbidity and cloudiness. This arises in one of two ways. Any mixing of interfacial growth can often produce particles of such a density that they remain in components. The hydrocarbon range of gasoline, C_5-C_9 is less likely to be degraded, but kerosene with a hydrocarbon range of C_{10}-C_{18} is more susceptible (Hill, 1984)[20]. The preferential removal of C_{15}-C_{35} n-alkanes relative to branched and cyclic alkanes is recognized as a standard feature in biodegradation of crude oils (Cannan, 1984 and 1987)[21-22]. Degradation normally proceeds by a mono terminal attack wherein usually a primary alcohol is formed followed by aldehyde and a mono carboxylic acid. Further degradation proceeds via β-oxidation but extensive methyl branching interferes with this process. Microorganisms convert aromatic hydrocarbons, by an initial dioxygenase attack, to dihydrodiols that are further oxidized to dihydroxy products. Condensed polycyclic aromatic are degraded, one ring at a time, by a similar mechanism but biodegradability declines with a number of the rings and the degree of condensation (Higgins and Gilbert, 1978)[23].

SCOPE AND AIM OF THE STUDY

Most hydrocarbons are stored above ground in steel or lined concrete tanks. However, this type of storage is seldom used in the long term basis. In the long term in fuel contamination, filter plugging and corrosion of tanks and souring of stored products. In order to prevent the effects of microbial growth, several lines of approach have been used, namely good housekeeping practices, treatment with biocides to limit growth and the use of special tank linings etc. Recently Jana (1999)[24] noticed a failure in an oil pipeline at Mumbai offshore. They suggested that the

combined effect of CO_2, SRB, and chloride in the low velocity area caused the severe corrosion and the failure of the pipeline. Besides, they commented that premature failure of machinery, equipment structural breakdown and explosion due to leakage of inflammable gases or liquids from corroded pipelines, contamination of products / water etc. lead to losses of around Rs. 8000 crores / annum in India, which should be protected to avoid the economic loss.

Microbial contamination of fuels has been the cause of intermittent operational problems throughout the world for many years and more recently the frequency and severity of the cases appear to have been increasing dramatically. This increase may be due to several factors, or a combination thereof such as: changes in fuel souring and quality; increasing use of additives which may provide the much required limiting micro-nutrients for biological growth; alterations in housekeeping practices and storage techniques / requirements and lastly, an increased awareness of the possibilities of microbiological growth or education to petroleum engineers, chemists and operators.

Hence, knowledge of microbial problems occurs in this type of storage and pipeline and it is important to develop methods or propose designs so as to minimize oil degradation / corrosion problems.

The petroleum industries are universally facing a tough time due to degradation and corrosion in the pipeline due to sludge petroleum product accumulation. Genetic diversity and molecular evolution among microbial consortia makes them more potent enough to transform or utilize the hydrocarbons before starting corrosion. The above mentioned diesel impacts and its degradation laid the foundation for this research on similar fields and the present study had the following pattern of work as objectives:

- ❖ Isolation and identification of microbes from the oil pipeline.
- ❖ Studies on the ability of the organisms to degrade the hydrocarbons based on
- ➢ Effect of incubation for 15 days.
- ➢ Regarding the control aspect, quaternary ammonium compound was selected to study the biocidal effect.
- ➢ FTIR analysis of the degraded diesel for fraction studies.
- ➢ NMR analysis of the degraded diesel for fraction studies.

MATERIALS AND METHODS

Sampling area

Muck sample was collected using sterilized conical flasks at various sites of the oil pipelines, northeast of India.

Strains employed for diesel degradation

From a total number of 45 bacterial strains (isolated and identified, previously by CECRI scientists as oil degraders), 5 identified genera which ways considered as highly efficient (from the degradation studies through turbid metric analysis) were chosen for the current diesel degradation studies and they were *Moraxella* sp., *Thiobacillus* sp., *Sulfobacillus* sp., *Gallionella* sp. and *Brucella* sp.

Media employed for diesel degradation

The medium used for detecting the oil degrading process by bacteria was Bushnell-Haas broth medium composed of: magnesium sulphate – 0.20g, calcium chloride – 0.02g, mono potassium phosphate – 1.0g, dipotassium phosphate – 1.0g, ammonium nitrate – 1.0g, ferric chloride – 0.05 g, deionised water – 1000ml, final pH – 7.0 ± 0.2 (Hi-Media, Mumbai) and Bushnell-Haas agar medium composed of: magnesium sulphate – 0.20g, calcium chloride – 0.02g, mono potassium phosphate – 1.0g, dipotassium phosphate – 1.0g, ammonium nitrate – 1.0g, ferric chloride – 0.05 g, agar agar – 20.0g, deionised water – 1000ml, final pH – 7.0 ± 0.2 (Hi-Media, Mumbai).

Experimental work of the degradation process

Two sets of bottles were used for the diesel degradation studies using the selected bacterial strains. To one set of the bottles containing volumes of 5ml of each of the bacterial strains was added to 10ml of diesel and 100ml of media. Total viable count inoculated as the first day was enumerated in each set of bottles. The control was identically maintained but without the inoculums.

To another set of bottles containing 5ml of each bacterial culture was added 100ml of media without adding diesel. The total viable count inoculated on the first day was enumerated in each

set of the bottles. All the bottles were maintained at room temperature for an incubation period of 15 days, after which the hydrocarbon utilizing bacterial population was enumerated.

Confirmative test for diesel degrading bacterial strains

To confirm the diesel degrading effect by the strains, after the fifteenth day of the incubation period, one lapful of each culture from respective bottles were taken and streaked on Bushnell-Haas agar plates and were incubated for about 15 days at 37^0C temperature and the results were observed for growth.

Extraction of diesel

After incubation, the degraded samples were separated for analytical purposes using a separating funnel. Much care was taken to see that the extracted oil was a clear solution without any water content.

Biocidal effect of diesel degradation

Cetyl trimethyl ammonium bromide (CTAB) which is used to control oil degradation was selected as a biocide. One gram of CTAB has been dissolved in 100ml of ethylene glycol monobutyl ether as a stock solution (1000ppm). After addition of 5ppm and 10ppm of the stock solution was used to test their efficiency on the growth of hydrocarbon degrading bacterial population which was detected by pour plate technique. Another set was compared identically without the biocide.

Analysis of the degraded diesel by Fourier transmission infra-red spectroscopy (FTIR)

FTIR was used to detect the functional group of a compound. Perkin Elmer, UK make, Paragon 500 model FTIR was used for the analysis of the oil samples. The spectrum was taken in the mid IR region of 400-4000cm^{-1} with 16-scan speed. The samples were mixed with spectroscopically pure chloroform in the ratio of 1:100 and pellets were fixed in the sample holder and the analyses were carried out.

Analysis of the degraded diesel by nuclear magnetic resonance spectroscopy (NMR)

NMR analysis was used to detect the protons of the nuclei in the compound. This analysis was made under the following conditions:

Model	-	Perkin Elmer spectrometer NMR (90 MHz)
Solvent	-	Carbon tetra chloride
Reference standard	-	Tetra methyl silane
Volume of injected	-	20 µl samples

RESULTS AND DISCUSSION

Microbial contamination of fuel has been the cause of intermittent operational problems throughout the world for many years and more recently, the frequency and severity of the cases appear to have been increasing dramatically. The degradation problem arises where excellent food sources (hydrocarbon fuels) for a wide variety of microorganisms are allowed to remain at stimulating temperatures, in contact with water. Roffey (1989)[25] reported that aerobic and anaerobic degradation of crude oil / refined products of diesel occurred in storage tanks.

Total viable count in the presence and absence of oil with Bushnell-Haas broth medium

Table – 1 and Table – 2 portrays changes of the bacterial population in the presence and absence of diesel from Bushnell-Haas broth medium.

Bacterial load in Bushnell-Haas broth medium supplemented with diesel over a period of 15 days was recorded and presented in Table – 1. From this, it evident that all the 5 bacterial cultures were able to utilize / degrade diesel resulting in a corresponding increase in population. Irrespective of the initial load, all the 5 bacterial inoculants exhibited 10 fold increases in the population density at the end of the experimental period (15th day of incubation).

Fig. 1: Sampling bottles indicating the level of hydrocarbon degradation at the end of fourth day`of incubation

Legend: A. *Moraxella* sp. with 100ml media and 5ml diesel, B. *Thiobacillus* sp. with 100ml media and 5ml diesel, C. *Sulphobacillus* sp. with 100ml media and 5 ml diesel, D. *Gallionella* sp. with 100ml media and 5ml diesel, E. *Brucella* sp. with 100ml media and 5ml diesel, F. Control with 100ml media and 5ml diesel.

Fig. 2: Sampling bottles indicating the level of hydrocarbon degradation at the end of fifteenth day of incubation

Legend: A. *Moraxella* sp. with 100ml media and 5 ml diesel, B. *Thiobacillus* sp. with 100ml media and 5ml diesel, C. *Sulphobacillus* sp. with 100ml media and 5ml diesel, D. *Gallionella* sp. with 100ml media and 5ml diesel, E. *Brucella* sp. with 100ml media and 5ml diesel, F. Control with 100ml media and 5ml diesel.

Davis, 1967; Higgins and Gilbert, 1978; Atlas, 1981[26, 23 and 5] were stated that the types and ability of microorganisms to degrade petroleum hydrocarbons have been widely documented. Water is essential for biodegradation but, less than 0.1% of it is enough for microbial activity. Most degrading microbes prefer a near neutral pH except that some organisms can tolerate extreme values.

Table – 1: Changes in bacterial population in diesel supplemented Bushnell-Haas broth medium

Sr. No.	Bacterial strains	Duration of incubation		Difference in population CFU/ml
		1st day CFU/ml	15th day CFU/ml	
1.	*Moraxella* sp.	6.9×10^7	5.6×10^8	4.91×10^1
2.	*Thiobacillus* sp.	3.10×10^7	3.00×10^8	2.69×10^1
3.	*Sulfobacillus* sp.	3.2×10^6	3.20×10^7	2.88×10^1
4.	*Gallionella* sp.	3.02×10^7	3.22×10^8	2.91×10^1
5.	*Brucella* sp.	3.12×10^6	3.12×10^7	2.80×10^1

On the other hand, examining the data presented in Table – 2 clearly reveals the inability of the bacterium to survive in Bushnell-Haas broth without diesel supplemented as all of them load exhibited remarkable reduction is viable cells that ranged from -2.21×10^1 to -5.31×10^3.

Table – 2: Changes in bacterial population in Bushnell-Haas broth medium

Sr. No.	Bacterial strains	Duration of incubation		Difference in population CFU/ml
		1st day CFU/ml	15th day CFU/ml	
1.	*Moraxella* sp.	6.0×10^6	6.9×10^3	-5.31×10^3
2.	*Thiobacillus* sp.	3.00×10^5	2.86×10^4	-2.71×10^1
3.	*Sulfobacillus* sp.	2.5×10^5	2.9×10^4	-2.21×10^1
4.	*Gallionella* sp.	2.8×10^6	2.72×10^4	-2.52×10^2
5.	*Brucella* sp.	2.78×10^6	2.85×10^3	-2.49×10^3

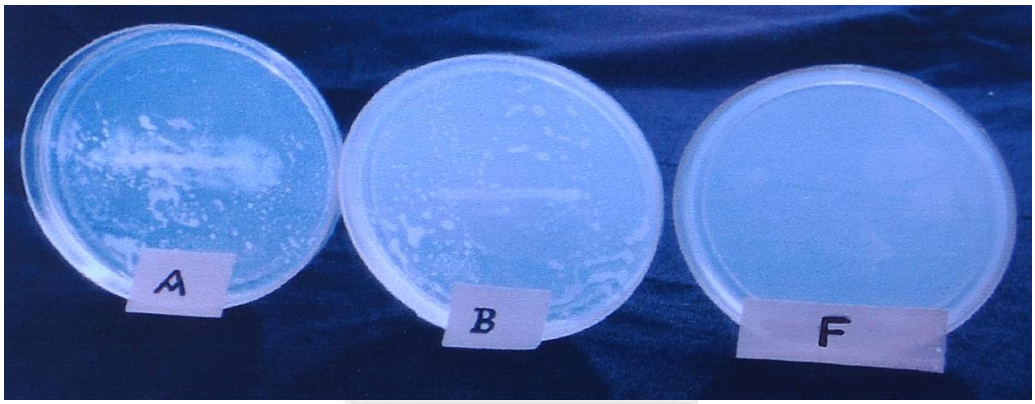

Fig. 3: Portraying growth of diesel degrading colonies at the end of fifteenth day of incubation

Legend: A. *Moraxella* sp., B. *Thiobacillus* sp. and F. Control

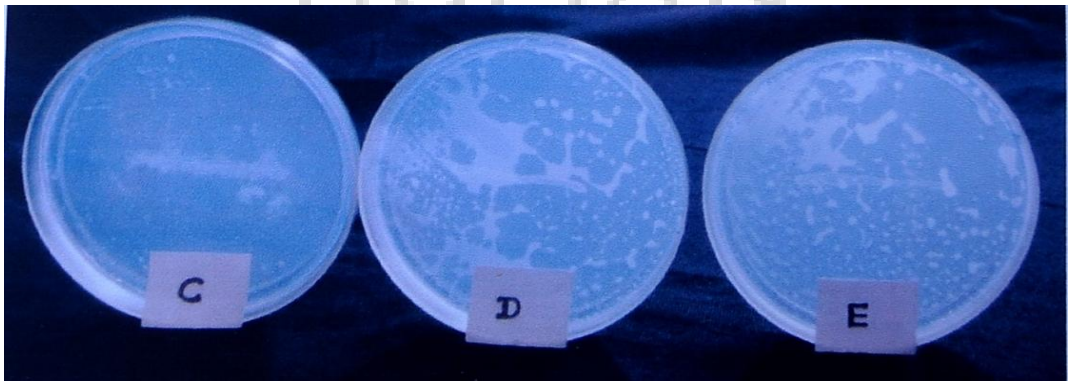

Fig. 4: Portraying growth of diesel degrading colonies at the end of fifteenth day of incubation

Legend: C. *Sulphobacillus* sp., D. *Gallionella* sp. and E. *Brucella* sp.

Biocidal effect of diesel degradation

One gram of cetyl trimethyl ammonium bromide (CTAB) was dissolved in 100ml of monobutyl ethylene glycol (1000ppm). 5ppm and 10ppm were selected for experimental studies. Table – 3 shows that the count of mixed bacterial culture in the presence of CTAB was about 10^1 on the seventh day. But there were no bacteria on the third and fifth day. But in control system bacterial count was in the range between 10^6 and 10^9. It reveals that CTAB act as a good biocide up to seven days. Since it is a cationic biocide, it disturbs the arrangement of negatively charged phospholipids of bacteria and makes the bacteria lyse (Denyer, 1990)[27].

CTAB a biocide has been checked for it biocidal activity against 5 bacterial isolate in the presence of diesel at two concentrations of 5ppm and 10ppm and the results are presented in Table – 3. From this, it evident that all five bacterial isolates were exhibited by CTAB even at 5ppm. But low recovery of bacterial population was recorded at the first day of incubation. This indicates that CTAB is probably a bacteriostatic agent in the population of bacterium that was recorded at the end of the seventh day probably is composed of CTAB resistant population.

Jack and Westlake (1995)[28] reported that oil production facilities can be routinely treated with biocides to control or eliminate microbes held responsible for souring (the formation of H_2S) or microbially influenced corrosion (MIC). Biocides can be divided into two groups: the oxidizing and non-oxidizing biocides. The latter are generally used to control microorganisms in systems with a high content of organics and include aldehydes, amine type, halogenated and sulphur compounds and quaternary phosphoric salts. The amines compounds can be divided into quaternary amine and amine and diamine biocides. The general formula of diamine biocides is R_1-NH-R_2-NH_2 with aliphatic chains of 12 to 16 (R_1) or 3(R_2) carbon atoms reported by Voordouw *et al*., 1993[29].

Table – 3: Biocidal effect of CTAB on diesel degrading bacterial strains

No. of days	Bacterial strains with diesel CFU/ml	Bacterial strains, diesel and CTAB	
		5ppm	10ppm
1st day	10^4	No growth	No growth
3rd day	10^5	No growth	No growth
5th day	10^8	No growth	No growth
7th day	10^9	10^1 CFU/ml	10^1 CFU/ml

FTIR analysis of diesel degradation

In fig. 5, the IR spectroscopy of pure oil showed the characteristics bands at 2954 cm^{-1}, 2923 cm^{-1} and 2854 cm^{-1} (C-H aliphatic stretch); 1604 cm^{-1}, 1557 cm^{-1} (C=C stretch in aromatic nuclei); 781 cm^{-1}, 699 cm^{-1} (meta disubstituted benzene) and 810 cm^{-1} (disubstituted benzene).

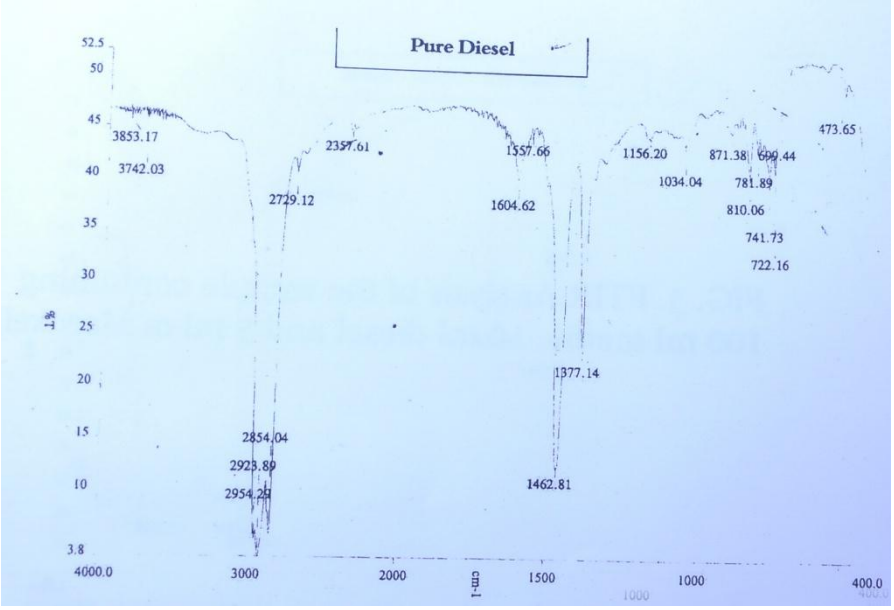

Fig. 5: FTIR analysis of pure diesel

In fig. 6 of the control system, the spectrum showed bands of Bushnell-Haas broth medium with diesel (without bacteria) at 2954cm^{-1}, 2923cm^{-1} and 2854cm^{-1} (C-H aliphatic stretch). 1604cm^{-1}, 1463cm^{-1} (C=C aromatic nuclei), 781cm^{-1}, 699cm^{-1} (meta substituted benzene) and 810cm^{-1} (disubstituted benzene).

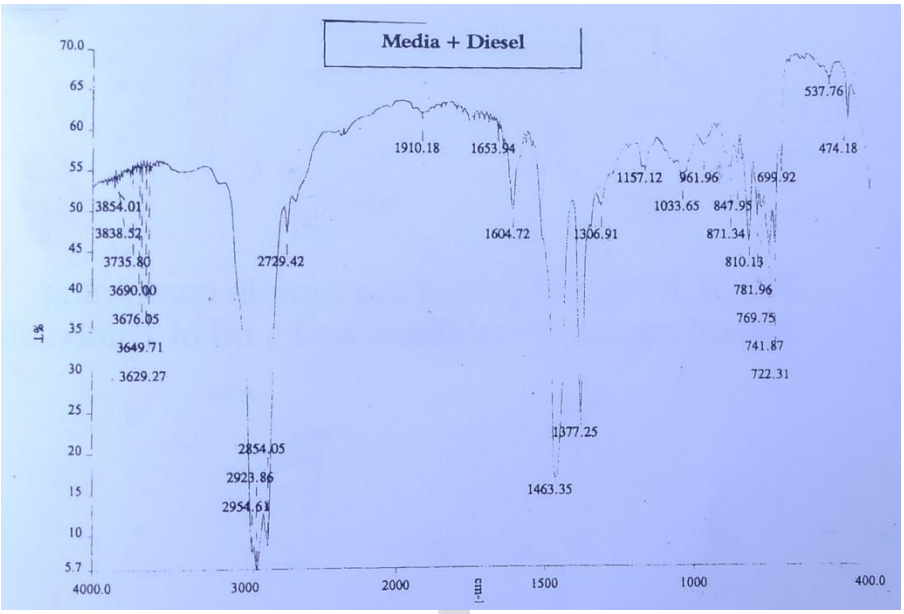

Fig. 6: FTIR analysis of the sample containing 100ml media and 10ml diesel

The spectrum of *Moraxella* sp. with free OH peaks at 3435cm^{-1}, CH aliphatic stretch at 2955cm^{-1}, 2924cm^{-1}and 2854cm^{-1}, C=C conjugated diene at 1633cm^{-1} and CH def. in methyl at 1463cm^{-1} and 1377cm^{-1} were noticed. In the presence of *Thiobacillus* sp. also the OH peak, CH aliphatic stretch, conjugated diene and CH def. in methyl group were noticed (Fig. 7 & 8).

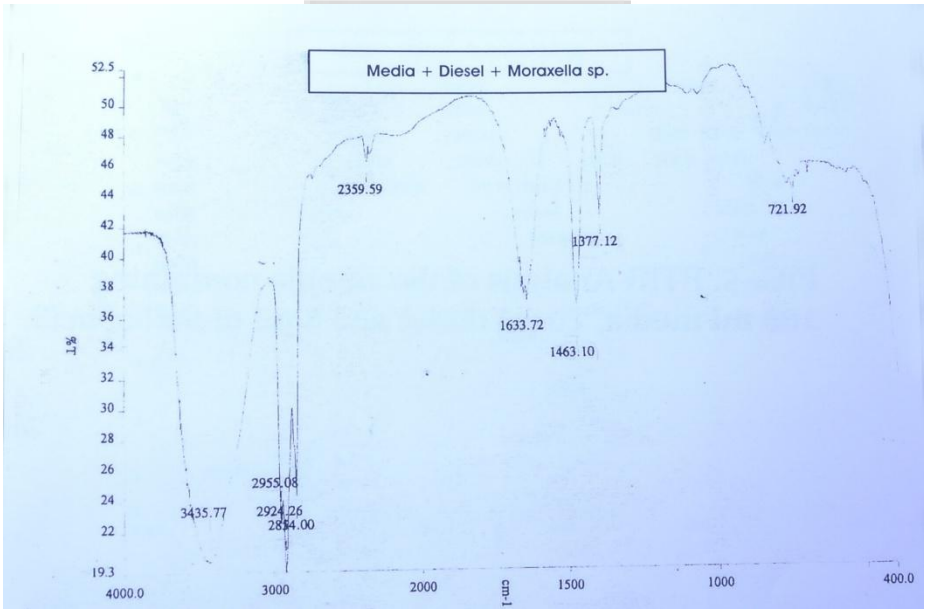

Fig. 7: FTIR analysis of the sample containing 100ml media, 10ml diesel and 5ml of *Moraxella* sp.

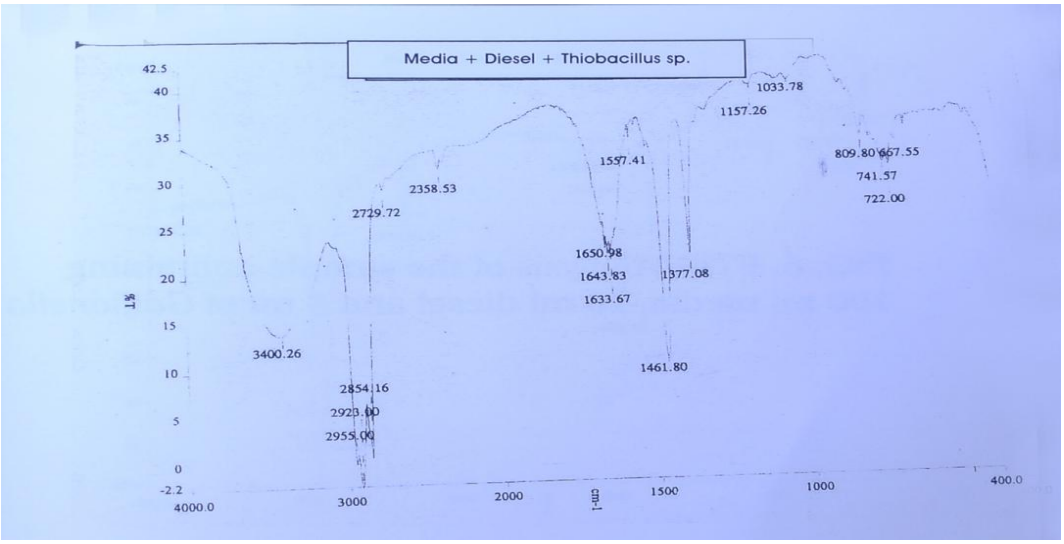

Fig. 8: FTIR analysis of the sample containing 100ml media, 10ml diesel and 5ml of *Thiobacillus* sp.

Spectrum for *Sulphobacillus* sp. showed peaks of OH, CH aliphatic stretch, conjugated diene, CH def. in methyl, meta disubstituted and disubstituted benzene (Fig. 9).

While adding *Gallionella* sp. CH aliphatic stretch, C=C aromatic nuclei, conjugated diene were observed. Besides, it was noticed that the presence of transmittance at 1456cm^{-1} and 1377cm^{-1} were higher when compared to control (Fig. 10).

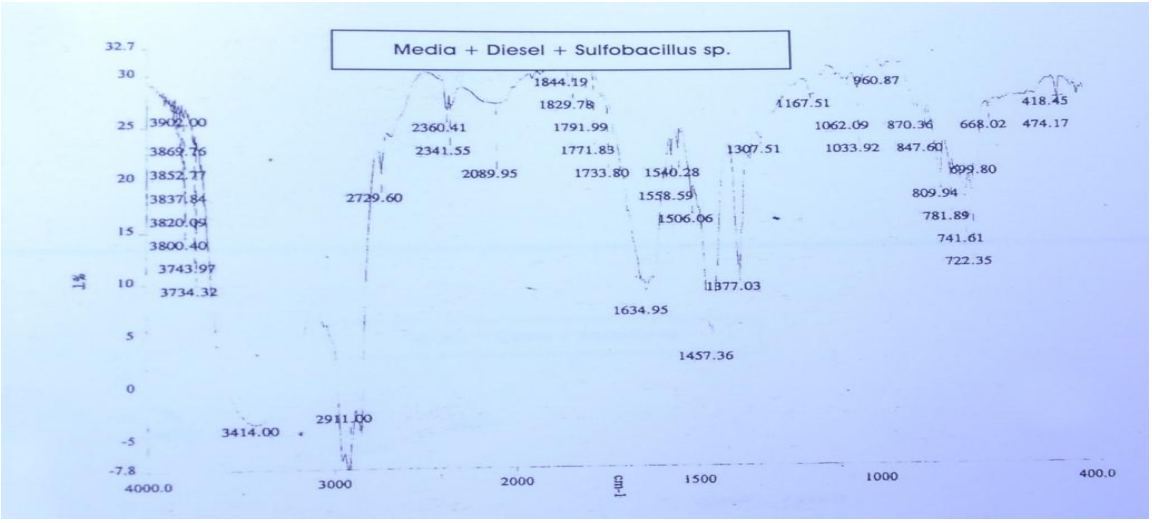

Fig. 9: FTIR analysis of the sample containing 100ml media, 10ml diesel and 5ml of *Sulphobacillus* sp.

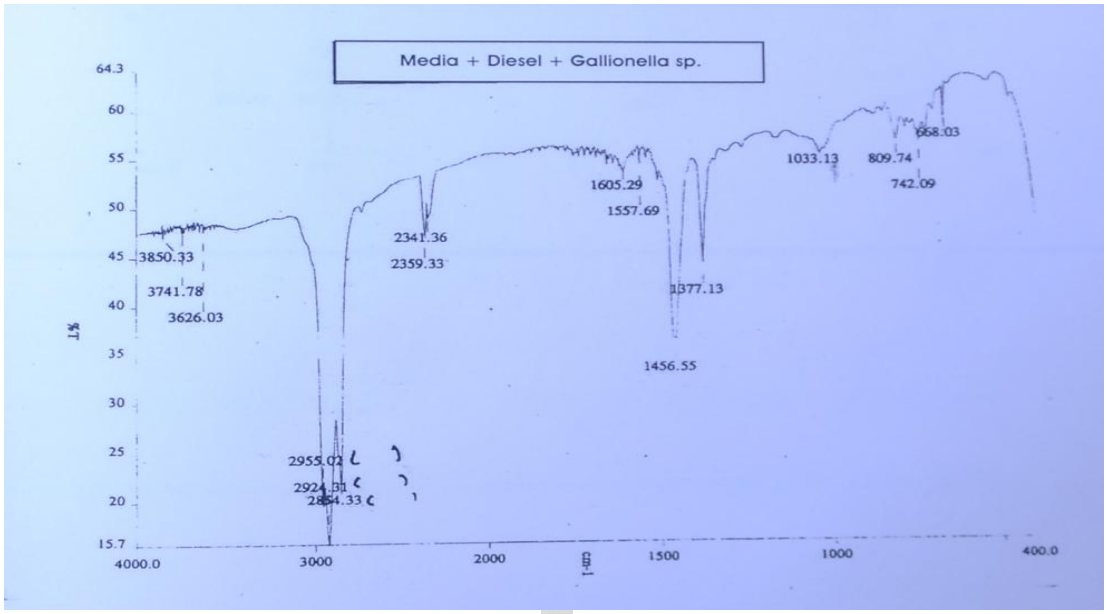

Fig. 10: FTIR analysis of the sample containing 100ml media, 10ml diesel and 5ml of *Gallionella* sp.

In FTIR spectrum of *Brucella* sp. it was seen that the observed peaks were OH group, CH aliphatic stretch, conjugated diene, CH def. in methyl and meta disubstituted benzene (Fig. 11).

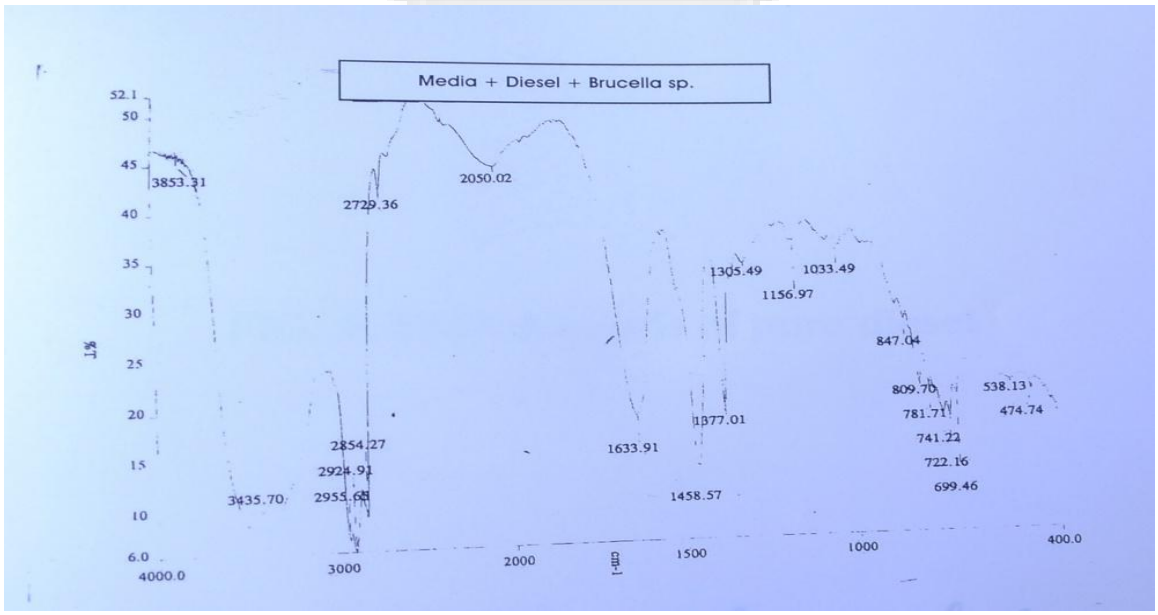

Fig. 11: FTIR analysis of the sample containing 100ml media, 10ml diesel and 5ml of *Brucella* sp.

The spectrum of pure oil indicated the presence of carbon and hydrogen stretching bond and carbon hydrogen def. in methyl. When bacterial strains were added new peaks mostly occurred at 1633cm^{-1} which could be identified as carbon=carbon double bond. From FTIR spectrum, it can be concluded that the bacterial strains played a major role on oil degradation. In the presence of selected strains, aromatic nuclei peaks disappeared because of degradation. It can be assumed that C=C aromatic bonds were broken by *Moraxella* sp. and converted as aliphatic C=C stretching bond. In the presence of *Gallionella* sp. benzene degradation could not be observed. The low percentage of transmittance in *Brucella* sp. at 1633cm^{-1} revealed the formation of aliphatic bond stretch at higher level than the other strains. So it can be assumed that the *Brucella* sp. takes more energy from aromatic than the aliphatic chains.

The increased transmittance at 1456 and 1377 cm^{-1} show that the stretch of C=C aliphatic bond is weakened by *Gallionella* sp. The following mechanisms can be explained in the presence of *Gallionella* sp.

Diesel contamination has a more complex effect. The effects of diesel and crude oil have been studied in oil-spilled soils by Delille (2000)[30]. Hydrocarbon degrading bacterial abundance has been found to increase after diesel oil addition. Lloyed-Jones and Trudgill (1989)[31] noticed that the isolated alicyclic hydrocarbon utilizing consortia (*Rhodococcus* sp., *Flavobacterium* sp. and *Pseudomonas* sp.) from oil refinery soil and found that it was unstable, rapidly losing the ability to grow with methyl cyclohexane when placed on non-selective media due to the effect of a wide range of alicyclic hydrocarbons and related compounds in soil.

NMR analysis of diesel degradation

The NMR spectrum of pure diesel showed some major peaks at 0-3 δ. It indicated the presence of aliphatic protons only. The presence of another peak at 7 δ indicated the presence of benzene (Fig. 12). The peaks were saturated after the degradation process by bacterial strains.

The new peaks obtained at 4-5 δ after the degradation by bacterial strains, indicated the addition of oxygen with carbon atoms. The present study also revealed that the aliphatic hydrogen was consumed by the degrading bacterial carbon-hydrogen bond and was cleaved into carbon-oxygen bond (Fig. 13).

$(CH_2\text{-}CH_2)_n$ Bacterial Metabolism $(CO\text{-}CH_2)_n$

The NMR spectrum of culture filtrate of *Moraxella* sp. showed less of ($CO\text{-}CH_2$) groups. This could be, due to very slow addition of oxygen (consumption of hydrogen) by this strain, which meant that small amount of hydrogen group was consumed by *Moraxella* sp. (Fig. 14).

Thiobacillus sp. produced less ($CO\text{-}CH_2$) group similar to that of *Moraxella* sp. (Fig. 15).

The NMR spectrum of *Sulphobacillus* sp. showed less ($CO\text{-}CH_2$) groups. This could be due to slow addition of oxygen by this strain, which means the minimum amount of hydrogen group was consumed by *Sulphobacillus* sp. (Fig. 16).

From the NMR spectrum it could be explained that the addition of oxygen was the common mechanism for all strains. *Gallionella* sp. produced more ($CO\text{-}CH_2$) groups. This could be due to a very rapid addition of oxygen by this strain, which meant that large amount of hydrogen group was consumed by *Gallionella* sp. (Fig. 17) when compared to other strains (Fig. 12-18).

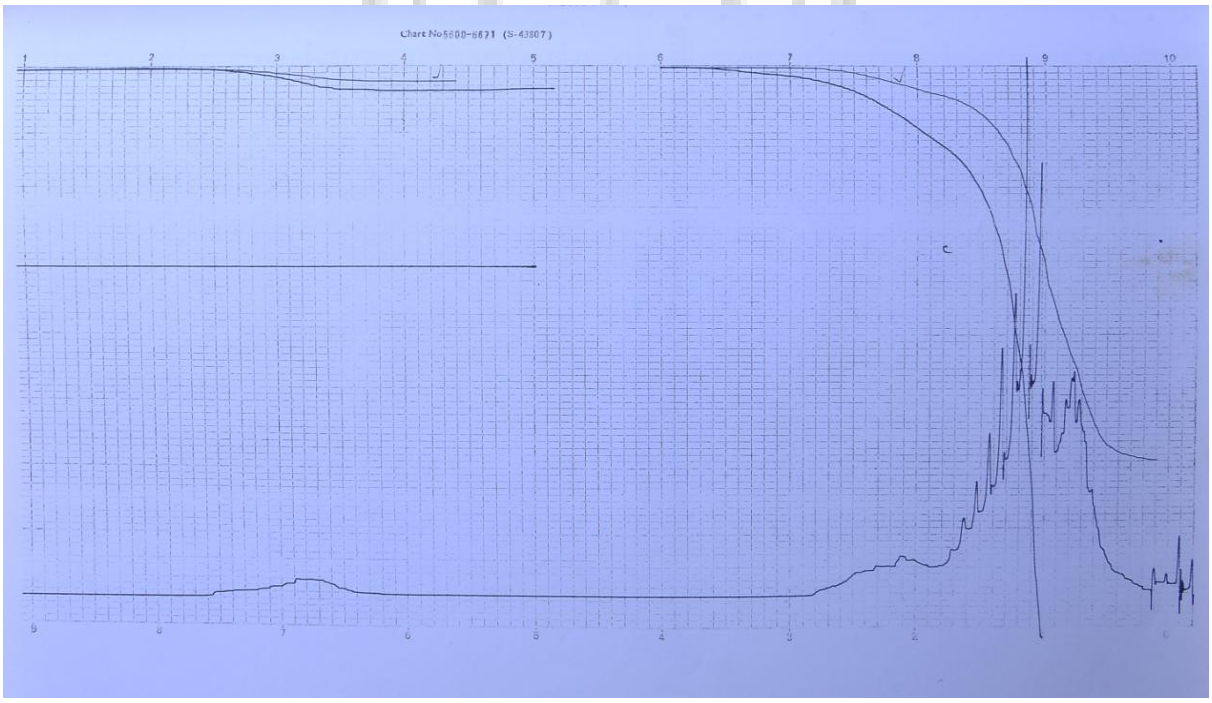

Fig. 12: NMR analysis of pure diesel

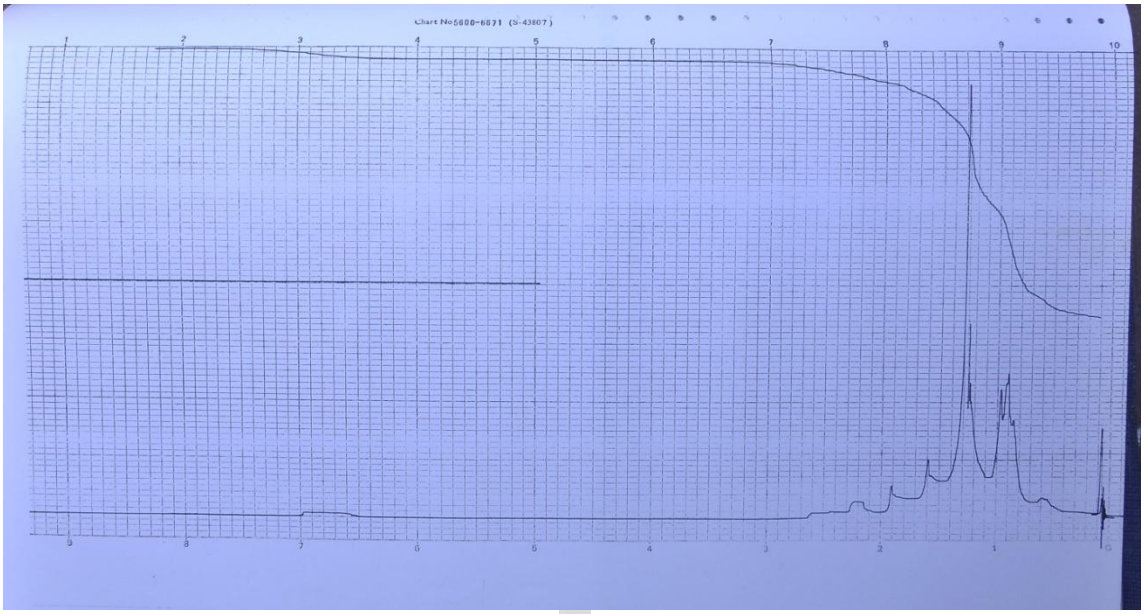

Fig. 13: NMR analysis of the sample containing 100ml media and 10ml diesel

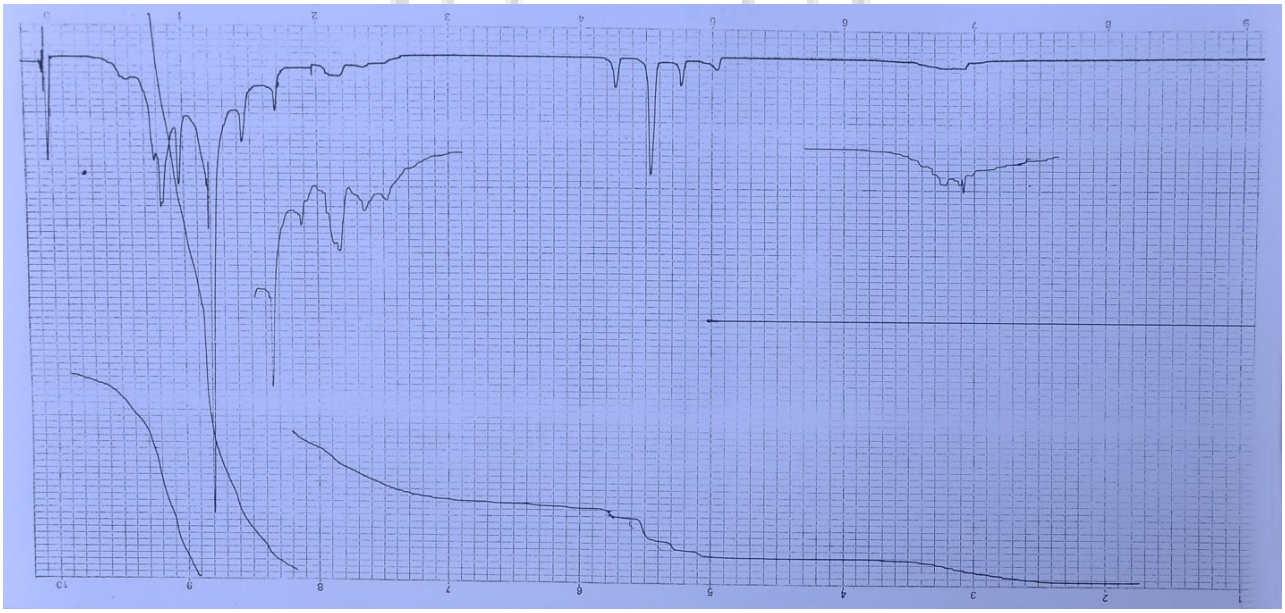

Fig. 14: NMR analysis of the sample containing 100ml media, 10ml diesel and 5ml of *Moraxella* sp.

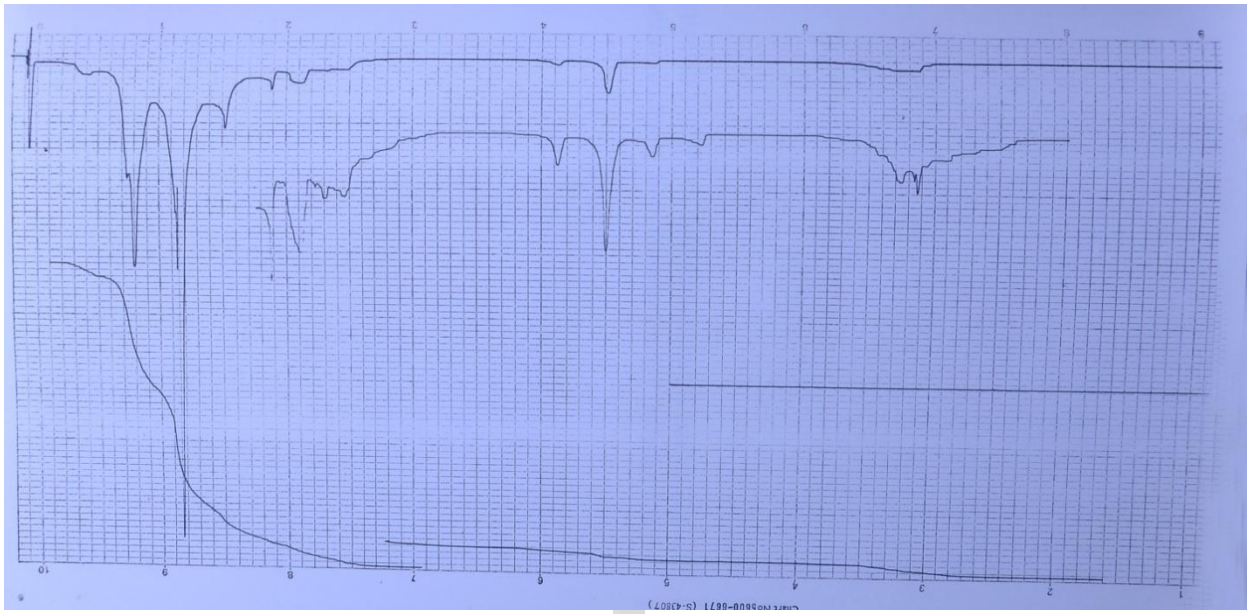

Fig. 15: NMR analysis of the sample containing 100ml media, 10ml diesel and 5ml of *Thiobacillus* sp.

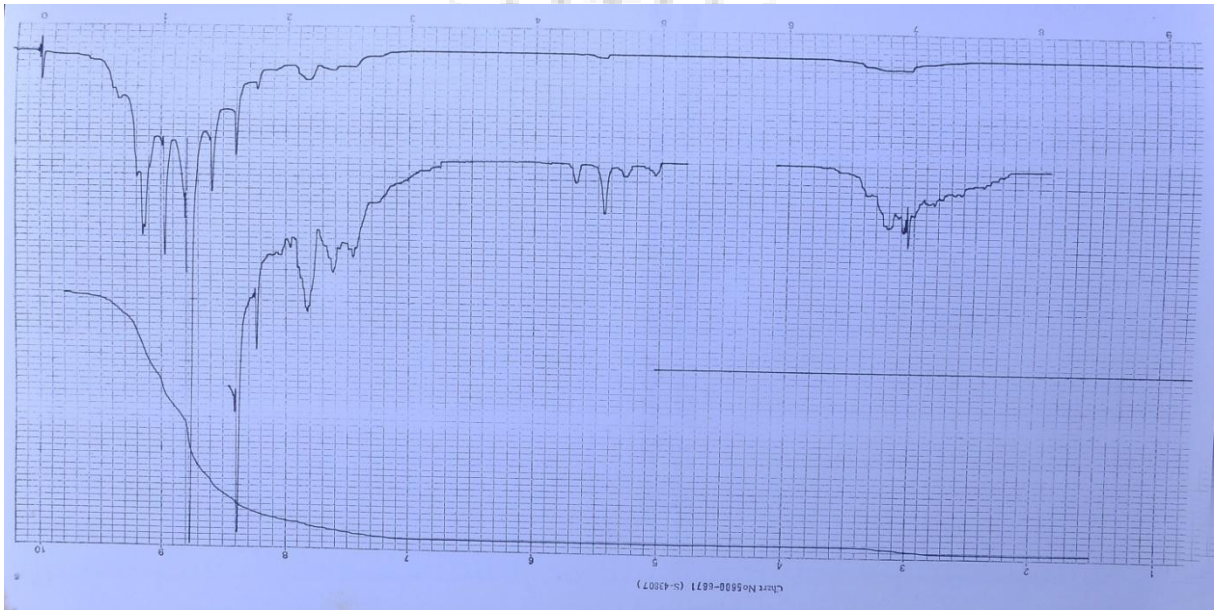

Fig. 16: NMR analysis of the sample containing 100ml media, 10ml diesel and 5ml of *Sulphobacillus* sp.

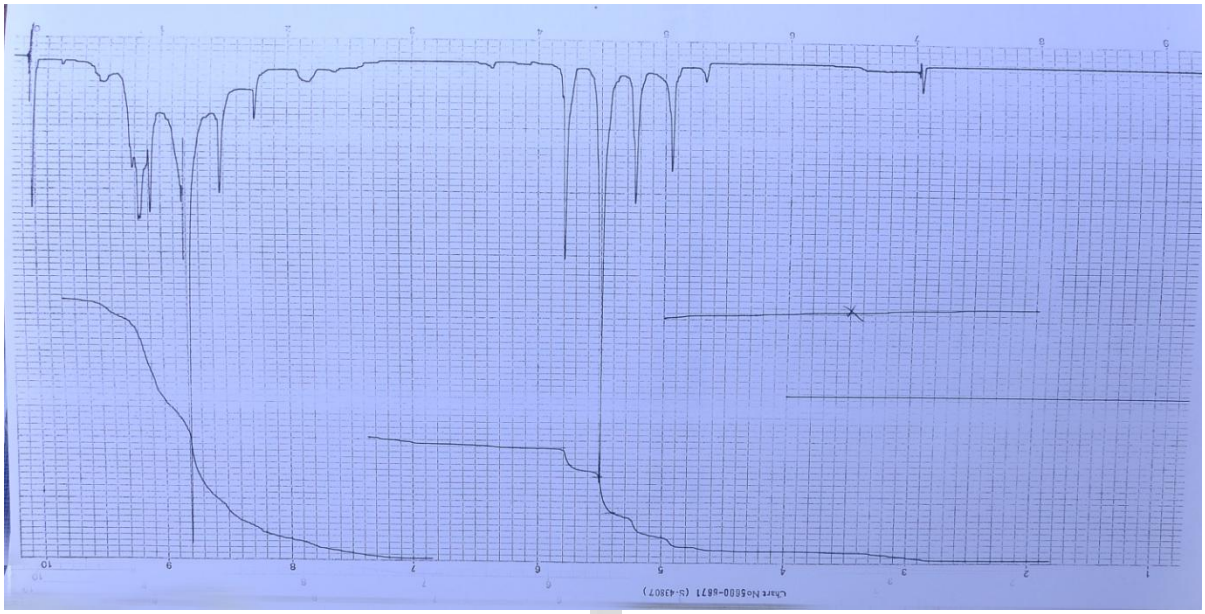

Fig. 17: NMR analysis of the sample containing 100ml media, 10ml diesel and 5ml of *Gallionella* sp.

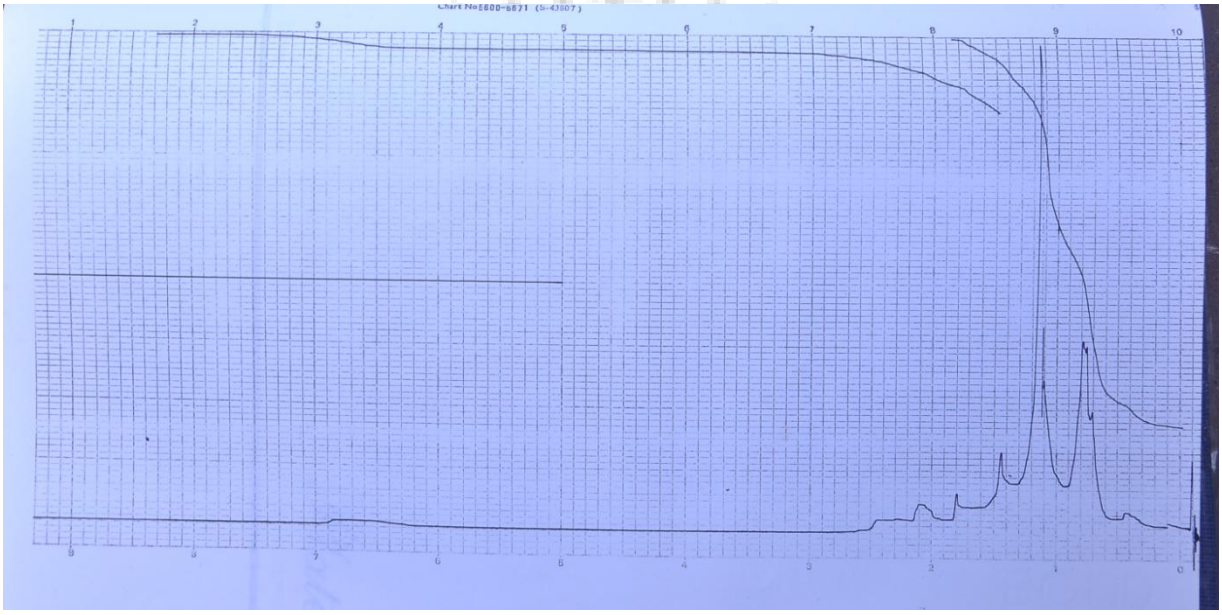

Fig. 18: NMR analysis of the sample containing 100ml media, 10ml diesel and 5ml of *Brucella* sp.

Langbehn and Steinhart (1995)[32] went on to study the microbial degradation of diesel fuel and lubricating oil in artificial soils. Soon after one week, an alicyclic and branched chain aliphatic organic acids, as well as diacids and aromatic ketones, were formed by degradation. Predominantly, the aliphatic hydrocarbons were found to be degraded. Similarly, we were able to observe in our study that within 15 days of incubation, both by single and microbial consortia, the aliphatic hydrocarbons had undergone degradation.

The biodegradation of aliphatic and aromatic hydrocarbons present in diesel oil by *Pseudomonas* sp. was studied by Sepic *et al*. (1999)[33] who observed and reported that the degradation of aliphatic was quicker than aromatic compounds with 20 days of incubation. Identically, in this experimental work too, the rates of degradation of aliphatic hydrocarbons were found to be high when compared to the rates of degradation of aromatic hydrocarbons.

April (2000)[34] noticed that sixty-four species of filamentous fungi from five flare pits in northern and western Canada were tested for their ability to degrade crude oil using gas chromatographic analysis of residual hydrocarbon following incubation. Gas chromatography indicated that species capable of hydrocarbon degradation attacked compounds within the aliphatic fraction of crude oil, nC_{12}-n-$C_{26}$and degradation of compounds that in the aromatic fraction was not observed.

NMR spectrum for *Brucella* sp. too showed the absence of $CO\text{-}CH_2$ formation at 4-5 δ value (Fig. 18). It can be explained that there is no conversion of aliphatic hydrocarbons. The addition of oxygen (consumption of hydrogen) by used strains is as given below:

Brucella sp. <*Sulfobacillus* sp. <*Thiobacillus* sp. <*Moraxella* sp. <*Gallionella* sp.

Similarly, the works of Dagher *et al*. (1997)[35] on the polycyclic aromatic hydrocarbon degrading bacterial strains showed *Pseudomonas putida*, *Pseudomonas fluorescens* and *Pseudomonas aeruginosa*, to be isolated from the oil contaminated soil which was further characterized by specific features regarding PAH degradation. Of all the most efficient strain in terms of substrate specificity and rapidity to degrade PAH's was the *Pseudomonas aeruginosa*. During the current study *Brucella* sp. was the most prevalent and dominant aromatic hydrocarbon degrading bacteria.

Temperatures also play a vital role in the physical nature and chemical composition of the oil, rate of hydrocarbon / inhibitor metabolized by the microorganism and on the microbial community (Atlas, 1981)[5]. Rates of degradation of oil have generally been observed to decrease with decreasing temperatures (Sadhushyamala, 1998)[36]. Higher temperatures in the range of 30^0C to 40^0C increase the metabolic activity to a maximum (Arumugam, 1999)[37]. Considering this factor, the effect of temperature as the parameter was worked out at room temperature for the preferential purpose.

Smart (1992)[38] also suggested that corrosion could occur when stratification of the pipeline liquids prevents the corrosion inhibitor from reaching the upper wall of the pipe. In oil pipelines, water can stratify also at the bottom of the line if the velocity is less than the required to entrain the water and sweep it through the system. Hence, biodegradation of oil is also possible in transporting pipeline also.

CONCLUSION

This investigation clearly indicates that, the possibility of a breakdown of diesel by various bacterial strains. Even though these isolates could be useful in the bioremediation of diesel polluted habitat, their presence in diesel storage and transportation facilities would lead to the reduction in the quality of diesel and in turn economic loses. More experiments need to be carried out with a different biocide to ensure the effective inhibition of the bacteria in diesel.

ACKNOWLEDGEMENT

We have great pleasure in expressing our deep sense of gratitude to our guide Dr.S. Ramesh M.Sc., Ph.D., Reader, PG Department of Microbiology, Sri Paramakalyani College, Alwarkurichi, Dr.S. Mohanan M.Sc., Ph.D., Scientist and Dr.S. Maruthamuthu M.Sc., M.Phil., Ph.D., Scientist, Corrosion Science and Engineering Division, Central Electro-Chemical Research Institute, Karaikudi for suggesting us this problem, their untiring guidance and constant encouragement which has made this work a possibility. We are highly grateful to Dr.N. Palaniswamy M.Sc., Ph.D., Head, Corrosion Science and Engineering Division, Central Electro-Chemical Research Institute, Karaikudi for having permitted us to utilize the facilities of their institute.

REFERENCES

1. Zobell CE. Action of microorganisms on hydrocarbons. Bacteriol Rev. 1946; 10(1-2): 1-49.
2. Gibson DT. Microbial degradation of aromatic compounds. Science. 1968; 161(3846): 1093-1097.
3. Zobell CE. Microbial degradation of oil. Bacteriol Rev. 1973; 2: 3-16.
4. Vander Linden AC. Degradation of oil in the marine environment. Appl Environ Microbiol. 1978; 165-200.
5. Atlas RM. Microbial degradation of petroleum hydrocarbons: An environmental perspective. Microbiol Rev. 1981; 45(1): 180-209.
6. Bartha R, Atlas RM. The microbiology of aquatic oil spills. Adv Applied Microbiol. 1977; 22: 225-266.
7. Walker JD. Utilization of mixed hydrocarbon substrate by petroleum-degrading microorganism. J Gen Appl Microbiol. 1975; 21(1): 27-39.
8. Walker JD. Petroleum degradation by estuarine microorganisms. Indian J Microbiol. 1976; 197-204.
9. Chakrabarty AM. Genetic basis of the biodegradation of salicylate in *Pseudomonas*. J Bacteriol. 1972; 112(2): 815-823.
10. Chakrabarty AM, Chou G, Gunsalus IC. Genetic regulation of octane dissimilation plasmid in *Pseudomonas*. Proc Natl Acad Sci USA. 1973; 70(4): 1137-1140.
11. Dunn NW, Gunsalus IC. Transmissible plasmid coding early enzymes of naphthalene oxidation in *Pseudomonas putida*. J Bacteriol. 1973; 114(3): 974-979.
12. Austin B, Calomiris JJ, Walker JD, Colwell RR.Numerical taxonomy and ecology of petroleum-degrading bacteria. Appl Environ Microbiol. 1977; 34(1): 60-68.
13. Lepetit J, Barthelemy MH. Les hydrocarbon on miracle problem del' epuration des zoneslittarates par les microorganisms. Ann Inst Pasteur Paris. 1968; 114: 149-158.
14. Kinney D. Biodegradation of hydrocarbons in the marine environment. Appl Environ Microbiol. 1968; 27: 125-130.
15. Colwell RR, Mills AL, Walker JD, Garcia P, Tello, Campos V. Microbial ecology studies of the medulla spill in the straits of Magellan. J Fish Res Board Can. 1978; 35: 573-580.
16. Senez JC, Azoulay E. Dehydrogenation of paraffinic hydrocarbon by resting cells and free extract of *Pseudomonas aeruginosa*. Biochem Biophys Acta. 1961; 47: 307-316.
17. Widdel E. Degradation of hydrocarbons by sulphate, reducing bacteria. J Appl Bacterial. 1980; 271-284.
18. Schwarz JR, Walker JD, Colwell RR. Deep-sea bacteria: Growth and utilization of n-hexa decane at insitu temperature and pressure. Can J Microbiol. 1975; 21(5): 682-687.
19. Degray RJ, Killian LN. Life is essentially non-aqueous hydrocarbons. Dev Ind Microbiol. 1962; 3: 296-303.
20. Hill EC. Biodegradation of petroleum products. In: Atlas RM (eds.). Petroleum Microbiology. New York: Macmillan; 1984.
21. Cannan J. Biodegradation of crude oil in reservoirs. In: Brooks J and Welbe DH (eds.). Advance in Petroleum Geochemistry. London: Academic Press; 1984.
22. Cannan J. Biodegradation of crude oil in reservoirs. In: Hirl EC (eds.). Microbial Problems in the Offshore Oil Industry. UK: Academic Press; 1987.
23. Higgins IJ, Gilbert PD. The biodegradation of hydrocarbon. In: Chater KA and Somerville HJ (eds.). The Oil Industry and Microbial Ecosystem. London: Heyden and Sons; 1978.
24. Jana J. Failure analysis of oil pipelines. Bull Electrochem. 1999; 262-266.
25. Roffey R. Microbial problems during long-term storage of petroleum products underground in rock caverns. Int Biodet. 1989; 25(1-3): 219-236.
26. Davis JB. Petroleum microbiology. New York: Elsevier Publisher; 1967.
27. Denyer SP. Mechanisms of action of biocides. Int Biodet. 1990; 26: 89-100.
28. Jack TR, Westlake DWS. Control in industrial settings. In: Barton LL (eds.). Sulphate Reducing Bacteria. New York: Plenum Press; 1995.

29. Voordouw G, Sheny, Harrington CS, Telang AJ, Jack TR, Westlake DW. Quantitative reverse sample genome probing of microbial communities and its application to oil field production waters. Appl Environ Microbiol. 1993; 59(12): 4101-4114.
30. Delille D. Response of Antarctic soil bacterial assemblages to contamination by diesel fuel and crude oil. Microb Ecol. 2000; 40(2): 159-168.
31. Lloyed-Jones G, Trudgill PW. The degradation of alicyclic hydrocarbons by a microbial consortium. Int Biodet. 1989; 25(1-5): 197-206.
32. Langbehn A, Steinhart H. Biodegradation studies of hydrocarbons in soils by analyzing metabolites formed. Chemosphere. 1995; 30(5): 855-868.
33. Sepic E, Leskovsek H. Isolation and identification of fluoranthene biodegradation products. Analyst. 1999; 124: 1765-1769.
34. April TM, Foght JM, Currah RS. Hydrocarbon-degrading filamentous fungi isolated from flare pit soils in northern and western Canada. Can J Microbiol. 2000; 46(1): 38-49.
35. Dagher F, Deziel E, Lirette P, Paguette G, Bisaillon JG, Villemur R. Comparative study of five polycyclic aromatic hydrocarbon degrading bacterial strains isolated from contaminated soils. Can J Microbiol. 1997; 43(4): 368-377.
36. Sadhushyamala S. Degradation and desulphurization of diesel by *Pseudomonas* sp. M.Sc. dissertation, Sri Paramakalyani College, Alwarkurichi, Tamilnadu, India. 1998; pp. 45.
37. Arumugam M. Microbial desulphurization of heavy duty oil. A challenge with the perspective approach. M.Sc. dissertation, Sri Paramakalyani College, Alwarkurichi, Tamilnadu, India. 1999; pp. 26.
38. Smith GL, Smart JS. Pigging and chemical treatment for oil and gas pipelines. Environ Effects. 1992; 1(22): 47-52.