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Bacterial Degradation of Crude Oil by Gravimetric Analysis

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ABSTRACT

Microbial degradation of petroleum hydrocarbons is one of the major practices in natural decontamination process. The present study investigated about the isolation of bacteria from crude oil contaminated site and gravimetric analysis of degradation in which, two bacterial isolates formed maximum clearing zone on mineral salt medium. Among these isolate S_2 showed maximum growth (0.85mg/ml) and degradation on seventh day of incubation, followed by S_{10} that showed maximum growth (0.92mg/ml) and degradation. Isolate S_2 was identified as Bacillus subtilis and S_{10} as Pseudomonas aeruginosa, were optimum for both growth and degradation. The total viable count of Bacillus subtilis and Pseudomonas aeruginosa were $257x10^{-6}$ Cfu and $248x10^{-3}$ Cfu respectively. An increase in oil degradation was correlated to an increase in cell number indicating that the bacterial isolates were responsible for the oil degradation. Our results obtained demonstrate the potential for biodegradation of these isolates in situ and/or ex situ.

Key word: Decontamination, biodegradation, *Bacillus subtilis*, *Pseudomonas aeruginosa*, gravimetry.

INTRODUCTION

Presence of Petroleum hydrocarbons has been reported to influence the biodiversity, distribution and pollution of microorganisms in an environment. Biodegradation is defined as the biologically catalyzed reduction in complexity of chemical compound. Biodegradation of petroleum hydrocarbons in the environment may be limited by a large number of factors. An important limiting factor in the biodegradation polluted soils is often the low bioavailability and solubility of the hydrocarbon. Crude oil, because of its characteristics is one of the most significant pollutants in the environment as it is capable of causing serious damages to humans and the ecosystem. Prolonged exposure and high oil concentration may cause the development of liver or kidney disease, possible damage to the bone marrow and an increased risk of cancer [1-2]. The environment of microorganisms in the degradation of petroleum and its products has been established as an efficient, economic, versatile and environmentally sound treatment. The search for effective and efficient methods of oil removal from contaminated sites has intensified in recent years, because microbial degradation that in responsible for clearing untreated oil spills is slow [3]. Microbial remediation of a hydrocarbon–contaminated site is accomplished with the help of a diverse group of microorganisms, particularly the indigenous bacteria present in soil. These microorganisms can degrade a wide range of target constituents present in oily sludge [4]. A large number of *Pseudomonas* strains capable of degrading PAHs have been isolated from soil

and aquifers [5,6]. Other petroleum hydrocarbon-degraders include *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. [7].

Microorganisms have enzyme systems to degrade and utilize diesel oil as a source of carbon and energy [8]. The growth and proliferation of oil utilizing microorganisms in polluted soil is greatly influenced by the availability of nutrients and their hydrocarbonoclastic property. Mechanical method to reduce hydrocarbon pollution is expensive and time consuming. Mass spectrometry is usually used to provide type analysis of petroleum products, which gives the percentages of hydrocarbon types such as alkanes and cycloalkanes in the oil. The type analysis provides the relative composition, the total amounts has to be determined by another method, called gravimetric method. Gravimetric would give satisfactory accuracy in experiments that employ fairly large amounts of petroleum. So, the present study was designed to gravimetric analysis of crude oil degradation by bacteria.

MATERIALS AND METHODS

Sample Collection

Oil samples were collected from Crude Oil Indian Corporation Pvt., Ltd., KK Nagar West, Chennai. Soil sample were used to analyse the physico-chemical parameters and to isolate the bacteria .Samples were collected at a depth of 5cm from the surface of the soil. 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

R_2B broth, mineral salt medium, and Bushnell Haas Broth were used for screening and isolation of bacteria from crude oil.

Selection method

Screening of crude oil degrading Bacteria

5gms of soil sample was inoculated in R_2B 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.

Isolation and Enumeration of Bacteria

Isolation and enumeration of bacteria were performed by soil dilution plate technique using Bushnell Haas agar media [9]. 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 7 days on a rotary shakes at 175rpm.

Estimation of Growth & Whole Cell Protein

For estimating growth in terms of whole cell protein [10] 0.5ml of medium was centrifuged at 3000rpm for 10min. The cell pellet was washed twice with Ringer's solution and the pellets were resuspended 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 [11] method. 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 [12]
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. 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

Identification of the isolates:
The colony characteristics and cellular morphology of the isolated, their pigmentation, staining reactions, physiological and biochemical characteristics were examined by standard methods and the isolates were identified
.

RESULT AND DISCUSSION

In mineral salt medium, it showed maximum clearing zone in plate 1&2. 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 S_1 to S_{10} were selected for further screening of biodegradation rates. Among the 10 isolates, S_2 and S_{10} formed maximum clearing zone on mineral salt medium.

Screening these isolates for oil degradation rates by observing temporal effects on growth and degradation (plate 3). S_2 showed maximum growth (Gr – 0.85 mg/ml), degradation (G-218) on 7^{th} day of incubation, followed by isolate S_{10} growth (Gr-0.25mg/ml) degradation (G-360) (table 1 & plate 4). Hence, these 2 are most efficient isolates S_2 and S_{10} that showed maximum growth and degradation (plate 5 & 6). Nwaogu *et al.* [13] reported that *B.subtilis* to utilize and degrade oil of 0.63 in 6^{th} day of incubation. Mandri and Lin [14] reported that the *P. aeruginosa* had degraded 90% in 4 weeks.

Based on various morphological, physiological and biochemical characterization, isolate S_2 was identified as *Bacillus subtilis* and S_{10} as *Pseudomonas aeruginosa*, the results presented in (table: 2&3).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. In Blood agar plates showed the heamolysis (plate 7).

In total viable counts of *Bacillus subtilis* $257x10^{-6}$ and *Pseudomonas aeruginosa* $248x10^{-3}$ the results presented in table: 4. from this table that the number of viable bacteria especially *B.subtilis* is greater than the other isolates of *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 [8]. 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 [15].

PLATE 1

SOIL SAMPLE

PLATE 2

ZONE FORMATION ON MINERAL SALT MEDIUM

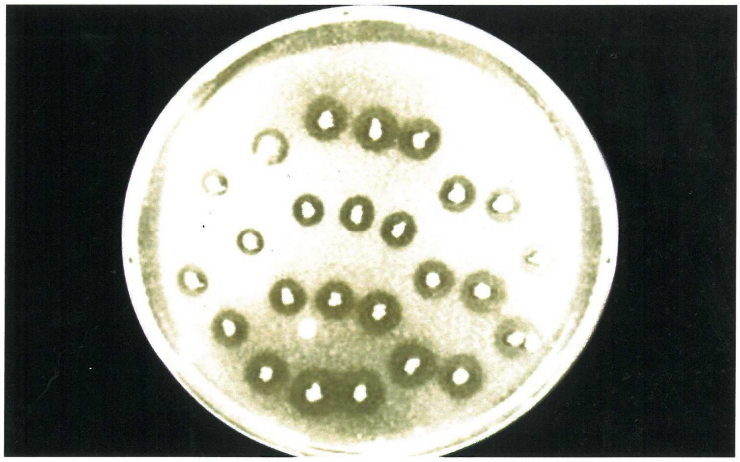


Table: 1 Crude oil degradation by bacterial isolates

Bacterial isolates	Crude oil degradation	
	Degradation rates (%) of isolates	Growth (mg/ml)
S_2	218	0.85
S_{10}	360	0.92

Table: 2 Morphology of nutrient agar plate

Character	Isolates	
	Bacillus subtilis	***Pseudomonas aeruginosa***
Colony morphology	Creamy, big spreading, finely wrinkled and slimy	Large, opaque irregular colonies with earthy odour

PLATE 3

PRECULTURE PREPARATION

PLATE 4

EXTRACTION OF CRUDE OIL

PLATE 5

AFTER DEGRADATION

PLATE 6

DIFFERENT BETWEEN BEFORE DEGRADATION AND AFTER DEGRADATION OF CRUDE OIL

Table: 3 Biochemical characteristics of bacterial isolates

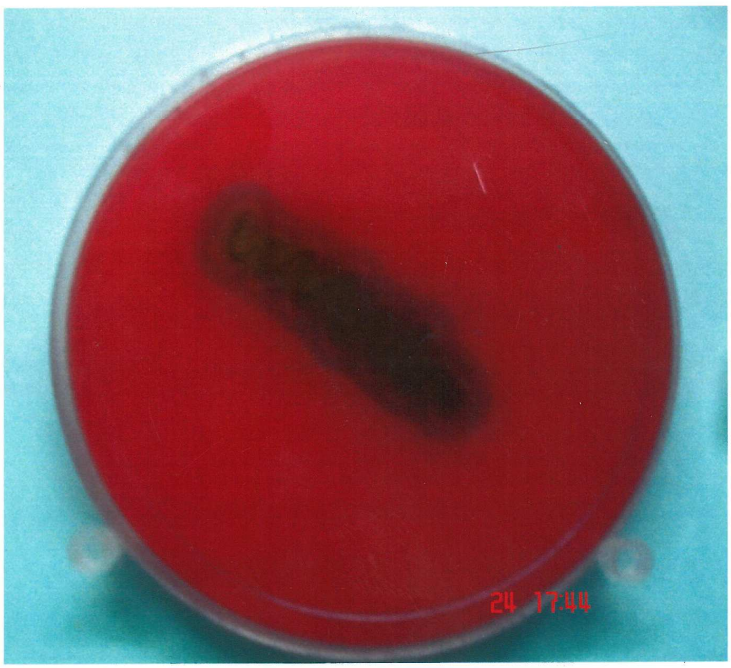
Character	Isolates	
	Bacillus subtilis	***Pseudomonas aeruginosa***
Gram staining	Gram positive rod	Gram negative rod
Motility	Positive	Positive
Catalase	Positive	Positive
Oxidase	Positive	Positive
Citrate	Positive	Positive
Indole	Positive	Positive
Methyl red	Positive	Positive
Voges – Proaskauer	Positive	Positive
Nitrate	Positive	Positive
Urease	Negative	Negative
TSI	K/A	K/K

Table: 4 Isolation and Enumeration of Bacteria

Isolates	Colony Forming units (Cfu)
Bacillus subtilis	$257x10^{-6}$
Pseudomonas aeruginosa	$248x10^{-3}$

PLATE 7

***P.aeruginosa* HEAMOLYSIS IN BLOOD AGAR PLATE**



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