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ISOLATION OF BIO-SURFACTANT PRODUCING ACTINOMYCETES FROM TERRESTRIAL AND MARINE SOILS

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Keywords:

Bio-surfactants, Emulsification index, Surface tension, L-rhamnose, Antibacterial activity

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ABSTRACT: Bio-surfactants, produced by microorganisms are important biotechnology products, owing to their modes of action, low toxicity, relative ease of preparation, and widespread applicability as an industrial, medicinal and in food industries. The present study was done to isolate the bio-surfactant producing Actinomycetes from three different types of soil samples contaminated with petroleum spillages from Naval Dockyard (NDYS), deep marine sediment from sea-bed (DMS), and with poultry litter (PLS), collected from around Visakhapatnam. Thirty isolates of soil Actinomycetes were initially screened in Humic-acid vitamin-salts agar-plates, to identify their bio-surfactant activity by Oil-displacement method. Only seven of these thirty isolates, namely, PLS-1, PLS-2, PLS-4, PLS-9, NDYS-3, NDYS-4, and DMS-1, showed promising bio-surfactant activity, compared to sodium-laryl-sulfate as standard. These seven isolates were further subjected to preliminary screening methods (modified drop collapse method, Para film-M method, and lipase activity method), to identify bio-surfactant production. Three of these seven isolates, namely, PLS-1 from poultry littered soil, NDYS-4 from petroleum contaminated soil from the naval dockyard, and DMS-1 from deep marine sediment, showed maximum bio-surfactant production. Of these three, only two isolates showed a positive result for phenol H_2SO_4 and blue agar plate methods. This indicated that the isolated bio-surfactant was Rhamnolipid in nature. This result was further confirmed by orcinol assay, taking L-rhamnose as standard. Bio-surfactant production from these two isolates was further evaluated by surface-tension measurement (Drop-count) method and emulsification index. It was concluded that this Rhamnolipid has permeabilizing effects on Gram-positive and Gram-negative human bacterial strains, reinforcing their potentiality in antibacterial activity.

INTRODUCTION: Bio-surfactant is a structurally diverse group of a surface-active molecule, synthesized during microbial growth. These bio-surfactants are extracellular products released by microorganisms growing on decaying hydrocarbons.

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The influence of these biosurfactants on the interfaces, especially on the surface tension of the liquid-vapor interface, is increasingly gaining attention in recent years. The capacity of bio-surfactants in reducing surface and interfacial tension with low toxicity and high specificity and bio-degradation lead to an increasing interest on these microbial bi-products as alternatives to chemical surfactants [1, 2].

Bio-surfactants are interesting amphiphilic molecules of microbial origin, whose hydrophobic and hydrophilic domains depend on the carbon

substrate and the strain of the organism. They have various biological functions/properties and have potentials in commercial applications in the food, microbiological, pharmaceutical and therapeutical agents in biological industries, as a bi-control agent in agricultural applications and health and beauty products for the cosmetic industry [2].

Almost all the surfactants that are being used in these days are chemical and are mainly petroleum derivatives. These are toxic and are the potential sources of pollution. The hazards associated with these synthetic emulsifiers have resulted in increasing interest towards bio-surfactants in recent years [3]. Till today, many bio-surfactant producing microbes were isolated from soils or water samples which are contaminated with hydrophobic organic compounds like refinery wastes. In contrast, an undisturbed environment also supports bio-surfactant producing microbes. The marine environment has also been reported as successful sampling sites for the isolation of bio-surfactant producing actinomycetes [4].

However, very little work was done so far on terrestrial soils, and some work was done on marine ecosystems. Since, the terrestrial soils are rich in bio-surfactant producing actinomycetes, and the marine ecosystems with diversified actinomycetes communities, two terrestrial soil samples, and one marine soil sample, were selected for the screening of bio-surfactant producing actinomycetes in this study [5]. As microorganisms from different ecosystems produce different groups of bio-surfactants with diverse properties and physiological functions, three different soils, i.e., oil-contaminated soil, natural organic soil, and marine soil, were taken. The bio-surfactant activity was further confirmed by identification and physicochemical characterization.

MATERIALS AND METHODS:

Isolation of Bio-surfactant Producing Actinomycetes: Three different soil samples was selected for the isolation of bio-surfactant producing actinomycetes. One sample was from poultry soil, rich in fats and oils (PLS), the second was the soil mixed with petrochemicals from Naval Dockyard (NDYS), and the third was from deep marine soil, contaminated with oils spilled from tankers (DMS). Humic acid supplemented actinomycetes-selective-medium was used for especially obtaining bio-surfactant producing actinomycetes. 100 µl aliquots of appropriate dilution were applied to humic acid- salts vitamin agar plates with the pH adjusted to 7.0 ± 0.2 [6].

These plates were supplemented with Rifampicin (50µg/ml) and Cycloheximide (50µg/ml) and were incubated at 28 ºC for 7 days for the growth of actinomycetes colonies. Humic acid-salts-vitamin agar medium was used to isolate specifically bio-surfactant producing actinomycetes [6]. These isolates were then transferred to Hickey-Tresner agar slants for maintenance of pure cultures. These pure cultures were subcultured for every forty-five days and were stored in the refrigerator at 4 ºC. From these slants, the isolates were further screened for the production of bio-surfactant activity [7].

Screening for Bio-surfactant Activity: From the working stocks, isolates were sub-cultured and incubated at 28 ºC for 7 days. After incubation, microbial growth from each slant was suspended in 2 l of sterile distilled water and was transferred into 50 ml of Kim's medium contained in 250 ml Erlenmeyer flasks [8]. These flasks were then incubated on a rotary shaker for 7 days at 28 ºC. After incubation, the contents of each flask were centrifuged at 7000 rpm for 20min and then were filtered. This cell-free supernatant was used for the production of the biosurfactant activity by conducting the following tests.

Drop Collapsing Method: In drop collapsing method, 2 µl of mineral oil was added to 96-well micro titer plate, and 5 µl of the culture supernatant was added to the surface of the oil. After 1 min, the shape of the drop on the surface of the oil was observed [9].

Oil Spreading Method: Ten µl of crude oil was added to the surface of 40 ml of distilled water in a Petri dish to form a thin layer of oil. Then, 10 µl of culture supernatant was gently placed on the center of this oil layer. If bio-surfactant is present in this supernatant, then the oil is displaced, and a clearing zone is formed. The diameter of this clearing zone on the oil correlates to surfactant activity [10].

Para Film-M Test: One drop of Bromophenol blue indicator was added to 2 ml of cell-free

supernatant. 10 µl of the sample solution was carefully added on parafilm-M with a micropipette like a drop. The shape of the drop on the surface was inspected after 1 min. The diameters of the droplets were evaluated [11].

Lipase Production: Lipase production by the actinomycetes-pure-culture was determined by using tributyrin agar plates [12]. Tributyrin-agar-medium was sterilized and was poured into the Petri-dish. After solidification of this medium, a loopful of culture was streaked and incubated at 28 ºC for 7 days. Clear-zone around the organism indicates the production of lipase, which is the characteristic feature of the bio-surfactant producing organisms.

Physico-chemical Characterization of Bio-Surfactants: Two types of physical tests, namely, surface tension measurement and emulsification index, were carried out for the evaluation of bio-surfactant activity on the sample isolates. Further, three types of chemical tests, namely, phenol-sulphuric acid method, biuret test, and phosphate test, were carried out for the characterization of the bio-surfactants. With these chemical tests, identification of the typology of the bio-surfactants was done.

Surface Tension Measurement: Surface tension of the liquid was measured with a stalagmometer by Drop count method [13]. Surface tension was determined based on the number of drops which fall per volume, the density of the sample, and the surface tension of the reference liquid (water).

$$\sigma_L = \sigma_W * \frac{\rho_L}{\rho_W} * \frac{N_W}{N_L}$$

Where σ_L is the surface tension of the liquid under investigation, σ_W is the surface tension of water, N_L is the number of drops of the liquid, N_W *is* the number of drops of water, ρ_L is the density of the liquid, and ρ_W is the density of water.

Emulsification Index: The E24 of culture samples was determined by adding 6 ml of kerosene, 4 ml of water and 1 ml of the cell-free broth in a test tube, vortexes at high speed for 2 min, and allowed to stand for 24 h. The E24 index is given as a percentage of the height of the emulsified layer (cm) divided by the total height of the liquid column (cm). The percentage of emulsification index was calculated by using the following equation [14].

$$E24 = \frac{\text{Height of emulsion formed}}{\text{The total height of the solution}} * 100$$

Phenol Sulphuric Acid Method: To 1 ml of cell-free supernatant, 1 ml of 5% phenol was added. To this mixture, 2-5 ml of concentrated Sulphuric acid was added drop by drop, until the characteristic color was developed. Development of orange color indicated the presence of glycolipids [15].

Biuret Test: Two ml of cell-free supernatant was heated at 70 ºC for 10 min; to this solution, 10 drops of 1M NaOH solution was added. To this mixture, 1% copper sulfate was added drop by drop, to observe a violet or pink ring, which indicates the presence of lipopeptides [16].

Phosphate Test: To 2 ml of cell-free supernatant, 10 drops of 6M Nitric acid was added, and then is heated at 70 ºC for 10 min. To this mixture, 5% ammonium molybdate was added drop by drop, slowly until the yellow color is formed, and then the formation of a yellow precipitate, which indicates the presence of phospholipids [17].

Confirmation Tests for Rhamnolipid: Following tests were conducted to confirm that the bio-surfactant produced was rhamnolipid.

CTAB/Methylene Blue Agar Plate Method: The CTAB agar plate method is a semi-quantitative assay for the detection of extracellular glycolipids or other anionic surfactants [18]. The microbes of interest were cultivated on a light blue mineral-salts-agar-plate, containing the cationic surfactant cetyltrimethylammonium bromide and the basic dye methylene blue. If anionic surfactants are secreted by the microbes growing on the plate, they form dark blue insoluble ion pair with cetyltrimethyl-ammonium bromide and methylene blue [19]. Thus, the productive colonies are surrounded by dark blue halos.

Orcinol Assay: This method was used for the direct assessment of the amount of Rhamnolipid in the sample. 400 µl of cell-free supernatant was taken, and its pH was adjusted to 2 by adding 2N HCl, which resulted in the separation of

Rhamnolipid. To this, 750 µl of diethyl ether was added to extract the Rhamnolipid into an organic layer. Solvent addition and extraction were repeated twice. Ether fractions were dried by evaporation. To the left out precipitate, 400 µl of pH 8 phosphate buffer was added. 2.7 ml of Orcinol was added to 300 µl of the sample. The sample tubes were boiled for 20 min. The solutions were left in dark for 35 min to cool to room temperature. Optical density was measured at 421 nm. The Rhamnolipid concentrations were calculated from standard graphs taking L-rhamnose as standard [20].

Determination of Antimicrobial Activity: The antimicrobial activity was determined by cup plate method. The partially purified extract obtained by the evaporation of the ethyl acetate extract was dissolved in 1 ml of 1M phosphate buffer (pH 7.0). Then, 50 µl of this extract was loaded into each well in solidified-agar-medium, previously inoculated with 25 µl of test organism against which, the activity has to be determined. The plates were incubated at 37 ºC for 18-24 h and were examined. The diameter of the zones of inhibition was measured [21].

Time Course of Bio-surfactant and Antibiotic Production: The time course of bio-surfactant and Antibiotic production was followed in batch cultures at optimum conditions. The experiment was designed for 10 days, starting from log phase to stationary phase under submerged culture conditions [22]. The resultant cell-free supernatant was removed by filtration, followed by cold centrifugation at 10,000 rpm at 4 ºC for 20 min. The supernatant was analyzed for bio-surfactant production by Orcinol assay, for antibiotic production by Cup-Plate method [23, 25].

RESULTS AND DISCUSSION:

Isolation and Screening of Bio-surfactant producing Actinomycetes: A total of 30 actinomycetes colonies were isolated from poultry litter, naval dockyard, and deep marine soils. The screening was carried by spread plate technique. According to Haykawa *et al*., Humic-acid-supplemented actinomycetes - selective-medium is selective for bio-surfactant producing actinomycetes [6]. Colonies which have white powdery appearance are mostly streptomycetes. Hence, 30 actinomycetes colonies with white powdery appearance were isolated and streaked on Hickey-Tresner medium and were incubated for 7 days. Based upon the growth and texture, only 7 isolates out of 30, were selected for the screening of bio-surfactant production [26, 28, 29]. These isolates were named PLS-1, PLS-2, PLS-4, PLS-9, NDYS-3, NDYS-4, and DMS-1.

These isolates were fermented in Kim's medium for 7 days in Erlenmeyer flask [8]. This cell-free supernatant was used for the detection of bio-surfactant production by modified Drop collapse method, Oil spreading test, Parafilm-M test, and Lipase production tests [9-12]. Sodium lauryl sulfate (SLS) was used as standard. Compared to the standard, PLS-1, NDYS-4, and DMS-1 showed maximum bio-surfactant production. These methods have several advantages such as the small volume of samples required, rapid and easy to carry out, *etc*. Therefore, these isolates were used for further studies. The results are shown in the **Table 1**.

TABLE 1: DETECTION OF BIOSURFACTANT PRODUCING ACTINOMYCETES

Test	Control	PLS-1	PLS-2	PLS-4	PLS-9	NDYS-4	NDYS-3	DMS-1	SLS
Drop collapsing method (a)	-	++	-	-	-	+++	-	++	+++
Oil spreading test (b)	-	7.5	0.5	1	4	7.5	2.5	4	10
Parafilm-M test (c)	-	++		-	-	+++	+	++	+++
Lipase test (d)	-	++	-	-	-	++	+	++	+++

(a). (+) spreading of the drop, (-): dome shape. (b). The diameter of the zone of oil displaced in 'cm'.
(c). (+) spreading of the drop, (-): dome shape. (d). (+) the clear zone around the streak, (-): no zone.

Chemical Characterization of the Type of Bio-Surfactant: As Glycolipids, lipopeptides, phospholipids are the three types of bio-surfactants, to identify the type of bio-surfactant. Phenol-H_2SO_4 test (glycolipids), Biuret test (lipopeptides), Phosphate test (phospholipids) was performed with cell-free supernatant of PLS-1, NDYS-4, DMS-1 [15-17]. The results are shown in **Table 2**. PLS-1, NDYS-4, DMS-1 showed a positive result for the phenol-H_2SO_4 test. This indicated that these isolates produced are glycolipids type of bio-surfactant [31-34]. Rhamnolipid, Sophorolipids, Prehaloselipids are

different types of glycolipids [23]. Among these, Rhamnolipid is most abundantly synthesized by microbes. Hence, CTAB/methylene blue agar plate method and orcinol assay were performed for the identification of rhamnolipid producing actinomycetes [19, 20, 30].

TABLE 2: DETECTION OF TYPE OF BIOSURFACTANT PRODUCED BY ISOLATES

Name of the isolate	Tests		
	Phenol-H_2SO_4 test	Biuret test	Phosphate test
PLS-1	+	-	-
NDYS-4	+	-	-
DMS-1	+	-	-
Control	-	-	-

Phenol-H_2SO_4 test (+): orange color to yellowish orange; Biuret test (+): violet or pink ring; Phosphate test (+): yellow precipitate.

The results for the orcinol assay and methylene blue agar plate method are shown in **Table 3**. From these results, it was confirmed that PLS-1 and NDYS-4 isolates are potential producers of glycolipids and the type of glycolipids are rhamnolipid. Therefore, these two isolates were selected for quantification of rhamnolipid and evaluation of their bio-surfactant activity. The quantification was done by orcinol assay taking L-rhamnose as standard for rhamnolipids, and the evaluation was done by surface-tension measurement and emulsification index.

TABLE 3: IDENTIFICATION TESTS FOR RHAMNOLIPID PRODUCING ACTINOMYCETES

Isolates	Tests	
	Methylene blue agar plate method (a)	Orcinol assay (b)
PLS-1	+	+
NDYS-4	+	+
DMS-1	-	-
SLS	+	+
CONTROL	-	-

(a) (+): dark blue colour halos
(b) (+): yellowish orange colour

Evaluation of Bio-Surfactant Activity: In this study, bio-surfactant activity was evaluated by measuring the surface tension by stalagmometer, and the emulsification index [13, 14]. These tests were performed with cell-free supernatant of the isolates PLS-1 and NDYS-4, un-inoculated medium (control), and 10% sodium lauryl sulfate (SLS), taken as standard [24]. The results are shown in **Fig. 1.**

From the above results, it was concluded that the isolates PLS-1 and NDYS-4 are potential bio-surfactant producers.

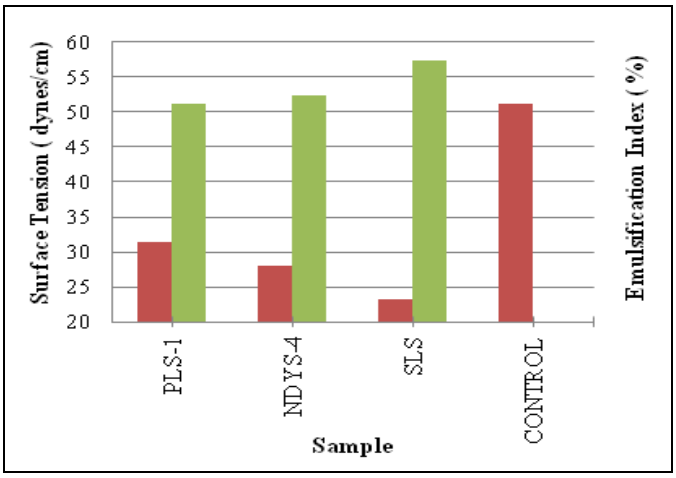

FIG. 1: EVALUATION OF BIOSURFACTANT ACTIVITY

Quantification of Rhamnolipid Content by Orcinol Assay: According to Chandrasekharan and Bemillar, total Rhamnolipid quantity was estimated by measuring the concentration of rhamnose, released by hydrolysis of Rhamnolipid on treatment with orcinol and concentrated H_2SO_4. PLS-1 and NDYS-4 were cultured in Kim's medium for 7 days for Rhamnose production. After incubation, cell-free supernatant was acidified and extracted with ethyl acetate. The extracted organic layer was dried. For this dried residue, Orcinol assay was performed to estimate rhamnose content [20, 27]. The results are shown in **Table 4**.

TABLE 4: ORCINOL ASSAY RESULTS OF PLS-1, NDYS-4

Sample	Optical density at 421nm	
	Control	Absorbance
PLS-1	0.000	0.454
NDYS-4	0.000	0.421

The amount of Rhamnolipid produced was obtained by interpreting the standard graph of L-rhamnose (Himedia). The standard graph was obtained by performing Orcinol assay for various concentrations of L-rhamnose shown in **Fig. 2.** The experiment was performed in triplicate. From the results of the obtained graph, the quantity of rhamnolipid produced was interpreted. From this, the amount of rhamnolipid produced by PLS-1 and NDYS-4 isolates was found to be 216 µg/ml, and 200 µg/ml respectively.

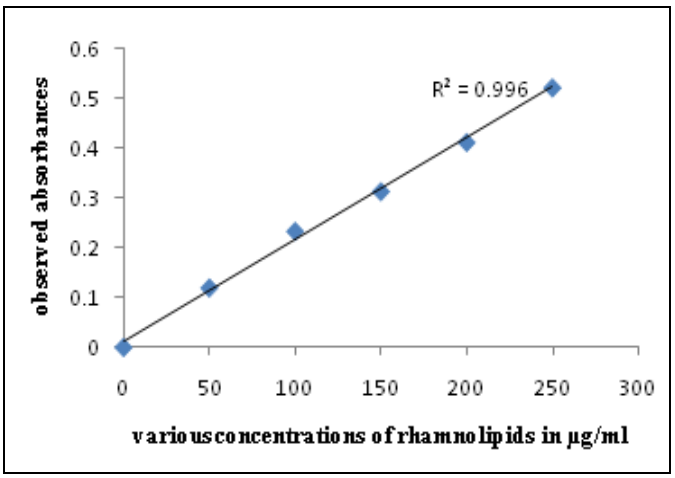

FIG. 2: STANDARD GRAPH OF L-RHAMNOSE

Time Course of Bio-Surfactant Production and Antimicrobial Activity: The bio-surfactant production was dependent on the growth of culture in the fermentation medium at about 3rd day of growth. The surfactant concentration started to increase, reaching its maximum after the about 7th day. As antimicrobial activity depends on the concentration of Rhamnolipid, maximum activity was exhibited on the 7th day.

The decrease in antimicrobial activity after the 8th day of incubation showed that the biosynthesis of the bio-surfactant was stopped, and was probably due to the production of secondary metabolites. This indicated that the bio-surfactant production occurred predominantly in an exponential growth phase, suggesting that the bio-surfactant produced was primary metabolite from the results shown in **Fig. 3** and **Fig. 4** [21, 22, 25].

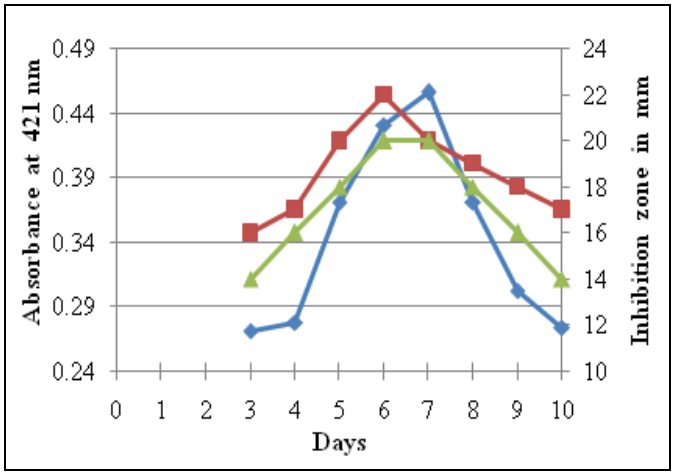

FIG. 3: BIOSURFACTANT PRODUCTION AND ANTIMICROBIAL ACTIVITY OF PLS-1

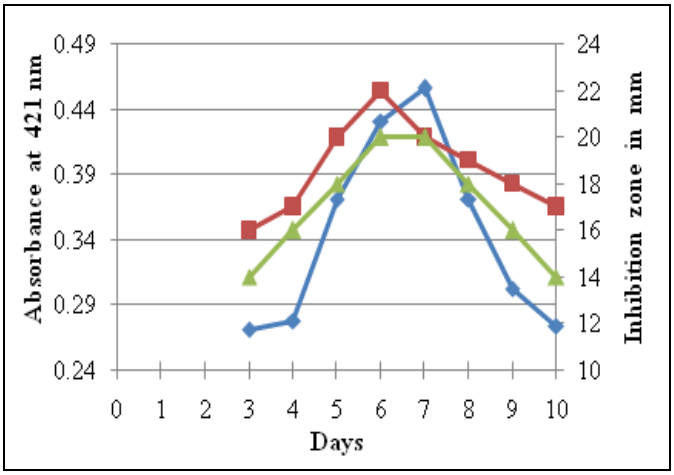

FIG. 4: BIOSURFACTANT PRODUCTION AND ANTIMICROBIAL ACTIVITY OF NDYS-4

CONCLUSION: The present study was undertaken to isolate the bio-surfactant producing microorganisms from three different ecosystems, namely, poultry soil, deep marine soil, and oil contaminated soil. The importance of these bio-surfactants for industrial use is shown by their physical properties like significant emulsification activity and surface tension. The functional or chemical characterization of the isolated bio-surfactants indicated that the bio-surfactants produced were rhamnolipid in nature, and belonged to glycolipids class. The isolated bio-surfactants had shown significant antibacterial activity. The antimicrobial spectrum of the compounds indicated very good activity against Gram positive and Gram-negative bacteria. This suggests that further studies may show the possibility for the potential use as an alternative to chemical surfactants for bioremediation of spills in oil-contaminated terrestrial environments and for the potential use of the compound as an alternative to the antibacterial agent. This was further purified by column chromatography to obtain pure rhamnolipid. These two isolates were sent to Institute of Microbial Technology, Chandigarh, India (IMTECH), for biochemical tests and 16S rDNA sequencing for the identification of the isolated strains.

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CONFLICT OF INTEREST: Nil

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