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Biodegradation of diesel by bacterial isolates and effect of diesel on seed germination in *Vigno mungo*

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Abstract: Diesel- degrading bacteria has been isolated from a diesel polluted site. Of the three isolates, the best degrader was selected on the basis of growth in liquid and solid media supplemented with diesel as sole carbon source. The isolates were identified as a *Pseudomonas aeruginosa, Staphylococcus aureus* and *Serratia marcescens* were further studied in batch culture for its diesel biodegradation potential under aerobic condition and was observed for seven days, where loss in diesel constituent was up to 75%. Germination and growth of *Vigno mungo* was significantly enhanced in presence of the diesel degrading bacteria.

Key words: *Vigno mungo*, Diesel degradation, *Pseudomonas aeruginosa, Staphylococcus aureus*, *Serratia marcescens* and seed germination

Introduction

Soil pollution by petroleum products is also a wide spread problem, and mineral oil hydrocarbons are the most frequently occurring environmental contaminants (Riffaldi *et al.,* 2006). Individual microorganisms are capable of degrading only a limited number of crude oil depends on the presence of metabolically diverse microbial communities (Atlas, 1981). The possibility of employing microorganism for the degradation of petroleum and it derivatives in minimizing contamination due to oil leaks and spills has prompted a number of investigators to study the process in the laboratory (Claus, 1964).

The various types of hazards posed by the diesel constituents include short term hazards of water soluble compounds such as toluene, ethyl benzene and xylene show acute toxicity to aquatic life in the water column and long term potential hazards including contamination of ground water, chronic effects in the liver and harmful effects on the kidneys, heart, lungs and nervous system. Increase rates of cancer, immunological reproductive fetotoxic and genotoxic effects have also been associated with some of the compounds found in diesel fuel (Irwin, 1997).

Biodegradation of petroleum hydrocarbon in oil and other minimal media has been reported by various scientists (Mohanty and Mukerji, 2007). Degradation of crude oil in the environment by autochthonous microbial communities have been well documented and individual microorganisms capable of metabolizing components of crude oil have been isolated from a variety of ecosystem (Atlas, 1981). The parameters typically measured in laboratory tests of bioremediation efficacy include enumeration of microbial population. Determination of fate of hydrocarbon degradation disappearances of individual hydrocarbons and or total hydrocarbon (Okora, 2008). Important variables affecting the success of bioremediation contaminated sites are identified as duration of contamination available or applied consortia and their predominance, nutrients applied and other environmental parameters.

Many bacterial species are involved in the degradation of hydrocarbon such as *Pseudomonas, Aeromonas, Moraxella, Beijerinickia, Corynebacteria, Mycobacteria, Streptomyces, Achromobacter, Arthrobacter, Acinetobacter, Alcaligenes, Bacillus, Flavobacterium, Nocardia, Rhodococcus* and fungal species such as *Oomycetes, Zygomycetes, Basidiomycota, Deuteromycota* and Micro-algae such as *Porphyridium, Petalonia, Diatoms, Chlorella, Dunaliella, Chlamydomonas, Volvox* (Cerniglia, 1992).

Keeping all these information, the present study was undertaken with the isolation of organisms from diesel contaminated soil and their diesel degrading ability and checking the impact of diesel on seed germination and growth of *Vigno mungo.*

Materials and Methods

Collection and processing of soil sample: 100 g of soil samples were collected randomly from the subsurface after removing the upper 3 cm of the surface soil, from diesel fuel oil polluted soil at an automobile workshop in Koradachery, Thiruvarur district,Tamil Nadu, India. It was thoroughly mixed, sieved through a 2 mm pore size sieve and placed in polyethylene bags closed tightly and then stored in specific container. After sample collection, serial dilution was performed for isolating microbial growth from the collected samples. The nutrient agar medium plates inoculated with 10^{-5}, 10^{-6}, 10^{-7} dilution for bacteria and incubated at 37°C for 24 hr. Isolated bacteria were identified based on morphological, physiological and biochemical characteristics (Cappuccino, 2004).

Screening of diesel degraders -

Inoculum preparation: Three isolates were selected on basis of distinct morphology were grown on nutrient agar slant at 37°C for 24 hrs were washed thrice in physiological saline, OD adjusted to

0.1 and streaked on nutrient agar plates with diesel. Three isolates showing maximum growth in medium with diesel as sole carbon source.

Growth efficiency in diesel as carbon source in liquid medium: Inoculums for three selected isolates were prepared as above, cells were inoculated into 250 ml flask containing 50 ml sterile nutrient broth with 2% diesel concentration with initial OD adjusted to obtain approximately 10^7 cells/ml and incubated at 37°C under shaker condition at 120 rpm for 7 days with control. .

The growth parameters were studied include cell turbidity was measured by determining the optical density at 540 nm using colorimeter (Kumar *et al.,* 2006; Lin *et al.,* 2005; Oboh *et al.,* 2006; Ziad *et al.,* 2005).Protein content of the cell biomass in the medium determined by Folin's -Lowry's method *(* Kumar *et al.,* 2006).

Well diffusion assay method (Saddoun *et al.,* 2002): Growth response of the selected isolates to diesel was also determined in solid medium by bulk seeding 20 ml of nutrient agar with the 1ml of test culture (as prepared in the above segment) and assayed by adding 50 µl of sterile diesel (1-6% prepared in hexane) in 6 mm diameter wells punched in the medium. The control wells were filled with hexane. The plates were incubated at 37°C and growth response was monitored by measuring the diameter of zone of exhibition around the wholes (Saddoun, 2002; Ziad *et al.,* 2005).

Biodegradation of diesel by isolates: 2 ml culture suspension from the stock was added to a 250 ml Erlenmeyar flask containing 100 ml of nutrient broth and diesel oil (2%).Cells were allowed to grow for 7 days at 120 rpm,30°C, and then washed thrice in physiological saline. The final suspension was prepared in 10 ml of nutrient broth to yield a cell concentration which was used as inoculums. Sampling was done on the first and seventh day to determine the amount of the initial and final concentration of diesel and its hydrocarbon.

Gravimetric analysis of degradation -

Diesel extraction from control flask: 100 ml of the flask medium was acidified with 0.1 to 0.2 ml of concentrated HCl to bring down the pH to less than 2 units. 10 ml hexane was added and flask was placed on shaker at 120 rpm for 20 min to enhance the mass transfer rate of diesel from H_2O to Hexane. The lower layer of H_2O was drained back into the conical flask and the extraction was repeated with 10 ml of solvent. The combined solvent extract was drained through a funnel containing 1g anhydrous Na_2So_4 in an ordinary filter paper. The oven at the pre set temperature at 72°C for a predetermined period to evaporate the hexane completely.

Diesel extraction from test flask: For a batch culture with microbial growth it was extremely difficult to filter the hexane water interphase through anhydrous Na_2SO_4. Medium with cells and diesel was centrifuged at a speed of 5000 rpm for 35 min. The diesel oil entrapped within and sorbed on the biomass pellet was extracted by adding 2 ml of hexane, extraction was enformed as for the batch cultures without biomass. The reduction in amount of diesel due to microbial action and due to a biotic losses were determined.

Standardizing time required for evaporation of Hexane: Removing hexane by evaporation in a heated oven is critical. To determine the time for complete evaporation of hexane, two beakers of 50 ml capacity were weighted individually .In the first beaker, 20 ml of pure hexane was taken while in other 20 ml 0f hexane along with 1ml diesel was taken and both were placed in an oven set at a constant temperature of 72°C.The weight of both beakers was measured overtime until there be no further in weight. At 72°C, 20 ml of hexane is evaporated in 126 min.

Effects of inoculum on germination of vigno mungo in the presence of diesel: Effects of diesel on the growth of plant were analyzed by pot culture experiment. The following treatment was made for this study.

1. Control – (sterile soil + seeds).
2. Effect of Diesel – (sterile soil + Diesel + seeds).
3. Degradation ability of microbes -(sterile soil+Diesel+microbes+ Seeds).
4. Effect of microbes – (sterile soil + microbes + seeds).

Results

In this study, diesel degrading bacteria were isolated from the diesel contaminated soil sample. The bacterial isolates were identified and used for biodegradation of diesel by various methods.

Isolation of bacteria: Number of colonies were isolated from one gram of diesel contaminated soil. In the plate containing three different bacterial colonies were isolated based on the cultural characteristics and Gram's staining variation. The numbers of colonies were 44 × 10^6 CFU g^{-1}.

Identification of selected bacterial isolates: There are three different diesel degrading bacterial (DDB) isolates were identified based on the biochemical characteristics. Different bacterial colonies (DDB1, DDB2 and DDB3) were compared with Bergey's manual of systematic Bacteriology. Bacterial isolates were confirmed as *Pseudomonas aeruginosa, Staphylococcus aureus* and *Serratia marcescens* and also has been screened in the presence of the diesel degrading bacteria was identified (Table 1).

Growth efficiency in diesel as carbon source in liquid medium: The selected three different bacterial isolates were diesel degrading ability also screened by liquid medium.All three bacterial isolates showed good growth in liquid medium, Initial growth rate measured by turbidity and protein content of the biomass were also recorded. Bacterial growth rate significantly increased at 7th day measurement of turbidity. Similarly the protein content of biomass increased in all bacterial isolates of DDB1, DDB2 and DDB3. After 7 days of incubation period, the optical density and protein content were noted for DDB1(0.82 and 0.34 µg), DDB2(0.77and 0.30 µg) and DDB3 (0.50 and 0.28 µg) respectively by using calorimeter at 680 nm (Table 2)

Well diffusion assay method: Although isolate three showed best growth as well as metabolic activity in broth culture, to prove

Table - 1: Morphological and biochemical characteristics of test isolates

S.No	Growth ability (%)	Control	Diesel+seed	Diesel+ seed *Pseudomonas aeruginosa*	*Pseudomonas aeruginosa*+seed
1	Germination	92%	50%	75%	100%
2	Shoot length	5.4±0.67	2.5±0.49	2.8±0.60	5.7±1.40
3	Leaf length	4.4±0.35	2.1±0.15	2.7±0.5	3.6±0.10

DDB1- *Pseudomonas aeruginosa* A-Acid ± = Variable reaction
DDB2- *Staphylococcus aureus* K- Alkaline
DDB3 -*Serratia marcescens* G-Gas
Values are Mean ± Standard deviation

Table – 2: Germination and growth of *Vigno mungo* after 7 Days

S.No	Characters	DDB1	DDB2	DDB3
1.	Colour of the colony	Green	White	White
2.	Shape of the cell	Rod	Cocci	Cocci
3.	Grams staining	Gram negative	Gram positive	Gram positive
4.	Motility	Motile	Non-motile	Non-motile
5.	Indole test	Negative	Negative	Negative
6.	Methyl red	Negative	Positive	±
7.	Vogues proskauer test	Negative	±	Positive
8.	Citrate utilization test	Positive	Negative	Positive
9.	Triple sugar iron test	K/A	A/A	K/A
10.	Urease hydrolysis	V(variable)	Negative	Negative
11.	Oxidase	V(variable)	Negative	Negative
12.	Catalase	Negative	Negative	Positive
13.	Carbohydrate			
	Glucose	Positive	Negative	Positive
	Sucrose	Negative	Negative	Positive

Table - 3: Cell turbidity and protein content of the biomass grown in presence of diesel

S.No	Test organisms	Incubation (days)	Optical density at 680 nm	Concentration of protein (µg)
1.	*Pseudomonas aeruginosa*	1st day	0.12	340±0.90
2.	*Staphylococcus aureus*		0.11	300±0.88
3.	*Serratia marcescens*		0.10	280±0.72
		7th dayIncubation	0.82	
			0.77	
			0.50	

Values are expressed as Mean ± Standard Deviation

Table-4: Well diffusion assay method

S.No	Diesel degrading bacteria	Zone of inhibition (mm in diameter)
1.	*Pseudomonas aeruginosa*	10±1.02
2.	*Staphylococcus aureus*	8±0.92
3.	*Serratia marcescens*	6±0.19

Values are expressed as Mean ± Standard Deviation

the versatility of the growth was studied in solid media in response to a range of diesel concentration and as clearly evident from the zones *Pseudomonas aeruginosa* (10 mm), *Staphylococcus aureus (8 mm)* and *Serratia marcescens* (6 mm) (Table 3)

Gravimetric analysis of diesel degradation: Diesel degrading ability was measured by gravimetric analysis. Flask containing liquid medium of diesel o.1ml of bacterial culture. After the incubation period, diesel was extracted by using hexane. The diesel containing hexane was evaporated using oven. Remain only diesel in flask. Diesel degrading ability in control (2 ml) *Pseudomonas aeruginosa* (0.8 ml) *Staphylococcus aureus* (1ml) *Serratia marcescens* (1.8 ml).

Pot culture experiment: Seed germinations were observed in pot culture, no need the diesel treated seed showed only 50% germination and diesel along with microbes showed considerable raising seed germination of 75% when compared with control. Seeds treated with microbes were seen equal germination of 100% with control. Like wise Shoot length and leaf length analysis showed that 50% reduced level in diesel treated seeds and 75% in Diesel along with microbes treated seeds and there are slightly variations in microbes compared with control. The values of

seed germination, shoot length and leaf length were tabulated. (Table 4).

Discussion

Earlier reports showed a lot of variations in number of diesel degraders in soil. Saddoun (2002) reporting a range from 7×10^7 to 2.8×10^9 CFU g^{-1} and Mishra *et al.* (2001) noted significantly low count of 10^4 CFU g^{-1}.

Jae Jun Jeong *et al.* (2003) was also isolated more than 100 species representing 30 genera have been shown to be capable of utilizing hydrocarbons. The association of various bacteria with different hydrocarbon systems *viz*. petrol, diesel and kerosene appear to vary with reference to soil and water characteristics, also the utilization potentials of these hydrocarbons by bacteria.

Bhadauria (1999) also collected agricultural soil irrigated with petroleum refinery effluent, 15 species of bacteria have been identified where the bacterial count range between 66 and 860×10^6. In addition to the complexity of the constituents of crude oil, the effectiveness of bioremediation is affected by several factors including temperature, pH, water content, aeration, nutrient status *etc*.

Rahaman *et al. (*2004) reported that *Pseudomonas* species degradation of hydrocarbon was achieved at 5% oil concentration. A fungal strain which showed high potentiality to adsorb and degrade crude petroleum oil has been isolated. Nutritional and environmental factors affecting petroleum degradation have been evaluated by applying Placketl-Burman design, where K_2HPO_4, inoculum size and pH were the most significant variables. The degradation of hydrocarbon was achieved at 5% oil concentration and proved a maximum petroleum oil degradation of 98.8%.

Microorganisms possess mechanisms by which they degrade the diesel compounds by utilizing them as carbon and nitrogen sources. The pattern of degradation varies for different degrading microorganisms because different microorganisms possess different catabolising enzymes (Penet *et al.,* 2006).

Ziad *et al.* (2005) reported that a noticeable decline in seed germination percentage of *Alfalfa* in the presence of the diesel in soil and the similar results were obtained in this study, which reveals that the diesel have inhibition of *Vigno mungo* seed germination potential in it.

The pollution problems caused by the various process industries are the major concern from environment point. The petroleum based products are the major source of energy for industry and daily life. From the study, concluded that diesel contaminated soil were treated by using microorganisms, it give a better yield for cultivation process. Hence those microbes degrade the diesel contaminated soil, then the soil was reused for cultivation purpose.

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