Isolation and Identification of Hydrocarbon Degrading Bacteria From Oil Sludge in Oil Producing Area of Basrah, Iraq

Maytham A. Hamdani , Astabraq A. AL Ghazi

***Abstract*— The research aims to isolate indigenous bacteria present in oil sludge from oil tanks and oil pits from Basrah Refinery Factories and study their ability to degrade oil sludge by Gas Chromatographic Analysis. The research started on October 2013 till November 2014. Soil sampling was taken on several places with contaminated soil location from oil tanks and oil pits in Basrah, south of Iraq. The bacteria were isolated by using an enrichment culture and a single colony isolation technique. All isolates were screened for bacterial oil degradation using 0.5% diesel in Bushnell-Hass Mineral Salt medium. The ability of to degrade oil sludge was identified by using Gas Chromatographic Analysis. The predominant bacteria belonged to the genera Pseudomonas spp., Arthrobacter spp., Rhodococcus spp., and Bacillus spp. The most active degraders of oil sludge Pseudomonas sp. and Rhodococcus sp. All the tested isolates show good activity to degrade n-alkanes in oil sludge.**

***Index Terms*— Hydrocarbon Degrading Bacteria, Oil Sludge, Biodegradation.**

I. INTRODUCTION

Sludge oil contains hydrocarbon substance and non hydrocarbon which posses' physical and chemical character that has the potential to decrease the quality of environment, poisoning biological life [1]. During the activities of petroleum industries large quantities of oil sludge generated, resulted in contamination of soil and ground water. Petroleum products contain many hazardous organic chemicals some of which are recognized carcinogenic [2].

Iraq considered as one of the oil – producing countries. Crude oil constitute an important source of energy at raw material for many industries and important source of national income for the country.

In Iraq, petroleum refineries generate solid wastes during refining process. Oil sludge usually contains a considerable quantity of heavy oil [3]. The heavy ends that separate from the crude oil are deposited on the bottoms of storage vessels are known as "tank bottoms", or "sludge" [4]. This stability is enhanced by the presence of polar fractions in the oil, especially resins and asphaltenes that are also responsible for the high viscosity of such sludge. Furthermore, this protective layer creates a favorable environment for microbial corrosion and may shelter a high and divers microbial community [5]. So a thick viscous mixture of sediments, water, oil, and hydrocarbons are called oil sludge, encountered during crude oil refining, cleaning of oil storage vessels and waste treatment [6]. Most of the storage tanks and other vessels in a refinery contain bottom sediments which accumulate over time. These sediments contain solids settled along with the hydrocarbons and water [7], [8], [9].

Common sources of these sludge are storage tank bottoms, oil-water separators, flotation and biological waste water treatment units cleaning of processing equipment, and soil from occasional minors spills on refinery grounds [10], [11], [12], [13], [14], [15]. Both the upstream and downstream operations in petroleum industry can generate a large amount oily wastes. The upstream operation includes the processes of extracting, transporting, and storing crude oil, while the downstream operation refers to crude oil refining processes [16].

Sludge usually accumulates in refineries because of pump failures, desalter failures oil draining from tanks and operation units, periodic cleaning of storage tanks and pipeline ruptures that during cleaning operation, all these wastes area removed and pumped in a nearby pit [9]. The petroleum industry in its production refining, transport, and storage processes, generate oil residues, which have been stored in pits, marshes or open earth pools for many years, causing a high degree of contamination, not only in the storage areas but also in nearby areas [17]. Advanced analysis on oily sludge showed that is composed of 40 – 52% alkanes, 28 – 31% aromatics, 8 – 10% asphaltenes, and 7 – 22.4% resins [18]. When oily sludge accumulation reduces tank storage capacity and this, together with the possibility of corrosion, makes it necessary to periodically remove these deposits [4], [5], [9]. It is a general knowledge that the activities of oil producing companies affect the environment and the health of the people living within the immediate vicinity of the crude oil processing plant.

The attendant hazards may trigger processes that may have adverse effects on the ecosystem of such areas [19]. They have cumulative effect on the Central Nervous System (CNS) leading to dizziness, tiredness loss of memory and headache, and the effect depends on duration of exposure. In severe cases, PAH metabolism in human body produces epoxide compound with mutagenic and carcinogenic properties that affects the skin, blood, immune system, liver, spleen, kidney, lungs, developing fetus, it also causes weight loss [6]. And therefore, for the restoration of oil polluted environments

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(Bioremediation) has become an important method by the use of indigenous or selected microbial flora [20], [21].

Bioremediation functions basically on biodegradation, which may refer to complete mineralization of organic contaminants into carbon dioxide, water, inorganic compounds, and cell protein or transformation of complex organic contaminants to other simpler organic compounds by biological agents like microorganisms. Many indigenous microorganisms in water and soil are capable to degrading hydrocarbon contaminants [22]. The purpose of the present study is to isolate indigenous bacteria present in oil sludge from oil tanks and oil pits from Basrah Refinery Factories and study their ability to degrade oil sludge by Gas Chromatographic Analysis.

II. MATERIALS AND METHODS

A. Collection of oil Sludge

Oil sludge sample (500 g) collected from five sites of South Oil Company, Basrah, Iraq were used for isolation of hydrocarbon utilizing microorganisms. The samples were collected in pre-sterilized sample bottle following aseptic conditions. The samples were stored at 4 °C for further analysis.

B. Medium Used for Screening and Isolation of Hydrocarbon degraders

Microbes were isolated from the oil sludge by using the Bushnell Hass Mineral Salts (BHMS) medium comprising crude oil in 0.1 ml concentrations. Bacteria were maintained on both liquid and solid mineral salt medium with crude oil as the sole carbon source. BHMS contained (per liter of distilled water) 0.2 g of $MgSO_4.7H_2O$, 0.02 g of $CaCl_2$, 1 g of KH_2PO_4, 1 g of K_2HPO_4, 1 g of NH_4NO_3, 2 drops of $FeCl_3$ 60%. The pH was adjusted to 7.0-7.8. the bacteria were isolated by using an enrichment culture and a single colony isolation technique. The isolated cultures were preserved in Nutrient agar and Potato Dextrose agar slants and stored at 4°C for further use.

C. Screening, Isolation and Maintenance of Hydrocarbons degraders

For screening 1 gm of oil sludge sample were suspended and vortexed in 10 ml of sterile distilled water, 1 ml of this sample was used as an inoculum for isolation of hydrocarbons degrading bacteria. Erlenmeyer flasks (250 ml) was taken and 100 ml of BHMS broth medium [23], [24], was transferred to each flask and sterilized 0.1% crude oil was used as the sole carbon source and incubated in shaker orbital incubator at 30 °C at 125 rpm for 5 days, 10 days and 15 days respectively for screening of hydrocarbons degraders. After respective days of incubation the hydrocarbons degradation was studied by gravimetric assay. All these screening experiments have in triplicate.

According to the screening, test the mixed culture that showing highest hydrocarbons degradation was taken for isolation of potent hydrocarbon degraders. The potent hydrocarbon degrading culture (mixed) was diluted (serial dilution) one time (10^{-1}) to decrease down the microbial cell density by which single isolated colonies obtained. From the dilution 1 ml of sample was used as an inoculums and spread over (0.03 g) glucose supplemented BHMS agar plates containing 500 µl of crude oil in it. The plates were incubated at 35 °C for 14 days and observed. Pure representative colonies were isolated the basis of colony morphology and the isolates were preserved in Nutrient agar slants at 4 °C.

D. Identification of Hydrocarbons Degraders

The most potent bacterial hydrocarbon degrader was identified by observing morphological characters, by doing several basic biochemical tests. Different types of biochemical tests were done such as Gram's staining, Indole test, Methyl Red test, VP test, Citrate utilization test, Urease test, Nitrate Reduction test, Triple Sugar Iron test, Gelatinase test, Starch Hydrolysis test, Catalase test, Oxidase test and II2S Production test, growth at 4 °C, growth at 41°C, spore forming, acid fast stain, mannitol fermentation, novobiocin sensitivity, hemolysis, glucose fermentation, motility, Na required for growth, fluorescent, lactose fermentation, morphological features include cell morphology, colony morphology and structural appearance etc .

E. Study of Oil Sludge degradation

2 ml of broth culture as well as was incubated separately Erlenmeyer and flasks (250 ml) containing 100 ml of BHMS at 30°C for 27day with shaking at 120 rpm 0.5 gm of oil sludge as a source of carbon and energy. All the experiments were carried out in three duplicates; each flask was taken out from the incubator in 9 days interval for estimation of residual oil sludge. Hydrocarbon degradation was studied by gravimetric analysis [25]. After desired interval of time, the flasks were taken out and bacterial activities were stopped by adding 1% N-HCl. For extraction of oil sludge 50 ml of culture broth was mixed with 50ml petroleum ether: acetone (1:1) in a separating funnel and was shaken vigorously to get a single emulsified layer. Acetone was then added to it and shaken gently to breaks the emulsification, which resulted in three layers. Top layer was a mixture of petroleum ether, oily from sludge and acetone, clumping cells make middle layer and the bottom aqueous layer contains acetone, water and biosurfactant in soluble form. The lower two layers were spread out while top layer containing petroleum ether mixed with oily sludge and acetone was taken in a preweighed clean breaker. The extracted oily sludge was passed through anhydrous sodium sulphate to remove moisture. The petroleum ether and acetone was evaporated on a water bath. The gravimetric estimation of residual oily sludge left after biodegradation was made weighing the quantity of oily sludge in trade breaker.

III. RESULTS

The present study was undertaken to isolate and identify bacteria indigenous to oil sludge from oil tanks and oil pits in

oil producing areas and refineries in Basrah, the main producing governorate of oil, South Iraq and study the biodegradation activity of these isolates against oil sludge.

Incubation of mineral salt broth medium (Bushnell and Haas broth medium with a sample of oil sludge from oil tanks and oil pits Pseudomonas spp., Arthrobacter spp., Rhodococcus spp., and Bacillus spp. Identification of these genera was done using morphological and biochemical reactions.

The highest number of isolates was achieved from oil sludge samples taken from oil pits was represented by Pseudomonas spp. (23 isolates) from liquid medium and 18 isolates from solid medium.

Highest number of Pseudomonas spp. Isolates were obtained from the oil sludge samples taken from the bottom of oil tanks (table. 1). The second bacterial isolates following Pseudomonas spp. found to belong to the genus Rhodococcus spp. followed by Bacillus spp. and anthrobacter spp. isolates in considering to the number of total isolates (table. 1 & 2).

Statistically, there is a significant difference between the numbers of isolates among the bacterial genera at ($P< 0.05$) and there is a significant difference among solid and liquid media at ($P< 0.05$).

Table. 1. No. of bacterial isolates from oil sludge (Bottom of Tank)

Bacterial species	Number of isolates	
	Solid	Liquid
Pseudomonas spp.	15	17
Bacillus spp.	8	13
Arthrobacter spp.	9	0
Rodococcus spp.	10	17
Total	42	47

$P< 0.05$; $X^2= 33.73$

Table. 2. No. of bacterial isolates from oil pits sludge samples

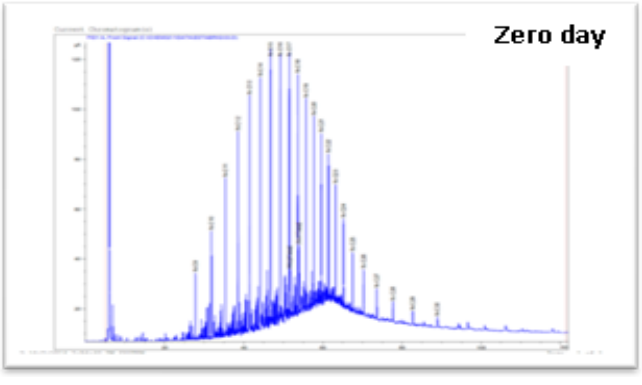
Bacterial species	Number of isolates	
	Solid	Liquid
Pseudomonas spp.	18	23
Bacillus spp.	9	13
Arthrobacter spp.	11	0
Rodococcus spp.	13	14
Total	51	50

$P< 0.05$; $X^2= 36.778$

Gas of chromatography analysis of the fate of oil sludge after inoculating broth medium with isolated bacterium and following degradation of oil in sludge by time (up to 27 days) showed that the loss in n-alkanes was highest by *Pseudomonas* sp. Isolates froim oil sludge taken from oil pits (79.9%) and (73.7) reduction in n-alkanes from oil sludge sample taken from the bottom of oil tank (fig 1 & 2, Table. 3) followed by *Rhodococcus* sp. Isoaltes reduction in n-alkanes by 74.8% of oil pit sludge and 55.6% of oil sludge from the bottom of oil tank samples (Fig. 3 & 4, Table. 3).

Tabl. 3. Loss of n-alkanes (ng/g) and percentage of degradation at different incubation time from oil tank sludge and oil pit sludge

Days	Pseudomonas sp			
	Oil pit		Bottom of tanks	
	BD%	Total con	BD%	Total con
Zero	0.00	264.783	0.00	360.009
Day 9	43.37	149.925	28.18	258.526
Day 18	65.02	92.594	51.05	176.221
Day 27	79.97	53.019	73.79	94.345
Days	**Rhodococcus sp.**			
	Oil pit		Bottom of tanks	
	BD %	Total con	BD%	Total con
Zero	0.00	264.783	0.00	360.009
Day 9	37.40	165.749	31	248.398
Day 18	53.72	122.523	54.03	165.487
Day 27	70.42	78.322	70.28	106.967
Days	**Arthrobacter sp.**			
	Oil pit		Bottom of tanks	
	BD%	Total con	BD%	Total con
Zero	0.00	264.783	0.00	360.009
Day 9	30.79	183.236	31.32	247.241
Day 18	49.39	133.986	54.80	162.700
Day 27	64.81	93.176	65.71	123.423
Days	**Bacillus sp.**			
	Oil pit		Bottom of tanks	
	BD%	Total con	BD%	Total con
Zero	0.00	264.783	0.00	360.009
Day 9	21.91	206.726	26.29	265.492
Day 18	38.41	163.059	38.76	220.437
Day 27	55.05	119.002	51.53	174.467



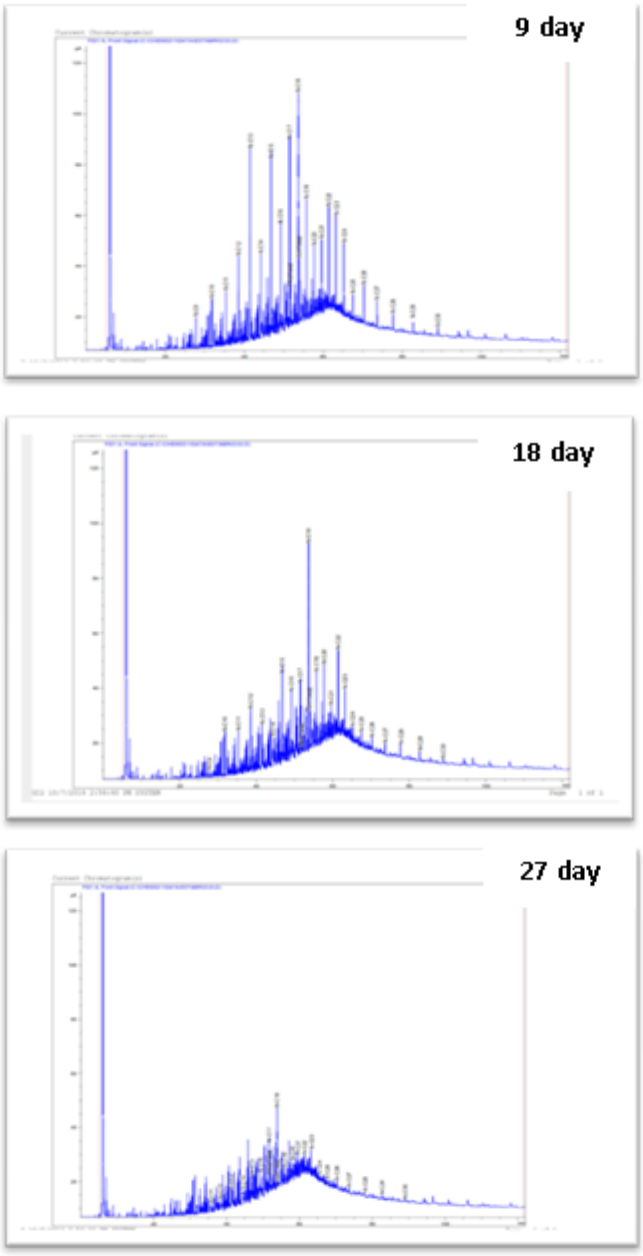


Fig.1: Gas chromatography analysis of oil in oil pit sludge treated with Pseudomonas isolate at different incubation time

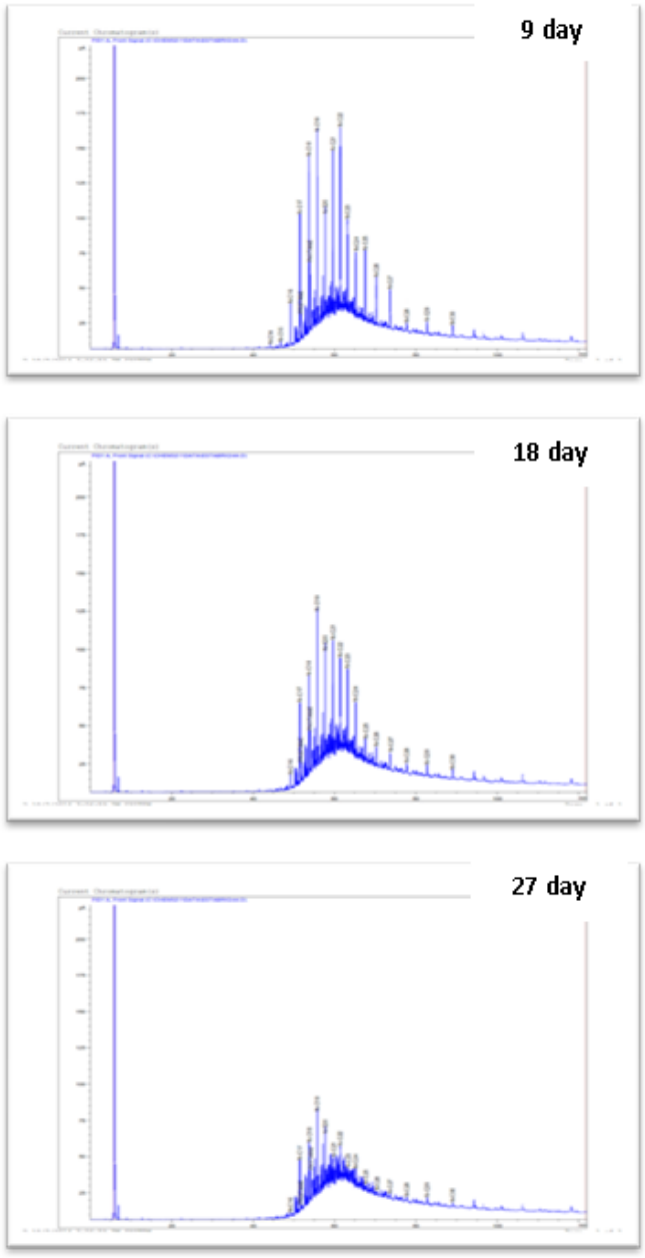
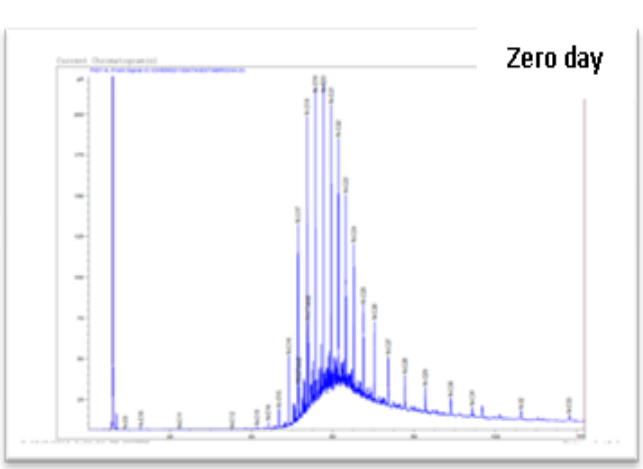
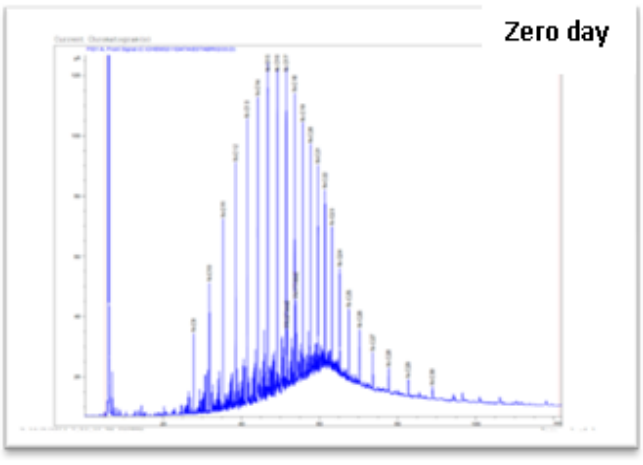


Fig.2: Gas chromatography analysis of oil in oil pit sludge from bottom of oil tank treated with Pseudomonas isolate at different incubation time





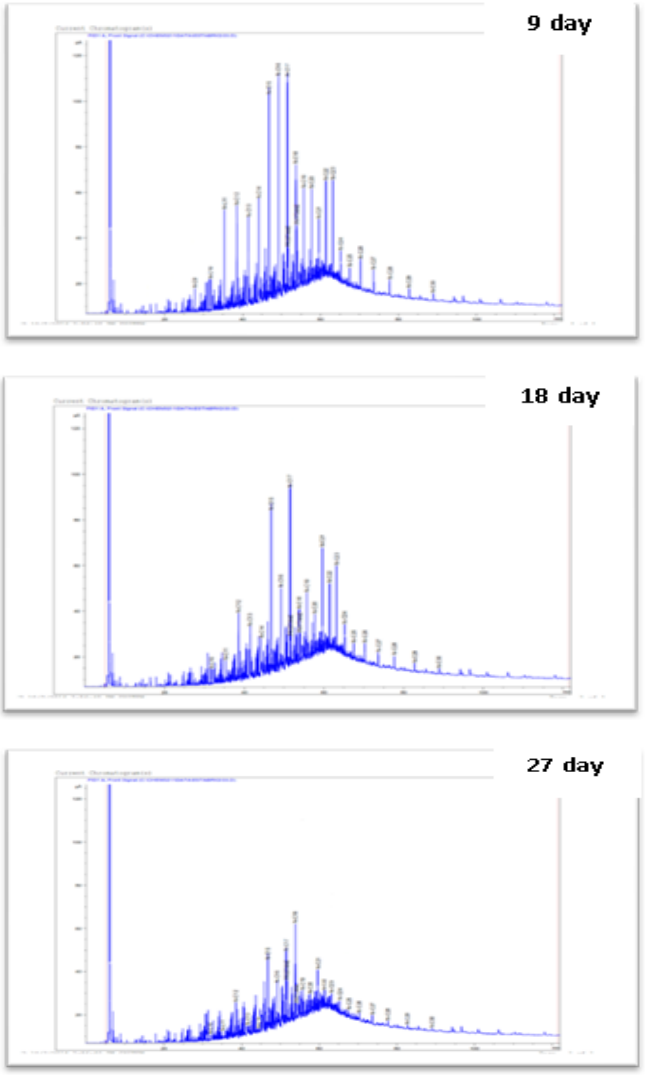
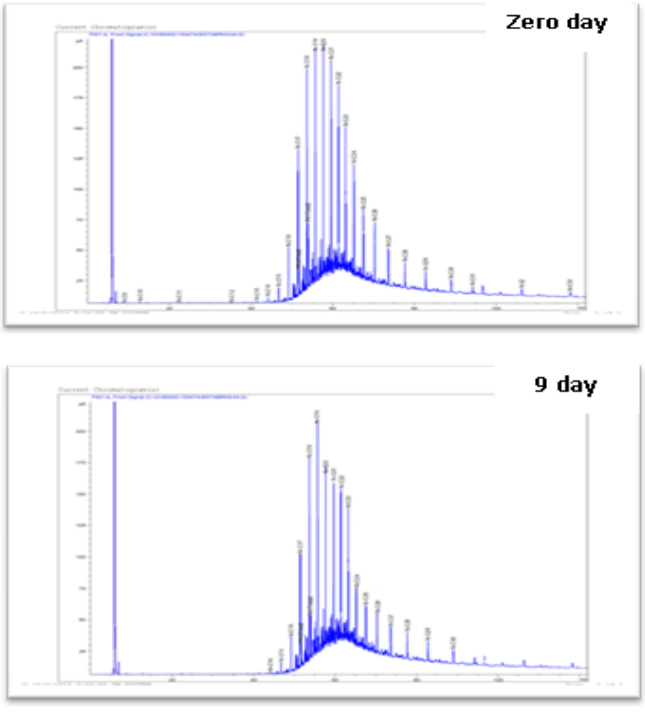


Fig.3: Gas chromatography analysis of oil in oil pit sludge treated with Rhodococcus isolate at different incubation time



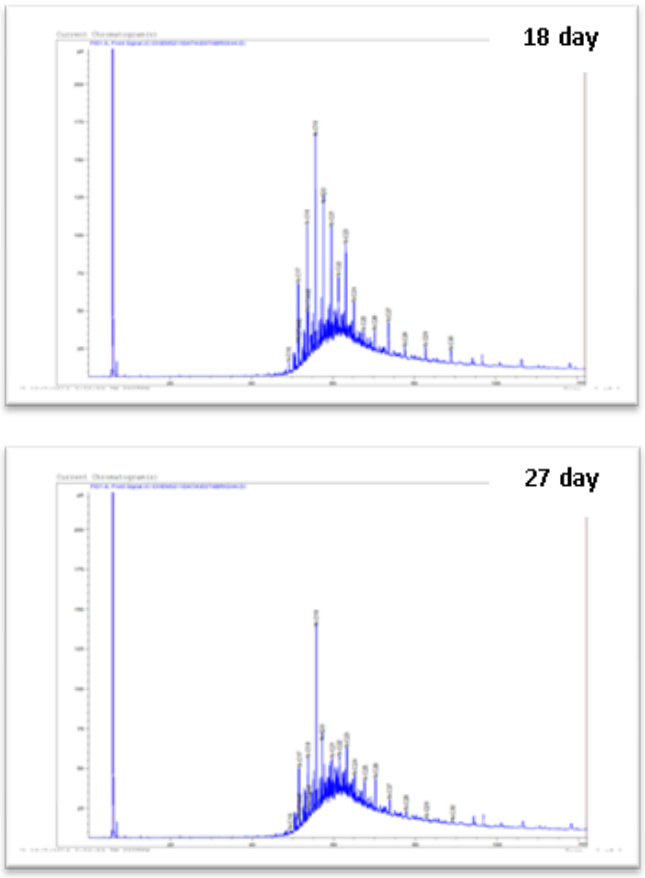


Fig.4: Gas chromatography analysis of oil in oil pit sludge from bottom of oil tank treated with Rhodococcus isolate at different incubation time

IV. DISCUSSION

Despite that Iraq is one of oil producing countries with many refineries distribution in the country with other petrochemical industries, as a result accumulating large quantities of waste – products one of these, oil sludge which is well known to be harmful to human life and to local biota. No previous study focus on oil sludge degradation in Iraq. The present study was carried out on oil sludge samples taken from oil tanks and oil pits revealed the isolation of many bacteria belonging to four genera dominated by Pseudomans and Rhodococcyus isolates. It seems that these groups of bacterial isolates is well adapted to these hard environments and can thrive in petroleum wastes.

Definitely, this ability to survive in these environments depending on that they possess diverse metabolic pathways that able them to use carbon and energy. Previous studies mentioned the isolation of oil sludge degrading bacteria from oil tank, the isolates were identified as Arthrobacter aurescns and Pseudomonas aeroginaosa it has been found that these resident bacteria in oil sludge are able to degrade heavy petroleum compounds [26]. In another study, many genera of bacteria were also isolated from sludge oil samples among them to Bacillus and Pseudomonas [27]. It seems that Pseudomonas and Rhodococcus isolates, in the present study

are considered as active degraders of oil sludge, both attack n-alkanes efficiently.

Previous work on oil sludge mentioned that strains of Pseudomonas putida and Rhodococcus sp. have been shown to be successful biodegraders of petroleum sludge located in Czech republic and found that there is a changing values of C10 – C40 hydrocarbons detected in liquid culture was observed [28]. Pseudomonas aeruginos that isolated from oil sludge in Nigeria was found to have the highest rate of oil degradation and Bacillus substilis was the least one [29].

V. CONCLUSION

We wish to emphasize that the our bacterial isolates can be used for degradation of oil sludge and represent promising solutions to the problems of oil pollution.

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