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Molecular characterization of bioactive biosurfactant metabolite producing isolate *StreptomycesLitmocidinivar*-DFS5.

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Abstract

The present study deals with the crude biosurfactant produced from DFS5 (*Streptomyces litmocidini var*) from Diary farm soils collected from various regions of Andhra Pradesh. This isolate, was screened and identified by The National Collection of Industrial Microorganisms (NCIM), Pune by comparing its phylogeny with other strains and analyzing the sequence of 16S rRNA nucleotide. For this study, a total of 18 isolates were initially screened in starch casein agar medium plates. The isolates were nurtured in Kim's medium supplemented with olive oil as the exclusive source of carbon and subjected to incubation at a temperature of 27°C for 7 days on a rotary shaker at 150 rpm for the biosurfactant production extracellularly into the medium. Out of these eighteen isolates only five isolates namely DFS1, DFS2, DFS3, DFS4, and DFS5 have been selected for further studies based on overall good growth patterns. All these isolates were subjected to preliminary screening for biosurfactant activity. Among these isolates, DFS5 was found to be a promising isolate and has shown considerable biosurfactant activity in comparison against standard sodium lauryl sulfate, when further subjected for their biosurfactant activity by using various methods like oil spreading test, and parafilm M method. The surface tension and emulsification index were tested to evaluate the presence of bioactive metabolite in the supernatant broth which is free from cells against Sodium Lauryl Sulphate as the control. The secretion of the extracellular lipase enzyme has been demonstrated during the growth of these isolates by employing a tributyrin agar medium. Isolate DFS5 has exhibited antibacterial activity against *B. subtilis, P. aeruginosa, S .aureus, and E. coli*. Following the extraction process using ethyl acetate, the resulting crude extract underwent analysis for the identification of bioactive metabolites using the TLC method and Bioautography methodology. With all the above results it is concluded that the actinomyces isolate DFS5 was a potential producer of biosurfactant metabolites.

Keywords: Actinomycetes, Kim's medium, 16S rRNA sequence, phylogenetic tree analysis, oil drop method.

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1. Introduction

Soil contamination caused by various oil products is a significant geo-environmental issue that harms the quality of soil, groundwater, and the atmosphere. The majority of hydrocarbon contamination on our planet is caused by oil spilled on land. When oil products are unintentionally spilled on the ground, they seep through the unsaturated zone. Some of the oil is trapped in this zone, while the rest reaches the water table, leading to contamination of the groundwater. Microorganisms are crucial in breaking down hydrocarbons that are not soluble in water. Biosurfactants are secondary bioactive metabolites that show surfactant properties. These substances are mostly generated by biological surfaces, mainly on microbial cell surfaces, or released into growth media. It possesses both hydrophilic and hydrophobic components and can collect at the interface between the two distinct fluid phases [1]. Surfactants are composed of hydrophobic and hydrophilic parts, which enable them to decrease surface and interfacial tensions and develop microemulsions. These microemulsions can dissolve hydrocarbons in water or vice versa [2]. Biosurfactants are naturally occurring substances that are produced in a sustainable and environmentally friendly manner. Furthermore, Biosurfactants exhibited reduced toxicity in comparison to synthetically generated surfactants [3]. Biosurfactants have gained popularity as a desirable alternative to synthetic surfactants. Consequently, their usage and market share in the surfactant industry have been steadily growing over the last 15 years [4]. Biosurfactant-producing microbes are found not just in soils contaminated with oil, but

also in undisturbed soils like dairy farm soils, cattle shed areas, and pasteurization areas which are rich in organic matter and provide a good niche for the growth of diverse microorganisms. These soils are rich in bacteria, fungi, and actinomycetes. The fats and milk solids present in these soils also contribute to the biosurfactant-producing microorganisms [5]. Actinomycetes are filamentous gram-positive bacteria with resemblance to fungus. They are derived from both aquatic and terrestrial samples. The actinomycete isolate *Streptomyces litmocidini* var. DFS5 strain was isolated from the dairy farm soil samples, in Andhra Pradesh, and assessed for its ability to produce biosurfactants and exhibit antibacterial properties.

2. Materials and methods

2.1 Screening of actinomycete isolate for biosurfactant-production

The isolate selected DFS5 was investigated for its ability to produce biosurfactants using Kim's medium. The medium consisted of NaNO3 (1g/L), KH_2PO_4 (0.1g/L), $MgSO_4$ (0.1g/L), CaCO3 (0.1g/L), Yeast extract (0.2g/L), and distilled H_2O (1000ml). Olive oil (30ml/L) was used as the only possible carbon source. The pH was maintained at 6.0±0.2, and the incubation was carried out at 27ºC for one week with continuous shaking at 150rpm using an orbital shaking incubator [6]. Following 7 days of incubation, the flasks containing the biosurfactant were taken off from the orbital shaker. They were then subjected to centrifugation at a speed of 4000 rpm for 20 minutes at a temperature of 4ºC. Upon centrifugation, supernatant free from cells was collected. The primary tests for identification of biosurfactant production were performed using the isolate DFS5.

2.1.1 Preliminary tests for identification of biosurfactant production:

2.1.1.1 Oil spreading test

The oil spreadingmethod serves as an initial evaluation for detecting biosurfactant synthesis. It is a relatively simple and time-efficient procedure [7]. A volume of 40 milliliters of distilled water was added to a Petri plate with a diameter of 90 millimeters. Following that, 15 microliters of oil was placed on the surface of the water to create an oily layer. A 10-microlitre sample of supernatant free from cells was dropped into the layer of oil causing a rapid displacement of the oil. The resulting displaced zone was measured and compared to a standard solution of SLS. Additionally, Kim's medium without any inoculation was used as a negative standard.

2.1.1.2 Parafilm-M test

The parafilm-M test is employed to ascertain the hydrophobic or hydrophilic nature of the biosurfactant. The procedure involved combining 10µl of supernatant free from cells with bromothymol blue (1%) containing biosurfactant. About 10µl of this mixture was placed onto a hydrophobic surface coated with Parafilm-M. Additionally, positive standard SLS, water, and uninoculated Kim's media were included in the experiment [8]. The diameter of the drop was examined and recorded one minute later. If the drop's shape is collapsed, it shows an indication of the production of biosurfactant bioactive metabolite.

2.1.1.3 Extracellular Lipase activity

The isolate DFS5 was screened for lipase activity using a 1% Tributyrin agar medium. The composition of this medium consisted of 1% tributyrin, 5 grams per liter of peptone, 3 grams per liter of yeast extract, 20 grams per liter of agar, and one liter of distilled water. The pH was set to 7.5±0.2. The isolate DFS5 was subcultured on the solidified tributyrin agar plates and thereafter placed in an incubator at a temperature of 27ºC for 7 days [9]. The appearance of a distinct transparent zone surrounding the selected isolate DFS5 was found after 7 days of incubation, indicating the production of the extracellular lipase enzyme.

2.1.2 Evaluation of Biosurfactant properties.

2.1.2.1 Surface tension

The bioactive matabolite surface tension has been assessed using a stalagmometer [10]. 5ml of the chosen isolate DFS5 was introduced into a 250ml Erlenmeyer flask that already contained Kim's medium of forty-five ml at a pH of 6±0.2. The mixture was then placed in an incubator for 7 days at a temperature of 27ºC while being agitated on a rotary shaker at a speed of 150 rpm. Following a 7-day incubation period, the cultured broth was subjected to centrifugation at a speed of 4000rpm for 20 minutes at a temperature of 4°C. The resultant was then further filtered to get a clear filtrate. This filtrate was utilized to measure the surface tension of the bioactive metabolite. Sodium Lauryl Sulfate was the reference, while H_2O served as a negative standard. The values of these samples was calculated using the provided formulas.

Surface tension of sample = $\rho 2n1/\rho 1n2 \times$surface tension water

$\rho 1$=density of distilled water

$\rho 2$=density of sample

n1=no of drops of water

n2=no of drops of samples.

2.1.2.2 Emulsification index

This parameter is crucial in assessing the performance of biosurfactants. It is both simple to execute and provides an intuitive way of computing the emulsification index values [11]. The emulsification index was evaluated using the cell-free supernatant for this test. A volume of 6 milliliters of kerosene was put into the test tube, to which 4 milliliters of centrifuged and filtered broth containing biosurfactant was added. The mixture was then vigorously mixed for 5 minutes using a vortex mixer, after which it was left undisturbed for a period of 24 hours. The positive control for this test was the standard SLS, while the negative control was the un-inoculated Kim's medium. The emulsification index findings for the samples were observed comparatively. If the biosurfactant was detected in the clear filtrate, it would create a cream-colored emulsified layer when mixed with the kerosene. The value was then determined with the below-mentioned formula. In the absence of biosurfactant production, the presence of two distinct layers are seen.

E (24) = (Height of emulsion formation)/ (Total height of solution) ×100.

2.1. 3 Morphological characteristics of isolate DFS5

Actinomyces display remarkable morphological differences compared to other Gram-positive bacteria. Actinomycetes feature filamentous hyphae with branching

patterns, resembling fungus, and cell structures resembling rod shapes analogous to Grams reaction positive bacteria. [12]. The morphological characteristics of the strain were examined by using scanning electron microscopy. The cells of the isolates DFS5 were placed in vials and treated with a 2.5% solution of glutaraldehyde in a 0.1 M phosphate buffer (pH 7.2) for 24 hours at a temperature of 4ºC. They were then further treated with a 2% solution of osmium tetroxide in water for 4 hours. Then, the sample underwent dehydration using a progressive succession of alcohols, each with a higher concentration and were then dried using a critical point drying apparatus. The prepared sample was attached to the stubs using double-sided carbon conductivity adhesives. An automated sputter coater was used to apply a thin layer of gold onto the samples. (FE-SEM-Model –ULTRA 55 by Carl Zeiss) and examined under a scanning electron microscope at the specified magnifications, following the normal technique.

2.1. 4 Genotypic characterization of the isolate DFS5

The sequence of 16S rRNA was submitted to the GenBank database at NCBI for identification. A BLASTN search was conducted to find closely related sequences and determine their percentage similarity. The phylogenetic tree was then constructed using the neighbor-joining method in MEGA software (version 6.0) [13].

2.1.5 Fermentation and solvent extraction of biosurfactant

Following the positive results obtained from the primary screening tests for biosurfactant synthesis using Kim's medium with olive oil as the source of carbon, the biosurfactant fermentations were performed using submerged type. A volume of 5ml of culture inoculum was introduced into forty-five ml of the production media in a two fifty ml conical flask. The flask was then placed on a rotary shaker and incubated at a temperature of 27ºC for 7 days, with the shaker set at a speed of 150 rotations per minute [14]. Following a 7-day incubation period, the conical flask was taken out of the shaking incubator and subjected to centrifugation at a speed of 4000rpm at a temperature of 4ºC for a duration of 20 minutes. Subsequently, the cells were separated by filtration, and the resultant filtrate was collected. The biosurfactant was extracted using the cell-free filtrate. This filtrate was subjected to extraction using ethyl acetate in a 1:1 ratio. This was done at room temperature using a separating funnel. The organic layer was carefully separated from the aqueous layer, and this extraction process was repeated thrice.[15]. The organic layer underwent evaporation using a rota evaporator to eliminate the organic layer. The resulting pale brown-colored crude extract was then collected in sterile containers and stored in a freezer for future analysis.

2.1. 6 Bioautography technique

The bioautography technique is a highly effective and established method for determining antibiotics in crude extracts. In this experiment, the crude extract that was obtained was utilized to carry out the bioautography procedure [16]. Four bacterial strains, including both Gram-negative and Gram-positive bacteria (*E.coli, Staphylococcus aureus, P aeruginosa, B.subtilis*), were employed in this method. Nutrient agar media for bacterial growth were prepared and sterilized. Suspensions of Bacteria were prepared and 25 micro liter of each were inoculated onto plates of nutrient agar media. The plates were left for solidification for about 10 minutes. A minute quantity of crude extract was carefully applied to the lower section of the thin layer chromatography plate with silica gel impregnation using a capillary tube. Following that, the TLC plate was placed in a mobile phase consisting of a mixture of 25% ethyl acetate and hexane. Subsequently, the Thin layer chromatography plates were taken out from the stabilizing chamber and left to dry in the air. Each Thin layer chromatography plate was then positioned on the corresponding solidified media, in contact with the bacteria. Following this, petri plates were refrigerated for one hour to facilitate the transfer of antibacterial compounds from the thin layer chromatography plates to the plated media. Within an hour, the Thin layer chromatography plates were carefully handled using sterile forceps from the top of the media plates and they were then left for incubation for 24h at 32°C. Then the areas of inhibition were documented [17].

3. Results and discussion

The strain DFS5 was subcultured and stocked on the YEME agar medium. The isolate is depicted in Figure 3. The media used for screening biosurfactant, which contained 30% olive oil as a carbon source, resulted in a milky frothy emulsified liquid in the production flask. In contrast, no such observations were found in the uninoculated control flask of the same medium. These results are presented in Figure 4 (A and B). The production of biosurfactant was assessed through preliminary oil spreading tests. These tests revealed a zone of displacement on the water's surface, indicating that the biosurfactant was present. The biosurfactant exhibited a displacement zone of 6.5cm, whereas the control SLS showed a displacement zone of 9.5cm. The control uninoculated Kim's did not exhibit any displacement. These results are depicted in Figure 5. The hydrophilic character of the supernatant was demonstrated by the parafilm-m test, indicating the presence of biosurfactants. The supernatant droplet on the parafilm-m strip exhibited a collapse, which was then compared to the standard SLS. The water and uninoculated broth did not exhibit any drop collapse and maintained a consistent dome form on the surface of parafilm M, as depicted in Figure 6. In the case of lipase activity, a transparent area around the streaked area of the isolate DFS5 in the tributyrin agar plate indicates that the DFS5 can catalyze triacyl glycerols by producing lipases in due course of their growth. The transparent area zone result is shown in Fig: 7. Surface tension of the crude biosurfactant has shown the values for DFS5 were 38.55 dynes/cm, Sodium lauryl sulphate showed 24.14 dynes/cm, and water was 54.35 dynes/cm. These are shown in Fig.8. The selected isolate DFS5 formed a creamy color emulsion with the kerosene and showed 58 %, and standard Sodium lauryl sulphate was an 81.03% emulsification index whereas no such emulsified layers are formed in the case of control (un-inoculated broth).These are given in fig: 9 (A, B, and C).

3.1 Morphology of isolate DFS5

The chosen strain of DFS5 exhibited morphological traits such as a purple color during grams reaction like Gram-positive bacteria. Additionally, it displayed spirals that are open and long, hyphae structures like hairs, along with the development of spore chains and filaments that are branched,

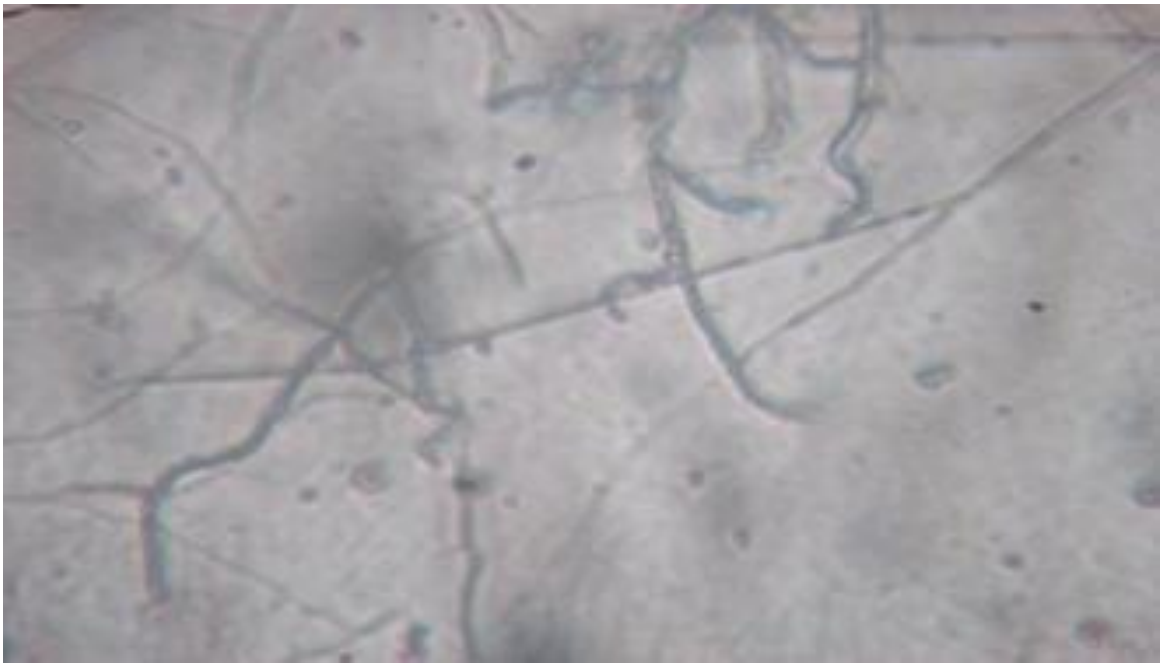


Fig 1: Morphology of DFS5 under 4000X magnification

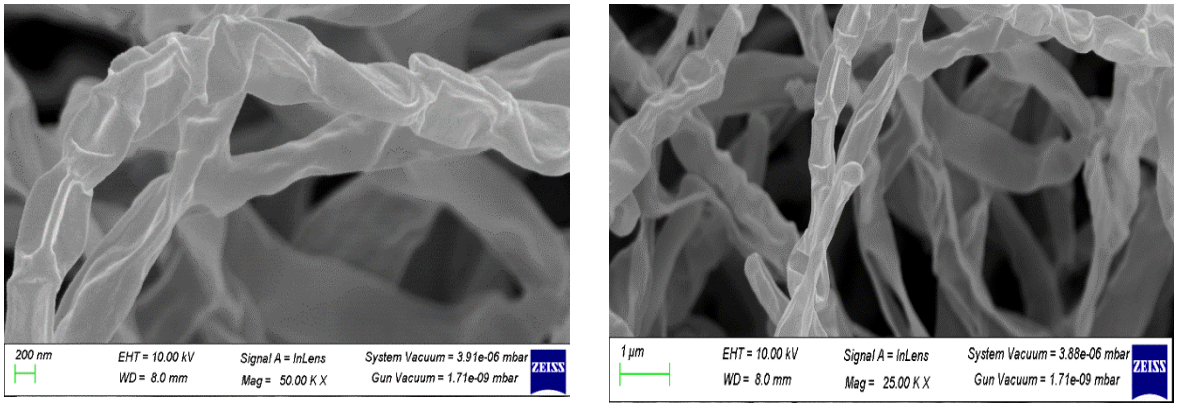
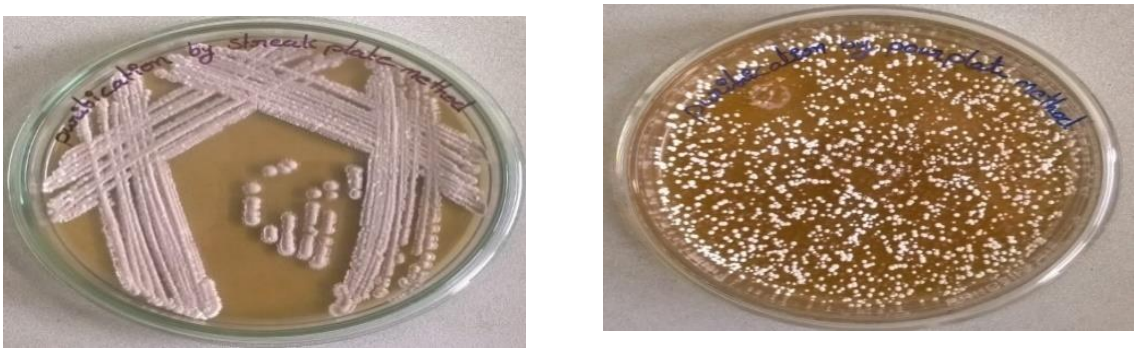


Figure 2: SEM images of selected strain DFS5 at different magnifications of 5.00 K X and 25.00

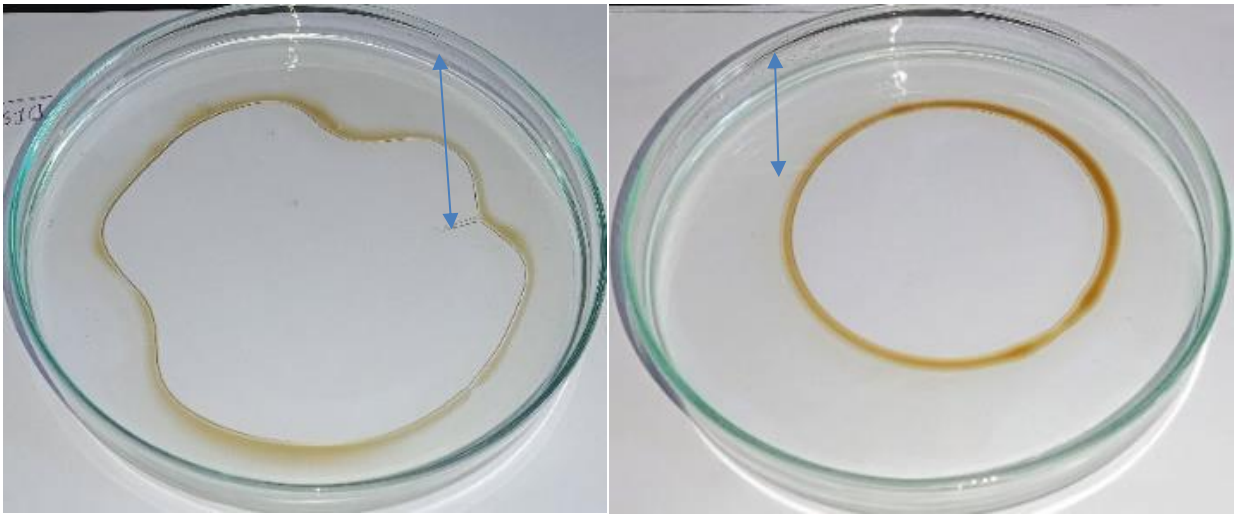


(A) (B)

Fig3: Selected isolate of DFS5 A) Streak plate Method B) Pour plate method

(A) **(B)**

Fig 4: Fermentation flasks A) control flask B)DFS5 flask.



(A) **(B)**

Fig: 5 Oil spreading test (displaced zone A) Control and B) DFS5

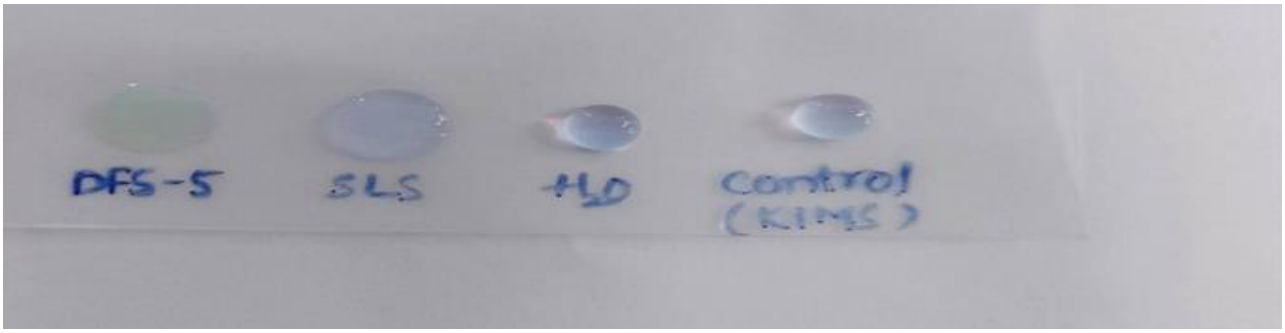


Fig 6: Hydrophilic nature of biosurfactant against negative controls by parafilm M test

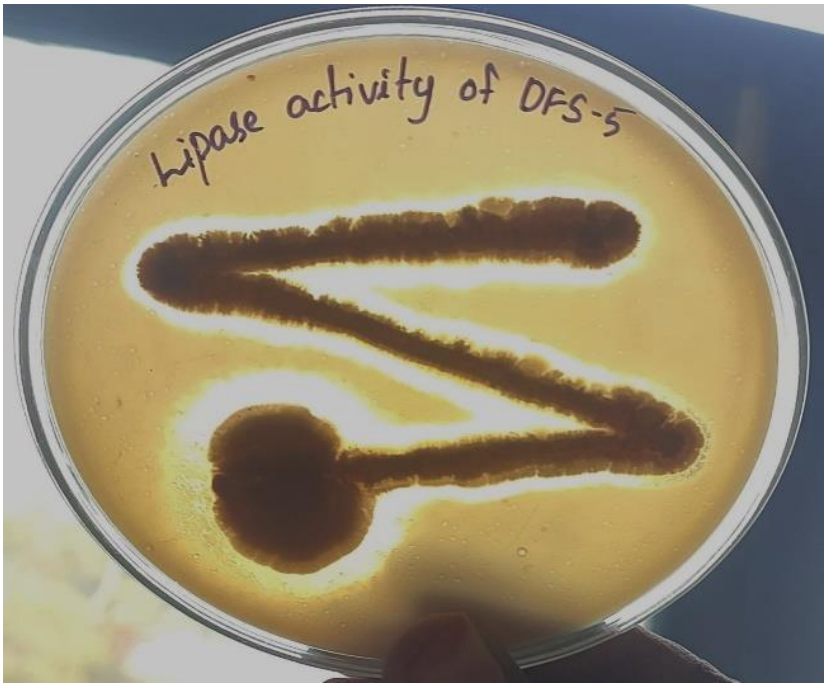
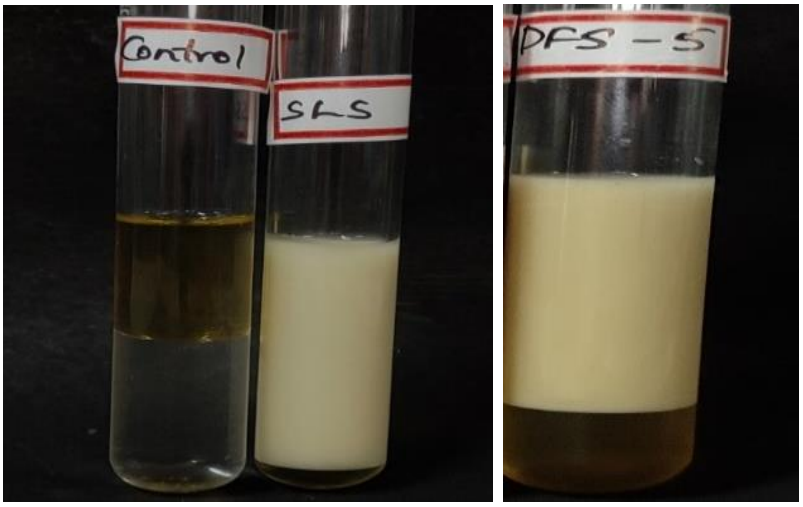


Fig 7: Transparent zone around the growth of DFS5 isolate for lipase enzyme production

(A) SLS (B)Negative control (C) DFS5

Figure 8: Surface tension.



(A) **(B)** **(C)**

(A)Control (B) SLS (C) DFS5

Figure 9: Emulsification index

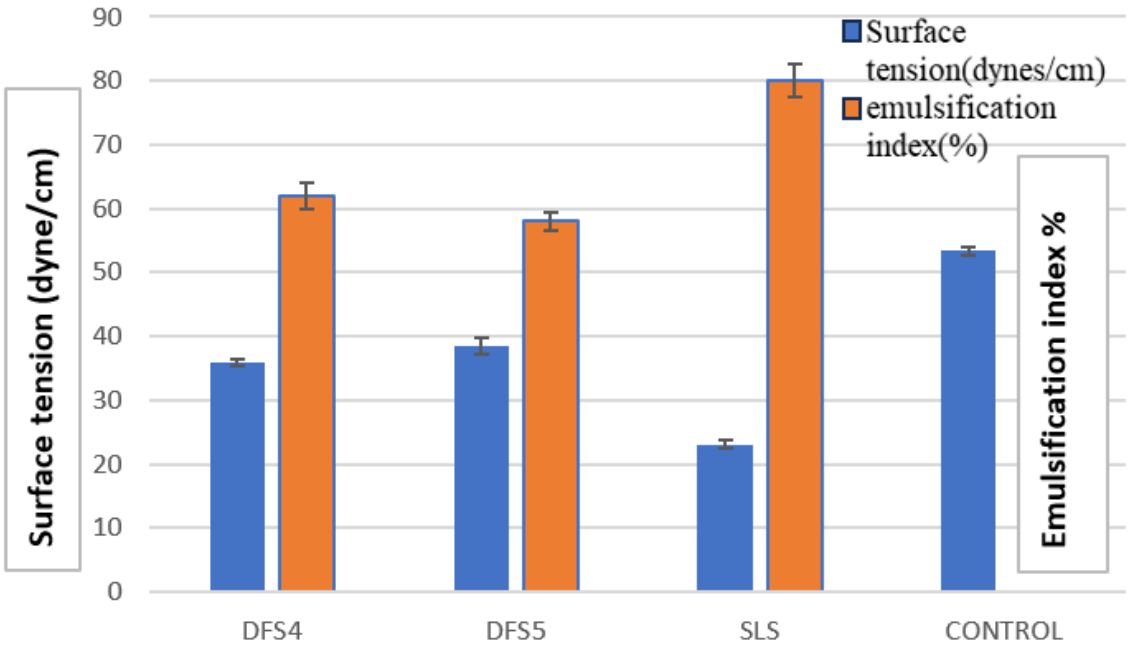


Figure 10: Surface tension and % emulsification index

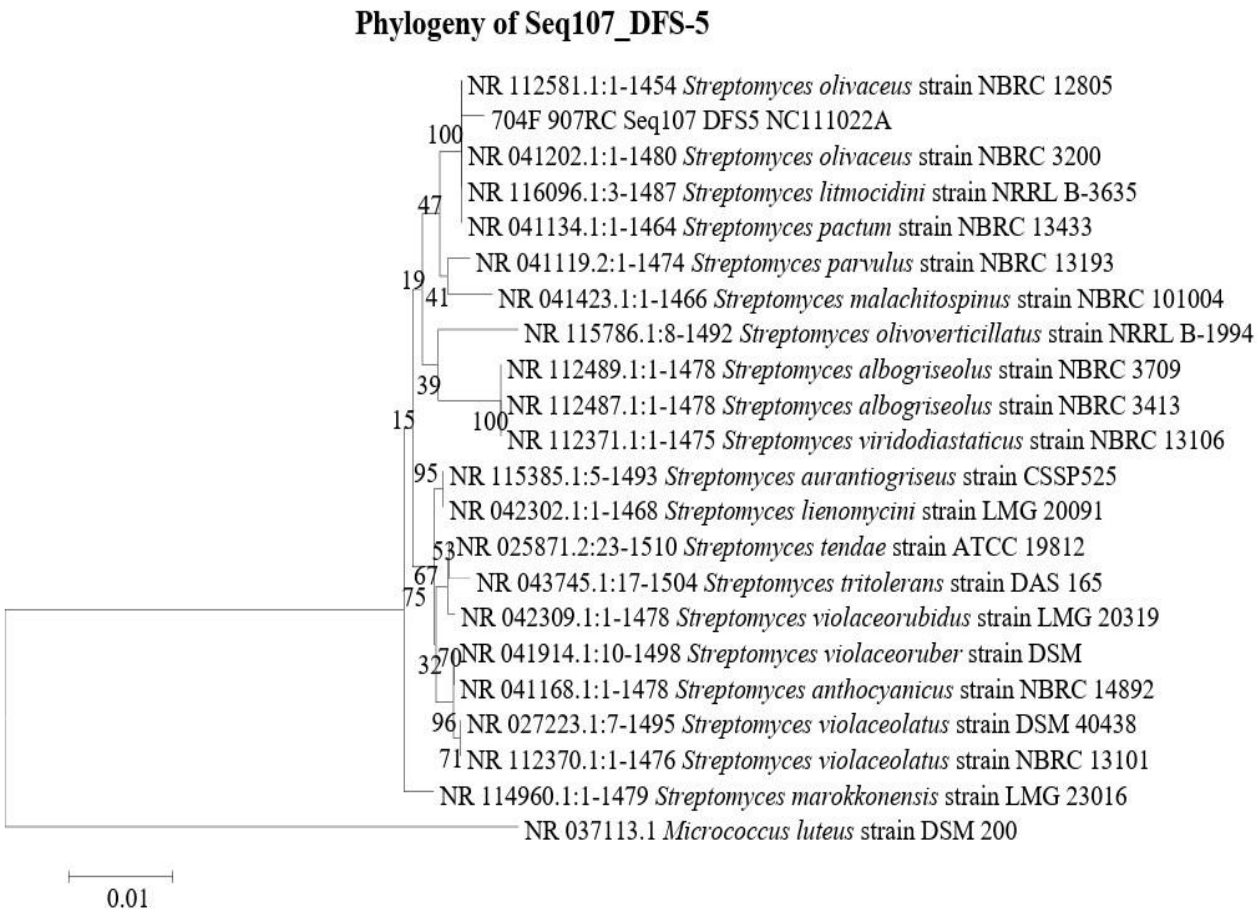


Figure11: Phylogenetic tree 16sRNA of *Streptomyces litmocidini* var. DFS5

Fig 12: Extraction of biosurfactant

Figure 13: Zones of Inhibition of DFS5 around the diffused areas

Similar to fungus in the trinocular microscope at magnification of 4000X and the image was shown in fig: 1. The isolate DFS5 displayed massive sporulation with branched mycelium and abundant aerial hyphae. Spore chains in Section Rectiflexibiles with some spore chains that are straight and primitive spirals or occasional hooks suggestive of Section Retinaculiaperti. Typically, developed spore strands include many spores per chain; it is common to see longer chains as well. The surface of the spore is devoid of any irregularities or roughness. The color of the colony is a gray hue, like the color of aerial mass. The opposite surface of the colony appears colorless or has a distinct grayish-yellow hue.

3.2. Genotypic characterization of the isolate DFS5

The chosen strain DFS5 was identified as *Streptomyces litmocidini var*. by NCIM, Pune. 16S rRNA sequence was analyzed. The accession number for the sequence is OR994261.1. given by GenBank. The phylogenetic tree analysis of the isolate *Streptomyces litmocidini var.* DFS5 is displayed in Figure 11.

3.3. Fermentation and solvent extraction of biosurfactant

Upon many trials with various organic solvents for biosurfactant extraction, Ethyl acetate was chosen due to its superior recovery rate compared to other solvents such as Hexane, Chloroform, and Methanol. The extraction process involved utilizing the supernatant filtrate of of DFS5 (*Streptomyces litmocidini var*.) and pure ethyl acetate in a equal ratio, as depicted in Figure 12. We obtained a pale brown colored crude, which was then analyzed using chromatographic technique (TLC) with a solvent system consisting of 25% ethyl acetate and hexane. The extract was exposed to an iodine chamber, which has a high vapor pressure. As a result, the TLC plate became saturated and turned light brown. After a short period, several aromatic and unsaturated compounds were detected as colored spots on the plate. Subsequently, the compound's spots were subjected to analysis by applying a methanol-Sulphuric acid mixture through spraying. The Thin layer chromatography plate was then maintained at a temperature of 110ºC for 30 minutes, while carefully observing and recording the distinct Rf values of the various compounds.

3.4Bioautography technique

Identified the antibacterial substances during production of biosurfactant by employing the bioautography approach to test their effectiveness against both Gram-negative and Gram-positive bacteria. Following a 24-hour incubation period, the petri dishes were observed. All bacteria exhibited inhibition zones surrounding the area where the antibiotic diffused on the media surface. The Gram-positive

bacteria displayed a diameter of 12-15mm, while the Gram-negative bacteria had a diameter of 15-17mm. The zones of inhibition in both petri plates are depicted in Figure 13. This suggests that the semi purified bioactive metabolite acquired during fermentation produces antimicrobial compounds.

4. Conclusion

Increasing environmental concern leads to the consideration of biological surfactants as an alternative to chemically synthesized surfactants. The most important advantage of biosurfactants when compared to synthetic surfactants is their ecological acceptance, owing to their low toxicity and biodegradable nature. Biodegradation of hydrocarbons by the natural population of microorganisms represents one of the primary mechanisms by which petroleum and other hydrocarbon pollutants are eliminated from the environment. In this study isolate DFS5 obtained from terrestrial soil samples, produced bioactive metabolites (biosurfactants) and it also produced a considerable amount of antibacterial metabolites. Indeed, biosurfactants are expected to become known as the "multifunctional materials" of the 21st century. Currently, the major market for biosurfactants is the petroleum industry, in which these compounds can be used in the cleanup of oil spills, the removal of oil residue from storage tanks, microbial-enhanced oil recovery, and bioremediation of soil and water. Therefore, from the environmental standpoint, simultaneous biosurfactant production by microbial species is a great interesting field for scientific research. So a lot of researchers have reported that *Streptomyces sp*. is an incredible producer of the release of high amounts of antibacterial compounds in the course of the biosurfactant fermentation process.

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Conflict of interest

No conflict of interest was encountered during the research as well as in the publication of the article.

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