Biodegradation of Crude Oil in Contaminated Water by Local Isolates of *Enterobacter cloacae*

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Abstract

This paper study the ability of *Enterobacter cloacae* for degrading crude oil in contaminated water. Six isolates of *E. cloacae* were isolated from hydrocarbon contaminated soil and water of different sites. The isolate *E. cloacae* E1 showed the highest emulsification index (E24%) reached 62% thus it was chosen for further study. Biosurfactant produced by *E. cloacae* E1 reduced the surface tension of the medium from 64 to 36 mN/m. pH range 6.5 – 7 and temperature range 30˚C - 35˚C were the optimal conditions for maximum degradation. After 30 days of incubation, *E. cloacae* E1 degraded 70.00 ± 0.40% of the crude oil. GC-MS analysis revealed that *E. cloacae* E1 was able to degrade aromatic compounds. This study proved that *E. cloacae* E1 consider being an efficient in crude oil degradation.

Keywords: biodegradation of crude oil, *Enterobacter cloacae*

التفكك الحيوي للنفط الخام في المياه الملوثة بواسطة عزلات محلية من *Enterobacter cloacae*

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الخلاصة

يدرس هذا البحث في قدرة بكتريا *Enterobacter cloacae* على تفكيك النفط الخام في المياه الملوثة. تم عزل ستة سلالات من بكتريا *E. cloacae* من ترب ومياه ملوثة بالهيدروكربونات ومن أماكن مختلفة . أظهرت العزلة *E. cloacae* E1 اعلى دليل استحلاب (E24%) وصل الى 62% ولذلك تم اختيارها لدراسات أخرى. المستحلبات الحياتية المنتجة من قبل العزلة *E. cloacae* E1 قامت بخفض الشد السطحي للوسط من 64 الى 36mN/m. الظروف المثلى لأعلى تفكك كانت عند مدى الرقم الهيدروجيني 6.5-7 ومدى درجة الحرارة 30°م-35°م. فككت العزلة *E. cloacae* E1 النفط الخام بنسبة 70.00± 0.40% بعد ثلاثين يوما من الحضن . كشف تحليل GC-MS بان العزلة *E. cloacae* E1 كانت قادرة على تفكيك المركبات الاروماتية. اثبتت هذه الدراسة بان العزلة *E. cloacae* E1 كفؤة في تفكيك النفط الخام.

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Introduction

Iraq considered being one of the main crude oil exporters. The oil industry on continuous progress, in 2014 the Iraqi government aims to export four million barrels per a day. The amount of crude oil exported from January 2013 to August 2013 was 596.5 million barrels [1]. Therefor oil contamination in Iraqi water environment is inevitable. The oil spill can happened through oil transporting tankers accident, leakage from oil transporting pipes or by human mistakes during lauding the oil into the tankers at Iraqi harbors. For instance in 1983, 80 million gallons was spilled in Arabian Gulf from Nowruz oil field spill, another example is the Gulf War oil spill in 1991, it was one of the largest oil spills in history and caused considerable damage to wildlife in the Arabian Gulf especially in areas surrounding Kuwait and Iraq. The volume spilled ranged around 11 million barrels [2]. Clean-up and recovery of hydrocarbons from an oil spill is difficult. Bioremediation has emerged as one of the most promising alternative treatment options for oil removal because of the low cost and effectiveness. Bioremediation is a method where microorganisms are used for the clean-up process. Bioremediation can be described as the conversion of chemical toxic and complicated organic compounds into nontoxic and simple inorganic compounds, such as CO2 and H2O along with microbial biomass accumulation, through oxidation under aerobic conditions [3] .Many scientists have identified various microbial species that are effective hydrocarbons degraders in natural environment such as bacteria, fungi, yeast and microalgae [4, 5]. However, bacteria have the main role in hydrocarbon degradation. To enhance the bioremediation process by the bacteria, the addition of nutrients (N and P) is very essential. Microorganisms utilize nutrients for the synthesis of organic compounds (Proteins) and intracellular metabolism [3]. Microbes, which can utilize hydrocarbons, are found naturally in the marine habitats; however, they grow and develop after an oil spill. Environmental factors like temperature and pH were considered as important factors as they have a significant effect on the rate of microbial growth and hence on the degradation [6]. Microorganisms have developed ways by which they increase the bioavailability of many water immiscible substrates. Two types of hydrocarbon interaction during biodegradation has been described earlier by [7] (1980) which are adhesion to oil and a hypothesized pseudosolubilization in which the hydrocarbon degrading bacteria assimilate small droplets of emulsified oil. [8] (2009) throw more light on the uptake mechanism of hydrocarbon by *Pseudomonas aeruginosa*. By reporting a new and exciting line of research for hydrocarbon uptake involving internalization of biosurfactant-covered hydrocarbon inside cell for subsequent breakdown. The bacteria produce biosurfactants to increase the bioavailability of hydrocarbon resulting in enhanced growth [9].

Materials and methods

Collection of samples

Hydrocarbon contaminated water and soil samples were aseptically collected from different sites, including Al Dura oil refinery, gas stations, local power generators and oil changing garages. All samples were kept in sterile plastic bags and transferred to the laboratory for bacterial isolation.

Cultural medium

Basal salt medium (BSM) was prepared from two solutions (solution A+ solution B), solution (A) consist of 0.001g of each of the following compounds CaSO4, FeSO4 and MgSO4, dissolved in 100 ml distilled water. Solution (B) consist of 1g K2HPO4H2O, 0.8g KH2PO4H2O and 0.5g NH4NO3, dissolved in 1000ml distilled water. 1ml from solution (A) was added to solution (B) with 1% wt/v crude oil. This medium was modified from [10] (2007).

Isolation

For soil samples, suspension was prepared by adding 0.5g soil sample to 4.5ml distilled water in sterile tube. Flask containing 100ml of BSM with 1% wt/v crude oil was inoculated with 1ml of soil suspension or water samples. The culture was incubated in shaker incubator at 120 rpm at 30˚C for seven days.

Identification

Colonies developed on nutrient agar were studied in term of their shape, color, odor and their margin. Microscopic examination is done for gram-stained slides to characterize gram-negative bacteria from others. Then biochemical tests were done for bacterial identification according to the identification scheme described by [11] (2013).

Screening of hydrocarbon degrading bacteria

To test the ability of bacterial isolates for degrading hydrocarbons, BSM medium containing crude oil as a sole carbon and energy source with 2% agar-agar was inoculated with tested isolates and incubated at 30˚C for seven days. Clearance zone around the colonies was taken as an indicator for biodegradation as well as change in the consistency of oil droplets as seen under microscope [5].

Emulsification index E24%

A flask containing 100ml of the medium was inoculated with the tested pure bacterial culture (1ml inoculum), then incubated in shaker incubator at 120 rpm at 30˚C for three days. For measuring the emulsification capacity, two equal volumes of cell free culture and substrate (kerosene) is added in a test tube. The mixture is vortexed at high speed for two minutes. After 24 hours, the height of the stable emulsion layer is measured as described by [12]. The emulsion index E24 is calculated as the ratio of the height of the emulsion layer and the total height of liquid:

$$E24 = \frac{h\ emulsion}{h\ total} * 100\%$$

Surface tension reduction test

The surface tension measurement was done at room temperature using Kruss tensiometer K6. The surface tension of free cell culture was measured in a 45mm diameter flat bottom container with approximately 40ml of sample. The platinum-iridium ring used for the measurements was cleaned with ethanol followed by a distilled water rinse after each sample. The measured values was directly read off the instrument in mN/m [13].

pH effect

Oil content analyzer (Horiba OCMA-350) was used to study the effect of pH on bioremediation by the isolates. A flask containing 50ml of the medium was prepared at pH 6, 6.5, 7, 7.5 and 8 using NaOH (0.1N) or HCl (0.1N). The media were inoculated with tested bacteria (1ml inoculum) and incubated in shaker incubator at120 rpm at 30˚C for seven days.

Temperature effect

Oil content analyzer (Horiba OCMA-350) was used to study the effect of temperature on bioremediation. A flask containing 50ml of the medium was prepared. The media were inoculated with tested bacteria (1ml inoculum) and incubated in shaker incubator at 120 rpm at 25˚C, 30˚C, 35˚C and 40˚C for seven days.

Petroleum hydrocarbons extraction

The petroleum hydrocarbons were extracted from the medium by liquid-to-liquid extraction method as fallowing: HCL was added to the medium to bring the pH to <= 2, then the extraction was done with a solvent (hexane) in ratio three volumes of solvent to 10 volumes of sample [14].

Analysis of hydrocarbon degradation

The total petroleum hydrocarbons in the sample were quantified using a Hewlett Packard 438A series gas chromatograph equipped with flame ionization detector (GC-FID). The carrier gas was helium and the column used for separation was SE-30 packet column, three m long and 1/8mm diameter. The operating conditions were as follows: initial oven temperature 100°C for 0.5 min and temperature ramp at a rate of 10°C min−1 to a final temperature of 300 °C. The injector temperature was 300 °C; the FID detector was at 325 °C; the flow rate of carrier gas was 40 ml/min−1 [15].

Identification of metabolites

The total petroleum hydrocarbons in the sample were analyzed and identified using Shimadzu GCMS-QP2010 Plus. The carrier gas was helium and the column used for separation was a quartz capillary column 30 M, The GC-MS operation conditions were as follow: carrier gas, helium 40 mL/min, injector and detector temperature, 300°C. The temperature program was 0 min at 90°C, then ramped to 300°C at 6°C/min, and held for 30 min at 300°C. The injected quantity was 1μL of 2% volume solution in hexane of the sample. The selective ions that are characteristic of the different compound types were chosen [16].

Statistical analysis

The Statistical Analysis System- [17] (2010) was used to test the effect of different factors in study parameters. Least significant difference -LSD test was used to test the significant difference among percentages in this study.

Results and discussion

Isolation and identification

Isolation results came up with 23 isolates capable of hydrocarbon degradation, which is an indicator for intrinsic bioremediation, whereas [18] (2010) mentioned that in most environments, the indigenous organisms have been exposed to the contaminant for extended periods of time have adapted or even naturally selected. Many contaminants, especially organic compounds are naturally occurring or have natural analogs in the environment. Rarely the environment does not have a number of organisms already present that can degrade or transform any contaminant present. According to colonies morphology, gram stain and biochemical tests, six isolates were identified as gram-negative bacteria belong to the genera *Enterobacter* and identified to species level as *E. cloacae* according to the identification scheme described by [11] (2013), table-1.

Table 1- Colonies morphology, gram stain and biochemical tests results.

Characteristics	*E. cloacae*
Colony shape	Smooth mucoid
Colony color	creamy
Cell shape	rod
Gram stain	-
Catalase test	+
Oxidase test	-
Methyl red test	-
Voges – Proskauer test	+
Citrate utilization test	+
Urease production test	+
Indole production test	-
Motility test	+
Lipase production test	-
Lactose fermentation test	+
Kligler iron test	A/A

(+) = positive result. (-) = negative result. (A) = acid

The degradation efficiency of these bacteria is due to their metabolic enzyme system, [19] (2007) mentioned that the cytochrome P450 alkane hydroxylases, which constitute a super family of ubiquitous Heme-thiolate Monooxygenases, play an important role in the microbial degradation of oil, chlorinated hydrocarbons, fuel additives, and many other compounds. Depending on the chain length, enzyme systems are required to introduce oxygen in the substrate to initiate biodegradation.

Emulsification index E24%

The results of E24% for isolates table-2 were varied from low to high emulsification. The statistical analysis showed a significant difference among the isolates, whereas the LSD value was 15.742. *E. cloacae* E1 showed the highest emulsification reached 62% thus it was chosen for further study.

Table 2- Emulsification index E24% for hydrocarbon degrading isolates (*E. cloacae)*

Isolates	E24 %
E. cloacae E1	62.0
E. cloacae E2	58.0
E. cloacae E3	38.0
E. cloacae E4	37.5
E. cloacae E5	32.5
E. cloacae E6	27.2

For hydrocarbon emulsification, the bacteria have produced biosurfactants to increase the bioavailability of hydrocarbon resulting in enhanced growth and degradation of contaminants by hydrocarbon-degraders [9]. [20] (2012) mentioned that biosurfactants are well known and well

documented for their role in enhancement of the emulsification of hydrocarbons, potentially solubilizing the hydrocarbon contaminants and increasing their availability for microbial degradation. The result of E24% test for *E. cloacae* E1 is an indicator for the efficiency of this strain for biosurfactant production. [21] (2008) indicated that the type of biosurfactant produced by *E. cloacae* is a glycoprotein and contains 45% total sugar, 18.75% protein, 7.0% sulfate and 9.23% uronic acid. [22] (2010) mentioned that biosurfactant of *E. cloacae* consists of glucose and galactose in its carbohydrate moiety. [23] (2012) obtained a similar result of E24 test for *Enterobacter* sp. MS16; she mentioned that the emulsification index for kerosene was 60.10%.

Surface tension reduction test (ST)

The results of ST test table-3 for the isolates were varied from low to high ST. The statistical analysis showed a significant difference among the isolates, whereas the LSD value was 6.426. ST of the control was 64 mN/m. *E. cloacae*. E1 showed the best reduction in ST, which is 36.0 mN/m.

Table 3- Surface tension reduction by hydrocarbon degrading isolates (*E. cloacae*)

Isolates	Surface tension mN/m
E. cloacae E1	36.0
E. cloacae E2	38.0
E. cloacae E3	41.0
E. cloacae E4	42.0
E. cloacae E5	39.0
E. cloacae E6	43.5
Control	64.0

The biosurfactant which produced by the isolates caused a reduction in ST, [24] (2010) mentioned that biosurfactants are surface- active compounds capable of lowering the surface tension and the interfacial tension of the growth medium. *E. cloacae* E1 showed a good result of ST reduction. [25] (2006) studied biosurfactant produced by *P. aeruginosa*. They found a marked reduction of the surface tension of the medium occurred, presenting values of approximately 35 mN/m. they also reported that critical micelle concentration (CMC) has the main role in surface tension reduction however; they made two critical micelle dilutions (CMD). The data demonstrated that the CMD–1 revealed a slight increase of the surface tension of the medium, whereas the CMD–2 caused a remarkable increased of the surface tension, which is an indicator of the elevated quantity of surfactants in the respective media. [26] (2011) reported that a new microbial consortium of *Enterobacter cloacae* and *Pseudomonas* sp. (ERCPPI-2) could reduce surface and interfacial tensions to 31.7 and 0.65mN/m from the original values of 58.3 and 16.9 mN/m, respectively. However, [23] (2012) found that glycolipids produced by *Enterobacter* sp. lowered the surface tension of the culture medium to 34 mN/m.

pH effect

The highest degradation for *E. cloacae* E1 was at pH range 6.5 - 7 with degradation capacity of 34.00 ± 0.20% and 37.00 ± 0.75% respectively table-4. The statistical analysis showed a significant difference among different pH cultures, whereas the LSD value was 8.830.

Table 4- Effect of pH on crude oil degradation by *E. cloacae* E1 within seven days

pH	Degradation %
6	33
6.5	34
7	37
7.5	33
8	23

In this study, neutral pH was the optimum level for oil utilization by the strain *E. cloacae* E1. This result agree with [27] (2010) who obtained the highest rate of hydrocarbon degradation by *Enterobacter* strain at pH 7. [28] (2011) confirmed this result when they found that the maximum degradation percentage was at pH 7. [29] (2013) studied heavy crude oil degradation by *E. cloacae*

strains; the initial pH level was 6.8. They noticed a fast reduction in pH level in first days of incubation, pH reduced from 6.8 to 6 relating to increase in microbial growth, which made medium acidy. Electric potential increased at the beginning of incubation periods but when microbial growth approached stationary phase, changes in pH and electric potential was not observed. The reason for pH reduction or increase in electrical conductivity of medium could be due to increase of production and consumption of different materials, which are the results of increase in biomass and rate of degradation.

Temperature effect

The highest degradation for *E. cloacae* E1 was at temperature range 30˚C - 35˚C with degradation capacity of 34.00 ± 0.03% and 36.00 ± 0.05% respectively table-5. The statistical analysis showed a significant difference among isolates of *E. cloacae* E1 incubated at different temperature, whereas the LSD value was 6.938. Temperature is very essential for hydrocarbon degradation; the rate of biodegradation generally decreases with the decreasing temperature [30]. Moreover, low temperature causes to increase oil viscosity [31]. [32] (2012) mentioned that increase in temperature cause to decrease viscosity, which is affecting the degree of distribution, and increase in diffusion rates of organic compounds. This means that higher biodegradation rates are expected at elevated temperatures.

Table 5- Effect of temperature on crude oil degradation by *E. cloacae* E1 within seven days

Temperature	Degradation %
25˚C	31
30˚C	34
35˚C	36
40˚C	21

The result of the present study is in agreement with [27] (2010) who reported that the maximum hydrocarbon degradation by *E. cloacae* was at 35˚C. [28] (2011) obtained different result. They mentioned that 40˚C was the optimal temperature for hydrocarbon degradation by *E. cloacae.* They concluded that at slightly increased temperature the physical nature and chemical composition of the oil would be effected favorably for degradation. Crude oil utilization is decreased at extreme temperatures, probably due to the change of physical and chemical composition of the oil or through the inactivation of metabolic enzyme system

Analysis of hydrocarbon degradation

The chemical analysis of basic organic contents in the control (untreated sample) by GC-FID revealed a very complex mixture of hydrocarbons figure-1.

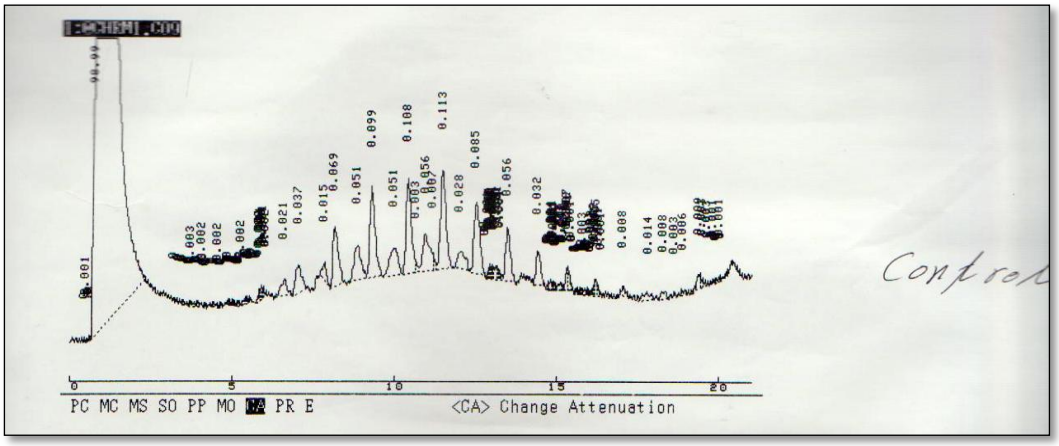

Figure 1- The chromatogram of the hydrocarbons in the control (untreated)

The axenic culture of *E. cloacae* E1 showed good degradation rate. However, the axenic cultures could utilize many hydrocarbon compounds as shown in figure-2. *E. cloacae* E1 reduced 70.00 ± 0.40% of total petroleum hydrocarbon. [28] (2011) reported that *E. cloacae* achieved 76% degradation of crude oil. On the other hand, [29] (2013) studied the potential of *E. cloacae* to degrade heavy crude

oil within 21 days; the result obtained was 53% degradation at 2500ppm, moreover biodegradation was improved with gradually addition of substrate. Substrate addition allowed bacteria to gradually adapt to a heavy hydrocarbon medium. Gradually adaptation raised biodegradation rate, increasing biomass.

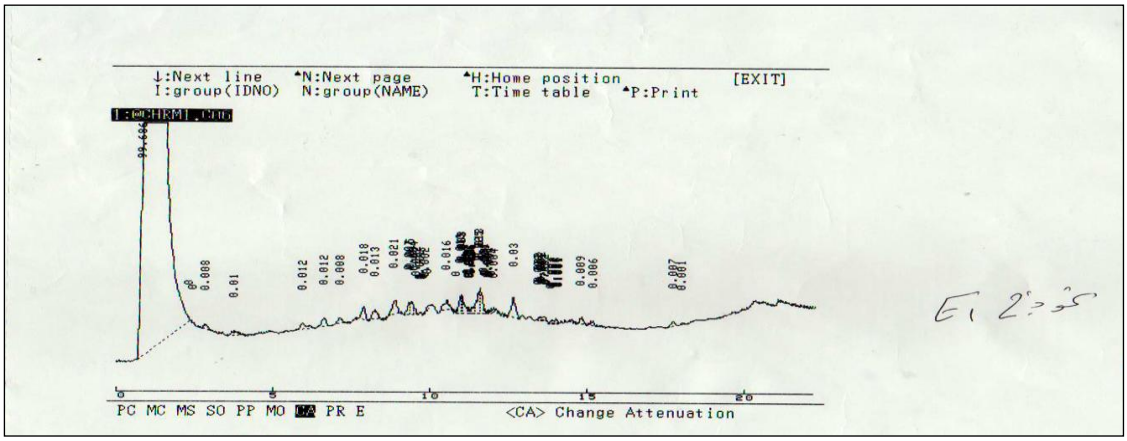

Figure 2- The chromatogram of the hydrocarbons in the sample treated by *E. cloacae* E1 within 30 days

Identification of metabolites

The identification of metabolites was done by GC-MS. In general, the results showed that many compounds were completely degraded or transformed into less complicated compounds due to the action of the axenic culture of *E. cloacae* E1. However, *E. cloacae* E1 was not effective against higher alkains. Although, the isolate could completely degrade bicyclic aromatic hydrocarbon (simple PAH) as shown in table-6. [33] (1999) mentioned that lower molecular weight PAH were substantially depleted through biodegradation by microorganisms indigenous to natural seawater under aerobic conditions. [34] (2009) reported that low molecular weight PAHs were 100% degraded by a consortium of *Enterobacter cloacae*, *Ochrobactrum* sp. and *Stenotrophomonas maltophilia*.

Table 6- Polycyclic aromatic hydrocarbons that completely degraded by *E. cloacae* E1 within 30 days

Compound	Structure	M wt.
Azulene, 4,6,8-trimethyl	C13H14	170
Naphthalene, 1,4,5-trimethyl	C13H14	170
Naphthalene, 1,4,6-trimethyl	C13H14	170
Naphthalene, 1,6,7-trimethyl	C13H14	170
3-(2-methyl-propenyl)-1H-indene	C13H14	170
5-isopropylidene-6-methyldeca-3,6,9-trien-2-one	C14H20O	204
4-(2-p-Tolyloxy-ethylsulfanyl)-quinazoline	C17H16N2OS	296
2,4-Bis(hydroxylamino)-6-methylpyrimidine	C5H8N402	156
Benzenamine, 2-methyl-4-[(2-methyl phenyl)azo]	C14H15N3	225
Benzenamine, 4-methyl-2-[(4-methylphenyl)azo]	C14H15N3	225
N-benzylbenzensulfonamide	C13H13NO2S	247
Beta-(4-aminophenyl)propionic acid	C9H11NO2	165
4-(phenylmethyl)benzenemethanamine	C14H15N	197
4,7-methanoazulene,1,2,3,4,5,6,7,8,-octahydro-1,4,9,9- tetramethyl	C15H24	204

Aromatic compounds such as BEX (Benzene, Ethylbenzene and Xylene) were completely degraded by *E. cloacae* E1 table-7. However, some aromatic compounds such as Mesitylene, pseudocumene, Hememellitene, M-ethyltoluene and O-ethyltoluene were hardly metabolized. In another local study, [35] (2009) had isolated a strain of *E. cloacae* with the ability of phenolic compounds degradation.

Table 7- Aromatic compounds that are completely degraded by *E. cloacae* E1 within 30 days

Compound	Structure	M wt.
o-xylene	C8H10	106
p-xylene	C8H10	106
Benzene, 1,3-dimthyl	C8H10	106
Ethylbenzene	C8H10	106
Benzadehyde o-benzyloxime	C14H13NO	211
N-[1-(Azepan-1carbonyl)-3-oxophenylpropyl]carbamic acid,benzyl ester	C24H28N2O4	408
Benzyl oxy tridecanoic acid	C20H32O3	320
Benzene, (1,3,3-trimethyl inonyl)	C18H30	246

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