

ISOLATION OF BIOSURFACTANT PRODUCING BACTERIA FROM ENVIRONMENTAL SAMPLES

R.C. Jaysree, Subham Basu, Priyanka P. Singh, Twinkle Ghosal, Pragya A. Patra, Yekala Keerthi, and Rajendran, N*

Molecular and Microbiology Research Lab, Environmental Biotechnology Division, School of Bio Sciences and Technology, VIT University, Vellore-632014, Tamil Nadu, India

*Corresponding author:

Dr. N. Rajendran

Professor

Molecular and Microbiology Research Laboratory,

Environmental Biotechnology Division,

School of Bio Sciences and Technology, VIT University, Vellore-632014, TN, India

Tel: + 91 9488338557, E-mail: rajendran.n@vit.ac.in

Summary

Biosurfactants are amphiphilic compounds produced extracellularly by microorganisms on cell surfaces, or excreted extracellularly. They contain hydrophilic and hydrophobic moieties that reduce surface and interfacial tension between molecules at the surface and interface respectively. The present study was focused on isolation of biosurfactant producing bacteria from environmental samples (contaminated soil and uncontaminated water) and assessing the potential of these isolates by various standard method. Oil displacement and emulsification index methods were used to screen the capability of isolates for producing biosurfactant. Studies were also carried out using diesel and engine oil as hydrocarbon source. The identified bacteria *Bacillus subtilis* and *Bacillus cereus* which were isolated from water and soil respectively were found to possess biosurfactant producing capabilities. The biosurfactants produced by *B. subtilis* had an emulsification capacity (E_{24}) of 20% and 15%, and that by *B. cereus* was 30% and 20% for diesel and engine oil respectively. Results of the present study suggested that these isolates have potential to use in oil degradation studies.

Keywords: Biosurfactants, bacteria, oil displacement, emulsification index, engine oil and diesel oil

Introduction

Surfactants, a short form for “surface-active-agents”, are basically chemical compounds which lower the surface tension of a liquid, the interfacial tension between two liquids, or that between a liquid and a solid. These surfactants are produced by a variety of microorganisms such as yeasts, bacteria and filamentous fungi¹ and thus are called biosurfactants. Biosurfactants have different properties such as

they act as detergents, wetting agents, emulsifiers, foaming agents, and dispersants². These are usually organic compounds that are amphiphilic in nature, which contains both the hydrophobic and hydrophilic component. The hydrophobic (non-polar) part of the biosurfactant is insoluble in water and may have a long-chain of fatty acids, hydroxyl fatty acids or α -alkyl- β -hydroxy fatty acids. The hydrophilic (polar) end can be a carbohydrate, amino acid, cyclic peptide, phosphate, carboxylic acid or alcohol³.

Biosurfactants can be characterized in two categories i.e. low molecular weight and high molecular weight molecules in which the first one shows lower surface and interfacial tensions and the later one bind tightly to surfaces⁴. Micro-organisms producing biosurfactants help to amplify the bioavailability of hydrocarbons by enhancing the contact between pollutant and the micro-organisms in the presence of the biosurfactant which helps in the accelerated bioremediation of hydrocarbon contaminated sites. The present study was carried out to know the biosurfactants producing potential of bacteria isolated from two different environmental samples i.e. contaminated soil and uncontaminated water samples.

Materials and methods

Sample collection

The surface water samples were collected from the artificial pond in the campus of VIT University (12°58'09"N 79°09'21"E). Two contaminated soil samples one was 1 m and the other one was about 5 m distance from the petrol pump that was located near Vellore, Tamil Nadu, India. The samples collected in vials and were immediately transported to the Molecular and Microbiology Research Laboratory and stored at 4°C until further analysis.

Media composition

In order to isolate the biosurfactant producing bacteria nutrient broth and agar medium were used. The composition of liquid medium was as follows (gram per liter of distilled water) peptone, (10.0); meat extract, (10.0); sodium chloride, (5.0); without agar medium and solid medium was prepared by adding 1.5% agar and pH was adjusted up to 7.2 ± 0.2 and autoclaved at 121 °C for 15 min at 15 lbs pressure.

Inocula preparation

Samples were serially diluted and plated by spread plate method on nutrient agar medium and incubated under aerobic conditions at 37°C for 24 hours. Diesel was used as hydrocarbon source in each of the petri-dish with the help of cotton buds and control with no diesel was also maintained. The medium was enriched with hydrocarbon source in the form of vapors. These 24 hours grown colonies were inoculated in 100 ml nutrient broth medium in 250 ml Erlenmeyer flask containing 1% water soluble fraction⁶ of diesel at 200 rpm at 30°C for 7 days. After 1 week of incubation, 1 ml of inoculum was transferred to 99 ml of nutrient broth containing 1% diesel for a incubation period of 2 days. The process was continued by transferring 1 ml of inoculum to a 99 ml broth kept for one day.

Oil displacement Test

Oil displacement is a method used to determine the diameter of the clear zone, which occurs after adding surfactant-containing solution on an oil-water interphase. The diameter evaluation allows the surface tension reduction efficiency of a given biosurfactant. In this test, 15 ml distilled water was added to a petri dish which is 90 mm in diameter. 100 μ l of diesel was added to the water surface, followed by the addition of 20 μ l of cell culture supernatant on to the oil surface. The diameter and the clear halo visualized under visible light was measured after 30 s ⁷.

Emulsification capacity (E₂₄)

Emulsification capacity of the biosurfactant towards two hydrocarbons i.e. diesel and engine oil was done using the Cooper and Goldenberg method ⁵. A mixture of 2 ml hydrocarbon and 1 ml cell free extract obtained after the centrifugation of sample culture were taken in a test tube and homogenized by vortexing for 2 min. The emulsion activity was investigated after 24 hours and the emulsification index (E₂₄) was calculated by the total height of the emulsion by the total height of the aqueous layer and then multiplying by 100. The results were compared with SDS as positive control.

Growth curve studies

Growth studies were also conducted with the isolates one from contaminated soil and another from uncontaminated water sample. The study was performed in 250 ml side arm flask using 120 ml of liquid medium kept in orbital shaker at 200 rpm at a temperature of 30°C for 78 hrs and reading was taken at regular intervals of 2 hrs at absorbance 600 nm. One was maintained as test with carbon source of 2% water soluble fraction of diesel and the control without carbon source. Oil displacement studies were performed at regular intervals of 6 hours for 54 hours.

Identification of the strain

Identification and characterization of isolates by Bergey's manual of determinative bacteriology⁷ using IMViC test kit from Hi-media company. Different staining methods such as Gram, capsular and endospore staining were also done.

RESULTS AND DISCUSSION

Of a total of 5 sample isolates, 3 were from uncontaminated water and 2 isolates were from contaminated soil. This study was only focused on one strain (W1) from the uncontaminated water and one strain (S1) from contaminated soil for characterizing these strains for biosurfactant production. W1 and S1 were identified by different staining, culture morphology and biochemical characteristics as *Bacillus subtilis* and *Bacillus cereus* respectively. Table 1 shows the different characteristics of the two strains identified using Bergey's manual of determinative bacteriology. The strain S1 showed more emulsifying ability comparing to W1 when both of the hydrocarbons i.e. diesel and engine oil

were used are shown in Table 2. According to the emulsification index, both strains showed good emulsifying ability when diesel was used as hydrocarbon source than the engine oil.

Table 1: Identification and characterization of the isolated strains

Isolate	W1	S1
Gram stain	+	+
Endospore stain	+	+
Colony morphology	White, large, undulate and rough	Cream, raised, circular and undulate
Catalase	+	+
Indole production	-	-
Glucose fermentation	-	+
Lactose fermentation	-	-
Starch hydrolysis	+	+
Nitrate reduction	+	+
Motility	+	+
Mannitol fermentation	+	-

Table 2: Emulsification indexes (E_{24}) of water isolate (W1) and soil isolate (S1)

Description	Diesel oil	Engine oil
Isolate	E_{24} (%)	E_{24} (%)
W1	20	15
S1	30	20
SDS	50	53.3

Bacterial growth studies were done for both W1 and S1 with control having no diesel. Figure 1 and 2 shows the graphical representation of both W1 and S1 readings taken at regular intervals of 2 hours up to 78 hours. The growth curve reveals that the S1 showed a better growth in the presence of hydrocarbon source compared to W1 isolated from uncontaminated water samples. This marked variation is obvious due to the difference in the sample sites, environmental condition, nutrient availability and also difference in time of exposure with the pollutants. Oil displacement readings were taken at intervals of 6 hours regularly and have been represented in Figure 3 & 4. Oil displacement was seen to be higher in the pollutant exposed (soil) isolates than that in the uncontaminated isolates (water), but both these isolates showed great difference with their respective controls.

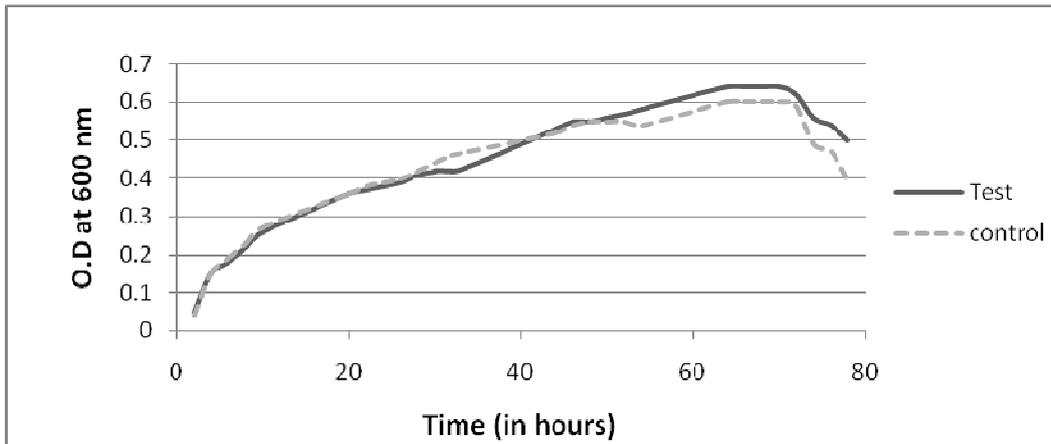


Fig. 1: Growth curve studies of Bacillus cereus (S1)

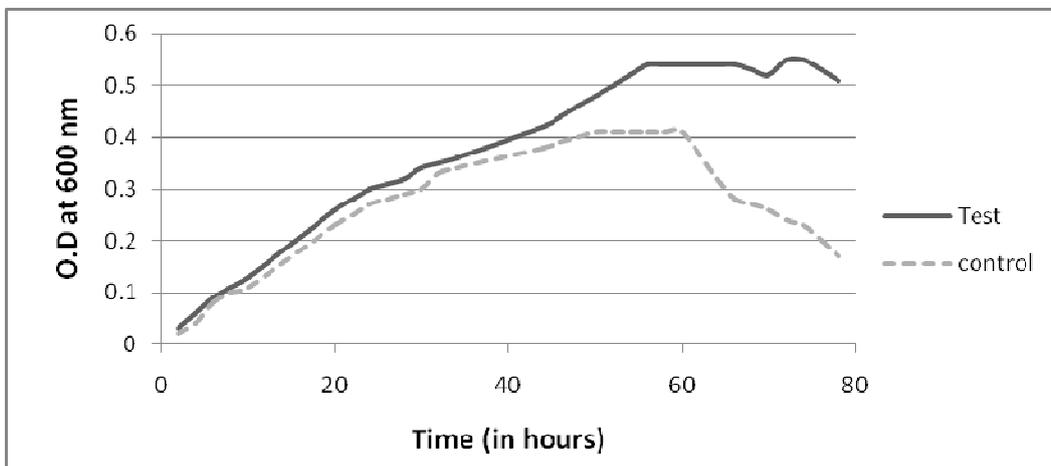


Fig. 2: Growth curve studies of Bacillus subtilis (W1)

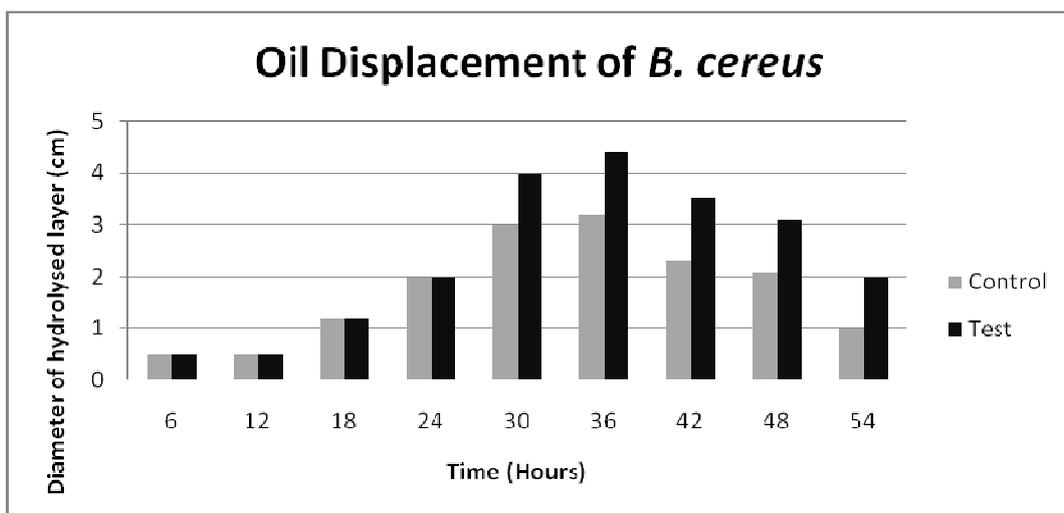


Fig. 3: Oil displacement by Bacillus cereus (S1)

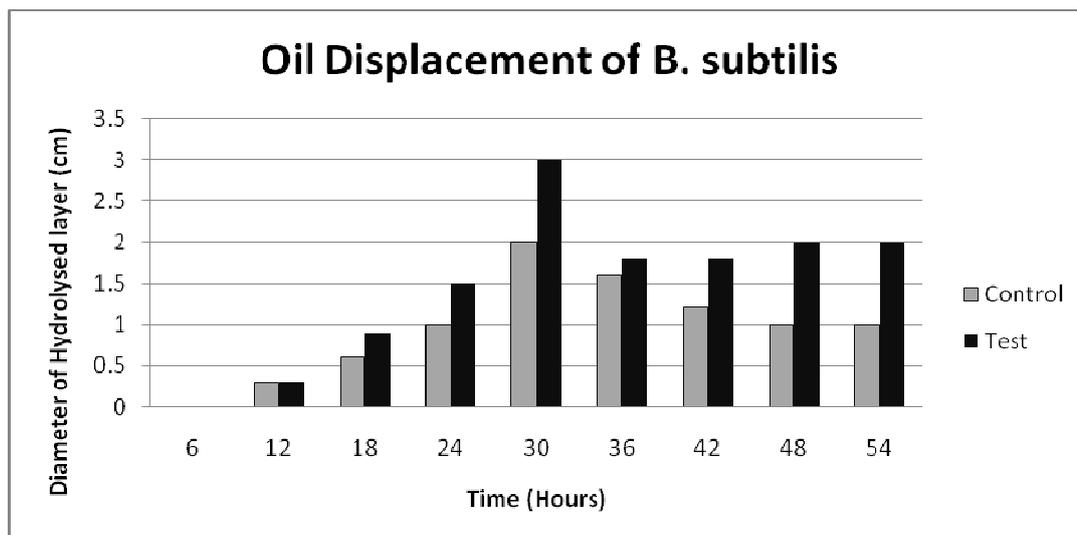


Fig.4: Oil displacement by Bacillus subtilis (W1)

Conclusion

The bacteria *Bacillus subtilis* (W1) and *Bacillus cereus* (S1) are found to produce ample biosurfactants. *B. cereus* was obtained from contaminated site and hence was expected to be a biosurfactant producer. However, *B. subtilis* was isolated from uncontaminated water sample but showed biosurfactant activity. Marked difference in the biosurfactant activity between experiment and control suggested that the biosurfactant producing activity of *B. subtilis* may be induced in the presence of pollutant. Isolation of the responsible gene and subsequent PCR amplification may prove useful to artificially produce environmentally friendly surfactants. In future studies involving biosurfactants and bioremediation technologies, these two strains may be helpful. It may be concluded that *B. subtilis* and *B. cereus* are capable of producing biosurfactants that would help in degrading oil and other hydrocarbon pollutants to the environment.

Acknowledgement

The authors wish to thank the management and staff of VIT University, Vellore, Tamil Nadu, India for their unending help and support extended to complete this study.

References

1. Chen SY, Wei YH, and Chang JS, Repeated pH-stat fed-batch fermentation for rhamnolipid production with indigenous *Pseudomonas aeruginosa* S2. *Applied Microbiol. Biotechnol.* 2007; 76 (1):67-74.
2. Makkar RS, Cameotra SS, An update on the use of unconventional substrates for biosurfactant production and their new applications. *Applied Microbiology Biotechnology* 2002;58: 428–434.

3. Van Ginkel CG, Complete degradation of xenobiotic surfactants by consortium of aerobic micro-organisms. *Biodegradation*. 1989; 7 (2): 151-164.
4. Rosenberg E and Ron EZ, High and low-molecular-mass microbial surfactants. *Applied Microbiol. Biotechnol.* 1999; 52 (2): 154-162.
5. Cooper DG, Goldenberg BG, Surface active agents from two *Bacillus* species. *Appl. Environmen. Microbiol.* 1987; 53(2):224 -229.
6. Dunlap LE and Beckmann DD, Soluble hydrocarbons analysis from kerosene / diesel. In proceedings of the conference on Petroleum hydrocarbons and Organic chemicals in ground water 1988: *Prevention, Detection and Restoration, Dublin, Ohio, National Water Well Association.*
7. Rodrigues LR, Teixeira JA, Van der Mei HC, Oliveira R, Physiochemical and functional characterization of a biosurfactant produced by *Lactococcus lactis* 53 . *Colloid surface B* 2006; 49(1):79 – 86.
8. Buchanan RE, Gibbons NE, *Bergey's manual of determinative bacteriology*, eighth edition. The Williams and Wilkins C, Baltimore, pp.1974;1146