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Isolation of Oil Degrading Strains from Soil Samples

Mrs. Pritha Ghosh[1], J. Deepa[2], R.S.Shravani[3], Maria Angelina[4], Celestina[5]
[1]*Lecturer, St. Pious X Degree & PG College for Women, Nacharam, Hyderabad.*
[5]*St.Pious X Degree & PG College for Women, Nacharam, Hyderabad.*

Abstract: ***A study was conducted to identify the micro organisms present in oil contaminated soil samples from different service stations and gas stations in Hyderabad. Ten fold serial dilutions of the samples were done with subsequent spreading on basal nutrient agar medium and incubated at 37°C for 24 hours. Bacterial isolates obtained were maintained in pure culture. Further, the isolates were cultured on an enrichment medium Bushnell Hass agar to narrow down the type of organisms capable of using the hydrocarbons as the carbon source for oil degradation. Determination of oil degradation by the isolates was done by DCPIP reagent and the variation in color change of the medium was observed. Morphological and biochemical identification revealed to be Bacillus and Pseudomonas.***

Keywords: Oil contaminated soil samples, Basal nutrient agar medium, Bushnell Hass agar, Oil degradation, DCPIP reagent, Bacillus, Pseudomonas, Bioremediators.

I. INTRODUCTION

Oil spillage and oil pollution in water environment have been a major threat to the ecosystem and human being through the transfer of toxic organic materials including polycyclic aromatic hydrocarbons (PAHs) into the food chain (Sei A. and Fathepure B. Z., 2009).With continuous increase in the industrial development there occurs a serious environmental pollution. Environmental contamination by petrol derivatives has been a subject of study over the past four decades. The leakage of these derivative oils, such as lubricant oils, is capable of harming the environment in many ways (Atlas, 1995). Thus, in order to prevent the hazardous effects of oil pollution control and treatment strategies are required. However, conventional physical-chemical treatments are very costly when the contamination is high and can produce toxic residues to the environment. Adapting “bioremediation” process which is highly efficient, less costly, and easy way of regaining the contaminated site as compare to other physiochemical processes (Bhupathiraju *et al.*, 2002). The process of bioremediation, defined as the use of microorganisms to detoxify or remove pollutants owing to their diverse metabolic capabilities is an evolving method for the removal and degradation of many environmental pollutants including the products of petroleum industry. In addition, bioremediation technology is believed to be noninvasive and relatively cost-effective (Nilanjana Das and Preethy Chandran, 2011)Bioremediation is a modern method in which the natural ability of microorganisms is employed for the reduction of the concentration and/or toxicity of various chemical substances, such as petroleum derivatives, aliphatic derivatives, aliphatic and aromatic hydrocarbons, industrial solvents, etc. A number of microbial agents (bacteria and fungi) which are capable of biodegrading petroleum and its derivatives have been identified (Atlas and Bartha, 1972; 1993). Fungi are an important component of the soil micro biota. (Ainsworth, 1995).N Environmental contamination by petroleum and its derivatives is a serious problem worldwide. Considerable amounts of hydrocarbons have been released into water and soil as a result of pipeline leaks, transport accidents, and storage tank ruptures. Apart from incidental contamination of the ecosystem, vast amounts of oily sludge generated in water–oil separation systems at oilfields and accumulation of waste oily materials in crude-oil storage tanks also causes problems to the environment. Many compounds of oily sludge are toxic, mutagenic and carcinogenic. Therefore, they are classified as priority environmental pollutants by the US Environmental Protection Agency (Liu *et. al.*, 2010). Microbial remediation of a hydrocarbon–contaminated site is accomplished with the help of a diverse group of microorganisms, particularly the indigenous bacteria present in soil. (Mandri, T. & Lin, J., 2007). At present, bioremediation (use of microorganisms to remove pollutants) is often the most suitable method for remediation of especially petroleum hydrocarbons, because it is cost effective and it converts the petroleum hydrocarbons into the harmless by-products such as carbon dioxide and water. (Prakash B, Irfan M 2011). Bioremediation makes use of indigenous oil–consuming microorganisms, called petrophiles, by enhancing and fertilizing them in their natural habitats. Petrophiles are very unique organisms that can naturally degrade large hydrocarbons and utilize then as a food source. Microorganisms degrade these compounds by using enzymes in their metabolism and can be useful in cleaning up contaminated sites. (Mandri, T. & Lin, J., 2007).

Petroleum-contaminated soil is currently treated using three methods: physical, chemical, and biological. Physical or chemical methods include incineration, chlorination, ozonation, and combustion. (Ting *et al.* 1999). Many of these technologies, however, are either costly or do not completely remove contaminants. On the other hand, biological treatment (bioremediation) appears to be among the most promising methods for dealing with a wide range of organic contaminants, particularly petroleum hydrocarbons. The technology is also environmentally sound since it simulates natural processes and can result in the complete destruction of hazardous compounds into innocuous products. (Balba *et al.* 1998)The biodegradation of crude oil by microorganisms is one of the primary ways to remove crude oil from contaminated area. It has been studied that bacterium that grows in oil contaminated soil are much capable of degrading oil when compare with those bacteria which are found on non-contaminated soil. The natural biodegradation process can be enhanced by addition of nutrients and optimizing the growth parameters. In order to remediate the crude oil pollution, crude oil biodegradation is necessary to isolate and characterize unique microbial species for evolution of their eficacy in utilization of crude oil before application of the contaminated sites. (Selvakumar S. *et. al.,* 2014).Bacteria are the most active agents in petroleum degradation, and they work as primary degraders of spilled oil in environment (Rahman, K. S. *et. al.,* 2003), (Brooijmans, R. J. W, *et. al.,* 2009).Several bacteria are even known tofeed exclusively on hydrocarbons(Yakimov, M. *et. al.,* 2007). The persistence of petroleum pollution depends on the quantity and characteristics of hydrocarbon mixture and on the properties of the affected ecosystem. The ability to isolate high numbers of certain oil degrading microorganisms from oil polluted environment is commonly taken as evidence that these microorganisms are active degraders in that environment. (Darsa, K.V. *et. al.,* (2014).The purpose of this studywas to isolate and identify the organisms from soils near petrol and diesel pumps, servicing stations, oil laden mud to isolate and detect the presence of oil degrading organisms present in the above said environments.

II. MATERIALS AND METHODS

A. *Sample Collection*

Four types of samples were used to isolate the hydrocarbon degrading bacteria were collected from petrol and diesel pumps, servicing stations, oil laden mud. These samples were stored in sterile zipper bags and maintained at 4°c for microbial analysis.

B. *Isolation of Bacterial Cultures*

Bacterial species were isolated from the collected soil samples by10 fold serial dilution and agar plating method. The inoculated plates were incubated at 37° C for 24 hours. The isolates were sub cultured and the pure cultures were stored at 4^{o} C and preserved for further analysis.

C. *Enrichment of Hydrocarbon Degrading Bacteria*

Bushnell- Hass(BH) medium was used as enrichment media supplemented with 2% of engine oil and petrol as a sole carbon source. The BH broth contained Magnesium Sulfate 0.2g/l, Calcium Chloride 0.02g/l, Monopotassium Phosphate 1.0g/l, Dipotassium Phosphate 1.0g/l, Ammonium Nitrate 1.0g/l and Ferric Chloride 0.05g/l, Agar-Agar 20g and Distilled water 1000ml. (Arpita Gupte, Sheetal Sonawdekar, 2015). BH medium was found to be more suitable for the growth of the isolates and therefore used as enrichment media for the growth of hydrocarbon degrading organisms.

D. *Identification of Hydrocarbon Degrading Organisms*

The bacterial isolated were identified based on morphological and biochemical tests according to Bergey's Manual of determinative Bacteriology(Buchana RE and Gibbons NE 1974).

E. *Determining the ability of Hydrocarbon Degrading (HDO) Organisms*

Screening of HDB was carried out by method using DCPIP as redox indicator. During the microbial oxidation of hydrocarbons, electrons are transferred to electron acceptors DCPIP to the culture medium, and it is possible to ascertain the ability of the microorganism to utilize hydrocarbons by observing the colour change from blue(oxidized) to colorless (reduced), which is monitored at 600 nm wavelength. (Varjani Sunita J. *et. al.,* 2013).

III. RESULTS

A. *Isolation of Bacterial Cultures*

The isolates were obtained on nutrient agar by serial dilution technique. A total of 36 colonies were isolated. The colonies were sub cultured and preserved as pure cultures for further investigation.

B. Enrichment of Hydrocarbon Degrading Bacteria

Bushnell- Hass (BH) medium was used as enrichment media supplemented with 1% of engine oil and petrol as a sole carbon source BH medium was found to be more suitable for the growth of the isolates as out of 36 isolates 9 isolates showed positive growth on BH medium out of which two isolates were taken into consideration for identification studies as they exhibited good growth characteristics.

C. Identification of Hydrocarbon Degrading Bacteria

Preliminary identification of the gram nature of the nine isolates revealed seven as gram negative and two as gram positive. The two bacterial isolates were identified based on morphological and biochemical tests according to Bergey's Manual of determinative Bacteriology (Buchana RE and Gibbons NE 1974)and were found to be as Bacillus and Pseudomonas species

D. Determining the true ability of Hydrocarbon Degrading (HDO) Bacteria

Screening of HDB was carried out by method using DCPIP as redox indicator. Significant change in the colour of the medium was observed in the flask inoculated with the two bacterial isolates. In the present research work the bacterial isolates efficiently utilized the hydrocarbons that resulted in the colour change from blue(oxidized) to colorless (reduced), which was monitored at 600 nm wavelength. (Varjani Sunita J. et. al., 2013).

IV. DISCUSSION

Removal of wide range of pollutants from the natural environment is an absolute requirement to enhance a sustainable development of our society with low ecological impacts. Applications of microorganisms play a major role in the removal of contaminants and they take advantage of the astonishing catabolic versatility of micro organisms to degrade or convert such compound. Among the micro organisms bacteria are usually the choice because, they have more rapid metabolic rate, numerous metabolic pathways of various organic pollutants have been determined in bacteria. Bacteria can be genetically manipulated to improve their bioremediation capabilities. Bioremediation is a process that uses naturally occurring microorganisms to transform harmful substances to non toxic compounds. Bioremediation exploits this natural process by promoting the growth of microbes that can effectively degrade specific contaminants (Selvakumar S et. al., P.M. 2014)Bacteria are the most active agents in petroleum degradation, and they work as primary degraders of spilled oil in environment. Several bacteria are even known to feed exclusively on hydrocarbons Remediation of petroleum contaminated sites could be achieved by either physicochemical or biological methods. Due to negative consequences of the physicochemical approach, more attention is now given to the exploitation of biological alternatives (Okoh, 2006). A strategy for screening of potential hydrocarbon utilizers / degraders involves sampling, isolation and screening of strains. Several methods of isolating and enumerating petroleum utilizing / degrading bacteria have been reported. (Varjani Sunita et. al., 2013).Hanson et al., (1993) and Biodia et al.,(2010) have reported a number of techniques evolved for screening of hydrocarbon degrading bacteria such as the use of liquid medium with hydrocarbons, oil containing mineral agar, measurement of turbidity in microtiter plates, O_2 consumption, MPN technique and colorimetric method. Colorimetric methods are referred as cost- effective and rapid in detecting microbial metabolism, occurrence, both in aerobic or anaerobic instances. These catalytic microbial based methods are rapid, in which the natural co-substrate (which can be oxygen, sulfates or nitrates) is substituted by a synthetic mediator. Alternative means of determining when reducing conditions exist are provided from redox indicators. (Bidoia et al.,2010).Low molecular weight hydrocarbons were found to be removed most quickly due to abiotic factors, while the removal of intermediate molecular weight hydrocarbons was attributed to both biotic and abiotic factors. Losses in the higher molecular weight hydrocarbons were mainly due to biodegradation. For screening of crude oil degrading bacteria, DCPIP redox indicator colorimetric technique provide rapid, easy and low cost method for detecting microbial metabolism from carbon and other nutrients sources including hydrocarbons. To ascertain microbial ability to utilize hydrocarbon substrate by simple observing the color change of DCPIP in which the quickest decolourization time represents the best oil biodegradation is a major breakthrough in biodegradation studies (Bidoia et al., 2010). In our study out of the thirty six isolates, nine exhibited growth on BH medium giving an indication for their possible role in hydrocarbon degradation. To confirm and determine their utilization or degradative capabilities DCPIP as redox indicator was used which clearly revealed their above said ability. This feature can also be explained in terms of nutrient availability because nutrient source serves as an indispensable factor for the growth and survival of microorganisms. In our case engine oil and petrol served as the sole carbon source and can be considered as one of the important factors governing the utilization or degradative capabilities of the selected isolates.

The other factor triggering the degradation ability is the degree of saturation. Oil laden mud and soils near petrol and diesel pumps, servicing stations have varied proportions of complex hydrocarbons exposed to environmental conditions which also affect the structural and functional integrity of the oils and hydrocarbons. Microorganisms are versatile in terms of metabolism. They exhibit varied biochemical function. The complex hydrocarbon degrading microorganisms play an important role and microbe aided bioremediation and allied technologies are widely used to clear and minimize hydrocarbon pollution. Increase in oil degradation is directly proportional to an increase in cell count indicating that bacterial isolates were capable for oil degradation (Rahman et. al., 2002; Mandri et. al., 2007).Several bacterial species have been identified as having the ability for oil degradation. In general, Bacillus genera have been identified as petroleum hydrocarbon degrader. The ability of Bacillus species in hydrocarbon degradation has consistently been observed and associated competent hydrocarbon enzyme system of the organism and its ability to form spores(Darsa, K.V. et. al., (2014). Pseudomonads are the best known bacteria capable of utilizing hydrocarbons as carbon and energy sources(Nilanjana Das and Preethy Chandran, 2011).In the present investigation also, the above features were observed thus correlating with the literature available. By this preliminary study it was found that the isolates had the potential to degrade the hydrocarbons. This study gives us an insight that the organisms can be used as potential bioremediations. Thus the cleanup approach mediated by microbial degradation, added to the use of native organisms and specifically exploiting their metabolic capabilities can serve as an important component in for hydrocarbon degradation.

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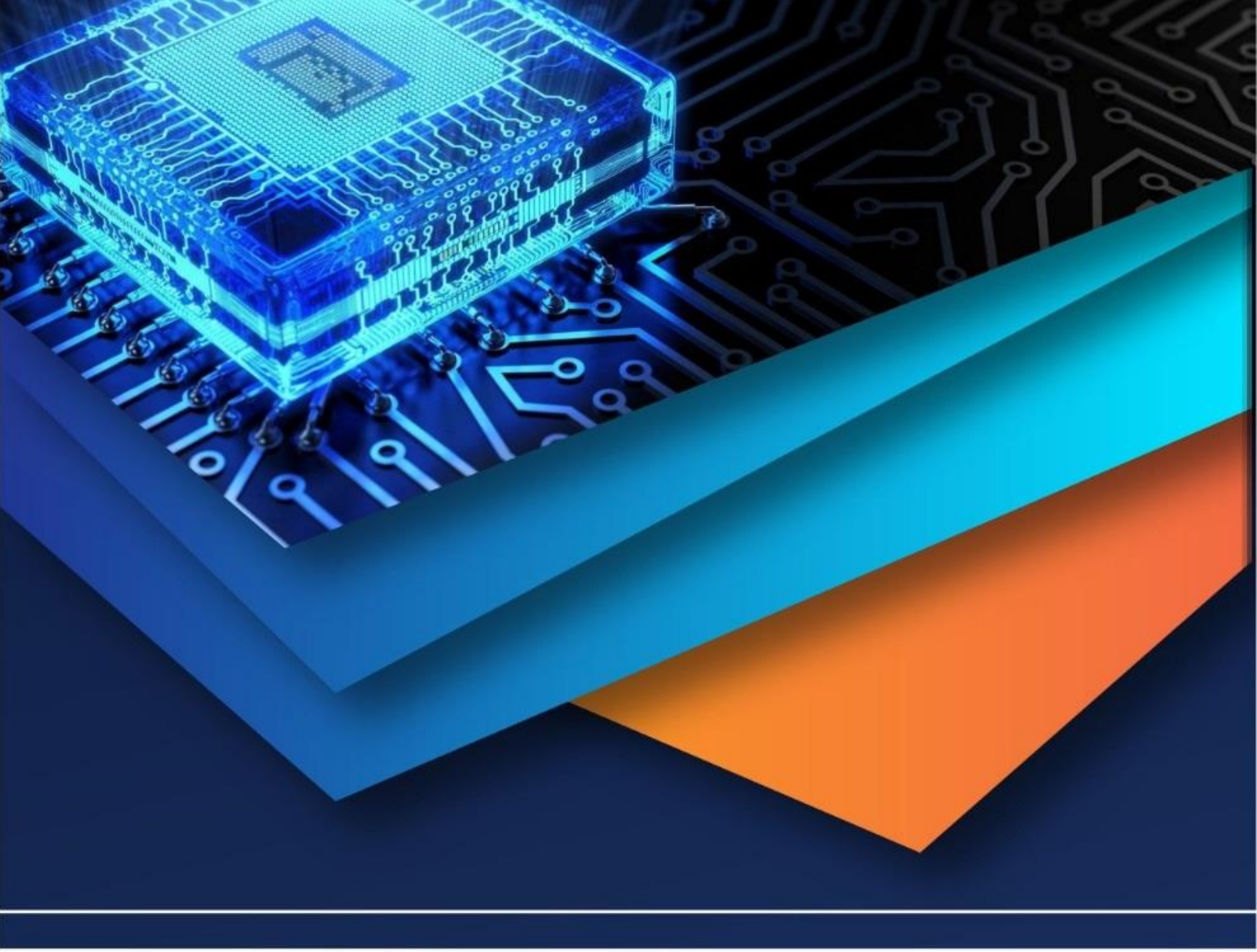

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