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Hydrocarbon Biodegradation Efficiency by Four Indigenous Bacterial Strains Isolated From Contaminated Soils

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Abstract*— Nineteen hydrocarbon degrading microorganisms were isolated from Ten hydrocarbon contaminated sites and were identified on the basis of morphological, biochemical and molecular characteristics as Acenetobacter junii, pantoea dispersa, Bacillus spizizenii, and Pseudomonas aeruginosa. The study illumined high density of bacteria acclimatized for biodegradation of hydrocarbon in soil. The isolates were examined for other hydrocarbon degradation in media supplemented with Diesel, Benzene, Petrol 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 Acenetobacter junii 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.

Keywords: Biodegradation, Benzene, Diesel, Petrol, Cyclohexane.

I. INTRODUCTION

Petroleum hydrocarbons are ubiquitous pollutants, found naturally in fossil fuels like as coal and petroleum. Several polluting anthropogenic activities particularly incomplete combustion of organic materials such as coal, diesel, fat, wood and vegetation, urban runoff and industrial activities such as coal liquefaction, coke production, petroleum refining, spillage of petroleum products and waste incineration have led to significant accumulation of PAHs in the environment especially those near to industrial sites **(Kanaly and Harayama, 2000; Haritash and Kaushik, 2009; Plaza *et al.*, 2009; Zhao *et al.*, 2009; Bakeas *et al.*, 2011; Singh and Tiwary, 2017).** 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 **(Shekhar *et al.*, 2015).**

Crude oil is components from thousands of constituents such as saturated hydrocarbons, aromatic hydrocarbons, resins and asphaltenes. Saturated hydrocarbons are the major pollutants; especially those of smaller molecular weight are readily biodegraded in soil environment. The aromatic hydrocarbons with one, two or three aromatic rings are also efficiently biodegraded in soil environment. However, those with four or more aromatic rings are quite resistant to biodegradation. The asphaltenes and resin fractions contain higher molecular weight, whose chemical structures have not yet been resolved. The biodegradability of these compounds is not yet known **(Gopinathan *et al.*, 2012)**. Petroleum-based products are the major source of energy for industry and daily life. Moreover, petroleum is the major source of raw material for many chemical products such as plastics, paints and cosmetics. The transport of petroleum across the world is frequent. The oil spill is heavily concentrated around offshore production sites, major shipping routes, and refineries and frequently exceeds the self- purification capacity of the receiving waters. Oil floating on water is technically difficult to contain and collect. Oil pollution is destructive to birds and various forms of marine life. Oil spills pollute ground water and are destructive to vegetation due to lack of oxygen and evolution of H2S, which kills the roots of most plants. **(Oudot J 1994, Prince *et al.*,1994 and Obire *et al.*, 2001; Gopinathan *et al.*, 2012).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 petroleum products also on the amount of other hydrocarbon products **(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).**

Bioremediation, bio-stimulation and bio-augmentation, is defined as the process that utilizes the metabolic capabilities of microorganisms for TPH uptake and degradation into less

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Vol 9, Issue 10, October 2022

toxic substance, or their removal from polluted soil. Bio-stimulation and bio-augmentation aim at increasing the level of TPH biodegradation by microorganisms. The difference of both techniques is bacteria used, where bio-stimulation and bio-augmentation use indigenous and specific competent exogenous bacteria introduced to the process, respectively **(Xu and Liu, 2010; Asquith *et al.*, 2012)**.[4,5,6,7] Both bacteria were stimulated through environmental factors (nutrient, oxygen, moisture, pH, and temperature) monitoring to enhance the effectivity of TPH degradation **(Xu and Liu, 2010; Das and Chandran, 2011; Sari *et al.*, 2019).[8,9,10,11,12,13]**

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, identification and optimizing condition of hydrocarbon degrading bacteria associated with petrol and diesel oil contaminated sites in chhattisgarh, India and also to test their capabilities to degrade different hydrocarbons. The expected elucidation of this study will provide information on the bacterial population, hydrocarbon-degrading microorganisms and their degrading capability of diesel because these bacteria can use the hydrocarbons as carbon source. Biodegradation by indigenous populations of microorganisms is one of the primary mechanisms by which petroleum and other hydrocarbon products can be removed from the environment **(Ulrici *et al.*, 2000)** and this process is also cheaper than the other pollution control technologies **(Leahy *et al.*,1990).** [18]

II. MATERIALS AND METHODS

Sources of sample collection: Samples were collected randomly from automobile workshop of Durg-Bhilai Contaminated 10 different sites 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.

2.1. 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).[1]** Culturally different colonies were selected and streaked over Bushnell HassAgar 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 agarslants which were subcultured at 15 days interval and were incubated at 37°C for 24- 42 hours and then stored at 4°**C (Shekhar *et al.*, 2015)**.[14]

2.2. Identification of hydrocarbon utilizing bacteria

The identification was done bycultural (margin, colour, texture and elevation), morphological and biochemical as per Bergey's Manual of Systemic Bacteriology **(Holt *et al.*, 1994).** [16] And molecular identification was done by analysis based on 16S r-RNA gene sequence analysis **(Parikh *et al.*, 2018).[17]**

2.3. 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 Diesel (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 un-inoculated tubes will be serve as control. Growth and degradation of the organism will be assayed by optical density (O.D) measurement at 620nm. **(Rahman and Rahman, 2002; Shekhar *et al.*, 2015).**

2.4. Evaluation of the specific degradation capacity of selected hydrocarbons

Biodegradation capability of the organism were determined the method given by **Santhini *et al.*, (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[22] were incubated at 37°C. Un-inoculated 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 un-inoculated tubes were incubated at 37°C for 5-15 days and examined regularly for growth in Petrol, Diesel, Benzene, and Cyclohexane.

III. RESULTS AND DISCUSSION

Isolation of Hydrocarbon degrading Bacteria The samples of hydrocarbon contaminated soil supplemented with various hydrocarbons showed the growth of Acenetobacter junii, Pantoea dispersa, Bacillus[21] spizizenii, and Pseudomonas aeruginosa. These organisms were found to be actively growing in Diesel during the study of the 30 hydrocarbon contaminated soils. A total of nineteen positive isolates.

3.1. Identification of highly efficient hydrocarbon utilizing bacteria

After evaluation of colony morphology, cell morphology, utilization of carbon source, biochemical and molecular characteristics the isolates were identified as Acenetobacter junii, Pantoea dispersa, Bacillus spizizenii, and Pseudomonas aeruginosa.

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Vol 9, Issue 10, October 2022

3.2. Effect of Temperature on hydrocarbon degrading bacteria

Hydrocarbon degrading bacteria grow optimally in a wide range of temperature ranging from 27 ^{0}C to 37^{0}C. Growth decreases dramatically at higher temperature. Acenetobacter junii showed highest growth at 30°C temperature at media supplemented with 5% Diesel while Pantoea dispersa and Bacillus spizizenii [23] showed maximum growth at 30°C whereas Pseudomonas aeruginosa showed high growth at 30°C. All these bacteria show less growth at low as well as high temperature.

3.3. 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 7.5Acenetobacter junii, Pantoea dispersa, Bacillus spizizenii, Pseudomonas aeruginosa showed highest growth at pH 7.5 at media supplemented with 5% Diesel. All these bacteria show very low growth at low as well as high pH.

Hydrocarbon degradation capacity of *Acenetobacter junii*

The degradation capacity of *Acenetobacter junii* was observed maximum with Diesel (0.84) followed by other hydrocarbon Cyclohexane (0.83), Petrol (0.81) and benzene (0.76). The degradation was found to gradually increase up to 10 days of incubation. A decreasing trend in optical density was observed. Even with *Acenetobacter junii*5% hydrocarbon concentration was found to be optimum for hydrocarbon degradation at 10 days. Significant increase in the degradation of hydrocarbon by *Acenetobacter junii* 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 *Pantoea dispersa*

The degradation capacity of *PantoeaDispersa* was observed maximum with Diesel (0.81) followed by other hydrocarbon viz petrol (0.70), Benzene (0.65), Cyclohexane (0.63). A decreasing trend in optical density was observed. Even with ***Pantoea dispersa*** 5% hydrocarbon concentration was found to be optimum for hydrocarbon[19] degradation at 10 days. Significant increase in the degradation of hydrocarbon by ***Pantoea dispersa***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 Bacillus spizizenii.

Among the hydrocarbons used for the evaluation of the degradation ability for Bacillus spizizenii, Diesel (0.69) was maximally degraded, followed by the hydrocarbons petrol (0.67), cyclohexane (0.63), Benzene (0.30). The concentration of 5% was found to be more suitable for the degradation of the hydrocarbons by Bacillus spizizenii as compared to 10% and 15% concentrations. There was significant increase in the degradation of hydrocarbons by Bacillus spizizenii with respect to the time, i.e. from 0 days towards 15 days.

Hydrocarbon degradation capacity of Pseudomonas aeruginosa.

Among the hydrocarbons used for the degradation studies by Pseudomonas aeruoginosa degradation was observed maximum in petrol (0.67) followed by cyclohexane (0.63), Diesel (0.46), Benzene (0.19) with a minimum hydrocarbons degradation with benzene (0.19)[20] 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.

Table 1: Colony and cell morphology

Colony and Cell Morphology	**Acenetobacterjunii**	**Pantoea dispersa**	***Bacillus* spizizenii**	***Pseudomonas* aeruginosa**
Margin	Entire	Regular	Undulate	Entire
Elevation	Dome shaped	Convex	Unbonate	Flat
Shape	Circular	Regular	Circular	Irregular
Opacity	Translucent	Translucent	Opaque	Opaque
Pigmentation	Greyish white	Yellow	Creamy white	Blue-green
Gram reaction	Gram negative	Gram Negative	Gram Positive	Gram negative
Cell shape	Rod	Rod	Rod	Rod

Table 2: Biochemical characteristics

Biochemical characteristics	**Acenetobacter junii**	**Pantoea dispersa**	**Bacillus spizizenii**	**Pseudomonas aeruginosa**
Catalase test	+	-	-	+
Oxidase test	-	-	+	+
Indole test	-	-	-	-
MR	-	-	-	-
VP	+	-	-	-
Citrate utilization test	+	-	-	-
Urease activity	+	-	-	-

ISSN (Online) 2456 -1304

International Journal of Science, Engineering and Management (IJSEM)

Vol 9, Issue 10, October 2022

H_2S production	-	-	-	-
Starch hydrolysis	-	-	-	-
Glucose	+	+	+A	-
Sucrose	+	+	+A	-
Lactose	+	+	-	-

Table 3: Effect of temperature on growth of bacteria in 5% diesel

Temperature	**10^{0}C**	**20^{0}C**	**30^{0}C**	**40^{0}C**	**50^{0}C**
Acenetobacter junii	0.30	0.48	**0.84**	0.58	0.32
Pantoea dispersa	0.22	0.38	**0.81**	0.61	0.34
Bacillus spizizenii	0.17	0.37	**0.69**	0.58	0.42
Pseudomonas aeruginosa	0.18	0.34	**0.45**	0.42	0.28

Fig 1: Effect of temperature on growth of bacteria in 5% diesel

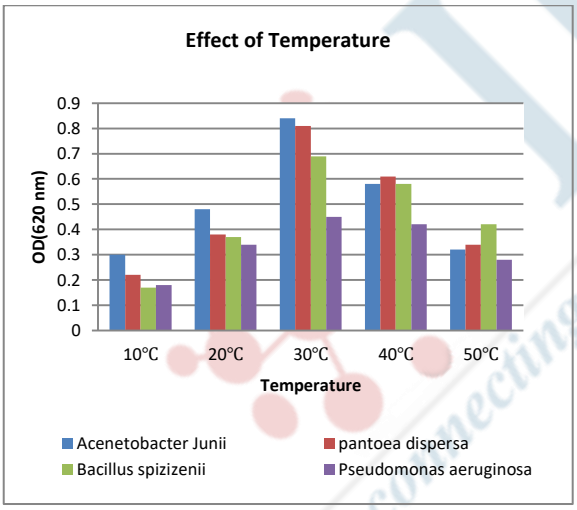


Table 4 : Effect of pH on growth of bacteria in 5% diesel

pH	**5.5**	**6.5**	**7.5**	**8.5**	**9.5**
Acenetobacter junii	0.42	0.57	**0.83**	0.38	0.5
Pantoea dispersa	0.35	0.55	**0.71**	0.31	0.8
Bacillus spizizenii	0.36	0.49	**0.65**	0.30	0.13
Pseudomonas aeruginosa	0.28	0.42	**0.46**	0.40	0.15

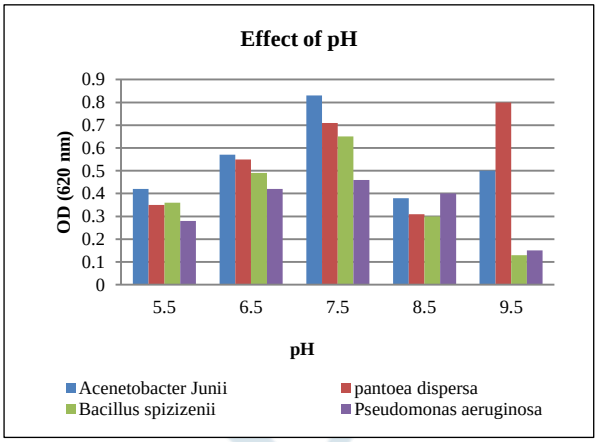
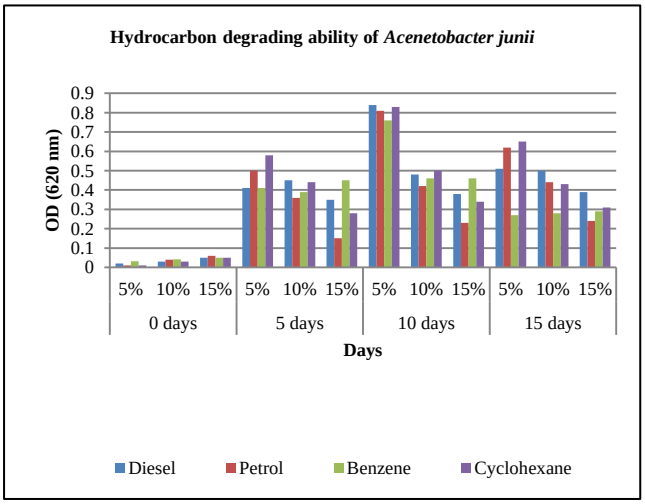


Table no 5: Hydrocarbon degrading ability of Acenetobacter junii

	0 days			5 days			10 days			15 days		
	5%	10%	15%	5%	10%	15%	5%	10%	15%	5%	10%	15%
Diesel	0.02	0.03	0.05	0.41	0.45	0.35	**0.84**	0.48	0.38	0.51	0.50	0.39
Petrol	0.01	0.04	0.06	0.50	0.36	0.15	**0.81**	0.42	0.23	0.62	0.44	0.24
Benzene	0.031	0.042	0.050	0.41	0.39	0.45	**0.76**	0.46	0.46	0.27	0.28	0.29
Cyclohexane	0.01	0.03	0.05	0.58	0.44	0.28	**0.83**	0.50	0.34	0.65	0.43	0.31

Hydrocarbon degradation ability of Acenetobacterjunii



ISSN (Online) 2456 -1304

International Journal of Science, Engineering and Management (IJSEM)

Vol 9, Issue 10, October 2022

Table no 6: Hydrocarbon degrading ability of Pantoea dispersa

	0 days			5 days			10 days			15 days		
	5%	10%	15%	5%	10%	15%	5%	10%	15%	5%	10%	15%
Diesel	0.02	0.03	0.05	0.41	0.45	0.35	**0.81**	0.48	0.36	0.41	0.50	0.39
Petrol	0.01	0.04	0.08	0.40	0.36	0.15	**0.70**	0.42	0.23	0.62	0.44	0.24
Benzene	0.031	0.03	0.05	0.41	0.39	0.35	**0.65**	0.46	0.46	0.27	0.28	0.29
Cyclohexane	0.01	0.03	0.05	0.58	0.44	0.28	**0.63**	0.50	0.34	0.65	0.43	0.31

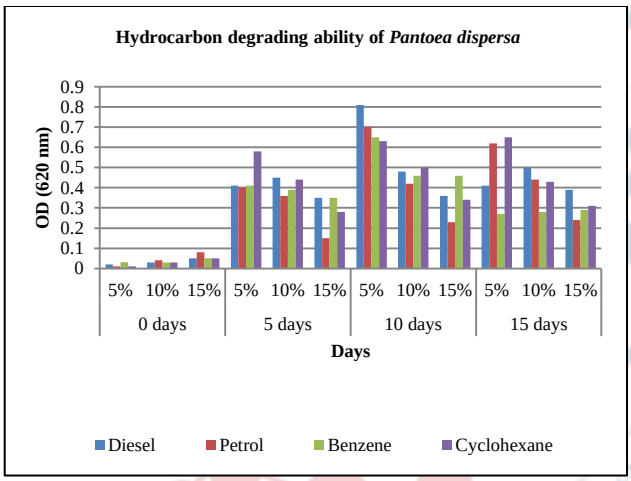


Table no 7: Hydrocarbon degrading ability of Bacillus spizizenii.

	0 days			5 days			10 days			15 days		
	5%	10%	15%	5%	10%	15%	5%	10%	15%	5%	10%	15%
Diesel	0.02	0.03	0.05	0.31	0.35	0.35	**0.69**	0.47	0.36	0.51	0.50	0.39
Petrol	0.01	0.04	0.06	0.50	0.36	0.15	**0.67**	0.41	0.23	0.61	0.43	0.22
Benzene	0.031	0.042	0.050	0.41	0.39	0.45	**0.30**	0.45	0.45	0.26	0.28	0.29
Cyclohexane	0.01	0.03	0.05	0.53	0.44	0.28	**0.63**	0.50	0.34	0.65	0.43	0.31

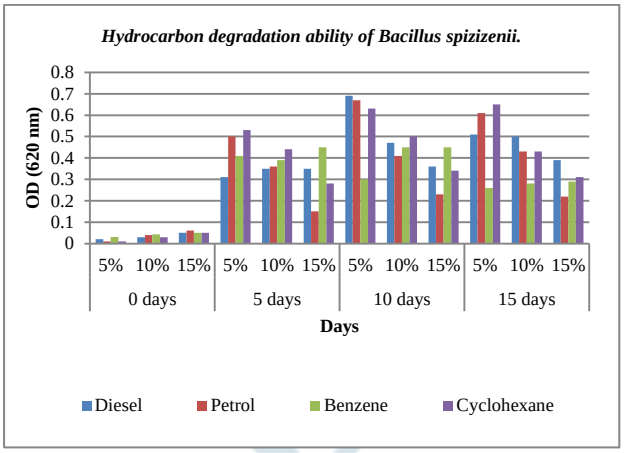
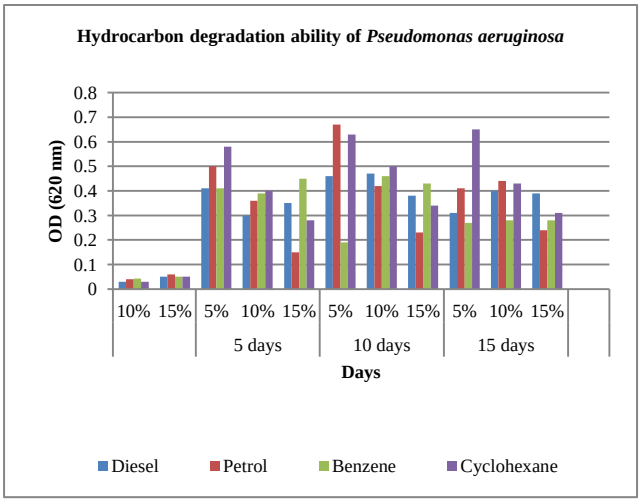


Table no 8 : Hydrocarbon degradation ability of Pseudomonas aeruginosa

	0 days			5 days			10 days			15 days		
	5%	10%	15%	5%	10%	15%	5%	10%	15%	5%	10%	15%
Diesel	0.02	0.03	0.05	0.41	0.30	0.35	**0.46**	0.47	0.38	0.31	0.40	0.39
Petrol	0.01	0.04	0.06	0.50	0.36	0.15	**0.67**	0.42	0.23	0.41	0.44	0.24
Benzene	0.031	0.042	0.050	0.41	0.39	0.45	**0.19**	0.46	0.43	0.27	0.28	0.28
Cyclohexane	0.01	0.03	0.05	0.58	0.40	0.28	**0.63**	0.50	0.34	0.65	0.43	0.31



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Vol 9, Issue 10, October 2022

IV. SUMMARY AND CONCLUSION

For the investigationThirty soil samples each from Ten 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 andenergy at 5% concentration of petrol. The highly efficient bacteria found positive for growth on screened plates were identified on the basis of cultural, morphological Biochemical and molecular 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 19 positive isolates were obtained, out of which Acenetobacter junii and Pantoea dispersa showed maximum occurrence while Pseudomonas aeruginosa and Bacillus spizizenii 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 10 days incubation and 5 % concentration and also at 15 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.

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