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ISOLATION AND CHARACTERIZATION OF HYDROCARBON DEGRADING BACTERIA

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ABSTRACT

Petroleum refineries around the world have played major role in generating the solid wastes during the refining process and stocking of crude oil. Oily sludge leads to critical effects in the environment. The ecology of hydrocarbon degradation by microbial populations in the natural environment is analysed, highlighting the physical, chemical, and biological factors that cause the biodegradation of petroleum and individual hydrocarbons. Therefore, in the present research work ventures to isolate hydrocarbon degrading bacteria from the contaminated soil with petrol and diesel oil. In the present work study was conducted in order to decode the microorganisms from oil contaminated sites for oil degradation abilities. Ten soil samples were isolated from oil contaminated sites. One isolate MS 9 showed maximum oil degradation ability. The isolate was characterized for staining and biochemical activities based on Bergey's Manual. The strains were preliminarily identified based on morphological observation, physiological and biochemical tests.

KEYWORDS: **Mycobacterium oxidants, Arthrobacter sp , Bacillus spp, LB Agar, Oil.**

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INTRODUCTION

Hydrocarbons are the world's most widely used primary energy and fuel resources, due to the energy they produce. Evidently inevitable spillages, which occur during routine operations of crude oil production, refining, distribution and as a consequence of acute accidents, have generated continuous research interest in this field[1]. Crude oil is a complex mixture of hydrocarbons and other organic compounds mainly composed of alkanes, cycloalkanes and aromatic alkanes, which constitute about 50 % to 80 % of the oil content. The ability of microorganisms to utilize hydrocarbons in oil contaminated environments has been documented[2]. Microbial degradation process aids the elimination of spilled oil from the environment after critical removal of large amount of oil by various physical and chemical methods[3]. This is possible because microorganisms have an enzyme system to degrade and utilize oil as a source of carbon and energy[3]. The most important characteristic of the lubricating oil for automotive use is its viscosity[4]. More than 230 hydrocarbons have been identified in oil [5]. As an important energy source, oil has played an indispensable role in industrial production and therefore, 20th century was named the "oil century". However, the oil exploration and transportation has contaminated the soil of various degrees. It is well known that a lot of soil bacteria and fungi can utilize petroleum hydrocarbons as a carbon source. At the same time, some aboriginal microbes have gradually adapted to the long-term oil contaminated soil and developed a superior community which can make use of oil contaminants through special substrate enrichment. Therefore, bioremediation of oil contaminated soil has broad prospects because of its low cost, no secondary pollution, processing in situ and so on [6,7,8]. It's a very costly approach to treat oil contaminated site by conventional methods such as use of chemicals or peat moss (a plant which absorbs hydrocarbons). These conventional methods can be replaced by modern methods such as micro-organism or engineered micro-organism which can detoxify the contaminants in to lesser toxic compounds. Bioremediation method is contemplated to be more economical and safe method for the treatment of oil contaminated site. It has been observed that micro-organism that grows on oil contaminated soil are much capable of degrading oil than those microorganisms which are found on non-contaminated site of oil. Prevailing evidence on bioremediation suggests that[9], a numbers of alkali spots, which have formed, limit the vegetation cover and crop growth. Moreover, petroleum hydrocarbons are absorbed by plant roots and accumulated. Thereby, they could potentially get into ahuman's body through the food chain and pose a threat to human health. One of the most significant impact associated with oil includes lose of soil fertility, water holding capacity, permeability and binding capacity.The work aims to isolate novel bacterial strains capable of biodegradation of used engine oil in order to decipher the cultures found in oil contaminated soil for their oil degradation abilities. In this study, we report isolates are capable of degrading a wide spectrum of hydrocarbons efficiently. Degradation studies with different isolates at varying interval of time will help to find out the most potent hydrocarbon degrading strains, which can be used for any bio augmentation studies during bioremediation.

MATERIALS AND METHODS

SAMPLE COLLECTION

Soil samples were collected from 10 different oil contaminated sites in sterile bottles and polythene bags (Work shop, Petrol pump, Service Centre). Samples were collected in 5 cm depth from the surface of soil to avoid surface contamination. Collected samples were transferred to the laboratory under sterile condition and stored at 4˚C until processed for analysis[10]

ISOLATION OF BACTERIA FROM SOIL SAMPLE

Bacterial species were isolated from the collected soil samples by serial dilution and

agar plating method where in the soil sample was diluted from 10-1 to 10-5 dilutions, and the diluted soil samples were spread on sterile Nutrient agar plates. The inoculated plates were incubated at 37° C for 24 hours. Mixed cultures obtained after incubation were named as MS1 to MS10 tentatively and were purified by quadrant streaking on sterile NA plates. The purity of cultures was cross checked by gram staining procedure.

STAINING AND BIOCHEMICAL ACTIVITIES OF PURIFIED CULTURES

In order to identify the purified cultures tentatively on the basis of Bergey's manual[11] various staining and biochemical tests were performed namely Gram staining, Endospore staining, Catalase test, Mannitol fermentation, Glucose fermentation, fructose fermentation, and Lactose fermentation Thirteen biochemical tests were performed in order to identify the two unknown bacteria. Some of the tests provided immediate results while others had to incubate for a period of time.

Mineral salt medium

NaCl10g·L^{-1}, MgSO40.5g·L^{-1},NH4CL 0.5g·L^{-1}, $CaCL_2$0.2g·L^{-1}, $K_2HP_4$1.0g·L^{-1}, $KH_2PO_4$0.5g·L^{-1}, KCLl0.1g·L^{-1}, $FeCl_3$. $6H_2O$ 0.03g· L^{-1}, pH 7.0.

Enrichment medium

1% Inorganic salt medium with 1% crude oil; LB solid medium: Yeast extract 5g·L^{-1}, Peptone 10g·L^{-1}, NaC10g·L^{-1}, Agar 15g·L^{-1} ~ 20g·L^{-1}, pH 7.4 ~ 7.6.

ISOLATION OF OIL-DEGRADING BACTERIAL STRAINS

One gram of oil-contaminated soil sample was added in a flask containing 100mL of sterile water as 10^{-1} diluents, which was then shaken for 30 min at 160 r·min^{-1} at 30°C. And then the solution was also diluted 5 times with sterile water to 10^{-7} diluents concentration, and then 0.1 ml was loaded onto LB solid medium plates and incubated for 1 or 2 days at 30° C. Microbial colonies with different colour and form were transferred with an inoculation loop onto solid LB medium again for separation and purification. The purified strains were pre-cultivated 3 to 4 times on enrichment medium, and were used as the petroleum-degrading bacteria for further study. The selected oil-degrading bacteria with superior growth were propagated on test tube slants and stored at 4 °c. Morphological observation, physiological and biochemical tests the strains were plated on LB solid medium and cultured for 1 or 2 days at 30° C. The morphological characteristics of the colonies were observed, e.g. colony colour, form and size, elevation, gloss, viscosity, medium color[12]. Gram staining [12] and capsule staining [13]were also performed. Ten physiological and biochemical assays were carried out [14] including Methyl red (MR) test, V.P. test (Vogex-Proskauer), oxidase test, catalase test, H_2S production, Indole test, starch hydrolase test, Glucose, Lactose and Mannitol fermentation.

OPTIMIZATION OF STRAIN GROWTH

The strain growth at different pH values and salt concentrations was evaluated by single-factor analysis. There were three replications for each treatment. The strains were cultivated in LB liquid medium to the logarithmic phase; then the cells were collected by centrifugation and washed three times with sterile water to remove residual culture medium. The cell density was measured in a UV-1800 UV-Vis spectrophotometer.

The isolated strains were inoculated in inorganic salt medium adjusted to pH in 6, 7, 8, and 9, with 0.1 mol·l-1HCl or NaOH, and were cultivated on a shaker for 3 days at 30 °c, 160 r·min-1. Then, the absorbance of the culture broth was measured at 258 nm in order to determine the optimum ph range for strain growth. The strains were inoculated in medium with 1 %, 3 %, 5 %, and 7 % NaCl, and cultured at 30 °c, 160 r·min-1 for 7 days.[15,16,17] . To determine the optimal salt concentration for strain growth, the OD of the culture medium was measured in a UV spectrophotometer at 258 nm.

CRUDE-OIL DEGRADATION ABILITY OF THE STRAINS

Crude oil hydrocarbon degradation in liquid medium. each strain was cultured in LB liquid medium containing 1 % crude oil at 30 °c, 160 r·min-1. The degradation rate of crude oil hydrocarbons was measured by the weighting method every week for a month, and non-inoculated liquid medium was used as the control[18,19,20,21].

STATISTICAL ANALYSIS

Pearson correlation analysis between crude oil degradation rate and degradation related enzymes of the strains were done using the Statistical Package for the Social Sciences statistical software.

RESULTS AND DISCUSSION

Ten oil-degrading bacteria were isolated from 3 soil samples from different contaminated field. Morphological features, physiological and biochemical characteristics of the strains.

Table 1

Staining And Biochemical Activities Of Purified Cultures.(Note: "+" Is Positive, "-" Is Negative. *Oxidase Test: "++" Is Positive, "+" Is Slow-Positive, "-" Is Negative.)

Strain characteristics	MS1	MS2	MS3	MS4	MS5	MS6	MS7	MS8	MS9	MS10
MORPHOLOGICAL FEATURES										
COLONY COLOUR	WHITE	WHITE	WHITE	WHITE	WHITE	WHITE	WHITE	WHITE	WHITE	WHITE
CELL SHAPE	ROUND	ROD	ROUND	ROUND	ROD	ROD	ROD	ROD	ROD	ROD
COLONY FORM	IRREGULAR	CIRCULAR	CIRCULAR	IRREGULAR	CIRCULAR	CIRCULAR	CIRCULAR	CIRCULAR	CIRCULAR	CIRCULAR
PHYSIOLOGICAL CHARACTERISTICS										
GRAM STAINING	+	+	+	-	+	+	+	+	+	+
ENDOSPORE STAINING	+	+	+	+	+	+	+	+	+	+
CAPSULE STAINING	+	+	+	+	+	+	+	+	+	+
BIOCHEMICALCHARACTERISTICS										
CATALASE	+	+	+	-	+	+	+	+	+	+
CITRATE	-	+	-	+	+	+	+	+	+	+
GLUCOSE	+	+	+	+	+	+	+	+	+	+
LACTOSE	+	+	+	+	+	+	+	+	+	-
MANNITOL	-	-	-	-	-	-	-	-	-	-
M.R	+	-	+	-	-	+	-	+	+	+
OXIDASE	+	++	-	++	-	++	++	+	-	+
INDOLE	+	+	+	+	+	+	+	+	+	+
V.P	-	+	+	+	+	-	+	+	+	+
H2S PRODUCTION	-	+	+	-	+	-	-	+	+	-
STARCH HYDROLASE	+	-	+	-	-	-	+	-	-	+

Table 2
Width of oil degradation by microbial isolates.

OIL DEGRADATION STUDIE		
ISOLATES	WIDTH OF OIL ON ZERO DAY (mm)	WIDTH OF OIL in 7th DAY(mm)
MS 1	6	1.4
MS 2	6	1
MS 3	6	2.5
MS 4	6	3.0
MS 5	6	1.9
MS 6	6	2.0
MS 7	6	2.9
MS 8	6	1.0
MS 9	6	0.9
MS10	6	1.0

Figure 1
Graphical representation on oil degradation study on microbial isolates

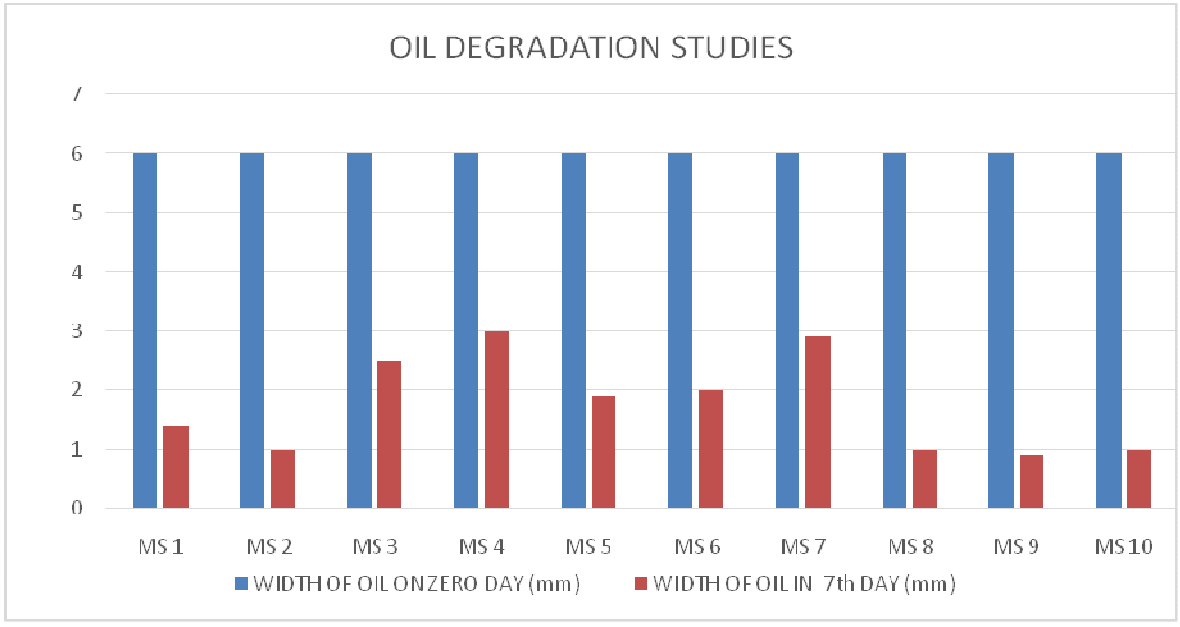


Table 3
Recovered oil after degradation study by microbial isolates

OIL DEGRADATION STUDIES(OIL RECOVERY)		
ISOLATES	**VOLUME OF OIL ON ZERO DAY (ml)**	**VOLUME OF OIL in 7th DAY(ml)**
MS 1	25	1.8
MS 2	25	1.9
MS 3	25	2.8
MS 4	25	7.3
MS 5	25	2.6
MS 6	25	2.8
MS 7	25	2.9
MS 8	25	1.3
MS 9	25	1.0
MS 10	25	1.3

Figure 2
Graphical representation on recovered oil study on microbial isolates

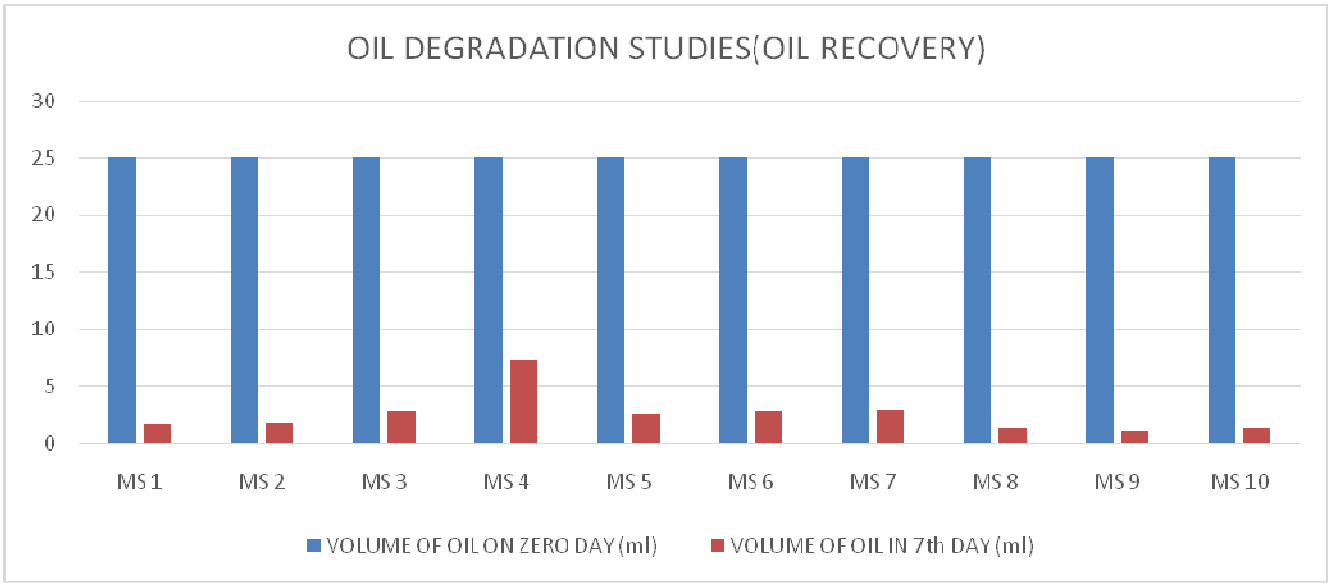
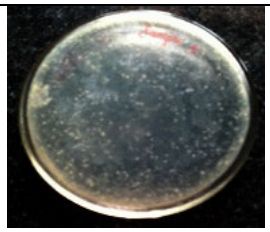
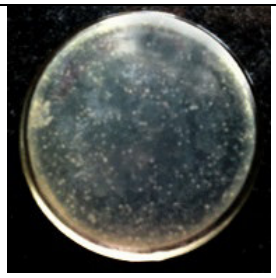
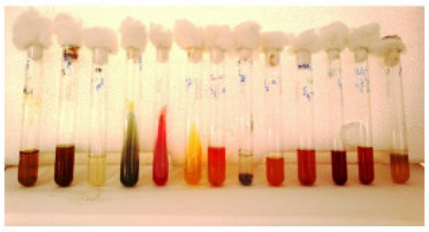
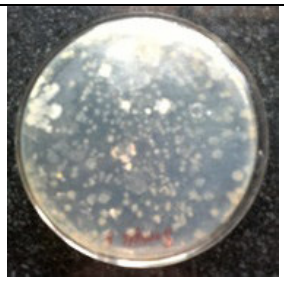
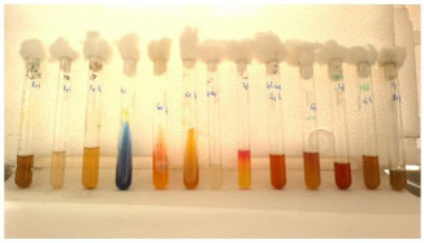
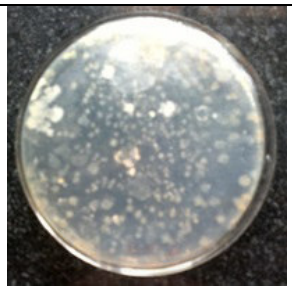
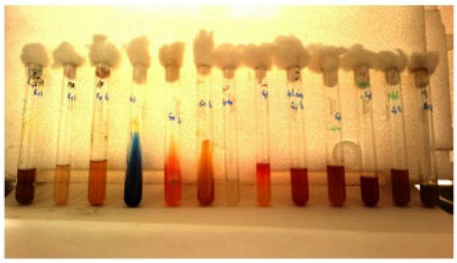
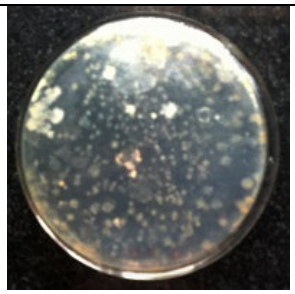
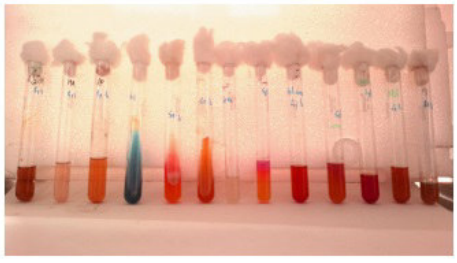
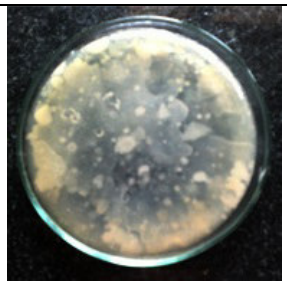
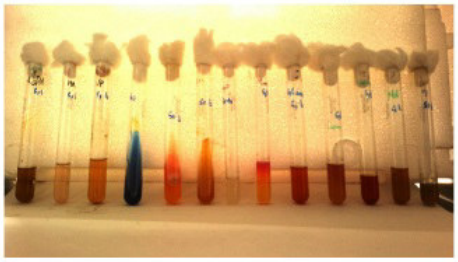
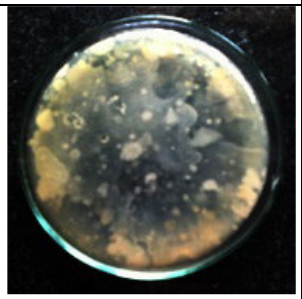
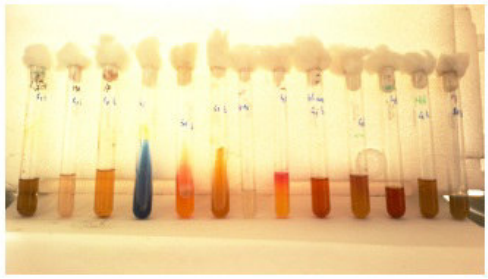
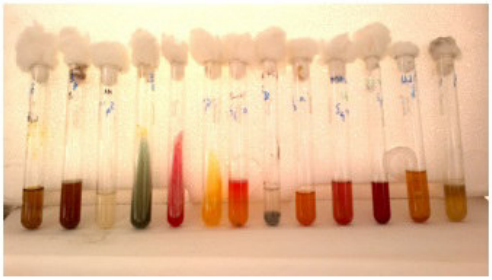
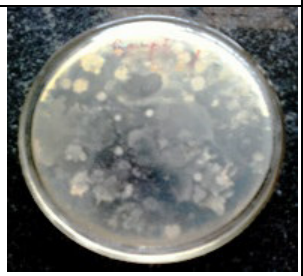
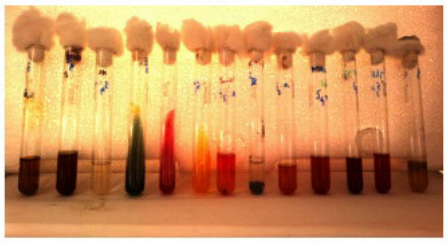
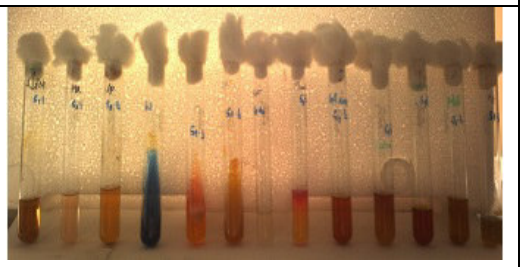


Table 4

Isolated plates and biochemical study on isolates (Refer Table 1 –Morphological features, physiological and biochemical characteristics of the strains)

SI NO	SAMPLE	PLATE	BIOCHEMICAL TEST
1	MS1		
2	MS2		
3	MS3		
4	MS4		
5	MS5		

6	MS6		
7	MS7		
8	MS8		
9	MS9		
10	MS10		

RESULTS AND DISCUSSION

In this study, 10 oil-degrading bacterial strains were isolated from long-term petroleum contaminated soil from different part of cochin, Kerala(India). One isolate MS 9 showed maximum oil degradation abilities, only 1.0 ml oil was recovered out of 25 ml after seven days incubation period and there was a decrease in width of oil layer from 6mm to 1mm after seven days incubation. MS8 and MS9 isolate also showed good degradation abilities, only 1.3ml oil was recovered out of 25 ml after seven days incubation period and there was a decrease in width of oil layer from 6mm to 1mm after seven days incubation.MS4 isolates hawed less oil degradation abilities,7.3 ml of oil was recovered out and there was a decrease in width of oil layer from 6mm to 3mm after seven days incubation.The isolates were identified as *Bacillus spp., Bacillus pumilus, Rhizobium sp., Microbacterium oxydans*, and *Arthrobacter sp.*, based on morphological, physiological and biochemical characteristics[12,13]. It has been postulated that Bacillus spp. are more tolerant to high levels of hydrocarbons in soil due to their resistant endospores. There is growing evidence that isolates belonging to the Bacillus spp. could be effective in clearing oil spills[22,23]. Introductory screening of purified culture was also done by retrieving oil from the flask and estimating the amount of oil left after degradation. This is one of the few reports on this method of quantifying oil degradation abilities. Washing up of petroleum hydrocarbons in the subsurface environment is a real world issue. A better interpretation of the mechanism of biodegradation has a high ecological consequence that depends on the indigenous microorganisms to convert or mineralize the organic contaminants. Microbial degradation process aids the elimination of spilled oil from the environment after critical removal of large amounts of the oil by various physical and chemical methods. This is possible because microorganisms have enzyme systems to degrade and utilize deferent hydrocarbons as a source of carbon and energy. The use of genetically modified (GM) bacteria represents a research boundary with wide suggestion. The future advantage of using genetically modified bacteria are remarkable. But the need for genetically modified bacteria may be controversial for many cases, considering that indigenous species often perform adequately but we do not tap the full prospective of wild species due to our limited understanding of various phytoremidiation mechanisms, including the regulation of enzyme systems that degrade pollutants. Therefore, based on the present review, it may be concluded that microbial degradation can be examine as a key component in the cleanup strategy for petroleum hydrocarbon remediation.

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