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Screening and Isolation of Gasoline Degrading Bacteria by Enrichment Technique

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Abstract: Gasoline a, major petroleum hydrocarbon used frequently in automobiles is a complex mixture of aliphatic, aromatic and allicyclic hydrocarbons. The general population is exposed to gasoline or gasoline vapors during automobile refueling procedures, refueling of gasoline-powered equipment and using the gasoline-contaminated surface water or groundwater. Biodegradation of gasoline holds a promising approach for handling the problems due to gasoline pollution. In the present study the soil samples from the regions around petrol stations and petroleum refinery industry located at Mangalore, Karnataka, India were collected and subjected to screening by enrichment technique. Two Gram-negative bacilli C1 and C3 were isolated from the soil samples of petrol filling stations. The gasoline biodegradation was confirmed by the gas chromatography technique. Both the organisms showed significant reduction in the peak area as compared to that of control. Both the organisms showed a good adherence to hydrocarbons.

Key words: Gasoline · Biodegradation · Enrichment · Screening · MIC · Growth kinetics · Adherence

INTRODUCTION

Petroleum hydrocarbon is a natural product resulting from anaerobic conversion of biomass under high temperature and pressure. Once purified, it is extensively used as gasoline for the automobile and combustion purposes. In nature, it is subjected to biodegradation, but at a slow rate because of its structural complexity. Microbial degradation is becoming popular as they can utilize complex chemicals as carbon sources for their growth. Enrichment of samples with the compound of interest results in better screening of the microorganisms. Various reports on enrichment isolation indicate that inducing the growth of microorganisms in the presence of the target compound results in the isolation of organisms with desired characteristics [1]. Petroleum hydrocarbon is a very complex carbon source and its biodegradation is slow and complex process [2]. Hence several approaches of biodegradation are considered. One of the approaches gaining importance is the use of biosurfactants. These biosurfactants enable a physical contact between the microbes and the hydrocarbons by breaking the interfacial tension between the same, hence aid the biodegradation. In the present study we discuss the isolation of organism degrading gasoline by enrichment method, their growth kinetics in gasoline substituted medium, biodegradation of gasoline and adherence of the microbes to gasoline.

MATERIALS AND METHODS

The chemicals used were the analytical grade from Merck, India Ltd. Gasoline was obtained from Sapthagiri Petrol filling Station, Mangalore, Karnataka, India.

Screening by Enrichment Technique: The soil samples were collected from sites namely A-Rhizosphere region (plant root exudates comprise of phenolic compounds which, are very similar in their composition to that of petroleum hydrocarbons and the organisms found in this region are expected to degrade the same), B-control (Soil free from traces of gasoline), C-petrol filling station and D-petrol refining industry. 10g of surface soil sample was

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Table 1: Modifications of Mineral Medium

Medium	Major ingredients	Concentration of Gasoline (%)	Concentration of Glucose (g/L)
MM1	Mineral nutrients, gasoline, glucose.	1	8
MM2	Mineral nutrients, gasoline, glucose	1	2
MM2c	Mineral nutrients, glucose	0	2
MM3	Mineral nutrients, gasoline	1	0
MM3c	Mineral nutrients,	0	0

collected to a depth of 10 cm from each site using sterile spatulas, transported in sterile polythene bags to the laboratory and stored at 4°C until used for analysis. The soil samples collected were subjected to screening by enrichment technique. For enrichment, each of the soil sample (10g) collected was suspended in 100ml of mineral salts medium (MM) [3] modified as MM1 (Table 1) and incubated at 32°C for 30 days in an incubator shaker (Rotek, India) at 150rpm. Isolation of organism was carried out at the interval of two and four weeks. The organisms selected from MM1 agar based on the colony characteristics were screened in MM2, MM2c, MM3 and MM3c broth (Table 1). The conical flasks were incubated at 32°C for one week. The growth of the organisms was determined by standard plate count method [4].

Determination of Minimal Inhibition Concentration (MIC) of Gasoline: Twenty four hours culture of the selected organisms were inoculated into conical flasks with 100 ml of MM2 broth modified by adding gasoline at the concentration ranging from 0.1% to 5% and was incubated for one week at 32°C in an incubator shaker rotating at 150rpm. The growth of the organism was determined by standard plate count method.

Growth Kinetics in the Presence and the Absence of the Gasoline: Twenty four hours culture of the selected organisms C1 and C3 was inoculated into conical flasks with 100ml of MM2 broth (Table 1) with and without gasoline and maintained at 32°C in an incubator shaker rotating at 150rpm. The growth profile was determined by dry weight method [4] at the interval of two days for two weeks.

Assessment of Gasoline Biodegradation by Gas Chromatography (GC): The sample extraction and GC was performed as according to [2]. In brief, twenty four hours culture of the organisms was inoculated into conical flasks with 100ml of MM2 and MM2c broth and maintained at 32°C in an incubator shaker rotating at 150 rpm. The samples were extracted for GC using dichloromethane (DCM) at zero time and at regular intervals of two days for two weeks.

Quantification of Gasoline Biodegradation: The quantification of percentage degradation of gasoline by the organisms was determined by UV spectrophotometric method as per the protocol of Environmental Protection Agency (EPA)*. Concentration of the oil Cx (Total mass of the oil in the flask at λx = 228, 260, 280, 300, 320 and 340nm) was calculated using the equation 1 and 2.

$$Rfx = C/Ax \quad (1)$$

Rfx = Response factor at λx
C = Standard oil concentration, in mg of oil/ml of DCM
Ax = Spectrophotometer readings at λx

$$Cx = (Ax)\times(RFx)\times(VDCM)\times(Vtw/Vew) \quad (2)$$

Cx = Concentration of oil
V_{DCM} = Volume of DCM extracts (50ml)
Vtw = olume of mineral medium in the flask (100ml)
Vew = Volume of mineral medium used for extraction (100ml)
Vtw/Vew = 1

Bacterial Adherence to Hydrocarbons (BATH): The BATH test was done according to Rosenberg *et al.* [5] method. In brief, cells were washed twice and suspended in phosphate buffer to an initial absorbance at 600nm of 0.6 optical density (O.D.) and mixed with hydrocarbon and vortexed and the phases were allowed to separate. The absorbance of the lower aqueous phase of each tube was measured at 600nm. Adherence was calculated as the percent loss in absorbance relative to that of the initial cell suspension using equation 3.

BATH index =
1-OD600 after mixing/OD600 before mixing (3)

* EPA App C, pt 300

RESULTS AND DISCUSSION

Screening by Enrichment Technique: Enrichment of the soil samples for four weeks resulted in the isolation of microorganisms, which could grow in the presence of gasoline. All the samples showed a hundred fold decrease in the number of colonies in the four weeks isolates compared to two week isolates (Table 2). This indicates increase of only those organisms which are tolerant to gasoline. This confirms the successful isolation of desired organisms by enrichment technique. The samples C and D showed a three fold and two fold increase respectively in the CFU/ml value compared to samples A and B. Total nine colonies were selected based on the colony characteristics. Screening of these nine colonies (Table 3) in different MM broths resulted in the selection of C1 and C3 as these showed nearly two fold increase in the medium containing gasoline (MM2 and MM3) compared to medium without gasoline (MM2c and MM3c). Reports state that successive subculturing of the organisms in mineral medium; the culture becomes incapable of growing in the absence of the target compound [6]. In our study we found that growth was taking place in MM2c and MM3c very slowly at a negligible rate. The growth in MM2 and MM3 were almost the same. Hence for further studies the MM2 medium was used. This is because of the presence of an easily utilizable carbon source like glucose enhances the growth and reduces the lag phase [7]. Since gasoline is a complex carbon source, its biodegradation is likely to be by cometabolism process [8] in which the microbe degrades the target compound while utilizing the simple carbon sources for its growth. Gram staining studies showed that C1 and C3 are Gram-negative rods and hanging drop motility test showed that they are motile in nature. Gram negative bacteria have been reported to be involved in the biodegradation of the hydrocarbons more than the Gram positive bacteria [8-10]. This may be due to the surface structure complexity of the Gram negative bacteria.

Minimal Inhibition Concentration of Gasoline: C3 bacteria showed growth from 0.1% to 3.5% gasoline concentrations (Fig. 1). Maximum growth was found at the concentration of 2.5% and the growth was inhibited at the concentration of 4%. Maximum growth of C1 was seen at 0.1% though growth was observed till 1%. Growth of C1 was inhibited at the concentration of 1.5%. Growth of organism was observed in a mineral salt medium containing 20% naphthalene [11]. Report suggests inhibition of growth of bacterium at 6% of crude oil [12]. However, no reports were obtained on the effect of different concentration of gasoline on the growth of the organisms.

Growth Profile and Kinetics of Bacteria in Gasoline: The kinetics of the growth in the petroleum component has been reported by Serena *et al.* [2]. The report shows the kinetics of the degradation in an artificially synthesized

Table 2: Effect of Enrichment on the Isolation

Sample	Medium	Duration of enrichment	CFU/g#
Sample A	MM1	2 weeks	10^5
		4weeks	10^3
Sample B	MM1	2weeks	10^5
		4 weeks	10^3
Sample C	MM1	2weeks	$3x10^5$
		4 weeks	3×10^3
Sample D	MM1	2weeks	2×10^5
		4 weeks	2×10^3

CFU/g-colony forming unit/g of soil sample
A: Rhizosphere region, B: Control, C: Petrol filling station, D: Effluents from refineries

Table 3: Screening of Bacteria in MM2 and MM2c

Sample	CFU/ml in MM2	CFU/ml in MM2c	CFU/ml in MM3	CFU/ml in MM3c
C3	3.7×10^6	1×10^5	3.5×10^6	1×10^5
C1	1.9×10^6	2×10^4	2×10^6	1.9×10^4
A1	1.5×10^3	1.5×10^3	10^3	10^2
A2	1.8×10^3	2.7×10^3	1.5×10^3	10^3
B1	2.5×10^2	10^2	--	--
D3	2.5×10^4	2×10^3	1.5×10^3	1×10^3
C2	1.5×10^4	2×10^3	2×10^3	1×10^3
D1	1.8×10^3	1×10^3	3×10^2	10^2
D2	2×10^3	2×10^3	2×10^2	10^2

A: Rhizosphere region B: Control, C: Petrol filling station, D: Effluents from refineries

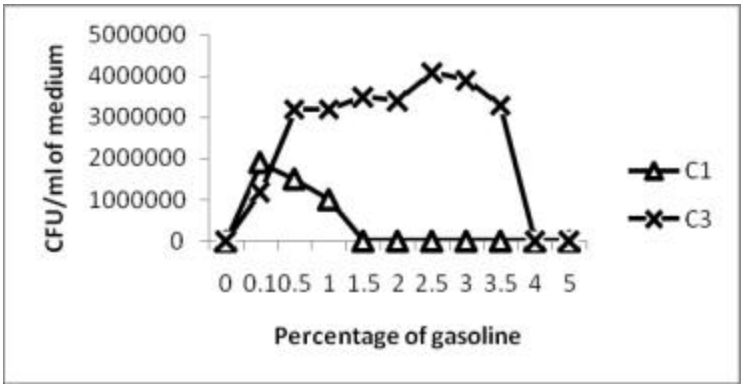

Fig. 1: Minimal Inhibition Concentration of Gasoline for C1 and C3

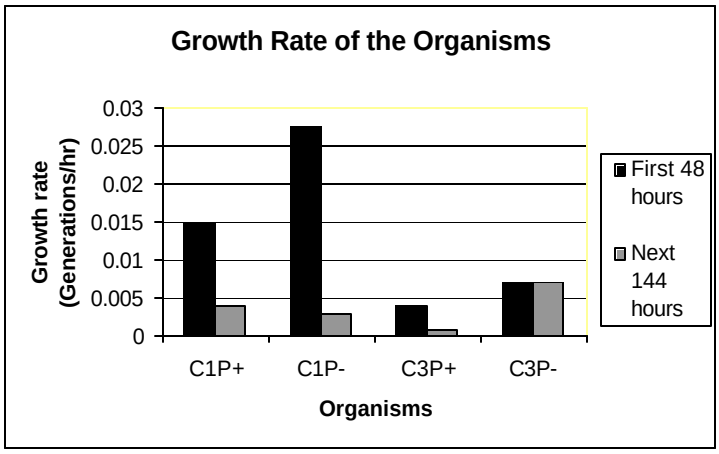

Fig. 2: Growth profile of C1 and C3

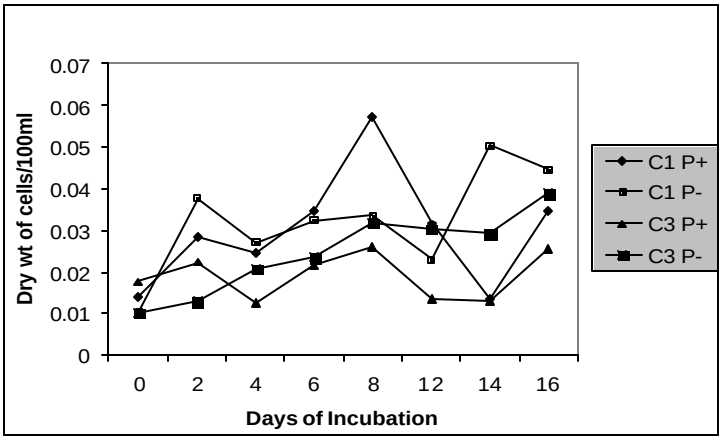

Fig. 3: Growth Rate of C1 and C3

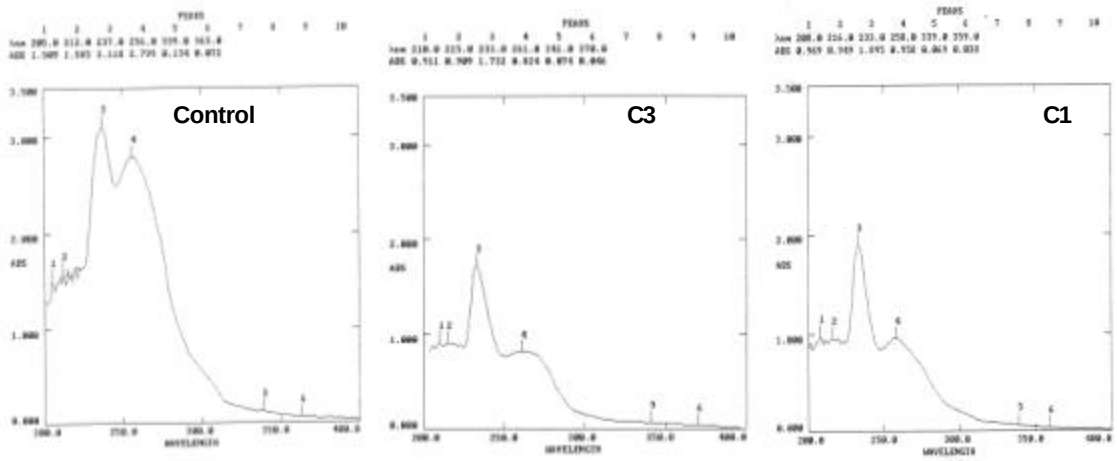

Fig. 4: UV Spectrophotometric Quantification of Gasoline Biodegradation
C3 and C1 Treated samples show reduction in the peak area very clearly compared to that of control

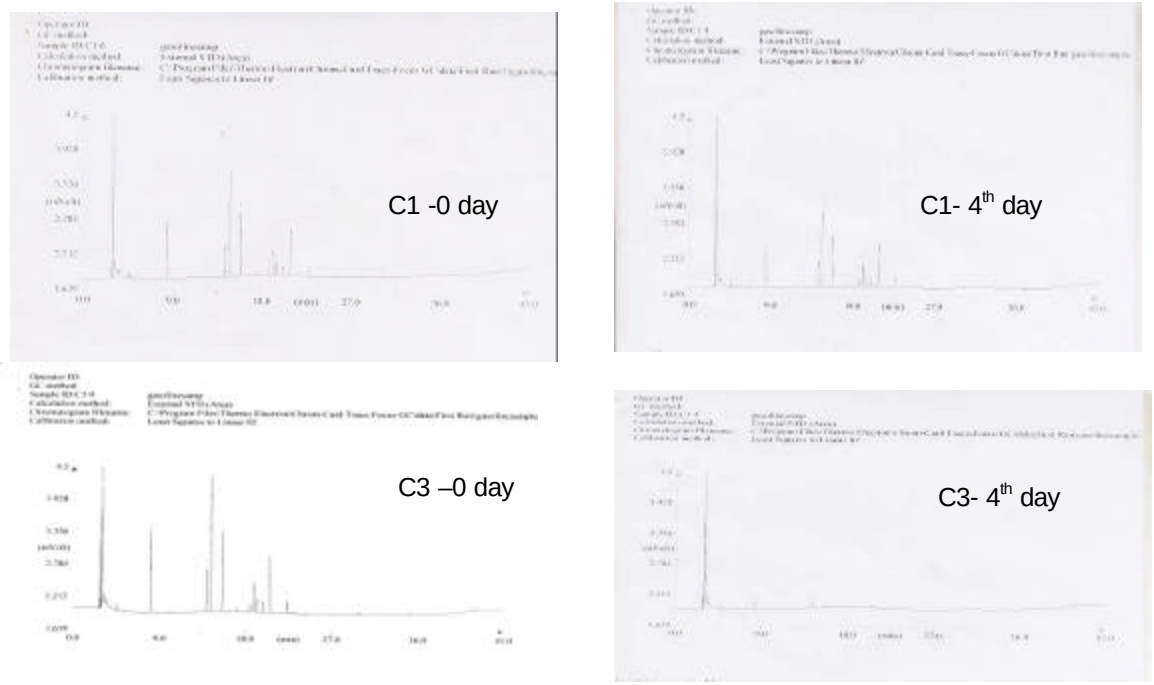

Fig. 5: GC analysis of Biodegradation

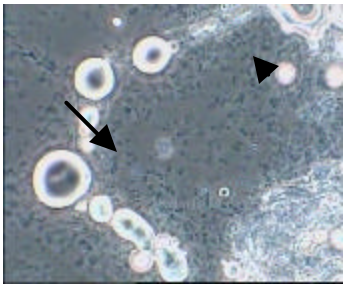

Fig. 6: Phase contrast Microscopic Images of Bacterial Adherence to Hydrocarbon (1000X)

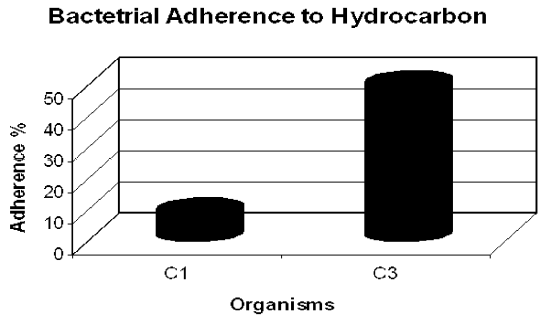

Fig. 7: BATH Test

gasoline consisting of the 23 components in an activated sludge process. The present study makes an attempt to analyze the growth kinetics of the organisms in whole gasoline in liquid cultures. The whole gasoline consists of more than 200 components and it is not easy to assess the biodegradation of the whole gasoline [2]. In the present study the growth profile and the kinetics of the growth of the organisms was studied. The bacteria are known to exhibit a diauxic growth when grown in the presence of complex carbon sources like naphthalene [11]. In the present study the organism C1 clearly demonstrate a diauxic growth (Fig. 2). Organism C1 showed an increase in the growth for the first two days followed by a relatively stationary period for the next four days and again an increase in the growth for the next two days. The growth was observed to continue in the same pattern. C1 when grown in the absence of gasoline showed the regular sigmoid growth pattern. C3 showed an increase and decrease in the growth at regular intervals of time. The decrease in the growth may be due to the toxic byproduct during the biodegradation process. C3 in the absence of gasoline showed sigmoid growth pattern. There are also reports on *Bacillus* and *Proteus* exhibiting a typical Monads kinetics and sigmoid growth in presence of crude oil [13]. The growth rate is given in the graph (Fig. 3).

Determination of Percentage of Degradation of Gasoline: The UV spectrophotometric scan (Fig. 4) and GC results showed significant reduction of gasoline by fourth day (Fig. 5). Similar reduction in the peak area was reported by Serena *et al.* [2]. It is necessary to assess the efficiency of the bacteria to degrade the toxic component in terms of percentage degradation. Serena *et al.* [2] report 74% degradation of gasoline in two days by the micro flora in the activated sludge. There are reports on the degradation of the gasoline components like benzene, toluene, ethyl benzene, xylenes (BTEX) by 99.9% by the micro flora in a bioreactor [14]. Our study emphasizes the percentage of degradation by individual organisms. The organisms C1 and C3 show a significant reduction in the gasoline concentration. The organism C3 degrades around 60% of the gasoline by fourth day and C1 degrades 56% of gasoline by sixth day.

Bacterial Adherence to Hydrocarbons: The phase contrast microscopic image shows a clear adherence of microbes to the oil droplets (Fig. 6). In BATH test the organism C3 showed 50% adherence and organism C1 showed 9% adherence to the hydrocarbon (Fig. 7). The importance of cell adherence in the growth physiology of many bacterial species is well documented [5]. Tuleva *et al*. [15], reported an adherence of 60% to hexadecane. The bacterial surface being hydrophilic, its attachment to the hydrocarbon indicates a modification of the surface structure of the bacteria in the presence of hydrocarbons. Bacteria are known to produce biosurfactants which modify the outer hydrophilic lipopolysaccharides enabling them to adhere to the hydrophobic hydrocarbons [16, 17]. Biosurfactants hence are gaining importance in the biodegradation of the hydrocarbons [18, 19]. In cases in which the carbon or energy source is a water-insoluble material such as cellulose or chitin, cell adhesion can facilitate growth but cell contact is not an absolute requirement because extracellular enzymes can degrade these polymers into water-soluble substrates. However, the growth of microorganisms on hydrocarbons presents a special problem, since not only are hydrocarbons immiscible in water, but also their breakdown cannot occur extracellularly [5]. Biosurfactants are amphipathic molecules with a hydrophilic and a hydrophobic domain. Because of these traits, biosurfactants accumulate at interfaces, can form micelles, lower the surface tension and thereby enhance the solubility of poorly soluble compounds in water and enable the bacteria to utilize these hydrocarbons as nutrients [5, 20]. These surfactants not only have role in biodegradation of hydrocarbons but also play in role the removal of heavy metals like arsenic [21].

CONCLUSION

The enrichment of the soil sample successfully resulted in the isolation of organisms, which were tolerant to gasoline for longer durations and for higher concentrations. The organisms C1 and C3 selected after screening show-promising results in the biodegradation

of gasoline. The minimal inhibition concentration of gasoline was 1.5% and 4% for C1 and C3 respectively. The growth studies indicated that these organisms were capable of utilizing gasoline as the carbon source. The isolated organisms showed degradation up to 56 to 60% by first week. They also showed significant adherence to the hydrocarbons. One of the reasons for the adherence of bacteria to hydrophobic hydrocarbons is the biosurfactant production by microorganisms. Some biosurfactants can also stimulate the attachment to and/or detachment of bacteria from the surface of xenobiotics by influencing the hydrophobicity of the bacterial cell surface or the surface of the xenobiotic compound. These biosurfactants enhance the bioavailability of hydrophobic soil pollutants. By reducing the surface tension between water and hydrophobic surfaces the formation of emulsions of hydrophobic xenobiotics in water is enhanced, thereby increasing the growth surfaces for bacteria. The importance of bacterial adherence to hydrocarbons has gained increasing recognition in recent years. Experiments are in progress to characterize the biosurfactant components responsible for the adherent properties.

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