Journal of Advances in Biology & Biotechnology
8(1): 1-9, 2016; Article no.JABB.27376
ISSN: 2394-1081

SCIENCEDOMAIN *international*
www.sciencedomain.org

Diesel Oil Degradation Using Biosurfactant Produced by *Pseudomonas* sp.

Harleen Kaur[1], Samiya Khan[1], Sanjay Gupta[1] and Nidhi Gupta[1*]

[1]Department of Biotechnology, Jaypee Institute of Information Technology, Noida, Uttar Pradesh, India.

Authors' contributions

This work was carried out in collaboration between all authors. Author NG designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors HK, SK, SG and NG managed the analyses of the study. Authors HK and NG managed the literature searches. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/JABB/2016/27376
Editor(s):
(1) Laura Pastorino, Laboratory of Nanobioscience and Medical Informatic, Department Informatics, Bioengineering, Robotics and Systems Engineering (DIBRIS), University of Genoa, Italy.
Reviewers:
(1) Ioana Stanciu, University of Bucharest, Romania.
(2) Wirojne Kanoksilapatham, Silpakorn University, Thailand.
(3) Yordanka Tsankova Tasheva, University "Prof. Dr. A. Zlatarov", Burgas , Bulgaria.
Complete Peer review History: http://sciencedomain.org/review-history/15458

Original Research Article

Received 30th May 2016
Accepted 15th July 2016
Published 20th July 2016

ABSTRACT

Aim: To evaluate the effect of biosurfactant produced by *Pseudomonas* sp. GBS.5 on degradation of diesel oil.
Study Design: Shake flask studies were carried out with alkanes and diesel oil.
Place and Duration of Study: Deptt of Biotechnology, Jaypee Institute of Information Technology, Noida, from June 2014 – 2015.
Methodology: Shake flask studies were carried out to study the degradation of alkanes and diesel oil. The degradation was studied using Gas Chromatography.
Results: Biosurfactant was extracted by growing *Pseudomonas* sp. cells on minimal media with oil after 168 hrs. The production was confirmed by various qualitative and quantitative assays. The biosurfactant extracted was used to study the degradation of alkanes (n-tetradecane, n-hexadecane, n-heptadecane, n-docosane) and oil. It was observed that biosurfactant enhanced the degradation rate of all alkanes except n-docosane. Similar effect was also observed on the degradation of alkanes in presence of oil.
Conclusion: The results indicated that the bacterial isolate *Pseudomonas* sp. strain GBS.5 exhibit

**Corresponding author: E-mail: nidhi.gupta@jiit.ac.in, nidhi112000@yahoo.com;*

potential for bioremediation of petroleum-contaminated soils. GC analysis led to the identification and degradation of certain long chain alkanes degraded by *Pseudomonas* sp. *GBS.5* associated with biosurfactant production. Biosurfactants produced by the oil degrading bacteria facilitate the uptake of oil hydrocarbons by bacterial cells thereby improving the degradation of the hydrocarbons which leads to a keen interest on these microbial products as alternatives to chemical surfactants.

Keywords: Alkane; biosurfactant, oil; pseudomonas.

1. INTRODUCTION

The demand of petroleum as an energy source has been increasing with increasing worldwide industrialization. Environmental pollution with petroleum and its products has been recognized as a significant and a serious problem. They are one of the most widespread contaminants in the environment due to their wide distribution, persistence, complex composition and toxicity and are therefore considered to be potentially mutagenic and carcinogenic [1]. Presently, the technologies commonly used for remediation of these petroleum hydrocarbons include evaporation, mechanical burying, dispersion and washing. These remedial measures are not only cost intensive and time consuming, but also laborious, and generally not very effective. Microbial degradation has been recognized as an economic, efficient and versatile alternative by which these contaminants can be removed from the environment. Many microorganisms like *Bacillus subtilis*, *Pseudomonas aeruginosa, Acinetobacter calcoaceticus, Pseudomonas fluorescens, Rhodococcus erythropolis, Bacillus licheniformis* etc. have been reported for oil degradation studies [2]. These oleophilic microorganisms have the capability to utilize oil as their carbon source and energy and further degrade hydrocarbons present in it. However, due to lack of functional group and low water solubility these hydrocarbons require solubilization before being degraded by microbial cells which can be achieved by the use of biosurfactants.

Biosurfactants are diverse group of surface-active molecules. They enhance the oil mobility by increasing the solubility of the water-insoluble organic compounds thereby improving the degradation of the hydrocarbons. Till now it has been seen that the application of biosurfactant is used to enhance the biodegradation of pollutants but there are reports which have demonstrated decreased degradation in presence of biosurfactants due to various reasons like surfactant toxicity, reduce bioavailability of micelle bound hydrocarbon contaminant and by surfactant enhanced contaminant toxicity [3,4].

The present study was taken with a view to elucidate the biosurfactant production and oil degradation ability of *Pseudomonas* sp. GBS.5, a native isolate of our lab reported for the degradation of carbazole [5].

2. MATERIALS AND METHODS

2.1 Chemicals and Culture Media

Crude Oil was purchased from Indian Oil Corporation (IOC) Petrol Pump Sector-24 Noida (U.P., India). Potassium phosphate, disodium hydrogen phosphate, sodium sulphate, potassium chloride, magnesium sulphate, manganese chloride, iron chloride, calcium chloride, carbazole and aliphatic hydrocarbons (hexane, n-tetradecane, n-hexadecane, heptadecane and docosane,), used in degradation studies, were of high purity (96-99%) purchased from Sigma-Aldrich (St Louis, MO, USA). Anthrone reagent, Bradford reagent and other chemicals used were of analytical grade from Qualigen and Hi Media Laboratories (India). Basal salt medium (BSM) was composed of (per liter of media) 2.44 g of KH_2PO_4; 5.57 g of Na_2HPO_4; 2 g of Na_2SO_4; 2 g of KCl; 0.2 g of $MgSO_4$; 0.001 g of $FeCl_3.6H_2O$; 0.02 g of $MnCl_2.4H2O$; 0.003 g of $CaCl_2.2H_2O$ and NH_4Cl (2 g) was added to the BSM as nitrogen source. The pH of the media was 7.0 ± 0.05.

2.2 Bacterial Growth in Presence of Crude Petroleum Oil Hydrocarbons

For growth curve experiment, LB-grown bacterial isolate *Pseudomonas* sp. GBS.5 was inoculated into 100 mL BSM supplemented with 1% oil in 250 mL Erlenmeyer flasks. Culture flask was then incubated at 37℃ and with a shaking speed of 180 rpm in rotary shaker for seven days. To measure cell growth, 2 mL culture was aliquoted at regular time intervals. Uninoculated flask was served as negative control. Bacterial growth was

assessed by measuring the protein concentration in the culture media using Bradford assay [6].

2.3 Extraction of Biosurfactant

The bacterial isolate was inoculated into 100 mL BSM supplemented with 1% oil. Culture flask was incubated at 37℃ at 180 rpm in rotary shaker for seven days. After incubation culture supernatant was precipitated by acidifying with conc. HCl (pH 2) overnight. The precipitate was collected and biosurfactant was extracted using equal volume of chloroform and methanol (2:1). The mixture was then evaporated to dryness and weight of the extract was measured [7]. Pellet was dissolved in water and was further used for biodegradation studies.

2.4 Biodegradation Studies

2.4.1 Biodegradation of alkanes and crude oil

Pseudomonas sp. GBS.5 was inoculated into 100 mL BSM supplemented with 1% alkane and biosurfactant (64 µg/ml) in 250 mL Erlenmeyer flasks. Degradation of n-alkanes, such as n-tetradecane (1%), n-hexadecane (1%), n-heptadecane (1%) and n-docosane in a concentration of 100 mg/l, was examined individually. n-tetradecane, n-hexadecane and n-heptadecane were added directly into the BSM media and n-docosane was dissolved in acetone and then added to the BSM media. In case of oil degradation, 1% oil was added into the media. Culture flask was incubated at 37℃ at 180 rpm in rotary shaker for seven days. For degradation studies, 2 mL culture was aliquoted at regular time intervals, acidified to pH 2 using 2 N HCl. Equal volume (1:1 *v*/*v*) of n-hexane was added. After 24 h, upper n-hexane layer was separated from aqueous phase [8]. Dried sample was then dissolved in n-hexane and used for analysis.

2.5 Analytical Methods

2.5.1 Biosurfactant analysis

Various qualitative and quantitative assays were used to estimate biosurfactant, produced by *Pseudomonas sp.* GBS.5. Samples were prepared by inoculating overnight LB-grown bacterial isolate in to the BSM supplemented with crude oil (1%). Biosurfactant was analyzed using standard test such as drop collapse assay [9], microplate assay [10], emulsification test [11] and anthrone test [12]. As surfactants are well known to reduce surface tension of liquids, these qualitative tests are indicative of surface activity and wetting properties. Further, to calculate the emulsification activity, emulsification assay was performed with n-hexadecane. Emulsification index (E24) was calculated by measuring the percentage of height occupied by the emulsion after 24 h: [%E24 = ($h_{emulsion}/h_{total}$)×100].

2.5.2 Gas chromatography (GC) analysis

Alkane degradation was analyzed using GC (Thermo Scientific 1100) equipped with a flame ionization detector fitted with 30 m TR-5 (5% Phenyl Methylpolysiloxane) capillary column. Nitrogen was the carrier gas with a constant flow rate of 1.5 mL/min. Injector and detector temperatures were 270℃ and 280℃, respectively. The oven temperature program started from 60℃ which further increased to 280℃ at a rate of 22℃/min.

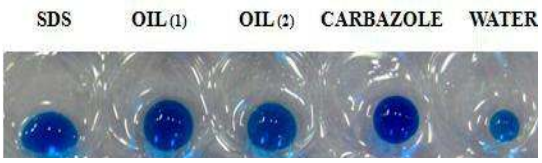
2.5.3 Gas chromatography- mass spectrometry (GC-MS) analysis

Identification of oil hydrocarbons, formed during oil degradation, was carried out using GC-MS (Shimadzu model GC-MS QP2010, Japan) equipped with quadrupole mass analyzer. Helium was the carrier gas /with a constant flow rate of 1.21 mL/min. A 2 µL of derivatized or underivatized sample was injected in splitless mode. For oil metabolite identification, 30 m DB-5 MS capillary column (J&W Scientific, Folsom, CA) was used. The oven temperature program started from 80℃ (3 min isothermal hold) and then was ramped to 280℃ held for 17 min. For the identification of oil hydrocarbons a 60 m Rtx®-5Sil MS capillary column (Restek, Bellefonte, PA) was used. The oven was heated to 280℃ at a rate of 5℃/min, followed by a 10℃/min increment to 320℃ and held for 30 min. Detector and Injector temperatures were 270℃ and 250℃ respectively. Biodegradation of oil was analyzed using GC (Thermo Scientific) equipped with a flame ionization detector fitted with 30 m TR-5 (5% Phenyl Methylpolysiloxane) capillary column. 0.1 µL sample was injected in split less mode. Nitrogen was the carrier gas with a constant flow rate of 1.5 mL/min. Injector and detector temperatures were 270℃ and 280℃, respectively. The oven temperature program started from 60℃ which further increased to 280℃ at a rate of 22℃/min.

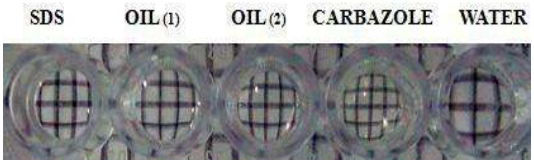
3. RESULTS AND DISCUSSION

3.1 Screening of Biosurfactant Production

The production of biosurfactant, in the presence of crude oil, by *Pseudomonas* sp. GBS.5 was primarily screened by drop collapse and microplate assay (Fig. 1). Emulsification index was calculated after 24 hours, by measuring the height of the stable emulsion layer. (Fig. 2). Emulsification index of 51.61 was obtained after 24 hrs of incubation. Higher the emulsification index, higher will be the emulsification activity of a surfactant. These tests indicate that GBS.5 has the capability of producing biosurfactant in the presence of crude oil. The maximum concentration of biosurfactant produced was 6 µg/ml.



(A)



(B)

Fig. 1. Preliminary screening of biosurfactant from 24 h grown culture of *Pseudomonas* sp. GBS.5; (A) Drop-collapse test (B) Microplate analysis; sodium dodecylsulphate (1%, w/v) and culture supernatant of strain GBS.5 growing in carbazole containing BSM medium. were used as positive controls and Milli-Q water was used as a negative control. Oil (1) and oil (2) represent two different samples. All experiments were performed in triplicates

3.2 Growth Characteristics and Biosurfactant Production

To analyze the relationship between the growth and production of biosurfactant, *Pseudomonas* sp. GBS.5 was inoculated into BSM supplemented media with 1% oil and incubated for 7 days. Fig. 3. shows profile of growth along with biosurfactant production by *Pseudomonas* sp. GBS.5. Biosurfactant production was found to be associated with the growth. Increase in biosurfactant production was seen with increase in growth. No growth and production was observed when bacterial strain reached stationary phase. Maximum growth and production was observed after 140 hrs showing proportional relationship between the growth and biosurfactant production. *Pseudomonas* sp. GBS.5 utilized oil hydrocarbons as their source of carbon and energy which was evident from its protein content after 140 hrs of incubation.

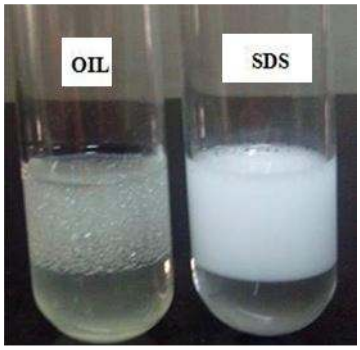


Fig. 2. Emulsification activity from 24 hr grown culture of *Pseudomonas sp.* GBS.5 in oil containing BSM; sodium dodecyl sulfate (1%, *w/v*) was used as positive control

Assay was performed with n-hexadecane. Emulsification index (E24) was calculated by measuring the percentage of height occupied by the emulsion after 24 h: [%E24 = ($h_{emulsion}/h_{total}$)×100]. E24 for SDS and Biosurfactant were 54.8 and 51.6 respectively

3.3 Degradation Studies

3.3.1 Degradation of n-alkanes

Effect of biosurfactant on the degradation of n-alkanes viz. tetradecane ($C_{14}H_{30}$), hexadecane ($C_{16}H_{34}$), heptadecane ($C_{17}H_{36}$) and docosane ($C_{22}H_{46}$) was examined in aqueous media. The biodegradation capability of *Pseudomonas* sp. GBS.5 in the presence of these alkanes as a sole of carbon and energy sources was studied using Gas chromatography. Cell-free controls served as negative controls.

As shown in Fig. 4, the degradation rate of tetradecane (C_{14}), hexadecane (C_{16}), heptadecane (C_{17}), docosane (C_{22}) after seven days of incubation were 64, 82, 63 and 81% respectively and when biosurfactant was added

to the media the rate of degradation increased to 80, 92, 76 and 57% respectively.

During oil spill degradation the short chain alkanes are easily volatilized, however, as the chain length increase the rate of degradation decreases. Metagenomic studies have revealed that the area is inhabited by oil degrading microorganisms but due to decreased bioavailability, no degradation is observed [13]. *n*-Alkanes are immiscible in water and form a layer at the top of the aqueous medium. The low solubility of alkanes in water potentially undermines the capacity of microorganism to degrade them. Biosurfactants are reported to aid in degradation by increasing the bioavailibity of these compounds. However, there are reports which suggest that biosurfactants can have negative impact on the degradation. Some of the mechanisms for the inhibition of microbial degradation at critical micelle concentration levels include surfactant toxicity due to lysis of cell membrane, reduce bioavailability of micelle bound hydrophobic contaminants and surfactant-enhanced contaminant toxicity due to high concentration of biosurfactant.

Biosurfactant produced by *Pseudomonas* sp. showed a positive impact on degradation by increasing the degradation rate of the long chain hydrocarbons. *Pseudomonas* sp. GBS.5 completely utilized n-tetradecane, n-hexadecane and n-heptadecane as its sole source of carbon and energy and degradation increased in the presence of biosurfactant (Fig. 4a, b, c). However it was observed that degradation of n-docosane was slightly slower in presence of biosurfactant (Fig. 4d) than that of which might be due to its higher molecular weight and hydrophobicity. Variation in degradation of n-alkanes is linearly correlated with inverse of carbon chain length [14].

3.3.2 Biodegradation of oil

Crude oil is a complex hydrocarbon mixture of hydrocarbons and alkanes are the major fraction representing 70-80% of it. To examine the oil degradation potential, cells of *Pseudomonas* sp. GBS.5 was grown on BSM media supplemented with 1% oil as sole source of carbon and energy. Effect of biosurfactant on oil hydrocarbon degradation was examined. The rate and extent of biodegradation was interpreted based on the GC chromatograms of the test sample (with and without biosurfactant) for the residual oil extracted with n-hexane. Uninoculated flask served as negative control.

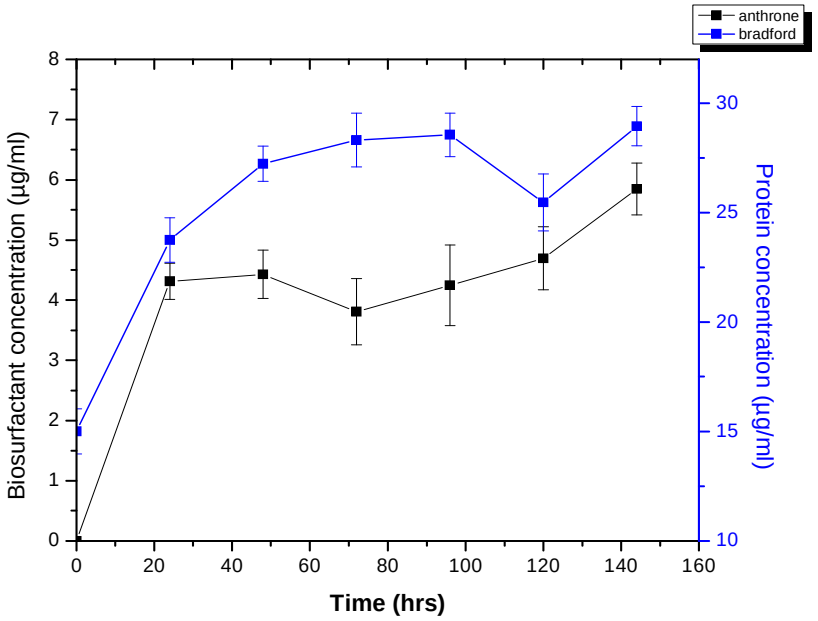
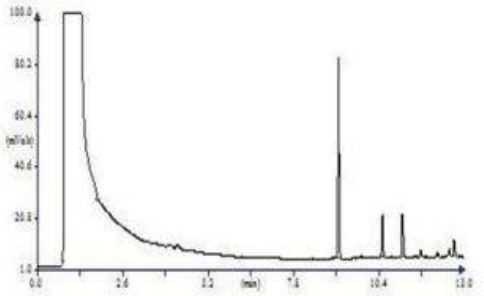


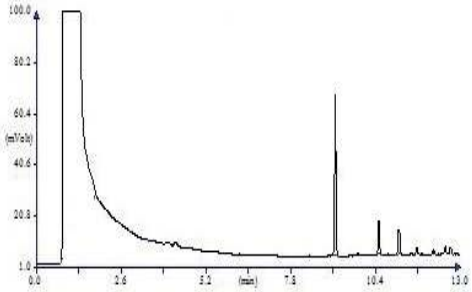
Fig. 3. Time course of increase in protein concentration and biosurfactant production by *Pseudomonas* sp. GBS. 5. The values are means of three independent replicates. SD was within the acceptable ranges

(A) Tetradecane - $C_{14}H_{30}$

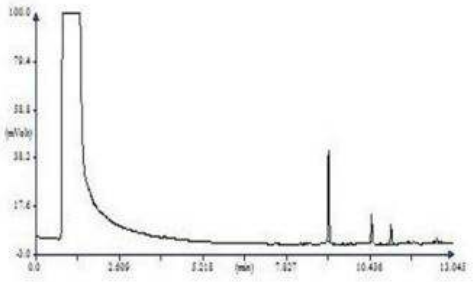
0 hr with biosurfactant



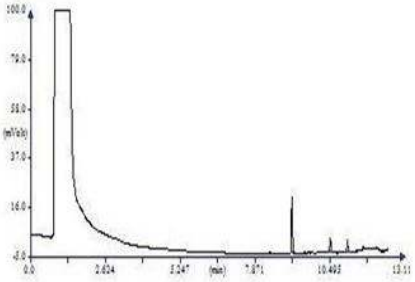
Negative control- 7th day with biosurfactant



7th day without biosurfactant

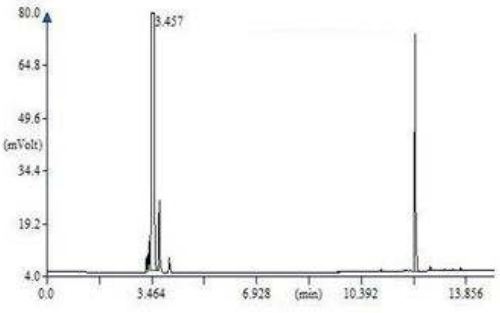


7th day with biosurfactant

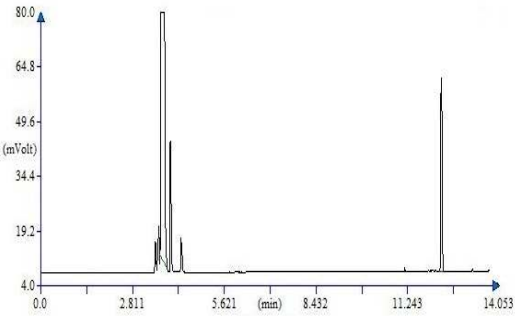


(B) Hexadecane -$C_{16}H_{34}$

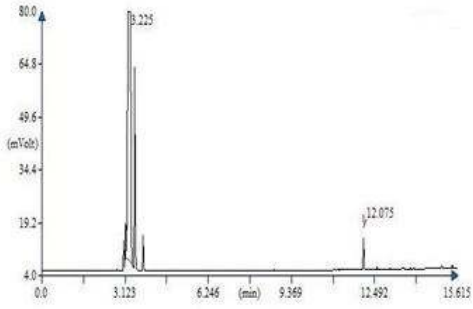
0 hr with biosurfactant



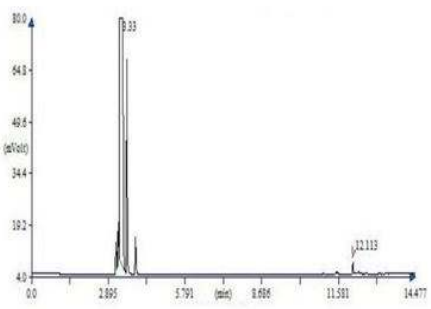
Negative control- 7th day with biosurfactant



7th day without biosurfactant

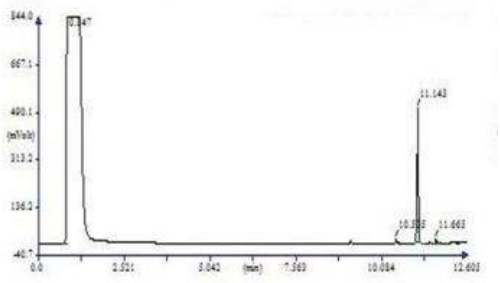


7th day with biosurfactant

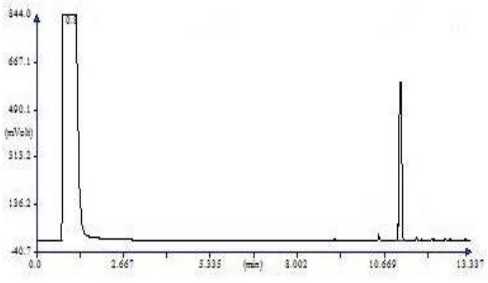


(C) Heptadecane - $C_{17}H_{36}$

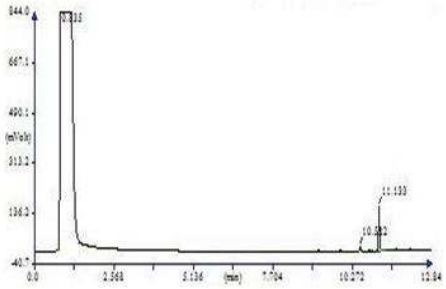
0 hr with biosurfactant



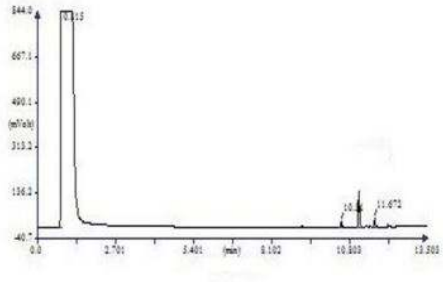
Negative control- 7th day with biosurfactant



7th day without biosurfactant

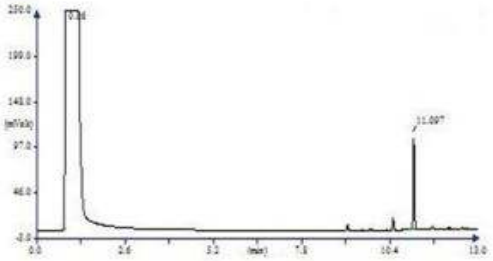


7th day with biosurfactant

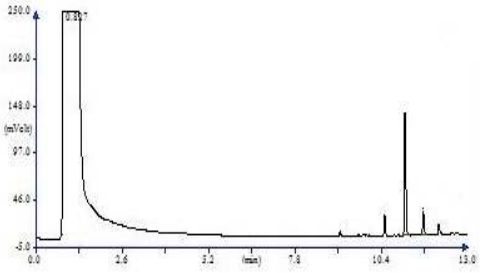


(D) Docosane -$C_{22}H_{48}$

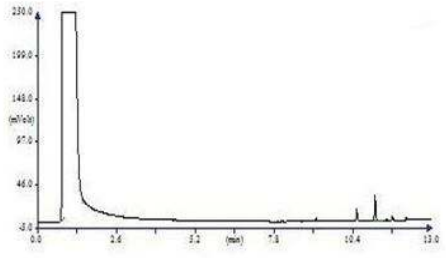
0 hr with biosurfactant



Negative control- 7th day with biosurfactant



7th day without biosurfactant



7th day with biosurfactant

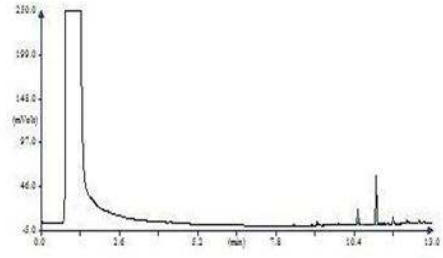


Fig. 4. GC chromatogram of different alkanes: Tetradecane, hexadecane, heptadecane and docosane. Effect of biosurfactant on degradation of alkanes at 0hr with Biosurfactant, Negative control- 7th day with biosurfactant 7th day without biosurfactant and 7th day with biosurfactant respectively- (A) tetradecane ($C_{14}H_{30}$) (B) hexadecane ($C_{16}H_{30}$) (C) heptadecane ($C_{17}H_{36}$) (D) docosane ($C_{22}H_{48}$)

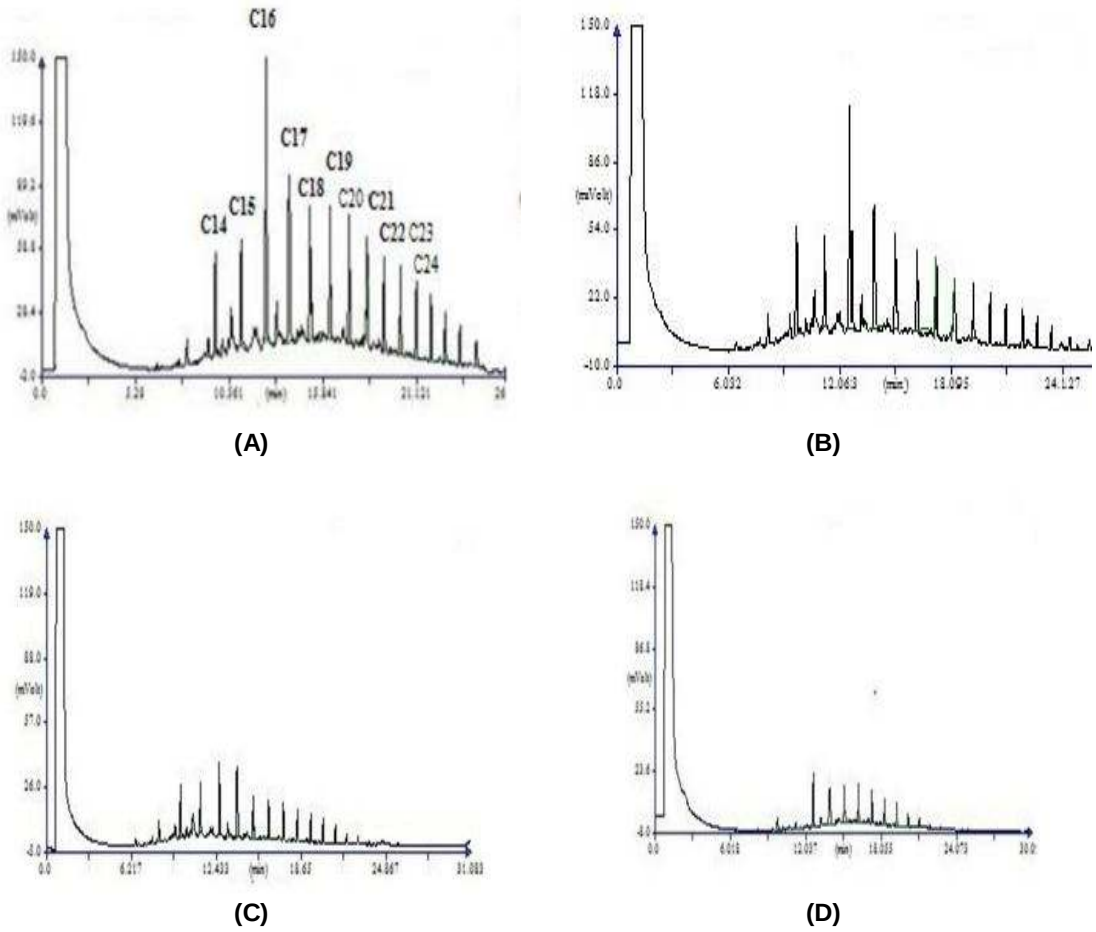


Fig. 5. GC chromatogram of oil degradation (a) 0 hr Sample (b) Negative control after 7 days of incubation (c) sample without biosurfactant after seven days of incubation (d) sample with biosurfactant after seven days of incubation

The resolved n-alkane peaks in the oil chromatograms were found to be in the carbon number range C_{13}–C_{24} (above Fig. 5a) various fractions of oil were efficiently degraded. The rate and extent of degradation of individual n-alkanes in the oil was also confirmed in oil based on variation in their peak area observed in GC chromatograms. C_{16} was one of the most abundant component in the residual oil. It was observed that degradation at the faster rate commenced immediately for compounds with carbon number less than C_{16}. This might have occurred because the bacteria consumed the lighter alkanes first or may be due to a decrease in solubility with an increase in molecular weight. The consumption of crude oil and its components was increased substantially in the culture on addition of biosurfactant (Fig. 5c). Its production contributed to the good dispersion and efficient degradation of oil by increasing the bioavailability thereby improving the rate of microbial utilization. Therefore, the solubilization of contaminants was markedly increased which further enhanced degradation of oil hydrocarbons.

4. CONCLUSION

Oil contamination with petroleum and petrochemical products has led to innumerable environmental and health problems. Biosurfactants help in the degradation of these compounds by promoting the bioavailability. In this study, the effect of biosurfactant produced by *Pseudomonas* sp. GBS.5 in bioremediation of crude oil hydrocarbons was evaluated. Incorporation of biosurfactant in minimal media with each alkane and oil independently, enhanced the degradation of all alkanes except n-docosane. Similar effect was also observed on

the degradation of alkanes in presence of oil. The enhanced degradation of various alkanes independently and in the presence of oil make this biosurfactant a promising candidate in bioremediation of oil spills or other hydrophobic pollutants.

ACKNOWLEDGEMENTS

This work, in part was supported by generous financial grant received from Department of Science and Technology, Government of India (Project No. SB/YS/LS-223/2013).

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Mrozik A, Piotrowska SZ, Labuzek S. Bacterial degradation and bioremediation of polycyclic aromatic hydrocarbons. Pol. J. Environ. Stud. 2003;12:15-25.
2. Nilanjana D, Preethy C. Microbial degradation of petroleum hydrocarbon contaminants: An overview. Biotech Res Int. 2011;2011:1-13.
3. Chrzanowski L, Wick LY, Meulenkamp R, Kaestner M, Heipieper HJ. Rhamnolipid biosurfactants decrease the toxicity of chlorinated phenols to *Pseudomonas putida* DOT-T1E. Lett Appl Microbiol. 2009; 48:756-762.
4. Sagarika M, Jublee J, Supara M. Practical considerations and challenges involved in surfactant enhanced bioremediation of oil. Biomed Res. Int. 2013;2013:1-16.
5. Gajendra BS, Sanjay G, Nidhi G. Carbazole degradation and biosurfactant production by newly isolated *Pseudomonas sp*. strain GBS.5. Int Biodet & Biodeg. 2013;84:35-43.
6. Bradford MM. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein dye binding. Anal Biochem. 1976; 72:248-254. Babita K, Singh SN, Kumar DP. Characterization of two biosurfactant producing strains in crude oil degradation. Process Biochem. 2012;47: 2463-2471.
7. Mohammad H, Akio U, Hitoshi I, Yukia I, Yusuke I, Isao Y, et al. Degradation of long-chain *n*-alkanes (C_{36} and C_{40}) by *Pseudomonas aeruginosa* strain Wat G. Int Biodet & Biodeg. 2007;59:40-43.
8. Jain DK, Collins DL, Lee H, Trevors JT. Drop-collapsing test for screening surfactant producing microorganisms. J. Microbiol. Method. 1991;13:271-279.
9. Cottingham MG, Bain CD, Vaux DJ. Rapid method for measurement of surface tension in multiwell plates. Lab. Invest. 2004;84:523-549.
10. Heyd M, Kohnert A, Tan TH, Nusser M, Kirschhöfer F, Brenner-Weiss G, et al. Development and trends of biosurfactant analysis and purification using rhamnolipids as an example. Anal Bioanal Chem. 2008;391:1579-1590.
11. Mahesh N, Murugesh S, Mohana VS. Determination of the presence of biosurfactant produced by the bacteria present in the soil samples. Res J Micro. 2006;1:339-345.
12. Chioma BC, Gideon CO, Blaise OC. Monitoring of microbial hydrocarbon remediation in the soil. 3 Biotech. 2011;1: 117-138.
13. Gita M, Suparna M. Biodegradation rate of diesel range *n*-alkanes by bacterial cultures *Exiguobacterium aurantiacum* and *Burkholderia cepacia*. Int Biodet & Biodeg. 2008;61:240-250.
14. Rojo F. Degradation of alkanes by bacteria. Environ Microbiol. 2009;11:2477-2490.

Peer-review history:
The peer review history for this paper can be accessed here:
http://sciencedomain.org/review-history/15458