Isolation and Characterization of Biosurfactant Producing Bacteria from Oil Sludge for Bioremediation of oil contaminated sites

Veeranna Channashettar[1], Shaili Srivastava[2], Banwari Lal[3]
[1]*Amity School of Earth and Environmental Sciences, Amity University, Gurgaon, Environmental and Industrial Biotechnology Division, The Enrgy and Resources Institute (TERI), New Delhi*
[2]*Amity School of Earth and Environmental Sciences, Amity University, Gurgaon, Haryana-122413, India*
[3]*Environmental and Industrial Biotechnology Division, The Enrgy and Resources Institute (TERI), New Delhi*

Abstract- The amount of leakage of oil is increasing due to increase in anthropogenic activities and natural disaster, causing hydrocarbon pollution in environment. Bacterial strains present in the oil sludge may have enormous capacity to produce biosurfactant, a tool for bioremediation of oil contaminated sites. The present study aimed to isolate and characterize bacterial strain from three oil refinery locations to obtain potential bacterial strains which can be explored for bioremediation of oil contaminated sites. The results of surface tension , emulsification activity (E24), total petroleum hydrocarbon (TPH) reduction , oil spreading and drop collapse test showed that two bacterial isolates viz. TER-4 and TER-7 are capable in production of significant amount of biosurfactant to degrade the oil, therefore these isolated bacterial strains can be used for bioremediation of oil contaminated sites.
Keywords – Isolation, Biosurfactant, Bacteria, Oil sludge, Bioremediation

I. INTRODUCTION

The amount of natural crude oil seepage was estimated to be 600,000 metric tons per year [1]. Soil contamination with hydrocarbons causes extensive damage by accumulation of pollutants in flora and fauna tissue, which may cause death or mutations [2] because of its carcinogenic nature. The technology commonly used for the remediation of hydrocarbon contaminated soil includes physical, chemical and biological. Physical and chemical methods are expensive, non-environment friendly and can lead to incomplete decomposition of contaminants. However bioremediation (biological) is the only method that allows for complete transformation of hydrocarbon pollutants into harmless compounds in a natural way. The key mechanism of any bioremediation technology is utilizing the oil degrading microorganism (bacteria, yeast, fungi, etc.) and/ or their metabolic product. Bacteria are the most active agents in petroleum degradation, and they work as primary degraders of spilled oil in environment [3-4]. Most of the oil degrading bacteria are producing biosurfactant, which is the key metabolite responsible for degradation of hydrocarbon pollutant.

Biosurfactants are microbially produced surface active compounds [5]. Biosurfactants are of increasing interest for commercial use because of the continually growing spectrum of available substances. There are many advantages of biosurfactants compared to their chemically synthesized counterpart. Unlike synthetic surfactants, microbial-produced compounds are easily degraded [6] and particularly suited for environmental applications such as bioremediation and dispersion of oil spills [7]. Microbial surfactants are complex molecules, comprising a wide variety of chemical structures, such as glycolipids, lipopeptides, fatty acids, polysaccharide-protein complexes, peptides, phospholipids and neutral lipids [8].

Surfactants can help to release hydrocarbons sorbed to soil organic matter by solubilization or emulsification, and increase the aqueous concentrations of hydrophobic compounds, resulting in higher mass transfer rates [9]. Recent studies indicate that biosurfactants can enhance hydrocarbon biodegradation by increasing microbial accessibility to insoluble substrates. Several researchers have investigated the addition of biosurfactants to enhance the biodegradation of hydrocarbons [10-11]. The bioremediation of soil contaminated with aromatic hydrocarbons and fossil fuels is limited by the poor availability of these hydrophobic contaminants to microorganisms [12]. Biosurfactants have been tested in environmental applications such as bioremediation and dispersion of oil spills, enhanced oil recovery and transfer of crude oil. Usually the microorganisms capable of utilizing aromatic and aliphatic hydrophobic compounds produce biosurfactant. On the other hand, catabolism of alkane and aromatic compound is not a prerequisite for biosurfactant production in all bacteria. However biosurfactants known today need to be studied with consideration of economic feasibility, practical adoptability of process know how and have to possible technical applications. The present study is aimed to identify and characterise the biosurfactant producing bacteria from oil sludge, which can be used for bioremadiation of oil contaminated sites.

II. MATERIALS AND METHODS

2.1. Soil sample collection

Oil contaminated soil samples were collected from oil refineries viz. Indian Oil Corporation Ltd. (IOCL), Panipat, Haryana, IOCL., Mathura, UP and Hindustan Petro Chemical Ltd., Mumbai, Maharastra from 10 different sites of each refinery in sterile glass containers and stored at 4-5˚C for further study.

2.2. Enrichment and Isolation of bacteria

The bacterial strain capable of degrading oil were isolated from oil contaminated soil by enrichment technique and serial dilution technique. The minimal salt medium (MSM) was prepared by adopting composition as described by [13]. The media was sterilized for 15 minutes by autoclaving at 121˚C at 15 lb pressure. The cultures were grown in 250 ml conical flask containing 100 ml sterile medium at 37˚C and 150 rpm. For enrichment, 10 g soil sample collected from oil contaminated sites of refineries were inoculated into 90 ml of MSM containing sterilized crude oil (1.0%, w/v) as carbon source and incubated at 37°C on a rotary shaker (150 rpm) for 5 days. Based on the cell growth in enriched culture, three cycles of re-inoculation in fresh MSM with 5 ml of enriched culture were repeated under similar conditions to ensure the utilization of crude oil as carbon source. After that, 1 ml of growth culture was diluted by serial dilution technique up to 109 fold. 100µL of all dilutions were plated on MSM agar plates with crude oil (1.0 % w/v) and were incubated at 37˚C. After an incubation period of 72 h, the bacterial colonies obtained were further purified on the MSM agar plates (with crude oil 1.0 % w/v).

The bacterial strains that could degrade total petroleum hydrocarbons were maintained on MSM agar plates with crude oil (1.0% v/v). Frozen stock cultures were stored in 25% glycerol at - 70˚C. Cultures were sub-cultured at an interval of 15 days. The glycerol stocks of all the bacterial strains were thawed and streaked over MSM containing crude oil (1.0% v/v). The plates were incubated at 37˚C. Liquid cultures of bacterial strains required for degradation studies were grown at 37˚C for 72 h in 5 ml MSM with 1.0 % (w/v) sterilized crude oil. For all molecular biology, biochemical characterization and colony morphology studies, the bacterial strains were grown either on Luria Bertani broth (LB) or Luria Bertani agar (LA) plates.

2.3 Characterization and screening of biosurfactant producing bacteria

The shape, size and colour of the colony were observed in the culture plates with MS media. The observations were noted down. The cells were identified to be Gram positive or Gram negetive by Gram staining technique. The surface tension was measured by following the De Nöuy platinum ring method described by Bodour and Miller-Maier [14]. Emulsification activity (E24) was measured by following the method described by Cooper and Goldenberg [15]. Residual crude oil was extracted from the media by using solvent extraction method (USEPA 9071B) using Soxhlet extractor and the amount of TPH recovered was quantified by gravimetric method [16]. Drop-collapse test was performed as described by Bodour and Maier [14]. Oil spreading method as described by Morikawa et al. [17] was followed in this study to correlate the surfactant activity.

III. EXPERIMENT AND RESULT

The oil sludge samples collected from three different oil refineries were enriched with 1% crude oil as a sole carbon source in MSM media to allow the bacteria present in oil sludge to degrade the crude oil (Figure 1). The turbidity due to growth of bacteria was observed in enriched media. The turbidity indicated that isolates were capable of utilization of crude oil as sole carbon source and able to degrade the crude oil.

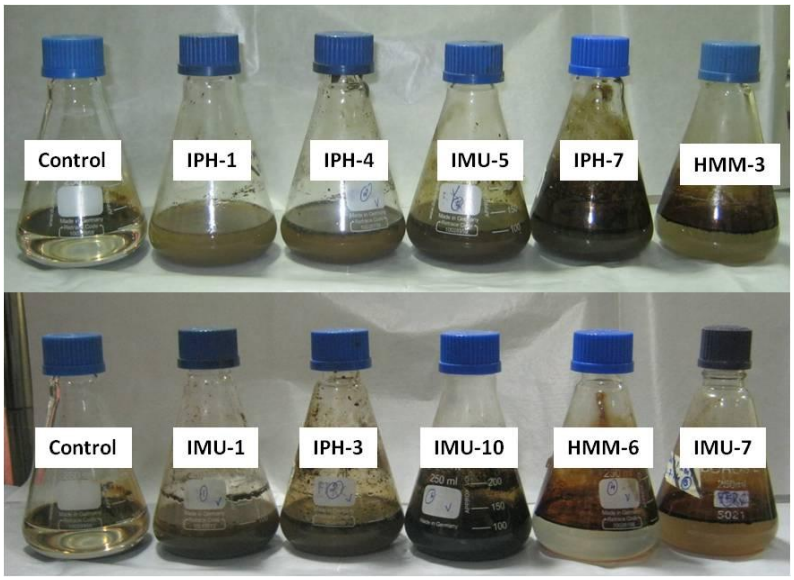

Figure 1: Enrichment of soil samples with crude oil for isolation of oil degrading bacteria. (IPH- IOCL, Panipat, Haryana; IMU-IOCL, Mathura, UP and HMM- HPCL, Mumbai Maharastra)

Based on the turbidity of culture grown in conical flask (Figure 1), eight bacterial strains were isolate by serial dilution technique and strains were grown in sterilized MSM agar solid media enriched with 1% crude oil as sole carbon source in petri plate (Figure 2). The growth of bacterial strains were observed after 5 days of incubation at 37˚C. The clear zone observed in petri plates indicated that isolates of bacterial strains were capable of degrading crude oil.

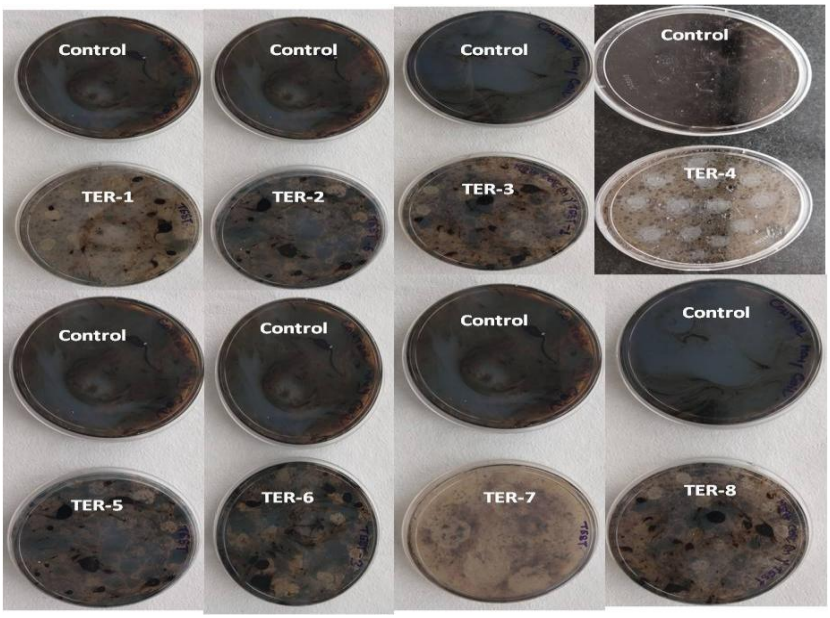

Figure 2: Bacterial isolates grown on crude oil plates (IPH- IOCL, Panipat, Haryana; IMU-IOCL, Mathura, UP and HMM- HPCL, Mumbai Maharastra).

The bacterial isolates grown in sterilized petri plates containing solid MSM agar media were observed for morphological characteristics after 72 h incubation at 37˚C as recorded in Table 1. Single isolated colonies of bacterial strains were gram stained using cristal violet and safranin dyes and recorded as gram '+' positive for the culture turned as violet colour with cristal violet dye and gram '-' negative for the culture turned as pink or red with safranin dye.

Table 1: Morphological characterization of biosurfactant producing isolated bacterial strains.

Isolate	Colony Colour	Colony size	Colony Form/shape	Gram Stain
TER-1	Whitish	Small	Circular/ Rod	+
TER-2	Whitish Yellow	Medium	Circular/Cocci	+
TER-3	Light yellow	Small	Irregular/Rod	-
TER-4	Creamy White	Medium	Filamentous/Rod	+
TER-5	Whitish yellow	Small	Circular/Rod	+
TER-6	Yellow	Small	Circular/Cocci	-
TER-7	Light yellow	Large	Filamentous/Rod	-
TER-8	Yellow	Small	Circular/Rod	-

The isolated bacterial strains were grwon in MSM liquide media in triplicate and supernatent of grown cultures were colected after incubation of 72 h at 37˚C. The surface tension and emulsification activity of supernatent solutions were observed and presented in Figure 3 and Figure 4, respectively. The results were statistically analyzed to observed the significant differance among isolated bacterial strains.

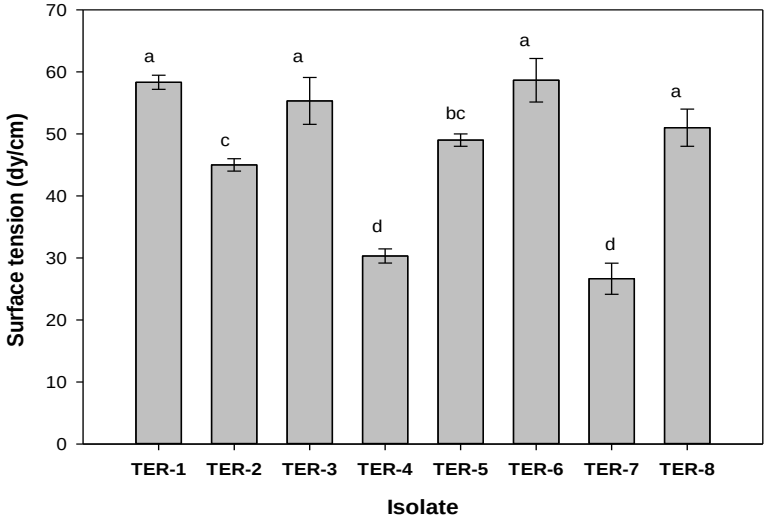

Figure 3: Surface tension during biosurfactant synthesis by different isolated bacterial strains (Mean ±SD; Bars not followed by same letter are significantly different at p≤0.05).

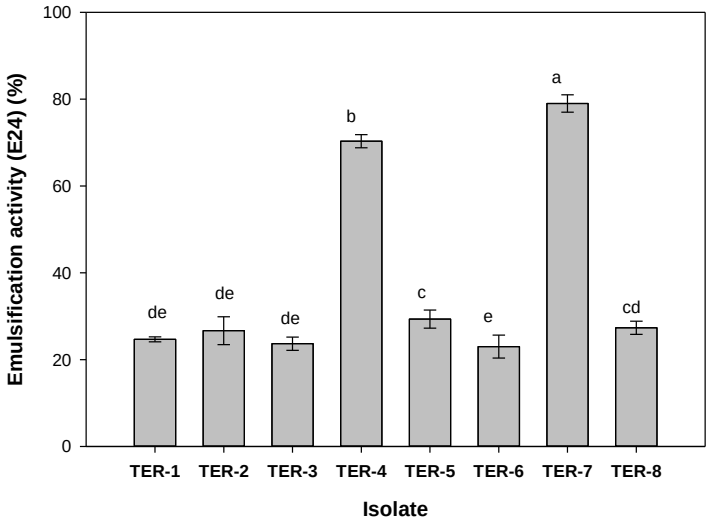

Figure 4: Emulsification activity of biosurfactant produced by different isolated bacterial strains (Mean ±SD; Bars not followed by same letter are significantly different at p≤0.05).

The isolated bacterial strains TER-7 showed maximum reduction of surface tension and TER-6 isolate showed least reduction of surface tension. The TER-7 and TER-4 were significantly reduced the surface tension (Figure 3). The emulsification activity (E24) was recorded significantly high in the supernatent of isolated bacterial strains TER-7 and significantly low E24 was recorded in the supernatent of TER-6 bacterial strains (Figure 4).
Biodegradation studies with isolated bacterial strains were performed in 250 ml conical flask containing 100 ml of sterilized MSM media with 1% (w/v) of crude oil as sole carbon source in triplicate and incubated on a rotary shaker at 150 rpm and 37 °C. The isolates were grown in standardized MSM for 24 h and were inoculated into the medium with 5 % (v/v) as inoculum. Uninoculated controls were kept to monitor natural weathering of crude oil. Residual crude oil was extracted from the media by using solvent extraction method (USEPA 9071B) using Soxhlet extractor and the amount of Total Petroleum Hydrocarbon (TPH) recovered was quantified by gravimetric method. The maximum TPH reduction of 92% was recorded by using TER-4 bacterial isolate followed by 90.66% by using TER-7 bacterial isolate, while least reduction of TPH was found 50.66% by using TER-1 bacterial isolate. The significant differance among the results of TPH reduction by different bacterial isolates were observed (Figure 5).

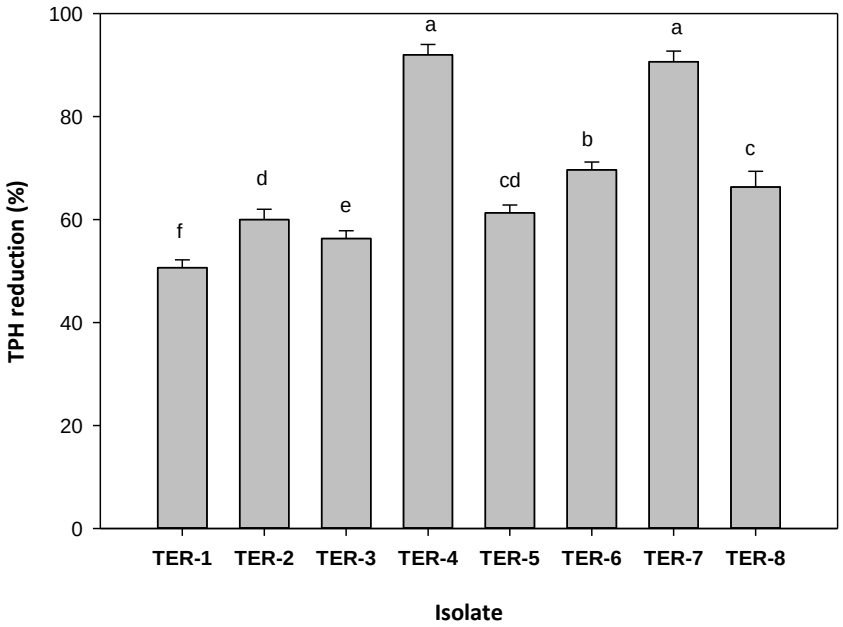

Figure 5: Total petroleum hydrocarbon (TPH) reduction by biosurfactant producing different isolated bacterial strains (Bars not followed by same letter are significantly different at $p \leq 0.05$).

Supernatant solution of different bacterial isolates, obtained after incubation of 72h at 37˚C, grown in MSM media was used for oil spreading test and drop collapse test. The results were presented in Table 2 as positive '+' or negative '-' for a clear zone of more than 0.5 mm and less than 0.5 mm, respectively in oil spreading test and for drop collapse test positive '+' results were recorded for drop collapsed within 2 minutes and negative '-' for drop not collapsed within 2 minutes. The TER-4 and TER-7 were shown positive results for both oil spreading and drop collapse test.

Table 2: Oil spreading test and Drop collapse test of cell free supernatant of biosurfactant producing different isolated bacterial strains. [Oil spreading testa: '-' - oil spreading with a clear zone of <0.5 mm; '+' - oil spreading with a clear zone of 0.5-1.5 mm, '++' - oil spreading with a clear zone of 1.6 to 2.5 mm; Drop collapse testb: '++'- drop collapse within 1minute, '+' - drop collapse within 2 minutes and '-' - drop not collapse within 2 minutes of biosurfactant addition]

Isolate	Oil spreading testa	Drop collapse testb
TER-1	-	-
TER-2	+	-
TER-3	-	-
TER-4	++	+
TER-5	+	-

TER-6	-	-
TER-7	++	++
TER-8	+	-

IV. CONCLUSION

The results of surface tension, emulsification activity, TPH reduction, oil spreading test and drop collapse test were indicating that TER-4 and TER-7 are producing biosurfactants and can be utilized for bioremadiation of oil contaminated sites.

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