



RESEARCH ARTICLE

SCREENING, PRODUCTION AND OPTIMIZATION OF BIOSURFACTANT BY *BACILLUS SPECIES* ISOLATED FROM PETROLEUM CONTAMINATED SOIL

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ABSTRACT

In present investigation, *B. subtilis* and *B. cereus* was isolated and Identified from the petroleum contaminated site soil and identified by gram's staining, motility and biochemical tests. After the isolation and identification, biosurfactant production from bacterial organisms was screened using mineral salt medium, by oil spreading, drop collapse and hemolysis test. From the blood agar plates, the biosurfactant producing bacterial culture were obtained and maintained as stock cultures. Organisms were inoculated into the mineral salt broth with petroleum oil, diesel oil, kerosene oil and crude oil as a carbon source for biosurfactant production. Biosurfactant was extracted by acid precipitation method. Biosurfactants production was optimized by using different pH, temperature, carbon and nitrogen sources. Emulsification activity of biosurfactant was analyzed. The extracted biosurfactant was characterized by using Thin Layer Chromatography (TLC). *B.subtilis* was able to produce the biosurfactant using pH (4), temperature (20°C), Carbon source (glucose), nitrogen source (NaNO₃). *B.cereus* was able to produce the biosurfactant by using pH (6), temperature (35°C), carbon source (Starch), nitrogen source (urea). Among this study, *Bacillus subtilis* produced higher amount of biosurfactant than *Bacillus cereus*.

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INTRODUCTION

Biosurfactants (BS) are amphiphilic compounds produced on living surfaces mostly microbial cell surfaces or excreted extracellularly and contain hydrophobic and hydrophilic moieties that reduce surface tension and interfacial tensions between individual molecules at the surface and interface respectively (Fiechter, 1992). Biosurfactants are surface active organic compounds synthesized by many microorganisms during their growth that cells for the utilization of hydrocarbon compounds (Cooper and Zajic, 1980). Biosurfactants can be divided into low molecular mass molecules, which efficient lower surface and interfacial tension and high molecular mass polymers, which are more effective as emulsion stabilizing agents (Rosenberg *et al.*, 1979). The term "Biosurfactant" refer to any compound obtained from microorganism which has striking influence on interfaces, further it brings down the interfacial tension between the two liquids. They are complex molecules covering a wide range of chemical types including peptides, fattyacids, phospholipids, glycolipids, antibodies and lipopeptides. They are produced as metabolic products on membrane components by microbes (Cooper, 1986).

Biosurfactants find applications in environmental protection and management, crude oil recovery, as antimicrobial agents in health care and food processing industries (Banat *et al.*, 2000). The biosurfactant production is reported and studied well in *Pseudomonas aeruginosa* (Robert *et al.*, 1989), *Bacillus licheniformis* (Javaheri, 1985), *Arthrobacter sp.* (Rosenberg *et al.*, 1979), *Staphylococcus sp.* (Nweke *et al.*, 2003) and *Flavobacterium sp.* strain MTN 11 (Bodour *et al.*, 2004). Oil pollution is relatively new problem for the environment. It is only recently since the discovery of crude oil that nations have refined it into different types of field and use oil as an engine lubricant (Alexander, 1967). Hence, the present study was undertaken to isolate and identify *Bacillus* species from Petroleum contaminated soil, to screen, extract and characterize the biosurfactants by standard methods.

MATERIALS AND METHODS

Collection of Sample

Soil samples were collected from petroleum contaminated site at Mannargudi, Tiruvarur District, Tamil Nadu.

Isolation and identification of Bacteria from soil samples

The microbial strains were isolated from the collected sample by serial dilution technique. Morphological, cultural and

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biochemical characteristics were identified by (Aneja, 2002) Gram's staining, motility test (Bailey and Scott, 1966) biochemical test by using Bregey's manual of systemic bacteriology.

Screening of biosurfactant production

I.Oil Spreading Method (Youssef *et al.*, 2004)

30ml of distilled water was taken in the petriplate and 1ml of used frying oil was added to the centre of the plate. Now 20 μ l of the culture broth was also added to it. The biosurfactant producing organisms can displace the oil and spread in the water.

II.Drop Collapse Method (Jain *et al.*, 1991)

This assay relies on the destabilization of liquid droplets by surfactants. Therefore, drops of a cell suspension or of culture 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. If the liquid contains surfactants, the drops spread or even collapse because the force or interfacial tension between the liquid drop and the hydrophobic surfactant concentration and correlates with surface and interfacial tension.

III.Blood Haemolysis Test (Rashedi *et al.*, 2005)

The fresh single colony from the isolated culture were taken and inoculated into blood agar plates. The plates were incubated for 48-72 hrs at 32°C. Then the plates were observed for presence of clear zone around the colonies. The clear zone indicates the presence of biosurfactant producing organisms.

Biosurfactant Production (Subramaniyam *et al.*, 2011)

Bacillus subtilis and *Bacillus cereus* culture were inoculated on the mineral salt broth containing 2% of petroleum oil, kerosene oil, diesel oil and crude oil and it was incubated at an optimized condition for 24-48 hrs in a shaker operating at 120 rpm/min. After incubation, the broth was screened for the production of biosurfactant.

Extraction of Biosurfactant (Cooper *et al.*, 1987)

Biosurfactant produced by *Bacillus subtilis* and *Bacillus cereus* were extracted by acid precipitation method. After incubation, the bacterial cells were removed by centrifugation at 5000 rpm, 4°C for 20 min. The surfactant was taken and pH of the supernatant was adjusted by using HCl. Equal volume of chloroform, methanol 2:1 ratio was added and mixed well. It was left for overnight

Dry weight of Biosurfactants (Subramaniyam *et al.*, 2011)

Sterile petriplate was taken and the weight of the plate was measured. Now the sediment was poured on the plates. They were placed on the hot air oven for drying at 100°C for 30 min. After drying, the plates were weighed. The dry weight of the biosurfactant was calculated by the formula,

Dry weight biosurfactant = Weight of the plate after drying – Weight of the empty plate.

Optimization by physical parameters

Effect of Incubation periods

To find the optimum condition for biosurfactant production, the media were prepared and sterilized. After sterilization 1% of inoculum was added into different flasks containing medium and the flasks were incubated at various incubation periods (3rd, 6th, 9th and 12th day).

Effect of pH

100ml of nutrient broth were prepared and separated into different conical flasks were adjusted to different pH such as (2,4,6 and 8). After sterilization, 1% of inoculum was added into different flasks containing medium and the flasks were incubated for 48-72 hrs.

Effect of Temperature

100 ml of nutrient broth were prepared and separated into different conical flasks, each flasks were adjusted to different temperature level such as (10°C, 20°C, 30°C and 40°C). After sterilization. 1% of inoculum was added into different flasks containing medium and the flasks were incubated for 48-72 hrs.

Optimization by Chemical parameters

Effect of carbon source

100ml of nutrient broth was prepared and separated into different conical flasks, each flasks were added with 1% of inoculum. Carbon sources like glucose, starch and galactose was added. Then the broth was incubated for 24-72 hrs at 37°C.

Effect of Nitrogen source

100ml of nutrient broth was prepared and separated into different conical flasks, Each flasks were added with 1% of inoculum. Nitrogen sources like urea, NaCl and NaNO₃ was added. Then the broth were incubated for 24-72 hrs at 37°C.

Estimation of Emulsification activity (Thavasi *et al.*, 2009)

Partially purified biosurfactant (5mg) was dissolved in 5ml of Tris buffer (pH 8.0) in 30ml test tubes. Hydrocarbons like waste petroleum oil, diesel oil, kerosene oil and crude oil were tested for emulsification activity. 5mg of hydrocarbon was added to above biosurfactant solution and shaken well for 20 min and the mixture was allowed to stand for 20 min. The optical density of the emulsified mixture was measured at 610nm and the results were expressed as D₆₁₀.

Analytical Method

Thin layer Chromatography (Thavasi *et al.*, 2011)

Preliminary characterization of the biosurfactant was done by TLC method. A portion of the crude biosurfactant was separated on a silica gel plate using chloroform: methanol: water (10:10:0.5 v/v/v) as developing solvent system with different colour developing reagent. Ninhydrin reagent was sprayed to detect rhamnolipid biosurfactant as red spot produced by *Bacillus subtilis* and *Bacillus cereus*.

RF value is calculated by using following formula

$$RF = \frac{\text{Distance moved by analyte from origin}}{\text{Distance moved by solvent front from origin}}$$

Statistical Analysis (Gupta, 1977)

Random sampling was used for the entire test. The data of all values, were statistically analysed and expressed as mean and standard deviation, by using the formula. Data obtained were subjected to the following analysis.

Mean

The mean for the data was calculated using formula given below.

$$\text{Mean} = \frac{\sum X}{N}$$

Standard deviation

It is used to get accurate value and to estimation of values between various data. By statistical analysis of standard deviation all the obtained data of the experiment was computed by using the formula given below.

$$\text{Standard Deviation } \sigma = \sqrt{\frac{\sum (X-X)^2}{N-1}}$$

RESULTS AND DISCUSSION

Collection of sample

For the isolation of bacteria, soil samples were collected from the petroleum contaminated site. Biosurfactant or bioemulsifiers play a key role in emulsifying hydrocarbons. Biosurfactant and bioemulsifiers are thought to be very suitable alternatives to chemical surfactants due to their properties like eco friendly, less or not toxicity, biodegradability, high specificity, selectivity at temperature, pH, salinity and synthesis from cheaper renewable substrates.

Isolation of bacteria

The isolation of bacteria was done by serial dilution method using Nutrient Agar medium. The isolated bacteria were subjected to morphological characteristics.

Identification of Bacteria

The selected two bacterial colonies were identified by Gram staining, motility test and biochemical characteristics. The results were compared Bergey's manual of systematic bacteriology classification. Based on the results the isolated colonies were confirmed as *Bacillus subtilis* and *Bacillus cereus*.

Oil Spreading Technique

Similar results were communicated in literature for different strains of *B.subtilis* species. The strains were able to displace vegetable oil, kerosene, petrol and diesel (Priya and usharani, 2009)

Table 1. Biochemical characteristics of isolated microbes

S.No.	Tests	<i>Bacillus subtilis</i>	<i>Bacillus cereus</i>
Isolated organism based on colony morphology			
1	Colony morphology	Dry, flat, irregular with lobate margins	Rough and dry texture
2	Gram's staining	Gram positive	Gram positive
3	Shape	Rod	Rod
4	Motility	Motile	Motile
Biochemical characters			
5	Indole	-	-
6	Methyl red	-	-
7	Voges proskauer	+	+
8	Catalase	+	+
9	Oxidase	-	+
10	Citrate	-	+
11	Urease	-	-

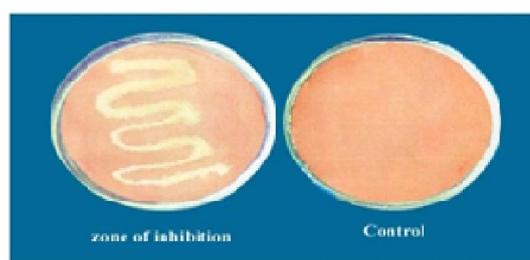
(+) – indicate positive, (-) – indicate negative

Blood Haemolysis Test

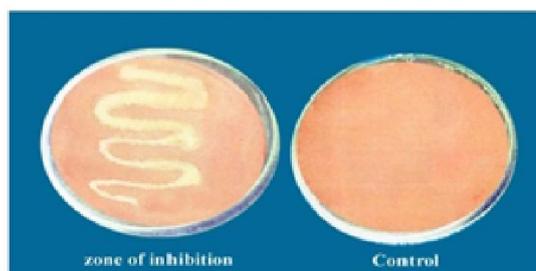
Biosurfactant isolated from *B.subtilis* and Triton X – 100 showed maximum emulsification activity against Petroleum contaminated soil. In our study reports similar to the biosurfactant producing organisms showed beta – haemolytic activity on blood agar plate (Rashedi *et al.*, 2005). The primary screening of biosurfactant producing bacteria was carried out using hemolytic activity, oil collapse and oil spreading techniques. Selection of these methods was due to their strong advantages including simplicity, low cost, quick implementation and use of relatively common equipment that is accessible in almost every microbiological laboratory; however, as expected, these methods are not perfect or flawless (Youssef *et al.*, 2004)

Plate - I

Screening of Biosurfactant Production



Bacillus subtilis



Bacillus cereus

Production of Biosurfactants

Biosurfactant produced by *B.subtilis* inferred that, biosurfactant produced with one carbon source like contaminated petroleum soil or peanut cake could be used to remediate different hydrocarbon pollution.

Table 2. Optimization by Physical Parameters

pH	<i>B. subtilis</i>		<i>B. cereus</i>		Temperature °C	<i>B. subtilis</i>		<i>B. cereus</i>	
	OD Value	g/100 ml	OD Value	g/100 ml		OD Value	g/100 ml	OD Value	g/100 ml
2	0.43 ± 0.02	0.36 ± 0.01	0.43 ± 0.02	0.32 ± 0.02	10	0.36 ± 0.01	0.24 ± 0.01	0.26 ± 0.01	0.14 ± 0.01
4	0.55 ± 0.04	0.49 ± 0.03	0.40 ± 0.02	0.30 ± 0.01	20	0.45 ± 0.02	0.36 ± 0.02	0.32 ± 0.02	0.23 ± 0.02
6	0.42 ± 0.02	0.31 ± 0.01	0.46 ± 0.04	0.35 ± 0.02	30	0.36 ± 0.01	0.24 ± 0.01	0.31 ± 0.02	0.21 ± 0.01
8	0.51 ± 0.03	0.26 ± 0.01	0.36 ± 0.01	0.34 ± 0.02	40	0.21 ± 0.01	0.16 ± 0.01	0.21 ± 0.01	0.11 ± 0.01

Values are expressed as Mean±Standard Deviation

Table 3. Optimization by Chemical Parameters

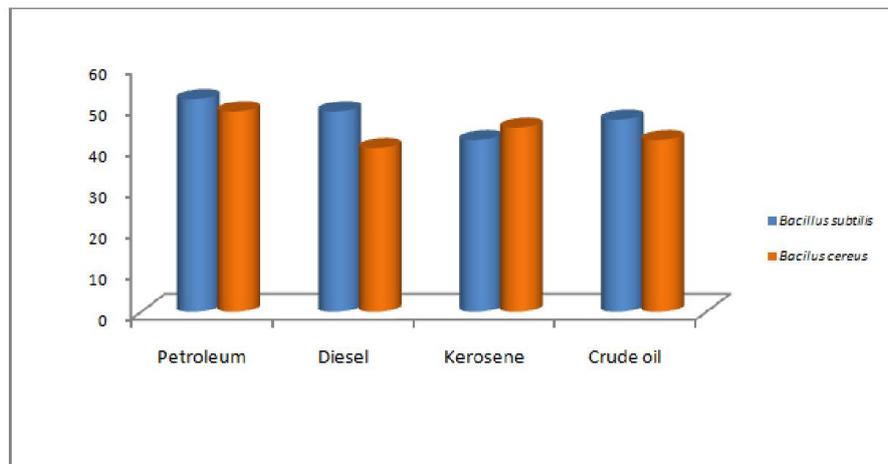
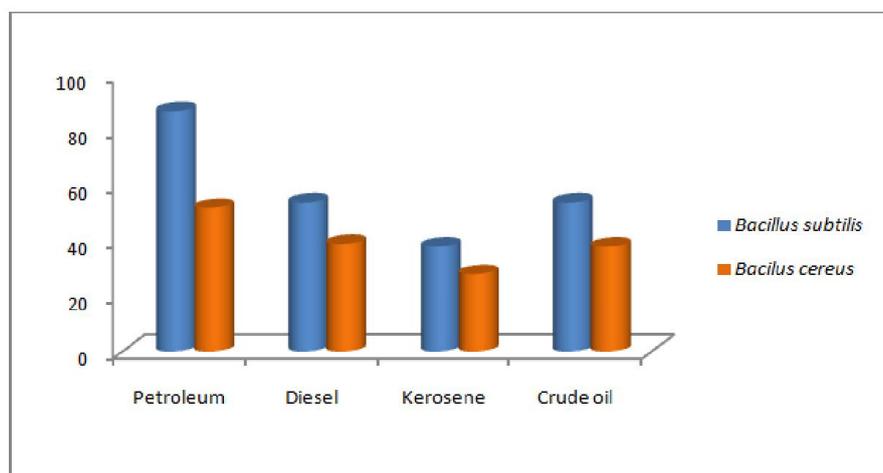
Carbon Sources	<i>B. subtilis</i>		<i>B. cereus</i>		Nitrogen Sources	<i>B. subtilis</i>		<i>B. cereus</i>	
	OD Value	g/100 ml	OD Value	g/100 ml		OD Value	g/100 ml	OD Value	g/100 ml
Glucose	0.56 ± 0.03	0.47 ± 0.02	0.3 ± 0.01	0.27 ± 0.01	Urea	0.36 ± 0.02	0.24 ± 0.01	0.56 ± 0.04	0.43 ± 0.02
Starch	0.31 ± 0.01	0.25 ± 0.01	0.45 ± 0.02	0.31 ± 0.02	NaCl	0.40 ± 0.03	0.31 ± 0.01	0.35 ± 0.01	0.21 ± 0.01
Galactose	0.43 ± 0.02	0.36 ± 0.01	0.33 ± 0.01	0.24 ± 0.01	NaNO ₃	0.45 ± 0.03	0.36 ± 0.02	0.41 ± 0.02	0.30 ± 0.01

Values are expressed by Mean ± Standard deviation

Table 4. Analysis of biosurfactants using thin layer chromatography

S.No.	Sources	RF Value (mm)	
		<i>B. subtilis</i>	<i>B. cereus</i>
1	Petroleum	0.61 ± 0.05	0.58 ± 0.04
2	Diesel	0.57 ± 0.02	0.47 ± 0.03
3	Kerosene	0.48 ± 0.02	0.50 ± 0.03
4	Crude oil	0.59 ± 0.03	0.53 ± 0.04

Values are expressed by Mean ± Standard deviation

**Figure 1. Zone displacement of oil spreading technique by *B. subtilis* and *B. cereus*****Figure 2. Emulsification activity on Biosurfactant production**

In the present study, the attempt made on biodegradation of crude oil in a laboratory scale experimental setup revealed that maximum biodegradation rate was found with biosurfactant and fertilizer addition. Above information, obtained in this study may be useful for the hydrocarbon polluted environments (Thavasi *et al.*, 2011). Identification of biosurfactant producing bacteria can be further confirmed by measurement of surface tension. Reduction of surface tension measurements by isolated bacteria from Iranian crude oil reservoirs indicates the production of surface active compounds (Banat *et al.*, 1991). Biosurfactant production in mineral salt broth with petroleum oil, diesel oil, kerosene oil and crude oil as carbon sources showed a colloidal thin white layer formation on the surface of the broth.

Extraction of Biosurfactants

Biosurfactants in pollution and environmental control are microbial enhanced oil recovery. Hydrocarbon degradation in soil environment and hexachloro cyclohexane degradation, heavy metal removal from contaminated soil and hydrocarbon in aquatic environment (Singh *et al.*, 2007). The surfactants was extracted by acid precipitation, method with chloroform, methanol and solvent. White sediment was retained when the mixture was placed in the rotor.

Dry Weight of Biosurfactants

The dry weight of the biosurfactants was measured and estimated. The result revealed that the maximum dry weight was in crude oil for *B.subtilis* and *B.cereus* respectively, when compared with other oils.

Effect of pH

The optimization of environmental conditions is very important for the enhancement of bacterial growth and for designing effective bioremediation strategy (Davey, 1994). Isolated *Bacillus licheniformis* BAS50 which grow and produced a lipopeptide surfactant when cultured on a variety of substrate at salinities of 13 NaCl.

Depicted results showed that the biosurfactant production was optimal at 5 NaCl. Environmental factors and growth conditions such as pH, temperature, and oxygen availability also effect biosurfactant production through their effects of cellular growth or activity (Kim *et al.*, 2010). Biosurfactant was analyzed in various pH ranges such as (2,4,6 and 8). The maximum biosurfactant production was recorded in pH (4) when compared with other pH range.

Effect of Temperature

The low temperature optimum exhibited by isolate and suggests that its good candidate for biosurfactant of polar regions contaminated sites (Ma Stalic, *et al.*, 2006).

Effect of carbon sources

Potato substrates were evaluated as a carbon source for surfactant production by *B. subtilis* ATCC 21332 (Thompson *et al.*, 2000).

Effect of Nitrogen sources

The molasses and cornsteep liquor as the primary carbon and nitrogen source to produce rhamnolipid biosurfactant from *B. subtilis* GS3 (Desai *et al.*, 1997).

Estimation of Emulsification activity

Biosurfactant used in this study was higher than the emulsification activity recorded with Triton X – 100 against waster motor lubricant oil, crude oil and peanut oil. Compare to Triton X – 100 emulsification activity of the biosurfactant was low against kerosene, diesel, xylene, naphthalene and anthracene. Biosurfactant over chemically synthesized surfactants, such as lower toxicity, biodegradability and ecological acceptability, the possibility of replacing the chemical surfactant in oil pollution (Kuiper *et al.*, 2004).

Characterization of Biosurfactants

Lipopeptide were obtained as rhamnolipid i.e. a glycolipid while sprayed at ninhydrine reagent on the TLC plate (Anandraj *et al.*, 2010). In our study similar to the findings of rhamnolipid produced from *B.cereus* in TLC (Rashedi *et al.*, 2005). Biosurfactants was to be more effective than chemically synthesized surfactants. It was used for the recorded of lead removal from the water and it was easily biodegraded in environment. This study should be progressed in future in such a way to find more applications of biosurfactants in bioremediation. Microbially produced compounds are easily degraded suited for environmental applications such as bioremediation and dispersion of oil spills (Mulligan *et al.*, 2005). The biosurfactant production was characterized by using TLC plate. On TLC plate, the biosurfactant production by *B.subtilis* and *B.cereus* were detected as red spot. Larger red spot on TLC plate was observed for crude oil. The RF values for the biosurfactants production for *B.subtilis* in petroleum oil was (0.61±0.05) which was higher than the diesel oil (0.57±0.02) kerosene oil (0.48±0.02) and crude oil (0.59±0.03) and RF values for *B.cereus* in petroleum oil was (0.58±0.04) which was higher than the diesel oil (0.47±0.03) kerosene oil (0.50±0.03) and crude oil (0.53±0.04) (Table – 7). The maximum biosurfactant production by *Bacillus cereus* observed in petroleum oil. From this study, *B.subtilis* was able to produce the biosurfactant using pH (4), temperature (20°C), Carbon Source (glucose), nitrogen source (NaNo3). *B.cereus* was able to produce the biosurfactant by using pH (6), temperature (35°C), carbon source (Starch), nitrogen source (urea). Among this study, *Bacillus subtilis* produced higher amount of biosurfactant than *Bacillus cereus*.

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