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RESEARCH **P**APER

Efficiency of bacterial isolates from oil contaminated soil for biodegradation of diesel

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Diesel degrading bacterial isolates Gh1 and Gh15 were isolated from crude oil contaminated soil, oil refinery of Guwahati (Assam, India) located at 26 ^{0} 11'0'' North, 91^0 44' 0'' East. Isolates showed optimized growth pattern at 35^{0}C, pH 6.5 and 100 ppm diesel using as the sole carbon and energy. Optical density and gas chromatography were used as evaluation experiment to check the degradation of aromatic hydrocarbons by strain. GC-FID chromatograms indicated the highest degradation efficiency of bacterial strains for aromatic hydrocarbons after 72 hours of incubation. This native microbial isolate could be considered as a powerful approach for the *in-situ* bioremediation of diesel contaminated soil.

Key words : Hydrocarbons, Bacteria, Bioremediation

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INTRODUCTION

Oil spill have become a universal problem particularly in industrialized and developing countries. Extensive hydrocarbon exploration activities often results in the pollution of the environment, which could lead to disastrous consequences for the biotic and abiotic components of the ecosystem if not restored (Jyothi *et al.,* 2012). Soil and water are often contaminated with diesel oil leakage from pipelines, storage tanks, accidental oil spills, etc. (Gallego Jose *et al*., 2001). Diesel fuel pollution affects the ecological and social catastrophes (Burger, 1993 and Burns *et al*., 1993) also exerts bio hazardous effects to the surrounding environment (Maola *et al*., 2002). A number of diesel spilled accident occurred in world for example, diesel spilled from an overturned lorry tanker in Seremban, polluting surrounding soils (The New Straits Times, 2000) whilst more than one ton of diesel was spilled into the soils in Gelugor, Penang from a 1,000 kw-mobile generator unit (The New Straits Times, 2001).

Diesel is complex petroleum hydrocarbon derivative their biodegradability depends on their bioavailability, which is directly related to composition, chain length (Gomes *et al.,* 2009). The native microbial strain of each environment are more efficient than the others because of their adaptability with the environmental conditions of the area such as temperature, pH, salinity etc. (Chang *et al.,* 2011). A number diesel degrading bacteria has been isolated from the oil contaminated soil and found to be potent degraders (Shukor *et al.,* 2009). Efficient diesel-degrading capacity of a locally-isolated bacterial consortium has been reported (Ghazali *et al.,* 2004 and Nisha *et al.,* 2013). For the proper exploitation of the bioremediation potential of indigenous microflora, the study of microbial diversity of site contaminated with petroleum hydrocarbons with a polyphasic approach become imperative. The parameters typically measured

in laboratory tests for bioremediation efficacy include enumeration of microbial populations, fate of hydrocarbon degradation. In present work, diesel degrading bacteria has been isolated that is able to grow in different concentration with minimum time to prevent direct and indirect adverse effect of diesel on environment.

RESEARCH METHODOLOGY

Collection of soil :

The soil sample was collected from the oil refinery of Guwahati. The soils were collected from the surface to a depth of about 0 to 5 cm with sterile spatula. Samples were then transported to laboratory under sterile conditions.

Isolation of bacterial isolates :

The bacteria were isolated by inoculating the soil sample on enrichment medium that contains the autoclaved mineral salt medium (MSM) supplemented with 1 per cent diesel. The medium contains K_2HPO_4 (1.8 g/lit); NH_4Cl (4 g/lit); $MgSO_4.7H_2O$ (0.2 g/lit); NaCl (0.1 g/lit); KH_2PO_4 (1.3 g/lit); carbon source (1% diesel); and distilled water (1L) with pH 6.5. The medium without hydrocarbons was sterilized by autoclaving at 121°C for 15 min followed by supplemented with 1 per cent (v/v) filter sterilized diesel and incubated at 37 ^{0}C for 5-10 days. After the incubation period, serial dilution-agar plating technique on mineral salt medium was carried out (Boboye *et al*., 2010). The isolated bacterial cultures were characterized by their morphological and biochemical characteristics (Cappuccino and Sherman, 2004).

Screening of isolates :

Bacterial cultures (24 hours old) were inoculated in mineral salts medium with the range (0 .006%-0.05 %) of diesel as a carbon source. They were kept in a shaker at 200 rpm at 35^{0}C for a period of 96 hours (Kim *et al*., 2001). The growth was monitored through culture densities, measuring the absorption at 595 nm, spectrophotometrically (Kumar *et al*., 2010). The isolate showed the maximum utilization (in terms of O.D. by spectrophotometer) at the concentration of 0.01 per cent of diesel. The isolates with highest rate of hydrocarbon utilization were selected for the further study.

Extraction of residual hydrocarbons :

For the extraction of secondary metabolites from the degradation of diesel by Gh1 and Gh15, mineral salt medium was prepared in three flasks (200ml in each) and inoculated with four ml of selected isolates with 100 ppm of diesel. These three flasks were subjected to treatment for 24, 48 and 72 hours, incubated in incubator shaker at 35^{0}C at 100 rpm. After desired interval of time, the flasks were taken out and bacterial activities were stopped by adding1 per cent N-HCL. For extraction of intermediates of diesel, culture broth was mixed with equal volume of petroleum ether and acetone (1:1) in a separating funnels and 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. The lower two layers were separated out while top layer containing petroleum ether mixed with hydrocarbon and acetone was taken out in a clean beaker. The extracted oil was passed through anhydrous sodium sulphate to eliminate moisture (Mittal and Singh, 2009).

Analysis of extracted diesel by GC :

Extracted diesel was measured by direct injection in to a gas chromatograph equipped with a FID and 30m x 0.25 um x 250 um (diameter) fused silica capillary column (BP-5). The nitrogen was carrier gas at a flow rate of 6psi and the sample size was 5µl. The injection and the detector were maintained at 250° C and the oven temperature was programmed to rise from 10° C/min to 280° C/min and to hold at 280° C for 10 min (Naggar *et al.*, 2012).

RESEARCH FINDINGS AND ANALYSIS

Both potent isolates Gh1 and Gh15 (Plate 1) were found to be Gram positive bacilli, this corroborates with the previous reported by Jyothi *et al.* (2012) where who have stated that hydrocarbon contaminated site is dominated by Gram positive bacteria.

Growth study :

Bacterial growth in medium was evaluated by measuring the light transmittance in liquid medium spectrophotometrically based on the increase of bacteria. Screening of these isolates was done by measuring the O.D. at 600nm in presence of diesel as a substrate at graded concentration (60-100 ppm). On the basis of growth it was found that isolate Gh1 (Fig.1a) and Gh15 (Fig.1b) have the ability to utilize the diesel up to 100 ppm as a source of carbon and energy. Morphological

Plate 1 : Microphotograph of bacterial isolates Gh1 and Gh15 (12Mpx)

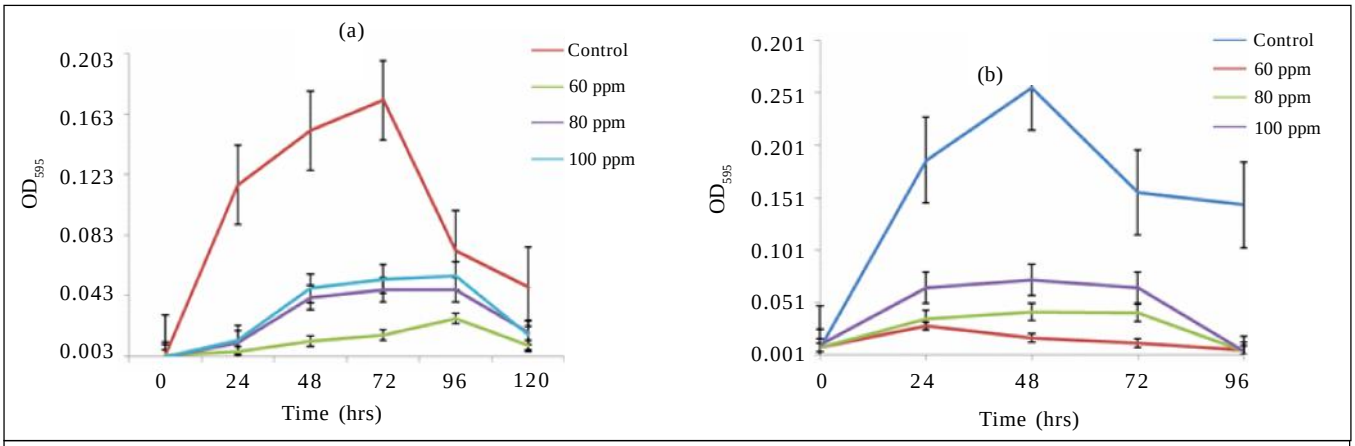

Fig. 1 : Growth pattern of Gh1 (a) and Gh15 (b) in presence of graded concentration of diesel

identification (Table 1) and number of biochemical tests of selected isolates were carried out.

Table 1: Morphological and biochemical characteristics of isolated bacteria

Features	Gh1	Gh15
Gram stain	+	+
Shape	Rod	Bacilli
Catalase	+	+
Citrate	-	-
Amylase	-	-
Urease	-	+
Hydrogen sulphide	-	-

Biodegradation of hydrocarbon using gas chromatography :

The ability of utilizing diesel as a carbon source was achieved quantitatively by GC-FID the efficiency of degradation was indicated by calculating the biodegradation efficiency percentage (%) of hexane extract of diesel. The GC profiles evidenced that Gh1 and Gh15 on diesel resulted in a substantial disappearance of the fraction of substrates as compared to control. Degradation of diesel revealed that Gh1 and Gh15 exhibited the biodegradation efficiency (B.E.) 93 per cent and 90 per cent, respectively with in 72 hours. Growth studies shown previously also indicated that the end of log growth phase was reached in about 96 hours, indicating that diesel degradation efficiency is linked with cellular growth of isolates. Fig. 2 and Fig. 3 showed an almost complete removal of diesel components as seen from the reduction in peaks after 72 hours of incubation by Gh1 and Gh15, respectively.

Biodegradation efficiency of various individual strains of microbes ranges between 8 to 26 per cent in

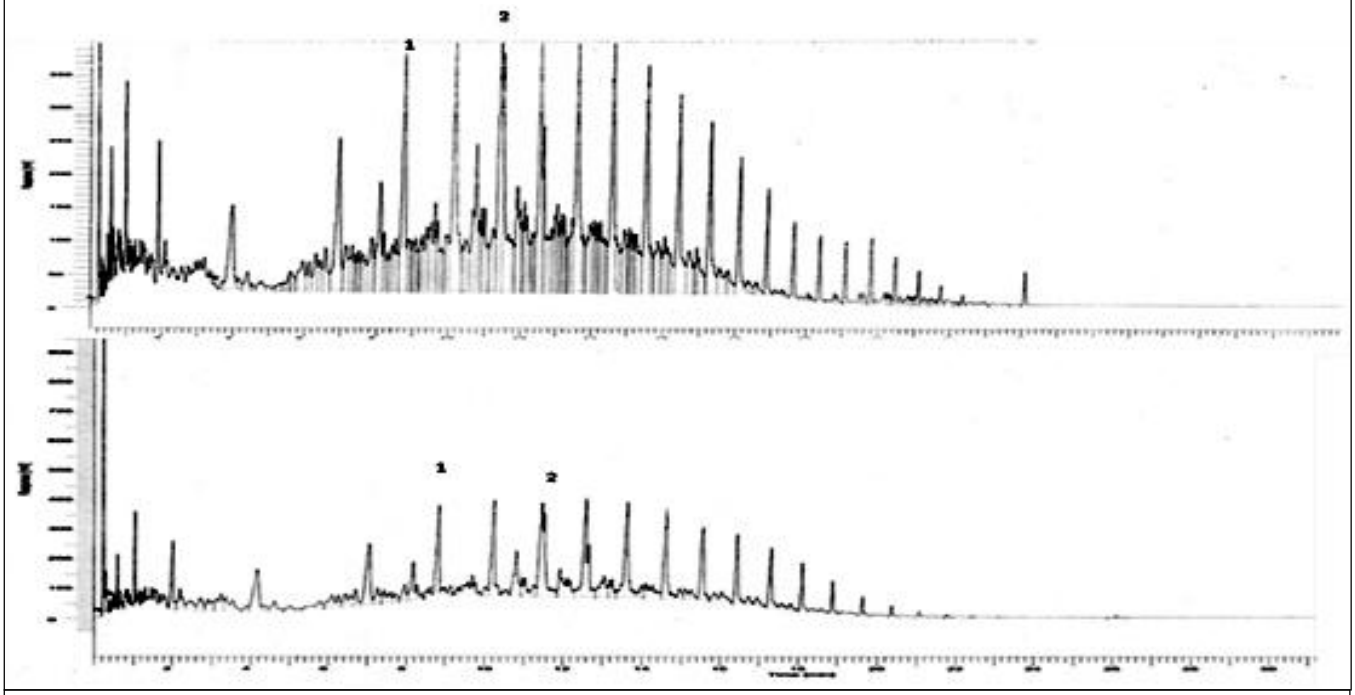

Fig. 2 : GC profiles of extracted diesel from aqueous phase of the medium after 72 hours of incubation with 0.01 per cent of substrate with and without inoculation of Gh1

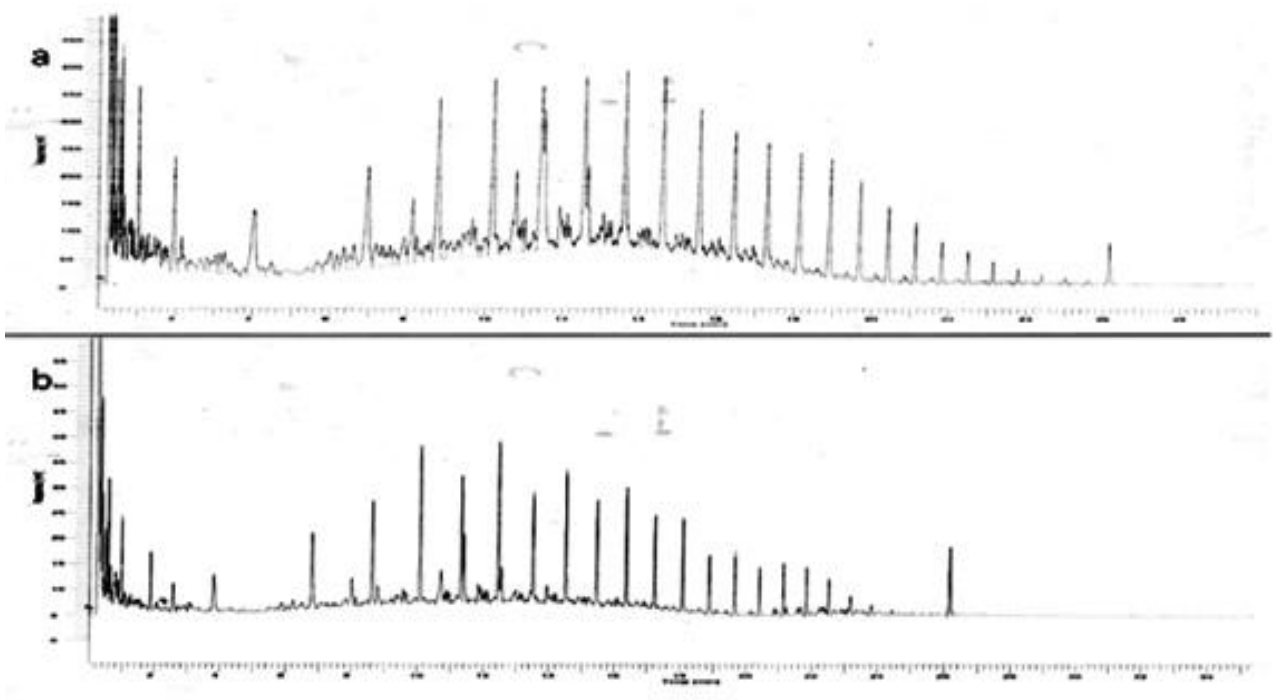

Fig. 3 : GC profiles of extracted diesel from aqueous phase of the medium after 72 hours of incubation with 0.01 per cent of substrate with and without inoculation of Gh15

10 days (Baryshnikova *et al.,* 2001). The biodegradation efficiency of two diesel-degrading arctic isolates shows a biodegradation efficiency of approximately 20 per cent on day ten for both isolates using 1 per cent diesel (v/v) as a carbon source (Michaud *et al.*, 2004). Diesel is used by bacteria as a carbon source upto only 1 per cent, above 1 per cent due to its solvent effect destroyed the cell membrane of bacteria (Rajasekar *et al.*, 2007). Ijah (1998) reported that a bacterium and a yeast isolate from tropical soils have BEs of 52 and 69 per cent, respectively, after sixteen days of incubation period. Lal and Khanna (1996) reported 58 per cent overall degradation of Indian crude oil samples by *Acinetobacter calcoaceticus* and *Alcaligenes odorans* over a fifteen day period. Dussan and Numpaque (2012) reported that bacterial strain TRI at 95 per cent. PRIII and TRIV isolates from coal mine also had high BE values of 70 per cent and 69 per cent, respectively, followed by TRIII with a BE of 52 per cent, and TRII with the lowest BE of 10 per cent for the biodegradation of diesel in 43 days.

Conclusion :

On the basis of above findings it can be concluded that Gh1 and Gh15 have shown high BE per cent in lesser time as compared to other reported work, so they could be a potential candidate in probable bioremediation of sites highly contaminated with petroleum hydrocarbons. It is also important to study chlorine degradation efficiency of isolates in future works since it is well known that in crude oil degradation, chlorine is accumulated in media as a byproduct which could inhibit bioremediation and also toxic for the environment.

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