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Biodegradation of Petroleum Oil by a Novel *Bacillus cereus* Strain DRDU1 from an Automobile Engine

Borah, D.* and Yadav, R. N. S.

Centre for Studies in Biotechnology, Dibrugarh University, Dibrugarh-786004, India

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ABSTRACT: Contamination of soil and air due to hydrocarbon is a global issue and bioremediation provides probably the best way to remediate the contaminants. The current study shows the biodegradation of crude oil, diesel and used engine oil by a newly isolated *Bacillus cereus* strain DRDU1 from an automobile engine. Hydrocarbon degrading strains were screened on BH (Bushnell and Haas broth) agar supplemented with 2% (*v/v*) of used engine oil as sole carbon source. The strain was found to be degrading 77%, 67%, and 16% of diesel, crude oil, and used engine oil respectively after 28 days under nitrogen and phosphorus stressed condition. It was increased significantly up to 99%, 84% and 29% in presence of nitrate and phosphate supplements. Degradation was confirmed both gravimetrically and by gas liquid chromatographic analysis. The strain proves itself a stress tolerating bacteria by withstanding 7% of salinity, 37% of glucose concentration and 52% of relative humidity. The thermal death point of the strain was found to be 86°C. The significance of the study is that the percentage degradation of the complex petroleum supplements used in the study was found to be far higher than some of the previously reported values.

Key words: *Bacillus cereus* strain DRDU1, Complex hydrocarbon, Degradation of complex petroleum oil

INTRODUCTION

Nowadays deliberate use of petroleum hydrocarbon products, such as diesel and engine oil increases the chance of soil pollution and gradually it is proving itself as a major environmental problem (NRC, 1985). The spillage also has severe health-related impacts on human and aquatic animals. It may cause severe risks to the workers associated with the cleaning up of oil spillage areas when exposed to oil fumes, volatile organic compounds (VOCs) (Bach, *et al*., 2005; Biddle, *et al*., 2006; Campbell and Cary, 2001), polycylic aromatic hydrocarbons (PAHs) (Chang, *et al*., 2002; Chang, *et al*., 2005), particulate matter from controlled burns, and heavy metals (NRC, 1985). Chemically crude oil contains paraffin (15-60%), naphthalene (30-60%), aromatics (3-30%) and asphaltic (6%), but the relative percentage of each varies from oil to oil (Mabro, 2006). Though the major constituents of crude oil may vary, but the percentage composition of the constituents may be given as, carbon (83-87%), nitrogen (0.1-2%), hydrogen (10-14%), oxygen (0.05-1.5%), sulphur (0.05-6%) and metals (<0.1%) (Hyne, 2001). Petroleum oil and PAH (poly aromatic hydrocarbon) has a wide spread effect on human body, as prolonged exposure to petroleum oil may induce liver and kidney diseases, bone marrow damage, or may lead to the development of cancer (Mandri, 2007).

Microbial bioremediation of hydrocarbon contaminated soil and water has emerged as a promising technology in recent years (Mandri, 2007). Most of the study has shown *Pseudomonas aeruginosa*, *Pseudomonas putida*, *Acinetobacter spp*., *Flavobacterium* spp., *Yokenella* spp., *Alcaligenes* spp., *Roseomonas* spp., *Sphingo-bacterium* spp., *Capnocytophaga* spp., *Moraxella* spp., *Corynebacterium* spp., *Streptococcus* spp., *Providencia* spp., etc. as common hydrocarbon degraders (Mandri, 2007; Juwarkar, 2012; Etkin, 1998). Biodegradation of complex hydrocarbons, naphthalene and pyrene with the help of *Bacillus spp.*, has been shown in many literatures and the degradation was found to be ranging from 20 to 60% (Ghazali *et al*., 2004; Das & Mukherjee, 2007; Bujang *et al*., 2013). Mukherjee and Bordoloi, 2012; has evaluated the degradation patterns of benzene, toluene, and xylene with the help of a consortium of *Bacillus subtilis* and *Pseudomonas aeruginosa*. The study was carried out both in presence and absence of external nitrate and phosphate supplements. Nwaogu *et al*., 2008; studied the biodegradation of diesel oil by *B. cereus* and it

*Corresponding author E-mail: dborah89@gmail.com

was found to be degrading upto 80% of diesel oil after 28 days of incubation at 30°C. But so far no report has been found studying the growth of *Bacillus cereus* in hydrocarbon media in presence and absence of external N and P supplements in detail.

The present study was initiated to assess and to compare the degradation of crude oil, diesel and used engine oil in liquid media by *Bacillus cereus* strain DRDU1 (isolated from automobile engine) in presence and absence of N and P supplements. Further, bacterial growth was evaluated in various stressed conditions (relative humidity, salinity, glucose concentration) in addition to its thermal death point (TDP).

MATERIALS & METHODS

Crude oil, used engine oil, and diesel oil used in the study were procured from Research and Development Laboratory of Oil India Limited, Duliajan, Assam. Mineral salts and other chemicals were purchased from Merck India Ltd. and all the media used in the study were purchased from HiMedia India Pvt. Ltd. Engine oil marketed by Honda India (P) Ltd. has been used during the study. Hydrocarbon residues from various parts of automobile engine (from the vehicles that are currently in use) were collected and inoculated on BH agar plates (composition g l^{-1}: $MgSO_4$-0.2, $CaCl_2$-0.02, KH_2PO_4-1.0, K_2HPO_4-1.0, NH_4NO_3-1.0, $FeCl_3$-0.05, agar-agar-20.0, pH-7.0 at 25°C) and incubated (Bacteriological incubator, Sciegenics Biotech India (P) Ltd) at 37°C for 36 hrs. Bacterial isolates obtained were subsequently sub cultured twice on Bushnell and Haas (BH) agar plates spread with 200µL (±2.0 µL) used engine oil to obtain pure colonies of hydrocarbon degrading microorganisms. Pure strains obtained were inoculated in 100 ml BH broth supplemented with 2% (*v/v*) (2000±2.0 µL) used engine oil as sole carbon source. These were kept in 250 ml air tight Erlenmeyer flask to minimize the evaporation of hydrocarbon supplements. The flasks were allowed to incubate at 37°C for 5 days at 135 rpm on rotary shaker (CERTOMAT®BS-1 shaker incubator, Sartorious Germany Ltd.). The bacterial isolates were identified on the basis of various staining techniques and their biochemical characteristics prescribed by Bergey's Manual of Systematic Bacteriology and finally by 16S rDNA sequencing.

DNA was isolated from the best potential bacterial isolate. Its quality was evaluated on 1.2% Agarose Gel, a single band of high-molecular weight DNA has been observed. Fragment of 16S rDNA was amplified by PCR from the above isolated DNA. A single discrete PCR amplicon band of 1500 bp was observed when resolved on Agarose Gel. The PCR amplicon was purified to remove contaminants. Fragment of 16S rDNA was amplified. Forward and reverse DNA sequencing reaction of PCR amplicon was carried out with 8F (52 - AGAGTTTGATCCTGGCTCAG-32) and 1492R (52 - GGTTACCTTGTTACGACTT-32) primers using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer. Consensus sequence of 1262 bp 16S rDNA was generated from forward and reverse sequence data using aligner software. The 16S rDNA sequence was used to carry out BLAST with the nr database of NCBI genbank database. Based on maximum identity score first ten sequences were selected and aligned using multiple alignment software program Clustal W™. Distance matrix was generated using RDP database and the phylogenetic tree was constructed using MEGA 4. The 16S rDNA sequence obtained was submitted to GenBank. The evolutionary history was inferred using the Neighbor-Joining method (Saitou And Nei, 1987). The evolutionary distances were computed using the Kimura 2-parameter method (Felsenstein, 1985). Phylogenetic analyses were conducted by using MEGA4 software (Kimura, 1980; Tamura *et al.*, 2007). The potential isolate was inoculated in 100mL BH broth (N and P supplements were added or eliminated as per the requirements) in 250 mL air tight Erlenmeyer flask, supplemented with 2 % (v/v) used engine oil, crude oil, and diesel separately. The growth of the isolate was evaluated both in presence and absence of nitrate (1 g NH_4NO_3 l^{-1}, *v/v*) and phosphate (1 g KH_2PO_4 l^{-1}, *v/v*). 250 mL air tight Erlenmeyer flasks devoid of inoculums maintained at same condition were used as control for each case. Each experiment was performed in triplicate. The colony forming unit (CFU) and the protein content were monitored in every 7 days till 28 days of incubation at 37°C and 135 rpm. The cell pellet obtained after centrifuging the broth at 8000 x *g* for 10 min (Sigma Germany was re suspended in 1 mL of distilled water and sonicated (Sartorius Stedim Labsonic, Germany Ltd.) for 10 sec at 100 % amplitude for one cycle to lyse the cells. Protein content was determined by the method described by Lowry *et al.* 1951.

The remaining oils from each isolate were extracted using n-hexane (HPLC grade), then the dry weight was determined and the oil degradability was calculated based on the weight loss as follows (Shirai *et al*., 1995):

(%) oil degradation =

$$\frac{\text{weight of the oil in negative control} - \text{weight of the oil in sample}}{\text{weight of the oil in negative control}} \times 100$$

Hydrocarbon degradation was further confirmed by GLC (gas liquid chromatography) analysis.

The effect of relative humidity on the growth of the isolates was analyzed by preparing relative

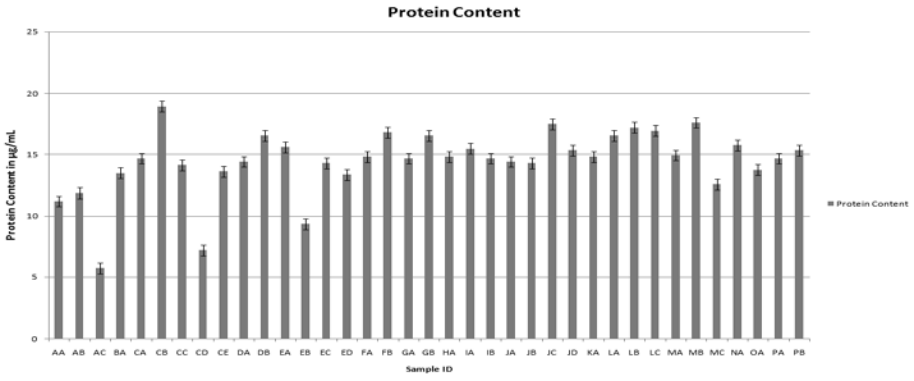
humidity chambers using saturated solutions of chemicals. Where $CaSO_4.5H_2O$ gives 98% R.H., KH_2PO_4 gives 96.6% R.H, $Na_2HPO_4.12H_2O$ gives 95% R.H., $NH_4H_2PO_4$ gives 93% R.H., $ZnSO_4$ gives 88.5% R.H., KCl gives 85% R.H., NH_4Cl/KBr gives 79% R.H., NaCl gives 76% R.H. and $CaNO_3.4H_2O$ gives 52% (Aneja, 2010). The isolates were incubated at 37°C for 3-5 days on separate nutrient agar plates containing 0.5% to 50% glucose for the determination of the effect of osmotic pressure on the bacterial isolates. The effect of saline on the isolates was determined by growing the isolate in nutrient agar plates containing NaCl (concentration ranging from 1 to 20%). The thermal death point (TDP) of the isolate was also determined. Student's *t*-test was performed. Each experiment was performed in triplicate and results were presented in mean ± S.D.

RESULTS & DISCUSSION

The work presented was conducted for the isolation of hydrocarbon degrading bacteria from various parts of automobile engine, as it was expected that an automobile engine would provide comparatively unfavourable conditions for the growth of microbes. A total of 71 hydrocarbon degrading bacterial isolates were screened. These were isolated from hydrocarbon residues from various parts of a total of 25 automobile engines. Each isolate was provided an identification code (IDs), *viz*, AA, AB,...YD (Fig. 1a, b), prior to the identification of the best isolate. No fungal strain was obtained during the study. Survival of microorganisms in a medium supplemented with petroleum hydrocarbon after their inoculation is a key deciding factor in the rate of biodegradation of hydrocarbon (Ramos *et al*., 1991). The bacterial sample XI was found to be shown most promising isolate for further studies on the basis of protein content 20 µg/mL just after 5 days of incubation (Fig. 1a, b) in BH broth supplemented with used engine oil. An increase in protein content signifies the increase in cell number and utilization of hydrocarbon supplement as a sole carbon source by the isolates (Mandri and Lin, 2007).

Presence of protein after incubation in the media, where only used engine oil acts as a carbon source clearly indicates the cell division, proliferation and hydrocarbon degradation in the media. Isolate XI shows maximum protein content (Fig. 1 b), followed by the isolate CC (Fig. 1 a).

(a)



(b)

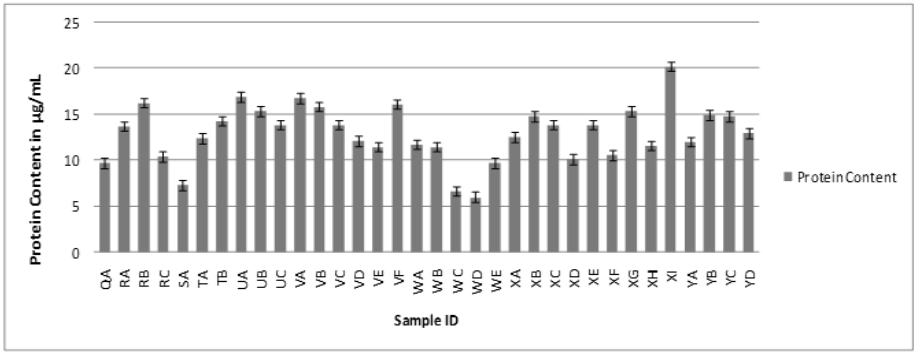


Fig. 1. protein contents (in µg/ml) of 71 bacterial isolates after 5 days of incubation in BH broth supplemented with 2% v/v used engine oil as sole carbon source. Sample IDs provided to the isolates and the protein content after incubation in the media has been shown on X and Y- axis respectively

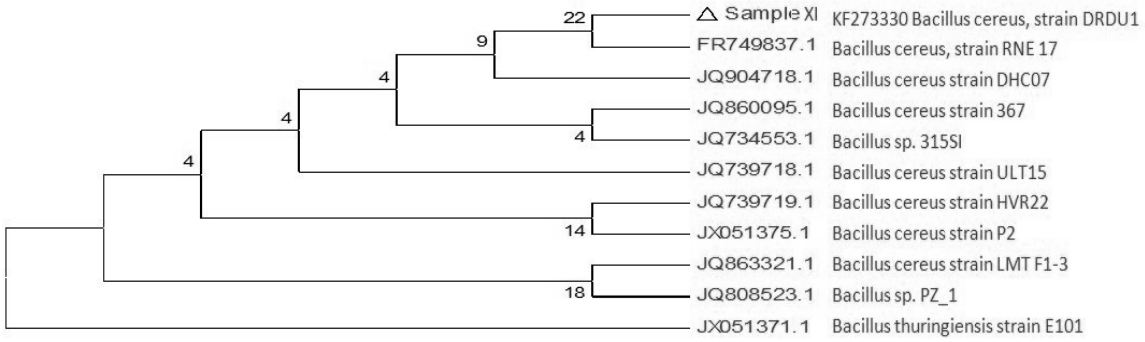


Fig. 2. Phylogenetic relationships of *Bacillus cereus* strain DRDU1 (sample XI, GenBank accession no. KF273330) and other closely related *Bacillus* species based on 16S rDNA sequencing. The tree was generated using the neighbour-joining method. The data set was resampled 1,000 times by using the bootstrap option, and percentage values are given at the nodes

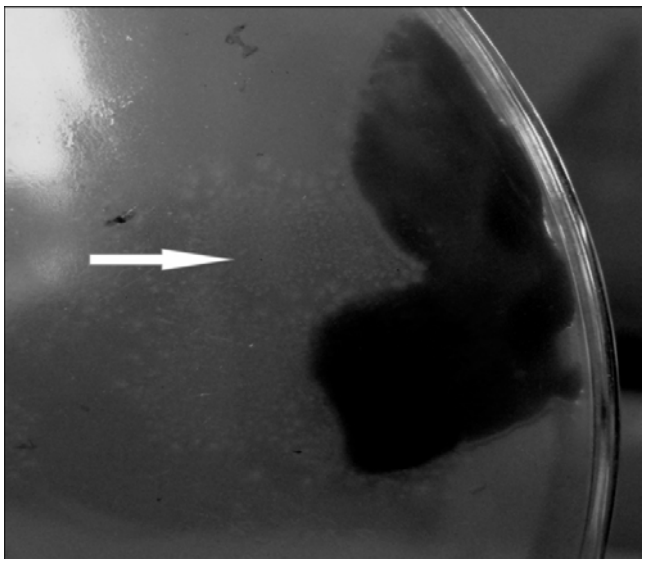


Fig. 3. Colonies of the newly isolated *Bacillus cereus* strain DRDU1 showing chemotexis towards hydrocarbon supplement (used engine oil) on BH agar plates

The potential isolate XI was identified as *Bacillus cereus* strain DRDU1 (GenBank Accession Number: KF273330) (Fig.2) based on biochemical characterizations and 16S rDNA sequencing. The strain was found to be fermenting sucrose, trehalose, Arabinose, glucose and mannitol. Sequence producing significant alignments and the optimal tree is shown (Fig. 2). The percentage of replicate trees in the bootstrap tests (500 replicates) was shown next to the branches (Saitou and Nei, 1987).

The bacterial isolate *Bacillus cereus* strain DRDU1 utilized crude oil, diesel and used engine oil as a sole source of carbon and energy. This was evident from the simultaneous increase in CFU and protein content in the medium after each 7 days of incubation till 28 days. The increase in the CFU count and the respective protein content with the increase in incubation time clearly indicates the hydrocarbon degradation by the potential isolate (Mandri and Lin, 2007; Das and Mukherjee, 2007). Colonies of the novel isolate *Bacillus cereus* strain DRDU1 showing chemotexis towards hydrocarbon supplement (used engine oil) on BH agar plates has been shown in Figure 3, and it also confirms the strain a potential hydrocarbon degrader.The maximum CFU count was found to be 7.47×10^9 at 2% (*v/v*) of diesel supplement in absence of N and P supplements after 28 days of incubation. It was found to be increasing upto 3.67×10^{10} in presence of N and P

Table 1. Detailed growth profile of the isolate *Bacillus cereus* strain DRDU1 in liquid medium containing 2% (v/v) hydrocarbon source and in presence and absence of N and P supplements (values are in Mean ± SD):

Name of the hydrocarbon supplemented (2%*v/v*)		cfu and protein content of the isolate in different hydrocarbon without P and N				cfu and protein content of the isolate in different hydrocarbon with P and N			
		1st week	2nd week	3rd week	4th week	1st week	2nd week	3rd week	4th week
Crude oil	cfu	(5±1)x10^4	(3.43±0.31) x10^6	(3.33±1.15) x10^8	(4.3±2.52) x10^5	(4.67±1.15) x10^5	(6.67±2.08)x10^5	(6.33±2.08) x10^8	(13.67±2.52) x10^8
	Protein content (µg/mL)	19.733±0.02	62.267±0.02	114.067±0.08	71.467±0.08	32.946±0.06	83.644±0.04	146.248±0.09	192.468±0.09
Diesel	cfu	(2.67±0.58) x10^5	(4.33±1.53) x10^5	(4±2)x10^8	(7.47±0.35) x10^9	(3.33±1.53) x10^5	(3±1)x10^6	(13±2)x10^8	(3.67±0.58) x10^{10}
	Protein content (µg/mL)	30.933±0.09	40.8±0.04	119.933±0.04	268.4±0.08	29.424±0.09	68.946±0.03	184.624±0.06	326.342±0.04
Used engine oil	cfu	(5.67±1.15) x10^4	(3.33±1.53) x10^5	(5.33±0.58) x10^8	(6±2.65)x10^6	(3±1)x10^5	(1.47±0.25) x10^6	(6±2)x10^8	(11.33±2.52) x10^8
	Protein content (µg/mL)	20.667±0.04	31.6±0.09	129.2±0.08	97.56±0.05	28.762±0.04	56.832±0.02	142.642±0.04	164.348±0.09

supplements (Table 1). The gradual increase in CFU count and the respective protein content has been observed in all the hydrocarbon supplements used. The detailed growth profile of the isolate in 2% (*v/v*) diesel, crude oil, kerosene and used engine oil has been shown in Table 1.

The strain was found to be degrading maximum 77%, 67%, and 16% of diesel, crude oil, kerosene and used engine oil respectively in absence of additional N and P sources in the medium containing 2% (v/v) hydrocarbon supplements. The degradation was increased upto 99%, 84% and 29% in addition of N and P supplements (Fig. 4).

The degradation was confirmed by both gravimetrically and also by GLC analysis (Fig. 5). The enhancement in the degradation may be explained by the fact that hydrocarbons exist in a reduced state and they are oxidized by microbes using electron acceptor. Nitrate possesses high oxidation potential for the removal of hydrocarbon contamination (Mukherjee and Bordoloi, 2012). Moreover additional N supplements acts as macronutrient for the synthesis of amino acids and nucleic acids for the rapid cell growth in the medium. Phosphorus on other hand helps in the synthesis of ATP and DNA.

The stress tolerant potential of the isolate was determined by growing the isolate at 37°C for 3-5 days on separate nutrient agar plates containing 0.5% to 50% glucose, 2% to 20% NaCl. It was done for the determination of the effect of osmotic pressure and salinity on the bacterial isolate *Bacillus cereus* strain DRDU1. The isolate was found to be tolerating 7.5% saline (moderately halophilic) and 37% sugar content, 52% RH. The strain was inoculated in nutrient broth maintained at temperature 40, 50, 60, 70, 80, 90 and 100°C for a duration of 10 min for the determination of its thermal death point (TDP). When no growth was observed at 90°C and above, the above procedure was repeated again from 80 to 90°C with an increase in 1°C for the determination of TDP of the isolate. The TDP of the isolate was found to be at 86°C. Hence the strain may be considered as a thermo tolerant strain.

The degradation potential and the cell growth were found to be decreasing with the increase in hydrocarbon supplements. This may be due to the increase in cytotoxicity in the medium with the increase in hydrocarbon supplements in the medium (Borah and Yadav, 2012; Borah and Yadav, 2014).

The current study showed detailed growth profile of *Bacillus cereus* (isolate XI) in hydrocarbon containing media, both in presence and absence of external N and P supplements. Also the isolate was found to be showing its potential to grow under stressed conditions such as less relative humidity (52%), higher osmotic pressure (37%) and moderate salinity (7%), with a TDP 86°C. These features may

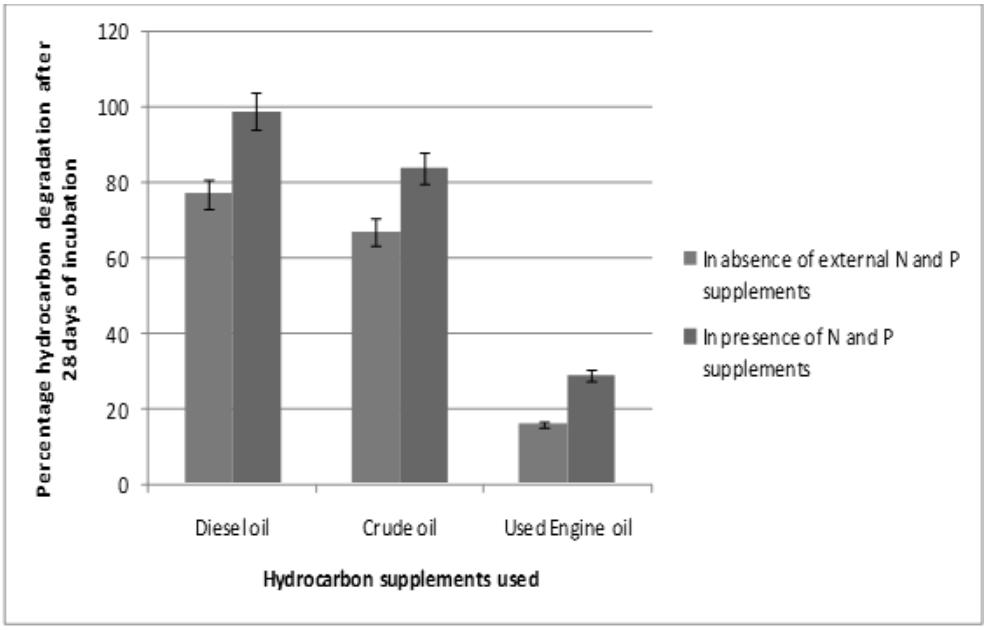


Fig. 4. Percentage hydrocarbon degradation after 28 days of incubation, both in presence and absence of N and P supplements

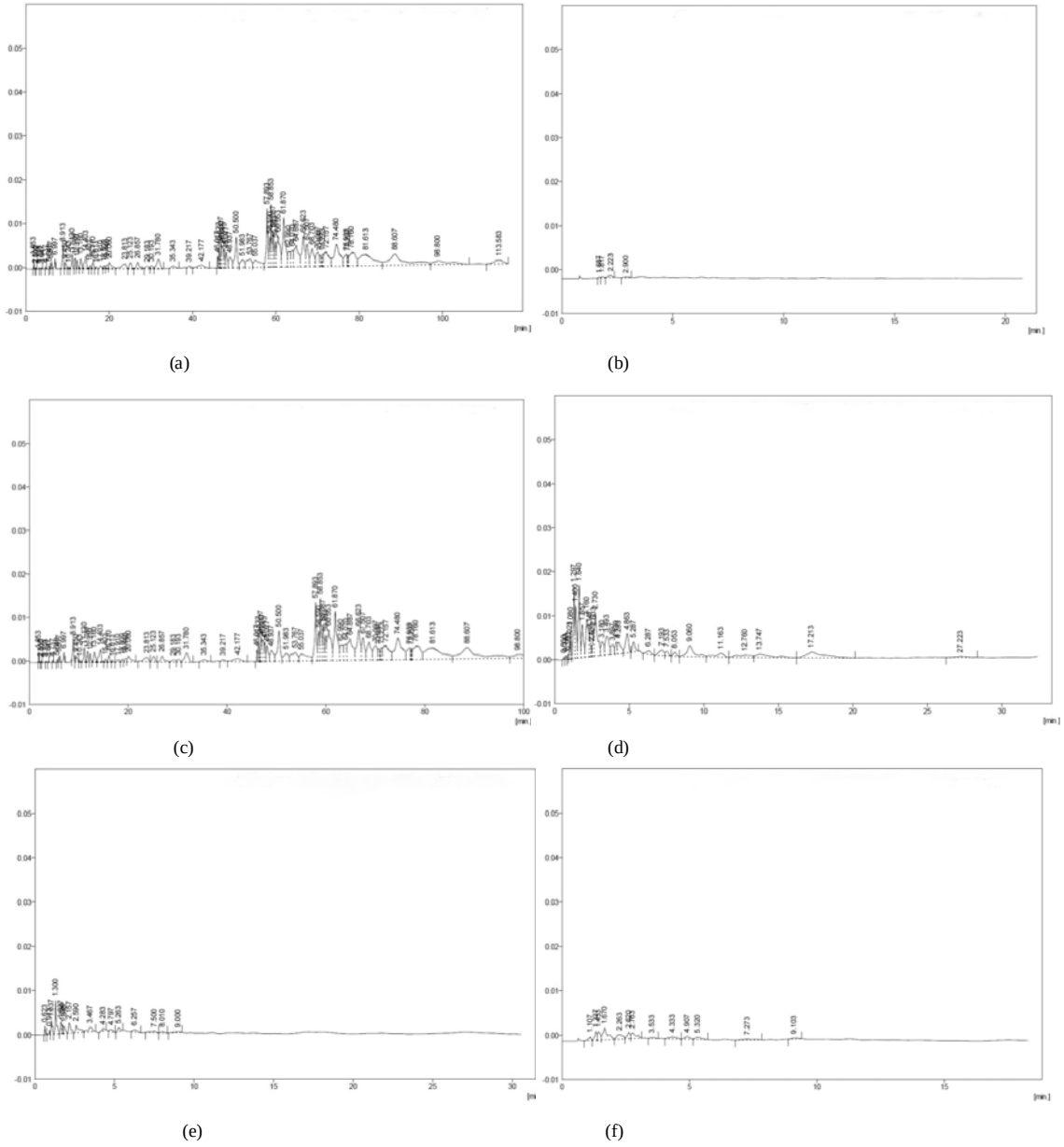


Fig. 5. Fig. a-b, c-d, and e-f shows GLC chromatogram of control sample for diesel oil, crude oil and used engine oil before and after degradation respectively.

play a key role for proving its survival in wide range of climatic conditions. Moreover, till date, no report is published on the detailed study of the growth of *Bacillus cereus* in liquid media supplemented with used engine oil, crude oil, and diesel oil both in presence and absence of N and P supplements. Therefore the current study had shown the newly isolated *Bacillus cereus* strain DRDU1 as a potential tool for bioremediation.

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