Isolation and identification of petroleum hydrocarbon degrading bacteria from oil contaminated soil samples

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Abstract
The present study was undertaken to isolate and characterize the hydrocarbon degrading microbes from oil contaminated soil samples in Coimbatore, Tamilnadu, India. Totally 113 bacterial isolates were obtained from 60 soil samples. Four were finally selected through secondary screening by an agar well diffusion method using Benzene, Toluene and Xylene (BTX) as carbon source. They were provisionally identified and found closely related to Bacillus cereus, Micrococcus spp, Pseudomonas aeruginosa and E. coli. These isolates selected based on the highest zone of the inhibition on agar well diffusion method. Furthermore, 4 potentials isolate were subjected to gravimetric analysis to determinate the degrading capacity of hydrocarbon degraders. in this study, Bacillus cereus were degrade the highest amount of BTX, especially Benzene was highly degrade and followed by Toluene. This study clearly demonstrates that Gram-positive bacteria are effective in BTX degradation.

Key words : Biodegradation, BTX, Bacillus cereus, Gravimetric analysis, PAHc

INTRODUCTION
Hydrocarbon pollution is widely recognized as a serious environmental problem since its not only serious damage to living things, it also causes adverse effect on the natural environment and ecosystem. Release of hydrocarbons into the environment, whether accidentally or due to human activities is a main cause of water and soil pollution. Hydrocarbon components have been known to belong to the family of carcinogens and neurotoxic organic pollutants [1]. All petroleum products are originated from crude oil whose major constituents are hydrocarbons. Crude oil is composed of complex mixtures of paraffinic, alicyclic and aromatic hydrocarbons [2]. Benzene, Toluene and Xylene (BTX) are major aromatic hydrocarbon in many petroleum products [3], which contaminated environment was hazardous to human and animals and decreases the agricultural productivity of the soil [4]. Prolonged exposure of BTX may cause the lung, heart, liver and kidney disease, bone marrow damage and benzene has been cause cancer [5]. These illnesses were affected by direct contact with the contaminated soil, vapors from the contaminants, and from secondary contamination of water supplies within and underlying the soil.

Multiple techniques have been developed to resolve the problem of petroleum pollution. Physical and chemical method to reduce hydrocarbon pollution is expensive than biological methods [6]. Among these procedures bioremediation is currently gaining importance. The biodegradation of crude oil by microorganisms is one of the primary ways to remove crude oil from contaminated area by use of microorganisms or by their enzymes [7]. Some types of bacterial isolates are able to degrade the hydrocarbons and use of them as a source of carbon and energy [8]. Among the different types of hydrocarbons, benzene is of major concern as it is a stable, water-miscible, highly mobile, poisonous, and cancer-causing aromatic compound. Successful degradation of benzene by microorganisms in an aerobic environment has been reported; however, under anaerobic conditions its rate of biodegradation is observed to be very slow and poor, but same time successful in aerobic condition [9].

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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 [9]; [10]. 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
Surface sediment samples were collected from oil contaminated sites in Coimbatore area. Surface sediment samples were collected over a period of one year at 60 different points in the oil contaminated site using a sterile stainless steel spatula. The collected samples were pooled and transferred to pre-sterilized, labeled, plastic container and transported at 4°C to the laboratory and maintained at 4°C until analysis.

Screening of bacterial isolates from oil contaminated soil
The serially diluted soil samples were spread on BHA plates overlaid with 100 μl of Hydrocarbon (Benzene, Toluene and Xylene) and were incubated at 25°C for 14 days. Any isolate which grow on BHA plate were confirmed as degraders. After, same colonies were inoculated into NA plates and incubate at 24hrs to 48hrs, which grow on the NA plates were confirmed as non hydrocarbon degrading isolates.

Screening of potential isolates
The confirmed isolates were inoculated into BH liquid media and incubated at 25°C for 7days. Prepare BH Agar plates and spread 100 μl of oil (Benzene, Toluene and Xylene), then prepare wells (8mm) and load 50 μl of broth culture and incubate plate at 37°C for 48hrs. The zone of clearance around the wells and measured the diameter of the zone.

Secondary screening by gravimetric analysis
To obtain more potent strains for crude oil degradation, secondary screening was performed by potential isolates. About 100ml of Bushnell Hass broth media was prepared in 3 different conical flasks and add one gram of hydrocarbon such as Benzene, Toluene and Xylene. Oil degrading isolates were added as an inoculums and the flask was incubated at 30°C for 7 days with 120rpm. After incubation, Diethyl ether was added to the flask and mix well. The complete mixed broth was poured into separate flask, which was left for at least 20 min. Collect the oil containing solvents from the upper portion of the flask and poured into pre weighted petriplate. After the complete evaporation of the solvent the plate was weighed. The estimation of residual oil left after degradation was made by the amount of oil in a pre weighted plate [11]. The percentage of oil degradation was calculated as {1-(Xo-X1)/Xo}100%(%)< where Xo- initial amount of crude oil, X1- amount of crude oil after degradation [12] and [13].

RESULTS AND DISCUSSION
Soil and water contamination with hydrocarbons caused extensive damage of the local system, this contamination are crisis to plants and animals. An efficient way of remediation the oil-contaminated sites could be employment of special microorganisms, such as bacteria, microalgae, and fungi [14] Bacteria are the most important microbes in this process because they break the dead materials into organic matter and nutrients [15]. Petroleum hydrocarbon is composed of carbon and hydrogen and many microorganisms have the ability to utilize the hydrocarbon as a sole source of carbon energy and these isolates are widely present in soil. In this present study, 113 isolates were observed from 60 oil contaminated soils, which were able to utilize BTX as a sole source of carbon and energy. The previous study of Marcelo [16] and Wang [17] were observed the hydrocarbon degrading bacteria with BTX containing BH media. In this study most of the isolates tolerate and grown in xylene containing media and followed by benzene (Fig.1). Totally 35% of isolates were observed from all 3 hydrocarbons containing media, which were subjected to biochemical studies for identification of species.
Among the 40 isolates, 44% of were belongs to gram positive and 32% were gram negative. In case of Gram negative, 4 types of genera were obtained and 3 were in gram positive (Fig.2). Quatrini et al. [18] and Navdeep et al. [19] 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 are more tolerant to high levels of hydrocarbons due to their resistance endospores. Recently in chennai, Mamitha et al observed a higher number of gram negative bacteria than gram positive bacteria, which were observed from oil contaminated sites.

**Fig 1. Screening of hydrocarbon degrading bacteria**

![Benzene, Toluene, Xylene](image1)

**Fig 2. Prevalence of hydrocarbon degrading bacteria**

![Bar chart showing percentage of isolates](image2)

**Fig 3. Determination of potential isolates by agar well diffusion method**

![Micrococcus spp, E.coli, Pseudomonas spp, Bacillus spp](image3)
Table 1. Gravimetric analysis for determine the potential of hydrocarbon degraders

<table>
<thead>
<tr>
<th>Isolates</th>
<th>Degradation of Hydrocarbons</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Benzene</td>
</tr>
<tr>
<td>E.coli</td>
<td>0.586</td>
</tr>
<tr>
<td>P.aeruginosa</td>
<td>0.363</td>
</tr>
<tr>
<td>Micrococcus</td>
<td>0.592</td>
</tr>
<tr>
<td>Bacillus cereus</td>
<td>0.648</td>
</tr>
</tbody>
</table>

In this current study, the next part of the investigation was agar well diffusion method. Among the 40 isolates, 4 were identified as potential isolates such as Bacillus cereus, Micrococcus spp, Pseudomonas aeruginosa and E. coli, these potential isolates obtained based on the highest zone of inhibition (Fig.3). Among the 3 types of hydrocarbons Benzene was highly suppressed by those isolates. In recently Amit and Chandra observed the potential of hydrocarbon degraders by agar well method. To date, genera belonging to Pseudomonas, Micrococcus, Bacillus cereus and E.coli have been characterized and reported in the literature as hydrocarbon degrading strains. In south India, Gomathi et al [20] also observed the crude oil degrading Pseudomonas spp from Cuddalore. Previous studies of Brindhalakshmi [21] also observed the Bacillus spp from oil contaminated area. In this study, 4 potentials isolate were subjected to gravimetric analysis for determine the degrading capacity of hydrocarbon degraders.

Gravimetric analysis

Among the 40 isolates 4 were degraded the 3 types of hydrocarbon. The results were tabulated in Table 1. As illustrated in this study, the Bacillus cereus was better than other isolates. This isolate was degrading the highest amount of BTX. Especially Benzene was highly degraded by Bacillus cereus and followed by Toluene. The anaerobic degradation of petroleum hydrocarbons by microorganisms has been occurring at negligible rates. The efficient degradation of toluene by a wide variety of microbes has been reported in aerobic environments [22].

In this study, Bacillus cereus was degrading the toluene under aerobic conditions. This method is simple and consumes the coast and very few authors only quantify the oil degradation ability of the isolates with aerobic method [23]. In this study, it was not used mixed hydrocarbon in the medium. For the reason, that previous study of Chang et al. [24] were not observed the better degradation, when using the mixed hydrocarbons. The Marcelo et al was demonstrated that the presence of benzene inhibited toluene and xylene degradation, irrespective of whether the microorganism grew in two or three component mixtures.

The wide varieties of aromatic compounds released into the environment through different human activities are metabolized by soil bacteria. This is of great importance in environmental cleanup technologies [25]. Degradation of hydrocarbons by environmental microflorae involves microorganisms having specialized metabolic capacities. In polluted environments, specialized microorganisms are abundant because of the adaptation of the microflorae to pollutants. It is evident from this study that, hydrocarbon degrading organisms are ubiquitous in the environment and they can be isolated from hydrocarbon polluted sites. It has also been shown that four bacterial strains isolated from contaminated soil. Further studies are needed to clarify their exact taxonomic position and explore their biodegradation potential for other polycyclic aromatic hydrocarbons.

REFERENCE


