Abstract for Thesis

Exploration of Bacterial Diversity from High Temperature Oil Reservoirs For The Degradation of Hydrocarbons at Elevated Temperature

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Microorganisms from oil reservoirs are able to survive in extreme conditions, and are gaining importance in areas like industrial waste-stream clean up, synthesis of useful chemical products, bioremediation etc. It has been generally accepted that only about 1% of environmental microbial flora has been cultivated in laboratory due to the limitations in the enrichment and isolation techniques. Oil reservoirs were previously thought to be unsuitable environment for the group of microorganisms due to extreme high temperature and pressure. However, increasing number of novel species are being isolated and reported from this environment with the advent of sophisticated molecular and culturing techniques.

Petroleum reservoir is a complex ecosystem with a wide range of ecophysiological parameters. The environment is almost always oxygen limiting, however, presence of dissolved oxygen or in the form of oxyanions may not be ruled out. A wide range of petroleum hydrocarbons as well as dissolved organics from the sediments are available for the microorganisms to be used as sources of carbon and energy. Also presence of sulfates, phosphates, nitrates, etc. may serve as a terminal electron acceptor. There has been paucity of information about the microbial communities associated with Indian oil reservoirs.

Conventional oil recovery processes retrieve up to 40% of the available oil in the area. Developing new technologies to recover even a fraction of residual oil (ca. 50% or more of the initial reserve) is of great interest. Conversion of this oil into easily extractable methane is one of the approaches for efficient and maximum utilization of energy potential of residual oil. Various hydrocarbons from the crude oil are hydrolyzed and fermented by diverse group of bacteria to yield a multitude of compounds including volatile fatty acids, CO₂, hydrogen, etc. which may serve as substrates for the production of methane by methanogens.
The present investigation was carried out to explore the diversity of bacteria associated with petroleum reservoirs for the taxonomic novelty and energy recovery. The first part of the thesis dealt with the physico-chemical analysis of formation water samples. Physico-chemical analysis of formation water samples was done in order to check whether formation water alone can support the growth of the native bacteria. The phylogenetic diversity of bacteria based on 16S rRNA gene analysis of formation water samples using nested PCR-DGGE was studied. The second part of thesis dealt with the enrichment and isolation of thermophilic hydrocarbon degrading bacteria from the formation water. This part also includes phylogenetic identification and biochemical and molecular characterization of isolates. Third part involves the comprehensive evaluation of hydrocarbon degrading properties of the isolates, optimization of process parameters for crude oil and alkane degradation. The final part dealt with the selected consortium to produce methane in sand pack trials.

Following are the major findings of the present investigation.

- Formation water is the great source for the isolation of thermophilic petroleum hydrocarbons degrading bacteria. The information like sampling sites, oil well designations, depth of the oil well, and temperature of the formation water was obtained from ONGC-IRS Ahmadabad. The range of the depth of oil wells was 850-1500 meters and the temperature of the formation water was in the range of 65-85°C.

- Upon physico-chemical analysis it was observed that salt and the pH of the formation water samples were in the physiological range of the bacteria. Nitrogen concentration was not enough to support microbial growth in any of the formation water tested. Total solids and volatile solids, both were low in concentration. Low VS content indicated insufficient organic carbon content to support microbial growth. CHNS analysis revealed all samples have low percentage of carbon and nitrogen indicating oligotrophic environment for bacteria growth.

- The bacterial populations of these formation water samples were analyzed by denaturing gradient gel electrophoresis (DGGE) with PCR-amplified 16S rRNA fragments. Bacteria identified by DGGE analyses reported in the literature as hydrocarbon degraders. In the phylogenetic tree it was revealed that formation water has different groups of bacteria.
like proteobacteria, actinobacteria, firmicutes etc. Sequence analysis of the DGGE bands revealed that Proteobacteria were a major component of the bacterial population of formation water samples.

- After studying culture independent diversity attempts were made to enrich the thermophilic, microaerophilic hydrocarbon degrading bacteria. By enrichment technique total eleven thermophilic hydrocarbon degrading bacteria were isolated. Based on INT assay total five crude oil degrading isolates (MCM B-882—MCM B886) were selected for further studies.

- Upon 16S rRNA analysis it was observed that out of five bacteria, three were strains of *Aeribacillus pallidus* and remaining are *Hydrogenophilus hirschii* and *Bacillus pumilus*, respectively. However, the polyphasic identification approach also revealed the significant differences in the DNA base composition, FAME profile, carbohydrate fermentation profiles of MCM B-882, MCM B-883, MCM B-885 and MCM B-886 when compared with their closest phylogenetic affililates. These observations indicated that MCM B-882, MCM B-885 and MCM B-886 were probably new sub species of genus *Aeribacillus*. Similarly, MCM B-883 was probably new species of genus *Hydrogenophilus*.

- The evaluation of potential of petroleum hydrocarbon degradation of the isolates was assessed by various techniques like INT assay, GC and spectrophotometric analysis of residual hydrocarbons. It was observed that all isolates are appeared to be the good degraders and degraded 7-9% of the crude oil as estimated by measuring absorbance at A\textsubscript{420}. These thermophilic bacteria were also displayed crude oil degradation potential when evaluated in terms of chemotaxis studies, INT assay and visual observation of oil dispersion. These cultures were further characterized and assessed for their ability to degrade n-alkanes. It was found that all isolates could utilize mixture of alkanes (C12-C18) as their sole source of carbon and energy.

- Production of surface active agents like biosurfactants is a desirable property of hydrocarbon degrading bacteria. In the present study all isolated bacterial cultures could produce biosurfactant in a complex as well as minimal medium. Production of biosurfactant was confirmed qualitatively and by its extraction with Chloroform: Ethanol.
The hydrocarbon emulsifying ability of individual isolate was assessed in terms of emulsion index. The emulsion index revealed good emulsifying ability of all isolates when compared with published data.

- The optimization of process parameters revealed that all isolates could effectively degrade crude oil as well as n-alkanes at elevated temperatures, at alkaline pH range when ammonium chloride provided as a nitrogen source under anoxic conditions. Residual energy recovery potential of these isolates was further confirmed by sand pack studies.

Therefore, conclusions of the present study were:

- Bacterial diversity associated with Indian oil reservoirs was investigated using PCR based DNA fingerprinting technique i.e. PCR-DGGE. So far, there has not been any report in the published literature of the description of microbial diversity associated with Indian oil reservoirs investigated by molecular tools.

- Many of the bacterial sequences shared less than 95% homology with the reference sequences available in GenBank database. Many of these could represent novel species/genera. Thus, Indian oil reservoirs could be effectively used as a source for isolation and identification of novel bacterial species. This could be extremely important from the point of view of bacterial taxonomy.

- To the best of our knowledge, this is the first report of isolation of *H. hirschii* from oil reservoirs. All the isolates obtained in the present study could effectively degrade crude oil as well as n-alkanes at elevated temperature and under oxygen limiting conditions. To the best of our knowledge this is the first report of isolation, identification and characterization of thermophilic crude oil degrading bacteria from Indian oil reservoirs.

- Generation of methane was detected in sand-pack trials where crude oil was used as a sole source of carbon and energy and a consortium of thermophilic anaerobes as microbial inoculum. This observation provided the proof of a concept that a microbial
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