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MORPHOLOGICAL AND MOLECULAR CHARACTERIZATION OF HYDROCARBON DEGRADING BACTERIA AND ITS ASSOCIATED GENES

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Abstract:

Objectives: To isolate the effective hydrocarbon degrading bacterial species from hydrocarbon contaminated site and analyse the efficacy of isolated hydrocarbon degrading bacterial species and their degrading sequence.

Methods:

The cultures were morphologically and biochemically identified by staining and biochemical tests. The diesel dependent growths of these isolates were assessed for 15 days by monitoring the gradient fluxes in the pH and Optical density OD of the media. Evolutionary relationship was demonstrated and rendered with phytogenetic tree using BLAST tree tool.

Results: Based on their morphological, physiological and biochemical traits, strains DC1- DC7 belong to *Flavobacterium sp*., *Citrobacter freundi*, *Citrobacter intermedius*, *Enterobacter aerogenes*, *Bacillus cereus*genera, respectively. Results showed an increase in OD as well as fluctuations in pH values. Microorganisms, capable of utilizing hydrocarbon as sole carbon source, were 16S rDNA analysis of the best degraders was found to belong to the Bacillus species. DC7 was identified as Bacillus cereus with 1414bp. The sequence shows 94% similarity for Bacillus thuringiensis and *Bacillus bombysepticus*, and 95% similarity for *Bacillus toyonensis BCT-7112*.

Conclusion: These findings demonstrated that the use of Hydrocarbon degrading bacteria along with nutrient supplements could revive hydrocarbon contaminated soil effectively in large scale. The use of native bacterial consortium with diesel utilizing capabilities as seed onto oil contaminated environment could prove a more

environmentally – friendly approach to bioremediation which would run enhance sustainable development rather than the use of exotic bacterial strains and chemicals.

Keywords: Biodegradation, Contaminated soil, Bacteria, Genomic DNA, and 16S rRNA.

Introduction

Bioremediation functions mainly on biodegradation, which may refer to complete mineralization of organic contaminants into carbon dioxide, water, inorganic compounds and cell protein or conversion of complex organic contaminants to other simpler organic compounds by biological agents like microorganisms. In addition, bioremediation technology is supposed to be non-invasive and moderately cost-effective. Indigenous oil consuming microorganisms, which have the ability to degrade organic compound play a significant role in the disappearance of oil from soil. This microbiological decontamination (bioremediation) of the oil-polluted soils is claimed to be a competent, economic and adaptable alternative to physiochemical treatments [1,2]. Hydrocarbon pollutants in contaminated soils can potentially be degraded by microbial activity. The potentiality of microbes as agents of degradation of several compounds thus indicates biological treatment as the major promising alternative to attenuate environmental impact caused by pollutants [3]. Microbial breakdown of hydrocarbon pollutants is generally a very slow process, but it could be optimized to enable the rate of microbial transformation proceed more rapidly. Optimum biodegradation can only occur if the right environmental conditions such as pH, temperature, nutrients and relevant microbial consortia are present. Conditions such as temperature and microbial composition cannot be influenced in real practical bioremediation situations except on ex-situ bioremediation programs.

Hydrocarbons are the world's most commonly used primary energy and fuel resources, due to the energy they produce. Apparently inevitable spillages, which follow during routine operations of crude oil production, refining, distribution and as a moment of acute accidents, have engendered continuous research interest in this field [4]. Oil spills have become a global problem in industrialized and developing countries. The amount of natural crude oil seepage was expected to be 600,000 metric tons per year with a range of uncertainty of 200,000 metrictons per year [5]. This study is an initial approach to evaluate the feasibility of applying a bioremediation process in the contaminated site. This study is an initial approach to evaluate the feasibility of applying a bioremediation process in the contaminated site and was to investigate possible methods to enhance the rate of biodegradation of hydrocarbon in soil.

Materials and Methods

Sample collection

The 200 gm of hydrocarbon polluted soil samples were collected (5-10 cm breath on surface) from different locations in and around Salem town. It was stored at 4^0C until using.

Isolation of microorganism from soil sample

The number of living microorganisms was estimated by viable count by serial spread plates. The colonies were counted using formula:

Population of microorganism present in 1gm of soil sample= Average no. of colonies x plate detection factor.

Identification of morphological and biochemical characteristics of microorganism were staining and different biochemical test.

Isolation of hydrocarbon degrading bacteria

Isolation of Hydrocarbon Degrading 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).

Turbiodmetry measurement: Determination of Bacterial Biodegradative Activity by Turbidometry method was to determine the bacterial growth by utilizing the hydrocarbons (1% diesel) given as carbon source in MSM broth. 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 + microorganism without hydrocarbon (Diesel) as blank.

Antibiotic sensitivity test: Isolates were tested for its sensitivity to antibiotics by means of a disc diffusion method. The isolates were investigated using antibiotics disc containing amoxillin (25µg), cloxacillin (15µg), cephalexin (30µg), erythromycin (15µg), vancomycin (30µg) and kanamycin (15µg). The different zones of inhibition were measured to the nearest millimeter and interpreted as sensitive, moderate sensitive and resistant based on the interpretation table recommended by the disc manufacturer (Whong and Kwaga, 2008).

Analyses of 16s rRNA sequence

Genomic DNA Extracted from the Hyrdrocarbon degrading bacteria with a GenElute DNA extraction kit from Sigma.The 16S rRNA gene of isolate was amplified using theuniversal primer 8F (5′-AGAGTTTGATCCTGGCTCAG)

and 1541R(50AAGGAGGTGATCCAGCCGCA-3′) [23]. The gene sequences of the isolate obtained in this study were compared with known 16s rRNA gene sequences in the GenBank database. Phylogenetic Tree Analysis was constructed using BLAST tree tool.

Result: Isolation of microorganisms

The contaminated soil sample were serially diluted and plated on a nutrient agar plate using the spread plate technique. The results of the bacterial count show that Diesel, Kerosene and Crude oil contaminated soil had the colony count of $224x10^{-6}$ CFU/ml, $80x10^{-5}$ CFU/ml and $248x10^{-5}$ CFU/ml.(Table. 1) (Fig. 1)

Morphological and biochemical characterization of microorganisms

The isolated organisms from the Diesel (7), Kerosene (5) and Crude oil (5) contaminated soil were morphologically and biochemically characterized. **(7 Isolates from Diesel contaminated soil is taken for further studies).**

Morphological and biochemical characterization (diesel)

The morphological and biochemical characterization of microorganism isolated from diesel contaminated soil as follows. DC1 showed gram negative rod shaped, Positive for Methyl red, Voges prosakeur, Catalase, Oxidase, TSI, Citrate, Urease. Nitrate. Negative for indole, gelatin, and carbohydrate fermentation. DC2 showed Gram negative rod shaped, positive for Methyl red, Voges prosakeur, urease, TSI, Glucose, lactose, Negative for Urease, Catalase, indole, Oxidase, Nitrate, and Fructose. DC3,DC4, showed gram negative rod shaped, positive for indole, citrate, urease, catalase, oxidase, TSI, fructose. Negative for Methyl red, Voges prosakeur, nitrate, glucose, lactose. DC5 and DC6 showed gram negative rod shaped, positive for citrate, urease, catalase, oxidase, TSI, glucose, lactose and fructose, negative for indole, Methyl red, Voges prosakeur, nitrate. DC7showed gram positive rod shaped, positive for, Methyl red, urease, catalase, TSI, oxidase, and carbohydrate fermentation Negative for indole, Voges prosakeur, citrate, nitrate.(Table 2) (Fig. 2,3,4,5)

Hydrocarbon by Turbidometry

The Table 4 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 gives the degrading activity on hydrocarbons by bacteria. The results demonstrated that Controls have 30% ability to degrade diesel. The results showed that all the organisms utilized maximum diesel as a substrate 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 was DC3 and DC4 which shows 57% ability to degrade Diesel. DC2, DC5, DC6 shows 72% degrading ability. DC1 shows 70% degrading ability, while DC7 shows greatest ability of 98 % to degrade Diesel. These degrading capabilities on hydrocarbons revealed that the microorganisms DC 1 to 7 isolated from the soil 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 soil 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 takes place between days 5 and 15. (Fig. 7)

Hydrocarbon degradation by pH value for 15 days of incubation

The pH during the degradation by Minimal Salt medium using turbidometry method showed the different pH values of the inoculated medium of isolates from soil, with hydrocarbon (diesel) over the 15 days period. The pH difference between day 5 and day 13 was at a high rate, this implies that the degradation probably occurred during these days while the rise in pH slightly reduced between day 14 and 15. This is probably because the microorganisms had utilized the hydrocarbon substrate in the culture medium, hence reducing the acid produced. Table 5 &Fig. 8 shows graph of pH of isolates from soil during the 15 day course degradation. *Bacillus cereus* (DC7) obviously having the highest value at the end of the growth period followed by *Enterobacter aerogenes* (DC5 and DC 6) and *Citrobacter freundii* (DC2) and *Flavobacterium* (DC1) respectively. *Citrobacter intermedius* (DC3,4) has the least ability to degrade Diesel.

Antibiotic susceptibility of *B. cereus* (DC 7) isolated from soil sample contaminated with Diesel

The antibiotic susceptibility of *B. cereus* (DC7) isolated from contaminated soil sample was summarized in table 6. It was found that the isolate exhibited resistance to most of the tested antibiotics. Where the isolates were resisting to amoxillin, cloxacillin and cephalexin, also it was sensitive to erythromycin, vancomycin and kanamycin. (Fig .9)

16s rRNA sequencing

Since *Bacillus cereus* has a highly degrading potential on diesel. It was identified by 16s rRNA sequencing, and identified as *Bacillus cereus* gram positive bacteria with 1414bp. (Fig.10). For the 16s rRNA gene sequence, highly homologous sequences were identified by BLASTIN results and were downloaded and phylogenetic tree was

constructed. The sequence shows 94% similarity for Bacillus thuringiensis and *Bacillus bombysepticus*, and 95% similarity for *Bacillus toyonensis BCT-7112*. (Fig.11).

Discussion:

The ability of the microorganism to degrade the diesel isolated from the diesel contaminated soil was studied. The organisms isolated from study (contaminated Soil) in this study have been confirmed to have hydrocarbon degrading abilities. Whereas, organic materials serve as a source of nutrition for some bacterial species, it could be toxic to other species. The number of living microorganisms was estimated by viable count on serial spread plates. In which the microbial count of DS1 and CS1 had the highest count (224×10^5cfu / ml, 248×10^{-5}cfu/ml). KS1 had the least bacterial count (80×10^{-5}cfu/ml). This is because not all microorganisms can utilize hydrocarbon as substrate for growth. Furthermore, the presence of hydrocarbon can inhibit the growth of many microorganisms.

Morphological (colony & cell morphology) and Biochemical tests like indole, methyl red, vp, citrate,oxidase, catalase, nitrate, urease production, TSI, carbohydrate fermentation test were analysed .The bacteria were identified by Bergey's Manual of Determinative Bacteriology (7th Edition) as a reference. Based on these test the 7 isolates were identified and included for further analysis.

The oil degrading bacteria has adopted to grow in high salt environments [6]. A study by Malkawi *et al.*, 2009identified effective oil degrading microorganism by UV irradiation onto hydrocarbon key enzyme (Catechol 2,3 dioxygenase and monooxgenase) [7]. The radiated bacterial organisms were *Pseudomonas aeruginosa*, *Pseudomonas putida*, *Pseudomonas mallei* and *Moraxella sp.* In which *Pseudomonas mallei* showed an increased monoxygenase activity.

The maximum oil degradation ability was gram positive *Bacillus* sp. are catalase positive. Few studies have been reported on the roles of *Bacillus sp*.in hydrocarbon bioremediation; although there are several reports on bioremediation of pollutants by the action of *Bacillus sp*. occurring in extreme environments [8,9,10,11,12]. Ijah and Antai reported *Bacillus sp*. as being the predominant isolate of all the crude oil utilizing bacteria characterized from highly polluted soil samples (30 and 40% crude oil) [9].

Based on their capabilities of microbes to grow on crude oil, kerosene and Diesel and/or individual hydrocarbons as their sole carbon source, 5 bacterial isolates were isolated in Diesel, Kerosene, Crude oil contaminated soil of which organism isolated from diesel were taken for further studies which shows maximum concentration of degradation of hydrocarbon.

Each of these isolates was selected based on the criteria that they were able to display good growth in crude oil, Kerosene and Diesel and on individual hydrocarbon compounds or both.

The growth dynamics was determined by the optical densities. The utilization of the hydrocarbons resulted in increase in cell densities with a visual gradual reduction in hydrocarbon. Interestingly, least lag phase was observed in the growth profiles of all the organisms used for the degradation experiments. This could be explained by the fact that the organisms have a previous exposure to hydrocarbon present in the soil and hence have developed enzymes capable of degrading hydrocarbon.

The optical density readings based on the turbidity of the minimal salt medium at regular intervals of 2 days show the degradation activities of the bacteria on hydrocarbons. The level of utilization differs between the bacterial species. The gradual increase in the concentration of the broth (turbidity) indicates bacterial growth, hence the degradation of hydrocarbons mostly between days 5 and 15. The increase in cell densities as a result of hydrocarbon utilization showed a gradual and visible disappearance of hydrocarbon and gradual decline in the turbidity of the broth suggests decrease in the bacterial population indicating that the hydrocarbon has been degraded.

From Table 4 and fig. 7, substrate specificity of *Bacillus cereus*. appeared to be maximum restricted to the hydrocarbon compounds, while the *Enterobacter aerogenes* shows Narrow range on degrading hydrocarbon and *Citrobacter intermedius*, *Citrobacter freundii*, and *Flavobacterium sp.*,shows moderate growth on a variety of hydrocarbon groups tested in this study. The organisms were able to utilize the available nutrients, and grew steadily from days 5 to 15. In addition, the pH of the culture media remained acidic, within the range of 5.36 – 7.76, as shown in the results. The difference between the pH values was highest between days 5 and 12, indicating that the rate of biodegradation was highest between these days. Microbial degradation of hydrocarbons often leads to production of organic acids and other metabolic products [13]. The initial pH of the culture medium was adjusted to 5.30, which eventually increased above 6 for all the organisms. This indicated the production of weak acids. In a study by Akvopetva *et al.*, 2011the crude oil contaminated soil has reduced pH in comparing to the normal soil [14]. The reduction in pH was due to the increased in acidity which makes a problem in agricultural soils because of metal cations are more soluble and available in the soil solution [15]. *B.cereus* had shown the sensitivity against three organic solvents, like erythromycin, vancomycin, and kanamycin and highly resistance for three classes of antibiotics namely amoxycilin, cloxacillin and cephalexin. This may

be due to the selective emergence of isolates from diesel contaminated environment in the presence of high concentration of hyrocarbons. Many investigators have been reported that the association between heavy metals and antibiotic resistance [16,17,18]. The bacteria which subjected to organic solvent can exhibit high antibiotic and heavy metals resistance due to the siphon out antimicrobials by efflux pump [19]. The majority of isolated strains were able to degrade different aromatic and aliphatic hydrocarbons also exhibited multiple resistances against antimicrobial agents [20].

The availability of molecular techniques for fast and reliable genotypic characterization should increase our knowledge of ecology, structure and dynamics of microbial communities in contaminated ecosystems. Documentation of microbial diversity at diesel-impacted sites will help to formulate novel strategies for efficient and effective reclamation of contaminated sites. From this highly degrading diesel strain (DC7) was 16srRNA sequenced and identified the organism. DC7 was identified as Bacillus cereus with 1414bp. The sequence shows 94% similarity for Bacillus thuringiensis and *Bacillus bombysepticus,* and 95% similarity for *Bacillus toyonensis BCT-7112*.

Diesel degrading microorganism from the diesel polluted region of Iranian, the 16s RNA sequence strain has the close relationship *Bacillus cereus* and *Bacillus thurigenesis* [21]. Bacterial 16S-rRNA is a common target for taxonomic purposes and identification, largely due to the mosaic composition of phylogenetically conserved and variable regions within the gene [22,23]. The seven different species like *Micrococcus, Pseudomonas, Flavobacterium, Serratia, Moraxella, Bacillus* and *Klebsiell*s has a potential hydrocarbon degrading organism which utilizes hydrocarbon has a sole carbon source for their growth was identified from hydrocarbon contaminated soil collected in Mexico [24]. Some of the researchers have reported that degradation of soil bacteria ranges from 0.13 [25] to 50% [26], and marine bacteria ranges from (0.003%) [27] to 100% [28]. *Bacillus Sp.,* was effective hydrocarbon degradation [29,30,31,32,33,34]. *Bacillus Sp*., identified from hydrocarbon contaminated soils has a potential to degrade benzene, crude, decanol, ethyl-benzene, n-tetradecanol and xylene [35]. 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) [36]. *Pseudomonas* strain has the ability to degrade the crude oil in in-vitro condition, it can degrade alkanes (70.69%) and aromatics (43.37%) [37]. A total of 24 bacterial strains from 14 genera were isolated from oiled beach sands and confirmed as oil-degrading microorganisms. Isolated bacterial strains were primarily Gammaproteo bacteria, including

representatives of genera with known oil degraders (*Alcanivorax, Marinobacter, Pseudomonas,* and *Acinetobacter*) [38]. *Pseudomonas sp*., *Micrococcus sp*. and mixed consortium of this has been used has bioremediation of diesel oil [39]. *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 [40].

Conclusion

Hydrocarbon based fuels are one of the most prevalent pollutants particularly in industrialized and developing countries. Isolate DC7 was one of the potential microorganism for bioremediation of diesel contaminated sites. This efficiently degrading strain was characterized by 16s RNA sequencing and confirmed as *bacillus cereus.* The use of native bacterial consortium with diesel utilizing capabilities as seed onto oil contaminated environment could prove a more environmentally-friendly approach to bioremediation which would run enhance sustainable development rather than the use of exotic bacterial strains and chemicals. This study reveals that bacterial species isolated from contaminated soil can be harnessed in an attempt at developing strains that will be useful in environmental bioremediation of contaminated sites.

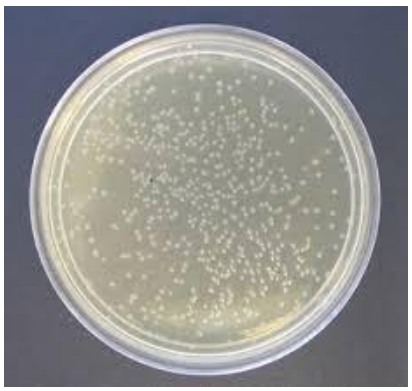


Fig. 1: Colony Count by Spread Plate method (DS1 -

Fig. 2: DC7 Gram negative rod

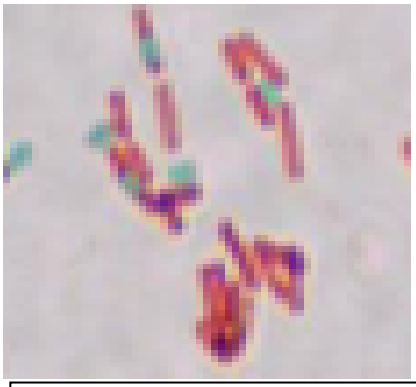


Fig. 3:DC7 Spore Staining

Fig. 4: DC7 Catalase Test

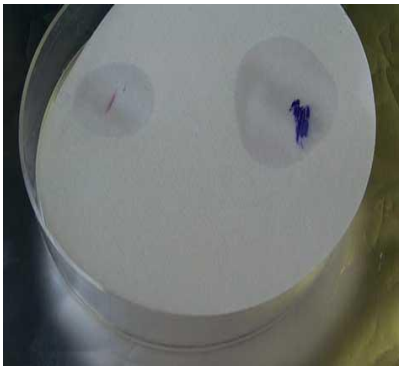


Fig. 5: MR VP Test (+), IndoleTest (-), Urea Test(+)

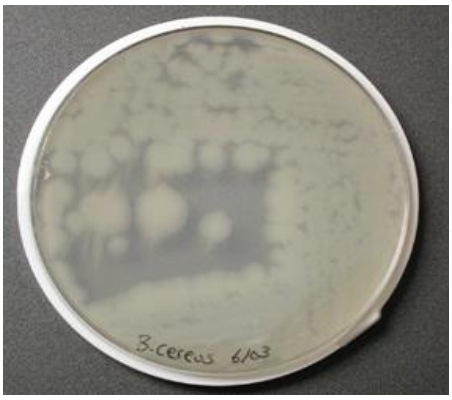
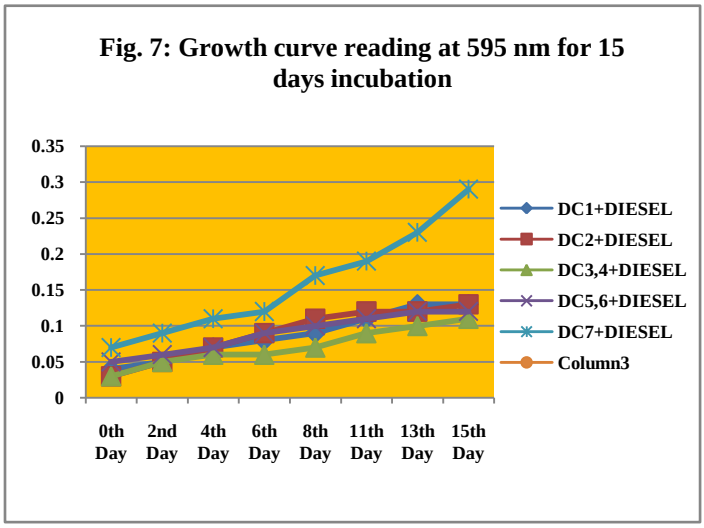
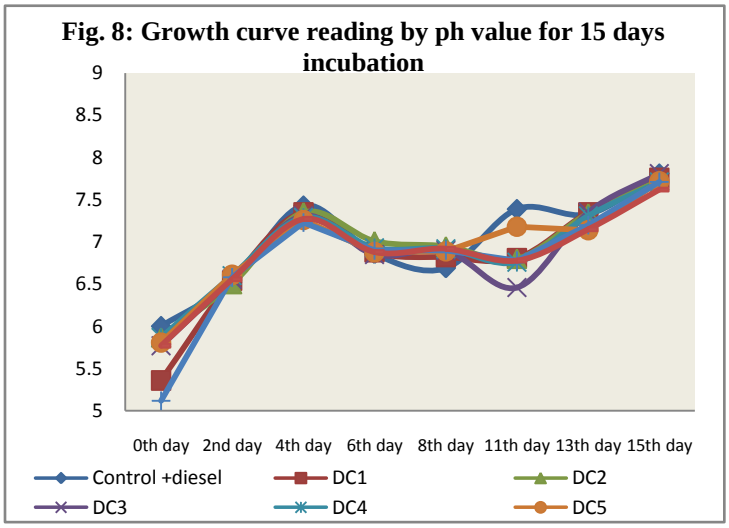


Fig. 6: Bacillus cereus (DC 7) growth on MSM





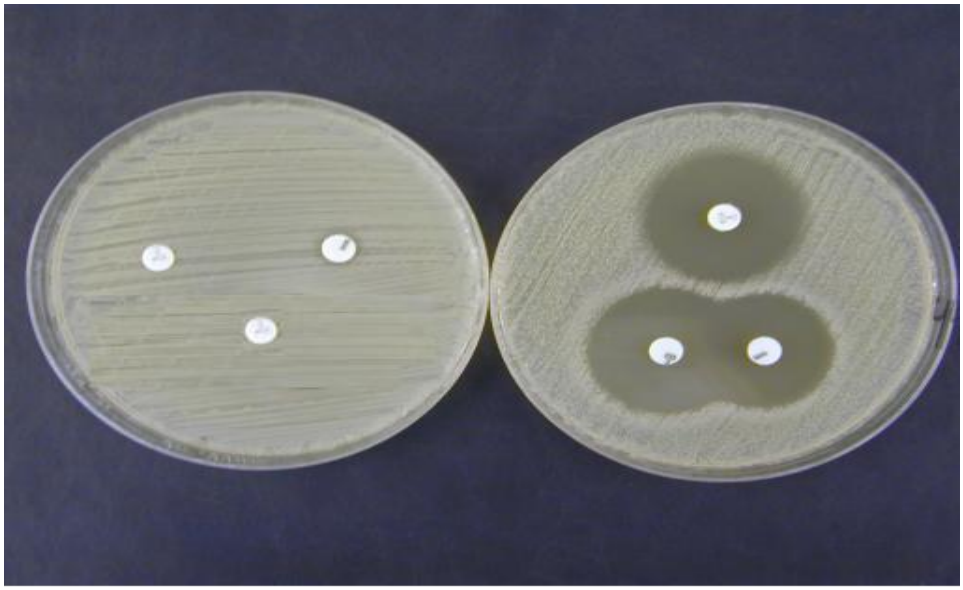


Fig. 9: *Antibiotic* sensitivity test

PLATE -1 SHOWS SENSITIVE FOR ERTHROMYCIN (15 µg),VANCOMYCIN (30 µg) AND KANAMYCIN (15 µg)

PLATE-2 SHOWS RESISTANCE FOR AMOXYCILLIN (25 µg), CLOXACILLIN (15 µg) AND CEPHALEXIN (30 µg)

Fig. 10: 16 s rRNA sequencing analysis for DC7

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>130520-14_E19_K_518F.ab1  1414
CGGGAAAATTATTGGGGCGTAAAGCGCGCGCAGGTGGTTTTTTACAGTCT
GGATGTGAAATGCCCAGGGGCTCAACTGCGGGGCGCCTAGGGAACGCGGC
GACTCGAGTGGCAAGGACGGCGGCGGATATCGAGGGGATGCGGCGAAAC
CGGAGAGATATGGAGGAACACCAGTGGCGAAGGCGACTTTCTGGTCTGTA
ACTGACACTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCC
GGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGAGGGTTTCCGCC
CTTTAGTGCTGAAGTTAACGCATTAAGCACTCCGCCTGGGGAGTACGGCC
GCAAGGCTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGA
CATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACAT
CCTCTGAAAACCCTAGAGATAGGGCTTCTCCTTCGGGAGCAGAGTGACAG
GTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCC
CGCAACGAGCGCAACCCTTGATCTTAGTTGCCATCATTAAGTTGGGCACT
CTAAGGTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAA
TCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGACGGTA
CAAAGAGCTGCAAGACCGCGAGGTGGAGCTAATCTCATAAAACCGTTCTC
AGTTCGGATTGTAAGCTGCAACTCGCCTACATGAAGCTGGAATCGCTAGT
AATCGCGGATCAGCATGCCGCCGGTGAATACGTTTCCGAGGCCTTGTACA
CACCGCCCGTCACACCACGAGAGTTTGTAACACCAGAAGTCGGTGGGGTA
ACCTTTGTGGACCCAACCGCCTAAAGTGAGACAGATGATTGGGGTGAATT
CGCACAAAGTATCCGATCGGAGGTGCGCTGAAACACTCCTAAGCGGGCTC
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TTTCCCCAAATTTTTTCTTAATATGTTCCAAATAGGGGGTCTTATTATTT
TTTATAAAAAAACACGGGTGGCCCAAAAGTGTGCCTTGACAGGGGTGGGC
ACACATCGAGAAATCCACACGGTGGGGCTCCACACATATGGGGGGTAGTA
AGCACAGTTTTTTAAAGGAAAAAGGAAACCCCCCCCCCCCGGGGTGACTC
CGCTTAACTATTCGGATGGGGGTTGACACACACACACAAAACAAAAAACAGA
TATTTCCACGAAATATACTAACACACAGCAGACGAGTGAGACATTAATAC
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Fig. 11: Blast tree rendering results for dc7.

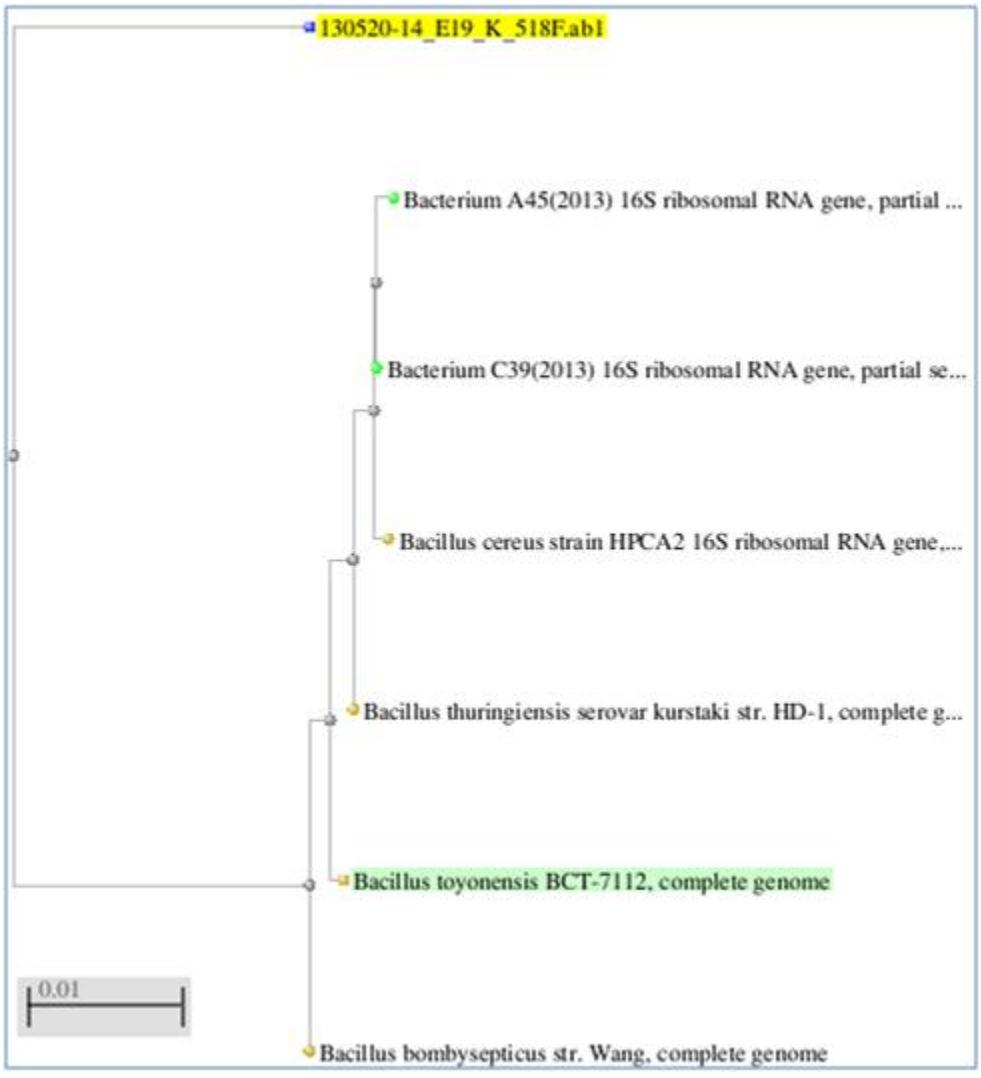

Table-1: Colony counting.

S.NO	SAMPLE	COLONIES	TOTAL PLATE COUNT(Cfu/ml)
1	*DS1*	*56×4*	*224×10-6*
2	*KS1*	*20×4*	*80×10-5*
3	*CS1*	*62×4*	*248×10-5*

Table-2: Morphological and Biochemical characterization (Diesel).

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	-	-	+	+	+	-	+

Positive(+), Negative (-)

Table-3: List of identified of isolates.

S.NO	ISOLATED COLONIES	ORGANISMS
1	DC1	*Flavobacterium sp.,*
2	DC2	*Citrobacter freundi*
3	DC3,DC4	*Citrobacter intermedius*
4	DC5,DC6	*Enterobacter aerogenes*
5	DC7	*Bacillus cereus*

Table4: OD value showing the organism grown in diesel containing medium.

NAME OF THE ORGANISM	OD at 0 DAY	OD at 2^{ND} DAY	OD at 4^{TH} DAY	OD at 6^{TH} DAY	OD at 8^{TH}DAy	OD at 11^{TH}DaY	OD at 13^{TH} DAY	OD at 15^{TH} DAY
Flavobacterium	*0.04*	*0.05*	*0.07*	*0.08*	*0.09*	*0.11*	*0.13*	*0.13*
Citrobacter freundi	*0.03*	*0.05*	*0.07*	*0.09*	*0.11*	*0.12*	*0.12*	*0.13*
Citrobacter intermedius	*0.03*	*0.05*	*0.06*	*0.06*	*0.07*	*0.09*	*0.1*	*0.11*
Enterobacter aerogenes	*0.05*	*0.06*	*0.07*	*0.09*	*0.1*	*0.11*	*0.12*	*0.12*
Bacillus cereus	*0.07*	*0.09*	*0.11*	*0.12*	*0.17*	*0.19*	*0.23*	*0.29*

Table 5: Degradation of pH value for 15 days of incubation

ORGANISMS	**pH VALUE**							
	0 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

Table 6: Antibiotic susceptibility of *B. cereus* (DC7) isolated from soil sample contaminated with Diesel

Antibiotic (conc. µg/ disc)	***B. cereus* state of susceptibility**
AMOXILIN (25)	R
VANCOMYCIN (30)	S
TRIMETHOPRIM (15)	R
KANAMYCIN (15)	S
CEPHALEXIN (30)	R
CLOXACILLIN(15)	R

R = RESISTANCE, S= SENSITIVE

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