Aerobic bacterial deterioration of gas oil by Pseudomonas oleovarans isolated from hydrocarbons contaminated soil

Wesam A.Z.Al-taher*

atu.edu.iq@inb.wsm

Technical University of alfurat alawsat / Iraq

Abstract ;

A total of (198) isolates belong to (10) different genera of microorganisms were isolated from (30) fuel stations contaminated soil samples . The dominant genus was Pseudomonas and represents (23.7%) of the total isolates and the most frequently isolated strains among them were diagnosed as being *Ps. oleovarans* and *Ps. formicans* . The effect of these two strains on gas oil was studied by determining the relative viable counts of cells and hydrocarbon utilization using G.L.C. apparatus . A definitive changes were noticed in the percentage of C13, C12 , C11 and C10 components with an increase of 10.6% , 11.3 % , 8.1% and 16.2 % respectively . The worthy finding was the formation of a new lighter components of C7 , C8 and C9 as a result of bacterial action on gas oil , while the most affected components were C17, C18 and $C19^{+}$ which showed a decreased in percentage of 13.7 % , 10.27 % and 12.01 % respectively .

*Prof.Dr. at Babylon technical institute , e-mail ; drwesamaltaher@gmail.com

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Introduction :

More than 400 million gallons (1.5 billion liters) of gas oil are consumed every day in North America, and more than twice that worldwide , the vast majority is burned in internal combustion engines, but a small fraction is emitted as fugitive vapor, leaks, or is spilled , two fates are most likely: evaporation followed by photochemical oxidation in the atmosphere and biodegradation (1) , Gas oils are very complex mixtures of hydrocarbons sold based on their combustion properties and specific volatility requirements rather than as chemically defined mixtures (2) . The biodegradation of several individual gas oil components has been studied both aerobically and anaerobically, often with pure microbial cultures and numerous isolates have been found that can grow at the expense of individual gas oil hydrocarbons , but little work has been reported on the biodegradation of the whole commercial product, or with inocula representing mixed consortia likely to be relevant in the environment (3,4) , Later on , there were a lot of information about the use of microorganisms in the removal of environmental pollution with oil or its derivatives , and in the use of hydrocarbons as a nutritional source to produce many useful materials in fermentation also studying the damage of various oil products as well as the mechanism and conditions leading to it and methods or ways of controlling them (5) . Many microorganisms of different species have the ability to attack hydrocarbons, this attack varies according to type of oil derivative depending on chain length , degree of branching and its paraffinic content (6) . There was a great deal of interest in the problems caused by microorganisms facing the use of hydrocarbons especially after the huge use of kerosene as a plane fuels . The two main reasons for that are kerosene attacks faster and with a wider range of microorganisms compared to other oil derivatives as well as the role of microorganisms in the formation of viscous substances that were not recognized to their responsibility in that (7) , considering the importance of gas oil as one of the important oil derivatives in its wide uses , whether in diesel engine oils or general use as in the projects that use gas with oil in combustion or as a solvent for many gases and due to the limited local studies in this field this research was done .

Materials and methods ;

1- Sampling : Samples of gas oil were obtained from Al-Dora refinery in Baghdad . The collected samples were pooled and transferred to pre-sterilized, labeled, glass bottles and transported at 4°C to the laboratory and maintained at 4°C until analysis.

2 - Bacterial isolation from soil and identification : Bushnell & Hass medium described by Jurgensen *et al* (8) was used . Methods for isolation of bacterial species utilizing hydrocarbon (3% gas oil) was summarized as follows ; 50 ml of sterile mineral salts liquid medium was inoculated with 3 ml of a mixture of three samples of solutions for the same medium of soil contaminated with hydrocarbons collected from different sites and solutions made from them by taking 10 g of soil collected from 30 different locations near gas stations with 90 ml of mineral salts medium , filtered through Whatman No.1 filter paper . The flasks were incubated in a rotating incubator for week at a temperature of 32 C , then 100µl (O.D. 0.5 at 600nm) from each flask was taken and transferred to a new flask having the same medium but with 3% gas oil as a sole source of carbon and energy , These steps were repeated for another two weeks under the same conditions to ensure isolation of pure colonies . The flasks were examined for growth by spectral absorption and bacterial isolates which exhibited growth > 1.0 O.D at 660 nm were selected to isolate pure cultures on sold mineral salt medium containing the same proportion of gas oil , Growth of colonies on plates demonstrates the ability of microbes to utilize gas oil . Hydrocarbon-degrading bacteria were later diagnosed and identified to genus level according to specific standard microbiological procedures through standardized API identification system, the most active strains were selected on the bases of their abundance and growth intensity on sold mineral salt medium with 3% gas oil and further identified to the species level on the basis of their colonial morphology, cellular morphology and biochemical characteristics according to Bergey's Manual (9) complemented by using the API 20E rapid test kit phenotypic typing .

3 - Sample study: the method indicated by francis & peter (10) was followed , the concentration of bacterial cells was evaluated by replica

plate dilution frequency technique to assay the number of viable cells in cultures at the indicated time intervals , as well as determining chemical changes in gas oil composition .

4 - Gas chromatographic analysis ; The residual oil was extracted twice from culture media with equal volume of n-hexane as described by Adebusoye *et al*. (11) . The hexane extract was subsequently analyzed using a Pye. Unicam series 304 Chromatograph equipped with flame ionizing detector (F.I.D.) , using a general type ES-30 column and nitrogen gas as a transport gas generated from Pye. Unicam HG 10 hydrogen generator , the recorder is of type PM8252 dual pen recorder with a capacity of millivolt , peaks were recorded on a paper sheet of 30 mm/ minute speed , 0.1 ml. of gas oil was injected in each turn , changes in gas oil composition were determined by comparing the total peaks areas and their values for each component of gas oil before and after exposure to bacterial attack .

5 - Use of standard compounds ; A high purity (analar) chemical compounds were used as standard materials including benzene, toluene , xylene , naphthalene , phenanthrine and anthracene were obtained from Aldrich Chemical Company to achieve machine standardization accuracy and determine the corresponding compounds in the gas oil during testing and analyzing by G.L.C. .

Results and discussion ;

1 – bacterial strains that showed the ability to utilize gas oil were isolated by the method mentioned earlier, the number of isolated strains were 198 distributed in ten different genera as shown in Table 1 , genus Pseudomonas was dominant and represents 47 (23.7%) of the total isolates, followed by Corynebacterium 38 (19.1%) and Micrococcus 29 (14.6%) . Many researchers differed in determining the most prevalent genus of microorganisms found in soil having the ability to utilize hydrocarbons . Some stated them as bacteria belongs mainly to the genera of Pseudomonas , Enterobacter and Acinetobacter, others arranged them as Pseudomonas , Burkholderia and bacillus being the predominant (5,6) different species of Bacillus , Burkholderia ,

Micrococcus , Proteus and Pseudomonas have been reported to utilize hydrocarbon through oxidation (12,13) , Recently, Roy *et al.* (14) reported bioremediation of crude oil contaminated soil by bacterial strains of genera Lysinibacillus, Brevibacillus, Bacillus, Paenibacillus, Stenotrophomonas, Alcaligenes, Delftia, Achromobacter and Pseudomonas. Researchers attributed these differences to the types of microbiological strains , ability of microbe to attack hydrocarbons , type of hydrocarbons or its branching and its ease for being utilized (4 ,15) . In this study genus Pseudomonas was chosen because of its dominance , two types of colonies which showed a higher frequency in isolation were selected as a representative strains of the genus, this selection was based on its high ability for initial growth in mineral salt medium containing 3% gas oil in the primary isolation by calculating the total viable number as shown in Figure 1 , the two strains were further identified as *Ps. oleovarans* and *Ps. formicans* and used to conduct the tests for utilization of gas oil , but their subsequent attack on gas oil was relatively similar by comparing analysis results from GLC where no significant differences between them , so we limited to mention the results of analysis by GLC apparatus of only *Ps. oleovarans* to prevent recurrence , this corresponds to what other researchers had mentioned that it appears the character of hydrocarbons utilization is almost a general characteristic description of species members of this genus (16) .

2 – Chromatographic study; The analysis of gas oil by the GLC device before it was subjected to the action of bacteria reveals it is a component of hydrocarbon substances of length from C10 to $C19^{+}$ as shown in Table 2 , the initial ratio of C10 components is 6.1% of the total hydrocarbons of gas oil and the proportion of other components increased as the chain length increased from C10 to C14 , where the percentage in C14 is 12.4% but proportion of hydrocarbons decreased for components between C15 to C18 and it was in C18 only 4.5% , while the ratio of $C19^{+}$ was 11.5%, which represents components of C19 and other parts of any hydrocarbons having more than 19 carbon atoms that may exist even in small amount as revealed by GLC analysis . Figure 3 shows physical changes in gas oil compared to the control model . An upper layer of emulsified material is clearly shown representing the new

mixture of paraffins after bacterial attack mixed with bio- surfactants products and other microbial byproducts , microbial contamination of hydrocarbon fuels is a serious problem that can lead to costly and dangerous operational problems in fuel storage tanks , engine systems and costly fuel quality degradation (7) . Table 2 and Figure 4 shows significant changes in gas oil components ratios after bacterial attack . What is interesting here is the appearance of new paraffins of C7, C8, and C9 in a ratio of 0.108%, 0.394% and 0.571% respectively that were not previously exist in gas oil , Significant role of native microbial flora has been reported in biodegradation / bioremediation processes and indicated that microbial action on hydrocarbons is usually by attacking alkanes or light aromatic fractions while the high molecular weight aromatics , resins and asphaltenes are considered to be recalcitrant or exhibit very low rates of biodegradation (14,17) , but the local isolates used in this research showed a great potential in attacking gas oil and achieved success in destruction , so one can benefit from their ability in other studies related to environment clean-up such as removal of contamination of oil derivatives in soil or water by bioremediation which is the use of microorganisms to detoxify or remove pollutants using their diverse metabolic capabilities and it is preferred over physico-chemical methods due to its eco-friendly and cost effective nature or even study their harmful effects of its presence in fuel storage tanks as fuel microbial testing can be used as part of a regular monitoring and maintenance program to give early warning of contamination issues (18) . Table 2 and Figure 4 shows that there is an increase in ratios of paraffinic components of C10 , C11 , C12 & C13 by 16.2% , 8.13% , 11.32% and 10.62% respectively. The utilization of gas oil began with a decrease in quantities of components from C14 to $C19^{+}$. Table 2 indicates the percentage of loss of paraffins of gas oil after bacterial attack with more loss noticed as chain length increased . The analysis showed that bacteria consumed compounds of C17 and C19 more than other components , with a loss rate of 13.7% and 12% respectively although other paraffins of C15, C16 and C18 were also attacked in significant ratios of 7.8%, 8.6% and 10.2% respectively , but the attack on C14 was quite poor and loss proportion is only 1.9% . The results

obtained are in consistent with what others have pointed in that C15 and C17 compounds are most widely utilized by bacteria and differences in paraffins that have been attacked is often due to the differences in bacterial species (4) . Rocha *et al.* (19) reported that 6 - 100% degradation of C11 to C21 hydrocarbons in heating oil at 20 days by biosurfactant producing *Ps. aeruginosa* , while bacteria used in this research showed its inability to attack paraffins from C10 to C13 on the contrary percentage was increased due to the breakdown of higher paraffins to a low ones , some researchers pointed to the same results in that the broadest attack of microorganisms would be for compounds with carbon atoms C10 to C16 or C18 (20 , 21 , 22) .

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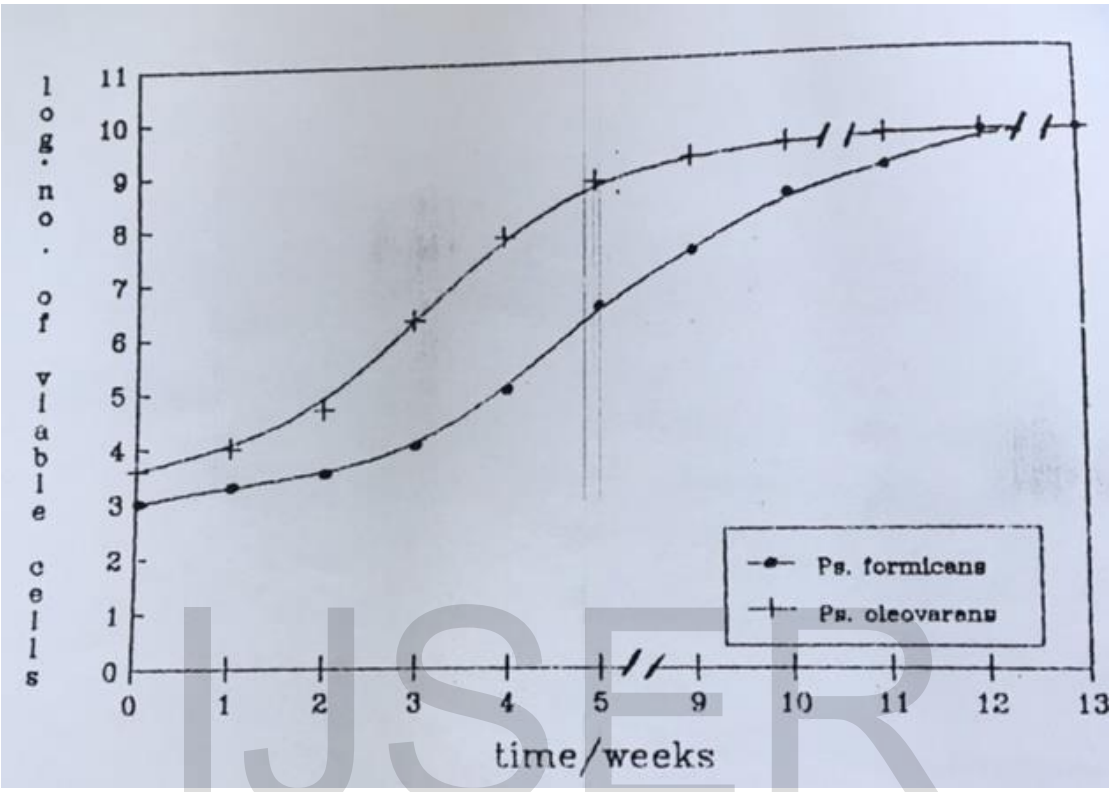
Table (1) shows the isolated bacterial genera and the number of isolates for each genus on solid mineral salts medium containing 3% gas oil at 37 C .

Genus name	No. of isolates	Percentage %
Pseudomonas	47	23.7
Corynebacterium	38	19.1
Micrococcus	29	14.6
Bacillus	22	11.1
Enterobacter	17	8.5
Acinetobacter	15	7.5
Mycobacterium	13	6.5
Vibrio	11	5.5
Alkaligenes	4	2.0
Nocardia	2	1.0
Total	198	99.9

Table (2) ; Changes in composition of gas oil components according to number of carbon atoms before and after bacterial attack as shown by GLC analysis

Carbon atoms no.	Ret. Time (min.)	GLC reading before attack % ($P.A.cm^2$)	GLC reading after attack % (P.A.cm2)	Increase value	Decrease value	Percentage of increase or decrease
C7	0.23	Zero	0.108	0.108	___	100
C8	0.34	Zero	0.394	0.394	___	100
C9	0.51	Zero	0.571	0.571	___	100
C10	0.68	6.160	7.162	1.002	___	16.20
C11	0.77	12.410	13.420	1.010	___	8.13
C12	0.90	9.005	10.025	1.020	___	11.32
C13	1.10	11.236	12.430	1.194	___	10.62
C14	1.37	12.420	12.180	___	0.240	1.93
C15	1.68	12.305	11.338	___	0.967	7.85
C16	2.03	10.959	10.010	___	0.949	8.65
C17	2.40	9.359	8.076	___	1.283	13.70
C18	2.77	4.574	4.104	___	0.470	10.27
$C19^+$	3.13	11.572	10.182	___	1.390	12.01

TOTAL		100	100	5.29	5.29	



Fig(1) viable bacterial cell count grown in liquid mineral salt medium at 32 C with 3% gas oil as a sole source of carbon and energy

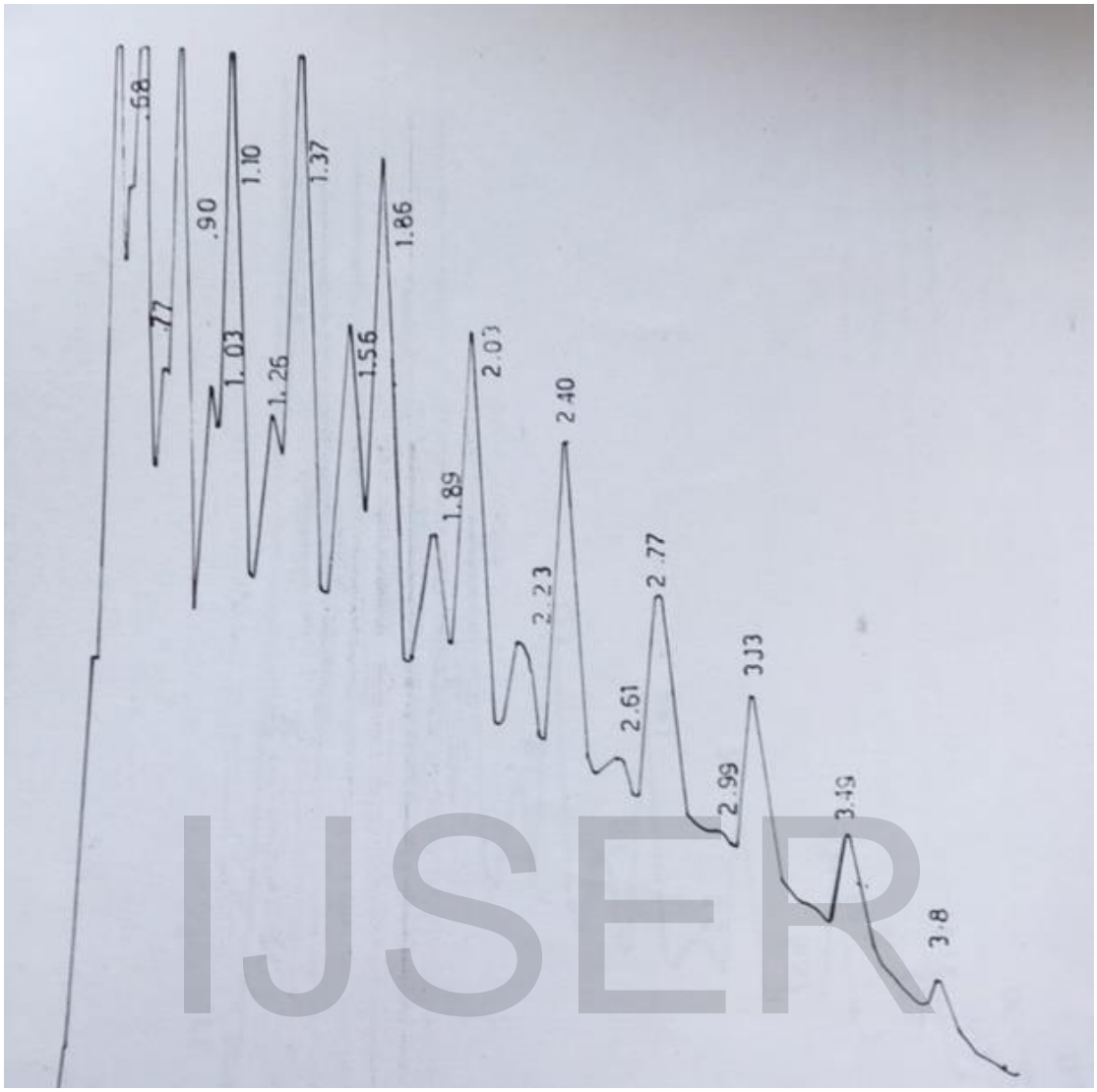


Fig (2) Analysis of gas oil components peaks (control sample) from C10 to C19 before bacterial attack as shown by GLC apparatus .

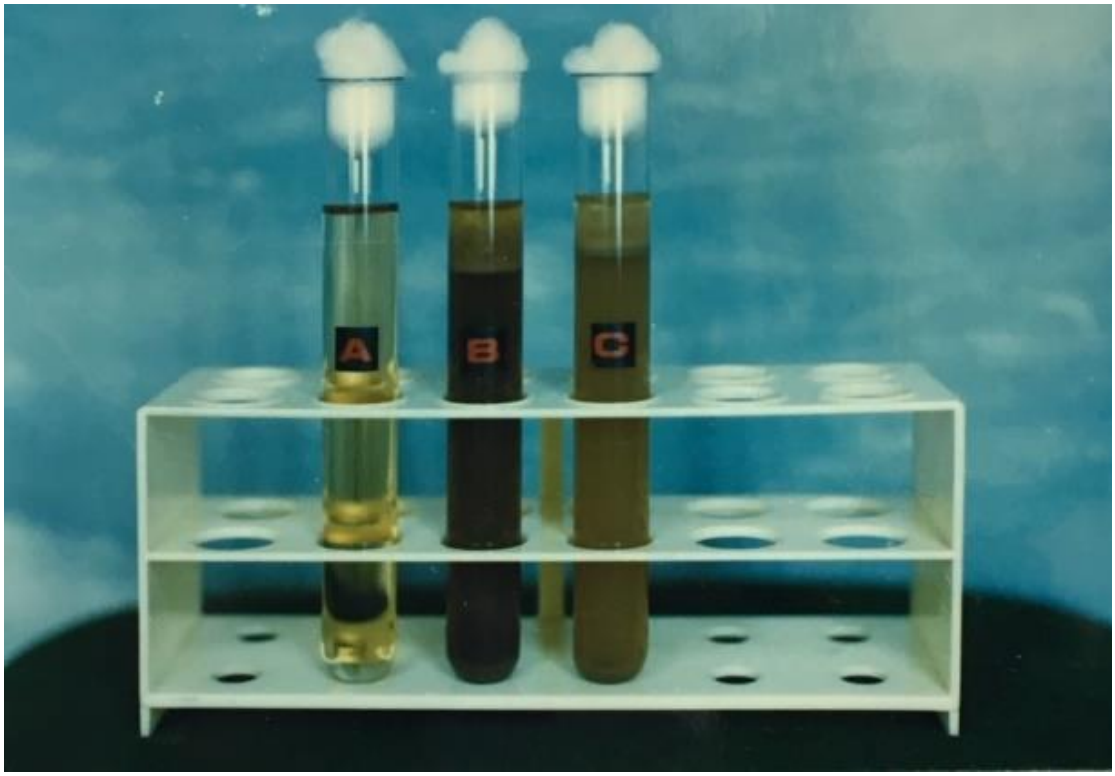


Fig (3) Physical changes occurred in gas oil after bacterial attack

A ; untreated control

B ; gas oil after attack by *Ps. oleovarans*

C ; gas oil after attack by *Ps. formicans*

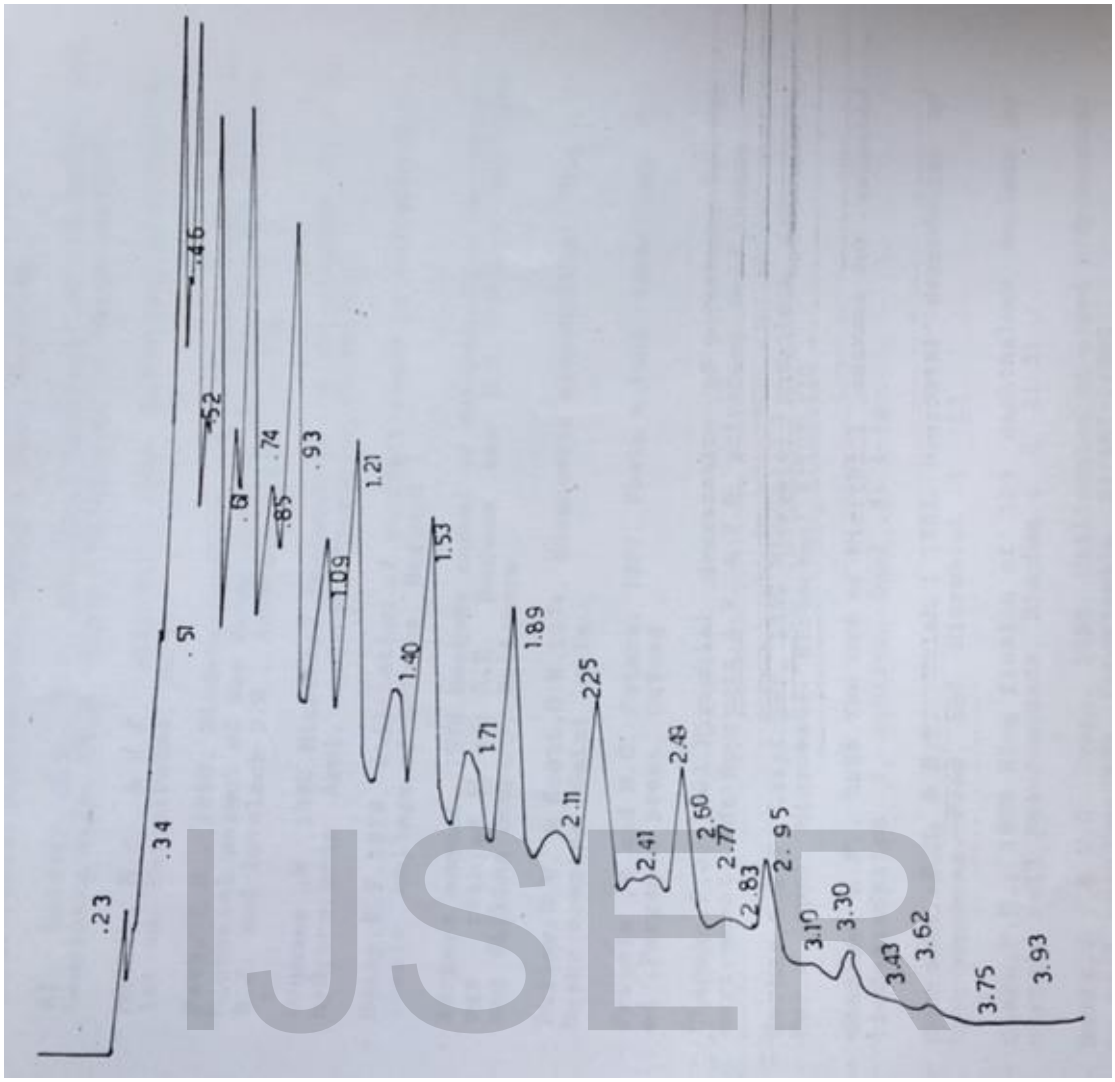


Fig (4) Analysis of gas oil components peaks from C7 to $C19^{+}$ after bacterial attack as shown by GLC apparatus .

References ;

1 - U.S. Department of Energy. 2007 . Energy Information Administration. ;http://www.eia.doe.gov/oil_gas/petroleum

2 - Ritter, S. 2005 . What's that stuff? Gasoline's ubiquity belies its complex blend of hydrocarbons and additives. C & E News, 83 ; 8 , 37 – 45 .

3 - Prince, R. C., C. C. Walters . 2006 . Biodegradation of oil hydrocarbons and its implications for source identification. In Environmental Forensics: Spill Oil Fingerprinting and Source Identification , Wang, Z., Stout, S.A., Eds.; Elsevier: New York, pp 349 - 379.

4 - Van Hamme, J. D., Ward, O. P. 2001 . Volatile hydrocarbon biodegradation by a mixed bacterial culture during growth on crude oil. J. Ind. Microbiol. Biotechnol. 26, 356-362.

5 - Saadoun, I., 2002, Isolation and characterization of bacteria from crude petroleum oil contaminated soil and their potential to degrade diesel fuel . J Basic Microbiol. , 42 (6) 420 - 428 .

6 – Judith W. , J. Gilbert and E. Mahenthiralingam . 2011 . Culture independent analysis of bacterial fuel contamination provide insight into the level of concordance with the standard industry practice of aerobic cultivation , Appl. Environ. Microbiol . 77 (13) ; 4527 – 4538 .

7 - Allsopp D., K. Seal ,C.C. Gaylarde . 2004 . Introduction to biodeterioration , 2nd ed. Cambridge University Press , UK .

8 – Jurgensen, K.S., J. Puustinen, j. Suortti . 2000. Bioremediation of petroleum hydrocarbon contaminated soil by composting in biopiles . Environ. Pollu. 107 ; 245 – 254 .

9 – Holt JG, Krieg NR, Sneath PHA, Staley JT, Williams ST. Bergey's Manual of Determinative Bacteriology 9th ed. Baltimore: Williams & Wilkins, 1994.

10 – Francis ,W. , M. C. Peters . 1987 . Fuels and fuel technology. 2nd ed. , Pergamon press , Oxford .

11 - Adebusoye S. A., M. O. Ilori, O.O. Amund , O.D. Teniola and S.O. Olatope . 2007. Microbial degradation of petroleum in a polluted tropical stream. World J. Microbiol. Biotechnol., 23: 1149–1159.

12 - Patil T.D., S. Pawar , P.N. Kamble and S.V. Thakare . 2012.

Bioremediation of complex hydrocarbons using microbial consortium isolated from diesel oil polluted soil. Der Chemica Sinica. 3:953-958 .

13 - Adegbola G.M., K.I.T. Eniola , O.A. Opasola . 2014. Isolation and identification of indigenous hydrocarbon tolerant bacteria from soil contaminated with used engine oil in Ogbomoso, Nigeria, Advances in Applied Science Research. 5(3):420-422.

14 - Roy, A.S., R. Baruah, M. Borah, A.K. Singh, A. Boruah, H.P.D. Saikia, M. Deka, N. Dutta, T.C. Bora . 2014. Bioremediation potential of native hydrocarbon degrading bacterial strains in crude oil contaminated soil under microcosm study. Int. Biodeterior. Biodegrad. 94, 79 - 89.

15 - Veerapagu M, K.R. Jeya , R. Kalaivani , K.A. Jeyanthi and Geethanjali S. 2019 . Screening of hydrocarbon degrading bacteria isolated from oil contaminated soil . The Pharma Innovation J. 8(6) : 69-72 .

16 – Oluwafemi S., O. Sunday , A. Adebusoye , O. Adewale and O. Oyetibo . 2009 . Differential degradation of crude oil (Bonny Light) by four Pseudomonas strain . J. Environ. Sciences 21; 243 –248

17 - Sepahi, A.A., I.D. Golpasha, M. Emami, A.M. Nakhoda . 2008. Isolation and characterization of crude oil degrading Bacillus spp. Iran. J. Environ. Health Sci. Eng. 5, 149 - 154.

18 - Sunita J. , P. R. Dolly , K.J. Ajay , B. Surendra , N.U. Vivek . 2015 . Synergistic ex-situ biodegradation of crude oil by halotolerant bacterial consortium of indigenous strains isolated from on shore sites of Gujarat, India . int. Biodeterior. Biodegrad. 103 ; 116 – 124

19 - Rocha, C.A., A.M. Pedregosa and F. Laborda . 2011. Biosurfactant-mediated biodegradation of straight and methyl-branched alkanes by *P. aeruginosa* ATCC 55925. AMB Express 1 , 1 - 10.

20 – Farinazleen, M.G., R.N.Zaliha, A.B.Salleh and M.Basri . 2004 . Biodegradation of hydrocarbons in soil by microbial consortium . Int. biodeterior. Biodegrade. 54 ; 61 – 67 .

21 - Bao, M.T., L. Wang, P. Sun, L. Cao, L. Zou and Y. Li . 2012. Biodegradation of crude oil using an efficient microbial consortium in a simulated marine environment. Mar. Pollut. Bull. 64, 1177 - 1185.

22 - Das, K., A.K. Mukherjee . 2007. Crude petroleum-oil biodegradation efficiency of Bacillus subtilis and *Ps. aeruginosa* strains isolated from a petroleum oil contaminated soil from North-East India. Bioresour. Technol. 98, 1339 - 1345.