ISOLATION AND SCREENING OF DIESEL OIL DEGRADING BACTERIA USING REDOX INDICATOR

T. SIVAGAMASUNDARI[1], Dr. N. JEYAKUMAR[2]

[1]Assistant Professor, Department of Microbiology and Biotechnology,
N.M.S.S.Vellaichamy Nadar College, Madurai – 625019. Tamilnadu. India.
[2]Assistant Professor, Department of Microbiology, Government Arts College, Surandai, Tamilnadu. India.

***ABSTRACT*: Petroleum hydrocarbons (PHC) represent one of the main ecological pollutants. The main causes of PHC pollution are Leaks and accidental spills occur regularly during the exploration, production, refining, transport, and storage of petroleum and petroleum products. Bioremediation is the biological process involving living microorganisms to remove contaminants or pollutants from soil or water. It is used to degrade toxic hydrocarbons into harmless products such as organic acids, aldehydes and ultimately carbon dioxide and water. The present study aims to, isolate and screen the diesel oil degrading bacteria from five different study localities like mechanic workshops in and around Madurai District. The concerned six isolates were subsequently confirmed as the degraders of the same of diesel oil in Bushnell Hass medium. Redox indicator 2, 6-dichlorophenol indophenol (DCPIP) was used to screen for efficient diesel oil degradation by bacteria. Degradation efficiency was measured by optical density at 540nm.**

***Keywords*: Petroleum hydrocarbon, Bioremediation, Redox indicator, DCPIP**

INTRODUCTION

Petroleum Hydrocarbons (PHC) are a combination of hydrocarbons obtained from reservoirs of crude petroleum. The petroleum hydrocarbons contain aliphatic and aromatic hydrocarbons. The most common petroleum hydrocarbons polluting environment are the gasoline, diesel and fuel oils. Hydrocarbon degraders may be expected to be isolated from petroleum oil associated environment. Petroleum hydrocarbon is composed of carbon and hydrogen. Many microorganisms have the ability to utilize petroleum as sole source of carbon as energy for metabolic activities, and these microorganisms are widely distributed in natural ecosystem. ZoBell (1946) reported that nearly 100 species of bacteria, representing 30 microbial genera had hydrocarbon oxidizing properties and many species have been found to have biodegradation ability and to be widely distributed in soils.

Oil contamination is one of the most important pollution factors known today. It can cause hazardous to the environment. It is very much threatened the environmentalists and very difficult to control. Oil contamination can be either at sea or soil. The most important aspect of environmental microbiology is the efficient management of hazardous and lethal pollutants by bioremediation.

Bioremediation involves the use of microbes to detoxify and humiliate environmental contaminants. It is used to degrade toxic hydrocarbons into harmless products such as organic acids, aldehydes and ultimately carbon dioxide and water (Atlas and Bartha, 1981).

Bacteria are the most important microbes in bioremediation process because they break the hydrocarbons into organic matter and nutrients. Biodegradation of hydrocarbons by environmental microorganisms having specialized metabolic abilities. In polluted environments, expertise microorganisms are abundant and they have the ability to utilize the hydrocarbon as a sole source of carbon energy (Marcelo *et al*., 2005).

MATERIALS AND METHODS

Isolation and Identification of Diesel oil Degrading Microorganisms

Collection of PHC contaminated soil samples

Petroleum hydrocarbon contaminated soil samples were collected from five different study localities like mechanic workshops in and around Madurai District. The samples were collected aseptically from the surface layer (5 - 30 cm in depth). Samples were then transported in an ice box to the laboratory under sterile condition for bacteriological analysis and stored at 5 - 10^0C for further studies. The bacteriological analysis was performed within 24 hrs of collection.

The diesel oil used in this experiment was purchased from a local oil filling station and stored in dark at ambient temperature throughout the study. The diesel oil was sterilized using 0.2µm membrane filter before use (Adam Sawadogo *et al*., 2016).

Enrichment of diesel oil degraders

For the enrichment of diesel degrading bacteria, 1g of oil polluted soil sample was inoculated onto Bushnell Haas (BH) medium (Bushnell and Haas, 1941) containing 1% (w/v) diesel as the sole carbon source and incubated at 37°C under shaking (180

rpm) for 7 days. After incubation, the culture medium was serially diluted and spreaded onto Mineral Salt Medium (MSM) agar plates with 1% Diesel oil. The plates were incubated for 48hrs at 37^0C. Microorganisms capable of degrading diesel oil were isolated from MSM supplemented with hydrocarbon compound as sole carbon source (1% of Diesel oil). Each bacterial isolates were sub cultured repeatedly in nutrient agar plates to obtain a pure culture (Anupama Mittal *et al*., 2009). Then the bacterial isolates were characterized based on cell morphology, cultural characteristics and biochemical characteristics according to Bergy's manual of systematic bacteriology (1994).

Determination of Total Heterotrophic Bacteria in the oil spilled soil samples using serial dilution agar plating method.

Total heterotrophic bacteria present in the soil samples were enumerated by Total viable count method. 5g of oil spilled soil samples were inoculated in 50 ml of nutrient broth and incubated at 37^0 C for 72 hrs. After incubation, the samples were serially diluted. One ml of the broth culture was suspended in 9ml of 0.85% (w/v) sterilized saline and vortexed for 1 min. This gives a 10 times dilution (10^{-1}dilution). The 0.1 ml of serially diluted samples from 10^{-1} to 10^{-6} was inoculated into sterilized nutrient agar plates labelled as 10^{-1} to 10^{-6} dilution. The plates were incubated at 37^0C for 24 hrs and an uninoculated agar plate was considered as control.

After incubation, the total number of bacterial colonies on the each plate were counted with the help of a Quebec colony counter and expressed as CFU/gm (Collins and Lynes, 1998). This was carried out in triplicates.

$$\text{CFU/gm} = \frac{(\text{Average number of colonies/plate})}{(\text{Dilution plated}) \times (\text{Volume plated in millilitres})}$$

The number of colonies present in the nutrient agar plates was calculated by the above mentioned formula and results were tabulated.

Determination of Hydrocarbon Degrading Bacteria from oil polluted Soil sample

The diesel oil (hydrocarbon) degrading bacteria were isolated using Enrichment culture technique. Five grams of the air dried oil polluted soil samples were transferred into 100ml conical flasks containing 50 ml of BH medium with 1 ml of filter sterilized diesel oil as carbon source and incubated in a shaking incubator with 150 rpm at 37^0C for 7 days. After seven days of incubation, 1ml of enriched culture were serially diluted up to 10^{-6} and inoculated onto sterilized Mineral Salt Medium (MSM) agar using spread plate method. The plates were incubated at 37^0C for 24 hrs. Distinct bacterial colonies were counted after incubation and sub-cultured on sterile nutrient agar slant for further studies. This was carried out in triplicates.

The number of colonies present in the MSM agar plates was calculated by the above mentioned formula (Collins and Lynes, 1998) and results were tabulated.

SCREENING AND SELECTION OF POTENTIAL BACTERIAL ISOLATES FOR PHC DEGRADATION

The bacterial isolates were inoculated into 7.5 ml of BH broth with 40 µl of redox indicator 2, 6 – dichlorophenol indophenol (DCPIP) and 50 µl of sterile diesel oil and incubated at 37^0C for 7 days (Hanson *et al*., 1993; Bidoia *et al*., 2010; Joshi *et al*., 2011). After 7 days of incubation, 5 ml of culture was taken. Centrifuged and supernatant was collected to measure the Optical Density (OD) at 540nm for biodegradation ability of the bacterial isolates (Selvakumar *et al*., 2014).

STATISTICAL ANALYSIS

Three replicates were used throughout the experiments and the mean values with standard deviation were calculated using Microsoft Excel 2013.

PRESERVATION AND MAINTENANCE OF THE STRAINS

The isolated strains were preserved in 25% v/v glycerol solution at -70^0 C. For day to day experimentation strains were maintained on nutrient agar slants at 4^0 C in refrigerator and sub cultured at an interval of thirty days (Anupama Mittal and Padma Singh, 2009).

RESULTS

Isolation of Diesel oil degrading bacteria

In this present study, there are five different diesel oil contaminated soil samples were collected for obtaining efficient diesel oil degrading bacterial strains. Bacterial species were isolated by spread plate method. Then pure cultures were isolated by streak plate method. There are ten bacterial isolates were identified by staining and biochemical tests. Among these isolates, six isolates have the potency to degrade diesel oil was isolated by primary screening (DCPIP method) based on optical density.

Determination of total heterotrophic bacteria in diesel oil contaminated soil samples

The heterotrophic bacteria present in the diesel oil contaminated soil samples were enumerated on nutrient agar medium by total viable count method. The range of total heterotrophic bacterial counts observed from 3.8×10^7 cfu/gm to 5.6×10^7 cfu/gm (Table 1).

Table 1: Total heterotrophic bacterial count on Nutrient Agar Medium

S.No.	Samples	Number of colonies 10^7 (cfu/gm)
1	Site A	5.6 ± 0.29
2	Site B	3.8 ± 0.13
3	Site C	4.8 ± 0.21
4	Site D	4.4 ± 0.17
5	Site E	4.5 ± 0.25

Determination of hydrocarbon degrader

The hydrocarbon degrading bacteria present in the oil contaminated soil samples were enumerated by viable count method on mineral salt agar medium. The range of total hydrocarbon degrading bacterial counts observed from 3.1 x 10^7 cfu/gm to 4.2 x 10^7 cfu/gm. (Table 2).

Table 2: Total Hydrocarbon degrading bacterial count on Mineral Salt Agar Medium

S.No.	Samples	Number of colonies 10^7 (cfu/gm)
1	Site A	4.0 ± 0.26
2	Site B	3.7 ± 0.20
3	Site C	4.2 ± 0.17
4	Site D	3.8 ± 0.15
5	Site E	3.1 ± 0.10

Screening of potential bacterial isolates for biodegradation of diesel oil

For primary screening, the isolates were grown in BH medium containing 1% diesel oil as sole carbon source and DCPIP. The growth of the potential bacterial isolates were observed based on the colour change from blue (oxidized) to colourless indicated that the ability of bacterial strains to utilize hydrocarbon substrate. The results were observed by optical density at 540nm (Table 3).

Table 3: Screening of potential bacterial isolates for biodegradation of PHC on BH medium with DCPIP.

S.No.	Strain No.	OD at 540 nm
1	S1	0.416 ± 0.12
2	S2	0.398 ± 0.15
3	S3	0.321 ± 0.21
4	S4	0.295 ± 0.19
5	S5	0.347 ± 0.17
6	S6	0.354 ± 0.13

DISCUSSION

In bioremediation process, isolation and screening of efficient hydrocarbon degrading microorganisms from diesel oil contaminated soil is the first step. Screening may be defined as the isolation and identification of microbes with high degrading ability among a large microbial population. It is also used to remove many valueless microorganisms, while at the same time used to select some proportion of useful microorganisms. Soil polluted with hydrocarbon (Diesel oil) is the clear choice to look for efficient strains.

By selective enrichment technique, the bacterial isolates were isolated from diesel oil contaminated soil. The culture enrichment method was adapted to isolate the hydrocarbon degrading bacterial strains for enrichment technique, Bushnell-Hass broth was supplemented with diesel oil as carbon source. 100ml of broth medium containing 1ml of diesel oil and 1gm of the soil contaminated with diesel oil was inoculated and incubated at 28^0C under shaking at 180 rpm for 7 days. After incubation, the enriched culture sample was serially diluted and transferred on to nutrient agar plates. After 48 hrs, the cultural characteristics were examined. Pure cultures were maintained by transferring representative colonies on nutrient agar slants as hydrocarbon degrading bacterial isolates (Udgire *et al*., 2015).

The bacterial isolates were subjected for the primary screening of potential isolates for petroleum hydrocarbon degradation. The degrading efficiency of isolated organisms was studied in BH medium supplemented with diesel and DCPIP. The experimental set up was incubated for 14 days in order to study the colour change of DCPIP, which is blue in colour and reduced to produce colourless form. This is due to reduction of the indicator by the oxidized product of hydrocarbon degradation which supports the facts that the isolates are potential hydrocarbon oxidizers (Selvakumar *et al*., 2014). Roy *et al*., 2002; Joshi and Pandey, 2011; Patil *et al*., 2012; Adegbola *et al*., 2014 reported that species of *Pseudomonas, Bacillus, Micrococcus* and *Proteus* isolated from hydrocarbon contaminated site and have been found to utilize hydrocarbon through oxidation of DCPIP. The oxidation of DCPIP supports the facts that the isolates were potential hydrocarbon degraders. Absorbance at a wavelength of 540 nm was monitored for the organisms because a peak in absorbance was observed at 540 nm as reported by Selvakumar *et al*., (2014).

CONCLUSION

Petroleum is a viscous liquid mixture that contains thousands of compounds mainly consisting of carbon and hydrogen. Microbes play a vital role in the weathering process microbial degradation is the major mechanism for the elimination of spilled oil from the environment. In this paper, petroleum tolerant and degrading bacterial were isolated and screened from diesel oil contaminated soil samples. Native predominant bacterial strains have more ability to degrade the diesel oil was proved by our study. Bushnel Hass medium serve as a significant growth media for determination of hydrocarbon degrading bacteria which provide all nutrient sources except carbon source, by our study diesel oil used as a sole source of carbon. A primary screening was performed to assess the indicator dye (2, 6-DCPIP) decolourization efficiency of selected strains for confirmation of diesel oil biodegradation.

REFERENCE

[1] Adama Sawadogo, Harmonie, C, Otoidobiga, Leon Nitiema, Alfred Traore & Dayeri Dianou, 2016, 'Optimization of Hydrocarbons Biodegradation by Bacterial strains isolated from wastewaters in Ouagadougou, Burkina Faso: Case study of SAE 40/50 used oils and Diesel', Journal of Agricultural Chemistry and Environment, Scientific Research Publishing, vol. 5, no.1, pp.1 – 11.

[2] Adegbola, GM, Eniola, KIT & Opasola, OA, 2014, 'Isolation and identification of indigenous hydrocarbon tolerant bacteria from soil contaminated with used engine oil in Ogbomoso, Nigeria', Advances in Applied Science Research, vol. 5, no. 3, pp. 420 - 422.

[3] Anupama Mittal & Padma singh, 2009, 'Isolation of hydrocarbon degrading bacteria from soils contaminated with crude oil spills', Indian Journal of Experimental Biology, vol.47, pp. 760 - 765.

[4] Atlas, RM & Bartha, R 1981, 'Microbial Ecology Fundamentals and Applications', Philippines: Addison-Wesley.

[5] Bergey, DH, John, G & Holt, 1994, Bergey's manual of determinative bacteriology, Baltimore: Williams & Wilkins, 9th Edition.

[6] Bidoia, Montagnolli & Lopes, 2010, 'Microbial biodegradation potential of hydrocarbons evaluated by colorimetric technique: a case study', Current Research, Technlology and Education Topics in Applied Microbiology and Microbial Biotechnology, pp. 1277 - 1288.

[7] Bushnell, LD & Haas, HF 1941, 'The Utilization of Certain Hydrocarbons by Microorganisms', Journal of Bacteriology, 1941, vol.41, No.5, pp. 653 – 673.

[8] Collins & Lyne's, 'Microbiological Methods', Eighth edition, 1998, Oxford University Press Inc.,

[9] Hanson, KG, Desai, JD & Desai, AJ, 1993, 'A Rapid and Simple Screening technique for potential crude oil Degrading microorganisms', Biotechnology Techniques, vol. 7, issue. 10, pp. 745 - 748.

[10] Joshi, RA, & Pandey, GB, 2011, 'Screening of petroleum degrading bacteria from cow dung', Research Journal of Agricultural Sciences, vol. 2, pp. 69 - 71.

[11] Marcelo Henrique Otenio1, Maria Teresa Lopes da Silva, Maria Luiza Oliveira Marques, José Carlos Roseiro & Ederio Dino Bidoia, 2005, 'Benzene, toluene and xylene biodegradation by *pseudomonas putida* CCMI 852', Brazilian Journal of Microbiology, vol. 36, pp. 258 - 261.

[12] Patil, TD, Pawar, S, Kamble, PN, & Thakare, SV, 2012, 'Bioremediation of complex hydrocarbons using microbial consortium isolated from diesel oil polluted soil', Pelagia Research Library, Der Chemica Sinica, vol. 3, no. 4, pp. 953 - 958.

[13] Roy, Hens, Biswas & Kumar, 2002, 'Survey of petroleumdegrading bacteria in coastal waters of Sunderban Biosphere Reserve', World Journal of Microbiology and Biotechnology, vol. 18, pp. 575 - 581.

[14] Selvakumar, S, Sekar, P, Rajakumar, S & Ayyasamy, PM, 2014, 'Rapid screening of crude oil degrading bacteria isolated from oil contaminated areas', The Scitech Journal , vol. 1, pp. 24 - 27.

[15] Udgire, M., Shah, N & Jadha, 2015, 'Enrichment, Isolation and Identification of Hydrocarbon Degrading Bacteria' International Journal of Curent Microbiology and Applied Sciences, vol. 4, no.6, pp.708 - 713.

[16] ZoBell, CE, 1946, 'Action of microörganisms on hydrocarbons', Bacteriology Review, vol. 10, pp. 1 - 49.