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RESEARCH ARTICLE

Isolation, screening and characterization of asphalt biodegrading bacteria isolated from damaged paved roads in Wasit province / Iraq

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ABSTRACT

Eleven soil samples were collected randomly from different locations in Wasit Governorate.15 bacterial isolates were isolated from soil samples by using BHM with 1% asphalt as carbon source. In primary and secondary screening (11) isolates of bacteria can biodegrading asphalt. Among the eleven isolates, bacterial isolate named A8 was most effective in biodegradiation process. The results of biomass, optical density, and percentage of of asphalt degradiation A8 were (0.21,0.325 and 42%) respectively. The results of morphological and biochemical tests showed that the bacterial isolate named A8 belongs to the species Pseudomonas aeruginosa

INTRODUCTION

Asphalt is a dark cementitious material that is mostly composed of bitumen derived from the distillation of crude oil is a dense viscous form of petroleum that is used to create asphalt when mixed with aggregates such as sand, stone, it can be found in naturally weathered geological deposits or as a heavy residual result of the petroleum refining process in crude oil processing plants (*Anupam et al*., 2023). According to Esmaeil *et al*. (2009), the process of biodegradation of petroleum hydrocarbons originating from bitumen and asphalt requires a combination of distinct bacterial groups or consortia in order to break down a broader range of petroleum hydrocarbons, is a complicated process that entails microorganisms breaking down these materials. Although bitumen and asphalt are known for their durability, under some circumstances, they can be biodegraded by particular microorganisms, the environment's temperature, pH, and other factors, as well as the presence of oxygen, water, and nutrients, can all affect how quickly bitumen and asphalt decompose (Zhang & Sun, 2020). Both aerobic and anaerobic processes can cause the breakdown of hydrocarbons, including asphalt (Wang *et al*., 2023). Many species of diverse microorganisms break down certain aromatics and alkanes, while others break down compounds of paraffinic and aromatic

hydrocarbons, producing carbon dioxide, water, biomass, and other less toxic byproducts (Kumar and Baul, 2010).The pollution from bitumen and asphalt arises from various sources and can take multiple forms, including air, water, and soil pollution during the production, transportation, and application of asphalt, volatile organic compounds (VOCs) and particulate matter are released into the air, contributing to air pollution and negatively affecting air quality furthermore, asphalt roads and pavements, over time, undergo wear and tear due to vehicular traffic and weather conditions, resulting in the release of microplastics and other pollutants into the environment (Azadgoleh *et al* .,2022).

Bitumen and asphalt are indispensable for modern infrastructure, their production, use, and degradation have significant environmental implications, the pollution from these materials can take various forms, including air, water, and soil pollution, and can have far-reaching impacts on ecosystems, therefore, it is crucial to develop and implement strategies to mitigate the environmental impact of bitumen and asphalt, such as recycling, using eco-friendly alternatives, and improving production and application processes to minimize pollution (Meng *et al* .,2023).

2. MATERIALS AND METHODS

2.1 Collection of soil samples

Eleven soil samples were collected randomly from different locations at a depth of 5-10 cm .The soil sample used in this study was collected by using a soil auger, properly labeled, and transported in polythene bags to the lab. These soil samples contaminated remains of damaged paved roads were used to isolate the bacteria that degrade asphalt.

2.2 Enhancement and isolation asphalt biodegrading bacteria from soil samples

The study area's asphalt served source of carbon and energy for bacteria isolated from soil samples that contaminated the remnants of damaged paved roads. (50ml) Bushnell-Hass medium broth (KH_2PO_4 (1 g), K_2HPO_4 (1 g), $MgSO_4$ (0.2 g), $CaCl_2$ (0.02 g), $(NH_4)_2SO_4$ (1 g), and $FeCl_3$ (0.05 g) . After adjusting the pH of the medium to a range of 7-7.2, these ingredients dissolve in 1 L of distilled water and are supplemented with 1% asphalt as a source of hydrocarbons. (1 g) from the original soil sample was added to each flask. The control flask contains BHM broth and asphalt without soil sample. All flasks were incubated in shaker incubator at 30 ˚C for 14 days at 150 rpm/min (Udgire *et al*.,2015). Measured optical density with spectrophotometer (600 nm) was measured for each flask and loop full of each flask cultured on plates of MSM agar ($NaNO_3$ (1.0 g), KH_2PO_4 (0.5 g), K_2HPO_4 (0.5 g), $MgSO_4$ (0.1 g), $CaCl_2$ (0.01 g), and $FeSO_4$ (0.001 g). These components were dissolved in (1 L) distilled water and the pH was changed to 7.2. Then, 2 % (v/v) agar - agar was added, and all plates were incubated at 30 ˚C for (1) week (Udgire *et al*.,2015).

2.3 Screening of bacterial isolation:

There are several techniques employed as follows to choose the most effective bacterial isolate for Hydrocarbon degradative activity

2.3.1 Preliminary screening

50 mL of liquid BHM, with the pH set at 7.0 and 1 % asphalt added as a substrate, the flasks were then inoculated with 5 mL of each reactivated bacterial isolate, and they were incubated at 30 °C for 7 days at 150 rpm in a shaker incubator. The percentage of hydrocarbon decomposition, biomass, and optical density (600 nm) were measured in order to identify the most effective bacterial isolates for decomposition (Abdulla *et al*.,2019).

2.3.2. Secondary screening

Two ways were used to evaluate the degradation.

a-Formation of clearance zone

Another way to verify the results of the previous experiment was to dilute the asphalt with hexane, spread the hexane solution uniformly over the surface of solid MSM plates, and then wait for the asphalt to evaporate, leaving a thin layer on the agar surface. A loop full of pure bacterial isolates was then cultured by spreading technique over a 1 cm area on the middle of solid MSM plates, and the plates were then incubated at 30 ˚C for 24 to 144 hours (Kiyohara *et al* .,1992).

b-The growth on mineral salt medium agar plates

The resulting pure isolates were cultivated on mineral salt medium agar supplemented with 1% v/v asphalt at pH-7 ± 0.2 , this medium was then inoculated with a loop full of each bacterial isolate in the center of the agar plate, and it was kept in an incubator set to 30 °C for seven days (John and Okpokwasili , 2012).

2.4 Identification of the bacterial isolates that degrade hydrocarbons.

The bacterial isolates were identified according biochemical characteristics (methyl red test, Voges Proskauer test, indole test, starch hydrolysis, catalase, oxidase, urease test, gelatin utilization test , motility test and Simon citrate test). according to the guidelines in Bergey's Manual of Determinative Bacteriology .

3. RESULTS AND DISCUSSION

3.1 Isolation of asphalt degrading bacteria

Table 1 shows that fifteen (15) bacterial isolates were found in soil that had been polluted with remains of damage roads at (11) distinct sites of the study area in Wasit province/ Iraq. Numerous studies have demonstrated that the most potent bacteria for asphalt bioremediation were isolated from contaminated soil these isolates included various species of bacteria.

Table 1: Enumeration of microbes in asphalt contaminated soil.

Asphalt sample	Isolates (n)
A1	**1**
A2	**1**
A3	**2**
A4	**1**
A5	**2**
A6	**1**
A7	**1**
A8	**1**
A9	**1**

A10	**1**
A11	**1**
Total number isolates	**15**

Enrichment of microorganisms capable of breaking down asphalt was carried out in BH medium with (1 %) asphalt as the only carbon source and by the addition of the soil were incubated in shaker incubator at 30 ˚C for 14 days at 150 rpm/min. 15 bacterial isolates were obtained using spread plating on MSM agar. Microorganisms capable of breaking down different petroleum hydrocarbons, such as polycyclic aromatic hydrocarbons (PAHs), naphthalene, monoaromatic hydrocarbons like toluene, or aliphatic hydrocarbons like the n-alkanes, can easily be isolated from the environment, especially from remains of damage roads contaminated sites (Geetha *et al*.,2013). Almansoory *et al*,(2019). Reported that from soil and water samples polluted with hydrocarbons, 15 bacterial isolates were isolated and cultivated for 48 hours on nutrient agar with 1 % (vol/vol) asphalt. Also 19 bitumen-degrading bacteria were found in enrichment cultures from various sampling sites (Anupam *et al*.,2023). Data showed that bitumen soil, which was predicted to have been exposed to hydrocarbon pollution, from garage sites from which also bitumen-degrading bacteria was collected. These bacterial isolates (15) were subjected to a primary and secondary examination to determine the most effective isolate in bioremediation of pollutants.

3.2 Result of clear zone formation

Experiments of clear zone formation have been done to 11 (A1-A11) isolates that showed highest degradation capacity in primary screening. The clear zone that formed on solid MSM plates served as a gauge for the effectiveness of asphalt degradation by 11bacterial isolates as shown in (table 2)

Table 2: The ability of bacterial isolates to degrade asphalt on MSM agar (30˚C for 7 days) by using diameter of a clear zone (mm) as indicator.

Bacterial isolate	clear zone (mm) An BHM plates
A1	8
A2	15
A3	22
A4	11
A5	17
A6	14
A7	12
A8	27
A9	18
A10	7
A11	8

The bacterial isolate A8 having a clear zone with the largest diameter of (27 mm) and the isolates A10 having a clear zone with the lowest diameter of 7 mm for each isolate after 7 days of incubation. The organisms that developed clear zones around the colonies were regarded as asphalt degraders.

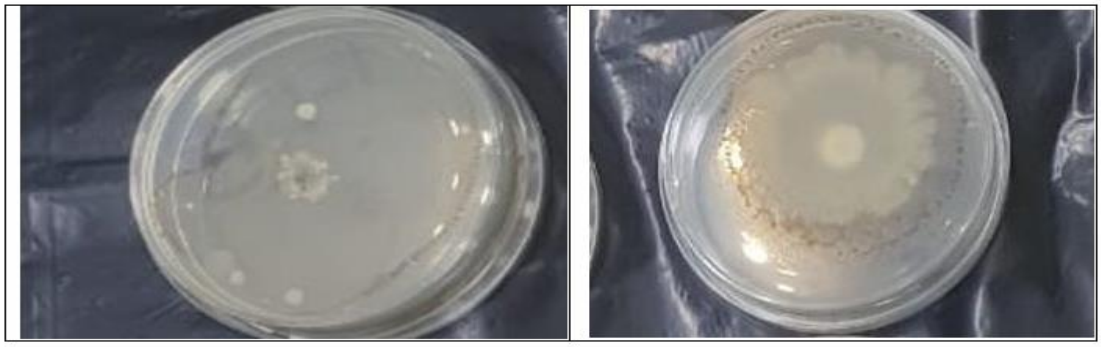

Figure 1: The formation of clear zone as result to degrade asphalt by bacterial isolate A8 that appeared the highest diameter of clear zone (27 mm) on MSM agar (Latha and Kalaivani ,2012).Demonstrated that on mineral salt media, the largest clearance zone was formed by two bacterial isolates. On the seventh day of incubation, S2 (Bacillus subtilis) among these isolates displayed the highest growth (0.85 mg/ml) and degradation, followed by S10 (Pseudomonas aeruginosa) (0.92 mg/ml). By using the plate assay method, the capacity of different bacterial strains to degrade asphalt was examined. Pseudomonas aeruginosa (27 mm in diameter) had the highest zone of exhibition in this investigation compared to Bacillus cereus (6 mm in diameter) (Sahu *et al* .,2019).

3.3 Screening of bacterial isolates that degrade hydrocarbons.

3.3.1 Preliminary screening for asphalt degradation

The ability of bacteria that isolated from soil samples to degrade asphalt was accomplished through growth of bacterial isolates in liquid BHM with 1% of asphalt as only carbon source incubated at 30˚C for 7 days at 150 rpm in shaker incubator. Table (2) showing eleven isolates from 15 hydrocarbon degrading bacteria were shown to have the highest ability for asphalt bioremediation ,while the other bacterial isolates showed weak results in degradation. The capacity for decomposition of these isolates was noted based on optical density (600nm), biomass and the percentage of hydrocarbon decomposition.

Table 3: The variations between bacterial isolates in primary screening.

Isolates	Optical density 600nm	Biomass g/L	Hydrocarbon degradation %
A1	0.121	0.08	10%
A2	0.132	0.25	17%
A3	0.313	0.44	39%
A4	0.218	0.22	16%
A5	0.168	0.08	28%

A6	0.129	0.34	23%
A7	0.232	0.27	19%
A8	0.325	0.21	42%
A9	0.301	0.48	30%
A10	0.088	0.55	1%
A11	0.17	0.16	7%

Results in (table 3) revealed the ability of (11) bacterial isolates to degrade asphalt as carbon source and the bacterial isolates named as A8 and A3 were more effective, especially A8 was extremely effective. The optical density, biomass, and hydrocarbon degradation % for bacterial isolate A8 were 0.325, 0.21, and 42 % respectively. Local microorganisms that have been isolated from locations where asphalt has been contaminated are more effective at degrading bitumen (Zhang and Sun,2020). Demonstrated that thirteen bacteria strains were found in samples of bitumen-contaminated soil and seawater from the research location after 24 hours of incubation. The results of the screening test revealed that the bacteria with the codes EL and CT had the best resistance to bitumen pollution and the highest growth rates. It was demonstrated that both may be more able than the others to use bitumen as a carbon and energy source. Furthermore, various hydrocarbon-contaminated areas, seventeen (17) bacterial isolates that could flourish on asphalt were found (Prathyusha *et al* 2016). The 0.5 % bitumen in Bushnell-Hass medium was used to screen for bacterial asphalt degradation. Six isolates were further chosen for a consortium that demonstrated highest hydrocarbon usage based on their ability to breakdown hydrocarbons

3.4 The growth on mineral salt agar plates

The ability of pure bacterial isolates to grow in mineral salt agar supplemented with asphalt (1 % v/v) is used to determine which isolates can grow in this condition. The results are shown in the table (4) below. It shows the increase in the mass and number of cell of an organism's in conjunction with the passage of time that the highest value of bacterial growth was in (120,144) hours of the incubation period, where the results for the living mass were respectively (0.81,1.13) and the number of cells was (5.5×10^5 , 7.6×10^5) The protein content(0.537, 0.833) respectively. Compared to the first and second days, it was mass of the organism (0.12, 0.35) the number of bacterial cells, respectively (1.4×10^5, 3.2×10^5), as well as the protein content, respectively (0.091, 0.172).

Grow time Hrs)	Dry biomass (g/L)	Cell count	Protein content (mg/L)
0	0.00	1×10^5	ND
24	0.12	1.4×10^5	0.091

72	0.35	3.2×10^5	0.172
120	0.81	5.5×10^5	0.537
144	1.13	7.6×10^5	0.833

Table (4):- The ability of bacterial isolates to grow in MSM agar supplemented with asphalt (1 % v/v).

4 CONCLUSIONS

fifteen bacterial isolates were isolated from soil samples polluted with remains of damage roads in Wasit Province, Iraq. Microorganisms in the soil would be able to utilize asphalt as their only source of carbon and energy. These organisms could then be used as possible bioremediation agents for soil that has been contaminated by remains of damaged paved roads.

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