

Bioremediation Potential of Hydrocarbonoclastic Bacteria in Cuddalore Harbour Waters (India)

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Abstract :

The present study was undertaken to evaluate the biodegradation potential of the indigenous bacterial population of Cuddalore fishing harbour waters against oil pollution. 14 strains identified up to the species level were screened for emulsification activity (D_{610}) against crude oil, kerosene, diesel, anthracene and naphthalene. All the strains showed emulsification activity in the order of crude oil > kerosene > diesel > anthracene > naphthalene. *Corynebacterium kutscheri* showed highest activity in crude oil (0.971) and diesel (0.581), where as in kerosene, anthracene and naphthalene the highest emulsification activity was shown respectively by *Escherichia coli* (0.969), *Pseudomonas aeruginosa* (0.112) and *Lactobacillus casei* (0.033). The emulsification activity value and the rate of degradation of crude oil coincided in most of the strains. Statistically highly significant positive correlation was obtained ($r = 0.248312$; $p < 0.10$). The maximum degradation activity was shown by *Branhamella catarrhalis* (91.37%), followed by *Enterobacter aerogenes* (91.22%), *Bacillus megaterium* (90.13%) and the lowest degradation activity was shown by *Citrobacter intermedius* (79.33%). When fertilizer was used only in 4 strains the rate of degradation was slightly improved (1.3%-3.4%). The study revealed the occurrence of highly potent strains and the possibility of using them in bioremediation of polluted areas.

Key words: Bioremediation, Biodegradation, Emulsification, Hydrocarbonoclastic bacteria, Hydrocarbons, Crude oil, Diesel, Kerosene, Anthracene, Naphthalene.

Introduction :

Oil pollution in the marine environment is reaching at an alarming rate and it receives the attention of the scientific community as a major problem, concerning the health of the marine environment. The sources of oil pollution in the marine environment are tanker operations and accidents, offshore oil exploration, fishing, industrial discharge and river run off⁶. There are so many conventional physical and chemical methods employed in cleaning of petroleum hydrocarbons spilled in the marine environment. Many of them are not satisfactory to the expectations and produce undesirable side effects to the living systems. Hence, the recent focus is on "Bioremediation"³. The ability of the bacteria to degrade the hydrocarbon depends on the emulsification of the hydrophobic substrate¹⁴. The present study includes biodegradation of crude oil and emulsification

of five different hydrocarbons.

Experimental :

Hydrocarbonoclastic Bacteria: Bacteria were isolated in water sediment samples collected from Cuddalore fishing harbour (Lat. 11° 42' N; Lon. 79° 46' E).

Culture Conditions : Serially diluted water and sediment samples were inoculated in to the Bushnell Hass agar (with 1% diesel) and spread plate technique was used for isolation of hydrocarbonoclastic bacteria. Plates were incubated at 28±2°C for 2 - 4 days. Results were expressed as CFU/ml/g. Colonies were isolated based on their colony morphology and pure cultures were obtained by adopting phase streaking method². Isolated strains were identified to the species level following the scheme of Cappuccino and Scherman⁷ and the results were cross checked with Bergey's Manual of determinative bacteriology⁶. Isolated pure cultures were maintained in Bushnell Hass agar for further investigations and 14 strains identified to the species level were used for the experimental works.

Separation of Emulsification factor : Emulsification factor (ES) was separated from the 14 strains by culturing them in seawater medium with 8.5mg of hydrocarbon (crude oil, diesel, kerosene, anthracene and naphthalene) for 7 days in a shaker. Then the broth was centrifuged at 12000rpm for 30 minutes and cell free aqueous broth was treated with three volumes of chilled acetone and allowed to stand for 10hrs at room temperature. The precipitate was collected by centrifugation, purified again using chilled acetone and vacuum dried at room temperature¹⁵.

Estimation of Emulsification activity: 3mg of the precipitate was dissolved in 1000ml of distilled water. pH was adjusted to 5.5. The solution was then diluted to 10⁵ times. To 10ml of the diluted solution, 0.1g of hydrocarbon was added. The mixture was taken in a glass stoppered flask, shaken vigorously by hand and allowed to stand for 10minutes. The turbidity of the stabilized emulsion was measured at 610nm in a spectrophotometer and emulsification activity was expressed as D_{610} ¹⁵.

Estimation of Crude oil degradation : The crude oil degradation was estimated by culturing the organisms in the same seawater medium with and without fertilizer (450mg urea) for 7 days in a shaker¹⁵ and crude extraction was made by using n-hexane and the degradation was estimated

fluorimetrically¹⁸ (Varian, Cary Eclipse, Fluorescence Spectrophotometer, Australia). The sample was measured at 310 nm excitation and at 374 nm emission wavelength. Crude oil was used as standard. Content of hydrocarbon in the sample after degradation was calculated from their OD value. From these values, % degradation was calculated. Uninoculated controls were maintained to elucidate the natural weathering of hydrocarbons.

Results and Discussion :

Emulsification activity : Five different hydrocarbons (crude oil, diesel, kerosene, anthracene and naphthalene) were used in this study. Among the five hydrocarbons tested invariably all the strains showed the emulsification activity in the order of crude oil > kerosene > diesel > anthracene > naphthalene. *Corynebacterium kutscheri* showed highest emulsification activity in crude oil (Fig.1) and diesel (Fig.3), where as the highest activity in kerosene (Fig.2), anthracene (Fig.4) and naphthalene (Fig.5) was shown respectively by *Escherichia coli*, *Pseudomonas aeruginosa* and *Lactobacillus casei*.

All the 14 strains experimented, produced emulsifiers against all the five hydrocarbon sources. Biodegradation of oil depends on its degree of spreading. Degradation occurs when the substrate is either dissolved or in the form of emulsions. Emulsions greatly increase the contact area between oil and microorganisms, thus favouring oxygen transfer and the supply of nutrients required for growth of the hydrocarbonoclastic microorganisms. They are often dispersed in the surrounding medium or they can be located on outer-cell surfaces and in that case, the cells themselves become a surfactant²⁰. In the present study it was possible to separate the emulsification factor so that the emulsifiers might have either released in to the medium or they might have located on the outer cell surface. As the experiment dealt with cell free extracts, the possibility of cells themselves acting as surfactants was ruled out in these bacteria.

Bioemulsifiers play an important role in regulating the attachment-detachment of microorganisms to and from surfaces¹⁹. They improve the bioavailability of hydrophobic water insoluble substrates by increasing their solubility. Emulsification made the insoluble to disperse in water and made it available to not only to the organism that produced that emulsifier but also to other organisms⁸. The highest emulsification activity against crude oil by all the 14 strains may be due to the higher content of alkanes in crude oil¹². The emulsification activity expressed as D_{610} was found to be higher compared to the results obtained by Bhosely and Mavinkurve⁵ in Goa waters. Actually, the value was found to be 3 times more for crude oil.

In the present study, it was evident that bacterial strains emulsified the crude oil to a greater extent than the individual hydrocarbons. From the results, it was evident

that *Enterobacteriaceae* played a major role in emulsification. Good emulsification activity in coliforms was also found in Mangalore harbour areas²¹. The present work contradicted their finding regarding *Pseudomonas* sp. as a poor emulsifier producer. However they didn't identify the bacteria up to the species level and also the method they followed to justify that emulsification activity was crude and based on opacity.

The observations made in the present study were highly encouraging and production of biopolymers has a greater advantage in oil industries and marine oil spill problems. Chemically synthesized emulsifying substances cause ecological problems owing to their resistance to biodegradation and due to their toxicity to other organisms in the natural ecosystem. On the other hand, these biopolymers are biodegradable, more efficient and more selective.

Degradation of Crude oil : The maximum degradation activity (Fig.6) was shown by *Branhamella catarrhalis* (91.37%), followed by *Enterobacter aerogenes* (91.22%), *Bacillus megaterium* (90.13%) and the lowest degradation activity was shown by *Citrobacter intermedius* (79.33%). When fertilizer was used only in 4 strains, the rate of degradation was slightly improved (1.3%-3.4%) and in other strains, there was absolutely no improvement (Fig. 7).

The emulsification activity (D_{610}) value and the rate of degradation of crude oil of most of the strains coincided. Statistically highly significant positive correlation was obtained ($r = 0.248312$; $p < 0.10$). The first step in microbial degradation is to convert the hydrocarbon molecule to a fatty acid. This results in the formation of so-called chocolate "mousse" and this colloidal effect acts further to enhance the rate of microbial degradation and to disperse the oil in the sea¹³. Pure and mixed culture of *Klebsiella pneumoniae* and *Bacillus pumilus* isolated from Goa waters degraded 80 - 85% of crude oil¹¹, which were comparable to the values obtained in the present study (79.33-91.37%). However varying results were obtained in different studies worldwide (i.e.) the versatility of bacterial strains in degradation of crude oil varies. The higher degradation rate of the indigenous population is a consequence of "adaptation" of the community to elevated levels of hydrocarbons⁴. Bacteria isolated from oil polluted environments have previously shown to be more effective in degrading crude oil than bacteria isolated from unpolluted environment⁹.

The degradation of hydrocarbons in the marine environment is dependent on the availability of two most important nutrients, nitrogen and phosphorus, which are generally limiting^{1, 17, 22, 24}. Often it had been shown that the microbial seeding in marine environment with fertilizer improves the rate of biodegradation²³. On the contrary in the present study addition of fertilizer did not improve the

degradation rate among 10 strains used and in the rest of the 4 strains it was only slightly improved. This may be due to the fact that these strains were isolated from the nutrient rich organically polluted water.

Bacteria isolated from oil polluted environments have previously shown to be more effective in degrading crude oil than bacteria isolated from unpolluted environment⁹. Plasmid frequency has also been shown to increase in various hydrocarbon contaminated environments¹⁰. Though in the present study plasmid frequency was not estimated, the abundance of multiple antibiotic resistant strains of various pathogens had been already reported in this study area¹⁴, which suggested the possibility for the rich plasmid frequency in the indigenous bacterial population.

Conclusion :

Experimental studies revealed that emulsification activity of bacterial strains was in the order of crude oil > kerosene > diesel > anthracene > naphthalene. *Corynebacterium kutscheri* showed highest emulsification activity in crude oil and diesel, whereas *Escherichia coli*, *Pseudomonas aeruginosa* and *Lactobacillus casei* showed highest activity respectively in kerosene, anthracene and naphthalene. In crude oil all the strains showed highest emulsification activity. It showed that bacteria prefer a mixture of hydrocarbons like crude oil than the individual hydrocarbon. The emulsification activity on crude oil confirm the existence of strong correlation between emulsification activity of bacteria and their potential to degrade this hydrocarbon source. The maximum degradation activity was shown by *Branhamella catarrhalis* (91.37%), followed by *Enterobacter aerogenes* (91.22%), *Bacillus megaterium* (90.13%) and the lowest degradation activity was shown by *Citrobacter intermedius* (79.33%). When fertilizer was used only in 4 strains, the rate of degradation was slightly improved (1.3%-3.4%). Among the strains tested, the degradation activity was in the range of 79.33% - 93.37%. The results of the present study clearly indicated the possibility to develop an environmentally friendly mitigation technology against oil pollution using the indigenous bacterial flora of this ecosystem.

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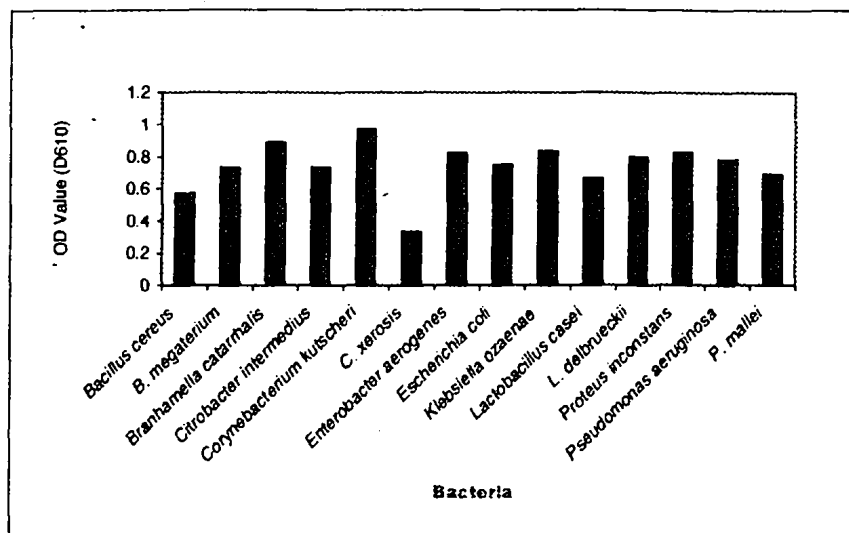


Fig. 1 : Emulsification of crude oil

Fig. 2 : Emulsification of kerosene

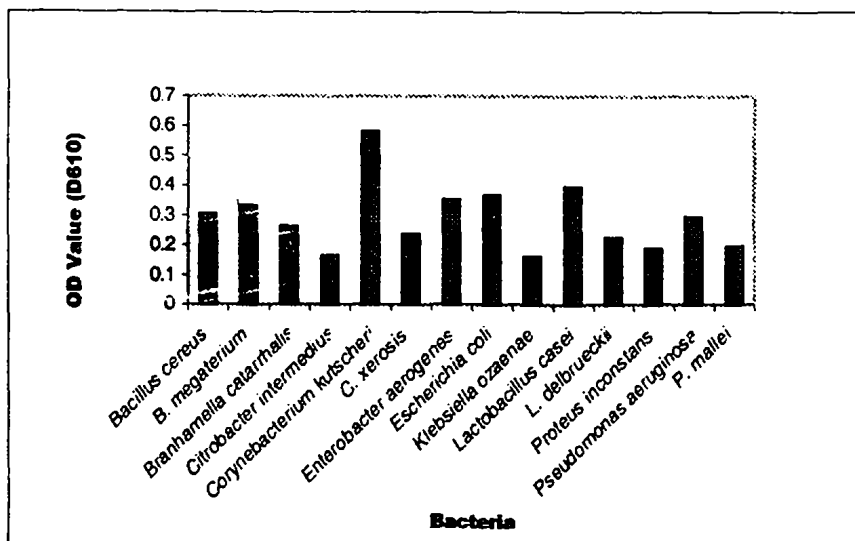
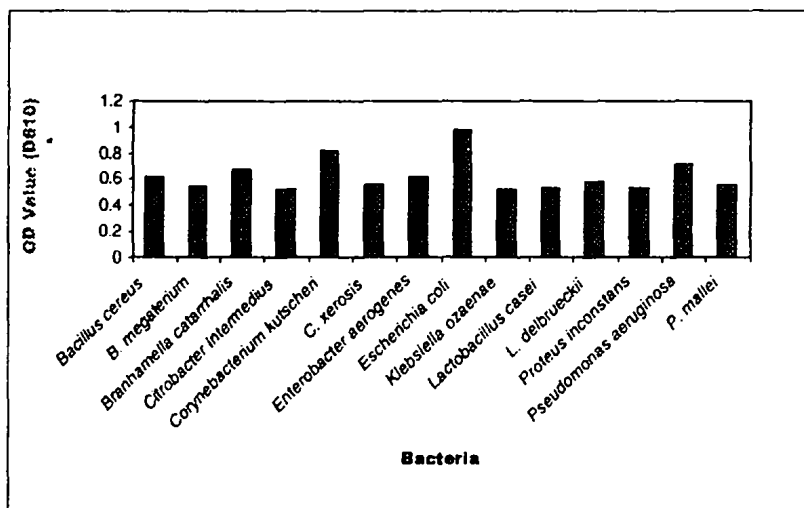


Fig. 3. Emulsification of diesel

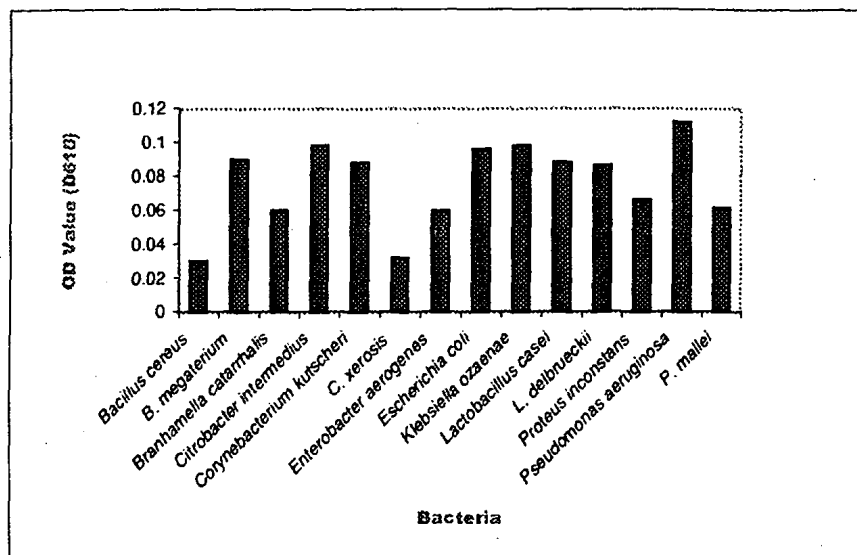


Fig. 4 : Emulsification of anthracene

Fig. 5. Emulsification of naphthalene

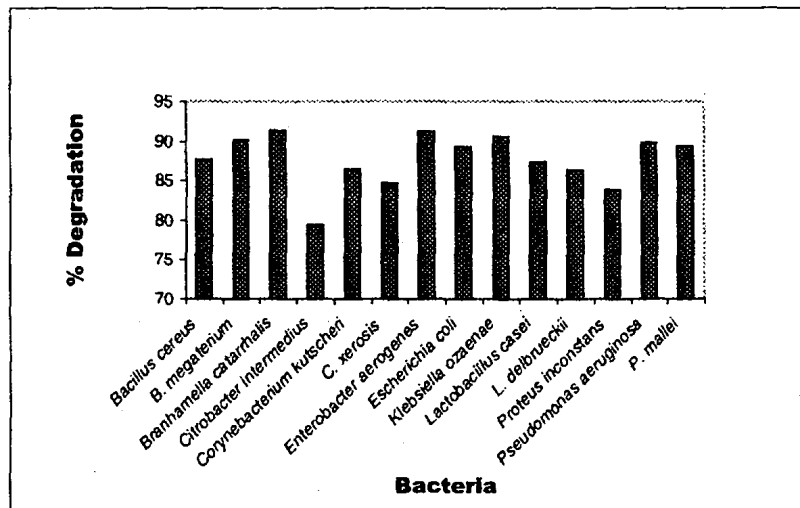
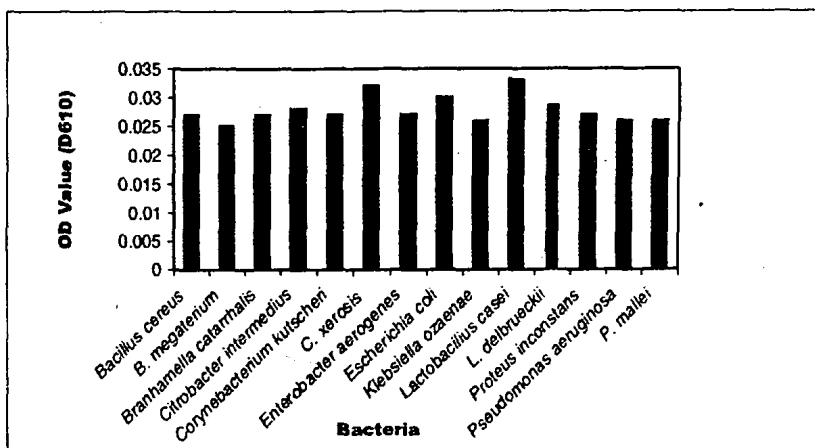


Fig. 6. Degradation of Crude oil

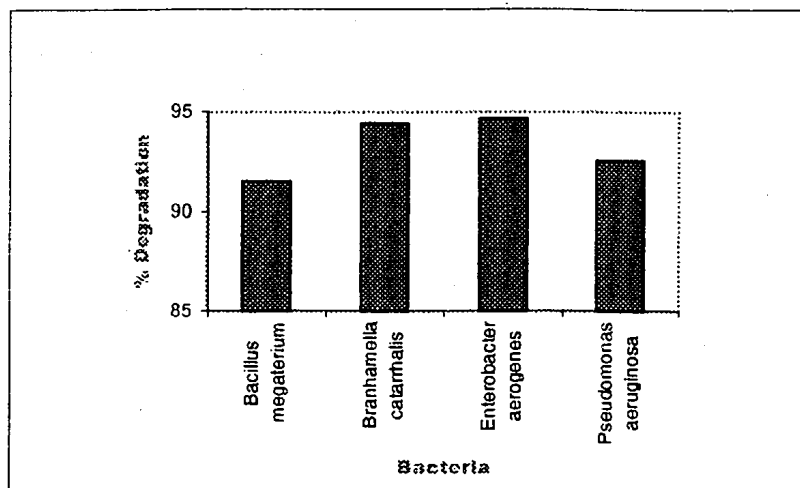


Fig. 7. Degradation of crude oil with fertilizer

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