Scholars Academic Journal of Biosciences (SAJB) **ISSN 2321-6883 (Online)**
Sch. Acad. J. Biosci., 2014; 2(3): 208-213 **ISSN 2347-9515 (Print)**

(An International Publisher for Academic and Scientific Resources)
www.saspublishers.com **DOI:** 10.36347/sajb.2014.v02i03.008

Research Article

Study of Adaptations in Bacterial Isolates Inhabiting Petroleum Contaminated Soil

Sanjeev Ranjan*, Sravani Pathula, Kapil Dixit, Glory Basumata, Rajesh Matcha
Orange Life Sciences Pvt Ltd., Gopalapatnam, Visakhapatnam-530027, Andhra Pradesh, India

***Corresponding author**
Sanjeev Ranjan
Email: sanjbt@gmail.com

Abstract: Oil spills and petroleum based pollutions are very common which cause a potential threat to both terrestrial as well as aquatic life. Bioremediation has become an alternative way to remedy. These microorganisms can degrade a wide range of target constituents present in oil sludge. An ability to isolate high numbers of certain oil degrading microorganisms from an environment is commonly taken as evidence that those microorganisms are the active degraders of that environment. A number of well-known microorganisms are responsible for the biodegradation of oil hydrocarbons. Bacteria have evolved regulatory systems that ensure the synthesis of enzymes so that the initial attack on these compounds is induced only when required. Some of these organisms have evolved an additional and highly effective system for responding to a variety of potential growth substrate. Few petroleum degrading bacteria were isolated from petroleum contaminated soil. The isolates were shown to be capable of degrading petrol at higher rates. Moreover they were very fast growing in petroleum contaminated soil as compared to non-indigenous bacteria belonging to the same type. The protein profile by SDS PAGE indicated presence of few new protein and also some critical modifications in some protein bands of isolated bacteria.
Keywords: Bioremediation , microorganisms, petroleum, Bacteria

INTRODUCTION

Environmental pollution with petroleum and petroleum products (complex mixture of hydrocarbons) has been recognized as one of the most serious current problems, hydrocarbons may reach the water table before becoming immobilized in the soil. They spread horizontally on the ground water surface and continue to partition into ground water, soil pore space, air and to the surface of soil particles. The vast range of substrates and metabolites present in hydrocarbon impacted soils surely provides an environment for the development of a quite complex microbial community [1]. Bioremediation has become an alternative way to remedy oil polluted sites, where the addition of specific microorganism (bacteria) or enhancement of microorganism already present can improve biodegradation efficiency [2]. These microorganisms can degrade a wide range of target constituents present in oil sludge [3, 4]. Petroleum products such as engine oil, petrol, diesel and kerosene are used daily in various forms in mechanic workshops. These products tend to harden and change the colour of the soil.

Crude oils are composed of mixtures of paraffin, alicylic and aromatic hydrocarbons. Microbial communities exposed to hydrocarbons become adapted, exhibiting selective enrichment and genetic changes resulting in increased proportions of hydrocarbon-degrading bacteria and bacterial plasmids encoding hydrocarbon catabolic genes [5]. Adapted microbial communities have higher proportions of hydrocarbon degraders that can respond to the presence of hydrocarbon pollutants. The measurement of biodegradation rates under favorable laboratory conditions using 14C-labelled hexadecane has led to the estimation that as much as 0.5 – 60 g oil/m^3 seawater convert to carbon dioxide, depending on temperature and mineral nutrient conditions. The principal forces limiting the biodegradation of polluting petroleum in the sea are the resistant and toxic components of oil itself, low water temperatures, scarcity of mineral nutrients (especially nitrogen and phosphorous), the exhaustion of dissolved oxygen and in previously unpolluted areas, the scarcity of hydrocarbon-degrading microorganisms [6].

Low winter temperature can limit rates of hydrocarbon biodegradation increasing resident time of oil pollutant [7]. Microbial degradation of oil has been shown to occur by attack on the aliphatic or light aromatic fractions of the oil. Although some studies have reported their removal at high rates under optimal conditions [8, 9], high molecular weight aromatics,

resins and asphaltenes are generally considered to be recalcitrant or exhibit only low rates of biodegradation.

In aquatic ecosystems, dispersion and emulsification of oil in slicks appear to be prerequisites for rapid biodegradation. Large masses of mousse, tar balls or high concentrations of oil in quiescent environments tend to persist because of the limited surface areas available for microbial activity. Petroleum and petroleum fractions containing asphalt components are not degraded quantitatively. The residues, along with polymerization products formed from free radical degradation intermediates with each other, forming tar globules. The tar is a partially oxygenated high molecular weight material resistant to further microbial degradation. Floating tar globules are encountered in the marine environment in increasing quantities [10].

An ability to isolate high numbers of certain oil degrading microorganisms from an environment is commonly taken as evidence that those microorganisms are the active degraders of that environment. A number of well-known microorganisms are responsible for the biodegradation of oil hydrocarbons. Bacteria have evolved regulatory systems that ensure the synthesis of enzymes so that the initial attack on these compounds is induced only when required. Thus, for an organism with the genetic information for utilizing benzene as carbon source, the enzyme for degrading benzene is induced when benzene reaches the bacterial environment. Some of these organisms have evolved an additional and highly effective system for responding to a variety of potential growth substrate. The essential genes of bacteria are carried on a single chromosome but genes specifying enzymes required for the catabolism of some of these unusual substrates may be carried on plasmids.

Oil spills and petroleum based pollutions are very common which cause a potential threat to both terrestrial as well as aquatic life. Moreover petroleum based pollution also include some xenobiotic compounds like long chain alkanes and other POPs (persistent organic pollutants). The study of petroleum degrading bacteria therefore becomes very important in tackling problems of oil and petroleum based pollutions. Keeping these facts in mind, the present study was intended towards following aims:

- To isolate & identify bacteria growing in petroleum contaminated soil.
- To test the petroleum degrading capability of these bacteria.
- To study the protein expression pattern in these bacteria in comparison to bacteria growing in normal conditions.

MATERIALS AND METHODS

Materials

Chemicals used for the present study were obtained from Merck India Ltd and Finar Chemicals, Glasswares were from Borosil and Qualigens, India except stated otherwise.

Pure cultures

Pure bacterial strains were purchased from MTCC, IMTech, Chandigarh.

Collection of samples

Top soil sample was collected from oil spilled area of an automobile repair shop located in Visakhapatnam. The location had no grass growing on them and the color of the soil was black. This soil is supposed to be rich in petroleum degrading bacteria. The sample was collected in a sterile plastic box and carried immediately to the laboratory.

Isolation & characterization of bacteria

The soil sample was subjected to 10 folds serial dilution, spread over petrol NA plates and incubated at 37°c for 24-48 hours. The isolated bacteria were then characterized by staining, colony characteristics, some biochemical tests and growth on few selective and differential media. The identified strains were isolated and cultured on agar plates and incubated for 24 hours to obtain the pure cultures. These cultures were further grown in L B medium.

Test for petroleum degradation

Basal salt agar plates were prepared. One set of plates was labeled as +ve control which were composed of glucose in them, another set was labeled as –ve controls which do not compose glucose and third set was labeled as tests. The both +ve and –ve controls were cultured with the five isolated strains separately by pour plate method. In all test plates two wells were punched for each after inoculating the cultures. One of the wells was filled with petroleum oil soaked cotton ball and the other with phenol soaked cotton. These plates were incubated for 48 hours under room temperature.

Study of growth under contaminated soil

Fresh top fine sieved soil (25 g each in two 150ml conical flasks for all the isolates) was sterilized by autoclaving for 20 min at 121^0C and 15 lb pressure and then keeping in hot air oven for 1 hr at 160^0C. 5 ml petroleum oil was added to each flask to contaminate them. 5 ml of bacterial consortia were added to each flask (one flask containing isolated bacteria and the other flask containing pure culture), mixed thoroughly and incubated. The growth of bacteria was studied by total viable count in each sample at an interval of 5 days for a period of 30 days.

These cultures grown in soil were cultured on the nutrient agar plates and were sub cultured. These were gram stained to re-confirm the cultures. These cultures were incubated for 48 hours for protein isolation.

Bacteria isolation and culturing

The soil cultured strains were isolated and cultured on agar plates and incubated for 24 hours to obtain the pure cultures. These cultures were further grown in LB broth medium.

Protein extraction/isolation

The proteins were extracted from the LB broth cultures of the isolated strains and their corresponding pure cultures by centrifugation. Simultaneously proteins from pure cultures of these strains were also extracted to make them run gel along with the isolated strains from polluted source.

48 hours broth culture was taken and centrifuged at 4000rpm for 15 minutes at 4°C. The pellet was washed three times with 10 mM TE buffer, p^H-7 for 10 minutes. The obtained pallet was suspended in 5 ml of ice cold acetone, vortexed and allowed to stand for 5-10 minutes in ice. This was centrifuged at 7000g, supernatant was discarded and pallet was air dried for ten minutes. The obtained pallet was vortexed in 1ml of samples were kept in boiling water bath at 90°C for 15 minutes this was done for the denaturing of proteins extracted. Later samples were centrifuged for 10 minutes at 4000rpm, supernatant was saved and pallet was discarded. The supernatant was run for electrophoresis. Before running gel these isolated proteins were denatured by heating them in hot water bath.

The sample was loaded in the well; the gel was run at 250V for 5 hours. After the run, stacking gel was removed and stained in silver stain and followed by destaining using a mixture of methanol, glacial acetic acid and water.

RESULTS & DISCUSSION

Isolation and characterization of bacteria

The five identified bacterial strains were as follows:

Table 1: Isolation and characterization of bacteria

Isolate no	Organism name
I	*Staphylococcus aureus*
V	*Streptococcus agalactis*
VI	*Streptococcus mutans*
VII	*Bacillus subtilis*

Test of petroleum degrading capability

All the –ve controls didn't show any growth. All the +ve controls showed good. In the test samples, the petroleum coated wells showed good growth but the phenol coated samples showed a zone of inhibition. This confirmed that the bacteria can digest petroleum oil but not phenol.

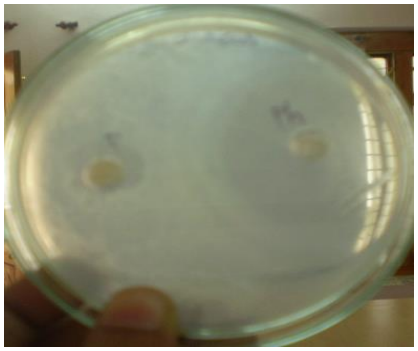

Fig. 1: zone of inhibition of *Staphylococcus aureus* (Isolate no. I)

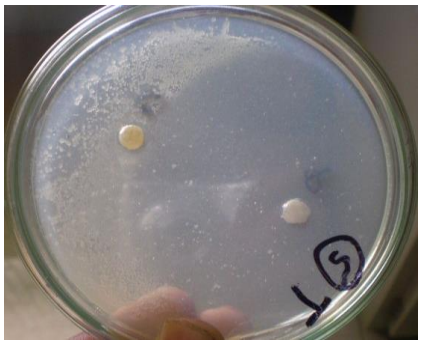

Fig. 2: zone of inhibition of *Streptococcus agalactis* (Isolate no. V)

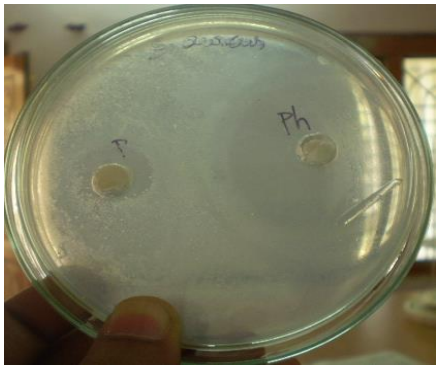

Fig. 3: zone of inhibition of ***Streptococcus mutans*** (Isolate no. **VI**)

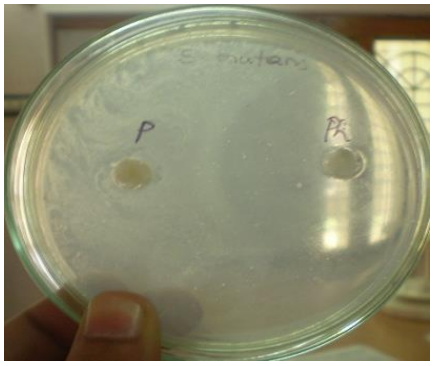

Fig. 4: zone of inhibition of *Bacillus subtilis* (Isolate no. VII)

Growth pattern in petroleum contaminated soil
For isolated bacterial consortium

Table 2: Day of incubation and Calculation organisms in contaminated soil/gm

Day of incubation	Calculation	Total no of organisms in contaminated soil/gm (TVC×10^3 (cfu/ml))
Day 0	27 X1000	27000
Day 5	8 X1000	8000
Day 10	5 X1000	5000
Day 15	23X1000	23000
Day 20	45X1000	45000
Day 25	56 X1000	56000
Day 30	59 X1000	59000

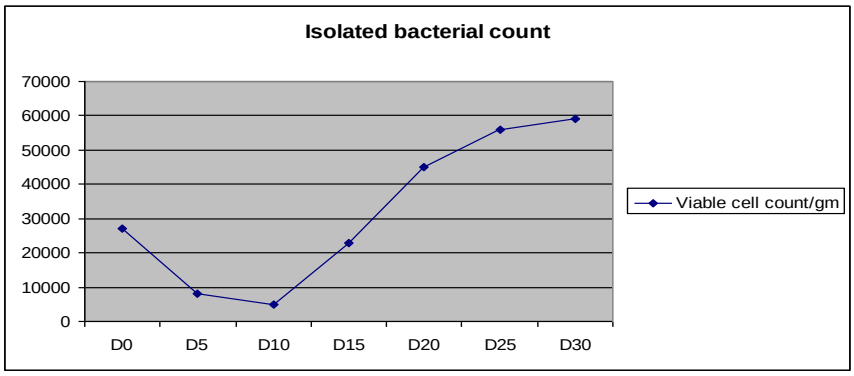

Fig. 5: Isolated bacterial count during day of incubation

For pure bacterial consortium

Table 3: Day of incubation and pure no of organisms in contaminated soil/gm

Day of incubation	Total no of organisms in contaminated soil/gm (TVC×10^3 (cfu/ml))
Day 0	27000
Day 5	8000
Day 10	6000
Day 15	5000
Day 20	2000
Day 25	2000
Day 30	2000

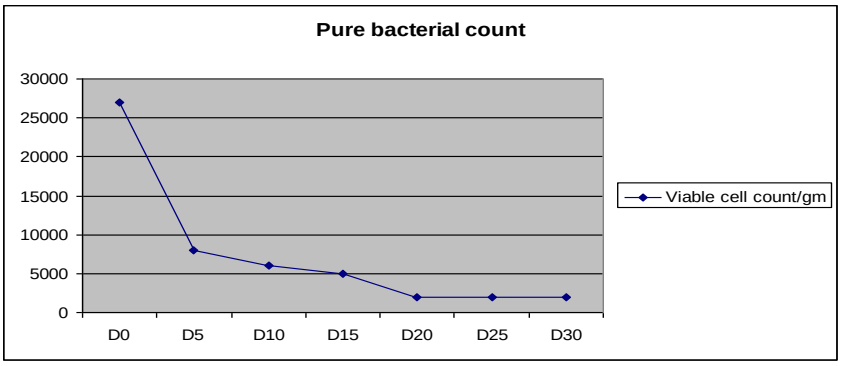

Fig. 6: Pure bacterial count during day of incubation

SDS-PAGE

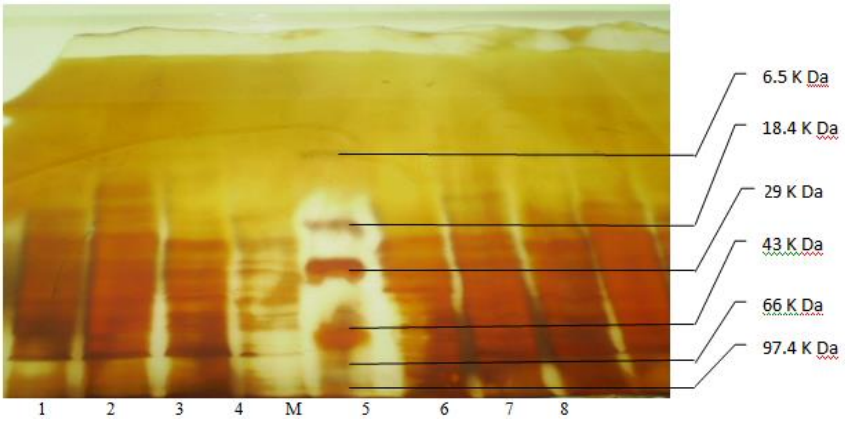

Fig. 7: SDS-PAGE bands

[Band 1: Pure culture of Staphylococcus aureus, Band 2: isolated culture of Staphylococcus aureus, Band 3: pure culture of Streptococcus agalactis, Band 4: isolated culture of Streptococcus agalactis, Band 5: pure culture of Streptococcus mutans, Band 6: isolated culture of Streptococcus mutans, Band 7: pure culture of Bacillus subtilis, Band 8: isolated culture of Bacillus subtilis, Band 8: isolated culture of Bacillus subtilis]

Molecular weights of isolated proteins in Pure and petroleum degrading bacteria

Table 1: Molecular weights of Pure and isolated petroleum degrading bacteria

Strains (Pure & Isolated cultures)	Proteins with modified molecular weights (KDa)	New protein found in isolated cultures (KDa)	Proteins present only in pure cultures (KDa)
Staphylococcus aureus (Pure)	47, 32		74, 72, 70
Staphylococcus aureus (Isolated)	48, 31	60, 35, 36, 35, 24	
Streptococcus agalactis (Pure)	38, 32		63, 62, 45, 40, 35, 28
Streptococcus agalactis (Isolated)	37 31	56, 47, 26	
Streptococcus mutans (Pure)	64, 62 46	50	
Streptococcus mutans (Isolated)	65, 63, 45		54, 44, 35, 30
Bacillus subtilis (Pure)	37		64, 63
Bacillus subtilis (Isolated)	38	44, 41, 39, 35, 27, 21	

CONCLUSION

Petroleum is a very common cause of pollution in both developed as well as developing countries like India. Coastal cities are always on a high risk of oil spill and related pollutions because of regular transport of petroleum and related products. The pollution of this type can contaminate both soil and water including ground water, hence making it unfit to consume. Also the soil and water of this type is inhabitable for plants and other aquatic lives, respectively. These contaminations are very difficult to treat and the process is time consuming. Moreover petroleum contains many xenobiotic compounds that are novel to biological systems and can not be digested by normal degradative microorganisms.

Selective bioremediation is therefore the best method considered for the above described problems. For this purpose, indigenous bacteria are considered the most fit. This is because bacteria are highly adaptive in nature and the bacteria growing in such polluted soil is supposed to be expressing few special proteins that help them survive in polluted environment.

In the present study, five different bacterial species identified were found to be degrading petroleum and were shown rapidly growing in oil contaminated soil under laboratory conditions. Also the protein expression studies showed the presence of few unique low molecular weight proteins that were found absent in bacteria growing in normal conditions. These special stress protein expressions can be further manipulated by using genetic engineering and other biotechnology approaches and an effective fighting strategy can be developed using, these bacteria, against petroleum contamination.

The following important conclusions can be made on the basis of the present study:

- The bacteria isolated were dominated by Gram positive cells and were capable of degrading petroleum.

- The bacterial consortium of isolated community showed initial decline in growth but then a sudden rise in growth was observed in petroleum contaminated soil inoculated with bacterial consortium.
- The pure culture consortium however showed a consistent decline and then stationary trend. This indicated that the indigenous bacteria are best for petroleum contamination treatment.
- New low molecular weight proteins were observed to be expressed in bacterial isolates and the same were absent in pure culture of bacteria. Few high molecular weight proteins were observed to be absent or change in their molecular weights were observed.
- Difference in expression levels were also observed in case of few proteins.
- The above listed information can be of extreme use for the purpose of bioremediation and study of adaptations in bacteria.

ACKNOWLEDGEMENTS

The authors thank Orange Life Sciences Pvt Ltd for providing all the infrastructure, literature and chemical support to complete the study.

REFERENCES

1. Butier CS, Mason JR; Structure function analysis of the bacteria aromatic ring hydroxylating dioxygenase. Advanc Microbiol Physiol., 1997; 38: 47-84
2. Hagwell IS, Delfino LM, Ras JJ; Partitioning of Polycyclic Aromatic Hydrocarbon from oil into water. Environ Sci Technol., 1992; 26: 2104-2110.
3. Barathi S, Vasudevan N; Utilization of petroleum hydrocarbons by pseudomonas flourescens isolated from petroleum contaminated soil. Environ Int., 2001; 26(5-6): 413-416.
4. Mishra SJ, Joyat RC, Kudad B; Evaluation of innoculum addition to stimulate in situ bioremediation of oily sludge contaminated soil. Appl Environ Microbiol., 2001; 67(4): 1675-1681.
5. Leahy JG, Colwell RR; Microbial degradation of hydrocarbons in the environment. Microb and Mol Bio Rev., 1990; 54(3): 305-315.
6. Atlas RM; Microbial Degradation of petroleum hydrocarbons: an experimental perspective; Microbiol Rev., 1981; 45(1): 180-209.
7. Bodennec GJ, Desmarquest P, Jensen B, Kantin R; Evolution of hydrocarbons and the activity of bacteria in marine sediments contaminated with discharge of petroleum. Int J Environ Anal Chem., 1987; 29:153-178.
8. Rotani JF, Bosser-Joulak E, Rambeloarisoa JE, Bertrand G, Fiusti G, Faure F; Analytical study of Ashart crude oil asphaltenes biodegradation. Chemosphere, 1985; 14: 1413-1422.
9. Shiaris LR, Dragun J; Seasonal biotransformation of naphthalene, phenanthrene, and benzoate [a] pyrene in surficial estuarine sediments. Appl Environ Microbiol., 1984; 55(6): 1391-1399.
10. Butler JN, Morris BF, Sass J; Pelagic tar from Bermuda and the Sargassosla. Bermuda Biological Station special publication No 10, Bermuda, 1973: 1-12.