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Measuring The Viability Of *Bacillus cereus* Bacteria On The Biodegradation Of Crude Oil In Soil And Incubated At Varying Temperatures And Concentrations

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Abstract

Forty-four isolates were isolated from soils of crude oil collected from different contaminated sites of crude oil refineries and reservoirs in Al-sulaymaniyah province on (MYP) medium. They were found to be *Bacillus cereus* bacteria. After phenotypic and biochemical tests, primary and secondary screening was performed on solid (BHM) medium covered with etheric solution of crude oil, 15 isolates were selected from the primary screening on the basis of growth density. The isolate marked *Bacillus cereus* (B28) out of 15 isolates as the most efficient depending on the diameter highest rate of the translucent ring which reach (3.1cm). the detection of isolation was confirmed by vitek2 device with detection kits BCL ID card.

Isolate viability (B28), which decomposes the hydrocarbons of crude oil, was studied by growing it on the liquid medium (BHM) that contains different concentrations for the two types of crude oil (average-kirkuk, heavy- Al-Qayyarah) (1,2,3%) and a different PH value (6,7,8). It was the highest rate for the consumption and preparation of living cells for Kirkuk oil (36.2%) and ($29.3x10^6$) respectively within 144 hours and at concentration of (1%) v/v, PH (7) and temperature $35C^o$.

Key words: Crude oil, *Bacillus cereus*, Biodegradation.

1-Introduction:.

Crude oil is a complex set of hydrocarbon aromatic, aliphatic and paraffinic compounds as well as compounds containing sulfur, nitrogen and oxygen and also other materials containing organic and inorganic metals [1].

Crude oil refineries and petrochemical industry factories are important for the national development and improvement of life quality. In recent years there has been a great demand for crude oil being a major source of energy in civilized societies, which led to dramatic increase in crude oil production, to the extent of ignoring the effect of these activities on the environment and human life [2].

Liquid wastes, produced by crude oil refineries and resulting from oil refining operations and fuel and lubricants manufacturing and petrochemical operations [3], are the main source of pollution of the environment, and a result of human activities in this domain [4]. Due to the lack of sufficient treatment systems, industrial waste water might become hazardous, leading to accumulation of toxic substances in water areas and have serious consequences for the ecosystem [5] [6].

Crude oil will remain the main source of energy during the next few decades, as there was no credible alternative found so far, and that pollution problem during crude oil production and

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transportation remains the major problem. It has been recognized as the significant problem in the world's pollution [7].

Hydrocarbons have susceptibility to move in food chains. So, the treatment of the polluting hydrocarbons is considered to be one of the important goals to avoid the damage to the biosphere caused by these compounds which afflict most of organisms including human beings. In order to eliminate contamination by hydrocarbons compounds or reduce it, many methods of treatment have emerged; an example is the use of micro-organisms. This method is called Biodegradation [8].

Biodegradation is the environmentally safest means to remove oil pollution; and there are several other ways and means like incineration, etc., which let enter more toxic compounds into environment. Various hydrocarbons biodegrading organisms, which are capable of organic compounds recycling, are important and effective to dispose of dangerous wastes such as hydrocarbons; and these hydrocarbons-degrading organisms are widely spread in ecosystems [9]. Most of these crude oil- decomposing and hydrocarbons- decomposing organisms are bacteria whose enzymatic systems have evolved, and these enzymes enable them to consume such compounds [10]. So, we note the bio- treatment concept link, for recent decades, with the pollution of environment by oil hydrocarbons in the first place, and secondly by manufactured hydrocarbons [11]. Diagnosis was confirmed by using vitek2 system with diagnosis kits BCL ID card.

2- Materials and Method

2-1 Crude oil samples:.

Samples of average Kirkuk crude oil were collected from the refinery of Baiji, which is supplied with Kirkuk crude oil. However, Al- Qayyarah heavy crude oil was obtained directly from the crude oil well after being extracted. A sample was tested chemically and physically for the purpose of study. Glass, sterile, opaque, marked and well-closed bottles were used for the collected samples. Bottles were kept in a cool place until the time of usage.

2-2 Soil samples:.

Soil samples were collected from a depth of (2-10cm) and put in sterile plastic bags, then taken to the laboratory from different locations of refineries and local storage tanks in Al-Sulaimaniya city as mentioned respectively: (Qiwan tanks, Hawin tanks, Soran tanks, Khawin tanks).

2-3 Isolation and detection of bacteria:.

One gram of each soil sample was put in a glass beaker containing 99 ml of the sterile physiological salt solution of concentration (0.85), and put in water bath at a temperature of 80C^{o} for 30 minutes to get rid of vegetative cells, then a series of dilutions (10^{-1}-10^{4}) was done. (0.1ml) of the latter dilution was drawn and distributed over three petri dishes containing (20ml) of the culture medium MYP prepared by dissolving (43gm) of *Bacillus cereus* selective agar base medium in a liter of distilled water, the PH was adjusted to (7.1±0.2) at a temperature 25C^{o}. The medium was cooled to 45-50C^{o} as per instructions of the manufacturing company. Then, (100ml) of egg yolk emulsion and (2ml) of polymixin B solution were added [12]. Dishes were incubated in the incubator at temperature of 37C^{o} for 24 hours [13].

A study and preview took place for the phenotypic characteristics of *Bacillus* bacteria colonies to dermine the size and colour of the colony, and to studyits texture. These colonies, which have the same characteristics as the *Bacillus cereus*, were tested by taking a samear of culture ring and diffusing it on a sterile glass slide, then installing and staining it by gram's method and

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examining the shape of the bacterial cell and the spore location in it, Then it was diagnosed by applying biochemical tests which included catalase, oxidase, indole, red methyle and vox-proskauer tests; consumption of nitrates, urease, motility test, sugar fermentation, starch hydrolysis, production of lecithinase enzyme and blood hydrolysis [14] [15].

2-4 Screening the efficient isolates in the degradation of crude oil:.

Isolated bacterial isolates were tested and diagnosed on the solid medium dishes (BHM) which is composed of KH_2PO_4 (1g), KH_2HPO_4 (1g), $(NH_4)_2SO_4$ (1g), $MgSO_4$ (0.2g), $CaCl_2$ (0.02g). $FeCl_3$ (0.05g), (2%) agar. Agar was dissolved in a liter of distilled water and PH was adjusted to (7) [16], crude oil was added as a sole hydrocarbon source at (1%) rate. Then dishes were incubated in an incubator at temperature of $35C^o$ for 144 hours. This medium was used for the purpose of selecting the efficient isolates depending on growth. Secondary screening followed where efficient isolates were cultured on the solid medium (BHM) which was covered with a solution of ethereal crude oil (v/v10%); and then, each isolate was stabbed in the dish center, by a transplant needle. Efficiency of the isolates was measured by forming the largest clear zone [17].

2-5 Study of the optimum environmental conditions for the growth of hydrocarbon-breaking bacteria:.

A study was conducted of the effect of the various environmental factors on the growth of *Bacillus cereus* bacteria and their capability of consuming crude oil, by using (100ml) of a liquid mineral salt medium (BHM) which is composed of the following salts: KH_2PO_4 (1g), KH_2HPO_4 (1g), $(NH_4)_2SO_4$ (1g), $MgSO_4$ (0.2g), $CaCl_2$ (0.02g). $FeCl_3$ (0.05g). These salts are dissolved, one by one, in a liter of distilled water and PH is adjusted to (7) [16], in conical flasks with a capacity of (250ml); and different concentrations (1,2,3%) of crude oil were added, and various PH (6,7,8) in a rocking incubator at speed of 120 RPM/minute and at temperature $35C^o$ for 144 hours.

2-6 Quantitative measurement of the loss of crude oil:.

Gravimetric method was applied to calculate the ratio of hydrocarbon consumption according to [18], during the calculation of the remainder amount of hydrocarbons, by during sterile filter paper type Whatman (No.1) in the oven for 24 hours at $45C^o$, and then weighted it. The medium was filtered on the filter- paper into Buechner funnel using negative pressure, then dried by oven for 24 hours at $45C^o$. After that, it was weighted to see the difference in weight before and after filteration ; as the sediment weight represents the remainder of hydrocarbon, taking into consideration that the entire process is done under sterile conditions by the following equation : $R=(A-B/A)x100\%$

R=Consumer proportion of hydrocarbons

A=The quantity of hydrocarbons added

B=The remaining amount of hydrocarbons

2-7 Viable count for crude oil- decomposing bacteria:.

A count was made on living numbers after(24,48,72,96,120,144) hours of incubation by spreading 0.1ml at concentration of $1x\ 10^{-6}$ cell/ml on the surface of nutrient agar dishes. All dishes were incubated at varying incubation temperature used (25,30,35) as well as various PH (6,7,8) and different concentrations of crude oil (1,2,3). Viable bacteria count was done for colonies that live on crude oil (Colony Forming Unit) (CFU)/ml, according to the following

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equation: The number of colony forming unit (cfu/ml) = number of colonies/ the size of the sample taken x dilution. [19] [20].

3- Results and Discussion:.

3-1 Isolation of *Bacillus cereus* bacteria from soils contaminated with crude oil:.

Forty-four isolates obtained on (MYP) medium were prepared primarily to be *Bacillus cereus* bacteria from various samples of crude oil- contaminated soils Figure(1). The isolates were given the symbol (B) and a serial number (1-44) each as per priority of isolation (tablel 1). All isolates consisted of internal spores were obtained from crude oil- contaminated soils; while only one isolate was selected from these isolates for the current study depending on the density of growth and the ability of bio- disassembly of crude oil by forming the translucent zone around the colony on the solid BHM medium surface. Abou Shanab *et al* could, in 2015, isolate 20 bacterial isolates from soils contaminated with oil hydrocarbons; they selected from among them 4 bacterial isolates depending on high growth in broth basal salt medium with 4% crude oil added, as a sole source of energy [21]. Subathra *et al*., 2013 mentioned that 113 crude oil- decomposing isolates which were isolated from oil derivaties- contaminated environment; 15 cultured isolated were selected from among them on BHM with crude oil (1%) as a sole source of energy and carbon[22].

Table(1): Number of isolates isolated as Bacillus cereus from soils local sulaimaniyah province.

Samples sites	Number of isolates	Isolates signs
Giwan tanks	16	B1- B16
Soran refinery	9	B17- B25
Haoan tanks	7	B26- B32
Hiwa refinery	7	B33- B39
Khaoan refinery	5	B40-B44
Total	44	B1- B44

Figure(1): growth on (MYP) medium and lecithinase hydrolysis.

3-2 Diagnosis of isolation:.

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All the 44 bacterial isolates were gram- positive and non- motile bacilli and forming internal spores and decomposing type B blood. Through physiological and biochemical tests for isolates shown in tables (2) and (3), and good growth on (MYP) medium and the formation of pink colonies, it became obvious all of them were related to *Bacillus cereus* type.

Table (2): Culture and microscopic characteristics isolated for 44 isolated first on the MYP medium.

Isolates	The nature of the colonies on the nutrient solid medium						Cell morphology	Com bines cell	Gram stain
	Morphology	Color	Textures	The edge	Transparency	height			
B1-B44	circular	white	viscous	zigzag	Opaque	regular flat	bacillary forming spores	bilateral serial	positive

Table (3): Biochemical tests isolates for (44) isolated first on the MYP medium.

N.	testes	B1- B44
1	Motility	-
2	Spores	+
3	Indole	-
4	Methyl red	+
5	Voges– Proskaoer	+
6	Starch hydrolysis	+
7	Oxidase	+
8	Catalase	+
9	Urease	-
10	Simmon Citrate	+
11	Glucose	+
12	Growth on MacConkey medium	-
13	Lecithinase enzyme production	+
14	Hymolysis	B
15	Growth on MYP medium	+

+:the result were positive for examination, _: the result were negative for examination, β: complete hydrolysis of blood.

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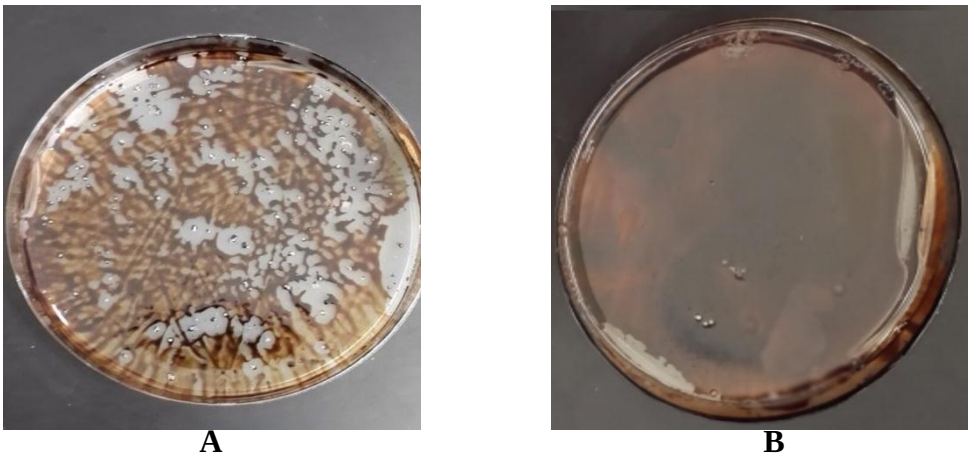
3-3 Primary screening, test the ability of bacterial isolates on dismantling of hydrocarbon compounds in solid medium:.

After isolating and diagnosing 44 isolates of *Bacillus cereus* bacterium from contaminated soils, primary screening was done for the isolates about their ability to dismantle hydrocarbon compounds in solid medium. Isolates were cultured on BHM medium, and grown in dishes from 24 to 144 hours. 15 bacterial isolates consumed (34.1%) of crude oil as a sole source of carbon and energy out of 44 isolates Figure(2), while 18 isolates (40.1%) grew weak and were of little ability for crude oil consumption out of the total 44 isolates. This may be caused by lack of colonies growth and crude oil consumption as they do not possess the ability to dismantle the hydrocarbon compounds because of the lack of a specialized enzymatic system; or they consume simple oxidation products of other bacteria that have the ability to oxidize hydrocarbon compounds [23] [24]. Fifteen efficient isolate were selected for their crude oil consumption in order to perform secondary screening on them.



A **B**

Figure(2): isolates growth on solid (MYP) medium with crude oil at temperature (35C^{o}) and PH(7). (A): growth with crude oil consumption. (B): no growth without crude oil consumption.

3-4 Secondary screening and choosing the most efficient isolates:.

Secondary screening depended on diameters rate of the translucent zone around the bacterial colony for their consumption to crude oil layer which covers the dish surface. As Kiyohara *et al*., 1999 explained that the utmost limit of the translucent zone is 2-3cm, the average limit is 1-1.9cm and the minimum is 0.5-0.9cm. isolate (B28) was chosen for aubsequent experiments, as result showed that isolate (B28) which was isolated from Hawin tanks soils were the most efficient in crude oil consumption and in formation of the largest translucent zone on the dish surface by diameter rate of (3.1cm) Figure(3). Subathra *et al*., 2013 explained that they were assured of the efficiency of crude oil consumption by the bacterial isolates by way of forming translucent zones around the bacterial colonies developing on BHM agar medium with 100 µ/L added of crude oil at temperature 37C^{o} for 24-48 hours [22].

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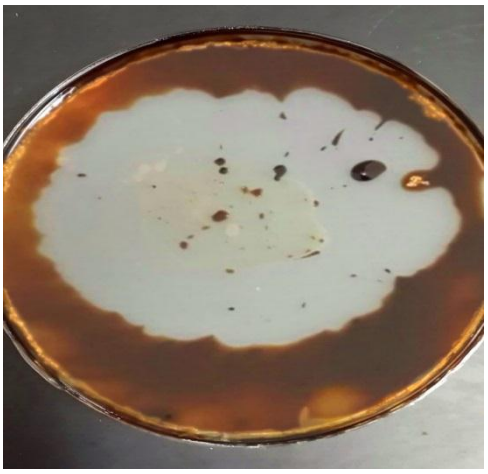

Figure(3): formation translucent zone on (BHM) medium with ethereal solution of crude oil (10v/v) at (35C^{o}) at period 24-144 hours for isolate (B28).

3-5 study of the different concentrations effect on the process of degradation:.

The cultural medium (BHM) incubated the liquid provided with different concentrations of the two types crude oil (Kirkuk, Al-Qayyarah) (1,2,3%) and inoculated with the isolate (B28) at a temperature 35C^{o} and PH (7). The results showed different ability at dismantling the various concentrations of two types of crude oil used in the study. The percentage of average Kirkuk oil at concentration (1%) for the first 24 hours of incubation (8.9%). Consumption continued to increase until reaching (36.2%) after 144 hours. As for Al-Qayyarah heavy oil, the percentage of consumption reached (6.2%) at concentration (1%) during 24 hours until it reached (28.5%) after 144 hours (figure, 4-A). At concentration of 2% for average Kirkuk oil, the consumption ratio was (5.1%) within 24 hours after 144 hours incubation, consumption increased to (22.1%), while the proportion of Al-Qayyarah heavy oil consumption at concentration of (2%) within 24 hours was (4.3%); and after incubation for 144hours it reached (18.4%) (figure, 4-B). As for consumption at (3%) for average Kirkuk oil, within 24 hours, it was (3.7%); then the ratio of consumption was growing up gradually until reaching (12.9%) after 144-hour-incubation; while the consumption ratio for Al-Qayyarah heavy oil at concentration (3%) was (2.6%) with 24 hours of incubation. Then, after 144 hours period of incubation it reached (9.4%) (figure, 4-C). The causes of variation may be due to that the degradation of crude oil is related to the nature of pollutants and chemical compounds within the composition [25]; since other studies have indicated that these chemical compounds are linked to coherence or correlation between oil hydrocarbons [26]; as average crude oil degrades at a higher rate than heavy crude oil [27]. The results showed, as in figures(5-A),(5-B), (5-C), that cells numbers-using concentrations of the two crude oil types (average Kirkuk and heavy Al-Qayyarah) (1,2,3%) were in average Kirkuk oil (14.2, 22.6, 29.3 x 10^{6}) successively after a 144 hour incubation; while the developing bacteria numbers for heavy Al-Qayyarah oil, they reached- at the same crude oil concentration (9.1, 12.6, 16.3 x $10^{6)}$ respectively within 144 hours of incubation. Results showed that concentration (1%) gave the best growth for bacterial cells and highest consumption ratio for the two crude oil types used in the experiment, as in figures (6-A),(6-B). In most cases, the types of *Bacillus cereus* prevail in the soil especially in crude oil-contaminated soil. This might be because of the bacteria's capacity to produce and form spores that could protect them from the toxic effects of hydrocarbons [28].

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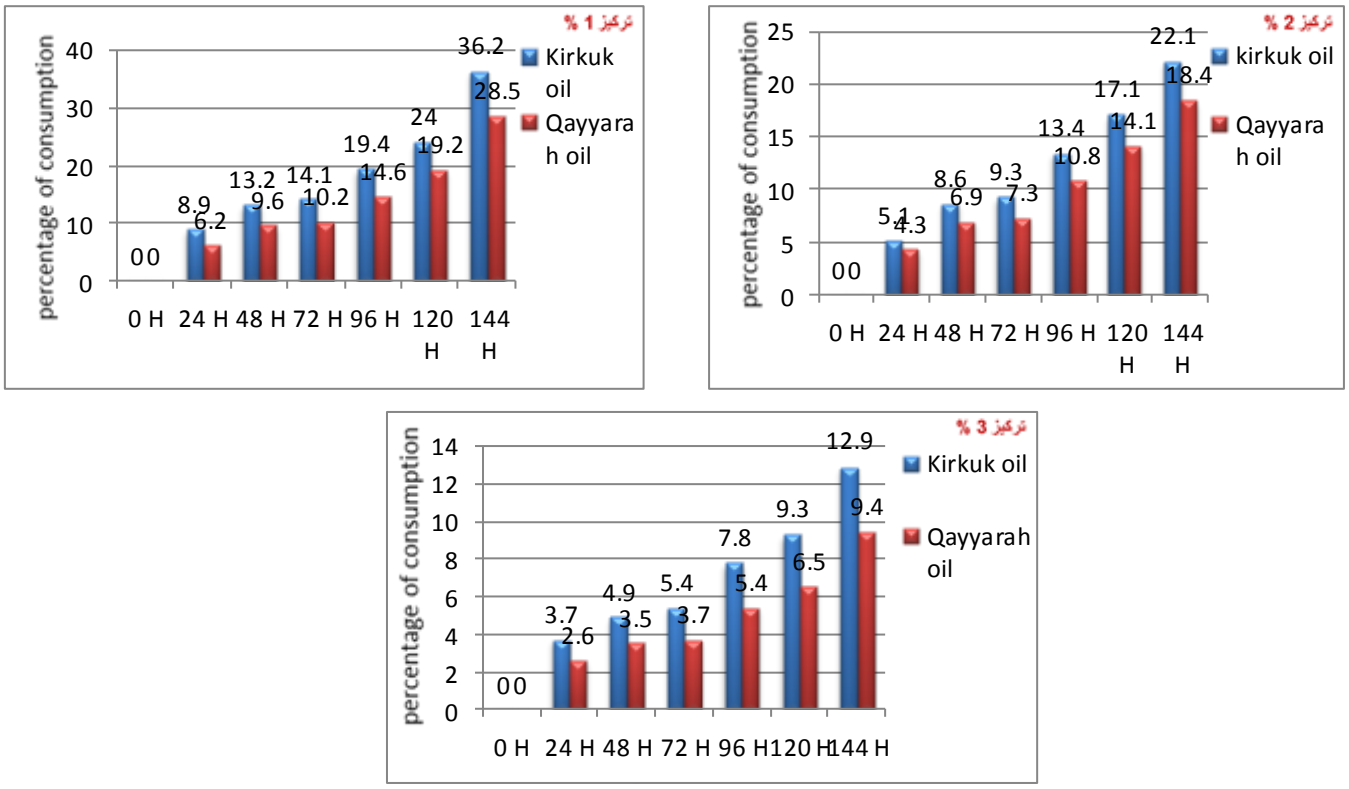
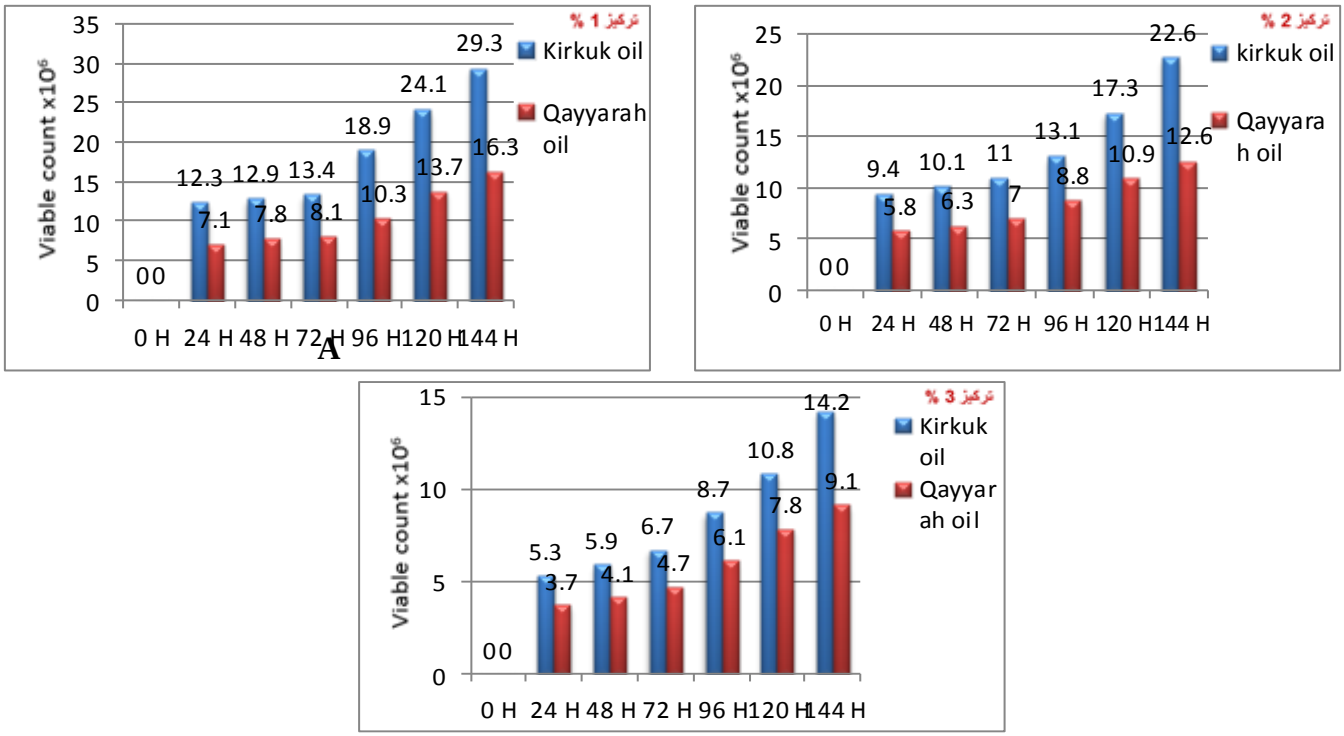


Figure(4): the percentage of consumption of the two types of crude oil (Kirkuk,qayyarah) at temperature (35C°) and PH (7) by *Bacillus cereus*. (A): 1% concentration, (B): 2% concentration, (C): 3% concentration.



Figure(5): viable count of *Bacillus cereus* growth on different concentrations of two types of crude oil from (24-144) hours of incubation at temperature (35C°) and PH (7). (A): 1% concentration, (B): 2% concentration, (C): 3% concentration.

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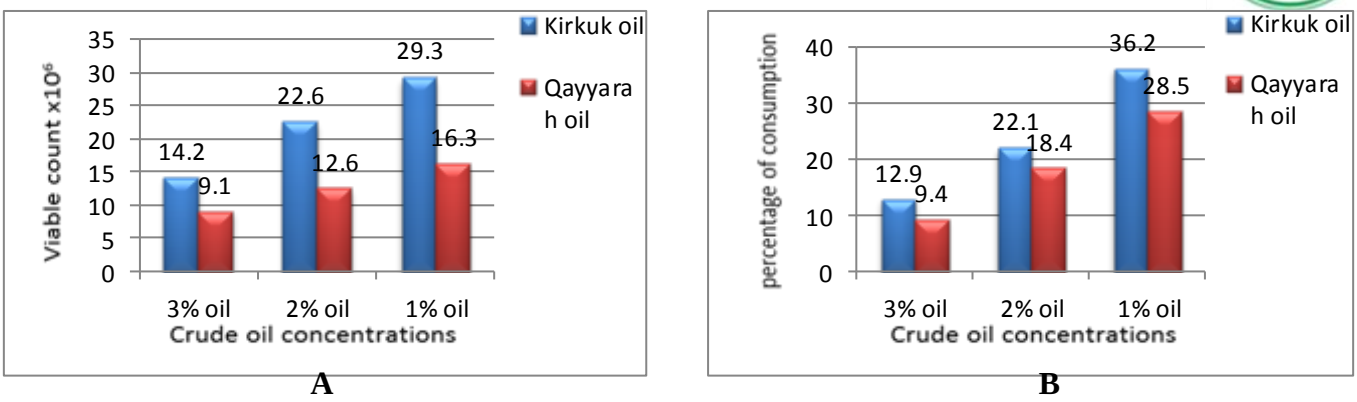

Figure(6): viable count of *Bacillus cereus* bacteria and the percentage of consumption at concentrations (1%, 2%, 3%) for two types of crude oil within (144) hours of incubation at temperature (35C^{o}) and PH (7). (A): viable count of *Bacillus cereus* bacteria. (B): the percentage of consumption.

3-6 the effect of the different PH on the process of degradation:.

The isolate (B28) was grown at temperature (35C^{o}) on a liquid medium BHM containing (1%) for each of the two types of crude oil unilaterally and by different hydrogenic numbers (6-7-8). Figure (7-A) shows the consumption ratio for the two types of crude oil at PH (6); as the results showed the proportion of Kirkuk average oil consumption during the first 24 hours (8.1%). Degradation continued until the period of incubation reached 144 hours (30.1%). The proportion of Al-Qayyarah heavy oil within 24 hours of the period of incubation (5.7%), consumption continued to reach (23.5%) after 144 hours. As for Kirkuk average oil consumption ratio at PH (7) it reached (8.9%) within the first 24 hours; and at 144 hours of incubation duration it reached (36.2%). The consumption rate of Al-Qayyarah heavy oil at this PH, during the first 24 hours, was (6.2%); and after 144 hours of incubation, the consumption continued to reach (28.5%) figure (7-B). At pH (8), proportion of oil consumption for Kirkuk average oil was (6.8%) after 24hours of incubation, and crude oil consumption continued to reach (23.2%) within 144 hours of incubation; while Al-Qayyarah heavy oil consumption ratio was (4.6%) at the first 24 hours; and the consumption reached (4.6%) after 144 hours of incubation figure (7-C) . Through these results we infer that the effectiveness of the bacterial consumption is around PH (6-8), as results recorded the optimum PH for the consumption of crude oil which was (7). The results of this study agree with what [29] [30] had done, who confirmed that the best growth for the bacteria lies between PH (6-8). figures(8-A), (8-B),(8-C) show the ability of *Bacillus cereus* bacteria to grow in the liquid medium BHM and in different numbers of PH (6,7,8), and at temperature (35C^{o}). Their numbers increase significantly by their consumption of crude oil as a sole source of energy and carbon. Results recorded the numbers of bacteria developing on Kirkuk average oil after 24 hours of incubation so that they amounted to (7.8, 12.3, 10.6 x 10^{6}) respectively according to PH order, while they reached (20.4, 29.3, 25.4 x 10^{6}) respectively within 144 hour incubation period. The results showed that the best PH for the two types of crude oil degradation is PH (7) as shown in figures(9-A)(9-B). Al-Busodh (2004) noted that PH affects the hydrocarbons- dismantling enzymes by micro-organisms, as the found that the optimum PH for the production of the fat (lipase)- dismantling enzyme was at PH (7) [31]. Moreover, Annadurai *et al*., 2007 pointed out that the best bio-dismantling for phenol by bacteria was at the optimal PH between 6.8 and 7 at 30C^{o} and by using phenol as an energy source at concentration of (0.2g/l) [32].

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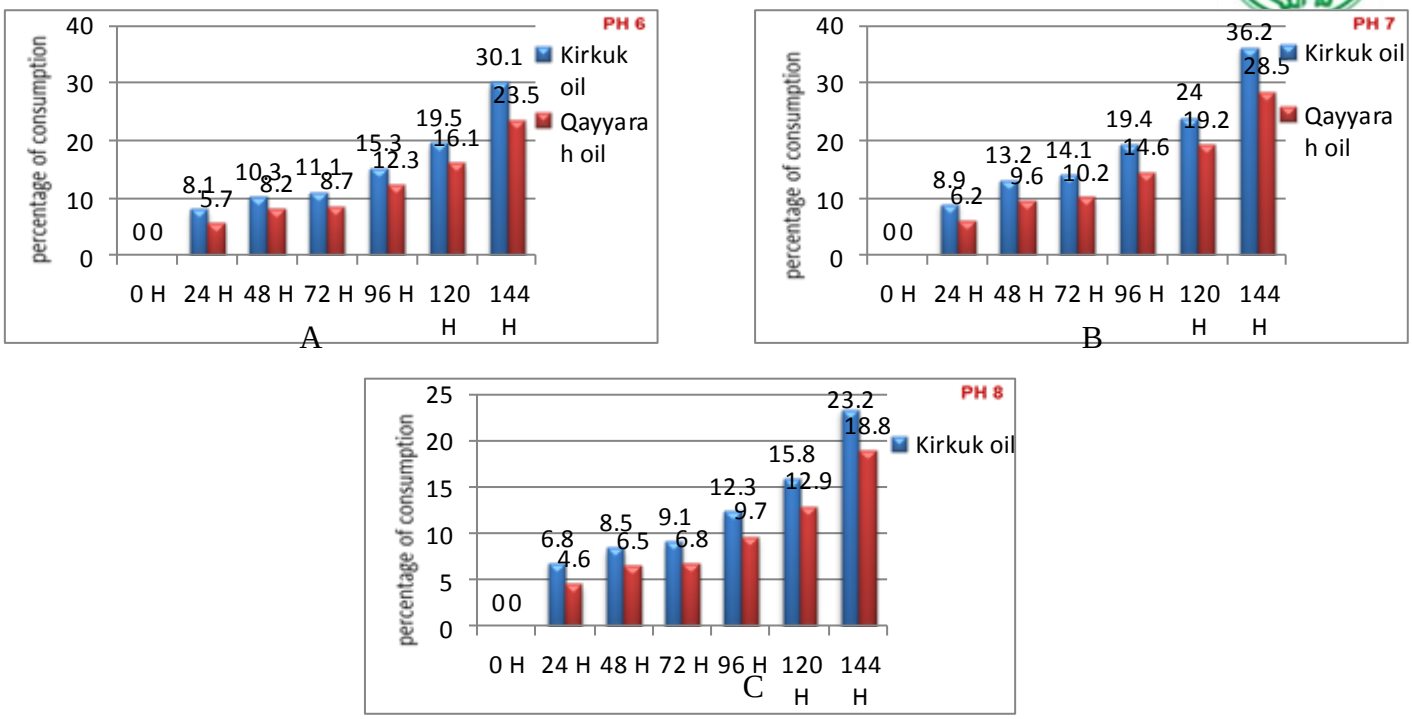
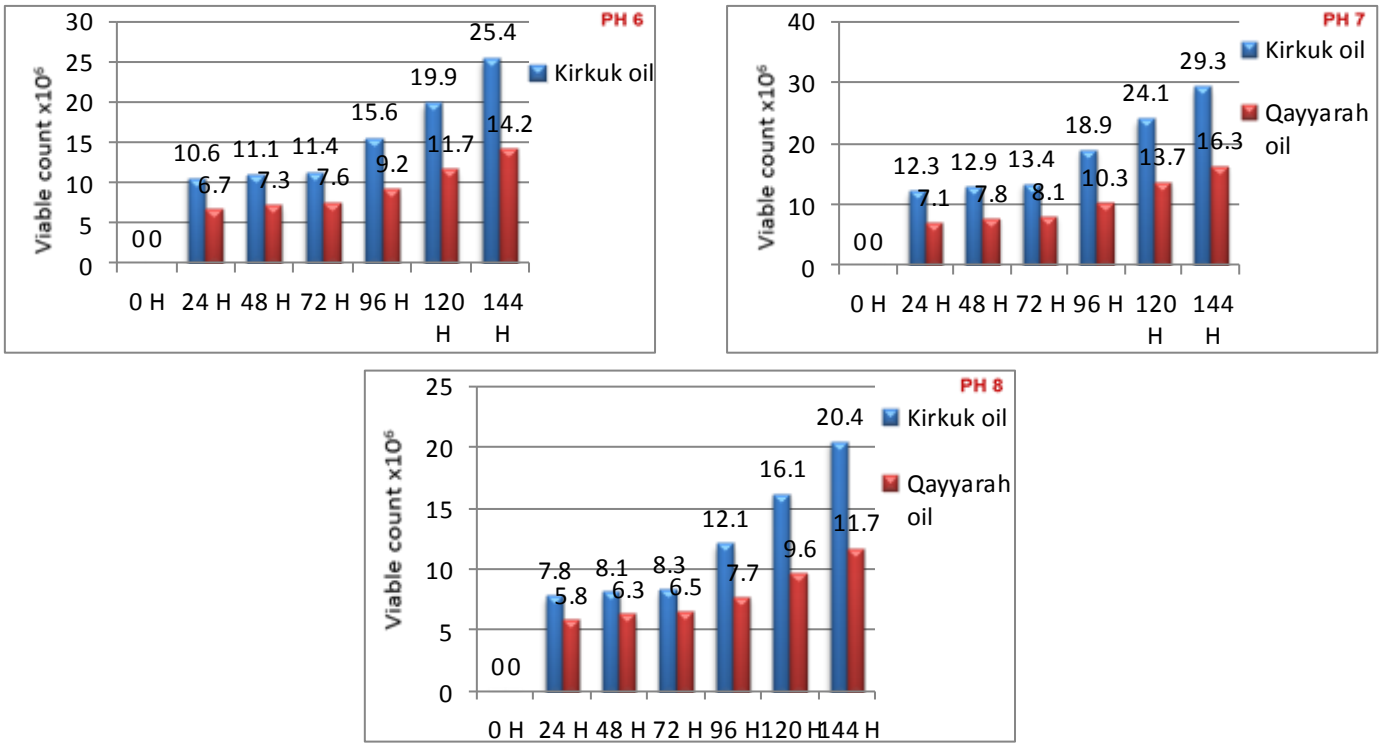


Figure(7): the percentage of consumption of the two types of crude oil (Kirkuk,qayyarah) at temperature (35C°) and (1%) concentration by *Bacillus cereus*. (A): PH (6), (B): PH (7), (C): PH (8).



Figure(8): viable count of *Bacillus cereus* growth on (1%) concentration of two types of crude oil from (24-144) hours of incubation at temperature (35C°). (A): PH (6), (B): PH (7), (C): PH (8).

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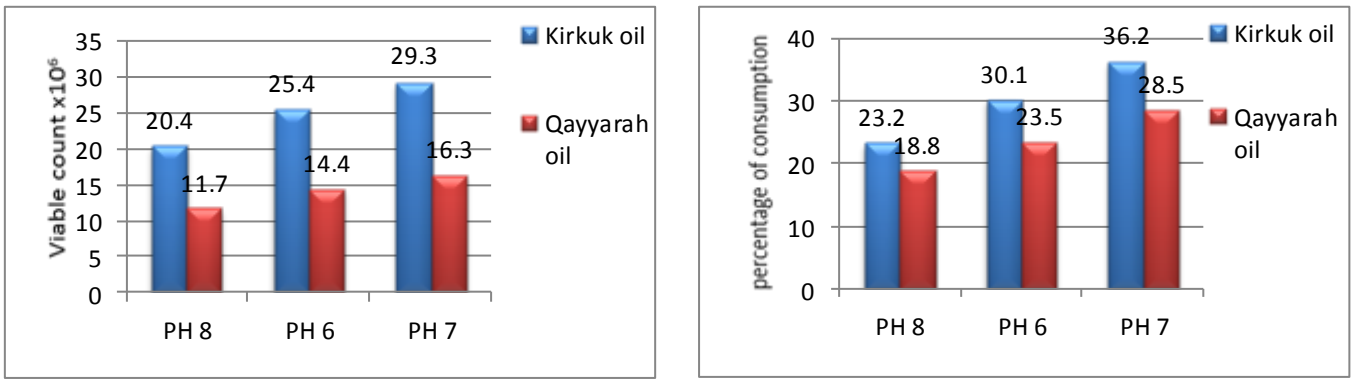

A B

Figure(9): viable count of *Bacillus cereus* bacteria and the percentage of consumption at (1%) concentration for two types of crude oil within (144) hours of incubation at temperature (35C^{0}) and PH (6,7,8). (A): viable count of *Bacillus cereus* bacteria. (B): the percentage of consumption.

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