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Comparative study of isolates to degrade high molecular weight hydrocarbons.

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ABSTRACT

An aerobic, rod-shaped gram-negative bacteria *Brevibacillus bortelensis* isolated from the cold region above 4003 feet of Rajasthan. It was identified and characterized for evaluating its comparative biodegradation ability with one of the known degrader MTCC culture (MTCC code-7199) *Pseudomonas aeruginosa*. The growth profiles were determined by monitoring optical density, Total petroleum hydrocarbon, Gas chromatography and mass spectroscopy etc. Bushnell Hass broth was used as selective enrichment medium with 2T engine oil as a sole source of carbon and energy. Maximum oil degradation capacity of isolated *Brevibacillus bortelensis* was found to be 53% after 14 days during fermentation process whereas for *Pseudomonas aeruginosa* it was 41% after 28 days. Petroleum hydrocarbons in residual oil were analyzed using Gas chromatography-Mass spectroscopy. Low molecular weight compounds were degraded and breakdown of high molecular weight hydrocarbons by *Brevibacillus bortelensis* suggested that this isolate can be used widely in the oil recovery treatments.

Keywords: 2T engine oil, poly aromatic hydrocarbons, toxicity, microorganisms, GC-MS, Gravimetric method, etc.

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INTRODUCTION

Pollution by petroleum hydrocarbons had become a serious problem since years due to their ubiquitous presence in nature and their carcinogenic and mutagenic characteristics. Environment contamination with oil derivatives is a serious problem. Oil has become a well known major source of environment pollution since many years. Petroleum is a complex structure of different hydrocarbons. They are in linear or branched, mono and polyaromatic which are considered as priority pollutants by USEPA (1). These Polyaromatic hydrocarbons (PAHs) are a large group of organic compounds. PAHs found in oil (2), soil (3, 4, 5, 6), air (7, 8, 9), water (10). They enter into the human body directly or indirectly through breathing or by eating the food (11). These compounds contain 2 or more than 2 benzene rings in linear, angular and cluster arrangements (12). Many PAHs and their epoxides have been found with exerting carcinogenic and neurotoxic effects apart from increasing environmental hazards. The toxicity of PAHs is directly proportional to the benzene rings. PAHs apart from being persistent in the environment are also carcinogenic and mutagenic, which are harmful not only to humans but also for both flora and fauna (13).

On the basis of molecular weight, they may be classified as low molecular weight (LMW) compounds which consist of two or three benzene rings and high molecular weight compounds (HMW) having four or more fused benzene rings. The higher the molecular weight, the lesser will be the rate of degradation (14). They are less soluble in water and hence are hydrophobic in nature (15). In other words, the solubility decreases with the increase in hydrophobicity by the increasing number of fused rings.

The rate of contamination is high as comparative to its solution methods. Cleaning of the polluted environment by chemical methods would upset the balance of living organisms but bioremediation is a technique which refers the cleanup of toxic pollutants by the means of biological methods by using non pathogenic microorganisms. This method established as an efficient, versatile, economic and environmentally sound treatment method which provides a promising opportunity to make a better environment. Microbial community is responsible at an enormous level in the process of biodegradation. Use of indigenous microbial community in the biodegradation process suggests reducing the risk of contamination associated with hydrocarbon contamination of soil. Several microorganisms are capable of degrading these hydrocarbons as they used them as an energy source for their metabolic activity and therefore can be applied to rehabilitate hydrocarbon contaminated site (16, 17) Microbial degradation in soil is a complex process and has been revived in the past (18, 19, 20). Microorganisms have their own enzyme system which can lead to the initial attack on the compounds and convert them to their lower forms or completely utilize (21, 22). Initially, by the oxidation process degradation occurs with the help of oxygenase and peroxidases enzymes (23).

In the process of bioremediation, also there is complex structure of factors are also involved. Not only the presence of microorganisms is good enough for the degradation but also the composition of hydrocarbons, microbial population, oxygen availability, temperature, pH, organic nutrients etc. are the other major components required for booming bioremediation. Bioavailability is one of the foremost factors which influence the extent of hydrophobic compounds which is found to be a priority research objective in bioremediation field (24). Moreover, these factors alone are not responsible for the desirable results but the manipulations of related factors with good microbial growth assume importance. In the extreme conditions such as low temperatures, high salinity, acidic or alkaline pH etc. these hydrocarbons have been shown to be degraded (25, 26). The supply of minerals in the form of carbon and nitrogen ratio to the contaminated site boosted the rate of degradation (27). The success has attained by many researchers by using microbial community in various stress conditions and several environmental factors which make them more effective and suitable in degradation of toxic hydrocarbons.

In the present investigation, the capability of isolated soil microbe from Mount Abu, (Cold region in Rajasthan situated at high altitude) and MTCC culture (purchased from IMTECH, Chandigarh) to degrade 2T engine oil was studied. Various factors were examined for the process of biodegradation. The ability of an isolate to utilize 2T engine oil was examined by using gravimetric assay. The GC-MS analysis was done for the estimation of degradation of hydrocarbons. Breakdown of high molecular hydrocarbons of 2T engine oil was examined by GC-MS data.

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MATERIAL AND METHODS

Collection of soil samples

The petroleum hydrocarbon contaminated soil samples were collected from the various automobile workshops of Mount Abu (Rajasthan). It was collected from 3 cm depth of the surface and kept in the sterile poly bags. The 2 stroke engine oil was obtained from the local petrol pump. Pre-preparation of samples by drying crushing and sieving through different mesh size sieves was done.

Isolation and screening of hydrocarbon degrading microorganisms-

Petroleum hydrocarbons degrading microorganisms were isolated using enrichment culture plate technique using Bushnell Hass media (28, 29). 1.0 g of soil sample was weighed and mixed with 100 mL of autoclaved Bushnell Hass media (BH medium). It was supplemented with 1% of 2T engine oil which was served as sole carbon and energy source. These flasks were kept on a shaker at 150 rpm for 7 days. After 7 days of incubation 10 ml of enriched media was transferred to freshly prepared BH media and kept on a shaker in same conditions as described. This cycle was repeated for 4 to 5 times. After every enrichment cycle, 1 mL of culture media was diluted to 10^3 to 10^8 fold and these diluted cultures were plated on BH agar plates supplemented with 2T engine oil at 37°C. The hydrocarbon degrading microorganisms colonies were counted by colony counting method by using colony counter. By culturing and sub-culturing of colonies onto BH agar plates pure culture of hydrocarbon degrading bacteria was obtained. At the end, these microbial strains were kept in 40% glycerol at 70^0C for future use.

Evaluation of degradation potential

Biodegradation assay

5 copies of 100 mL BH medium flasks were prepared by adding l mL of 48 hours old bacterial inoculums and 5 g of 2T engine oil. These flasks were kept on a rotatory shaker for 7-14-21-28 and 35 days at 25^0C temperature. The concentrations of residual oil were determined by gravimetrically and chromatographic analysis (30).

Gravimetric analysis

After every 7 days of incubation, 1 flask was taken out for gravimetric analysis. Absorbance was analyzed at 620 nm by using a spectrophotometer. After that, 5 mL of n-hexane (petroleum fraction) was added to the flask for the separation of a mixture of oil and broth and shaken vigorously. It was allowed to stand for 5 minutes. Two layers were formed, upper layer contained oil mixed with n-hexane and the lower layer was of broth medium. The residual oil was separated using separating funnel in pre-weighed petri-dish after passing through sodium sulfate to remove the moisture. The petri-dish allows to stand for 24 hours to evaporate n-hexane and the weight of residual oil was noted. 500µL of the residual oil was used for GC-MS analysis (31, 32, 33).

Percentage of oil degraded was calculated using the formula:

Weight of residual oil = weight of beaker containing extracted oil – weight of empty beaker
Amount of oil degraded = weight of oil added in media – weight of residual oil

$$\% \text{ degradation} = \frac{\text{Amount of oil degraded}}{\text{Amount of oil added in the media}} \times 100$$

GC-MS analysis

By chromatographic determination, the hydrocarbons present in the residual oil was separated and identified. The GC-MS analysis was performed using Shimadzu model QP-2010 plus, column-Rtx-5MS, 30-meter x 0.25 mm i.d. x 0.25 um film thickness.

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Characterization and Identification of Bacterial strain

The morphological and biochemical characterization of isolated microbial strain was identified and characterized on the basis of Bergey's Manual of Determinative Bacteriology (34, 35). 16sRNA sequencing was performed at Xcelris Labs Ltd. India for molecular identification of hydrocarbon degrading microorganisms.

RESULTS AND DISCUSSION

From the previous data it has been observed that bioremediation is a method which is used to remediate petroleum contaminated land, thus underscoring the importance of microorganisms in the remediation technologies (36). The identity of the isolated isolate was confirmed by morphological properties, biochemical tests, and molecular identification (Table 1, Fig.1). The isolate was showing the maximum similarity to *Brevibacillus borstelensis* strain SRDTh1, **(**GenBank Accession Number: EU714902.1) based on nucleotide homology and phylogenetic analysis was performed at Xcelris Labs Ltd. India. By using gravimetric analysis the rate of 2T engine oil degradation was evaluated which was obtained rapidly for first 14 days for *Brevibacillus bortelensis* whereas for *Pseudomonas aeruginosa* it was slow and showed maximum degradation after 28 days. The pace of engine oil biodegradation obtained considerably swift for seven days with 51% for *Brevibacillus bortelensis*. The utmost degradation was obtained after 2 weeks with 53% of degradation of 2T engine oil then it was reduced. While for *Pseudomonas aeruginosa* the maximum degradation of 2T engine oil was obtained after 28 days with 41%. The growth (cell density) was measured using absorbance at 620 nm (Table 2, and graphically representation of absorbance and degradation rate in Fig 2, Fig. 3, Fig. 4 and Fig 5).

Table 1: Morphological and biochemical properties of *Brevibacillus borstelensis*

	S.No.	Biochemical test	Reaction
Morphological characters	1	Morphology	Rod
	2	Opaque	+ ve
	3	Convex	+ ve
	4	Diffusible pigment	-ve
	5	Color	Pink
	6	Gram's Reaction	-ve
Biochemical characters	8	Catalase	+ve
	9	Oxidase	+ve
	10	Indole	-ve
	11	MR	+ve
	12	VP	-ve
	13	Citrate	-ve
	14	Starch	-ve
	15	Phenylalanine Deaminase	-ve
	16	Gelatin	+ve
	17	Motility	+ve
	18	TSI agar	-ve
	19	Spirit blue agar	+ve
	20	Klinger Iron agar	±ve

Footnotes- + ve=Positive, -ve=Negative

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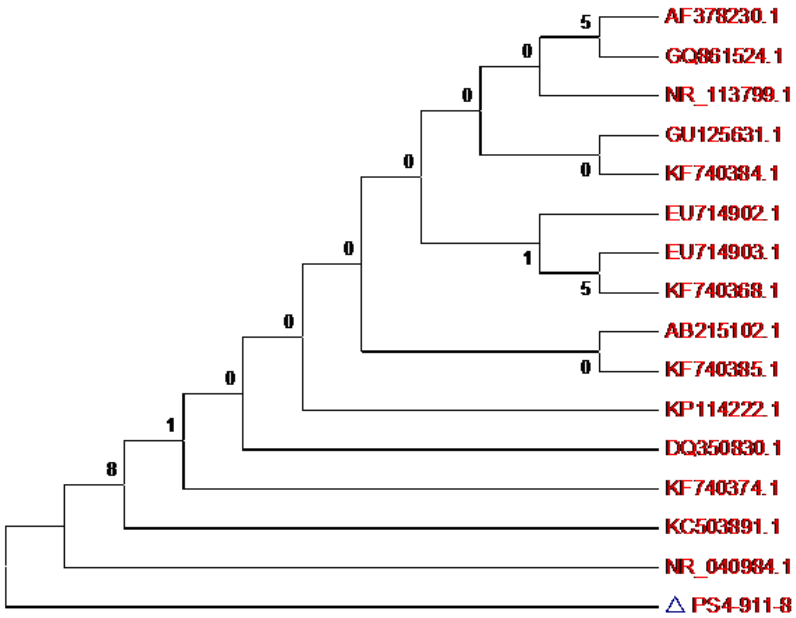

Fig 1: The phylogenetic analysis of *Brevibacillus bortenlsi*s (16s rRNA sequencing)

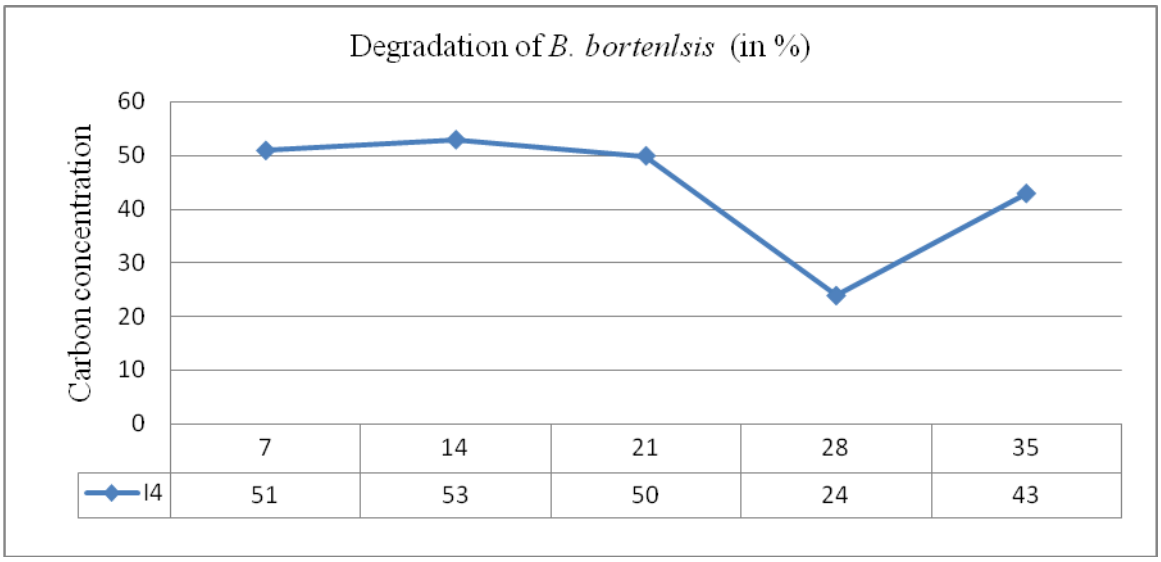

Fig 2: Graphically representation of degradation rate of *B. bortenlsis*

Table 2: Rate of Biodegradation (in %age) of *Brevibacillus bortenlsis* and *Pseudomonas aeruginosa* grew in Bushnell-Hass medium amended with 2T engine oil as sole carbon and energy source and their microbial growth at 620nm

		7 days	14 days	21 days	28 days	35 days
Brevibacillus borstelensis	Biodegradation potential in (%)	51	53	50	24	43
	O.D. at 620nm	0.14	0.16	0.74	1.06	1.27
Pseudomonas aeruginosa	Biodegradation potential in (%)	25	15	29	41	38
	O.D. at 620nm	1.69	0.46	1.25	1.02	1.21

Footnotes-O.D.=optical density

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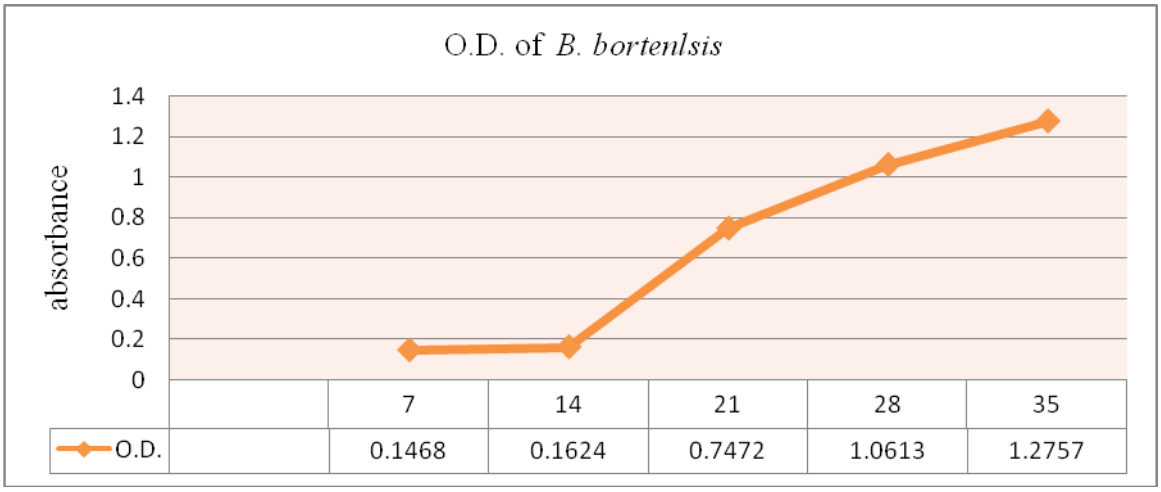

Fig 3: Graphically representation of O.D. at 620nm of *B. Bortenlsis*

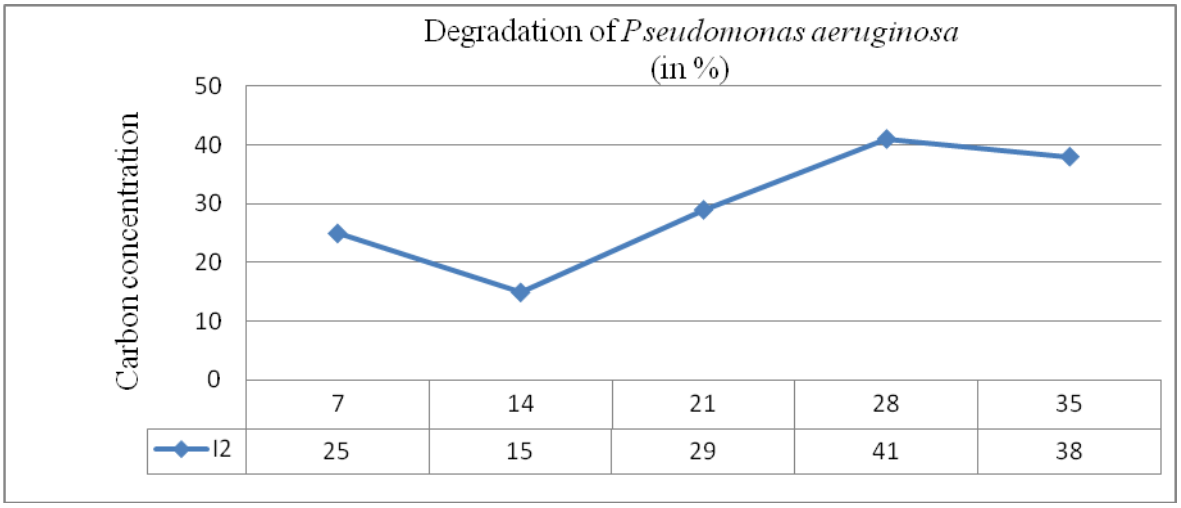

Fig 4: Graphically representation of degradation rate of *Psuedomonas aeruginosa*

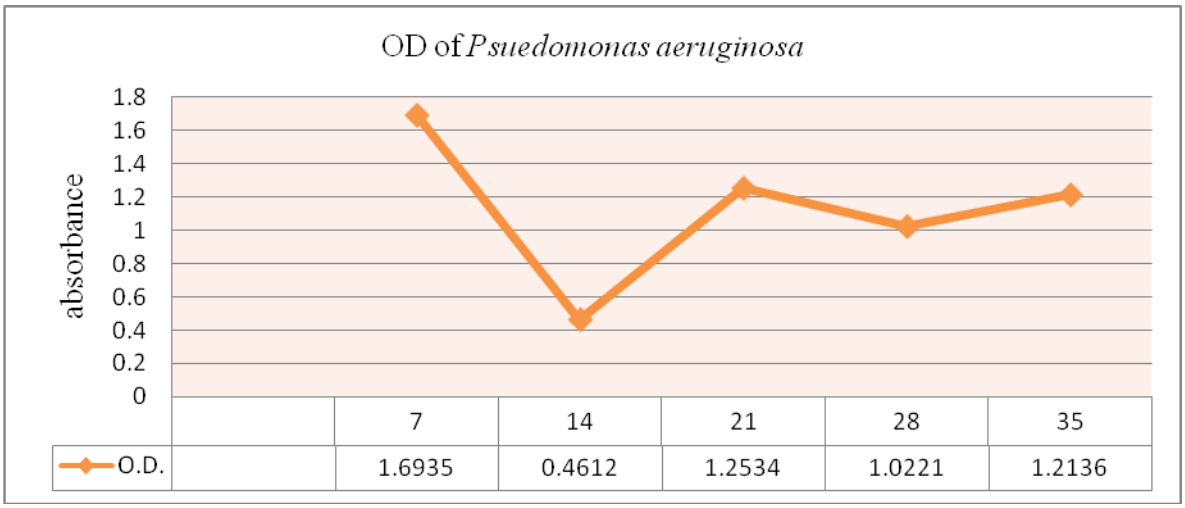

Fig 5: Graphically representation of O.D. at 620 nm of *Psuedomonas aeruginosa*

The concentration of crude oil and the microorganisms involved are some conditions on which the process of biodegradation depends (37). Several microorganisms are capable of utilizing these highly toxic hydrocarbons. Microorganisms can produce spore, which may shield them from the toxic effect of hydrocarbons (38). Bacteria belonging to *Pseudomonas* genera, *Acinetobacter, Flavobacterium* were reported to utilize engine oil up to 90% degradation (39). Biodegradation of diesel oil by *Pseudomonas* have been studied by various researchers (40, 41, 42, 43, 44).

GC-MS was used to directly examine the compounds of residual 2T engine oil in each cycle of 7-14-21-28 and 35 days of incubation period. Engine oil is a mixture of biodegradable hydrocarbons. These hydrocarbons were categorized according to the carbon atom ranges and the percentage of each fraction was calculated (C1-C_{10}, C_{11}-C_{21}, C_{21}-C_{30}, C_{31}-C_{40} etc) of 7-14-21-28 and 35 days (Table 3 and 4 and their graphical representations in Fig 6 and 7). From Table 3 and 4 is has been clearly shown that the percentage of higher alkanes were decreased and lower alkanes were increased which means the conversion of higher alkanes into lower alkanes occurred during the fermentation process of 7-35 days. The total ion chromatogram of 2T engine oil degradation shows the majority of higher alkanes were significantly biodegraded. Some of the major hydrocarbons found Hexacontane ($C_{60}H_{122}$), Tetracosane ($C_{24}H_{50}$), Docosane ($C_{22}H_{46}$), Dotriaconatne ($C_{32}H_{66}$) converted into some lower forms such as Tetratetracontane ($C_{44}H_{90}$), Tricosane ($C_{23}H_{48}$), Heneicosane ($C_{21}H_{44}$), Octacosane ($C_{28}H_{58}$), Pentatriacontane ($C_{35}H_{72}$) to be found by GC-MS data.

Table 3: The carbon atom number (range-) wise degradation of compounds obtained from GC-MS analysis of *Brevibacillus borstelensis*

RANGE /DAYS	C_1-C_{10} Peak % area	C_{11}-C_{20} Peak % area	C_{21}-C_{30} Peak % area	C_{31}-C_{40} Peak % area	C_{41}-C_{50} Peak % area	NSO Peak % area	UI Peak % area
7-Days	5.57	61.53	13.96	1.71	0.24	10.25	6.7
14-Days	0.62	67.33	17.44	1.61	Nil	9.32	3.65
21-Days	0.99	72.27	15.12	nil	Nil	10.36	1.29
28-Days	1.54	69.48	7.44	1.15	0.82	17.49	3.24
35-Days	2.88	54.44	21.81	0.95	Nil	18.82	1.07

Table 4: The carbon number (range-) wise degradation of compounds obtained from GC-MS analysis of *Pseudomonas aeruginosa*

RANGE /DAYS	C1-C10 Peak % area	C11-C20 Peak % area	C21-C30 Peak % area	C31-C40 Peak % area	C41-C50 Peak % area	NSO Peak % area	UI Peak % area
7-Days	2.37	66.02	20.25	1.16	1.52	5.97	2.7
14-Days	0.46	56.41	31.6	2.58	0.19	7.26	1.48
21-Days	0.23	53.52	37.17	0.33	0.8	7.4	0.52
28-Days	nil	41.06	34.79	1.75	0.41	6.63	15.39
35-Days	0.13	78.96	17.81	0.32	nil	2.77	nil

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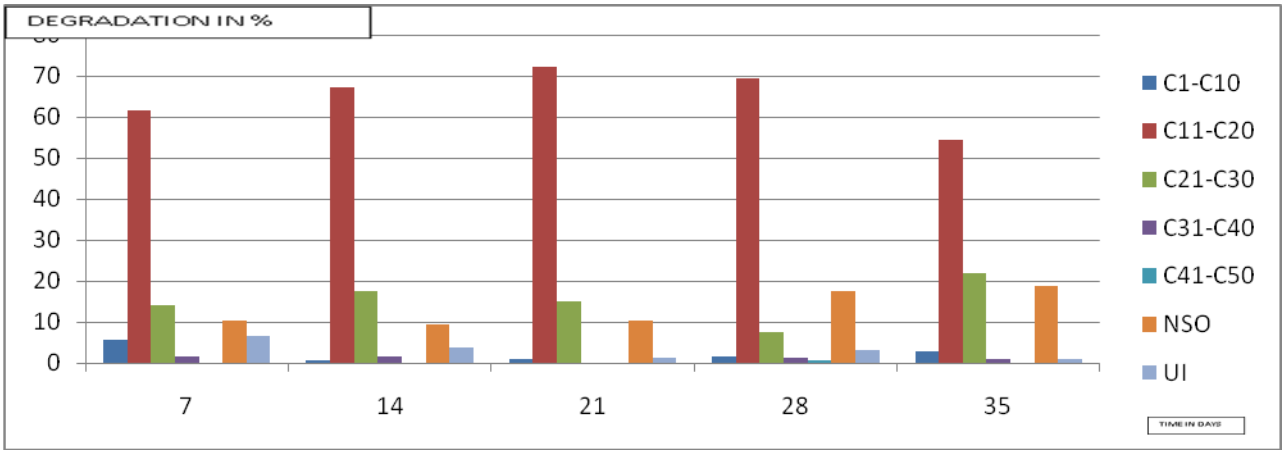

Fig 6: The graphical presentation of carbon range wise peak percentage area (% area) of *Brevibacillus borstelensis*

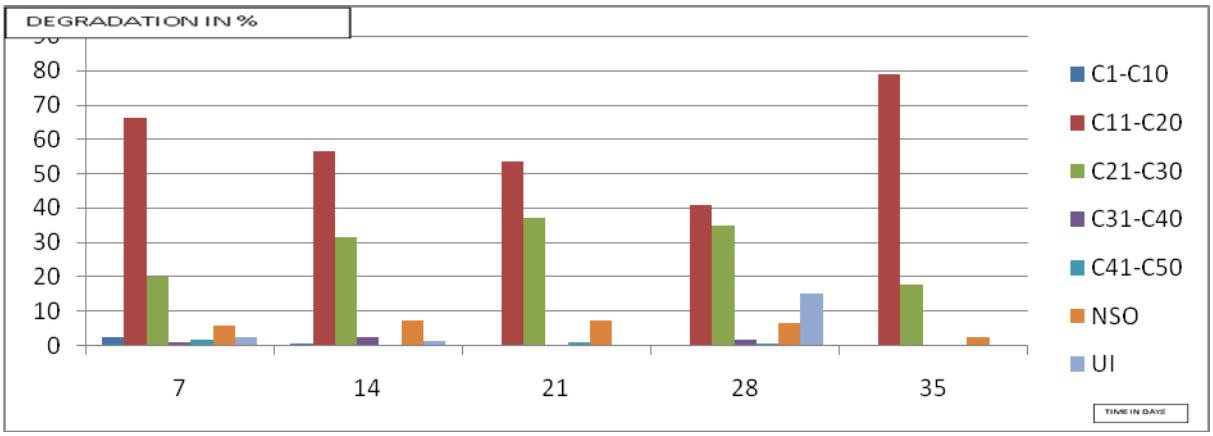

Fig 7: The graphical presentation of carbon range wise peak percentage area (%area) of *Pseudomonas aeruginosa*

From the studies, it has been concluded that aliphatic fractions were degraded more rapidly than the branched aromatic hydrocarbons (45). In the microbial degradation of petroleum hydrocarbons, n-alkane chain length is one of the important factors because the short chain length hydrocarbons oxidized rapidly as comparative to higher chain length hydrocarbons (46, 47, 48). The complex structure of the hydrocarbons is oxidized slowly as comparative to simple structures. In addition to the chain length, petroleum hydrocarbon degradation efficiency also determined by the organisms involved, the medium in which it was developed and structure of oil hydrocarbons. For this reason, the longer enrichment period was selected for the fresh medium of toxic metabolites, which enhanced the proliferation of bacteria to utilize more complex compounds. The ability of an isolate to degrade engine oil is taken as evidence that these microorganisms are the active degraders of the environment (49, 50). From the chromatograms obtained by GC-MS, the reduction in the height and density of peaks confirmed that there is a reduction in the 2T engine oil from 7 to 35 days (Fig 8 and 9).

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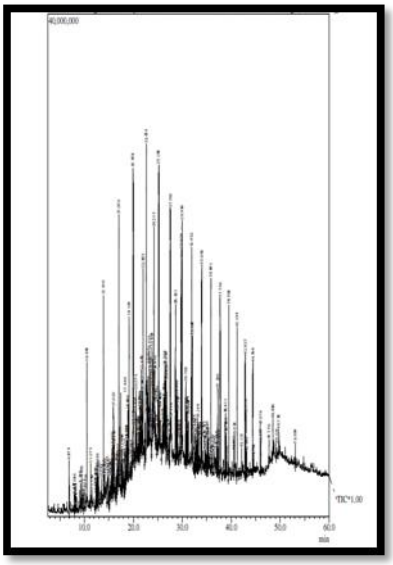
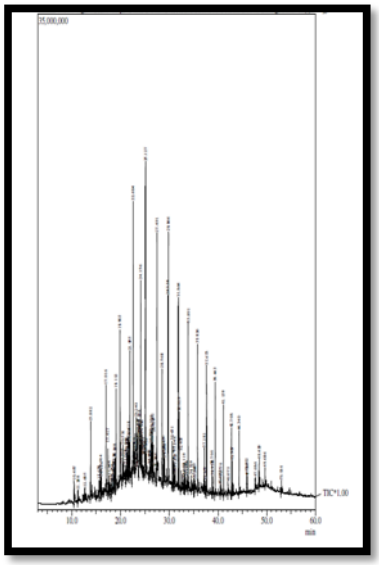
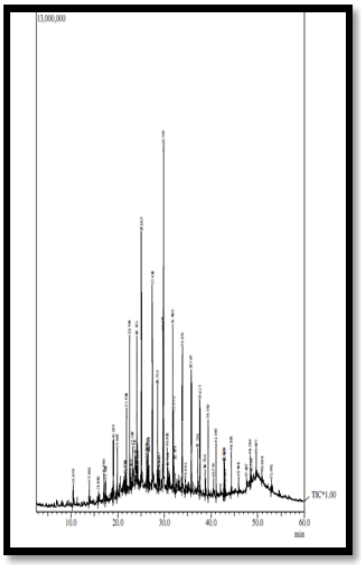


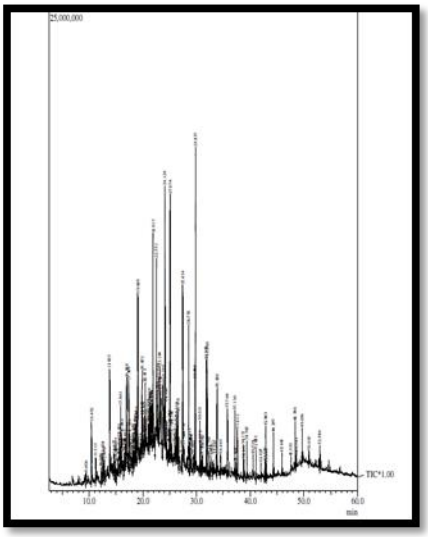
7 Days Chromatogram



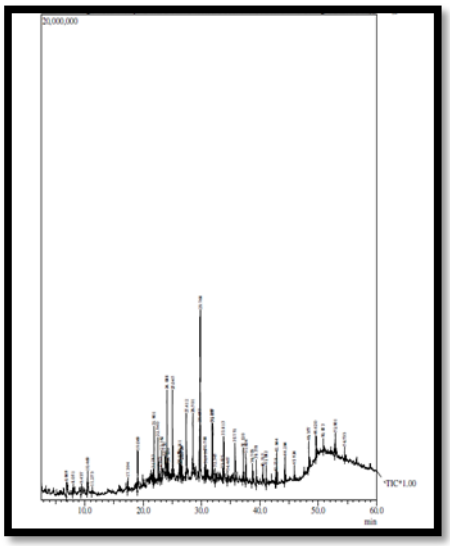
14 Days Chromatogram



21 Days Chromatogram

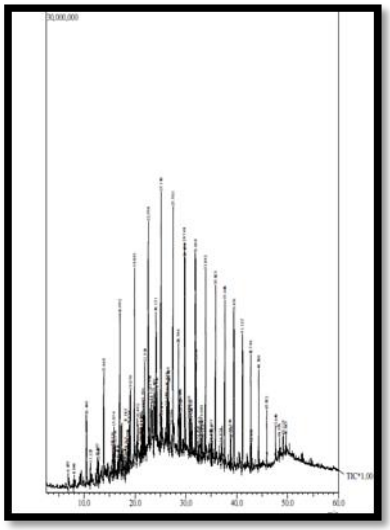


28 Days Chromatogram

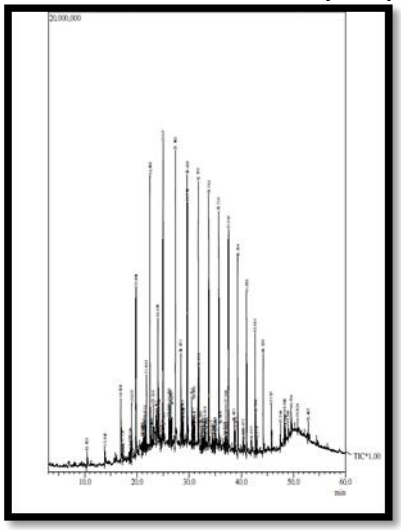


35 Days Chromatogram

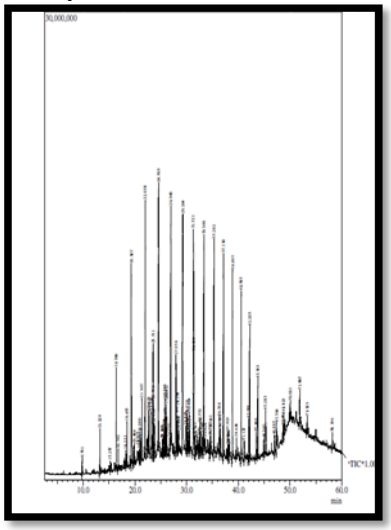
Fig 8: The chromatograms obtained by GC-MS analysis of biodegradation by *Brevibacillus Borstelensis* of 7-14-21-28 and 35 days respectively



7 Days Chromatogram



14 Days Chromatogram



21 Days Chromatogram

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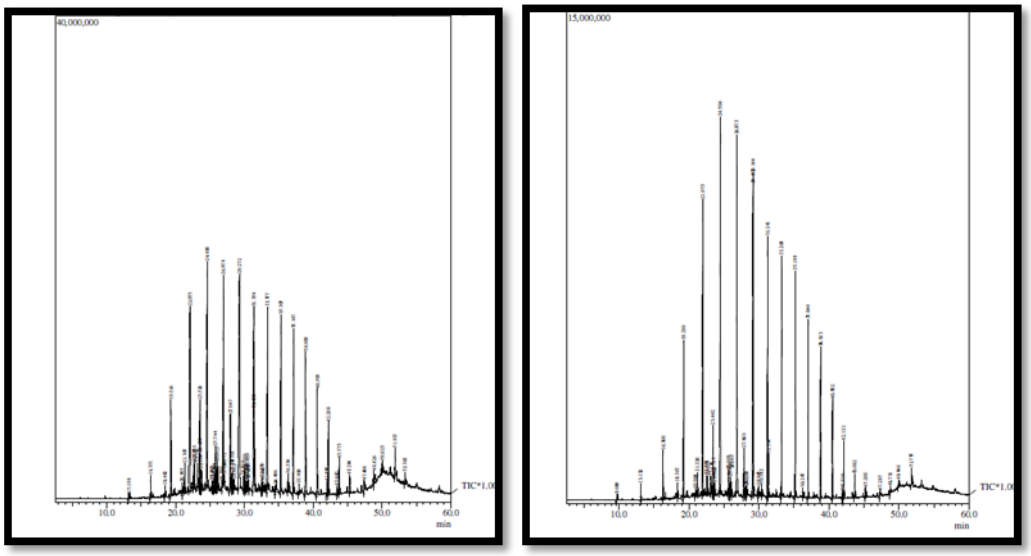

28 Days Chromatogram **35 Days Chromatogram**

Fig 9: The chromatograms obtained by GC-MS analysis of biodegradation by *Pseudomonas aeruginosa* of 7-14-21-28 and 35 days respectively

The main goal of this research was to judge the potential of isolated isolates to utilize 2T engine oil by gravimetric method and their comparison with the best-known degrader purchased. The decrease in the weight of oil and n-hexane removal of residual oil was conducted to determine biodegradation and concentrations of residual engine oil. The conversion of higher molecular weight hydrocarbons to lower molecular weights hydrocarbons from GC-MS data suggests the microorganism has the efficient capability to utilize petroleum hydrocarbons. An effort was designed to understand the ability of cold region isolate to degrade hydrocarbons.

CONCLUSION

It has been stated that the bioremediation is one of the prominent methods which converts the highly toxic compounds to lower toxic compounds and also, may remove completely. The hydrocarbons degradation can be enhanced in many types of environments, have contributes to the development of oil bioremediation technique (51). Various factors are involved in the process of degradation such as the nutrient availability, concentration of hydrocarbons, physical factors etc. (52).

The knowledge on the ability of microorganisms to utilize toxic hydrocarbons has accumulated considerably in the past two decades. These observations suggest that the isolated bacterial strain is capable of utilizing 2T engine oil as a carbon source. This capability to utilize engine oil as a carbon source makes it a potential biodegrader however. A mixture of the microbial population gives successful results for the complete biodegradation of complex hydrocarbons (53).

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REFERENCES

[1] Piola RF, Johnston EL. Diversity Distributors 2008; 14: 239-242.
[2] Nishioka M, Chang HC, Lee V. Environment Science & Technology 1986; 20: 1023-1027.
[3] Jones KC, Stratford JA, Tidridge P, Waterhouse KS, Johnston AE. Environmental Pollution 1989; 56:337-351.

[4] Jones KC, Stratford JA, Waterhouse KS, Furlong ET, Giger W, Hites RA, Scha€ner C, Johbston AE. Environmental Science & Technology 1989; 23:95-101.
[5] Jones KC, Stratford JA, Waterhouse KS, Vogt NB. Environmental Science & Technology 1989; 23:540-550.
[6] Wilson SC, Jones KC. Environmental Pollution 1993; 81:229-249.
[7] Freeman DJ, Cattell FCR. Environmental Science & Technology 1990; 24:1581-1585.
[8] Sexton K, Liu K, Hayward SB, Spengler JD. Atmospheric Environment 1985; 19:1225-1236.
[9] Greenberg A, Darack F, Harkov R, Lioy P, Daisey J. Atmospheric Environment 1985; 19(8):1325-1339.
[10] Cerniglia CE, Heitkamp MA. 1989. Microbial degradation of polycyclic aromatic hydrocarbons (PAH) in the aquatic environment, p. 41-68. In U. Varanasi (ed.), Metabolism of polycyclic aromatic hydrocarbons in the aquatic environment, CRC Press, Boca Raton, Fla.
[11] Samanta SK, Singh OV, Jain RK. Trends Biotechnology 2002; 20:243–248.
[12] Sims RC, Overcash MR. Residue Reviews 1983; 88:1–68.
[13] Mrayyana B, Battikhi MN Journal of Hazardous Material 2005; 120(1-3):127-134.
[14] Harvey RG. Polycyclic aromatic hydrocarbons, 1997 Wiley-VCH. New York.
[15] Cerniglia CE. Biodegradation of polycyclic aromatic hydrocarbons 1992 pp. 351-368. *In* E. Rosenberg (ed.), Microorganisms to combat pollution. Kluwer Academic Publishers, Dordrecht, the Netherlands. Biodegradation,3
[16] Chaudhry Q, Blom-Zandstra M, Gupta S, Joner EJ. Environmental Science and Pollution Research International 2005; 12(1):34-48.
[17] Singh OV, Jain RK. Applied. Microbiology and Biotechnology 2003; 63(2)**:**128-135.
[18] Atlas RM. Microbiology Reviews 1981; 45:80-209.
[19] Bartha R. Microbial Ecology 1986; 12:155–172.
[20] Morgan P, Watkinson RJ. Critical Reviews in Biotechnology 1989; 8(4): 305-333.
[21] Dhaker AK, Jain PK. Sewage Pollutants and their Bioremediation by Using Different Microorganism', In: Recent Advances in Environmental, 2011:189-204
[22] Jain PK, Gupta VK, Gaur RK, Lowry M, Jaroli DP, Chauhan UK. Research Journal of Environmental Toxicology 2011a; 5:1-26.
[23] Ron E, Rosenberg E. Current Opinion Biotechnology 2002; 13:249–252.
[24] Bardi L, Mattei A, Steffan S, Marzona M. Enzyme and Microbial Technology 2000; 27(9):709-713.
[25] Pieper DH, Reineke W. Current Opinion in Biotechnology 2000; 11(3): 262-270.
[26] Margesin R. Schinner F. Applied Microbiology and Biotechnology 2001; 56(5-6): 650-663.
[27] Namkoong W. Hwang EY. Park JS Choi JY. Environment. Pollution 2002; 119(1):23-31.
[28] Hemalatha S. Veeramanikandan P. Journal of Environmental Protection 2011; 2: 243-254.
[29] Subathra MK, Immanuel G, Suresh AH. Bioinformation 2013; 9(3):150-157.
[30] Obuekwe CO, Al-Zarban SS. Environment International 1998; 24 (8): 823–834.
[31] Mittal A. Singh P. Indian Journal of Experimental Biology 2009; 47:760-765.
[32] Mukherjee S. Jagadevan S, Mohapatra G, Vijay A. Bioresource Technology 2005; 95(3): 281-6.
[33] Raed SAl-W, Shimaa RH. 2014 International Journal of Bacteriology.
[34] Chaudhary VK, Borah D. International Journal of Plant Animal and Environmental Sciences 2011; 1(2):36-49.
[35] Khan JA, Rizvi SHA Advances in Applied Science Research 2011; 2(3): 455-460.
[36] Holden PA. LaMontagne MG, Bruce AK, Miller WG, Lindow SE. Applied and Environmental Biotechnology 2002; 68(5):2509-2518.
[37] Zhang G, Yue-ting W, Xin-ping Q, Qin M. Journal of Zbejian University Sc. 2005; 6B (8):725-730.
[38] Onifade AK, Abubakar FA. Research Journal of Biological Sciences 2007; 2:36-40.
[39] Mandri T, Lin J. African Journal of Biotechnology 2007; 6(1): 023-27.
[40] Emtiazi G, Shakarmi H, Nahvi I, Mirdamadian SH. Africa Journal Biotechnology 2005; 4 (2):172 – 176.
[41] Davies JI, Evans WC Biochemical Journal 1964; 91:251.
[42] Das K, Mukherjee AK. Bioresource Technology 2007; 98(7): 1339-1345.
[43] Teli N, Verma D, Gavankar R, Bhalerao S. International Research Journal Environmental Science 2013; 2(2):48-52.
[44] Jayanthi R, Hemashenpagam N. International Journal Of Novel Trends In Pharmaceutical Sciences 2015; 5(3).
[45] Donaldson EC, Chilingarian GV, Yen TF. Microbial Enhanced Oil Recovery book 1989.
[46] Mohanty G, Mukherji S. International Biodeterioration and Biodegradation 2008; 61:240-250.
[47] Seklemova E, Pavlova A, Kovacheva K. Biodegradation 2001; 12:311–316.

[48] Jonge DH, Freijer JI, Verstraten JM, Westerveld J, van der Wielen FWM. Environmental Science and Technology 1997; 31:771-775.
[49] Farag M.S. Abdelhamid HE Ahmed I. International Journal of Current Microbiology and Applied Sciences 2015; 4(2):920-927.
[50] Van HJD, Singh A, Ward OP. Microbiology and Molecular Biology Review 2003; 67(4):503-549.
[51] Jain PK, Gupta VK, Pathak H, Lowry M, Jaroli DP. World Journal of Microbiology and Biotechnology 2010; 26:1419-1426.
[52] Nweke CO, Okpokwasili GC. African Journal of Biotechnology 2003; 2(9): 293-295.
[53] Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. Molecular Biology Evolution 2011; 28(10):2731-1739.