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Isolation and identification of petroleum hydrocarbon degrading microorganisms from oil contaminated environment

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ABSTRACT

Oil contaminated soil, sea water and sediment samples were collected from old oil jetty of Paradip port, Orissa. These samples were screened for bacterial oil degradation using 0.5% diesel in Bushnell-Hass Mineral Salt medium. Samples were incubated separately in shaking orbital incubator at 37^0C at 125rpm up to 15days. The level of petroleum hydrocarbon degradation was determined by gravimetric assay at each 5 days interval. One potent Gram negative bacillus bacteria was isolated on the basis of petroleum hydrocarbon degradation efficiency and identified as *Pseudomonas sp*. The bacterial strain had shown 49.93% of diesel oil degradation in 20days against 0.5% of diesel in 100ml BHMS.

Keywords: Biodegradation, Bacteria, Diesel oil, Marine environment

1. Introduction

Hydrocarbons are the world's most widely used primary energy and fuel resources, due to the energy they produce. Apparently inevitable spillages, which occur during routine operations of crude oil production, refining, distribution and as a consequence of acute accidents, have generated continuous research interest in this field (Okoh, 2003). Oil spill have become a global problem in industrialized and developing countries. Attention has been focused on the marine environment, because of the largest and most dramatic spills (Cooney, 1984). Undoubtedly, the most direct measure of bioremediation efficacy is the monitoring of hydrocarbon disappearance rates (Song and Bartha, 1990). Hydrocarbons are considered to be of biological origin, since short and long chain hydrocarbons (alkanes: C_{10} - C_{20}; C_{20} - C_{40}) appear to be exclusively the origin of biological processes (Surridge, 2007). These Hydrocarbon contaminations are hazardous to the health of plants and are also carcinogenic, mutagenic and potent immuno-toxicants posing a serious threat to human and animal health (Atlas, 1981; Zhou Crawford, 1995; Liebeg and Cutright, 1999, Ting and Hutan, 1999, Vasudevan and Rajaram, 2001). Petroleum and coal contain a class of molecules known as hopanoids, which are commonly found in bacterial cell walls (Gold, 1985). Gold (1985) indicated that these fuels at some point originated at least in part from microorganisms and that biodegradation of these fuels has always been occurring to some extent. Biodegradation of hydrocarbons by natural populations of microorganisms allows for the conversion of hazardous substances into forms that are less or non-toxic and represents one of the primary mechanisms by which petroleum and diesel products are removed from the environment inexpensively (Atlas, 1981; Floodgate, 1984; Leahy and Colwell, 1990; Lidderdale, 1993). One of the most important characteristics of hydrocarbon degrading bacteria is the ability of emulsifying hydrocarbons in solution by producing surface active agents such as biosurfactants (Hommel, 1990; Neu, 1996; Desai and Banat, 1997; Bredholt et al., 1998). Biosurfactants are directly involved in the process of hydrocarbon removal from the environment through increased bioavailability and subsequent biodegradation of the

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hydrocarbons by direct cell contact (Hommel, 1990; Leahy and Colwell, 1990; Deleu et al., 1999; Banat et al., 2000). Addition of biosurfactants increases the availability of long chain hydrocarbons to microbes and renders them more accessible to microbial enzyme system for utilization (Banat et al., 2000; Hommel, 1990). In certain cases when intrinsic bacteria are unable to meet the required demand, specific degrader(s) that have been selected for degrading various hydrocarbons are used (bioaugmentation) (Thomassin- Lacroix et al., 2002; Vogel, 1996). However, inhibition of inoculum growth due to nutrient limitation and competition with indigenous microorganisms, poor availability of contaminants (because of their low water solubility) and the difficulties of mixing the inoculated organisms with the contaminated soil (Vogel, 1996) are the pitfalls of bioaugmentation.

The presence of oil degrading microorganisms such as bacteria and fungi is not restricted to a particular ecosystem and has been found in the Arctic, Antarctic and temperate region but little work has been reported in high temperate ecosystem (Prince, 1993). The ability of microorganisms to utilize hydrocarbons in oil contaminated environments has been documented (Atlas and Bartha, 1972; Atlas, 1981; Obuekwe et al., 2005). Microbial degradation process aids the elimination of spilled oil from the environment after critical removal of large amount of oil by various physical and chemical methods (Ljah and Okang, 1993). This is possible because microorganisms have enzyme system to degrade and utilize diesel oil as a source of carbon and energy (Ljah and Antai, 1998; Antai and Mgbomo, 1993).

The present work has been focused on this approach, aiming to isolate novel bacterial strains capable of petroleum hydrocarbon degradation *in situ* conditions. In this study, we report isolates capable of degrading a wide spectrum of hydrocarbons efficiently. Degradation studies to be carried out with different isolates at varying interval of time will help to find out the most potent hydrocarbon degrading strains, which can be used for any bioaugmentation studies during bioremediation.

2. Materials & methods

The diesel fuel used in this experiment was purchased from a local oil filling station and stored in dark at ambient temperature throughout the study. Before use the diesel was sterilized using 0.2μm membrane filter.

Oil contaminated Sea water, sediment and Soil sample (500g) collected from Oil Jetty, Paradip Port (Orissa) were used for isolation of hydrocarbon utilizing microorganisms. The samples were collected in pre-sterilized sample bottle following aseptic conditions. The samples duly labeled were stored at 4^0C for further analysis.

2.1 Medium used for Screening and isolation of diesel degraders

Microbes were isolated from seawater, sediment and oil contaminated soil by using the Bushnell Hass Mineral Salts (BHMS) medium comprising diesel oil in various concentrations. Bacteria were maintained on both liquid and solid mineral salt medium with diesel oil as the sole carbon source. The medium supplemented with 2% NaCl was used for the growth of marine bacteria. BHMS contained (per liter of distilled water) 0.2g of $MgSo_4$.$7H_2O$, 0.02g of $CaCl_2$, 1g of KH_2PO_4, 1g of K_2HPO_4, 1g of NH_4NO_3, 2 drops of $FeCl_3$ 60%. The pH was adjusted to 7.0-7.8. The bacteria were isolated by using an enrichment culture and a single colony isolation technique. The isolated cultures were preserved in Nutrient agar and Potato Dextrose agar slants and stored at 4^0C for further use.

2.2 Screening, Isolation and maintenance of diesel degraders

For screening 1gm of oil contaminated soil and sediment samples were separately suspended and vortexed in 10ml of sterile distilled water, 1ml of this sample was used as an inoculum for isolation of oil degrading bacteria. 1ml of sea water sample was directly used as an inoculum. 15 Erlenmeyer flask (250ml) was taken and 100ml of BHMS broth medium (Bushnell and Haas, 1941; Atlas and Bartha, 1992) was transferred to each flask and sterilized. 0.5% Diesel oil was used as the sole carbon source and incubated in shaker orbital incubator at 37^0C at 125rpm for 5days, 10days and 15days respectively for screening of diesel degraders. After respective days of incubation the diesel degradation was studied by gravimetric assay. All these screening experiments have done in triplicate. The average value of triplicate and standard error was calculated by Microsoft XL 2007.

According to the screening test the mixed culture that showing highest diesel degradation was taken for isolation of potent diesel degraders. The potent oil degrading culture (Mixed) was diluted (Serial dilution) one time (10^{-1}) to decrease down the microbial cell density by which single isolated colony will obtain. From the dilution 1ml of sample was used as an inoculum and spread over glucose supplemented BHMS-Agar plates containing 500µl of diesel oil in it. The plates were incubated at 37^0C for 72hours and observed. Pure representative colonies were isolated on the basis of colony morphology and the isolates were preserved in Nutrient Agar slants at 4^0C.

To study the efficiency test of the isolates, eight Erlenmeyer flask (250ml) were taken, 100ml of BHMS broth was added to each flasks and sterilized. 0.5% diesel oil was added to each flask and incubated for 5, 10, 15 and 20days respectively in shaker orbital incubator at37^0C at 125rpm. The diesel degradation was studied by gravimetric assay in desired time interval.

2.3 Study of diesel degradation

Diesel oil degradation was studied by gravimetric analysis (Chang, 1998, Marquez-Rocha et al., 2001). After desired interval of time, the flasks were taken out and bacterial activities were stopped by adding 1% 1*N*-HCL. For extraction of diesel oil, 50ml of culture broth was mixed with 50ml petroleum ether : acetone (1:1) in a separating funnel and was shaken vigorously to get a single emulsified layer. Acetone was then added to it and shaken gently to break the emulsification, which resulted in three layers. Top layer was a mixture of petroleum ether, diesel oil and acetone; clumping cells make a middle layer and the bottom aqueous layer contains acetone, water and biosurfactant in soluble form. The lower two layers were spread out while top layer containing petroleum ether mixed with diesel oil and acetone was taken in a preweighed clean beaker. The extracted oil was passed through anhydrous sodium sulphate to remove moisture. The petroleum ether and acetone was evaporated on a water bath. The gravimetric estimation of residual oil left after biodegradation was made by weighing the quantity of oil in a tared beaker.

The percentage of diesel oil degraded was determined as per the standard method. (Ganesh A., and Lin J., 2009).

2.4 Identification of diesel degraders

The most potent bacterial diesel degrader was identified by observing morphological characters, by doing several basic Biochemical tests and fatty acid profiling test (Balows et al., 1992). Different types of biochemical tests were done such as Gram's staining, Indole test, Methyl red test, VP test, Citrate utilization test, Urease test, Nitrate reduction test, Triple sugar iron test, Gelatinase test, starch hydrolysis test, Catalase test, Oxidase test and H_2S production test etc. in Biochemical test kit (HIMEDIA). Morphological features include cell morphology, colony morphology and structural appearance.

3. Results and discussion

3.1 Screening test

Among screening test experiments of soil (Table 1), sea water (Table 2) and sediment samples (Table 3) against 0.5% of diesel oil over various time intervals, soil sample inoculated flasks showed maximum percentage (53%) of diesel oil degradation after 15days in comparison to water and sediment samples (Figure 1).

Table 1: Percentage of oil degradation after different days of incubation with oil contaminated soil against 0.5% of diesel

Incubation period in days	% of degradation in Triplicate			Average	Standard error
5	46.7	51.84	49.8	49.39	±0.94
10	51.9	52.18	52.6	52.23	±0.35
15	54.3	53	52.8	53.37	±0.81

Table 2: Percentage of oil degradation after different days of incubation with oil contaminated water against 0.5% of diesel

Incubation period in days	% of degradation in Triplicate			Average	Standard error
5	7.65	8.59	8.42	8.22	±0.50
10	29.52	30.28	30.01	29.94	±0.39
15	31.3	30.76	30.8	30.95	±0.30

Table 3: Percentage of oil degradation after different days of incubation with Oil contaminated sediment against 0.5% of diesel oil

Incubation period in days	% of degradation in Triplicate			Average	Standard error
5	18.21	18.07	17.69	17.99	±0.27
10	32.76	33.39	33.25	33.13	±0.33
15	42.5	41.74	40.86	41.70	±0.82

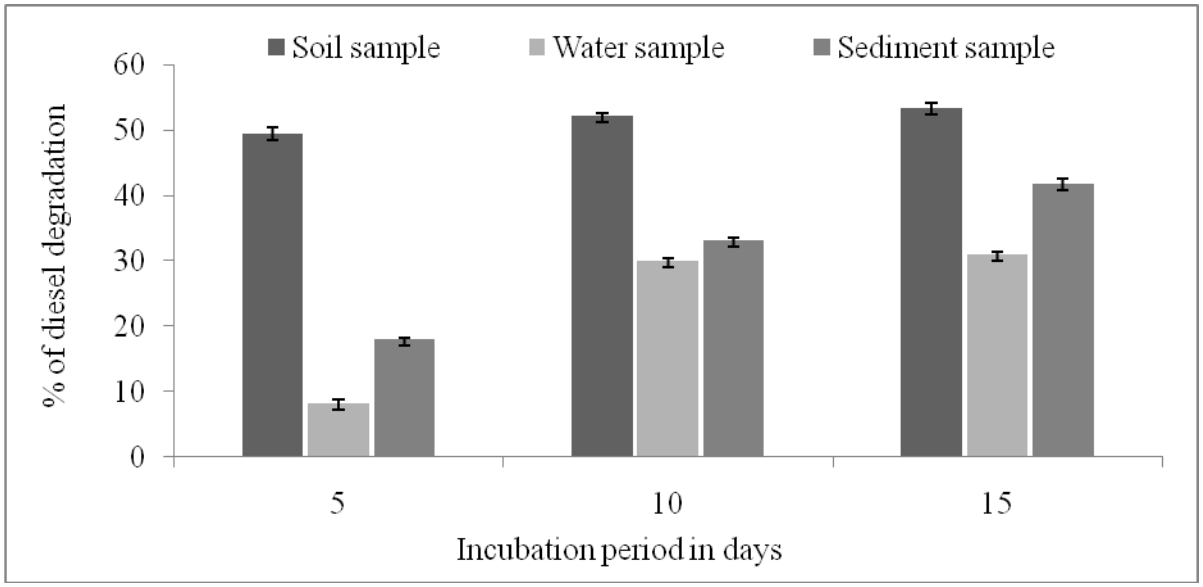

Figure 1: % of diesel degradation by microbial consortium present in three oil contaminated samples at different incubation period

3.2 Efficiency test of isolates

On the basis of screening test result, 15days incubated soil sample mix culture was selected for isolation of diesel oil degraders. After plating the mixed culture of soil sample over glucose supplemented BHMS agar plates, two microbial strains were isolated on the basis of their colony morphology i.e. one white color circular convex colony (strain-I) and one cream color, regular, sticky and convex colony (strain-II). The isolates were separately inoculated in BHMS broth containing diesel as sole of carbon source to test the oil degrading efficiency. According to the efficiency test strain – II was shown 49.93% of oil degradation and strain – I was shown 48.02% of oil degradation against 0.5% of diesel oil in 20days (Figure 2). Hence, according to the diesel degradation efficiency the potent oil degrader was identified.

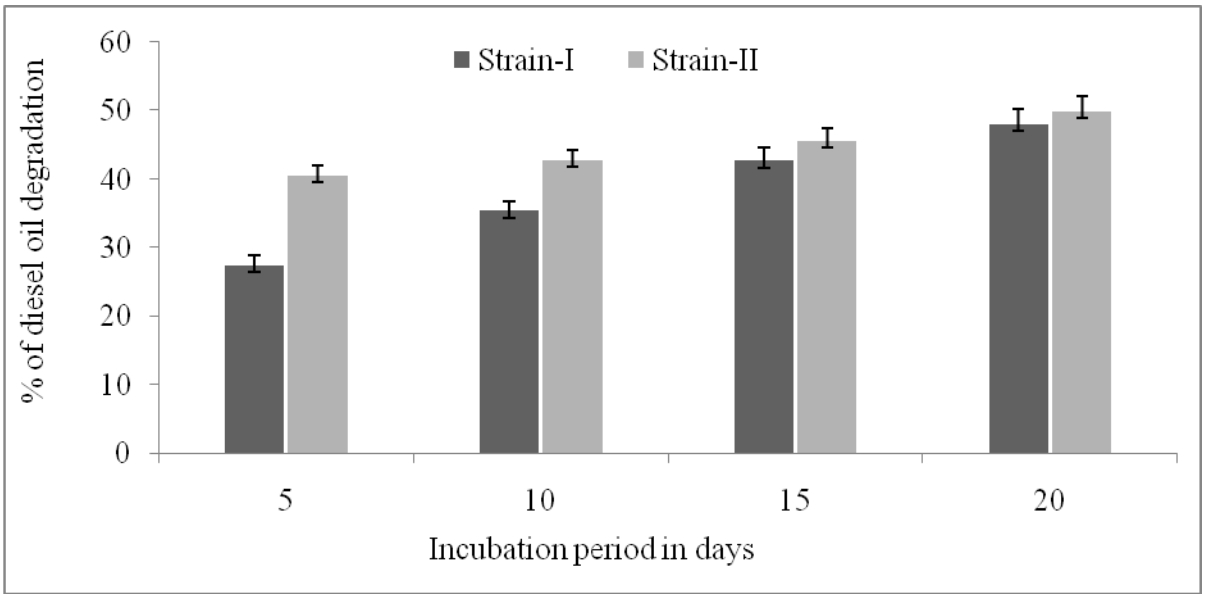

Figure 2: % of oil degradation by two isolates against 0.5% of diesel oil in various time intervals

3.3 Identification of the efficient oil degraders

Colony morphology- Microscopic observation reveled that the microbial colony was small in size, round, convex, regular in shape and the color of the colony was creamy. From the Morphological (colony & cell morphology) and Biochemical characterization (Table 4) point of view strain II was identified as Gram's negative rod shaped Bacillus *Pseudomonas sp.* and by fatty acid profiling test it was confirmed that the potent oil degrading bacteria was *Pseudomonas aeruginosa*.

Table 4: Biochemical test results of Strain-II (Bacteria)

Biochemical Tests	Results	Biochemical Tests	Results
Gram staining	Grams –ve (Rods)	Lactose	-ve
Catalase	-ve	Nitrate reduction	+ve
Indole	-ve	Adonitol	-ve
Citrate	+ve	H_2S gas production	-ve
Oxidase	+ve	Sorbitol	-ve
Urease	-ve	Glucose	+ve
Methyl Red	-ve	Lysine decarboxylase	+ve
VP	+ve	Glucorinidase	-ve
U.V Fluorescence	+ve	Inulin	-ve
Gelatinase	+ve	Esculin	-ve
Growth at 37^0C	+ve	Malonate	-ve
Motility test	+ve, (Motile)	Cellobiose	-ve
Kovac's oxidase	+ve	Rhamanose	-ve
Glucose dissimilation	Oxidative	Lysine decarboxylase	+ve

4. Conclusion

According to the biodegradation studies it was concluded that the *pseudomonas aeruginosa* had shown 49.93% of diesel oil degradation in 20days against 0.5% diesel oil. So *pseudomonas aeruginosa* is the natural occurring most potent oil degrading bacteria.

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