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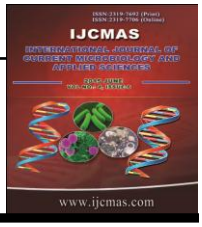

Original Research Article

Enrichment, Isolation and Identification of Hydrocarbon Degrading Bacteria

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A B S T R A C T

A preliminary study was undertaken to evaluate the capability of native bacterial strains to utilize the petroleum oils as the sole carbon source under in vitro conditions. Total of four isolated bacterial strains from the oil spill contaminated areas were assessed for their oil degradation efficiency. Two biodegradation experiments were performed in low and high (1% and 10%) concentration of crude oil for 21 days using selected bacterial cultures. At temperature 22 ^{0} C out of the four isolated strains, only one was demonstrated the maximum oil degradation capacity 66% and 58 %, respectively for two concentration of crude oil , after 21 days of incubation. Based on biochemical characterization, the isolate was identified as *Bacillus sp*p. The results of the present study demonstrated the isolation and identification of the bacteria from the contaminated oil spills, and its significance in biodegradation of oil spills and oil contaminated sites.

Keywords

Bacterial strains, Biodegradation, Crude oil, *Bacillus*

Introduction

Oil spills are one of the most damaging forms of water pollution, and is one of the significant problem in the industrialized and developing world today (Song H G and Bartha R, 1990). Biodegradation is being used as a treatment option at many hydrocarbons contaminated sites (Braddock at al 1997), which exploits the ability of microorganisms to degrade and/or detoxify organic contaminants.

Petroleum (crude) oil is complex mixture of many thousands of compounds. Petroleum hydrocarbons are the major constituents of crude oil (50-98%) and alkenes represent 20-50% of oil, depending on the source of the oil. Petroleum hydrocarbons can be degraded by microorganisms such as bacteria, fungi, yeast and microalgae. However, bacteria play an imperative role in hydrocarbon degradation. Microorganisms are endowed with metabolism machinery to use petroleum products as a carbon and energy source. The extent of biodegradation of hydrocarbons in contaminated sites is dependent on several factors such as molecular composition of the hydrocarbons, the type of microbial population, and optimal environmental conditions to stimulate the bioavability of the contaminants to microorganisms (Huesemann, 1995).

There are quite a few reports of isolation of petroleum hydrocarbon degrading bacteria from oil overlie areas (Kasai *et al*., 2001). Biodegradation of individual hydrocarbon compounds by pure bacterial strains has been studied in depth with their metabolic pathways (Tazaki, 2005, Das N. *et al*. 2011)). The ability to isolate high numbers of certain oil-degrading microorganisms from petroleum-contaminated sites is commonly taken as evidence that these microorganisms being the active degraders of that environment (Okerentugba and Ezeronye, 2003).

The aim of the present study was conducted to isolate and identify the bacterial population and access their crude oil biodegradation potential under *in vitro* conditions. Degradation studies carried out with the most promising isolates at predefine set up to explore the biodegradation capability of hydrocarbon degrading bacterial strain.

Materials and Methods

Sample collection

The oil contaminated soil samples were collected from oil spill site near seashore in Mumbai, India. The soil samples were collected in pre-sterilized sample bottles with aseptic conditions. The collected samples were labeled and stored at -4°C till further analysis.

Culture enrichment and isolation of hyrdrocarbon degrading bacteria (HDB)

Microorganisms were isolated by selective enrichment technique. The commercial petroleum (crude) oil used for enrichment and biodegradation experiments as the sole carbon and energy source for microorganisms. Bushnell-Hass broth was used in enrichment technique supplemented with hydrocarbon source. The broth medium containing soil and crude oil was incubated at 22ºC with orbital shaking (120 r/min). Enrichment of microbial culture was carried out in 300 ml erlenmeyer flasks containing 100 ml of media. The pH was adjusted to 7.3. The broth medium was sterilized by autoclaving (121 ºC for 20 min). The original soil sample (1 g) was added to 100 ml of broth medium containing 1 g of crude oil. At weekly intervals sample from primary enrichment (1/100 v/v) were transferred to the fresh Bushnell-Hass broth containing same hydrocarbon source. After six transfers, 10 ml of sample was removed aseptically and serially diluted with phenol dextrose broth (Beef extract-1gm, peptone -10gms, phenol red -0.018gms, dextrose-5gms, Nacl-5gms, distilled water-1000ml). The nutrient agar was used for isolation, enumeration and maintenance of pure strains. After serial dilution the diluted sample was spread on sterile agar plates. The inoculated plates were incubated at 37°C for 48 h. After incubation the plates were observed for bacterial growth.

Identification of the bacterial isolates

Morphological and biochemical characteristics of the all isolated strain were studied either on nutrient agar or in nutrient broth as described earlier (Claus D *et al*., 1986). Gram reaction, motility, shape and color of colony and acid / gas production from carbohydrates and sugars fermentations were performed as recommended by (Ventosa *et al*. 1982). Biochemical tests catalase, urease, oxidase activities, nitrate reduction, Indol production etc were tested as recommended by (Smibert and Krieg 1994). Based on the test results the preliminary identification of the isolated bacterial strain was done.

Preliminary screening and selection of efficient crude oil degrader

The hydrocarbon degraders which are stored as pure cultures on slants were subjected to its efficiency of crude oil degradation assay. The equal volume of culture (25µl) of each isolates were spot inoculated on sterile filter paper placed on a nutrient plates overlaid with 100 µl of petroleum crude oil and were incubated at 37°C for 48 h. The zone of clearance around the bacterial growth on nutrient agar plate with varying diameters was observed and recorded. Test for each bacterial strain isolated was performed in triplicate. The final zone of clearance was taken as an average of the three readings. The isolate with the maximum zone of clearance was subjected for further analysis.

Crude oil biodegradation assay

The most promising bacterial strains (I 3, and I 4) were evaluated for their crude oil biodegradation ability. Total of a six reactions were subjected in 500 ml Erlenmeyer flasks with 100 ml of nutrient broth with oil sample and culture was inoculated by transferring 3 ml of (about 2×10^8 cells/ml) of the each bacterial isolate. The biodegradation assays were performed in two low (1.0%) and high (10%) concentrations of commercial crude oil as the sole carbon and energy source. A set of six Erlenmeyer flasks were subjected for biodegradation assay including one control without addition of an inoculum. All flasks were incubated under aerobic condition with shaking on an orbital shaker (rotation speed of 120 rpm) at room temperature (22 ± 2 °C) for 21 days. Samples were removed periodically at 7, 14 and 21 day of incubation to assess the final concentration of the petroleum hydrocarbons, while the bacterial growth was monitored with viable counts on nutrient agar plates (Amund and Igiri, 1990).

Estimation of crude oil degradation

Estimation of crude oil degradation was done by gravimetric analysis as performed in earlier studies (Pirnik *et al*., 1974). The residual crude oil was extracted in a preweighed flask with petroleum ether in a separating funnel. Extraction was repeated 3-4 times to ensure complete extraction. After extraction, petroleum ether was evaporated in a hot air oven. The flask was cooled down in desiccators and weighed. The percentage of degradation was calculated and documented. Control and 21 day biodegraded test samples were also analysed by GC MS. The GC-MS analysis was performed with GC equipped with a cool-on-column inlet and capillary direct interface. The instrument conditions were as following: Capillary helium column with flow 1ml/min, pressure 18.5 psi and split ratio 20:1, The initial temperature was 70°C kept for 5mins with a temperature ramp of 14°C per minute and final temperature of 280°C kept for 10 minutes with total run time 30 minutes.

Results and Discussion

Four microorganisms were isolated (I-1 to I-4) by selective enrichment technique from petroleum contaminated-area obtained from the oil spill sites near seashore in Mumbai, India. The culture enrichment method was adapted to isolate the hydrocarbon degrading bacterial strains. Bushnell-Hass broth was used in enrichment technique supplemented with hydrocarbon source. The original soil sample (1 g) was added to 100 ml of broth medium containing 1 g of crude oil. After predefine transfers and incubation of six week, the enriched culture sample was serially diluted and transfer on nutrient agar plates. After 48 h the colony morphology and characteristics were documented. Pure cultures were maintained by transferring representative colonies on nutrient agar

slants as hydrocarbon degrading bacterial isolates (HDB).

All four bacterial isolates I-1, I-2, I-3 and I-4, respectively were subjected for a preliminary assessment of their crude oil degradation efficiency. After 48 h of incubation, on nutrient plates overlaid with 100 µl of petroleum crude oil, the zone of clearance were observed as shown in figure 1. Each bacterial isolate the average zone of clearance was observed as I-1 (10mm), I-2(7.67mm), I-3(9mm), I-4 (11mm), respectively. Among all the isolates the strain I-4 was observed with the maximum oil degradation ability.

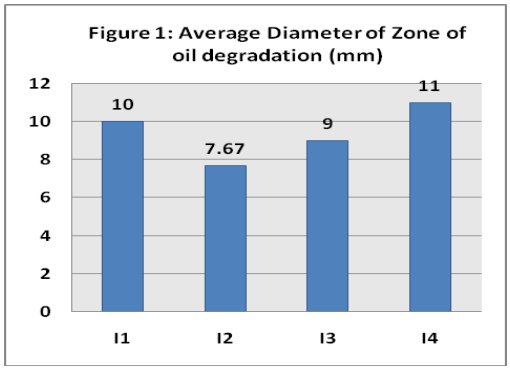
Each hydrocarbon degrading bacterial strain (HDB) was subjected for the Gram-reaction, and colony, biochemical and physiological characteristics studies. The results of each of the biochemical test were recorded after incubation along with the colony characteristics. Results obtained were summarised below in Table 1. The results of the biochemical tests performed were analysed based on the Bergey's manual of systematic bacteriology for identification of each bacterial isolate. Out of the four bacterial isolates three were Gram negative while one isolate I 4, was Gram positive bacilli. As represented in the Table 1 the strains earlier labeled as I-1, I-2, I-3 and I-4 were identified as *Enterobacter* sp. and *Bacillus* sp., respectively. These strains will be identified further using 16s rRNA sequencing.

Estimation of crude oil degradation by gravimetric analysis for the two isolates at two different oil concentrations was represented in Figure 2. The high oil degradation rate, almost 66% was observed in a reaction with low (1.0%) concentration of the oil where as the high (10%) concentration oil was observed with low 58% rate of oil degradation at the end of the assay reaction with bacterial isolate I 4. The bacterial isolate I 3 showed the less oil degradation capability, almost 52% was observed in a reaction with (1.0%) concentration of the oil and 47 % oil degradation with high (10%) concentration oil at the end of the assay reaction, respectively. Both the isolated bacterial stains I 3, and I 4 confirmed promising oil degradation ability even at high oil concentration.

The Gas Chromatography analysis of the oil degraded sample with the observed Gas Chromatography data profiles confirmed the maximum oil degradation (66%) with the test sample with Isolate 4 (*Bacillus* sp.) as compared to that of control sample. This confirms that biodegradation of hydrocarbon was exhibited by this isolates, however the type of hydrocarbon being degraded was not being investigated under this study.

Biodegradation has been considered as efficient, economic, versatile and environmentally sustainable treatment. Degradation of petroleum hydrocarbons by environmental micro flora involves microorganisms having specialized metabolic capacities. It has also been a known fact that bacteria are the most predominant microorganism among other microorganisms in biodegradation of environmental pollutants. The ability of native bacteria to utilize crude oil as the sole carbon and energy source was investigated in this study. This paper describes the study on the isolation and characterization of petroleum (crude) oil-degrading bacterium from contaminated oil region. Out of the total four bacterial isolates, only one isolate was confirmed as a promising oil degradation capability and was identified as (*Bacillus* sp.). In conclusion, the selected bacterial isolates could be effective in clearing oil spills or oil contaminated soils.

Fig.1 Average diameter of zone of oil degradation (mm)



(I-Bacterial isolate)

Fig.2 Estimation of oil degradation

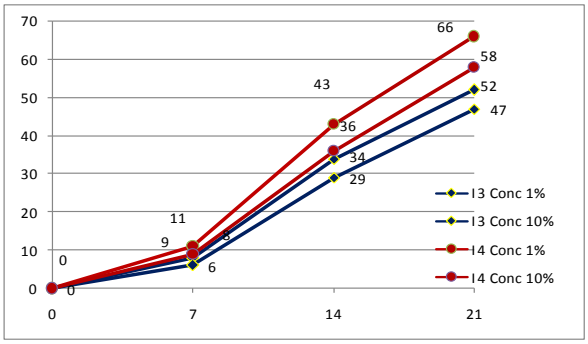


Fig.3 GC MS Spectra Control

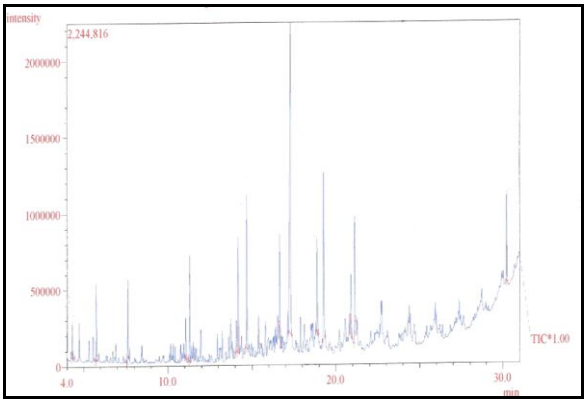
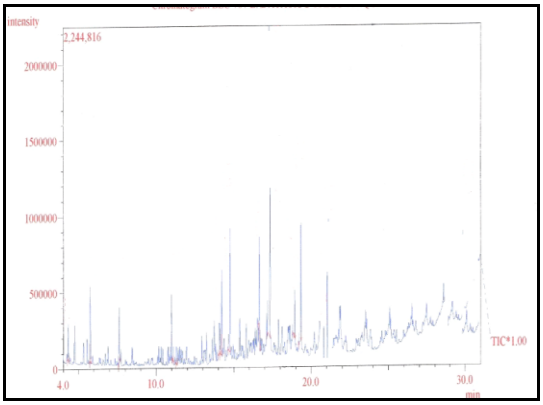


Fig.4 GC MS Spectra Sample (I 4)



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