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Isolation and Identification of Toluene and Pentachlorophenol Degrading Bacteria and Fungi from Engine Oil Contaminated Soil

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Oil pollution is a serious environmental problem throughout the world. This pollution may be caused due to various activities in oil exploration that include geophysical explorations, drilling of wells, pressure control and management of oil and natural gas gushing from the well, transportation and refining of engine etc. Engine oil is a homogenous but complex mixture of hundreds of different hydrocarbons which widely very in their characteristics. Petroleum industry is responsible for the generation of high amount of residues, as well as for the pollution of soils, rivers and seas. Soil pollution is a major problem in the world. In this present work, the engine oil containing hydrocarbons toluene and pentachlorophenol was biologically degraded using *Pseudomonas alcaligenes* , *Serratia marcescens,, A.niger* and *C.mansoni* The degrading bacteria was isolated and identified from engine oil contaminated area. Compared with other organism *P.alcaligenes* gave better degradation of toluene.

Key words: Biodegradation, Hydrocarbon, Toluene, Pentachlorophenol, *P.alcaligenes.*

Polycyclic aromatic hydrocarbon are a group of compounds containing carbon and hydrogen, composed of two or more fused aromatic rings in linear angular and relatively insoluble in water. Polycyclic aromatic hydrocarbons are ubiquitous pollutants have been identified as hazardous chemicals because of their toxin carcinogenic effects on living body[1].

Engine oil contains metals and heavy polycyclic aromatic hydrocarbons that could contribute to chronic hazards including mutagenecity and carcinogenicity[2]. Prolonged exposure to high oil concentration may cause the development of liver or kidney disease, possible damage to the bone marrow, and an increased risk of cancer[3]. Biodegradation has been successful for clean up of pentachlorophenol (PCP), a wood preservative and polycyclic aromatic hydrocarbon the advantages associated with fungal bioremediation lay primarily in the versatility of the technology and its cost efficiency compared to other degradation technologies[4].Most recently, the deuteromycete *Cladosporium* species was isolated from a biofilter that had been used to remove toluene from contaminated air. This fungus can use toluene as the sole source of carbon an energy. These findings demonstrate that it is possible to isolated fungi that grow on aromatic hydrocarbons, provided adequate enrichment techniques are used[5]. Hydrocarbon compounds such as petroleum are essential elements of lift. In Iran is the first country in the oil – rich Middle East region to start oil operations with current production capacities of over 4 millions barrels of

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crude oil 80,000 millions m3/day diesel fuel. There are up to 1,500,000 cubic meters of soil contaminated with engine oil around Tehran refinery Iran. Spills and leaks of the petroleum hydrocarbons from storage facilities and distributions systems results in contaminations of soil and water systems world wide[6].Oil biodegradation of subsurface does not require oxygen, it does require certain essential nutrients (e.g., nitrogen, phosphorus, potassium), which can be provided by dissolution of minerals in the water lake. Empirically, it has been noted that biodegraded oil accumulations occur in reservoirs that are at temperatures less than 80^0C.

Toluene is a aromatic hydrocarbon that is widely used as a industrial feed stock and as a solvent. Toluene sulphonyl iodide, in the presence of copper powder. Like other solvents, toluene is sometimes also used as an inhalant drug for its properties[7]. Pentachlorophenol is a chlorinated hydrocarbon insecticide and fungicide. It is used primarily to protect timber from fungal rot and wood – boring insects, this soil was contaminated with wood preserving wastes including composed primarily of polycyclic aromatic hydro carbons of Pentachlorophenol [8].

MATERIAL AND METHODS

Sample Collection

Soil samples were collected randomly from engine oil contaminated soil from an automobile workshop in Madukkur , Thanjavur District. The sample was exposed to fuel spills for more than 3 years . 100g of soil samples were obtained from the subsurface after removing the upper 3cm of the surface soil . It was thoroughly mixed , sieved through a 2mm pore size sieve and placed in polythene bags closed tightly and then stored in specific container .

Isolation of bacteria and fungi [9]

After sample collection, serial dilution was performed for isolating microbial growth from the collected samples. For this, 1g of soil was added . The tube was vigorously vortexed for 3 minutes to obtain uniform suspension of organisms. A series of tubes labeled as 10^{-1} up to 10^{-8} were filled with 9ml sterile distilled water and serial dilution was performed. The bacterial plates were incubated at 37^0C for 24 hrs and the fungal plates were incubated at 28 for 3 days. After collection of the incubation period Isolated colonies were observed and used for further use .

Identification of bacteria

The colonies grown in the nutrient agar medium where subjected to staining and other biochemical procedure for identifying the bacteria[10].

Isolation of efficient engine oil degraders of Fungi [11]

0.1 ml of fungal culture were spread on potato dextrose agar plates. Then the plates were incubated at 28^0C for 2 days same type of mycelium colonies were developed on potato dextrose agar plates on third day. The plates were sprayed with 1% toluene and1% pentachlorophenol using acetone. After spraying all plates appeared in mycelium growth. The pure culture of each of the two best potential strains were inoculated into 10 ml Bushnell – hass medium containing 1% toluene and 1% pentachlorophenol in conical flasks and incubated at 28^0C on rotatory shaker for 7 days.

Isolation of efficient engine oil degraders of bacteria [12]

0.1 ml of bacterial culture were spread on peptone glycerol phosphate agar plates. Then the plates were incubated at 37^0C for 3 days same type of colonies was developed on peptone glycerol phosphate agar plates on third day. The plates were sprayed with 1% toluene and 1% pentachlorophenol using acetone.After spraying all plates appeared buff white color. The plates were further incubated at 37^0C for the observation of zone of clearance around the colonies. After 2 days zone of clearance were observed. Colonies which shows maximum diameter of zone of clearance in 2 days were further inoculated into 10 ml Bushnell – hass medium containing 1% toluene and pentachlorophenol in conical flasks and incubated at 37^0C on rotatory shaker for 9 days to determine percentage of degradation and bacterial density.

Estimation of bacterial density

Cell biomass concentration was determined by optical density using photo colorimeter.

Estimation of degradation percentage

The percentage of degradation was calculated with initial absorbance value, after inoculation by using the formula at 600 nm

spectrophotometrically . Blank was made without addition of hydrocarbon[13] .

$$\text{Percentage of degradation} = \frac{\text{(initial absorbance) - (final absorbance)}}{\text{Initial absorbance}}$$

Physico-Chemical properties [14]

Determination of P^H and Temperature

100 ml of Nutrient broth were prepared and separated into different conical flasks. Each flasks were adjusted to different p^H such as 4,5,6,7 and Temperatures at at 25^0c, 35^0c, 45^0c and 55^0c . After sterilization, 1% Bushnell-hass broth culture was added into different flasks containing medium. And the flasks were incubated for 48-72 hrs.

Determination of Alkalinity

5ml culture sample was taken and few drops of phenolphthalein indicator were added . Pink color solution was titrated against 0.01N H_2SO_4. To this colorless solution 2,3 drops of methyl orange indicator was added and titrated against 0.01N H_2SO_4 until orange colour was turned to yellow.

Determination of Chloride

Determination of chloride was also done by[14]

Statistical Analysis

Rondom sampling was used for the entire test. The data of all values, were statistically analzsed and expressed as mean ± standard deviation [15].

RESULTS AND DISSCUSSION

In the present study hydrocarbons degrading bacterial and fungal species were isolated from the engine oil contaminated soil. There are engine oil degrading bacterial isolates were identified based on the biochemical characteristics. Different bacterial colonies were compared with Bergey's manual of systemic bacteriology. Bacterial isolates were confirmed as *P.alcaligenes,* and *Serratia marcescens*.The fungal colonies strains were identified by lacto phenol cotton blue staining.

After 9 days of incubation the optical density of toluene degraders such as *Pseudomonas alcaligenes* was 0.32 IU/ml and *Serratia marcescens* 0.30 IU/ml. optical density of pentachlorophenol degraders such as *Pseudomonas alcaligenes* was 0.35 IU/ml and *S.marcescens* was 0.36U/ml. (Table 1)

Toluene degradation was carried out in 9 days by *Pseudomonas alcaligenes* and *Serratia marcescens*. The percentage of degradation of toluene was *P.alcaligenes* 52.36% and *S.marcescens* 48.66%. Pentachlorophenol degradation was carried out in 9 days by *Pseudomonas alcaligenes* and *Serratia*

Table 1. Density of Toluene and Pentachlorophenol Degraders (IU/MI)

S.No.	Days	Compound Toluene	*P.alcaligenes*	*S.marcescens*
1.	3	Toluene	0.42	0.53
2.	6		0.35	0.46
3.	9		0.32	0.30
4.	3	Pentachlorophenol	0.50	0.51
5.	6		0.43	0.32
6.	9		0.35	0.36

Table 2. Percentage of Toluene and Pentachlorophenol Degraders (%)

S.No.	Days	Compound Toluene	*P.alcaligenes*	*S.marcescens*
1.	3		32.11	22.10
2.	6	Toluene	45.13	35.42
3.	9		50.27	44.61
4.	3	Pentachlorophenol	35.67	32.71
5.	6		40.62	39.27
6.	9		49.39	42.78

marcescens. The percentage of degradation of Pentachlorophenol was *P.alcaligenes* 51.48% and *S.marcescens* 47.88%. There organism degrade toluene and Pentachlorophenol with clear zone on liquid media with in 2 days(Table 2)

Physico-chemicals Parameters

Determination of P^H and Temperature

Among the four different P^H and Temperature , maximum hydrocarbon degradation was observed in *P.alcaligenes* at pH 7 (8.2±0.01

Table 3. Determination of different P^H for toluene and Pentachlorophenol degraders

S.No	Compound	Organisms	P^H (IU/ml)^{HHHh}			
			4	5	6	7
1.	**Toluene**	*P.alcaligenes*	4.0±0.02	2.5±0.01	3.5±0.01	8.2±00.01
2.		*S.marcescens*	2.5±0.01	3.7±0.01	4.2±0.01	6.1±0.01
3.		*A.niger*	5.2±0.02	4.2±0.01	3.5±0.01	3.1±0.01
4.		*C.mansoni*	4.7±0.01	3.8±0.01	3.2±0.01	4.5±0.01
5.	**Pentachlorophenol**	*P.alcaligenes*	2.6±0.02	3.2±0.02	5.6±0.02	4.5±0.01
6.		*S.marcescens*	4.8±0.01	5.8±0.01	6.7±0.01	6.2±0.01
7.		*A.niger*	5.2±0.03	2.6±0.02	3.2±0.02	1.5±0.02
8.		*C.mansoni*	4.2±0.03	4.2±0.05	7.2±0.02	5.7±0.02

Values are mean ± standard deviation

Table 4. Determination of different temperature for toluene and pentachlorophenol degraders

S.No	Compound	Organisms	Temperature^{HHHh}			
			25°C	35°C	45°C	55°C
1.	Toluene	*P.alcaligenes*	8.5±0.02	3.2±0.02	2.1±00.02	3.1±0.01
2.		*S.marcescens*	3.3±0.02	2.3±0.02	6.7±0.01	3.9±0.01
3.		*A.niger*	5.7±0.01	3.2±0.01	4.5±0.02	8.7±0.02
4.		*C.mansoni*	6.8±0.01	3.1±0.01	4.1±0.02	3.2±0.01
5.	Pentachlorophenol	*P.alcaligenes*	4.2±0.01	7.5±0.01	4.8±0.01	3.8±0.02
6.		*S.marcescens*	5.7±0.01	3.1±0.01	4.2±0.01	2.4±0.02
7.		*A.niger*	4.2±0.02	6.2±0.01	3.8±0.01	4.6±0.01
8.		*C.mansoni*	5.6±0.01	4.8±0.02	5.2±0.02	6.6±0.01

Values are mean ± standard deviation

Table 5. Determination of Chloride and Alkalinity (mg/l)

S.No	Test Organisms	Chloride Toluene	Pentachlorophenol
1.	*P.alcaligenes*	2.6	3.2
2.	*S.marcescens*	1.7	2.8
3.	*A.niger*	2.4	1.5
4.	*C.mansoni*	1.5	1.2
		Alkalinity	
1.	*P.alcaligenes*	1.8	2.7
2.	*S.marcescens*	2.0	1.8
3.	*A.niger*	1.7	2.2
4.	*C.mansoni*	2.5	1.9

IU/ml) (Table 3) and Temperature was *A.niger* at 55^0C (8.7±0.01 IU/ml) (Table 4).

Determination of alkalinity and chloride

Alkalinity was estimated in *Pseudomonas alcaligenes* (3.2 Mg/l) and Chloride was estimated in *Pseudomonas alcaligenes* (2.7 Mg/l) (Table 5).

Polynuclear Aromatic Hydrocarbons (PAHs) degrading microbial strains were isolated from oil contaminated soil and characterized for specific features regarding toluene and pentachlorophenol degradation. The most efficient strains in terms of rapidity to degrade toluene and pentachlorophenol were identified was *Pseudomonas* species and *Serratia* species[16].

Our study reports similar to the findings was also isolated more than 100 species representing 30 genera have been shown to be capable of utilizing hydrocarbons, the association of various bacterial and fungi with different hydrocarbon system viz. petrol, diesel, engine oil and kerosene appear to vary with reference to soil and water characteristics also the utilization potentials of these hydrocarbons by bacteria and fungi[17].

To identify the degraded compounds of hydrocarbon further investigation is necessary. Moreover, bacterial and fungal stain can be used to recovery of hydrocarbons from oil contaminated site which field trial is also necessary.

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