International Journal of Environment and Health Sciences (IJEHS)
2022, VOL. 4, Issue 3, 11-20
https://doi.org/10.47062/1190.0403.03

ISOLATION, IDENTIFICATION AND MOLECULAR CHARACTERIZATION OF CRUDE OIL DEGRADING NOVEL *PSEUDOMONAS AERUGINOSA* STRAIN MR21 FROM OIL POLLUTED MUMBAI HARBOR

Rajamani Mohanram[1&2], C. B. Jagtap[1], Murlidhar J. Mendki[1] and Susan Titus[1]

[1]Department of Marine Biotechnology, Naval Materials Research Laboratory, DRDO, Ambernath 421506, India.
[2]Defence Bioengineering and Electromedical Laboratory, DRDO, Bangalore 560093, India.

Received on: 20.02.2023 **Revised on:** 13.03.2023 **Accepted on:** 25.03.2023

Abstract
Marine oil spill affects the marine flora and fauna to a greater extent in the marine ecosystem. Many marine bacteria have the ability to degrade petroleum hydrocarbons by producing biosurfactants and alkane hydroxylase enzymes. Biosurfactant enhances bioavailability of crude oil increasing the dispersion of hydrocarbons in the water by expanding the contact surface area due to emulsification. In this study, we have isolated and characterized crude oil-degrading *Pseudomonas aeruginosa* from oil-polluted Mumbai harbor water. The biosurfactant-producing ability of the strain MR21 was tested by different biosurfactant screening methods like the blue agar method, hemolysis, drop collapse assay, oil displacement method, surface tension reduction, and emulsification index. The strain MR21 showed a positive response for all these methods. Biochemical characterization indicated that this strain is able to hydrolyze casein and Tween 80 by producing proteases and lipases. The gas chromatography analysis indicated complete degradation of crude oil by strain MR21 after 30 days. The catabolic genes responsible for alkane degradation such as alkane hydroxylase gene (*alkB*) and flavin-binding monooxygenase gene (*almA*) were detected in this strain MR21 by polymerase chain reaction. This strain could be useful for bioremediation of marine oil spills in the environment.

Keywords
Biosurfactant, alkane hydroxylase, flavin-binding monooxygenase, proteases, lipases, emulsification.

1. INTRODUCTION

Man depends on petroleum hydrocarbons as the main source of energy for transportation and industry. Maritime transport of crude oil throughout the world by cargo is indispensable because many non oil producing countries like India imports crude oils from oil producing countries like Saudi Arabia and Iran. Oil spill caused by the accidents of oil carrying cargos are frequently happening throughout the world. Crude oil spills into the marine environment causes serious ecological damage to coastal fauna and flora. Crude oil is chemically complex in nature they are separated into saturates, aromatics, resins and asphaltenes [1]. Crude oil immediately undergoes transformation of its constituents when it's released into the marine environment. Microbial degradation is considered to be a major route for the breakdown of hydrocarbon components in the marine environment [2].

In 1946, Zobell recognized that many microorganisms have the ability to utilize hydrocarbons as a source of energy. After the supertanker Torrey Canyon sank in the English Channel, it triggered the scientific community's attention toward the problem of oil pollution in marine environment [3]. Diverse bacterial species capable of degrading crude oil have been isolated and characterized from oil polluted marine environment. Among the crude oil-degrading bacterial species the genus *Pseudomonas* plays an important role in degradation of spilled oil in sea. Oil degrading microorganisms have different mechanisms for uptake of these hydrocarbon molecules and diverse catabolic pathways to degrade hydrocarbons [4]. Bacteria have developed different hydrocarbon accession mechanisms to metabolize hydrocarbons, which include direct contact with these compounds in solid or liquid state, contact with pseudo-solubilized hydrocarbons by biosurfactant excretion and oil-water emulsions [5].

Biosurfactants are amphiphilic molecules that accumulate at interfaces, decrease interfacial tensions and form aggregate

CONTACT *Corresponding author: *mohanmarinemicrobiol@gmail.com*
Color versions of one or more of the figures in this article can be found online at www.stenvironment.org

structures called micelles. Bacteria produce wide variety of high and low molecular weight Surface Active Agents (SAAS). The low molecular weight SAAS called biosurfactants and the higher molecular weight SAAS called bioemulsifiers. The best examples for low molecular weight biosurfactants are rhamnolipids produced by *Pseudomonas aeruginosa*. Rhamnolipids are more effective in lowering the interfacial and surface tension [6]. The genus *Pseudomonas* has different species producing diverse biosurfactant molecules [7-8].

Alkanes are a major fraction (>50%) of the crude oil. The ability of bacteria to degrade alkanes is generally determined by the presence of enzyme alkane hydroxylase which catalyze the initial step in the degradation of alkane. These alkane hydroxylase are widely present in gram-positive and gram-negative oil degrading bacteria and the encoding genes are usually denoted as *alkB* genes [9]. The alkane hydroxylase genes present in bacteria are classified into three groups based on phylogenetic analysis. The group (I) alkane hydroxylase encoded by *alkB1* catalyze the degradation of short chain n-alkanes (C_6-C_{12}). The groups (II) alkane hydroxylase encoded by *alkB2* catalyze the degradation of medium chain n-alkanes (C_8-C_{16}). The group (III) alkane hydroxylase encoded by *alkB3* catalyze the degradation of long chain n-alkanes (>C_{16}) [10].

Many long chain n-alkane degrading bacteria such as *Acinetobacter* and *Alcanivorax* genera produces an enzyme called flavin-binding monoxygenase which is responsible for long chain n-alkane degradation. The gene encoding for this flavin-binding monoxygenase named as *almA* gene [11]. In this study we have isolated and characterized a crude oil degrading *Pseudomonas aeruginosa* strain MR21 from oil polluted Mumbai harbor water and analyzed the presence of the alkane degrading genes in them.

2. MATERIALS AND METHODS

2.1 Sampling

Sea water sample was collected from Gateway of India-(GI) (18.921836°N 72.834705°E) in Arabian Sea near Mumbai. The samples were collected from the depth of 15cm in sterile 100 ml bottles and transported within 4 hours on ice to the Laboratory at Ambernath for isolation of crude oil degrading bacteria.

2.2. Isolation of crude oil degrading bacteria by enrichment culture method:

Modified Bushnell-Hass broth (MBHB) was used for isolation of biosurfactant producing bacteria. The chemical components of MBHB are (gl^{-1}) 1 g of KH_2PO_4, 0.2 g of K_2HPO_4, 0.2 g of $MgSO_4.7H_2O$, 0.02g of $CaCl_2$, 1g of NH_4NO_3, and 2 droplets of $FeCl_3$ 60% and 30g of NaCl. The pH was adjusted to 8.2 and autoclaved at 121^{0}C for 15 minutes. After the MBHM was cooled down to 40^{o}C, it was supplemented with 1% crude oil (v/v) (Bharat Petroleum Corporation, India) as the sole carbon and energy source. For solid modified Bushnell-Hass agar medium (MBHA) Agar, (Himedia, India) ($20g/l^{-1}$) was added to the solution [12]. 1ml of oil polluted seawater collected from Gateway of India in Arabian Sea near Mumbai, was added to 250ml Erlenmeyer flasks containing 50ml of MBHB & 1% crude oil (v/v) as carbon source and the flasks were incubated for 7 days at 30^{o}C on a orbital shaker (Orbitek Shaker, Scigenics, India) operating at 180 rpm. After a series of four subcultures, inoculum from the MBHB with 1% crude oil were streaked out to MBHM agar containing 1% (v/v) crude oil and phenotypically different colonies isolated on MBHM agar medium. One strain, which exhibited fast growth rate on crude oil was purified and designated as MR21 and stored in glycerol stock at -80 ^{0}C for further characterization [13].

2.3 Morphological and Biochemical characterisation of strain MR21

The morphological characterizations of the strain MR21 was carried out by Gram staining and hanging drop method. The colony characterisation was carried in *Pseudomonas* agar (fluorescein) media. In order to characterize the biochemical enzyme profile of the strain biochemical tests were carried out such as utilization of pure alkanes like hexadecane, tetradecane, octadecane, pristine, eicosane and utilization of polyaromatic hydrocarbon (PAH) like naphthalene, hydrolysis of casein, tween 80 and carbohydrate fermentations tests.These tests were done according to the Bergey's manual for identification taxonomy [14].

2.4 Biodegradation of hydrocarbons by DCPIP assay:

The conical flask containing 100 ml of Modified Bushnell Haas Broth (MBHB) with 1% diesel as carbon source and 1% 2, 6- dichloro phenol indophenol (DCPIP) redox indicator was inoculated with strain MR21 and incubated at 37°C on a rotary shaker The color change of MBHB containing DCPIP from blue to colorless indicated the biodegradation of hydrocarbon by bacterium. The bacterium which decolorizes DCPIP within 48 hours is considered to be efficient in degrading oil [15].

2.5 Biosurfactant screening methods for strain MR21

The strain MR21 was screened for their SAA-producing ability with nine different methods. The MR21 was grown in 500ml Erlenmeyer flasks with 100 ml of MBHB containing 1% (v/v) HSD as the carbon source. Flasks containing sterilized MBHB with 1% (v/v) HSD were inoculated with a loop full of bacterial culture grown in HSD containing marine agar plates, and the culture flasks were incubated in a rotary shaker for 3 days at 180 rpm and 37°C. After 3 days of incubation, the culture broth from each flask was centrifuged at 6000 rpm and 4°C for 15 min, and the supernatant was passed through a membrane filter paper of 0.22-µm pore size (Millipore). This cell-free culture broth was used to perform the drop-collapse assay, oil spreading assay, microplate assay, penetration assay, stable emulsification assay, and measurement of reduction in surface tension. The bacterial cells were used to perform the blue agar method, hemolytic assay, and bacterial adhesion to hydrocarbon (BATH) assay. All screening experiments were performed in triplicate, and the mean values were used as results. These tests were carried out as described below.

2.6 Blue agar plate method

The MBHM agar medium supplemented with 20 g l^{-1} hexadecane or diesel as carbon sources and cetyl trimethyl ammonium bromide 0.2 g l^{-1}, methylene blue 0.005 g l^{-1} were prepared. Single colony of MR21 was inoculated at the centre of blue agar plate and incubated at 37^{o}C for 24 hrs. A dark halo

around the colony was considered as positive for biosurfactant production. Biosurfactants that belongs to anionic class like rhamnolipids are detected by this method [16].

2.7 Hemolytic assay

The isolated single colony of strain MR21 was inoculated on Zobell marine agar (Himedia, India) containing 5% (v/v) blood and incubated at 37^{0}C for 24-48 hours. Hemolytic activity by bacterial culture was detected by clear halo zone around the colonies [12]

2. 8 Oil spreading assay

In 100mm glass Petri dishes (Borsosil, India) 40 ml of distilled water was added followed by addition of 100 µl of crude oil to the surface of the water, Then 10 µl of the cell free culture filtrates was added on the centre of the crude oil surface. The diameter of the clear zone formed by the oil displacement was measured [17].

2.9 Drop collapse assay

The drop collapse technique was carried out in the polystyrene lid of a 96-microwell plate (Nunc, USA) with slight modification [18]. The culture supernatant 100 µl was mixed with 5µl of methylene blue and added to the centre of wells of a 96-well microtiter plate lid. Then 5µl of diesel was added to the surface of the culture supernatant. Biosurfactant producing culture gave flat drops. For clear visualization of drop collapse and photographic purpose 5µl of methylene blue was added, which had no influence on the shape of droplets. The tests were carried out in triplicates in three separate microtiter plate lids.

2.10 Microplate assay:

The 100 µl of cell free supernatant of strain MR21 was added to a 96-well microplate (Nunc, USA). The plate was viewed underneath a paper with a grid. If biosurfactant is present in the cell free supernatant, the concave surface distorts the image of the grid below. The optical distortion of the grid provided a qualitative assay for the presence of biosurfactants [19].

2.11 Penetration assay:

Penetration assay was carried out as described in the literature [20]. The principle of the assay is the contacting of two insoluble phases which leads to a color change. The wells of 96-well microplate were filled with a 150 µl of a hydrophobic phase consisting of oil and silica gel. The paste was covered with a 10 µl of oil. Then, the 90 µl supernatant of the strain MR21 was colored by adding 10 µl of a red staining solution. The colored supernatant was placed on the surface of the paste. The presence of biosurfactants in the cell free supernatant facilitates silica entering the hydrophilic phase and the upper phase would change from clear red to cloudy white within 15 min.

2.12 Surface Tension

The surface tension (ST) of the culture supernatants was measured with a digital surface tensiometer (K12–Kruss Tensiometer, Germany) by Wilhelmy plate method [21]. The validity of the surface tension readings was checked with pure double distilled water (72mN/m) before each reading. The surface tension readings were taken in triplicate.

2.13 Emuslification Index

An equal volume of hexadecane was added to the same volume of cell free culture broth, mixed with a vortex vigorously for 2mins and left to stand for 24hrs. The emulsification activity was determined as the percentage of height of the emulsified layer (mm) divided by the total height of the liquid column (mm) [22].

2.14 Bacterial Adhesion to Hydrocarbon (BATH Test)

The strain MR21 was grown at 37°C with shaking at 150 rpm in Zobell marine broth for 24 h. The cells of strain MR21 was harvested at early logarithmic phase and washed twice with and resuspended in phosphate urea magnesium sulphate buffer (PUM). The chemical components of PUM buffer pH 7.1, are (g l-^{1}) 22.2 g of K_2HPO_4 $3H_2O$, 7.26 g of KH_2PO_4, 1.8 g of urea, 0.2g of $MgSO_4.7H_2O$. Bacterial cell suspensions were prepared in PUM buffer, 1.2 ml of suspension dispensed into 10 mm round bottom test tubes and hexadecane 0.2 ml was added to it. Following 10 min pre-incubation at 30°C, the tubes were vortexed uniformly on a vortex mixer (Mo Bio Laboratories, USA) for 2 min. After 15 min incubation the hydrocarbon phase accumulated at the upper phase, the aqueous phase was carefully removed with a micropipette and transferred to a 1 ml cuvette. The turbidity of the aqueous phase was determined at 400 nm using multimode microplate reader (Biotek Laboratories, Germany), before and after treatment. Results were recorded as the percentage absorbance of the aqueous phase after treatment relative to the initial absorbance of the bacterial suspension [23].

2.15 Determination of biodegradation of crude oil (ASTM) by *P. aeruginosa* strain MR21

The crude oil biodegradation potential of strain MR21 was quantitatively determined using crude oil quantitative mixture (ASTM-D5307). The biodegradation was carried in 500ml capacity conical flask containing 100ml of MBHB with 1% (v/v) crude oil quantitative mixture (ASTM-D5307) with 1% MR21 inoculum. The test and control were incubated for 30 days at 37 ^{0}C in orbital shaker (180RPM). After 30 days to determine the biodegradation rate the test and control flasks were extracted with equal volume of hexane thrice. The extracts were concentrated by rotary evaporator (Rotavapor – R110, Buchi, Switzerland). The residue was dissolved in 10ml of HPLC grade hexane and analysed by GC-FID[24].

2.16 Molecular identification of crude oil degrading strain MR21

The bacterial strain MR21 was identified by analysis of 16S rRNA for the taxonomic characterization. Total DNA extraction of bacterial strain MR21 was performed using a Ultraclean Microbial DNA isolation kit (Mo Bio Laboratories, USA). The complete bacterial 16S rRNA gene 1.5kb fragment was amplified by PCR from genomic DNA of strain MR21 using bacterial 16S rRNA gene universal forward primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and universal reverse 1492R (5'- ACGGCTACCTTGTTACGACTT-3') and species specific *P. aeruginosa* primers PA-SS-F and PA-SS-R [25]. The polymerase chain reaction (PCR)

amplification was performed in the total volume of 25µl in 200µl capacity thin wall PCR tube consisting of 7.5 µl of PCR grade water, 12.5 µl of 2X PCR master mix (MBI, Fermentas), 1 µl (10 pm) of each primer, and 3 µl of template DNA (50 pm).

Amplification was performed in a thermal cycler Mastercycler Epgradient PCR machine (Eppendorf, Hamburg, Germany). The PCR condition was, 94°C for 2 min, 94°C for 0.5 min, 52°C for 0.5min, 72°C for 1.5min, 30cycles and final extension at 72°C for 10 min. The species specific amplification of 16S rRNA for *P. aeruginosa* was performed as per recommended conditions of species specific *P. aeruginosa* primer[25]. The amplified 16S rRNA PCR product was purified using Invitrogen Pure Link Quick Gel Extraction and PCR Purification Combo Kit. The purified PCR products was sequenced using BigDye® Terminator V3.1 Cycle sequencing kit on automated capillary sequencer (ABI 3730xl Genetic Analyzer, Applied Biosystems). Partial 16S rRNA gene sequences were initially compared with sequences in the GenBank database using BLASTN [available at http://blast.ncbi.nlm.nih.gov] to identify their approximate phylogenetic affiliation and closest relatives. The 16S rRNA sequences of strain MR21was aligned with closest GenBank matches using CLUSTALW [26]. A phylogenetic tree was constructed using 1200 bp long aligned sequences with the neighbor-joining algorithm (P-Distance Method) in Molecular Evolutionary Genetics Analysis 2.1 software (MEGA, version 5) [27]. Bootstrapping was used to estimate reliability of the phylogenetic reconstructions (500 replicates).

2.17 Detection of alkane hydroxylase (*alkB*), flavin binding monoxygenase (*almA*) and rhamnosyl transferase I (*rhlB*) genes in strain MR21 by PCR:
The purified DNA of MR21 was screened to detect two catabolic genes that encode enzymes involved in alkane degradation pathway alkane hydroxylase gene *alkB* and flavin binding monoxynase gene *almA*. The *alkB* gene was amplified using two different reported primer sets (Mon F401 & MonR820) and *alkBf* and *alkBr* [28-29]. The *almA* gene was amplified using reported degenerate primer specific for *almA* (*alm*Adf & *almAdf*) [11]. The gene coding for rhamnosyl transferase I (*rhlB*) was detected in strain MR21 with *rhlB* specific primers KPD1 and KPD2 as per reported PCR condition [30].

3. RESULTS

3.1 Isolation, identification and characterisation of MR21

The bacterium was isolated by enrichment in the MBHB media with 1% crude oil. The morphological characterisation by Gram's staining and hanging drop method showed MR21 as motile rods (Figure. 1 & 2). The green fluorescent colonies were observed on *Pseudomonas* fluorescein agar. The isolated MR21 strain was able to utilize the different types of hydrocarbons. The hydrolytic enzyme assay revealed the production of hydrolytic enzymes like protease and lipase. The carbohydrate fermentation test showed that only few sugars like glucose, xylose and mannose were utilized by MR21 (Table1).

Table 1: Biochemical characterization of strain *Pseudomonas aeruginosa* MR21.

S. No	Biochemical Test	Results	S. No	Biochemical Test	Results
1	Gram staining	Gram –ve rods	30	Casein Hydrolysis	+
2	Motility	Motile	31	Tween 80 hydrolysis	+
3	Growth on Crude Oil	+		Carbohydrate Fermentation	
4	Growth on Diesel	+	32	Glucose	+
5	Growth on Kerosene	+	33	Mannitol	-
6	Growth on Petrol	+	34	Xylose	+
7	Growth on Lubricating oil	+	35	Inositol	-
8	Growth on Tetradecane	+	36	Sorbitol	-
9	Growth on Hexadecane	+	37	Rhamnose	-
10	Growth on Octadecane	+	38	Sucrose	-
11	Growth on Pristane	+	39	Lactose	-
12	Growth on Eicosane	+	40	Arabinose	
13	Growth on naphthalene	+	41	Adonitol	-
14	Growth on Sodium succinate	+	43	Salicin	-
15	Growth in the absence of sodium chloride	+	44	Maltose	-
16	Optimum temperature for Growth	30-45oC	45	Fructose	-
17	Pigment production	Pyoverdine	46	Galactose	-
18	Indole	-	47	Melibiose	-

19	Voges-Proskauer	-	48	Inulin	-
20	Citrate utilization	+	49	Sodium gluconate	-
21	Lysine utilization	-	50	Glycerol	-
22	Ornithine utilization	-	51	Dulcitol	-
23	Arginine utilization	+	52	Arabitol	
24	Nitrate reduction	-	53	Erythritol	
25	Malonate	+	54	Cellobiose	-
26	Urease	-	55	Melezitose	
27	Phenylalanine deamination	-	56	Raffinose	
28	H2S Production	-	56	Mannose	+
29	ONPG	-	57	Esculin hydrolysis	+

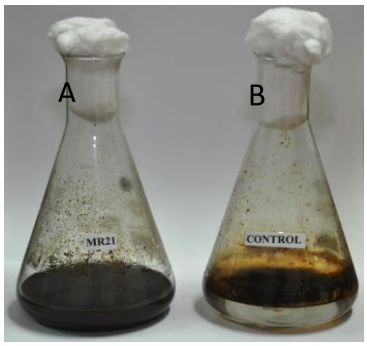


Figure 1. Enrichment of the Pseudomonas aeruginosa MR21 in crude oil.

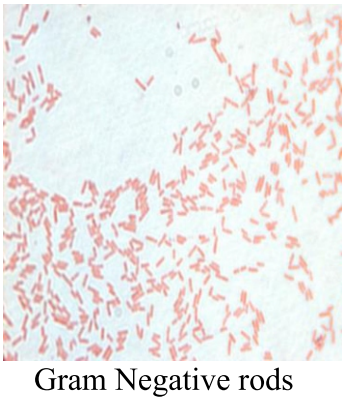


Figure 2. Gram's staining of Pseudomonas aeruginosa MR21.

3.2 DCPIP assay for hydrocarbon degradation:

The strain MR21 was subjected to hydrocarbon degradation by DCPIP assay. The assay showed the change in colour from blue to colourless within 48 hrs, indicating the utilisation/degradation of diesel as source of carbon (Figure 3a).

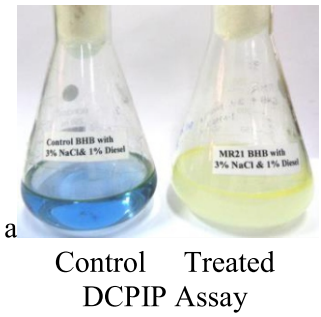


Fig.. 3: DCPIP assay for oil degradation by Pseudomonas aeruginosa MR21.

3.3 Biosurfactant screening of strain MR21:

The biosurfactant property was tested by various assays such as blue agar, hemolytic, drop-collapse, oil spreading, microplate, penetration, stable emulsification, ST measurement and BATH. The blue agar assay showed dark blue clear halo around the colonies, the hemolytic assay exhibited the zone of beta-hemolysis around the colonies. The MR21 showed positive results for the remaining assays as shown in Table 2 & Figure. 3b & c.

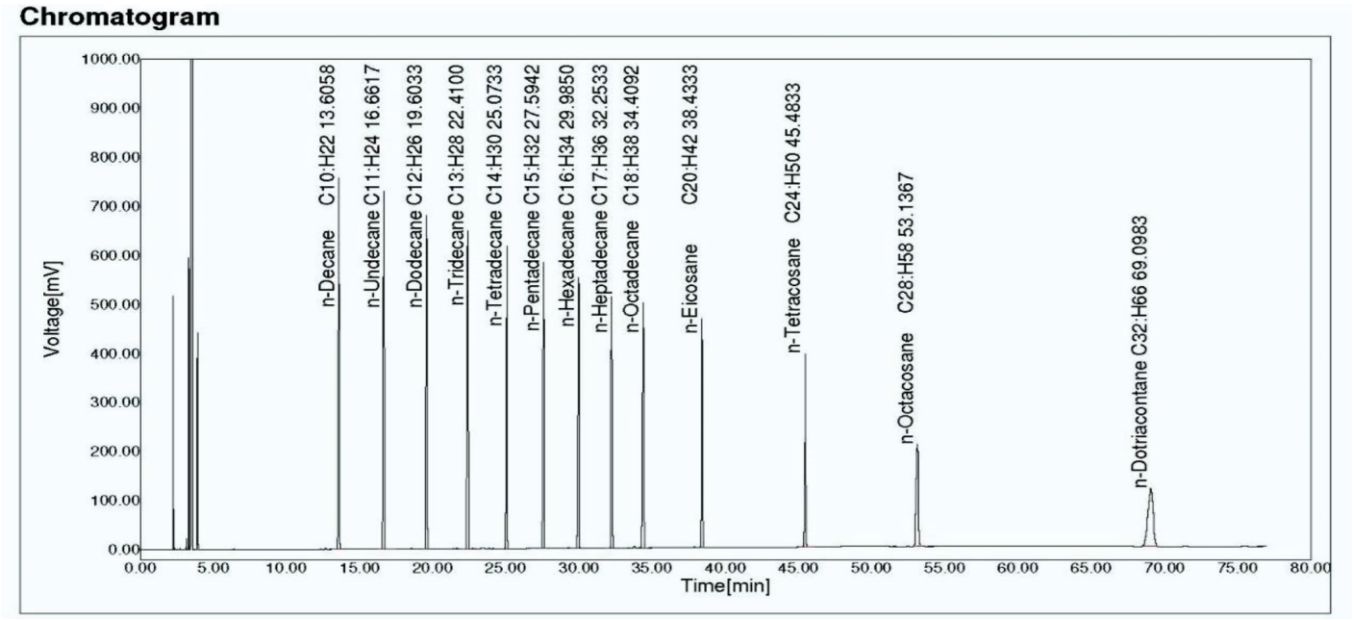
Blue agar plate	Hemolytic Test	Drop Collapse	Oil displacement (mm)	Microplate assay	Penetration assay	Surface Tension (mN/m)	BATH (%)	E24 (%)
+	+	+	48	+	+	28.2	65	70

Abbreviations: +, Positive response; -, Negative response

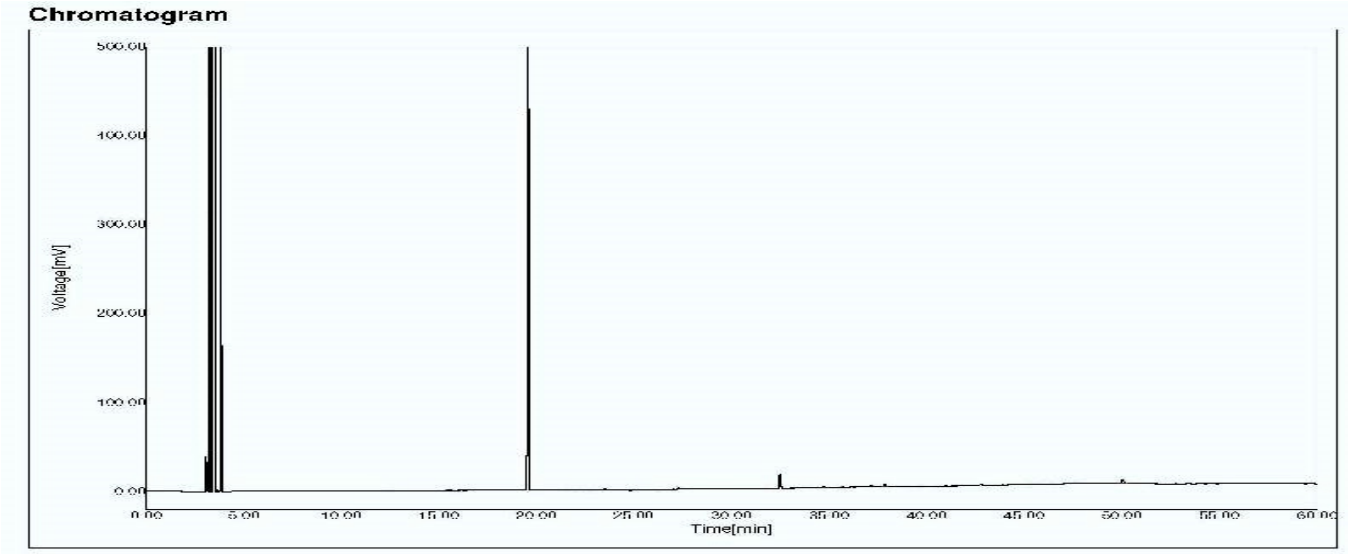
3.4 Determination of biodegradation of crude oil (ASTM) by *P. aeruginosa* strain MR21

The biodegradation of the crude oil was analysed by GC-FID showing the complete disappearance of the chromatogram peaks of the hydrocarbons of the crude oil ranging from C_{10} to C_{32}. This clearly indicated the complete biodegradation property of the MR21 strain. (Figure. 4a & b).

Fig. 4: GC -FID analysis of biodegradation of Crude Oil Quantitative Standard Mixture (ASTM-D5309) after 30 Days.



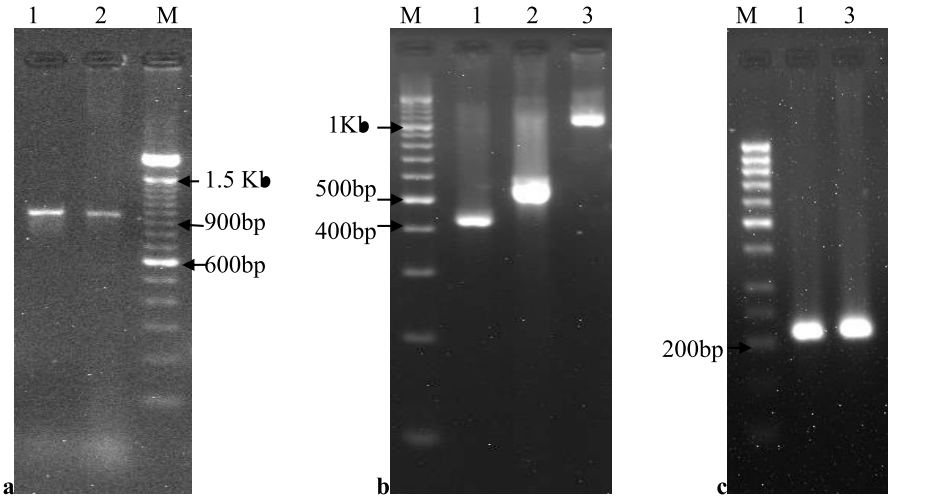
(a) Control



(a) Treatment

3.5 Molecular identification of MR21 strain:

The molecular identification was carried by the PCR amplification of universal 16S rRNA gene and species specific primers. The universal 16S rRNA gene amplification resulted in ~1500bp product and subsequently sequence characterisation revealed the homology with *P. aeruginosa* type strain (Figure 6a). The *Pseudomonas aeruginosa* strain MR 21 16S rRNA ribosomal RNA gene nucleotide was deposited in the GenBank database at the National Center for Biotechnology Information (NCBI), USA. The nucleotide accession numbers obtained was KY651215. This was further confirmed by *P. aeruginosa* species specific primers with amplification of 956bp PCR product (Figure 5a).



a	b	c
M-DNA ladder	M- DNA Ladder	M- DNA Ladder
Lane 1- ATCC10415	Lane 1- 420bp *alkB2* Gene	Lane 1- ATCC10415
Lane 2- MR21	Lane 2- 550bp *alkB1* Gene	Lane 2- MR21
Lane 1 & 2-956bp	Lane 3- 1100bp *AlmA* Gene	Lane 1&2- 226bp

Figure 5. (a) Molecular characterisation of *Pseudomonas aeruginosa MR21by P. aeruginosa species specific primer, (b) PCR amplification of alk1, alkB2 and AlmA Gene in P. aeruginosa strain MR21, (c) PCR amplification of* rhamnosyl transferase I (*rhlB*) in strain MR21 by PCR

3.6 Detection of alkane hydroxylase (*alkB*) gene, flavin binding monoxygenase gene (*almA*) and rhamnosyl transferase I (*rhlB*) in strain MR21 by PCR:

The bacterial DNA of MR21 was subjected for the detection of various genes responsible for the hydrocarbon degradation and biosurfactant production. The PCR amplification with two different *alkB* primer sets showed 420 and 550 bp products specific for the alkane hydroxylase gene. The PCR amplification of flavin binding monoxygenase specific gene (*almA*) resulted in 1100bp species specific product (Figure 5b). The gene coding for biosurfactant rhamnopilid was detected by amplification of *rhlB* gene of 226 PCR product (Fig 5c). The analysis of sequence characterisation has been described in Fig. 6. The PCR products of *alkB2* and *almA* were sequenced and their phylogenetic relationship with other reported gene was analysed with MEGA5. Both the genes were found clustered with reported *alkB /almA* genes of *P. aeruginosa* species (Figure 6b & c). The nucleotide sequence of *alkB2* and *almA* gene were deposited in the GenBank database at the National Center for Biotechnology Information (NCBI), USA. The nucleotide accession numbers obtained were LC179843 (*alkB2*) and LC179844 (*almA*).

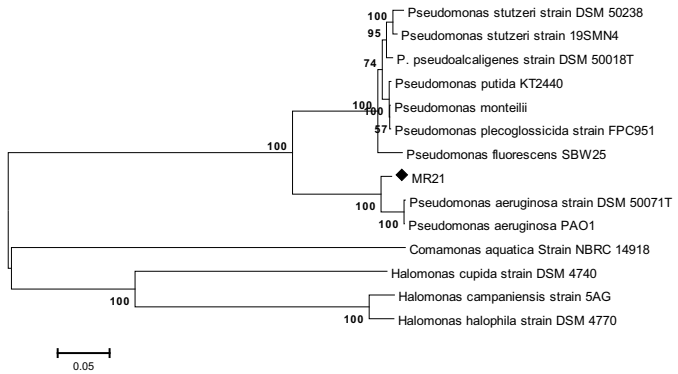
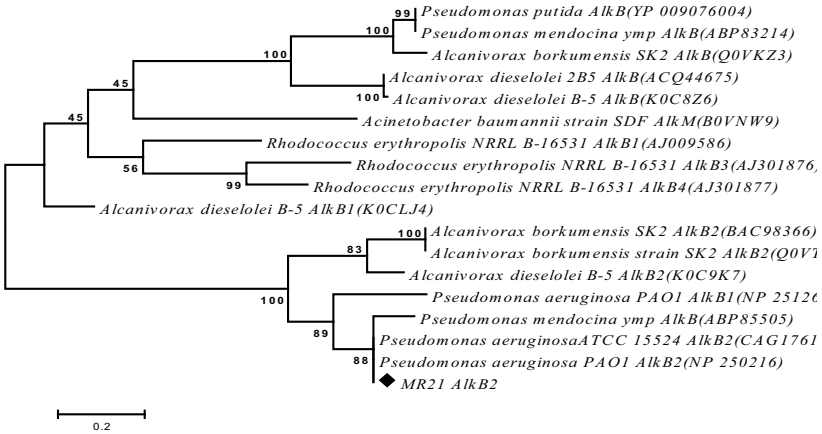
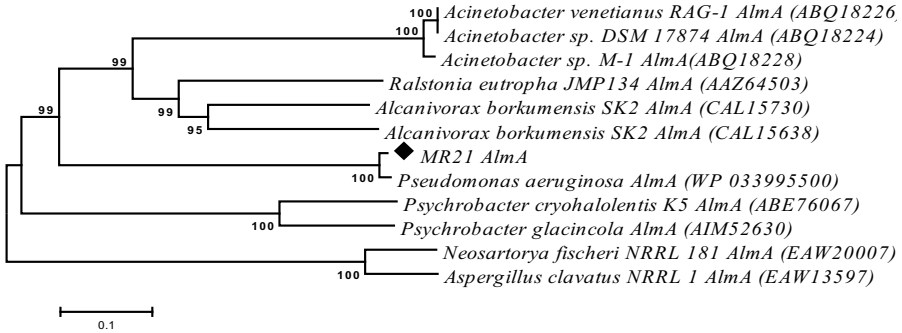


Figure 6: Evolutionary relationship of strain *P. aeruginosa* Mr21
a. Phylogenetic relationship of MR21 with related taxa based on 16S rRNA gene sequences. The evolutionary history was inferred using the Neighbor-Joining method in MEGA5. Bootstrap values (expressed as percentage of 500 replications) are shown at branch points. Bar indicates number of base differences per site.



b. Phylogenetic relationship of protein sequences for the partial alkane hydroxylase, AlkB (120 aa in total) from strain MR21 and other bacteria. The alignment was carried out with ClustalW and tree was generated with MEGA 5. Scale Bar, 0.2 substitutions per amino acid site.



c. Phylogenetic relationship of protein sequences for the partial flavin-binding monooxygenase, AlmA (333 aa in total) from strain MR21 and other bacteria. Scale Bar, 0.1 substitutions per amino acid site.

4. DISCUSSION

The *P. aeruginosa* MR21 strain a new marine isolate from Mumbai harbor, India was able to degrade medium and long chain alkanes (C_{12}–C_{32}), present in crude oil. In present study we have observed complete degradation (100%) of the wide range of alkanes where as the *P. aeruginosa*-AS, isolated from Caspian Sea has ability to degrade 12–100% of alkanes ranging from C_9-C_{25}. As compared to MR21 strain, the *P. aeruginosa*-AS has low oil degradation capability for medium and long chain alkanes [10]. Based on the 16S rRNA sequence alignment and phenotypic analysis it was understood that the MR21 strain is homologus with type strain *P. aeruginosa* DSM-50071T and PAO-1. The biosurfactant (rhamnolipids) producing property of MR21 was confirmed by blue agar method [16, 31-32]. This was further confirmed by detection of rhamnosyl transferase (*rhlB*) gene, a part of the rhamnolipids biosynthesis pathway unique to *P. aeruginosa* [10]. The oil degradation property of MR21 by DCPIP a redox indicator resulted in the change of blue colour to colourless [15, 33-34]. The biosurfactant produced by MR21 strain showed better oil displacement of 48mm, emulsification index value 70% and surface tension reduction to 28mN/m, than *P. aeruginosa-AS* which showed oil displacement of 21mm, emulsification index value 64% and surface tension reduction to 56mN/m [10].

Molecular characterisation of *P. aeruginosa* revealed presence of *alkB1*,*alkB2* and *almA* genes. The alkane hydroxylase enzyme catalyses the first step in aerobic degradation of alkanes by introducing the oxygen atoms derived from molecular oxygen into the alkane substrates which plays an important role in oil bioremediation. The presence of the *alkB1* and *alkB2* genes are responsible for the degradation of short and medium chain alkanes, while *almA* gene in MR21 is responsible for degradation of long chain alkanes [10-11]. The presence of these multiple alkane monoxygenase genes in the MR21 strain corroborates the efficient degradation of crude oil as confirmed by the GC-FID analysis. The present study revealed that the *P. aeruginosa* MR21 strain is a potential microorganism for bioremediation of oil spills in marine environment.

5. CONCLUSION

A crude oil-degrading strain, *P.aeruginosa* MR21 was isolated from oil polluted Mumbai Harbor efficiently degraded all major alkanes present in the crude oil. The multiple alkane degrading genes present in chromosome of strain MR21 completely metabolizes the alkanes in crude oil. The strain also produced anionic biosurfactant rhamnolipid which increases the bioavailability of crude oil for biodegradation in seawater. Thus, isolating functional microorganisms from oil polluted seawater can provide useful microbial resources for development of effective bioremediation technology for marine oil spills.

ACKNOWLEDGEMENT
The authors thanks Director, NMRL, Ambernath for his keen interest in the work, guidance, providing necessary facilities and encouragement.

CONFLICT OF INTEREST
The authors declare that there is no conflict of interest.

REFERENCES

1. **Harayama S, Kishira H, Kasai Y, Shutsubo K.** Petroleum biodegradation in marine environments. *Journal of Molecular Microbiology and Biotechnol*ogy. 1999; 1:1: 63-70.
2. **Hara A, Syutsubo K, Harayama S.** *Alcanivorax* which prevails in oil-contaminated seawater exhibits broad substrate specificity for alkane degradation. *Environmental Microbiology.* 2003; 5:9: 745-753.
3. **Atlas R M.** Microbial degradation of petroleum hydrocarbons as environmental perspectives. *Microbiology Reviews*.1981; 45:1: 180-209.
4. **Olivera NL, Nievas ML, Lozada M, Del Prado G, Dionisi HM.** Sineriz, F. Isolation and characterization of biosurfactant producing *Alcanivorax* strains: hydrocarbon accession strategies and alkane hydroxylase gene analysis. *Research in Microbiology*. 2009; 160:19-26.
5. **Ron E, Rosenberg E.** Biosurfactants and oil bioremediation. *Current. Opinion in Biotechnology.* 2001; 13:249-252.
6. **VanHamme JD, Singh A, Ward OP.** Surfactants in microbiology and biotechnology: Part 1. Physiological aspects. *Biotechology Advances*. 2006; 24:604–620.
7. **Bonilla M, Olivero C, Corona M, Vazcuez A, Soubs M.** Production and characterisation of new bioemulsifier from *Peudomonas putida* ML2. *Journal of Applied Microbiolology.* 2005; 98:456-463
8. **Hye-Joon M, Lim YY, Kim HS, Kwon DY, Chung WJ**. Glycolipid biosurfactants produced by *Pseudomonas aeruginosa* D2D2 from diesel contaminated soil. *Jounral of Microbiology and Biotechnology.* 2002; 12:3, 371-376.
9. **Smits TH, Rothlisberger M, Witholt B, VanBeilen JB.** Molecular screening for alkane hydroxylase genes in Gram negative and gram positive strains. *Environmental Microbiology.*1999; 1:307-317.
10. **Hassanshahian M, Emtiazi G, Cappello S.** Isolation and characterization of crude-oil-degrading bacteria from the Persian Gulf and the Caspian Sea. *Marine. Pollution Bulletin.*2012; 64: 7-12.
11. **Wang W, Shao Z.** Diversity of flavin-binding mnonooxygenase genes (*almA*) in marine bacteria capable of degrading long chain-alkanes. *FEMS Microbiology Ecology*. 2012; 80:523-533.
12. **Mohanram R, Jagtap C, Pradeep K.** Isolation screening and characterisation of surface active agent producing oil degrading marine bacteria of Mumbai Harbour. *Marine Pollution Bulletin.* 2016; 105:131-138.
13. **Hassanshahian, M.** Isolation and characterization of biosurfactant producing bacteria from Persian Gulf (Bushehr provenance). *Marine Pollution Bulletin*. 2014; 86: 361-366.
14. **Holt SG, Kriey NR, Sneath PHA, Staley JT, Williams ST.** Bergey's manual of determinative for bacteriology. Williams and Wilkins, 1998, New York.
15. **Hanson KG, Desai JD, Desai AJ**. A rapid and simple screening technique for potential crude oil degrading microorganisms. *Biotechnology Techniques.* 1993; 7:10: 745-748.
16. **Satpute SK, Bhawsar BD, Dhakephalkar PK, Chopade BA**. Assessment of different screening methods for selecting biosurfactant producing bacteria. *Indian Journal of Marine Sciences*. 2008; 37:3: 243–250.
17. **Youssef NH, Duncan KE, Nagle DP, Savage KN, Knapp RM, McInerney MJ.** Comparison of methods to detect biosurfactant production by diverse microorganisms. *Journal of Microbiology Methods.* 2004; 56:339-347.
18. **Bodour A, Miller-Maier R.** Application of modified drop collapse technique for surfactant quantitation and screening of biosurfactant producing microorganisms. *Journal of Microbiolpgy Methods*.1998; 32:273-280.
19. **Vaux D, Cottingham M.** Methods and apparatus for measuring surface configuration. 2001. *Patent number WO 2007/0329729 A1*.
20. **Walter V, Syldatk C, Hausmann R.** Biosurfactants: screening concepts for the isolation of biosurfactant producing microorganisms. *Advance in Experimental Medicine Biololgy*. 2010; 672:1-13.
21. **Joshi PA, Shekhawat** D. Screening and isolation of biosurfactant producing bacteria from petroleum contaminated soil. *European Jounral of Experimental Biology*. 2014; 4(4): 164-169.
22 **Panjiar N, Sachan SG, Sachan A.** Screening of bioemulsifier producing microorganisms isolated from oil contaminated sites. *Annals of Microbiol*ogy. 2015; 65:2: 753-764.
23. **Rosenberg M, Gutnick D, Rosenberg E.** Adherence of bacteria to hydrocarbons: a simple method for measuring cell surface hydrophobicity. *FEMS Microbiology Letters.* 1980; **9**:29–33.
24. **Wongsa P, Tanaka M, Ueno A, Hasanuzzaman M, Yumoto I, Okuyama H.** Isolation and characterisation of novel strain of *Pseudomonas seruginosa and Serratia marcescens* possessing high efficiency to degrade, kerosene, diesel oil, and lubricating oil. *Current Microbiology*. 2004; 49:514-422.
25. **Spilker T, CoenyeT, Vandamme P, LiPuma J J.** PCR-based assay for differentiation of *Pseudomonas aeruginosa* from other *Pseudomonas* species recovered from cystic fIrbrosis patients. *Journal of Clinical Microbiology*. 2004; 42:5: 2074 - 2079.
26. **Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, Valentin F, Wallaee**

IM, Wilm A, Lopez R, Thompson JD, Gibson TJ, Higgins DG. Clustal W and Clustal X version 2. *Bioinformatics*.2007; 23**:**21: 2947-2948.

27. **Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S.** MEGA5: Molecular Evolutionary Genetics Analysis using Maximum Likelihood, Evolutionary distance, and Maximum Parsimony methods. *Molecular Biology and Evolution*. 2011; 28: 2731-2739.

28**. Liu C, Shao Z.** *Alcanivorx dieseloli* sp. nov., a novel alkane-degrading bacterium isolated from sea water and deep sea sediments. *International Journal of Systematic and Evolutionary Microbiology*. 2005; 55**:** 1181-1186.

29. **Bak F, Bonnichsen L, Jorgensen NOG, Nicolaisen HM, Nybore O.** The microbial surfactant viscosin transiently stimulated n-hexadecane mineralisation by a bacterial consortium. *Applied Microbiology and Biotechnology*. 2015; 99:1475-1483.

30. **Bodour AA, Drees KP, Maier MR.** Distribution of biosurfactant-producing bacteria in undisturbed and contaminated arid Southwestern soils. *Applied and Environmental Microbiology*. 2003; 69:6: 3280-3287.

31. **Siegmund I, Wagner F.** New method for detecting rhamnolipids excreted by *Pseudomonas* spp. During growth on mineral agar. *Biotechnology Techniques*. 1991; 5: 265-268.

32. **Rebello S, Asok AK, Joseph SV, Joseph BV, Jose L, Mundayoor S, Jisha MS.** Bioconversion of sodium dodecyl sulphate to rhamnolipids by *Pseudomonas aeruginosa*: a novel and cost-effective production strategy. *Applied Biochemistry and. Biotechnology* 2013. **169**:418-430.

33. **Varjani S J, Upasani VN.** Biodegradation of petroleum hydrocarbons by *Pseudomonas aeruginosa-NCIM 5514. Bioresource. Technology*. 2016; 222:195-201.

34. **Bidoia ED, Montagnolli RN, Lopes RPM.** Microbial biodegradation potential of hydrocarbons evaluated by colorimetric techniques: a case study. *Current Ressearch in Applied Microbiology and Biotechnology. FORMATEX*. 2010; 1277- 1288.