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EXPERIMENTAL ARTICLES

Isolation and Characterization of Biosurfactant Producing *Bacillus* sp. from Diesel Fuel-Contaminated Site[1]

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Abstract—Among hydrocarbon pollutants, diesel oil is a complex mixture of alkanes and aromatic compounds which are often encountered as soil contaminants leaking from storage tanks and pipelines or as result of accidental spillage. One of the best ecofriendly approaches is to restore contaminated soil by using microorganisms able to degrade those toxic compounds in a bioremediation process. In the present study, nineteen bacteria were isolated by enrichment culture technique from diesel spilled soil collected from electric generator shed of NBAIM, Mau. All the isolates were subjected to screening for lipase production and twelve isolates were found to be positive for lipase. When the isolates were screened for biosurfactant production using CTAB-methylene blue agar plates, only one isolate viz. 2NBDSH3 was found positive which was found to be phylogenetically closely related with *Bacillus flexus*. Despite having low emulsification index, the bacterium could degrade 88.6% of diesel oil in soil. Biosurfactant from the isolate was extracted and characterized through infra-red spectroscopy which indicated its possible lipopeptide nature which was further supported by strong absorption in UV range in the UV-Vis spectrum. The results of the present study indicated that the isolate either does not produce any bioemulsifier or produces very low amount of emulsifier rather it produces a lipopeptide biosurfactant which helps in degradation of diesel oil by lowering the surface tension. The bacterium thus isolated and characterized can serve as a promising solution for ecofriendly remediation of bacterium diesel contaminated soils.

Keywords: Bacillus, biosurfactant, degradation, diesel oil, emulsification, lipopeptide

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Life in our planet is sustained in a complex but delicate biochemical balance which started getting more fragile since the industrial revolution marked its entry in the human civilization. Continuous anthropogenic intervention and mechanization to exploit the natural resources have cursed the earth with numerous maladies in the form of pollution almost everywhere. Release of contaminants and pollutants including petroleum and petroleum by-products to the environment, is one of the major causes of global contamination (Rahman et al., 2003). These also pose serious threat to human and animal health, as many of these contaminants have been reported to be toxic and carcinogenic (Kanaly and Harayama, 2000; Langworthy et al., 2002). Hydrocarbon molecules that are released into the environment are difficult to remove, since they adsorb to surfaces of soil particle and are trapped by capillarity in a water-immiscible phase (Viramontes-Ramos et al., 2010). Diesel fuel, also known as automotive gas oil (AGO), is a complex mixture of alkanes and aromatic compounds which are often encountered as soil contaminants leaking from storage tanks and pipelines or as result of accidental spillage. Its harmful impacts include reduction of plant growth by its toxic effect, reduced germination and pore space between the soil particles. Further, the microbial communities responsible for the biogeochemical cycles are being truncated (Kayode-Isola et al., 2008).

Decontamination and remediation of oil spilled ecosystems, is a must which may otherwise create a serious ecological imbalance. One of such remediation process is bioremediation, using microorganisms, which due to their extremely flexible physiology and metabolism can easily colonize contaminated habitats followed by subsequent clean up. Microbes are known to be involved in degrading a considerable number of organic pollutants like aliphatic compounds, *n*-alkanes, diesel fuel and tetrachloroethylene, monoaromatic compounds, toluene, benzene, xylene, ethylbenzene and polycyclic aromatic hydrocarbons (Lal and Khanna, 1996). Bacteria like *Pseu-*

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domonas, Bacillus, Acinetobacter, etc. have been well recognized as agents for biological remediation of hydrocarbon contaminated soils.

Biosurfactants are polymers, totally or partially extracellular, amphipathic molecules containing polar and non polar moieties which allow them to form micelles that accumulate at interphase between liquids of different polarities such as water and oil (Desai and Banat, 1997). Hydrocarbon degrading microorganisms produce biosurfactants of different chemical nature and molecular size that decreases the surface tension of the hydrophobic water-insoluble substrates and thereby enhancing their bioavailability and the rate of bioremediation (Pekdemir et al., 1999). Other advantages of biosurfactants include selectivity and specific activity at extreme temperatures, pH and salinity (Kosaric and Sukan, 1993). Because of these properties, biosurfactants have potential application in food, pharmaceutical and cosmetic industries (Desai and Banat, 1997).

In the present study, we isolated a diesel degrading bacilli through enrichment culture technique and subsequently characterized the biosurfactant produced by the bacterium.

MATERIALS AND METHODS

Enrichment and Isolation

Diesel spilled soils were collected from diesel generator house (25°53′50.65″ N and 83°29′26.37″ E) of National Bureau of Agriculturally Important Microorganisms (NBAIM), Mau, India. Bacteria were isolated from the soil using liquid enrichment cultures technique with diesel as sole carbon source. The enrichment culture technique was performed using mineral salt (MS) medium containing: $NaNO_3$ (7 g/L), K_2HPO_4 (1 g/L), KH_2PO_4 (0.5 g/L), KCl (0.1 g/L), $MgSO_4 \cdot 7H_2O$ (0.5 g/L), $CaCl_2$ (0.01 g/L), $FeSO_4 \cdot 7H_2O$ (0.01 g/L), yeast extract (0.1 g/L) and 0.05 mL of trace elements solution containing H_3BO_3 (0.25 g/L), $CuSO_4 \cdot 5H_2O$ (0.5 g/L), $MnSO_4 \cdot H_2O$ (0.5 g/L), $MoNa_2O_4 \cdot H_2O$ (0.06 g/L), $ZnSO_4 \cdot 7H_2O$ (0.7 g/L). The pH of the medium was adjusted to 7.5 before autoclaving. The filter sterilized (thrice with 0. 0.45 μm syringe filters, HiMedia) diesel [1% (v/v)] was added aseptically to the sterile MS medium. One gram of soil sample was added aseptically to 50 mL of MS medium supplemented with diesel and kept on incubator shaker (140 rpm) at 37°C for 10 days. 20 μL of the enriched suspension was spread on nutrient agar (NA) (HiMedia, India) and tryptic soy agar (TSA) (HiMedia, India) plates followed by incubation at 37°C for 24 hours. Subsequently, bacterial colonies with different morphology were purified on NA and TSA and preserved with 20% glycerol at –80°C.

Screening for Lipase Production and Biosurfactant Production

The bacterial isolates were screened for lipase production following the methods described by Samad et al. (1989). Briefly, bacterial suspension was placed in the wells made on the agar plates containing 2% Tween 20 and incubated for 48 hours at 37°C. Subsequently plates were stained with 0.01% methyl red (aqueous) and observed for halo zone.

To screen for biosurfactant production, blue agar plate was used as described by Satpute et al. (2008). The bacterial suspensions were placed into the wells made on MS agar medium containing cetyltrimethylammonium bromide (CTAB) (0.5 mg/mL) and methylene blue (0.2 mg/mL). Then plates were incubated at 37°C for 48–72 hours. Formation of dark blue ring around the well indicated positive results.

For the above experiments, bacterial inoculums were prepared in MS medium containing 1% (v/v) diesel.

Determination of Emulsification Index (E_{24} value)

The emulsification index (E_{24}) was expressed as the height of the emulsion layer to the total height of the mixture, multiplied by 100 (Cooper and Goldenberg, 1987). This was performed for the bacterial isolate which showed positive result for biosurfactant production. Loopful of overnight grown bacterial culture was inoculated in nutrient broth and incubated at 37°C under continuous shaking (140 rpm) for 24 hours. From this, 1.5 mL of fresh bacterial suspension was transferred to 15 mL Falcon's tube and to it 1 mL of filter sterilized diesel was added. Subsequently, the suspension was mixed vigorously for 2 minutes and left undisturbed for 24 hours. Then the total height of the suspension and the height of the emulsified layer were determined by a measuring scale. The emulsification index (in %) was calculated using the following formula: (Height of emulsified layer/Total height) × 100.

Extraction and Purification of Crude Biosurfactant

For extracting biosurfactant, the bacterial isolate was grown in 100 mL MS medium containing 1% (v/v) filter sterilized diesel for 7 days at 35°C under continuous shaking (140 rpm). After incubation, the bacterial suspension was centrifuged at 10000 rpm at 4°C for 30 minutes to remove the bacterial cells. To the supernatant thus obtained, 1 M H_2SO_4 was added to adjust the pH at 2. To this, equal volumes of chloroform: methanol (2 : 1) was added. These mixtures were shaken well to ensure proper mixing and were left overnight for evaporation. White coloured precipitate if seen at the interface between the two liquids proved the presence of biosurfactant (Mukherjee et al., 2009).

The precipitate thus formed was carefully taken out with the help of separator funnel and kept in micro-

centrifuge tube. One millilitre distilled water was added to the microcentrifuge tubes containing biosurfactant and was thoroughly vortexed to ensure uniform mixing. The tubes were centrifuged at 10000 rpm at 4°C for 30 minutes. The supernatant was discarded and the pellet was allowed to dry for 24 hours. The dry pellet thus obtained was the crude biosurfactant.

Spectral Characterization of Crude Biosurfactant

Infrared spectrum of the crude biosurfactant pellets was recorded using a Bruker Alpha FT-IR spectrophotometer (Germany) and the UV-Visible spectrum was recorded using a Shimadzu UV-500 spectrophotometer at wavelengths ranging from 250 to 500 nm.

Gravimetric Analysis for Diesel Degradation

The diesel degradation by the bacterial isolate in soil was carried out in the laboratory. Soils were collected from the fields of Directorate of Seed Research (DSR), Mau and sterilized twice and then left overnight. The next day, soil was again autoclaved and used for the experiment. In the experiment, the soil was mixed with 1% (w/w) filter sterilized diesel. Twenty five grams of soil: diesel mix was seeded with 100 mL of bacterial suspension in sterile flasks (250 mL). The flasks were then incubated for 10 days under continuous shaking (140 rpm) at 37°C. The experiment was performed in triplicates with a control containing only soil: diesel mix and MS medium only. Residual diesel was extracted by hexane following United States Environment protection Agency (USEPA, 1986). The degradation rate was determined gravimetrically by measuring the weight of the residual diesel. The calculations were performed as given below:

Diesel degraded (g) = Weight of diesel added in soil (g) − Weight of residual diesel (g).

Degradation rate = (Weight of degraded diesel/Weight of diesel added in soil) × 100.

Phenotypic Characterization and Molecular Identification of the Isolate

Colony and cell morphology of the bacterial isolate was determined after microscopy with stereo microscope (Olympus™ SZX7) and light microscope (Olympus™ BX41). Gram reaction was confirmed by KOH test. Biochemical tests and sugar utilization pattern was performed with Hi25™ Enterobacterial Identification kit (HiMedia, India) following the manufacturer's protocol.

Genomic DNA from the overnight grown bacterial isolates was extracted using methods described by Ausubel et al. (1992). 16S rRNA gene was amplified using the universal primers (Edwards et al., 1989) and the amplification was performed in a Thermal Cycler (G-STORM, United Kingdom) following the procedure described by (Singh et al., 2015). Amplified products were extracted from 1.2% agarose (Amresco, United States) gel and purified using Gel Extraction kit (GeNei, Bangalore, India). Purified PCR products were sequenced by ABI-Prism Sequencer (ABI-3130). Identity of the bacterium was revealed by performing search in EzTaxon database (Kim et al., 2012) . Phylogenetically related bacterial rDNA sequences were retrieved from NCBI GenBank database and phylogenetic tree was constructed using neighbor joining method with bootstrap sample size 1000 by MEGA 5.0 after alignment with inbuilt CLUSTAL W option in MEGA 5.0 (Tamura et al., 2011).

RESULTS AND DISCUSSION

Isolation and Characterization of Biosurfactant Producing Bacteria

Enrichment techniques to isolate oil-degraders using diesel as sole carbon and energy source have been employed to isolate various microorganisms from several genera (Jirasripongpun, 2002). A total of 19 different bacterial morphotypes were isolated by enrichment culture technique from diesel spilled soil collected from generator house of NBAIM, Mau. All the isolates were subjected to screening for lipase production and twelve isolates were found positive for lipase (table).

Biosurfactants are heterogeneous group of surface active compounds which enhances the solubilisation and removal of chemical contaminants and are produced by wide variety of microorganisms (Bai et al., 1997; Ilori et al., 2008). Production of surface active compounds is one of the most important properties of hydrocarbon degrading bacteria. Among the isolates screened for biosurfactant production using CTAB-methylene blue agar plates, only one isolate viz. 2NBDSH3 was found positive for anionic biosurfactant production (table). Hence, the isolate 2NBDSH3 was taken for further studies as it was the only isolate which showed biosurfactant activity among all the isolates. It was found to be a gram positive, rod shaped, oxidase and catalase positive, methyl red positive, citrate utilizing bacteria.

Based on partial 16S rDNA sequence (504 bp, partial) homology (Fig. 1), it was identified as *Bacillus* sp. and showed 100% similarity to *Bacillus flexus* IFO 1571 (T). Partial sequences (500 bp) of the 16S rDNA sequence have been submitted at NCBI and the sequence has been allotted accession number KR011151. Most of the bioremediation studies has reported the *Bacillus* sp. as the predominant genera prevalent in the hydrocarbon contaminated soil and has been termed as more tolerant bacteria to high levels of hydrocarbon contaminants in soil due to the presence of its resistant endospores (Viramontes-Ramos et al., 2010). The study carried out by Adeyemo et al. (2013) by isolation of microbial flora from the

Screening of isolates for lipase production and biosurfactant production

Isolates	Lipase	Biosurfactant production on CTAB agar
1NBDSN1	+	–
1NBDSN2	+	–
1NBDSN3	–	–
2NBDSN1	+	–
2NBDSN2	–	–
2NBDSN3	+	–
1NBDSH1	–	–
1NBDSH2	–	–
1NBDSH3	–	–
1NBDSH4	–	–
1NBDSH5	+	–
1NBDSH6	+	–
2NBDSH1	+	–
2NBDSH2	+	–
2NBDSH3	**+**	**+**
2NBDSH4	+	–
2NBDSH5	+	–
2NBDSH6	+	–
2NBDSH7	+	–
2NBDSH8	–	+

soil contaminated by diesel oil around power generators in various universities of Nigeria revealed that among the isolates, *Bacillus* species were the most predominant bacteria with 57% abundance. Further they suggested that it can be developed as seed microflora for bioremediation of oil spilled contaminated soil. Bento et al. (2005) isolated *Bacillus sphaericus*, *B. fusiformis*, *B. cereus* and *B. pumilus* from diesel contaminated soils of Honkong, China and Long Beach, United States. Khanna et al. (2012) isolated *B. flexus*, *B. pumilus* and *B. firmus* from crude oil contaminated soils of Patiala, Punjab. However, we did not come across many reports of *B. flexus* from oil contaminated soils.

Microbial surface-active compounds (SACs) are amphiphilic metabolites consisting of both hydrophobic and hydrophilic moieties allowing to partition at the interface between liquid phases with varying polarity. These compounds can be divided into two major categories: low molecular weight and high molecular weight SACs. Low molecular weight molecules decrease surface and interfacial tension and generally include lipopeptides and glycolipids. On the other hand, high molecular weight compounds are called bioemulsifiers, and are more effective in forming and stabilizing oil in water or water in oil emulsions, but do not necessarily reduce surface or interfacial tension. Although, E_{24} value has been used as a screening method and a direct correlation has been reported by many workers (Cooper and Goldenberg, 1987; Denger and Schink, 1995; Makkar and Cameotra, 1998) between surface activity and emulsification activity, however, the ability to form a stable emulsion is not universally related with surface tension reducing activity (Willumsen and Karlson, 1996). When emulsification assay was performed for the isolate, the E_{24} value was found to be 12% which was quite low as compared to E_{24} value of 33% reported for *Bacillus subtilis* MTCC 1427 (Makkar and Cameotra, 1998).

Spectral Characterization of Crude Biosurfactant

Fourier transformation infra-red spectroscopy of the crude biosurfactant revealed N–H stretching (3452 cm^{-1}), C=O (amide) stretching (1655 cm^{-1}), and C–H stretching (aromatic, 801 cm^{-1}) indicating its peptide component (Fig. 2), C–H stretching (2966 cm^{-1}) indicated aliphatic chains while C–O (of COOH) stretching (1263 cm^{-1}) indicated the presence of ester groups. Its peptide nature was further sup-

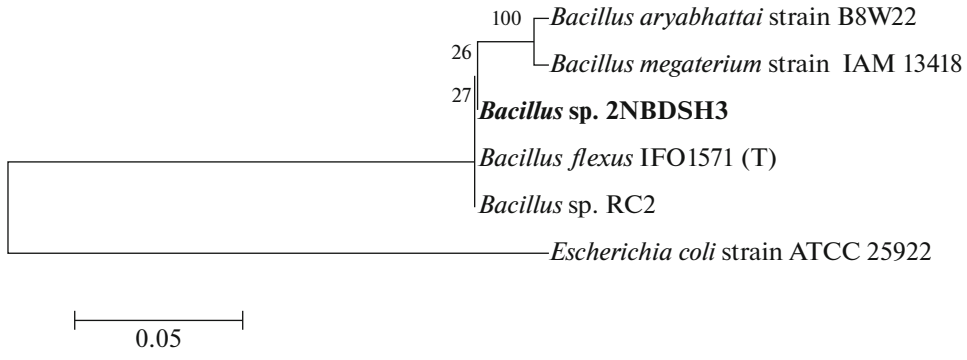

Fig. 1. NJ phenogram of the isolate 2NBDSH3 showing its phylogenetic position.

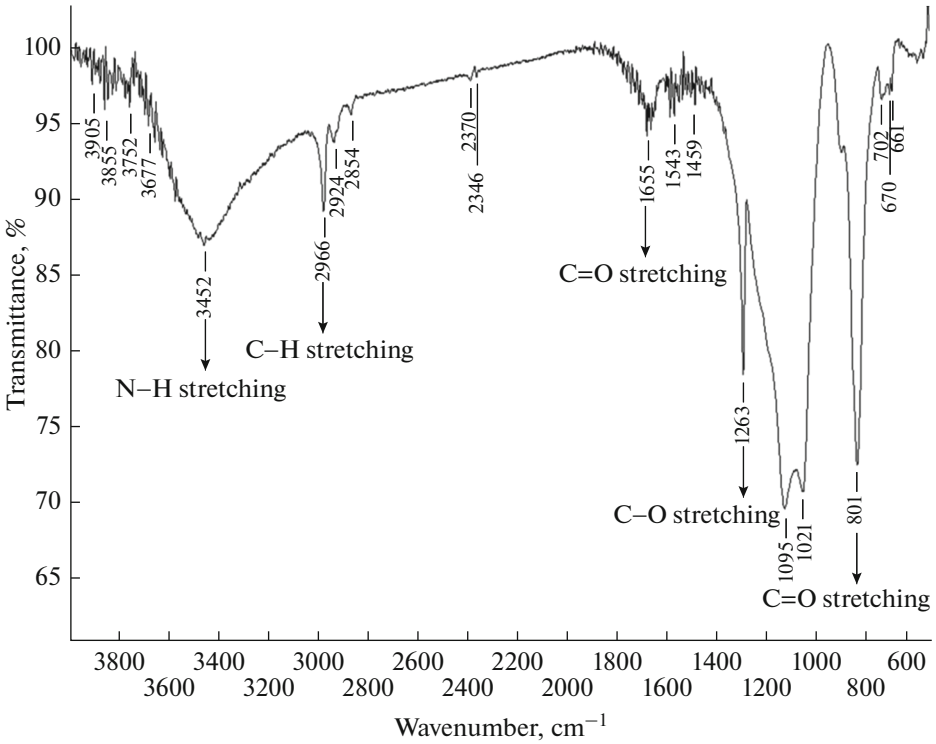

Fig. 2. Infra-red spectrum of the crude biosurfactant isolated from the bacterium.

ported by strong absorption of the crude biosurfactant at ultra violet range (280, 310 and 340 nm) deduced from the absorption spectrum (Fig. 3). The IR and UV spectra indicated the possible lipopeptide nature of the crude biosurfactant. Similar spectral characters for lipopeptide surfactants from *Bacillus clausii* (Hazra et al., 2015), *Bacillus pumilus* (Morikawa et al., 1992) and *Bacillus licheniformis* (Yakimov et al., 1995) have been reported.

Degradation of Diesel Oil by the Bacterial Isolate

Gravimetric analysis for diesel degradation in soil under controlled condition revealed that 88.6% of the diesel could be degraded by the isolate in 10 days. Kebria et al. (2009) reported a strain capable of degrading 85.20% of diesel fuel (10000 ppm) in 15 days. Wu et al. (2012) has reported the degradation of 20% by inoculation of strain name of the bacterium BF40 and 59.1% by addition of biosurfactant which shows that the addition of biosurfactant removes the significant amount of contaminants especially under saline conditions. Marquez-Rocha et al. (2001) reported 91% degradation efficiency for a bacterial consortium consisting of *Pseudomonas*, *Serratia*, *Acinetobacter* and *Flavobacterium*. When the same consortium was used in combination with *Pleurotus ostreatus,* the efficiency increased to 96%. Rahman et al. (2002) reported that at 1% crude oil concentration, consortium of *Pseudomonas* sp. DS10-129, *Bacillus* sp. DS6-86, *Micrococcus* sp. GS2-22, *Corynebacterium* sp. GS5-66 and *Flavobacterium* sp. DS5-73 degraded 78% of BH crude oil. Individually, *Pseudomonas* sp. DS10-129, *Bacillus* sp. DS6-86, *Micrococcus* sp. GS2-22, *Corynebacterium* sp. GS5-66 and *Flavobacterium* sp. DS5-73 could degrade 66, 59, 49, 43 and 41% of crude oil, respectively. However the efficiency of degradation (78%) by the consortium decreased when the concentration of crude oil was increased to 10%.

In the present study, low emulsification index indicates that the isolate either does not produce any bioemulsifier or produces very low amount of emulsifier. The isolate produces a lipopeptide biosurfactant which in turn helps in degradation of diesel oil by lowering the surface tension which is well supported by considerably high amount of diesel degradation. Results obtained in this study also revealed that emulsifying ability is not always related with the biosurfactant production or hydrocarbon degradation.

CONCLUSIONS

The bacterium isolated in the present study holds considerable potential for bioremediation of diesel contaminated soil. However, further studies are required to validate its efficacy under varying soil conditions and varying degree of contamination. Furthermore, studies to elucidate the actual mechanism of degradation and the identity of the biosurfactant could

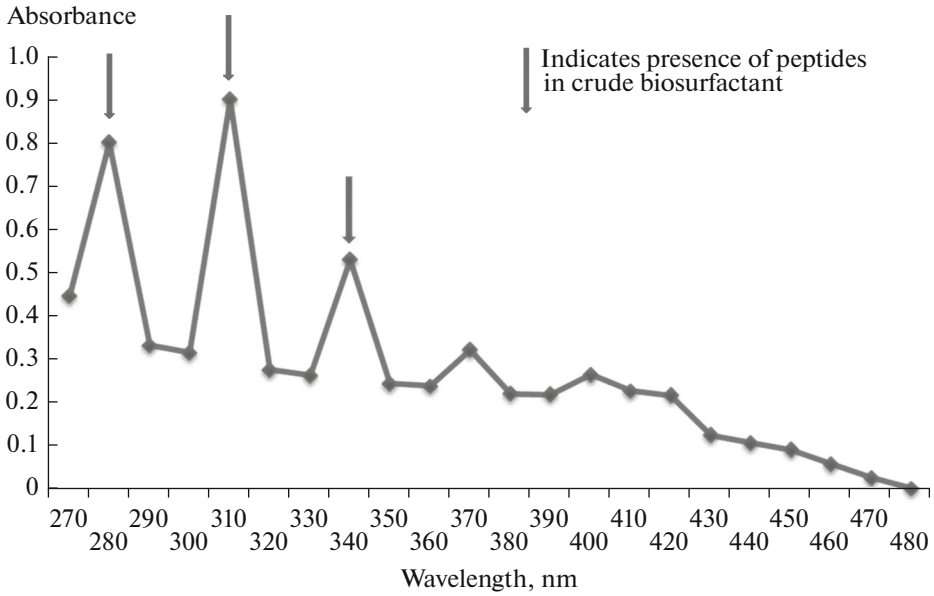

Fig. 3. UV-Vis spectrum of the crude biosurfactant isolated from the bacterium.

also be useful to improve our understanding about such unique low emulsifying diesel degrading bacteria.

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