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(RESEARCH ARTICLE)

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Study of petrol degrading bacteria and screening for biosurfactants

Pratibha Jinesh Shah *, Jaya Naresh Israni and Insiya Zulfeqar Lokhandwala

Department of Microbiology, Kishinchand Chellaram college, Mumbai-400 020, India.

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Abstract

Petroleum-based products when mixed with soil, air and water can lead to environmental pollution which can be dangerous for humans. In the current study, soil samples from the petrol unloading area of a petrol pump were collected and nine isolates of petrol degrading bacteria were isolated from the soil by enrichment technique using Bushnell and Hass media. The isolated bacteria could degrade up to 5% petrol and the best degraders were chosen after evaluating their cell mass when grown in the presence of petrol. The percentage of petrol degraded after different intervals of incubation was compared using a UV-vis double beam spectrometer at 228 nm. It was found that the two bacteria - *Pseudomonas aeruginosa* and *Burkholderia cepacia* degraded petrol 94.96% and 94.74%, respectively, by day 15 of incubation. The percentage degradation gradually decreased by day 20. Both bacteria were also screened for the production of biosurfactants through haemolysis. It was found that both the bacteria could produce biosurfactant along with them being a potent petrol degrader.

Keywords: Petrol Degraders; Bioremediation; *Pseudomonas aeruginosa; Burkholderia cepacian;* Biosurfactants

1. Introduction

Our planet Earth, hosts many different environments where different forms of life can be found. Not all organisms can adapt and survive in diverse environments; hence they inhabit specific environments according to their characteristics. However, microorganisms are ubiquitous; they have colonized diverse environments for centuries, including those that are considered “extreme.” Microorganisms have a great metabolic diversity, which allows them this ubiquity. Because of their ubiquitous nature, the biotechnological potential of microorganisms is virtually endless, with many possible applications, especially in bioremediation. [1].

Wide-scale production, transport, use and disposal of petroleum, have made it a lead contaminant in the environment. In gasoline and diesel stations, oil is spilled during transfer and servicing operations. In the present scenario, major environmental pollution of soil and water is due to hydrocarbon contamination resulting from the industrial activities. It can be caused by accidental liberation of petroleum industries discharged in the environment or it can also be caused by human activity [2].

Petroleum and oil residues are complex mixtures of many compounds with a high proportion of hydrocarbons, which have different solubility and microbial resistances to biodegradation. There are lots of methods for treating petroleum-contaminated sites such as mechanical and chemical methods, but these methods generally have limited effectiveness and can be expensive. The bioremediation on the other hand is a promising technology for the treatment of this petroleum polluted areas since it is cost-effective and will result in complete mineralization. Bioremediation functions basically on biodegradation by microbes. During bioremediation, absolute mineralization of hydrocarbon contaminants into carbon dioxide, water, inorganic compounds, may take place by bio-degraders. The biodegradation of petroleum hydrocarbons in the environment may be limited by a large number of factors [3].

* Corresponding author: Pratibha Shah

An important hindrance in the biodegradation of polluted soils is the low bioavailability and solubility of the hydrocarbon. Since most hydrocarbons are hydrophobic, the supply of the carbon is limited by diffusion from the liquid or solid state into the cell surface. Microbial surface-active agents or biosurfactants are extracellular products which will interact with surfaces of different polarities and reduce the surface and interfacial tension of solutions, facilitating hydrocarbon uptake and dispersion [4]. Biosurfactants are considered as an important microbial product with industrial and medical applications. They can improve the bioavailability of hydrocarbons to the microbial cells by increasing the area of contact at the aqueous–hydrocarbon interface. This increases the rate of hydrocarbon dissolution and their utilization by microorganisms. Biosurfactants have low toxicity, high specificity of action, simplicity of preparation and extensive applicability. Biosurfactants have many advantages over synthetic ones, including bioavailability, structural diversity, specific activity at extreme salinity, temperatures and pH [5].

The biodegradation of petrol by microorganisms is one of the prime ways to remove petrol from contaminated areas. Application of efficient biodegradation technologies through discovery of novel microbes and biodegradation pathways are in great demand. The current study aims to isolate petrol-degrading bacteria, study the rate of degradation and check its degradation efficiency. Since the hydrophobicity of hydrocarbons limits their entry into microbial cells, biosurfactants have received considerable attention in the field of biodegradation. Hence, this study will also screen for biosurfactants in the microbial degraders which would enhance the degradation of petrol.

2. Materials and Methods

2.1. Collection of soil sample

Soil was collected in a pre-sterilised test tube from the grounds of the petrol unloading area of a petrol pump in south Mumbai.

2.2. Isolation of degraders from soil

5 g of petrol-contaminated soil sample was inoculated in 50 ml of sterile Bushnell and Hass media (BHM) with 0.5% unleaded petrol as the sole carbon source. The contents were mixed properly and incubated on a shaker at room temperature for seven days. For the second enrichment, 2% of the inoculum from the first enrichment broth was added to sterile Bushnell and Hass media containing 0.5% petrol and incubated on a shaker for seven days. For subsequent enrichments, the amount of petrol being added was gradually increased to 1%, 3%, 5% and 7%. Since, the enrichment broth containing 7% petrol did not show any growth, subsequent isolations were carried out from the enrichment broth containing 5% unleaded petrol as the sole source of carbon [6].

2.3. Isolation on solid media

The isolation on solid media was performed as described by Latha and Kalaivani [7]. 1 ml of the enrichment broth containing 5% petrol was surface spread on BHM agar plates, and petrol was provided as the sole carbon source by evaporative technique i.e. adding 2 ml of petrol on the lid of the petri plate and inverting the plate during incubation. These petri-plates were incubated at room temperature (~25 °C) for 48 hours. To estimate the growth of petrol degraders, and thereby estimate the extent of degradation, the optical density of the isolates was measured at 600 nm. The isolates showing high dry cell mass, estimated by gravimetric method and a high absorption at 600 nm, at the end of incubation were selected for further experiments [8].

2.4. Petrol degradation study

To study the petrol degradation by the isolates, the amount of unused petrol left after the incubation for growth, was measured spectrophotometrically through a UV-Vis double beam spectrometer at 228 nm. 5 ml of sterile BHM broth with 5% petrol (as the sole source of carbon), was inoculated with 2% inoculum. To account for the loss of petrol due to evaporative loss, a blank without culture was used as a control. All tubes were incubated at room temperature on a rotary shaker for 5 days. After the period of incubation, 5 ml of petroleum ether was added to the medium and vortexed for proper mixing with the media. The unutilised petrol is thus extracted in the organic layer. The absorption of the organic phase to measure the amount of petrol left was carried out at 228 nm. The higher the absorption reading, the more is the amount of petrol left in the media; and the lesser is the degradation [4].

2.5. Identification of selected isolates

The isolates to be selected for performing further tests in the study were first studied in the laboratory. Macroscopic and microscopic characters were studied. For further confirmation of the genus and species of the isolates they were sent to Sunflower Laboratories, Mumbai. The identification was done using the VITEK 2 system.

2.6. To study the rate of petrol degradation

5 ml of BHM broth, containing 5% petrol was inoculated with 48 hr old culture suspension (O.D- 0.1 at 600 nm) and incubated for 2,5,10,15, and 20 days. The amount of petrol utilised was measured indirectly by measuring the amount of petrol left in the inoculated broth using the same method as described above [4].

2.7. To screen the isolates for the production of biosurfactants

The isolates which degraded petrol to a greater extent were screened for the production of biosurfactants. The preliminary screening tests included haemolysis. - Haemolytic assay was performed with sterile 5% sheep blood agar plates. 0.1 ml of bacterial culture, grown in a sterile BHM agar plate containing 2 ml of petrol in its lid as the sole source of carbon was used. This culture was used to prepare the culture suspension (0.1 O.D. at 600 nm), and was spot inoculated onto blood agar plates and incubated for 24 h at room temperature. The plates were visually inspected for the clear zone (haemolysis) around the colony [9-10].

3. Results and Discussions

The present study was aimed at the exploration of the indigenous microflora of the petrol unloading area of the petrol pump and the investigation of their biosurfactant-producing potential. Nine microbial strains capable of utilizing petrol as the sole carbon source were isolated after the enrichment broth was plated on BHM agar plates. The occurrence of petrol degrading and biosurfactant producing bacteria in hydrocarbon-polluted environments has been reported by many researchers [9-10].

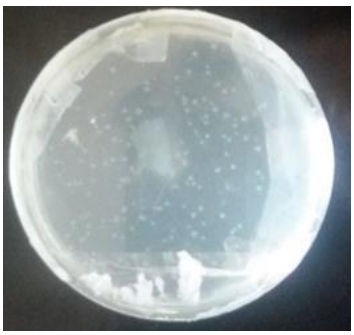

Figure 1 Isolated colonies on BHM agar plates

To determine which isolates were more efficient in petrol degradation, the growth of micro-organisms in the presence of 5% petrol was analysed by studying the absorbance at 600 nm. The dry cell mass of the isolates was measured by gravimetric method (Fig 2). ROR3 and BOR3 had more dry cell mass, 211 mg and 227 mg respectively, when estimated by gravimetric analysis.

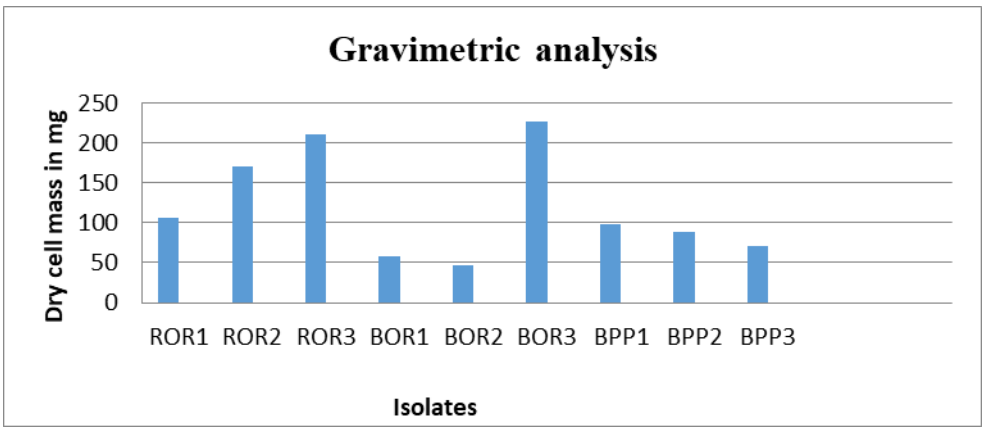

Figure 2 Gravimetric analysis to detect cell mass

Isolates with a higher dry cell mass indicated that they can grow luxuriously in 5% petrol. Two isolates were selected on the basis of growth and petrol degradation capacity and were sent to Sunflower Laboratories, Mumbai for

identification. The identification was done using VITEK 2 systems. Isolate 1(ROR 3) was identified as *Burkholderia cepacia* group, while 2 (BOR 3) was identified as *Pseudomonas aeruginosa. Pseudomonas* has been previously documented to grow in the presence of 0.5% crude oil [7] or 1% of another hydrocarbon source like pyrene [11]. Both *Pseudomonas aeruginosa* and *Burkholderia cepacia* were able to degrade 5% petrol.

Burkholderia cepacia (ROR3) and *Pseudomonas aeruginosa (*BOR3) showed maximum growth when allowed to grow in presence of petrol, indicating better tolerance and petrol utilising ability. Hence, they were used for further analysis. The amount of petrol consumed by the isolates was indirectly determined by measuring the amount of petrol left in the media after 5 days of incubation. Both the isolates have a lower optical density as compared to blank, indicating degradation of petrol by the microorganisms (fig. 3).

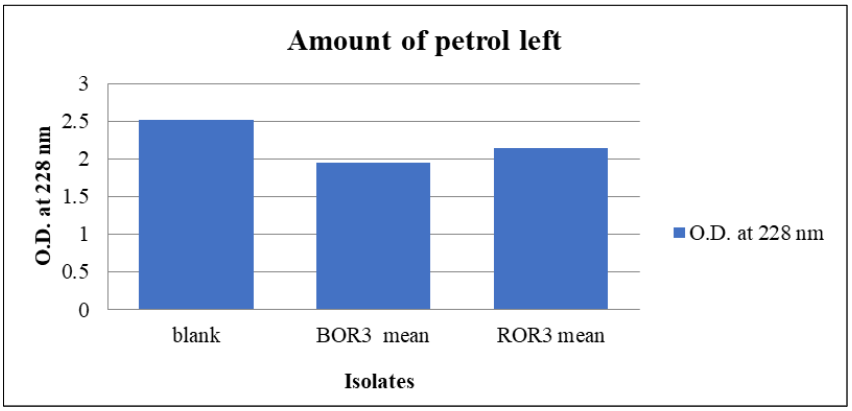

Figure 3 Amount of petrol left after utilisation by the degraders

A standard graph containg unleaded 5% petrol was plotted to estimate the petrol utilised by the degraders and amount of petrol left after utilisation (fig. 4).

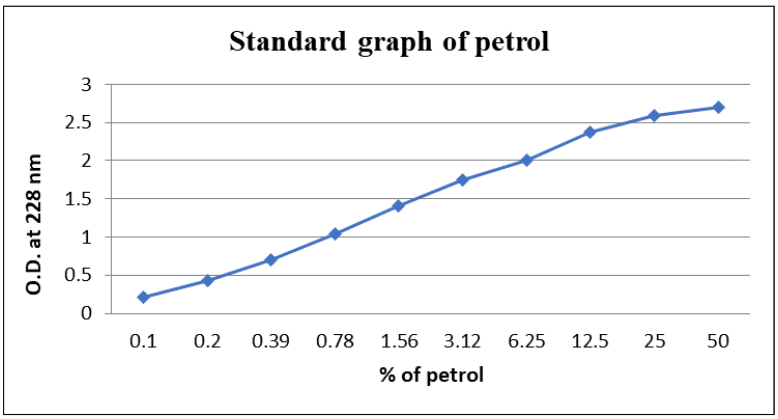

Figure 4 Standard Graph using 5% petrol

The amount of petrol utilised was measured indirectly by measuring the amount of petrol left in the inoculated BHM broth and using uninoculated BHM broth as the control. Both had 5% petrol as the sole source of carbon.

Table 1 Percentage of petrol degraded by the two degrader isolates in 20 days

Isolate	**% Petrol Degraded In**				
	2 days	**5 days**	**10 days**	**15 days**	**20 days**
Blank	Nil	Nil	Nil	Nil	Nil
ROR3	47.4%	79.84%	76.92%	94.96%	92.91%
BOR3	58.4%	68.44%	83.2%	94.74%	87.91%

From the degradation studies carried out over 20 days, it can be concluded that *Pseudomonas aeruginosa* (ROR 3) showed 94.74% of petrol degradation in 15 days with 5% of petrol as sole carbon source, while *Burkholderia cepacia* (BOR 3) group showed 94.96% of petrol degradation in 15 days. The percentage of petrol degraded was maximum in 15 days of incubation and decreased slightly by day 20.

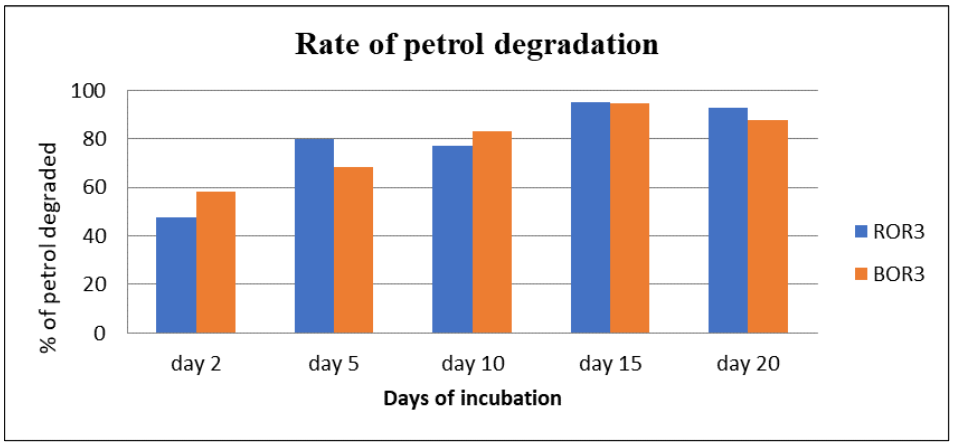

Figure 5 Graphical representation of petrol degraded by the two isolates in 20 days

Screening for the production of Biosurfactants (preliminary tests) was carried out by haemolytic tests. The clear zone around the colonies indicates that the organisms are haemolytic, which is a general feature of most of the biosurfactant producing organisms.

Figure 6 Haemolysis by BOR3 and ROR3

4. Conclusion

Petroleum hydrocarbons are alarming pollutants due to their high toxicity. Bioremediation with petroleum hydrocarbon-degrading bacteria is widely regarded as an eco-friendly and efficient technology. The microbial degradation process supports the eradication of spilled petrol from the environment after the removal of the petrol. Two efficient petrol-degrading isolates were identified and screened in the present study. The two isolates exhibited promising petrol-degradation activity. Microbial hydrocarbon degradation may happen because microorganisms have enzyme systems to degrade hydrocarbons and utilize different hydrocarbons as a source of carbon and energy. Hence, the isolates from this study should be screened further for enzymes. From the results obtained in this study, it may be suggested that microbial degradation can be considered as a key component in the bioremediation for petroleum products.

Compliance with ethical standards

Acknowledgement

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Disclosure of conflict of interest

The authors declare that they have no competing interests with whomsoever.

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