Effect of time, temperature and pH on crude oil biodegradation by bacterial isolates from Al- Dora refinery-Baghdad

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Abstract

Bacterial isolates *Pseudomonas aeruginosa* , *Bacillus cereus* and *Bacillus subtilis* were isolated from wastewater treatment plant of Al- Dora refinery and used to study the optimal conditions (time , temperature and pH) for biodegradation of crude oil . Liquid BH medium with 1% crude oil was used in these experiments , 25 ml Erlenmeyer flask with 1% crude oil at different pHs (4 - 10) were inoculated by the three bacterial isolates and incubated at different period (1 - 10) days and different temperature (25 - 45˚C). Results shown that *P. aeruginosa* degraded a maximum of (72%) of crude oil when grown in the medium at initial pH of 8 and incubated at 30 ˚C for 6 days , while *B. subtilis* and *B. cereus* shown (65%) and (55%) of crude oil degradation respectively when grown in BH medium, pH 8 and incubated at 30 ˚C for 6 days . Results also indicated that the three isolates gave a significant reduction in surface tension of medium and the *P. aeruginosa* showed the high ability to decrease the surface tension from (73 - 24 mN/m) compared with other two isolates .

Key words : Biodegradation ,crude oil , refinery ,surface tension , bacterial isolates.

Microbiology Classification QR75-99.5

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Microorganisms survive in contaminated habitat because they are metabolically capable of utilizing its resources and can occupy a suitable niche (10) . Many bacteria like *Bacillus subtilis* and *Pseudomonas aeruginosa* and others have been reported by various workers for petroleum oil degradation (25) . *Bacillus cereus* was demonstrated by (6) as hydrocarbon degrading organism can be isolated from hydrocarbon polluted area and its degrading ability is a clear indicator that this bacteria can be applied in the bioremediation techniques.

Microbial action is directly influenced by several biotic and abiotic parameters, amongst these, special attention must be given to temperature, pH as well as incubation period (11). Extreme pH and temperature conditions are expected to have a negative influence on the ability of microbial populations to degrade the hydrocarbons Since the fate of hydrocarbon degradation is largely determined by the local environmental conditions, which influence the microbial growth and activities (12). The purpose of this study is to investigate the ability of native bacterial strains to degrade crude oil

Introduction :

Petrochemical industry , crude oil production and oil refinery serve as the major contributor to the environmental problems especially in soil and water. The discharges of oily wastewater to the environment have potential to cause significant environment harm through the release of contaminants to the environment and considered as hazardous industrial wastewater. This is because, this kind of wastewater contain toxic substances such as petroleum hydrocarbons, phenols, polyaromatic hydrocarbon which are inhibitory to animal and plant growth and also are mutagenic and carcinogen to human being (29 , 6)

In the last three decades more intensively studied focused on founding a cost–effective, natural and efficient clean-up treatment of hazardous waste water known as bioremediation technologies (7).which can be described as the conversion of chemical compounds by living organisms, especially microorganisms, into energy, cell mass, and biological waste products (8) .These microorganisms may be indigenous to contaminated area or they are isolated from other area and brought to the hydrocarbon contaminated area (3).

by autoclave at 121˚C/15 psi for 15 min. . After sterilization 1 ml from each reactivated bacterial strains was inoculated to one Erlenmeyer flask , control flask was inoculated with 1ml of sterile distill water flasks were incubated at 30˚C for 3 days at 120 rpm. (14) .

After incubation period ,the cultures were harvested by centrifugation at 10000 rpm , 4˚C for 15 min . cells were washed twice in phosphate buffer (PH7.2) and resuspended in appropriate volume of liquid BH medium to obtain a bacterial suspension with an optical density at 600 nm wavelength (OD 600) around 0.5 ,this suspension was used as a bacterial inoculum (15) .

Optimal conditions of crude oil biodegradation :

Bacterial strains were optimized under different growth parameters such as temperature , pH and incubation period :

1- Effect of incubation period :

25 ml of liquid BH medium were prepared and dispensed in 100 ml Erlenmeyer flasks , pH adjusted at 7.0 and supplemented with 1% crude oil as a substrate (duplicate and control for each bacterial isolate). Flasks were under optimized environmental conditions .

MATERIALSAND METHODOS:

The optimal conditions for crude oil biodegradation were examined for three bacterial isolates, isolated from waste water treatment plants of Al-Dora refinery. These bacteria based on biochemical and morphological tests as described in Bergey's Manual of Determinative Bacteriology (13) were *Pseudomonas aeruginosa* , *Bacillus cereus* and *Bacillus subtilis* .

Preparation of the bacterial inoculums :

Each of the three bacterial isolates was reactivated in L- broth medium composed of (g/l) **:** tryptone 15 , yeast extract 5.0 , NaCl 5 and glucose 1.0 at pH 7.0 and incubated at 30˚C for 18 hours .

Fifty ml of liquid Bushnell- Hass medium which compose of (g/l) : $MgSO_4$ 0.2, $CaCl_2$ 0.02 , KH_2PO_4 1, $(NH_4)_3PO_4$ 1 , KNO_3 1 and $Fe\ CL_3$ 0.05 were dispensed in 250 ml Erlenmeyer flasks , pH adjusted at 7.0 and supplemented with 1% crude oil as a substrate . Flasks were sterilized

NaOH solutions for adjusting. The flasks were sterilized by autoclave at 121˚C/15 psi for 15 min .After sterilization the media was inoculated with 1% of bacterial inoculums in each flask and incubated at 30 ˚C for 6 days . After incubation period the bacterial growth , crude oil biodegradation and surface tension were determined for each flask (20).

Crude oil degradation studies under optimal conditions :

A- Estimation of growth :

Optical density at 600 nm by spectrophotometer was used to evaluated the growth on zero time and after 6 days incubation period for each bacterial isolates and control (17 , 2 , 6).

B-Evaluation percentage of degradation :

For estimation of oil degradation rates by added 5 ml of n - hexane to the prepared flasks ,the content were transferred to a separating funnel and extracted . Extraction was carried out twice to ensure complete recovery of oil ,the extract was treated with 0.4 g of anhydrous sodium sulphate to remove the moisture and transferred in to a beaker leaving behind sodium sterilized by autoclave at 121˚C/15 psi for 15 min .After sterilization the media was inoculated with 1% of bacterial inoculums in each flask and incubated at 30˚C for different time period (0 , 1, 2 ,3 , 4 , 5 , 6 , 7 , 8 , 9 and 10) days at 150 rpm .After incubation period the bacterial growth , crude oil biodegradation and surface tension were determined for each flask (10) .

2- Effect of temperature :

Sterilized BH media , pH7.0 were used to study the effect of temperature .The flasks were sterilized by autoclave at 121˚C/15 psi for 15 min. . After sterilization the media were inoculated with 1% of bacterial inoculums in each flask and incubated at several temperatures (25, 30, 32, 35 , 40 and 45 ˚C) for 6 days at 150 rpm . After incubation period the bacterial growth , crude oil biodegradation and surface tension were determined for each flask(16 , 20) .

3-Effect of PH:

The effect of PH was determined by preparation of liquid BH medium with different PHs value from 5.0 - 9.0 (5.0 , 6.0 , 7.0 , 8.0 and 9.0) respectively using 1N HCL and 1N

and after 6 days of enrichment was measured by tension meter (Sigma 703D, Finland) at room temperature 30 ˚C (2 , 18) .

RESULTS AND DISCUSSION:

Biodegradation of waste water which contain hydrocarbons and their derivatives is based on the ability of microorganisms to increase their biomass growing on these substrates and to degrade them to non-toxic products, such as H_2O and CO_2 (5). Most potential bacteria for petroleum hydrocarbon degradation have been isolated from areas contaminated with oil (4).

The application of bacterial isolates in degrading crude oil involves the manipulation of environmental parameters such as temperature , pH and incubation period to allow microbial growth and degradation to proceed at a faster rate (3).

1- Effect of incubation period:

Result in Figure (1) showed that all the three individual isolates gave maximum growth rate after 6 days incubation period, which indicated faster utilization of crude oil as carbon and energy source(12) .

sulphate . this was evaporated to dryness at 40 ˚C under reduced pressure (19, 20)

Percentage of oil degraded was calculated as follows:

The % of degradation was calculated as follows ;(19) .

Weight of Residual crude oil= Weight of beaker containing extracted crude oil – Weight of empty beaker.

Amount of crude oil degraded = Weight of crude oil added in the media – Weight of residual crude oil.

% degradation = Amount of crude oil degraded / Amount of crude oil added in the media x 100.

C- Surface tension:

Bacterial isolates were enriched in 20 ml BH medium supplemented with crude oil (1%, w/v) for 6 days in an orbital shaker at 30 ˚C and 150 rpm pH was adjusted at 8 . Cells were harvested by centrifugation and re-suspended in sterile BH medium. Crude oil was added to cell suspension and vortexes for 3 min. . Change in O.D on 600 nm was recorded for cell suspension after allowing the crude oil and aqueous phase to separate. Surface tension of cell free BH medium before

medium with 1% crude oil at 30 ˚C and initial pH 7.0.

P. aeruginosa showed a significant reduction in surface tension (24 mN/m) as a result of its higher bio-surfactant production at30 ˚C , pH 7 after 6 days while *B. subtilis* and *B.cereus* showed less value in surface tension 34 mN/m 29 mN/m respectively when grown at the same conditions ,Figure(3) .

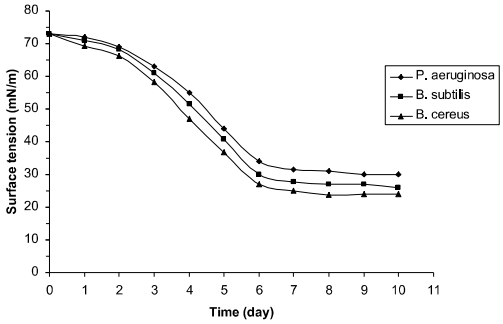

Figure (3): Effect of incubation period on surface tension of culture media that inoculated of isolates *P. aeruginosa* , *B.subtilis* and *B. cereus* . Bacteria were grown in BH medium with 1% crude oil at 30 ˚C and initial pH 7.

2- Effect of temperature:

The three bacterial isolates *P. aeruginosa* , *B.subtilis* and *B. cereus* showed high ability to grown in BH medium pH 7.0 with 1% crude oil after 6 days incubation at30 ˚C. Figure (4) .

Also results in Figure (5) indicated that *P. aeruginosa* gave the higher

P. aeruginosa showed maximum percentage of crude oil degradation 72% when inoculated in BH medium ,pH 7.0 and incubated at 30 ˚C for 6 days , Figure(2).

Also *B. cereus* and *B. subtilis* showed 65%, biodegradation respectively under the same conditions.

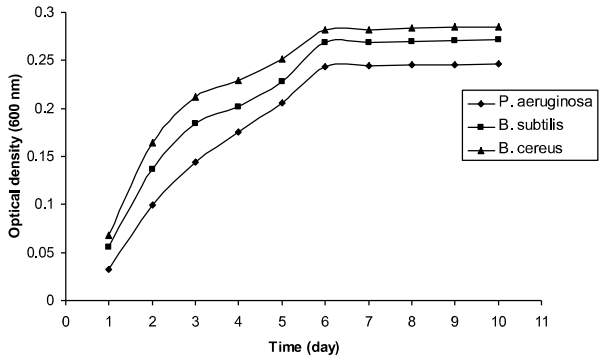

Figure (1): Change in bacterial growth by (O.D 600nm) of *P. aeruginosa* , *B.subtilis* and *B. cereus* isolates in BH medium with 1% crude oil , pH 7.0 and incubated at 30 ˚C at different incubation period .

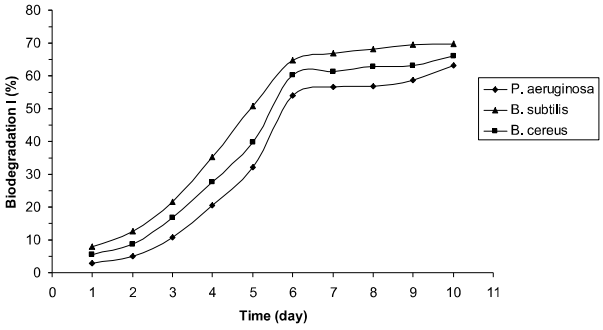

Figure (2): Influence of incubation period on the crude oil degradation of isolates *P. aeruginosa* , *B.subtilis* and *B. cereus* . Bacteria were grown in BH

temperature on the crude oil degradation for isolates *P. aeruginosa* , *B.subtilis* and *B. cereus* isolates in BH medium with 1% crude oil , pH 7 for 6 days at different incubation temperature.

Figure (6) indicate that *P. aeruginosa* showed High Reduction in surface tension of medium (24 mN/m) when inoculated in BH medium with 1% crude oil , pH 7 for 6 days at30 ˚C . While *B.subtilis* and *B. cereus* showed less ability to reduce surface tension (29 mN/m) , (34 mN/m) respectively.

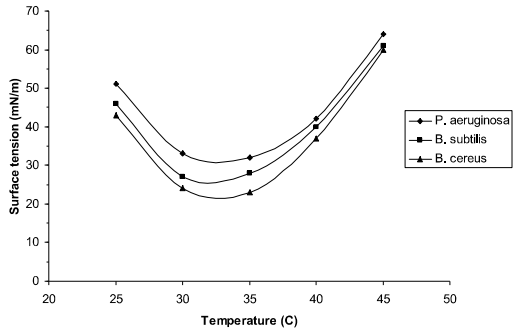

Figure (6): Effect of incubation temperature on surface tension of culture media that inoculated of isolates *P. aeruginosa* , *B.subtilis* and *B. cereus* . Bacteria were grown in BH medium with 1% crude oil for 6 days and initial pH 7.

3-Effect of pH:

P. aeruginosa , *B.subtilis* and *B. cereus* showed maximum growth ability to consume crude oil 72% compared to 65% , 55% for *B.subtilis* and *B. cereus* respectively , when grown at the same conditions.

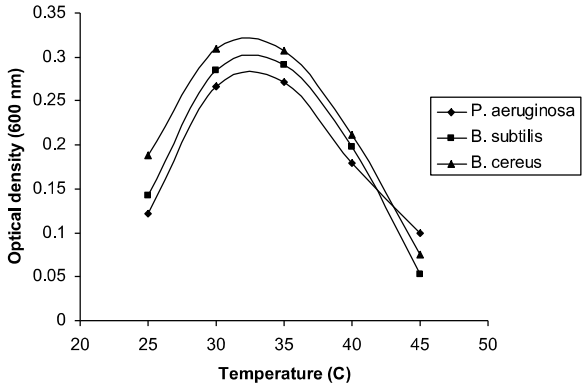

Figure (4): Effect of incubation temperature on bacterial growth by (O.D 600nm) of *P. aeruginosa* , *B.subtilis* and *B. cereus* isolates in BH medium with 1% crude oil , pH 7 for 6 days at different incubation temperature.

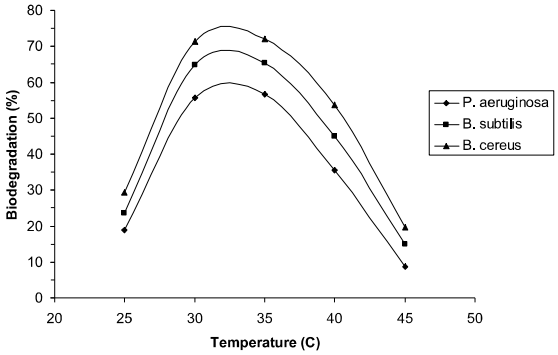

Figure (5): Effect of incubation

medium from (73 - 24 mN/ m) for isolates *P. aeruginosa* , and from (73 mN/m to 29 and 34 mN/m) for *B.subtilis* and *B. cereus* respectivly , when incubated at pH value of 8 for 6 days at 30 ˚C.

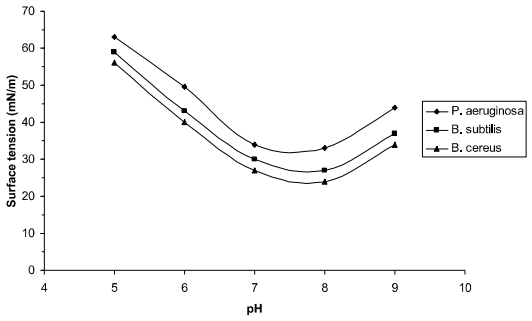

Figure (9): Influence of initial pHs value of the culture medium (BHM) on the surface tension of the medium when inoculated with *P. aeruginosa* , *B.subtilis* and *B. cereus* isolates . Bacteria were grown in BH medium with 1% crude oil for 6 days at 30 ˚C.

P. aeruginosa had the highest growth in sterilized media supplemented with crude oil followed by *B.subtilis* after 4-8 days of incubation period at (28 ± 2°C) not only because it was isolated from oil contaminated environment but also because it is known to possessed a more competent and active hydrocarbon degrading enzymes than other biodegraders (26 , 27) . Similar to our result (1) reported that *B.subtilis* had the ability to utilize and degrade Figure (7) and crude oil biodegradation Figure (8) when incubated at pH 8 for 6 days at 30 ˚C .

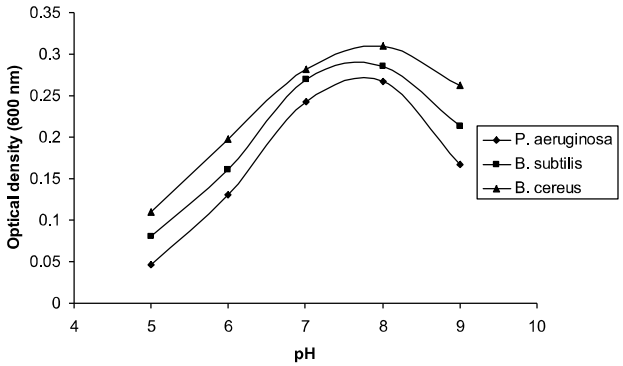

Figure (7): Influence of initial pHs value of the culture medium (BHM) on bacterial growth for isolates *P. aeruginosa* , *B.subtilis* and *B. cereus* . Bacteria were grown in BH medium with 1% crude oil for 6 days at 30 ◦C.

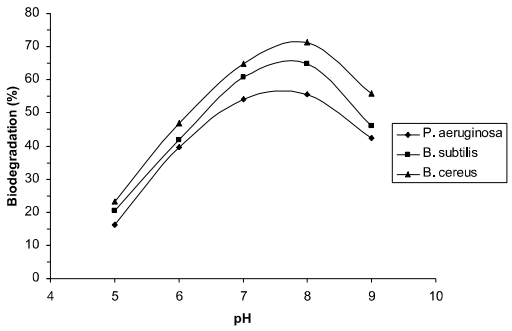

Figure (8): Influence of initial pHs value of the culture medium (BHM) on the crude oil biodegradation for isolates *P. aeruginosa* , *B.subtilis* and *B. cereus* . Bacteria were grown in BH medium with 1% crude oil for 6 days at 30 ˚C.

Resultes in Figure(9) showed reduction in surfactant tension of

All the three bacterial isolates showed high absorbance value at pH 8 , this could be as a results of natural selection and evolution (25) . A pH 8 reported to gave optimum growth and enzyme activity for *bacillus sp*. and *pseudomonas sp.* (22 , 23).

These results are agreed with those found by (15) which found that the biosurfactant produced by *Pseudomonas aeruginosa* has higher emulsification stability to wide range of pH 'between' 4 to 10, with decrease the surface tension values of the media from 31 to 34 mN/m. similar to our work (24) mentioned that surface tension shows that biosurfactant molecules of two isolated bacillus was different and that reduction of surface tension by isolated *bacillus* indicates that these bacillus could produce surface active compounds .

Reduction in surfactant tension of medium was a result of emulsification of crude oil by the surfactant produced by inoculated strains both *Pseudomonas aeruginosa* and *Bacillus subtilis* (2).

Microbial community caused reduction in surface tension during the experiment, indicated that these strains could produce sufficient biosurfactant andThere was a positive correlation between the reduction of surface crude oil in 6 th day of incubation . *Pseudomonas sp.* gave maximum growth rate after 6 days incubation period, which indicated faster utilizatation of crude oil as carbon and energy source and degrade amaximum of 69% of crude oil followed by Bacillus sp. 64% at 1% crude oil concentration when incubated at 35°C and pH 7 (12) .

(12) reported that *P. aeruginosa* degraded 97.2% of the oil introduced into the medium followed by *B.subtilis* with 72% degradation after 4 days of incubation period at (28 ± 2°C) .

Temperature influences petroleum biodegradation by its effect on physical nature and chemical composition of crude oil .At low temperature the viscosity of oil increased and water solubility decreased which delaying and decreasing biodegradation (28 , 2). Similar to our results (8) reported that 30 ˚C is the optimum temperature for degradation of crude oil by individual bacterial strain .

(21). reported that 30 ˚C to be the optimum temperature for microbial growth and crude oil degradation.

Decrease in temperature decreased the percentage of degradation and increase in temperature increased the rate of hydrocarbon metabolism(12).

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تأثير الوقت , الحرارة والدالة الهايدروجينية على تفكيك النفط الخام بواسطة عزلات بكتيرية من مصفى الدورة - بغداد

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[4,3] كلية العلوم / جامعة بغداد

الخلاصة

العزلات البكتيرية *Pseudomonas aeruginosa* , *Bacillus cereus* و *Bacillus subtilis* عزلت من منظومة معالجة المياه في مصفى الدورة وأستخدمت لدراسة الظروف المثلى من (وقت , حرارة ودالة الاس الهيدروجيني) لتفكيك النفط الخام . أستخدم و سط الاملاح السائل BH medium المضاف له النفط الخام بنسبة 1% كمصدر وحيد للكاربون في هذه التجارب . لقحت الدوارق الحاوية على 25 مل من وسط الاملاح المذكور المضاف له النفط الخام بنسبة 1% وبقيم مختلفه من دالة الاس الهيدروجيني (4- 10) بالعزلات البكتيرية الثلاث وحضنت على فترات زمنية مختلفة من (1-10) أيام وبدرجات حرارة مختلفة تراوحت من (25-45) درجة مئوية . أظهرت النتائج أن العزلة *P. aeruginosa* فككت 72% من النفط الخام عندما نميت في الوسط المذكور عند قيمة الدالة الهيدروجينية 8 عندما حضنت بدرجة حرارة 30 مئوية لمدة 6 أيام بينما أظهرت العزلات *B. subtilis* و *B. cereus* 65% و 55% كنسبة تفكيك للنفط الخام على التوالي عندما لقحت على نفس الوسط الزرعي وتحت نفس ظروف التحضين . وأظهرت النتائج أيضا أن العزلات البكتيرية الثلاث سببت أنخفاضا ملحوظا في قيمة الشد السطحي للوسط الزرعي وأن العزلة *P. aeruginosa* أظهرت أعلى قدرة على خفض الشد السطحي من (73-24)mN/m مقارنة مع العزلتين الاخريين .

الكلمات المفتاحية : التفكيك الحيوي , النفط الخام , المصفى , الشد السطحي , العزلات البكتيرية .

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