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RESEARCH ARTICLE

ISOLATION, IDENTIFICATION AND CHARACTERISATION OF DIESEL DEGRADING BACTERIA

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ABSTRACT

Hydrocarbons such as diesel fuel, crude oil and petroleum distillates are some of the world's most widely used primary energy and fuel resources (Watanabe, 2001). Biodegradation of hydrocarbons by natural populations of microorganisms allows for the conversion of hazardous substances into forms that are less or non-toxic and represents one of the primary mechanisms by which petroleum and diesel products are removed from the environment. The present study was designed to identify the diesel degrading microorganism from the diesel polluted soil and study their degradation capacity. The different isolates were identified from the contaminated soil belongs to *Bacillus species, Klebsiella species, Citrobacter and Pseudomonas species*. The isolates identified were analysed for biodegradation potential of diesel in Minimal salt medium by turbidometry method OD values measured at 595nm. The result showed that DC7 has highly diesel degrading organism in compared to other isolates, the organism was identified as *Bacillus sp.*

INTRODUCTION

Soil contamination with hydrocarbons caused extensive damage to local ecosystems since accumulation of pollutants in animals and plant tissues may cause progeny's death or mutation. The carbon number of diesel oil hydrocarbons is between 11 and 25 (2000 to 4000 hydrocarbons) and the distillation range is between 180 to 380 Ω (Durand *et al.,* 1995). Hydrocarbons such as diesel fuel, crude oil and petroleum distillates are some of the world's most widely used primary energy and fuel resources (Watanabe, 2001). Biodegradation of hydrocarbons by natural populations of microorganisms allows for the conversion of hazardous substances into forms that are less or non-toxic and represents one of the primary mechanisms by which petroleum and diesel products are removed from the environment inexpensively (Leahy and Colwell, 1990). The ability to isolate high numbers of oil degrading microorganisms from an environment is commonly taken as evidence that those organisms are the active degraders of the environment (Okerentugba and Ezeronye, 2003). Many oil degrading microorganisms produced extracellular surface active products to enhance the utilization of oil substrates effectively via formation of extracellular or cell membrane bound bioemulsifiers (Noordaman and Janssen, 2002). It's a great challenge to remove or degrade oil compounds that cover the water surface or deposit on solid supports (Thomassin *et al*., 2002).

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Bioremediation has been recognized as an economically feasible and effective means for treatment of oil contaminations (Thomassin-Lacroix, 2002; Vinas, *et al*., 2002). Diesel was chosen as the model oil substrate due to its extensive applications as industrial fuels and transportation. Some microorganisms, though, cannot produce bio-surfactants but are still able to degrade oil substrates effectively via formation of extra cellular or cell membrane-bound bio-emulsifiers (such as exo-polysaccharides, EPS) (Hino, *et al*., 1997). The constituents of these contaminants such as diesel oil, are carcinogenic, mutagenic and are potent immune toxicants, thus posing a serious threat to human and animal health (Boonchan, *et al*., 2000). Oil spills, especially in soil contamination have prompted research on cost-effective, environmentally cleanup strategies (Margesin and Schinner, 2001). Therefore the present research aims at isolating diesel degrading organisms from diesel polluted soil.

MATERIALS AND METHODS

Sample Collection

The soil samples were collected from four different diesel polluted area situated at different location of the town. These locations were around Salem (district). 200 gm of soil samples were aseptically collected randomly 5-10 cm beneath the surface using spatula and were packed in sterile polythene bags and transferred to the laboratories.

Physiochemical Characterization

The physiochemical caharacters the pH of the soil was determined by using Potentiometric method. The electrical conductivity of the soil was determined by the conductivity of the salts present in the soil by EC meter. The Macronutrient such as nitrogen by Alkaline Permanganate method (Subbiah and Asija, 1956). phosphorous by using Olsen's method. (Olsen *et al.,* 1954 and Watanable and Olsen 1965), potassium by Boiling Nitric acid method using Flame Photometer. Micronutrients are analysed using Atomic Absorption Spectrometer Standards.

Isolation of microorganism from soil sample

One gm of soil sample were taken and serially diluted from 10^{-1} to 10^{-8} dilutions. The diluted sample was inoculated on nutrient agar plates by spread plate method. The plates were incubated for about 24 hours and the growth of microorganisms was noted. The colonies were counted. Population of microorganism present in 1gm of soil sample = Average no. of colonies x plate detection factor.

Identification of Microorganism

The cultures were morphologically and biochemically identified by staining and biochemical tests.

Morphological Characterizatrion of Microorganism by Staining Method

Identification of selected isolate was studied based on different staining methods like simple staining and gram staining.

Biochemical Characterization

Identification of selected isolate was studied based on different biochemical characteristics like indole test, methyl red test, voges-proskauer test, citrate utilization test, triple sugar iron-agar test, catalase test, oxidase test, nitrate reduction test, litmus milk reaction test, urease test, carbohydrate fermentation test, starch hydrolysis test, gelatin hydrolysis test.

Hydrocarbon degradation

Isolation of hydrocarbon degrading bacteria

The bacteria were isolated by inoculating the soil samples on enrichment medium that contains the autoclaved Bushnell-Haas agar supplemented with single hydrocarbon compound as sole carbon source (1% diesel). The medium without hydrocarbons was sterilized by autoclaving at 121°C for 15 minutes. The medium was supplemented with 1% filter sterilized hydrocarbons (diesel) to serve as the only source of carbon and energy. The medium was incubated at 37°C for 10-15 days and observed.

Determination of bacterial biodegradative activity by turbidometry method

Turbidometry is to determine the bacterial growth by utilizing the hydrocarbons (1% diesel) given as carbon source in MSM broth. The medium contains K_2HPO_4 (1.8g/l); NH_4Cl (4g/l); $MgSo_4.7H_2O$ (0.2g/l); NaCl (0.1g/l); $Na_2So_4.7H_2O$ (0.01g/l); Carbon source (1% diesel); and distilled water (1L) with P^H 7.2. The medium without hydrocarbons was sterilized by autoclaving at 121°C for 15 min. The degrading activities of each isolates were obtained by using mineral salt broth (MSB) in which 1% of hydrocarbon (diesel) was added and incubated at room temperature for 15 days. The growth of the bacterium was measured by taking the O.D readings at 595 nm from 0 hrs – 15 days at regular intervals of 2 days against mineral salt medium as blank.

RESULTS

Physicochemical analysis

The physicochemical characteristics of the soil influenced by the impact of diesel as shown in table 1 are substantiated below, the pH value of soil sample is Control contains pH 7.7 and DS3, and DS4 contain pH 7.1 and DS1 contain pH 7.4 and DS2 contain pH. The electric conductivity of the Control sample is 0.5 kg/Ac, DS1 and DS4 contain 0.4µs/cm, DS2 contain 0.5µs/cm, and DS3 contain 0.6µs/cm. The diesel soil sample and control sample contain lime. Macronutrients of the polluted Soil. The soil containing macronutrients are Nitrogen, Phosphorus and Potassium. The high amount of Nitrogen was present in the Soil sample DS4 with 73 kg/ac, DS3 contain 67kg/ac which is Sandy Loam (SL) soil. The Control sample contain an amount of nitrogen is 70 kg/Ac. In other soil samples Loamy Sand (LS) contain 62kg/ac in DS2 and, 59kg/ac in DS1 sample. Phosphorus content of the Control, DS1contain 4kg/ac, DS2contain and 6kg/ac, DS3 contain 7kg/ac and DS4 contain 8kg/ac. The potassium content of the Control sample is 104kg/ac, DS1 and DS4 contain 86kg/ac, DS2 contain 104kg/ac and DS3 contain 78kg/ac. (Table.1)

Table 1. Soil sample analysis

SOIL SAMPLE		DS1	DS2	DS3	DS4
WEIGHT (gm)		305	205	475	215
TEXTURE		SL	SL	LS	LS
LIME STATUS		M	P	M	N
P^H		7.4	7.3	7.1	7.1
ELECTRIC CONDUCTIVITY		0.4	0.5	0.6	0.4
MACRO NUTRIENTS kg/ac	NITROGEN	59	62	67	73
	PHOSPHORUS	4	6	7	8
	POTASSIUM	86	104	78	86
MICRO NUTRIENTS CONTENT (PPM)	FERROUS	4.8	7.0	8.4	8.0
	MANGANESE	2.4	2.6	2.4	2.0
	ZINC	0.6	1.4	1.6	1.2
	COPPER	1.0	1.2	0.8	0.6

Micronutrients of the Polluted Soil

The micronutrient content of the Diesel polluted Soil (Control, DS1, DS2, DS3, and DS4) is Ferric, Manganese, Zinc and Copper. Ferric Content was 5.6ppm, 4.8ppm, 7.0ppm, 8.4ppm and 8.0ppm. Manganese content was 2.6ppm, 2.4ppm, 2.6ppm,

2.4ppm and 2.0ppm. Zinc content was Control, DS1, DS2, DS3 and DS4 contain 1.0ppm, 0.6ppm 1.4ppm, 1.6ppm and 1.2ppm. Copper content was 1.0ppm, 1.0ppm, 1.2ppm, 0.8ppm and 0.6ppm. (Table.1)

Isolation of microorganisms

Diesel polluted soil sample were serially diluted and plated on a nutrient agar plate using the spread plate technique. The result of the bacterial count show that Diesel polluted soil had the highest count of $224x10^{-6}$ CFU/ml, $80x10^{-5}$ CFU/ml and $248x10^{-5}$ CFU/ml. (Table. 2)

Table 2. Colony counting

S.NO	DILUTION	COLONIES	TOTAL PLATE COUNT
1.	10^{-4}	56×4	$224×10^{-6}$
2.	10^{-5}	20×4	$80×10^{-5}$
3.	10^{-6}	62×4	$248×10^{-5}$

Morphological characterisation of microorganisms

The isolated organism from the Diesel polluted soil was named as DC1 to DC7. The morphological characterization of the all the isolates shows gram negative rod shaped bacteria. (Table. 2)

Biochemical test

Indole test shows positive cherry red color in the isolates DC3 and DC4, DC7, other organisms are negative. Methyl red test shows a positive result a red or pink in color in the isolates DC1, DC2, DC3 and DC7, negative in DC4, DC5, DC6. Voges-Proskauer test shows a positive result brown or red color in the isolates DC2, DC4, and negative in DC1, DC23, DC5, and DC6, DC7, (Plate 6).

Table 3. Morphological and biochemical characterization

ISOLATES		DC1	DC2	DC3	DC4	DC5	DC6	DC7
SIMPLE STAINING		Rod	Rod	Rod	Rod	Rod	Rod	Rod
GRAM STAINING		-	-	-	-	-	-	-
INDOLE		-	-	+	+	-	-	+
MR		+	+	+	-	-	-	+
VP		-	+	-	+	-	-	-
CITRATE		+	-	+	+	+	+	-
UREASE		+	+	+	+	+	+	+
CATALASE		+	+	+	+	+	+	+
OXIDASE		+	-	+	-	+	-	+
TSI		+	+	+	+	+	+	+
NO_3 REDUCTION TEST		-	-	-	+	-	-	-
LITMUS MILK REACTION		Acid	Acid	Acid	Acid	Acid	Acid	Acid
GELATIN		-	-	-	-	-	-	-
STARCH HYDROLYSIS		-	-	-	-	-	-	-
CARBOHYDRATE FERMENTATION	GLUCOSE	-	-	-	-	-	+	-
	LACTOSE	-	-	-	+	+	+	+
	FRUCTOSE	-	+	+	+	+	-	+

Citrate utilization test shows a positive result deep Prussian blue in the isolates DC1, DC3, DC4, DC5 to DC6, negative in DC2, DC7. Urease test shows a positive result pink color in the isolates DC1 to DC7 and, negative in no (Plate 8). Triple sugar iron agar test shows a positive result red color in the isolates DC1 to DC7, (Plate 9). Nitrate reduction test shows a positive result red color in the isolate, negative in DC1 and DC7. Litmus milk reaction test acid observed in the isolates, DC1 toDC7 (Plate 11). Catalase test shows a positive result in air bubbles to adding of hydrogen peroxide in the isolates DC1, to the DC7, Oxidase test shows a positive result blue color in the isolates DC1 to DC3, DC5, DC7, negative in DC2, DC4, DC6. Starch hydrolysis and gelatin hydrolysis shows a negative result of all the organisms. Carbohydrate fermentation test fructose shows a positive result in the isolates DC1, DC3, DC4, DC5, DC6 to, negative in DC2. Glucose and sucrose show a positive result in acid and gas formation in the isolates DC6 negative DC1 to DC7. Lactose shows a positive result in the isolates DC4, DC5, DC6 to DC7, negative in DC1, DC3 (Table 3).

Identification of isolated microorganisms

According to Bergey's manual of determinative of bacteriology, 90% of results showed the similarity in characteristics with *Citrobacter Sp*. *Enterobactor Sp.* and *Citrobacter Sp.* Using a specific medium to confirm the species. *Citrobacter species* are inoculated to blood agar medium showed positive results Isolated microorganisms are DC1 Pseudomonas, DC3, DC4, *Citrobactor intermediates*, DC2 *Citrobacter freundi*, DC5, DC6 *Enterobactor aerogenes*, DC7 *Bacillus cereus* (Table. 4).

Table 4. List of identified of isolates kc1 to kc8

S.NO	ISOLATED COLONIES	ORGANISMS
1	DC1	*Pseudomonas*
2	DC2,	*Citrobactor freundi*
3	DC3,DC4	*Citrobactor intermedius*
4	DC5,DC6	*Enterobactor aerogenes*
5	DC7	*Bacillus cereus*

Hydrocarbon degradation

Soil contaminated by diesel are the most potent source to isolate high performed diesel degrading microorganisms using Bushnell-Haas agar medium the inoculated sample to form a clear zone formation surrounding the hydrocarbon (Diesel) degrading microorganisms. DC1, DC2 and DC7 degrading microorganisms to form a zone formation around the organisms.

Hydrocarbon by turbidometry

The Table 5 shows the OD readings of biodegrading activity of each isolates on hydrocarbon (Diesel). The OD readings based on the turbidity of MSM broth at regular intervals of 2 days give the degrading activity on hydrocarbons by bacteria. The results demonstrated that DC1 and DC6 have the greatest ability to degrade diesel. Our results showed that all the organisms utilized maximum hydrocarbon substrate (Diesel)

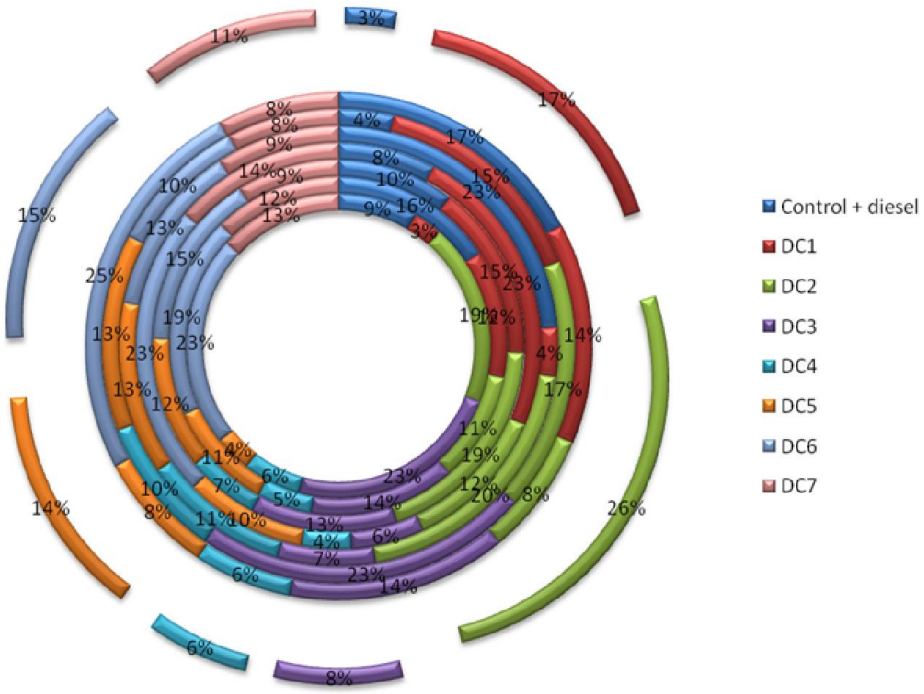
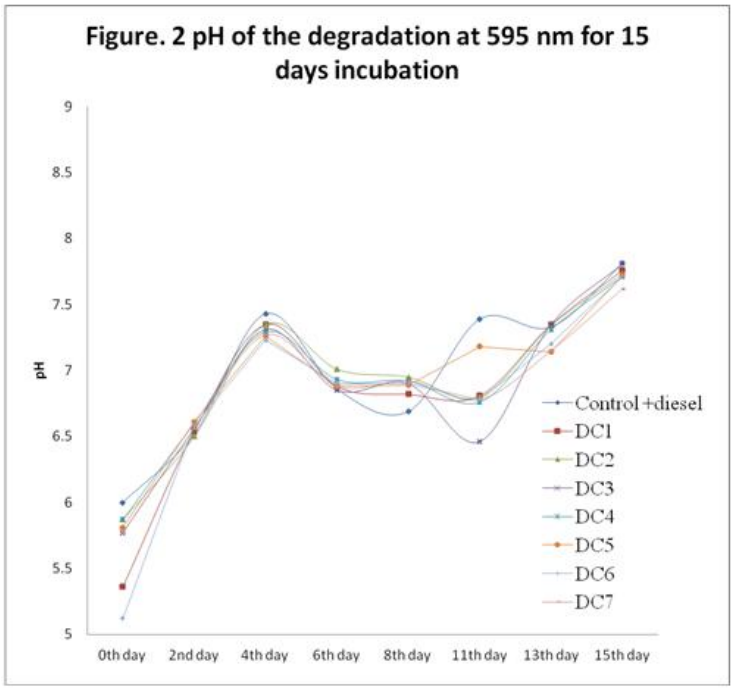
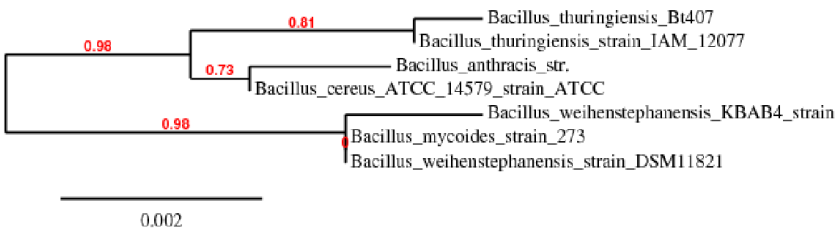


Fig. 1. Degradation percentage of all isolates for 15 days OD at 595 nm



Blast Tree Rendering results for DC7



when supplied as the sole source of carbon and energy, although the level of utilization differs from one microbe to another (due to differences in their growth) and from one hydrocarbon substrate to the others, due to the obvious differences in their molecular sizes. The bacterium with the least degrading activities on Diesel was DC2 and DC4 respectively. These degrading capabilities on different hydrocarbons revealed that the microorganisms isolated from the soil and water samples were able to degrade hydrocarbons. The cells were able to multiply within the days of study, indicating that they were able to degrade and utilize the oil for their growth and development, hence the concomitant increase in the concentration of the broth (turbidity). This gradual increase in the concentration of the broth indicates bacterial growth, hence degradation of hydrocarbons, mostly between days 5 and 12 and gradual decline in the concentration of the broth suggests a decrease in the bacterial population and that the hydrocarbon has been degraded, mostly between days 13 and 15 (Figure1).

The pH during the diesel degradation by Minimal Salt medium using turbidometry method showed the pH from 5 to 8.3 in all the 15 days of treatment (Figure 2, Table 6).

Table 5. Growth curve readings at 595nm for 15 days of incubation

ORGANISMS	GROWTH CURVE READING AT 595 NM FOR 15 DAYS INCUBATION (O.D)							
	0^{th} day	2^{nd} day	4^{th} day	6^{th} day	8^{th} day	11^{th} day	13^{th} day	15^{th} day
Control + diesel	0.11	0.09	0.08	0.04	0.13	0.02	0.11	0.02
DC1	0.04	0.07	0.12	0.12	0.02	0.08	0.09	0.11
DC2	0.25	0.06	0.15	0.06	0.11	0.09	0.05	0.17
DC3	0.30	0.08	0.10	0.03	0.04	0.12	0.09	0.05
DC4	0.08	0.03	0.05	0.02	0.06	0.05	0.04	0.04
DC5	0.05	0.06	0.09	0.05	0.07	0.07	0.05	0.09
DC6	0.29	0.11	0.12	0.12	0.07	0.05	0.16	0.10
DC7	0.17	0.07	0.07	0.07	0.05	0.04	0.05	0.07

Table 6. Degradation of ph value for 15 days of incubation

ORGANISMS	pH VALUE							
	0^{th} day	2^{nd} day	4^{th} day	6^{th} day	8^{th} day	11^{th} day	13^{th} day	15^{th} day
Control +diesel	6.0	6.52	7.43	6.86	6.69	7.39	7.33	7.81
DC1	5.36	6.54	7.35	6.88	6.82	6.81	7.35	7.76
DC2	5.87	6.50	7.35	7.01	6.95	6.80	7.34	7.73
DC3	5.77	6.56	7.31	6.85	6.90	6.46	7.35	7.81
DC4	5.87	6.60	7.29	6.93	6.92	6.76	7.31	7.71
DC5	5.81	6.61	7.26	6.88	6.89	7.18	7.14	7.72
DC6	5.12	6.58	7.23	6.90	6.90	6.78	7.20	7.71
DC7	5.77	6.56	7.27	6.88	6.92	6.78	7.15	7.62

DISCUSSION

The ability of the microorganism to degrade the diesel isolated from the diesel contaminated soil was studied. The physiochemical characters of the polluted soil were studied in which soil sample belongs to sandy loam and loamy sand soil. Carbon is most important for the growth of any living organism, it helps to stimulate the growth 50% of carbon is needed for the growth of microbial cell. The bacteria need micronutrients like nitrogen and phosphorous for effective degradation of the oil. The optimum nutrient balance required for hydrocarbon remediation is Carbon: Nitrogen: phosphorous equal 100:10:4. The nitrogen, phosphorous and potassium of the collected polluted soil sample ranged from the nitrogen of 73kg/acre, Phosphorous of 8kg/acre, and 104kg/acre. They were also differing in the micronutrient in the soil. The 5 different isolated, identified from the diesel polluted soil; the isolates were *Pseudomonas, Bacillus, Citrobacter, Enterobacter* and E. coli. Highly degrading potential organism of diesel was identified has *Bacillus cereus* by 16s rNA sequencing. *Bacillus cereus* was able to degrade. The seven (*Micrococcus, Pseudomonas, Flavobacterium, Serratia, Moraxella, Bacillus and Klibesella*) different species (*Bacillus species, Klebsiella species, Citrobacter and Pseudomonas species*) of potential hydrocarbon degrading organism which utilizes hydrocarbon has a sole carbon source for their growth was identified from hydrocarbon contaminated soil collected in Mexico (Santhini *et al.,* 2009). Some of the researchers have reported that degradation of soil bacteria ranges from 0.13 (Jones *et al.,* 1970) to 50% (Pinholt *et al.,* 1979), and marine bacteria ranges from (0.003% (Hollaway, *et al.,* 1980) to 100% (Mulkins and Phillips 1974). *Bacillus Sp* was effective hydrocarbon degradation (Amund and Adebiyi, 1991; Atlas, 1992; Nwachuku and Ugoji, 1995; Nwachuku, 2001; Benkacaker and Ekundato, 1997; Diaz *et al.,* 2000). *Bacillus Sp* identified from hydrocarbon contaminated soils has a potential to degrade benzene, crude, decanol, ethyl-benzene, n-tetradecanol and xylene (Ghazali *et al.,* 2004). The hydrocarbons from the environment has the following bacteria such as Bacillus megaterium, Bacillus cereus, Micrococcus luteus, Staphylococcus aureus, Lactobacillus acidophilus, Neisseria fluorescence and Corynebacterium xerosis were the potent degraders of hydrocarbons (gasoline and diesel) (Jyothi *et al*., 2012).

Diesel degrading microorganism from the diesel polluted region of Iranian, the 16s RNA sequence strain has the close relationship *Bacillus Cereus* and *Bacillus thurigenesis* (Kebria *et al.,* 2009). All of five isolates that was identified as Ochrobactrum oryzae M2292, Bacillus subtilis M128, Bacillus subtilis C318, Bacillus subtilis C19 and Bacillus pumilus C15 have the capacity to degrade PAHs pyrene and phenanthrene because those strains possessed the dioxyrenese nidA and nahAc gene which are responsible for the initial attack of PAHs degradation (Yulani *et al*., 2012). *Staphylococcus Sp* identified has hydrocarbon- degrading bacteria found in many hydrocarbon-polluted sites (Shukor *et al.,* 2009). *Pseudomonas sp, Micrococcus sp*. And a mixed consortium of this has been used has bioremediation of diesel oil (Nikhil 2013). *Pseudomonas aeruginosa* had shown 49.93% of diesel oil degradation in 20days against 0.5% of diesel oil. So *Pseudomonas aeruginosa* is the natural occurring most potent oil degrading bacteria (Panda *et al.,* 2013). Microbial consortia was prepared for identifying the difference between the using of single and group of organism in degrading diesel oil. The isolates used as microbial consortia were *Rhodococcus Sp., Pseudomonas Sp., Psychrobacter Sp., and Achromobacter Sp* (circa 2009).

Conclusion

The present study was designed to identify the diesel degrading microorganism from the diesel polluted soil and study their

degradation capacity. The 1 gm of diesel contaminated soil sample was serially diluted to find the microbial colonies. The isolated colonies were biochemically characterized and identified. The different isolates were identified from the contaminated soil belongs to *Bacillus species, Klebsiella species, Citrob acter and Pseudomonas species.* The isolates identified were analyzed for biodegradation potential of diesel in the Minimal salt medium by turbidometry method OD values measured at 595nm

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