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Biosurfactant Production and Hydrocarbon Degradation Activity of Bacteria Isolated from Petroleum Contaminated Soil

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ABSTRACT

In this study bacterial strain*B4A* isolated from petroleum hydrocarbon contaminated soil showed potential to produce bio-surfactant in the presence of 2% (v/v) diesel oil as a carbon source. The growth rate was determined using spectrophotometer at 600nm. Identification based on 16S r-RNA sequencing proved *B4A* isolate as *Pantoeadispersa*. Bio surfactant production by this strain was found to be growth-linked. Maximum emulsifying activity and Fourier-transform infrared spectroscopic analysis revealed the glycoprotein nature of the bio-surfactant. Results indicated that the isolated strain had effectively utilized crude oil as sole carbon source. Linear increase in optical density was observed between 1-12 days. *Pantoeadispersa B4A* showed the highest growth of 81% in medium with diesel oil. This study held in isolation of potential hydrocarbon degrading bacteria from contaminated soil and it can further be used for bioremediation of oil contaminated soil. The strain showed production of bio-surfactant which will assist in further effective remediation of oil polluted sites.

Key words*: Hydrocarbon, Pollution, Bioremediation, Bio-surfactant*

Introduction

Hydrocarbons are the foremost broadly utilized and necessary form of primary energy resources in the world. It is one of the most important energy resources and raw materials for various industries. These are organic compounds made primarily of the elements hydrogen and carbon. They moreover contain little amounts of non-hydrocarbon compounds like sulfur, nitrogen and oxygen etc., (Varjani *et al.*, 2013). The term petroleum comes from the Latin word Petra=rock and ileum =oil (Vieira *et al.*, 2007). Petroleum is a term from "rock oil," and it is a liquid that is sticky and dark in color (Varjani *et al*., 2013). It is oil of rock but today the definition of petroleum has been enlarged to include gasoline also and defined as a mixture of dominantly of hydrocarbons and subordinately non-hydrocarbons and inorganic constituents. Hydrocarbon products like kerosene diesel, fuel oil, crude oil, LPG gases, coals dust particles, asphalts are complex mixture of hydrocarbon compounds (Mittal and Singh, 2009; Singh and Lin, 2008; Vieira *et al*, 2007). Petroleum, often known as crude oil, is a naturally occurring substance produced when organic matter is converted anaerobically at high temperature and pressure (Balba *et al.*,

1998). Numerous millions of aliphatic, branching, and aromatic hydrocarbons make up its composition and some organometallic constituents (Yuniati, 2018; Butler and Mason, 1997). Petroleum is heterogeneous liquid called hydrocarbons comprise entirely and almost of "Hydrocarbon" is a compound made of hydrogen and carbon (Kvenvolden, 2006; Okoh, 2006). Both industrialized and developing countries experience frequent oil spills the volume of crude oil seepage was estimated to be 600,000 metric tones per year (Prathyusha *et al*., 2016). Hydrocarbon products leakages accidental spills and anthropogenic activities are common during the periods of exploration, production, refining, usage, transportation, and storage time (Darsa and Ramya, 2014; Das and Chandran, 2010). All living things are at risk from these hydrocarbon contaminations, which are also highly immunotoxic, mutagenic, and carcinogenic (Pham and Jeong, 2014). Theoretically, preserved prehistoric zooplankton and algae that have gathered in considerable proportions at sea or lake bottoms under anoxic conditions are what turn into crude oil (Kvenvolden, 2006). As a result of this process, organic matter is transformed into a waxy material known as kerogen that, through a procedure known as catagenesis, can be heated further to form liquid and gaseous hydrocarbons. These petroleum hydrocarbons from the air accumulate on the foliage of plants, the surface (Braun and Burnham, 1993).

This work was concentrated on properties and characterization of bio-surfactant produced by hydrocarbon degrading bacteria isolated from petrol hydrocarbon contaminated sites of Durg, Chhattisgarh.

Materials and Methods

Sample Collection: Petroleum hydrocarbon contaminated soil samples were collected from identified site at a depth of 1-2 cm below soil surface using a sterile spatula in sterile polythene bags in triplicates. The samples were immediately transferred to the laboratory for further examination and kept at 4°C after collection.

Estimation of physicochemical properties of hydrocarbon contaminated soil: pH, Electrical Conductivity, Carbon and Moisture content **was determined by** (Jackson, 1958; Walkey and Black, 1934; Ezeigbo *et al*., 2013).

Isolation of Petroleum Hydrocarbon Degrading Bacteria

One gram (1 g) of dried soil sample was dissolved in 9 ml of distilled water, serial dilution was carried out, and the pour plate method was used for inoculation. After serial dilution, 1 ml of 10^{-1} to 10^{-7} dilution were added to different sterile Petri plates , than 20 ml of nutrient agar media was poured onto it. Inoculated plates were cultured for 24 hours at 37 °C (Santhini *et al*., 2009).

Screening and quantitative analysis of petroleum hydrocarbon degrading bacterial isolates

the indirect and direct methods of evaluating bacterial growth in the presence of hydrocarbons were used for primary screening. In the indirect method, the ability of all isolates to grow in the presence of 1% diesel and petrol as the sole carbon source in the Bushnellhass broth was evaluated by spectrophotometric method after 1weeks of incubation. Individual bacterial strains were cultured for 16 hours to measure the quantitative degradation of PHCs. 250 ml conical flask that already contained 100 ml of sterile defined Mineral salt broth (MSB) and 1% diesel were inoculated with bacteria selected by primary screening. All of these flasks were incubated in shaking incubator at 200 rpm for 12 days at 30 °C. A spectrophotometric technique was employed in all treatments to gauge the total hydrocarbon degradation (Prakash *et al*., 2014).

Identification of Highly Efficient Petroleum Hydrocarbon Degrading Bacterial Isolates

Different microscopic and biochemical tests were performed for the characterization of highly efficient petroleum hydrocarbon degrading bacteria. Than the confirm identification of selected bacterial isolates was done by molecular characterization through 16S r-RNA sequencing (Prescott and Harley, 2002; Aldisi *et al*., 2016; Tamura *et al*., 2021).

Screening for Bio-Surfactant Production of the Selected Efficient Bacterial Strain

Various screening test oil displacement, drop collapse test, CTAB agar (Cetyl-tri-methyl ammonium Bromide Agar) plate method, emulsification index test (E24 %) was used (Rodrigues, 2006; Tugrul and Cansunar, 2005; Satpute *et al*., 2010).

Emulsification index test (E24 %)

the technique described bywas used to assess the

bio-surfactants emulsification capacity. By measuring the emulsification index, the emulsification capacity of the bio-surfactant was identified (E24). After 72 hours of fermentation, 4 ml of cell-free culture was added to 6 ml of hydrophobic substance (1% diesel) in a 10 ml screw-capped glass tube. The mixture was then agitated vigorously for 2 minutes to determine the amount of emulsifying activity. The height of the emulsion layer was measured and E24 was determined after 24 hours of standing (Sahu and Shrivastava, 2022; Deepak and Jayapradha, 2015).

$$\text{Emulsification Index (\%)} = \frac{\text{Total Height of the Emulsified Layer}}{\text{Total Height of the Liquid Layer}} \times 100\%$$

Production and Extraction of Bio-surfactant

Bio-surfactant production was carried out using a method described by and Mishra *et al.* (2014). After being streaked on a nutrient agar slant, the bacterial strains were cultured for 24 hours at 30°C.An aliquot of 2 ml of inoculum was transferred to 100 ml of Bushnell Haas broth and cultured for 96 hours at 150 rpm and 30^0C in a temperature controlled shaker incubator. The final concentration was made to be 1% (w/v) by adding the carbon sources carbohydrate (glucose). At 1% (v/v), the hydrocarbon (n-hexadecane) was added. Samples were gathered extracted, and used for further analysis through TLC, GCMS, and FTIR (Mishra *et al.*, 2014).

Characterization of Bio-Surfactant was done by TLC, FTIR and GCMS

Thin Layer Chromatography (TLC)

On silica 60 gel, thin layer chromatography was performed in order to examine the purified bio-surfactant (Priya and Usharani, 2009) with few modifications. The TLC plate was filled with a solvent solution consisting of chloroform, methanol, and water in the ratio 81:17:2 (v//v/v). The TLC plate was then carefully placed in the chamber after being spotted with the bio-surfactant, by exposing it to iodine vapor, the chromatogram was detected. The plate was then heated for 15 mint at 100^0 until the appearance of the particular color. The carbohydrates were detected after spraying 5% concentrated H_2SO_4.

Anthrone reagent (1 g anthronein 5 ml sulfuric acid mixed with 95 ml ethanol) were used to detect glycolipid as yellow spots and ninhydrin reagent (0.5 g ninhydrin in 100 ml anhydrous acetone) were used to detect lipopeptide as red spots.

Fourier Transform Infrared Spectroscopy (FTIR) Analysis

In order to characterize the components of biosurfactant, various types of chemical bonds and functional groups were identified through FTIR spectroscopy. Dried bio-surfactant samples was mixed with spectroscopic grade potassium bromide (KBr) sample 1: KBr 100 (w/w) and the spectra was taken in absorption mode in the range of 4000-400 cm^{-1} at a resolution of 4 cm^{-1} using a FTIR spectrophotometer, In order to identify various types of chemical bonds and functional groups formed (Saravanan and Vijayakumar, 2012) with slight modifications.

Analysis of Gas Chromatography and Mass Spectroscopy (GC-MS)

Sample was injected into an Agilent 7890B GC connected to a 5977A MSD, Column HP_5MS, after being dissolved in a solution of methanol and ethyl acetate. 5% Phenyl Methyl Silox, 30 m x 250 m x 0.25 m, 60 ºC-325 ºC.injected in splitless mode. The GC-MS used for the analysis was run under the following conditions Oven temperature 45 °C for 2 minutes, followed by 140 °C at 5 °C/min, then 280 °C, which was maintained isothermally for 10 minutes. The carrier gas was helium at a rate of 1 ml/min, and the sample injection volume was 2 L. The sample's constituent parts were ionised at a 70 eV energy level. The GC was operational for 54.0 minutes. The structures of the compounds were then compared to those in the NIST database using a search of the NIST14.L library (2020). Then, using retention times and mass spectra of known compounds from the NIST library (C: Database NIST MS2011.L), compounds were identified (Singh and Tiwary, 2017).

Results and Discussion

Physico-chemical Characteristics of Contaminated Soil

The physico-chemical characteristics of soil sample collected from different automobile shops used for the study was analyzed and tabulated in (Table 1). The various characteristics like texture, temperature, pH, Electrical conductivity, Carbon content, Nitro-

gen content, TDs, Moisture content were analyzed.

Table 1. Physico-chemical properties of contaminated soils

Texture	Sandy loamy
pH	8.60
Temperature	50^0C
Electrical conductivity (µs/cm)	108.7
Carbon (in%)	29.9
Moisture (in %)	2.5
Total nitrogen (kg/hect)	40.6
TDS (in ppm)	54.21

Isolation of Bacterial isolates from Hydrocarbon Contaminated soil

In this work, 45 bacterial isolates were isolated from petroleum hydrocarbon contaminated sites. After pure culturing, all these bacterial strains were checked for the petroleum hydrocarbon degradation capability. Total of 45 hydrocarbon contaminated soil bacterial isolates were isolated from all the ten sites. Out of 45, 22 bacterial isolates were isolated from various automobile workshop of Durg and 23 bacterial isolates were isolated from the hydrocarbon contaminated sites of Bhilai.

Table 2. Growth of hydrocarbon utilizing bacterial isolates B4A in Petrol and Diesel

Organisms	Petrol	Diesel
Pantoeadispersa	+++	+++

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

Growth Potential of Hydrocarbon-utilizing Bacterial Isolates

The growth potential of B4A on petrol and diesel were tested and results were observed and shown in Table 2.

Identification of Petroleum Hydrocarbon Degrading Bacterial Isolates

Bacterial isolates B4A was selected for further studies identified as *Pantoea dispersa.* It showed 99.23% similarity to *Pantoea dispersa* strain DSM 30073 (Accession no. OQ120600), sequence similarity check was analyzed by BLAST analysis (Table 3, 4 and 5) and phylogenetic tree was constructed by MEGA X software (Figure 1).

Biodegradation Efficiency: The efficiency of

Table 3. Morphological characteristics of isolate B4A

Morphological characterization	B4A
Colony Count CFU/ml	50×10^5
Gram staining	Negative coccobacillary
Shape	Cocci in cluster
Acid fast staining	Negative
Motility test	Motile
Form	Circular
Size	Large
Elevation	Raised
Margin	Entire
Opacity	Opaque
Surface	Smooth
Pigmentation	Cream colour

Table 4. Biochemical characteristics of isolate B4A

Biochemical characteristic	B4A
Catalase test	+
Oxidase test	-
Indole test	-
MR	-
VP	-
Citrate utilization test	+
Urease activity	+
TSI	-
H_2S production	-
Starch hydrolysis	-
Glucose	+
Sucrose	+
Lactose	+
Mannitol	-

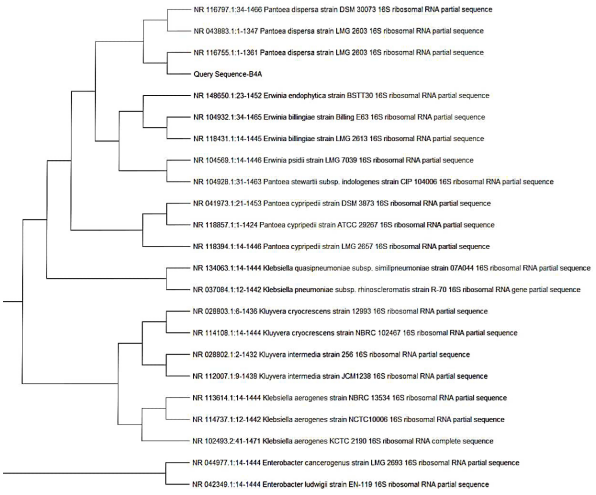

Fig. 1. A phylogenetic tree of *Pantoea dispersa* (B4A).

Pantoea dispersa to degrade diesel was also noted and presented in (Table 6) selected bacterial isolates B4A *Pantoea dispersa* showed 81% of degradation.

Bio-surfactant production: The strain *Pantoea dispersa* B4A 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 B4A showed 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 biosurfactant (Youssef *et al*., 2004). 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 were an indicative of 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 *Pantoea dispersa*was depicted in Table 7.

Characterization of bio-surfactant: The biosurfactant produced from *Pantoea dispersa B4A* was characterized by Thin Layer Chromatography

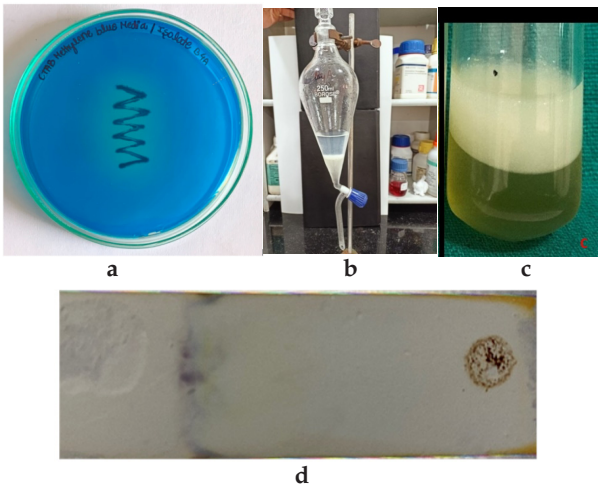

Fig. 2. a) CTAB, b) Biosurfactant extraction, c) Emulsification index, d) TLC

Table 5. BLAST search result of *Pantoea dispersa* (B4A)

S. N.	Name	Strains	Accession num	Pairwise similarity (%)
1	*Pantoeadispersa*	strain DSM 30073	NR_116797.1	99.23
2	*Pantoeadispersa*	strain LMG 2603	NR_116755.1	99.63
3	*Pantoeadispersa*	strain LMG 2603	NR_043883.1	99.55
4	*Pantoeacypripedii*	strain DSM 3873	NR_041973.1	97.63
5	*Pantoeacypripedii*	strain LMG 2657	NR_118394.1	97.28
6	*Pantoeacypripedii*	strain ATCC 29267	NR_118857.1	97.69
7	*Kluyveracryocrescens*	strain 12993	NR_028803.1	97.49
8	*Kluyveracryocrescens*	strain NBRC 102467	NR_114108.1	97.42
9	*Erwiniaendophytica*	strain BSTT30	NR_148650.1	97.35
10	*Erwiniapsidii*	strain LMG 7039	NR_104569.1	97.22
11	*Kluyveraintermedia*	strain 256	NR_028802.1	97.21
12	*Enterobactercancerogenus*	strain LMG 2693	NR_044977.1	97.00
13	*Klebsiellaaerogenes*	strain NBRC 13534	NR_113614.1	97.14
14	*Erwiniabillingiae*	strain Billing E63	NR_104932.1	97.14
15	*Pantoeastewartii*	strain CIP 104006	NR_104928.1	97.14

Table 6. Degradation potential of *Pantoeadispersa* 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	B4A		1.450	0.269	**81.473**

(TLC) and Fourier Transform Infrared analysis (FTIR) and GCMS.

Thin Layer Chromatography (TLC): Thin layer chromatography was performed to analyze the biochemical nature of extracted bio-surfactants. Extracted bio-surfactant showed single yellow colour spot when they were exposed in iodine vapour by TLC. The yellow spot with Rf values of 0.72 confirms the presence of lipid molecules in extracted biosurfactants by *Pentoeadispers*. Subsequently, when the extracted bio-surfactants were heated with orcinol reagent, purple colour appeared which showed the presence of sugar moieties in the extracted biosurfactant from the bacteria. The presence of lipid and carbohydrates confirms the glycolipid nature of extracted bio-surfactants.

Fourier Transform Infra-red (FTIR) analysis

FTIR analysis of the extracted bio-surfactant of *P. dispersa* showed the presence of polysaccharide moiety and aliphatic hydrocarbon chains (lipid) (Figure 2). The presence of free -OH groups due to the hydrocarbon bonding of polysaccharides and –OH stretching of carboxylic acid groups are revealed through the absorption bands of *P. dispersa* at 3783.04 $cm^{-1,}$ and 3385.59 cm^{-1}. These absorption spectra confirms the glycolipid nature of bio-surfactants. The stretching vibrations of –CH bond of respective sugar moiety is suggested by the absorption peak of *P. dispersa* at 2852.94 and 2922.41 cm^{-1} respectively. The C = O stretching in ester groups of lipids and fatty acids was evidence by the absorption peak 1736.66 and 1509.82 cm^{-1}in the extracted bio-surfactants produced by *P. dispersa* confirms the presence of the deformation vibration of alkyl group. The stretching bonds of *P. dispersa*, it was associated with the absorption bands at 1455.02 and 1154.34 cm^{-1}. absorption peaks at 1038.37 and 820.63 cm^{-1} indicated the stretching vibrations of glycosidic linkage which proves the glycolipid nature of biosurfactant of *P. dispersa*. The hydrophilic characteristics in the bio-surfactant are due to the presence of sugar residue while the hydrophobic characteristics are due to the presence of lipid residue. Johnson

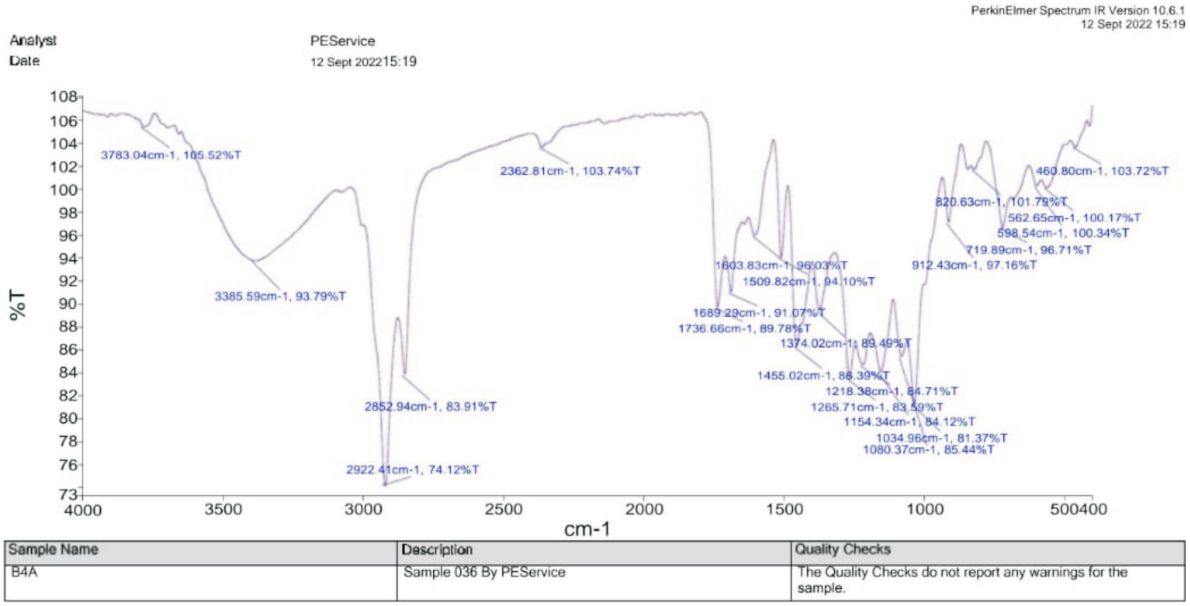

Fig. 3. Fourier transform infrared spectra (FTIR) of the biosurfactant produced by P. dispersa

Table 7. Screening for Bio-surfactant production *Pantoea dispersa:* (B4A)

Isolates	Oil displacement test	Drop collapsed test	Emulsification index (EI 24%)	CTAB (Methylene blue agar test)
Control	Negative	Negative	7-11	Negative
B4A	Positive	Positive	39	Positive

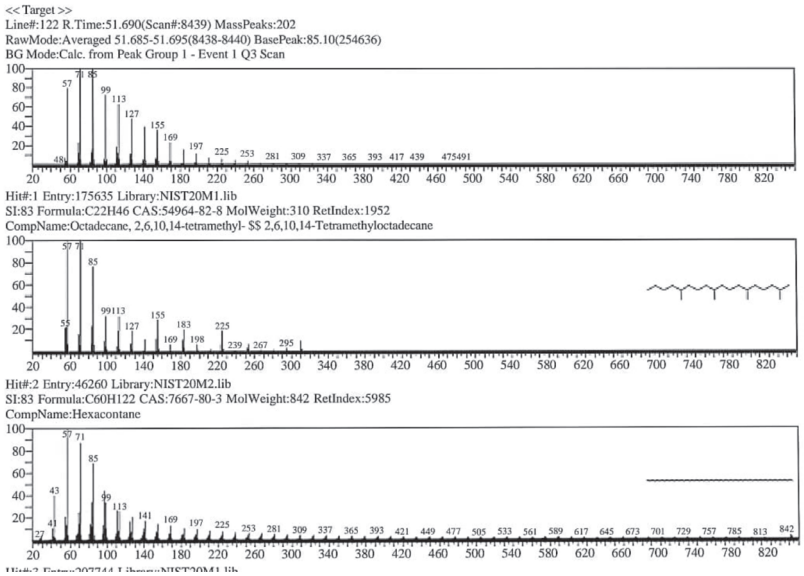

Fig. 4. Analysis of GC-MS extracted bio-surfactant produced by *P. dispersa*

(1949) characterized the rhamnolipids of the bio-surfactants. They suggested that one or two rhamnose molecules is connected to one or two -hydroxy-decanoic acid molecules in the rhamnolipids produced by *P. aeruginosa*. Many researchers like Rahman *et al*. (2007), Da Rosa *et al.* (2010) Toribio *et al*. (2010) and Saravanan and Vijayaumar (2012) also reported rhamnolipid nature of bio-surfactants and almost all rhamnolipids bio-surfactants were obtained from *Psedomonas aeruginosa*.

A GC-MS analysis of bio-surfactants produced by *P. dispersa* was carried out to further support the findings of this investigation and composition of the bio-surfactants was identified. GCMS of bio-surfactants produced by three major peaks *i.e*., Eicosane, Hexacontane, Octadecane2,6,10,14tetramethyl were identified in the GCMS analysis of bio-surfactants produced by *P. dispersa* through the NIST library. Bhardwaj *et al.* (2013) identified the hydroxyl acid methyl ester which is liberated by the methanolysis of the sophorolipids produced by *Candida bombicola*.

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 above bacterial strain as alternative technology for effective and ecofriendly way for the degradation of PHCs. In the present study glycolipid biosurfactant production from indigenous *Pantoea dispersa* isolated from hydrocarbon contaminated soil was reported. The Bioemulsifier demonstrated higher emulsifying activity in the presence of hydrocarbon diesel oil this bio emulsifiersre presents a good candidate for various industrial and environmental applications, especially in the remediation of oil-contaminated soil.

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Conflict of Interest: None

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