Biodegradation of 2T engine oil using soil microbe and gravimetric analysis

Shallu Sihag and Hardik Pathak

Abstract – The rod shaped gram negative, an aerobic, motile hydrocarbon degrader *Rhodococcus rhodochrous* was isolated from the petroleum contaminated soil of Mount Abu, the high altitude region of Rajasthan at 4003 feet. Poly Aromatic Hydrocarbon degrading bacterial strain was isolated by enrichment culture technique in Bushnell Hass medium in the presence of 2T engine oil as a sole source of carbon and energy. The evaluation parameters like Optical density (OD), total petroleum hydrocarbon (TPH) and gas chromatography and mass spectroscopy were used as the major indicator of microbial degradation of 2T engine oil at concentration of 1% (v/v). The physico-chemical analysis of the petroleum contaminated soil samples was analyzed and their effect on the rate of degradation was determined. By GC-MS analysis it was observed that the 2T engine oil and the contaminated soil was highly polluted with the hydrocarbons of LMW as well as HMW ranges from C_1-C_{50}. The maximum height of peaks were of medium fractions ranges of C_{11}-C_{20} on day 14 which were with 78.07%, which were 58.55% on day 7. They appeared due to the reduction of peaks of C_{21}-C_{30} from 22.24 % to 3% on day 7-14 respectively. The maximum degradation of 2T engine oil hydrocarbons with 62% was observed within 14 days of incubation period by the conversion of higher alkanes to their lower forms by gravimetric method. Apart from the degradation, also the secondary metabolites were produced by the *Rhodococcus rhodochrous* which have a wide application in the field of medicines, dyes etc. The organism *Rhodococcus rhodochrous* showed remarkable potential for use in degradation of 2T engine oil.

Keywords- 2T engine oil, bioremediation, Poly aromatics hydrocarbons, gas chromatography mass spectroscopy, secondary metabolites etc

1 INTRODUCTION-

The eminence of earth is highly related with its environment. Nowadays, the organic and inorganic both natures can be considered as a major source of pollution. The widespread use and improper disposal of organic pollutants have resulted in the persistent soil contamination, which ultimately become a major issue because of its adverse effects on human health as well as potential risk to the various ecosystems. These organic pollutants enter into the various atmospheres by the various anthropogenic activities. The most common are petroleum derivatives which include alkanes and other aliphatic and aromatic compounds [1], [2]. The rate of increasing usage of petroleum and related products, refineries, high temperature treatment of toxic substances (incineration), mining, oil spills, incomplete combustion etc has increased the fear of getting exposure to toxic substances [3]. Crude oil is one of the examples of highly complex mixture of organic compounds. The numerous oil shipping disasters such as Exxon Valdez, Erika and the Prestige have got public attention to this type of environmental problem.

The occurrence of Poly aromatic hydrocarbons (PAHs) in the soil has a great concern of carcinogenicity, toxicity and mutagenecity [4]. These PAHs are a class of toxic pollutants which have chemicals-related organic compounds of diverse structures and wide-ranging toxicity. On the basis of rings present they are classified as low molecular weight (LMW) compounds and high molecular weight compounds (HMW) The LMW compounds consists of two or three benzene aromatic rings which are readily degraded by bacteria while the HMW compounds consists more than three benzene ring are recalcitrant to biodegradation and persist in the environment [5], [6]. The benzene rings can be in linear, angular and cluster arrangements with a pair of carbon and hydrogen atoms [7]. The toxicity of PAHs can be measured by the rings present in it. The presence of PAHs in environment is of great concern due to their hydrophobic properties, low volatility and high affinity for sediment particles. PAHs are readily adsorbed to surfaces in soil environment, dust particles, which could get evenly distributed through air [8], [9]. These PAHs compounds are present in the mixture of compounds rather than single. Man gets contacted intentionally or un-intentially to these toxic substances by the various routes such as food, water, air, occupational exposure etc. [10], [11]. The adverse effects to health

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and environment by these toxic pollutants are well known [12], [13], [14]. Benzo (a) pyrene is considered as human carcinogenic. They have detrimental effects on the various ecosystems, as well causes serious health problems or genetic defects in humans [15], [9], [16]. Moreover, by the USEPA the ecotoxicity of 16 PAHs have been listed [17], [18]. Also, the IARC (International Agency for Research on Cancer) has identified 15 PAHs including 6 of the 16 USEPA- regulated PAHs, as potential carcinogens.

Current physical and chemical treatment of wastes is generally expensive and is not able to remove trace quantities of pollutants [19]. To remediate these toxic substances from the environment, bioremediation is a method which detoxifies and converts them into lower toxic forms and sometimes completely degrade [20]. This is the most effective management tool for environmental cleanup [3]. In this method the use of naturally occurring bacteria, fungi etc. are used to remove hazardous substance which are harmful for environment and human both [21]. The hydrocarbon degradation by microorganisms is the best way to produce non toxic end products such as water and carbon dioxide which are environmentally safe [22].

There is a complex system of various environmental factors which may increase or may decrease the rate of remediation by microorganisms [23]. Factors like pH of soil, nutrient availability in the soil, concentration of PAHs and microbial community, heavy metals, porosity, salinity, water holding capacity of soil etc. are the key factors which have a significant role in bioremediation [24]. It is very difficult to get rid of pollution produced by PAHs due to their high hydrophobicity which increases with increasing molecular weight which ultimately results in higher toxicity and complexity however may persist in environment for longer duration [17]. But there are variety of microorganisms that are capable of degrading certain PAHs therefore there is a considerable interest in studying microorganisms. [25]. Various microorganisms such as *Gordonia, Achromobacter, Bacillus, Enterobacter, Escherichia, Mycobacterium, Pseudomonas, Rhodococcus, Serratia and Staphylococcus* have been found their potential to degrade hydrocarbons[26], [27], [28], [29], [30], [31], [32]. Also a thorough list of microorganisms which can utilize PAHs is provided by Muller et al., [33]. Microorganism have their own system for adapting to the various environmental conditions in which they are surviving such as sensing and responding systems, regulation, metabolism, transportation system and so on. Different and abundant genes and enzymes in these types of systems may suggest the strong ability of the cells to adapt to their habitat.

The processes of degradation of PAHs by microorganisms have been found by either metabolism or co-metabolism (Aerobic and anaerobic metabolisms). Co-metabolism is required for the degradation of mixtures of PAHs. Aerobic metabolisms have been considered for the metabolism of PAHs. The common aerobic metabolic pathways, their rate of degradation, the enzymatic regulation, the genetic metabolism involved are quite well understood [34], [35], [36]. The aerobic catabolism of PAHs can be carried out by the enzymatic actions. The monooxygenase or dioxygenase enzyme incorporates the atoms of molecular oxygen into the aromatic nucleus which further oxidized the aromatic rings [35], [36], [37]. Two hydroxyl groups positioned according to the substituents on the original molecule, either ortho or para to each other. Now, by the aromatic dihydroxy compounds and the ortho- or meta cleavage pathways the cis-dihydrodiols was further oxidized which was formed in the reaction [38], [39].After this the reaction leads to the precursors of tricarboxylic acid cycle (TCA) intermediates. This is the basic mechanisms of degradation process. The kinetics efficiency of the pathways and the type of reaction intermediates produced depends on the number on the aromatic rings.

However, the most of the information of the degradation process, metabolic pathways, enzymes involved and genes has been restricted to LMW PAHs [40], [41], [42]. The information on genes and their encoded enzymes should help to the researchers for enhancing the performance of PAHs degrading bacteria as bioremediators.

Considerable attention have been focused on the potential of hydrocarbon degrading microorganisms to degrade 2T engine oil, which was isolated from the petroleum contaminated soil of high altitude low temperatures region of Rajasthan (Mount Abu).

2 Experimental-

2.1 Sample collection – The petroleum contaminated soil samples were collected with the aid of sterile spatula in sterile plastic bags from different locations of high altitude region of Rajasthan (Mount Abu). The petroleum contaminated soil samples were kept at 4^0C until proceed for further manipulations.

2.2 Physico-chemical analysis- The determination of pH of the soil, moisture, bulk density, heavy metal detection by Atomic absorption Spectroscopy (AAS), carbonate and

bicarbonate estimation, chloride estimation etc. physical properties were performed. For chemical analysis of oil contaminated soil samples, column chromatography was performed by using n-hexane and toluene (petroleum fractions) respectively for the separation of the aliphatic and aromatic fractions of petroleum hydrocarbons present. The petroleum contaminated soil sample and activated silica gel of mesh size of 60-120 was weighed in 1:2 ratios respectively and packed in column. The residual oil obtained was analyzed by Gas Chromatography-Mass Spectroscopy (Shimadzu model QP-2010 plus, column-Rtx-5MS, 30 meter x 0.25 mm i.d. x 0.25 um film thickness).

2.3 Isolation of hydrocarbon degrading microorganisms- 2T engine oil degrader was isolated by enrichment culture plate technique method by using Bushnell Hass medium (BH medium) [4], [43]. 1 g of soil sample was added in 100 ml of autoclaved Bushnell Hass media supplemented by 1% of 2T engine oil as a carbon source. The flask was kept on shaker at 150 rpm for one week. After 1 week 10 ml of enriched media was transferred to freshly prepared media and kept for incubation. This cycle was repeated for 5-6 cycle. After every enrichment cycle 1 ml of culture was diluted to 10^8 fold and diluted culture was plated out on BH agar plates which was supplemented with 1 ml of 2T engine oil as carbon source and kept for incubation at 37^0C. These colonies were streaked onto BH agar plates to achieve a pure culture. At the end these microbial strains were kept in 40% glycerol and stored for future use.

2.4 Evaluation of degradation potential by gravimetric method- Biodegradation Potential was determined by Gravimetric method. l ml of 36 hours old bacterial culture supplemented with 5 g of 2T engine oil was added in 100ml BH medium. These flasks were kept on shaker for 7-14-21-28 and 35 days at 25^0C temperature. After every 7 days of incubation, 1 flask was taken out for gravimetric analysis. O.D. was taken on UV spectrophotometer at 620 nm for bacterial growth. For separating the residual 2T engine oil from the medium, 5 ml of n-hexane was added to flask and the mixture was separated using separating funnel. Two layers were formed, the upper layer was of oil and lower layer was of broth. The oil from the upper layer was collected in the pre-weighed petriplate. 500 µl from the residual oil was used for GC-MS analysis.

Percentage of oil degraded was calculated using the formula:

Weight of residual oil = weight of beaker containing extracted oil – weight of empty beaker

Amount of oil degraded = weight of oil added in media – weight of residual oil

$$\% \text{ degradation} = \frac{\text{Amount of oil degraded}}{\text{Amount of oil added in the media}} \times 100$$

2.5 Identification of microbial strain- The morphological and biochemical properties of the isolate was done on the basis of the identification scheme of Bergey's manual of Determinative Bacteriology [44], [45]. For molecular identification 16sRNA sequencing was performed at Yaazh Xenomics Tamilnadu, India. By using bio-informatics tools the identification of isolate was done and phylogenetic tree was constructed by using MEGA 5.

3 Results and Discussions

3.1 Physico chemical analysis of petroleum contaminated soil samples

One of the most vital parameter for biodegradation is the physico chemical properties of soil where organisms survive. From table 1 the pH of the petroleum contaminated as well as normal soil was found slightly alkaline in nature with 7.8 pH and optimum pH for the biodegradation is assumed as 6.5 to 8.0. There was not a healthy amount of moisture in both contaminated and normal soil were obtained but however the soil holds the more moisture with 0.23 % and therefore, the water holding capacity was less of the contaminated soil. The higher bulk density lowers the porosity of the soil. The contaminated soil and the normal soil were obtained with high chloride amount with 8.6 mg/100 g, 18 mg/100 g respectively. The petroleum contaminated soil samples and the normal soils sample were also having the macro and micronutrients like zinc, copper, phosphate, carbon, potassium etc. The availability of the nutrients definitely impacts the biodegradation rate and effectiveness like other biotic factors such as temperature, pH and oxygen supply etc. The nutrient balance for the microbial growth and reproduction is adjusted by adding the nutrients and the aerobic conditions are maintained by supply of oxygen for the hydrolysis and microbial oxidation of hydrocarbon compounds. The hydrolysis and oxidation are the primary procedures to breakdown the higher and complicated hydrocarbons to be utilized by the microorganisms. Therefore, the H_2O and CO_2 are the end products for the aerobic degradation. These soil samples were traces with the heavy metals present in them. Chromium, nickel, iron and manganese are the metals required by the microorganisms as essential micronutrients for

their growth and development. For example- Fe^{3+} is required by all types of bacteria whereas Fe^{2+} is required by the anaerobic bacteria. Iron and manganese were traced during the Atomic absorption spectroscopy in all contaminated and normal soil samples.

Table 1- Shows the Physico-chemical properties of the petroleum contaminated soil samples

S.No.	Test Name / Sample name	PCS 1	NS 1
1	pH	7.8 ± 0.1	8 ± 0.2
2	Electric Conductivity µs cm^{-1}	0.6 ± 0.002	0.34 ± 0.002
3	Moisture content %	0.23±0.40	0.07±0.53
4	WHC %	54.34±0.31	59.13±0.32
5	Bulk density g mL^{-1}	0.88±0.03	0.92±0.12
6	Porosity %	65.34±0.12	66.29±0.31
7	Organic matter %	6.34	6.12
8	C/N	121.31±0.67	97.32±0.52
9	Chloride mg *per*100g	8.6±0.03	18±0.08
10	Carbonate mg *per* 100g	0.21±0.01	0.34±0.04
11	Bicarbonate mg *per* 100 g	1.67±0.35	1.21±0.15
12	Zinc mg kg^{-1}	0.19±0.01	0.24±0.01
13	Copper mg kg^{-1}	1.6±0.12	0.84±0.21
14	Phosphate	42±0.09	36±0.03
15	Carbon	0.14±0.07	0.3±0.02
16	Potassium mg kg^{-1}	206±0.01	198±0.04
17	Iron mg kg^{-1}	2.62±0.08	1.62±0.21
18	Cadmium mg kg^{-1}	-	-
19	Nickel mg kg^{-1}	138.06	-
20	Lead mg kg^{-1}	12.78	-
21	Chromium mg kg^{-1}	21.8	-
22	Manganese mg kg^{-1}	2.2±0.03	0

The chemical analysis of 2T engine oil showed that oil is a mixture of various compounds such as hydrogen, carbon, nitrogen, sulphur, oxygen etc. These hydrocarbons are categorized as saturated and unsaturated compounds. These compounds have different no of carbon atoms ranges from carbon atom number 1 to carbon atom number 50 or more. The various peaks indicate the various compounds of various ranges of carbon and hydrogen atoms found at different retention time. These hydrocarbons act as source of carbon and energy for the various microorganisms.
The morphological characteristics and biochemical properties are shown in Table 2. By molecular identification the isolate was identified as *Rhodococcus Rhodochrous*. The phylogenetic tree is shown in Fig 1.

Table no 2- Showing the morphological characteristics and biochemical properties of *Rhodococcus Rhodochrous*

	S.No.	Biochemical test	Reaction
Morphological characters	1	Morphology	cocci
	2	Opaque	+ ve
	3	Convex	- ve
	4	Diffusible pigment	-ve
	5	Color	Yello/Pink
	6	Gram's Reaction	-ve
Biochemical characters	8	Catalase	+ve
	9	Oxidase	+ve
	10	Indole	-ve
	11	MR	-ve
	12	VP	-ve
	13	Citrate	-ve
	14	Starch	-ve
	15	Phenylalanine Deaminase	-ve
	16	Gelatin	+ve
	17	Motility	-ve
	18	TSI agar	+ve
	19	Spirit blue agar	+ve
	20	Klinger Iron agar	+ve

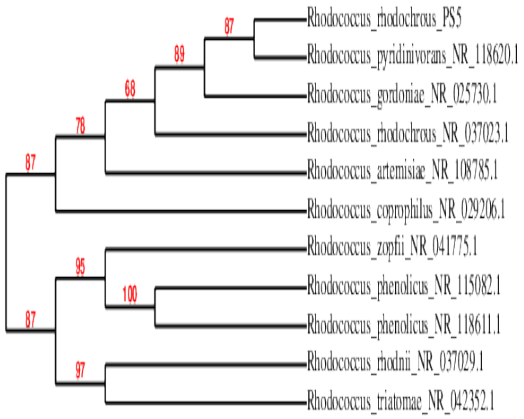

Fig 1- Showing the phylogenetic tree of *Rhodococcus rhodococcus*

3.2 Biodegradation potential of hydrocarbon degrading microorganism

The main objective of this study was to assess the potential of isolated microorganism to degrade the PAHs. The quantitative estimation of residual 2T engine oil amount in culture medium suggests that 2T engine oil was utilized by the microorganism. The rate of degradation was considerably high at initial 7 days with 60%. The maximum degradation with 62% was obtained on 14 days of incubation after that the rate of degradation was decreased. The capability to degrade the 2T engine oil (Table 3, Fig 2) was calculated. During the gravimetric analysis the microbial growth was also estimated for the degradation process. The turbidity in the flasks was obtained and was analyzed for the spectrophotometer analysis at 620 nm (Table 3, Fig 3). By the increasing number of days the rate of microbial growth was increased which means the microorganisms were utilizing the compounds of 2T engine oil and used as source of carbon and energy. It has been observed that the concentration of substrate only affect the multiplication rate of microorganisms while there is no effect on the growth profile by increasing the PAHs concentrations. The enrichment substrate significantly affected the microbial population. It is observed that large and complex the structure of hydrocarbons the more slowly is oxidized. This might also be depends on the organisms involved and the medium in which it was developed. For this reason, the longer enrichment period was selected for fresh medium of toxic metabolites, which enhanced the proliferation of bacteria to utilize more complex compounds.

Table No. 3 - Showing the rate of Biodegradation in percentage of *Rhodococcus Rhodochrous* grown in Bushnell-Hass medium amended with 2T oil as sole carbon and energy source and the microbial growth at 620nm

	Days	Biodegradation potential in (%)	(O.D. at 620 nm)
Rhodococcus Rhodochrous	7	60	0.13
	14	62	0.09
	21	22	0.33
	28	54	0.43
	35	1.48	0.21

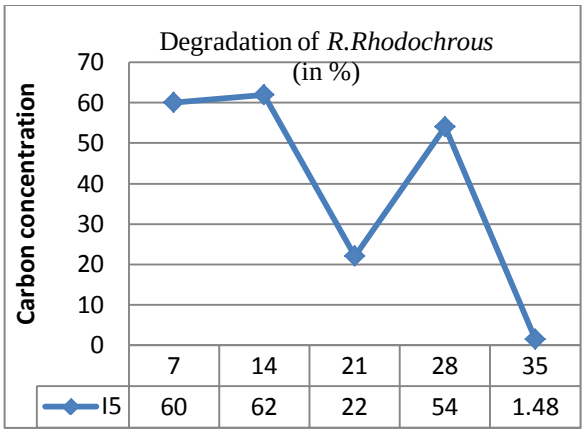

Fig 2- Showing the biodegradation potential of *R. Rhodochrous*

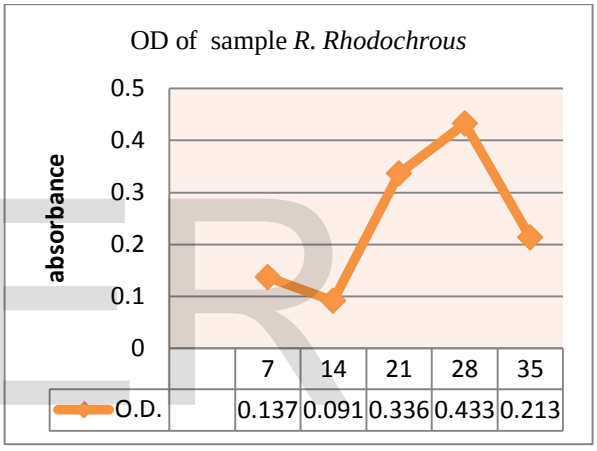

Fig 3- Showing the O.D. at 620nm of *R. Rhodochrous*

After getting the data of GC-MS analysis it was found that there was a number of compounds were identified having different range of carbon atom numbers. These compounds were categorized on the basis of carbon atom numbers to determine the amount of different range of compounds. The heights of peaks of C_1-C_{10} were recorded as 4.45%, 1.02%, 1.56%, 2.98% and 4.18 on day 7,14,21,28 and 35 respectively whereas of C11-C20 were 58.55%, 78.07%, 76.34%, 71.93% and 60.5 on day 7,14 21,28 and 35 respectively (Table 4 and Fig 4) The reduction in the height of peaks further confirmed the degradation by the microorganism (Fig. 5)

Table 4 – Showing the carbon number (range-) wise degradation of compounds obtained from GC-MS analysis of *R. Rhodochrous*

RANGE /DAYS	C_1-C_{10} Peak % area	C_{11}-C_{20} Peak % area	C_{21}-C_{30} Peak % area	C_{31}-C_{40} Peak % area	C_{41}-C_{50} Peak % area	NSO Peak % area	UI Peak %
7-Days	4.45	58.55	22.24	1.9	nil	9.94	2
14-Days	1.02	78.07	3	0.91	0.9	12.01	4
21-Days	1.56	76.34	11.4	1.25	nil	8.13	1
28-Days	2.98	71.93	11.73	1.99	nil	8.19	
35-Days	4.18	60.5	19.82	2.36	0.57	10.1	2

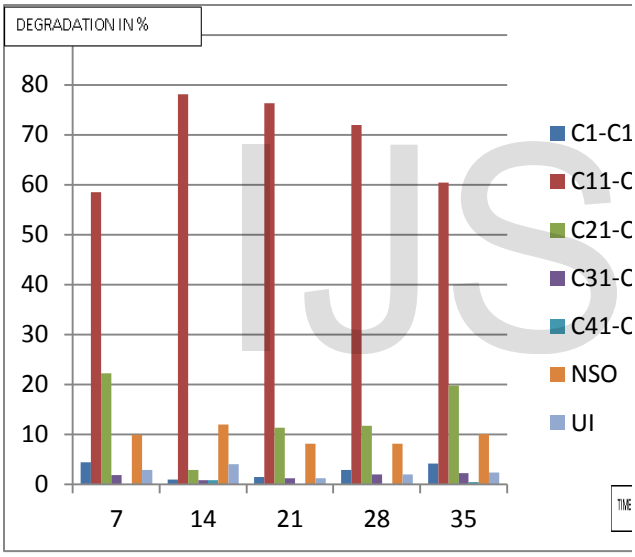

Fig no. 4 - Showing the graphical presentation of carbon range wise peak percentage area of *R. Rhodochrous*

The main principle of bioremediation is to convert highly toxic compounds to their lower toxic forms and completely degrade the compounds having very low molecular weight and lesser toxicity. By the GC-MS analysis, the compounds were screened which were partially degraded by the isolates. By comparing the results of 7-14-21-28 and 35 days of chromatograms the derivatives of compounds were determined. Nonacosane, tetracosane docosane tetrapentacontane and Hexacontane defined as HMW compounds and found converted into their lowers forms such as to octacosane, eicosane, Heneicosane, tetratetracontane, Tetracontane and tetratriacontane etc.

In the process of degradation of PAHs the partial degradation was occur but apart from this some secondary metabolites was also obtained through analysis of data of GC-MS. These secondary metabolites are the compounds which were produced by the microorganisms. Octane, 2,6-dimethyl, Undecane, 2,5-dimethyl, 6-Tridecene, 2,2,4,10,12,12-hexamethyl-7-(3,5,5-trimethylhex, Tridecane, 2-methyl, Tetradecane, 5-methyl, Octane, 3,6-dimethyl, Cyclohexane, octyl for drug for disorders of urinary system, urolithiasis, prostate,kidneys etc., in diagnosis of chronic obstructive pulmonary disease, cell dysplasia, perfume composition, in preparation of primary alcohols, breath test , antiandrogenic, antivirals, for HIV, DNA viruses, immune-stimulants, immune suppressants and synthetic diesel fuel composition respectively (Table 5).

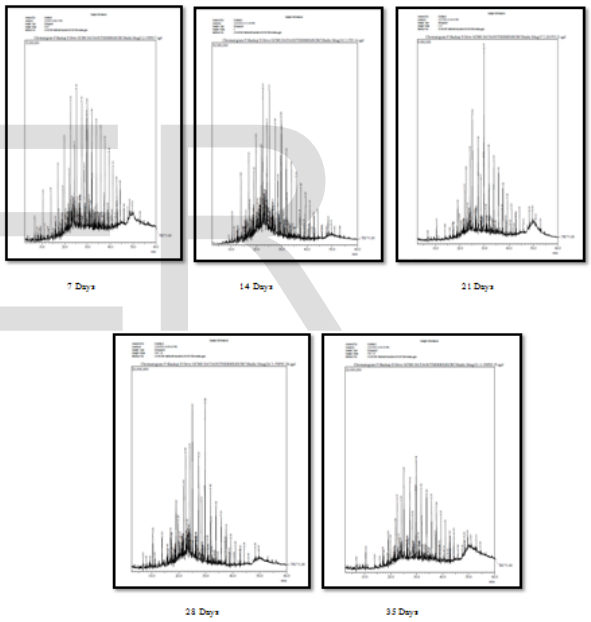

Fig 5- Showing the chromatograms obtained by GC-MS analysis of biodegradation by *R. Rhodochrous* of 7 days, 14 days, 21 days, 28 days and 35 days respectively

Table no 5 - Showing the secondary metabolites of *R. Rhodochrous* [46].

Peak#	R.Time	Area	Area %	Name	M.F.	M.W.	Structure	Activity
4	9.45	4572455	0.28	2-Undecene, 5-methyl-	C12H24	168		not identified
6	11.275	4696263	0.3	Decane, 4-methyl-	C11H24	156		Antiandrogenic ag antioxidants, antidepre for cataracts, antiinfe antivirals, for HIV, DNA
19	19.137	29211246	1.86	Octane, 3,6-dimethyl-	C10H22	142		immunostimulan immunosuppressa
22	20.552	7709034	0.49	Naphthalene, 1,2,3,4-tetrahydro-2,7-dimethyl-	C12H16	160		intermediates used in a
23	21.093	2549790	0.16	Cyclohexane, octyl-	C14H28	196		synthetic diesel fuel com

4 CONCLUSIONS-

Bioremediation is one of the methods which have a great importance in the field of removal of toxins and hazardous substances from the environment in a natural way. It is an enhancement of the natural fate of biodegradable pollutants and therefore a green solution to the problem of the environment pollution. Soil is the ultimate sink for the various types of pollutants. In the present study it has clearly stated that the *Rhodococcus rhodhocorus* was isolated from an oil polluted environment and could utilize numerous compounds derived from 2T engine oil as its sole carbon sources. By the GC-MS data it was clearly confirmed that these toxic hydrocarbons were present in the soil samples out of which most compounds were HMW compounds. These PAHs are of toxicological concern due to which the interest of scientist was evoked during last many years. This observation suggests that the isolate bacteria strain is capable to utilize 2T engine oil as a carbon source. This capability to utilize engine oil as a carbon source makes it a potential biodegrader however; every microorganism has a limitation to metabolite petroleum hydrocarbons. Mixture of different microbial population is always required for the complete biodegradation of complex hydrocarbons. Microorganism are not able to degrade pollutants in natural environment so further research is required for the betterment in the field of biodegradation.

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