Bioremediation of Polluted Water with Crude Oil in South Baghdad Power Plant

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Abstract

Pseudomonas aeruginosa and *Klebsiella pneumoniae* appears good growth when replicate to culture with heavy crude oil. *K. pneumoniae* was less ability to biodegrade the heavy crude oil (66.22 wt.%) compare with *P. aeruginosa* 74 wt.%). Also the emulsion percent were about 64.8 % and 62.5 % for *K. pneumoniae* and *P. aeruginosa*, respectively. The results showed that the emulsions produced from both the strains decrease the surface tension of the media from 68.43 Mn/m (for control sample) to 44.50 and 43.30 Mn/m for *P. aeruginosa* and *K. pneumoniae*, respectively. The optimum temperature and pH for the hydrocarbons biodegradation were 28 °C and 7, respectively. The incubation period of 28 days of the isolated increased hydrocarbons biodegradation, which were (78 %) for *P. aeruginosa*, and (83 %) for *K. pneumoniae.* While, *P. aeruginosa* recorded heavy oil biodegradation percents were 76.94 and 71.73 % for oil concentrations 1000 and 2000 mg/l, respectively. While the percentage of biodegradation by *K. pneumoniae* to heavy oil were 74.87 and 69.92 % for 1000 and 2000 mg/l, respectively.

Keyword: Bioremediation, Heavy crude oil, *P. aeruginosa*, *K. pneumonia*, Emulsification, Surface tension, Biodegradation.

المعالجة الحيوية للمياه الملوثة بالنفط الخام في محطة جنوب بغداد لإنتاج الطاقة الكهربائية

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الخلاصة:

أظهرت العزلتان *Pseudomonas aeruginosa* و *Klebsiella pneumoniae* نموا" جيدا" بوجود النفط الخام الثقيل عند تكرار زرعها على نفس الوسط وكانت بكتريا *K. pneomoniae* الاقل في تفكيك النفط الخام الثقيل مقارنة ببكتريا *P. aeruginosa* والتي سجلت نسبة تفكيك % 66.22 كما سجلت البكتريا *K. pneumoniae* نسبة استحلاب بلغت % 64.8 بينما كانت النسبة % 62.5 لبكتريا *P. aeruginosa*. أظهرت النتائج ان المستحلبات المنتجة من قبل العزلتين قامت بتخفيض قيمة الشد السطحي للوسط من (68.43 Mn/m) بالنسبة لعينة الكونترول (من غير لقاح بكتيري) الى (44.50 Mn/m) و (43.30 Mn/m) لكل من *P. aeruginosa* و *K. pneumoniae* على التوالي. وكانت درجة الحرارة المثلى 28° م والرقم الهيدروجيني (pH) الأمثل هو 7 لنمو البكتريا واستهلاك الهيدروكاربونات، و تبين ان فترة الحضن 28 يوم للعزلات البكتيرية قد أعطت زيادة في نسبة استهلاك الهيدروكاربونات حيث سجلت بكتريا *P. aeruginosa* نسبة استهلاك بلغت % 78 بينما كانت النسبة 83% لبكتريا *K. pneumoniae*. سجلت بكتريا *P. aeruginosa* نسبة استهلاك للنفط الخام الثقيل بلغت % 76.94 و% 71.73 للتراكيز 1000 mg/l و 2000 mg/l على التوالي. بينما سجلت بكتريا *K. pneumoniae* نسبة استهلاك بلغت % 74.87 و % 69.92 للتراكيز 1000 mg/l و 2000 mg/l على التوالي.

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Introduction

Petroleum hydrocarbon continues to be used as the principle source of energy, leaks and accidental spills occur regularly during the exploration, production, refining, transport, and storage of petroleum and petroleum products, made it an important global environment pollutant [1]. Oils contain many aliphatic and aromatic hydrocarbons which when present in any environmental system can cause a detrimental effect directly or indirectly to human and to many organisms and its habitat [2]. Bioremediation is the use of living organisms to reduce or eliminate environmental hazards resulting from accumulations of toxic chemicals or other hazardous waste, usually as contaminants of soil, water, or sediments [3]. Aerobic bioremediation processes are very effective in treating hydrocarbon contamination [4]. Microorganisms such as bacteria, fungi and yeast, can break down complex chains of hydrocarbons into smaller chains [5]. Most workers consider bacteria to be the most important group of petroleum degrading organisms while the most prominent species include *Pseudomonas, Achromobacter, Aicaligenes, Flavobacterium, Mycobacterium, Acinetobacter, Corynebacterium, Bacillus* and *Arthrobacter* [6].

Materials and methods

Water and soil samples

Oily water samples that used in the bioremediation experiments were collected from the washing unit of fuel oil, and soil from the South Baghdad Gas Power Plant. Two samples, where taken from the tanks in the first and last stage of the washing unit prior to discharge to Tigris river.

Cultures medium

- **Inorganic basal medium (IBM)**
  Was prepared according to [7].
- **Modified mineral salt medium (MMSM)**
  Was prepared according to [8].
- **Modified mineral salt medium with agar (MMSM-Agar)**
  Was prepared according to [9].
- **Modified mineral salt medium with Agar and solid hydrocarbon (MMSM-Agar-H)**
  Was prepared according to [10].

Bacterial identification

The identification was according to morphological and biochemical tests [11], also by VITEK 2 compact device.

Isolation of bacteria

Liquid cultures were prepared by first transferring 100 ml of Mineral Salt Medium (MSM) to 500 ml Erlenmeyer flask containing (1%) heavy crude oil as sole carbon source was stoppered with cotton wool bungs, wrapped in aluminum foil and autoclaved at 121°C for 15 min. Under aseptic conditions, 2 ml of each oily water sample was added to the flask and incubated at 30 $^{\circ}$C on a shaker incubator at 150 rpm. After 7 days of incubation, 1 ml of liquid culture were aseptically transferred to a Petri dishes with the addition of MMSM-Agar-H, incubated at 30 $^{\circ}$C for 7 days. The bacterial isolates were purified by streaking method on nutrient agar plates. This method was repeated to get pure isolated colonies [12].

Soil samples

Soil samples were achieved by adding 1 g of oil-contaminated soil to mineral salt medium containing 1 % sterilized crude oil as only carbon source and incubated for 7 days at 30 $^{\circ}$C on a shaker incubator at 150 rpm. After a week, 2 ml of cultured medium suspension transferred to new medium with the same conditions, and the transfer was repeated twice. The growing bacteria in the last medium culture were transferred to a Petri dish [12].

The ability of bacterial isolates to degrade heavy crude oil

- **Determination of biomass**

Bacterial isolates were inoculated in 100 ml of sterilize Modified Mineral Salt Medium (MMSM) (containing 1% ml of heavy crude oil) in 500 ml Erlenmeyer flask and incubated at 30 $^{\circ}$C on a shaker incubator at 150 rpm for 7 days. Solvent of hexane : acetone (1:3) was added to separate hydrocarbons from the liquid culture. Medium poured in centrifuge tubes and centrifuged at 10000 rpm for 30 minutes to precipitate the cells. The cells then transported to a weighted aluminum plates, dried in oven for 24 hours at 70 $^{\circ}$C and weighing. The hydrocarbon loss percentage was the organic layer determine in the top layer solution.

- Determination of quantitative hydrocarbon loss percent

The bacterial isolates were inoculated in 100 ml of sterilize MMSM (containing 1% ml of heavy crude oil) in 500 ml flask and incubated at 30 °C on a shaker incubator at 150 rpm for 7 days. Hexane (50 ml) was added, the solution was shaken vigorously. The solvent phase was evaporated and the residue was weighed to determine the amount of total heavy crude oil. the same steps for control flask was performed [13].

- Emulsification test

Was done according to [14].

- Measurement surface tension

Isolated bacteria was inoculated in 100 ml modified mineral salt medium containing 200 mg/l heavy crude oil, pH was adjusted to 7.2 and sterilized by autoclave.After 8 days of incubation using shaker incubator at 30 °C, the surface tension was measured using a Sigma 703D Du-Nouy-Ring tensiometer. All measurements were taken at room temperature 27 °C [14].

Study the optimal conditions for the bacterial isolates growth which degrading hydrocarbons

Temperature:

Bacterial isolates were reactivated using nutrient broth at 37 °C for 24 hours and transferred to 100 ml of MMSM containing 200 mg/l heavy crude oil, in 500 ml flask. The sterilized flasks incubated in a shaker incubator at 150 rpm, at different temperatures 28, 37, 40 and 45 °C. After 8 days, optical density of the aqueous phase and the amount of hydrocarbon biodegradation were measured by spectrophotometer. The measure of bacterial growth by optical density (O.D) was carried out by using spectrophotometer (Thermo Spectronic 20D, USA), at wave length 600 nm [15].

Measuring the amount of degraded hydrocarbons

Hydrocarbon biodegradation amount was measured by spectrophotometer (Shimadzu UV-1800 Japan). Standards were prepared with different heavy crude oil concentrations using solvent *n*-hexane. Crude oil were extracts from flasks according to EPA method 1664 [16], which used *n*-Hexane as extractable material. Extracted Samples were stored in sealed vials in refrigerator at 4 °C. The amount of hydrocarbon degradation of samples and control were measured at wave length 469 nm.

Results and discussion

Identification of isolated bacteria

P. aeruginosa and *K. pneumoniae* were identified on the base of morphological characteristics, the biochemical tests results and by using VITEK 2 compact device.

Ability of isolated bacteria to degrade heavy oil in contaminated water

Four methods were used to test and detect the ability of *P. aeruginosa* and *K. pneumoniae* to degrade heavy oil by using MMSM media:

- Cell biomass test and the percentage of quantitative hydrocarbons loss

Cell biomass quantity of *P. aeruginosa* was 12.56 g/l, while it was 7.314 (g/l) for *K. pneumonia*, Figure 1. *P. aeruginosa* performed the highest percentage of hydrocarbon degradation, which represents, depending on original hydrocarbons used, of 74 wt.%, while it was 66.22 wt.% for *K. pneumoniae.* These results insure that the *P. aeruginosa* is more affective in the biodegradation of heavy oil compounds as compared with other species of bacteria, these results are close to the results of previous study [17].

Emulsions

K. pneumoniae showed the highest amount of emulsion production, where the emulsion percent was 64.8 % as compared with *P. aeruginosa*, whereas its emulsion percent was 62.5 %, figure 2. The high emulsification activity due to the production of extracellular water soluble biosurfactants by isolates during the incubation that is utilizing the crude oil as sole carbon source [18].

Surface tension

Figure 2- showed the surface tension of media that treated with *K. pneumoniae* was 43.30 Mn/m due to the presence of emulsions produced by the bacteria. This result was less than the surface tension of the media that treated with *P. aeruginosa*, (44.50 Mn/m). The surface tension of control was 68.43 Mn/m.

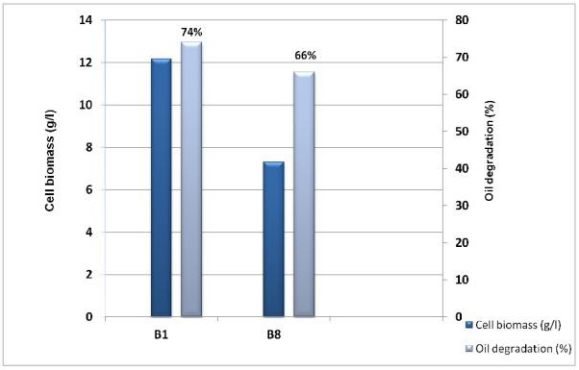


Figure 1- Oil degradation by *P. aeruginosa* and *K. pneumoniae*.

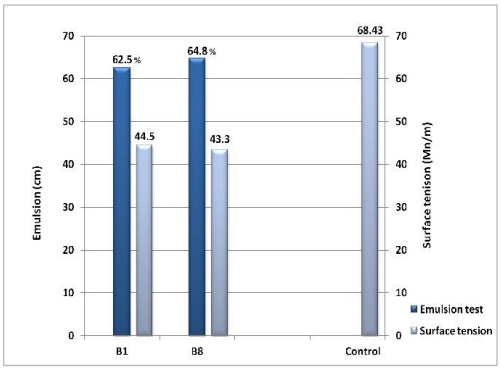


Figure 2 Emulsion test and surface tension of the bacterial isolates.

Optimum conditions for the growth of bacterial isolates

1- Optimum temperature

Two methods where used to detect the optimum temperature, optical density method using spectrophotometer 20D, and measuring the hydrocarbons degradation by UV-1800. Figure 3 A, showed that, *P. aeruginosa* appeared the highest optical density at temperature 28 °C and the lower optical density was observed at 45 °C. The optical density at 28 °C was 0.311 on day 5, and 0.269 on day 8. This was the highest values as comparing with the growth in other temperatures. Significant decrease in optical density was observed on day 8, (0.171), (0.158) and (0.141) at temperature 37, 40 and 45 °C, respectively. The optical density of *K. pneumoniae* growth was 0.398 at 28 °C , while it was 0.314, 0.290 and 0.271 at temperatures 37, 40 and 45 °C, respectively. This is clearly shows that temperature 28 °C resulted the larger optical density (bacterial growth) (figure 3 B). These results are identical to the previous results of Rezende, *et al*. 2012 [19], who suggested that the optimum temperature for bacterial growth was 28 °C.

Using UV-1800 spectrophotometer, percentage of hydrocarbons degradation was 77.1 % at 28 °C, while they were 73.3 %, 66.1 % and 56.7 % at temperatures 37, 40 and 45 °C, respectively for bacteria *P. aeruginosa.* (figure 4). The percentage of hydrocarbons degradation by *K. pneumoniae* was 74.2 % at 28 °C, while they were 71.7 %, 67 % and 61 % at temperatures 37, 40 and 45 °C, respectively (figure 4). These results showed that 28 °C was the optimum temperature for the *P. aeruginosa*, and *K. pneumoniae.* Similar results of Hasanuzzaman, *et al*. 2007 [20], observed 75% and 85% degradation of total crude oil by *P. aeruginosa* strain at 20 and 30 °C, respectively.

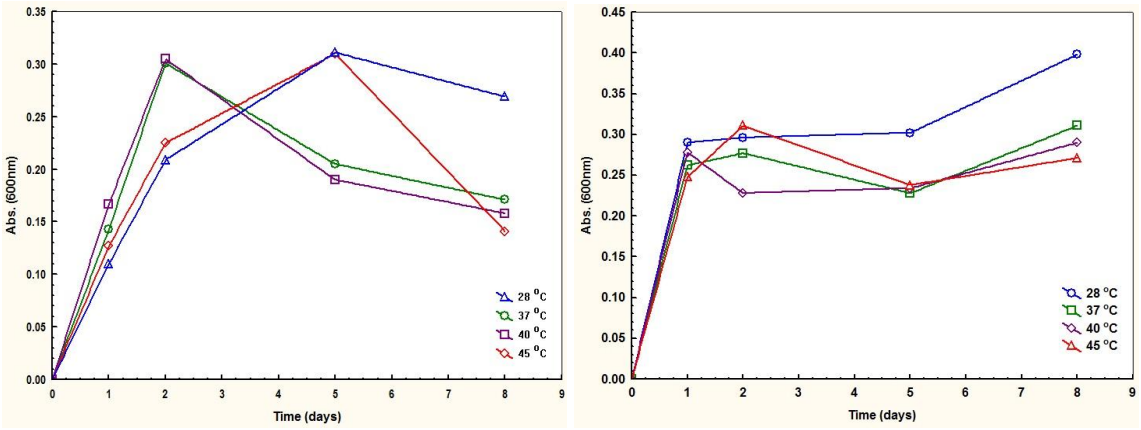
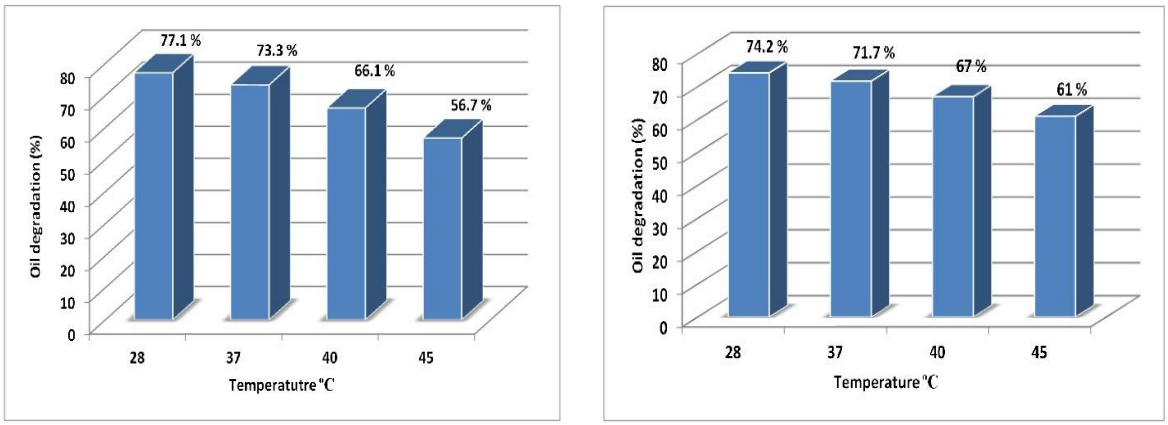


Figure 3 Optical density (600 nm) of bacterial growth at different time intervals.
A= *P. aeruginosa* B= *K. pneumoniae*.



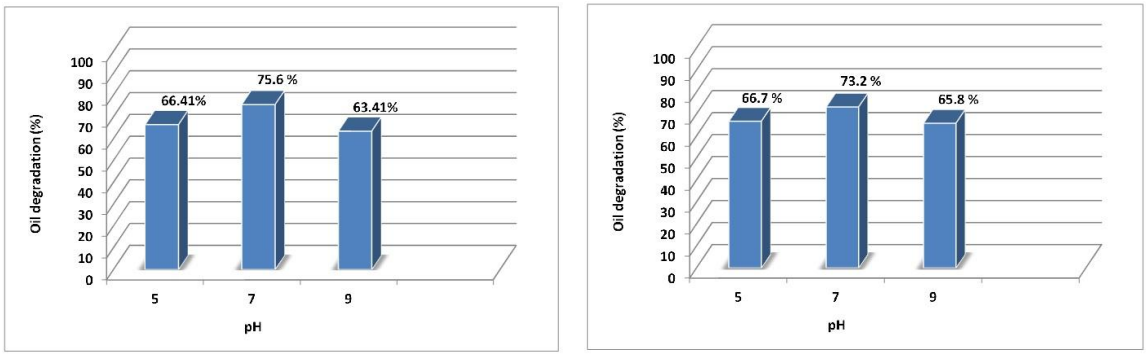
A (*P. aeruginosa*) **B** (*K. pneumoniae*)

Figure 4- Optimum temperature for bacterial isolates.
A= *P. aeruginosa*, B= *K. pneumoniae*.

2- Optimum pH

The hydrocarbons degradation percentage for *P. aeruginosa* at pH 5 was 68.41%, while it was 75.62 % and 63.41 % at pH 7 and 9, respectively (figure 5).

The percentage of hydrocarbons degradation by *K. pneumoniae* was 66.73 % at pH 5, while it was 73.26 % and 65.81 % at pH 7 and 9, respectively (figure 5). These results showed that pH 7 was the favorable pH for the bacterial isolates *P. aeruginosa* and *K. pneumonia* to degrade hydrocarbons Similar results were suggested by Salmon, *et al*. 1998 [21], who reported that, neutral pH of 7.0 is the optimal pH for biodegradation.

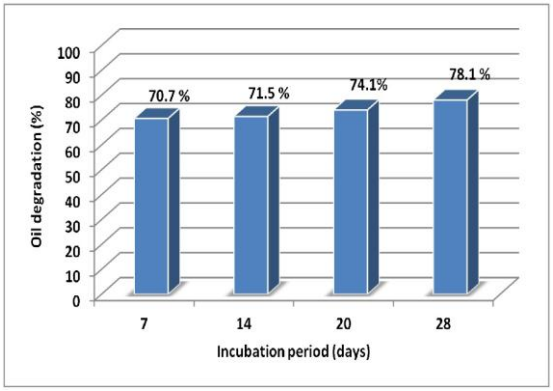


A (*P. aeruginosa*) **B** (*K. pneumoniae*)

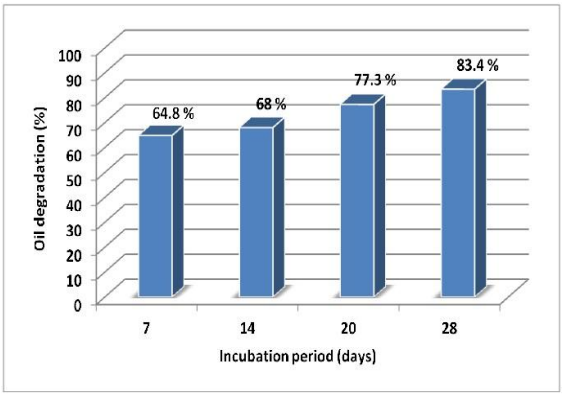
Figure 5- Percentage of oil degradation at different pH values.
A= *p. aeruginosa*. B= *K. Pneumoniae.*

3- Incubation period

The present result showed that the incubation period of 28 days for the bacterial isolates increased hydrocarbons consumption percentage. The percentage of hydrocarbons consumption of *P. aeruginosa* after 7 days of incubation was (70.7 %), while it was 71.5 %, 74.1 %, and 78.11 % for incubation period of 14, 20, and 28, respectively (figure 6). The hydrocarbons consumption percentage for the bacteria *K. pneumoniae* after 7 days of incubation, which was 64.8 %. The degradation past of 14 days of incubation was 68 %, while it was 77.3 % and 83.48 % after 20 and 28 days, respectively (figure 6). Similar results were suggested by Płaza, *et al.* (2008) [18], they found that the degradation of crude oil was above 80 % after 20 days of incubation.



A (*P. aeruginosa)*

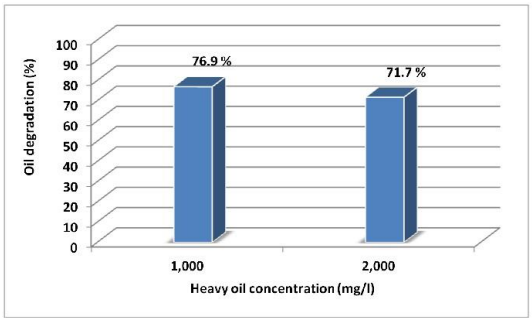


B (*K. pneumoniae*)

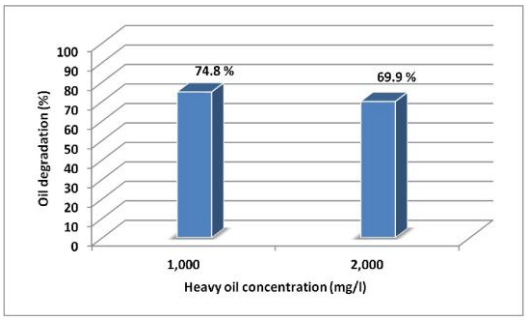
Figure 6- The percentage of oil degradation at different time intervals.
A= *P. aeruginosa*. B= *K. pneumoniae.*

4- Effect of the heavy oil concentration

Figure 7 A showed that, total petroleum hydrocarbons in MMSM media that inoculated by *P. aeruginosa* was degraded and the percentage was 76.94 % at the concentration of 1000 mg/l. While it was 71.73 % in the concentration of 2000 mg/l. *K. pneumoniae*, gives 74.87 ad 69.92 of degradation for the concentrations of 1000 and 2000 mg/l, respectively (figure 7 B). It is clear that there is a slight difference in the degradation percentage between two concentrations, where the degradation percentage increased when the concentration decreased.



A (*P. aeruginosa***)**



B (*K. pneumoniae*)

Figure 7- Total petroleum hydrocarbons concentrations (1000 and 2000 mg) in liquid medium that inoculated with A= *P. aeruginosa.* B= *K. pneumoniae.*

Study of Infra-Red spectroscopy

The liquid medium inoculated by *P. aeruginosa* and *K. pneumoniae* respectively, after 7 days of incubation (figure 9 and 11). Comparing with the control sample (figure8), a new absorption bands were appeared in the range of wave number (2362-2340 cm−1), which refer to the C-O stretching and accompanied by release of CO2 from the oxidation of hydrocarbons related to bioremediation processes [22]. In case of *P. aeruginosa*, after 14 days of incubation (figure 10), a significant increase in the area of the peak of this bands with the increase in the incubation time, also there is a changing in the absorption bands that correspond to C-H deformation and bending vibrations at wave number (1460-1378 cm−1) increase the transmittance percent indicating the transforming and consuming of some compounds by bacteria during bioremediation. The region close to (1750-1600 cm-1) was the carbonyl functional group C=O of oil due to formation of the free fatty acids [23]. Moreover there is a significant decrease in the area of the peak at wave number (2960-2850 cm−1) that was corresponding to aliphatic hydrocarbons stretching, with the appearance of a new absorption bond at wave number (3726-3590 cm−1) for hydroxyl group and hydrogen bonding [24]. In case of *K. pneumoniae,* after 14 days of incubation (figure 12) the increase in the peak of (2362-2340 cm−1) was appeared, that indicating those bacteria biodegrade the aromatic compounds to stretch chains insuring the involvement of this bacteria in the bioremediation process, also there was a simple changes in the

transmittance percents at wave numbers (1465-1378 cm−1), (2960-2850 cm−1) and (3726-3590 cm−1) as comparing with *P. aeruginosa*.

It is clear that from the previous explanation the difference in absorption bonds and its appearance/disappearance gave a good indication of the biodegradation process [25].

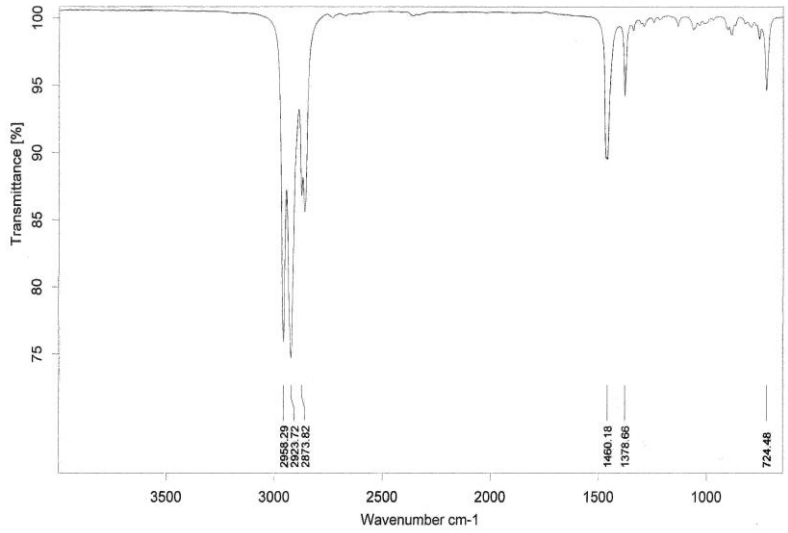


Figure 8- FT-IR spectrum of heavy oil for control.

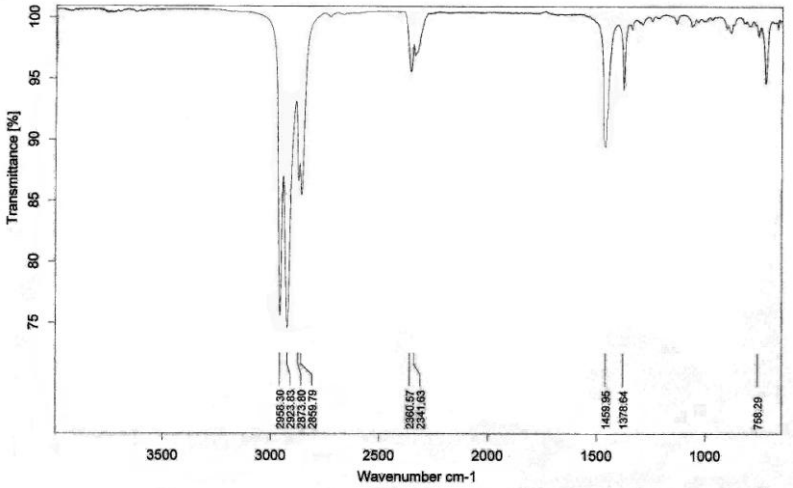


Figure 9- FT–IR spectrum of heavy oil after biotreatment for 7 days with *P. aeruginosa.*

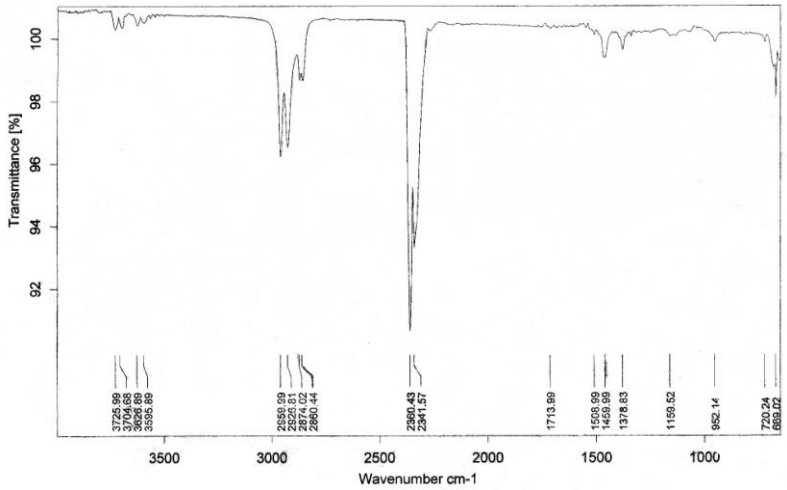


Figure 10- FT-IR spectrum of heavy crude oil after biotreatment for 14 days with *P. aeruginosa.*

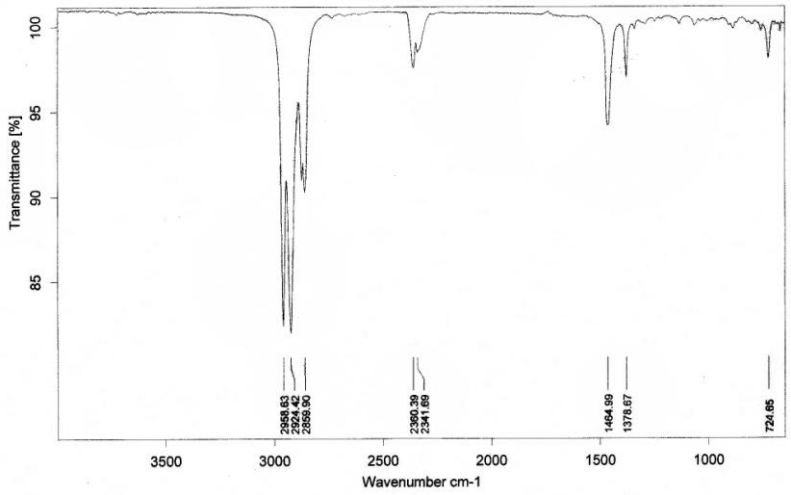


Figure 11- FT-IR spectrum of heavy oil after biotreatment for 7 days with *K. pneumoniae***.**

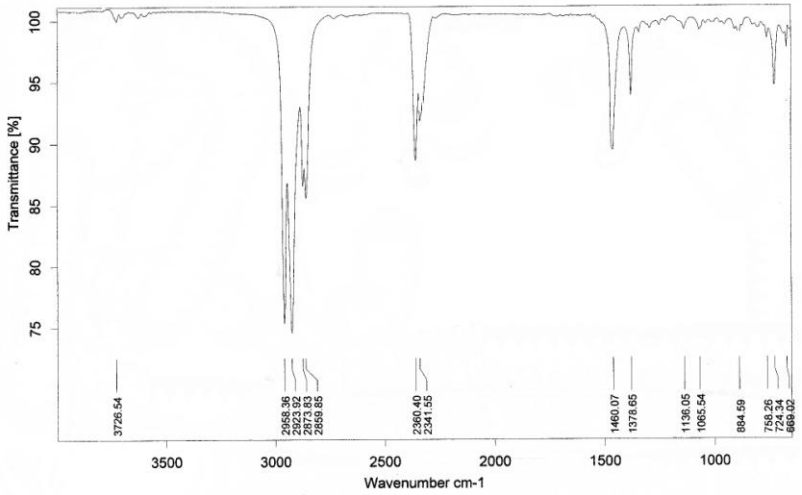


Figure 12- FT-IR spectrum of heavy crude oil after biotreatment for 14 days with *K. pneumoniae*

Conclusions

Selective media of modified mineral salt medium with agar and solid hydrocarbon (MMSM-agar-H) had an efficiency in the isolation of bacteria for hydrocarbons degradation, through the multiple re-cultured on the same media. Thus, these bacteria able to adapt to this media and using hydrocarbons as a sole source of carbon. *P. aeruginosa* and *K. pneumoniae*, were characterized by producing emulsion materials when treated with heavy oil, the emulsions decreased the surface tension of the medium. The optimum temperatures for bacterial isolates to degrade hydrocarbons was 28 $^{\circ}$C with optimum pH 7, while Incubation period for 28 days gave high level of the hydrocarbon biodegradation. It is clear that there is a slight difference in the consumption percentage between two concentrations 1000 and 2000 mg/l.

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