OIL DEGRADING EFFICACY OF MICROBES ISOLATED FROM OIL CONTAMINATED WORKSHOP SOIL OF MANKADU, KANYAKUMARI DISTRICT, TAMIL NADU, INDIA

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Abstract

The main petroleum hydrocarbons (PHs) found in motor oil, such as asphaltenes and aliphatic and aromatic resins, are thought to be significant environmental and health hazards. Used engine oil contaminated soil samples were collected from the subsurface soil layers at a depth of 5 to 30 cm from four different service stations and motor garages located at Mankadu in the Kanniyakumari District. Using tributyrin agar (TBA) and rhodamine agar media, the microbial colonies were separated using mineral salt agar medium and then tested for lipolytic (lipase/esterase-producing) activity. Using drop collapse assays, emulsifying activity, oil surface tension decrease, and surfactant adhesion experiments, the effectiveness of lipolytic activity was assessed. The time for complete lapse of oil by the biosurfactant was observed on 48 hours to a period of 60th days. Four *Proteus* and one *Pseudomonas* were among the five bacteria that were isolated from the soil. The screening tests confirm the presence of biosurfactant. *Pseudomonas* sps showed the most highly significant lipolytic activity, followed by *Proteus*. *Pseudomonas* and *Proteus* species are excellent common species detected in oil-contaminated soil, according to the current research.

Keywords: Engine oil, Petroleum hydrocarbons, Biosurfactant

Introduction

Polycyclic aromatic hydrocarbons (PAHs) with long-chain saturated and unsaturated hydrocarbons with carbon lengths of C16 to C36, cyclic alkanes, and additives including anticorrosive, anti-wearing, and anti-tearing chemicals make up the engine oil (Koma et al. 2003; Shahida et al. 2015). Engine oil contamination can have many different effects, but they all have the same negative effects. Hydrocarbon buildup in the environment has been

linked to long-term health risks and has been demonstrated to have poisonous, mutagenic, and cancerous effects on people (Lin and Mandri 2007). Because of the wide range of organic chemicals that microorganisms may degrade, bioremediation technologies have been found to be efficient means of accelerating the biodegradation of polluted soil and water (Udeani et al. 2009). As a sustainable remediation technique for contaminated soil, bioremediation involves biological activity that breaks down an organic contamination at a low cost (Ismail 2008; Stephen et al. 2013). The primary method for removing spent petroleum products from the environment is microbiological deterioration (Bartha 1992). According to Namkoong et al. (2002), bacteria and fungus may degrade a variety of complex compounds in soil due to the abundance of hydrocarbons they use as food (Boonchan et al. 2000). The main goal of a bioremediation design should be the creation of the most favourable conditions for microbial growth and activities which would further enhance the breakdown of contaminants (mostly hydrocarbons) that need to be broken down (Balba et al. 1998).

Materials and method Sample collection

In sterilised bags, samples of soil polluted with engine oil were taken from the subsurface soil at a depth of between 5 and 30 cm near the service stations in Mankadu, Kanniyakumari District. The soil is kept at 4°C after being sieved to remove surface debris. Each sample of soil contained one gram of soil suspended in 1ml of distilled water. The supernatant was inoculated on Mineral Salt Agar medium (MSA) supplemented with 1% engine oil after the soil particles had settled. As a negative control, the medium is employed without 1% engine oil. In soil samples, heterotrophic bacteria were counted using the spread plating technique. For 24-48 hours, the plates were incubated at 37ºC. Colonies that differed morphologically were isolated and named as M1- M5. The identified bacteria were cultured in MSA broth for further analysis.

Drop Collapse Assay

Utilizing 96-well microtitre - plates with 100 µl of used engine oil that had been equilibrated for an hour at room temperature, drop collapse assays were conducted (Bodour and Miller-Maier 2003). Different concentrations of cell-free culture broth (10, 30, 50, 70, 90, and 110 µl) were added to the surface of engine oil. It was noted when the engine oil would completely lapse. The experiment was repeated on a period of 7th, 14th, 21st, 30th and 60th days. The broth without microbes is the negative control and the broth with 1% Tween is the

positive control. The length of time it takes for oil to spread throughout the broth reveals the presence or absence of a biosurfactant. More effective surfactant is demonstrated by a shorter oil lapse period, and vice versa.

Estimation of Surface Tension

Using the Stallagmometer or drop weight method at 37°C, the surface tension of the cell free culture broth containing biosurfactant was determined (Podlogar et al. 2004). In order to calculate the change in surface tension, it is necessary to know how many drops fall per minute. The volume of cell-free broth with time indicates the sample's density. Surface tension was calculated using the formula,

$$\text{Surface tension } (\sigma) = mg / 2\pi r$$

mg - gram weight of sample fall / one minute;

r – radius of the tube of stallagmometer.

Emulsification Index (E$_{24}$ Index) Assay

The emulsification activity was assessed on 24 hours, 7th, 14th, 21st, 30th, and 60th day of study period. Isolated colonies of pure culture were inoculated in test tubes containing 2 ml of MSA, 2 ml of used motor oil was added after 48 hours of incubation. The mixture was vortexes at high speed for 1 min and allowed to stand for 24 hours (Walter et al.2010).

$$\text{Emulsification index} = H / T \times 100$$

H- Height of the emulsion layer; T- Total height

The results were compared with positive control and negative control.

Surfactant Adhesion Assay (SAA)

The Surfactant Adhesion Assay was used to determine the extracellular biosurfactant binding activity with hydrocarbon. Selected isolates were cultured for one week in 20 ml of broth with 1% (v/v) engine oil. 10 ml of the broth was centrifuged for 30 minutes at 4°C at 5,000 rpm after one week. The surfactant adhesion assay was conducted using the cell free culture broth. 5ml of cell-free suspension were added to 500 µl of engine oil in a clean test tube, and the mixture was left for 30 minutes to phase separate. The optical density (OD) was measured using a spectrophotometer at wavelength 600nm after vortexing for approximately 10

minutes. The OD was recorded after 30 min at the same wavelength. The whole procedure was repeated in triplicate. The percentage binding of Extracellular Enzyme Binding with Hydrocarbon (EEBH) was calculated using the formula,

$$EEBH = 1 - A_1 / A_0$$

A_0: absorbance at wavelength 600 nm of initial suspension.

A_1: absorbance at wavelength 600 nm of suspension after 30 minutes

Statistical analysis

Using MS Excel software, statistical analysis of variance was performed on the data to find significant differences between the treatments.

Results

The five morphologically different bacteria species were isolated from the oil contaminated soil of Mankaduworkshop, and are named as M1–M5. Of those M1, M2, M3, M5 and M4 were identified as *Proteus* and*Pseudomonas* species (M4) respectively. Of these five, *Proteus* (M1) and *Pseudomonas* (M4), were shown to possess lipolytic activity (Jenisha&Brisca Renuga 2021). Drop collapse assay was used to demonstrate the presence of biosurfactant, and further analysis showed that *Proteus*sps. and *Pseudomonas*sps showed lipolyticactivity at rates of 1.75 and 1.6 respectively (Figure 1).

Figure 1. Rate of activity of cell free culture broth
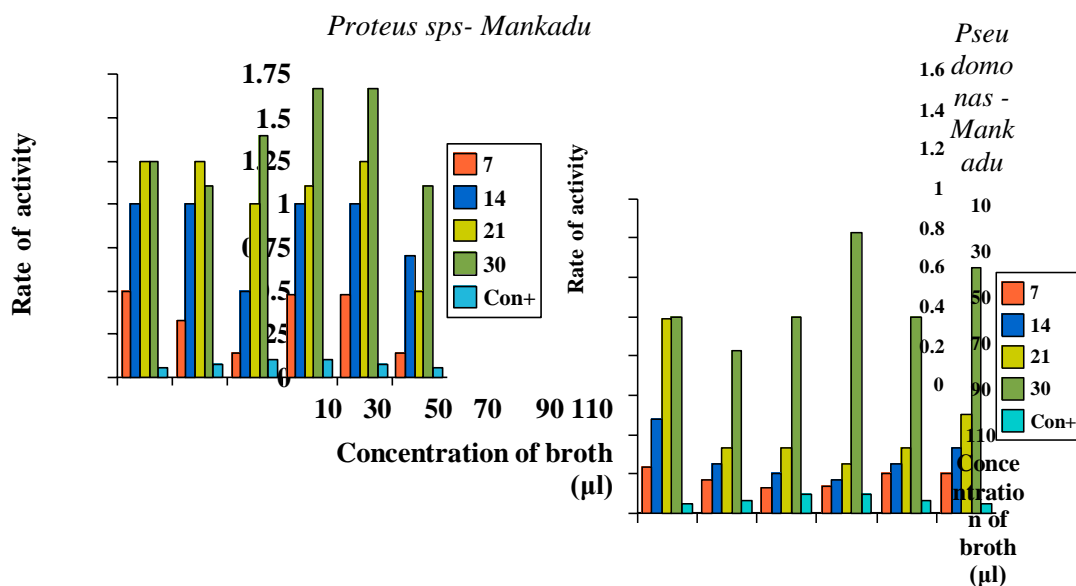
Surface tension reduction is a sign that biosurfactant, is being secreted. On the 60th day of exposure, the most significant decrease in surface tension was noted (Figure 2). In contrast to the positive control, where the surface tension was reduced after 14th days of exposure, the surface tension was stable in all control categories. A decreasing trend of surface tension was observed in all experimental categories (coloured dots & horizontal lines blue and pink) against

the positive control (Rectangular checked boxes). On the 60th day, the *Proteus* species (PRO M1) showed the greatest decrease in surface tension. (Violet dots and lines). Statistically significant reduction in surface tension was noted on 60th day of exposure in all the species ((*df= 1; F= 33.05; p < 0.05 (0.0004); F crit= 7.71*).

Figure 2 Reduction in surface tension by *Proteus* (PRO M1) and *Pseudomonas* (PSE M4) species isolated from contaminated soil of Mankadu
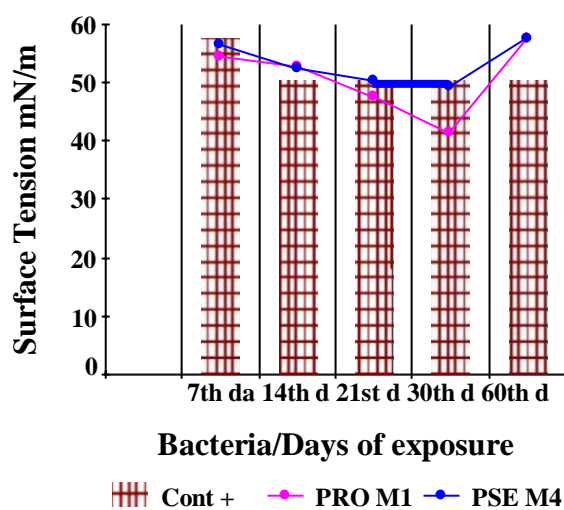

Figure 3 displayed the findings of Emulsification Index (EI24). After 24 hours, the emulsification activity of both bacterial strain increases. In the *Proteus* species, the highest Emulsification Index (EI 24) was found to be 37.4 and 40 in *Proteus* and *Pseudomonas* respecively.Highly significant emulsifying activity was observed in *Pseudomonas*sps. (PSE M4) (*df = 1; F= 33.33; p > 0.05 (0.001); F crit = 6.61*) followed by *Proteus* species of Mankadu (PRO *M1)* exhibits (*df = 1; F= 88.02; p > 0.05 (0.002); F crit = 6.61*) on 60th day of exposure (Figure 3).

Figure3. Emulsification activity of cell free culture broth of *Proteus* (PRO M1) and

Pseudomonas (PSE M4) of Mankadu
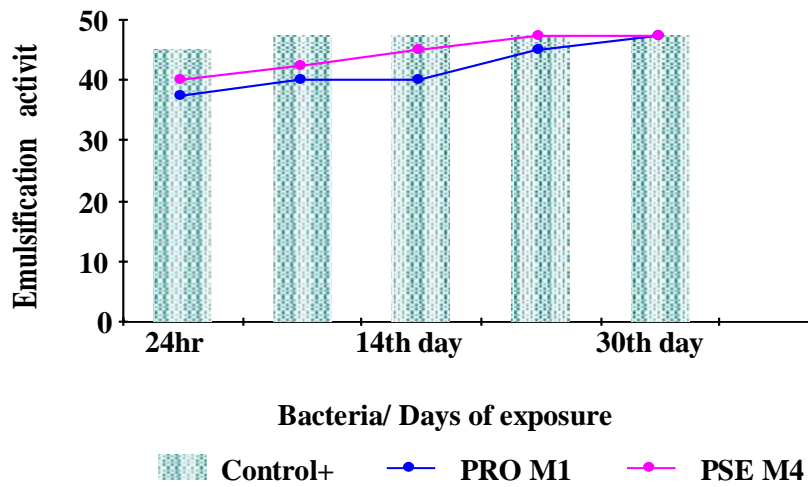
The difference in optical density between the start and final readings of the cell-free culture broth for all experimental and control categories provided as an indicator of the pace at which the biosurfactant adhered to the hydrocarbons. The *Pseudomonas* species isolated from Mankadu (PSE M4) had the highest binding levels on the seventh day of exposure and remained active until the 21st day of exposure, but the activity decreased after the 30th day of exposure (Figure4). Statically significant binding activity was observed on 7th day (*df= 4; F= 55.46; p < 0.05 (0.000054); F crit 4.28*) and 14th day (*df= 4; F= 10.30; p < 0.05(0.0060); F crit= 4.28*) of exposure.

Figure4. Rate of adsorption of cell free culture broth with used engine on different days of exposure
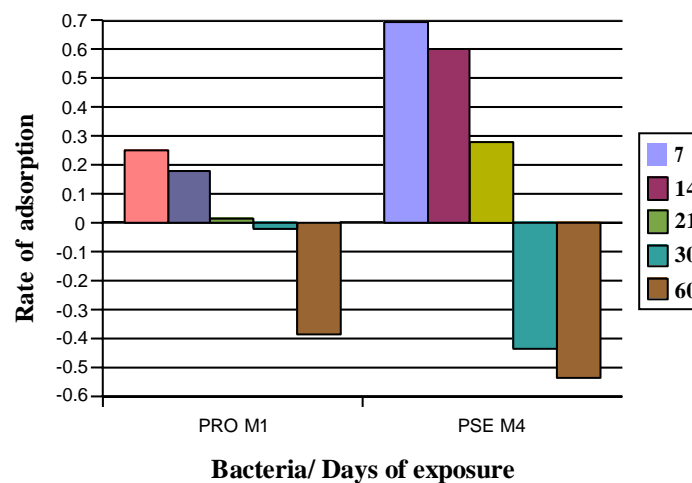
Discussion

Due to their extreme toxicity to both human and environmental health, petroleum hydrocarbons are one of the contaminants that should concern us the most. The most environmentally friendly method of cleaning up oil spills is bioremediation, which uses microorganisms to break down harmful macromolecules into safer, simpler parts. As a result, a green alternative strategy based on the idea that contaminants can be removed through bioremediation by using native or foreign microorganisms to break down petroleum hydrocarbons is gradually emerging (Frutos et al. 2012). Five bacterial strains were found in the oil-contaminated workshop soil used in this study. Only two bacterial strains, out of the five species, were recovered from oil-contaminated workshop soil.The most widely utilised *Peudomonasaeruginosa* species have affinity for a variety of hydrocarbon petroleum products (Okoh and Trejo-Hernandez 2006; Obayori et al. 2008).

The drop collapse method is a practical way to find the strains which produce extracellular biosurfactantor bioemulsifier (Jain et al. 1991), because this method requires a small volume (5-10 µl) of culture broth or biosurfactant solution to test the surfactant property (Tugrul and Cansunar 2005). As very little broth is needed this method is applied several times for screeningthe purposes (Batista et al. 2006; Plaza et al. 2006). The cell free culture broth should be used instead of using culture broth with cells.Drop collapse activity will be favourable for extracellular biosurfactant-producing cultures, but the outcomes were unfavourable for non-producing strains. The drop collapse species of the Mankadu*Pseudomonas* genus has the highest rate of activity. Glycolipids and rhamnolipids are biosurfactant that is very effective at reducing the surface tension of oil, are produced by *Proteus*and *Pseudomonas* (Eslami et al 2020).

The accuracy and dependability of the results obtained in this study's drop collapse assay were comparable to those published by KebboucheGana et al (2009). Direct correlation between drop collapse, oil spreading and reduction in surface tension was reported by (Bodour and Miller-Maier 1998; Youssef et al. 2004). Emulsification Assay is an indirect method used to screen biosurfactant production.It was assumed that if the cell free culture broth contains biosurfactant will emulsify the hydrocarbons (Walter et al.2010).

The hydrophobicity of the cell encourages microbial adhesion to hydrophobic substrates, making them more receptive to nutrient intake. Adhesion is the first phase of hydrocarbons biodegradation. The biodegradation of hydrophobic hydrocarbons can be

accelerated by bacterial adhesion (Abbasnezhad et al. 2011). The characteristics of bacterial surface and their adhesion processes are necessary for the efficient biodegradation of hydrophobic hydrocarbon substrates (Zhang et al. 2015). A number of studies reports supported that the biosurfactants enhance the bioavailability of polycyclic aromatic hydrocarbons with low degree of solubility (Mulligan 2005; Singhet al. 2007).

Conclusion

The emulsification activity, surfactant adhesion assay, and reduction in surface tension were all positive results for the cell-free culture broth of the two isolated species. Surface tension reduction enhanced emulsification index, while *Pseudomonas* and *Proteus* species demonstrated considerable oil binding activity as an indication of biosurfactant secretion.

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