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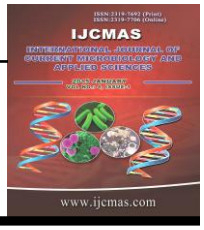

Original Research Article

Hydrocarbon Bioremediation Efficiency by five Indigenous Bacterial Strains isolated from Contaminated Soils

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Keywords

Bioremediation, Petrol, Benzene, Toluene, Xylene and Cyclohexane

A B S T R A C T

Twenty hydrocarbon degrading microorganism were isolated from four hydrocarbon contaminated sites and were identified on the basis of morphological and biochemical characteristics as *Bacillus cereus*, *Bacillus subtilis, Escherichia coli, Staphylococcus aureus* and *Pseudomonas aeruginosa*. The study revealed high density of bacteria acclimatized for biodegradation of hydrocarbon (Petrol) in soil. The isolates were examined for other hydrocarbon degradation in media supplemented with Benzene, Toluene, Xylene and Cyclohexane at three different concentrations *viz* 5%, 10% and 15% incubated for 3 different time intervals 5, 10 and 15 days. The results indicated that all the isolates possessed potential to degrade the wide variety of hydrocarbons. The most efficient among them was *Pseudomonas aeruginosa* which degraded all tested hydrocarbon showing maximum growth at 5% concentration and 10 days incubation. It could be concluded that native flora of hydrocarbon contaminated site adapt to the environmental condition and could be implicated to remove hydrocarbons.

Introduction

In the last years, a large number of ecosystems have been changed by the growing influence of human activity. As a result, many people have become aware of the need to protect ecosystems as well as to evaluate the damage caused by contamination. During the previous years, the frequency and risk of oil pollution has lead to extensive research. Most of the petroleum goes in the ecosystem via leak of coastal oil refineries. Approximately five million tons of crude oil and refined oil enter the environment each year as a result of anthropogenic sources such as oil spills (Hinchee and Kitte, 1995).

Hydrocarbon pollutants are amongst the most reported pollution worldwide (Shukor *et al*, 2009). Polyaromatic hydrocarbons in the environment originate from anthropogenic source like mineral oil. Numerous bacteria, fungi and algae have been isolated for the breakdown of aromatic hydrocarbons as carbon and energy sources (Cerniglia, 1992; Lal et al., 2004; Pathak et al. 2008). The degradation pathways have been -elucidated

PAHs present as natural constituents in fossils fuels, are formed during the incomplete combustion of organic materials (Lee et al., 1981; Wang et al., 1999; Desche Anes et al., 1996). PAHs can exert toxic effects or possess mutagenic, teratogenic, or carcinogenic properties (Heitkamp and Cerniglia, 1987).

Some microorganisms can utilize the hydrocarbons as sole carbon sources for getting their energy and metabolic activities (Jyothi et al.,2012). Biodegradation is a complex process that depends on the nature of petroleum and also on the amount of the hydrocarbons present (Das et al.,2011). The microbes can utilize the hydrocarbons depending on the chemical nature of the compounds within the petroleum mixture (Adeline et al.,2009). Hydrocarbon degrading microorganisms usually exist in very low abundance in aquatic environments (Sivaraman et al., 2011). Biodegradation of bacteria is considered as the most active process in petroleum degradation and they are the primary degraders of spilled oil (Rahman et al.,2003; Brooijmans et al.,2009) and this is specially carried out largely by diverse bacterial populations, mostly by Pseudomonas species (Boboye et al.,2010; Dubey et al.,2009).

There are so many known consortia of microorganisms which can degrade mineral oil hydrocarbons under laboratory or field conditions (Ratajczak et al.,1998; Wikstrom et al.,1996). This work is concentrated on isolation and identification of hydrocarbon degrading bacteria associated with petrol and diesel oil contaminated sites in India and also to test their ability to degrade different hydrocarbons. The expected interpretation of this study will provide information on the bacterial population, hydrocarbon-degrading microorganisms and their degrading ability of diesel because these bacteria can utilize the hydrocarbons as carbon source. Biodegradation by indigenous populations of microorganisms is one of the primary mechanisms by which petroleum and other hydrocarbon pollutants can be removed from the environment (Ulrici et al.,2000) and this process is also cheaper than the other remediation technologies (Leahy et al.,1990).

Materials and Methods

Samples were collected randomly from Fuel oil pumps of Mathura oil refinery, Barauni oil refinery, Haldia oil refinery, Paradip oil refinery at a depth within 1-5cm from the surface of the soil using sterile spatula and were placed in pre sterilized polythene bags and tightly packed. Samples were immediately transferred to the laboratory for analysis and stored at 4°C for further processing.

Isolation of Hydrocarbon degrading Bacteria

One gram of dried soil sample was dissolved in 9ml of distilled water and agitated vigorously. A 10 fold serial dilution was done followed pour plate method. Soil sample was serially diluted upto 10^{-7} dilution and 1 ml from each dilution poured in Petri plate followed by addition of 20ml of molten Bushnell Haas-Agar medium at around 50°C. After gently rotating, the plates were incubated at 37°C for 24 hours and uninoculated plate was serve as media control and then enumeration of different isolates were carried out (Santhini*et al.*, 2009). Culturally different colonies were selected and streaked over Bushnell Hass-Agar medium supplemented with 5% petrol. Uninoculated media plate was serve as control. Incubation was done at 28˚C for upto7 days and growth were examined. Isolates were maintained on Nutrient agar

slants which were subcultured at 15 days interval and were incubated at 37°C for 24-42 hours and then stored at 4°C.

Identification of hydrocarbon utilizing bacteria

The identification was done by cultural (margin, colour, texture and elevation), morphological and biochemical analysis as per Bergey's Manual of Systemic Bacteriology (Holt *et al*., 1994).

Effect of Temperature and pH

The effect of temperature and pH on the growth and degradation will be studied by using Bushnell-Haas broth supplemented with petrol (5%) will inoculate with the isolates and incubate at different temperatures (10°C, 20°C, 30°C, 40°C, 50°C) and different pH (5.5, 6.5, 7.5, 8.5 and 9.5) for this uninoculated tubes will be serve as control. Growth and degradation of the organism will be assayed by optical density (O.D) measurement at 600nm. (Rahman and Rahman, 2002).

Evaluation of the specific degradation capacity of selected solid and liquid hydrocarbons

Biodegradation capability of the organism were determined the method given by Santhini*etal*., (2009). In order to monitor the liquid hydrocarbon degradation, overnight cultures were inoculated on Bushnell-Haas medium at pH 7.0 supplemented with hydrocarbon (5-15%v/v) then the tubes were incubated at 37°C. Uninoculated medium with hydrocarbon were served as a control. Growth of the organism was assayed by optical density (O.D) measurement at 620nm. The inoculated and uninoculated tubes were incubated at 37°C for 5-15 days and examined regularly for growth in Benzene, Toluene, Xylene, Cyclohexane.

Results and Discussion

Isolation of Hydrocarbon degrading Bacteria

The samples of hydrocarbon contaminated soil supplemented with various hydrocarbons showed the growth of *Pseudomonas aeruoginosa, Bacillus subtilis, Bacillus cereus, E.coli and Staphylococcus aureus.* These organisms were found to be actively growing in petrol during the study of the 40 hydrocarbon contaminated soils. A total of twenty positive isolates of bacterial species were identified out of which 9 (45%) were isolated from Mathura oil refinery, 6 (30 %) from Barauni oil refinery, 4 (20 %) from Haldia oil refinery and 1 (5 %) from Paradip oil refinery. Maximum isolates were of *Psuedomonas aeruginosa* 9 (45%) followed by *Bacillus subtilis* 6 (30 %), *Bacillus cereus* 3 (15 %), *E. Coli* 1 (5 %) and *Staphylococcus aureus* 1 (5%).

Microbial distribution in Hydrocarbon contaminated soil samples.

The highest population was observed from the soil sample of Mathura oil refinery. The bacterial counts were recorded as 4.26×10^6 in this sample. Plate count of viable bacteria from Barauni oil refinery and Haldia oil refinery was determined to be in order of 3.4×10^6 and 3.04×10^6. The least count of bacteria was determined in the soil sample from Paradip oil refinery as 2.58×10^6**.**

Identification of hydrocarbon utilizing bacteria

After evaluation of colony morphology, cell morphology, utilization of carbon source and biochemical characteristics the isolates were identified as *Pseudomonas aeruoginosa, Bacillus subtilis, Bacillus cereus, E.coli and Staphylococcus aureus.*

Effect of Temperature on hydrocarbon degrading bacteria

Hydrocarbon degrading bacteria grow optimally in a wide range of temperature ranging from 27^0C to 37^0C. Growth decreases dramatically at higher temperature. *Pseudomonas aeruoginosa* showed highest growth at 30°C temperature at media supplemented with 5% petrol while *Bacillus cereus* and *Bacillus subtilis* showed maximum growth at 40°C whereas *E.coli* and *Staphylococcus aureus* showed high growth at 30°C. All these bacteria show less growth at low as well as high temperature.

Effect of pH on hydrocarbon degrading bacteria

Maintenance of pH in bacterial medium is important since pH strongly affect bacterial growth. The optimal pH that supported growth of bacteria was range between 6.5 to 7.5 *Pseudomonas aeruoginosa Bacillus subtilis, Bacillus cereus, Staphylococcus aureus and E. coli* showed highest growth at pH 7.5 at media supplemented with 5% petrol. All these bacteria show very low growth at low as well as high pH.

Hydrocarbon degradation capacity of *Pseudomonas aeruoginosa.*

Among the hydrocarbons used for the degradation studies by *Pseudomonas aeruoginosa* degradation was observed maximum in petrol (0.68) followed by cyclohexane (0.65), toluene (0.64), xylene (0.62) and with a minimum hydrocarbons degradation with benzene (0.53).

The degradation process was observed to gradually increase with the peak value at 10 days and then a gradual decrease in the optical density was observed. While testing the concentrations of hydrocarbons, 5% hydrocarbons concentration was found to be most effective for degradation as compared to 10% and 15% hydrocarbons concentration.

Hydrocarbon degradation capacity of *Bacillus subtilis.*

Among the hydrocarbons used for the evaluation of the degradation ability for *Bacillus subtilis*, benzene (0.67) was maximally degraded, followed by the hydrocarbons toluene (0.65), cyclohexane (0.54), xylene (0.51) and petrol (0.47). The concentration of 5% was found to be more pronounced for the degradation of the hydrocarbons by *Bacillus subtilis* as compared to 10% and 15% concentrations.

There was significant increase in the degradation of hydrocarbons by *Bacillus subtilis* with respect to the time, *i.e.* from 0 days towards 15 days.

Hydrocarbon degradation capacity of *Bacillus cereus.*

The degradation capacity of *bacillus cereus* was observed maximum with petrol (0.52) followed by other hydrocarbon *viz* xylene (0.5), cyclohexane (0.48), toluene (0.47) and benzene (0.46). The degradation was found to gradually increase up to 15 days of incubation.

A decreasing trend in optical density was observed. Even with *bacillus cereus* 5% hydrocarbon concentration was found to be optimum for hydrocarbon degradation. Significant increase in the degradation of hydrocarbon by *bacillus cereus* was found with respect to time, *i.e.*, from 0 hour to 15 days, then a decrease towards 15 days at 10% and 15% concentration was observed.

Hydrocarbon degradation capacity of *E.coli*

The degradation capacity of *E.coli* was observed maximum with cyclohexane (0.49) followed by other hydrocarbon *viz* petrol (0.43), toluene (0.43), xylene (0.42) and benzene (0.41). A decreasing trend in optical density was observed. Even with *E.coli 5*% hydrocarbon concentration was found to be optimum for hydrocarbon degradation at 10 days. Significant increase in the degradation of hydrocarbon by *E.coli was* found with respect to time, *i.e.,* from 0 hour to 15 days, then a decrease towards 15 days at 10% and 15% concentration was observed.

Hydrocarbon degradation capacity of *Staphylococcus aureus.*

Among the hydrocarbons used for the degradation studies by S*taphylococcus aureus* degradation was observed maximum in petrol (0.39) followed by xylene (0.35), benzene (0.34), cyclohexane (0.33) and toluene (0.26). While testing the concentrations of hydrocarbons, 10% hydrocarbons concentration was found to be most effective for degradation as compared to 5% and 15% hydrocarbons concentration. A significant increase in the degradation of Hydrocarbon by *Staphylococcus aureus* was observed with increase in time (*i.e.* from 0 days to 10 days) and a decrease at 15 days at 10% and 15% concentrations.

Summary and Conclusion

For the investigation ten soil samples each from four different hydrocarbon contaminated sites were obtained and used for isolation and enumeration of bacteria after which the culturally different colonies were purified and screened for hydrocarbon utilization as sole source of carbon and energy at 5% concentration of petrol. The isolate found positive for growth on screened plates were identified on the basis of cultural, morphological and biochemical analysis and were further assessed for their growth potential for selected hydrocarbons at varying concentrations (5%, 10%, and 15%) incubated for three different time intervals *i.e*. 5 days, 10 days, and 15 days.

The present investigation revealed that indigenous bacterial population isolated from hydrocarbon contaminated sites could be used for *insitu* bioremediation purpose. A total of 20 positive isolates were obtained, out of which *Psuedomonas aeruginosa* showed maximum occurrence while *E. coli* and *Staphylococcus aureus* showed least occurrence. Analysis of temperature and pH optimization showed that all the bacterial species were most active at 40^0C-50^0C and pH 7.5 respectively. Upon analyzing the growth potential of isolates at different concentration (5%, 10%, and 15%) of hydrocarbons and different time of incubation (5, 10 and 15 days) it was found that the bacterial species showed maximum growth at 15 days incubation and 5 % concentration and also at 10 days incubation and 5 % concentration

The study highlighted the potential of bacterial population isolated from hydrocarbon contaminated soil for bioremediation of hydrocarbon polluted area, spills as it offers effective degradation of various fractions of hydrocarbons at wide range of concentration and time duration. Therefore, bioremediation of toxicant hydrocarbons in soil or spill have a better option of environmentally adopted microflora that effect detoxification and stabilization of processes of biological degradation with low economical expenses and of no danger for environment.

Table.1 Incidence of hydrocarbon degrading bacteria

Sample Sites n=40	No of positive isolates	Individual Incidence				
		P. aeruginosa	***B. subtilis***	***B. cereus***	***E. coli***	***S. aureus***
Mathura oil refinery (n=10)	9	4(45%)	2(22.2%)	1(11.1%)	1(11%)	1(11%)
Barauni oil refinery (n=10)	6	2(33.3%)	3(50%)	1(16.6%)	0(0%)	0(0%)
Haldia oil refinery (n=10)	4	2(50%)	1(25%)	1(25%)	0(0%)	0(0%)
Paradip oil refinery (n=10)	1	1(100%)	0(0%)	0(0%)	0(0%)	0(0%)
Total sample (n=40)	20	9(45%)	6(30%)	3(15%)	1(5%)	1(5%)

Table 2: Microbial distribution

Sample sites	Soil sample size n=40	Average cfu g/ml(10^6)
Mathura oil refinery	n=10	4.26
Barauni oil refinery	n=10	3.4
Haldia oil refinery	n=10	3.04
Paradip oil refinery	n=10	2.58

Fig 1: Effect of temperature on growth of bacteria at 5% petrol

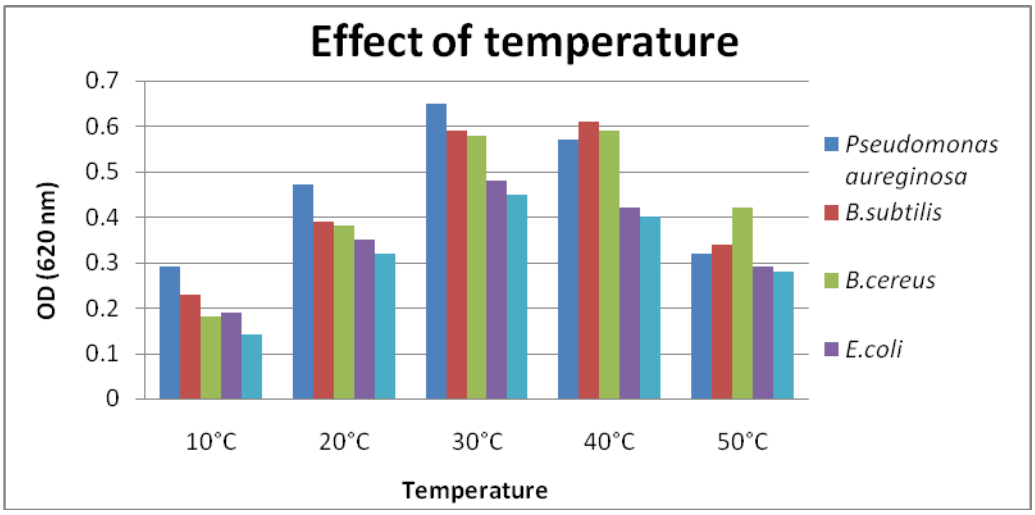


Table 3: Biochemical chart

Characteristics		Hydrocarbon degrading bacteria from soil				
		Pseudomonas aeruginosa	***Bacillus subtilis***	***Bacillus cereus***	***E. coli***	***Saphylococcus auries***
Colony morphology	Color	White	Creamy white	White waxy	White	Shiny yellow
	Margin	Entire	Undulate	Undulate	Regular	Entire
	Elevation	Flat	Unbonate	Flat	Convex	Convex
	Shape	Irregular large	Circular flat	Irregular large	Circular large	Circular
	Opacity	Opaque	opaque	Opaque	Smooth Glistening	Opaque
	Pigmentation	Blue-green	No	No	No	Golden yellow
Carbon source	Glucose	_	+ A	+ A	+ AG	+ A
	Sucrose	_	+ A	+ A	+ A	+ A
	Mannose	+	+	_	+	+
	Xylose	_	+	_	+	_
	Arabinose	_	+	_	+	_
	Lactose	_		_	+ AG	+ A
Cell morphology	Gram stain reaction	_	+	+	_	+
	Cell shape	Cylindrical rods	Rods	Rods	Rods	Coccus
Biochemical characteristics	Oxidase test	+	+	+	_	_
	Catalase test	+	_	_	+	+
	Starch hydrolysis test	–	–	+	–	–
	M.R	_	_	_	+	+
	V.P	_	_	_	_	_
	Indole test	_	_	_	+	_
	Citrate utilization test	–	–	–	–	–
	Gelatin liquefication	+	+	+	_	+
	Lipid Hydrolysis	+	–	+	–	+
	H_2S Reduction	_	_	_	_	_
	Nitrate Reduction	+	+	+	+	+
	Urease Activity	_	_	_	_	_

A = Acid, AG = Acid with gas

Table 4: Effect of temperature on growth of bacteria in 5% petrol

Temperature	**10°C**	**20°C**	**30°C**	**40°C**	**50°C**
P. aeruginosa	0.29	0.47	**0.65**	0.57	0.32
B. subtilis	0.23	0.39	0.59	**0.61**	0.34
B. cereus	0.18	0.38	0.58	**0.59**	0.42
E. coli	0.19	0.35	**0.48**	0.42	0.29
S. aureus	0.14	0.32	**0.45**	0.40	0.28

Table 5: Effect of pH on growth of bacteria at 5% petrol

pH	**5.5**	**6.5**	**7.5**	**8.5**	**9.5**
Pseudomonas aeruginosa	0.43	0.58	**0.65**	0.38	0.2
B. subtilis	0.48	0.56	**0.64**	0.32	0.10
B. cereus	0.45	0.50	**0.62**	0.30	0.14
E. coli	0.3	0.45	**0.59**	0.42	0.18
S. aureus	0.28	0.43	**0.58**	0.48	0.19

Fig 2: Effect of pH on growth of bacteria at 5% petrol

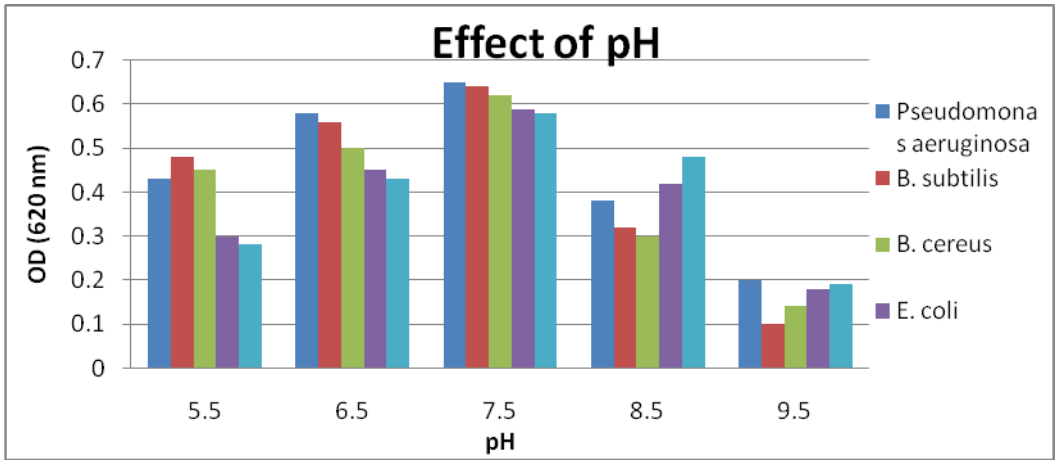


Table 6: Hydrocarbon degrading ability of *Pseudomonas aeruginosa*

	0 days			5 days			10 days			15 days		
	5%	10%	15%	5%	10%	15%	5%	10%	15%	5%	10%	15%
Petrol	0.02	0.05	0.06	0.52	0.29	0.3	0.67	0.57	0.39	**0.68**	0.51	0.44
Benzene	0.01	0.04	0.06	0.39	0.48	0.39	0.49	0.52	0.43	**0.53**	0.50	0.45
Toluene	0.02	0.04	0.06	0.57	0.49	0.36	0.59	0.56	0.46	**0.64**	0.59	0.42
Xylene	0.02	0.04	0.06	0.5	0.41	0.25	0.59	0.49	0.45	**0.62**	0.57	0.42
Cyclohexane	0.02	0.04	0.05	0.59	0.46	0.45	0.64	0.48	0.48	**0.65**	0.53	0.54

Fig 3: Hydrocarbon degradation ability of *Psuedomonas aeruginosa*

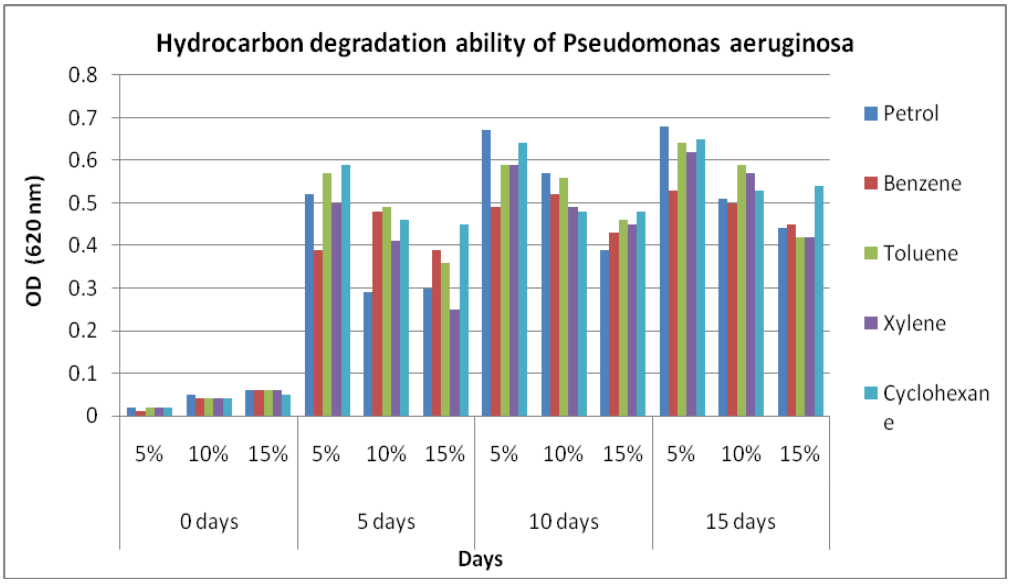


Table 7: Hydrocarbon degrading ability of *Bacillus subtilis*

	0 days			5 days			10 days			15 days		
	5%	10%	15%	5%	10%	15%	5%	10%	15%	5%	10%	15%
Petrol	0.03	0.04	0.06	0.39	0.42	0.19	0.46	0.46	0.27	0.44	**0.47**	0.28
Benzene	0.01	0.04	0.06	0.50	0.36	0.15	**0.67**	0.42	0.23	0.62	0.44	0.24
Toluene	0.01	0.03	0.05	0.58	0.44	0.28	0.63	0.50	0.34	**0.65**	0.43	0.31
Xylene	0.02	0.03	0.05	0.41	0.43	0.35	0.48	0.48	0.38	**0.51**	0.50	0.39
Cyclohexane	0.02	0.04	0.05	0.45	0.37	0.30	**0.54**	0.39	0.33	0.52	0.40	0.35

Fig 4: Hydrocarbon degradation ability of *Bacillus subtilis*

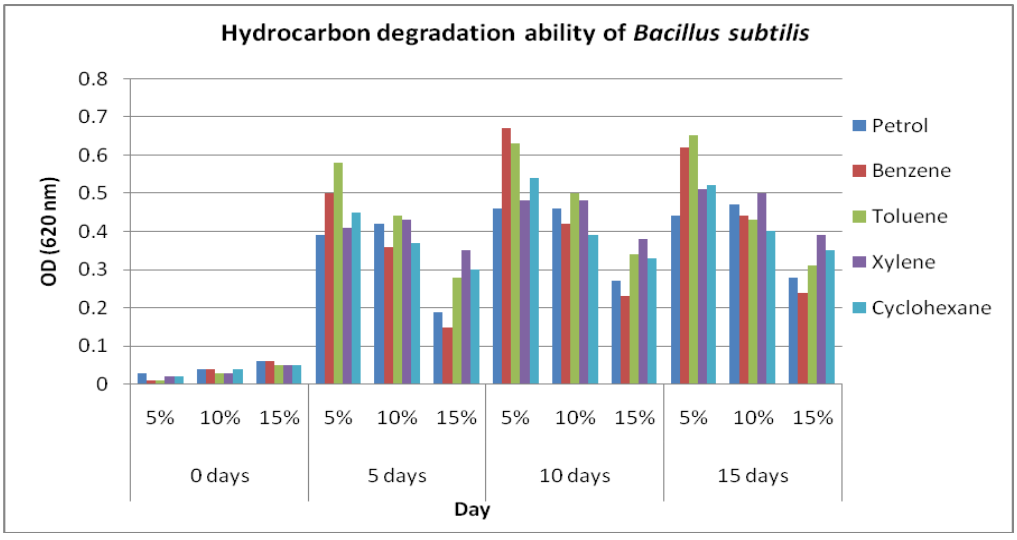


Table 8: Hydrocarbon degrading ability of *Bacillus cereus*

	0 days			5 days			10 days			15 days		
	5%	10 %	15 %	5%	10 %	15 %	5%	10 %	15 %	5%	10 %	15 %
Petrol	0.03	0.04	0.05	0.4	0.43	0.34	0.49	0.48	0.38	**0.52**	0.5	0.44
Benzene	0.02	0.04	0.06	0.31	0.27	0.05	0.43	0.35	0.21	**0.46**	0.43	0.26
Toluene	0.02	0.05	0.06	0.32	0.28	0.1	**0.47**	0.34	0.18	0.44	0.27	0.22
Xylene	0.02	0.05	0.06	0.3	0.28	0.06	0.49	0.37	0.16	**0.5**	0.29	0.34
Cyclohexane	0.03	0.04	0.05	0.37	0.4	0.24	0.44	0.44	0.31	**0.48**	0.46	0.3

Fig 5: Hydrocarbon degradation ability of *Bacillus cereus*

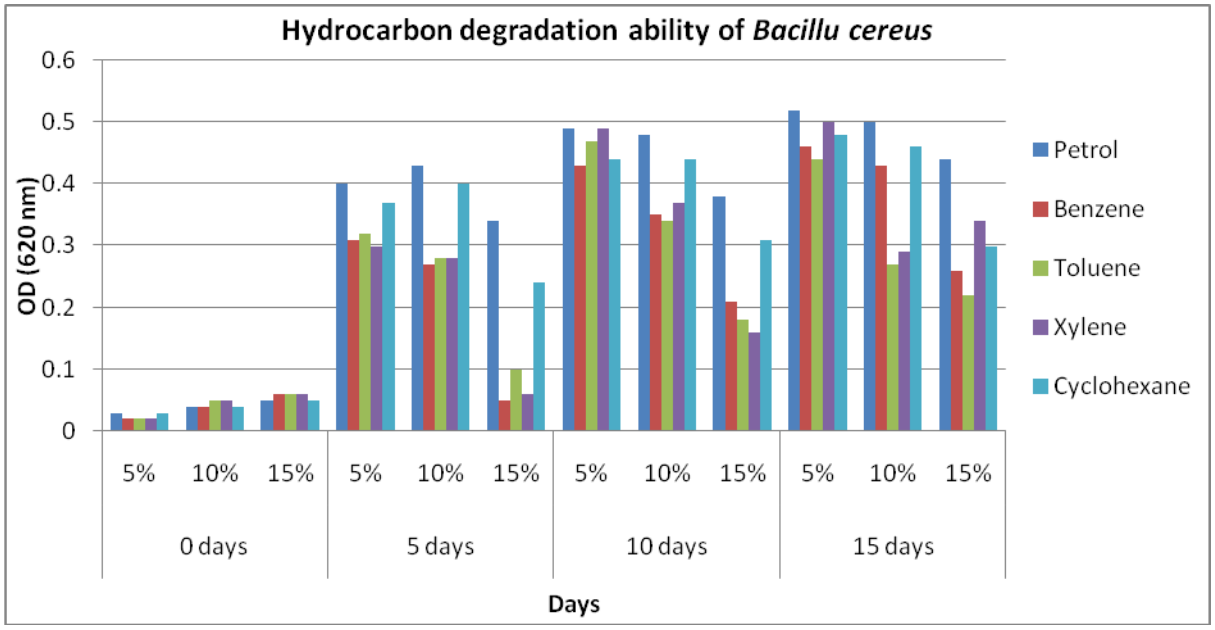


Table 9: Hydrocarbon degrading ability of *E. coli*

	0 days			5 days			10 days			15 days		
	5%	10%	15%	5%	10%	15%	5%	10%	15%	5%	10%	15%
Petrol	0.02	0.04	0.06	0.41	0.28	0.11	**0.43**	0.31	0.17	0.36	0.22	0.20
Benzene	0.02	0.04	.05	0.37	0.29	0.18	0.39	0.29	0.22	**0.41**	0.24	0.25
Toluene	0.02	0.03	0.04	0.38	0.35	0.18	**0.43**	0.37	0.26	0.41	0.29	0.29
Xylene	0.02	0.04	0.05	0.37	0.26	0.22	**0.42**	0.30	0.25	0.35	0.29	0.29
Cyclohexane	.02	.04	.06	0.36	0.22	0.21	**0.49**	0.35	0.20	0.44	0.42	0.31

Fig 6: Hydrocarbon degradation ability of *E. coli*

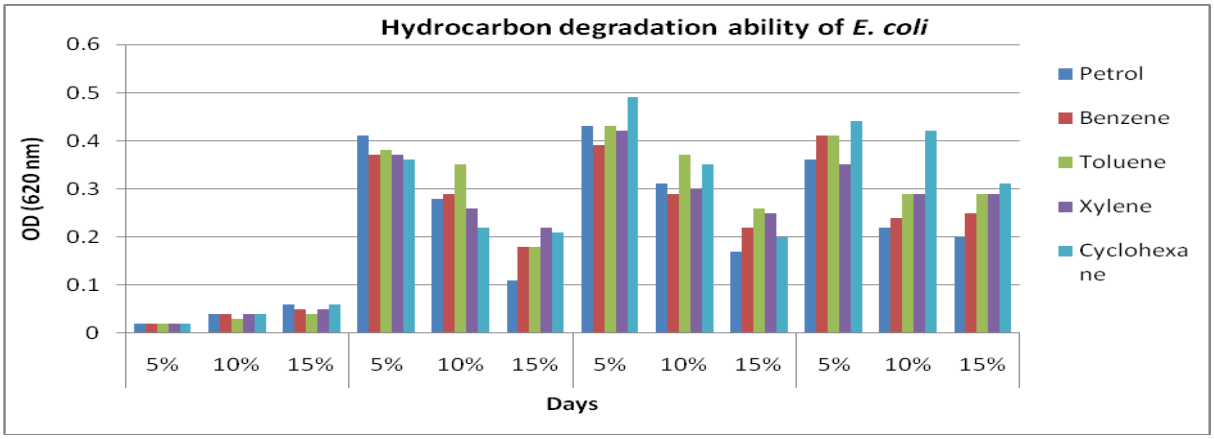
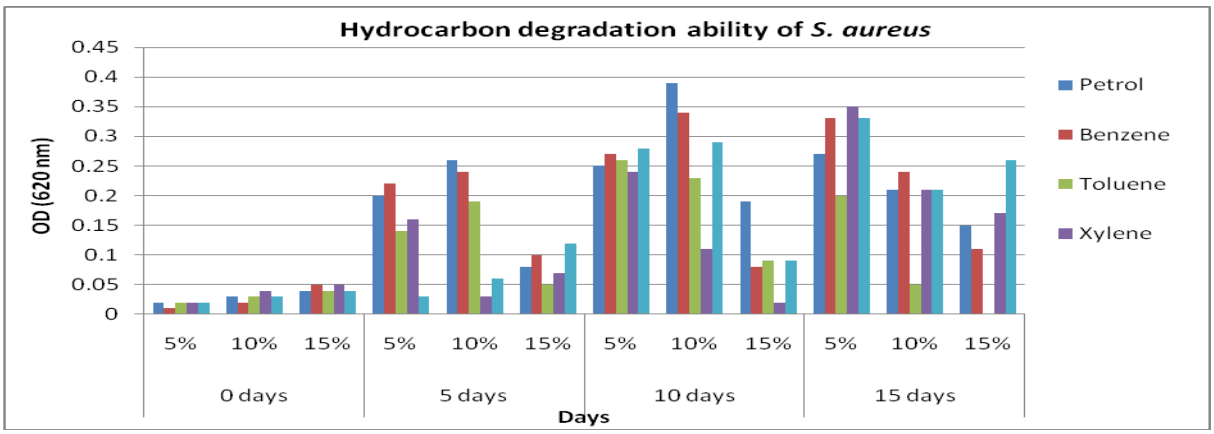


Table 10: Hydrocarbon degrading ability of *Staphylococcus aureus*

	0 days			5 days			10 days			15 days		
	5%	10%	15%	5%	10%	15%	5%	10%	15%	5%	10%	15%
Petrol	0.02	0.03	0.04	0.2	0.26	0.08	0.25	**0.39**	0.19	0.27	0.21	0.15
Benzene	0.01	0.02	0.05	0.22	0.24	0.1	0.27	**0.34**	0.08	0.33	0.24	0.11
Toluene	0.02	0.03	0.04	0.14	0.19	0.05	**0.26**	0.23	0.09	0.2	0.05	0
Xylene	0.02	0.04	0.05	0.16	0.03	0.07	0.24	0.11	0.02	**0.35**	0.21	0.17
Cyclohexane	0.02	0.03	0.04	0.03	0.06	0.12	0.28	0.29	0.09	**0.33**	0.21	0.26

Fig 7: Hydrocarbon degradation ability of *Staphylococcus aureus*



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