Oil Spill Bioremediation by *Pseudomonas* sps and its Potential in Energy Recovery using Microbial Fuel Cells

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Abstract:-

Oil contamination in soils poses a significant environmental challenge, necessitating effective and sustainable remediation strategies. This study investigates the bioremediation potential of *Pseudomonas* species for the degradation of oil contaminants in soils collected from various anthropogenic sources. Pure cultures of *Pseudomonas* were isolated, screened, and evaluated for their efficiency in degrading oil under controlled conditions using oil-spiked minimal media. The degradation efficiency was assessed based on microbial growth and reduction in oil content. Among the isolates, the strains demonstrating the highest adaptability and degradation rate were selected for further application. These selected strains were subsequently employed in a microbial fuel cell system to evaluate its potential for simultaneous bioremediation and energy recovery. The study highlights the feasibility of utilizing *Pseudomonas* species in integrated systems for environmental cleanup and sustainable energy generation, with prospects for future scale-up and practical applications.

Aim:-

The objective of this experiment is to study the bioremediation potential of *Pseudomonas* species for the degradation of oil contaminants present in soils from varied sources. In addition, the efficiency of oil degradation by isolated pure cultures of *Pseudomonas* is evaluated. The strain exhibiting the highest level of adaptation to oil-contaminated conditions, as determined by its growth and degradation rate in oil-spiked media, is subsequently selected for application in a microbial fuel cell. This approach is used to assess its potential for energy recovery and to evaluate the feasibility of scaling up the process.

Introduction:-

Oil, a long-used fossil fuel, causes severe environmental damage when spilled, releasing approximately 7 million tonnes from major incidents (1907–2024) and through land leaks that contaminate soil, water, vegetation, and ecosystems, with lasting ecological and economic impacts.

Bioremediation employs microbes to break down pollutants into non-toxic products. *Pseudomonas* spp. metabolically versatile, gram-negative bacteria are especially effective, using enzymes (e.g., lipases, oxygenases) and biosurfactants to degrade hydrocarbons into CO_2, water, and biomass. Key species such as *P. putida*, *P. aeruginosa*, and *P. mendocina* can degrade diverse oil components, including aromatic compounds.

This study screens native soil *Pseudomonas* isolates to identify the most efficient oil degrader while integrating a microbial fuel cell (MFC) for simultaneous bioelectricity generation. In MFCs, bacteria oxidise organic matter at the anode, releasing electrons that generate current, enabling concurrent bioremediation and energy recovery.

Principle:-

Cetrimide agar: It is a selective and differential medium used to isolate *Pseudomonas species*. Cetrimide (cetyltrimethylammonium bromide), a quaternary ammonium salt, that acts as a cationic detergent when it comes in contact with the bacterial cells, causing the release of nitrogen and phosphorus. This has denaturing effects on membrane proteins of the bacterial cell. *Pseudomonas* is resistant and grows well due to its low outer membrane permeability and highly efficient multidrug efflux pumps, which actively pump out the toxic cetrimide. Cetrimide enhances the production of pyocyanin and fluorescein pigment, which gives *Pseudomonas spp.* a bright green colour. The other components of agar, like gelatin peptone, provide necessary nutrients; sodium chloride maintains osmotic equilibrium; and magnesium chloride and potassium sulphate stimulate pyocyanin production. Glycerol is the carbon source, and agar is the solidifying agent.

Biochemical tests: -

- **Gram Staining –** This test differentiates bacteria into Gram-positive and Gram-negative based on their cell wall structure. When the bacteria are stained with the primary stain, crystal violet, and fixed by the mordant (iodine), some of the bacteria are able to retain the primary stain, and some are decolorized by alcohol. The cell walls of gram-positive bacteria have a thick layer of protein-sugar complexes called peptidoglycan, and lipid content is low. Decolorizing the cell causes this thick cell wall to dehydrate and shrink, which closes the pores in the cell wall and prevents the stain from exiting the cell. So the ethanol cannot remove the crystal violet-iodine complex that is bound to the thick layer of peptidoglycan of Gram-positive bacteria and appears blue or purple in colour. In case of gram-negative bacteria, the cell wall also takes up the CV-iodine complex, but due to the thin layer of peptidoglycan and thick outer layer, which is formed of lipids, the CV-iodine complex gets washed off. When they are exposed to alcohol, the decolouriser dissolves the lipids in the cell walls, which allows the crystal violet-iodine complex to leach out of the cells. Then, when stained again with safranin, they take the stain and appear red in colour.

- **Catalase test –** The enzyme catalase (produced by *Pseudomonas*) mediates the breakdown of hydrogen peroxide into oxygen and water. When a small inoculum of the bacteria is introduced to hydrogen peroxide, rapid oxygen bubbling occurs. Catalase uses a heme-bound iron at its active site to capture one H_2O_2 molecule, breaking it into water

and leaving an oxygen atom on the iron. A second H_2O_2 then enters to react with that bound oxygen, releasing oxygen gas (bubbling) and another water molecule, which resets the enzyme.

- **Oxidase test –** The oxidase test identifies *Pseudomonas*, which contains cytochrome c oxidase, an enzyme that transfers electrons to oxygen in the respiratory chain. Using the Gordon and MacLeod reagent (N,N-dimethyl-p-phenylenediamine dihydrochloride) as an artificial electron donor, the enzyme catalyzes an oxidation reaction in the presence of atmospheric oxygen. This process converts the colourless reagent into a coloured compound called indophenol blue, resulting in a distinct deep purple or blue-black colour change that signifies a positive result.

Bushnell-Haas media: Bushnell-Haas (BH) medium is designed for the cultivation of microorganisms capable of degrading hydrocarbons, such as fuels, oils, and grease, by providing essential nutrients while lacking a carbon source. It acts as a selective medium where only hydrocarbon-oxidising bacteria, actinomycetes, and fungi can grow. The mineral composition of Bushnell-Haas medium supports microbial metabolism by providing essential nutrients like magnesium sulphate, calcium chloride, and ferric chloride. To facilitate protein and nucleic acid synthesis, ammonium nitrate is included as the primary nitrogen source. Additionally, a buffering system consisting of monopotassium phosphate and potassium phosphate is used to maintain a stable pH, ensuring an environment conducive to the growth of hydrocarbon-degrading microorganisms.

Optical Density (OD): It is an indirect measure of microbial concentration based on light scattering. When a beam of light passes through a microbial suspension, the cells scatter the light; a spectrophotometer measures the portion of light that is not scattered.
Reporting it as OD or absorbance. While spectrophotometers express measurements as "absorbance", the value actually reflects turbidity, which correlates to the total density of the microbial population (cells/mL). This relationship is only linear at lower densities, generally up to an OD600 of approximately 0.5 to 1.0. In high-density cultures, light can be re-scattered back toward the detector, requiring samples to be diluted to stay within the instrument's accurate range. Finally, because light scattering is heavily influenced by a cell's specific size, shape, and structure, a calibration curve (comparing OD to actual cell counts or dry weight) is essential for converting these arbitrary units into precise concentration data.

Solvent Extraction: The solvent extraction principle in this process relies on differential solubility and the use of immiscible solvents to isolate oil from a complex mixture. After centrifugation removes the solid bacterial pellets, the liquid supernatant (media and emulsified oil) is mixed with hexane. Because oil is non-polar, it preferentially dissolves into the hexane rather than the water-based media, according to Nernst’s distribution law. Since hexane and

water do not mix, they form two distinct layers in the separatory funnel. This allows the denser aqueous media to be drained from the bottom, leaving the oil-rich hexane layer behind. The final step uses the principle of volatility; the hexane is evaporated in a fume hood, leaving the non-volatile oil as a concentrated dry residue.

Fuel cell: A microbial fuel cell (MFC) degrades oil by using hydrocarbon-eating bacteria as a biological catalyst to turn oil into electricity. In the anode chamber, these specialized microbes break down complex oil molecules through oxidation. The oil also serves as a source of carbon for microbial growth. As they consume the oil, they strip away electrons and protons. The electrons are deposited onto the anode and travel through an external circuit to create an electric current, while the protons migrate through a membrane to the cathode. At the cathode, these components reunite with oxygen to form water, completing the circuit. The voltage can then be measured using a multimeter.

Procedure:-

1) Collection of soil samples with oil contamination (the oil that contaminated the soil is also sourced) from different sources.
    a) Temple soil, Home soil (lamp oil)
    b) Generator soil (Diesel)
    c) Restaurants, home soil (used cooking oil)
    d) Farm soil - control (no oil exposure)
2) Make a master plate in nutrient media agar.
3) Selective Cetrimide agar base culture plates are prepared for *Pseudomonas*
    a) Prepare cetrimide agar
        i) 8 plates with 20 ml each – 7.47 g in 160 ml distilled water
        ii) Autoclave-prepared media and plates
        iii) Pour the media into the plates in a sterile environment (LAF)
    b) The soil sample is serially diluted (10^{-2} dilution). Incubate for 24 hr at 37 degrees.
        i) Serially dilute twice to a 10^{-2} dilution
        ii) Using spread plate method, inoculate 20 microlitres of each sample on the corresponding plates with solidified cetrimide agar
    c) Incubate for 24 hr at 37 degrees.
    d) Growth of microbial colonies observed.
        - Overgrowth observed in 2 plates – hotel 1 & home cooking oil samples
        - Discarded due to no isolated colonies
4) Biochemical tests for confirming the presence of *Pseudomonas sps*
    a) Gram Staining
        i) Procedure:-

(1) Prepare a thin smear of microbial culture on a clean slide and air-dry.
(2) Heat-fix the smear gently.
(3) Flood with crystal violet for 1 minute → rinse with water.
(4) Add Gram's iodine for 1 minute → rinse.
(5) Decolourise with 95% ethanol/alcohol for 10–20 seconds → rinse immediately.
(6) Counterstain with safranin for 30–60 seconds → rinse and air-dry.
(7) Observe under a microscope.

ii) Observation: pink-coloured (Gram-negative), rod-shaped (bacillus) bacterium observed and identified as *Pseudomonas*

b) Catalase test - 3% H_2O_2

i) Procedure:
(1) Place a clean glass slide on the work surface.
(2) Add 1–2 drops of **3% hydrogen peroxide (H_2O_2)** on the slide.
(3) Using a sterile loop or toothpick, transfer a small amount of *Pseudomonas* culture onto the drop.

ii) Observation: Intense bubbling with peroxide is a positive test, characteristic of *Pseudomonas*

c) Oxidase test – Gordon-McLeod reagent

i) Procedure:
(1) Take a clean slide and add 1–2 drops of **Gordon & McLeod oxidase reagen**t.
(2) Using a sterile glass rod, pick a small portion of the test culture.
(3) Smear the culture onto the reagent-soaked area.
(4) Observe the colour change within **10–30 seconds**.

ii) Observation: purple-coloured colonies – positive test, characteristic of *Pseudomonas.*

5) Spot the tested bacterial colonies onto freshly prepared and sterile cetrimide agar plates to obtain a pure culture.
6) Prepare LB broth – 30 ml each for the *Pseudomonas* in 6 plates.
7) Inoculate the *Pseudomonas* pure culture from the plates into the broth. Incubate at 37 °C in a shaker incubator.
8) Using a spectrophotometer, measure the OD value at ~1.0 at 600 nm. Dilute with LB broth to standardise the OD across all samples.
   - Fill a cuvette with 1 mL of sterile LB broth to "zero" or blank the instrument.
   - If the culture is visibly cloudy (overnight growth), start with a **1:10 dilution** (100 µL culture + 900 µL sterile LB) for an accurate initial reading.
   - Mix Thoroughly**:** *Pseudomonas* can settle or form aggregates. Vortex the sample immediately before every reading to ensure the bacteria are evenly suspended.

- Since *Pseudomonas* grows quickly in LB, keep samples on **ice** if you are processing many at once to "freeze" their density at that moment.

1. The Dilution Calculation

If your culture is too dense (OD > 1.0), it will fall outside the linear range of the spectrophotometer and give inaccurate results. Use the standard $C_1V_1 = C_2V_2$ formula to target an OD within the linear range (typically **0.1 to 0.8**).

- C_1: Starting (high) OD of your sample.
- C_2: Target OD (e.g., 0.5).
- V_2: Total final volume needed for your cuvette (e.g., 1000 µL).
- V_1: Volume of culture to add ($V_1 = \frac{C_2 \times V_2}{C_1}$).

9) Prepare Bushnell Hass media.
10) Once the OD value is the same, inoculate the bacteria into Bushnell's media:
    - ➔ Centrifugation: Transfer your culture (at the desired OD) into sterile centrifuge tubes. Spin them at approximately 4,000–6,000 rpm for 10 minutes to pellet the bacteria.
    - ➔ Discard Supernatant: Carefully pour off or pipette out the LB broth without disturbing the bacterial pellet at the bottom.
    - ➔ Add sterile BH broth into the centrifuge tube and vortex to resuspend the cells.
    - ➔ Centrifuge again and discard the liquid. Repeating this wash step twice ensures no traces of LB remain.
11) Add the required volume of this suspension to the final BH flasks. A common inoculation ratio (0.5 ml of culture into 100 ml of BH broth).
12) Add a carbon source: add the oils to the broth. Let the bacteria grow
13) Check OD after 1 week.
14) Take 20 ml of media and perform the solvent extraction method.
    - Add the required amount of BH media, oil, and *Pseudomonas* into a centrifuge tube and centrifuge at 10,000 rpm.
    - The pellets formed are bacteria and debris; the pellets form at the bottom.
    - Pour the clear liquid into a clean tube – emulsified oil.
    - Pour the supernatant into a separatory funnel and add (3-6 ml) hexane.
    - Let the layers settle, then drain the water phase, i.e., the media. Pour out the oil emulsion into a separate dish.
    - Put the oil emulsion into a glass dish under a fume hood – hexane evaporates, and the brown dry spot remaining is the oil residue.
    - Quantify all 4 oil types to be tested.

- Calculation: % OIL eaten by *Pseudomonas* = 100 x (starting weight of oil - dried residue weight) / starting weight of oil.

MFC Construction:-

1. Anode – Bushnell media (200 ml) + *Pseudomonas* inoculum + oil sample (2 ml)
2. Cathode - 200 ml PBS
3. Salt bridge – 3% KCl and Agar
4. Electrodes - Graphite connected to copper wire
5. Seal the anode chamber completely
6. Provide air supply to cathode – air pump/spargers
7. Place setup on orbital shaker – distribution of oil over media
8. + Methylene blue in anode – enhance ion exchange (done on last 2 days)

Results:-

1) **LB broth Set 1 – following 2 days of growth at 37 degrees in a shaker incubator**
   - **Before inoculation into BH broth**

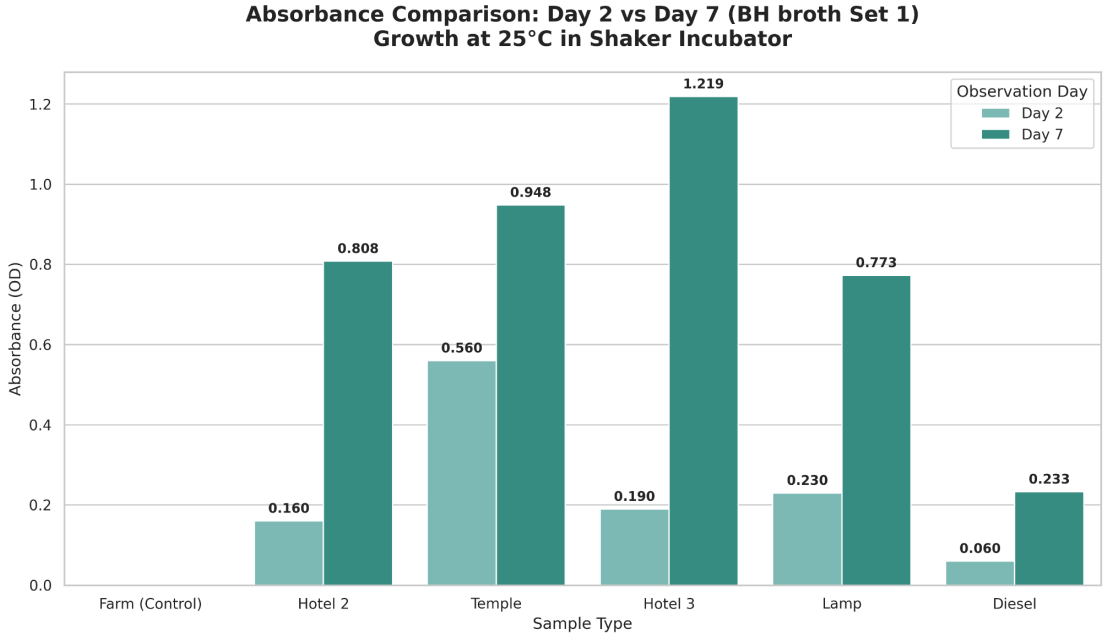
SAMPLE	Absorbance (@ 600 nm)
Hotel 2	0.61
Temple	0.57
Farm	0.53
Hotel 3	0.59
Lamp	0.47
Diesel	0.57

→ Dilute to 0.5 using fresh LB broth before inoculation to ensure equal concentration of inoculum introduced into BH broth.

2) **BH broth Set 1 - growth at 25 degrees in shaker incubator**
   - **200 microlitres of oil supplied as a carbon source at the time of inoculation**

SAMPLE	Absorbance at Day 2	Absorbance at Day 7	Δ Absorbance	% Growth
Farm	0.00 (no corresponding oil - Control and	0.00 (no corresponding oil - Control and	0.00	–

	Blank)	Blank)		
Hotel 2	0.160	0.808	+0.648	+405%
Temple	0.560	0.948	+0.388	+69.3%
Hotel 3	0.190	1.219	+1.029	+541.6%
Lamp	0.230	0.773	+0.543	+236.1%
Diesel	0.060	0.233	+0.173	+288.3%



Inoculum density: 0.5 (OD)

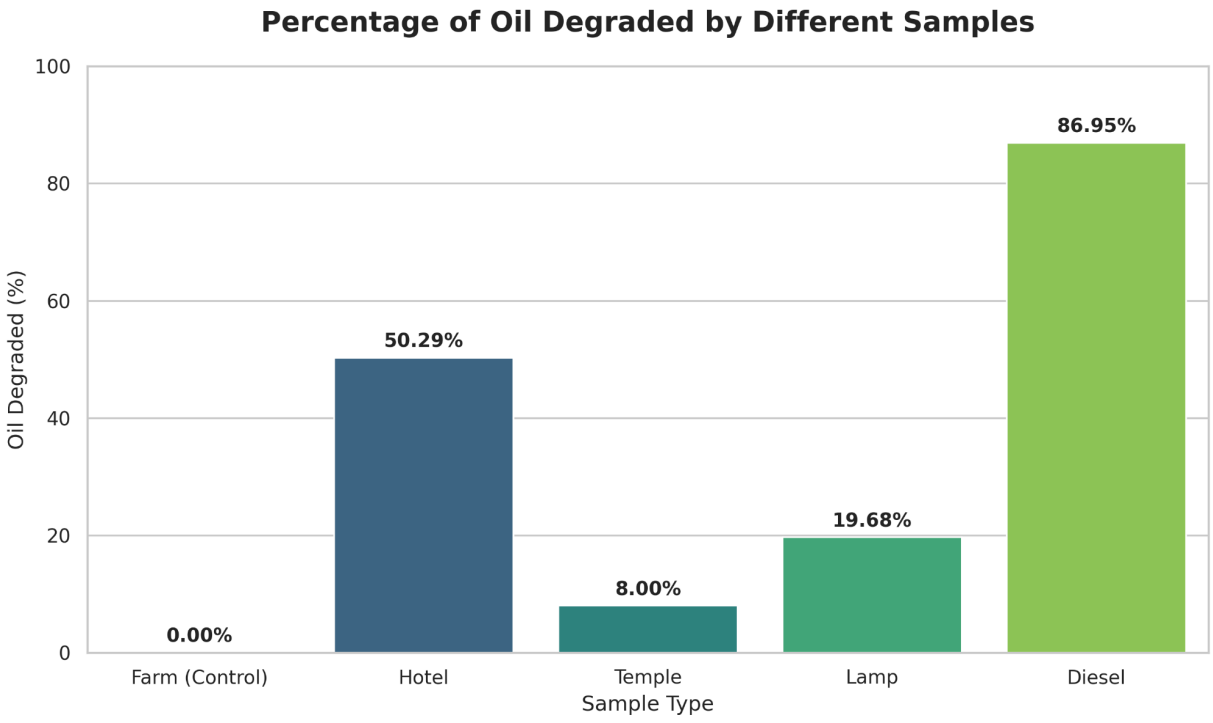
In BH Broth set 1:

- **Highest growth (Δ):** Hotel 3 with an increase in OD from 0.190 on day 2 to 1.219 on day 7, indicating it had the strongest hydrocarbon-degrading ability in this set.
- **Strong performer:** Temple, OD increased from 0.560 to 0.948.
- Diesel showed the smallest increase among the samples. from OD 0.060 to 0.2333, suggesting weaker degradation ability.

3) **Percentage of oil consumed by *Pseudomonas* in BH medium in 7 days of growth estimated by the solvent extraction method – BH SET 1**

SAMPLE	Percentage of Oil degraded
Farm	0 (no corresponding oil - Control)
Hotel	50.29%
Temple	8%
Lamp	19.68%
Diesel	86.95%

* Possible errors in using solvent extraction for a small starting volume (200 microlitres) include low recovery due to small volume, evaporation loss, volatility of oil (diesel is especially volatile, leading to excess evaporation and oil sample loss at the time of measurement), and incomplete extraction or emulsification in the solvent.



- **Highest oil degradation:** Diesel (86.95%), likely influenced by high volatility and possible evaporation losses during extraction.
- **Moderate degradation:** Hotel (50.29%), indicating effective oil utilisation.
- **Low degradation:** Lamp (19.68%) and Temple (8%), suggesting limited oil breakdown.

- **Control:** Farm showed 0% degradation, confirming the validity of the control.

Apparent degradation may be overestimated due to methodological limitations (small volume, evaporation, and emulsification), particularly affecting volatile samples like diesel.

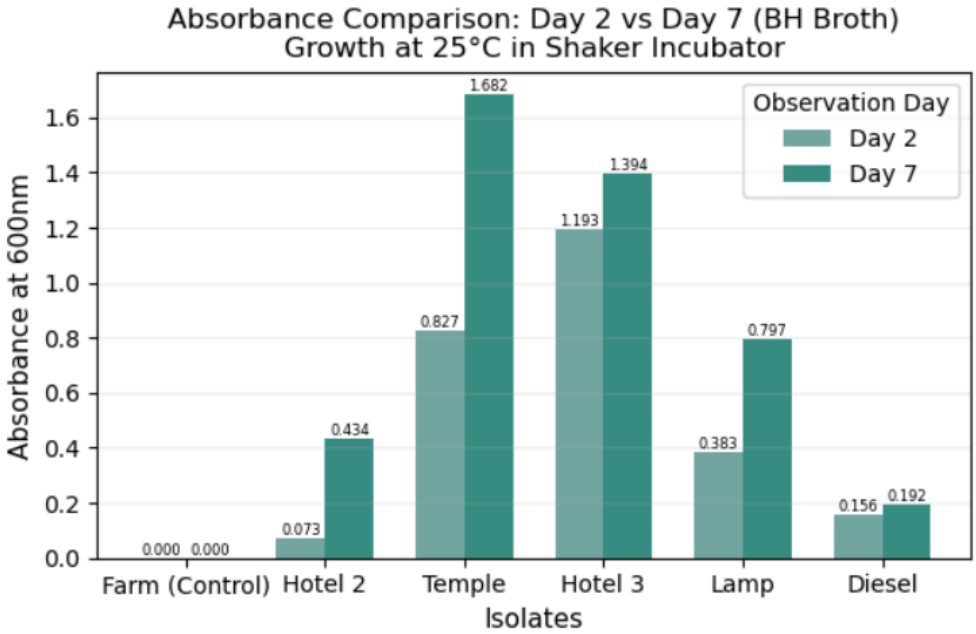
4) **LB broth Set 2 – following 2 days of growth at 37 degrees in a shaker incubator**
   - **Before inoculation into BH broth**

SAMPLE	Absorbance
Hotel 2	0.70
Temple	0.76
Farm	0.70
Hotel 3	0.65
Lamp	0.78
Diesel	0.14

→ Dilute to 0.6 using fresh LB broth before inoculation to ensure equal concentration of inoculum introduced into BH broth.

5) **BH broth Set 2 - growth at 25 degrees in shaker incubator**
   - **210 microlitres of oil supplied as a carbon source at the time of inoculation**

SAMPLE	Absorbance at Day 2	Absorbance at Day 7	Δ Absorbance	% Growth
Farm	0.00 (no corresponding oil - Control and Blank)	0.00 (no corresponding oil - Control and Blank)	0.00	–
Hotel 2	0.073	0.434	+0.361	+494.5%
Temple	0.827	1.682	+0.855	+103.4%
Hotel 3	1.193	1.394	+0.201	+16.8%
Lamp	0.383	0.797	+0.414	+108.1%
Diesel	0.156	0.192	+0.036	+23.1%



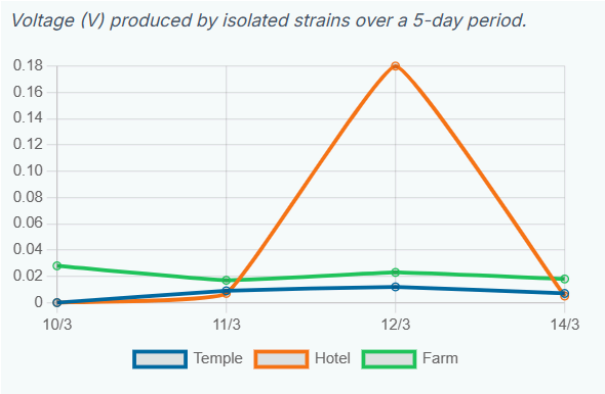
- **Highest absolute growth (Δ):** Temple
- **Strong performers:** Temple and Hotel 3
  → Hotel 3 shows high growth within the first 2 days of incubation.
  → Temple and Hotel 3 isolates show the highest overall growth by turbidity in media.
- **Moderate growth:** Lamp
- **Weak growth:** Diesel and Hotel 2
- **No growth (control):** Farm

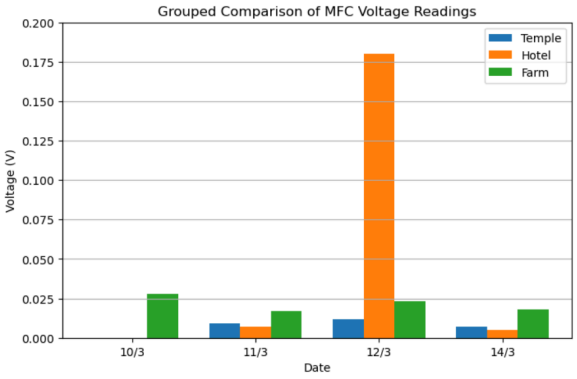
Variability in OD results of the two trials appears due to differences in cell density of inoculum; temperature fluctuations in the shaker incubator from the target 25°C; inconsistent shaking speed, affecting oxygen availability; oil dispersion throughout the media; and microbial growth.
OD values vary for different oils due to differences in oil composition – some oil components may **absorb or scatter light**, interfering with OD measurements. Diesel, being completely clear, does not absorb, but other oils, with variations in viscosity and colouration, may give higher absorbance.

Consistent strong performers across both sets were Temple and Hotel, demonstrating high overall growth under both conditions. Hotel 2 showed moderate but consistent performance. Hotel 3 exhibited variable growth between the two sets, while Diesel showed the least consistent and overall weakest performance.
→ Temple strain picked for MFC construction (similar oil composition of both lamp and temple)
→ Hotel 3 strain also picked for MFC construction
The farm was also set up as a basis for comparison by providing sucrose in the media.

6) Voltage readings for MFC

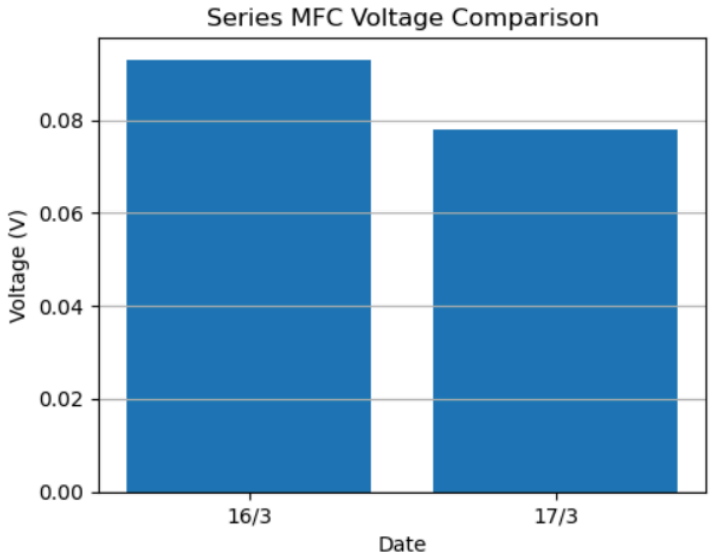
Date	Voltage reading (Individual cells) in V			Voltage in Series Circuit (V)
	Temple	Hotel	Farm	
10/3/26	0.00	0.00	0.028	-
11/3/26	0.009	0.007	0.017	-
12/3/26	0.012	0.180	0.023	-
13/3/26	-	-	-	-
14/3/26	0.007	0.005	0.018	-
15/3/26	-	-	-	-
16/3/26	No individual reading – Cells connected in Series			0.093
17/3/26	No individual reading - Cells connected in Series			0.078





- **Hotel** shows a sharp voltage spike on 12/3 (the highest individual output)
- **Farm** maintains a low but relatively stable voltage
- **Temple** shows consistently low output

A series connection of MFCs in the last 2 days gives a higher voltage than individual cells. Relatively stable voltage output observed with a peak of 0.093V on the first day of connection.



Discussion:-

Pseudomonas species show significant potential in the bioremediation of oil contamination in soil. They are capable of degrading hydrocarbons often found in oil-contaminated soil. Degradation is influenced by various factors, like concentration of bacteria, temperature, pH, and the specific carbon source used. *Pseudomonas* employs a variety of enzymes, including mono- and dioxygenases, for hydrocarbon and aromatic compound oxidation. Moreover, it secretes biosurfactants like rhamnolipids, which enhance the bioavailability of hydrophobic substrates like oil by emulsification. This study evaluates *Pseudomonas spp*. isolated from anthropogenic soils for their oil-degrading potential using oil-spiked minimal media.

Microorganism growth is evidence that the biodegradation process is occurring, and microorganisms can survive by utilizing the nutritional supplies in their living media. The biodegradation of pollutants in oil blends by *Pseudomonas* sps results in the production of biomass, carbon dioxide (CO_2), and water (H_2O). The increase in optical density, which denotes higher cell proliferation, is evidence of hydrocarbon elimination during the study period. In this study, microbial growth is demonstrated in minimal Bushnell-Haas media, with the only carbon source being the supplied oil that was to be tested, for pure isolates of *Pseudomonas*. An increase in optical densities is observed at days 2 and 7, of a 7-day incubation period for both sets of test samples, proving that the bacteria are able to break down the hydrocarbons efficiently and grow in the minimal medium.

The optical density (OD) measurements obtained from the two experimental trials exhibited variability in microbial growth assessment. This variation can primarily be attributed to differences in initial inoculum cell density between trials, incubation conditions - temperature

and shaker, and oil composition and distribution. Even minor deviations in the experimental parameters can significantly influence growth kinetics, thereby affecting absorbance readings.

In an MFC, Pseudomonas functions as an oxoelectrogen that produces its own electron shuttles called phenazines which allow electron transfer from internal metabolism to external anode. This is known as Mediated Electron Transfer. Key electron shuttles include pyocyanin, phenazine-1-carboxamide (PCA), pyoverdine. Metabolism within the cells generates excess electrons which are transferred to an oxidized phenazine molecule, which once reduced diffuses out of the cell and through the biofilm to the anode. At the anode, reduced phenazine gives up electrons creating an electric circuit.

The strains that performed well consistently – Temple and Hotel isolates – were selected for a microbial fuel cell to test energy recovery. The farm isolate, with no corresponding oil, was set up in similar media but using sucrose as a carbon source, for comparison of growth based on nutrient availability. The constructed MFCs were able to generate small amounts of voltage individually. Peak voltage was obtained after 2 days of inoculation, with the hotel strain outperforming the others, reaching a peak output of 0.180V on 12/3/26, while Temple and Farm exhibited lower outputs. Connecting multiple microbial fuel cells in series shows a stable power output and a higher combined voltage than individual cells, validating the feasibility of scaling up for bioremediation sites. However, a slight decline in voltage over time suggests possible substrate depletion, reduced microbial metabolic activity, or increased internal resistance within the system.

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