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Isolation, Characterization and Identification of Diesel Engine Oil Degrading Bacteria from Garage Soil and Comparison of their Bioremediation Potential

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Abstract

The rate of biodegradation of diesel engine oil by microorganisms isolated from garage soil (petroleum contaminated soil) was studied. Modified diesel engine oil medium was used and two most abundant microorganisms were isolated from garage soil – Micrococcus sp. and Pseudomonas sp. were found to be hydrocarbon degraders and these two bacteria's were selected for the degradation test. The degradation of diesel engine oil was monitored at a five day interval up to twenty five day period, using gravimetric method. After 25 days of incubation period, Pseudomonas sp. degraded 67.57 % of the oil and Micrococcus sp. with 52.95 %. But the mixture of Micrococcus sp. and Pseudomonas sp. were found to have great potential to degrade diesel engine oil i.e. 89.98 % after 25 days. The rate of degradation of diesel engine oil by Micrococcus sp. was found to be 7.48 x 10^{-4}gm/hr and that of Pseudomonas sp. was 9.55 x 10^{-4}gm/hr while the mixture of both bacterial isolates showed highest rate of degradation of diesel engine oil i.e. 1.27 x 10^{-3}gm/hr.

Keywords: Bioremediation, Diesel engine oil, oil spills, hydrocarbon degraders, Micrococcus sp., Pseudomonas sp.

Introduction

As we dig deeper into the modern industrial age of technologies, several aspects of human life change. People benefit largely from life development and many live in prosperity, but prosperity has a price. This price is paid by our environment that suffers daily from all kinds of pollutants and destruction. People now have to find ways to cure this destruction.

Oil contamination is one of the most dangerous pollution factors known today. It can cause a threat to the environment. It is very feared by environmentalists and it's very hard to control if it gets out of hand.

Oil spills have been a major issue across decades. One of the famous oil spills which are also ongoing is in Taylor Energy Well in Gulf of Mexico, U.S.A caused due to Hurricane; Sept 16, 2004 till present date and almost 0.03- 0.05 tones oil/per day is estimated to leak. Another recent oil spill was in Mumbai (India) and caused due to the leakage in Mumbai-Uran pipeline dated January 21, 2011 and about 55 tons of oil was leaked in Arabian Sea. Various such accidents occur throughout the years and it causes damage to our surrounding.

Diesel engine oil, which is one of the major products of crude oil, constitutes a major source of pollution in our environment. With the combined dependence on diesel engine oil by some vehicles and generators, greater quantities are being transported over long distances. Therefore diesel engine oil can enter into the environment through wrecks of oil tankers carrying diesel oil, cleaning of diesel tanks by merchants, war ships carrying diesel oil and motor mechanics[1]. Diesel oil spills on agricultural land generally reduce plant growth. Suggested reasons for the reduced plant growth in diesel oil contaminated soils range from direct toxic effect on plants[2] and reduced germination to unsatisfactory soil condition due to insufficient aeration of the soil because of the displacement of air from the space between the soil particles by diesel engine oil[3].

Among several clean–up techniques available to remove petroleum hydrocarbons from the soil and groundwater, bioremediation processes are gaining ground due to their simplicity, higher efficiency and cost–effectiveness when compared to other technologies[4]. This study was therefore designed to monitor the rate of biodegradation of diesel engine oil (hydrocarbon) by microorganisms isolated from garage soil (petroleum contaminated soil), by using gravimetric method.

Material and Methods

Preparation of modified diesel oil medium: The modified diesel oil medium comprised of 0.7 gm K_2HPO_4**,** 0.1 gm $(NH_4)_2SO_4$**,** 0.3 gm KH_2PO_4**,** 0.3 gm $MgSO_4$ $7H_2O$**,** 2.2 gm agar – agar[5]. The mineral components of the medium were dissolved in 100 ml of distilled water and mixed with 2 ml of Gulf diesel engine oil. The medium was autoclaved at 121^oC for 15 min.

Enrichment of microorganisms: Microorganisms capable of degrading diesel engine oil were enriched in sterile modified diesel engine oil medium by inoculating soil (which was collected from Maharashtra garage, 65 years old garage at Sewri) in to the medium in 250 ml conical flask. 0.5 gm of this garage soil was inoculated in to the 100 ml of sterile modified diesel oil broth and allowed to incubate at 37^{o}C for 1 week.

Isolation of microorganisms: After 1 week of incubation period, 1 drop of enriched culture was spread on to the sterile modified diesel oil agar plate. The plate was incubated at 37^{o}C for 48 hr. After 48 hr incubation; two different bacterial colonies were selected from incubated plate. Each bacterial colony type was sub cultured repeatedly onto sterile nutrient agar plates to obtain a pure culture. Pure cultures of bacterial isolates were identified on the basis of their colonial morphology, cellular morphology and biochemical characteristics according to the taxonomic scheme of Bergey's Manual of Determinative Bacteriology[6].

Determination of microbial colony numbers for degradation studies: 5 ml of sterile Nutrient broth was aseptically inoculated with a loopful of pure culture of Colony 1(C1) in first test tube and Colony 2 (C2) in second test tube and incubated both the tubes at 37^{o}C for 24 hr. After incubation, the numbers of organisms present in one ml of nutrient broth were determined by spread plate method. The numbers of organisms were adjusted in both the tubes in such a way that both the isolates contain approximately equal numbers of microorganism in one ml of sample by using sterile Nutrient broth as a diluent[7].

Soil sample collection and preparation: Top surface soil sample was collected from the premises of the Shahid Bhagatsingh Ground, Kalachowki; in sterilized plastic containers. Soil sample meant for degradation studies was sterilized using autoclave at 121^{o}C for 15 min, after which it was allowed to cool to room temperature for further treatments.

Description and treatment of samples: Test: i. 12 samples of 15 gm sterilized soil mixed with 1 ml (0.848 gm) of Sterile Gulf diesel engine oil + 0.2 ml culture of C1, ii. 12 samples of 15 gm sterilized soil mixed with 1 ml (0.848 gm) of Sterile Gulf diesel engine oil + 0.2 ml culture of C 2, iii. 12 samples of 15 gm sterilized soil mixed with 1 ml (0.848 gm) of Sterile Gulf diesel engine oil + 0.1 ml culture of C1+ 0.1 ml culture of C 2

Control: 12 samples of 15 gm sterilized soil mixed with 1 ml (0.848 gm) of Sterile Gulf diesel engine oil + 0.2 ml of sterile distilled water.

Diesel oil degradation studies: The ability of C1, C2 and mixture of both the bacterial isolates to degrade diesel oil was monitored on the first day (day zero) of the study and subsequently at 5-day interval for 25 days. Carbon tetrachloride was employed as an extractant. On each day, two samples per single treatment were analyzed for the quantity of residual diesel oil[7]. Each of the 15gm soil treatment samples was mixed with 40 ml of carbon tetrachloride, placed in a separating conical flask, shaken vigorously for 3 min and allowed to settle for 5 min. The liquid phase was separated by allowing the supernatant (diesel oil – carbon tetrachloride) to pass gradually through a funnel fitted with filter paper (Whatman No 1). Anhydrous sodium sulphate spread on the filter paper was employed to remove any moisture in the mixture. The liquid phase was collected in a 50-ml pre-weighed beaker. The beaker containing the extract was placed in an oven and the extractant allowed to evaporate at 50^{o}C. The beaker with the residual diesel oil was allowed to cool to room temperature and weighed to determine the quantity of residual diesel oil by difference[8].

Results and Discussion

In this study, the soil samples were gathered from the garage (oil contaminated site) because the capability of native bacterial population to mineralize crude oil hydrocarbons in oil contaminated sites was confirmed before by many scientists[9].

The rate of biodegradation of Diesel engine oil by hydrocarbonoclastic organisms isolated from garage soil were assessed. Table - 1 and table - 2 shows that, using cultural characteristics and biochemical characteristics, two bacterial isolates; *Micrococcus* sp. and *Pseudomonas* sp. were identified by compairing it with the Bergey's manual of determinative bacteriology.

The number of CFU/ml of both the bacterial isolates was adjusted to 7.88 x 10^{7} CFU/ml for degradation studies. The biodegraders which were *Micrococcus* sp., *Pseudomonas* sp., and Mixture of both the culture showed different abilities in the breakdown and utilization of the diesel engine oil.

Table-1
Colony characteristics of bacterial isolates on Nutrient agar plate

Character	Size	Shape	Elevation	Colour	Consistency	Opacity
Colony 1	1-2 mm	Circular	Convex	Yellowish	Butyrous	Opaque
Colony 2	2-3 mm	Irregular	Flat	Fluorescent green	Mucoidal	Translucent

Table-2
Biochemical characteristics of bacterial isolates

Character	C1	C2
Gram stain	Positive	Negative
Morphology	Cocci	Rods
Arrangement	Clusters	Solitary
Endospore	No spore	No spore
Motility	Non motile	Sluggishly Motile
Catalase	Positive	Positive
Oxidase	Negative	Positive
Citrate	Negative	Positive
Indole	Negative	Negative
Gelatin	Positive	Positive
Glucose fermentation	No Fermentation	No Fermentation
Lactose fermentation	No Fermentation	No Fermentation
Sucrose fermentation	No Fermentation	No Fermentation
Mannitol fermentation	No Fermentation	No Fermentation
Tripple sugar iron	Acidic, No gas, No H_2S	Alkaline, No gas, No H_2S
Methyl red	Negative	Negative
Voges proskauer	Negative	Negative
Nitrate reduction	Negative	Negative
Urea	Positive	Negative
Organism	*Micrococcus* sp.	*Pseudomonas* sp.

Table-3
Weight of diesel engine oil extracted (on various days) from 15 gm soil samples polluted with 1 ml (0.848 gm) of Sterilized diesel oil and 0.2 ml of culture

Day	Sample	Weight of diesel oil extracted (gm)	Weight of diesel oil degraded (gm)	Rate of degradation (gm/hr)
0	I	0.848 gm	0.000	0.00
	II	0.848 gm	0.000	0.00
	III	0.848 gm	0.000	0.00
	IV	0.848 gm	0.000	0.00
5	I	0.807 gm	0.041	3.42×10^{-4}
	II	0.801 gm	0.047	3.92×10^{-4}
	III	0.830 gm	0.018	1.50×10^{-4}
	IV	0.848 gm	0.000	0.00
10	I	0.787 gm	0.061	2.54×10^{-4}
	II	0.639 gm	0.209	8.71×10^{-4}
	III	0.639 gm	0.209	8.71×10^{-4}
	IV	0.848 gm	0.000	0.00
15	I	0.663 gm	0.185	5.14×10^{-4}
	II	0.348 gm	0.500	1.39×10^{-3}
	III	0.483 gm	0.365	1.01×10^{-3}
	IV	0.848 gm	0.000	0.00
20	I	0.545 gm	0.303	6.31×10^{-4}
	II	0.290 gm	0.558	1.16×10^{-3}
	III	0.271 gm	0.577	1.20×10^{-3}
	IV	0.848 gm	0.000	0.00
25	I	0.399 gm	0.449	7.48×10^{-4}
	II	0.275 gm	0.573	9.55×10^{-4}
	III	0.085 gm	0.763	1.27×10^{-3}
	IV	0.848 gm	0.000	0.00

*values are means of twice determinations.
Key: i. Sterilized soil + Sterilized diesel oil + *Micrococcus* sp. ii. Sterilized soil + Sterilized diesel oil + *Pseudomonas* sp. iii. Sterilized soil + Sterilized diesel oil + *Micrococcus* sp. + *Pseudomonas* sp. IV. Sterilized soil + Sterilized diesel oil

Diesel engine oil degradation study by Micrococcus sp: It was seen that the rate of diesel oil degradation by *Micrococcus* sp. was slow as compared to the rate of degradation of diesel oil by *Pseudomonas* sp. and mixture of *Micrococcus* sp. and *pseudomonas* sp. But the diesel oil degradation potential of *Micrococcus* sp. was continuously increasing as the time of contact between oil and organism increased.

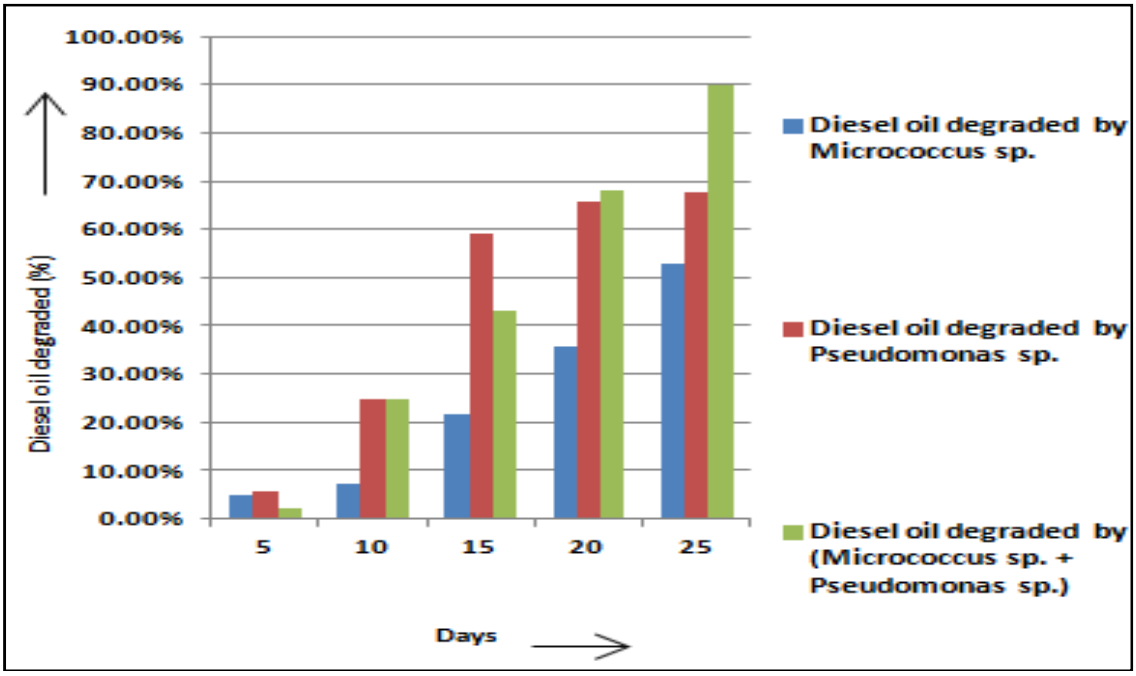
Diesel engine oil degradation study by Pseudomonas sp: It can be seen that the efficiency of Pseudomonas sp. to degrade diesel engine oil is faster than that of *Micrococcus* sp. As the incubation period increases the rate of degradation of diesel engine oil also increases. But it was seen that till 15th day, the rate of degradation was much faster. This was probably due to the exponential phase of the cell growth but after that the rate of degradation was slightly decreased. It was possibly because of cells of the *Pseudomonas* sp. were near to its stationary phase of cell growth.

Diesel engine oil degradation study by mixture of Micrococcus sp. and Pseudomonas sp: The weight of diesel oil extracted from soil containing diesel engine oil and mixture of both bacterial isolates i.e. *Micrococcus* sp. + *Pseudomonas* sp. showed continuous weight loss till the 25th day of incubation period. After 5th day of incubation period it was seen that there was a drastic increase in the rate of diesel oil degradation till the 25th day of incubation period which was quite higher than that of the single culture of *Micrococcus* sp. as well as that of the *Pseudomonas* sp. In this case it was found that around 90% of the diesel engine oil was degraded after 25th day and rate of degradation of diesel oil was found to be continuously increasing i.e. 1.50 x 10^{-4}gm/hr after 5th day to 1.27 x 10^{-3}gm/hr after 25th day.

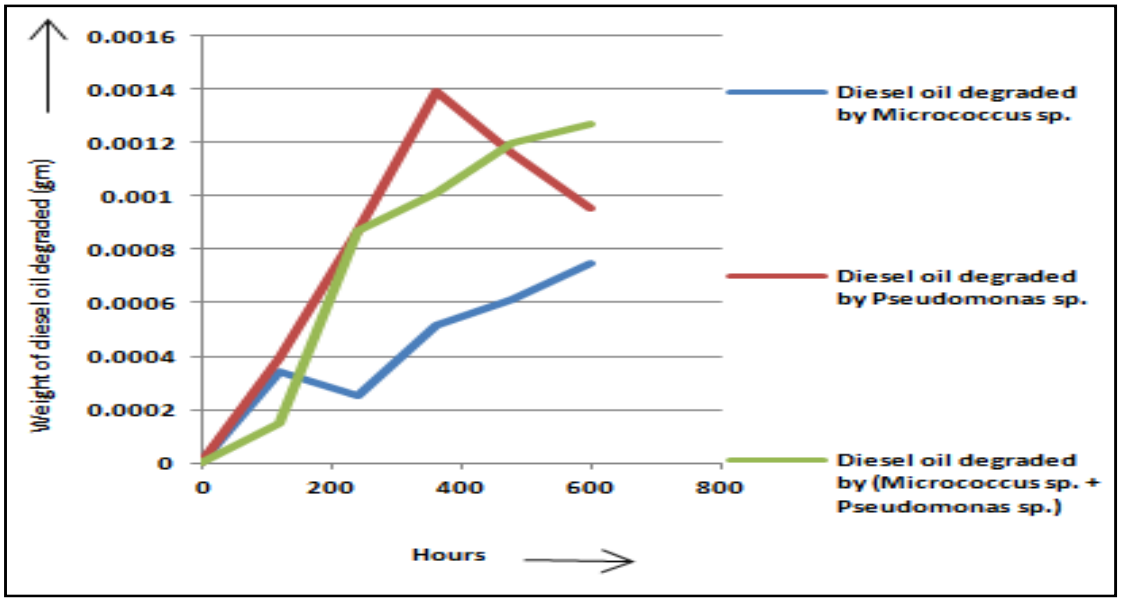
Conclusion

When *Micrococcus* sp. is used in combination with *Pseudomonas* sp. it showed a great potential to diesel oil degradation. This was probably due to the different enzyme system from two different bacterial isolates that acts on hydrocarbon at a time which proved to be an excellent option to degrade that hydrocarbon if both the bacterial enzyme system posses considerable efficiency to act upon it and to degrade it[10]. This was followed by single culture of Pseudomonas sp and then *Micrococcus* sp.

The oil degradation by *Pseudomonas* sp. was not surprising not only because it was isolated from garage soil which was already contaminated by oil and grease but also because it is known to possess a more competent and active hydrocarbon degrading enzyme system than *Micrococcus* sp. It is known to be fast growing and is capable of degrading a wide variety of organic compounds[11]. In the case of *Micrococcus* sp. which is also known to posses the considerable efficiency to use it as an oil degrader, but it requires more time compared to that of the *Pseudomonas* sp.



Figure–1
Comparison of % Diesel engine oil degradation



Figure–2
Comparison: Rate of Diesel engine oil degradation (gm/hr)

By using biological processes, as in the case of bioremediation, usually lowers the costs as compared to chemical treatment processes for various contaminated sites. It is also less disturbing to the environment. However, because it is a natural process, it requires time.

The above experiment shows that bioremediation can be used effectively to treat oil contaminated soil. The remarkable rate of diesel oil degradation by bacterial isolates shown by this method allows for the safe and convenient use of this microorganism in the oil contaminated area. Moreover the results obtained from the comparison between the diesel oil degrading ability of *Pseudomonas* sp., *Micrococcus* sp. and mixture of both helps them to use in different bioremediation processes based upon their efficiencies. And the advantages of employing mixed cultures as opposed to pure cultures in bioremediation have been demonstrated.

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