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# An ecofriendly approach for removal of oil contaminated soil: A biosurfactant study

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## Abstract

Oil sludge which is entrapped within soil where its spills. It causes serious problems to the environment as well as both animal and human who interact with them. The hydrocarbons in the sludge penetrate from the top soil into the subsoil slowly, presenting a direct risk of contamination to subsoil and groundwater. On the other hand, the light hydrocarbons in the oil sludge vaporize, leaving behind a layer of oil containing dust of soil which blows upwards to pollute the air. Therefore, the oil sludge should be treated to prevent harm to environment. In this present study, oil contaminated soil was taken for the research work. The soil samples were isolated and their growth characteristics, DNA isolation and DNA estimation was done by using electrophoresis and diphenylamine method. From the results the growth characteristics and DNA quantification confirms that *Bacillus subtilis* will act as a good surfactant and ecofriendly microorganism. This study will enhance the growth of biosurfactant (bioremediation) for our sustainable environment.

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## Introduction

Microorganisms can only alter the speciation of metal contaminant and convert them into nontoxic forms (Lovley, 1997). Recent advances in the field of microbial surfactants are largely attributed to the development of quick, reliable methods for screening biosurfactant-producing microbes and assessing their potential (Desai, 1997).

Van der (Vegt *et al.,* 1991) developed an asymmetric drop shape analysis by profile for the assessment of potential biosurfactant- producing bacteria. A list of industrially important biosurfactants produced by microorganisms is given in Table 1. Out of all these, three biosurfactants sophorolipids, surfactin and rhamnolipids are most important biosurfactants, which are studied, in detail in this work.

While bioventing has been shown to remove soil pollutants ranging from light to middle distillate oil (Lee and Swindoll, 1993; Mosco and Zytner, 2017). Most remediation experts believe the most effective remediation method will depend on the type of oil spilled, quantity spilled, and the environmental medium impacted (Samanta *et al.*, 2002; Pugazhendhi *et al.*, 2018). The aim of this work is design after number of literature survey done and to remove oil from contaminated soil for oil free environment.

## Materials and methods

The samples were collected from oil contaminated areas such as, petrol bunk, Automobile workshop (Tractor workshop), oil mill, and Engine pump set around Thanjavur district. The sample was collected in a sterile polyethylene bag with the help of sterile tea spoon and it was taken to the laboratory immediately and analyzed for the isolation of biosurfactant producing bacteria (Fig. 1).

*Isolation of bacterial species from soil samples*

Bacterial species were isolated from the collected soil samples by serial dilution and agar plating method. The soil sample was diluted from $10^{-1}$ to $10^{-6}$ dilutions, and the diluted soil samples were spread on sterile nutrient agar plates.

**Fig. 1.** Soil samples

*pH*

The isolates (1ml each) were inoculated into six tubes of 10 ml nutrient broth of varying pH values (6, 7 and 8). NaOH (1M) and HCl (1M) were used to adjust the pH in the broths. The tubes were then incubated at 37°C for 160 hours, after which the cultures were analysed for further analyses.

*Growth characteristics*

The inoculated microbes on the selective medium are tend to grow during the incubation period from 24 hours to 48 hours at 22°C. The growth is observed and the colony morphology is interpreted. The bioluminescent bacteria give a characteristic circular, rod shaped colony with white translucent appearance.

*Molecular analysis - DNA isolation*

The old nutrient broth culture is transferred into fresh nutrient broth. Then incubated at 37°C for 24 hours in shaker. On the next day, 1.5 ml of grown culture was taken in a centrifuge tube and centrifuged at 8000 rpm for 5 minutes. Supernatant was discarded and pellet was suspended in 100 µl of CTAB and 20µl of 10% SDS added and vortex mixing. The tube is placed in a water bath at 65°C for 15 minutes. Equal volume of phenol: chloroform (1:1 ratio) was added and vortexes. Centrifuge 8000 rpm for 10 minutes. Collect the supernatant and add 100 µl of ice cold ethanol and mixed vortexes. Centrifuged 8000 rpm for 10 minutes and air dried for 15 minutes, and ethanol in discarded. Then add 15µl of 1X TAE buffer stored in refrigerator at 4°C. Then the stored DNA will be electrophoresis.

*Agarose gel electrophoresis*

Electrophoresis is the technique used to separate and sometimes purify macromolecules especially proteins and nucleic acids. 0.8% of agarose gel was prepared in 1X TAE buffer ethidium bromide (1µg /M) was added into it and heated till Agarose gel fully suspended. The gel was cooled up to 55°C and casted in casting unit of electrophoresis assembly. The gel was run at 50 V for 30 minutes to equilibrate. The 9µl of isolated DNA sample was mixed with 2 µl of gel loading dye. 10 µl of mixture was loaded into wells and run at 80-100 V for 30 minutes.

*Quantitative analysis of genomic DNA*

To estimate the DNA, a convenient and easy method is colorimeter method, which is based on the quantitative reaction of deoxy sugar with the diphenylamine reagent. 1.0g of diphenylamine dissolved in 97.5ml of glacial acidic acid and added 2.5ml of concentrated sulphuric acid and stored in a room temperature. The 5ml of diphenylamine reagent was mixed with 0.5ml solution containing nucleic acid. The mixture was showed on a boiling water bath for 5 minutes. It was then estimated at 595 nm against blank. The blank was prepared with 0.5ml of distilled water once 5ml of a reagent in water both for 5 minutes and cooled. Absorbance was read out at 595 nm using UV spectrophotometer.

**Results and discussion**

The present study was conducted to determine the oil degrading activity of biosurfactant producing organism and all the isolated strains were screened for the confirmation of bio surfactant producing bacteria is *Bacillus subtilis* and the strains showed good result finally that strains taken for the further study.

**Table 1.** Industrially important biosurfactants produced by microorganisms

| Biosurfactant | Organisms | Reference(s) |
|---|---|---|
| Glycolipids | | |
| Sophorolipids | *T. bombicola*; *T. apicola*; *T. petrophilum* | Tulloch *et al.*, 1962; Spenser *et al.*, 1970; Inoue and Ito 1982; Asmer *et al.*, 1988; Cavelero and Cooper, 2003; Daverey and Pakshirajan, 2010 |
| Rhamnolipids | *P. aeruginosa*; *Pseudomonas* sp *Serratia rubidea* | Jarvis and Johnson, 1949; Reiling *et al.*, 1986; Matsufuji *et al.*, 1997; Wu *et al.*, 2007; Sarachat *et al.*, 2010 |
| Trehalolipids | *R. erythropolis*, *N. erythropolis* | Rapp *et al.*, 1979; Uchida *et al.*, 1989 |
| Lipopeptides | | |
| Surfactin | *B. subtilis*; *B. pumilus* | Arima *et al.*, 1968; Kakinuma *et al.*, 1969; Cooper *et al.*, 1981; Makkar and Cameotra, 1998; Liu *et al.*, 2009 |
| Viscosin | *P. fluorescens* | Neu and Poralla, 1990 |
| Gramicidins | *B. brevis* | Marahiel *et al.*, 1977 |
| Polymyxins | *B. polymyxa* | Suzuki *et al.*, 1965; Falagas *et al.*, 2005 |
| Glycolipids | | |
| Fatty acids, Phospholipids and Neutral lipids | | |
| Fatty acids | *A. parafineus*; *C. lepus*; *P. spiculisporum* | Cooper *et al.*, 1989; Makkar and Cameotra, 2002 |
| Neutral lipids | *N. erythropolis* | McDonald *et al.*, 1981 |
| Phospholipids | *T. thioxidans* | Beeba *et al.*, 1971; Lemke *et al.*, 1995 |
| Polymeric surfactants | | |
| Emulsan | *A. calcoaceticus* | Zosim *et al.*, 1982; Rosenberg *et al.*, 1993 |
| Biodispersan | *A. calcoaceticus* | Rosenberg *et al.*, 1988 |
| Mannan-lipid -protein | *Candida tropicalis* | Kappeli *et al.*, 1984 |
| Liposan | *Candida lipolytica* | Cirigliano and Carman, 1985 |
| Carbohydrate protein lipid | *Pseudomonas fluorescens* | Desai *et al.*, 1988 |
| Particulate biosurfactants | | |
| Vesicles and fimbriae | *A. calcoaceticus* | Gutnick and Shabtai, 1987 |
| Whole cells | Variety of bacteria | Fattom and Shilo, 1985 |

**Table 2.** Colony morphology of nutrient agar

| SL | Sample | Colony morphology on nutrient agar |
|---|---|---|
| 1 | Petrol bunk soil | Smooth whitish pin pointed colony |
| 2 | Tractor work shop soil | Smooth whitish pin pointed colony |
| 3 | Oil mill soil | Smooth whitish pin pointed colony |
| 4 | Engine oil soil | Smooth whitish pin pointed colony |

*Characterization of isolated bacteria*

Colony morphology: The shape, size, elevation, margin and cooler of the colony were observed in the culture plates with Nutrient Agar used as the nutrient medium. The observations were noted down (Table 2).

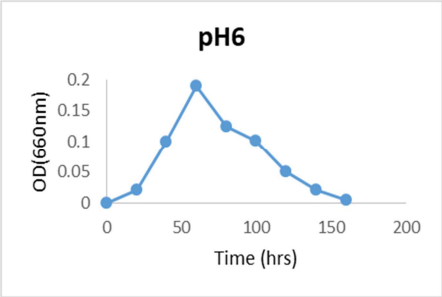


**Fig. 2A.** Growth study of *Bacillus subtilis* pH 6

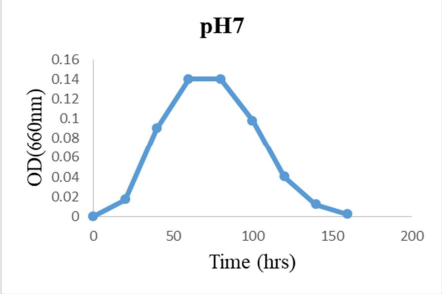


**Fig. 2B.** Growth study of *Bacillus subtilis* pH 7

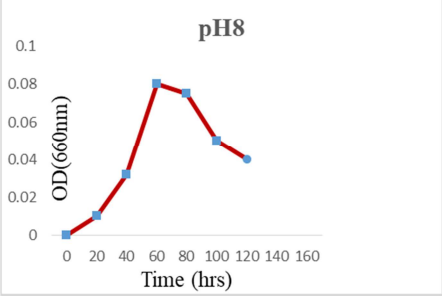


**Fig. 2C.** Growth study of *Bacillus subtilis* pH 8

The effect of temperature, pH and salinity on surface activity and emulsification index has been shown in (Fig. 2A, 1B & 1C), respectively. The results were comparable to that obtained for *B. subtilis*. The surface activity of the biosurfactant remained stable over the range of temperature and pH, studied. With respect to pH, the least surface tension was obtained from neutral pH, onwards. Such extreme stability was also reported by Abdel-Mawgoud *et al.,* 2009 and Gandhimathi *et al.,* 2009 for the *Pseudomonas aeruginosa* strain and *Brevibacterium aureum* MSA13 respectively. This suggests that the biosurfactant isolated may be used in microbial enhanced oil recovery processes where high temperatures prevail. The findings indicate the potential application of the biosurfactant over a wide range of temperature and pH. This finds application in situations where extreme conditions of temperature and pH prevail such as bioremediation of soil as well as marine environments. It can also be used for enhanced oil recovery operations.

In this present study, initially the growth pattern by the isolates on relatively simple hydrocarbon source (engine oil) was studied. Mitchell, 1965 has reported that growth can be taken as a parameter for microbial utilization of substrate. Eventually screening that about Arabinose, Salicin, Trebulose, Cellobiose, Maltose, Lactose, Sucrose, Fructose and Glucose carbon content of hydrocarbons present as bacterial protoplasm.

The ability of a surfactant to enhance the biodegradation of slightly soluble organic compounds depends on the extent to which it increases the bioavailability of the compound. Research has demonstrated that biosurfactants exhibit versatile properties such as excellent self-organization, protein-binding and antitumor action, Harvest the synthetic surfactants synthetic do have the properties which end up in causing a high environmental impact Mukherjee, 2006. In the present study, the partially purified biosurfactant exhibited maximum emulsification activity and decay constant when compared to other commercially available surfactants thus proving its efficiency. The estimation of DNA results were presented in the Table 3 and Fig. 2D.

**Table 3.** DNA Estimation by diphenylamine method

| SL | Particulars | B | S1 | S2 | S3 | S4 | S5 | T1 | T2 | T3 | T4 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | Volume of standard(ml) | _ | 0.4 | 0.8 | 1.2 | 1.6 | 2.0 | _ | _ | _ | _ |
| 2 | Concentration of standard(ml) | _ | 40 | 80 | 120 | 160 | 200 | _ | _ | _ | _ |
| 3 | Volume of test sample(ml) | _ | _ | _ | _ | _ | _ | 0.5 | 0.5 | 0.5 | 0.5 |
| 4 | Volume of purchloric acid(ml) | 2 | 1.6 | 1.2 | 0.8 | 0.4 | _ | _ | _ | _ | _ |
| 5 | Volume of diphenylamine(ml) | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 |
| 6 | Absorbance of DNA estimation 630nm | | | | | | | | | | |
| 7 | Optical density | 0.00 | 0.454 | 0.479 | 0.434 | 0.418 | 0.479 | 0.573 | 0.825 | 0.019 | 0.020 |

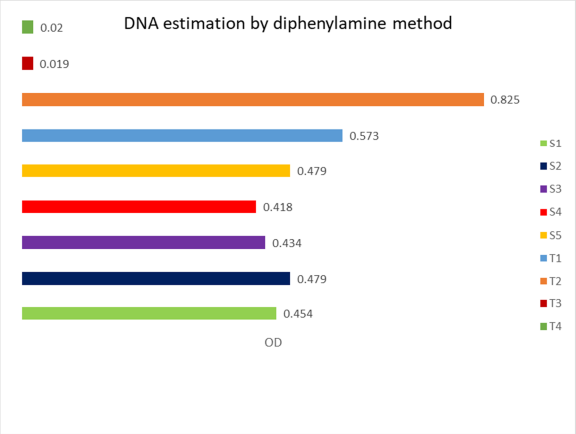


**Fig. 2D.** DNA estimation by diphenylamine method

The bacterial isolates viz. *Bacillus subtilis* were screened as hydrocarbon utilizers as they were capable of growing in petrol bunk soil in this present study. Members of the genus *Rhodococcus* are HC degraders Van Hamme, 2003. *Pseudomonas species*, ubiquitous in soil and water are of considerable scientific and technological importance and comprise a taxon of metabolically versatile organism capable of utilizing a wide range of simple and complex organic compound. Molecular techniques for the identification of hydrocarbon-degrading bacteria have been rarely used in environmental studies Chang, 2001 and Roling, 2002.

## Conclusion

Eco-friendly technologies must be used to clean the environment such as degradation by microorganisms. Bioremediation has been accepted as an important method for the treatment of oil pollution by biosurfactant produced by bacterial colonies. Under certain conditions, living microorganisms primarily bacteria can metabolize various classes of hydrocarbons compound. The results suggest that *Bacillus subtilis* is a good producer of biosurfactant and against hydrocarbon contaminated places. Thereby the oil contaminated soils can be degraded by introducing the biosurfactant producing organisms such as *Bacillus subtilis,* which is able to degrade the oil contaminated soil from the environment. This study has to be taken for further research for betterment work.

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