Biodegradation of Petrol Using the Fungus Penicillium sp.

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ABSTRACT

Background: Oil spills are considered as one of the critical problems which cause a decline in environmental health. Currently the biological solutions have become more familiar to remove hazardous substances from the environment.

Methods: Soil samples were collected from petrol bunks and automobile workshops at Madurai and used for the isolation of fungi. The isolated fungus was identified as Penicillium sp. using lacto phenol cotton blue staining method and cultural characteristics. The efficiency of the fungal strain on the degradation of different concentrations of petrol was studied using 2.5, 5, 7.5 and 10% of petrol in minimal medium.

Results: The parameters, pH, optical density and CO₂ released were determined. HPLC analysis exhibited a difference in the pattern of peaks between control and the treated sample confirming petrol degradation.

Conclusion: The ability of Penicillium sp. to tolerate oil pollutants and grow on them, suggest that it can be employed as bioremediation agent and can be used in restoring the ecosystem when contaminated by oil.

Key words: Penicillium sp, petrol concentration, HPLC, biodegradation

INTRODUCTION

The dominance of petroleum products in the world economy creates the conditions for distributing different hydrocarbon molecules and a huge volume of oily sludge, which are carcinogenic and potent immunotoxicants. Oil spillage is the accidental discharge or pouring of crude oil into the environment which involves the contamination of the environment with liquid hydrocarbons. These spills endanger public health, drinking water and natural resources and disrupt the economy. Crude oil is a naturally occurring complex mixture of hydrocarbons and non hydrocarbon compounds which at appropriate concentration possess a measurable toxicity to living organisms. The toxicity of crude oil or petroleum products varies widely depending on their composition, concentration, environmental factors and on the biological state of the organisms at the time of the contamination.

In the aquatic ecosystems, fungi play an important role with their ability in removing hazardous compounds from the water. Sediment particles contaminated with crude oil from oil spills is one of the desired ecological niches to fungi which inhabit such substrates and use them as carbon source. Fungi have been found to be better degraders of petroleum than traditional bioremediation techniques including bacteria. Although, hydrocarbon degraders may be expected to be readily isolated from a petroleum oil associated environment, the same degree of expectation may be anticipated for microorganisms isolated from a totally unrelated environment.

Several authors have made lists containing bacteria and fungi that are able to degrade a wide spectrum of pollutants. Recently, many researchers studied the role of fungi in the biodegradation of petroleum products and the most common fungi which have been recorded as a biodegrader belong to the following genera: Alternaria, Aspergillus, Candida, Cephalosporium, Cladosporium, Fusarium, Geotrichum, Gliocladium, Mucor, Penicillium, Pseudomonas, Rhizopus, Rhodotorula, Saccharomyces, Talaromyces and Torulopsis.

The ability of most fungi to produce extracellular enzymes for the assimilation of complex carbohydrates makes possible the degradation of a wide range of pollutants. They also have the advantage of being relatively easy to grow in fermenters, thus being suited for large scale production. Another advantage is the easy separation of fungal biomass by filtration due to its filamentous structure. In comparison to yeasts, filamentous fungi are less sensitive to variations in nutrients, aeration, pH, temperature and have a lower nucleic content in the biomass. In addition, several...
Penicillium strains have been shown to be able to live in saline environments, an advantage of these microorganisms over the others in the bioremediation field. Penicillium strains generally are halotolerant organisms and are able to grow either in the presence or in the absence of salt. Hypersaline wastes are generated in several industrial activities, such as chemical manufacture, oil and gas production and waste minimization practices. This waste, commonly designated as produce waters, are constituted by water containing high concentration of salts, oil, organic acids, heavy metals and radionuclides. Therefore, the ability of halotolerants to remediate pollutants in the presence of salt is useful for biological treatment without damage to the physically sensitive ecosystem.

The aim of the present study is to isolate a fungus from petrol bunk sands in Madurai and to test its ability in the biodegradation of petroleum compound.

**MATERIALS AND METHODS**

**Collection of samples:** The oil contaminated soil samples were collected from the workshops and petrol bunk in Madurai, in sterile containers and transported to the laboratory for analysis.

**Isolation of fungi:** The oil contaminated soil samples were serially diluted up to 10\(^{-6}\) dilution and 0.1 mL from the dilutions 10\(^{-6}\) and 10\(^{-7}\) were inoculated in Potato Dextrose Agar (PDA) plates, which were supplemented with 50 μL of streptomycin antibiotic solution and 10% of petrol, by spread plate technique. These plates were incubated at 37°C for two days.

**Determination of resistance:** The isolated colonies were inoculated in oil agar medium, which was prepared according to the Mineral Salts Medium (MSM) composition by Mills et al.\(^{19}\) as modified by Okpokwasili and Okorie\(^{19}\). The medium was used for isolation, enumeration and preliminary identification of petroleum utilizing fungi (oil degraders). The medium was prepared by the addition of 1% crude oil to sterile MSM, which was cooled to 45°C under aseptic conditions. Streptomycin was added to prevent bacterial growth. The MSM and crude oil were then mixed thoroughly and dispensed into sterile petri plates to settle. The plates were incubated at room temperature for one week and pure and representative colonies were transferred to PDA slants for preservation.

**Identification of the fungal isolate:** Morphological identification was done for the selected fungal strain by both microscopic (fungus wet mount lacto phenol cotton blue staining) and macroscopic (cultural characteristics) observations.

**Biodegradation studies:** The ability of the isolated strain to degrade petrol was studied by determining various parameters. The isolated fungal strain was inoculated into Bushnell Hass Broth containing various concentrations of petrol (2.5, 5, 7.5 and 10%) and incubated at 30°C at 100 rpm for sixteen days.

**pH estimation:** pH of the fermented broth was determined after 0, 4, 8, 12 and 16 days of treatment using a pH meter.

**Optical density (growth rate) determination:** The optical density of the fermented broth was determined after 0, 4, 8, 12 and 16 days of treatment.

**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 an indicator and appearance of stable pink colour was considered as the end point. The amount of CO\(_2\) was calculated using the following equation\(^{20}\):

\[
\text{Amount of free CO}_2 \text{ (mg L}^{-1}\text{)} = \frac{\text{Titre value} \times \text{Normality of NaOH} \times 1000 \times 44}{\text{Volume of sample}}
\]

**High pressure liquid chromatography (HPLC) analysis:** Samples from 10% petrol and the control were taken on 16 day of incubation and were subjected to HPLC analysis.

**Statistical analysis:** Two way ANOVA was performed for the parameters, 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 at P is less than or equal to 0.05.

**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 *Penicillium* sp. based on the microscopic and macroscopic observations (Table 1 and Fig. 1).

| Table 1: Microscopic and Macroscopic observation of the isolated fungus, *Penicillium* sp. grown on potato dextrose agar medium |
|---------------------------------|
| **Observations** | **Identified Organism** |
| **medium** | **M Microscopic** | **M Macroscopic** | **Organism** |
| PDA | Single celled spores in chains, stroma arising from the metabula of the conidiophore, branching conidiophores, arise from a septate mycelium | Yellow colored colonies with greenish black color inside | *Penicillium* sp. |
| PDA: Potato dextrose agar |

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Fig 1: Growth of *Penicillium* sp. in potato dextrose agar medium containing 10% petrol concentration compared to control condition.

![Graph showing growth of *Penicillium* sp.](image)

Fig 2: Changes in the pH of the medium during the degradation of petrol by the fungus *Penicillium* sp.

Changes in pH were recorded after 4, 8, 12 and 16 days of treatment with *Penicillium* sp. at various concentrations of petrol. Figure 2 depicts the variations in the pH of the medium during the treatment period and pH was found to be decreasing from 7.4-6 gradually, resulting in the acidic environment indicating the degradation of petrol.

Changes in the optical density at 600 nm were recorded after 4, 8, 12 and 16 days of treatment with *Penicillium* sp. for the various concentrations of petrol.

![Graph showing optical density](image)

Fig 3: Changes in optical density during the degradation of petrol by the fungus *Penicillium* sp. at 600 nm.

Figure 3 shows the variations in the optical density which seems to be fluctuating. But still an increase in growth rate was observed with the increase in the days of treatment while there was a decrease in optical density with increasing concentration of petrol.

The CO₂ released showed an increase during the degradation of petrol by *Penicillium* 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. 4).
Table 2: Two way analysis of variance (ANOVA) for the factors pH, optical density and CO$_2$ with the variables treatment period and petrol concentration for the treatment of petrol with Penicillium sp.

| Factor          | Source of variation | df | M.S   | Calculated F value | Table F value | Level of significance at 5% level |
|-----------------|---------------------|----|-------|--------------------|---------------|-----------------------------------|
| pH              | Treatment period    | 4  | 0.8045 | 79.13113           | 3.259167      | Significant                       |
|                 | Petrol concentration| 3  | 0.0438 | 4.114754           | 3.490295      | Significant                       |
| Optical density | Treatment period    | 4  | 0.0073 | 93.64516           | 3.259167      | Significant                       |
|                 | Petrol concentration| 3  | 0.0022 | 3.096774           | 3.490295      | Significant                       |
| CO$_2$          | Treatment period    | 3  | 1169667| 54.375             | 3.862548      | Significant                       |
|                 | Petrol concentration| 3  | 72600  | 3.375              | 3.862548      | Significant                       |

Fig. 4: Carbon dioxide released (mg L$^{-1}$) during the degradation of petrol by Penicillium sp.

Fig. 5(a-b): High pressure liquid chromatographic analysis report for 10% petrol concentration in minimal medium (a) Before and (b) After treatment with fungus Penicillium sp. for sixteen days

DISCUSSION

The increase in rates of fungal growth in the media containing crude oil might be due to the fact that fungi use crude oil as a substrate for their survival and growth using extra cellular enzymes$^{10}$. In a previous review, Bartha and Atlas$^{21}$ listed fourteen genera of fungi isolated from an aquatic environment which had been demonstrated to contain members which utilize petroleum hydrocarbons. The evolution of the hydrocarbon mixture depends on the nature of the oil, microbial community and environmental factors which impact microbial activities. Singh$^{22}$ also reported a group of terrestrial fungi, namely, Aspergillus, Cephalosporium and Pencillium which were found to be potential degraders of crude oil hydrocarbons.

This study represents the first step towards the generation of an efficient biodegradation process. First, a suitable microorganism was chosen for its degradation potential and then culture conditions were identified that optimize the growth of the microorganism$^{23}$. As expected, the present study confirmed that Penicillium sp. grew better in acidic conditions. Previous studies also reported that several fungal isolates such as Fusarium solani, F. oxysporum, Trichoderma viride and Aspergillus niger$^{24,25}$ cultured in MSM medium at pH 5.5 also showing good growth. However, Penicillium sp. was able to grow in a relatively wide range of pH from 6.0-7.5, suggesting that this isolate could degrade oil under not only acidic but also neutral conditions.

Higher concentrations of crude oil could have toxic effects on the cells and lead to decreased biomass production with the increment of concentrations of crude oil$^{26}$. The toxicity of crude oil or petroleum...
products varies widely depending on their composition, concentration, environmental factors and on the biological state of the organisms at the time of contamination. Different species and different life stages of organisms have been demonstrated to have different susceptibilities to pollution. The decrease in biomass production with increasing concentration of crude oil is often attributed to oil toxicity. Some microorganisms are killed or inhibited by toxic components in the oil, while other heterotrophic organisms degrading the oil are increasing in number. In the present study, a faster rate of growth 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 is observed to increase, which indicates that the degradation of petrol increases as the incubation period increases. The decrease in the optical density with the increase in the concentration of petrol may be due to the toxicity of pollutants. From the result, maximum optical density was observed for Penicillium sp. at 10% concentration. It indicated that Penicillium sp. as the efficient strain in the biodegradation of petroleum compound.

Liberation of carbon dioxide during the degradation of petrol can be used as an indication for the activity of fungus in the growth medium. Penicillium sp. showed more CO₂ production at both 7.5 and 10% petrol concentration. Penicillium sp. was found to degrade petrol more efficiently at higher concentrations. Penicillium sp. was capable of producing enzymes at a faster rate to decompose the substrate hydrocarbon and release more CO₂. Hence this fungus can be utilized effectively as an agent of petrol biodegradation process.

HPLC analysis for 10% petrol concentration before and after treatment with Penicillium sp. showed peaks with different retention time. The reports showed the difference in retention time between the control and experimental samples. The new peaks obtained on the sixteenth day of treatment indicated the mineralization of petrol into new unknown isomers. Only two new peaks were identified and when the height of the peaks and retention time for the isolate was compared with the control Penicillium sp. was found to be efficient petrol degrader. In pure cultures, specific aromatic hydrocarbons and PAH fractions have been removed by upto 90 and 75%, respectively.

CONCLUSION
The isolate Penicillium sp. has the ability to tolerate the tested petrol concentrations and grow on them. Changes in pH, OD and CO₂ levels along with the HPLC analysis reports confirm its potential of petrol degradation.

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