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Determination of hydrocarbon pollutants bioremediation using microbial consortium via Capillary Gas Chromatography

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Abstract:- Capillary gas chromatography (CGC) is a high sensitive and powerful analytical tool used for evaluating the hydrocarbons in the environmental pollution caused by crude oil spills. The weight percentage of the crude oil was evaluated quantitatively by CGC according to the internal standard method. The three studied strains Penicillium chrysogenum (RS-F7), Streptomyces rimosus (EPRI-A4) and Saccharomyces cerevisiae (Ferm-Bam-Y3) singly and in combination were selected from Forty one crude oil utilizing microbial isolates and subjected for the degradation of petroleum crude oil. The effect of both different pH values and nitrogen sources on the bioremediation was investigated. Complete degradation of petroleum pollutants was achieved using consortium between RS-F7 and Ferm-Bam-Y3 at pH 9 and Potassium nitrate as nitrogen source. Ammonium sulphate and PH 9 are the preferred conditions at which the consortium between RS-F7 and (EPRI-A4) exhibit high efficiency on the complete degradation of paraffinic hydrocarbons.

***Keywords:-*Capillary gas chromatography, internal standard method, Saccharomyces cerevisiae Penicillium chrysogenum, Streptomyces rimosus, pH values and nutrient addition.**

I. INTRODUCTION

Bioremediation is a technology using microorganisms (bacteria, fungi, actinomycetes and yeast) to clean up chemically contaminated soil. This technology is based on the activation of microbial degradation of pollutants in contaminated sites by optimizing environmental factors [1, 2]. Bioremediation is slow, but has advantages in comparison to chemical, physical and thermal remediation techniques [3]. The optimal pH for microbial activity is usually between 5.5 and 8.5. However, biodegradation at some sites with extreme pH values may occur as a result of the activity of unique microorganisms which grow in very acidic or alkaline environments [4]. Additions of organic and inorganic nutrients, for example, have been found to increase microbial metabolism of some target pollutants or decrease metabolism of others [5]. Also, addition of specific forms of nitrogen (NH4+, NO3-) have also been found to vary effects on the metabolism of organic compounds [6]. Individual microorganisms can metabolize only a limited range of hydrocarbons substrates, so assemblages of mixed populations with overall broad enzymatic co parities are required to bring the rate and extent of petroleum biodegradation further. Gas chromatography (GC) plays an important role in the qualitative and quantitative analysis of the hydrocarbon compounds in petroleum crude oil especially paraffinic hydrocarbons [7], concerning the types of hydrocarbons and their distribution. CGC gives a fingerprint for the studied crude oil before and after the effect of working conditions on biodegradation as significance for the change obtained in this oil [8-10]. The goal of the present paper is to examine the effect of nutrient addition and PH on microbial degradation of petroleum hydrocarbons. This the second study after the first study carried out in the effected of different Incubation Period on degradation of petroleum hydrocarbons.

II. MATERIALS AND METHODS

1- Microbial Consortium preparation.

Three microbial consortia were formulated by mixing equal proportions of pure microbial cultures that were isolated from hydrocarbon- contaminated soils and water. The first Consortium consisted of *Penicillium chrysogenum*-RS-F7 and *Saccharomyces cerevisiae*-Ferm-Bam-Y3 and the second Consortium consisted of *Penicillium chrysogenum*-RS-F7and *Streptomycin rimosus*-EPRI-A4.

2- Parameters controlling the growth of the most potent crude oil degrading organisms.

a- pH values:

Flasks each containing one hundred mls of Basal Salt (BS) broth medium supplemented with suez crude petroleum oil were sterilized, pH was adjusted by pH meter using 1 N NaOH or 1 N HCl at different pH values (4,5,6,7,8,9,10).Duplicates of flasks for each pH value were used, flasks were inoculated, and then incubated at 30ºC for 10 days.

b- Different nitrogen sources:

This experiment was carried out to investigate the effect of different nitrogen sources viz. sodium nitrate, ammonium chloride, ammonium mono hydrogen phosphate, ammonium sulphate and potassium nitrate as

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inorganic nitrogen and peptone and urea as organic nitrogen source on the growth of the most potent microbial isolates when allowed to grow on crude oil as the sole C- source. The nitrogen sources were added at equimolecular nitrogen content to that located in sodium nitrate (control). The nitrogen sources were added separately with Basal Salt broth medium supplemented with petroleum oil distributed into the flasks (100 ml for each flask). The sterilized flasks were inoculated separately with the most potent microbial isolates, and then incubated at 30ºC for the optimum incubation period (10 days). At the end of incubation period the loss of hydrocarbons was detected qualitatively and quantitatively (CGC analyses) to determine the optimum nitrogen source.

3-Extraction and analysis of residual oil

Conical flasks (250ml capacity) each containing 100ml Basal Salt (BS) broth medium supplemented with petroleum oil were inoculated by crude oil utilizing microbial isolates separately. The flasks were incubated at 30ºC for 21 days. At the end of incubation period the residual compounds were extracted from the culture medium by chloroform. The contents of the flasks with chloroform (3:1, sample: chloroform) were placed in a separating funnel, with continuous shaking. These lead to formation of three phases: chloroform containing the hydrocarbons (oil), biomass, and watery layer. The chloroform containing the hydrocarbons were analyzed chromatographically by Capillary gas chromatographic (CGC).

4-Gas chromatographic analysis

The analysis was achieved using Agilent 6890 plus HP gas chromatograph, equipped with flame ionization detector (FID), using the fused silica capillary column HP-1 of 30 meter in length and 0.35 mm int. diameter, The separation of the crude oil samples was achieved with temperature programming from 80 to 300 ^{o}C at a temperature rate 3 ^{o}C min^{-1}. The nitrogen flow rate is 2 ml min^{-1} that measured from the end of the column with a soap bubble flow rate. The quantitative analysis of the cud oil samples was achieved using normal octane as internal standard of known concentration and according to the standard ASTM method. The injector and detector temperatures are 250 and 300 ^{o}C respectively.

III. RESULTS AND DISCUSSIONS

Forty one crude oil utilizing microbial isolates were isolated from nine crude oil polluted soil and water samples, Collected from red sea. Twenty one bacterial, Fourteen fungal and four actinomycetes isolates were purified as well as well identified Saccharomyces cerevisiae. Qualitative and quantitative screening for crude oil biodegradation showed that fungal isolate RS-F7 and actinomycet isolate EPRI-A4, and Saccharomyces cerevisiae Ferm-Bam-Y3 were selected as the three most potent microbial isolates individually and in combination which have the highest degrading power of petroleum crude oil.

The physicochemical properties and composition of the crude oil before degradation

The physicochemical properties of the crude oil used in this study was given in Table 1 and the capillary gas chromatographic analysis of the crude oil exhibits n-paraffin's from C_{14} to C_{37}, and small quantity of iso-paraffines from C_{15} to C_{33} as given in Fig. (1). There are more than one peak maxima, and the highest maxima were obtained at C_{18} and C_{19} due to their high weight percents 17.448 % and 16.834 % respectively. Also, the chromatogram of crude oil contains unresolved complex mixture (UCM) as a hump under the peaks, representing the non eluted hydrocarbons like heavy naphthenic and heavy aromatic hydrocarbons.

1- **Different pH:**

Data recorded in Table (2) shows the preferred pH value on biodegradation of crude oil by the *Penicillium chrysogenum*-RS-F7. The data recorded emphasize that, this organism causes the degradation of crude oil at different pH values with different degrees but the optimum pH value 7 at which, C_{13}, C_{36}, and C_{37} were completely disappeared while other carbons were maximally degraded and presented in low concentration than control and other pH values. The percentage of n-C_{17}/pr equal (1.60) and n-C_{18}/ph (1.62) while area ratio of UCM/n+iso paraffins equal (0.24). Data recorded in Table (2) and the GC chromatogram given in Figs. (2-4) emphasize that, the optimum pH value for consortium between Penicillium chrysogenum-RS-F7 and Saccharomyces cerevisiae- Ferm-Bam-Y3 was pH 9 at which all carbon number completely disappeared except carbons C_{20}-C_{25} presented in low concentration than control and other pH values.

Table 1. Physicochemical properties of crude oil before degradation

The properties	The result	The properties	The result
Density	0.8456	pour point	18
specific gravity	0.8677	sulfur content (wt%)	1.77
API	30	asphaltene content	3.89
kinematic viscosity (cSt)	18.73	resin content	9.66

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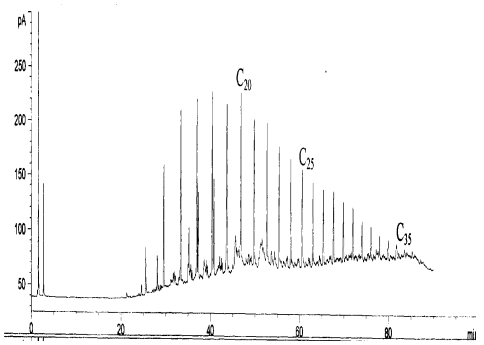

Fig. 1 the GC Chromatogram of the Studied Crude Oil before Degradation

Parameters controlling the growth of the three most potent microbial isolates

The bioremediation effect of consortium between Penicillium chrysogenum-RS-F7 and Saccharomyces cerevisiae-Ferm-Bam-Y3 was increased and considered to be higher compared with the effect of Penicillium chrysogenum-RS-F7 alone. These isolates together causes complete degradation of the main component C_{17} and C_{18} in the control sample of petroleum crude oil. The percentage of n-C_{17}/pr and of n-C_{18}/ph equal (zero). This combination not only causes the complete degradation of aliphatic (iso- and normal) but also causes the degradation of aromatic and other unresolved complex mixture so that area ratio of UCM/n+iso paraffin equal (zero). From the data recorded by CGC showed that the optimum pH value for Penicillium chrysogenum-RS-F7 was pH 7 at which this organism causes maximum degradation of normal and iso paraffin. The optimum pH value for the consortium between *Penicillium chrysogenum*-RS-F7 and *Saccharomyces cerevisiae*-Ferm-Bam-Y3 was pH 9 at which this combination causes maximum degradation of n- paraffin and complete degradation of iso- paraffin. The effect of consortium between *Penicillium chrysogenum*-RS-F7 and *Saccharomyces cerevisiae*-Ferm-Bam-Y3 as a consortium on the bioremediation of crude oil was also determined. Also, the bioremediation effect of consortium between *Penicillium chrysogenum*-RS-F7 and *Saccharomyces cerevisiae*-Ferm-Bam-Y3 isolates was increased and considered to be higher compared with the effect of *Penicillium chrysogenum*-RS-F7 alone. These isolates together causes complete degradation of the main component C_{17} and C_{18} in the control sample of Suez–Gulf petroleum crude oil. This consortium not only causes the complete degradation of paraffin (iso- and normal) but also causes the degradation of aromatic and other unresolved complex mixture so that UCM/n+iso paraffin equal zero. The consortium between Penicillium chrysogenum-RS-F7and Streptomyces rimosus-EPRI-A4 causes degradation of crud oil at different pH with different degree tell reached to maximum degradation at pH 9 at which this consortium causes complete degradation to iso-paraffin. And also this combination has the ability to degrade UCM more than Penicillium chrysogenum-RS-F7 alone.

2- Different nitrogen sources:

Hydrocarbon biodegradation can be increased by the addition of supplemental nutrients particularly nitrogen and to lesser degree of phosphorous [11]. This experiment was performed to examine the optimum nitrogen source for the three selected microbial isolates at which the highest degree of crude oil biodegradation can be obtained. The best nitrogen source for each organism was determined quantitatively. Table (2) show that, when peptone was used as nitrogen source the Penicillium chrysogenum-RS-F7 achieved degradation for only three carbons (C13, C14, and C15) from light fraction and two carbons from heavy fraction (C36, and C37) while other carbons presented in low concentration less than control. The percentage of n-C17/pr equal (1.31) and n-C18/ph (1.12) while the area ratio of UCM/n+iso paraffins (0.54).

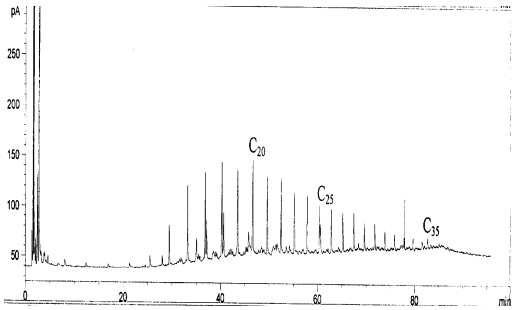

Fig.(2): Capillary gas chromatograms of crude oil sample after biodegradation by *Penicillium chrysogenum*-RS-F7 at pH value 7.

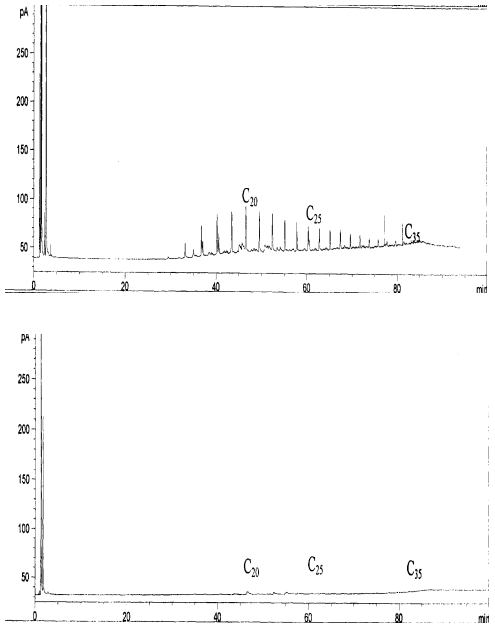

Fig.(3): CGC of crude oil samples after biodegradation by combination between Penicillium chrysogenum- RS-F7 and Saccharomyces cerevisiae-Ferm-Bam-Y3, at different pH values 8 and 9.

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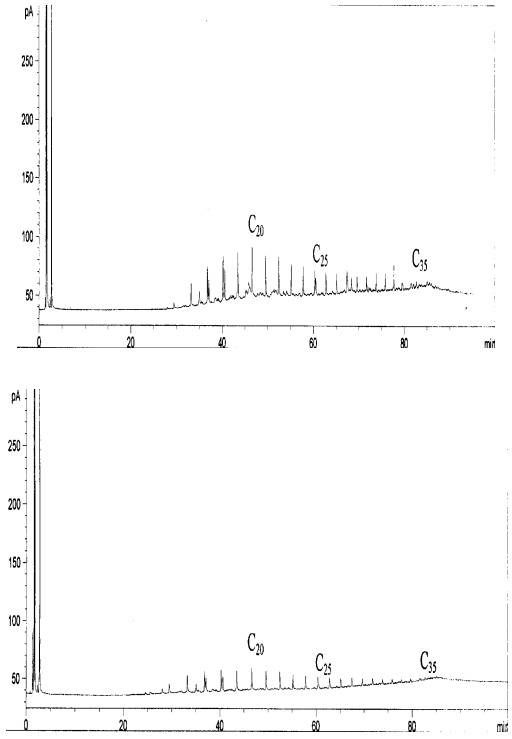

Fig.(4): CGC of crude oil samples after biodegradation by the combination between *Penicillium chrysogenum*-RS-F7and *Streptomyces rimosus*- EPRI-A4, at different pH values.

When $(NH_4)_2SO_4$ was used as nitrogen source the *Penicillium chrysogenum*-RS-F7 caused degradation of most carbon number (C_{13}, C_{14}, and C_{15}) from light fraction and (C_{33} - C_{37}) from heavy fraction, while others presented in low concentration . The percentage of n-C_{17}/pr equal (1.13) and n-C_{18}/ph (1.06) while the area ratio of UCM/n+isoparaffin (0.24). When KNO_3 was used as nitrogen source two carbons only (C_{13} and C_{14}) from light fraction and (C_{36} and C_{37}) from heavy fraction were completely degraded while other carbons were presented in low concentrations. The percentage of n-C_{17}/pr equal (1.45) and n-C_{18}/ph (1.23) while the area ratio of UCM/n+iso paraffins (2.42). When NH_4Cl was used as nitrogen source all carbons were completely degraded except (C_{19} - C_{28}) which maximally degraded and presented in less concentration than other nitrogen source. The percentage of n-C_{17}/pr equal (zero) and n-C_{18}/ph (zero) while the area ratio of UCM/n+iso paraffin (0.14). When $NaNO_3$ was used as nitrogen source three carbons (C_{13}, C_{14}, and C_{15}) were completely degraded while other carbons were presented in low concentration. The percentage of n-C_{17}/pr equal (1.11) and n-C_{18}/ph (1.07) while the area ratios of UCM/n+iso paraffin (0.39). The effect of different nitrogen sources on biodegradation of petroleum crude oil by the three most potent microbial isolates were studied in this investigation using quantitative analysis. When NH_4Cl used as nitrogen source the *Penicillium chrysogenum*-RS-F7 causes maximum degradation for normal and iso- paraffin and also has the ability to degrade UCM. Potassium nitrate was the optimum nitrogen source for consortium between *Penicillium chrysogenum*-RS-F7 and *Saccharomyces cerevisiae*-Ferm-Bam-Y3*;* this consortium causes the complete degradation for crude oil. This combination causes the degradation of C_{17} and pr so that percentage of C_{17}/pr equal (zero), and also C_{18}/ph equal (zero) this organism not only causes the complete degradation of aliphatic (iso- and normal) but also causes the complete degradation of aromatic and other unresolved complex mixture so that UCM/n+iso- paraffin equal (zero). The optimum nitrogen source for consortium between *Penicillium chrysogenum*-RS-F7 and *Streptomyces rimosus*-EPRI-A4*;* was $(NH_4)_2SO_4$ at which this consortium causes the complete degradation of C_{17}, and pr(pristine) so that the percentage of C_{17}/pr equal (zero) it also degrade completely C_{18} and ph so that the C_{18}/ph equal (zero). This combination not only causes the degradation of aliphatic but also aromatic so that the percentage of UCM/n+iso- paraffin (zero). In accordance to our results Chang and Weaver, 1997 [11] found that biodegradation of crude oil was carried out with different nitrogen and phosphorus sources. Interestingly the addition of nitrogen and phosphorous nutrients is a known mechanism aim to enhance the natural biodegradation process that will stay with oil rather than wash away into the marine environments were investigated by [12].

IV. CONCLUSION

Capillary gas chromatography is a powerful analytical tool used for evaluating the hydrocarbons in the environmental pollution caused by crude oil spills. Both different pH values and nitrogen sources have high effect on the bioremediation petroleum pollutants. Complete degradation of petroleum pollutants was achieved using consortium between RS-F7 and Ferm-Bam-Y3 at pH 9 and Potassium nitrate as nitrogen source. Ammonium sulphate and PH 9 are the preferred conditions at which the consortium between RS-F7 and (EPRI-A4) exhibit high efficiency on the complete degradation of paraffinic hydrocarbons.

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Table (2): Effect of pH values and different nitrogen sources on biodegradation of crude oil by the studied microorganisms using CGC technique

		Different pH values			Different nitrogen sources		
		RS-F7	RS-F7 and Ferm-Bam-Y3	RS-F7 and EPRI-A4	RS-F7	RS-F7 and Ferm-Bam-Y3	RS-F7 and EPRI-A4
Carbon number	Control	7	9	9	NH_4Cl	KNO_3	$(NH_4)_2SO_4$
C_{13}	1.231	0	0	0	0	0	0
C_{14}	1.671	0.06	0	0	0	0	0
C_{15}	7.255	0.202	0	0.043	0	0	0
C_{16}	10.397	0.37	0	0.056	0	0	0
C_{17}	12.99	0.481	0	0.092	0	0	0
C_{18}	17.448	0.518	0	0.115	0	0	0
C_{19}	16.834	0.49	0	0.066	0.035	0	0
C_{20}	12.303	0.511	0.012	0.072	0.046	0	0
C_{21}	9.694	0.402	0.005	0.051	0.048	0	0
C_{22}	9.126	0.433	0.031	0.051	0.051	0	0
C_{23}	10.524	0.332	0.006	0.042	0.043	0.032	0
C_{24}	7.332	0.265	0.01	0.036	0.048	0	0
C_{25}	9.221	0.17	0.006	0.034	0.041	0	0.006
C_{26}	6.323	0.161	0	0.031	0.037	0	0
C_{27}	6.406	0.169	0	0.03	0.029	0	0
C_{28}	9.599	102	0	0.029	0.034	0	0
C_{29}	4.168	0.122	0	0.025	0	0	0
C_{30}	3.983	0.113	0	0.024	0	0	0
C_{31}	4.753	0.103	0	0.019	0	0	0
C_{32}	2.045	0.099	0	0.017	0	0	0
C_{33}	2.086	0.095	0	0.016	0	0	0
C_{34}	1.201	0.072	0	0.013	0	0	0
C_{35}	0.881	0.03	0	0.011	0	0	0
C_{36}	0.445	0	0	0.005	0	0	0
C_{37}	1.15	0	0	0	0	0	0

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$n\text{-}C_{17}$/ pr	5.26	1.6	0	1.11	0	0	0
$n\text{-}C_{18}$/ ph	5.98	1.62	0	1.04	0	0	0
UCM/n-and iso-paraffin	3.74	0.24	0	0.05	0.14	0	0