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Characterization of *Pseudomonas* aeruginosa for Effective Bitumen Biodegradation under optimal condition

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ABSTRACT: This study aims to cultivate bacterial samples from soil acquired from bitumen-contaminated regions Kut City, Iraq's paved roadways are regularly exposed to chemicals, oil and other contaminants of the eleven bacterial samples tested A8 showed the best asphalt -breaking performance with a hydrocarbon breakdown percentage ranging from 42% its optical density of 0.325 nm and biomass generation rate of 0.21g/L according to the results of biochemical testing the bacterial isolate A8 was determined to be *Pseudomonas aeruginosa.* The tests for methyl red, Voges Proskauer, indol, starch hydrolysis, glucose fermentation, sucrose and lactose all yielded negative results while show positive result for every one (gelatin, catalase and oxidase). To temperature, pH and carbon source were the ideal variables for *Pseudomonas aeruginosa*-mediated asphalt bioremediation it was demonstrated that the ideal pH for *Pseudomonas aeruginosa* growth and proliferation to cure asphalt under the given experimental conditions is 6 and the organism's mass concentration was 1.2g/L, 25 C was the ideal temperature for asphalt biodegradation with an organism mass of 1.28 g/L glucose was the most efficient carbon source for asphalt bioremediation with an organism mass of 1.3g/L the purpose of bacterial characterization and isolation from paved roads is to find a bacterial isolate capable of decomposing bitumen.

Keywords: Biodegradation, Bitumen, *Pseudomonas aeruginosa*

1. INTRODUCTION

Bitumen, a viscous petroleum product derived from the distillation of crude oil, is combined with crushed stone, gravel and sand to create asphalt. Asphalt and bitumen are widely used and important for construction and transportation but they also present serious environmental problems, mostly because of pollution as the main building materials for roads and roofs, bitumen and asphalt are crucial parts of contemporary infrastructure (1). The mechanism breakdown of asphalt by microorganisms is a complicated process, however even though bitumen and asphalt are known to survive a long time, certain microbes can biodegrade them under the right condition's environmental variables like temperature and pH, as well as the availability of nutrients, water and oxygen can all affect how bitumen and asphalt biodegrade (2). A greater range of petroleum hydrocarbons can be broken down through the biodegradation process if a number of bacterial groups or consortiums cooperate (3). The basis of Bioremediation relies on the capacity of microorganisms to decompose oil hydrocarbons into elements that can be safely reintroduced into the environment and utilized by other microbes as a source of nutrition, on the other hand, decomposed organic materials can be transformed into water, carbon dioxide and other inorganic substances, the right environmental conditions will help the microorganisms eliminate the contaminants rapidly and multiply (4). Studies on biodegradation and bioremediation can result in the creation of novel technologies that use microorganisms' ability to clean up contaminated areas and reduce the need for conventional, resource-intensive techniques (5). Environmental conditions (such as pH, temperature, moisture, soil structure, water solubility, nutrients, site features, redox potential and oxygen content), the presence of trained personnel in the field and

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the physico-chemical bioavailability of contaminants (such as concentration, type, solubility, chemical structure and toxicity) are some of the factors that affect the rate of degradation (6).

2. MATERIALS AND METHODS

2.1 Sampling

In order to isolate the bacteria that would break down bitumen, consisting of damaged bitumen roads were randomly collected at a depth of 10 cm from various locations, the soil sample used in this study was collected using a soil auger and is accurately identified as such, it was then stored in a freezer and brought to the laboratory (7).

2.2 Isolation Bacterial method.

The Bitumen is the only carbon source used by isolated bacteria from soil samples contaminated by remaining paved-road debris Kut City, Iraq's add 1% bitumen 50 milliliters of Bushnell-Hass medium broth were put into 250 milliliters of Erlenmeyer flasks, which were then autoclave-sterilized for 15 minutes at 121 degrees Celsius. Following sterilization, 1 gram of the original soil sample was added to each Erlenmeyer flask and the control flask was left without a soil sample for 14 days at 150 revolutions per minute all of the Erlenmeyer flasks were then incubated at 30 degrees Celsius in a shaker incubator (8). A single colony of various isolates was repeatedly transferred onto Lauria agar plates using the streaking method to create pure cultures of bacteria then, the plates were incubated at 30°C for a full day pure culture plates were then stored in a refrigerator at 4 degrees Celsius the lauria agar and nutrient agar were used to maintain and isolate pure cultures (9).

2.3 Screening for bitumen Degradation

To ascertain which bacterial isolates were most effective for decomposition, 250 ml Erlenmeyer flasks containing 50 ml of pH 7.0 liquid Bushnell-Hass medium (BHM), as a substrate 1% bitumen were applied after pure bacterial isolates were reactivated on nutrient agar medium and incubated for 24 hours at 30˚C, the flasks were then shaker-incubated for seven days at 150 rpm and 30˚C (10).

2.4. Biochemical test (Reagents and stains and media)

2.4.1. Red methyl reagent

To create this reagent, 20 milligrams of methyl red dye were dissolved in 60 milliliters of 95% ethylene alcohol and the mixture was finished with 100 milliliters of distilled water (11). This reagent is used to determine whether bacteria are capable of producing acid as a byproduct of fermenting sugar.

2.4.2. Test reagent for oxidase

To generate 100 milliliters, one gram of tetramethyl p-phenylene diamine dihydrochloride was dissolved in a little amount of distilled water, it was created in order to determine whether bacteria were capable of producing the Oxidase enzyme (12)

2.4.3. Catalase Reagent

A catalyst that contains 3% hydrogen peroxide was created (13).

2.4.4. Vogues Proskauer VP-reagent

It was made using the two reagents listed below and was used to find out whether bacteria in broth culture could ferment glucose to create acetone (11).

1. Using Reagent -A (5%): 5 g of α-naphthol was dissolved in 100 ml of 100% ethanol to create the solution.

2. Reagent -B (40%): 100 milliliters of distilled water were mixed with 40 grams of sodium hydroxide to create the solution.

2.4.5. Pepton water medium

The 10 ml of medium, which was created by dissolving 10 grams of peptone and 5 grams of sodium chloride in 900 milliliters of distilled water was autoclaved to sterilize it, after all the ingredients were dissolved, the pH was adjusted to 7 and more distilled water was added to bring the volume up to 1000 milliliters (11). It was examining the capacity of bacterial isolates to generate indole.

2.4.6. Starch medium

It was made by adding 2% soluble starch to nutritional broth, heating it while stirring constantly, and adjusting the pH to 7.2 five milliliters of this medium were then transferred into test tubes covered and autoclaved to sterilize them (14). This media is used to show how bacterial isolates produce the enzyme amylase.

2.4.7. Urea agar medium

The procedure involved dissolving 2.4 grams of urea base agar in 100 milliliters of distilled water, setting the pH at 7 heating the medium to the boiling point, autoclaving it, cooling it to 50 degrees Celsius aseptically adding 50 milliliters of sterile 40% urea solution, mixing thoroughly and distributing the mixture in test tubes in a slant (14). Bacterial isolates that produced urease were identified using this medium.

2.4.8. Sugar fermentation medium

After 10 g of peptone was dissolved in 80 ml of distilled water, 0.5 g of phenol red was added and the volume was increased to 100 ml with distilled water the pH was then adjusted to 7.2 and 9 ml of this medium was transferred into test tubes with Durham tubes, the medium was then autoclave-sterilized once it had cooled to 50 °C one milliliter of the prepared glucose and sucrose solutions (final concentration of 1%) was added to each test tube (11). This medium was used to test the bacteria's capacity to ferment sugar.

2.5. Optimization for bitumen bioremediation

2.5.1 Temperature's Effect

Bushnell-Hass media (BHM) in liquid form (50 milliliters) was made, five milliliters of the bacterial suspension A8 were added to each flask after the medium's pH was brought down to 7.0 and 1% bitumen was added as a substrate for seven days, the flasks were incubated at 150 revolutions per minute (rpm) at various temperatures (20, 25, 30, 35 and 40°C) (15).

2.5.2 Estimation of Optimal pH

Each flask was infected with 5 milliliters of the bacterial inoculum A8 after 50 milliliters of liquid Bushnell-Hass medium (BHM) had been prepared and the pH of the medium had been adjusted to 4, 5, 6, 7, and 8 using 0.1 N HCl or 0.1 N NaOH solutions (15) . **2.5.3 Carbon Sources**

Fifty milliliters of liquid Bushnell-Hass medium (BHM) were produced after adjusting the medium's pH to 7.0, each flask was filled with various carbon sources including sucrose, fructose, xylose, glucose and maltose, each flask was then supplemented with 1% bitumen as a substrate and inoculated with the bacterial isolate A8.

3. RESULTS AND DISCUSSION

3.1. Bacterial Isolation from remains of damaged paved roads Contaminated Soil Samples

The bacteria in these samples might exhibit unique characteristics, such as resistance to particular chemicals or the ability to degrade hydrocarbons, due to the extreme environmental conditions. As shown in table (1), 15 bacterial isolates were obtained on BH agar using the spread plate method. Asphalt was utilized as the only carbon and energy source in a selective medium to extract bacteria that could degrade hydrocarbons although there is a diverse bacterial population present at the soil-asphalt interface, there is no obvious link between the amount of bacteria present and the

bituminous layer's physical deterioration, nevertheless, in both natural and artificial settings, these microbes have the physiological ability to change the physical characteristics of asphalt by degrading hydrocarbons, bacterial activity has been demonstrated to exacerbate asphalt deterioration and compromise the material's structural integrity certain types of bacteria release enzymes that break down asphalt, hastening the development of potholes and cracks. This microbial action has the potential to cause considerable surface wear over time, particularly in locations where chemical or oil spills occur often. The ongoing interaction between bacteria and asphalt not only impacts road durability but also highlights the potential for bioremediation applications in polluted environments (16).

Table (1): quantities of microorganisms in the soil tainted by the remnants of roads that had been damaged.

Asphalt sample	Isolates (n)
A1	**1**
A2	**1**
A3	**2**
A4	**1**
A5	**2**
A6	**1**
A7	**1**
A8	**2**
A9	**1**
A10	**2**
A11	**1**
Total number isolates	**15**

The statistical analysis's findings are displayed above the microbial population on naturally occurring soils that have been contaminated with hydrocarbons depends on nutrient accessibility because bacteria require resources for gro wth and metabolism biostimulation rises when the soil is contaminated with macronutrients or added to it, so it can be c oncluded that environmental factors affect how well the microbial isolates break down asphalt and hydrocarbons the ph ysicochemical characteristics of the pollutants (such as their chemical structure, concentration and bioavailability) and e nvironmental parameters (such as temperature, pH and nutrient availability) all affect the effectiveness and rate of biod egradation A8 was quite successful. This implies that this isolate has the ability and effectiveness to break down the largest percentage of hydrocarbon because bacteria have enzymes that can break down complicated compounds like the hydrocarbon that actually makes up asphalt.

According to (Table 1) 15 bacterial isolates demonstrated intrinsic bioremediation by being able to biodegrade hydrocarbons. According to (17), local organisms exposed to pollutants for prolonged periods of time either adapt or are naturally selected to survive in the majority of settings although it is uncommon for one ecosystem to have a wide variety of organisms that may degrade or transform all toxins present many pollutants especially organic ones have natural counterparts in the environment, it is well known that a broad range of pure and mixed hydrocarbons are suitable for the growth of these bacteria. Bacteria that break down hydrocarbons are more effective in contaminated environments because of their specialized enzymes and flexible metabolic pathways (18)

3.2 Screening for asphalt Degradation

The conclusion of this a study that aimed to identify distinct microbial isolates that were beneficial in hydrocarbon breakdown. The table (2)'s primary metrics are the indicator of the microorganisms' growth is determined by the optical density at 600 nm. Biomass (g/L), which is expressed in grams per liter, indicates the amount of biomass

produced by the isolates. Hydrocarbon Degradation (%): Based on the percentage attained, this shows how well each isolate breaks down hydrocarbons.

Table (2): variations among the bacterial isolates found during the screening procedure.

Isolates	OD $_{600nm}$	Biomass g/L	Hydrocarbon remaining %
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%

In order to assess the ability of bacteria isolated from soil samples to break down asphalt, they were grown in liquid BHM with 1% asphalt as the only carbon source the incubation period lasted seven days at 30˚C and 150 rpm in a shaker incubator and the findings are shown in table (2) with the highest percentage of hydrocarbon degradation ranging at 42% A8 proved particularly effective
this suggests that this isolate has the capacity and efficiency to degrade the highest percentage of hydrocarbon because bacteria they are endowed with enzymes that are capable of breaking down complex molecules for instance the hydrocarbon that constitutes asphalt in effect, it is intermediate in biomass production (0.21g/L) and optical density (0.325nm).

discovered that *P. aeruginosa* could degrade 46.4% of the crude oil in a mere seven days, they examined the bacteria's ability to emulsify, the reduction in surface tension and the biodegradation of crude oil, concluding that the biosurfactant produced by the bacteria was the main contributor to biodegradation (19).

3.3. Biochemical characteristics of bacterial isolate

Additional biochemical testing was necessary to accurately identify isolate A8, as shown in table (3), which shows negative findings from the(Methyl red ,Voges Proskauer test, indol test, Starch hydrolysis, glucose fermentation, sucrose, lactose) this shows that none of the bacteria that ferment glucose produce a lot of acidic end products the bacteria that do not hydrolyze urea produce negative urease results but the gelatin test shows a positive result because it shows that the bacteria can hydrolyze gelatin, the Catalase & Oxidase test shows a positive result because it shows that oxidase is present because it breaks down hydrogen peroxide into oxygen and water.

Table (3): Biochemical characteristics of bacterial isolate

Biochemical test	Result
"Methyl red"	–
"Voges-Proskauer test"	–
"Indol test"	–
"Gelatin hydrolysis"	+
"Starch hydrolysis"	–
"Catalase test"	+
"Oxidase test"	+
"Urease"	–
"Citrate utilization"	+
"Glucose fermentation"	–
"Sucrose"	–
Lactose	–

The isolates used for our hydrocarbon degradation were identified using morphological colony characteristics examination (motility, shape and color) and biochemical assays (carbohydrate acid/gas generation, sugar fermentation) and enzyme assays (urease, oxidase, catalase, nitrate reduction, idol production and so on) these tests were thought to be traditional identification methods, because oxidase and catalase tests came back positive while indole and urease tests came back negative for the isolates which relate to *Pseudomonas aeruginosa* (9).

3.4. The ideal conditions for *Pseudomonus aeruginosa*-mediated asphalt bioremediation

3.4.1. pH Effect

Utilizing modified BHM with a range of pH values (4,5,6,7,8) the optimal pH for *Pseudomonas aeruginosa* to remediate asphalt was determined. Figure1 (A) shows the connection between biomass (g/L) and PH values these bacteria grows and multiplies best at a pH of 6 and under the experimental conditions, the organism's mass concentration was 1.2g/L. However, biomass is found to decrease at pH values of 8 and 4 and to increase at pH values of 5 and 7. This shows that bacteria cannot thrive at pH values outside of the ideal range. The influence of pH on bacterial growth is depicted in Figure1 (B) the trend of OD values which peak at pH 6 corresponds to the biomass trend this indicates that pH 6 is the ideal growth environment in this experiment for *Pseudomonas aeruginosa*.

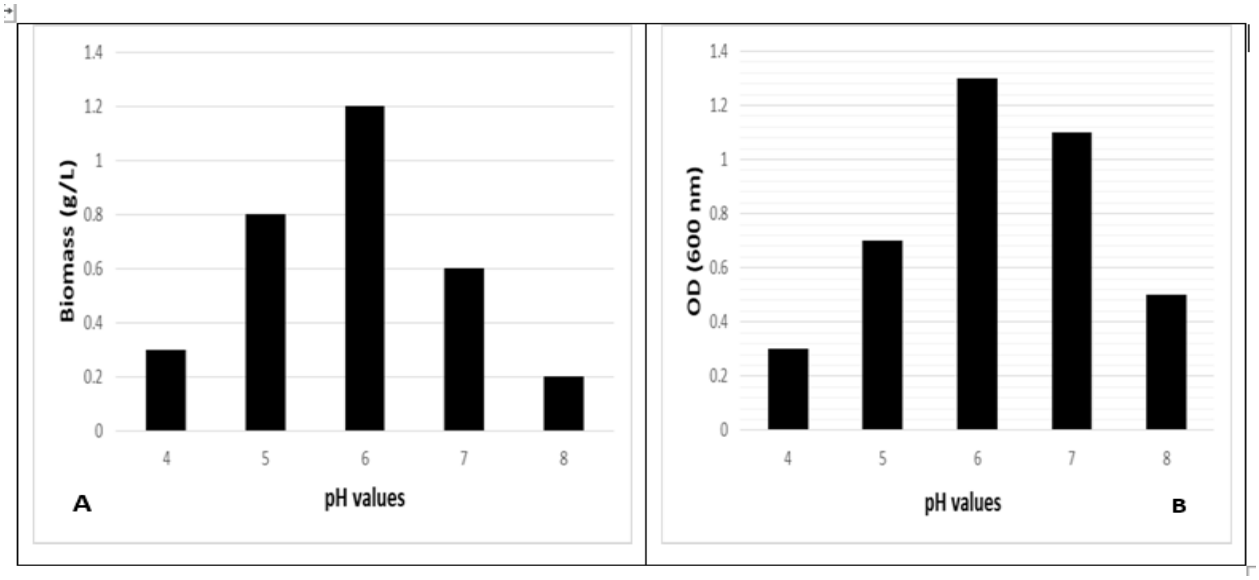

Figure (1): The impact of pH on *Pseudomonas aeruginosa's* biodegradation of asphalt A. The connection between biomass and pH. B. The connection between optical density and pH.

approximately neutral pH (5.2–7.0) allows for efficient hydrocarbon breakdown (20).This is due to the fact that maintaining the ideal pH of the soil is crucial so that the biodegradation of hydrocarbons, enzyme activity and microbial biomass can be controlled (21).

3.4.2. The Temperature Effect

Figure (2) illustrates the connection between *Pseudomonas aeruginosa* biomass and temperature, various temperatures (20–40°C) were used at 25°C the maximum biomass value of 1.28 g/L was recorded because of the presence of nutrients in BH medium that contains 1% asphalt, *Pseudomonas aeruginosa* thrives best at 25°C.

This means that the bacteria can grow and reproduce more effectively at this temperature, it is crucial to remember that the optimal temperature for *Pseudomonas aeruginosa* development can vary based on the particular growth conditions and the bacterial strain but 25°C is the typical.

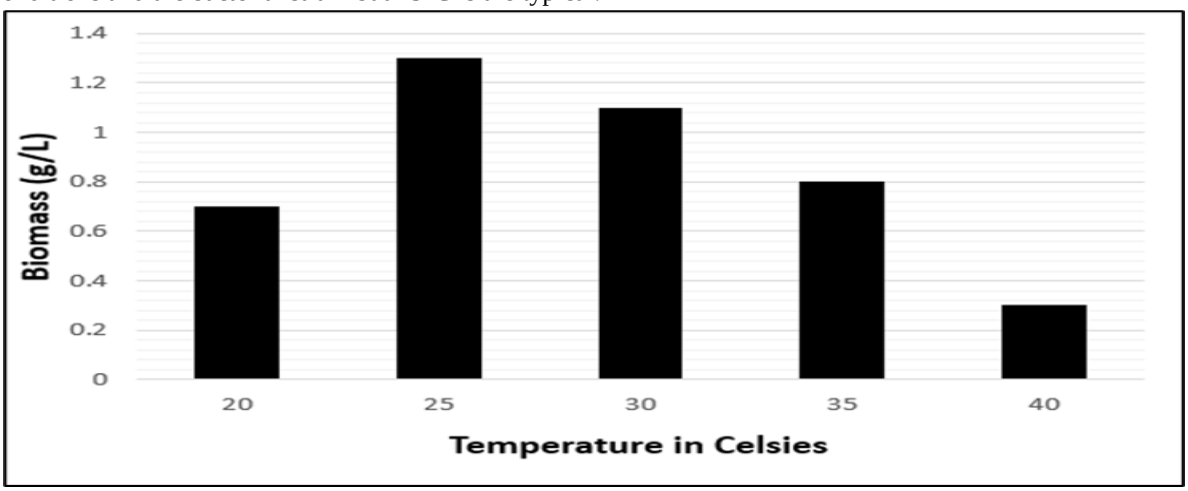

Figure (2): The impact of temperature on *Pseudomonas aeruginosa's* biodegradation of asphalt. the connection between biomass and temperature.

3.4.3. Effect of Carbon Source

According to the research, glucose is the best carbon source for increasing *Pseudomonas aeruginosa* biomass production we also show how various carbon sources affect *Pseudomonas aeruginosa* biomass production. Among the carbon sources studied glucose produced the most biomass reaching about 1.3 g/L as shown in Figure (3), which explains the link between carbon sources and biomass. This implies that in the experimental setting glucose is the most effective

carbon source for fostering *Pseudomonas aeruginosa* growth and biomass buildup Glucose produced more biomass than the other carbon sources that were studied biomass levels from xylose, maltose, sucrose and fructose ranged from roughly 0.6 to 0.9 g/L. the role of bacteria use carbon sources extend energy and for enhancing biomass production.

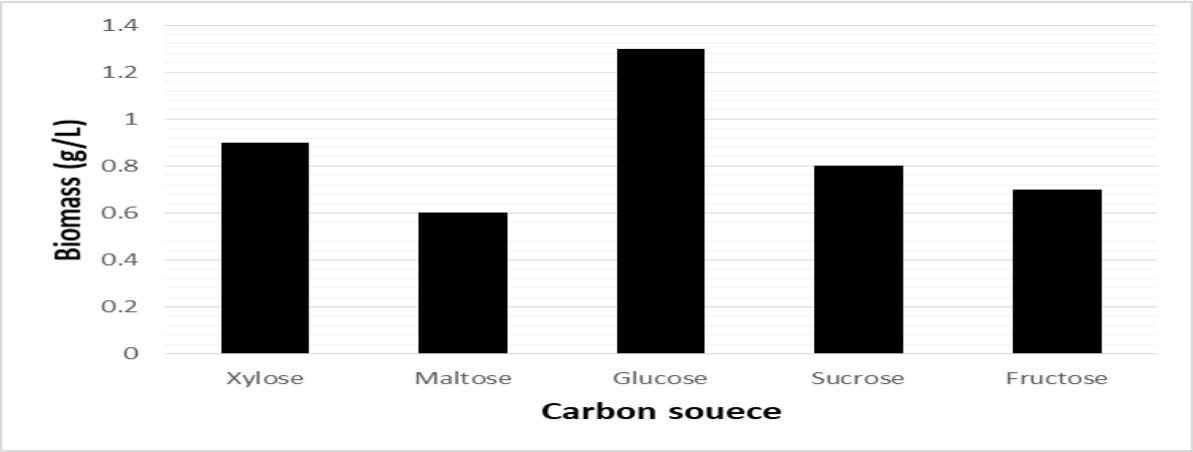

Figure (3): Impact of carbon sources on *Pseudomonas aeruginosa's* biodegradation of asphalt. the connection between biomass and carbon sources.

4. CONCLUSION

Pseudomonas aeruginosa was the most effective strain of the hydrocarbon-degrading bacteria that the study was able to extract and identify from bitumen-contaminated soils, significant hydrocarbon degradation was shown by the bacterial isolates with A8 exhibiting the highest rates of degradation. The biodegradation efficiency was found to be best at pH 6 and 25°C the most efficient carbon source for hydrocarbon breakdown and biomass synthesis was discovered to be glucose.

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