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# Efficiency of Oil Degrading Marine Bacteria

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**Oil spill poses a major problem to the marine environment throughout the world. In order to bioremediate the oil from the marine environment, a study was designed incorporating the bacteria which was isolated from the oil contaminated site of Tuticorin port. Among 28 strains isolated from sediments sample only 06 like *Bacillus* spk.1, *Bacillus* spk.2, *Bacillus* spk.3, *Staphylococcus* sp., *Pseudomonas* sp., *and Arthrobacter* sp. used for further experimental analysis based on the amount lipase produced. The efficiency of crude oil biodegradation was compared with two type strains such as *Acinetobacter calcoaceticus* and *Pseudomonas citronellosis.* In order to evaluate the effect of culture conditions on the biodegradation of crude oil, 6 strains were cultured at different temperatures (37 and 45ºC) and inoculum concentrations (0.5 and 1ml). Using a Fourier Transform Infrared Spectroscopy (FTIR), the results of degradation of crude oil was depicted by three ways: 1) Fingerprint region, 2) Number of compounds broken down, and 3) Number of compounds lost by percentage of transmittance. On this basis, it was found that *Staphylococcus* sp., and *Arthrobacter* sp. have the ability to breakdown the crude oil into a maximum number of compounds (18) while *Bacillus* spk. 2 and *Bacillus* spk. 3 possess the ability to degrade certain compounds completely.**

**Key words:** Biodegradation, oil, Marine, Bacteria.

It is estimated that about 8.8 million metric tons of oil are being released into the aquatic realm every year. Among those spills more than 90% are caused by human activities including deliberate waste disposal. Marine oil spills, particularly accidental spill of oil have received greater attention due to their catastrophic damage to the environment. Spill of 37,000 metric tons (11 million gallons) of North Slope crude oil into the Prince William Sound, Alaska, from the Exxon Valdez in 1989 led to the mortality of thousands of seabirds and marine mammals. Significant reductions in population of many intertidal and subtidal organisms were observed after the spill. Minor oil spills and oil contamination from non-point source discharges (e.g., urban runoff and boat bilge) are no less threats to public health and the environment, although they have received much less attention in the past. About 8.13 crores rupees spent on the recovery charges toward cleanup cost for 68 oil spills in India last ten years[1].

Oil spills are known to cause major stresses in the aquatic environment including estuarine ecology and damages the mangrove forest all over the globe[2]. Crude oil is complex mixture that consists of different components like aliphatics, aromatics, nephthelnics and asphaltic molecules. Few compounds of crude oil such as

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the smaller aromatics like benzene, toluene and xylene are considered as predominant water soluble fractions causing detrimental effect in the environment[3]. Benzene causes leukemia and also associated with other blood cancers in human and other animals.

Among the biological techniques bioremediation evolved as most promising method because of its economic, safe and environmental-friendly in which the complex organic compounds are transformed into a simple one. Biodegradation of oil involves several microbial processes, breaking hazardous substances either under aerobic or anaerobic conditions leading to formation of less toxic or non-toxic substances like organic acids, fatty acids, aldehydes, carbon dioxide and water[4]. The success of biodegradation depends on the various environmental factors. Although extensive research continues on oil degradation, the effectiveness of degradative processes are still a phenomenon warranting additional studies on factors affecting oil degradation that remains a quest.

## MATERIALS AND METHODS

### Enrichment of crude oil degrader

The sediment samples collected from oil spill site at Tuticorin harbor were used for the isolation of crude oil degrading bacterial populations. Enrichment of crude oil degrading population was done using minimal salt broth medium supplemented with crude oil as substrate at 2.0 % concentration. Seeded Erlenmeyer flasks were incubated for 20 days at 37ºC in shaker incubator rotating at 200 rpm.

### Isolation and Characterization of crude oil degrading bacteria

One milliliter of enriched bacterial culture was diluted serially up to $10^{-5}$. Each diluted samples were plated over the Minimal Salt Agar medium supplemented with 2% crude oil as sole carbon source. The seeded plates were sealed with parafilm and incubated at 37ºC for 72 hours. Morphologically different colonies were isolated. All isolates were identified based on their morphological, physiological and biochemical characteristics according to Bergey's Manual of Determinative Bacteriology[5].

### Selection of Strain

Efficient strains for crude oil degradation were identified on the basis of their ability to produce lipase on tributyrin agar plates[6]. Agar wells made in the plates using cork borer were filled with 100 µL of 16 hour old culture adjusted to 0.41 O.D and incubated at 30 ºC for 24 hours. The zone of clearance was measured in mm. Among 28 positive strains, only six strains showed wider zone of clearance and were used for further experimental study. Owing to verify the experimental setup *Acinetobacter calcoaceticus* and *Pseudomonas citronellosis* MTCC strains were used as positive control.

### Optimization of Crude Oil Degradation through catechol removal

Optimizations of crude oil degradation by all the six different cultures were done for various parameters affecting the crude oil degradation. Crude oil degradation was carried out at different pH (pH 4.0 to pH 10.0). Similarly it was also standardized for the temperatures. The rate of crude oil degradation was done with the range of temperatures (37 and 45ºC). Seeded crude oil flasks set accordingly different temperature and pH was maintained in an orbital shaker (200 rpm) at 37ºC for 20 days. Rate of crude oil degradation was assayed by drawing 5ml of culture.

### FTIR Analysis to assess the crude oil degradation

The rate of crude oil degradation was identified by following three methods: (1) Finger print region, (2) number of compounds broken down and (3) Percentage of transmittance. The analysis was done with 8400S Shimadzu FT-IR Spectrophotometer with the spectral range between 200 to 4000 cm-1 (50 to 2.5 mm) and Wave number: 7800 – 350 $cm^{-1}$ with 100% of transmittance.

### Estimation of catechol

In order to estimate the amount of unutilized catechol, 0.5 milliliter of culture was added and incubated at 30ºC for a period of 18 hours in a rotatory shaker with 200rpm and then 0.5ml of organic layer was taken to make up to 5ml with ethyl acetate. The result was read optically at 256 nm.

## RESULTS

All the 28 bacterial strains isolated from the oil contaminated site were identified as *Bacillus*

**Table 1.** Optimization of hydrocarbon degradation through catechol removal

| Parameters | | Percentage of catechol removal after 18 hours of incubation | | | | | |
|---|---|---|---|---|---|---|---|
| | | *Bacillus* spk.1 | *Bacillus* spk.2 | *Bacillus* spk.3 | *Staphylococcus* sp. | *Pseudomonas* sp. | *Arthrobacter* sp. |
| Temperature (°C) | 37 | 78 | 84 | 80 | 89 | 81 | 82 |
| | 45 | 75 | 76 | 71 | 79 | 75 | 81 |
| Inoculum size(ml) | 0.5 | 78 | 81 | 80 | 82 | 78 | 84 |
| | 1.0 | 84 | 84 | 86 | 81 | 82 | 96 |

spp., *Staphylococcus* spp., *Pseudomonas* spp., *and Arthrobacter* spp. As *Bacillus* (spk. 1, 2 and 3 isolate), *Arthrobacter* sp., *Staphylococcus* sp. And *Pseudomonas* sp.(Table. a) These six isolates were used for the further experimental analysis based on the efficiency in the production of lipase. The characteristics of absorption bands were analyzed by comparing with the known frequencies using SHIMADZU FTIR 8400 spectrophotometer. The results obtained confirmed the degradation of crude oil by the organisms.

Based on FTIR analysis 3 things were extrapolated: A) existence of the finger print region which occurred with less than 1300 $cm^{-1}$ in the control was not evinced in the oil degraded samples at both ambient temperatures (37°C and 45°C) (Figure. 1), B) number of compounds broken down in the crude oil by microorganisms was more from that of untreated crude oil (Figure. 2, 3), and C) finally the disappearance of certain compounds in crude oil based on the percentage of transmittance (Figure. 4, 5).

Finger print region gave the first evidence that the 6 isolates were best and even better than the type strains of these 6 isolates *Staphylococcus* sp. and *Arthrobacter* sp. Had the ability to break down the crude oil into a maximum of 18 compounds; But, *Bacillus* spk. 2 and *Bacillus* spk. 3 were able to degrade certain compounds of crude oil making them to disappear upto 80%. Catechol estimation proves that the breakdown of crude oil has done and *Staphylococcus* sp. And *Arthrobacter* sp. Best in crude oil degradation.

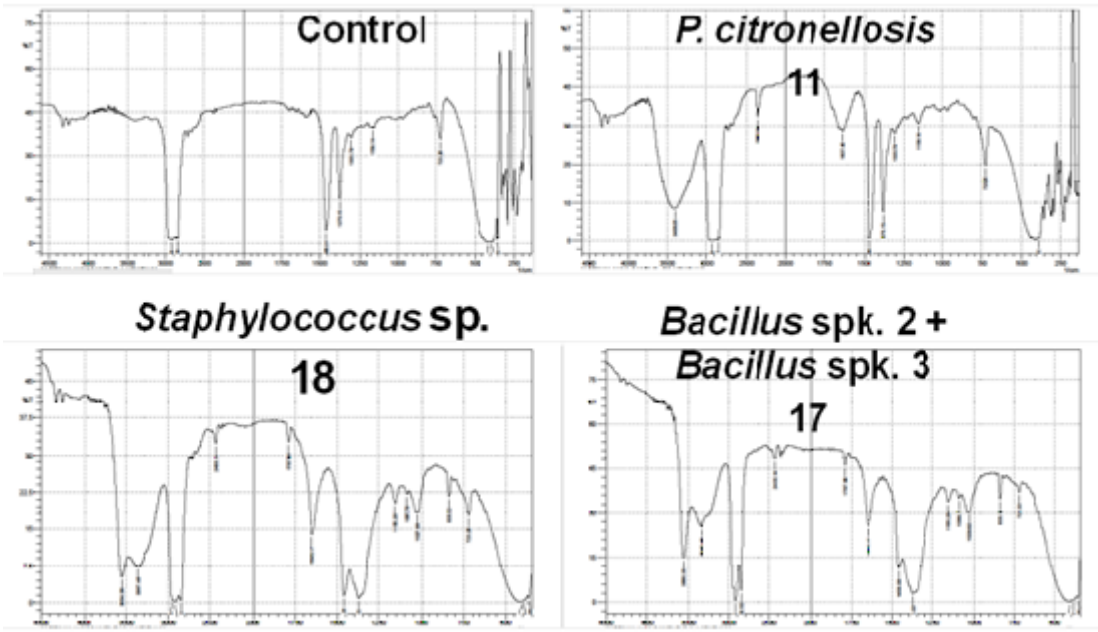



**Fig. 1.** Determination of Fingerprint region

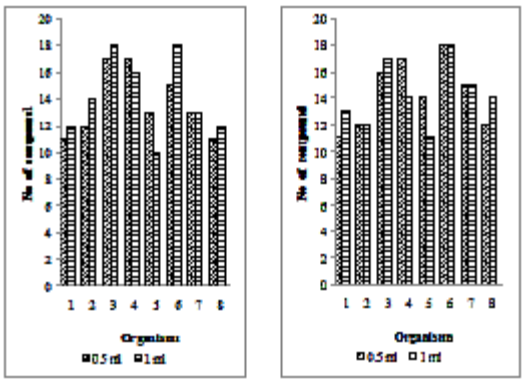


**Fig. 2.** Inoculum concentration Vs No of compounds brokendown at 30°C & 45°C

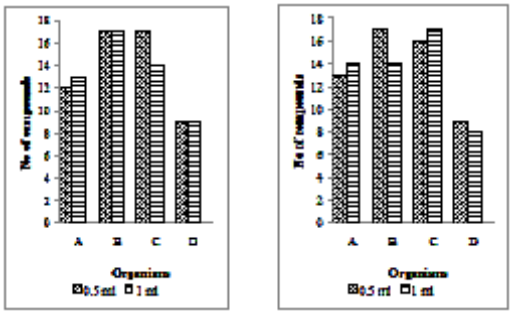


**Fig. 3.** Consortial study Vs No of compounds brokendown at 30°C & 45°C

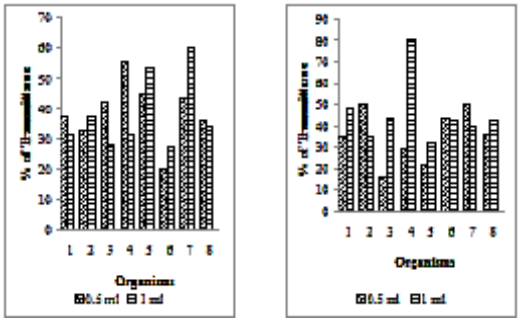
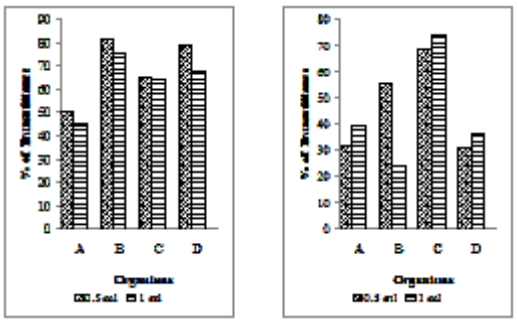


**Fig. 4.** Inoculum concentration Vs Percentage of transmittance at 30°C & 45°C

## DISCUSSION

Marine spills are now becoming a frequent and major source of water and coastal contamination (US Environmental Protection Agency 1990). The Gulf War in 1991 resulted in the worst man-made environmental disaster, with millions of gallons of crude oil being released from the destroyed oil wells into the waters and



[a] (1) *Bacillus* spk.1 (2) *Bacillus* spk.2 (3) *Bacillus* spk.3 (4) *Staphylococcus* sp. (5) *Pseudomonas* sp.(6) *Arthrobacter* sp. (7) *Acinetobacter calcoaceticus* (8) *Pseudomonas citronellosis.* [b] (A) *Bacillus* spk.1+ *Pseudomonas* sp. (B) *Bacillus* spk.2 + *Bacillus* spk.3 (C) *Staphylococcus* sp. +*Arthrobacter* sp. (D) *Acinetobacter calcoaceticus* + *Pseudomonas citronellosis*

**Fig. 5.** Consortial study Vs Percentage of transmittance at 30°C & 45°C

surrounding land, forming more than 330 oil lakes, covering an area of 49 square km. Such releases of large quantities of oil to marine and terrestrial environments present a long term threat to all forms of life. There is now an increasing need for cost-effective remediation technologies for hydrocarbon contamination[7].

Bioremediation has been evaluated in several studies as an option to treat the oil pollution resulting from the spillage or leakage of crude oil and fuels in the environment[8]. Current evidence suggests that in aquatic and terrestrial environments microorganisms are the chief agents for the biodegradation of molecules of environmental concern, including petroleum hydrocarbons[9, 10].

The microbial degradation of oil pollutants is a complex process. An understanding of this process in the environment would help to develop strategies for hydrocarbon degradation through microbial activities for the removal of hydrocarbon from contaminated areas. The present study was circumvented on the isolation and identification of indigenous bacteria from crude oil contaminated sample by the enrichment culture technique, using 6 different medium (ASW, BH, LB, MM, MSM with oil and NB without oil) from which 28 colonies were isolated based on their morphology[11,12] and showed MM agar medium to be the best medium. The presence of hydrocarbons reported that[13,14] in the environment selects the microorganisms capable of surviving

toxic contamination which may result in the degradation of such hydrocarbons. Similarly, the current study also indicated that the bacterial isolates from sample near Tuticorin harbor have the ability to degrade the hydrocarbons in the sea. Microbial degradation, a temperature dependent process, is a natural mechanism by which much of the polluting oil is eliminated. The influence of temperature on petroleum degradation affecting the physical nature and chemical composition of the oil, rate of hydrocarbon metabolism by microorganisms, and on the composition of the microbial community has already been outlined[15]. Considering this factor, biodegradation studies of crude oil at a concentration of 1 ml was carried out at various temperatures (37 and 45°C), inoculum load (0.5 and 1 ml) and using a consortial study at pH 8 after 30 days. FTIR analysis revealed that 37°C temperature and a inoculums load of 0.5 ml were the best for the degradation of crude oil.

Similarly[16] studied the biodegradation of crude oil using *Pseudomonas aeruginosa* at different culture conditions like temperature (28, 30, 32, 34, 36, 38, 40, 42, 44 and 46°C), substrate concentrations (crude oil 0.1, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0 and 4.5%), pH (5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0 and 9.5), salinity NaCl (0, 5, 10, 15, 20, 25, 30, 35 and 40%) and fertilizers - Urea and $K_2HPO_4$ at a ratio of 1:1 (w/w) at 0.1, 0.5, 1.0, 1.5 and 2.0% concentrations (w/v) and observed maximum growth and biodegradation to occur at 38°C, pH 8.0, 35% salinity, 0.1% fertilizer and 2.0% of crude oil using Gas chromatography.

The stated[17] that higher temperatures increase the rates of hydrocarbon metabolism to a maximum, typically in the range of 30 to 40°C, above which the membrane toxicity of hydrocarbons are increased. This is the reason behind our finding why the degradation of crude oil has been reduced at 45°C and been increased at 37°C. On the other hand, FTIR analysis also indicated that the degradation was best by the 6 isolates and was even better than the reference (MTCC) cultures (*Acinetobacter calcoaceticus* and *Pseudomonas citronellosis*) used.

Usually the effectiveness in the degradation of crude oil gets increased by mixed culture, which was also proven in our experiment [18] developed active microbial consortium (*Acinetobacter faecalis* WD2, *Staphylococcus.* sp. DD3 and *Neisseria elongate* TDA4 that exhibited higher degradation of crude oil contaminated groundwater, wastewater aeration pond and biopond at the oil refinery Terengganu Malaysia. Reports of bioremediation[19] also state that consortium may be much more useful for bioaugmentation of oil contaminated environments.

The biodegradation studies[20] carried out of crude oil with *Pseudomonas* at various incubation periods (12 and 25 days) and detected the rate of degradation using FTIR spectra obtained for untreated sample (0 day), treated samples on 12$^{th}$ day and 25$^{th}$ day. The result revealed the presence of new bands pertaining to aliphatic and polycyclic hydrocarbons including various alcohols, aldehydes and ketones. The studied[21] of biodegradation of palmrose oil with *Serratia marcescens*, detecting the rate of degradation using HPLC, FTIR and NMR analysis and found the presence of carboxyl group in the palmorasa oil which was utilised as a sole carbon sources. Similar to the above studies, the present study also showed stretching at 835 $cm^{-1}$ (substituted benzene), C=C stretching at 1645 $cm^{-1}$ (alkene, aromatic ring), C=O stretching at 1787 $cm^{-1}$ (amides, ketones, aldehydes, carboxylic acid and esters), P-H stretching at 2430 $cm^{-1}$, C-H stretching at 2852 $cm^{-1}$, 2921 and 2954 $cm^{-1}$ ($sp^3$ carbon), N-H stretching at 3367 and 3406 $cm^{-1}$ (amide or amine) and O-H (broad) stretching at 3554 $cm^{-1}$ (alcohol) indicating the conversion of hydrocarbon by *Bacillus* (spk 1, 2 and 3 isolate), *Arthrobacter* sp., *Staphylococcus* sp. and *Pseudomonas* sp. when compared to control.

## CONCLUSION

Overall, it could be concluded that the disappearance of finger print region is quite indicative of oil degradation as observed in most tested samples. Maximum degradation was exhibited by *Staphylococcus* sp. and *Arthrobacter* sp. (18 stretching) while others were able to present stretching frequencies in the range of 12-17 only.

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