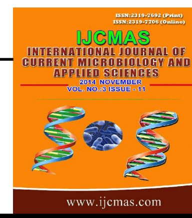

International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 3 Number 11 (2014) pp. 363-369**
http://www.ijcmas.com

Original Research Article

Diversity of diesel degrading bacteria from a hydrocarbon contaminated soil

S.K.Bhasheer, S.Umavathi*, D.Banupriya, M.Thangavel and Y.Thangam

PG and Research Department of Zoology, J. K. K. Nattraja College of Arts and Science, Kumaraplayam, Namakkal District, Tamilnadu.
**Corresponding author*

A B S T R A C T

Five bacterial strains were isolated from diesel contaminated soil in petroleum filling station at Nadapuram, Kozhikode District, Kerala. From the soil sample five morphologically different bacterial species were isolated and the active strains were *Acinetobacter* sp, *Moraxella* sp, *Bacillus* sp, *Vibrio* sp and *Alcaligenes* sp. The isolated hydrocarbon degrading bacterial strains were analyzed with gram staining, spore staining, motility, catalase, oxidase, penicillin sensitivity, Huge and Leifson's test and pigmentation test. The diesel degrading efficiency of isolated organisms was tested in BH medium supplemented with diesel and DCPIP for 21 days.

Keywords

Bioremediation
DCPIP,
Diesel,
Alcaligenes sp.,
Degradation rate

Introduction

Hydrocarbon contamination resulting from leaking above ground and underground storage tanks, spillage during transport of petroleum products, abandoned manufactured gas sites and various industrial processes is hazardous to soil and water ecosystem and is expensive to remediate. There is an increased interest in promoting ecofriendly methods in the process of cleaning oil-polluted sites. Biological and non-biological approaches are being used for remediation of oil pollution. Bioremediation is one of the principle strategies for remediation, wherein the pollution can be removed by use of microorganism or by any biological process that uses microorganisms or their enzymes to return the environment altered by contaminants to its original condition (Olu-Arotiowa *et al.,* 2007).

Many species of microorganisms including bacteria, yeasts and fungi obtain both energy and tissue-building material from hydrocarbons. A wide range of studies have dealt with biotransformation, biodegradation and bioremediation of petroleum hydrocarbons and interested in exploiting crude oil-degrading organisms for environmental cleanup has become central to petroleum microbiology. In the course of biological restoration of hydrocarbon contaminated soil, the main factors that affect the effect of remediation include the pH value, soil moisture, oxygen supply, the nutrient level, bacterial diversity and the

temperature, among which the impact of petroleum hydrocarbon degrading bacteria on the effect is critical (Venosa and Xuequing, 2003; Chaillan *et al.,* 2006). The ability of many microorganisms in order to biodegradation of hydrocarbons has been studied by Liangli and Hungchen, (2009); Sarikhani *et al.* (2011); Ebrahimi *et al.* (2012) and Geetha *et al.* (2013). These methods are less expensive and do not introduce additional chemicals to the environment.

Hence in this investigation indigenous bacteria which degrade diesel and hydrocarbons are isolated from diesel contaminated site and screened for their hydrocarbon degradation efficiency. They were further characterized by morphological, cultural and biochemical techniques.

Materials and Methods

Collection of soil samples

The diesel contaminated soil samples (at Nadapuram, Kozhikode District, Kerala) were aseptically collected at a depth of 20cm, stored in a sterilized container. The soil samples were placed in 4ºC and transported to the laboratory immediately and maintained at 4ºC until microbial analysis.

Substrates and indicators used for the degradation study

Diesel sample was collected from petrol bunk and the indicator 2, 6-dichlorophenol indophenol (DCPIP) used for the study was obtained from Lobachemie.

Isolation of hydrocarbon degrading bacteria

Bushnell-Hass (BH) medium (Atlas, 1995) was used as the enrichment media with 1% (v/v) diesel as the sole carbon source. 10 gm of the contaminated soil was added to 100 ml of BH medium and incubated at 30ºC at 170 rpm.

After 5 days of incubation, loopfull of inoculum from Bushnell-Hass medium was streaked onto the nutrient agar plate and incubated at 30ºC for 72 hours. Colonies with different morphological appearance were selected and purified in nutrient agar medium and were transferred to nutrient agar slants and stored for identification and further experimental studies. The cultures obtained were sub cultured monthly in nutrient agar medium.

Identification of hydrocarbon degrading bacteria

The bacteria isolated were identified based on physical characterization and the biochemical tests outlined in Bergey's manual of determination bacteriology (Holt *et al.,* 1994). The isolated hydrocarbon degrading bacterial strains were analyzed with gram staining, motility test, spore staining, catalase test, oxidase, penicillin sensitivity, Huge and Leifson's test and pigmentation test.

Estimation of diesel degradation

The identified seven bacterial isolates were tested for their ability of diesel degradation and to determine the most efficient degrader. Each of the five bacterial cultures was inoculated to 100 ml of nutrient broth medium and incubated to 37ºC for 24 hours at 150 rpm. The cultures were used as inoculum for the hydrocarbon degradation.

100ml of Bushnell-Hass (BH) medium was prepared with 1% diesel as sole carbon source. DCPIP at a concentration of 20 mg/l of the medium was used. The medium was

sterilized at 121ºC for 15 minutes. 1 ml each of the inoculum was added to each set of experiment and incubated at 150 rpm for 21 days. Control was also maintained.

Bacterial growth determination

The initial and final growth was determined by pour plate method using nutrient agar. The plates were incubated at 37ºC for 48 hours. After the incubation period, colonies were counted and total microbial count/ml was calculated.

Determination of diesel degradation

Diesel degradation was determined by the ability of microorganisms to utilize the diesel as sole carbon source which indicate the change in colour of DCPIP from blue to colourless. The indicator, when oxidized was blue and reduced was transparent. The reaction was observed visually till the end of incubation and also spectrophotometrically (600nm) at an interval of 4 days using Shimadzu model UV-3600 visible spectrophotometer according to Bharathi and Vasudevan, (2001).

Results and Discussion

Isolation and identification of diesel degrading bacteria

In the present study five diesel degrading bacterial species *Acinetobacter* sp., *Moraxella* sp., *Bacillus* sp., *Vibrio* sp. and *Alcaligenes* sp. were identified in the diesel contaminated sites (Table 1). Similarly Ebrahimi *et al.* (2012) reported the hydrocarbon degrading efficiency of some isolated bacteria from oil polluted sites of Bushehr provine, such as *Pseudomonas stutzeri, Pseudomonas alcaligenes, Pseudomonas aeruginosa, Stenotrophomonas maltophilia, Serratia odorifera, Achromobacter xylosoxidans, Acinetobacter johnsonii, Enterobacter cloacae, Ralstonia sp., Vibrio sp., Sphingobacterium sp., Zymomonas sp., Paracoccus sp., Pantoea sp.* and *Chryseobacterium sp.*

Biochemical identification of hydrocarbon degrading bacteria

The morphologically identified bacterial strains were confirmed with the biochemical tests and the experiments showed the presence of *Acinetobacter* sp*., Moraxella* sp., *Bacillus* sp., *Vibrio* sp. and *Alcaligenes* sp in the diesel contaminated soil samples (Table 2). Khan and Rizvi (2011) isolated the hydrocarbon degradation strains in the oil contaminated sites were tested with the gram staining, endospore staining, catalase staining, mannitol fermentation, glucose fermentation, sucrose fermentation and lactose fermentation test and prove the presence of *Bacillus (*MJH1101 and MJH1104*)* and *Coccus* (MJH1102 and MJH1103).

Quatrini *et al.* (2008) and Navdeep *et al.* (2013) have been isolated the hydrocarbon degrading bacteria based on the gram staining technique. The gram positive bacteria dominate in oil contaminated areas. Gram positive bacteria have a stronger cell envelope than gram negative bacteria and this allows them to thrive in highly variable intertidal sediment environment, where sediment temperatures are high in the day and osmotic pressures and nutrient supply may change periodically over a daily cycle. Endospores are resistant to unfavorable conditions like heat, desiccation, radiation, oxidants and proteases and allow the bacteria to persist and to be ubiquitous in the environment without losing the capacity of germination and outgrowth (Kaplan and Kitts, 2004).

Estimation of biodegradation of diesel by isolated organisms

The degrading efficiency of isolated organisms was studied in BH medium supplemented with diesel and DCPIP. The experimental set up was kept for 21 days of incubation, in order to study the colour change of DCPIP, which is blue in colour and its oxidized form and colourless in its reduced form. Among the five isolated species incubated and monitored in diesel supplemented medium for 21 days, the maximum diesel biodegradability was noticed in *Alcaligenes* sp at fourth day itself and a TPC $57 x 10^5$ CFU at a temperature of 30°C and pH 5. The degradation was also checked spectrophotometrically at an OD of 660nm; where in the OD reading was found to decrease in all the causes till the end of incubation period. This confirms that all the isolates were capable of degrading diesel. It has been shown that there is a high correlation between cellular growth and diesel assimilation in microbes (Petrikevich *et al.,* 2003). Kayoda-Isola *et al.* (2008) reported the *Alcaligenes paradoxus, Aeromonas* sp.*, Bacillus licheniformis* and *Pseudomonas fluorescens* were efficient in biodegradation of diesel oil.

Acinetobacter sp and *Moraxella* sp showed the diesel biodegradability at day 9 of incubation with a TPC of $63 x 10^5$ CFU and $56 x 10^5$ CFU, respectively. While *Bacillus* sp showed diesel biodegradability at day 14th of incubation of TPC $48 x 10^5$ CFU. The *Vibrio* sp produced biodegradability at day 17th of incubation with a TPC of $39 x 10^5$ CFU. All these were carried at a temperature of 30^oC and pH 5 (Table 3 and 4).

Generally, the entire carbon source is assimilated by the bacterium for growth and reproduction. For bacterial growth diesel acts as a carbon source but at certain concentrations, diesel can be toxic to microorganism due to the solvent effect of diesel which destroys cell membrane. Thus many biodegradation studies on diesel are carried out using lesser diesel concentrations ranging from 0.5 to 1.5% (Lee *et al.,* 2006; Hong *et al.,* 2005; Ueno *et al.,* 2007 and Rajasekar *et al.,* 2007).

Bacterial growth and hydrocarbon degradation were fluctuated by increase of incubation time (Table 4). Increasing and decreasing of viable recovered bacterial number was related to the kind of hydrocarbons and also to the bacterial species. Decreasing of bacterial population in some cases may be explained by composition of hydrocarbons and its concentration which at initial step bacteria faced with a shock, stress and gradually adaptation mechanism in bacteria and induction of some mechanism which helps to survive and growth in new condition (Abd-Elsalam *et al.,* 2009) causes an increasing trend in bacterial number. Reversely in some condition such as high concentration of hydrocarbons and its toxicity furthermore limitation of oxygen and nutrition causes to inhibit biodegradation (Leahy and Colwell, 1990). Increasing time of incubation usually causes to increase of bacterial growth (Shafiee *et al.,* 2006).

Thus, the distribution of bacterial isolates obtained from the sampling source indicates the common occurrence of metabolically active strains in areas that are contaminated with hydrocarbons strongly suggested the ability of these bacteria to utilize the hydrocarbons as their carbon and energy source. This study also proves that population of microorganisms found in a polluted environment degrades diesel at a different rate and clean up the environment. Hydrocarbon degrading bacteria can tolerate oil contaminated environments because they possess the capability to utilize oil as energy

sources (Afuwale and Modi, 2012). Other species may not and are gradually eliminated.

In nature most microbial process results are based on microorganism's activity. A bacterial population in pollution environment tends to dominate by the strains that can endure toxicity and are able to consume contaminated pollutants itself for growth. These populations might actively being degrading contaminants and cleaning the environment which leads to increasing bacterial communities in soil. Adaptation of microbial communities to hydrocarbons such as increase in rate of transformation of hydrocarbon associated with oil contaminated environment.

Table.1 Isolation and identification of hydrocarbon degrading bacteria from diesel contaminated soil

S. No.	Colony morphology	Identified species
1.	Cream, slight, irregular, raised, transparent colonies	*Acinetobacter* sp.
2.	White, Abundant, Circular, Raised, Opaque colonies	*Moraxella sp.*
3.	Cream, abundant, irregular, convex, irregular dry colonies	*Bacillus* sp.
4.	Light cream, moderate, irregular, flat, transparent	*Vibrio* sp.
5.	White, abundant, irregular, convex, transparent colonies	*Alcaligenes sp.*

Table.2 Biochemical identification of isolated bacteria

Test required	*Acinetobacter* sp.	*Moraxella sp.*	*Bacillus* sp.	*Vibrio* sp.	*Alcaligenes sp.*
Gram staining	- Rods	- Rods	+ Rods	- Rods	- Rods
Motility	NA	-	+	NA	+
Spore staining	NA	NA	+	NA	NA
Catalase	-	NA	+	+	+
Oxidase	-	+	+	+	+ Weak
Penicillin sensitivity	-	+	-	-	+
Hugh-Leifson's test	-	-	NA	+	NA
Pigmentation test	-	-	-	-	-

NA-Not Applicable; + Positive; - Negative

Table.3 Biodegradation of diesel by isolated organisms

S. No	Days of incubation	OD at 660nm				
		Acenetobacter sp.	*Moraxella* sp.	*Bacillus* sp.	*Vibrio* sp.	*Alcaligenes* sp.
1.	4th day	0.398	0.398	0.441	0.425	0.329
2.	8th day	0.390	0.386	0.432	0.418	0.302
3.	12th day	0.350	0.354	0.387	0.381	0.299
4.	16th day	0.311	0.320	0.305	0.257	0.287
5.	21st day	0.298	0.232	0.236	0.227	0.260

Table.4 Bacterial growth determination during biodegradation of diesel

S. No	Organisms	T.P.C/ml		Final day of incubation
		Initial	Final	
1.	*Acenetobacter* sp.	16×10^5 CFU	63×10^5 CFU	9th day
2.	*Moraxella sp.*	13×10^5 CFU	56×10^5 CFU	9th day
3.	*Bacillus* sp.	18×10^5 CFU	48×10^5 CFU	14th day
4.	*Vibrio* sp.	16×10^5 CFU	39×10^5 CFU	17th day
5.	*Alcaligenes sp.*	15×10^5 CFU	57×10^5 CFU	4th day

References

Abd-Elsalam, H.E., hafez, E.E., Hussain, A.A., Ali, A.G., Hanafy, A.A. 2009. Isolation and identification of three-rings polyaromatic hydrocarbons. *Amer. Euras. J. Agric. Environ. Sci.,* 5(1): 31–38.

Afuwale, C., Modi, H.A. 2012. Study of bacterial diversity of crude oil degrading bacteria isolated from crude oil contaminated sites. *Life Sci. Leaflets.,* 6: 13–23.

Atlas, R.M. 1995. Bioremediation of petroleum pollutants. *Int. Biodegrad. Biodegrad.,* 35: 317–327.

Bharathi, S., Vasudevan, N. 2001. Utilization of petroleum hydrocarbons by *Pseudomonas fluorescens* isolated from petroleum contaminated soil. *Environ. Int.,* 26: 413–413.

Chaillan, F., Chaineau, C.H., Point, V. 2006. Factors inhibiting bioremediation of soil contaminated with weathered oils and drill cuttings. *J. Environ. Poll.* 144(1): 255–265.

Ebrahimi, M., Fallah, R., Sarikhani, M.R., Taheri, M.T. 2012. Assessment of biodegradation efficiency of some isolated bacteria from oil-contaminated sites in solid and liquid media containing oil-compounds. *Int. Res. J. Appl. Basic Sci.,* 3(1): 138–147.

Geetha, S.J., Joshi, S.J., Kathrotiya, S. 2013. Isolation and characterization of hydrocarbon degrading bacteria isolate from oil contaminated sites. *APCBEE Procedia.,* 5: 237–241.

Holt, J.G., Krieg, N.R., Sneath, P.H.A., Staley, J.T., Williams, S.T. 1994. Bergey's manual of determinative bacteriology, 9th edn. Williams and Wilkins, Baltimore. Pp. 71–561.

Hong, J.H., Kim, J., Choi, O.K., Cho, K.S., Ryu, H.W. 2005. Ryu: Characterization of a diesel-degrading bacterium, *Pseudomonas aeruginosa* IU5, isolated from oil-contaminated

soil in Korea. *World J. Microbiol. Biotechnol.,* 21: 381–384.

Kaplan, C.W., Kitts, C.L. 2004. Bacterial succession in a petroleum land treatment unit. *Appl. Environ. Microbiol.,* 73(3): 1777–1786.

Kayoda-Isola, T.M., Eniola, K.I.T., Olayemi, A.B., Igunnungbemi, O.O. 2008. Response of resident bacteria of a crude-oil polluted river to diesel oil. *Am. Eurasian. J. Agro.,* 1(1): 06–09.

Khan, J.A., Rizvi, S.H.A. 2011. Isolation and characterization of micro-organism from oil contaminated sites. *Adv. Appl. Scien. Res.,* 2(3): 455–460.

Leahy, J.G., Colwell, R.R. 1990. Microbial degradation of hydrocarbons in the environment. *Microbiol. Rev.,* 54(3): 305–315.

Lee, M., Kim, M.K., Singleton, I., Goodfellow, M., 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.

Liangli, J., Hungchen, B. 2009. Surfactant mediated biodegradation of polycyclic aromatic hydrocarbons. *Materials.,* 2: 76–94.

Navdeep, S., Yuvneet, R., Maninder, K., Ashna, G. 2013. The isolation and characterization of several starins of Naphthaline degrading bacteria from oil contaminated sites. *J. Pharm. Biol. Scien.,* 5(2): 23–28.

Olu-Arotiowa, O.A., Aremu, M.O., Alade, A.O. 2007. Ex-situ bioremediation of diesel polluted waste water in trophical hot climate. *Asia J. Info. Technol.,* 6(9): 961–963.

Petrikevich, S.B., Kobzev, E.N., Shkidchenko, A.N. 2003. Estimation of the hydrocarbon-oxidizing activity of microorganisms. *Appl. Biochem. Microbiol.,* 301: 19–23.

Quatrini, P., Scaglione, G., De Pasquale, C., Riela, S., Puglia, A.M. 2008. Isolation of Gram-pasitive n-alkane degraders from a hydrocarbon contaminated Mediterranean shoreline. *J. Appl. Microbiol.,* 104(1): 251–259.

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

Sarikhani, M.R., Ebrahimi, M., Fallah, A.R. 2011. Comparison of hydrocarbon-degradation by isolates of *Pseudomonas fluorescens* Chao, *P. Putida* and *Pantoea agglomerans* P5 in presence of gas oil, toluene and phenanthrene. 4th International Conference of Environmental Industrial and Applied Microbiology, 14-16 September, Malaga, Spain.

Shafiee, P., Shojaosadati, S.A., Charkhabi, A.H. 2006. Biodegradation of polycyclic aromatic hydrocarbons by aerobic mixed bacterial culture isolated from hydrocarbon polluted solis. *Iran. J. Chem. Eng.,* 25(3): 73–78.

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

Venosa, A.D., Xueqing, Z. 2003. Biodegradation of crude oil contaminated marine shorelines and freshwater wetlands. *J. Spill Sci. Technol. Bullet.* 8(2): 163–178.