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# Lipopeptide Biosurfactant Production by Bacillus Cereus Mfs16 Isolated From Mangrove Forest Soil Using Pineapple Juice As Substrates

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## ABSTRACT

*In the present investigation, the pineapple juice production has been tested for its suitability for biosurfactant production using Bacillus cereus MFS16 isolated from the crude oil enriched mangrove soil. The strain recorded maximum biosurfactant production (9.43 g $L^{-1}$) when cassava wastewater was used as a medium. Biosurfactant production was confirmed by standard screening methods include Hemolytic activity, Drop collapsing test, Oil displacement method and Emulsification index. The isolate Bacillus cereus MFS16 showed lowest surface tension reduction potential of 26.6 mN $m^{-1}$, an interfacial tension of 0.97 mN $m^{-1}$ and a CMC of 33 mg $L^{-1}$. The active compound was extracted with diethyl ether and fractionated by TLC and FT-IR to confirm the presence of functional groups of a lipopeptide compound. FT-IR spectrum revealed that the important adsorption bands at 3423.04, 2958.90, 1630.84, 1350.12 and 1091.92 $cm^{-1}$ indicate the chemical structure of lipopeptide. The results demonstrated that the pineapple juice can be a suitable medium for the biosurfactant production, which can improve the process economical.*

**Keywords:** *Bacillus cereus* MFS16, Biosurfactant, pineapple juice, Lipopeptide, Mangrove forest soil.

## INTRODUCTION

Surfactants are surface-active compounds capable of reducing surface and interfacial tension between liquids, solids, and gases. The industrial need for surfactants is constantly growing. But many synthetic surfactants cause environmental problems due to their resistance to biodegradation and toxicity to ecosystems. Increasing environmental awareness has led to serious consideration of biological surfactants as possible alternatives to synthetic surfactants [1]. Biosurfactants or microbial surfactants are surface active molecules that are produced from a variety of microorganisms. Due to its amphiphatic nature, these biomolecules are capable of lowering the surface tension, interfacial tension and forming microemulsion to enable mixing of two immiscible solutions. Such properties exhibit excellent detergency, emulsifying, foaming and dispersing traits, which can be applied in various industries.

The features that make them commercially promising alternatives to chemically synthesized surfactants are their lower toxicity, higher biodegradability, better foaming properties, and greater stability towards

temperature and pH. However, biosurfactants have been paid increasing attention to replace the synthetic surfactants. Nowadays, the use of biosurfactants has been limited due to the high production cost. The production economy is the major setback in biosurfactant production, as the amount and type of raw materials can contribute considerably to the production cost. It is estimate that the raw materials account for 30% of the total production cost in most bioprocesses.

Thus to reduce this cost it is desirable to use low-cost raw materials for the production of biosurfactants [2]. One possibility explored extensively is the use of cheap and agro-based raw materials as substrates for biosurfactant production. A variety of cheap raw materials including, plant-derived oils, oil wastes and cashew apple juice and agriculture residues have been reported to support biosurfactant production [3-6].
The objective of the present study was to produce lipopeptide biosurfactant from *Bacillus cereus* MFS16 using pineapple juice as substrates.

## MATERIALS AND METHODS

**Isolation of heterotrophic bacteria from mangrove soil:** One hundred gram of freshly collected soil samples from ten different locations of Pichavaram Mangrove forest soil Tamilnadu, India (latitude 11°20' to 11°30' North and longitudes 79°45' to 79°55' East) were enriched with 10 ml of crude oil (obtained from Indian Oil Corporation, Chennai, Tamilnadu, India), incubated at room temperature (28 ± 2°C) for 30 days. After a month period of incubation the soils were analyzed for microbial population using standard microbiological procedures.

**Screening for biosurfactant production**

**Glass plate assay:** Glass plate assay is to ascertain the biosurfactant production by the bacterial isolates [7]. The drops on the glass plate were observed carefully. Because of the surfactant produced by the bacterial isolate the drops soon would collapse and spread on the glass plate. The larger the collapse area the greater is the activity of biosurfactant. A drop of distilled water, placed similarly on the plate served as control.

**Haemolytic activity:** Isolated strains were screened on blood agar plates containing 5% (v/v) sheep blood and incubated at room temperature for 24 h. Haemolytic activity was detected as the occurrence of a define clear zone around a colony [8]. The diameter of the zone of clearance is a qualitative method used as an indicator of biosurfactant production.

**Drop collapsing test:** Two microliters of mineral oil was added to each well of a 96 well microtitre plate lid. The lid was equilibrated for 1 h at room temperature and then 5µl of the culture supernatant was added to the surface of oil. The shape of the drop on the oil surface was inspected after 1 min. Biosurfactant producing cultures giving flat drops were scored as positive '+'. Those cultures that gave rounded drops were scored as negative '-', indicative of the lack of biosurfactant production [9].

**Oil displacement test:** Fifteen micro litre of weathered crude oil placed on the surface of distilled water (40 µL) in a petridish (150 mm in diameter). Then 10 µL of the culture supernatant was gently dropped on the centre of the oil film. The diameter and area of clear zone were measured and calculated after 30 seconds as described by [10].

**Cetyl Trimethyl Ammonium Bromide (CTAB) plate assay procedure:** CTAB plate assay procedure was performed according to [11]. The plates were incubated for 48 h at 34°C. In control plates only medium without inoculum was added in the wells. Appearance of blue halos in these plates indicated the production of biosurfactant. The blue halo zones indicated: '+'; whereas '_', as no halos.

**Identification of biosurfactant producer:** The isolate was identified by 16S rRNA gene sequence analysis. The 16S rRNA gene was amplified by PCR reaction using the primer 27F and 1492R. The

unincorporated PCR primers and dNTPs from PCR products are removed by using Montage PCR clean up kit (Millipore). Sequencing was performed in a MJ Research PTC-225 Peltier thermal Cycler using ABI PRISM® BigDeyTM Terminator cycle sequencing kits with AmpliTaq® DNA polymerase (FS enzyme) (Applied Biosystems).Single-pass sequencing was performed on each template using universal primer. The fluorescent-labelled fragments were purified from the unincorporated terminators with an ethanol precipitation protocol. The samples were subjected to electrophoresis in an ABI 3730xl sequencer (Applied Biosystems). The 16S r RNA gene sequencing was compared with sequences in GenBank using the BLAST search program and aligned by multiple alignments with the Clustal X 2.0 software.

**Preparation of pineapple juice medium:** 100mL of Mineral salt medium (MSM) broth was sterilized in an autoclave at 121$^{o}$ C for 15 min. The clarified pineapple juice with various concentrations i.e. 2, 4, 6 and 8 percent was added to the sterilized MSM broth. Same way PAJ was used as such without inorganic mineral salts to prove the effect of PAJ on the biosurfactant production. For the comparison, defined medium (MSM with 2% glucose) was included in this study. The sterilized MSM broth was inoculated with 5mL of the isolate MFS03 and incubated at room temperature over an orbital rotary shaker set at 129rpm min$^{-1}$ for 3 days.

**Extraction of Lipopeptide:** The culture broth was centrifuged at 10,000 × g for 30 min to discard the cells. The cell-free supernatant was acidified with 2 N HCl to pH 2 and extracted with diethyl ether (1:1 ratio, 5 times). The extracted fractions were combined and evaporated to dryness under reduced pressure in a rotary vacuum evaporator (Rotavapor R-205; Buchi, Bern, Switzerland). Lipopeptide from the extract were purified on a silica gel (100-200mesh, 30×2 cm) column washed with chloroform and then eluted with 3% methanol in chloroform to remove traces of contaminants.

**Chemical characterization of Lipopeptide**

**Surface activity:** Surface tension was measured with a du Nouy Tensiometer (Kruss Digital-tensiometer 10, Hamburg, Germany) at room temperature (28 ± 2°C). Twenty ml volume of each cell free culture broth was placed into a clean 50 ml glass beaker and placed onto the tensiometer platform. Cell free culture broth was equilibrated for 15 min in a small weighing dish prior to the surface tension determination. A platinum wire ring submerged into the solution was then slowly pulled through the liquid–air interface, to measure the surface tension (mN m$^{-1}$). Surface tension measurement values were recorded and expressed as mN/m. Between each measurement, the platinum wire ring was rinsed three times with water, followed by acetone and was allowed to dry. The surface tension value shown is the average of three replicates from the same culture. Control consisted of a sterile culture medium plus an inoculum, at initial at 28 ± 2°C. Distilled water and isopropanol were used as standards [12].The CMC value was used to measure the surface tension of dilutions of the cell free culture broth. The dilution reduces the biosurfactant levels below the CMC value at a point in which the surface tension of the media increase suddenly. The dilution at which the surface tension suddenly increases is the break point (CMC). The CMC value was used to measure the sudden increase in the surface tension of a series liquid the biosurfactant produced [13].

**Emulsification activity:** The emulsifying activity of the biosurfactant was determined by using the cell free culture broth [14]. The assay was carried out by adding hexane, heptanes, soybean oil and diesel oil and then vortex for 1 min individually. The emulsification was determined by measuring the height of emulsion layer after 24 h. Emulsion index $E_{24}$ (%) were determined by

$$E_{24}(\%) = \frac{\text{The height of emulsion layer}}{\text{The height of total solution}} \times 100$$

**Bacterial adherence to hydrocarbons (BATH):** BATH assay was carried out, *Bacillus cereus* MFS16 cell was harvested by centrifuging at 10,000 rpm for 15 min and washed twice with 4 mL PUM Buffer (pH 7.1) containing 16.9 g of $K_2HPO_4$, 7.3 g of $KH_2PO_4$, 18 g of urea and 0.2 g of $MgSO_4.7H_2O$, again resuspended

in same buffer. The initial density of cell suspension measured spectrophotometrically at 400 nm. Bacterial cell suspension (8ml) was then mixed with (2mL of hexadecane incubated at room temperature (28 ± 2°C) for 10 minutes and vortexed for 2 min, the mixture left undisturbed for the phase separation for 15 min, the bottom layer was recovered and the OD 400 was measured. Adherence percent to hexadecane was measured before and after the addition of hexadecane and multiple by 100 [15].

$$\text{Hydrophobicity index (\%)} = 100\left(\frac{1-\text{ODa}}{\text{ODc}}\right)$$

ODc - initial density of the cell suspension
(ODa) - Cell density remained in the aqueous phase after 15 minutes

**Analytical method**

**Thin layer chromatography: T**he active fractions were analyzed by thin-layer chromatography (TLC) on silica gel 60 plates (F254; Merck, Darmstadt, Germany) and developed in different solvent systems, namely, chloroform-methanol-acetic acid (85:10:5), n-hexane-ethyl ether-acetic acid ( 80:20:1), chloroform-methanol-5M ammonia (80:25:4). The silica containing the spot was scraped from the plate and extracted with chloroform-methanol mixture. The extract obtained in the chloroform-methanol-acetic acid solvent system demonstrated the highest surface activity. Detection of material was done by spraying the developed plate, after air-drying, with ninhydrin solution and heated at 100 ºC for 5 min.

**FTIR spectral analysis of biosurfactant:** The FT-IR spectra was recorded in a Thermo Niocolet, AVATAR 330 FT-IR system, Madison WI 53711-4495, in the spectral region of 4000-400 $cm^{-1}$ using potassium bromide (KBr) solid cells. The analysis was done in the Department of Chemistry, Annamalai University, India. The spectra were recorded and analyzed using the standard methods described by the previous authors [16].

## RESULTS AND DISCUSSION

**Isolation of biosurfactant producing bacteria:** Totally twenty five strains were isolated from the crude oil enriched mangrove soil. Of these strain only five shows higher activity; for the further studies *Bacillus cereus* MFS16 was selected and studied in detailed for the biosurfactant production.

**Screening of biosurfactant production**

**Haemolytic activity:** Haemolytic activity of *Bacillus cereus* MFS16 showed a clear zone diameter of 9 mm around the colony. Haemolytic activity is the widely used method to screen biosurfactant production. Literatures evidenced that biosurfactant productions of new isolates was preliminary screened by haemolytic activity. In the present study, a significant correlation was established between the haemolytic activity and surfactant production. Blood agar lysis has been used to quantify surfactin [17] and has been used to screen for biosurfactant production by new isolates [18].

**Drop collapsing and oil spreading test:** For the confirmation of surface activity, the drop collapsing and oil displacement method was performed. In drop collapsing test *Bacillus cereus* MFS16 with flat drop was observed, it shows positive results. The drop collapse technique depends on the principle that a drop of liquid containing a biosurfactant will collapse and spread completely over the surface of oil [7]. In oil spreading test *Bacillus cereus* MFS16 showed a positive result with a zone diameter of 3.2 cm. It was studied by the method described by [19]. Based on this observation, it was confirmed that the *Bacillus cereus* MFS16 was a biosurfactant producing bacteria.

**Identification of the selected isolate:** The genomic DNA of the isolate MFS16 was isolated and 16SrRNA gene sequencing was amplified. The 16SrRNA sequencing of the isolate MFS16 was further analyzed using NCBI BLAST tool with a query to limit the search for closest biosurfactant producing relatives. It was found that the isolate MFS16 showed clustering exclusively with biosurfactant producing

*Bacillus* sp. Based on the above analysis, the organism was identified as *Bacillus cereus*. The gene bank accession number was KC683828.

**Chemical Characterization of lipopeptide**

**Biosurfactant production:** Biosurfactant production and growth characteristics of *B. cereus* MFS16 using pineapple juice as substrate are illustrated in fig. 1. Maximal surfactant concentration (9.43 g $L^{-1}$) was attained after 48 h of cultivation. The lowest surface tension (ST) of 26.6 mN $m^{-1}$, an interfacial tension of 0.97 mN $m^{-1}$ and a CMC of 33 m $L^{-1}$l, characterizing the compound as a powerful surface-active agent. The CMC of biosurfactant obtained from *B. subtilis* was 33 mg $L^{-1}$ was reported by [20].

The *Bacillus cereus* MFS16 produced biosurfactant having emulsification ability on all the hydrocarbons and soy bean oil tested. It shows maximum emulsifying ability of 73.62% on soy bean oil, 16.73% on kerosene, 23.32% on heptanes and 25.26% on hexane. The emulsification activity of biosurfactant revealed that it could be used as an emulsion-forming agent for hydrocarbons and oils, given stable emulsions. A surfactant obtained from *Rhodococcus* strain ST-5 had lower emulsifying index towards short-chain hydrocarbons than for long-chain hydrocarbons [21]. The emulsification activity of *B. Cereus* MFS16 surfactant seemed not to be related to hydrocarbon chain length. The ability to form emulsions with vegetable oils and fats suggests potential application as cleaning and emulsifying agent in food industry.

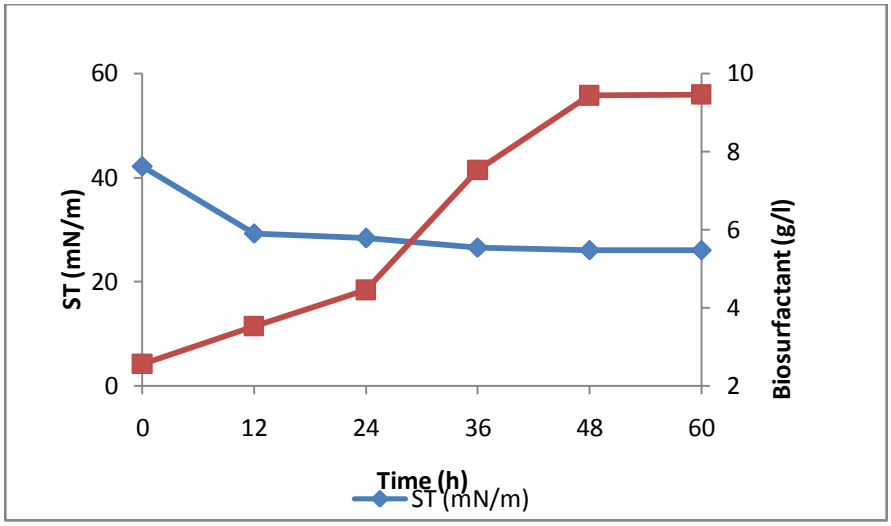


**Fig. 1.** Time-course of biosurfactant production by *B. cereus* MFS16 using pineapple juice (ST- surface tension; Biosurfactant production).

**Analytical method**

**Thin layer chromatography:** The active compound was enriched by the step-by-step liquid-liquid partitioning in chloroform methanol mixture and silica gel adsorption chromatography. The extract was identified as a lipopeptide based on its positive chromogenic reactions with ninhydrin-acetone solution spray reagent after development. The isolated biosurfactant after purification and biochemical characterization was found to contain about 56.2% lipid, 39.8% protein and 0.46% carbohydrate. The thin - layer chromatogram of the lipopeptide biosurfactant exhibited an RF value of 0.71. TLC revealed the presence of lipids in the crude biosurfactant. However, no reaction occurred with ninhydrin, which usually indicates the presence of amino acids, probably due to the absence of free amino groups. Alternatively, the amino groups might be blocked [22].

**FT-IR analysis:** The IR spectrum of the crude biosurfactant was investigated to gain insight into its chemical nature (Fig. 2). The results were compared with IR spectral data of some known biosurfactants. The observed peaks are those commonly found in the IR spectra of lipopeptide biosurfactants produced by several *Bacillus* species [23]. The broad strong band in the range of 3000-3700 $cm^{-1}$ with a maximum at

3423.04 $cm^{-1}$ represents –OH, –CH, and –NH stretching vibrations. This is characteristic of carbon-containing compounds with amino groups [24]. The presence of aliphatic chains was confirmed by the observation of peaks in the region 2850- 2975 $cm^{-1}$due to the –C –H stretching mode of $CH_3$ and $CH_2$ groups in alkyl chains. Another strong sharp band was observed at 1630.84 $cm^{-1}$, which signifies CO–N stretching vibration [24]. Moreover, absorption in the region 1600-1700 $cm^{-1}$ is characteristic for amide I vibrations in proteins, thus indicating the presence of peptide groups in the biosurfactant [23,25]. The weak band at 1350.12 $cm^{-1}$ is in the absorption range 1370-1470 $cm^{-1}$resulting from deformation and bending vibrations of –C–CH2 and –C–CH3 groups in aliphatic chains. The absorption at 1091.92 $cm^{-1}$ might be due to C–O–C vibrations in esters [23,26]. Therefore, it can be concluded that the biosurfactant produced by *Bacillus cereus* MFS16 is a lipopeptide structure.

## APPLICATIONS

The selected medium can be useful as low-cost medium for biosurfactant production thus can make the process economical.

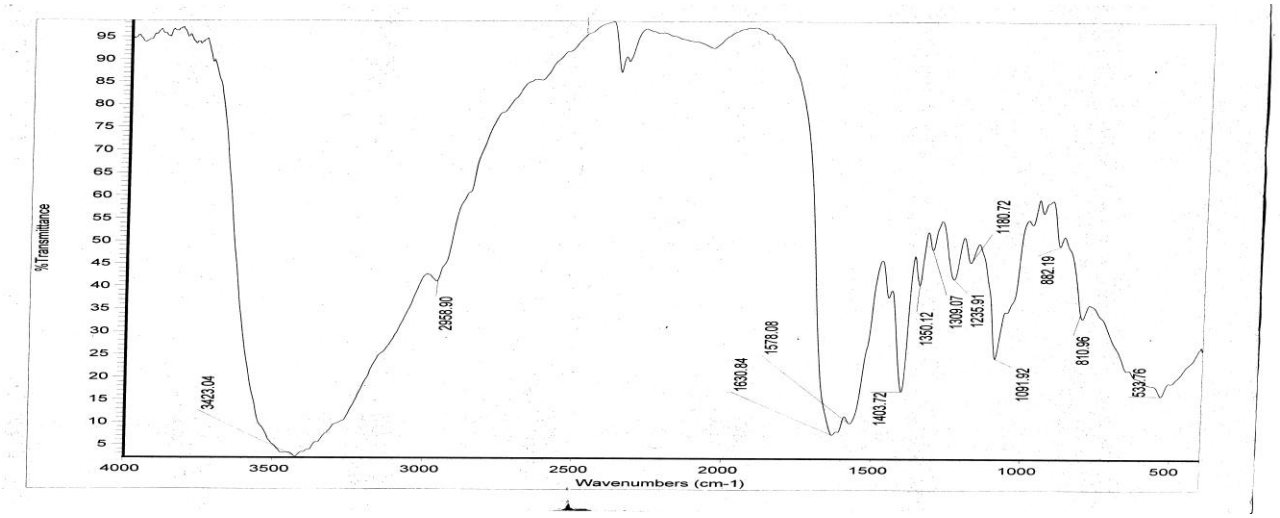


**Fig.2.** FT-IR spectral analysis of lipopeptide produced by *Bacillus cereus* MFS16.

## CONCLUSIONS

In conclusion, the present study is an attempt to find economically cheaper sources for the large scale production of microbial biosurfactants. The *Bacillus cereus* MFS16 used in this study were able to produce lipopeptide in pineapple juice as medium proved to be a suitable substrate for production, results obtained in biosurfactant production with pineapple juice suggested the possibility of industrial production of biosurfactants using economically cheaper sources. Satisfactory emulsification activity of the biosurfactant against different hydrocarbons indicated its diverse applicability against different hydrocarbon pollution. It is well known phenomenon that the amount and type of a raw material contribute considerably to the production cost in most of biotechnological processes, so the above medium can serve as low-cost medium for biosurfactant production thus can make the process economical.

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