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Comparative studies on biodegradation of petroleum Hydrocarbon by two effective microorganisms

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ABSTRACT

It is known that bacteria and fungi are the principle petroleum degrading microorganisms. Therefore, the study of the petroleum hydrocarbon degradation was accomplished by filamentous fungi and bacteria to evaluate the potential of strains, previously isolated from three different petrol bank soil, accidentally contaminated with crude oil. The results of such an evaluation allowed the selection of those microorganisms with ability to degrade petroleum, added as the only carbon and energy source to a mineral medium. The selected strains were submitted to identification, being the following genera detected: *Pseudomonas putida* and *Aspergillus niger*. Gravimetric analysis showed that *Pseudomonas putida* degraded the oil in 5 days at 40 °C and pH as neutral whereas *Aspergillus niger* degraded 30°c. Gas chromatographic analysis revealed that after 5 days the *Pseudomonas putida* strain was able to degrade hydrocarbon components more effectively than the other *Aspergillus* strains.

Key words: *Petroleum hydrocarbon, Pseudomonas putida, Aspergillus niger, Environmental factors, Black gram.*

Introduction

Interest in the microbial biodegradation of pollutants has intensified in recent years as mankind strives to find sustainable ways to clean up contaminated environments. These bioremediation and biotransformation methods endeavour to harness the astonishing, naturally occurring, microbial catabolic diversity to degrade, transform or accumulate a huge range of compounds including hydrocarbons (e.g. oil), polychlorinated biphenyls (PCBs), polyaromatic hydrocarbons (PAHs), pharmaceutical substances, radio nuclides and metals. Major methodological breakthroughs in recent years have enabled detailed genomic, metagenomic, proteomic, bioinformatics and other valuable analyses of environmentally elevant microorganisms providing unprecedented insights into key biodegradative pathways and the ability of organisms to adapt to changing environmental conditions (Meagher, 2000).

Hydrocarbons consisting of a "backbone" or "skeleton" composed entirely of carbon and hydrogen and other bonded compounds, and lack a functional group that generally facilitates combustion without adverse effects. The majority of hydrocarbons found naturally occur in crude oil, where decomposed organic matter provides an abundance of carbon and hydrogen which, when bonded, can catenate to form seemingly limitless chains. In organic chemistry, a hydrocarbon is an organic compound consisting entirely of hydrogen and carbon. (Clayden and Greeves, 2000).

Given favorable environmental conditions, all natural organic compounds degrade. If any organic

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compound produced in the ecosphere were inherently resistant to recycling, huge deposits of this material would have accumulated throughout the geological ages. Substantial organic deposits, such as fossil fuels, accumulate only under conditions adverse to biodegradation. The introduction of indigenous microorganisms isolated from a contaminated site after culturing seems to be a highly effective bioremediation method, especially when microbial growth is supplemented by oxygen and fertilizers (Harayama *et al.,* 1999). Thus, bioremediation is normally achieved by stimulating the indigenous microbiota (naturally occurring microorganisms). Stimulation is achieved by the addition of growth substrates, nutrients, terminal electron acceptor, electron donors, or some combination there in, resulting in an increase in contaminant biodegradation and biotransformation (Korda *et al.,* 1997).

The microbial communities are not static, and factors affecting the composition of the community will alter their degradation potential. Alterations in community degradation potential due to physicochemical changes (i.e., temperature, nutrient availability) have been characterized (Bartha, 1977). Oil biodegradation of subsurface does not require oxygen, it does require certain essential nutrients (e.g., nitrogen, phosphorus, potassium), which can be provided by dissolution of minerals in the water leg (Larter *et al*., 2006). Empirically, it has been noted that biodegraded oil accumulations occur in reservoirs that are at temperatures less than 80°C. At higher temperatures, it appears that many of the microorganisms involved in subsurface oil biodegradation cannot exist (Wilhelms *et al*., 2001).

Many bacterial species are involved in the degradation of hydrocarbon such as *Pseudomonas, Aeromonas, Moraxella, Beijerinickia, Corynebacteria, Mycobacteria, Streptomyces, Achromobacter, Arthrobacter Acinetobacter, Alcaligenes, Bacillus, Flavobacterium, Nocardia, Rhodococcus* and fungal species such as *Cbytidomycetes, Oomycetes, Zygomycetes, Basidiomycota, Deuteromycota* and Micro-algae such as *Porphyridium, Petalonia, Diatoms, Chlorella, Dunaliella, Chlamydomonas, Volvox* (Cerniglia, 1992).

Pseudomonas putida is a gram-negative rod-shaped saprophytic soil bacterium. Based on 16S rRNA analysis, *P. putida* has been placed in the *Pseudomonas* group, to which it lends its name (Anzai, 2000). It demonstrates very diverse metabolism, including the ability to degrade organic solvents. This ability has been put to use in bioremediation, or the use of microorganisms to biodegrade oil. *P. putita* is frequently isolated from petroleum-contaminated sites and is capable of producing metabolites (i.e., alginate, rhamnolipid, pyocyanin) that enhance its competitiveness and survival. Oil-degrading microbial communities were initiated with soil from a polycyclic aromatic hydrocarbon (PAH)-contaminated site (Morita, 1982).

Aspergillus niger is a saprophytic fungus. It is a common mold found in dump bread or almost any other organic matters. It is also involved in the production of some secondary metabolites. However, the *Aspergillus niger* posses some attributes that enable them as good potential agents of degradation and digesting it through the secretion of extracellular enzymes. Besides, it was capable to grow under environmental conditions of stress, for example: environmental with low pH values or poor in nutrients and with low water activity (Yateem *et al.,* 1998).

The specific aim of this research work is: (1) To compare of the two microorganisms involved in degradation, (2) To understand the mechanism behind the degradation, (3) To develop molecular tools to look into the changes of the population following exposure to crude oil and (4) To develop a system to treat highly polluted oil contaminated soil in a manner analogous of *Pseudomonas putida* and *Aspergillus niger* with black gram plants.

Materials and Methods

Sample collection

The soil samples were collected at a depth of 3 inches from oil contaminated area from the three petrol banks such as Thiruvanaikovil, Maambalasaalai. Tolgate, Trichy.

Isolation of microbes from soil samples

Microbial isolation was realized from the collected samples through successive dilutions, and inoculation in Petri dishes, where three different media were used (Nutrient, cetrimide, Saburoud agar medium) with the purpose of offering different nutritional options for the development of several bacteria and fungal species that could have been established in the samples, during the natural process of soil weathering.

Identification of microbes from soil samples

Isolated samples was examined many times for its size, shape, margin, consistency, opacity, elevation,

pigmentation, gram reaction and cell morphology as described by Connan (1984). The isolates were characterized as described by Hanson *et al.,* (1994). Diagnostic properties used include motility, production of cytochrome oxidase, catalase, indole and urease, gelatin liguefaction, starch hydrolysis, oxidation/ fermentation of sugars, methyl red, and Voges proskaeur test at 37 °C for 24hours.

Biodegradation test

Inoculum: A bacterial and fungal colony was taken from the Petri plate. It was streaked on the surface of nutrient agar and saburoud dextrose agar medium solidified with agar. It was incubated. Inoculums preparation was done by using nutrient broth and saburoud dextrose broth. Nutrient broth and saburoud dextrose broth was prepare and a loopfull culture from the pure form was transferred into two broth and incubated.

Hydrocarbon degradation: Mineral salts medium (100ml, pH-1.0) supplemented with trace elements solution (2.5 ml per liters) of Bauchop and Elsden (1960) were put into 500 ml Erlenmeyer flasks. The hydrocarbon substrates (0.1%, v/v) petrol was used as sole carbon sources. The media were inoculated with cells previously grown for 24h in nutrient broth and washed four times in phosphate buffer (pH 7.0) to remove traces of nutrient. Incubation was carried out at room temperature (30 ºC ± 2). Growths of the organisms were assayed after 48 h by optical density (0D) measurement at 560 nm. Inoculated minimal salts medium without hydrocarbon served as control. Cultures without any increase in turbidity over the initial OD of test and control were scored as no growth (-), cultures with slight increase over initial OD but significantly greater than the control OD were scored as poor growth (+). Cultures with growth well above the initial were scored as moderate (++) while cultures with luxuriant growth were scored as heavy growth (+++). The time-course growth was monitored by total viable count on nutrient agar from the day of inoculation and subsequently after 48 h for 14 days.

Various environmental factors: Nutritional and environmental factors affecting petroleum degradation such as inoculum's size, pH, concentrations of sodium chloride, temperature, energy source and different time intervals were the most significant variables. In order to optimize the level of the significant factors proved a maximum petroleum oil degradation of 98.8%. These results considered as an important practical application in the field of petroleum waste remediation (Bartha, 1977).

Role of Plasmid and Protein in Biodegradation: Many plasmid containing bacteria have been isolated from polluted areas, which are relative to those present in pristine control sites. Plasmids are important in the rapid transfer of genes among populations in a microbial community. Plasmids contain antibiotic resistance genes, as well as involved those degradative pathways. Plasmid which code for degradative enzymes, complete degradation of growth substrate is possible with the help of such plasmids alone (Whyte *et al.,* 1997). The cell proteins and total extra cellular proteins involved in the reduction of oil shale. The outer membrane proteins of *some species* grown on crude oil as sole source of carbon showed induction of two proteins/polypeptides (Chakrabarth *et al.,* 1973). So, such microbial community was characterized by agarose gel and SDS-PAGE electrophoresis.

Gas chromatography: Analyses the oil degradation processes done by high resolution gas chromatography method.

Extraction of samples: Oil was extracted from each culture medium by one-half volume of dichloromethane and the samples was kept into separating unit, the extraction procedure being repeated until the dichloromethane layer had become clear. The extract was then dried in vaccum, and the residual material was dissolved in chloroform. Each sample was subsequently analyzed by gas chromatography (Westlake, 1982).

Quantification of samples: The GC analysis was performed with a HP-5890A instrument fitted with a fused silica capillary column (HP-1701; 30 m long, 0.25 mm diameter; 0.25 mm thickness) and the detector is Flame ionizing detector. The operating temperature of the flame ionization detector was 300 °C and that of the injector was 230 °C. The column temperature was set at 50 °C for 2 min, increased to 300 °C at a rate of 6 °C/min, and then kept at 300 °C for 15 min. The carrier gas was nitrogen, air and hydrogen is a detector gas at a flow rate of 1 ml/min, and naphthalene was added to each sample as an internal standard. The GC-FID instrument was operated in the selected ion-monitoring mode (Ijahu, 1998).

Field application: The treatment highly polluted oil contaminated soil in a manner analogous of *Pseudomonas putida* and *Aspergillus niger* with black gram plants. Preparation of five pots containing

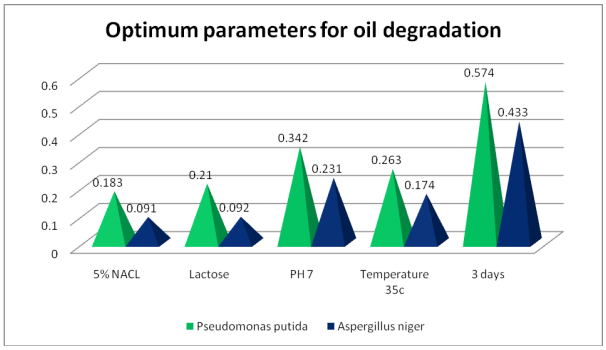
separate the pots are as follows:-1.One pots with seeds in oil-contaminated soil (control). 2. Two pots with black gram seeds in oil contaminated soil inoculated with *Pseudomonas putida* bacterial culture for 50 ml. 3. Two pots with black gram seeds in oil contaminated soil inoculated with *Aspergillus niger* fungal culture for 50ml. Measure the pH of each soil sample 5 times during the investigation. Count the bacterial population in each soil sample 3 times during the experiment by using dilution and plating-out procedures and test the toxicity of the soil (Paul A.Westhart, 2005).

Result and Discussion

Determination of hydrocarbon degradation capacity allowed the selection of the strains that actually contributed in the degradation of the petroleum hydrocarbons, diminishing the two different strains guided for identification. In the present study the heterogeneous bacteria obtained by serial dilution in cetrimide media contains below number of bacterial counts than nutrient agar media ranged from 4 to 9×10^6 g^{-1}. A total of 5 isolates were collected from 20 different colonies and *Pseudomonas putida* were characterized. Bhadauria, (1999) also collected agricultural soil irrigated with petroleum refinery effluent, then 15 species of bacteria have been identified where the bacterial count range between 66 and 860×10^6. In the present study the fungal obtained by serial dilution in hydrocarbon degradation medium contains less number of fungal counts than saburoud dextrose agar media ranged from 2 and 8×10^3 g^{-1}. A total of 3 isolates were collected from 10 different colonies and *Pseudomonas putida* were characterized. Judith *et al.* (1996) reported a total 9 different species were detected in guararema soil and the experiment allowed pointing out *Aspergillus niger* as bearing the highest potential to degrade petroleum hydrocarbon when compared to other fungal species.

Microorganisms that tolerate or required high salt concentration are called halotolerent or halophilic respectively. In the present study the increased salt concentration of the media above 10% sodium chloride result in absence of bacterial and fungal growth and utilization of oil (Figure 1). Atlas and Bartha (1991) reported that *Staphylococcus species* and *Halomonas species* characteristic feature is halotolerants. *Pseudomonas species* was not tolerates for high salt concentration and also *Holodurans species* show growth in salt concentration between 1.8 and 15.5%.

The present study also deals with the effect of growth with different carbon sources. *Pseudomonas putida* showed significant growth by utilizing glucose and sucrose while *Aspergillus niger* was maximum growth in glucose. Rogers *et al.,* (1965) reported that *Cladosporium resinae* is a filamentous fungus which acts as contaminant and deteriogen of fuel oils, utilizing them as a carbon source. All the bacteria are producing more surfactant when glucose is the carbon source.



The pH in soil is one of the important abiotic factors for soil microorganisms. In the present study the maximum growth of bacteria and fungi were noted in the pH 7 whereas in pH 4 and pH 5 there was no bacterial growth. The present study has been supported by the earlier finding by Alexander *et al.,* (1999) who reported that maximum degradation occurred at pH near to neutrality.

Another important abiotic factor which has more impact on the growth of bacteria and fungi is temperature. *Pseudomonas putida* was oil degrade for optimum temperature at the range on 35-45 °C. *Aspergillus niger* was oil degrade for optimum temperature at the range on 25-35 °C. Zobell (1946) reported hydrocarbon degradation at below 0°C. Zobell found that hydrocarbon degradation was over an order of magnitude faster at 25°C than at 5°C for *Aspergillus niger*.

The oil degradation microbes flourish in soil medium where as the oil concentration is less. Infact if the oil concentration is increased in the medium it will inhibit the microbial growth. In the present investigation the optimum growth of *Pseudomonas putida* in the medium is noted when the oil concentration range from 0.5% to 2.5%. *Aspergillus niger* in the medium is noted when the oil concentration range from 2.5% to 5%. Rahman *et al.* (2004) re-

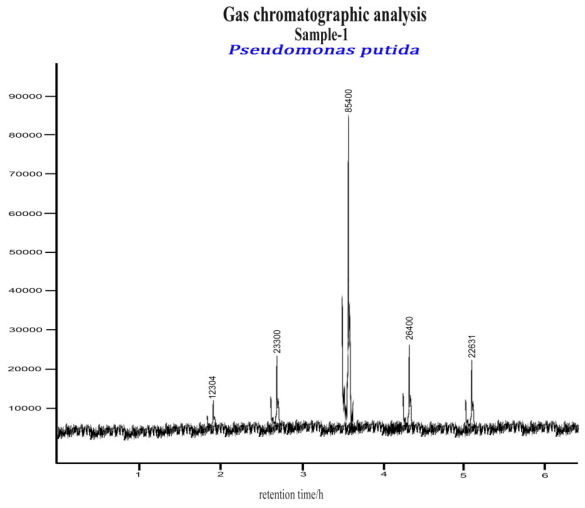
ported that *Pseudomonas species* degradation of hydrocarbon was achieved at 5% oil concentration. A fungal strain which showed high potentiality to adsorb and degrade crude petroleum oil has been isolated.

The oil degradation microbes flourish in soil medium where as the time interval is very high. In the present study the optimum growth of microbes in the medium is noted when the rate and percentage of biodegradation was improved during the increase incubation time. *Aspergillus niger* in the medium is noted when the oil concentration range from 2.5% to 5% for 10th day. *Pseudomonas putida* in the medium is noted when the oil concentration range from 0.5% to 2.5% for 5th day. Jan *et al.* (2001) reported that the fifth day of the maximum degradation was achieved at 5% (v/v) of petrol concentration for *Pseudomonas* species.

The result of the plasmid profile study shows absence of plasmid in *Pseudomonas putida* (pp-3) and *Aspergillus niger* which may also have efficiency to degrade oil and the result showed *Pseudomonas putida* (pp-3) 24kbp, 8kbp, 3kbp and 1.5kbp and the *Aspergillus niger* was 8kbp, 3kbp. This may increase the efficiency of oil degradation; plasmid frequency has been shown to increase in various hydrocarbons contaminated environments. Leonardo *et al.*, (2000) reported that some hydrocarbon degrading strains plasmids contain 44kbp, 28kbp, 13kbp and 6kbp, in such cases degradation coding genes are probably located in the chromosomal DNA.

The result of the protein profile of the isolates namely *Pseudomonas putida* and *Aspergillus niger* showed higher concentration of 120KD, 60KD, 40KD, 30KD and 10KD for *Pseudomonas putida* and 120KD, 60KD, 30KD, 17KD, 13KD and 11KD for *Aspergillus niger* respectively in nutrient media without hydrocarbon. This shows that protein encoded genes are responsible for hydrocarbon degradation in these organisms. A large number of *Pseudomonas sp.* has been isolated (110KD, 40KD, 20KD, 10KD) which are capable of utilizing petroleum hydrocarbons (Chakrabarth *et al.*, 1973).

The efficiency of hydrocarbon degradation through Gas chromatography (GC) analysis and the peaks reveals that maximum degradation was found in *Pseudomonas putida* (72.48%) when compared with *Aspergillus niger* (57%) (Figure 2). This GC spectral analysis gains solid support from the work of Raghavan and Vivekanandan, (1998). This GC spectral analysis showed that both the pollutants



were found to be decreased with increasing the concentration of *Pseudomonas* isolated from the field.

In the present investigation of the field application of soil, to support the hypothesis that the *Pseudomonas putida* and *Aspergillus niger* promotes the biodegradation of oil contaminated soil by increasing the composition of the microbial community. The symbiotic relationship between the soil microbes and *Pseudomonas putida* and *Aspergillus niger* may be responsible for the degradation of oil contaminates. The black gram sample with *Pseudomonas putida* worked best at removing hydrocarbon from the soil (Table 1). Paul A. Westhart (2005) supported this work, which reported on 10 pots of oil contaminated soil was prepared with tomato and alfalfa plants of *Pseudomonas putida*, to test

Table 1. Toxicity of soil samples (after the growth of plants)

S. No.	Name of the parameters	Sample details		
		Control	*P. putida*	*A. niger*
1	pH	8.91	7.01	7.44
2	Electrical conductivity (dsm^{-1})	0.24	0.29	0.27
3	Organic carbon (%)	0.94	0.32	0.42
4	Organic matter (%)	1.88	0.64	0.70
5	Total nitrogen (%)	1.26	0.74	0.74
6	Total phosphorus (%)	0.18	0.22	0.18
7	Total potassium (%)	0.82	0.91	0.86
8	Total zinc (ppm)	1.26	1.65	1.44
9	Total copper (ppm)	1.67	1.87	1.81
10	Total iron (ppm)	7.95	10.98	8.81
11	Total manganese (ppm)	4.23	5.89	4.76
12	Oil and grease (%)	58.94	0.16	1.82

on microbial community various time intervals. The symbiotic relationship between the soil microbes and in *Pseudomonas* may be responsible for the degradation of oil contaminates. The Alfalfa sample with *Pseudomonas putida* worked best at removing hydrocarbon from the soil.

Based on the above studies it appears that the oil degrading microbes depends on temperature, salinity, energy source, pH and oil concentration. If we are able to provide an environment with all these facilities then we can degrade the pollution of oil from the soil. These two indigenous microorganisms (*Pseudomonas putida* and *Aspergillus niger*) were capable to degrade the hydrocarbon. Especially *Pseudomonas putida* are capable to remove hydrocarbon such as petrol oil through oxidative pathway other than *Aspergillus niger*. The oil polluted soils cannot be utilized in agricultural activities; hence the degradation of oil in polluted soil will have positive impact in future in the field of agriculture.

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