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Bioremediation of Petroleum Polluted Soils using Consortium Bacteria

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Abstract

This study was carried out to isolate opportunistic hydrocarbons oil-degrading bacteria and develop a consortium or a mixture of bacteria with high biodegradation capabilities which can be used in biological treatment units of the contaminated water before release. The biological processes in general are environmentally friendly and cost effective, as they are easy to design and apply; as such they are more appropriate to the public.

The location of the study was in Al-Dora refinery sludge holes area. The samples were collected for three seasons (winter, spring and summer) each consisted of three months. The sludge samples were analyzed for various physical and chemical parameters. Temperature values of the sludge were at maximum in summer season, reaching 32˚C, whereas they were at minimum in winter (24 ˚C). The values of sludge pH were at maximum in summer (9.70) and minimum in winter (9.20). Turbidity levels were 382 NTU in spring and 353 NUT in winter. Biological oxygen demand (BOD5) was at maximum in summer (760) and (690 mg/l) in winter. The maximum dissolved oxygen (DO) value of 5.20 mg/l was recorded in winter, while the minimum was 3.80 mg/l recorded in summer. The maximum electrical conductivity (EC) was 17130 µs/cm recorded in summer, while the minimum was 16150 µs/cm recorded in winter. The maximum total dissolved solids (TDS) values were 10335 mg/l recorded in summer, while the minimum (10015 mg/l) was recorded in winter. The maximum total petroleum hydrocarbon (TPH) value (431 mg/l) was recorded in summer, while the minimum (367 mg/l) was recorded in spring. Finally, the maximum salinity value (9.90%) was recorded in spring, while the minimum (9.30%) was recorded in winter. Also, hydrocarbon compounds in sludge samples were measured using Gas Chromatography - Mass Spectrometry (GC-MS), and the result showed that they were composed of 31 hydrocarbon compounds.In the present work, nineteen sludge degrading bacterial strains were isolated from the soil near Al-Dora refinery hole by primary and secondary screenings using a modified mineral salt medium supplemented with 1% (v/v) sludge as a carbon source. The most efficient two sludge degraded isolates identified by VITIK 2 compact were Kocuria *rosea* and *Bacillus amyloliquefaciens*. The tow isolates and there mixture showed best growth at 30°C for 12 days, as shown by the measurement of the optical density of the liquid culture and the final oil concentration by spectrophotometer.

The bacterial isolates in liquid media with 2% (v/v) sludge showed best growth and the maximum biodegradation percentage after 12-day incubation period, as determined by gas chromatographic (GC). The degradation values were 68.9, 93.8 and 95.5% for *Bacillus amyloliquefaciens, Kocuria rosea* and the mixture of the tow isolates, respectively. In optimum conditions of pH 7, 40°C, 12 days incubation, the mixed bacterial consortium showed maximum sludge degradation.

Keywords: Bioremediation, Consortium Bacteria, Petroleum Hydrocarbons, Ir

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المعالجة البايولوجية للترب الملوثة بالنفط باستخدام خليط بكتيري

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الخلاصة

هدفت الدراسة عزل انواع من البكتريا الانتهازية المحللة للنفط وتطوير امكانية استخدام خليط من البكتريا في وحدة المعالجة البايولوجية للمياه الملوثة قبل طرحها. العمليات البايولوجية بصورة عامة صديقة للبيئة وذات تكلفة قليلة فهي سهلة التصميم والتنفيذ. موقع الدراسة كان في منطقة حفر الحمأة في مصفى الدورة , حيث تم جمع عينات الحمأة وتحليلها بواسطة العديد من الفحوصات الفيزياوية والكيمياوية اذ جمعت العينات خلال ثلاثة فصول (الشتاء و الربيع والصيف) وكانت النتائج كالتالي : اعلى درجة حراة كانت في فصل الصيف 32م˚ واقل درجة حراة في فصل الشتاء وكانت 24 م˚ , قيم الـ pH كانت اعلى قيمة في الصيف 9,70 و اقل قيمة في الشتاء كانت 9,20 ,نسبة العكورة اعلى قيمة في فصل الربيع كانت 382 NTU وفي الشتاء اقل قيمة وكانت 353 NUT , المتطلب الحيوي للاوكسجين (BOD5) اعلى قيمة في فصل الصيف وكانت 760 واقل قيمة في فصل الشتاء وكانت 690 ملغم/لتر, والاوكسجين الذائب (DO) كانت اعلى نسبة في فصل الشتاء 5,20 واقل نسبة في فصل الصيف 3,60 ملغم/لتر, اعلى قيمة توصيلية كهربائية غي فصل الصيف كانت 17130 واقل قيمة كانت في فصل الشتاء 16150 مايكرو سيمنز/سم, قيم الاملاح الذائبة الكلية (TDS) كانت اعلى قيمة في فصل الصيف 10335 و اقل قيمة كانت في فصل الشتاء10015 ملغم/لتر, اعلى قيمة الهيدروكاربونات كانت في فصل الصيف 431 وفي فصل الشتاء كانت اقل نسبة 367 ملغم/لتر, واخيرا نسبة الملوحة كانت 9,90 في فصل الصيف و9.30 % في فصل الشتاء, كذلك معرفة المركبات الهيدروكاربونية الموجودة في عينة الحمأه النفطية بواسطة استخدام جهاز Gas Chromatography – Mass Spectrometry (GC–MS). واظهرت النتائج وجود 31 مركب هيدروكاربوني.

في الدراسة الحالية تم عزل تسعة عشر سلالة بكترية من التربة القريبة من الحمأة النفطية بواسطة غربلة اولية وثانوية باستخدام 1% من الوسط الملحي مع الحمأة كمصدر كاربوني. تم تحديد نوعين من البكتيرية الفعالة في تحليل الحمأة بواسطه جهاز الـ2 VITICV وهما *Kocuria rosea* and *Bacillus amyloliquefaciens* هذه العزلتين وخليطهما اظهرت افضل نمو من خلال قياس الكثافة الضوئية وتركيز المحلول بواسطة جهاز المطياف الضوئي.

هذه العزلات اظهرت اعلى نسبة نمو في المحلول السائل عند درجة حرارة 40 م وpH 7 وبفترة حضن 12 يوم وتركيز الحمأه 2 مل وخليط البكتريا اعطى اكثر نسبة تفكك للحمأه . ان نسب التفكك البايولوجي للحمأه لثلاث عينات معالجة بعزلات بكتيرية مختلفة بعد مدة حضن 12 يوم قد حدد باستعمال جهاز gas chromatographic (GC) كانت النسب كالتالي 68,9 ’ 93,8 و 95,5 للعزلات *Bacillus amyloliquefaciens,* ’ *Kocuria rosea* ولخليط العزلات على التوالي .

Introduction

Petroleum hydrocarbon continues to be used as the main supply of energy. Accidental spills and leak occur frequently during the transport, exploration, refining, and storage of petroleum and petroleum products, making petroleum an essential global environmental pollutant [1].

Oily sludge is a solid after oil solidifying, usually at temperatures lesser than 100° C. Oil sludge can be a most important contributor to the problems internal combustion in engines, which can require the engine to be replaced if the damage is severe. Sludge is caused by the presence of water within oil, which can accumulate with time. Methods to reduce petroleum sludge formation and accumulation include performing frequent oil alteration, using synthetic oil, performing mechanized engine flushing, desludging, and following the manufacturer's engine maintenance routine [2].

Biodegradation is the main mechanism to decrease biodegradable contaminant. This technique offers low risk to polluted sites and it is an alternative with favorable cost-benefits [3]. Some kinds of microorganism are capable to degrade oil hydrocarbons and can be used as sources of energy carbon supply. The specificity of the degradation process is associated to the genetic potential of the particular microorganism to introduce hydrocarbons into molecular oxygen and to produce the intermediates that subsequently enter the common energy-yielding metabolic pathway of the cells [4]. Other species of bacteria have the efficiency of bioremoval the contaminated sites like *Pseudomonas aeruginosa* [5]. Bacteria, such as species from genera *Acinetobacter, Bacillus, Arthrobacter* and *Rhodococcus, Sphingomonas*… etc. tolerate diverse petroleum hydrocarbon concentrations and are able of their degradation [6].

Researchers showed the characteristics of *Kocuria spp*. in the bioremediation of hydrocarbons [7]. Another bacterium with remarkable metabolic capabilities, *B. amyloliquefaciens*, was used for new applications such as degradation of crude oil from petroleum contaminated soils [8].

The use of a consortium of microorganisms to eliminate petroleum contamination in a bioremediation process is highly recommended [9]. In fact, the consortium microorganisms have a high capacity in biodegradation of various types of hydrocarbons and can produce biosurfactants effectively. Besides, the verdict about the potential capabilities of a single isolate within a bioremediation process should not be considered independently from the whole biosystem. The use of indigenous bacterial mixtures requires to ensure that the organisms have a high tolerance to the toxic hydrocarbons and are resistant to changes in the environment [4].

Materials and Methods

Physicochemical and biological qualities of the major effluents to the hole area in AL-Dora refinery were investigated in sludge samples at different periods, as shown in Figure-1.

Temperature, hydrogen ion concentration, EC, TDS and salinity of the sludge were determined using WTW series pH/ºC /EC/TDS meter. These parameters were used to recognize the characteristics of Al-Dora refinery sludge release to the hole area.

Biological oxygen demand (BOD_5)

Dissolved oxygen was determined by using an azide modification of the Winkler method as described previously [10]. BOD_5 was determined after five days incubation at 20º C, according to the following equation:

$BOD_5 = DO_1 - DO_2$

where: DO_1= dissolved oxygen (mg/1) on the first day.

DO_2= dissolved oxygen (mg/1) after 5 days incubation.

Total petroleum hydrocarbon (TPH)

The hydrocarbon concentration in oil sludge samples was measured by liquid-liquid extraction as described previously [11].

Gas chromatography analysis

GC methods are in common use because of the broad range of hydrocarbons that are detected selectively and sensitively. The results of GC analyses are shown as Figures-(2, 3, 4 and 5). Also, the identification of individual compounds can be achieved by coupling a variety of detectors to GC analysis, including flame ionization detectors GC/FID. All the three treatment samples were extracted first, then pooled and dried at room temperature by evaporation of solvents under a gentle nitrogen stream in a fume hood. After solvent evaporation, the quantity of the residual total petroleum hydrocarbons was determined and analyzed by a GC device [12].

Bacterial isolation

Eight samples were aseptically collected from different locations at Al-Dora refinery. These samples included tanks soils, water polluted with refinery sludge, and refinery sludge soils. 60 % of the collected samples had solid and semisolid nature, which included the soil samples, while the other 40% of the collected samples had a liquid and oily sludge nature.

Soil samples were isolated from the overall collected sample by the serial dilution method. Selected bacterial colonies were identified by morphological, cultural and biochemical characteristics [13]. Further identification of the bacteria was performed using a VITEK 2 device, with the determination of the optimum conditions for bacterial degradation.

Bacterial efficiency

The primary screening was performed using a modified mineral salt medium agar supplemented with 1% (v/v) sludge as a carbon source with 1% of bacterial isolates from nineteen isolates.

The secondary screening was conducted in a flask containing 50 ml of modified mineral salt **(**MMS) medium supplemented with 1% sludge inoculated with selected bacterial isolates. The mixture was incubated at 30˚C for 12 days with 150 rpm. Cell turbidity was measured at 600 nm using a spectrophotometer[14].

Figure 1-Description of the hole area in AL-Dora refinery

Optimization of sludge biodegradation

Effect of incubation period

One hundred milliliters of modified mineral salt (MMS) medium were dispensed in Erlenmeyer flasks and the pH was adjusted at 7.0. The mixture was supplemented with 1% sludge as a substrate and 0.5 % of the bacterial isolate and incubated at 30˚C for different periods (3 , 7 , 10 , 11 and 12 days) at 150 rpm [15].

Effect of sludge concentration

The same steps above were followed with different concentrations of sludge (50,100,150,200, 300 ml \l) at pH 7.0 and incubation for 12 days in a shaker incubator [16].

Effect of pH

The effect of pH was determined by the preparation of 1% of sludge and liquid MMS with different pH values (5, 6, 7, 8 and 9) using HCl (0.1N) and NaOH (0.1N) solutions for adjusting [17].

Effect of temperature

The same steps above were followed at different temperatures (25, 30**,** 35**,** 40 and ˚C**)** for 12 days at 150 rpm.

Results and discussion

Table-1 shows the characteristics of Al-Dora refinery effluents to the sludge holes inside the refinery for three seasons.

Table1-Sludge quality parameters in AL-Dora refinery (holes area) in three seasons

Seasons	TDS (mg/l)	Salinity%	Tur (NTU)	Ec (µs/cm)	Temp (°C)	PH	Oil contain (mg/l)	BOD5 (mg/l)	DO (mg/l)
Summer	10335 a ± 72.17	9.50a ± 0.26	382 a ± 5.48	17130 a ± 92.38	32 a ± 1.50	9.70 a ± 0.38	431 a ± 4.10	760 a ± 4.10	3.80 c ± 0.05
Spring	10224 a ± 60.62	9.90a ± 0.40	360 b ± 2.89	17040 a ± 77.94	28 b ± 0.64	9.40 ab ±	367 b ± 1.91	725 b ± 1.91	4.50 b ± 0.13
Winter	10015 b ± 34.64	9.30a ± 0.35	353 b ± 6.93	16150 b ± 51.96	24 c ± 0.69	9.20 b ± 0.57	370 b ± 3.00	690 c ± 3.00	5.20 a ± 0.07
LSD (P ≤ 0.05)	**200.62**	**1.18**	**18.57**	**262.85**	**3.54**	**1.70**	**10.84**	**10.84**	**0.31**

- LSD: least significant difference

- Means with capital letters indicate significant difference in rows, while small letters indicate significant differences between means in columns.

The sludge samples were collected from Al-Dora refinery effluents to the sludge holes inside the refinery in three seasons. Among the tested parameters, EC showed high values of 17130, 17040 and 16150 µS/cm. All EC values were out of the range of the WHO limitation (500µs/cm). Salinity values were 9.50, 9.90 and 9.30%, whereas those for wastewater samples of the biological treatment unit, which included the biological tank (site 1), the final clarifier tank (site 2), and the final discharge unit in Al-Dora refinery, were 1.267, 1.234 and 1.277 %, respectively [18].

TDS values were 10335, 10224 and 10015 mg/l, whereas those for wastewater samples collected from the biological treatment unit were 1184, 1173 and 1030.3 mg/l, for sites 1 and 2 and the final discharge unit, respectively [18].

Turbidity values were 382, 360 and 353 NTU. The turbidity values for all of wastewater samples at diverse time periods were higher than the WHO standard of 5N/m [19].

TPH values were 431, 367 and 370 mg/l, while all of the values were higher than the WHO set limit (10 mg/l) of wastewater to be discharged into the aquatic environment [20]. BOD values were 760, 725 and 690 mg/l**.** The BOD concentration values obtained for all of sludge samples at different time periods were higher than WHO standards of 10m/l [19]. DO values in the three seasons were 3.80, 4.50 and 5.20 mg/l. DO values for wastewater samples before and after biological management in Al-Dora refinery were 5.94 and 6.80 (mg/l) respectively, as the lower values was recorded in June 4.56 and 5.88 (mg/l) respectively [21].

The height of these values maybe as a result of continue pumping with greater concentration of hydrocarbons and crude oil residue, untreated wastewater with sludge and evaporation of water in high temperature in summer.

The results indicated that the values of some properties were out of acceptable values, pH, temperature were within the limitation. temperature values were 32, 28 and 24 °C The value of wastewater temperature was in the acceptable limit of WHO values [19], which was between 25-30ºC. pH values were 9.70, 9.40 and 9.20 All values are in the World Health Organization values (WHO) set limit, 6.5 - 9.6 of wastewater which have to be discharged into the aquatic environment [20].

Determination of hydrocarbon compounds by GC-MS

After analysis of sludge sample, the result showed that they composed of 31 compounds showed in Table-2. The released from the refineries are characterized through the presence of large quantity of crude oil products, sulfides, polycyclic, metal derivatives, phenols, surface active substances, naphthalene acids and other chemicals [22].

Table 2-GC-MS analysis of sludge sample from AL-Dora refinery

Peak no.	Retention Time	Peak Area	Compound Name
1	2.148	1894233	Acetoin
2	2.320	429338	Formamide, N,N',N''-methylidynetris
3	2.567	372974	Carbonic acid, cyclic 1,2-dimethylethylene ester
4	2.857	3849828	2,3-Butanediol
5	2.986	5969436	1-Ethylpropyl methyl ether
6	3.203	4224408	Diisopropyl formal
7	3.277	597569	2-Nitroethanol
8	3.355	1965798	Ether, sec-butyl ethyl
9	4.003	29280	Formic acid, ethyl ester
10	4.388	34082	3-Hydroxy-2-butanone, acetate
11	5.018	94092	Propylene glycol methyl ether acetate
12	5.224	107968	Butyl isopropyl ether
13	5.472	165914	Propanoic acid, 2-hydroxy-, 2-methylpropyl ester
14	5.596	199561	2-Butoxypentane

15	6.270	44334	Propanoic acid, 3-(acetyloxy)-, anhydride
16	6.734	52881	2,4-Pentanediol, 3-methyl-
17	6.993	50883	Pyruvic acid
18	8.977	35235	Cyclohexanone, 5-methyl-2-(1-methylethyl)-, cis-
19	9.307	23464	Cyclohexanol, 5-methyl-2-(1-methylethyl)- ,[1S-(1.alpha.,2.alpha.,5.beta.)]-
20	10.928	17142	3,4,5,6-Tetramethyloctane
21	11.156	64237	Cyclopropanetetradecanoic acid, 2-octyl-, methyl ester
22	13.981	59112	2,9-Dimethyldecane
23	15.256	151031	11-Methyldodecane
24	16.460	292868	Tridecane
25	17.603	327573	2-Methylpentadecane
26	17.726	116920	2,6-Dimethylheptadecane
27	18.393	40263	Eicosane, 3-methyl-
28	18.690	293103	2-Methylnonadecane
29	19.725	225512	2,6-Dimethylheptadecane
30	20.712	110320	Bute hydrocarbon
31	21.656	78628	Tetradecyl iodide

Bacterial isolation

Nineteen isolates hydrocarbon degrading bacteria isolated from contaminated soil, there were shown the highest ability for sludge biodegradation, which was achieved by growing bacterial isolates on solid MMS with 1% of sludge.

Growth of isolates was detected by measured of optical density (OD) by spectrophotometer in 1% sludge at 12 day of incubation in liquid MMS media, the highest values of OD 1.44 was recorded for *B. amyloliquefaciens*, an increased in the growth rate from the beginning of experiment.The second isolate was *K. rosea* recorded growth rate 1.41 in 11^{th} day Table-3.

The *Bacillus* spp. were more tolerant to high concentrations of poly cyclic aromatic hydrocarbons (PAH) in soil due to their resistant endospores so the isolates belonging to the *Bacillus* sp.this bacteria might be effective in removal of oil hydrocarbons in the contaminated soils [23].

In the degradation of crude oil by *Kocuria* sp. fast changes of optical density in initial degradation phases (0 to 1 day) were not observed. Rapid increase of optical density was observed following three days in all treatments. At the end of incubation period, highest values of optical density in every treatments were observed. The similar increase of optical density during hydrocarbons degradation [24].

Table 3-Mainly active bacterial isolates in liquid MMS medium incubation, 1% of sludge at 30 ˚C at 150 rpm by spectrophotometer.

Incubation period bacteria	**s.p 11^{th} day**
Kocuria rosea	1.41
Bacillus amyloliquefaciens	1.44
Mix of *k.rosea* and *B. amyloliquefaciens*	1.63

The growth rate for the mix of tow isolates was higher growth rate in 1% sludge at 12 day of incubation in MMS media, this result indicated that, the isolate of *K. rosea* and *B. amyloliquefaciens* were degraded sludge more than consumption it compared with the other bacterial isolates. Numerous bacterial isolates were used for heavy oil biodegradation, number of these isolates were degraded the petroleum hydrocarbon in to other compound and the others bacteria were consumed the compounds as a source of energy [25], [10] evidence that, mixed culture of microorgansims community is essential to complete biodegradation of oil pollutants as the hydrocarbon mixtures vary markedly in

volatility, susceptibility andsolubility, to degradation and the necessary enzymes wanted cannot be found in a single organism.

Results showed that the best tow isolatesin primary and secondary screening were *K. rosea* and *B. amyloliquefaciens*, percentage of hydrocarbons degradation were maximum for the mix of two bacterial isolates to consume sludge *K. rosea* and *B. amyloliquefaciens* was recorded of 95.5 %, while the single bacteria *K. rosea*and *B. amyloliquefaciensm* were recorded respectively 93.8% and 68.9% was matured by GC devices in the optimum conditions for the growth rates of bacterial isolates and consuming hydrocarbons media were at optimum conditions, temperature was 40 ºC, pH= 7, incubation period of 12 days and optimum concentration of sludge was 2 ml.

[26] found that, a consortium bacteria givesafter 15 daysincubation a maximum of 98% degradation, while 100% of hydrocarbon removals are seen with themedium and shortchain alkaneswith compared to the longer chain alkanes.

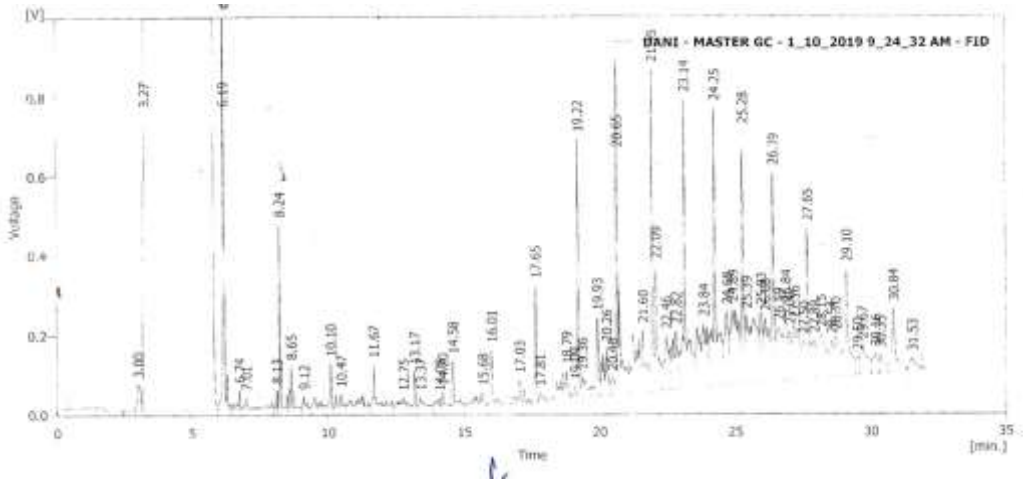

Figure 2-Gas chromatography for control sample.

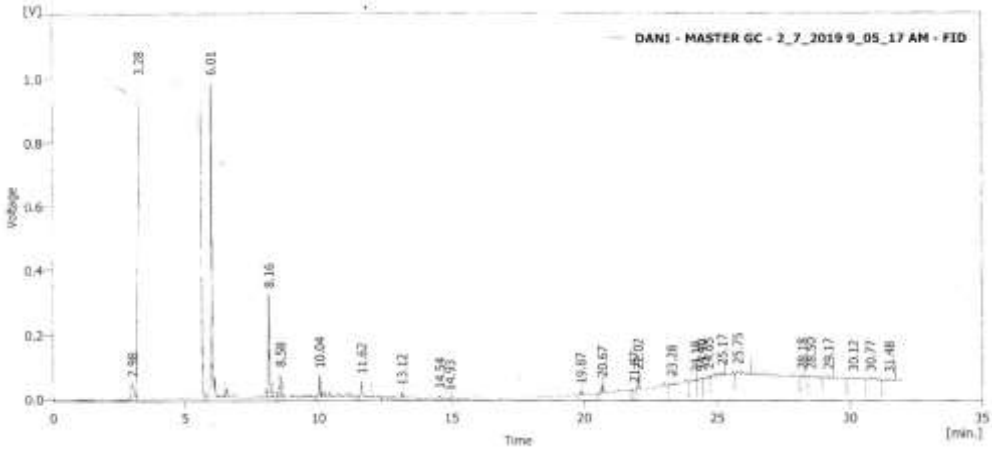

Figure 3-Gas chromatography for mix of tow isolatesbiodegrediation.

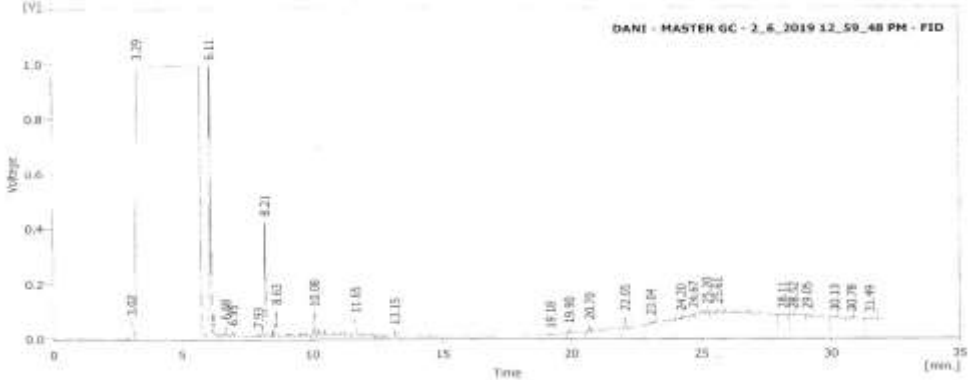

Figure 4-Gas chromatography for *Kocuriarosea*biodegrediation.

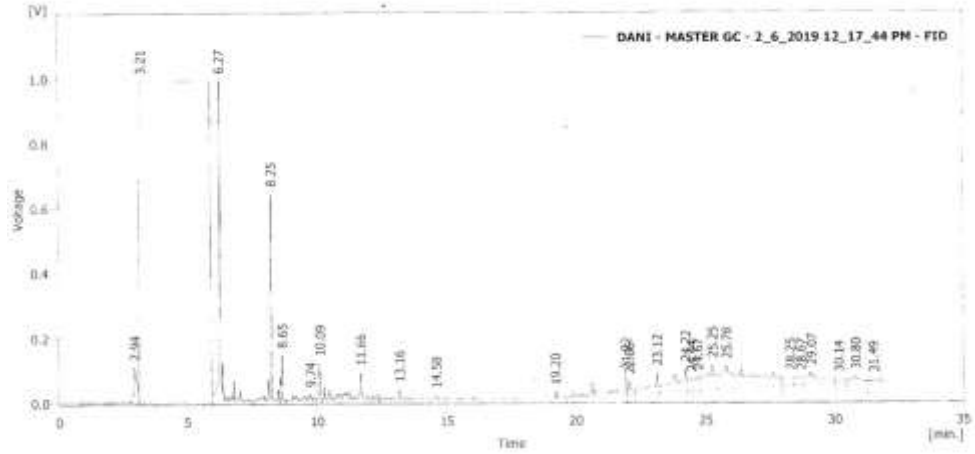

Figure 5-Gas chromatography for*Bacillus amyloliquefaciens*biodegrediation

Conclusion

In this study, nearly all of physicochemical parameters which were determined in sludge samples from AL-Dora refinery hole area were shown there is no treatment to wastewater and sludge and mainly of them out of the limited values and bacterial isolates *Kocuria rosea* and *Bacillus amyloliquefaciens* were the best isolates in sludge biodegradation.

The comparative study between single and consortium or mixed bacterial cultures of selected isolates for sludge biodegradation shown that the best results were obtained when use mix isolates compared with single isolates.

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