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FULL LENGTH ARTICLE

Bioremediation of petroleum based contaminants with biosurfactant produced by a newly isolated petroleum oil degrading bacterial strain

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Abstract Petroleum based hydrocarbon degrading and biosurfactant producing bacterial strain was isolated from an automobile engine. The strain was identified as *Bacillus cereus* DRDU1 on the basis of 16S rDNA sequencing analysis. The strain was found to be efficiently degrading 96% of kerosene making it a potential tool for bioremediation of petroleum based contaminants. Production and optimization of the biosurfactant produced by the isolate were also carried out. Surface hydrophobicity trait of isolate was found to be 60.67 ± 1.53% and foaming percentage of the crude biosurfactant was found to be 31.33 ± 0.58%. The presence of amino acids and sugar moieties in the biosurfactant was confirmed by biochemical tests and were further validated by FTIR (the Fourier transform infrared) spectrometric analysis revealing the presence of υ_{OH}, $\upsilon_{CO=O}$, υ_{COOH}, υ_{CH} (stretching), υ_{NH}, υ_{CH_2}, υ_{CH_3}, and υ_{CH} (bending), and $\upsilon_{C=O}$ (ester) in the surfactant. The decrease in contact angle of hydrocarbon oil from (30.67 ± 1.15)° to (21.3 ± 1.53)° respectively after 3 and 6 days of incubation reveals its potential to emulsify petroleum oil. Further, emulsification index (E_{24}) of biosurfactant against kerosene, crude oil, and used engine oil were determined to be 55.33 ± 1.53%, 29.67 ± 1.53%, and 20 ± 1% respectively which attracts its future application in MEOR (microbial enhanced oil recovery) process.

1. Introduction

The usage of petroleum hydrocarbon products increases the chances of soil contamination with diesel and used engine oil that becomes one of the major environmental problems. Statistical analysis, carried out by the International Tanker Owners Pollution Federation Ltd. (ITOPF) since the year 1970–2013, shows three oil spills of 700 tons or more occurred in year 2013 itself [1]. Large-scale marine oil spills, and oil spill accidents, have received great attention worldwide, due to their cataclysmic effect on the environment. Numerous physical and chemical techniques are practiced worldwide but bioremediation provides the most cost effective and eco-friendly measure for the remediation of petroleum contaminated soil and water to bring back its native environment. Although extensive

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research has been conducted on microbial bioremediation of oil contaminants during the last decade the effectiveness of stress tolerant hydrocarbon degrading microorganisms are less studied with the evidence of scientific publications [2–10]. Stress tolerant microbes are of great interest for their ability to survive in a wide range of geographic and climatic conditions. As it is known hydrocarbon residues are retained in various parts of automobile engines run on petroleum fuel, and release more or less converted form of hydrocarbon residues after combustion [10]. The circulating flow of lubricating oil can carry away a lot of heat and have the supplementary advantage of reducing the thermal stress on the part of automobile engine. Therefore, it is assumed that automobile engine facilitates the growth of stress tolerant hydrocarbon degrading microorganisms, which are capable of tolerating physical and nutritional stress. Earlier it was reported that a stress tolerant hydrocarbon degrading *Bacillus cereus* strain DRDU1 isolated from an automobile engine could degrade 77%, 67%, and 16% of diesel, crude oil and used engine oil respectively in the absence of external nitrate (N) and phosphate (P) supplement and the degradation rates were significantly increased up to 99%, 84% and 29% in the presence of N and P supplement [11]. The important requirement for the success of bioremediation is the existence of microorganisms with the suitable metabolic activities for the degradation of a specific type of petroleum oil. One of the most important features of hydrocarbon degrading bacteria is the ability to produce biosurfactants comprising various chemical structures, such as polysaccharides, fatty acids, glycolipids, peptides, and proteins with hydrophobic and hydrophilic moieties that diminish surface and interfacial tension between individual molecules making them potential candidates in emulsification and enhancing oil recovery [12].

Therefore, the study was extended for the production and characterization of biosurfactant produced by the isolate *B. cereus* strain DRDU1, which attracts its further application in microbial enhanced oil recovery (MEOR).

2. Materials and methods

2.1. Isolation, screening, and identification of hydrocarbon degrading microorganisms

Hydrocarbon degrading microorganisms were screened by spreading hydrocarbon residues from various parts of automobile engine on Bushnell and Haas (BH) media (composition g/L: $MgSO_4$-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) supplemented with 200 μL of used engine oil [11]. The best isolate was further screened on the basis of growth on BH broth supplemented with 2% (*v*/*v*) used engine oil as sole source of carbon. Best isolate was identified on the basis of 16S rDNA sequencing using:

Forward primer: 8F (5′-AGA-GTT-TGA-TCC-TGG-CTC-AG-3′) and

Reverse primer: 1492R (5′-GGT-TAC-CTT-GTT-ACG-ACT-T-3′)

BLAST was carried out with the consensus sequence hence generated and the phylogenetic analysis was carried out using MEGA-6 software by neighbor-joining method with 1000 bootstrap [13–15]. The evolutionary distance of the isolate with 10 most closely related strains on the basis of BLAST result was computed using the Kimura 2-parameter method by forcing *Bacillus amyloliquefaciens* ATCC-23350 as out group. The consensus sequence hence generated was deposited in NCBI GenBank database to receive the GenBank accession number [16].

2.2. Optimization of growth conditions

Hundred milliliter freshly prepared BH broth supplemented with 2% *v*/*v* petroleum oil (kerosene, crude oil, and used engine oil) was inoculated with 1 mL of overnight bacterial broth (O.D. $\geqslant$ 0.1) of the most potent isolate taken in separate air tight Erlenmeyer flasks. The optimum concentration of the salt components for the strain was determined by growing the isolate at different concentration of $MgSO_4$, $CaCl_2$, KH_2PO_4, K_2HPO_4, NH_4NO_3 and $FeCl_3$ individually, keeping the rest of the components constant as described earlier [17]. Bacterial growth in terms of cfu was monitored on the 7th day of incubation maintained at 37 ± 2 °C and 135 rpm. Optimum pH and temperature for the maximum growth of the isolate was determined by maintaining the media at a pH range from 1 to 14 and incubation temperatures at 25, 30, 35, 40 and 45 °C individually at 135 rpm for 7 days.

2.3. Biodegradation of kerosene by the isolate

Degradation of kerosene oil by the isolate was determined after 28 days of incubation at 135 rpm, 37 °C by the isolate in BH broth supplemented with 2% (*v*/*v*) kerosene oil by gravimetrically. Degradation was finally confirmed by gas liquid chromatographic (GLC) analysis [18].

2.4. Recovery of biosurfactant

The most potent isolate was inoculated in BH broth supplemented with 2% (*v*/*v*) of the most preferred hydrocarbon supplement *i.e.*, diesel oil as reported earlier under optimized condition for 7 days [11]. After incubation, the culture was centrifuged at 10,000*g* for 15 min at 4 °C to separate the biomass. Crude biosurfactant was precipitated by adding three volumes of chilled acetone to the cell free supernatant, maintained at 4 °C with vigorous stirring for 10 h on a magnetic stirrer [19]. The crude biosurfactant was recovered by separating the precipitate under 10,000*g* for 10 min followed by air drying.

2.5. Bacterial adhesion to hydrocarbon (BATH) test

Hydrophobicity assay of the isolate was carried out by the method described by Ramasamy et al. (2014) [18]. The isolate was grown in BH broth supplemented with 2% (*v*/*v*) diesel oil under optimized condition. The cell pellet was obtained after centrifugation at 8000*g* for 10 min and washed twice to remove the hydrocarbon and other biopolymer residues. The pellet was resuspended in buffer salt solution (composition g/L: K_2HPO_4-16.9, KH_2PO_4-7.3, urea-1.8, $MgSO_4{\cdot}7H_2O$-0.7, and pH-7.0) and adjusted the optical density (OD) at 1.0 at wavelength 600 nm [(A_0) = 1.0]. Four milliliter of cell suspension was mixed with 1.0 mL of hexadecane in a screw capped tube

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and vortexed for 60 s, and allowed to stand for 30 min to separate two phases. Turbidity of the aqueous phase was measured at 600 nm (A_1). Hydrophobicity percentage of adherence to hexadecane was calculated as below:

$[1 - (A_1/A_0)] \times 100$ [%].

2.6. Characterization of the biosurfactant

2.6.1. Foaming

Hundred milliliters of the cell free media was shaken for foam formation and the percentage foam formation was determined by the following formula [20]:

Foaming (%) = (height of the foam layer/total height) × 100

2.6.2. Determination of emulsification index (E_{24}) of the biosurfactant

The emulsification index (E_{24}) of the biosurfactant was determined by adding 2 mL of petroleum hydrocarbon (diesel, kerosene, crude oil, and used engine oil) individually to the same amount of cell free culture followed by vortexing for 2 min. These were then allowed to stand for 24 h at room temperature. The E_{24} index was determined by the formula [19]:

$$E_{24}\ \text{index}\ (\%) = [\text{height of emulsified layer (mm)}/\text{total height of the liquid column (mm)}] \times 100$$

2.6.3. Drop collapse assay

Ten microliters of cell free media was placed on a grease free surface to see whether the drop spread or collapsed [12].

2.6.4. Contact angle determination

The contact angle of a drop of used engine oil on a hydrophobic surface after 3 and 6 days of incubation with the most potent isolate was also determined.

2.6.5. Biochemical characterization of the biosurfactant

Biochemical characterization of the biosurfactant was carried out by standard techniques [21,22]. The presence or absence of amino acid and protein in the biosurfactant was evaluated by ninhydrin test. Anthrone and saponification tests were carried out respectively to evaluate the presence of carbohydrate moiety and lipid in the biosurfactant.

2.6.6. Fourier transform infrared (FTIR) spectra Infrared spectra

The FTIR spectra (4000–400 cm^{-1}) of the dry biosurfactant was recorded in KBr on a Brucker, UK IR-spectrophotometer with 500 scans taking KBr pellet as background reference [12,23].

2.7. Statistical analysis

Student's *t*-test was performed. Each experiment was performed in triplicate and results were expressed in terms of mean ± S.D.

3. Results and discussion

Potential hydrocarbon degrading microorganisms were isolated on the basis of their growth on BH agar medium supplemented with used engine oil. The most potent isolate was identified as *B. cereus* strain DRDU1 on the basis of 16S rDNA sequencing. The 1262 bp long consensus sequence generated by the forward and reverse sequencees was submitted to NCBI GenBank database and the accession number KF273330 was successfully obtained for the novel isolate. The isolate established itself as a potential hydrocarbon degrader in the previous report [11]. The phylogenetic relationship of the isolate *B. cereus* strain DRDU1 strain was shown with nine most closely related strains on the basis of BLAST hits taking *B. amyloliquefaciens* ATCC-23350 shown in Fig. 1.

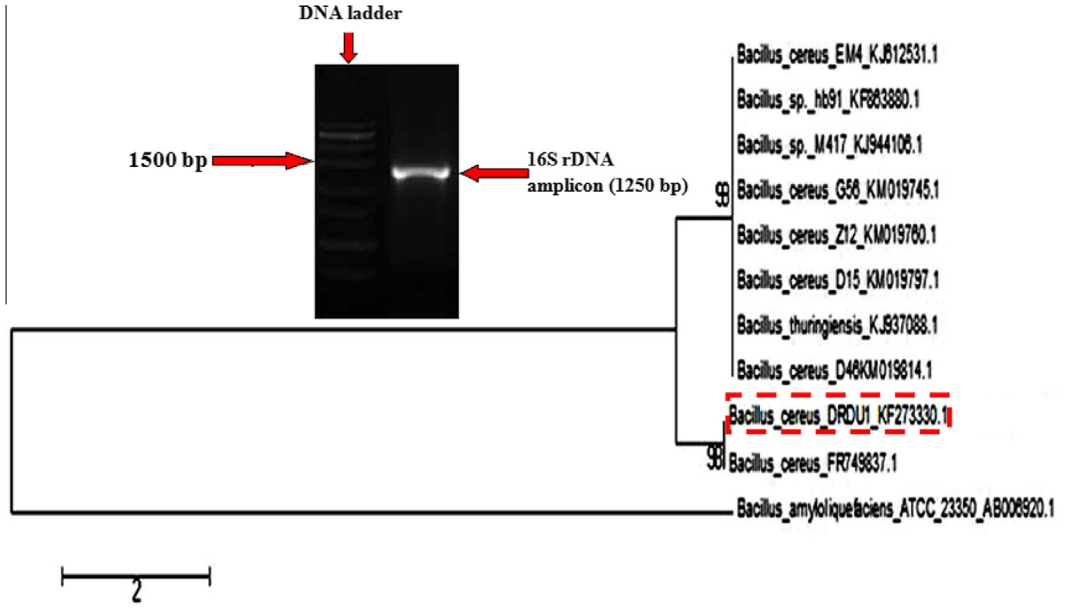

Figure 1 The tree shows phylogenetic relationship of the isolate *B. cereus* DRDU1 with 9 other most closely related strains constructed by neighbor-joining method with 1000 bootstrap values. The values on the nodes indicate the similarity percentage among the strains.

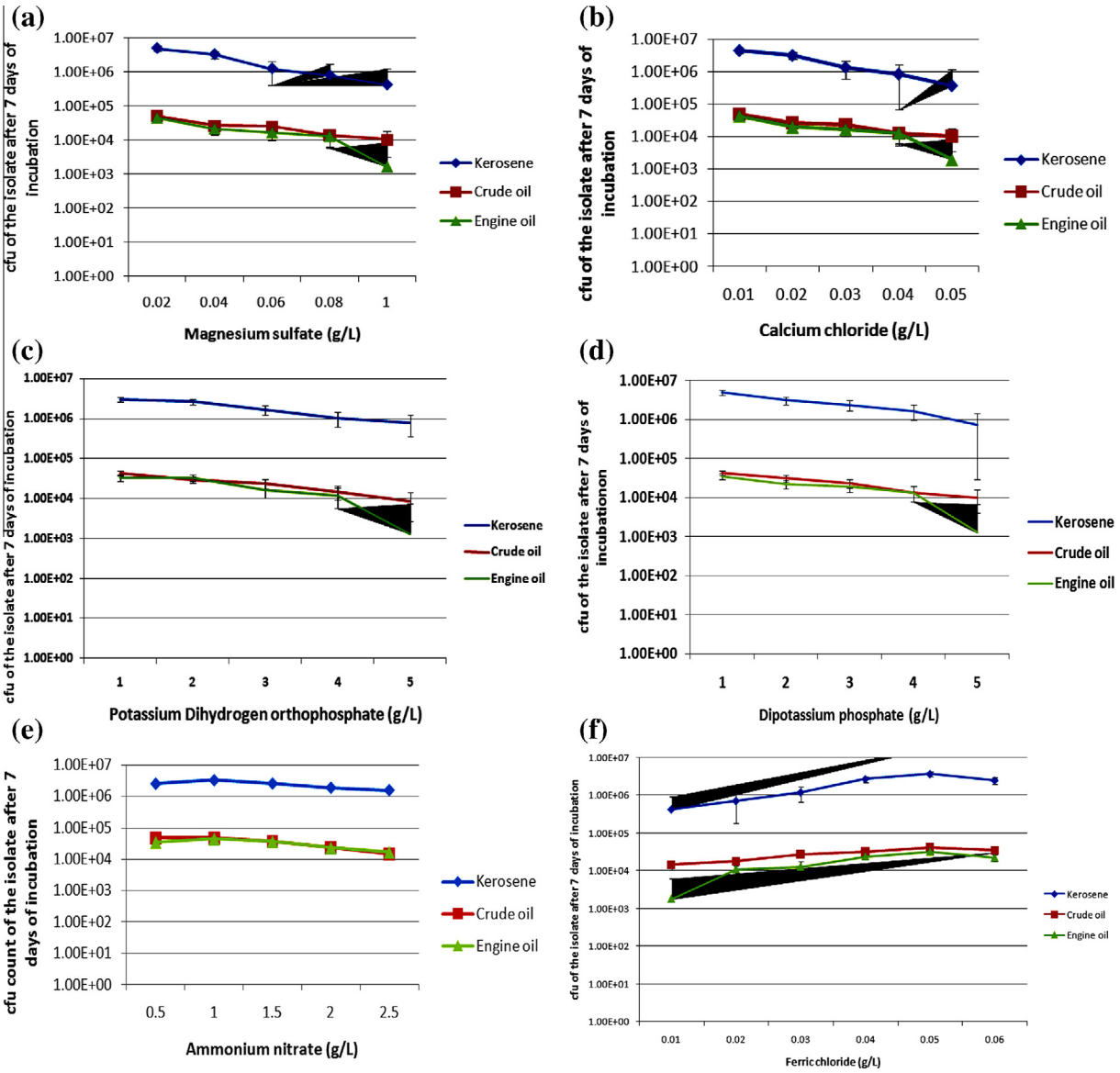

Figure 2 Growth of the isolate *B. cereus* strain DRDU1 at different concentrations of minimal salt supplemented with hydrocarbon oil after 7 days of incubation at 37 °C and 135 rpm.

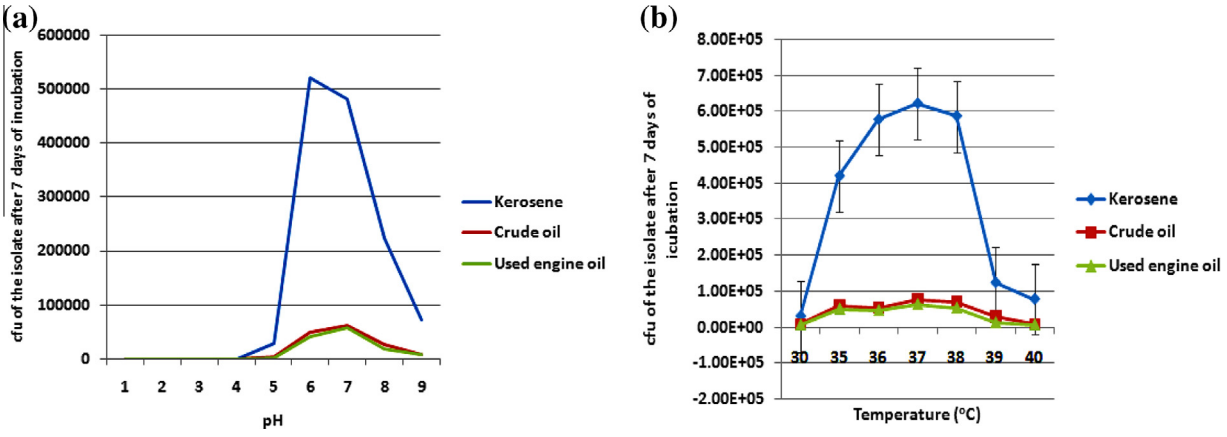

Figure 3 Growth of the isolate *B. cereus* strain DRDU1 in BH broth maintained at different pH, and temperature.

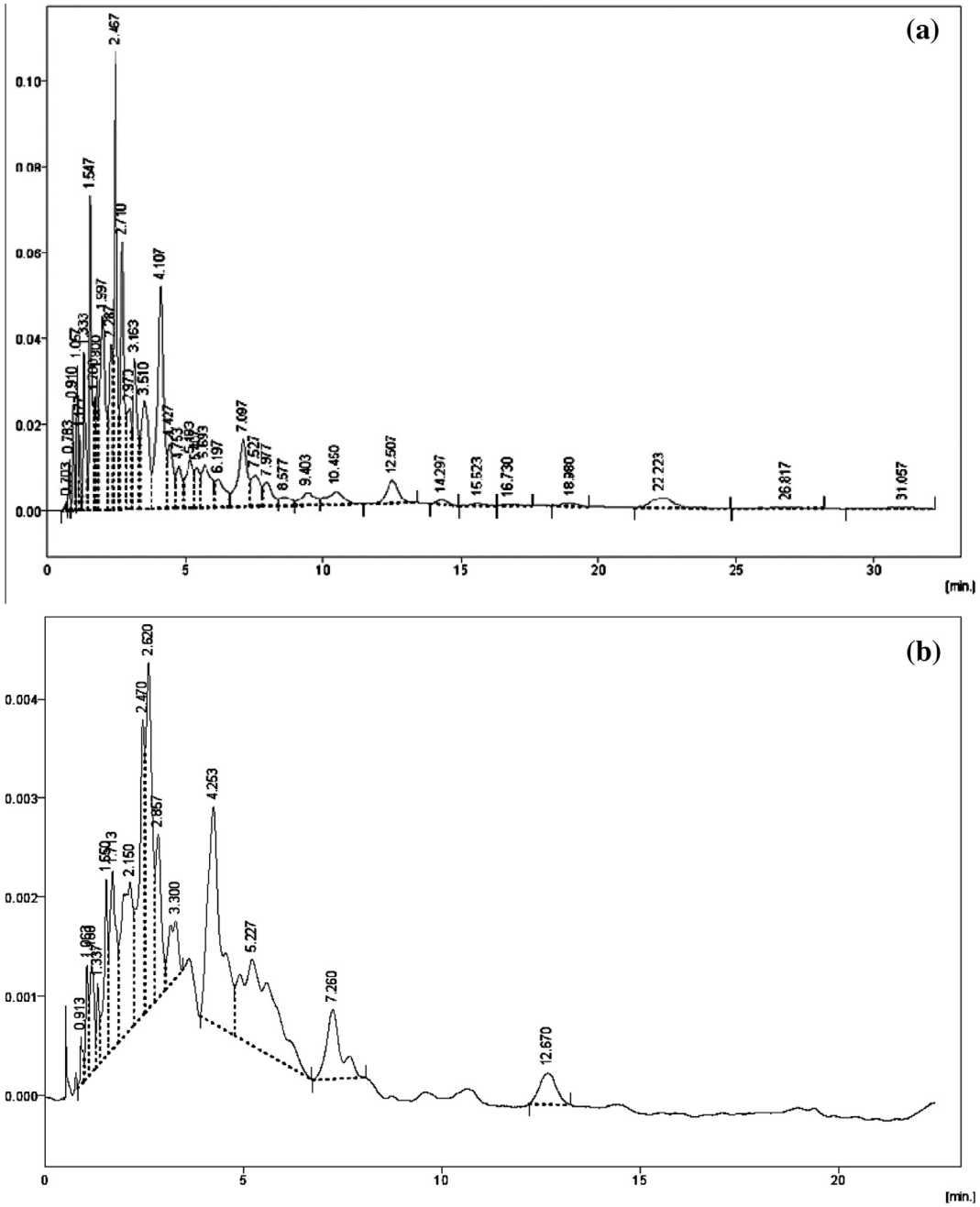

Figure 4 (a) Shows GC peaks of the kerosene extract in the abiotic control and (b) shows a decrease in peak area in the test sample after incubation.

The media components were standardized for the optimum growth of new isolate for the optimum production of biosurfactant. The optimum media composition was found to be, $MgSO_4$-0.2 (Fig. 2a), $CaCl_2$-0.02 (Fig. 2b), KH_2PO_4-1.0 (Fig. 2c), K_2HPO_4-1.0 (Fig. 2d), NH_4NO_3-1.0 (Fig. 2e), and $FeCl_3$-0.05 g/L (Fig. 2f), for all the hydrocarbon supplements used in the study. The optimum temperature and pH for the growth of the isolate was found to be 37 °C and pH 7 respectively (Fig. 3a and b). The hydrocarbon degradation by the potent isolate under optimum condition was determined initially by gravimetric analysis using kerosene as a sole source of carbon. The reduction of total peak area of 8218 in the control to 312 mV in GC analysis indicates 96% of kerosene degradation after 28 days of incubation (Fig. 4).

The hydrophobicity percentage of the isolate was found to be (60.67 ± 1.53)%. Foaming percentage of the cell free medium (crude biosurfactant) was determined to be 31.33 ± 0.58%. Crude biosurfactant was recovered from the cell free media and its emulsification index (E_{24}) was evaluated against kerosene, crude oil, used engine oil and was found to be 55.33 ± 1.53%, 29.67 ± 1.53%, and 20 ± 1% respectively (Fig. 5). The presence of biosurfactant in the cell free media was confirmed by observing the collapse of the drop immediately after placing on a grease free surface. The contact angle was found

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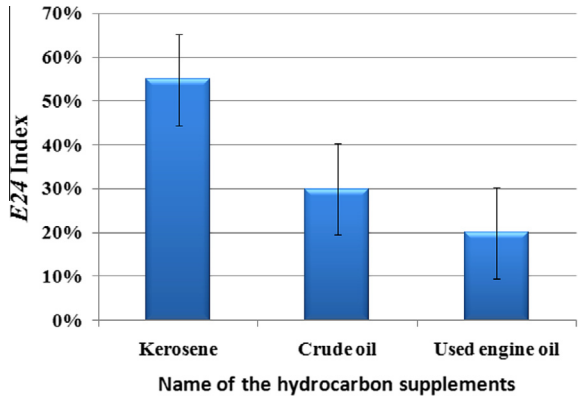

Figure 5 E_{24} index of the cell free extract against different petroleum hydrocarbons.

to be decreasing from (30.67 ± 1.15)° to (21.3 ± 1.53)° respectively after 3 and 6 days of incubation (Fig. 6).

The presence of amino acids and the carbohydrate moieties in the biosurfactant was confirmed by ninhydrin and anthrone tests respectively. The biosurfactant was further analyzed by IR spectroscopy. The FTIR spectra of the crude biosurfactant shows five very prominent peaks at 3742, 1741, 1679, 1646, and 1549 cm^{-1} which could be attributed to υ_{OH} (stretching), $\upsilon_{C=O}$ (ester), υ_{NH} (bending), $\upsilon_{CO=O}$ (stretching), and υ_{COOH} respectively. The spectrum also shows four more peaks at 2947, 1480, 1339, and 997 cm^{-1} which could be attributed to υ_{CH} (stretching), υ_{CH_2}, υ_{CH_3}, and υ_{CH} (bending) respectively (Fig. 7).

The success of bioremediation depends on the capacity to establish and maintain conditions that favor enhanced oil degradation rates in the contaminated environment. Microbial remediation of a hydrocarbon-contaminated site is accomplished with the help of a diverse group of microorganisms, and indigenous bacteria present in the soil but stress tolerant hydrocarbon degrading microbes are least explored [6,8,9,24–29]. In the current study, production, optimization and characterization of biosurfactant from a novel *B. cereus* strain DRDU1 was carried out, which was isolated from an unconventional source, *i.e.*, an automobile engine. The strain demonstrated its potential to degrade petroleum hydrocarbon (diesel, crude oil, and used engine oil) even in nutrient stressed condition [11]. The phylogenetic tree shows 99% similarity of the most potent isolate *B. cereus* DRDU1 with a *B. cereus* strain (GenBank accession FR749837.1), which was also reported as a potent hydrocarbon and radioactive waste degrader by Giacobone 2012 [30]. Numerous scientific reviews covered various factors that influence the rate of oil biodegradation [31–34]. The important requirement for the success of bioremediation is the presence of microorganisms with the appropriate metabolic activities for the degradation of specific type of petroleum oil. Biosurfactants are a major group of valuable natural microbial products with biochemical property, which can emulsify and degrade petroleum based hydrocarbons. Apart from its application in petrochemical industries, biosurfactants also

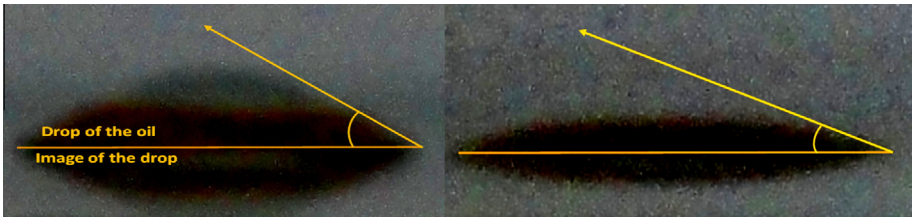

Figure 6 Figure shows the gradual decrease in contact angle of a drop of used engine oil after 3 and 6 days of incubation in the presence of the isolate.

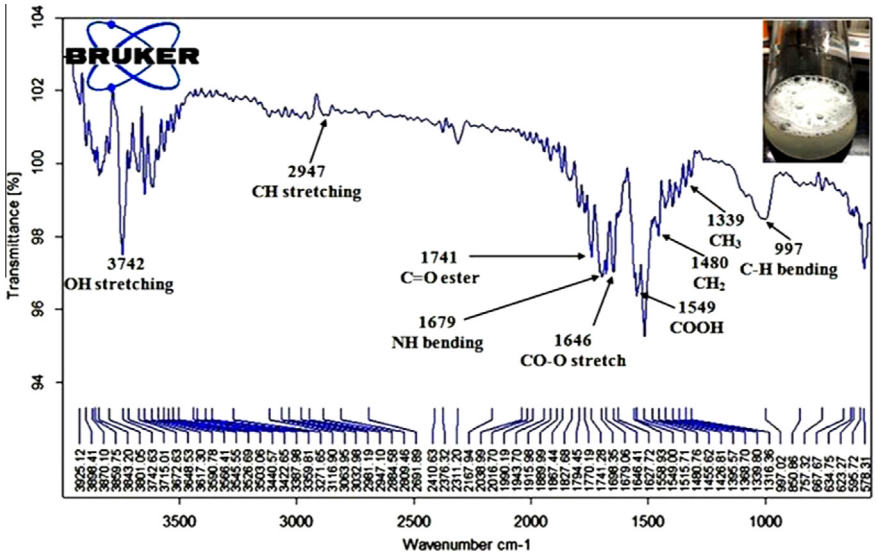

Figure 7 FTIR absorption analysis of the biosurfactant produced by the isolate *B. cereus* strain DRDU1 in BH broth. Figure in the inset shows foam formation in the cell free medium.

have a widespread application in the field of food processing, cosmetic, agricultural, and pharmaceutical industries [35]. Interest in microbial surfactants has progressively been increasing during recent years since they have higher biodegradability, higher specific activity at extreme temperatures, salinity, and pH levels and considered environmentally compatible with limited toxicity [36]. These kinds of natural microbial compounds are biologically surface-active agents and have various impending advantages over their synthetic counterparts. Microorganisms were considered to be detrimental to the petroleum industry in the earlier period but it is now well known that they can also be beneficial in terms of oil recovery. The low water solubility of various hydrocarbons, particularly the polycyclic aromatic hydrocarbons (PAHs), is believed to limit their accessibility to microorganisms, which is a potential problem for bacteria. It has been assumed that surfactants would augment the bioavailability of hydrophobic compounds [36]. Augmentation of microbes for enhanced hydrocarbon degradation primarily depends on the optimization of media components and growth parameters. Hence the importance of such optimization studies is advocated by many researchers [37–40]. Among the media components, $KH_2PO_4{\cdot}2H_2O$ and $K_2HPO_4{\cdot}2H_2O$ helps in buffering the media throughout incubation. Moreover, phosphorus is the major ingredient which helps in ATP and DNA. $CaCl_2{\cdot}2H_2O$, $MgSO_4{\cdot}7H_2O$, and $FeCl_3{\cdot}2H_2O$ provides Ca^{2+}, Mg^{2+} and Fe^{2+} ions, which helps in enhancing the enzymatic action for the degradation of hydrocarbons. $NH_4NO_3{\cdot}2H_2O$ provides free ions to the microbes for oxidizing hydrocarbons, which basically exist in reduced form. Moreover, nitrate is very much essential for the synthesis of amino acids and nucleotides [41].

The E_{24} values against kerosene, crude oil, and used engine oil were found to be correlating to some of the reported values but make it more preferable over others due to its ability to withstand physical and nutritional stress condition [18,19]. The cell surface hydrophobicity of the isolate was determined to be 60.67 ± 1.53% which is much higher than some of the previously reported microbial hydrocarbon degraders and commercial surfactants *viz*., Triton X-100, Tween 20 (31.4%), and Igepal CO-890 (28.5%) [18,42]. As described by Batista et al., 2006, surfactants possess both hydrophilic and hydrophobic moieties in its structure which gives rise to the capability to lower the surface tension between air–water interface (biosurfactants), and solid liquid interface (bioemulsifiers) and also can reduce interfacial tension between two immiscible liquids [43].

Contact angle less than 90° for surfactant containing liquid is considered to be a wet solid surface and a contact angle greater than 90° is said to be non-wetting. The alteration in wettability has been projected as one of the mechanisms of MEOR (contact angle). A drop of used engine oil (from the test media), being the most viscous hydrocarbon supplement used in the current study was considered to see the change in contact angle. The gradual reduction of contact angle of used engine oil in the test from (30.67 ± 1.15)° after 3 days to (21.3 ± 1.53)° after 6 days of inoculation the current study attracts the scope for its future application in MEOR studies. The strain was earlier reported to be an endospore former with a thermal death point of 86 °C [11]. Even though the strain optimally grows at pH 7, it could also withstand a wide range of pH 5–9. Moreover, the strain could significantly degrade 96% of kerosene oil in liquid culture. These features advocate its applicability for further commercial application in hydrocarbon bioremediation and also in MEOR.

4. Conclusion

The current work showed successful hydrocarbon degradation in liquid culture by *B. cereus* strain DRDU1 isolated from an unconventional source, *i.e*., an automobile engine. For the very first time, the production, optimization and characterization of biosurfactant from the isolate were carried out, showing its significant potential to emulsify petroleum hydrocarbon which may attract its future application toward MOER studies. These findings prove the isolated strain a potential tool for bioremediation and MOER purpose.

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