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Biodegradation of Petroleum Oil by a Novel *Bacillus megaterium* Strain isolated from contaminated soil of Neemrana, Alwar, Rajasthan, India

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Abstract

Bioremediation processes have been found to be an efficient method for remediation of petroleum byproducts, pesticides and other potential harmful chemical. The current study shows the biodegradation of crude oil, diesel and used engine oil by a newly isolated *Bacillus megaterium* from contaminated soil of Neemrana, Alwar, Rajasthan. Hydrocarbon degrading strain was screened on BHA (Bushnell Haas Agar) media supplemented with 2T engine oil as sole carbon source. The strain was found to be degrading at 1%, 4% and 10% of used 2T engine oil respectively after 14 days. Degradation was confirmed both gravimetrically and by Gas Chromatography Mass Spectroscopy analysis. The degradation was found very good at short term basis. The optimization of growth also studied at temperature and pH basis also. The significance of the study is that the percentage degradation of the complex petroleum supplements used in the study was found to be far higher than some of the previously reported values.

Keywords: *Bacillus megaterium*, Complex hydrocarbon, Degradation of complex petroleum oil, Bioremediation

INTRODUCTION

The use of microorganism to destroy or to reduce the concentration of hazardous wastes on a contaminated site is called "Bioremediation". Biodegradation of these compounds are common in nature. From an environment perspective, bacteria which grow on hydrocarbons and mineralize them may be especially useful for soil bioremediation. 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. This is possible because microorganisms have enzymes system to degrade and utilize diesel oil as a source of carbon and energy (Ljah *et al*., 1998; Antai *et al*., 1993). A

number of gram positive and negative microbes have been reported to be capable of utilizing a wide variety of hydrocarbons as carbon and energy (Fought *et al*., 1987).

The soil environment is the most dynamic site of interactions in nature and it is also the region in which many of the biochemical reactions concerned in the decomposition of organic matter and nutrition of plants particularly agricultural crops occur (Torstensson *et al*., 1998). Bioremediation involves the use of indigenous or introduced microorganisms to degrade environmental contaminants (Margesin and Schinner, 1997). The rate of microbial degradation of hydrocarbons in soils is affected by several physicochemical and biological parameters including the number and species of microorganisms present, the conditions for microbial degradation activity (e.g. presence of nutrient, oxygen, pH and temperature) the quality, quantity and bioavailability of the contaminants and the soil characteristics such as particle size distribution (Margesin and Schinner, 1997).

Microbial bioremediation of hydrocarbon contaminated soil and water has emerged as a promising technology in recent years (Mandri, 2007). Most of the study has shown *Pseudomonas aeruginosa*, *Pseudomonas putida*, *Acinetobacter spp.*, *Flavobacterium* spp., *Yokenella* spp., *Alcaligenes* spp., *Roseomonas* spp., *Sphingobacterium* spp., *Capnocytophaga* spp., *Moraxella* spp., *Corynebacterium* spp., *Streptococcus* spp., *Providencia* spp., etc. as common hydrocarbon degraders (Mandri, 2007; Juwarkar, 2012; Etkin, 1998).

The present study was initiated to assess and to compare the degradation of crude oil, diesel and used engine oil in liquid media by *Bacillus megaterium* strain (isolated from contaminated soil of motor workshop, Neemrana, Alwar, Rajasthan, India). Further, bacterial growth was evaluated in various conditions (i.e., temp., pH and media composition).

MATERIALS AND METHODS

Soil samples were collected from different motor workshop areas of Neemrana industrial region, Alwar, Rajasthan. Petroleum oil used in this study was obtained from Bharat oil petroleum Ltd. Sitapura, Jaipur.

Isolation, screening, morphology and biochemical characterization

Bacterial strain was isolated from petroleum contaminated soil from Neemrana, Alwar region, Rajasthan, India. Selective media as Bushnell Hass Agar (BHA) containing 2T oil as sole source of carbon and energy was used for their isolation. The bacterial strain was screened based on the ability of the bacterial species to degrade petroleum oil. The isolated bacterial colony was identified by morphological and biochemical characteristics. The biochemical test was applied to identify bacterial isolates upto generic level.

Degradation Capability to Petroleum oil (2T Engine oil) by *Bacillus megaterium* at different oil concentrations.

(1) By Gravimetric Analysis:

(2) By GCMS methods

Bacterial degradation of petroleum oil was done using the protocol of Mittal and Singh (2000). Petroleum hydrocarbon degradation at different oil concentrations at 1%, 4% and 10%. First of all prepared the Luria Bertani (LB) Broth media and then inoculate the bacterial culture into the LB Broth media at 37^0 C for 48 hours. Secondly, prepared the Mineral Salt Media containing pH 5.6 +_ 0.2. Then after inoculate the 1% 2T engine oil into the MS Media. At last inoculated 1% of the isolated inoculums from LB Broth into the respective flasks. Then proceed with the Gravimetric analysis on Day 0, Day 7 and Day 14.

Gravimetric analysis 1% 1N HCl: added in 25 ml media into each flask. 25 ml Acetone and Petroleum ether (in 1:1 ratio) was added and mixed properly. Then after 1ml Acetone was added and the funnel remain still for 15-20 minute. After 15-20different layers (3 layers) was observed. The 1^{st} and 2^{nd} layers were discarded and the 3^{rd} layer was collected in the weight beaker and kept at water bath at 100^0 C for 10-15 minutes for evaporation. After evaporation is complete, clean the beaker from outside properly to remove any water on the outer side and the again weight the beaker (final weight).

The amount of oil left in the beaker after evaporation was calculated as follows:

Amount of oil left = Final weight of beaker – Initial weight of beaker

Percent Degradation was calculated by the following formula:

Degradation = (Initial weight – Final weight)/ Initial weight × 100

(2) By GCMS methods:

Principle of GC-MS: GC/MS-a combination of two different analytical techniques, Gas Chromatography (GC) and Mass Spectrometry (MS), is used to analyze complex organic and biochemical mixtures (Skoog *et al*., 2007). The GC-MS instrument consists of two main components. The gas chromatography portion separates different compounds in the sample into pulses of pure chemicals based on their volatility by flowing an inert gas (mobile phase), which carries the sample, through a stationary phase fixed in the column (Skoog *et al*., 2007). Spectra of compounds are collected as they exit a chromatographic column by the mass spectrometer, which identifies and quantifies the chemicals according to their mass-to-charge ratio (m/z). These spectra can then be stored on the computer and analyzed.

Procedure:

The extract of each degraded oil samples was mixed in ethyl acetate and then these samples were filtrated. After filtration, remaining substance was ester. These samples were injected to the gas chromatography and mass spectroscopy. Then analyzed complex organic and biochemical mixtures.

RESULTS

Isolation, screening and biochemical characteristic of indigenous *Bacillus megaterium* from petroleum contaminated soil samples.

For their isolation selective media (BHA) containing 2T oil as sole source of carbon and energy was used. Based on varied colony characteristic (margin, elevation, color etc.) bacterial isolate was screened.

But on the basis of cell morphology and biochemical characteristics, this bacterial isolate was identified upto genus level and named *Bacillus* sp.. So, for identification upto specific level 16S rRNA sequencing was done. Through 16S rRNA sequencing it was identified and was named *Bacillus megaterium*.

Fig.1. Sample collection site and pure culture of *Bacillus megaterium*

Percentage Oil Degradation by *Bacillus megaterium* (BS-A1) at different oil concentrations.

Table: A1. Bacterial degradation of 1% 2T Engine Oil

Bacterial Isolates	**Sample code**	**Weight of oil (gm)**			
		0 Day	**7 Day**	**10 Day**	**14 Day**
Bacillus megaterium	BS-A1	1.202	0.312	0.140	0.119

Table: A2. Percent degradation of oil (At 1% oil concentration)

Bacterial Isolates	**Sample code**	**Percent degradation (%)**		
		0 to 7 Day	**0 to 10 Day**	**0 to 14 Day**
Bacillus megaterium	BS-A1	74.0433	88.3527	90.0998

Table A3. Bacterial degradation of 4% 2T Engine Oil

Bacterial Isolates	**Sample code**	**Weight of 2T oil (gm)**			
		Day 0	**Day 7**	**Day 10**	**Day 14**
Bacillus megaterium	BS-A1	0.812	0.652	0.339	0.328

Table: A4. Percentage of oil degradation at 4% 2T Engine oil

Bacterial Isolates	**Sample code**	**Percent degradation**		
		Day 0 to 7	**Day 0 to 10**	**Day 0 to 14**
Bacillus megaterium	BS-A1	19.7044	58.2512	59.6059

Table A5. Percentage of oil degradation: At 10% 2T Engine oil

Bacterial Isolates	**Sample Code**	**Percent degradation**		
		Day 0 to 7	**Day 0 to 10**	**Day 0 to 14**
Bacillus megaterium	BS-A1	4.10	13.54	18.35

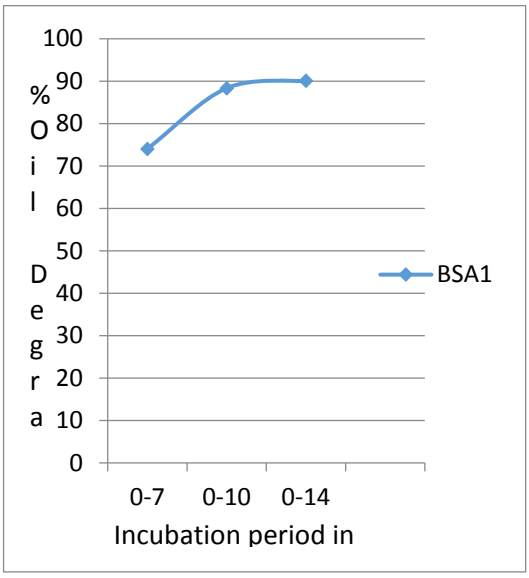

Fig: 2. % degradation of oil at 1% 2T oil cons.

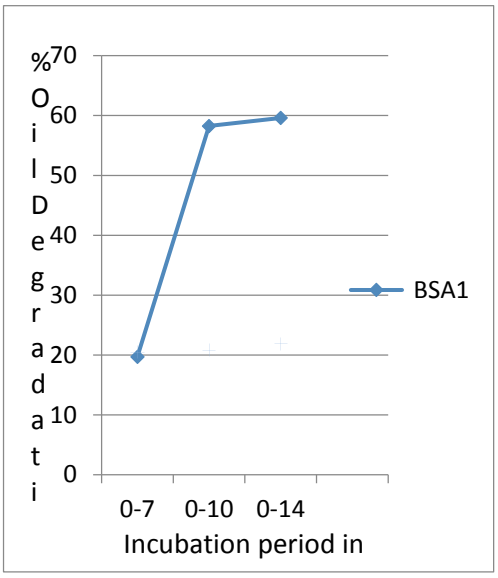

Fig: 3. % degradation of oil at 4% 2T oil cons.

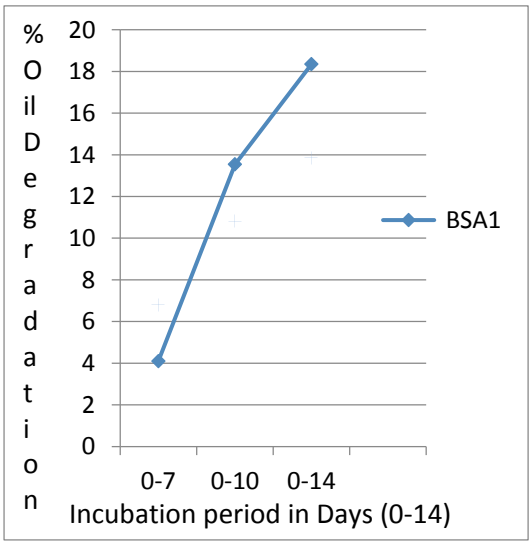

Fig: 4. % degradation of oil at 10% 2T oil cons.

Table: B. Percent Reduction of Peak Areas observed in GC-MS by *Bacillus megaterium* (BS-A1) indicating biodegradation

S. No.	Samples	Peak/ Retention time	Covered area of Retention time/Peak area	Difference of Peak area from control	Percent reduction of Peak area
1	Control	RT 6.34	13476280459		
	1% 2T oil	RT 6.50	4764225110		
		RT 6.66	3683993298		
		RT 6.84	7082295746		
		RT 6.95	4923405066		
		RT 7.07	13751150495		
2	Isolate BS-A1	RT 6.34	7743569917	5 732710542	42.38
		RT 6.49	1458446088	3305779022	69.41
		RT 6.65	1304001730	2379991568	64.60
		RT 6.83	3918610842	3163684904	44.67
		RT 6.94	2892145358	2031259708	41.26
		RT 7.06	7846030607	5905119888	42.95

Table: C. Chart of degradation of GC-MS Peaks at different Retention Time (RT) obtained from culture of *Bacillus megaterium* (BS-A1) for the study which was cultured for 15 days on 1% 2T oil supplemented medium

S. No.	Retention Time	BS-A1	Maximum Degradation Percentage (%)
1	RT 6.34/6.35	42.38	
2	RT 6.49/6.50	69.41	69.41/BS-A1
3	RT 6.65/6.66	64.60	
4	RT 6.83/6.84	44.67	
5	RT 6.94/6.95	41.26	
6	RT 7.06/7.07	42.95	

Figure: 5 – 6 Degradation capability of the *Bacillus megaterium* (BS-A1) studied by GC-MS (In all the graphs Horizontal axis→ Retention time and Vertical axis↑ represents Relative abundance).

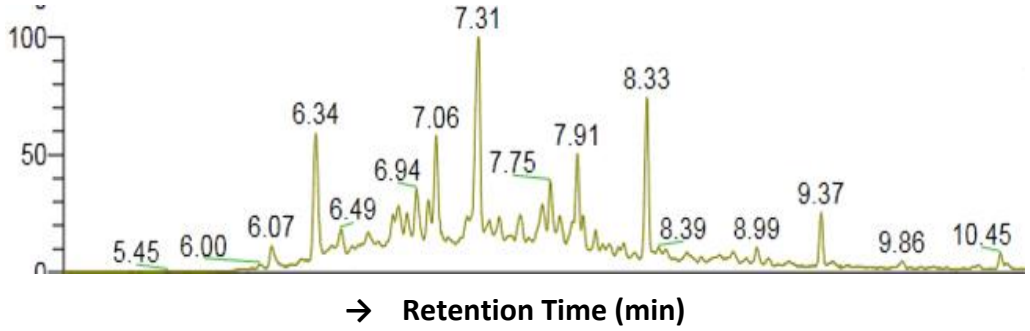

Figure: 5. Peaks of 1% 2T oil concentration (Control sample) at different Retention time

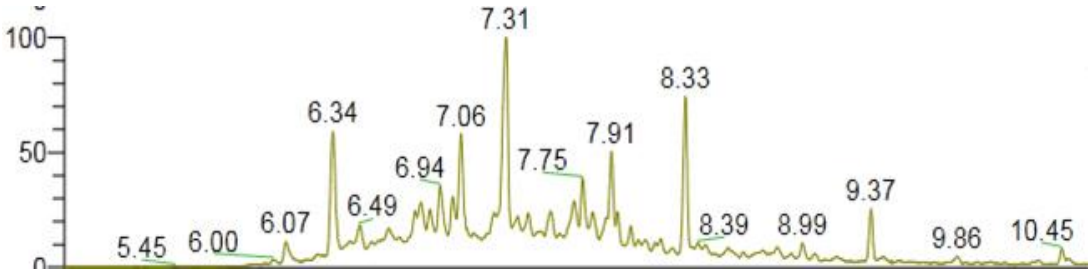

Figure: 6. Peaks of Sample BS-A1 (*Bacillus megaterium*) at different retention time

DISCUSSION

For sustainable development of the nation it should be focused on oil bioremediation using pure cultures or mixed bacterial consortia isolated from oil spilled soils. Microorganisms are diverse and are capable of utilizing petroleum hydrocarbons as energy and carbon source to survive in natural environment (Singh *et al.*, 2010). Elimination of wide ranges of hydrocarbon pollutants from the natural environment is required to enhance a sustainable development of the ecosystem with low ecological impacts (Selvakumar *et al*., 2014). Microorganisms with oil-degrading potential are widely dispersed across nature and have been isolated in multiple ecosystems (Yakubu M, 2007). Similar kinds of studies have been reported for microbial capability exploiting oil and its derivatives as a source of carbon and energy (Magot M, 2005; Khan and Asthana, 2011).

The research study was focused to test the degradation capacity of petroleum oil by the bacterial species isolated from the soil sample of petroleum contaminated site. The bacterial isolate (*Bacillus megaterium*) showed different hydrocarbon degradation capabilities at different (1%, 4% and 10%) oil concentrations in the culture medium.

However, highest hydrocarbon degradation occurred at **1% 2T engine oil** containing culture media. It ranged from 74.04% to 90.09% in 14 days, while after 10^{th} day it showed stationary growth phase. Upto 10^{th} days of culture growth showing fast growth and degradation. It means, it was found best degrader in short term basis. So we concluded that it is best degrader of petroleum hydrocarbons in short term basis and maximum degradation found at minimum 1% oil concentration.

When **4% 2T engine oil** was supplemented with BHA media then *Bacillus megaterium* showed upto 59.60% by 14^{th} days. After 14^{th} day its growth decreases and got stagnation.

When **10% of 2T engine oil** was supplemented in the medium, this shows its slow and steady degradation process. It ranged from 4.10% to 18.35% degradation by 14^{th} days. Hence with time it may give better petroleum oil degradation and will not die out early. It's true because of very high oil concentration the bacteria had to struggle hard for their survival and growth which resulted in poor degradation but continued.

Therefore, to understand in the present study, which isolate is the best degrader and up to what percentage the degradation has actually taken place, Gas Chromatography Mass Spectrophotometry (GC-MS) study was conducted. The peak areas at different Retention time of the isolate was compared with control and the difference in the peak areas between control and an isolate indicated the amount of degradation by that isolate. Hence through it, the difference between peak areas of the sample and control were calculated in terms of percentage to understand the highest degrading isolate.

However, when we comparing the results of the complete period from the 0-14 days at 1% 2T engine oil and GC-MS on the 15^{th} day, the *Bacillus megaterium* has showed good percent degradation upto 69.41%. Here, also proved by Gass Chromatography Mass Spectroscopy (GCMS) that it is very good biodegrader in short term basis. Hence, the identification at genetic level of bacterial isolate (*Bacillus* sp.) was conducted through 16S rRNA sequencing.

CONCLUSION

It can be concluded from the above research experiment that crude oil degrading potent bacteria could be easily isolated from crude oil contaminated sites and bioremediation could be make possible by using potential microbes obtained. Because bioremediation is ecofriendly, non-invasive, less expensive, do not producing additional compound to the environment.

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