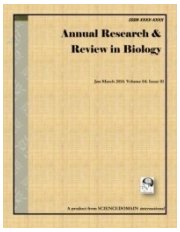

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Biodegradation of Petrol Using *Aspergillus* sp.

M. Vanishree[1], A. J. Thatheyus[2*] and D. Ramya[2]

[1]PG Department of Immunology and Microbiology, The American college, Madurai-625 002, Tamil Nadu, India.
[2]PG and Research Department of Zoology, The American college, Madurai-625 002, Tamil Nadu, India.

Authors' contributions

This work was carried out in collaboration between all authors. Author AJT designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Author MV managed the analyses of the study. Author DR managed the literature searches. All authors read and approved the final manuscript.

Original Research Article

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ABSTRACT

Soil samples collected from petrol bunks and workshops in Madurai were subjected to serial dilution and the development of fungal colonies in PDA plates. One of the colonies were selected and identified as *Aspergillus sp.* based on staining and cultural characteristics. Efficiency of this fungus on the degradation of 2.5, 5, 7.5 and 10% concentrations of petrol in minimal broth was studied for sixteen days. Decline in pH and increase in optical density and amount of CO_2 released was noticed indicating the degradation of petrol by the fungus indirectly. It was also confirmed by the appearance of new peaks in HPLC analysis after sixteen days of treatment. Hence this strain can be used in cleaning oil polluted sites.

Keywords: Aspergillus sp; Petrol; HPLC analysis; Biodegradation.

1. INTRODUCTION

With the rapid increase in human population worldwide, there is an increased demand for petroleum products such as diesel, petrol, kerosene and other industrial chemicals [1]. Although many of the chemicals are utilized or destroyed, a high percentage is released

**Corresponding author: Email: jthatheyus@yahoo.co.in;*

into the air, water and soil representing a potential environmental hazard [2,3]. Processing and distribution of petroleum hydrocarbons as well as the use of petroleum products lead to contamination of soil [4]. Changes in soil properties due to contamination with petroleum derived substances can lead to water and oxygen deficits as well as shortage of available forms of nitrogen and phosphorus [5].

Petroleum oil is a serious threat to the ecology [6]. According to Dorn et al. [7], hydrocarbons contain substances that are toxic to the flora and fauna found in the ecosystem. Petrol contains low molecular weight compounds and high proportion of saturated hydrocarbons that are usually more toxic than long chained hydrocarbons. Hence their removal becomes a necessity and several physical and chemical methods are available. But biological methods are cost-effective and ecofriendly. Hence biodegradation of petrochemicals with microbes is the best option.

Biodegradation is a process whereby compounds are broken down into smaller constituents or completely broken down into carbon dioxide and minerals by enzymatic or metabolic processes [8]. Biodegradation is a sustainable and inexpensive remediation method for contaminated soil [9]. However, the rate of biodegradation is influenced by factors such as soil type, pH, water holding capacity and nutrient limitations [10].

The most prevalent bacterial hydrocarbon degraders, in decreasing order, belong to the genera *Pseudomonas*, *Achromobacter*, *Flavobacterium*, *Nocardia*, *Arthrobacter* and other coryneforms, *Vibrio*, *Bacillus*, *Micrococus* and *Acinetobacter*. Other genera of bacteria that are able to degrade hydrocarbons include *Actinomyces, Aeromonas* and *Alcaligenes* [11]. Fewer fungi are known to degrade hydrocarbons, because of reduced fungal growth in the soil due to factors such as competition with bacteria and the toxicity of the pollutants; other mechanisms that limit fungal growth are antibiotic production, nutrient competition, and mycoparasitism [12]. Fungi are of interest because of their ability to synthesize relatively unspecific enzymes involved in cellulose and lignin decay that can degrade high molecular weight, complex and more recalcitrant toxic compounds, including aromatic structures [13].

Mycoremediation as a process focuses on the degradation of organic compounds by fungi and this is achieved through the production of extra-cellular and intracellular enzymes which catalyse various reactions [14]. It is practically established that fungi (mostly white rot fungi) are capable of using their mycelia to bioremediate hydrocarbon products due to their high production of organic acids, chelators, oxidative enzymes and extracellular enzymes that enable them to utilize the hydrocarbon products faster [15]. The ability of fungi to lower the pH of its environment also appears to be involved in the reduction of some of these compounds. The present work has focused on this approach, aiming to isolate and identify fungal strain capable of petrol degradation.

2. MATERIALS AND METHODS

2.1 Collection of Samples

The oil contaminated soil samples were collected in sterile containers from the workshops and petrol bunks at Madurai and transported to the laboratory immediately for analysis.

2.2 Isolation of Fungi

One gram of the oil contaminated soil samples were serially diluted with sterile distilled water up to 10^{-5} dilution and 0.1 ml from the dilutions 10^{-3} and 10^{-4} was inoculated in 25 ml Potato Dextrose Agar plates, which were supplemented with 50µl of streptomycin antibiotic solution and 10% of petrol, by spread plate technique. These plates were incubated at 37˚C for two days. One of the grown fungal colonies was selected for further experiments.

2.3 Identification of the Fungal Isolate

Morphological identification was done by both microscopic (fungal wet mount-Lacto phenol cotton blue staining) and macroscopic (cultural characteristics) observations.

2.4 Biodegradation Studies

The ability of the isolated strain to degrade petrol was studied by determining the following parameters. The isolated fungal strain was inoculated into Bushnell Hass Broth (Magnesium sulphate 0.2g, Calcium chloride 0.02g, Mono potassium phosphate 1g, Di potassium phosphate 1g, Ammonium nitrate 1g and Ferric chloride 0.05g) [16] containing various concentrations of petrol (2.5, 5, 7.5, and 10%) and incubated at 30ºC at 100 rpm for sixteen days.

2.5 pH Estimation

pH of the fermented broth collected from each petrol concentration was determined using a pH meter after 0,4,8,12 and 16 days of treatment.

2.6 Optical Density Determination

The optical density of the fermented broth from each petrol concentration was determined after 0, 4, 8, 12, and 16 days of treatment at 600 nm using a spectrophotometer.

2.7 CO_2 Estimation

One ml of the fermented broth was taken after 4, 8, 12 and 16 days of treatment and titrated against 0.05 N NaOH solution. Phenolphthalein was used as the indicator and appearance of stable pink colour was considered as the end point. The amount of CO_2 was calculated using the following formula [17].

$$\text{Free } CO_2 \text{ (mg/l)} = \frac{\text{Titre value x Normality of NaOH x 1000 x 44}}{\text{Volume of sample}}$$

2.8 High Pressure Liquid Chromatography (HPLC) Analysis

Fermented broth from 10% petrol and the control were taken on sixteenth day of incubation and were subjected to HPLC analysis (Model – Shimadzu, Pump – LC-20AD, PDA detector – SPDM20A and Injection - 20µl).

2.9 Statistical Analysis

Two way ANOVA was performed for the factors pH, optical density and CO_2 released using MS excel. Variability was considered significant only when the calculated F value was greater than the table F value when P is less than or equal to 0.05 [18].

3. RESULTS

One fungal strain was isolated from the oil contaminated soil and it was found to be effectively utilizing the petrol as a sole carbon source and hence it was selected for further studies of biodegradation of petrol. The isolated strain was identified as *Aspergillus sp.* based on the microscopic and macroscopic observations (Table 1). Changes in pH were recorded after 4,8,12 and 16 days of treatment with *Aspergillus* sp. at various concentrations of petrol. Figure1 depicts the variations in the pH of the medium during the treatment period and was found to be decreasing gradually, resulting in the acidic environment indicating the degradation of petrol. Variations in pH due to treatment period were statistically significant while they were not significant due to petrol concentration (Table 2).

Table 1. Observations for the identification of *Aspergillus* sp.

Medium	Microscopic Observation	Macroscopic Observation	Organism
PDA	Single celled spores in chains, sterigma arising from the terminal end of the conidiophore, the vesicle, long conidiophores arise from a septate mycelium.	White colored colonies	*Aspergillus sp.*

Changes in the optical density at 600nm were recorded after 4, 8, 12 and 16 days of treatment with *Aspergillus* sp. in various concentrations of petrol. Figure 2 shows the increase in optical density values with the increase in treatment period but a decline with the increase in petrol concentration. Variations in optical density values due to treatment period and petrol concentration were statistically significant at 5% level.

Table. 2. Two way analysis of variance for the factors with the variables, treatment period and petrol concentration

Factor	Source of variation	df	MS	Calculated F value	Table F value	Level of Significance at 5% level
	Treatment period	4	0.81325	53.32787	3.25916673	Significant
PH	Petrol concentration	3	0.024167	1.519126	3.49029482	Not Significant
Optical	Treatment period	4	0.005695	148.5652	37.25916	Significant
density	Petrol concentration	3	0.000272	7.086957	3.490295	Significant
	Treatment period	3	809691.7	29.74074	3.862548	Significant
CO_2	Petrol concentration	3	156291.7	5.7440741	3.862548	Significant

CO_2 released increased during the degradation of petrol by *Aspergillus* sp. This indicates that the biodegradation of petrol resulted in the production of carbon dioxide which was found to increase linearly with the increasing concentration of petrol (Fig. 3). Variations in the levels of CO_2 due to treatment period and petrol concentration were statistically significant.

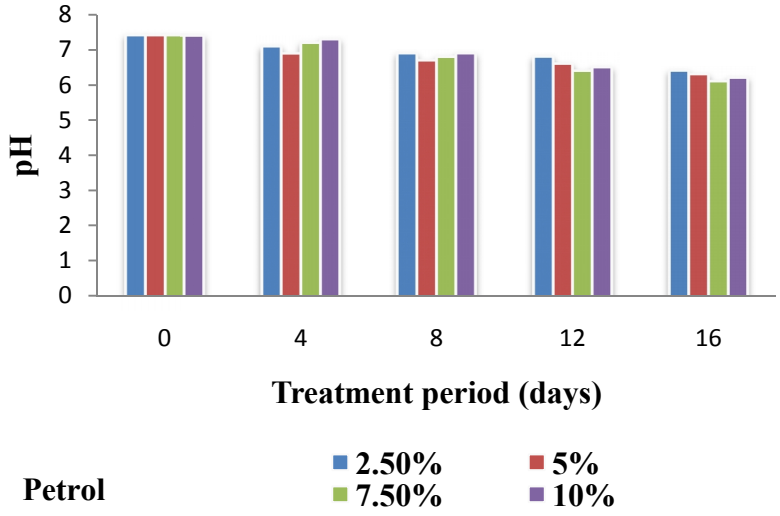

Fig. 1. Changes in the pH of the medium during the degradation of Petrol

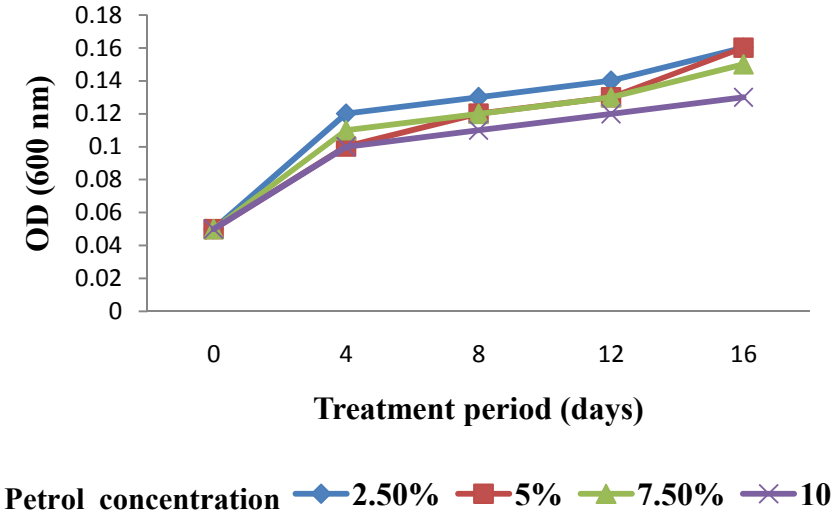

Fig. 2. Optical Density during the degradation of Petrol by *Aspergillus* sp. at 600 nm

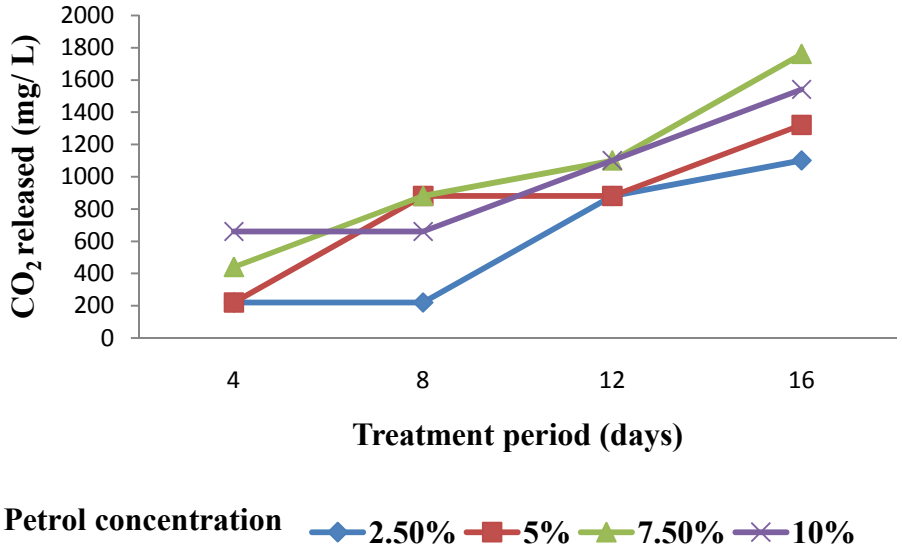

Fig. 3. Carbon dioxide released (mg/L) during the degradation of Petrol by *Aspergillus* sp.

Fig. 4. divulges the HPLC analysis for the control sample containing 10% of petrol. Two peaks were observed with the retention time of 1.930 and 2.283. These peaks observed here were missing in the HPLC analysis of the same concentration of petrol on the 16th day of degradation by *Aspergillus* sp. Two new peaks appeared with the retention time of 1.843 and 2.277. The two peaks with the retention time of 1.930 and 2.283 were missing (Fig. 5).

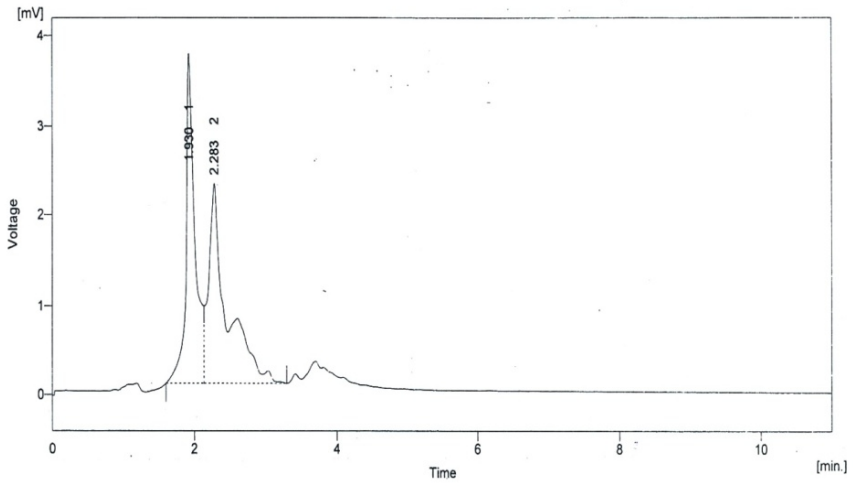

Fig. 4. HPLC analysis report for 10% Petrol (Control)

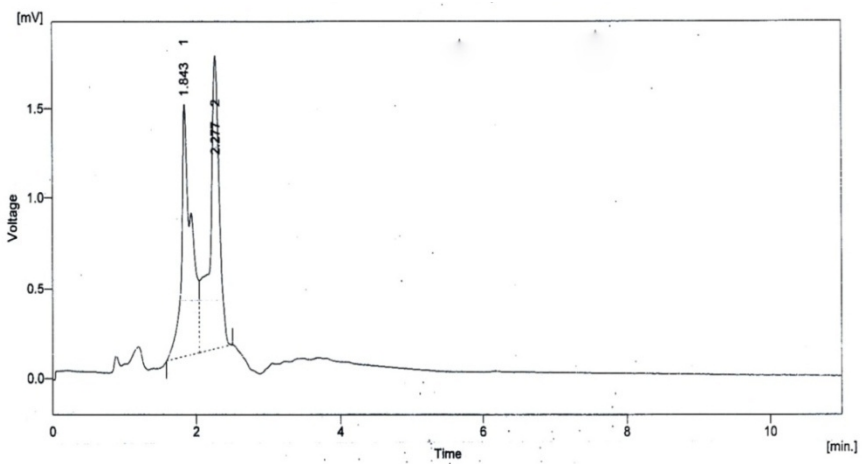

Fig. 5. HPLC analysis report for 10% Petrol treated with *Aspergillus sp.* for sixteen days

4. DISCUSSION

Fungi play a central role in the biodegradation or decomposition of organic compounds and are producers of an array of extracellular enzymes. In particular, filamentous fungi have been implicated in the biodegradation of a wide range of aromatic hydrocarbons and thus they could contribute significantly to bioremediation efforts [19,20]. Petroleum contamination is a global problem and in Polar regions these spills result in extensive damage to ecosystems as cold region ecosystem recovery is a much slower process than that of warmer regions [21].

Okerentugba and Ezeronye [22] demonstrated that *Penicillium spp*., *Aspergillus spp*. and *Rhizopus spp*. were capable of degrading hydrocarbons especially when single cultures were used. Oboh et al., [23]) have reported the abilities of bacterial species such as *Pseudomonas, Bacillus, Alcaligenes, Citrobacter* and fungi such as *Aspergillus, Penicillum, Rhizopus* and *Rhodotorula* which can grow on crude petroleum as the sole carbon and energy source when screened for hydrocarbon utilization. In this study, it was observed that there was growth and extension of the hyphae forming mycelium in the medium. Uzoamaka *et al*., [24] reported that some eight isolates of fungi showing potentials for hydrocarbon biodegradation including *Aspergillus versicolor, A. niger, A.flavus, Syncephalastrum spp*., *Trichoderma spp., Neurospora sitophila, Rhizopus arrhizus* and *Mucor spp*,.

In the present study, the fungal isolate *Aspergillus* sp. showed efficiency to degrade the petrol. In a taxonomic study of fungi, hydrocarbon assimilation is most common in the orders *Mucorales* and *Monilales,* as well as in the genera *Aspergillus* and *Penicillium* which come under the Order Eurotiales. [25]. During the degradation of petrol, pH was found to decrease gradually from the day of incubation. The optimum pH for biodegradation of hydrocarbons is around 6-8 [26]. Biodegradation of crude petroleum in an acid soil (pH 4.5) could be doubled by limiting to pH 7.4. In the present work, the decrease in pH may be due to the release of organic acids in the medium. Petroleum contaminated soil contains various hazardous materials such as aromatic hydrocarbons and polycyclic aromatic hydrocarbons; they are potentially toxic, mutagenic, and carcinogenic [27]. Microorganisms promoting fouling of oil can live in a wide range of pH from 4 up to 9, however, they tend to prefer a neutral pH [28].

Ekpenyong et al. [29] reported that in studies involving mixed microbial consortium, the pH depression was not as much as was observed in the yeast or mould consortial studies, but decreased from 7-6 gradually suggesting possible neutralizing effect by basic intermediate products mostly from organisms that utilize oxidative biodegradation pathways.

Increase in the optical density during the treatment period indicates the fungal growth due to the utilization of petrol as a source of carbon. Growth of the fungus was observed to increase, which indicated the degradation of petrol increasing with the incubation period. The decrease in the optical density with the increase in the concentration of the petrol may be due to the toxicity of pollutants. From the result, the maximum optical density was observed for *Aspergillus* sp. at 5% petrol concentration. Eja et al., [30] investigated the biodegradative potentials of the isolated fungal species from the soil polluted by petroleum products by measuring the optical densities (OD) of the fungal cultures spectrophotometrically and reported for the fungal isolates of the genera, *Saccharomyces, Aspergillus, Cladosporium, Rhizopus, Mucor, Penicillium* and *Cladosporium*.

Liberation of carbon dioxide during the degradation of petrol can be used as an indication for the activity of fungi in the growth medium. The maximum release of CO_2 was found during degradation of petrol at 7.5% for *Aspergillus* sp. Balba et al. [31] stated that mineralization studies involving measurements of total CO_2 production can provide excellent information on the biodegradability potential of hydrocarbons in contaminated soils. The approach, considered to be a preliminary step in the feasibility study, provided rapid, relatively unequivocal time-course data suitable for testing different biological treatment options, like the effect of nutrient supplementation, microbial inoculation, etc. The test can be useful for confirming active hydrocarbon degradation during full scale bioremediation.

HPLC analysis of 10% of petrol and 10% petrol after degradation showed peaks with different retention time. The new peaks obtained on the sixteenth day of treatment indicated the degradation of petrol into new unknown compounds. *Aspergillus* sp. was found to be efficient petrol degrader. In pure cultures, specific aromatic hydrocarbons and PAH fractions have been removed by up to 90% and 75%, respectively [11]. It is also known that these hydrocarbon removal percentages can diminish or increase depending on the fermentation type (solid, liquid or slurry and microorganisms or micro flora involved (pure cultures or co-cultures; bacteria-bacteria, fungi-fungi or bacteria-fungi), as well as on the characteristics and concentration of the pollutant involved.

5. CONCLUSION

Aspergillus sp. tested in the present study can be used in oil bioremediation programmes as it has the activity to grow in even 10% petrol concentration evidenced by the decline in pH and increase in CO_2 and optical density levels in the fermented broth. This is further confirmed by the disappearance of peaks found in the control and the appearance of new peaks after treatment.

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COMPETING INTERESTS

The authors have declared that there are no competing interests.

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