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Biodegradation of Petroleum Hydrocarbons by Biosurfactant Producing *Bacillus subtilis* SBM1

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Abstract

The cost of recovering heavy crude oil and cleaning up areas that have been contaminated by hydrocarbons is substantial. It is possible to restore the environment at a reasonable cost by using microorganisms for enhanced oil recovery and cleanup of contaminated areas. Biosurfactants make hydrocarbons more bioavailable, hastening the biodegradation and absorption processes. Seventeen strains isolated from oil contaminated soil/ oil cakes were used in this study. In a comparative study with hydrocarbon enrichment and without hydrocarbon enrichment was performed to detect the emulsification activity. *Bacillus subtilis* SBM1 producing biosurfactant, having high ability of adherence to hydrocarbon showed biodegradation of diesel. The degradation of diesel by SBM1 was further confirmed by the FTIR analysis indicating the strain can be used for the oil extraction and remediation of polluted sites.

Keywords: *Bacillus subtilis*,biodegradation, biosurfactant, emulsification, hydrocarbon

Introduction

A natural substance made of hydrogen and carbon is called a hydrocarbon. Crude oil naturally contains the majority of the hydrocarbons that may be found on Earth, whereas decomposing organic matter is a rich source of carbon and hydrogen. Group 14 hydride examples include hydrocarbons. There are various types of hydrocarbons, including alkanes, cycloalkanes, arenes, and alkyne-based compounds (McMurry 2000). Regarding hydrocarbons, there are two main problems. First, hot water and high pH are frequently employed to extract oil from sands, which results in generally high viscosity hydrocarbon leftovers. Following the application of standard oil extraction techniques, a sizable percentage of this valuable and non-renewable resource is left in the ground.

Second, oil spills have emerged as a major environmental hazard in industrialised and developing nations during the past few decades (Das and Chandran 2011). The Taylor energy well disasters in the Gulf of Mexico (USA), which were brought on by a hurricane on September 16, 2004, is one of several major ongoing oil spills in the world. There are many ways that oil can return to humans from accidental spills, including accumulation in fish and shellfish and consumption of contaminated groundwater. Crude oil and petroleum products are common water and soil pollutants resulting from marine and terrestrial spillages. Accidental spills of petroleum fuel, which are the most common organic pollutant of soil and ground water and are labeled as hazardous waste, pose a major threat to both human and animal health and are also carcinogenic and mutagenic (Connellan 2017).

Oil can be destroyed by microorganisms like bacteria, fungus, yeast, and microalgae depending on the source of the oil. Petroleum hydrocarbons make up 50–98% of crude oil, and alkenes make up 20–50% of oil. According to Shukor et al. (2009), a variety of microorganisms have the capacity to release enzyme systems that allow them to use hydrocarbons as a source of energy. Different hydrocarbons are more or less vulnerable to microbial assault. According to Wentzel et al. (2007), linear alkanes are more susceptible to microbial degradation than branched alkanes, tiny aromatics, and cyclic alkanes. The primary and most effective natural method for removing petroleum hydrocarbon contaminants from the environment is microbial degradation. At many hydrocarbon-contaminated locations, biodegradation—which takes use of microorganisms' capacity to break down or detoxify organic contaminants—is employed as a treatment option. One of the main methods for removing petroleum and diesel products from the environment on a budget is through the biodegradation of hydrocarbons by a natural population of microorganisms, which enables the conversion of toxic substances into less toxic or nontoxic

forms (Xu et al. 2018). The purpose of this study is to screen bacteria for the capacity to mobilise and/or degrade hydrocarbons in light of these two significant challenges connected to hydrocarbons.

Material and Methods

Bacterial strains

Seventeen bacterial isolates from samples taken from automobile workshops, kitchen waste dumping areas and mustard oil cakes procured from Department of Microbiology, SLSBT, CSJM University, Kanpur were used for the study. The bacterial strains were stored in 25 % glycerol at - 20 °C.

Preliminary screening for Hydrocarbon utilization

Bushnell Hass (BH) medium, a minimum medium that has been supplemented with diesel, was used to screen bacterial strains. BH medium contained (g/L) NaCl: 10.0 g, KCl: 0.29 g, $MgSO_4$.7H2O: 0.42 g, KH_2PO_4: 0.83 g, NH_4SO_4: 0.42 g, and K_2HPO_4: 1.25 g. It was supplemented with 1% of diesel as a sole source of carbon and energy. 100 µl of a log phase cultures in LB broth were inoculated into 25 ml of BH broth that had been amended with diesel (1%), and then incubated at 28 °C and 120 rpm. After incubation for 7 days, successive dilutions up to 10^{-5} and 10^{-8} were performed. BH agar plates with 100µl of diesel on top were inoculated by spreading with 100 µl of culture, and the plates were incubated at 28 °C for 48 hours. The number of colonies that had developed on the medium after 48 hours of incubation was counted. Calculated CFU was used to plot the graph.

Emulsification index

Bacterial strains were cultured in BH minimum media supplemented with diesel (1%) and biosurfactant media (Medium 5, Turkovskaya et al. 2001) separately, and after 7 days of incubation at 28° C, cultures were centrifuged and vortexed for 5 mins to test for the ability to produce biosurfactants. By mixing 3 ml of hexane into 2 ml of culture medium, the emulsification index of bacterial strains cultured in BH minimum media (with diesel) and biosurfactant media was independently assessed. The mixture was then allowed to stand for 24 hours. The emulsion stability was assessed after 24 hours. In order to calculate the emulsification index (E24), the measured height of the emulsion layer was divided by the mixture's total height, then multiplied by 100 (Cooper and Goidenberg 1987).

BATH Test (Bacterial adhesion to hydrocarbon)

Using the BATH test, cell hydrophobicity was evaluated according to Rosenberg et al. (1980). The culture was centrifuged and the cells were twice washed with distilled water after the inoculation period of two days, resulting in an optical density (OD) at 600 nm of 0.5. The crude oil (100 µl) was then combined with the cell suspension (2 ml) and vortexed in tubes for 5 minutes. After shaking, the aqueous phase and crude oil were allowed to separate for one hour. Then, using a spectrophotometer, the optical density (OD) of the aqueous phase was determined at 600 nm. The percentage of cell adhesion to crude oil used to express hydrophobicity is computed as follows:

100 × (1- OD of the aqueous phase/OD of the initial cell suspension)

Screening for hydrocarbon degradative activity

By using the hydrocarbons (petrol, diesel, benzene, toluene, and kerosene) provided as a carbon source in BH broth, turbidometry study was carried out to determine the bacterial growth. BH broth containing 1% of each hydrocarbon and cultured for 10 days was used to measure the degrading activities of each isolate. By measuring the OD at 600 nm at regular intervals of 2 days from 0 hours to 10 days against BH media as a blank, the bacterial growth was quantified.

Estimation of crude oil degradation

Gravimetric analysis was used to estimate the deterioration of crude oil (Subathra et al. 2013). In brief, the chosen strains (SBM1, PN22, and BAN4) were pre-cultured in 5 mL of LB broth medium and incubated for 24 hours at 28° C at 180 rpm. After that, strains were cultured for 7 days at 28° C on BH medium that had been supplemented with 1% diesel. The remaining crude oil was

extracted using acetone and hexane (1:1) in a separating funnel in a preweighed flask. To ensure full extraction, the extraction process was carried out twice. Following extraction, the solvent was evaporated at 60 to 70˚ C in a hot air oven there after the flask was cooled and weighed. The degradation % was determined as follows:

$$\text{Percentage of degradation} = \frac{\text{Amount of crude oil degraded}}{\text{Amount of crude oil added in media}} \times 100$$

Where,
Weight of residual oil = Wt. of the container with extracted crude oil - Wt. of empty container
Amount of crude oil degraded = Wt. of crude oil added in media - Wt. of residual oil

Fourier Transform Infrared Spectroscopy

Given that Fourier transform infrared spectroscopy (FTIR) is best suited for identifying specific chemical bonds (functional groups), it can be used to clarify how a particular strain SBM1 degrades diesel. Diesel samples (i) extracted diesel following SBM1 growth on BH medium + diesel, and (ii) pure diesel were used for the molecular characterization. The Bruker IFS113vFTIR-spectrometer was used to record the infrared (IR) spectra in the 4000-400 cm^{-1} spectral range. At the Indian Institute of Technology in Kanpur, this study was carried out.

Results

Preliminary screening for Hydrocarbon utilization

All bacterial strains showed growth on BH media amended with diesel indicated their ability of hydrocarbon utilization (Fig. 1a). Population counts of strains were ranging between 1.5×10^{-8} and 2.5×10^{-11} CFU/ml (Fig. 1b). *Bacillus subtilis* SBM1 (accession number MN428789, Khare and Verma 2020) followed by CPM2 showed maximum growth using diesel as carbon source.

Emulsification index

The strain LP2 had the highest emulsification activity of 87.5% on growing on media supplemented with diesel, followed by SBM1 (77.27%), and BAN4 (60%). Most of the strains showed better emulsification activity on growth in BH media amended with diesel over biosurfactant production media (Medium 5) (Table 1).

BATH Test (Bacterial adhesion to hydrocarbon)

Bacillus subtilis SBM1 showed best ability of adhesion to diesel (71%). Strains PCB2 (68%) and PN22 (62%) comes next to SBM1 (Table 1). Positive cell hydrophobicity was reported as an indication of biosurfactant production.

Screening for Hydrocarbon degradative activity

Among the hydrocarbons used for the evaluation of the degradation ability of bacterial strains, kerosene was maximally utilized as carbon source after 10 days, followed by the hydrocarbons toluene, diesel, benzene and petrol (Table 2). SBM1 and PN22 were among the best five strains that are utilizing petrol, toluene, diesel and kerosene (Fig. 2). Strain BAN4 showed maximum growth on benzene (Table 2, Fig. 2).

Estimation of crude oil degradation

Estimation of crude oil degradation by gravimetric analysis showed that selected strain SBM1 showed 18.33% degradation after 15 days of incubation. However strains PN22 and BAN4 not showed negligible reduction in weight of extracted diesel after 15days of growth.

Fourier Transform Infrared Spectroscopy

The FTIR spectrum of uninoculated diesel revealed bands at 2924.53 cm^{-1}, 2854.52 cm^{-1} indicating a CH stretch in aromatic and aliphatic compounds, and 1462.28, 1377.37 cm^{-1} peak showed –CH deformation and $–CH_3$ symmetrical deformation. The short peaks at 700-810 cm^{-1} represent presence of substituted benzene. Peak in the range of 1377-1600 cm^{-1} showed presence of C=O stretching and peaks in the range of 1400 cm-1-1000 cm^{-1} showed C=O stretching for primary alcohol. Presence of some peaks in the range of 550-850 cm^{-1} showed presence of alkylhalide

group (Fig. 5). Absence of characteristic peak in the region of 1605.79 and 810.73 cm^{-1} in treated sample indicates absence of ester linkage and benzene substitution (Fig. 3).

Discussion

Researchers have concentrated on determining the bioremediation capacity of bacteria that live in contaminated locations and break down hydrocarbons. The current study unequivocally shown that microbes living in oil-contaminated areas may use hydrocarbon. To test their capacity to mobilise and use hydrocarbons, all the bacterial strains that demonstrated growth on Bushnell Haas agar were submitted to a screening procedure for the generation of biosurfactants. An indirect technique for screening the generation of biosurfactants is the emulsification assay. According to numerous studies, the production of surfactants is directly connected with the deterioration of crude oil (Parthipan et al. 2017; Viesser et al. 2020). It was believed that, if biosurfactant was present in the cell free culture broth utilised in this assay, it would emulsify the hydrocarbons contained in the test solution. In this investigation, bacterial strains produced in media containing diesel had greater emulsifying abilities than those cultivated in media containing glucose (medium 5). Because cells attach to oil droplets by producing surface-active substances known as biosurfactants, cell adhesion with hydrophobic substances like crude oil is thought to be an indirect technique to screen bacteria for biosurfactant synthesis (Nayarisseri et al. 2018; Thavasi et al. 2011). The strongest ability for adherence to hydrocarbon was demonstrated by *Bacillus subtilis* strain SMB1. According to Volchenko et al. (2007), bacterial strains with high cell hydrophobicity have the ability to synthesise biosurfactants. Results suggested that the positive BATH activity seen with the strains showing negative emulsification activity is due to extracellular compounds other than biosurfactants.

The findings of the spectrophotometric examination showed that each screened strain has the ability to break down a wide range of hydrocarbons. The bacteria *Bacillus subtilis* SBM1 had the best ability to use fuel, diesel, benzene, toluene and kerosene, followed by PN22 and lastly BAN4. Erdoan et al. (2012) and Leahy et al. (1990) conducted studies that used spectrophotometry to ascertain the capacity of bacteria to break down hydrocarbons. Based on the aforementioned findings, SBM1, PN22, and BAN4 were chosen and individually inoculated in BH broth with diesel as the only carbon source to examine the effectiveness of oil degradation. After 10 days of treatment, strain SBM1 demonstrated a decrease in the weight of the extracted diesel. This result was confirmed by the FTIR analysis of SBM1 treated and non-treated diesel. According to Revathy et al. (2015) FTIR examination of degraded diesel, the extracted material lacked the characteristic signal between 1610 and 810.73 cm-1, which shows ester linkage. Thus, FTIR research revealed that strain SBM1 broke down the aliphatic and aromatic alkanes in diesel. This study reported a *Bacillus subtilis* strain SBM1 isolated from oil-contaminated soil, which can be used for oil recovery and bioremediation of polluted areas, spills, as provide high emulsification of hydrocarbons and their efficient breakdown.

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Table1. Characteristics for hydrocarbons mobilization and utilization.

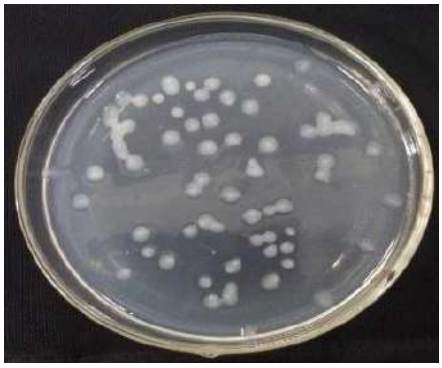
S.No.	Strains	Emulsification index(E_{24})		Hydrophobicity [Cell adherence to diesel (%)]
		BH media + Diesel	Medium 5	
1.	SBM1	77.27	40	71
2.	SN2	18.75	-	60
3.	SB2	37.50	-	38
4.	LP2	87.50	12.5	20
5.	PC14	25.00	25	39
6.	PN22	52.12	25	62
7.	SBM2	-	31.25	39
8.	SM2	70.58	-	47
9.	CM3	06.66	-	25
10.	BM1	-	-	51
11.	CPM1	-	33.33	38

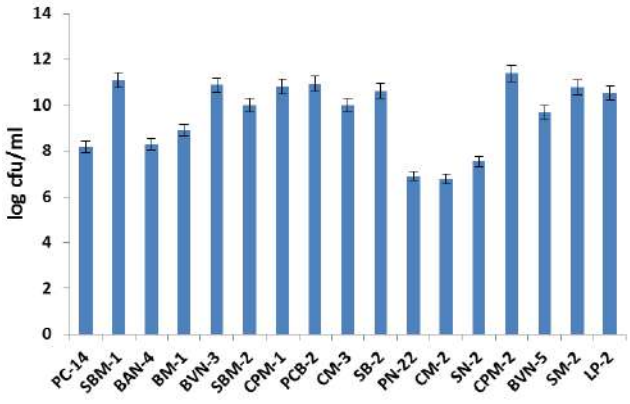
12.	BVN3	33.33	6.66	49
13.	BVN5	-	12.5	53
14.	CM2	31.25	41.17	55
15.	CPM2	37.50	12.5	48
16.	PCB2	43.75	37.5	68
17.	BAN4	60.00	75	57

Table 2. Utilization of various hydrocarbons by bacterial strains.

S. No.	Isolates	Growth on X Day (OD_{600})				
		Benzene	Diesel	Kerosene	Petrol	Toluene
1.	SBM1	0.323	0.495	0.967	0.365	0.779
2.	SN2	0.219	0.439	0.613	0.200	0.451
3.	SB2	0.236	0.211	0.563	0.133	0.361
4.	LP2	0.210	0.507	0.572	0.127	0.667
5.	PC14	0.195	0.416	0.502	0.178	0.399
6.	PN22	0.258	0.630	0.991	0.201	0.617
7.	SM2	0.210	0.451	0.582	0.109	0.632
8.	SM2	0.145	0.208	0.718	0.150	0.304
9.	CM3	0.198	0.458	0.912	0.165	0.315
10.	BM1	0.269	0.450	0.685	0.124	0.174
11.	CPM1	0.461	0.368	0.718	0.210	0.567
12.	BVN3	0.429	0.224	0.863	0.196	0.158
13.	BVN5	0.392	0.492	0.209	0.273	0.582
14.	CM2	0.315	0.480	0.629	0.247	0.528
15.	CPM2	0.559	0.422	0.583	0.167	0.667
16.	PCB2	0.632	0.772	0.666	0.107	0.429
17.	BAN4	0.696	0.526	0.798	0.195	0.481

Figure 1. Identification of hydrocarbon utilizing bacteria.
(a) Growth of bacterial colonies on BH Agar medium.
(b) Log cfu/ml of hydrocarbon utilizing bacteria on BH media amended with diesel.





(a) **(b)**

Figure 2.Time scale performance of selected hydrocarbon utilizing bacteria.

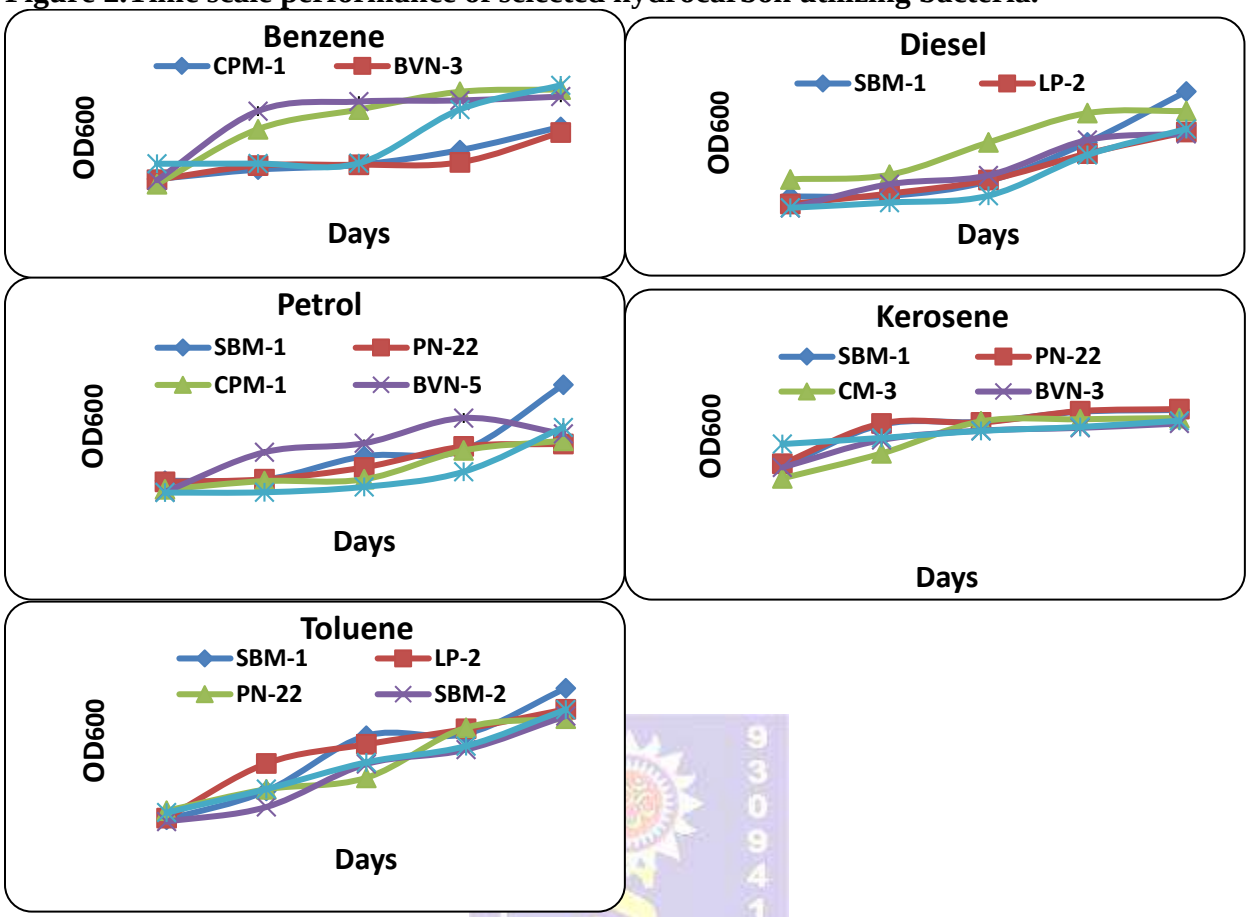
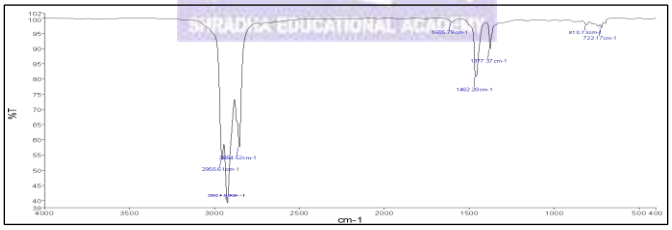
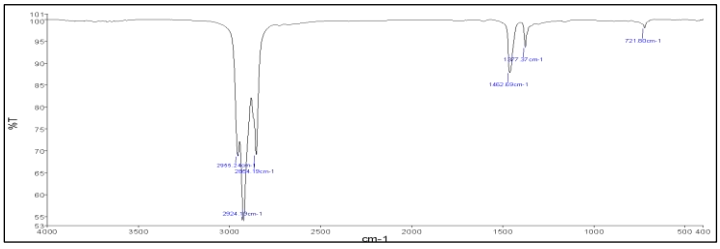


Figure 3. FTIR spectrum of diesel. (a) untreated diesel, (b) diesel extracted from BH media after treatment with strain SBM1.



(a)



(b)