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ORIGINAL ARTICLE **OPEN ACCESS**

Characterization of biosurfactant produced by hydrocarbon-degrading Bacteria *Acinetobacter junii* isolated from petroleum hydrocarbon-contaminated soil

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ABSTRACT
In this research bacterial strains D1A isolated from petroleum hydrocarbon-contaminated soil showed the potential to produce bio-surfactant in the presence of 2% (v/v) diesel oil as a carbon source. Using a spectrophotometer 600 nm the growth rates of the selected isolate were determined and molecular characterization based on 16S r-RNA analysis proved D1A isolate as Acinetobacter junii. Bio-surfactant production by this strain was found to be growth-linked. Maximum emulsifying activity by emulsifying index and FTIR spectroscopic analysis revealed the glycolipid nature of the bio-surfactant. Indicated the results of the isolated strain had effectively utilized crude oil as the sole carbon source. The growth was monitored initial concentration and the final concentration increase in optical density was observed between 1-12 days. Acinetobacter junii D1A showed the highest growth in mineral salt medium with diesel oil. This work help in the isolation of potential hydrocarbon-degrading bacteria from contaminated soil and it can further be used for the biodegradation of oil-contaminated soil. The strain showed the production of biosurfactants which will assist in further effective remediation of oil-polluted sites.
Keywords: Hydrocarbon pollution, Bioremediation, Biosurfactant.

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INTRODUCTION

Petroleum and petroleum product like kerosene, gasoline, crude oil, and diesel oil spill both offshore and onshore can lead to serious damage to environment pollution as well as large accidental spills in aquatic and terrestrial regions. [1].The contamination of the environment with petroleum and petroleum products hydrocarbons has significant effects on all living beings. Novel approaches for bioremediation of these oil-based contaminants the techniques of bioremediation are at present receiving encouraging promotion and exposure as effective approaches for hydrocarbon degradation. Furthermore, the biological approaches and techniques could have an advantage over the commonly applied physicochemical system of oil spills, as they are more cost-effective and eco-friendly for the degradation of petroleum hydrocarbons [1,2]. Biodegradation is a complex process that depends on the nature of hydrocarbon pollution and also on the amount of the hydrocarbons and their physicochemical environment [3].The microbes can utilize the hydrocarbons depending on the carbon source nature of the compounds within the petroleum mixture[4]. Biodegradation by the population of microorganisms is one of the primary mechanisms by which petroleum and petroleum products hydrocarbon pollutants can be removed from the environment [5]. This degradation process is also cheaper than the other remediation technologies [6]. Non-pathogenic, bacterial strain *Acinetobacter junii* was found to produce bio-surfactant with the potential for hydrocarbon degradation. This strain was capable for emulsify oil in water [7]. This work was concentrated on the properties and characterization of bio-surfactant produced by hydrocarbon-degrading isolate, isolated from the petrol and diesel oil contaminated sites of Durg, Chhattisgarh. The results of this study will provide information on the bacterial strains which prevalent in the hydrocarbon-contaminated area, their hydrocarbon degrading ability as well as bio-surfactant production of the bacterial strain.

MATERIAL AND METHODS

Soil samples were collected from five automobile work-shops in Durg Chhattisgarh used to analyze the Physico-chemical characterization, biochemical and molecular characterization of isolated bacterial strain, growth efficiency of hydrocarbon utilizing bacteria, and production of bio-surfactant by hydrocarbon-degrading bacteria.

Collection of Samples: Samples were collected at a depth within 5cm from the surface of the soil. They were collected in sterile containers and tightly packed carefully transferred to the laboratory for further analysis and stored at 4°C [8].

Physico-Chemical Properties of Contaminated Soil: The soil Physico-chemical properties were determined soil pH was analyzed using a pH meter. The temperature of the soil samples was determined using a mercury thermometer. The electrical conductivity and TDS of soil samples were determined by digital electrical conductivity meter [9,10,]. Total nitrogen was determined by kiedahl digestion and steam distillation method [11]. Organic carbon in the soil was estimated by using Walkley- black method [12] as described by [9].The moisture content of the soil samples were calculated immediately by oven drying method[9, 10].

Enrichment and isolation of petroleum hydrocarbon-degrading bacteria: One gram of dried soil sample was dissolved in 9ml distilled water and agitated vigorously. Soil sample was serially diluted upto 10-8 dilution and 1 ml from each dilution was poured in Petri plate followed by addition of 20ml of molten nutrient agar medium at around 50°C. After gently rotating, the plates were incubated at 37°C for 24 hours [8]. Selected different colonies were streaked over Bushnell Hass-Agar supplemented with 5% Diesel. Un-inoculated media plate was served as control. Incubation was done at 30 degrees C for 7 days and growth was examined [13]. The composition of Bushnell Hass-Agar (g/liter): 0.2 g $MgSO_4$, 0.02 g $CaCl_2$, 1.0 g KH_2PO_4, 1.0 g K_2HPO_4, 1.0 g NH_4NO_3, 0.05 g $FeCl_3$, and the pH was adjusted to 7 - 7.2 with 0.01N HCl [14, 15].

Characterization of Isolates: Each isolate was morphologically examined (size, shape, margin, consistency, opacity, elevation, pigmentation and Gram reaction) as described by [16]. The isolates were characterized biochemically (motility, production of catalase, indole, urease, oxidative fermentation of sugars, methyl red test, VogesProskauertest and citrate utilization [17].

Growth potential of Hydrocarbon Utilizing Bacteria: Mineral salts medium (100ml, pH 1.0) supplemented with trace elements solution (2.5 ml per litre) [18] were put into 500ml Erlenmeyer flasks. The hydrocarbon substrates like 10% v/v; diesel and petrol were used as sole carbonsources. The media were inoculated with cells previously grown for 24 hours in nutrient broth and incubated at room temperature (30°C ± 2). The growth of the organisms was assayed after 48 hours by optical density (O.D) measurement at 600nm[8]**.**

Quantitative estimation of the Degradation Potential of *Acinetobacterjunii*: The degradation activities of *Acinetobacterjunii* were obtained by using MSM broth with 1% of crude oil as the sole carbon source and incubated at 30°C for 12 days. The growth of the bacterium was measured by taking the optical density (OD) readings at 600nm initial concentration and final concentration were calculated for 12 days at regular 2-day intervals by a spectrophotometer, against sterile mineral salt medium as a blank [19,20].

Molecular characterization of Bacterial 16S rRNA gene was amplified using the following two primers forward primer 27F (5'- AGAGTTTGATCMTGGCTCAG-3') and Reverse Sequencing of 16S rDNA and phylogenetic analysis was done. The purified PCR product was sequenced by Sanger di-deoxy method. Sequences were then compared to those in Gen Bank database in the National Centre for Biotechnology Information (NCBI) using the website for BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The evolutionary history was inferred using the Maximum parsimony method and a phylogenetic tree was constructed by MEGAX [21].

Assays for Bio-surfactant Production: Bacterial isolates were assayed for biosurfactant production by applying the most commonly used assays like oil spreading test and drop-collapse assay, CTAB agar method, emulsification assay, TLC and FTIR **[22].**

CTAB agar (Cetyltrimethylammoniumbromide agar) Plate Method: The growth of isolates was checked on the CTAB methylene blue agar medium for the screening of bio-surfactants producing ability of the isolates. This screening test was performed for two isolates with CTAB supplemented medium**:** The composition of CTAB methylene blue agar medium for 1L is as follows; Glucose-20gm Peptone-10gm, Beef extract- 1gm, CTAB- 0.78gm, Methylene blue- 0.002gm, Yeast extract- 0.5gm and Agar-17gm. The culture medium was autoclaved and the medium was poured in sterile petri dish under laminar air flow. The medium was allowed to solidify and then inoculated with the pure culture of all three isolates by streak plate method. In this method, the loop full culture of isolated was taken from the pure culture plate and streaked on the center of CTAB medium with an inoculation loop. The plates were incubated at 37ºC

for 24 hrs and next day they were observed for the growth of isolates on CTAB methylene blue agar**.** To detect the production of extracellular glycolipids, methylene blue agar plates containing cetyltrimethylammonium bromide (CTAB) (0.2 mg ml-1) and methylene blue dye (5 mg ml-1) were used. The development of dark blue halos around the bacterial colonies indicated the production of anionic glycolipid biosurfactants [23].

Emulsification index test (E24 %)

The emulsification activity of the bio-surfactant was measured using the method described by [24]. The emulsification capacity of biosurfactants was determined with the measurement of the emulsification index (E24). The emulsifying activity was measured by adding 4 ml cell-free culture after 72 h fermentation into 6 ml hydrophobic compound (1% diesel) in a 10 ml screw-capped glass tube and shaking with a vortex for 2 min. After 24 h standing, the height of the emulsion layer was measured and E24 was calculated.

Equation: E24 (%) = total height of the emulsified layer/total height of the liquid layer ×100%

Biosurfactant Extraction: The cell-free supernatant was obtained by centrifugation at 10,000 rpm for 15 min at 4 ◦ C. After the pH was adjusted to 2.0 with 1 mol/L HCl, 200 mL of the samples were extracted with equal volume of ethyl ether. The solvent was removed by vacuum distillation to collect the dry product. The purified biosurfactants in chloroform were then spotted on a silica gel thin-layer chromatography. The compounds were separated using a mobile phase containing chloroform, methanol, acetic acid, and water in volume ratios of 65:25:1:1, all of the solvent components being of analytical grade purity. The dry plates were sprayed with a phenol sulfate solution and incubated at 105 ◦ C for 5 min for glycolipid detection.

Purification and Structural Analysis of the Biosurfactants The crude extracts of biosurfactant were dissolved in chloroform and were then loaded onto a column of silica gel The column was first washed with n-hexane and then with chloroform/methanol solvent systems to recover potential biosurfactants. The eluate was collected separately every 10 mL. The glycolipid was detected using Fourier-transform infrared (FT-IR) spectroscopic analysis of biosurfactant. Functional groups of the biosurfactant samples were elucidated by FT-IR spectroscopic analysis. The FT-IR spectra were generated in the wave number range of 3500–500 cm−1 all measurements were recorded at room temperature [25, 26].

RESULTS AND DISCUSSION

Physico-chemical Characteristics of Contaminated Soil: The Physico-chemical characteristics of soil samples collected from different automobile shops used for the study were analyzed and tabulated in (Table 1). The various characteristics like texture, temperature, pH, Electrical conductivity, Carbon content, Nitrogen content, TDs, and Moisture content were analyzed.

Isolation of Bacteria from Hydrocarbon-Contaminated soil: Bacteria were isolated from hydrocarbon-contaminated soil samples by serial dilution technique. Then the isolated bacteria were identified by morphological, biochemical, and molecular characteristics totally five species of bacteria were isolated, theyare*Acinetobacterjunii, Pantoeadispersa, Acinetobacterbaumanii, Bacillus spizizenii, Pseudomonas aeruginosa*in that *Acinetobacterjunii* was selected in the present study.The bacteria were different based on their growth pigmentation and colony morphology on nutrient agar and selective media at 37°c for 48 hours. The morphological, biochemical and molecular characterization of *Acinetobacterjunii* were shown in Tables 2, 3, and 4 and Figure-1)**.**

Growth Potential of Hydrocarbon-utilizing Bacteria *Acinetobacterjunii*: The growth potential of *Acinetobacterjunii*on petrol and diesel was tested and results were observed and shown in Table 5.

Biodegradation Efficiency: The efficiency of *Acinetobacterjunii* to degrade diesel was also noted and presented in Table 6

Table 1: Physico-chemical properties of contaminated soilS

Texture	Sandy loamy
pH	9.01
Temperature	50^0C
Electrical conductivity (µs/cm)	59.4
Carbon (in%)	23.9
Moisture (in %)	2.9
Total nitrogen (kg/hect)	40.6
TDS (in ppm)	29.69

Table 2: Morphological characteristics of isolate (D1A) *Acinetobacter junii*

Morphological characterization	*Acinetobacterjunii*(D1A)
Colony Count CFU/ml	66×10^5
Gram staining	Negative coccobacillary
Shape	Cocci in cluster
Acid fast staining	Negative
Motility test	Non-motile
Form	Circular
Size	Small
Elevation	Raised
Margin	Entire
Opacity	Opaque
Surface	Smooth
Pigmentation	Greyish white

Table 3: Biochemical characteristics of isolate (D1A) *Acinetobacter junii*

Biochemical characteristic	***Acinetobacter junii*(D1A)**
Catalase test	+
Oxidase test	-
Indole test	-
MR	-
VP	-
Citrate utilization test	+
Urease activity	+
TSI	-
H_2S production	-
Starch hydrolysis	-
Glucose	+
Sucrose	+
Lactose	+
Mannitol	-

-

Table 4: BLAST search result of *Acinetobacter junii* D1A

S.N.	Name	Strain	Accession num	Pairwise similarity (%)
1	Acinetobacterjunii	ATCC 17908	NR_117623.1	99.50
2	Acinetobacterjunii	DSM 6964	NR_026208.1	99.50
3	Acinetobacterjunii	Mannheim 2723/59	NR_119360.1	99.49
4	Acinetobactermodestus	NIPH 236	NR_148845.1	98.41
5	Acinetobacterbaumannii	DSM 30007	NR_117677.1	97.91
6	Acinetobacterbaumannii	ATCC 19606	NR_117620.1	97.91
7	Acinetobacterbaumannii	JCM 6841	NR_113237.1	97.91
8	Acinetobacterbaumannii	DSM 30007	NR_026206.1	97.91
9	Acinetobactervivianii	NIPH 2168	NR_148847.1	97.83
10	Acinetobacterbaumannii	CIP 70.34	NR_116845.1	97.84
11	Acinetobacterbaumannii	ATCC 19606	NR_119358.1	97.69
12	Acinetobactervenetianus	RAG-1 = CIP 110063	NR_042049.1	97.48
13	Acinetobactercourvalinii	ANC 3623	NR_148843.1	97.55
14	Acinetobacterparvus	LUH4616	NR_025425.1	97.26
15	Acinetobactergerneri	DSM 14967 = CIP 107464 = MTCC 9824	NR_117627.1	97.18
16	Acinetobactertjernbergiae	DSM 14971	NR_117629.1	96.90
17	Acinetobacterchinensis	WCHAc010005	NR_165666.1	96.90

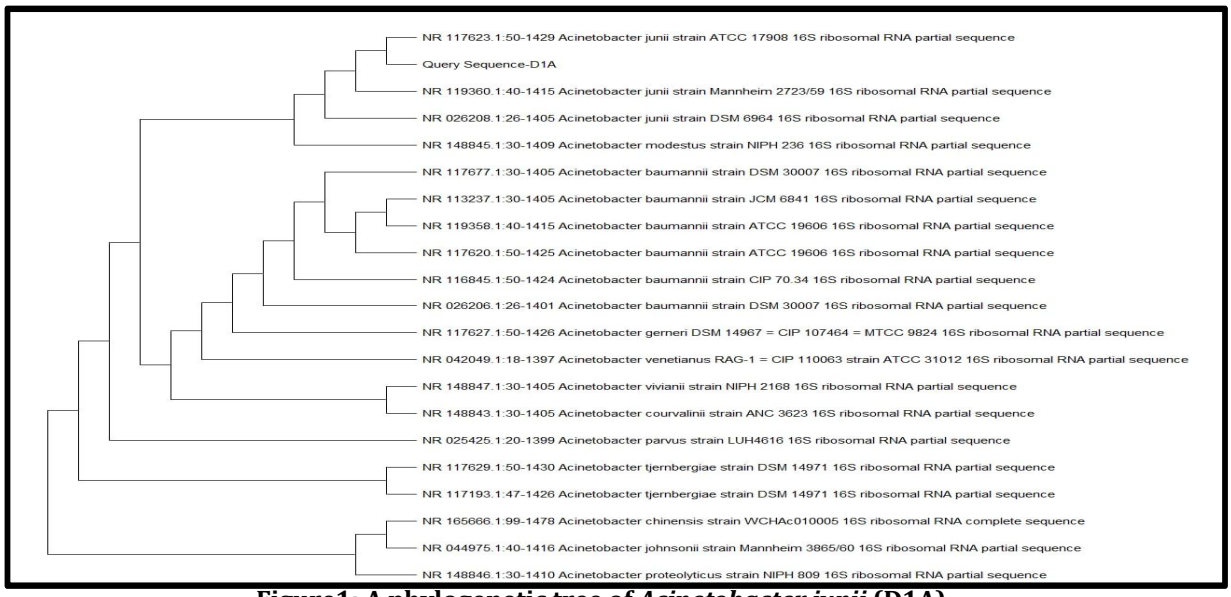


Figure1: A phylogenetic tree of *Acinetobacter junii* (D1A).

Table 5: Growth of hydrocarbon utilizing bacteria *Acinetobacterjunii D1A*

Organisms	Petrol	Diesel
Acinetobacterjunii	++	+++

(+ = poor growth, ++ = moderate growth, +++=higher growth)

Table 6: Degradation potential of *Acinetobacter junii* D1A during 12 days incubation

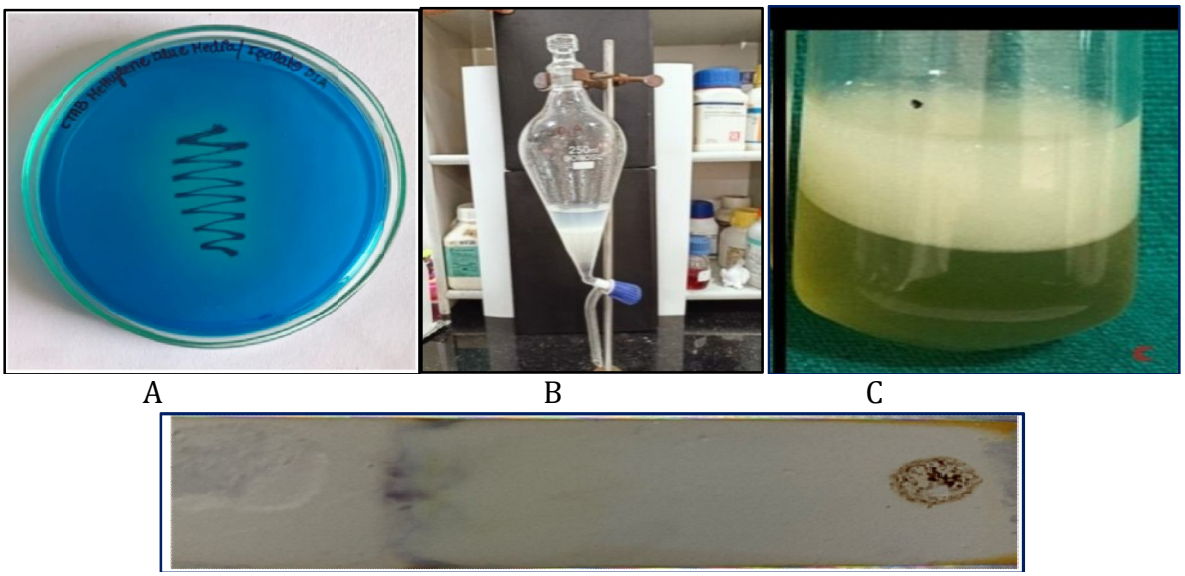
No of days	Sample absorbance	Blank absorbance	Initial concentration of diesel	Final concentration of diesel	Degradation Percentage (%)
2	0.33	0.36	1.450	1.318	9.053
4	0.13		1.450	1.012	30.175
6	0.22		1.450	0.837	42.245
8	0.21		1.450	0.794	45.263
10	0.13		1.450	0.444	69.403
12	**0.08**		**1.450**	**0.225**	**84.490**

Bio-surfactant production: The strain *Acinetobacterjunii* D1A was screened by several methods including oil displacement, drop collapse test, CTAB Methylene blue agar test, and emulsification index (EI 24%) for bio-surfactant production.

The bio-surfactant solution obtained from strain D1A showed a positive Oil displacement test, Drop collapsed test and a good Emulsification index (EI 24%) showed (Fig-2). These qualitative tests suggested the surface and wetting activities of the bio-surfactant. For the detection of anionic surfactants, CTAB–methylene blue agar test was performed. After 48 h of incubation at 37 °C on CTAB–Methylene blue agar medium, the isolates showed the presence of blue halos around the bacterial Colonies which was an indication of the anionic nature of biosurfactant. An indirect method of testing the surface activity of any solution is to test its emulsification activity. The results of screening of bio-surfactant production by *Acinetobacter junii* was depicted in Table 07.

Table 7: Screening for Bio-surfactant production *Acinetobacter junii* (D1A)

Isolates	Oil displacement test	Drop collapsed test	Emulsification index (EI 24%)	CTAB (Methylene blue agar test)
Control	Negative	Negative	7-11	Negative
D1A	Positive	Positive	47	Positive



A B C

D

Fig:2 A) CTAB method, B) Bio-surfactant extraction, C)Emulsification index, D) Thin layear chromatography

Characterization of bio-surfactant: The biosurfactant produced from *Acinetobacterjunii D1A* was characterized by Thin Layer Chromatography (TLC) and Fourier Transform Infrared analysis (FTIR) technique.

Thin Layer Chromatography (TLC): Bio-surfactants obtained from the isolate by acid precipitation solvent extraction method at pH-2 was identified by Thin Layer Chromatography (TLC) (27). Exposure of iodine vapor resulted in the formation of yellow and brown spot, indicated the presence of lipid whereas, dark brown spot after ninhydrin spray indicated the presence of peptide moiety the results are shown in (Figure-2) further confirmation of lipid and peptide moiety was done by FTIR.

Fourier Transform Infra-red (FTIR) analysis The chemical composition of the extracted biosurfactant of the isolate *Acinetobacterjunii (D1A*) was investigated using FTIR spectroscopy and the results are shown in Fig 3. Fourier transformation infra-red spectroscopy of the crude bio-surfactant produced by *Acinetobacterjunii* (D1A) revealed N–H stretching (3384 cm–1), C=O (amide) stretching (1637 cm–1) indicating its peptide component. Peak at 1014 cm-1 is probably because of C-O-C vibrations in the ester as observed in (Figure-3). The FTIR spectrum indicated the possible lipopeptide nature of the crude biosurfactant similar results were noted by [28, 29].

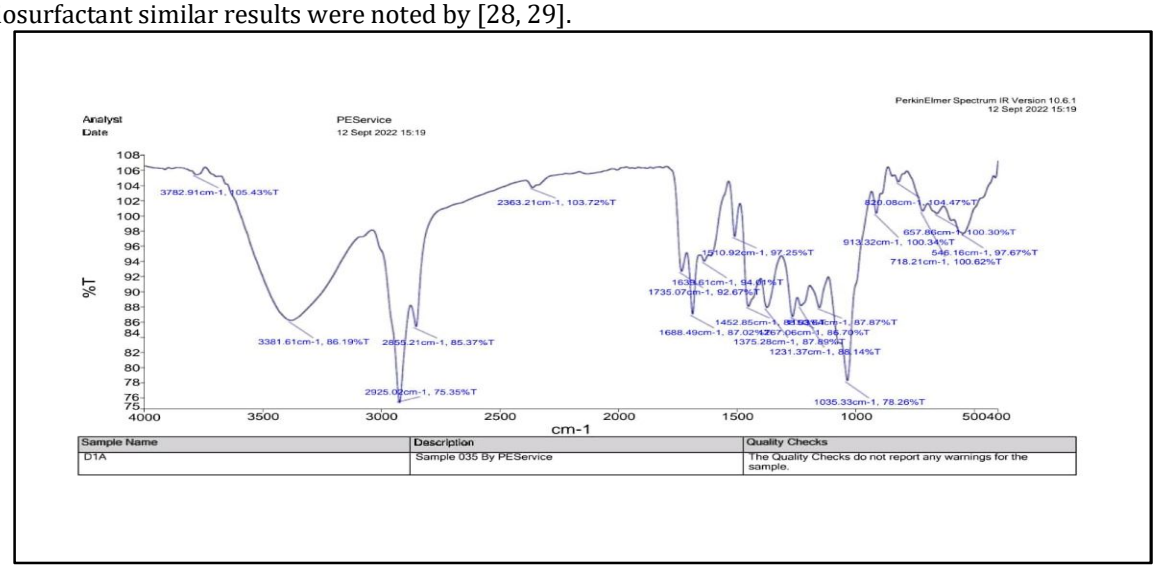


Figure 3: Fourier transform infrared spectra (FTIR) of the biosurfactant produced by *Acinetobacter junii* D1A

CONCLUSION

The strain was found to have effective hydrocarbon degradation potential as well as capable of producing bio-surfactant. Moreover, this strain formulation will be further explored for remediation applications for other recalcitrant pollutants. Hence, it is suggested that the use of the above bacterial strain is an alternative technology for an effective and eco-friendly way for the degradation of PHCs. In the present study lipopeptide biosurfactant production from indigenous *Acinetobacter junii* isolated from hydrocarbon contaminated soil was reported. The bioemulsifiers (*Acinetobacter junii*) demonstrated higher emulsifying activity in the presence of hydrocarbon diesel oil this bioemulsifiers represent a good candidate for various industrial and environmental applications, especially in the remediation of oil-contaminated soil.

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