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BIODEGRADATION OF CRUDE OIL AND BIOSURFACTANT PRODUCTION BY CRUDE OIL-DEGRADING BACTERIA STRAIN

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Abstract:
From soil samples collected from Al-Faw district in Basra governorate, oil-degradable bacterial strains were isolated, identified and described. Using common and well-established bacteriological techniques, five bacterial isolates have been described at the biochemical level. The Vitek-2 system was used to further identify the isolates. Five separate bacteria (*Staphylococcus lentus, Enterococcus casseliflavus, Staphylococcus haemolyticus, Staphylococcus vitulinus*, and *Bacillus pumilus*). Crude oil biodegradation was successfully demonstrated when the five bacterial isolates were cultured on mineral salt medium (MSM) filled with 1% crude oil for ten days.
The findings showed that the higher percentage of the total petroleum hydrocarbons (TPH) degradation rate by *Bacillus* sp. was 69%. And testing its ability to produce a biosurfactant, It was very effective in the emulsification coefficient test, where the percentage was 65%, in the oil spread test of 13 mm, and in the formation of foam by 35%, and the hemolysis was of the type beta, and it gave a positive result in the drop collapse test. We conclude that this type of bacteria can be used in bioremediation treatment of soil contaminated with crude oil.

Keywords: Biosurfactant, *Bacillus* sp, degradation, Foaming Activity, Emulsification

1. INTRODUCTION

Petroleum hydrocarbon spills from storage facilities and distribution systems have contaminated land and water systems all over the world(10). Numerous cleanup techniques for petroleum products have been developed due to the hazard they pose to public health and the requirement to restore renewable and non-renewable resources (4).
Because of the development in the use of petroleum as a source of energy in society, particularly in the form of crude oil, petroleum hydrocarbons are one of the most prevalent environmental contaminants in soil (14). Biological treatment methods for removing petroleum hydrocarbons from soil were created employing approaches to increase bacterial activity in pollutant breakdown by providing nutrients, ventilation, and moisture (10, 12). In order to create essential treatment systems and utilise petroleum hydrocarbons as a source of energy and carbon, numerous species of bacteria were investigated and identified (25). The degradation of crude oil is influenced by a number of parameters, including the bioavailability of crude oil, the type of bacteria present, temperature, the availability of nutrients and oxygen, salinity, and pH. Achieving a high removal efficiency will be very beneficial, and these parameters are strongly associated with the perfect conditions for bacteria to exist in contaminated environments (16). However, the primary factor determining adequate and precise bacterial performance in biological hydrocarbon biodegradation (1).

2. MATERIALS AND METHODS

2.1. Sample collection

For this investigation, samples of oil-contaminated soil were taken from the Al-Faw region in the Basra Governorate in December 2021. The depth at which soil samples were taken ranged from 5 to 15 cm. Samples of crude oil were taken from the Nahran Omar location.

2.2. measuring soil's temperature and pH

After samples were collected from the aforementioned places, the pH of the soil was determined in the lab using a pH meter. The pH of the soil was measured using the method of (19) by combining 10g of soil with 100ml of distilled water. Using a thermometer, the temperature of the oil-contaminated soil was determined at the time of sample.

2.3. Morphological characterizations of bacteria

Gram staining and morphological characterizations of the bacterial isolates based on color, size, and colony features were performed when bacterial strains were cultured on common enrichment, selective, and differential media such Nutrient agar, Blood agar, and MacConkey agar (Himedia/India) (margin, form, and elevation) (15).

2.4. Biochemical characterization

According to Bergey's Handbook of Systemic Bacteriology, biochemical tests were performed on bacterial isolates for identification and characterization, including Gram staining, oxidation/fermentation tests, acid production from carbohydrates, indole production, hemolysin production, and methyl red and citrate testing (2).

2.5. Tests used to study the ability of the isolate to produce biological surfactant materials

2.5.1. Emulsification index (E24)

The tubes intended for the crude oil emulsification experiment were prepared by washing them with methyl alcohol and drying them well. Add 5 ml of crude oil and 5 ml of crude oil culture filtrate to each tube and add water. Instead, the control well mixed for two minutes with a Vortex device (and quickly distilled a sample of the filtrate after that). maximum and left vertically for a period of 24 hours, after which the results were read according to the following equation: (23).

Emulsification percentage = (height of the highest emulsion height / total height) X 100

2.5.2.Drop collapse method

Determine the droplet collapse by adding 100 microliters of mineral oil (mineral oil)) on a glass slide. cleaned and incubated at room temperature for an hour ,Followed by the addition of 10 µl of culture filtrate. The same volume of distilled water was placed on the surface of a cell-free oil drop as a control sample, and left for a minute. The shape of the oil drop was examined, and the results were compared with the control sample. Total oil diffusion was evaluated as (+++) and partial surface diffusion (+)(26) .

2.5.3. Oil spreading method

To test the oil spread, 100 microliters of crude oil, 10 microliters of cell-free filtrate, and 50 ml of distilled water were placed to a petri dish(3). It was carefully dripped on the crude oil layer in the dish while ten microliters of distilled water were used. As a control sample, the diameter of the net zone was measured in mm on the surface of the crude oil compared to the control sample (27).

2.5.4. Foaming Activity

In order to conduct this test, 10 ml of cell-free filtrate were shaken quickly for two minutes, and the height of the foam that resulted was measured using the equation (3). Foam = height of foam formed / height of fever x 100

2.5.5. Hemolytic activity

To check whether the bacterial isolates could analyze blood, Blood agar dishes containing human blood were cultivated for 48 hours at 37 °C. The bacteria that could analyze blood were then identified by observing the corona that formed around the colonies (8).

3. RESULTS AND DISCUSSION

3.1. The physical and chemical characteristics of soil

As shown in Table 4, the soil utilized in the current study was obtained from the Al-Faw district in the Basra governorate and is characterized by some of the traits that have been seen and diagnosed, including color, temperature, and pH. The recent findings revealed gradations in soil sample color, ranging from black to dark brown. The morphological properties of the soil may change if the amount of petroleum hydrocarbons increases, according to past study (13, 18). Temperature values were noted; 28 ° C to spend Fao temperature increase over optimal would inhibit development; nevertheless, reducing the temperature will not cause the death of living things, just slows growth (6). The pH scale ranged from 7.84, which is barely alkaline, to neutral. According to our findings,(11). Because excessive levels of acidity or alkalinity have negative impacts on the soil's living organisms, the pH range for oil-contaminated soil that was discovered was neutral to slightly alkaline. They also affect the bacterial activity and the balance of the soil.

Table 1. The physical and chemical characteristics of soil

Characteristic of soil	Site Al-Faw
Color	Dark brown
Temperature C	30 C
Ph	7

3.2. Identification of bacterial isolates

According to the approach described in Materials and Methods, enrichment techniques were used to isolate several hydrocarbon-degrading bacteria. To verify that the bacteria obtained at the end of the enrichment cycle were able to use the petroleum compounds instead of just carrying them, hydrocarbon-degrading soil microbes were performed in several cycles. Fertilizing cultures were prepared at 30 °C for 10 days and five bacterial isolates were obtained from those cultures. One of the five strains isolated with the highest growth rate on crude oil was selected for further investigation.

3.2.1. Appearance definition

The sample was initially diagnosed based on the phenotypic characteristics, as the isolate showed the characteristics of the genus *Bacillus* sp. when it was grown on the nutrient agar culture medium at 37 °C for 24 hours. White to creamy colonies with a flat elevation in the middle, dense, sticky, slightly irregular in shape, with wavy edges, and smelling of old hay. Their colonies crowded on the surface of the dish

3.2.2. Biochemical identification

Table 2. shows the biochemical tests

	biochemical tests	*Bacillus* sp.
1	Gram stain	+
2	Catalase	+
3	Oxidase	+
4	VP	+
5	Citrate	+
6	MR	+
7	Indole	-
8	Urease	-

The sign (-) means negative results.The (+) sign means positive results

3.2.3. The Vitek-2 system

The Vitek-2 system was used to further identify the isolates. Five separate bacteria (*Staphylococcus lentus, Enterococcus casseliflavus, Staphylococcus haemolyticus, Staphylococcus vitulinus*, and *Bacillus pumilus*).

3.2. TPH degradation

The method [20,21] was used to evaluate the ability of bacterial species to decompose crude oil. Using 100 mL of MSM medium ((1 g) KH2PO4(NH4)2SO4, (1 g) KNO3, (0.2 g) MgSO4, (0.02 g) CaCl2 and (0.05 g) FeCl3), 1 mL of a single bacterial colony was added. Add 1% ml of crude oil next. All flasks were incubated for 10 days at 30 °C with 150 rpm in a shaker incubator. The results showed that the bacterium *Bacillus pumilus* recorded the highest percentage of biodegradation of crude oil among the five species, reaching 69% after the end of the 10-day incubation period, and *Staphylococcus vitulinus* recorded the lowest percentage of biodegradation of crude oil, reaching 27%, and the bacteria, *Staphylococcus haemolyticus* showed the percentage of biodegradation of oil Crude which amounted to 30% compared to the control sample amounted to 6%. While the two strains, *Staphylococcus lentus* and *Enterococcus casseliflavus*, did not show any rate of biodegradation.

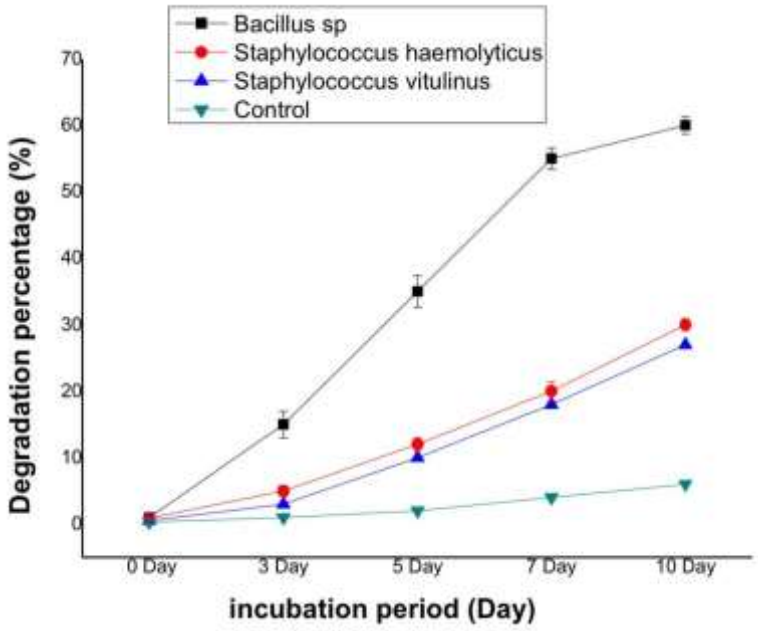
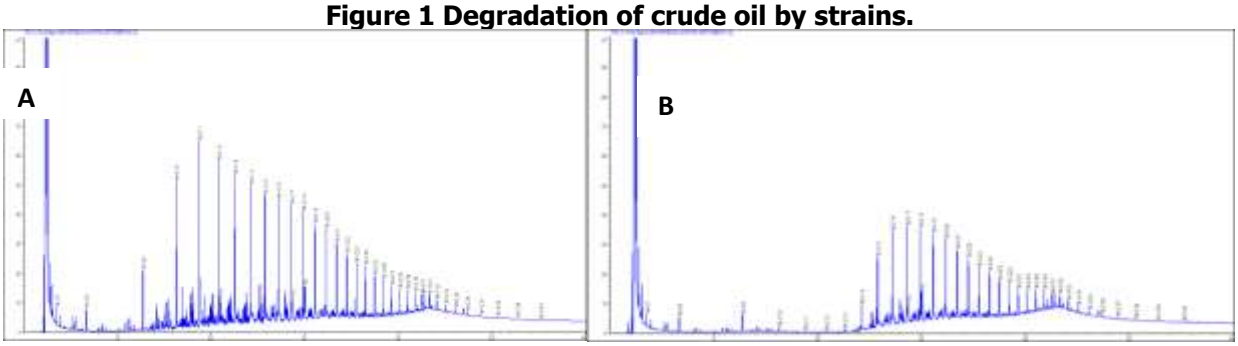


Figure 1 Degradation of crude oil by strains.



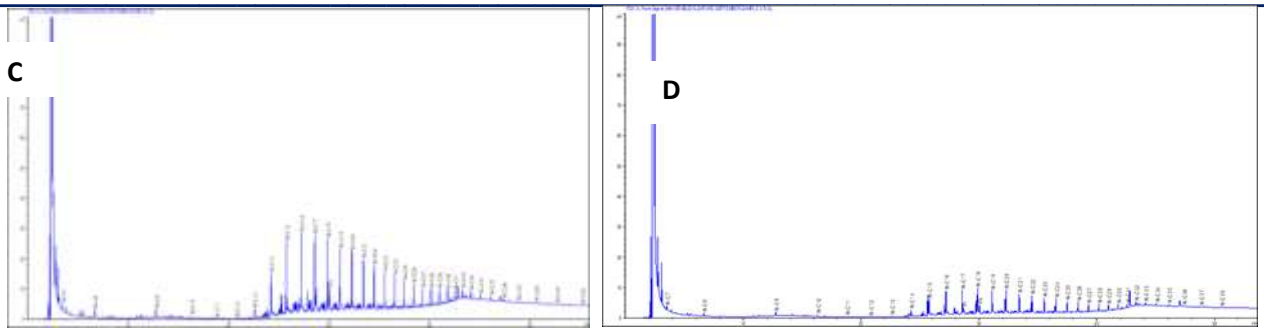


Figure 2. GC chromatogram of crude oil removal after 10 days soil treatment with *Bacillus pumilus*. A- control sample B- sample after an incubation period of 3 days C- sample after an incubation period of 7 days D- sample after an incubation period of 10 days.

3.3.1. Emulsification index (E24)

An experiment demonstrated the ability of bacteria to produce biodispersive compounds. Biosurfactants were able to emulsify crude oil through the ability of farm filtrate, as results showed. Emulsification *Bacillus pumilus* achieved the highest value of crude oil emulsification (65%). According to the study (22) the biological surfactant produced by *Bacillus Subtilis* had an emulsification coefficient of 75% for soybean oil and 83% for motor oil, respectively. According to (24). The best emulsifier is one that is stable for at least 24 hours and can emulsify with liquid hydrocarbon molecules at a rate greater than 50%.

3.3.2. Drop collapse method

The results of the droplet collapse test were shown after adding 10 microliters of filtrate to the surface of a droplet of mineral oil, with an estimated value of (++) for the dispersant produced by *Bacillus* sp. Where (27) showed that the droplet collapse test expresses the activity of surfactants and indicates the production of surfactant compounds, where the droplet will collapse from a liquid containing a surfactant and spread across the surface of the oil, according to the droplet collapse technique. Where this study agreed with (5), where the filtrate of the bacteria *Bacillus oceanisediminis* H2 was tested in droplet collapse using coconut oil, where it was noted that the average diameter was 4.1 mm.

3.3.3. Oil spreading method

When the isolated filter was placed on the plate containing distilled water, the effects of oil diffusion were visible. The diameter of the net circle (13 mm) compared to a control sample of 4 mm, and the crude oil is a net area by *Bacillus* sp. as a result of the spread of the oil due to the presence of dispersed elements. This test is thought to be quite sensitive for detection and has some additional benefits such only requiring a small volume of samples, being quick and simple to do, and not requiring any specific equipment (27).

3.3.4. Foaming Activity

The results showed that the ability of the bacterial isolate *Bacillus* sp. FB1 increased foam formation by 35% compared to the control sample in which water was used. The foam is an unstable dispersion of a large volume of gas in a continuous liquid phase (22), in a similar study by (17) the foam activity resulting from the production of vital compounds by *Bacillus Subtilis*DSM 15029 increased by 51%.

3.3.5. Hemolytic test

The results of this test showed that the colonies growing on the solid blood medium have the ability to analyze blood very efficiently, and the decomposition was of the beta type due to its complete decomposition of the medium (7). When Bacillus subtilis created a surfactant for red blood cells, they made the initial discovery of biological surfactant compounds' hemolytic activity(9). also found a correlation between hemolytic activity and the production of bioactive surfactants.

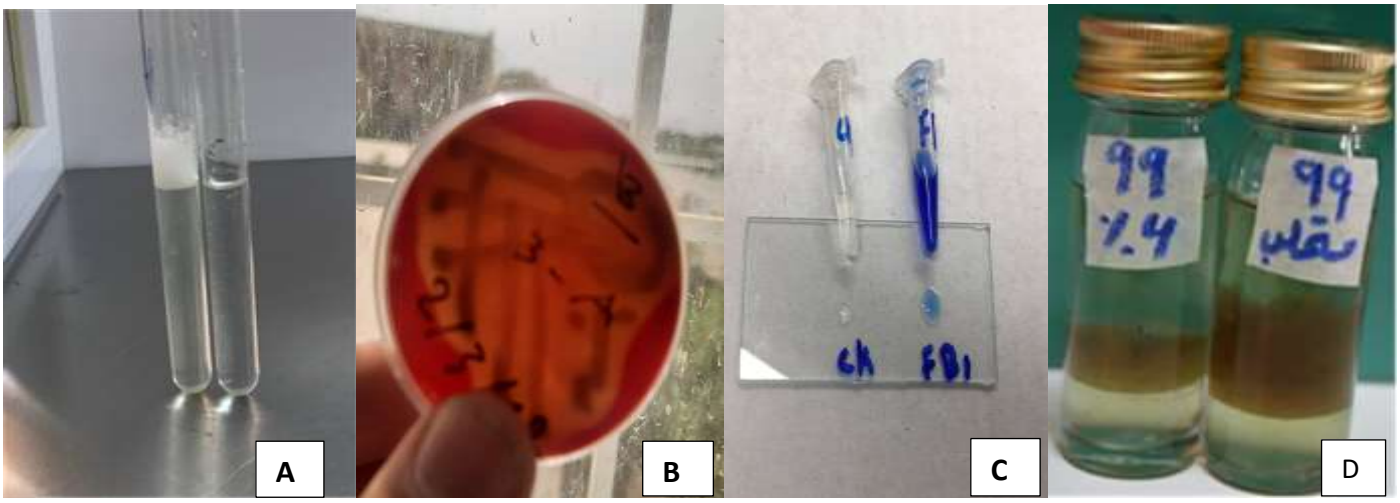


Figure 3: Biosurfactant production tests: (a) foaming activity test; (b) hemolytic activity; (c) Drop collapse (D)emulsification activity

4. CONCLUSIONS

Based on the results of this study, it can be concluded that the bacteria in Basra that biodegradation crude oil from contaminated soil produce plenty of surfactants, which enhance their diversity and biodegradability. It will be easier for researchers to take action against oiling Iraq if these bacteria are used in bioremediation.

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