ISOLATION AND CHARACTERIZATION OF MARINE BACTERIA FOR CRUDE OIL DEGRADATION

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Abstract:

Environmental spoilage of petroleum hydrocarbons handling is essential to safe our resources and health. The amount of natural crude oil seepage was estimated to be 600,000 metric tons per 2 year with a range of uncertainty of 200,000 metric tons per year. Oil spilled in water tends to spread and form a slick. From the findings wind and wave action, oil-in-water or water-in-oil emulsions may form. So the present study was carried out to isolate and characterize crude oil degrading microbes from crude oil contaminated marine water samples collected from Ennore, Tamil Nadu, India. The gravimetric analysis of degradation shows that two isolates, CDB1 and CDB2 among 5 isolates found to be potential for degradation of crude oil. The effective CDB1 and CDB2 isolates were further characterized morphological, physiological and Biochemical properties. The maximum crude oil degradation of 71.9% was achieved using CDB2 with 1% crude oil after 7 days of reaction. It is concluded that that the CDB2 isolate Such a bacterial species may be useful for preventing natural decontamination process.

Keywords: Hydrocarbon, Contamination, Environment, microbes.

1. Introduction

Crude oil is a heterogeneous liquid that consist of petroleum hydrocarbons in which the ratio of hydrogen and carbon is 2:1, it is highly contaminate environment. Formation of hydrocarbon is transport, storage and refinery of crude oil, the contamination of ground and surface water bodies, soils and sediments results from accidents and improper handling. Generally this petroleum hydrocarbon contaminate are saturates, the aromatics, the asphaltenes (phenols, fatty acids, ketones, esters and porphyrins), and the resins (pyridines, quinolines,

carbazoles, sulfoxides and amides) [1]. Hydrocarbons differ in their susceptibility to microbial attack and ranked in order of decreasing susceptibility: n-alkanes>branched alkanes>aromatics>cyclic alkanes. Due to its complicated composition, petroleum hydrocarbons have the potential to elicit multiple types of toxic effects. It can cause acute lethal toxicity, sub-lethal chronic toxicity, or both depending on the exposure, dosage, and the organism exposed. Petroleum hydrocarbons are also mutagenic and carcinogenic which causes serious environmental problems due to its persistence in the environment and bioaccumulation [2].

Oil spillage and oil pollution in water environment have been a major threat to the ecosystem and human being through the transfer of toxic organic materials including hydrocarbons into the food chain [3]. The amount of natural crude oil seepage was estimated to be 600,000 metric tons per 2 year with a range of uncertainty of 200,000 metric tons per year. Oil spilled in water tends to spread and form a slick. As a result of wind and wave action, oil-in-water or water-in-oil emulsions may form. Spread of contaminant affect the microbes and formulate bio surfactants. Microbial surfactants are useful to uptake the hydrocarbons. It was reported that 96% of hydrocarbon-utilizing bacteria isolated from freshwater lakes were able to emulsify kerosene, and it has been observed that mixed cultures of marine and soil bacteria which effectively degrade crude oil also exhibit strong emulsifying activity [2, 4].

Oil spillage occurred near Kamarajar port, Ennore on January 28, 2017 in which the BW Maple, a liquefied petroleum gas tanker collided with another tanker, MT Dawn Kanchipuram, laden containing 32,813 tonnes of petroleum lubricant. Initially, it was estimated that about one ton of Heavy Furnace Oil (HFO) Grade-IV was spilled; however, later it was confirmed by Indian Coast Guard that there is a probability of 20 metric tons of oil might have been spilled to the coastal waters. As soon as the oil spill event was made public, the Indian Coast Guard, Ministry of Defence, Govt. of India, deployed their contingent for cleaning, containment and recovery process in liaison with the district authorities, Tamil Nadu Pollution Control Board (TNPCB), Fisheries department and local volunteers.

Physical collection of the oil with booms, skimmers, sorbents, chemical barriers and dispersants is generally the first priority of responders during marine oil spillages, but this is rarely easy, nor very effective after a large spill. Amongst the most promising are those that aim to stimulate the natural process of oil biodegradation [5]. Bioremidial mechanism of

microorganisms to detoxify or remove pollutants owing to their diverse metabolic capabilities is an evolving method for the removal and degradation of many environmental pollutants including the products of petroleum industry [6]. Bioremediation epitomizes modern environmental technologies; working with natural phenomena to achieve a more rapid clean up while minimizing undesirable environmental impacts [7].

In this present study we experimented with isolation and characterization of crude oil degrading microorganism from the sample collected from natural sources.

2. Materials and Methods

Sample collection: Marine water samples were collected from Kamarajar port, Ennore, Tamil Nadu, India with geographic location 13.206524° N, 80.327225° E, since the history of oil spill reported in that area. The samples were collected in a pre-sterilized glass bottles and their physical characteristics were analyzed immediately. The samples were carefully transferred to the laboratory for further analysis [8]. Crude oil sample was collected from oil tanker, Kamarajar port, Ennore (Fig 1).

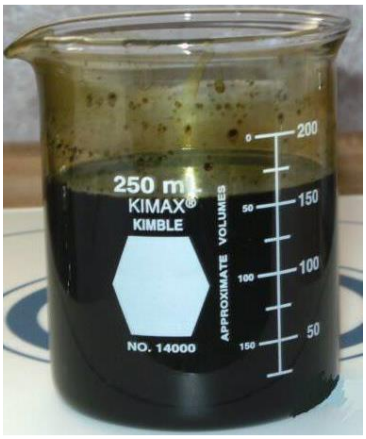

Fig 1: Crude oil collected from Oil tanker, Ennore Port.

Screening of crude oil degrading bacteria: The isolates were isolated from collected crude oil through serial dilution up to 10^{-6}. Each dilutions were plated onto agar plates containing 20ml Minimal Salt Medium (MSM). 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. These plates were incubated at 25°C for 2 days [8].

Isolation and enumeration of crude oil degrading bacteria: The colonies grown in agar plates were isolated, which has the potential to degrade crude oil. The isolated colonies were inoculated in test tube containing 9 ml MSM plus 1% crude oil. The medium was incubated for 7 days at 37ºC. After incubation, the grown crude oil degrading bacteria was maintained for further analysis [8].

Efficiency of crude oil degradation: The efficiency of degradation was calculated by analysing the residual crude oil present in the fermented medium by extraction. The extraction was carried out using chloroform with the ratio of 3:1, sample and chloroform respectively. It was placed in a separating funnel with 16 continuous shaking. After 5-10 mins, two layers were formed: watery layer and chloroform layer containing the residual hydrocarbons. The chloroform layer decanted and air dried, from which the residual oil was quantified gravimetrically [8].

Gravimetric analysis: The amount of residual oil was measured after extraction of oil.

The percentage 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
Percentage degradation = (Amount of crude oil degraded / Amount of crude oil added in the media) x 100

Characterization of Isolates: The isolated potential microbes were characterized Morphological, physiological (Temp. pH,) and biochemical properties. (Catalase, Methyl red, Citrate, Urease, Nitrate).

3. Results:

Physical characteristics of sample: The collected marine water sample was characterized using standard methods and identified that the pH of the sample is 8.1, colourless, has brackish odour with a salinity of 35g/l.

Screening of crude oil degrading bacteria: The colonies in different diluted plates were counted using colony counter. It was observed that maximum of 300 colonies were identified in 10-1 and 10-2 dilutions respectively (Table 1).

Table 1: Serial dilution of collected crude oil contaminated sample for counting colonies in each dilutions

Dilutions	**No. of colonies (CFU / ml)**
10^{-1}	TNTC
10^{-2}	TNTC
10^{-3}	150
10^{-4}	97
10^{-5}	38
10^{-6}	TFTC

*TNTC: Too numerous to count, TFTC: Too few colonies to count

Isolation of crude oil degrading bacteria: The clear zones were observed with colonies in 10^{-6}, 10^{-5}, 10^{-4} dilution plates. These bacterial colonies were isolated further for characterization. The zone forming colonies were considered as crude oil degraders. Based on morphological parameters, colonies were characterized and named the bacterial isolates as CDB1, CDB2, CDB3, CDB4, CDB5 respectively.

Table 2: **Colony morphology in MSM agar plate**

Character	Isolates				
	CDB 1	**CDB 2**	**CDB 3**	**CDB 4**	**CDB 5**
Size	Large	Medium	Small	Medium	Medium
Shape	Irregular	Oval	Pinpoint	Irregular	Circular
Colour	White dull	Pale yellow	Pale Yellow	Dull white	Dull white
Texture	Sticky	Mucoid	Dry	Dry	Dry
Elevation	Umbonate	Umbonate	Flat	Raised	Raised
Margin	Undulate	Smooth	Smooth	Lobate	Smooth

Efficiency of crude oil degradation: The efficiency of crude oil degradation was identified by using the two bacterial isolates. The bacteria degraded the crude oil to a varying extent (Fig 4.3). The extent of oil biodegradation ranged from species to species. It was observed that CDB1 degraded 64.8% and CDB2 degraded 71.9% of crude oil **(Fig. 2**). Remaining all strains observed that below 50% of degradation. Maximum of 7 days it shows the maximum effect. Thus, CDB2 was observed to be the potential crude oil degrading bacteria and further characterization was carried out with these 2 strains, since it was showing maximum above 50% of degradation (**Table 3**).

Table 3: the percentage of degradation by isolated strain on various days

Strain	**Day5**	**Day6**	**Day7**	**Day8**
CDB 1	24.8%	58.4 %	64.8%	64.8%
CDB2	21.3%	59.9%	71.9%	70.6%
CDB 3	12.1%	19.8%	23.2%	31.6%
CDB 4	42.1%	42.7%	31.2%	28.5%
CDB 5	23.3%	22.4%	25.8%	23.3%

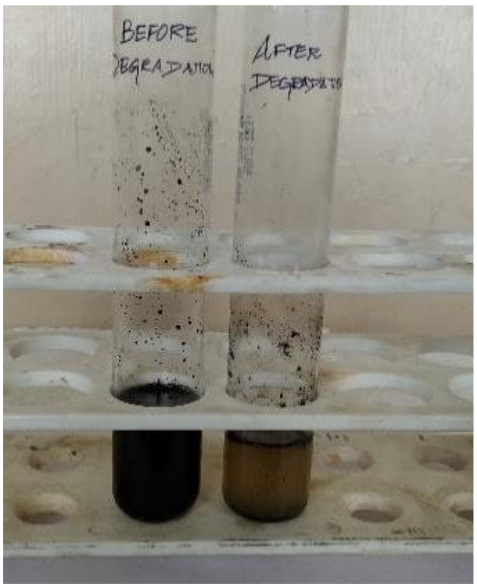

Fig 2: Crude oil degradation by microbes

Characterization of crude oil degrading bacteria: The effective crude oil degrading bacteria CDB2 were characterized as Gram positive and biochemical test reveals that the Catalase, Methyl red, citrate, nitrate shows positive. The urease test shows negative. The physiological characterization reveals the temperature suitable for the growth was optimized and it was found as 28oC. The suitable pH was optimized and identified as 8. The optimum incubation time for the growth was identified as 4 days.

4. Discussion:

The work had revealed that crude oil contaminated marine water harboured bacteria that can degrade crude oil. Growth of the bacteria and the weight loss of crude oil could be due to varying capabilities of the organisms to elaborate crude oil degrading capacity. The use of the four chemical dispersants, Corexit 8666, Gamlen Sea Clean, G. H. Woods Degreaser-Formula 11470, and Sugee 2 for crude oil degradation [9] showed toxicity when used in mixture. Thus, biological methods are effective for crude oil degradation. It is obvious that, a single hydrocarbon degrading bacteria cannot completely degrade all components of crude oil [7], thus two genera of bacteria, CDB1 and CDB2 were isolated from marine water contaminated with crude oil using MSM agar plate containing 1% crude oil. The biochemical characteristics of the bacteria varied between CDB1 and CDB2. Sherry *et al.*, [5] reported that the biochemical

reactions of the crude oil degraders, as a whole, were not uniform and no pattern of relatedness were discernible based on the reactions. The marine water also contributes to the crude oil degrading ability, due to it varied availability of nutrient, pH, salinity, and temperature which ensures the appropriate contact time between the bacteria and the crude oil. It is evident, however that not only bacteria take part in the degradation of these crude oil, but other organisms, such as fungi and protozoa. Freshwater and soil protozoa have degraded 41% of crude oil [10]. A hydrocarbon-degrading fungus, *Cladosporium resinae*, degraded 13% of crude oil when added to the inoculum (Joseph leahy *et al*.,1990). Maximum crude oil degradation was achieved by CDB2 with 71.4% followed by CDB1 with 64.8%. The future scope of this project suggests the use of this potential bacteria in oil fields such as soil washing, microbial enhanced oil recovery, and removal of heavy metal pollutants by measuring the level of toxicity with other marine vertebrates and ecosystem.

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