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Bioremediation of Petroleum Hydrocarbons from Crude Oil Contaminated Site by Gravimetric Analysis

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Abstract—*The bio-degradation of petroleum hydrocarbons by Microbes is major practices in natural decontamination process. This study investigated about the isolation of bacteria from crude oil contaminated site and bio-degradation analyzed by gravimetric method in which, three bacterial isolates formed maximum clearing zone on mineral salt medium. Among these isolate R2 showed optimum growth (0.84mg/ml) and degradation on six day of incubation, followed by R9 & R10 that showed optimum growth (0.75mg/ml & 0.65mg/ml) and also degradation. Isolate R2 identified as Bacillus subtilis, R9 as Acinetobacter & R10 as Pseudomonas aeruginosa, were maximum for both growth and degradation. The total viable count of Bacillus subtilis , Acinetobacter and Pseudomonas aeruginosa were 247x10-6 Cfu, 231 x10-6 Cfu and 228x10-3 Cfu respectively. An increase in total petroleum hydrocarbon bio- degradation was directly proportional to an increase in cell number showing that the bacterial strains were responsible for bio-degradation of petroleum hydrocarbon. Results obtained evidence the bio-degradation potential of these isolates.*

Keyword*: Decontamination, crude oil, biodegradation, Bacillus subtilis, Acinetobacter, Pseudomonas aeruginosa, gravimetry.*

1. INTRODUCTION

Existence of Petroleum hydrocarbons has been reported to enhance the biodiversity, distribution and pollution of microorganisms in an environment. Biodegradation is defined as the biologically catalyzed reduction in complexity of toxic compound. Biodegradation of petroleum hydrocarbons in the environment may be restricted by many limiting factors. The low solubility and bioavailability of the hydrocarbon are often a major limiting factor in the biodegradation contaminated site. As we know that Crude oil is one of the most significant pollutants in the environment. It is responsible for serious damages to humans and the ecosystem. Its long time exposure and higher concentration of crude oil may cause of kidney or liver disease, possible damage to the bone marrow and risk of cancer(Mishra et al.,2001)(Lloyd et al., 2001).The microorganisms in the petroleum degradation and its bio products have been noticed as an efficient, economic, versatile and ecofriendly. Generally microbial degradation is often slow for oil spills that are why search still going on for efficient and effective methods of oil removal from contaminated sites (Gragemard et al., 2001)(Latha et al., 2012). Many different microbial groups are involved in remediation of a petroleum hydrocarbon contaminated site. These indigenous microbes can degrade a wide range of selected constituent present in oily sludge (Barathi et al., 2001). Some Bacterial strains like *Pseudomonas* spp., *Yokenella* spp., *Alcaligenes* spp., *Roseomonas* spp., *Stenotrophomonas* spp., *Acinetobacter* spp, *Flavobacter* spp, *Corynebacterium* spp, *Streptococcus* spp.,*Providencia* spp., *Sphingobacterium* spp, *Capnocytophaga* spp, *Moraxella* spp, and *Bacillus* spp. are capable of degrading PAHs have been isolated from aquifers and soil(Johnson et al., 1996)(Kiyohara et al., 1992)(Bhattacharya et al., 2002). The utilization of crude oil as a source of carbon and energy by microbes become possible because of enzyme system having it self (Antai et al., 1990). The availability of nutrients in polluted soil influences the growth and proliferation of oil utilizing microorganisms. Other methods like mechanical method to decrease the hydrocarbon pollution generally expensive and time consuming. The analysis of petroleum products by mass spectrometry is usually used to provide the percentages of hydrocarbon types such as alkanes and cycloalkanes in the crude oil. The total amounts and relative composition can be determined by another method, called gravimetric method. The accuracy of gravimetric method is satisfactory in experiments that employ fairly large amounts of petroleum hydrocarbon. Thus, this study was constructed to analyze gravimetrically of crude oil bio-degradation by bacterial isolates.

2. MATERIALS AND METHODS

Sample Collection

Oily sludge sample was collected from Assam Oil refinery India. Oil sample was used to analyse the physico-chemical parameters and to isolate the bacteria .Sample was collected from a depth of 5cm. They were collected in sterile polythene bags and tightly packed. They were then carefully transferred

to the laboratory for the analysis and stored at 4°C aseptically before processing.

Media used

R2A medium, Luria Burtani (LB) medium, mineral salt medium (MSM) with sludge and MSM + 0.2% Yeast Extract were used for various experimental purpose. Some of the Instruments that have used shown below in table1.

Table 1: Instruments used

Name	Company
Glasswares, eppendrof tube, falcon tube & microtips	Borocil
Vertical autoclave	Test master
Analytical balance	A & D company Ltd D0008
Distillation column	Borocil
Laminar airflow	ESCO
pH meter	A & D company Ltd D0008
BOD Incubator & shaker	Digitech
Hot Air Oven	Digitech
Laboratory micro wave	Whirlpool magicook 20s(m)
Ultra centrifuge	ESCO
Hot waterbath	UNITECH

Screening of crude oil degrading Bacteria

10gms of soil sample was inoculated in R2B broth and was incubated at 37°C for 2 days. After incubation 0.1ml of broth culture was plated in mineral salt medium using spread plate technique. An ethereal solution of crude oil (10% w/v) was uniformly sprayed over the surface of the agar plate. The ether immediately vaporized and thin layer of oil remained on the entire surface. The plates were incubated at 25°C for 2 days. The organisms that formed clear zones around the colonies were considered as crude oil degraders.

Identification of isolated bacteria

The most potent bacterial diesel degrader was identified by observing morphological characters, by doing several basic Biochemical tests and fatty acid profiling test. Different types of biochemical tests were done such as Gram's staining, Indole test, Methyl red test, VP test, Citrate utilization test, Urease test, Nitrate reduction test, Triple sugar iron test, Gelatinase test, starch hydrolysis test, Catalase test, Oxidase test and H2S production test etc. in Biochemical test kit (HIMEDIA). Morphological features include cell morphology, colony morphology and structural appearance.

Enumeration of Bacteria

Isolation and enumeration of bacteria were performed by soil dilution plate technique using Bushnell Haas agar media (Bushnell et al., 1941). One gram of dried soil was dissolved in 9ml of distilled water and agitated vigorously. Different aqueous dilutions 10-1, 10-2…10-10 of the suspension were applied onto plates and 20ml melted medium at around 50°C was added to it. After gently rotating, the plates were incubated at 37°C for 24hrs. Enumeration of different isolates was carried out selected colonies of bacteria were transferred from mixed culture plates onto respective agar plates and incubated at 37°C for 24hrs plates containing pure cultures were stored at 4°C until the examination.

Oil Degradation

For examining the degradation of oil, Bushnell Haas medium (BHM) supplemented with 5g/l of crude oil was used. About 50ml medium was dispensed in 250ml conical flasks. The media was inoculated with 0.1ml of crude oil degrading bacteria (bacteria obtained by screening of crude oil degrading bacteria) and incubated at 28°C for 6 days on a rotary shakes at 175rpm.

Estimation of Growth & Whole Cell Protein

For estimating growth in terms of whole cell protein (Stanley et al., 2000) 0.5ml of medium was centrifuged at 3000rpm for 10min. The cell pellet was washed twice with Ringer's solution and the pellets were re suspended in 1.0ml of 4.6M NaOH to boiling temperature for 10min to obtain cell free extract protein concentration in cell free extracts was estimated by method (Lowry et al., 1951). Growth was also monitored by measuring optical density at 620nm.

Extraction of Crude Oil

For estimation of oil degradation rates by gravimetric analysis 5ml of n-hexane was added to above flasks. The contents were transferred to a separating funnel and extracted. Extraction was carried out twice to ensure complete recovery of oil. The extract was treated with 0.4g of anhydrous sodium sulphate to remove the moisture and decanted into a beaker leaving behind sodium sulphate. This was evaporated to dryness in a rotary evaporator under reduced pressure.

Gravimetric Analysis

The amount of residual oil was measured after extraction of oil from the medium and evaporating it to dryness in rotary evaporator at 40°C under reduced pressure(Saxena et al., 1990). The volume of extracted oil was deducted from the previously weighed beaker.

The % of degradation was calculated as follows;

Weight of Residual crude oil= Weight of beaker containing extracted crude oil – Weight of empty beaker.

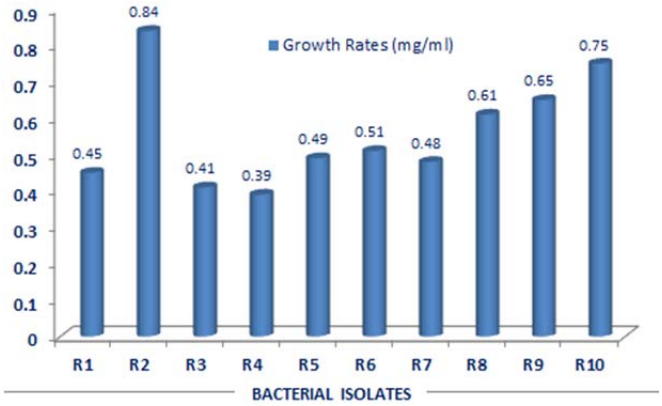
Amount of crude oil degraded = Weight of crude oil added in the media – Weight of residual crude oil

% degradation = Amount of crude oil degraded / Amount of crude oil added in the media x 100

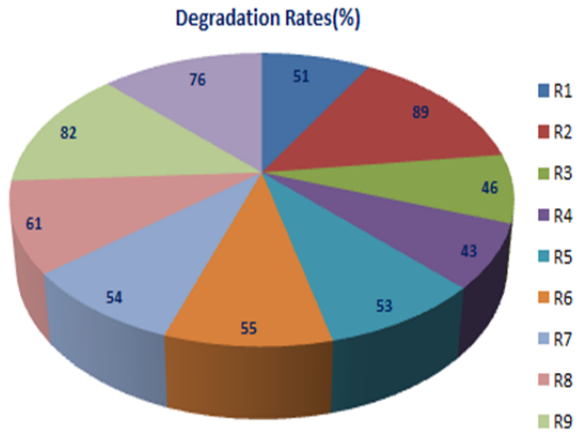
3. RESULT AND DISCUSSION

In mineral salt medium, it showed maximum clearing zone in plates. Clearing of crude oil in the medium showed the

bacterial growth. It indicates the degradation, may be due to production of emulsifiers, surfactants etc. Hence, these 10 isolated designated R1 to R10 were selected for further screening of biodegradation rates. Among the 10 isolates, R2, R9 and R10 formed maximum clearing zone on mineral salt medium. Screening these isolates for oil degradation rates by observing temporal effects on growth and degradation. R2 showed maximum growth (Gr – 0.84 mg/ml), degradation (89%)on 6th day of incubation, followed by isolate R9 growth (Gr-0.75mg/ml) degradation (82%)and isolate R10 growth(Gr-0.65mg/ml) degradation (76%). Hence, these 3 are most efficient isolates R2, R9 and R10 that showed maximum growth and degradation. Nwaogu *et al., (2008)* reported that *B.subtilis* to utilize and degrade oil of 0.63 in 6th day of incubation. Mandri and Lin (2006) reported that the *P. aeruginosa* had degraded 90% in 4 weeks.



Based on various morphological, physiological and biochemical characterization, Isolate R2 identified as Bacillus subtilis, R9 as Acinetobacter & R10 as Pseudomonas aeruginosa, were maximum for both growth and degradation the results presented in pie graph.

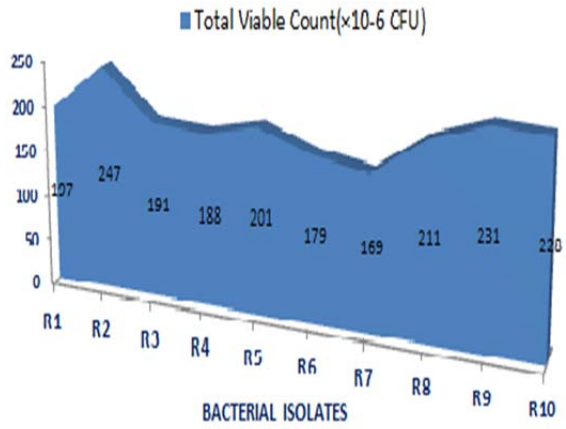


Colony Morphology on nutrient agar plate, *B.subtilis* showed Creamy, big spreading, finely wrinkled and Slimy. In *P. aeruginosa* showed large, opaque irregular colonies with earthy odour.

Table 2: Biochemical characteristics of bacterial isolates

Character	Isolates									
	R1	R2	R3	R4	R5	R6	R7	R8	R9	R10
Motility	+	+	+	+	+	+	+	+	+	+
Catalase	+	+	+	+	+	+	+	+	-	+
Oxitase	-	+	+	+	+	+	+	+	+	+
Citrate	+	+	-	+	+	+	+	+	+	-
Indole	+	+	+	-	+	+	+	+	+	+
Metyl red	-	+	+	+	+	+	+	+	+	+
Voges-p	+	+	+	+	+	+	+	-	+	+
Nitrate	+	+	+	+	+	-	+	+	+	+
Urease	-	-	-	-	+	-	-	-	-	-

The total viable count of Bacillus subtilis , Acinetobacter and Pseudomonas aeruginosa were 247x10-6 Cfu, 231 x10-6 Cfu and 228x10-3 Cfu respectively from this result that the number of viable bacteria especially B.subtilis is greater than the other isolates of Acinetobacter and P. aeruginosa. Biodegradation has been widely received by the public.



However a number of factors must be taken into consideration before *in situ* biodegradation can be applied. These includes, type and concentration of oil contaminated, prevalent climatic conditions, type of environment that has been contaminated and Nutrient content as well as pH of the contaminated site. The rate of crude oil biodegradation in the soil seems to be rapid. This may be due to the fact that the microorganisms in the soil have efficiency ability in utilizing the residual crude oil as a source of carbon and energy (Antai et al., 1990). Crude oil contains hydrocarbon and does not resist attack by microorganisms. The hydrocarbon utilizing microorganisms isolated from the soil were species of *Bacillus, Lactobacter, Arthrobacter, Pseudomonas, Micrococcus, Zoopage, and Articulosporium. Bacillus sp.* predominated, especially in the crude oil polluted soil. This may be due to the ability of the organisms to produce spores, which may shield them from the toxic effects of the hydrocarbons (Onifade et al., 2007).

REFERENCES

[1] Mishra S, Jyot J, Kuhad RC, Lal B, *Appl. Environ.Microbiol.*, 67, 1675,**2001**.

[2] Lloyd CA, Cackette TA, *Air and waste management Association*, 51, 805**, 2001**.

[3] Grangemard IJ, Wallach R, Marget-Dana F, Peypous, *Appl. Biochem. Biotechnol.*, 90, 199,**2001**.

[4] Barathi S, Vasudevan V, *Envion. Int.*, 26, 413, **2001**.

[5] Johnson K, Anderson S, Jacobson CS, *Appl. Environ.Microbiol.*, 62, 3818, **1996**.

[6] Kiyohara H, Takizawa N, Nagao K, *J. Ferment. Bioeng.*, 74, 49 **1992**.

[7] Bhattacharya D, Sarma PM, Krishnan S, Mishra S, Lal B, *Appl.Environ. Microbiol.*, 69, 1435,**2002**.

[8] Antai SP, *Waste Manage*, 10, 61, **1990**.

[9] Bushnell LD, Haas HF, *J.Bacteriol.*, 41, 653, **1941**.

[10] Stanley GA, Juhasz A, Britz ML, *J.Ind. Microbiol. Biotechnol.*, **,** 24, 277, **2000**.

[11] Lowry OH, Rosenbrough NJ, Farr AL, Randall RJ, *J. Biol. Chem.*, 193, 265, **1951** .

[12] Saxena MM, *Environmental analysis: Water, soil and air.* **1990.**

[13] Nwaogu LA, Onyeze GOC, Nwabueze RN, *African Journal of Biotechnology*, 7, 1939, **2008**.

[14] Mandri T, Lin J, *African Journal of Biotechnology*, 6, 22, **2006** .

[15] Onifade AK, Abubakar FA, *Research Journal of Biological Sciences*, 2, 36, **2007**.

[16] Latha R and Kalaivani R, *Advances in Applied Science Research*, 3 (5):2789-2795, **2012**.