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# Screening and isolation of biosurfactant producing bacteria from petroleum contaminated soil

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**ABSTRACT**

*Biosurfactant reduce surface tension of both aqueous solutions and hydrocarbon mixtures. In this study, isolation and identification of biosurfactant producing strain were assessed. Soil samples from 45 petrol pumps and 75 garages in Kalyan area were collected. To confirm the ability of isolates to produce biosurfactant, oil displacement test, measurement of surface tension and $E_{24}$ test were conducted. The isolate DB1 from Dombivli petrol pump was found to be the potent biosurfactant producer. This isolate gave a maximum biomass and biosurfactant yield of 1.72 g/L$^{-1}$ and 0.68 g/L$^{-1}$ respectively. With 16s rRNA analysis the isolate DB1 was identified as Pseudomonas stutzeri. The thin layer chromatography analysis revealed the polymeric nature of the biosurfactant. The biosurfactant was found to emulsify various hydrocarbon. This ability of the isolate can be utilized in environment clean up procedures.*

**Key words**: Biosurfactant, surface tension, emulsify, 16s rRNA, polymeric nature.

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## INTRODUCTION

Biosurfactants are the biologically synthesised surface-active agents (Nitschke and Pastore, 2006). They are amphiphilic compounds consisting of hydrophilic and hydrophobic domains. The hydrophilic domain can be carbohydrate, amino acid, phosphate group or some other compounds whereas the hydrophobic domain usually is a long chain fatty acid (Lang, 2002). The majority of known biosurfactants are synthesized by microorganisms grown on water immisible hydrocarbons,but some have been produced on water soluble substrate such as glucose,glycerol and ethanol (Abu-Ruwaida *et al.*,1991). Microorganisms have been reported to produce several classes of biosurfactants such as glycolipids, lipopeptides, phospholipids, neutral lipids or fatty acids and polymeric biosurfactants (Franzetti *et al*., 2010; Banat *et al*., 2010). Chemically synthesized surfactants have been used in the oil industry to aid clean up of oil spills as well as to enhance oil recovery from oil reservoirs. These compounds are not biodegradable and can be toxic to environment. Biosurfactant have special advantage over their commercially manufactured chemical surfactants because of their lower toxicity, biodegradable nature and effectiveness at extreme temperature, pH, salinity and ease of synthesis (Ilori and Amund, 2001; Ilori *et al*., 2005).

This study describes the screening and isolation of a potent biosurfactant producing microorganism, the biochemical characterization and emulsification ability of the biosurfactant.

## MATERIALS AND METHODS

**Isolation and screening of biosurfactant producing bacteria**
Biosurfactant producing bacteria were isolated by successive enrichment culture technique from the petroleum contaminated soil using Minimal Salt medium containing diesel oil (2%) as a sole source of carbon. The minimal salt media used consist of ($g/L^{-1}$):$MgSO_4$:0.2, $CaCl_2$:0.02, $KH_2PO_4$:1, $K_2HPO_4$:1, $NH_4NO_3$:1, $FeCl_3.6H_2O$:0.05 pH adjusted to 7.0 (Patel and Desai, 1997).The isolation was done on solidified minimal salt medium where diesel oil was introduced in vapour phase transfer technique as described by Raymond *et al*.,(1976). Incubation was carried out at room temperature for 5 days.

The isolates obtained were grown in liquid medium with diesel oil (2% w/v) for 5 days on shaker at room temperature. The cell free broth obtained after centrifugation (10,000 rpm for 30 min) was then studied for surfactant property as below:

1) Qualitative - Drop Collapse and oil displacement assay
2) Quantitative- $E_{24\%}$.

**Drop collapse test**- Drops of the cell free supernatant are placed on an oil coated, solid surface. If the liquid does not contain surfactants, the polar water molecules are repelled from the hydrophobic surface and the drops remain stable whereas if the drop contains surfactant it spreads or even collapse (Jain *et al*., 1991).

**Oil displacement assay**- 10 µl of crude oil is added to the surface of 40 ml of distilled water in a petridish to form a thin oil layer. Then, 10 µl of culture or culture supernatant are gently placed on the centre of the oil layer. If biosurfactant is present in the supernatant, the oil is displaced and a clearing zone is formed (Morikawa *et al*., 2000).

**Emulsification Index ($E_{24\%}$) -** Emulsification index of cell free broth was determined by adding 2 ml of fuel oil to 2 ml cell free broth, mixing with a vortex for 2 min, and leaving it undisturbed for 24 h. The $E_{24}$ index is given as percentage of height of emulsified layer (mm) divided by total height of the liquid column (mm) (Cooper and Goldenberg, 1987).

**Identification of biosurfactant producer-**The biosurfactant producers were identified on the basis of their morphological, cultural and biochemical characteristics as described by Holt *et al*., (1994).

**Selection of best biosurfactant producer**- The best biosurfactant producer was selected based upon the emulsification activity, biomass and biosurfactant yield and by measuring reduction in surface tension of the culture media.

**Emulsification activity assay-** 2.0 ml cell free broth sample obtained from each of the isolate were added in a tube containing 15 ml of 0.2 M Tris buffer pH 8.0 and 0.2 ml of fuel oil. The mixture was then vortexed for 10 min, allowed to rest for 1 min. Then extinction was read at 540 nm against blank containing buffer and fuel oil (Banat *et al*.,1990).

**Biomass determination-**The culture media was centrifuged at 10,000 rpm for 30 min to obtain pellet. Six volume of a mixture of petroleum ether and acetone (1:3 ratio) was mixed thoroughly with the pellet and centrifuged at 3000 rpm for 20 min. This was repeated till all the unutilised oil sample was removed leaving the solvent layer clear. Such cell mass free of oil settled down even at low speed centrifugation. The upper solvent layer was removed and cell mass was further treated with acetone. The cell mass was then washed with distilled water and dried at $60^0$C overnight in a preweighed crucible. The dry mass of cells was determined.

**Extraction of biosurfactant-**The pH of the cell free supernatant was adjusted to pH 2 using 6 N HCl and kept at $4^0$C for 24 hr. The cell free supernatant and chilled mixture of chloroform and methanol (2:1) in equal volume was added and mixed vigorously to obtain the biosurfactant within the organic layer. This layer was separated using a separating funnel and dried at $40^0$C for 4-5 hours to obtain dry mass. The yield as g/L was recorded (Desai and Banat, 1997).

**Surface tension-**The surface tension of the cellfree broth was measured by Kruss Processor Tensiometer K-12 (Wilhelmy plate) method as described by (Singh *et al.,* 1989, Tuleva *et al.,* 2005).

**16 s rRNA sequence analysis**: 16s rRNA genes were amplified using PCR with the universal primer FD1 and RP2. The sequence of FD1 and RP2 were 5'- AGAGTTTGATCTGGCTCAG-3'and5'AAGGAGGTCATCCAGCC-3'respectively. A search of the GenBank Nucleotide library for sequence similar to those determined was made by using BLAST (Altschul *et al*., 1990), through National Center for Biotechnology information (NCBI) internet site http://www.ncbi.nlmnih.gov/BLAST. Sequences with more than 98% identity with a Genbank sequence were considered to be of the same species as the highest score matching sequence on the public sequence databases.

**Analysis of component of biosurfactant by Thin layer Chromatography-**The crude extract was separated by TLC using aluminium sheets silica gel plates with chloroform:methanol:acetic acid and water (25:15:4:2). Ninhydrin reagent was used to detect free amino groups. The lipid components were detected as brown spots after spraying the plate with chromosulphuric acid. The carbohydrate compound were detected as red spots after spraying the plates with α-napthol in concentrated sulphuric acid.

**Emulsification properties of biosurfactant-**The emulsification index ($E_{24\%}$) of produced compound using different hydrocarbons like kerosene, petrol, diesel and hexadecane was tested.

## RESULTS AND DISCUSSION

**Isolation and screening of biosurfactant producing bacteria-** From petrol pump soil samples 78 isolates were obtained from these, 52 showed oil displacement test positive, of these 43 isolate showed drop collapse test positive. Further emulsification index test found that 36 isolates showed emulsification index ranging from 10% to 30%. 6 isolates showing emulsification index of more than 30% were selected. Isolate DB1 from Dombivli petrol pump gave maximum emulsification index of 40% and oil displacement zone of 22 mm (Fig 2.1).Similarly, from garage soil and garage pits, 121 isolates were obtained. 86 isolates showed oil displacement test positive, of these 60 isolates showed drop collapse test positive. Further emulsification index test found that 73 isolates could give emulsification index ranging from 08% to 38%. 8 isolates gave emulsification index of more than 30%.These 14 isolates exhibited good emulsification activity as shown in Table 2.1. Among the screening methods oil displacement test was found to be more sensitive than other methods for detection of biosurfactant in the supernatant from a culture medium. Similar findings are reported by many workers. Youssef *et al*. (2004), reported the oil displacement technique is more suitable than the drop collapse method in detecting low levels of biosurfactant production. Hamzah *et al*.,(2013), also suggested that oil displacement test was better in detection of small quantity of biosurfactant. Drop collapse method for screening of biosurfactant producers was used by many workers (Batista *et al*., (2006), Bodour *et al*., (2003), Plaza *et al*. (2006) and Youssef *et al*.(2004). However, Husain (2008), found that drop collapse test was not able to reliably detect low concentration of biosurfactants.

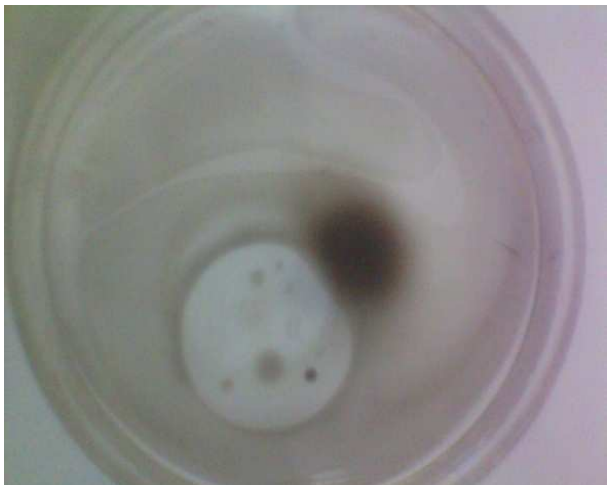

**Fig 2.1 Oil displacement zone exhibited by isolate DB1**

**Table 2.1: Isolates selected for biosurfactant production**

| Isolates | Oil displacement Test | Emulsification index (E24%) |
|---|---|---|
| Petrol pump | | |
| U1 | ++ | 30 |
| K1 | ++ | 32 |
| K2 | ++ | 34 |
| K4 | ++ | 34 |
| **DB1** | +++ | **40** |
| DB3 | ++ | 36 |
| Garage and garage pits | | |
| UG6 | ++ | 32 |
| MhG4 | ++ | 35 |
| BHG4 | ++ | 38 |
| MSG5 | ++ | 35 |
| ASG3 | ++ | 32 |
| KpG2 | ++ | 35 |
| KpG7 | ++ | 32 |
| KSG8 | ++ | 35 |

*(++ oil displacement zone of more than 10 mm, +++= oil displacement zone of more than 20 mm)*

**Identification of biosurfactant producer**

Among these 14 isolates 9 isolates were Gram positive and 5 were Gram negative. The gram negative isolates belonged to *Pseudomonas*, *Serratia* and *Azotobacter sp*. and among the gram positive isolates *Bacillus* sp were predominant (Table 2.2)

**Table 2.2: The isolates were identified as follows-**

| Isolate | Name of the organism |
|---|---|
| U1 | *Bacillus subtilis* |
| K1 | *Bacillus coagulans* |
| K2 | *Serratia* |
| K4 | *Bacillus polymyxa* |
| DB1 | *Pseudomonas stutzeri* |
| DB3 | *Pseudomonas aeruginosa* |
| UG6 | *Bacillus licheniformis* |
| MhG4 | *Bacillus megaterium* |
| BHG4 | *Arthrobacter* |
| MSG5 | *Bacillus cereus* |
| ASG3 | *Bacillus marcerans* |
| KpG2 | *Pseudomonas sp* |
| KpG7 | *Rhodococcus sp* |
| KSG8 | *Azotobacter sp* |

**Selection of best biosurfactant producer-** The isolates gave a biomass yield ranging from 0.76 g/L to 1.7 g/L and the biosurfactant yield from 0.08 g/L to 0.68 g/L. All the isolates lowered the surface tension of the media from 65 mN/m to 42 mN/m and below (Table 2.3).

**Table-2.3: Selection of best biosurfactant producer based on emulsification activity, surface tension, biomass and biosurfactant yield**

| Isolates | Biomass ($gL^{-1}$) | E.A(O.D-540nm) | Biosurfactant ($gL^{-1}$) | Surface tension(mN/m) |
|---|---|---|---|---|
| U1 | 0.9 | 0.14 | 0.11 | 41.3 |
| K1 | 0.81 | 0.11 | 0.09 | 42.0 |
| K2 | 0.9 | 0.15 | 0.10 | 41.6 |
| K4 | 1.3 | 0.32 | 0.43 | 39.2 |
| DB3 | 1.2 | 0.25 | 0.26 | 40.0 |
| KpG7 | 0.92 | 0.18 | 0.15 | 40.8 |
| UG6 | 0.96 | 0.29 | 0.21 | 39.9 |
| MhG4 | 0.79 | 0.13 | 0.09 | 41.8 |
| BHG4 | 0.85 | 0.15 | 0.11 | 40.4 |
| MSG5 | 0.76 | 0.08 | 0.08 | 42.8 |
| ASG3 | 0.85 | 0.12 | 0.10 | 40.8 |
| KpG2 | 0.92 | 0.18 | 0.12 | 41.5 |
| **DB1** | **1.72** | **0.52** | **0.68** | **36.0** |
| KSG8 | 0.90 | 0.17 | 0.15 | 40.0 |

**16s RNA sequence analysis**: Sequence alignment and comparison revealed 100% similarity with *Pseudomonas stutzeri*. The nucleotide sequence of 16S rRNA of isolate DB1 has been deposited in GenBank database under accesssion number JQ480622.

**Analysis of component of biosurfactant by Thin layer Chromatography**-The biosurfactant showed purple colour spot when sprayed with ninhydrin, indicating the presence of free amino groups. The lipid components were observed as brown spots when sprayed with chromosulphuric acid. Also, red spot was seen with α-napthol in concentrated sulphuric acid, this shows the presence of carbohydrate compounds. The thin layer chromatography result suggests that the biosurfactant contain protein, carbohydrate as well as lipid moieties. Polymeric nature of biosurfactant is reported by many workers. Adebusoye *et al*. (2007), found that *Pseudomonas aeruginosa* DDV4 produced a heteropolymer biosurfactant consisting of lipid, protein and carbohydrate moieties. Husain *et al*. (1997), studied the production of biosurfactant by *Pseudomonas nautica* and reported that the major constituents of the biosurfactant were proteins, carbohydrates and lipid at the ratio of 35:63:2, respectively. However, Pruthi and Cameotra (1995),found that the biosurfactant isolated from *Pseudomonas putida, Pseudomonas diminuta* and *Pseudomonas aeruginosa* were a mixture of only proteins and lipids.

**Emulsification property of biosurfactant**- As shown in Table 2.4, the biosurfactant was found to have strong emulsification ability.

**Table 2.4: Emulsification ability of biosurfactant**

| Hydrocarbons | (E24%) | Emulsification activity (OD at 540 nm) |
|---|---|---|
| Kerosene | 69 | 0.39 |
| Petrol | 66 | 0.24 |
| Diesel | 67 | 0.61 |
| Hexadecane | 62 | 0.20 |

## CONCLUSION

In the present study, 199 bacterial cultures were isolated from petroleum hydrocarbon contaminated soil. Among them, 14 strains were selected in terms of their emulsification ability. The potent biosurfactant producer *Pseudomonas stutzeri DB1* was selected as it gave maximum biomass and biosurfactant yield. The isolate also showed strong emulsification ability. Thus the ability of isolate DB1 (*Pseudomonas stutzeri DB1*) to produce biosurfactant with potent emulsification properties suggests its potential application in environment clean up procedures.

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