

## INTRODUCTION

The unprecedented population increase and anthropogenic activities such as industrialization and urbanization of the twentieth century in the name of modernization have not only increased conventional solid and liquid waste pollutants to critical levels but also produced a wide range of previously unknown contaminants in the form of xenobiotics for which society is un-prepared. Microbes, the oldest inhabitants of the earth that are versatile and adaptive to the changing environment, will be the cost-effective components to combat the present problems. Petroleum hydrocarbons belong to the most widespread contaminants of water and soil, even minute concentrations of these contaminants can have a very significant effect on the taste and odour of the polluted water and may be toxic and/or carcinogenic. The predominant factors influencing microbial community structure after contamination likely include (i) contaminant mixture type, (ii) soil/water type (i.e., physical, chemical, and biological characteristics), and (iii) time.

Complex petroleum hydrocarbon mixtures, including crude oil, diesel fuel, kerosene and creosote, consist of various concentrations of *n*- and branched alkanes, cycloalkanes, phenolics, aromatics, and polycyclic aromatic hydrocarbons. Although these mixtures contain similar constituents, the relative abundance of mixture components and toxic compounds (e.g., heterocyclics, chlorophenols) vary considerably, and these variations are potentially important in determining which microbial populations are involved in biodegradation. The physical/chemical/biological properties (e.g., temperature, pH, conductivity, nutrient status, texture, biota) are expected to further influence the selection of adapted microbial populations.

Kerosene is a colourless flammable hydrocarbon liquid and has many toxic effects on plants, animals and humans. It is obtained from the fractional distillation of petroleum at 150<sup>0</sup>C and 275<sup>0</sup>C. Kerosene contains paraffins (alkanes) cycloparaffins (cycloalkanes), aromatics, and olefines from approximately C<sub>9</sub> to C<sub>20</sub> (Irwin et al. 1997). Dermal exposure and inhalation of kerosene are reported to be toxic and to an extent

carcinogenic. Yoshio *et al.*, in 2005 reported of dermatitis in human on exposure to kerosene.

The reports on the alarming level of pollution of Vembanad Lake by kerosene gave the thought of making the lake as the sampling site. The samples were collected from the southern fresh water zone of the lake where majority of the houseboats ply. For degradation to occur, the right microbes should be utilized in the right place with the right environmental factors (Boopathy 2000), therefore the *in situ* bioaugmentation- mediated technique should be tailored specifically to each polluted site, as each site is thought to have inherent characteristics. In this study, we used the concept of bioaugmentation- ‘‘autochthonous bioaugmentation’’-, and proposes a consortium of kerosene degrading bacteria for the remediation of the pollution problem.

The objectives of the study were

1. Isolation, screening and characterization of efficient kerosene degrading bacteria from Vembanad Lake
2. To optimize the conditions of efficient degradation
3. Analysis for the production of biosurfactant and characterization of it.
4. To develop a consortium with maximum degradation ability
5. To check the role of amendments on the efficiency of developed consortia.

## **Materials and Methods**

### **Isolation and screening of potential kerosene degrading bacterial strains**

Water samples were collected from Vembanad Lake and were used for the isolation of bacteria. Water samples were inoculated on to mineral salt media (Raymond *et al.*, 1976) without any carbon source. Kerosene was added on to the media and the bacteria those are capable of utilizing kerosene as carbon source only will grow on the media making it turbid. Then from this liquid culture aliquots were plated on mineral salt agar media and kerosene wet filter paper were used as source of carbon for growth. The selected isolates were subjected to secondary screening by checking OD at 420nm to measure the total hydrocarbon.

### **Biochemical and Molecular characterization**

The selected four isolates were subjected to biochemical characterization using bioMerieux VITEK 2 compact system. 16 S rDNA analysis was done and the nucleotides were sequenced and deposited in Genbank, NCBI.

### **Optimization of degradation conditions**

Degradation parameters such as incubation period, pH, temperature, and concentration of nitrogen source were optimized. Degradation was assessed based on the measure of CO<sub>2</sub> evolved on biometric flask according to the method of Pochon and Tardieux (1962)

### **Growth profiles**

Growth profiles of the selected strains K1, K2, K3, and K4 were studied on both nutrient broth and mineral salt media. The media were inoculated with 50µL of 24h culture and incubated for 40 h and 60 h respectively at 37°C during which the optical density (OD) was read every 2 hour (nutrient broth) and 3 hour (Mineral salt media) at 600 nm. Colony forming units were counted after plating on nutrient agar plates. Each culture was inoculated in triplicate and the readings of the profiles were averaged.

### **Degradation analysis**

The four bacteria were subjected to degradation efficiency analysis using HPLC and GC at optimized conditions. Aliquots of culture were taken on definite intervals and extracted and concentrated to obtain the degradation profile of the isolates.

### **Analysis of plasmid DNA**

Small-scale preparation of plasmid DNA from the four selected strains was performed using rapid alkaline lysis procedure (Sambrook, J.1989). The curing of plasmid was performed as described by Trevor's (1986) and the role of plasmid in degradation was checked.

### **Biosurfactant production analysis**

The four bacterial strains namely K1, K2, K3 and K4 were subjected to preliminary screening of biosurfactant production using methods such as emulsification index (EI<sub>24</sub>) (Paraszkiewicz *et al.*, 2002) and blood agar hemolysis. Then the screened strains were inoculated to produce biosurfactant as described by Manreet, S and Pheetrong, K (2007). The extracted biosurfactant was analyzed using HPLC, MALDI-TOF, and LC-MS.

### **Cellular hydrophobicity measurement**

Cellular hydrophobicity of the organism was measured by BATH assay as per the method of Rosenberg *et al.*, (1980).

### **Adhesion, immobilization and scanning electron microscopy of the bacteria**

The bacteria were subjected to adhesion assay (S. Stepanovic' *et al.*, 2000) in microtitre plate and read the optical density at 570 nm. The bacteria were immobilized on sodium alginate and coir pith block pieces. The bacteria immobilized on glass plates and coir pith blocks were subjected to scanning electron microscopy according to the procedure of Radwan *et al.*, 2001.

### **Toxicity analysis of kerosene**

Toxicity of kerosene to plant and animal life was tested by seed toxicity test and fish toxicity study according to the guidelines of EPA. The fish toxicity was assessed according to the methods of OECD guidelines (1992)

### **Development of consortium**

5 different combinations were made out of the four cultures and tested for their ability to degrade kerosene. The degradation ability was analyzed by HPLC.

### **Transmission electron microscopy**

The consortia was grown on glass slides and subjected to transmission electron microscopic analysis. (Graham and Beveridge, 1990)

### **Amendments on consortium**

### **Immobilization of consortium**

Sodium alginate immobilized consortia were inoculated on mineral salt media with kerosene as source of carbon and subjected to degradation analysis.

### **Consortium amended with NPK (18:18:18)**

Consortium was inoculated on mineral salt media and amended with 1% NPK (18:18:18) and degradation percentage was analyzed.

### **Consortium immobilized on coir pith blocks and treated with NPK**

The bacteria were immobilized on coir pith blocks and these were used as seed for treatment. NPK was also added along this consortium adhered on coir pith block and rate of degradation was analyzed.

## **Results and Discussion**

### **Isolation and screening of potential kerosene degrading bacterial strains**

28 isolates were obtained on primary screening. 10 of the isolates were selected based on the vigor of growth and subjected to secondary screening and four isolates K1, K2, K3, and K4 were selected.

### **Biochemical and Molecular characterization**

The four isolates K1, K2, K3, and K4 were identified using bioMerieux VITEK 2 compact system as *Pseudomonas aeruginosa*, *Sphingomonas paucimobilis*, *Bacillus cereus*, and *Bacillus mycoides* respectively. They were characterized molecularly by 16S rDNA sequencing. The sequence was deposited at NCBI Genbank and accession numbers were obtained. The available numbers are K1-JN540024, K2-JN600441, K3-JN540025, and K4-JN600440.

### **Optimization of degradation conditions**

The condition optimums for efficient degradation were analyzed as pH 7, temperature 37, and 0.4% N<sub>2</sub> concentration.

### **Degradation analysis**

On HPLC analysis degradation rate was found to increase as days progress and goes up to 30 days. K1 showed a final degradation of 98%, K2 91%, K3 98%, and K4 96% by day 30. On GC analysis the final degradation was calculated as K1 79%, K2 75.6%, K3 82.4%, K4 79.2%.

### **Analysis of plasmid DNA**

All the four isolates K1, K2, K3, and K4 found to possess plasmid and were cured by acridine orange and an elevated temperature of 42°C.

### **Biosurfactant production analysis**

On primary screening of biosurfactant production of the three bacteria namely K1, K3, and K4 were found to produce biosurfactant. They were structurally characterized and found to be of different molecular nature such as rhamnolipid, and iturin.

### **Cellular hydrophobicity measurement**

The three organisms K1, K3 and K4 were found to show high cellular hydrophobicity which is a characteristic feature associated with hydrocarbon degrading bacteria.

### **Adhesion, immobilization and scanning electron microscopy of the bacteria**

The optical density measurements of the isolates proved that these bacteria were capable of adhering on to microtitre plate wells. The bacteria were immobilized on coir pith blocks and also they were entrapped on sodium alginate beads. The adhesion and biofilm formation of the strains were visualized on scanning electron microscope. Biofilm formation ability was also evident on glass slides under scanning electron microscopy.

### **Toxicity analysis of kerosene**

Even low concentration of kerosene was found to inhibit seed germination. The fish LC50 also showed the same result establishing the acute toxicity of kerosene on plant and animal life.

### **Development of consortium and Transmission Electron Microscopy (TEM)**

The third group named CK-3 with bacteria K1, K3, and K4 were found to co-exist and effectively did the degradation. This was confirmed by HPLC. The number of days taken for degradation came down to 10 from 30 as that with single bacteria. The TEM analysis revealed the co-existence of the three bacteria.

### **Amendments on consortium**

#### **Immobilization of consortium**

Sodium alginate immobilized consortia were subjected to degradation analysis and found to enhance degradation in shorter days than non-immobilized.

#### **Consortium amended with NPK (18:18:18)**

Amendment of consortia with NPK was found to enhance degradation by increasing its rate and reducing the number of days needed for degradation.

#### **Consortium immobilized on coir pith blocks and treated with NPK**

On degradation analysis of coir pith adhered bacteria in the presence of NPK exhibited a degradation percentage of 99.9% and the period needed for complete degradation has come down to 6 days.

The work gave an insight into the role of bacteria in kerosene degradation. The role of autochthonous flora on degradation was established in this study and gives idea to develop an *in situ* bioremediation strategy for the pollution of kerosene and similar hydrocarbons in water bodies. The bacterial biodegradation found to reduce the constituents of kerosene completely as compared to abiotic loss. Structural characterizations of the biosurfactants can be useful to develop biosurfactant mediated strategies to remediate pollution in place of chemical surfactants. The development of consortia and utilization of consortium in degradation makes the remediation programme more effective. Trials to develop consortia showed that only certain bacteria have the capacity to co-exist.

Immobilization protects the bacteria from toxicities of the compound so the bacteria can work better in immobilized condition. Viability of the strains was also increased on immobilization. Coir pith is a locally available low cost material which can be effectively used for immobilization of the culture. It acts as a stable support for the bacteria to adhere. This makes the substrate available for degradation. That may be the reason why the rate of degradation has increased to 99.9% in 6 days. So this can be modified and used as a low cost technology for remediating water bodies where heavy contamination of water vessel fuel occurs.

## **Conclusion**

We had performed isolation, characterization, and identification of four kerosene degrading strains namely K1, K2, K3, and K4. They were found to possess plasmid. Three of the bacteria such as K1, K3, and K4 were found to produce biosurfactant. The biosurfactant produced by the individual bacteria were characterized and found to have different structural properties.

The factors of degradation such as incubation period, pH, temperature, and N<sub>2</sub> source were optimized. Degradation rates were studied at optimum conditions. At optimum conditions it was found all fractions of kerosene such as long chain alkanes, aliphatics and naphthalenes degrade completely to CO<sub>2</sub>.

The consortia found to degrade kerosene completely in a short span of time. All the fractions of kerosene were also degraded. All the amendments used on consortia were found to be effective such as immobilization, adhesion on coir pith block and fertigation. Fertilizer along with biofilm formed on coir pith block enhances degradation nearly to 100%. This can be used as a cost effective technology for *in situ* remediation trials. So we feel these isolates have promising biodegradation capability for remediation of polluted sites. This study can be used as base for developing biosurfactant mediated remediation strategy. The other future perspective of this study is setting up field trials for the use of consortia in different hydrocarbon polluted sites.