The Bioscan 7(4): 719-722, 2012
AN INTERNATIONAL QUARTERLY JOURNAL OF LIFE SCIENCES
www.thebioscan.in

ISOLATION AND IDENTIFICATION OF DIESEL DEGRADING MICROORGANISM FROM BARDDHAMAN LOCO SHED POND, BURDWAN, WEST BENGAL, INDIA

SMARANYA HAQUE, SUBINOY MONDAL AND APURBA R GHOSH*
Department of Environmental Science, The University of Burdwan,
Burdwan - 713 104, West Bengal, INDIA
e-mail: apurbaghosh2010@gmail.com

KEY WORDS
Diesel Pond
Pseudomonas sp.

Received on :
10.08.2012

Accepted on :
26.11.2012

***Corresponding author**

ABSTRACT
Diesel fuel is considered as a common environmental pollutant comprised of a large number of both aromatic and aliphatic hydrocarbons. The present study was carried out to isolate hydrocarbon (diesel) degrading bacteria collected from the diesel fed pond known as Barddhaman Loco Shed Pond. The samples were analyzed microbiologically using standard microbiological techniques. These organisms were further studied to determine their biodegrading activities on hydrocarbons (diesel) as the sole carbon source using enrichment medium. Phenotype and phylogeny analysis of this strain was characterized and identified as diesel degrading bacteria, based on gram staining, 16S rRNA gene sequence analysis. These results indicate that this new strain was *Pseudomonas* sp. and showed 99% similarities with *Pseudomonas mendocina* and *Pseudomonas pseudoalcaligenes* with 99 % 16S rRNA gene sequence similarity.

INTRODUCTION

Oil spill is a global problem particularly in industrialized and developing countries (Mittal and Singh, 2009). Hydrocarbon pollution is the most important pollution worldwide (Ruberto *et al.,* 2005). Many highly concentrated toxic materials are present in diesel discharge which can negatively affect the surface water quality as well as the groundwater quality (Dillard *et al.,* 1997; Gold-Bouchot *et al.,* 1997). Petroleum is used as the principle source of energy; despite of its all importance it also acts as a global environmental pollutant (Plohl *et al.,* 2002). It has been considered as priority pollutants which causes biohazards on the living organisms in the environment (Kramer and Van, 1990; Refaat *et al.,* 2008; Richardson, 1996). Diesel oil is a complex mixture of normal, branched and cyclic alkanes and aromatic hydrocarbon compounds. It has low water solubility, high adsorption coefficient and high stability of the aromatic ring (Dean *et al.,* 2002; Kanaly and Harayama, 2002; Kropp and Fedorak, 1998; Van *et al.,* 2003). Diesel oil consists of mainly aliphatic hydrocarbons ranging from C9 to C23 as well as a number of aromatic compounds (Bacha *et al.,* 1998). Nearly five million tons of crude oil and refined oil enters into the environment each year due to anthropogenic sources such as oil spills (Hinchee and Kitte, 1995). Hydrocarbons enter into the environment through waste disposal and accidental spills. Some microorganisms can utilize the hydrocarbons as sole carbon sources for getting their energy and metabolic activities (Jyothi *et al.,* 2012). Biodegradation is a complex process that depends on the nature of petroleum and also on the amount of the hydrocarbons present (Das *et al.,* 2011). The microbes can utilize the hydrocarbons depending on the chemical nature of the compounds within the petroleum mixture (Adeline *et al.,* 2009). Hydrocarbon degrading microorganisms usually exist in very low abundance in aquatic environments (Sivaraman *et al.,* 2011). Biodegradation of bacteria is considered as the most active process in petroleum degradation and they are the primary degraders of spilled oil (Rahman *et al.,* 2003; Brooijmans *et al.,* 2009) and this is specially carried out largely by diverse bacterial populations, mostly by *Pseudomonas* species (Boboye *et al.,* 2010; Dubey *et al.,* 2009). There are so many known consortia of microorganisms which can degrade mineral oil hydrocarbons under laboratory or field conditions (Ratajczak *et al.,* 1998; Wikstrom *et al.,* 1996). This work is concentrated on isolation and identification of hydrocarbon-degrading bacteria associated with diesel oil contaminated Barddhaman Loco Shed Pond, Burdwan, West Bengal, India and also to test their ability to degrade different hydrocarbons. The expected interpretation of this study will provide information on the bacterial population, hydrocarbon-degrading microorganisms and their degrading ability of diesel because these bacteria can utilize the hydrocarbons as carbon source. Biodegradation by indigenous populations of microorganisms is one of the primary mechanisms by which petroleum and other hydrocarbon pollutants can be removed from the environment (Ulrici *et al.,* 2000) and this process is also cheaper than the other remediation technologies (Leahy *et al.,* 1990).

MATERIALS AND METHODS

The Loco Shed Pond, locally called Loco tank, is adjacent to

the Barddhaman locomotives used for aquaculture since 1994. It is managed by the Rupali Cooperative limited, Barddhaman. The total area of the pond is 27 acres (approx. 11 hectares) with a yearly catch at an average 22.72 metric tons. The main species cultured in this pond are tilapia, catla, rohu, mrigal, silver carps *etc.* Geographical location is at 23°14′42"N latitude and 87°52′38"E longitude. This pond is embraced by the local city buildings, housing and small shops *etc.* There are two main points situated in the southern and south-western parts from where the diesel oils are directly discharged into the pond, although there is a treatment plant "effluent treatment plant" on the southern part of the pond. During the rainy season due to heavy precipitation both the sides of the ponds become inundated and excess water drains off from the south-western part of the pond.

Isolation of diesel-degrading organisms from the study site

Diesel-contaminated water samples were collected from the Diesel oil contaminated Barddhaman Loco Shed Pond in a sterilized polythene bottle and kept at 4°C in refrigerator. An enrichment culture technique was used to isolate diesel-degrading bacteria. The enrichment culture media consisted of Bushnell-Haas agar media supplemented with 1% diesel as carbon source. The culture media was prepared by Bushnell-Haas broth by adding agar powder to it. 100 ml of Bushnell-Haas agar media was sterilized by autoclaving at 121°C, 15 lb pressure for 15 minutes and plated into sterilized petridishes, complemented with 1 mL autoclaved diesel oil which has been spread-over the BH-agar media. Then 100 μL of water sample was added to the media by spreading it by an autoclaved glass spreader. All the methods are performed aseptically under laminar air flow hood. Successive transfers of the bacterial colonies are done in each 72h interval and incubated in 37°C for 72h. These transfers were done to achieve the pure culture of the isolates. After 10-12 transfers the bacterial colony was checked for its purity morphologically by Gram staining method. After Gram staining, the bacterial cultures were sent for 16S rRNA analysis and sequencing.

Isolation of genomic DNA from bacteria

DNA was extracted from 1mL of bacterial culture.The culture was pelleted by centrifuging at 12,000 rpm for 2 min. The pellet was treated with lysis solution and proteinase K and incubated at 60°C for 30 min. Nucleic acids were precipitated with isopropanol by centrifuging at 10,000 rpm for 10 min, washed with 1mL of a 70% (v/v) ethanol solution and dissolved in 0.1 mL of a TE buffer. The purity and quantity of DNA were examined by recording its UV absorption spectrum and running on 1% agarose gel electrophoresis.

Sequence determination of 16S rRNA

The DNA isolated was amplified using 16S rRNA universal primers and sequenced for the identification of bacterial strain at molecular level. Amplification of the PCR products of expected size was confirmed by electrophoresis. The sequence of the 16S rRNA was determined with a Dye terminator sequencing kit (Applied Biosystems), and the product was analyzed with an ABI Prism DNA sequencer (ABI). The gene sequences of each isolate obtained in this study were compared with known 16S rRNA gene sequences in the GenBank database.

RESULTS AND DISCUSSION

The pure monoclonal bacterium was isolated from the diesel infected pond, Barddhaman Loco shed pond by using nutrient agar media followed by screening for the presence of hydrocarbon-degrading bacteria on Bushnell-Haas agar media with 1% of the hydrocarbons as the sole carbon source, *i.e.,* commercial diesel oil. Hydrocarbons are essential as a carbon source but it can be toxic to microorganisms due to the solvent effects of diesel that could destroy bacterial cell membrane. Some biodegradation studies were reported on diesel are carried out by using the diesel concentrations ranging from 0.5 to 1.5% (Mukherji *et al.,* 2004; Lee *et al.,* 2006; Hong *et al.,* 2005; Ueno *et al.,* 2007; Rajasekar *et al.,* 2007). According to Shukor *et al.* (2009) the optimum growth of microorganisms occurred at 3.5% (v/v) diesel oil concentration and according to Kwapisz *et al.* (2008) it was 6%. It was also noticed that degradation is generally unfavourable at concentrations higher than 1 or 1.5% (Espeche *et al.,* 1994; Gold-Bouchot *et al.,*

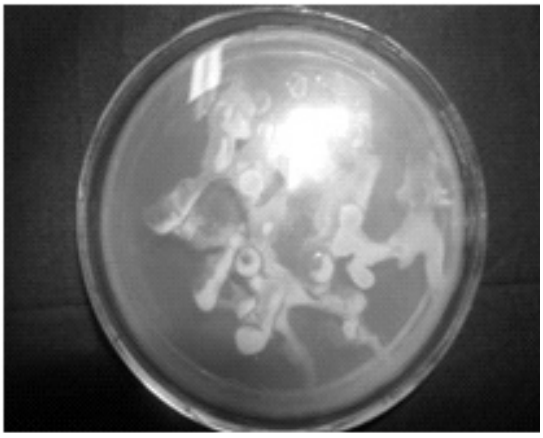

Figure 1: The culture of diesel-degrading microorganism on Bushnell-Haas Agar media plate

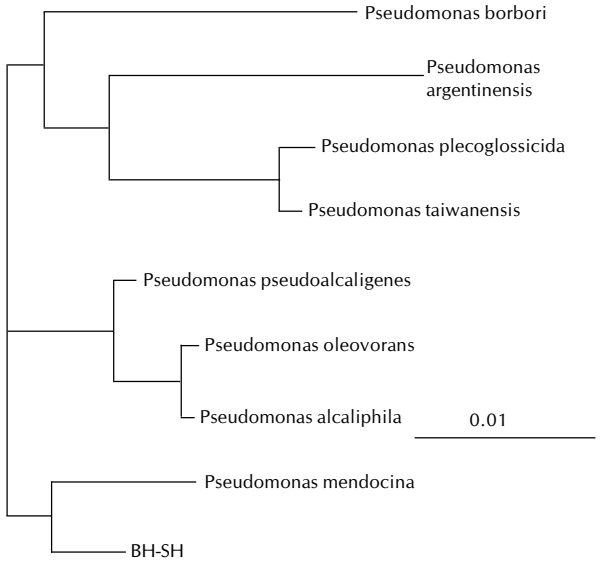

Figure 2: The dendogram analysis of the bacteria based on 16S rRNA sequencing

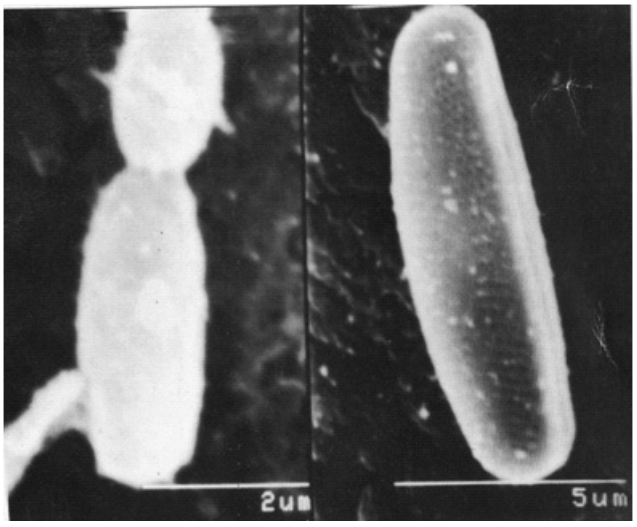

Figure 3: Figure showing the Scanning Electron Microscopic (SEM) photographs of the diesel-degrading bacteria

1997 and Bicca *et al.,* 1999). Number of colonies on Bushnell-Haas agar medium is lower when compared to the mother plate without hydrocarbons (Fig. 1). This result showed that the bacteria have grown on enriched medium and was able to degrade the hydrocarbon source. Gram staining study shows that the isolated bacteria are rod shaped and Gram negative.The isolates were further confirmed by 16S rRNA sequencing (Fig. 2). The bacterial 16S rRNA sequences were aligned with Blast search of NCBI databases. The sequence aligned gave 99% similarity with *Pseudomonas mendocina* and *Pseudomonas pseudoalcaligenes*. It is clear from this study that when the aquatic environment was contaminated with petroleum and diesel components then the growth of 'hydrocarbon-degrading microorganisms' increases proportionately (Jyothi *et al.,* 2012). The oil-degrading microorganisms are present in the polluted soil and water even within the gastro-intestinal tract of fish which depicts the clear indication that the indigenous microbes are carrying out their metabolic activity. Their presence and metabolic activity are also supported by Ojo (2006). The SEM study also reveals the presence of the same bacterial species within the gut of inhabitant fish species particularly in the surface and column dwellers exhibiting their dividing states (Fig. 3) High numbers of certain hydrocarbon-degrading microorganisms prove that those organisms are the active degraders of that environment (Okerentugba and Ezeronye, 2003). Most of the isolates are predominantly indigenous microorganisms of the polluted sites, which are constantly exposed to the different petroleum contaminants. *Pseudomonas mendocina* could grow on different aliphatic hydrocarbons namely tetradecane, hexadecane and octadecane (Sivaraman *et al.,* 2011).

REFERENCES

Adeline, S. Y., Ting Carol, H. C. and Tan and Aw, C. S. 2009. Hydrocarbon-degradation by isolate *Pseudomonas lundensis* UTAR FPE2. *Malaysian Journal of Microbiology*. **5:** 104-108.

Boboye, B., Olukunle, O. F. and Adetuyi, F. C. 2010. Degradative activity of bacteria isolated from hydrocarbon-polluted site in Ilaje, Ondo State, Nigeria. *African J. Microbio. Res.* **4:** 2484-2491.

Bacha, J., Blondis, L. and Freel, J. 1998. Diesel Fuels Technical Review (FTR-2). Chevron Products Company, Chevron USA Inc., San Ramon, CA.

Bicca, F. C., Fleck, L. C., Antonio, M. and Ayub, Z. 1999. Production of biosurfactant by hydrocarbon degrading Rhodococcus ruber and Rhodococcus erythropolis. *Rev de Microbiol.* **30:** 231-236.

Brooijmans, R. J. W., Pastink, M. I. and Siezen, R. J. 2009. Hydrocarbon-degrading bacteria: the oil-spill clean-up Crew. *Microbial Biotechnology.* **2:** 587–594.

Das, N. and Chandran, P. 2011. Microbial Degradation of Petroleum Hydrocarbon Contaminants: An Overview. SAGE-Hindawi Access to Research Biotechnology Research International.

Dean-Ross, D., Moody, J. and Cerniglia, C. E. 2002. Utilization of mixtures of polycyclic aromatic hydrocarbons by bacteria isolated from contaminated sediment. *FEMS Microbiol Ecol.* **41:** 1-7.

Dillard, L. A., Essaid, H. I. and Herkelrath, W. N. 1997. Multiphase flow modeling of a crude-oil spill site with a bimodal permeability distribution. Water Resources Research. **33:** 1617-1632.

Dubey, R. C. 2009. A text book of Biotechnology. S. Chand and Company Ltd. Ram Nagar, New Delhi – 110055.

Espeche, M. E., MacCormack, W. P. and Fraile, E. R. 1994. Factors affecting growth of an n- hexadecane degrader Acinetobacter species isolated from a highly polluted urban river. *Int Biodeterior Biodegrad.* **33:** 187-196.

Gold-Bouchot, G., Zavala-Coral, M., Zapata-Pérez, O. and Ceja-Moreno, V. 1997. Hydrocarbon concentrations in oysters (*Crassostrea virginica*) and recent sediments from three coastal lagoons in Tabasco, Mexico. *Bulletin of Environmental Contamination and Toxicology*. **59**: 430-437.

Hinchee, E. R. and Kitte, A. J. 1995. Applied Bioremediation of Petroleum Hydrocarbons. Columbus (OH): Battelle Press.

Hong, J. H., Kim, J., Choi, O. K., Cho, K. S. and Ryu, H. W. 2005. Characterization of a diesel-degrading bacterium, Pseudomonas aeruginosa IU5, isolated from oil-contaminated soil in Korea. *World J. Microbiol. Biotechnol.* **21:** 381-384.

Jyothi, K., Surendra Babu, K., Nancy Clara, K. and Kashyap, A. 2012. Identification and Isolation of Hydrocarbon Degrading Bacteria by Molecular Characterization. *Helix.* **2:** 105-111.

Kanaly, R. A. and Harayama, S. 2002. Biodegradation of highmolecular weight polycyclic aromatic hydrocarbons by bacteria. *J. Bacteriol.* **182:** 2059-2067.

Kramer, P. G. N. and Van der Heijden, C. A. 1990. Polycyclic aromatic hydrocarbons (PAH): Carcinogenicity data and risk extrapolations. *Toxicol Environ Chem.* **16:** 341-451.

Kropp, K. G. and Fedorak, P. M. 1998. A review of the occurrence, toxicity, and biodegradation of condensed thiophenes found in petroleum. *Can. J. Microbiol.* **44:** 605–622.

Kwapisz, E., Wszelaka, J., Marchut, O. and Bielecki, S. 2008. The effect of nitrate and ammonium ions on kinetics of diesel oil degradation by Gordonia alkanivorans S7. *Int. Biodeterior Biodegrad.* **61:** 214-222.

Leahy, J. G. and Colwell, R. R. 1990. Microbial degradation of hydrocarbons in the environment. *Microbiol. Reviews.* **54:** 305-315.

Lee, M., Kim, M. K., Singleton, I., Goodfellow, M. and Lee, S. T. 2006. Enhanced biodegradation of diesel oil by a newly identified *Rhodococcus baikonurensis* EN3 in the presence of mycolic acid. *J. Appl. Microbiol.* **100:** 325-333.

Mittal, A. and Singh, P. 2009. Isolation of hydrocarbon degrading bacteria from soils contaminated with crude oil spills. *Indian J. Experimental Biology.* **47:** 760-765.

Mukherji, S., Jagadevan, S., Mohapatra, G. and Vijay, A. 2004. Biodegradation of diesel oil by an Arabian Sea sediment culture isolated

from the vicinity of an oil field. *Bioresource Technol.* **95:** 281-286.

Ojo, O. A. 2006. Petroleum-hydrocarbon utilization by native bacterial population from a wastewater canal Southwest Nigeria. *Afr. J. Biotechnol.* **5:** 333-337.

Okerentugba, P. O. and Ezeronye, O. U. 2003. Petroleum degrading potentials of single and mixed microbial cultures isolated from rivers and effluents in Nigeria. *Afr. J. Biotech.* **2:** 288 –292.

Plohl, K., Leskovsek, H. and Bricelj, M. 2002. Biological degradation of motor oil in water. *Acta Chim Slovenica.* **49:** 279-289.

Rahman, K. S. M., Rahman, T. J., Kourkoutas, Y., Petsas, I., Marchant, R. and Banat, I. M. 2003. Enhanced bioremediation of n-alkane in petroleum sludge using bacterial consortium amended with rhamnolipid and micronutrients. *Bioreso. Technology.* **90**:159-168.

Rajasekar, A., Babu, T. G., Pandian, S. T., Maruthamuthu, S., Palaniswamy, N. and Rajendran, A. 2007. Role of Serratia marcescens ACE2 on diesel degradation and its influence on corrosion. *J. Ind. Microbiol. Biotechnol.* **34:** 589-598.

Ratajczak, A., Geißdo¨ rfer, W. and Hillen, W. 1998. Expression of alkane hydroxylase from *Acinetobacter* sp. strain ADP1 is induced by a broad range of n-alkanes and requires the transcriptional activator AlkR. *J. Bacteriol.* **180:** 5822–5827.

Refaat, A. A., Attia, N. K., Sibak, H. A., El Sheltawy, S. T. and El Diwani, G. I. 2008. Production optimization and quality assessment of biodiesel from waste vegetable oil. *Int. J. Env. Sci. Tech.* **5:** 75-82.

Richardson, M. 1996. Environmental Xenobiotics. CRC Press. pp.11-18.

Ruberto, L. A. M., Vázquez, S. C., Lobalbo, A. and Mac Cormack, W. P. 2005. Psychrotolerant hydrocarbon-degrading Rhodococcus strains isolated from polluted Antarctic soils. *Ant Sci.* **17:** 47–56.

Shukor, M. Y., Hassan, N. A. A., Jusoh, A. Z., Perumal, N. and Shamaan, N. A. 2009. Isolation and characterization of a Pseudomonas diesel-degrading strain from Antarctica. *J. Environ Biol.* **30:** 1-6.

Sivaraman, C., Ganguly, A., Nikolausz, M. and Mutnuri, S. 2011. Isolation of hydrocarbonoclastic bacteria from bilge oil contaminated water. *Int. J. Environ. Sci. Tech.* **8:** 461-470.

Ueno, A., Ito, Y., Yumoto, I. and Okuyama, H. 2007. Isolation and characterization of bacteria from soil contaminated with diesel oil and the possible use of these in autochthonous bioaugmentation. *World J. Microbiol Biotechnol.* **23:** 1739-1745.

Ulrici, W. 2000. Contaminant soil areas, different countries and contaminant monitoring of contaminants. Environmental Process II. Soil Decontamination Biotechnology H. J. Rehm and G. Reed, (Eds). **11:** 5-42.

Van Hamme, J. D., Singh, A. and Ward, O. P. 2003. Recent advances in petroleum microbiology. *Microbiol. Molecul. Bio. Rev.* **67:** 1092-2172.

Wikstrom, P., Wiklund, A., Andersson, A. C. and Forsman, M. 1996. DNA recovery and PCR quantification of catechol 2,3-dioxygenase genes from different soil types. *J. Biotechnol.* **52:** 107–120.