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Study of Oil Degrading Bacteria Isolated From Oil Contaminated Sites

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Abstract--- Hydrocarbon degrading microorganisms were isolated from ten oil polluted areas in and around Navi Mumbai. Bushnell and Hass broth was used as selective enrichment medium with engine oil as a sole carbon and energy source and 19 different isolates were obtained. The oil degradative capacity of these isolates was evaluated using Emulsification index and Gravimetric analysis. The oil emulsification index was found to be in the range of 25 to 54%. Amongst the isolates, culture 1 showed best emulsification index and hence was used for further study. Oil degradative capacity of this isolate was found to be 41%. GC-MS study showed the conversion of Heneicosane to hexadecane indicating that the oil is partially degraded by the organism. Biochemical tests and 16s RNA sequencing confirmed that the isolate is Bacillus cereus.
Key words---- Engine oil degradation, Bacillus cereus, GC-MS, EI24, Gravimetric analysis.

I. INTRODUCTION

The origin of oil and gas industry in India can be traced back to the year 1867 when oil was struck at Makum near Margherita in Assam. Since then the consumption and demand of different petroleum products has been increasing steadily. Along an increase in consumer demands for petroleum based products, a consequent increase in related environmental hazards has been observed that pose a great threat to human race 1. Some petroleum products have been found to exert carcinogenic and neurotoxic effects 2. Though several mechanical and chemical methods have been implemented for the degradation of these products, the rate of contamination is quite high and the cost involved is also high 3. In view of this situation bioremediation gives a better solution compared to the currently existing methods. It provides efficacy, safety on long term use, cost and simplicity of administration with promising opportunity for creating better environment 4, 5, 6. Bioaugmentation with external addition of such microorganisms is widely practiced and has been shown to facilitate *in situ* bioremediation of oil-contaminated sites 7. Bioemediation of affected areas can offer a cost effective solution for restoring the ecosystem and can provide cleaner groundwater supplies. Several researchers have reported microorganisms having abilities to degrade oil, isolated from natural habitats contaminated with oil 2, 8. Bacteria play a central role in hydrocarbon degradation. The driving force for petroleum biodegradation is the ability of microorganisms to utilize hydrocarbons to satisfy their cell growth and energy needs. Hydrocarbon contaminated soil and marine sources have been the common choice for the isolation of such hydrocarbon emulsifying and degrading bacteria. One of the most important characteristics of hydrocarbon-degrading bacteria is the ability to emulsify hydrocarbons in solution 9, 10, 11, 12. Such microorganisms possess the ability to produce different types of bioactive compounds which also includes bioemulsifiers. In day to day life use of synthetic emulsifiers is very common. But due their highly complex nature and environmental accumulation, there is extreme need of eco-friendly biological emulsifiers. Bioemulsifiers reduce the surface tension between oil and water interface. They emulsify oil to sub-droplet level which can be easily utilised by the microorganism as a substrate. Such hydrocarbon emulsifying bacteria can be considered as potential hydrocarbon degraders 13. The isolates which express better oil emulsifying capacity can be effectively used for treating oil or hydrocarbon contaminated soils. Use of bacteria in degradation of petroleum hydrocarbon pollutants and petrochemicals has been extensively investigated 14, 15 and Biodegradation process has been established as one of the efficient, economic, versatile and environmentally sound treatment 16. In the present work oil degrading microorganisms were isolated from oil polluted areas and degradative studies were undertaken.

II. MATERIALS AND METHODS

A. Collection of Samples

Soil samples from the different oil contaminated sites in and around Navi Mumbai were collected in sterile plastic bags. The samples duly labelled were stored at -4°C for further analysis. The four stroke engine oil was obtained from the local petrol pump.

B. Isolation of Microorganisms

Soil sample (1.0g) was aseptically suspended into a sterile test tube containing 9.0 ml of the st. saline and vortexed. The soil

particles were allowed to settle. The supernatant was used as inoculum to inoculate 100ml st. Bushnell-Hass (BH) broth and st. Nutrient broth (NB) with 2% v/v engine oil. The BH broth contained Magnesium Sulfate 0.2g/l, Calcium Chloride 0.02g/l, Monopotassium Phosphate 1.0g/l, Dipotassium Phosphate 1.0g/l, Ammonium Nitrate 1.0g/l and Ferric Chloride 0.05g/l, Agar Agar 25g and D/W 1000ml whereas st. NB contained Peptic digest of animal tissue 5g/l, Sodium chloride 5g/l, Beef extract 1.5g/l and Yeast extract 1.5g/l, Agar Agar 25g and D/W 1000ml. The flasks were incubated on rotary shaker at 100rpm, 37°C for 1week. Two subculturing were done with the same media with 2% oil 17, 18.

C. Screening of Oil Degrading Bacteria

After second subculturing oil degrading microorganisms were isolated on st. BH agar plate overlayed with 0.1ml oil. The plates were incubated at 37°C for 24 hours to 1week in an incubator. The pure isolates obtained were preserved on st. nutrient agar slants. Screening of petroleum degrading isolate was carried out by growing the isolates in 50ml nutrient broth and Bushnell-Hass (BH) broth overlayed with 2% v/v oil. Their ability to tolerate the oil content was accessed by measuring the turbidity using absorption spectrophotometer at 540nm.

D. Morphological and Physiological Characteristics

The isolated bacteria were characterised and identified by their morphological characteristics based on size, shape and colony morphology on nutrient agar plate 19. All isolates were examined by gram staining. The best oil emulsifying and oil degrading culture was identified using 16s RNA sequencing.

E. Preservation and Subculture of the Isolates

The isolates were preserved in 50% (v/v) glycerol at -80°C. For the daily requirement of the culture, the isolates were streaked on nutrient agar slants and stored at 4°C. The sub-culturing was done every 15-20 days.

F. Emulsification Index

The emulsification index (EI24) of culture samples was determined by adding 2 ml of oil to the same amount of culture media. The contents were mixed for 2 min and left to stand for 24 hours. The EI24 is given as percentage of height of emulsified layer (mm) divided by total height of the liquid column (mm) 11, 12, 20.

$$EI24 = \frac{\text{Height of emulsified layer in mm X 100}}{\text{Height of the total layer in mm}} \quad \text{(Eq. 1)}$$

G. Biodegradation Assays

Sterile 50 ml of Bushnell and Hass broth was inoculated with 1 ml of culture 1 suspension and overlayed with 1 ml (2% v/v) of oil (the weight of the oil was noted). The flasks were prepared in triplicates and were kept in an incubator shaker for 7 days at 37ºC. Residual concentrations of crude oil were determined gravimetrically and by gas chromatography 21.

H. Gravimetric analysis

After 7 days of incubation, the mixture was subjected to separation in a separating funnel. In the separating funnel the mixture was mixed with 40 ml of petroleum ether and was shaken vigorously. It was allowed to stand to get two layers separated. The top layer contained the oil mixed with petroleum ether and the bottom layer contained the broth. The oil layer was collected in a pre-weighed tube after passing through sodium sulphate to remove the moisture. The mixture of oil and ether was kept at 85ºC in a water bath, allowing the petroleum ether to evaporate. The weight of the remaining oil was determined for estimating oil degradation quantitatively. The same sample was used for Gas Chromatography analysis 18, 22, 23.

I. Gas Chromatography

The GC-MS analysis was performed using an Agilent model 7890 with mass spectrometer detector. The GC column was DB-5, 30m, 0.25 capillary size. The initial column temperature was 40^{o}C with a hold time of four minutes. The temperature was programmed to increase by 5^{o}C per minute with a final temperature of 300^{o}C. The same GC conditions were used for both the FID and the mass spectrometer detector analyses.

In a typical process, 1µl of the sample was injected into the port and immediately vapourised and moved down the column with hydrogen as the carrier gas. After the separation in the column, the components were identified and further analysed by FID

detector 24, 25.

III. RESULTS AND DISCUSSION

A total of nineteen hydrocarbon-utilizing microorganisms were isolated from the contaminated soil after the enrichment process. Seven isolates were found to be Gram-positive spore forming rods and the remaining isolates were Gram-negative rods. These isolates were screened using oil emulsification index. Results for the same are showed in Fig. 1. It showed that all isolates have a good potential to emulsify the oil. They expressed the oil emulsification index from 3-54%. Culture 17 had the least emulsification index (3%) whereas culture 1 had a highest percentage of emulsification i.e 54%. The culture 1 was further tested for oil degradation using gravimetric method and GC-MS. The gravimetric study for culture 1 showed approximately 41% degradation capacity. Gas chromatographic showed that the starting molecule in unprocessed oil sample was C27, Heneicosane and the molecule obtained after bacterial degradation was C16, Hexadecane. The GC-MS spectra are given in Fig. 2. Microorganisms utilize a variety of organic compounds as the source of carbon and energy for their growth. Some microorganisms possess the ability to produce different types of bioactive compounds which also includes bioemulsifiers. These compounds help in emulsifying the oil. In the study undertaken by Murray et al it was shown that different bacteria express the oil emulsification capacity in the range of 0-80% 26. The *Bacillus cereus* culture isolated during the present study showed 53% emulsification. Hydrocarbons in the environment are biodegraded primarily by bacteria, yeast, and fungi. The reported efficiency of biodegradation ranged from 6% 27 to 82% 28; for soil fungi, 0.13% 27 to 50% 28; for soil bacteria, and 0.003% 29 to 100% 30. In this work we have demonstrated the oil degrading potential of a *Bacillus cereus* isolated from polluted sites. A maximum degradation of 41% was observed under aerobic conditions over 7 days at 37°C. The engine oil is a mixture of alkanes and it contains heavy chains (C_{10}-C_{40}) (Lin J., 2009). The alkanes are the most abundant compounds and are simpler to oxidize. Aliphatic hydrocarbons are degraded faster but the key step involves oxidation of these molecules to increase their solubility (Silva et al, 2006). Biodegradation rates have been shown to be highest for the saturate compounds, followed by the light aromatics, with high-molecular-weight aromatics and polar compounds exhibiting lowest rates of degradation 31. The GC-MS study showed the conversion of Heneicosane to Hexadecane.

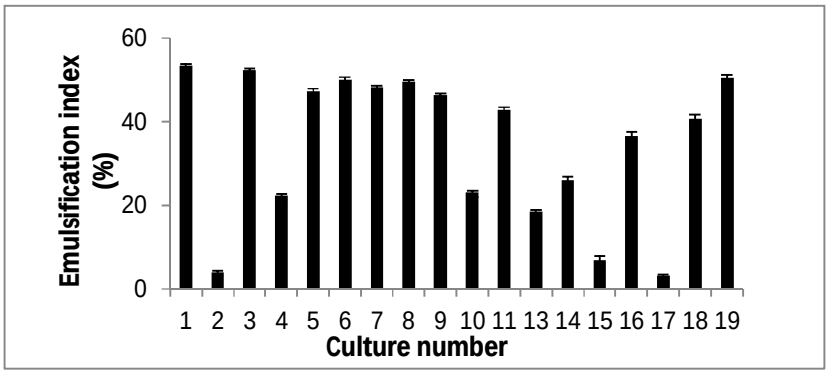
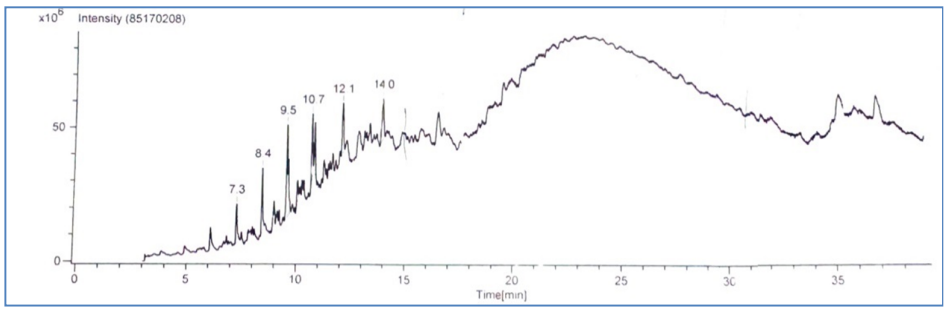


Fig. 1: Emulsification Index of different isolates.



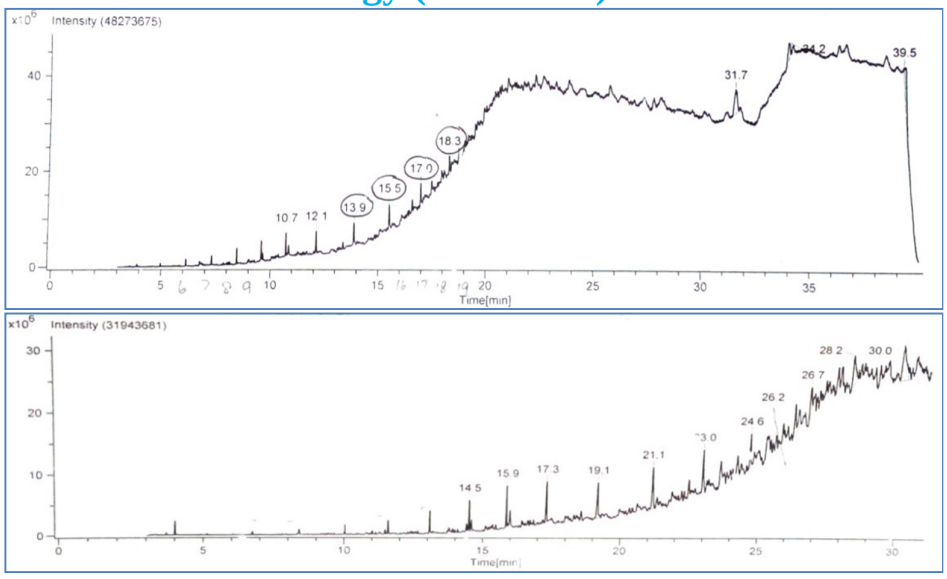


Fig. 2: GC-MS spectra of a) blank (engine oil) and b) Control (Engine oil in broth without culture) c) Engine oil in broth inoculated with culture 1.

IV. CONCLUSION

In conclusion, the present study provides the evidence that the oil degrading bacteria can be isolated from oil contaminated sites. Amongst the isolates obtained, the *bacillus cereus* culture expressed the better oil emulsification and degradation ability. This could be suggested that the *bacillus cereus* strain can be effectively used in the bioremediation of oil pollution.

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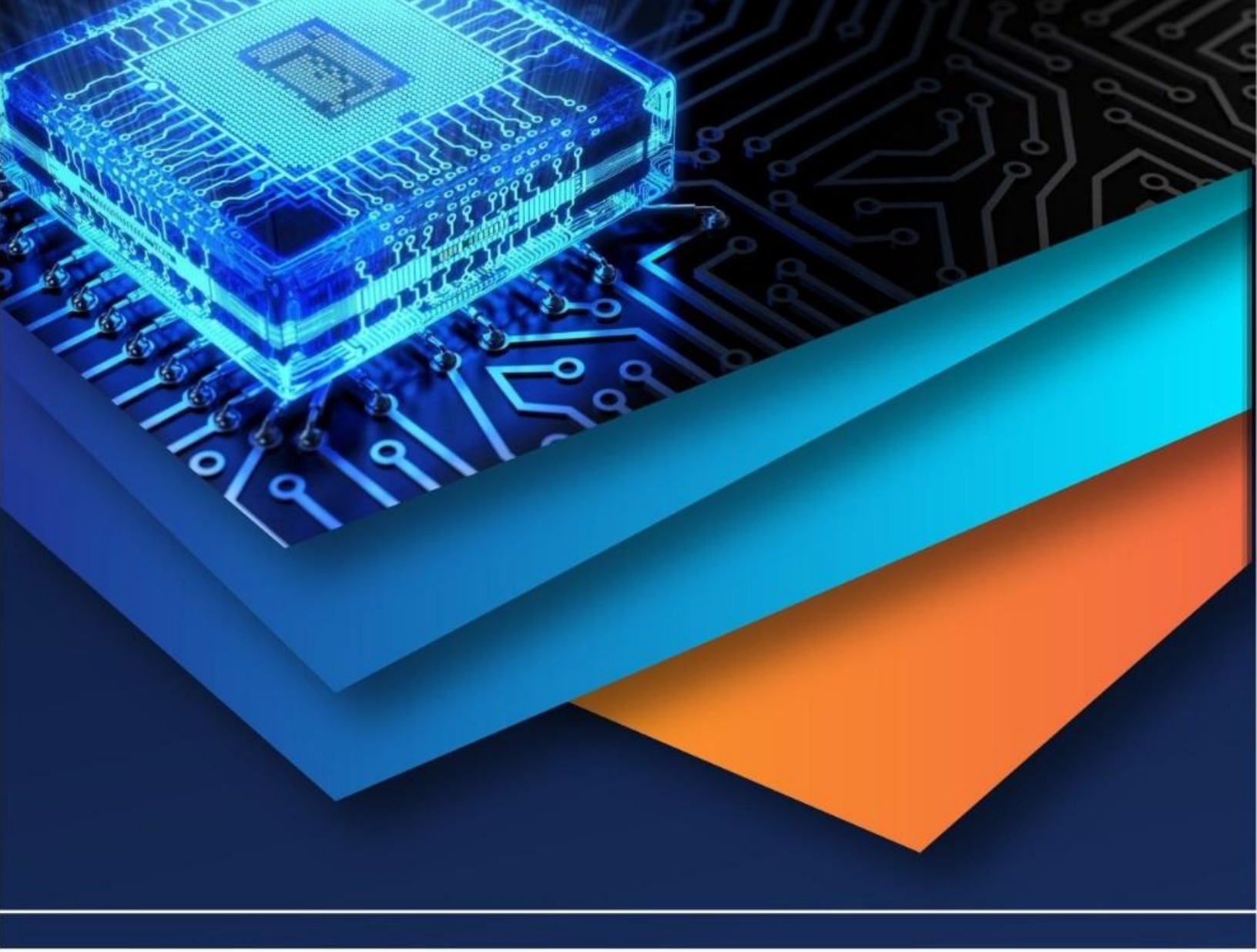

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