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Genetic diversity of microorganisms capable of degrading diesel as a pool of bioremediation

Snehita Chauhan✉, Manjula Saini, Neeraj Dubey and Ram Saran Chaurasiya

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Abstract

The ability to degrade the diesel by bacterial species (*Micrococcus* spp., *Staphylococcus* spp., and *Celibiosococcus* spp.) as a sole source of hydrocarbon was studied in the present work. In laboratory by identifying and assessing the potential of hydrocarbon degrader by micro titer plate method, the best diesel degrading bacteria was isolated. The similarity trait among the 25 bacterial isolates was also assessed. All the bacterial species utilized the hydrocarbons as sole carbon and energy sources showing increases in cell number and optical density with decreases in pH of the culture media. This study shown that *Micrococcus spp*., has maximum ability to degrade diesel. The banding pattern of RAPD showed that all the isolates share some similarity between them RAPD was chosen as it has been shown to be effective sub typing method for several other species.

Keywords: Bioremediation, bacterial diversity, RAPD, diesel degrading bacteria, Micrococcus.

Introduction

Microbial degradation of petroleum hydrocarbons is one of the major practices in natural decontamination process (Vinothini et al., 2015). Petroleum is a complex mixture of hydrocarbons and it is derived diesel composed of about 75% of saturated hydrocarbons (primarily paraffins including n, iso and cycloparaffins and 25% aromatic hydrocarbon (including naphthalenes and alkylbenzenes). The average chemical formula for common diesel is $C_{12}H_{33,}$ ranging from approx $C_{10}H_{20}$ to $C_{15}H_{28}$ (Riser-Roberts, 1992). Accidental releases of petroleum products are of particular concern in the environment and this has led to a concerted effort in studying the viability of using oil-degrading microorganisms for bioremediation (Sebiomo et al., 2010). Oil spills especially, soil contamination has prompted research on cost-effective, environmentally benign clean up strategies (Margesin and Schinner, 2001). Bioremediation functions basically on biodegradation, may refer to complete mineralization of organic contaminants into Carbon dioxide, water, inorganic compounds, and cell protein or transformation of complex organic contaminants to other simpler organic compounds by biological agents like microorganisms (Nilanjana and Preethy, 2010). Microbial degradation appears to be the most environmental friendly method of removal of oil pollutant since other methods such as surfactant, washing and incineration lead to introduction of more toxic compounds to the environment (Oboh et al., 2006).

The genetic diversity of soil microorganisms is an indicator of the genetic resource, which is the basis of all actual and potential functions. Genetic diversity of bacteria is most commonly studied by diversity of the 16S rDNA genes, which occur in all bacteria and which shows variation in base composition among species. 16S rDNA genes are thus used for phylogenetic affiliation of eubacteria and archaea and large database exist on sequences of 16S rDNA. It consists of variable and conserved regions, and this has facilitated the design of primers in the conserved regions for targeting the majority of members of defined groups of bacteria (Heller and Smalla 1997; Johnsen et al., 2001). Genomic fingerprinting assays using Random amplification polymorphism DNA are excellent technologies for differentiating and tracking Specific genetic elements within a complex genome or genomes (Roberts and Crawford, 2000).

Author's Address
PGT-Biology, Stepping Stone, ICSE Board School, Gorakhpur
Department of Zoology, Career College, Bhopal
E-mail: snehita.chouhan@gmail.com

This study was aimed at assessing the microbes degrading diesel and its similarity trait among them. Thus, keeping a hypothesis in mind the possible similarity and dissimilarity trait of biodegrading microorganism and made it possible by performing RAPD technique.

Material and Methods

Sampling

Sampling was done from different region of Satna, Madhya Pradesh. And selection of site was done on the basis of heavily contaminated diesel soil. Freshly collected soil samples were collected from four different locations from different depths, with the help of sterilized instrument. The soil samples were brought to laboratory under ice cold conditions.

Soil microbial counts

Heterotrophic plate count (HPC) was done by standard pour plate dilution agar technique using Bushnell Haas (BH) medium.

Identification of bacterial isolates (Mac Faddin, 1980)

Identification of bacterial isolates was on the basis of morphological, cultural and biochemical characteristic with the help of the Bergey's Manual of Systematic Bacteriology (Krieg and Holt, 1984) and Probabilistic Identification of Bacteria (PIB) computer software (Bryant, 2003).

Diesel Degrading Capability of microorganism by microtiter plate count method (Medhi and Giti, 2008; Bento et al., 2005)

Microtiter plate assay was used to screen out diesel degrading bacterial isolates and analyzed with the help of ELISA reader (Thermoelectron corporation multiskan Ex.) at 450 nm using a UV spectrophotometer (Systronics, model no. 118).

Assessment of potential of screened bacterial species (Whyte et al., 1998)

To determine the range of alkanes utilized by bacterial isolates, the viable count of the organisms on different concentration of diesel fuel, containing 1-10% of diesel oil as well as pH were observed spectrophotometrically at 660 nm using a UV spectrophotometer (Systronics, model no. 118).

Molecular characterization of bacterial isolates RAPD (Random amplified polymorphic DNA)

RAPD finger printing was done for accessing the diversity in the bacterial isolates on the basis of polymorphism. The major steps involved are:

Template DNA preparation (Van Soolinger and Hermans, 1995)

The isolation of DNA was done by phenol extraction method.

PCR Amplification

PCR amplification of isolated template DNA was carried out by using primers according to the method described by Williams et al., 1990. The thermal cycle profile was as follows: 4 min initial denaturation at 95ºC, 44 cycles of 1 min at 92ºC, 1 min at 30ºC, 1 min at 72ºC, followed by a final extension at 72ºC for 10 min.

Electrophoresis and pattern analysis

The aliquots (8 µl) of amplified products were loaded in 2% agarose gel stained with 0.5 µg/ml ethidium bromide and photographed under UV light in a transilluminator (Biotech, India). A 1 Kb DNA ladder (Bangalore Genei, India), which were depicted in a dendogram.

Primer sequence used in RADP analysis (El-Hanafy et al., 2007)

EZA1A13 – 5' CAG GCC CTT GCA GCA CCCAC 3'

Results and Discussion

Identification of bacterial isolates was on the basis of morphological and biochemical characterization obtained from different sites revealed the following genera: *Micrococcus varians, M. agilis, M. mucilaginosis, Staphylococcus saprophyticus, S. epidermidis, Celibiosococcus spp., Streptococcus sanguis.* All the isolates were non-motile, cocci shaped and gram positive. Most were Voges prouskauer positive, urease and citrate utilizers. Out of 25 isolates, 5 isolates with highest Optical Density value were screened out. These isolates were *Stapylococcus xylosus*, *Micrococcus agilis*, *Micrococcus varians*, *Staphylococcus saprophyticus*-3 and *Celibiosococcus* spp.

Diesel hydrocarbon utilization potential of five bacterial isolates was measured at different concentrations (1 to 10%) of diesel in the medium. *Staphylococcus xylosus, Staphylococcus saprophyticus* and *Celibisococcus* spp. showed maximum growth on 1% diesel oil while

Micrococcus agilis and *Micrococcus varians* on 8% diesel oil.The results shown that *Micrococcus agilis* could utilize a higher percentage of diesel oil while high percentage of diesel decreased the growth of all the other isolate The pH change in the culture growing medium was also studied for each isolates. The results showed pH decrease from 7.2 to 6.29.

Random amplified polymorphic DNA analysis revealed that isolates produced 7 different banding patterns with no. of bands ranging from 100 bp to 1000 base pair. All the isolates were analyzed by unweight pair group method with arithmetic mean (UPGMA) on the basis of RAPD analysis to know the similarity/distance among them. Microbial diversity offers an immense field of environment friendly options for mineralization of contaminants or their transformation into less harmful non-hazardous compounds. There is a general interest in studying the diversity if indigenous microorganisms capable of degrading different pollutants because of their varied effects on environment. RAPD is largely used for genetic variability analysis. The banding pattern of RAPD showed that all the isolates share some similarity between them RAPD was chosen as it has been shown to be effective sub typing method for several other species (Roberts and Crawford, 2000; Sharma et al., 2008). Bacteria show tremendous diversity and adaptability in utilization of different organic molecules as a carbon source however their abilities to degrade a specific hydrocarbon as a source of energy and/or biomass may differ. Since all the bacteria in the present study was isolated from a petroleum contaminated oil site, some of them survived and adapted the oil-contaminated solid/liquid environment very easily as also reported by other authors (Rahman et al., 2003; Das and Mukherjii, 2007). Twenty five bacterial isolates were obtained from diesel contaminated soil samples. The predominant flora was composed of *Micrococcus* spp., *Staphylococcus* spp., and *Celibiosococcus* spp. Bacteria belonging to these genera have been described as petroleum degraders or even as hydrocarbon degraders by (Marin et al., 1996, Chauhan and Chaurasiya, 2012). Soil bacterial diversity, as estimated by phenotype richness and diversity of all the soil variables examined, soil pH was, by far the best predictor of both soil. The lowest level of richness was observed in acidic soil. Microtiter plates have been already extensively used in applied research as well as less time consuming method for the reseachers (Muyzer and Smalla, 1999). Microbial diversity offers an immense field of environment friendly options for mineralization of contaminants or their transformation into less harmful non-hazardous compounds. There is a general interest in studying the diversity if indigenous microorganisms capable of degrading different pollutants because of their varied effects on environment. RAPD is largely used for genetic variability analysis. The banding pattern of RAPD showed that all the isolates share some similarity between them RAPD was chosen as it has been shown to be effective sub typing method for several other species (Roberts and Crawford, 2000; Sharma et al., 2008).

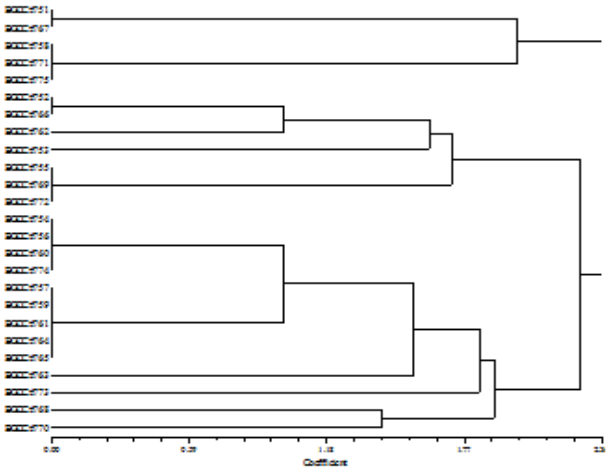

Fig 1: Dendrogram showing relatedness among 25 bacterial isolates isolated from diesel contaminated soil, on the basis of Random Amplified Polymorphic DNA analysis.

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