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BIOSURFACTANT PRODUCING MICROORGANISMS FROM OIL CONTAMINATED AREAS OF INDIAN THAR DESERT

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ABSTRACT

Biosurfactants are surface-active molecule produced by microorganisms and have several advantages over the chemical surfactants. The objective of this research was to obtain biosurfactant-producing bacteria from hydrocarbon contaminated soils from Barmer, diesel shed Jodhpur etc. to observe their biosurfactant activity through oil spreading test, emulsification test, haemolysis test and drop collapse assay. Overall, 14 isolates were screened for biosurfactant production. The result showed that 13 strains gave positive results on the screening tests and were determined as biosurfactant-producing bacteria. Details of the biosurfactant producing efficiency of various bacterial strains are described. The findings can be useful for removal of organic pollutants from oil contaminated areas.

Keywords: biosurfactants, microorganisms, emulsification, haemolysis, thar desert

INTRODUCTION

Remediation technology for oil pollution has become a global importance. Degradation of hydrocarbons using microorganisms has an important role in combating environmental pollution. Biosurfactants produced by hydrocarbon degrading microorganisms are of different chemical nature and molecular size. These biosurfactants increase the surface tension of the hydrophobic water insoluble substrates and thereby enhancing their bioavailability and the rate of bioremediation. Surface active compounds occurring naturally, derived from microorganisms viz. bacteria, yeast and fungi

are called as biosurfactants. Biosurfactants produced by bacteria are surface active compounds required in the degradation of hydrocarbons **[1]**. Microbial diversity in oil contaminated areas of thar desert **[2]** and seasonal fluctuations in its population has been reported **[3]**. Biosurfactants are amphiphilic, consisting of two parts, a polar (hydrophilic) moiety and a non polar (hydrophobic) group. The hydrophilic group consists of mono-, oligo-, or polysaccharides, peptides or proteins while the hydrophobic moiety usually contains saturated, unsaturated and hydroxylated fatty acids.or fatty alcohols **[4]**. Bacterial co-metabolism in composting process has been widely used to remove hydrocarbons, aided by in-situ production of bio-based surfactants **[5]**. For example, the biosurfactant rhamnolipid has been produced from *Pseudomonas aeruginosa* **[6]** while *Bacillus subtilis* is known to produce surfactin **[7]**. Biosurfactants are amphiphilis compounds produced by variety of microbes as extracellular compounds **[8]**. The advantageous characteristics of biosurfactants such as structural diversity, low toxicity, higher biodegradability, better environmental compatibility, higher substrate selectivity and lower CMC have led to several applications in the food, cosmetics and pharmaceutical industries **[9]**. Biosurfactants has received considerable attention in the field of environmental remediation processes such as bioremediation, soil washing and soil flushing. Although biosurfactants exhibit such important advantages, they have not yet been employed extensively in industry because of relatively high production costs **[10]**. Oil eating fungi from Indian thar desert has been reported **[11]**. Since the last decade increasing attention has been made to the development and implementation of biotechnology for cleaning up this contamination. The objectives of this study were to isolate and screen biosurfactant producing microorganisms from oil contaminated soil samples of The Indian Thar desert.

MATERIALS & METHODS

Collection of Soil Samples and Enrichment of Microorganisms

Samples were collected from oil contaminated areas near Barmer, Jodhpur (Rajasthan) and Gujarat. In all 14 samples were selected and were enriched by inoculating into McKeen Medium using 0.1% soyabean oil as a carbon source. One gram soil sample was incubated into 100ml of culture medium comprising of: (20 gL^{-1} glucose, 5.0gL^{-1} glutamic acid, 1.0 gL^{-1}

K_2HPO_4, 1.02 gL^{-1} $gMgSO_4$, 0.5 gL^{-1} KCl) supplemented with 1 ml of trace elements solution (0.5 gL^{-1} $MnSO_4$,.$7H_2O$, 0.16 gL^{-1} $CuSO_4$,.$5H_2O$ and 0.015 $gL^{-1}FeSO_4$,.$7H_2O$) adjusting to pH 7.0 was used as cultural medium. The cultures were incubated on rotary shaker (150 rpm) for 3 days at 45ºC. In all nine bacterial isolates viz. I, T1, X3, Z5, U1, F, C1, Q2 and B were maintained on nutrient agar plates and screened for the production of biosurfactants using various screening methods.

Screening for Biosurfactant Production

Oil Spreading Test

Nine bacterial strains were taken into consideration and were compared by measuring the diameter of the clear zone on an oil-water surface. The 50ml of distill water was added to a large petridish (15cm diameter) and 20µl of crude oil was added to the surface of water. By the addition of 10 µl of supernatant of culture broth the diameter of clear zones was determined. Respective readings were taken with *Soybaean oil, Petrol, Diesel and Engine oil.*

Emulsification Test

Selected strains were inoculated in Bushnell Haas broth with oil as the sole carbon source and incubated on a shaker for 7 days. Cultures were centrifuged at 6000 rpm for 25minutes and emulsification factor was precipitated by addition of chilled acetone and was vacuum dried. Precipitate was dissolved in Tris buffer with pH 8 in 30ml capped test tubes. 0.1 ml of oil was added to the tubes and were kept in shaker for 25mins at 120rpm and allowed to stand for 20mins. Emulsification capacity was evaluated spectrophotometrically at 620nm for the each strain.

Emulsification Index (E_{24})

Emulsification index is the emulsifying capacity of a particular strain. For measuring the E_{24}, two equal volume of culture supernatant and oil is added in a test tube. The mixture is vortexed at high speed for 2 minutes and allowed to stand for 24 hours. The E_{24} is given as percentage of the height of emulsified layer (cm) divided by the total height of the liquid column (cm) **[12]**.

$$E_{24} = \frac{\text{Height of emulsion formed/}}{\text{Total height of solution}} \times 100$$

Hemolytic Assay

Blood agar plate contains mammalian blood (usually sheep or horse), typically at a concentration of 5-10%. Medium is enriched, differential media used to isolate fastidious organisms & detect hemolytic activity. Blood agar is a general purpose enriched medium often used to grow fastidious organisms & to differentiate bacteria based on their hemolytic properties.

Blue Agar Plate Method (Bap Method)

This method aims at determination of anionic biosurfactant. Mineral salt agar medium supplemented with 2% glucose as carbon source, cetyltrimethylammonium bromide (CTAB-0.5 mg/ml) and methylene blue (0.2mg/ml) was prepared **[13]**. Wells were prepared in the methylene blue agar plate using a cork borer (4mm) and each of them was loaded with 30µl of the cell free supernatant. The plate was incubated at 37ºC for 48-72 hrs. A dark blue halo zone surrounding the culture was considered as a positive result.

RESULTS AND DISCUSSION

Biosurfactant consist of many types based on their chemical nature, such as glycolipids, lipopeptides,polysaccharide–protein complexes, phospholipids, fatty acids and neutral lipids **[14]**. Only single method was unsufficient to detect biosufactant producing bacteria. Therefore, combination of various screening methods is required to understand the ability of microbe in biosurfactant production. Based on screening tests performed, 13 from 19 isolates which give positive result in more than one screening methods considered as biosurfactant producing bacteria. Oil spreading test or sometimes referred to as an oil displacement assay has advantage that it can detect biosurfactants with low activity and quantity **[15]**. The oil spreading test results are stated positively when a clear zone is formed on the supernatant droplets in the oil layer **(Figure 1A-D)**. The clear zone is formed because the hydrophobic part of the oil and hydrophilic in biosurfactant fuses, then causes pressure between the hydrophobic and hydrophilic parts. This condition causes interface tension to decrease, the oil layer breaks and a clear zone is formed **[16]**.

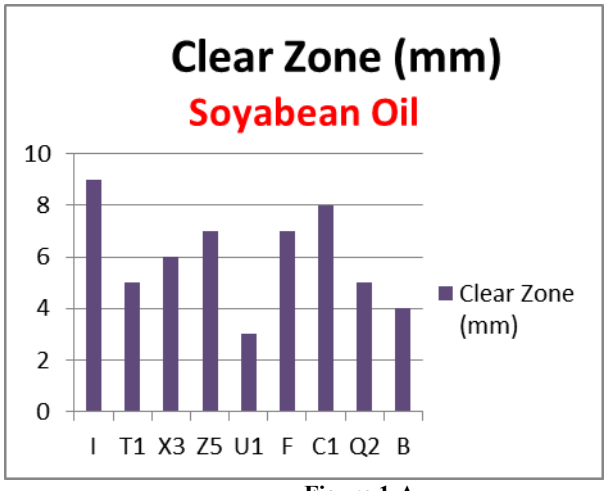

Figure 1-A

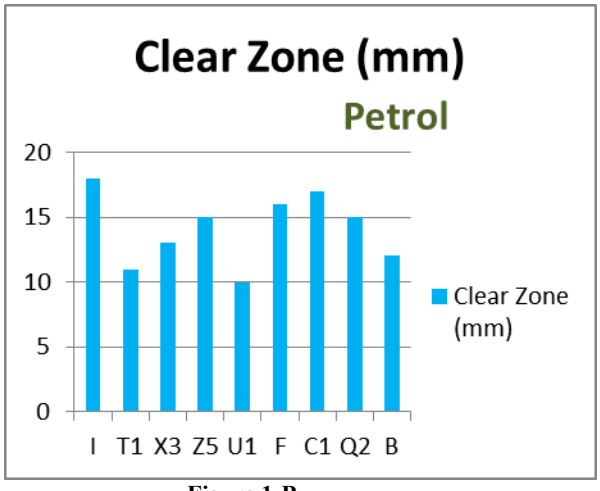

Figure 1-B

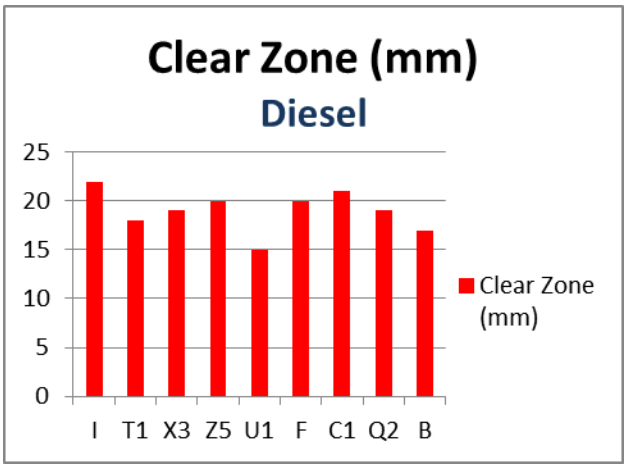

Figure 1-C

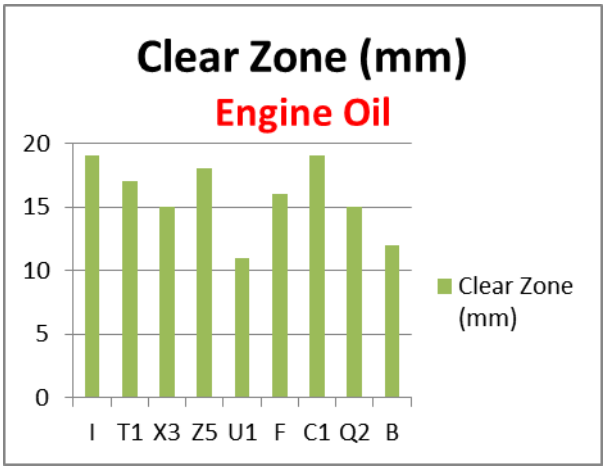

Figure 1-D

Figures 1: A-D Showing clear zone on an oil-water surface by different bacterial strains

Highest clear zone was noted for strains I, Z5, C1 & F with each of the oils. Strain I gave clear zones of 9mm (soya bean), 18mm (Petrol), 22mm (Diesel) & 19mm (engineoil) indicating diesel as an ideal source **(Figures 1 A-D)**. Biosurfactant isolated from strain I showed maximum emulsification activity against Diesel oil. Emulsification of different hydrocarbons by the biosurfactant was in the order of Diesel oil > Engine oil > Petrol> Soyabean oil **(Figure 2 A-D)**. The emulsion formed by the biosurfactant against each hydrocarbon was stable up to 48 h. Emulsification of four different hydrocarbons by the biosurfactant reflects the possibility of its application against different hydrocarbon pollution. This study revealed the possibility of biosurfactant production using Diesel oil and soyabean oil. The possibility of biosurfactant production using cheaper carbon sources was already reported by earlier workers; use of olive oil mill effluent, animal fat and frying oil **[17]**, molasses **[18]** and starch-rich wastes **[19]** supporting the present study on use of renewable carbon sources for biosurfactant production. Haba *et al* **[20]** reported that, biosurfactant produced from firing oil have low emulsifying properties, where as biosurfactant produced in the present study using diesel oil and engine oil showed good emulsification activity against four different hydrocarbons. Further, it encouraged the aim of the present study to produce biosurfactants from carbon sources with high emulsification property.

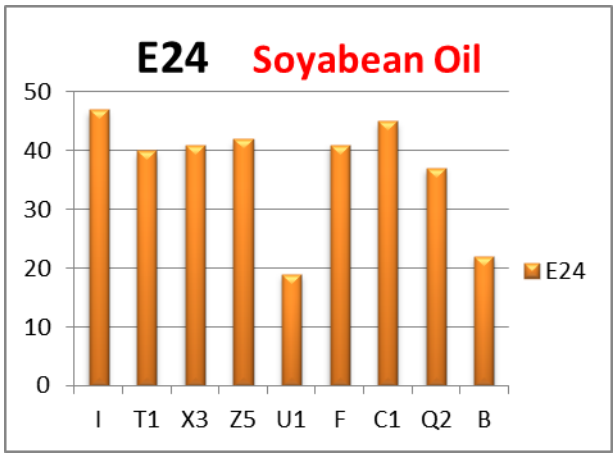

Figure 2-A

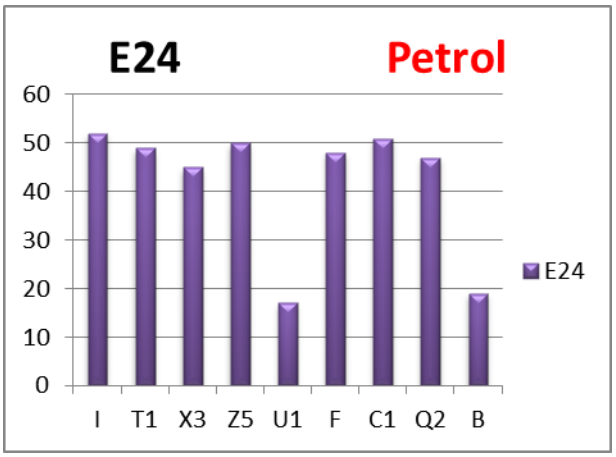

Figure 2-B

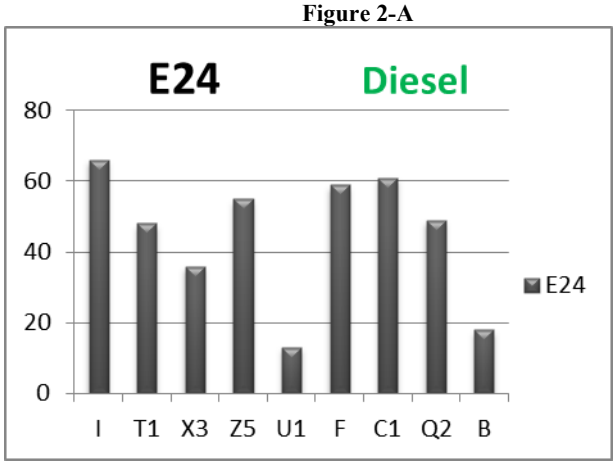

Figure 2-C

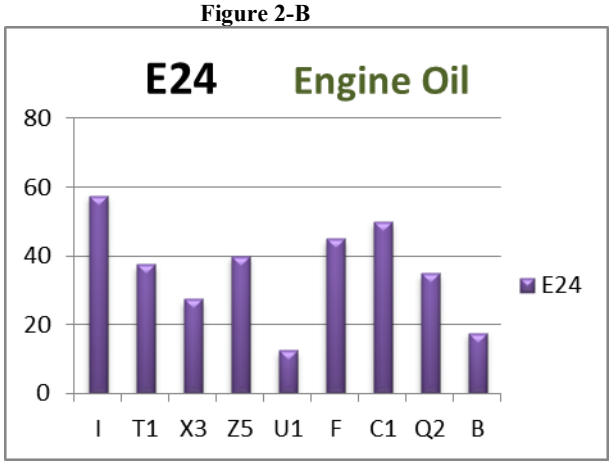

Figure 2-D

Figures 2: A-D Emulsification index of different oil sources by various bacterial strains

The highest E24 value for soybean oil, petrol, diesel & engine oil is observed in strain *I* as 47, 52, 66 & 57.5 respectively. Highest E24 value for diesel indicates that microorganism is utilizing diesel as the best carbon source **(Figures 2 A-D)**. The hemolysis test is generally carried out as a pre-elimination screening on bacteria to determine its ability to produce biosurfactant **[21]**. The hemolysis test was done because it was easily observed and did not require a long time. Biosurfactant producing bacteria will form a clear zone around the colonies on the blood agar media.

Plates Interpretation of Hemolysis on Blood Agar

Beta hemolysis (β):- It is defined as complete or true lysis of red blood cells. A clear zone, approaching the color & transparency of the base medium, surrounds the colony. Many species of bacteria produce toxic by- products that are capable of destroying red blood cells.

Alpha hemolysis (α):- It is the reduction of the red blood cell hemoglobin to methemoglobin in the medium surrounding the colony. This causes a green or brown discoloration in the medium. Gamma hemolysis (γ) :- It is somewhat self-contradictory. Gamma indicates the lack of hemolysis. There should be no reaction in the surrounding medium. Selected strains were streaked on fresh blood agar plates and incubated for 24-48 hrs at 37ºC. The bacterial colonies were interpreted for the biosurfactant property indicated by the formation of clear zone surrounding them **(Figure 3)**. *Strains I & F has shown the maximum beta hemolysis* **(Figure 4)**.

The BAP method is a highly special technique for detection of glycolipid producing microorganisms. Glycolipid production was detected in strain I during the present investigation. A dark blue halo zone surrounding the culture was observed **(Figure 5)** which was considered as a positive result.

Figure 3:β Hemolysis

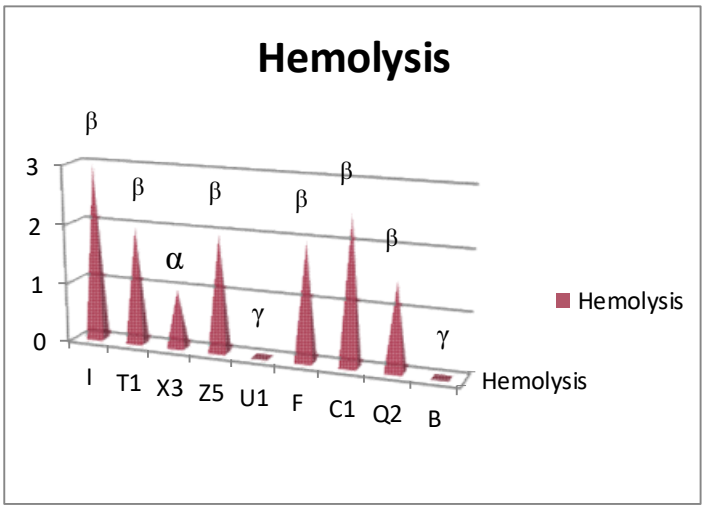

Figure 4: Showing hemolysis by different bacterial strains

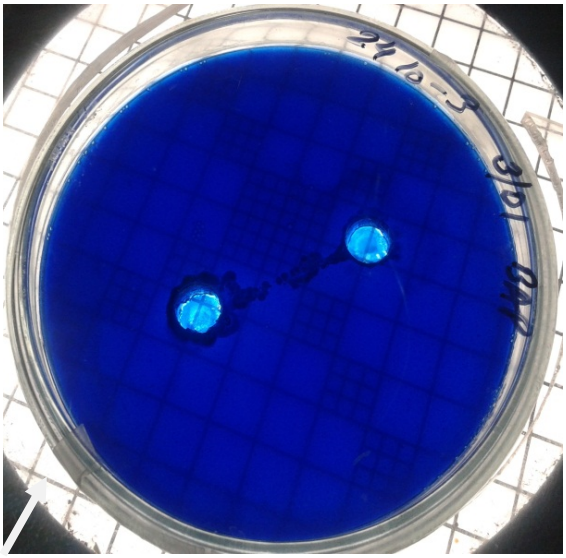

Figure 5: Showing dark blue halo zone surrounding the culture

CONCLUSION

In this era of green technology biosurfactants have led considerable interest for present and future application. of biosurfactant and biosurfactant producing bacteria in environmental cleaning. Present investigation clearly reveals potential use of microorganisms present in oil contaminated areas of Indian thar desert for biodegradation of pollutants. These microbes may be very promising for use in environmental biotechnologies.

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