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RESEARCH ARTICLE

Isolation, Screening, and Characterization of Biosurfactant-Producing Microorganisms from Petroleum-Contaminated Soil and Further Optimization of Parameters for Biosurfactant Production

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ABSTRACT

Introduction: Biosurfactants are amphiphatic in nature and are surface-active compounds produced by microorganisms. These molecules reduce interfacial surface tension between aqueous solutions and hydrocarbon mixtures. Unfortunately, oil spills and industrial discharges from petroleum-related industries have been identified as the major pollution sources. The hydrophobicity and low aqueous solubility of petroleum pollutant limit the biodegradation process. The features that make biosurfactants as an alternative to commercially synthesized surfactants are its low toxicity, higher biodegradability and, hence, greater environmental compatibility, better foaming properties, and stable activity at extreme pH, temperature, and salinity. **Objective:** Therefore, in this study, hydrocarbon-degrading bacteria were screened from petroleum-contaminated soil, characterized and optimization of the physical and nutrient parameters were done to enhance the production of biosurfactants. **Results:** Petroleum-contaminated soil was collected from different petrol pumps in Pune and screening was done on minimal salt medium media containing palm oil as carbon source using hemolytic activity, emulsification index, drop-collapse test, and oil displacement method. The most promising strain was isolated and identified using Bergey's Manual of Determinative Biology and 16s rRNA sequencing and was found to be *Staphylococcus epidermidis*. The optimization of various parameters, namely temperature, pH, carbon, and nitrogen sources on growth, and biosurfactant production was studied. The highest biosurfactant production was obtained when MSS media contains sucrose (carbon source) and urea (nitrogen source) at pH 10 and temperature 55°C. The Fourier transform-infrared (FT-IR) analysis of purified biosurfactant indicated the presence of lipopeptide biosurfactant when compared with reference FT-IR spectra.

Keywords: Biodegradation, biosurfactants, emulsification, petroleum, screening, soil

INTRODUCTION

Biosurfactants or microbial surfactants are surface-active molecules that are produced by a variety of microorganisms including bacteria, yeast, and filamentous fungus. Due to their amphipathic nature, these biomolecules reduce the interfacial tension between an aqueous phase and hydrophobic molecules, thereby enhancing the solubility and bioavailability of hydrophobic organic compounds.[1] However, biosurfactants have not yet been commercialized extensively due to low production yields and high feedstock and purification costs.[2,3] Due to environmental issues and restrictive laws, the demand for biodegradable surfactants is increasing. The use of these biocompounds as an alternative to the traditional chemical surfactants has been impelled by the fast progress of biotechnology and also by their interesting features including their lower toxicity, higher biodegradability, and effectiveness at extreme temperature, salinity, and pH conditions [4]

Biosurfactants are mainly categorized by their chemical composition and microbial origin. In general, their structure includes a hydrophilic moiety consisting of amino acids or peptides, mono-, di-, or poly-saccharides and hydrophobic moiety comprising saturated or unsaturated fatty acids. A major class of biosurfactants includes - glycolipids, phospholipids, fatty acid and natural lipids, peptides, polymeric biosurfactants,

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and particulate biosurfactant. Among the various classes of biosurfactants - rhamnolipids and surfactins are best-studied biosurfactants. Rhamnolipid is one of the types of glycolipids, in which one or two molecules of rhamnose are linked to one or two molecules of β-hydroxydecanoic acid while the -OH group of one of the acids is involved in glycosidic linkage with the reducing end of the rhamnose disaccharide, in ester formation. Rhamnolipids are produced by *Pseudomonas aeruginosa*, Gram-negative, motile, and non-spore-forming bacteria. Surfactin is a cyclic lipopeptide commonly used as an antibiotic. In the various course of studies of its properties, surfactin was found to exhibit effective characteristics such as antibacterial, antiviral, antifungal, antimycoplasma, and hemolytic activities. Surfactin is produced by *Bacillus subtilis*, Gram-positive, motile, and spore-forming bacteria. Surface activity in most hydrocarbon-degrading microorganisms is attributed to several cell surface constituents, which includes structures such as M protein and lipoteichoic acid in Group A *Streptococci*, Protein A in *Staphylococcus aureus*, Layer A in *Aeromonas salmonicida*, prodigiosin in *Serratia* spp., gramicidins in *Bacillus brevis* spores, and thin fimbriae in *A. calcoaceticus*.[5]

The oil and gas industry is one of the most important sectors in India. Unfortunately, oil spills and industrial discharges from petroleum-related industries have been identified as the two major sources of pollution. The biodegradation of petroleum pollutant is limited by its poor availability to the microorganisms, its hydrophobicity, and low aqueous solubility, [6] The applications of biosurfactants are not limited to bioremediation purposes, but they can be used in petroleum engineering to reduce heavy oil viscosity, clean oil storage tanks, increase petroleum transport in pipelines, and stabilize fuel water-oil emulsions.[7] The features that make biosurfactants as an alternative to commercially synthesized surfactants are its low toxicity, higher biodegradability and, hence, greater environmental compatibility, better foaming properties (useful in mineral processing), and stable activity at extreme pH, temperature, and salinity.[8] Biosurfactants also found to be important in therapeutic and biomedical field as they possess antimicrobial, antiviral, and antifungal properties also they inhibit fibrin clot formation and have antiadhesive properties. These biomolecules can replace the harsh surfactants, presently being used in million dollar pesticide industries.[9]

To reduce the production costs, different routes could be investigated such as the increase of yields and product accumulation, the development of economical engineering processes, and the use of cost-free or cost-credit feedstock for microorganism growth and surfactant production. The choice of low cost or waste substrates is important to overall economy of the process, and they account for 30–50% of final product cost and also minimize the expenses cost of waste treatment. [10-12] Hydrocarbons such as crude oil and diesel and various carbohydrates such as glucose, sucrose, and glycerol have been commonly used as substrates for the production of biosurfactants. Since the biological function of biosurfactant is related to hydrocarbon uptake, a spontaneous release occurs with these substrates, [13] It is well known that combination of different environmental factors such as temperature, pH, salinity, and hydrocarbon toxicity would affect growth of microorganisms, hence, the amount of their bioproducts. The combination of these constraints can be expected to limit the number of suitable organisms that would grow and produce bioproducts.[14] The objective of this study is, therefore, to screen hydrocarbon-degrading bacteria from petroleum-contaminated soil and to optimize the physical and nutrient parameters to enhance the production of biosurfactants.

MATERIALS AND METHODS

Isolation of organisms from soil

Soil samples were collected from different petrol pumps in Pune, India. All the samples were collected using polyethylene containers with utmost care to avoid contamination. 1 g of soil sample was taken and serially diluted in 0.85% sterile saline. Flasks were maintained in a shaker with 150 rpm at room temperature for 4 days. Screening was performed using serial dilutions of the sample and plating on minimal salt medium (MSM) with palm oil (1% w/v) as carbon source for the isolation of bacteria. The MSM of following composition was used (g/L) - 0.8 g dipotassium hydrogen phosphate (K_2HPO_4), 0.2 g potassium dihydrogen potassium (KH_2PO_4),

0.05 g calcium chloride ($CaCl_2$), 0.5 g magnesium chloride ($MgCl_2$), 0.01 g ferric chloride ($FeCl_2$), 1 g diammonium sulfate $(NH_4)_2SO_4$), and 5 g sodium chloride (NaCl). Plates were incubated at 37°C for 72 h. Different bacterial colonies were selected based on the colony morphology and then streaked on nutrient agar. Morphologically, distinct colonies were reisolated by transferring them to fresh palm oil containing agar plates at least 3 times to obtain pure cultures. The colonies were then screened for biosurfactant production.

Screening for biosurfactant activity

Hemolytic activity

Pure culture of bacterial isolates was streaked on the freshly prepared blood agar plates containing 5% sheep blood and incubated at 37°C for 48 h. Results were recorded based on the type of zone of clearance observed, i.e. α-hemolysis when colony was surrounded by greenish zone, β-hemolysis when colony was surrounded by clear white zone, and γ-hemolysis when there was no change in the medium surrounding the colony.[15] This is preliminary method for screening biosurfactant activity.

Emulsification index (%EI_{24})

Colonies of pure culture were suspended in test tube containing 2 ml of MSM and incubated at 37°C for 24 h. 2 ml hydrocarbon (oil) was added to each tube. Then, the mixture was vortexed at high speed for 2 min and allowed to stand for 24 h at room temperature and emulsification index was calculated. The $\%EI_{24}$ was calculated by dividing the height of emulsified layer (mm) by total height of the liquid in tube (mm) and then multiplying by100.[16]

$$\text{Emulsification index } (E24) = \frac{\text{Height of emulsion layer}}{\text{Total height}} \times 100$$

Drop-collapse test

The cultures that showed hemolytic activity and emulsification index were grown in MSM supplemented with 1% palm oil for 48 h at room temperature for drop-collapse test. Screening of biosurfactant production was performed using the qualitative drop-collapse test described by Bordour and Maier.[17] 2 µl of palm oil was added to each well of 96-well microtiter plate lid and these were left to equilibrate for 24 h. 5 µl of cultural supernatant was added to the surface of oil and drop size was observed after 1 min. The result was considered positive for biosurfactant production and flat rounded drops indicated lack of biosurfactant production.[18]

Oil displacement method

20 µl of palm oil was placed on surface of distilled water (20 ml) in a Petri dish, then 20 µl of culture supernatant was gently put on center of oil film. If the biosurfactant is present in the supernatant, the oil is displaced and diameter of the clear hallow area visualized under visible light was measured and calculated after 30 s.[19]

Identification of biosurfactant-producing bacteria

Based on the screening test results, the positive isolates were inoculated into the mineral salt medium for the biosurfactant production. Based on the quantification of biosurfactant produced, the best strain was selected and then identified by its microscopic appearance and biochemical tests based on Bergey's Manual of Determinative Bacteriology. In addition to morphological and biochemical tests, the phylogeny of strain is determined using sequence analysis of polymerase chain reaction (PCR) amplified 16s rRNA gene using the primer pair RPP2 (CCAAGCTTCTAGA CGGITACCTTGTTA CGACTT) and FDD2 (CCGGATCCGTCGACA GAGTTTGATCI TGGCTCAG). RPP2 and FDD2 are universal primers for 1.5 kb fragment amplification for eubacteria. The following PCR conditions were used: 95°C for 5 min, followed by 25 cycles of 95°C for 1 min, 50°C for 30 s, and 72°C for 5 min, followed by 72°C for 5 min. After completion of PCR amplification, the samples were purified and loaded on the sequencer - Avant 3100 Gene Analyzer. The sequencing output was analyzed using the accompanying DNA Sequence Analyzer computer software. The sequence was compared with National Center for Biotechnology Information GenBank entries using BLAST algorithm.

Effect of temperature, pH, carbon, and nitrogen sources on growth and biosurfactant production

Optimization study was conducted to find out the optimum conditions for the large-scale production of biosurfactant. The selected isolate was inoculated in 100 ml of minimal salt media in 250 ml flask, and the pH of the media was adjusted to 4, 6, 8, 10, and 12, and incubated for 48 h. The cells were removed by centrifugation, and supernatant was used for the estimation of biosurfactant production by measuring OD at 420 nm. Similarly, a range of different temperatures was used (15°C, 25°C, 35°C, 45°C, and 55°C) to find the optimum temperature.

Growth and biosurfactant production by the isolate was evaluated using mineral salt media (MSS) with different carbon and nitrogen sources. The MSS consisted of gl^{-1}:NaCl 10.0; Na_2HPO_4 5.0; KH_2PO_4 2.0; and $MgSO_4 7H_2O$ 0.2. The carbon sources used were sodium acetate, paraffin, sucrose, and starch using ammonium nitrate as the nitrogen source (2/g). The different carbon sources were added to MSS at a concentration of 10/gl. The different nitrogen sources used were - ammonium sulfate, ammonium nitrate, sodium nitrate, and urea while sucrose is used as carbon source (10/gl).The nitrogen sources added to MSS at a concentration of 2/gl. All media were adjusted to a pH of 7. Assays were performed in 100 ml flasks containing 50 ml of different media. Each flask was inoculated with 1% of preculture grown in same medium for 24 h. Cultures were incubated at 40°C without shaking for 120 h. After every 24 h, samples were taken, centrifuged at 10,000, 20 min, 20°C, and cell-free supernatants were used to measure biosurfactant production using various parameters.

Extraction and purification of biosurfactant

Biosurfactant for chemical composition analysis was extracted from cell-free supernatant using Folch extraction method that is commonly used to extract lipids from biomolecules.[20] Briefly, a chloroform/methanol mixture (2:1) was added to supernatant sample to a final chloroform/methanol/water ratio of 8:4:3. The mixture was centrifuged (9000 g, 5 min), the organic layer was collected, and samples were evaporated to dryness under N_2 at 37°C for 30 min.

Fourier transform-infrared (FTIR) Spectroscopy

FTIR is most useful for identifying types of chemical bonds (functional groups) and therefore can be used to elucidate some components of the unknown sample. 10 mg of freeze-dried pure biosurfacant was grounded with 100 mg of KBr and pressed with 7500 kg for 30 s to obtain translucent pellets. The FTIR spectra, with resolution of 1/cm, were collected from 400 to 4000 wavenumbers (cm^{-1}) and are an average of 128 scans using a Tensor 27 infrared spectrometer operating in attenuated total reflection mode.

RESULTS AND DISCUSSION

Biosurfactants play an important role in food industry, bioremediation, food industry, pharmaceuticals, oil industry, petrochemistry, paper, and pulp industry.[21] Furthermore, due to diversity of microorganisms and different niches, they inhabit that there is need of efficient isolation, screening, and optimization of biosurfactant-producing bacteria from the natural environment.

In the present study, the contaminated soil was collected from various petrol pumps. A total of 29 strains were isolated from the soil sample and screened for biosurfactant production using various screening tests, and it was found that only 17 colonies were positive in the primary screening through hemolytic activity in blood agar plate. The hemolytic activity was first studied by Bernheimer and Avigad[22] in *B. subtilis*. Carrillo *et al*.[15] found an association between hemolytic activity and surfactant production, and they recommended the use of blood agar lysis as a primary screening method. All the 17 strains were further screened by drop-collapse test to confirm biosurfactant production, and the results revealed only 10 colonies were potent biosurfactant producers. Bodour *et al*.[16] used drop-collapse assay and found that only biosurfactant-producing organisms formed microemulsion in this assay [Table 1]. For screening of biosurfactant-producing isolates, they emulsified the coated oil in culture broth and made microemulsion, because of the formation of microemulsion, the culture drops collapsed in coated oil. The presence of organisms present in the soil depends on the nature and nutrient content of the soil. These colonies were further screened to select the most potent biosurfactant producer. For the

Table 1: Comparison of emulsification, oil spreading, drop collapse, and hemolytic activity of the 10 isolated strains from petroleum-contaminated soils

Colony number	E_{24}%	Oil spreading (cm)	Drop collapse	Hemolysis
C1	24.24	0.25	+	α-hemolysis
C2	37.83	1.9	+	No hemolysis
C3	2.57	0	-	No hemolysis
C4	57.14	2	++	α-hemolysis
C5	34.28	1.2	+	No hemolysis
C6	21.21	1.65	-	No hemolysis
C7	43.75	0.3	+	No hemolysis
C8	77.77	1.7	+++	α-hemolysis
C9	37.14	1.5	+	No hemolysis
C10	45.71	1.9	++	γ-hemolysis

Table 2: Biochemical characterization of the selected strain (C8)

Characteristics/test	Result
Shape	Circular
Size	0.1 cm
Margin	Smooth
Elevation	Flat
Color	Pale yellow
Opacity	Translucent
Consistency	Soft
Gram stain	+
Catalase	+
Oxidase	-
Motility	-

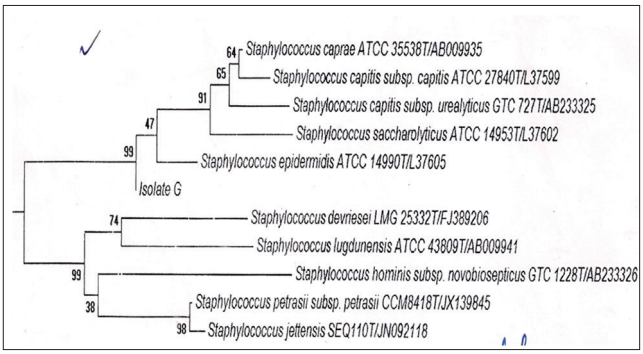

Figure 1: Unrooted phylogenetic tree based on 16s rRNA gene comparison of the biosurfactant-producing bacteria isolated from petroleum-contaminated soil and the nearest relative in Genbank, only values >50% are given. NCBI accession numbers are given

detection of potential strain, E24 value of the isolates was compared. It was found that strain C8 showed 77.77% emulsification, which was significantly high compared to other strains [Table 1]. From 10 selected colonies, therefore, only one colony was selected as a highly potent biosurfactant producer based on emulsification (77.77%), oil spread (1.7 cm), drop collapse, and hemolytic activity (α-hemolysis). The strains showed α, γ, or no hemolysis [Table 1]. The potent strain was characterized by standard bacteriological procedure through biochemical tests, and the results were interpreted with Bergey's Manual of Determinative Biology and were identified to be *Staphylococcus* spp. [Table 2]. For further identification and phylogenetic relation analysis, 16s rRNA sequencing was performed which shows that isolated biosurfactant-producing bacteria was *Staphylococcus epidermidis* [Figure 1].

Effect of pH and temperature on biosurfactant production

The applications of biosurfactants in various fields depend on their stability at different ranges of temperature and pH. The stability of biosurfactant was tested over a wide range of temperature ranging from 15°C to 55°C and pH 4–12. The pH of the medium was important characteristic for cell growth of organism and production of secondary metabolites. At pH 5, the biosurfactant production was severely decreased, and the cell growth was significantly retarded. This low pH created unfavorable growth conditions for the bacterial population. When the initial pH was set to 8, the emulsification activity increased (E24 = 45.45%). In case of *S. epidermidis,* the emulsification activity was 51.25% for pH 10 [Figure 2a]. The emulsification index decreased with any further increase in pH. Therefore, it was concluded that any change to both lower and higher pH values caused an appreciable drop in biosurfactant production.

Temperature is one of the critical parameters that greatly affected the culture growth and the biosurfactant production. The results in the present study revealed that the biosurfactant activity reached the highest for the isolate *S. epidermidis* grown at 45°C (E24 = 55%) followed by E24 =

50% at 25°C, and this clearly indicates moderately thermostable nature of biosurfactant [Figure 2b]. When the incubation temperature increased to 55°C, bacterial growth and biosurfactant production were totally inhibited, indicating that the biosurfactant production by *S. epidermidis* is greatly reduced at high temperature.

Effect of carbon and nitrogen sources on biosurfactant production

Biosurfactants can only act as substitutes of synthetic surfactants if the cost of the raw material is minimal. The use of various alternative substrates is one of the attractive strategies for economical biosurfactant production. The production of biosurfactant was found to be dependent on the composition of the medium. The MSS media optimization was carried out using various carbon sources, namely sodium acetate, paraffin, sucrose, and starch using ammonium nitrate as nitrogen source. The highest biosurfactant production was achieved using sucrose (10% w/v) being the sole source of the carbon [Figure 3a]. Various nitrogen sources selected were ammonium sulfate, ammonium nitrate, sodium nitrate, and urea using sucrose as carbon source, and highest biosurfactant production was observed with urea as nitrogen source [Figure 3b]. The highest biosurfactant production obtained when MSS media contains sucrose (carbon source) and urea (nitrogen source) at pH 10 and temperature 55°C.

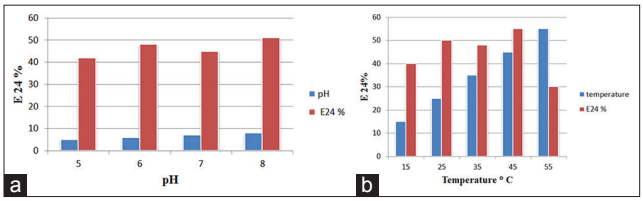

Figure 2: (a) Effect of pH on biosurfactant production as measured by emulsification index (E24%). (b) Effect of temperature on biosurfactant production as measured by emulsification index (E24%)

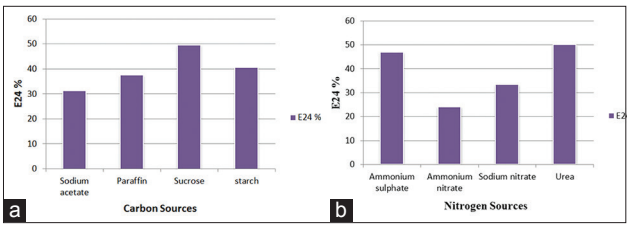

Figure 3: (a) Effect of different carbon sources on biosurfactant production as measured by emulsification index (E24%). (b) Effect of different nitrogen sources on biosurfactant production as measured by emulsification index (E24%)

FTIR analysis

Purification of biosurfactant was carried out using Folch extraction method and characterized using FT-IR analysis. It was observed that characteristic absorbance bands of peptides at 3435–3404.47/cm (NH stretching mode); C-O stretching mode (aromatic) at 1259.56/cm; and C=C stretching mode was observed at 1614–1642/cm. The presence of vinyl chains ($HC=CH_2$) was confirmed at 2975/cm [Figure 4].

The FT-IR analysis indicates the presence of aromatic hydrocarbons combined with a peptide moiety that is characteristic of lipopeptide

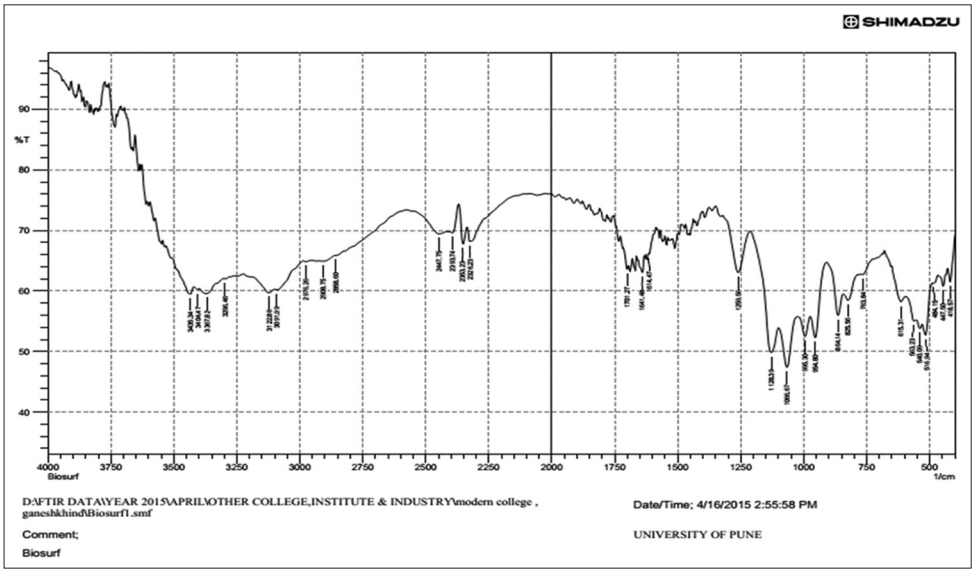

Figure 4: Fourier transform infrared spectra of crude biosurfactant extracted from ***Staphylococcus epidermidis***

biosurfactant when compared with reference FT-IR spectra.[23]

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