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THE EFFECT OF BIOSURFACTANT (RHAMNOLIPID) ON CONTAMINATED SOIL BY PETROLEUM HYDROCARBONS FROM OIL SPILLS IN EL-ZAWIA CITY –LIBYA

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ABSTRACT:

The petroleum contaminations are identified as challenges for environments and human health, where petroleum products have caused serious problems in the contamination of the soil and groundwater. Recently many technologies have been put into use to clean such pollution.

In this study, the biosurfactant has been used for washing contaminated soil to enhance biodegradation of the contaminants. The amount of contamination of soil was assessed by using Adenosine Tri-Phosphate (ATP) bioluminescence technique. The soil was treated with different concentrations of the Rhamnolipid solutions (0.5, 1, and 2%) for different incubation time and finally the effect of the biosurfactant (Rhamnolipid) in enhancing degradation of contaminated hydrocarbon soil was conducted by using gas chromatograph technique (GC) before and after treatment. The measurement of ATP showed an increase in the growth of the microorganism in the presence of different concentrations of rhamnolipid while a decrease in the control. The GC analysis confirmed that rhamnolipid enhanced the degradation of a major portion of sorbed hydrocarbon from contaminated soil relative to treatment without rhamnolipid. 1% and 2% rhamnolipid were the most effective concentration in hydrocarbon biodegradation.

INTRODUCTION:

In the last decades, tremendous development has occurred in all industrial fields and as a consequence, the appearance of new sources of pollution affecting the human health such as industrial wastes and oil spills where the yearly influx of petroleum pollutants has been estimated to be as high as 10 million tons and the petrol spills may contaminate groundwater well below the surface of the ground[1]. In general, the wastes are categorized into two main groups: liquid (liquid chemicals, paint, contaminated water, solvents, sludge, waste oil)[2,3] and solid wastes (scrap parts, paper and documents, wood, cardboard, plastic, pesticides, straw), varying in nature, composition and calorific value[4, 5].

Many compounds in crude oil are environmentally benign, but significant fractions are toxigenic, mutagenic or growth inhibitory compound. The last two are the ones we are most interested in removing or destroying in an oil spill. In fact, there are three major methods used for the "clean up" of oil spills namely; containment and removal method, application of dispersants method, and bioremediation method, when dealing with biotechnology, "bioremediation method" is the most recommended[6-8].

Bioremediation is a relatively cheap clean up technology that offers great pledge in converting the toxigenic compounds to nontoxic products without further disruption to the local environment[8,9]. *In-situ* bioremediation by indigenous microbial population is an increasingly popular option for clean-up of sites with readily degradable contaminants[8-10].

The effectiveness of this method depends upon a variety of factors including: physical and chemical properties of soil, seawater and oil, the environmental conditions and the biota itself[11,12]. The most limiting factors of biodegradation are probably the presence of bacteria with appropriate metabolic capabilities, the availability of moisture, and nutrients especially Nitrogen, Phosphorus and Potassium (NPK), appropriate pH (6.8-8.5), electron acceptors such as Nitrogen, Phosphorus and Oxygen. In view of the fact that the contaminants of concern in crude oil are readily biodegradable under appropriate conditions, the success of oil-spill bioremediation depends on our ability to establish those conditions in the contaminated environment[12,13].

In fact, soil bioremediation has many challenges[14]. One of those challenges is the difficulties in treating soil, especially when pollution is distributed over a large area because crude oil hydrocarbons are highly hydrophobic materials that can hardly be degraded or decomposed due to their poor availability to microorganisms[14,15]. The solubilization of hydrocarbons may be restrained by their existence in oil matrix and may be also dependent on the attachment of microorganisms to oil surface. Thus the direct contact of cells with the surface of oil is thought to be important in the bioremediation of contaminated area with crude oil[16]. Surface-active agents (microbial surfactants/ biosurfactants) can be used as alternative solutions where microorganisms have biosurfactant producing capabilities.

In this case, there is an increased mobility into the cell and localized effects where the bacterial coat creates an enhanced microbial habitat[17]. Some of the bacteria, such as *Pseudomonas aeuroginosa*, produce biosurfactant (rhamnolipid) from a carbon source feedstock, e.g. carbohydrates, hydrocarbons, oils and fats[10,16].

These biosurfactants are amphiphilic molecules, consisting of hydrophilic and hydrophobic domains, which tend to partition preferentially at the interface between fluids of different degrees of polarity and hydrogen bonding[18,19]. Our previous work has concluded that the isolated bacteria from the soil of local

site were found to have the ability of producing the biosurfactant (Rhamnolipid) (Figure 1) in the form of biological molecules[20]. In this study, the biosurfactant has been used for washing a hydrocarbon contaminated soil to enhance biodegradation of the contaminants.

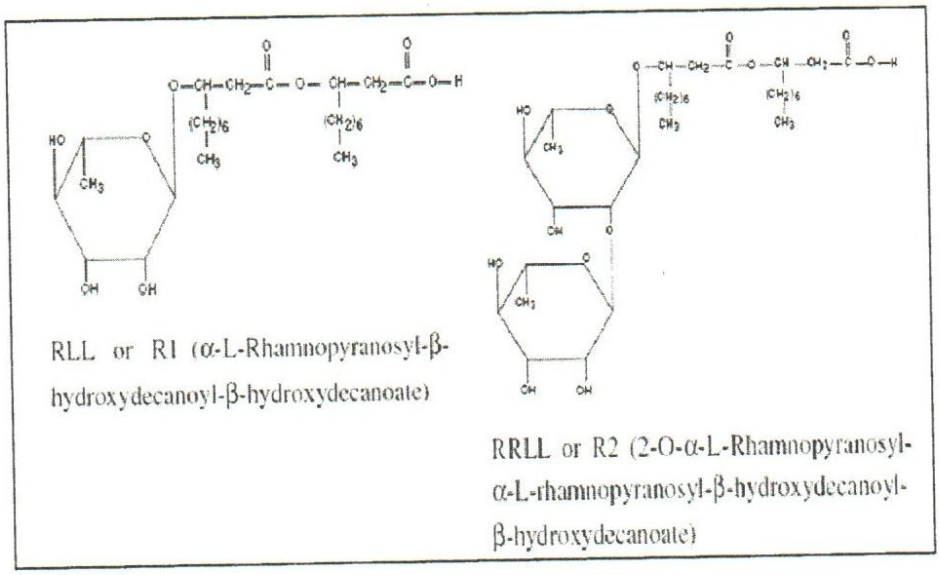

Figure 1: Structure of Rhamnolipid

MATERIALS AND METHODS:

1-The characteristic of soil samples and history of site:

The soil was sampled from a polluted site located near El-zawia city-Libya. Eight contaminated soil samples were collected from the surface in autoclaved bags and container. The contamination occurred due to a leakage from a pipeline transferring some of petroleum products (such as gas oil) from El-zawia refinery. The soil pH was 7.8 (determined by pH meter, after mixing 1 g of soil with 100 ml sterile water) and temperature was between 20-40°C. After removing plant residues and stones through sieving, 10 g of soil were kept in each flask. Soil physical characterization tests were carried out and the composition of soil mineralogy was determined by microscopic examination where the results showed the presence of clay and rock fragments and quartz, besides the plant residues, the soil was highly contaminated by hydrocarbons.

2-Adenosine Tri-Phosphate (ATP) bioluminescence technology:

The ATP tests were used for each treated soil samples after different period of incubation time. Adenosine Tri-Phosphate (ATP) was the most popular biochemical index as it was ubiquitous in cellular life forms and can be detected rapidly using bio-luminescence reactions. Kits from Biotrace (Aqua- Trace®) contained preparations of Firefly Luciferase, with a few simple steps, produced results within

seconds. The assay was based on the amount of light produced from the reaction of Luciferase together with its co-factors D-Luciferin and Oxygen in the presence of ATP according to the following reaction:

$$\text{Luciferase+D-Luciferin+}O_2\text{+ATP} \rightarrow \text{Luciferase+oxy-Luciferin+}CO_2\text{+AMP+PPi+Light}$$

The amount of light was proportional to the concentration of ATP in the original sample. The ATP concentration in a sample was in turn, related to the number and types of organisms within the sample. Thus, a relative index of the amount of contamination can be generated using Firefly bioluminescence within few minutes of sampling.

Due to the time consuming and less specificity of the "plate count" traditional technique, the ATP measurements technique being mostly recommended[21].

3- Solubilization of crude oil:

The effect of rhamnolipid on enhancing solubility of Libyan crude oil (ABUTAFIL CRUDE OIL) was visually examined by mixing 1 ml of crude oil and 1 ml of rhamnolipid solution at concentration of 2% in a covered test-tube. The content in the tube was vortex for 2 minutes at room temperature.

4-Soil washing study:

The soil washing study was conducted to observed hydrocarbon removal with different concentrations of rhamnolipid solutions (0.5, 1, and 2%). The soil washing methods described in (22,23), was applied for this study. The study was carried out at 30°C, shaken at 150 rpm from one to two weeks with different concentration of rhamnolipid. After that the samples was analyzed with Gas chromatography (GC).

5-Oil content in soil samples and Gas chromatographic (GC) analysis:

A-Sample Extraction:

To extract the petroleum oil from soil samples, two methods were used as following:

*Gravimetric method:

Each treated sample was filtrated with a filter paper (Waterrnan l) and the filtrate was collected and poured in a separating funnel[24-26]. After that 30 ml of dichloromethane was added and mixed thoroughly and the solution is left to settle. The lower layer was collected in cleaned-dried beaker while the upper layer was washed two more times with a 15 ml of dichlorornethane. The removal of water from extracted solution was done by using 15 gm of anhydrous sodium sulphate. To measure total petroleum hydrocarbons (TPH), pre-weighed cleaned dishes (placed in a moisture-free environment) were used to collect the lower layer and allowed to evaporate solvent (dichloromethane) completely. The dishes were weighed and the percentage of extractable organic material was calculated as the following:

$$\text{Extractable organic material \%} = \frac{\text{Difference in dishes weight}}{\text{Volume of the pre-treated filtrate}} \times 100$$

Finally; the residue was washed separately with 3 ml of dichloromethane and collected in different bottles and the analysis of hydrocarbons was detected using a gas chromatograph.

*Soxhlet Method:

Extraction procedure of organic material from the soil was also carried out using Soxhlet method[27,28], (SER 148 solvent extraction, VELP SCIENTIFICA) as the following; The soil was dried overnight in the oven at a temperature of 130°C and 7 g of dried soil folded in a filter paper and installed in a thimble. Cleaned beakers (specific to the Soxhlet system), (Sigma Aldrich, St Louis, MO, USA) were weighed and 70-l00ml of dichloromethane was added in each beaker. The idea is simplified into 3 steps: An immersion followed by washing and finally a 15 minutes recovery step where the overall temperature during this process may reach up to 130 °C (for the evaporation of the solvent).

*Detection of hydrocarbons distribution:

After TPH was extracted with dichloromethane which used as extraction solvent, following the procedure recommended in U.S.EPA Test Methods 351^0C[29], the quantity of TPH in extract was analyzed using a gas chromatograph with a flame ionization detector (GC-FID, Varian CP-3800, Palo Alto, CA, USA) equipped with a 30m capillary column (Supelco SPB™-5, 0.53 mm I.D., 1.5 µm film thickness). The temperature conditions of GC-FID were operated at 250°C for injection port, 300°C for detector, and an oven temperature program of 45°C (held for 3 min) to 300°C (held for 10min) at a rate of 12°C/min. Nitrogen was used as the carrier gas at a flow rate of 5 mL/min.

6-Statistical analysis:

Results were expressed as a mean±SD for triplicate samples. The results statistically analyzed and the difference among the groups was examined by analysis of variance using one-way analysis (ANOVA) and post-tests carried out using Fisher's pair wise comparisons *via* the statistical package Minitab TM 13 windows. Statistically significant differences were considered at $P < 0.05$.

RESULTS AND DISCUSSION:

1-Adenosine Tri-Phosphate (ATP) bioluminescence technology:

The measurement of ATP reflected the viability of microorganisms in each treated sample. Figure 2 shows that in control sample, the growth of microorganisms was high after one week and that probably due to the presence of the nutrients (the minerals in the media) which enhanced the growth and then dramatically decreased in the second week as a result of decreasing in nutrients. With respect to the effect of concentration of rhamnolipid on hydrocarbons biodegradation in soil-water system after one week comparing with

untreated sample, the activity of microorganisms was modestly increased and this is possibly due to effect of the rhamnolipid specially at 0.5% of rhamnolipid whereas the amount of ATP in presences of other concentration of rhamnolipid (1% and 2%) was not significant when compared with the control (Fig. 2).

The results of the second week, on the contrary, showed substantially an increase in the growth of the microorganism in the three different concentrations of rhamnolipid while a decrease in the control. The increase in the activity was an indicator of the hydrocarbons biodegradation as long as it was the sole source of carbon in the media. On the other hand, the microbial growth and hydrocarbons biodegradation in the presence of 1% and 2% rhamnolipid was 2-fold greater than that observed in the control (Mineral Salts Medium; MSM). The microbial growth and hydrocarbon biodegradation in the presence of 0.5% rhamnolipid was much better than that observed in the control. The highest growth level and biodegradation of hydrocarbon was seen in the presence of 1% rhamnolipid. Further increase of rhamnolipid concentration to 2%, however, does not improve hydrocarbons biodegradation. It seemed to be that the optimum rhamnolipid addition for microbial growth and hydrocarbons biodegradation exists at 1% concentration. Furthermore, the amount of ATP in the three different concentrations of rhamnolipid in the second week was high when compared with first week. This would probably explained on the bases that the incubation of rhamnolipid for longer time enhanced the biodegradation of hydrocarbons (Fig. 2).

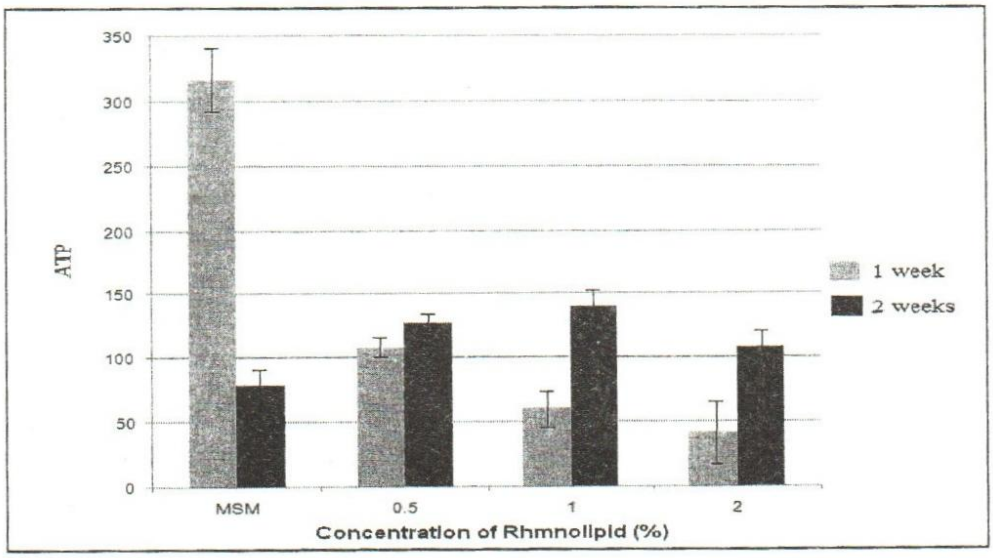

Figure 2: ATP measurement reflected the viable of microorganisms in each sample. Different concentrations of Rhamnolipid were tested [0.5%, 1% and 2%]. The soil with the mineral salts medium (MSM) was used as control

2-Solubilization effect of rhamnolipid on the crude oil:

The effect of rhamnolipid was tested on Libyan crude oil obtained from El-zawia refinery. Figure 3 shows the strong effect of 2% rhamnolipid at room temperature on the Libyan crude oil. The crude oil at room temperature sticks on the glass sides of the tube (Fig. 3,1) but upon the addition of rhamnolipid the status of the crude immediately changed indicating the solubilization effect (Fig. 3, 2).

3-Oil content in soil samples:

The laboratory analysis was performed on treated and untreated soil samples using gravirnetric method and gas chromatography. The total petroleum hydrocarbons (TPH), in water and soil, in each sample were calculated. From table 1 it can be seen that in 1% and 2% rhamnolipid, the concentrations of hydrocarbons in soil and water was significantly decreased when compared with the efficiency observed in 0.5% rhamnolipid and control. 1% rhamnolipid seemed to be the most effective on the solubility of the hydrocarbons as confirmed by the low concentration of hydrocarbons (Table 1). The results were in agreement with our previous results[20].

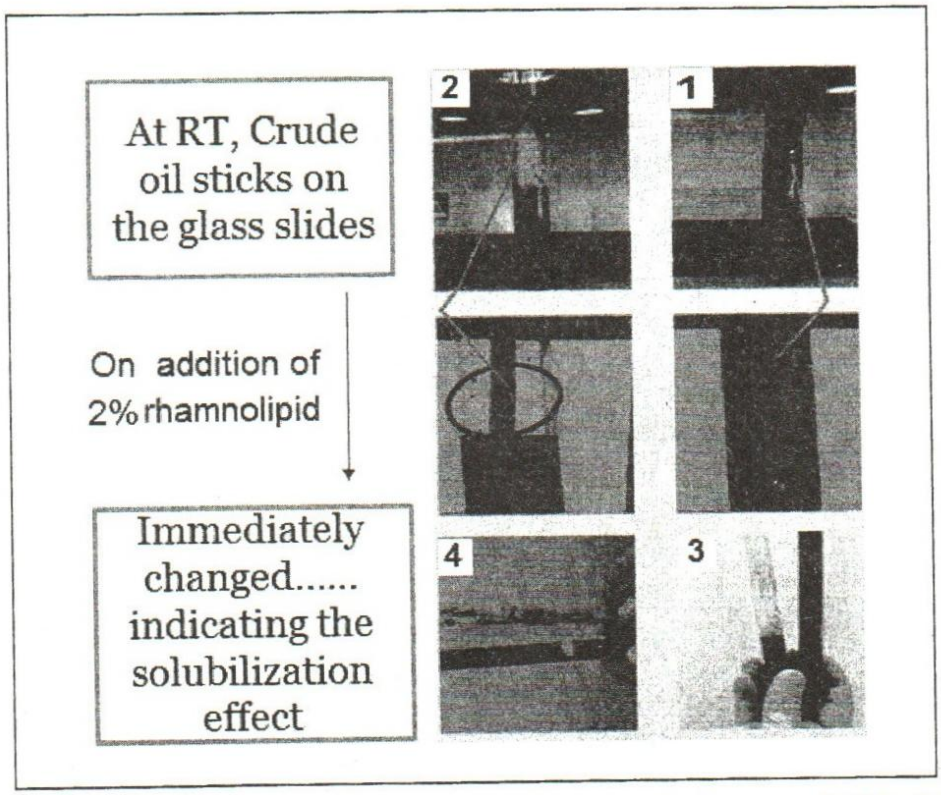

Figure 3: The solubility of crude oil increased after being treated with rhamnolipid for 1 hour; (1) Control, (2) treated oil, (3) the upside-down tube (4) horizontal tube showed that the viscosity of crude oil decreased after treatment

Table 1: Shows the concentration of hydrocarbons in soil and water before and after treatment with different concentration of Rhamnolipid and different incubation time

Concentration of Rhamnolipid	Concentration of hydrocarbons at first week			Concentration of hydrocarbons at second week		
	Water (ppm)	Soil (ppm)	Sum (ppm)	Water (ppm)	Soil (ppm)	Sum (ppm)
Control (untreated soil)	1741.924±11					
0.5%	1355.55±10	378.571±9	1734.12±9	1046.81±11	410.10±13	1456.91±12
1%	788.88±12	364.566±3	1153.45±7	393.75±9	352.44±11	746.19±10
2%	1150±15	278.571±7	1428.6±11	414.28±5	956.55±6	1370.83±5

1% rhamnolipid showed great effect on the solubility of the hydrocarbons.

4-Gas chromatography (GC) results:

The GC analysis has been used to assess the biodegradation effect of rhamnolipid on hydrocarbons chain. Figures 4 and 5 illustrated variable changes in the distribution of short and medium carbon chain hydrocarbons and a decrease in the long chains (C22 to C27).

From figure 4 it can be seen that after one week of treatment, the biodegradable effect of 2% rhamnolipid was statistically significantly (p ▯ 0.05) higher than the biodegradable efficiency of 1% and 0.5% rhamnolipid. The highest degradation level was seen at C19 and this was approximately 3-fold higher than the effect of 1% rhamnolipid and 4-fold higher than the effect of 0.5% rhamnolipid.

A statistically significant difference of p ▯ 0.05 was also observed between the 2% rhamnolipid and other concentration of rhamnolipid at C20, C21 and C22. Moreover, the biodegradable effect of 2% rhamnolipid at C14-C17 was statistically indistinguishable from the biodegradable effect of 0.5% and 1% rhamnolipid. For the 1% and 0.5% of rhamnolipid (fig4), the results also showed clearly reducing in the hydrocarbon chains of long chains but were less than that observed in 2% of rhamnolipid. Further proof of the hypothesis that rhamnolipid is crucial to the degradation of heavy hydrocarbons chain was found after two weeks of treatment (Fig. 5). The results of figure 5 showed that 2% of rhamnolipid were the most effective in removing hydrocarbons. The highest degradation level was seen at C17 and this was 2-fold higher than 1% rhamnolipid and 4-fold higher than 0.5% rhamnolipid. Comparing with the control (MSM only), the level of hydrocarbons in the presence of 2% rhamnolipid at the same carbon (C17) was significantly high, which was 2-fold higher than the control. Furthermore, for C14-C16 and C18-C22 the biodegradation effect of 2% rhamnolipid was significantly higher than that observed with 0.5% and 1% rhamnolipid. On the other hand, the presence of rhamnolipid at concentration of 0.5% and 1% was also effected on heavy hydrocarbons chain but it was less than 2% rhamnolipid. The highest degradation effect of 0.5% and 1% rhamnolipid (Fig. 5) was

also seen at C17. However the degradation effect of 1% rhamnolipid was significantly more than the effect that produced by 0.5% rhamnolipid. For the use of the biosurfactant in enhancing the solubility of hydrocarbon, it was found that the low concentration was the most recommended.

From Figs. 4 & 5, the results demonstrated that an increase degradation in long chain hydrocarbons was clear which might reflect the effect of rhamnolipid on the degradation of the heavy chains (longer chains, C25-C27) and accumulating them as lighter ones (short and medium hydrocarbons chain).

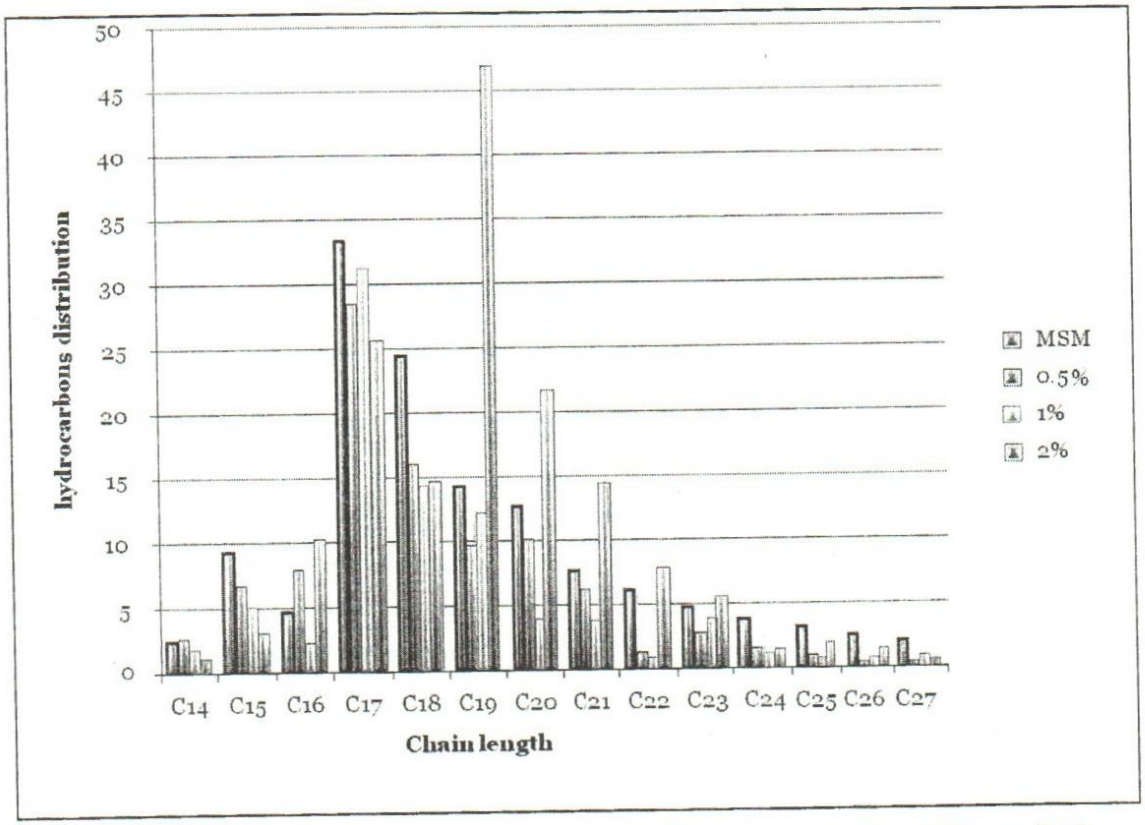

Figure 4: GC analysis after a week of treatment with different concentration of Rhamnolipid. MSM was used as a control. Increase in some fractions in long chain hydrocarbons is clear which might reflect its effect on the degradation of the heavy chains (longer chains, C25-C27) and accumulating them as lighter ones

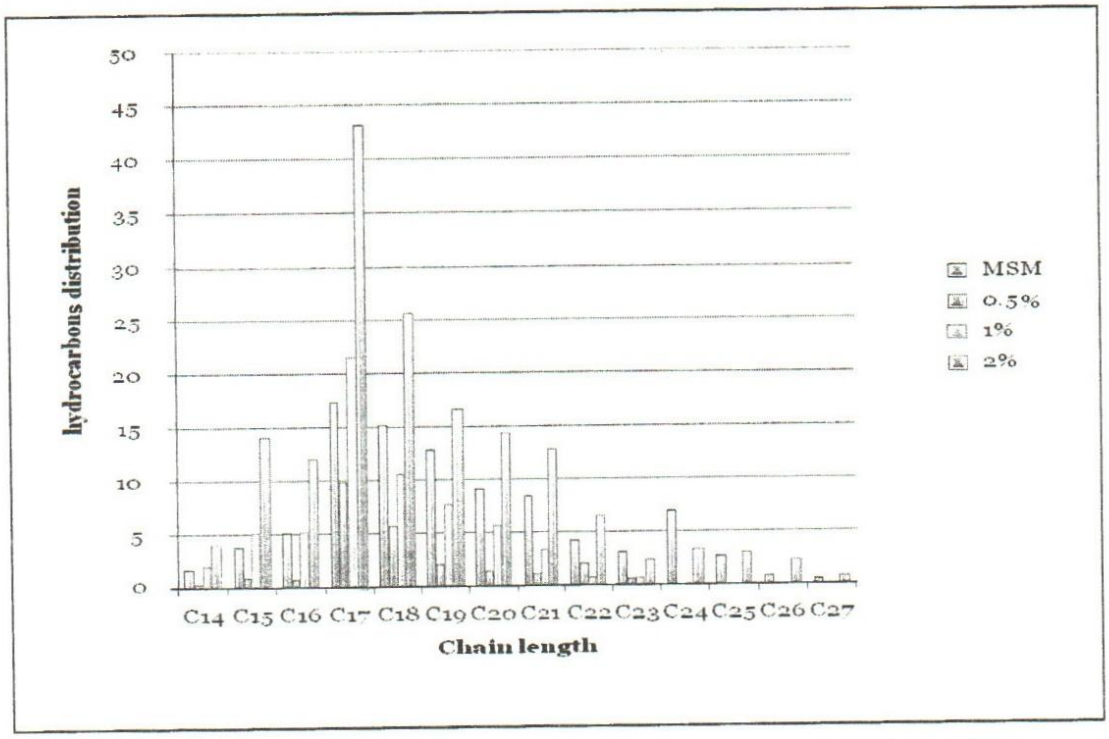

Figure 5: GC analysis after 2 weeks of treatment with different conc. of Rhamnolipid. MSM was used as a control. The results showed a variation in the distribution of carbon chain and a decrease in the long chains (C22 to C27). Where 1% was most effective on HC biodegradation than the control

CONCLUSION:

Current study presents experimental results that evaluate the capability of rhamnolipid on enhancing biodegradation of hydrocarbon-contaminated soil systems. It is concluded that rhamnolipid enhanced the removal of a major portion of sorbed hydrocarbons from contaminated soil compared to the experiments without biosurfactant addition. Adding rhamnolipid to hydrocarbon-contaminated soil systems at different concentrations, in general, benefits hydrocarbon emulsification and, therefore, enhances the degree and rate of hydrocarbons degradation. Total biodegradation of hydrocarbon is ultimately determined by the desorption rate of the contaminant, which is significantly increased by increasing rhamnolipid concentration. This is of significance for the use of rhamnolipid for soil remediation and the capability of rhamnolipid to increase the bioavailability of sorbed hydrocarbons.

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تأثير منشط السطوح البيولوجي (Rhamnolipid) على التربة الملوثة بالهيدروكربونات النفطية الناتجة من التسرب النفطي بالقرب من مدينة الزاوية – ليبيا

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**معهد النفط الليبي للأبحاث، قرقارش ، طرابلس- ليبيا

من المعروف أن التلوث البترولي من التحديات التي تواجه البيئة وصحة الإنسان، حيث أن المنتجات النفطية تسبب مشاكل خطيرة في تلوث التربة والمياه الجوفية. مؤخراً استخدم العديد من التقنيات لتنظيف هذا التلوث. في هذه الدراسة تم استخدام منشط السطوح البيولوجي (Rhamnolipid) لغسل التربة الملوثة بالهيدروكربونات النفطية، وذلك لتعزيز التحلل البيولوجي للملوثات. فلقد تم أولاً قياس مدى تلوث التربة، وذلك باستخدام تقنية التلألؤ البيولوجي لقياس كمية الادينوسين ثلاثي الفوسفات (ATP)، ثم من بعد ذلك تمت معالجة التربة بتركيزات مختلفة من منشط السطوح البيولوجي (٠,٥، ١، ٢%) وفي فترة حضانة مختلفة.

وأخيراً تم تحديد تأثير Rhamnolipid في غسل وتعزيز تكسير (Degradation) الهيدروكربونات الملوثة للتربة، وذلك باستخدام تقنيات كروماتوجرافياً الغاز (GC) قبل وبعد العلاج.

أظهرت نتائج قياس ATP زيادة نمو الكائنات الحية الدقيقة في وجود تركيزات مختلفة من Rhamnolipid في حين لوحظ انخفاض فى النمو العينات التى لم يتم إضافة منشط السطوح البيولوجي إليها (Control)، كما أكدت نتائج كروماتوجرافياً الغاز أن Rhamnolipid عزز من تكسير جزء كبير من الهيدروكربونات الممتص والملوثة للتربة بالمقارنة بالعينات التى لم يتم معالجتها. وقد تبين أيضاً أن Rhamnolipid عند تركيزي ١٪، ٢٪ كان أكثر فعالية في تحلل المواد الهيدروكربونية.