

Exploration of Organic Compound Degradation Capability of Indigenous Soil Bacteria and their Consortium from Oil Refinery of Assam

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Abstract

Present study was focused on the biodegradation capability of indigenous bacterial culture and their consortium. On the basis of GC-FID, Consortium Qs₁₅ was found to be potent consortium for the degradation of petrol, toluene, xylene, diesel, benzene and hexane. Gene which is responsible for the degradation is present on the plasmid. The amplification and sequencing of the 16S rRNA gene showed, Consortium Qs₁₅ comprised of two *Bacillus* strains namely, *Bacillus sp* E₁₄ and *Bacillus sp* E₈₇. Experimental data reveals that Qs₁₅ proven to be better option to eradicate wide range of hydrocarbon via bioremediation technique.

Keywords: Hydrocarbon; Intrinsic; Indigenous; Consortium

Introduction

Spillage and discharges of hydrocarbons occurred due to storage, transportation, refining and processing of petroleum compound, these activities include leakage from oil pipelines, storage tanks, tanker leakage accidents, oil well waxing, overhauls of refineries and production of petrochemical equipment. Petroleum hydrocarbon is a crude mixture of alkane, cyclic alkane, alkene, and aromatic hydrocarbon, which are found to be highly toxic and carcinogenic in nature [1]. Many normal and extreme bacterial species have been isolated and utilized as biodegraders for dealing with petroleum hydrocarbons for bioremediation. This is also indicating that the joint action of multiple functional bacteria requires to achieve the best environmental purification effect for the remediation of petroleum hydrocarbon contamination [2]. The advantages of microbial communities are presented because there are a variety of catabolic genes in a bacterial consortium, and the synergistic effects of these genes are beneficial to achieve the purification of pollutants [3]. In their finding, it was concluded that due to the abundance of alk B and nah genes responsible for the catabolism of hydrocarbons consortium show the maximum degradation as compared to individual isolates.

Materials and Methods

Isolation of bacteria

The bacteria were isolated by using standard protocol of Xiao and Zhang (2011) in mineral salt medium (MSM) supplemented with 1% of each hydrocarbon such as, diesel, petrol, xylene, benzene, toluene and hexane) [4]. The flask was incubated at 37 °C on a rotatory shaker at 100rpm for 48hrs. Three successive sub-culturing were done with 1% hydrocarbon. 0.1ml of culture was plated and incubated at 37 °C for 48hrs from the third sub-culturing. After over incubation times pure colonies were isolated by using single colony isolation methods [5].

Screening of isolates

Exponentially growing and pure bacterial culture (24hrs old) were inoculated in mineral salts medium with 0.006%, 0.008% and 0.01% concentrations of selected hydrocarbons (benzene, diesel, hexane, petrol, toluene and xylene). They were kept in a shaker at 200rpm at 35 °C for a period of 96hrs. The growth was monitored at regular interval of 24hrs up to 96hrs through culture densities, measuring the absorption spectrophotometrically at 595nm [6].

Plasmid curing

To check whether the oil degrading gene are plasmid encoded or chromosomal encoded modified method of Trevors [7] was used for the curing of plasmid. The bacterial isolates Gh₁ and Gh₁₅ were cultured in 50ml MSM containing ethidium bromide (20mgml⁻¹) and incubated at 37 °C for 24hrs [7]. Thereafter, the broth was agitated to homogenize the content and 1ml of cultures was sub cultured 50ml MSM medium containing 100ppm of each hydrocarbon individually. The flasks were then incubated at 37 °C for 24hrs and the optical density was taken. Isolates that failed to grow on MSM medium were considered as cured.

Consortium preparation

A loop full of overnight grown culture was used to inoculate 25ml sterile mineral salt medium. The flasks were kept in a shaker at 200rpm for 24hrs at 35 °C. Equal volumes of culture broth from the selected isolates were used to prepare the bacterial consortium. For the preparation of consortium only those strain were selected which have the capability to degrade all the selected hydrocarbons. Selection criteria of hydrocarbon is based on diverse range of hydrocarbon such as aromatic, aliphatic and complex hydrocarbon due to which selected consortium will be used as potent agent for degradation of wide spectrum of hydrocarbons. This bacterial consortium (1ml) inoculated in 50ml mineral salt medium with 0.01% of different hydrocarbon. The growth was monitored through culture densities, measuring the absorption at 595nm, spectrophotometrically Boboye et al. [5] at regular interval of 24hrs [5]. Quantitative estimation of protein was also performed [8]. The nomenclature of consortium have been illustrated which are as follows (Table 1).

Table 1: The nomenclature of consortium.

Codes of Consortium	Combination of Isolates
Qs ₁₉	Gh1+Gh6+Gh7+Gh8+Gh9+Gh10+Gh13+Gh15+Gh16
Qs ₆₈	Gh6+Gh7+Gh8+ Gh9+Gh10 +Gh13+Gh15+Gh16
Qs ₆₇	Gh6+Gh7+Gh8+ Gh9+Gh10 +Gh13+Gh15
Qs ₆₆	Gh6+Gh7+Gh8+ Gh9+Gh10 +Gh13
Qs ₆₅	Gh6+Gh7+Gh8+ Gh9+Gh10
Qs ₁	Gh7+Gh8+ Gh9+Gh10
Qs ₇	Gh7+Gh8+ Gh9+Gh10 +Gh13
Qs ₈	Gh8+ Gh9+Gh10 +Gh13+Gh15
Qs ₉	Gh9+Gh10 +Gh13+Gh15+Gh16
Qs ₁₀	Gh10 +Gh13+Gh15+Gh16
Qs ₁₃	Gh13+Gh15
Qs ₁₃	Gh1+Gh8+Gh15
Qs ₁₅	Gh1+Gh13

Identification of the Members of Selected Consortium

The molecular identification of bacterial isolates in which the genomic DNA was extracted using standard protocol [9]. The 16S rDNA was polymerase chain reaction (PCR) amplified using universal primer pair, 968F (5'-AGAGTTTGATCMTGG-3') and 1541R (5'-TACCTTGTACGACTT 3') (White et al. 1990). PCR was performed with final volume of 50µl in thermal cycler (Mastercycler Nexus gradient, Eppendorf, Germany) with a final concentration of 1X standard buffer, 1.5mmol⁻¹ MgCl₂, 0.25µmol⁻¹ each primer, 0.25mmol⁻¹ dNTPs and 1.0U Taq DNA polymerase (Sigma-Aldrich, USA) and 25ng of template DNA. The PCR reaction conditions consisted of initial denaturation at 95 °C for 7 minutes, followed by 35 cycles of denaturation at 94 °C for 1min, annealing at 51 °C for 1min, extension for 1min at 72 °C, and a final extension for 10min at 72 °C for PCR products were analyzed on 0.8% agarose gel and visualized under UV transilluminator. From PCR products, unincorporated PCR primers and dNTPs were removed by using Montage PCR clean up kit (Millipore). Sequencing of PCR product was done in 3730XL automated DNA sequencing system (Applied Bio Systems, USA). 16S rDNA consensus sequence was used for Basic Local Alignment Search Tool (BLAST) analysis against nr database in the National Centre for Biotechnology Information (NCBI) GenBank. Aligning of sequence data were done using Clustal W (Thompson et al. 1994) and phylogenetic relationship among the strains were determined by neighbor-joining method using MEGA 6 software [10].

Gas Chromatographic Analysis of Extracted Hydrocarbons by Individual Isolates

The hydrocarbons (Benzene, hexane, toluene and xylene) were extracted from liquid cultures with acetone. The extracts were then measured by direct injection into a Gas chromatograph equipped with an FID and 30mx0.25umx250um (diameter) fused silica capillary column (PE-FFA8). Flow rate of nitrogen was 6psi and the sample size was 5µl. The injection and the detector were maintained at 230 °C, and the oven temperature was programmed to rise from 10 °C/min to 210 °C/min and to hold at 210 °C for 10min. For the analysis of diesel and petrol BP-5 column was used with the following dimension: 15mx0.35umx2.50um. Nitrogen was the carrier gas at a flow rate of 6psi. The injection port was set at 250 °C. The oven temperature was programmed from 10 °C/min to 280 °C, holding the temperature for 10min [11].

Results and Discussion

On the basis of turbidity, it was concluded only 10 isolates have the ability to utilize hydrocarbons as carbon source (Figure 1) Gh₁ and Gh₁₅ showed the better efficiency towards the higher concentration of selected hydrocarbons. To check whether this hydrocarbon degrading ability is plasmid encoded or not, plasmid curing process was done (Figures 2 & 3). Curing experiments demonstrated that cured isolates was unable to grow in hydrocarbons supplemented media. Therefore, biodegradation

potential shown by the strain is plasmid mediated. Catabolic pathways involved in aromatic hydrocarbon degradation routes are located on large plasmids in most of the cases although degradative genes can be located on either chromosome or plasmid or on both [12]. The TOL plasmid pWWO of *Pseudomonas putida* mt-2 is a

117-kilobase (kb) catabolic plasmid which has ability to encode all enzymes necessary for bacterial utilization of toluene (Tol), m- and p-xylene (Xyl), 3-ethyltoluene, and 1,2,4-trimethylbenzene and carboxylic acid derivatives, via a meta cleavage pathway [13].

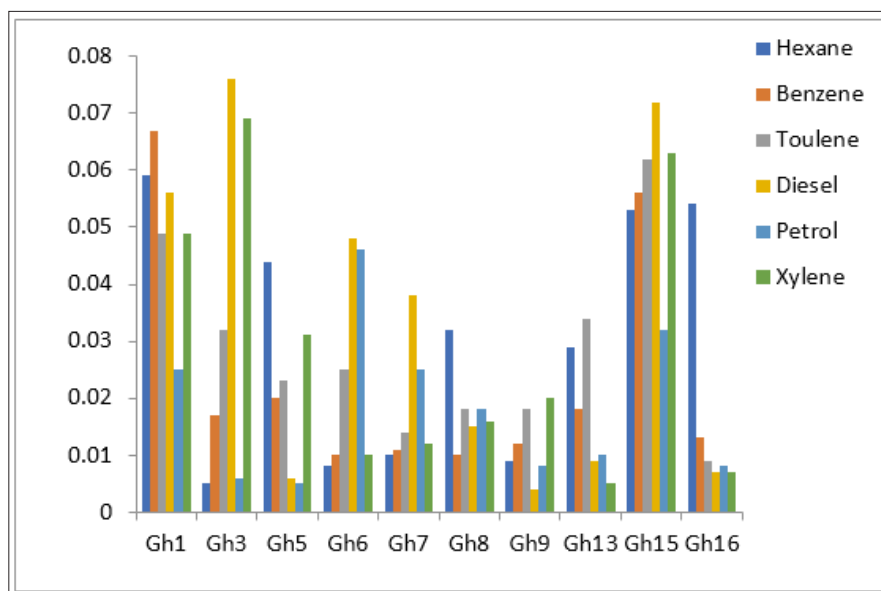


Figure 1: Growth of selected morphotypes in presence of tested hydrocarbons at 100ppm.

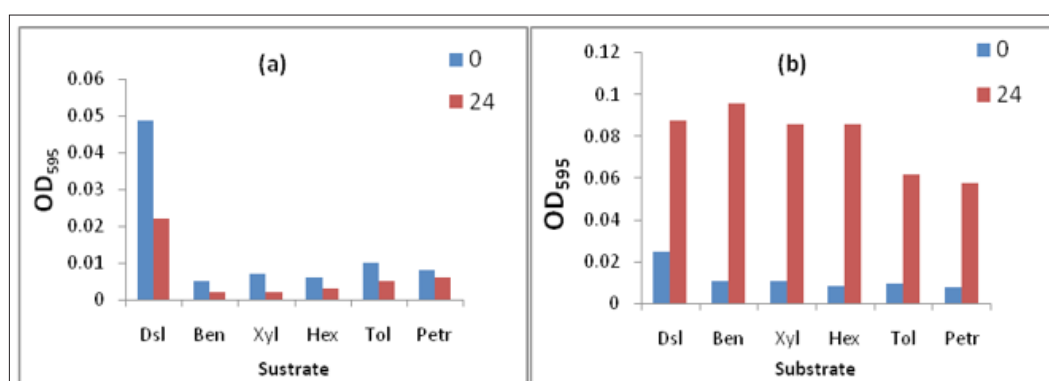


Figure 2: Growth pattern of Gh₁ with cured and a. uncured plasmid, b. In the presence of selected hydrocarbons.

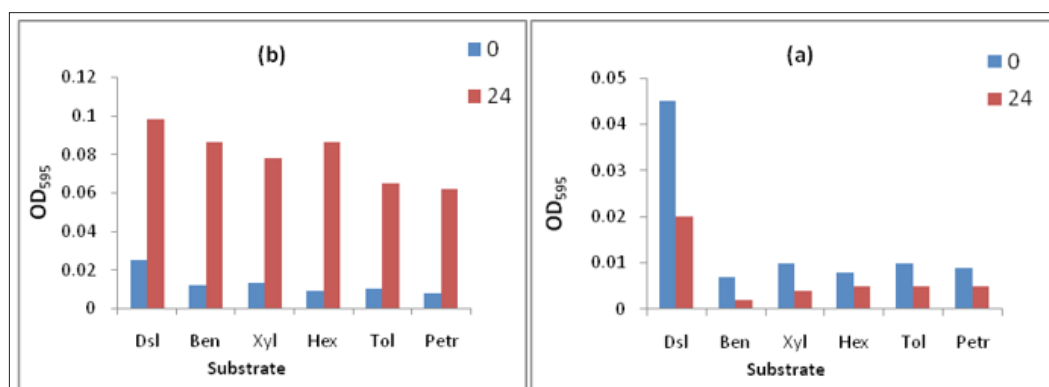


Figure 3: Growth pattern of Gh₁₅ with cured a. uncured plasmid and b. In the presence of selected hydrocarbons.

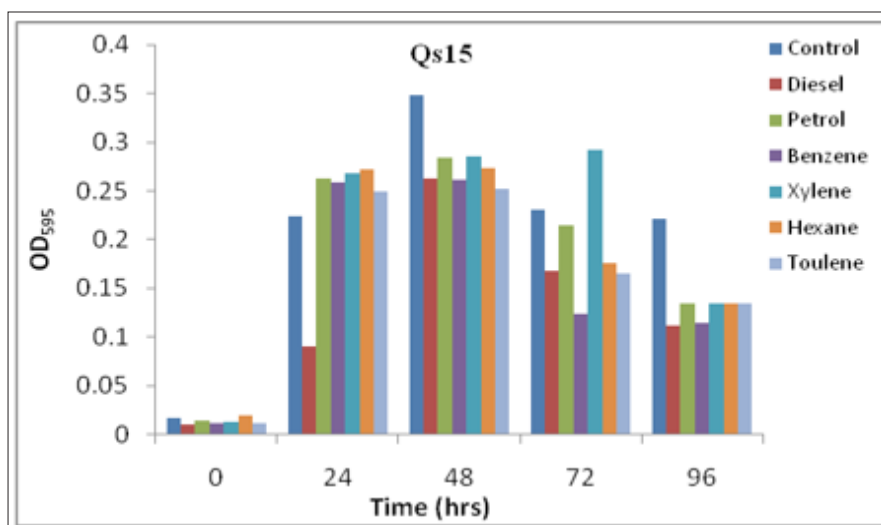


Figure 4: Growth pattern of consortium Qs₁₅ on different substrate (100ppm of each).

These two isolates selected for further study by the formation of consortium (Qs₁₅). Qs₁₅ showed the exponential phase up to 48hrs but in the presence of xylene it showed log phase up to 72hrs (Figure 4). Availability of nutrients is rate-limiting factor for successful bioremediation of hydrocarbons contaminated environments. Along with easily metabolized carbon source, microorganisms require various minerals like nitrogen, potassium, and iron for normal metabolism and growth. During the biodegradation of hydrocarbons as time increases, chemical changes in hydrocarbon, production of bio-products such as organic acids and depletion of minerals from the medium occurred, which indirectly affect the growth of bacteria [14].

Identification of the members of selected consortium

The BLAST search results depicted that the isolates Gh₁ and Gh₁₅ had the closest 16S rDNA sequence homology (99%) to *Bacillus weihenstephanensis* and *Bacillus pumilus* SAPR 032 respectively (Figure 5). The 16S rRNA gene partial sequence of strain Gh₁ and Gh₁₅ were submitted to the NCBI GenBank database under accession no KM385425 and KM385426 respectively. Thus, the constituents of the selected consortium were *Bacillus* sp. E₁₄ and *Bacillus* sp. E₉₇. The genus *Bacillus* is outstanding bacterial genus reported as petroleum hydrocarbon degrader. Their ability to form spores when nutrients are limiting makes species of *Bacillus* self-sustainable bioremediation tools [15].

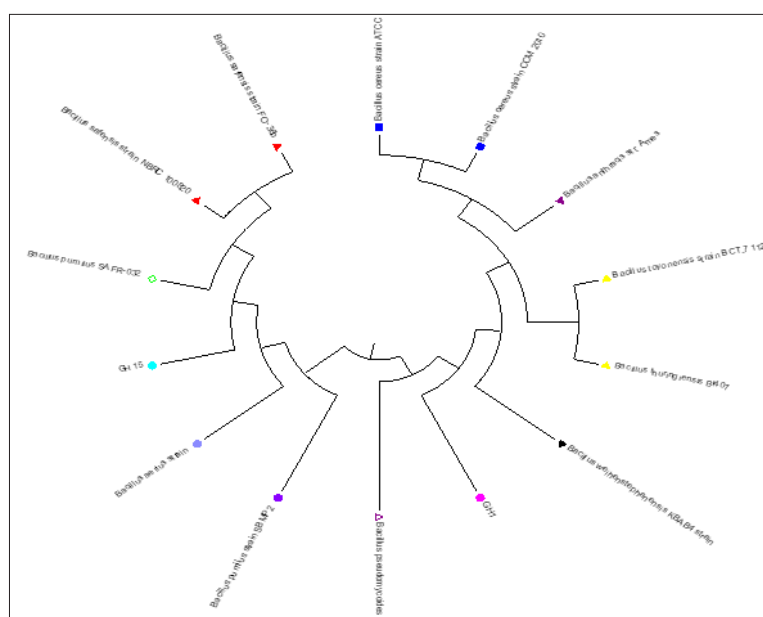


Figure 5: Dendrogram of Gh₁ and Gh₁₅ using PAST software showing 99% homology with *Bacillus weihenstephanensis* and *Bacillus pumilus* SAPR 032 respectively.

Biodegradation efficiency of Qs₁₅

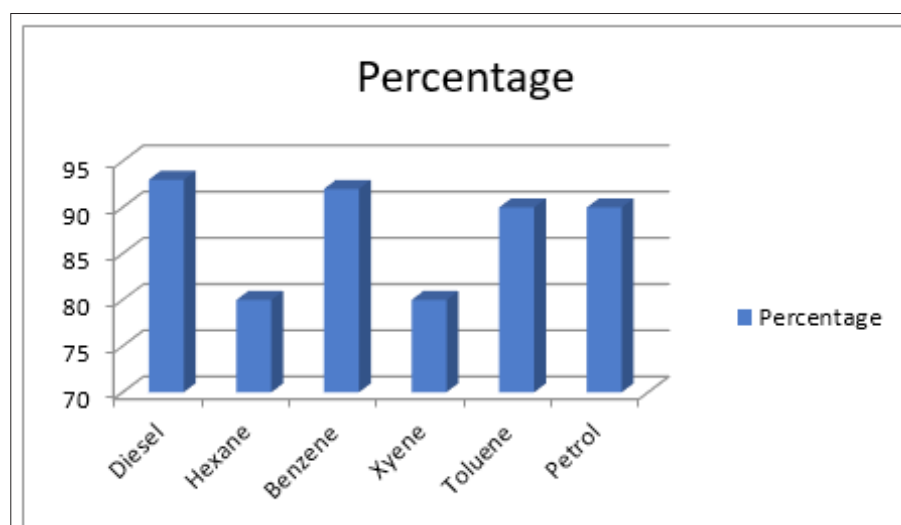


Figure 6: Degradation of selected hydrocarbon by Qs₁₅ as assessed by GC-FID.

According to the GC-FID results, Qs₁₅ exhibits the high biodegradation efficiency towards the hydrocarbons (Figure 6). Qs₁₅ showed the high degradation capability in aromatic hydrocarbons as compare to alkane in the medium. In presence of petrol and toluene in media, Qs₁₅ degrades these two hydrocarbons up to 90%. Similar finding was revealed in the study of Sumathy [16] in which *Pseudomonas* spp. degrade the 1% toluene and result of GC-MS confirm the presence of catechol. Presence of catechol indicates the m-cleavage pathway of toluene degradation. *Bacillus methylotrophicus* contain plasmid-encoded operons account for the entire metabolic process that leads to biodegradation of xylene. Enzymes encoded by upper operon convert m-xylene into 3-methylbenzoate or benzoate respectively, and the enzyme of lower operon convert 3MBz into 3-methylcatechol or benzoate into catechol and it is the resultant of meta ring cleavage, which ultimately leads to intermediates of the TCA cycle [13]. On the basis of GC profile, it was depicted that Qs₁₅ degrades 93% diesel within 48hrs as compared to control. Lima et al. [17] concluded that *Bacillus* sp. show the low degradation ability towards alkane present in diesel compared to higher alkane of diesel because short-chain n-alkanes dissolve the cellular membrane and are toxic to bacteria and long-chain n-alkanes have low solubility, inhibiting degradation by bacteria. In the degradation of benzene Qs₁₅ achieved the 94% biodegradation efficiency while in case of hexane Qs₁₅ showed 80% degradation that may be due to the induction of hexane-degrading enzymes that had already been induced during the preculture of Qs₁₅ in the hexane-containing minimal salt medium. The enzymes alkane 1-monooxygenase, alcohol dehydrogenase, cyclohexanol-dehydrogenase, methane monooxygenase and cyclohexanone 1,2 monooxygenase are involved in degradation of alkanes [18]. Lee et al. (2010) found the level of 14C was higher in the medium supplemented with hexane (liquid phase) as compared to control, possibly due to the conversion of hexane into soluble metabolites by bacterium, EH831.

Individual bacteria could metabolize only to a limited hydrocarbons and the complete biodegradation requires mixture of different bacterial groups. The broad mineralization capacity exhibited by the mixed culture in this study is not surprising and may be attributed either to the presence of different microbial species with a number of metabolic pathways and/or to interspecies interactions [19]. Mixed populations have broad enzymatic capacities, which are responsible for the degradation of hydrocarbon at greater rate [20]. Microbial communities present in natural ecosystems proliferate together in a synergetic relationship and produce a remarkable cocktail of primary and secondary bioactive molecules (metabolites) including oxidative and hydrolytic enzymes, which are direct or indirect support the degradation of hydrocarbon [21-23].

Conclusion

The results of this study indicated that hydrocarbon-degrading bacteria isolated from oil contaminated soil, oil refinery, Assam, India, two potential strain (*Bacillus* sp. E₁₄ and *Bacillus* sp. E₈₇) were selected for the consortium construction (Qs₁₅). have the highest potential to use different hydrocarbons (diesel, hexadecane, benzene, toluene and xylene) as the sole carbon source. This consortium Qs₁₅ has high levels of crude oil degradation and sufficient growth on mineral medium supplemented with hydrocarbons. From the data of present investigation, it can be concluded Qs₁₅ could be considered as good prospects for their application in bioremediation and restoration of hydrocarbon contaminated soil.

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