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ORIGINAL ARTICLE

Chemical Analysis of the Biosurfactant from Soil Derived Bacteria

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ABSTRACT
With the increase in the use of petroleum products worldwide, the environment has been facing a serious issue by the release of complex, harmful compounds such as hydrocarbons and PAHs. Unique compounds known as Biosurfactant which can degrade these are indeed a solution to this problem and therefore, an attempt was made to isolate a biosurfactant producing bacterium from sediment samples collected from the local mangrove ecosystem. The chemical analysis of the biosurfactant was carried out using TLC and FTIR. The bacterium was identified using the gene sequences of the amplified 16S rRNA.
Key words *- Hydrocarbons, environmental clean-up, biosurfactant, mangrove*

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INTRODUCTION
Biosurfactants are the compounds which have the degrading ability and are produced as metabolic by - products by certain microorganisms. Biosurfactants hold significant importance in environmental biotechnology as they can break down complex compounds such as polycyclic aromatic hydrocarbons as a part of the organisms' metabolic and energy gaining processes [1].
There are many microorganisms which are known as the producers of biosurfactants and studies are ongoing to find novel strains from less explored areas [2]. In view of this, the present study was undertaken to isolate a biosurfactant producing bacterium from the mangrove sediment sample and chemically analyze the biosurfactant produced.

MATERIALS AND METHODS
Sample collection
Sediment samples were collected from mangrove areas of Thane, Maharashtra (West coast of India) at low tide using a plastic spatula and immediately poured into a plastic bag and carried to laboratory in an icebox. Sediment sample were stored at 4°C till further used.
Media used for bacterial isolation
The media used for isolation were nutrient agar and nutrient broth (Himedia Laboratories, India) prepared in 50% seawater as per the instructions given by the manufacturer. The seawater was filtered through cotton and used in the media preparation.
Isolation of bacterial cultures
1g of soil sample was dried at 40°C for 5 hrs and then suspended in 9 ml of sterile distilled water. Sediment samples were diluted and spread plated on nutrient agar, prepared in seawater. Antibiotics were added to above media in order to eliminate bacterial and fungal growth. Plates were incubated at 30°C for 48 hrs.
Morphological characteristics
Colony characteristics of a 48 hr grown colony were noted for the isolated cultures. The smears prepared were observed under oil immersion and the cell type was noted.
Screening of Biosurfactant producing bacteria
Hemolysis test
A protocol to detect biosurfactant producing bacteria by using hemolysis test was used after slight modification from earlier reports [2]. The isolates were streaked onto sheep blood agar plates (Himedia, Mumbai) in a sterile environment. The plates were incubated at 37°C for 24-48 hrs. After incubation period, the plates were checked for zones of clearing (hemolysis) around the colonies, indicative of

biosurfactant production. Two bacterial isolates, which showed strong hemolysis were taken further for confirmation using oil spreading technique.

Oil spreading technique

Two bacterial isolates were tested for their oil displacement activity. This experiment was based on the ability of the biosurfactant to alter the contact angle at the oil-water interface. The surface pressure of the biosurfactant displaced the oil. 10 µl coconut oil was added to the surface of 50 ml distilled water in a petri plate. Oil layer was formed on water. On a thin oil layer 10 µl cultured supernatant was gently placed at the centre of oil layer. The displacement of oil and clear zone formation was the indication of presence of biosurfactant. The displaced diameter was measured after 30 seconds. Out of the two bacteria tested, MSB4 was taken further for the extraction of biosurfactant.

Fermentation of positive culture

The seed inoculum was prepared in 50 ml of sterile Nutrient broth (Hi-Media, Mumbai) prepared in 50% seawater, by inoculating MSB4 culture from the slant. The culture was incubated for 24 hrs at 30°C and 100 RMP. The production medium described by Makkar and Cameotra (1998) was used for the fermentation [3]. Crude coconut oil (from a local refinery) was used as the carbon source at 2% (v/v) concentration. 25 ml seed culture was inoculated in 200 ml production media in 500 ml conical flask. After inoculation, the flask was kept at 30°C at 100 RPM for 72 hrs.

Extraction of the biosurfactant

After 72 hrs, 200 ml culture media was centrifuged at 10000 rpm for 10-15 mins at cold (4°C) condition. The supernatant was transferred to a separate flask and the pH of it was adjusted to 2.0 using 6N hydrochloric acid. After adjusting pH, the supernatant was kept overnight at 4°C. The sample was then centrifuged at 15000 rpm for 15 mins in cooling centrifuge. The crude biosurfactant precipitate was extracted with a chloroform: ethanol (2:1) mixture. The extraction procedure was repeated three times. The organic layer was pooled together, filtered and concentrated to dryness by using rotary evaporator. This dried extract was kept in a refrigerator and used for further experiments.

Measurement of Emulsification index (EMI)

The ability of the extracted biosurfactant to emulsify hydrocarbons was determined as follows. 100 mg biosurfactant extract was dissolved in 1ml chloroform and to this 1ml biosurfactant, 1ml of a hydrocarbon (Hexane, Xylene and crude oil) was added in a glass test tube. The tube was vortexed for 5 mins. The emulsification activity was checked after being allowed to settle for 24 hr and the emulsification index (E_{24}) was calculated by measuring the emulsion layer, expressed as a percentage of the total height of the mixture in the tube, as described by Cooper and Goldenberg (1987) [4]. The emulsification power of a mixture of equal volumes of 1 mg/mL SDS and the hydrocarbon was used as the control. EI was calculated by using following formula:

Emulsification index E_{24} = Height of the emulsion layer / Total height X 100

Thin layer chromatography (TLC)

The crude biosurfactant was used for chromatographic studies. The TLC plate used was made up of aluminium sheet on to which silica gel was used as an absorbent. The dried biosurfactant was weighed (approximately 10 mg) and dissolved in 50 µl of chloroform. Sample of 2-3 µl was applied on the TLC plate with the help of capillary tube. The plate was run by using chloroform, methanol, and water (65 : 25 : 4) as the solvent system. After development, the solvent was evaporated and the dried plates were kept under UV to check UV visible compounds. TLC was developed by spraying 5% H_2SO_4 and ninhydrin for detection of the separated bands.

Fourier Transform Infra Red spectroscopy (FTIR)

Crude biosurfactant extract (5 mg) was taken and mixed with potassium bromide (KBR) and crushed to a fine powder using mortar and pestle. The homogenized sample was then placed in the moulds and pressed using mechanical strength for 20-30 sec using a clean alcohol sterilized spatula. The sample pellet with the mould was then placed on the sample pan and was ready for analysis. Analysis was done using Shimadzu FTIR system. The scanning was done at frequency wavelength 400-4000 cm^{-1} with resolution of 4 cm^{-1}.

Phylogenetic analysis of MSB4 biosurfactant producing isolate

DNA Extraction was carried out using HiPurA Bacterial Genomic DNA Purification Kit (Himedia, MB505). The DNA was stored at -20°C till further use. DNA isolated from bacteria was subjected to polymerase chain reaction (PCR) amplification using Biometra thermal cycler (T-Personal 48). The reagents used were procured from GeNei. Gel electrophoresis was performed using 1.0% agarose (Seakem, 50004L) to analyze the size of amplified PCR products. The size obtained was approx. 850bp for 16S rRNA region. The PCR product was sequenced using Applied Biosystems 3730xl DNA Analyzer USA and chromatogram was obtained. For sequencing of PCR product, 519F and 1385R sequencing primers were used.

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RESULTS

Bacterial isolation

From the mangrove sediment samples, a total of eight different bacterial isolates were obtained. The isolates were chosen based on their distinct colony morphology, obtained
streak plating technique (Table 1).

Table 1: Colony characteristics of bacterial isolates obtained from sediments

Culture No.	Pigment	Surface	Shape	Edge	Consistency
MSB1	Cream	raised	round	smooth	opaque
MSB2	Off white	folded	rough	uneven	pellet
MSB3	Light brown	Smooth, flat	round	smooth	translucent
MSB4	cream	raised	round	rough	opaque
MSB5	Off white	metallic sheen	round	uneven	opaque
MSB6	Light brown	metallic sheen	round	uneven	opaque
MSB7	cream	flat	uneven	uneven	Translucent
MSB8	Light brown	flat	round	smooth	transparent

Screening for biosurfactant

Hemolytic experiment

The haemolytic activity was observed in the bacteria isolated from the mangrove sediments. Out of the eight bacteria, two bacterial isolates (MSB1 and MSB7) showed weak haemolytic activity and two bacteria (MSB4 and MSB5) showed strong haemolytic activity. The results are shown in the table 2.

Oil spreading experiment

Out of the eight bacterial isolates, two isolates were positive for the oil-spreading assay and isolate MSB4 showed the highest oil-spreading activity (Table 2).

Table 2: Haemolytic activity of bacteria on blood agar

Bacterial isolate	Haemolysis Activity zone	Oli spreading zone
MSB1	1 cm	-
MSB2	-	-
MSB3	-	-
MSB4	2.8 cm	3.2 cm
MSB5	2.1 cm	2.6 cm
MSB6	-	-
MSB7	1 cm	-
MSB8	-	-

Results for Emulsification index

Three hydrocarbons (hexane, xylene, and crude oil) were used for emulsification test. Average emulsification units (EU/mL) results for hexane was 92.60± 3 EU/mL, for xylene of 110.30± 6 EU/mL, and for crude oil of 122.52±8 EU/mL were recorded.

Results of TLC

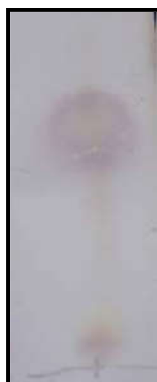

Figure 1: TLC profile of biosurfactant isolated from MSB4 culture

When the plate was sprayed with 0.2% ninhydrin, the biosurfactant component was observed as a single spot on the TLC plate (Fig. 1). The observation suggests the presence of amino acids in the sample.

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Results of FTIR

FTIR spectra showed a peak at 2933, which indicates presence of amine salts. Other peaks showed possible presence of the alkenes (Fig. 2). As this biosurfactant was crude (not purified), FTIR analysis could not give clear picture of functional groups.

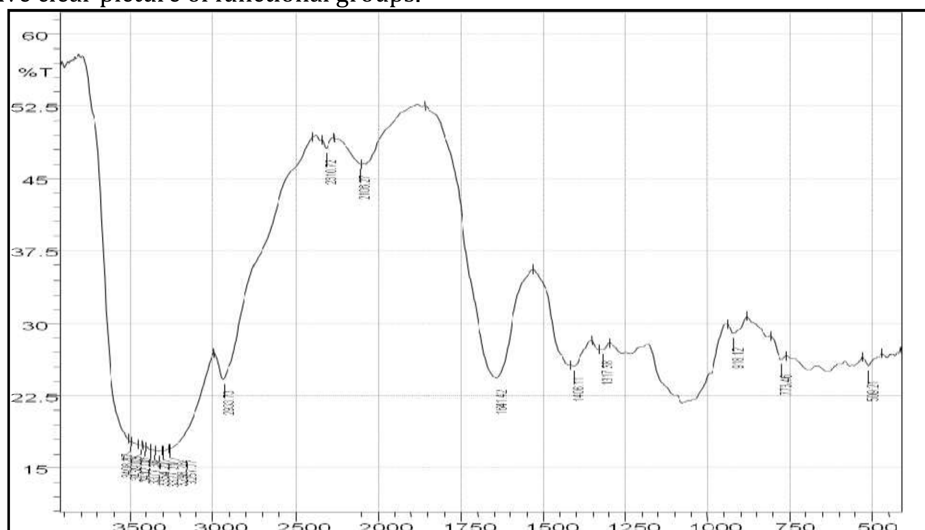

Figure 2: FTIR of crude biosurfactant isolated from MSB4 culture.

Phylogenetic analysis

Sequencing of the amplified 16S rRNA gene produced a product of 720 bp as shown in the fig. 3. This sequence when analysed using BLASTn tool indicated that the isolate was definitely a bacterium as it showed 99% sequence similarity with other bacteria of the genus *Micrococcus* which were present in the existing nucleotide database (Table 3).

AAGATTTATCGGTTTTGGATGGACTCGCGGCCTATCAGCTTGTTGGTGAGGTAATGGCTCAC
CAAGGCGACGACGGGTAGCCGGCCTGAGAGGGTGACCGGCCACACTGGGACTGAGACACG
GCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTGATGC
AGCGACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTCAGTAGGGAAGAAG
CGAAAGTGACGGTACCTGCAGAAGAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAAT
ACGTAGGGTGCGAGCGTTATCCGGAATTATTGGGCGTAAAGAGCTCGTAGGCGGTTTGTCGC
GTCTGTCGTGAAAGTCCGGGGCTTAACCCCGGATCTGCGGTGGGTACGGGCAGACTAGAGT
GCAGTAGGGGAGACTGGAATTCCTGGTGTAGCGGTGGAATGCGCAGATATCAGGAGGAACA
CCGATGGCGAAGGCAGGTCTCTGGGCTGTAACTGACGCTGAGGAGCGAAAGCATGGGGAGC
GAACAGGATTAGATACCCTGGTAGTCCATGCCGTAAACGTTGGGCACTAGGTGTGGGGACC
ATTCCACGGTTTCCGCGCCGCAGCTAACGCATTAAGTGCCCCGCCTGGGGAGTACGGCCGCA
AGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGCGTCG

Figure 3: Gene sequence of 16S rRNA of the bacterial isolate MBS4

Table 3: Phylogenetic neighbours of MSB4 isolate

Description	Ident	Accession
Micrococcus yunnanensis strain YIM 65004 16S ribosomal RNA gene, partial sequence	99%	NR_116578.1
Micrococcus aloeverae strain AE-6 16S ribosomal RNA, partial sequence	99%	NR_134088.1
Micrococcus luteus strain NCTC 2665 16S ribosomal RNA gene, partial sequence	99%	NR_075062.2
Micrococcus luteus strain ATCC 4698 16S ribosomal RNA gene, partial sequence	99%	NR_114673.1
Micrococcus luteus strain DSM 20030 16S ribosomal RNA gene, partial sequence	99%	NR_037113.1

DISCUSSION

The results of the present study indicate that less explored areas like mangrove ecosystem could act as a hot spot to find novel microbes having the ability to produce bioactive compounds and secondary

metabolites such as biosurfactants, enzymes, bioactive compounds which can have industrial applications. This observation suggests that this mangrove patch of Konkan needs to be further explored.
In the present study, biosurfactant was extracted from a bacterial isolate belonging to the genus Micrococcus. Many researchers from all over the globe have reported biosurfactants from different bacteria belonging to different genera such as *Pseudomonas* [1] , *Micrococcus*, *Acinetobacter* and *Vibrio* [5], *Virgibacillus* [6], and *Bacillus* [7] indicating that bacteria continue to serve as one of the best sources for extracting unique biosurfactants.

CONCLUSION

This research work shows that mangrove sediment could be an excellent source of bio-surfactant producing bacteria. We can also conclude that 16S rRNA gene sequencing studies can be used for identifying the unknown bacteria up to the genus level.

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