



## Isolation, Identification and Antimicrobial activity of Bio-surfactant from *Streptomyces matensis* (PLS-1)

Kalyani A.L.T\*, Naga Sireesha G, Girija Sankar G, Prabhakar T

AU College of Pharmaceutical Sciences, Pharmaceutical Biotechnology Division, Andhra University, Visakhapatnam, India.

\*Corresponding author's E-mail: [kalyani.lakshmitripura@gmail.com](mailto:kalyani.lakshmitripura@gmail.com)

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### ABSTRACT

This is a study of the crude bio-surfactant produced by a *Streptomyces matensis* PLS-1 isolate from a poultry littered soil near Vishakhapatnam. For this study, fifteen isolates of soil actinomycetes are initially screened in humic acid-salts vitamin agar plates. These isolates are then grown in Kim's medium, containing olive oil as the sole source of carbon, for extracellular bio-surfactant activity. All these isolates are primarily screened for their bio-surfactant activity by oil-displacement method. Out of these fifteen, only four isolates namely, PLS-1, PLS-2, PLS-4, PLS-9, showed promising bio-surfactant activity compared to the standard, sodium laryl sulphate. These four promising isolates are further screened for their bio-surfactant activity by using various methods like parafilm-M method, lipase activity, surface tension measurement (Drop count method), and emulsification index. Among these four promising isolates, one isolate, from the poultry-littered soil (PLS-1), showed maximum bio-surfactant activity for the above tests and also showed positive result for various methods like, Phenol H<sub>2</sub>SO<sub>4</sub> and Blue Agar plate method. This indicates that the bio-surfactant is Rhamnolipid in nature. This result is further confirmed by orcinol assay, taking L-rhamnose as standard. It is concluded that actinomycetes isolate PLS-1 has potential as source of new antibacterial compounds against pathogenic microorganisms (unicellular Gram positive and Gram negative bacteria and drug resistance microorganism) in human-beings.

**Keywords:** Actinomycetes, Antimicrobial activity, Bio-surfactants, Kim's medium, Orcinol assay, Rhamnolipid.

### INTRODUCTION

Oil pollution and remediation technology has become a Global phenomenon of increasing importance. Most of the hydrocarbons are insoluble in water and their degradation using microorganisms has an important role in combating environmental pollution. These bio-surfactants are extracellular products excreted by microbial cells grown on certain hydrocarbons, although it may be possible to produce them from other substrates such as carbohydrates.<sup>1</sup>

The main characteristic of these microbial cultures is their ability to excrete relatively large amount of surface active substances that emulsify or wet the hydrocarbon phase, thus making them available for absorption.<sup>2</sup> Bio-surfactant producing microbes are present not only in oil contaminated soils, but also in undistributed soils like poultry soil, which is rich in organic matter suitable for the growth of diverse organisms. This soil is rich in bacteria, fungi and actinomycetes. Hydrocarbon degrading micro-organisms produce bio-surfactants of different chemical nature and molecular sizes, which are surface active compounds, increase the surface tension of the hydrophobic water-insoluble substrates, and thereby enhancing their bioavailability and the rate of bioremediation.<sup>3</sup>

At present, almost all chemically synthesised surfactants are derived from petroleum. These synthetic surfactants are toxic and can be hardly degraded by microorganisms. Thus, these synthetic surfactants are a potential source of pollution and cause damage to the environment. Such

hazards associated with synthetic surfactants have, in recent years, drawn much attention to bio-surfactants.<sup>4</sup>

Bio-surfactants derived from micro-organisms have attracted much attention because of their advantageous characteristics such as structural diversity, low toxicity, higher biodegradability, better environmental compatibility, higher substrate selectivity, and lower CMC. These properties have led to several bio-surfactant applications in the food, cosmetic and pharmaceutical industries.<sup>5</sup>

Most commonly isolated bio-surfactants are glycolipids and lipopeptides. They include rhamnolipid, released by *Pseudomonas aeruginosa*<sup>6</sup>, glycolipids from *Rhodococcus erythropolis*, and *Rhodococcus aurantiacus*, and surface active lipid from *Nocardia erythropolis* strains.<sup>21</sup> Thus, based on their chemical composition, the bio-surfactants produced by microorganisms are of many types such as glycolipids, lipopolysaccharides, oligosaccharides, and lipopeptides.<sup>5,7</sup>

Bio-surfactants have many applications in environmental sector, like bio-remediation, soil washing, and soil flushing. Bio-surfactants influence these processes because of their efficacy as dispersion and remediation agents, environment friendly characteristics such as low toxicity and high bio-degradability. Although bio-surfactants exhibit such important advantages, they have not yet been employed extensively in industry because of relatively high production costs.<sup>8</sup>

Against these backdrops, this study is aimed at isolation and screening of microorganisms from poultry litter soils, that produce bio-surfactants and their identification methods.

## MATERIALS AND METHODS

### Isolation of bio-surfactant producing actinomycetes

#### Selection of soil samples

The soil sample was collected in sterile plastic bags from poultry near Sujatha nagar in Visakhapatnam. This soil sample was found to be rich in fats and oils, and hence was used for the screening of bio-surfactant producing organisms.

#### Selective isolation of actinomycetes

The collected soil sample was air dried at room temperature for one week and pre-treated at 55°C in a hot air oven for 3h, then stored at room temperature in sterile bags. This sample was labelled as PLS. One gram of soil sample was serially diluted and 100µl aliquot of appropriate dilution was applied to humic acid-Salts-Vitamin agar plates<sup>9</sup>, with pH adjusted to 7.0. Humic acid was synthesised in our laboratory by using modified Essington method.<sup>10</sup> These plates are then supplemented with 50µg/ml of rifampicin and cycloheximide each, incubated at 28°C for 7days for the growth of actinomycetes colonies. After 7days of incubation, actinomycetes colonies were preliminarily selected based on colony morphology, and, a small portion of the colony was streaked on starch-casein agar medium.

#### Screening for bio-surfactant activity

Seven day old actinomycetes were inoculated into 500ml Erlenmeyer flasks containing 100ml Kim's medium<sup>11</sup>, with 3% olive oil as the sole carbon source. The broth cultures were incubated at 30±2°C on a reciprocal shaker at 120 rpm for 5 days. The culture broth was then tested for the production of extracellular bio-surfactants.

#### Oil displacement method

Forty ml of distilled water was added to a petridish (15cm diameter), followed by the addition of 10µl of crude oil to the surface of the water, 10µl of sample was added onto the centre of this oil film. The diameter of the oil displaced zone on the surface were measured and compared with the zones produced by an uninoculated medium as negative control, and sodium lauryl sulphate as positive control. All these tests were carried out in triplicates.<sup>12</sup>

#### Lipase Activity

The isolates that produce lipase were screened using tributyrin agar plates. 1% tributyrin was added to YEME medium. The pH of the medium was adjusted to 7.2. A loopful of inoculum was streaked on to each of the tributyrin agar plates. These plates were then incubated at 28°C for 7 days. After incubation, these plates were

examined for the formation of clear zone around the colonies.<sup>12</sup>

#### Para film M test

Ten µl of isolate supernatants, mixed with 1% bromothymol blue, was added to the hydro-phobic surface of the parafilm M. The shape of this drop on the surface was inspected after 1 min. The diameters of these droplets are evaluated. Sodium lauryl sulphate and distilled water were used as positive and negative controls, respectively.<sup>13</sup>

#### Evaluation of bio-surfactants

Evaluation of bio-surfactants was carried out by measuring the changes in surface tension and stabilization of the emulsion.

#### Surface tension measurement

The surface tension of the liquid was measured with a stalagmometer. The surface tension was determined on the basis of the number of drops that fell per volume, the density of the sample and the surface tension of the reference liquid, e.g., water. The surface tension is given by<sup>14</sup>;

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

Whereas  $\sigma_L$  is the surface tension of the liquid under investigation,  $\sigma_W$  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,  $\rho_L$  is the density of the liquid and  $\rho_W$  is the density of water.

#### Emulsification index

The emulsifying capacity was evaluated by emulsification index (E24).<sup>15</sup> The E24 of culture samples was determined by adding 6ml of kerosene, 4ml water and 1ml of the cell-free broth in centrifuge tube, vortexed at high speed for 2 min and allowed to stand for 24h. The E24 index is given as percentage of the height of 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.

$$E24 = \frac{\text{Height of emulsion formed} \times 100}{\text{Total height of solution}}$$

#### Preliminary Identification Tests

##### Phenol: H<sub>2</sub>SO<sub>4</sub> Method

To 1ml of supernatant, 1ml of 5% phenol was added. To this mixture, 2-5 ml of conc. H<sub>2</sub>SO<sub>4</sub> was added drop by drop, until orange colour was developed. The development of orange colour indicated the presence of glycolipids containing biosurfactant.<sup>16</sup>

##### Biuret Test

This test was used to detect the lipopeptide containing bio-surfactant. 2ml of supernatant was heated at 70°C, and then was mixed with 1ml of 1M NaOH solution. Drops



of 1% CuSO<sub>4</sub> were added slowly until violet or pink ring was observed. Formation of violet or pink ring indicates the presence of lipopeptides containing biosurfactant.<sup>17</sup>

### Phosphate Test

Up to ten drops of 6M HNO<sub>3</sub> was added to 2ml of supernatant, and was heated at 70°C. 5%(w/v) ammonium molybdate was added to this mixture, drop by drop, slowly until the formation of yellow colour, and then the yellow precipitate. This indicates the presence of Phospholipids containing biosurfactant.<sup>18</sup>

### Conformation tests for rhamnolipid

#### CTAB/Methylene Blue agar plate method

Blue agar plates composing of mineral salts-agar-medium, supplemented with 2% carbon sources, N-Cetyl-N,N,N-trimethyl-ammonium-bromide (CTAB) 0.5mg/ml, methylene blue 0.2mg/ml were used to detect the extracellular glycolipids production. Dark blue colour zones indicate the Rhamnolipid bio-surfactant production.<sup>15,19</sup>

#### Orcinol Assay

This method was used for direct assessment of amount of rhamnolipid in the sample. 400µl of cell-free supernatant was taken. Its P<sup>H</sup> was adjusted to 2 by adding 2N HCl. Addition of HCl results in separation of rhamnolipid. To this, 750µl of diethyl ether was added, so that Rhamnolipid was extracted into an organic layer. Solvent addition and extraction were repeated twice. Ether fractions were dried by evaporation. To the left out precipitate 400µl of P<sup>H</sup> 8 phosphate buffer was added. 2.7ml of orcinol was added to 300µl of sample (left out ether fractions with phosphate buffer). The sample tubes were boiled for 20min. The solutions were left in dark for 35min to cool to room temperature. Optical density was measured at 421nm against blank. The Rhamnolipid concentrations were calculated from standard graph taking L-rhamnose as standard.<sup>20</sup>

### Time course of bio-surfactant production

The time course of bio-surfactant 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.<sup>21</sup> The resultant cell-free supernatant was removed by filtration, followed by cold centrifugation at 10,000 rpm at 4°C for 20min. The supernatant was analyzed for bio-surfactant production by orcinol assay.

### 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 1ml of 1M phosphate buffer (pH 7.0). Then 50µl of this extract was loaded into each well in solidified-agar-medium, previously inoculated with 25µl of test organism against which, the activity has to be determined. The plates were incubated at 37°C for 18-24hrs and were

examined. The diameter of the zones of inhibition was measured.<sup>22</sup>

### Time course of antibiotic production

The experiment was designed for 10 days starting from the log phase to stationary phase under submerged culture conditions. The resultant cell-free supernatant was removed by filtration, followed by cold centrifugation at 10,000 rpm at 4°C for 20min. The supernatant was analyzed for antibiotic production by cup-Plate method.<sup>23</sup>

### Identification of Strain PLS-1

Identification of strain was done by 16S r-DNA sequencing, biochemical and cultural characterizations.<sup>24,25</sup>

## RESULTS AND DISCUSSION

### Isolation and Screening of Biosurfactant Producing Actinomycetes

A total of 15 strains of actinomycetes were isolated from poultry litter soil named PLS. All these isolates were initially screened for extracellular bio-surfactant production grown in Kim's medium containing olive oil as sole source of carbon.<sup>11</sup> Out of these 15, only four isolates of PLS, namely, PLS-1, PLS-2, PLS-4, PLS-9, showed positive result for drop displacement test<sup>12</sup>. As per the results observed for these four isolates, the drop displaced zone for PLS-1 showed maximum displaced area with a diameter of 7.5 cm, indicating a good bio-surfactant production. These results are shown in the Table 1. Again, these four isolates were tested for bio-surfactant production by parafilm-M test and lipase production test. Only PLS-1 showed the flat drop in the parafilm-M test and a clear zone in lipase production test.<sup>12,13</sup> Figure 1 shows the oil spreading activity, parafilm-M test, and lipase production of PLS-1 isolate. Therefore, this isolate was used for physical and chemical characterization of the bio-surfactant.

### Evaluation of bio-surfactant activity

Bio-surfactant activity of PLS was evaluated by measuring the surface tension by stalagmometer and emulsification index. The emulsification of these isolates were detected from the first day of incubation period and showed that the highest of emulsion was formed at 60 hrs by PLS-1 compared with the standard sodium laryl sulphate.<sup>26</sup> These results are shown in Figure 2.

### Preliminary identification of bio-surfactant

Glycolipids, lipopeptides, phospholipids are three types of bio-surfactants. In order to identify the type of bio-surfactant, Phenol-H<sub>2</sub>SO<sub>4</sub> test (glycolipids), Biuret test (lipopeptides), and phosphate test (phosphate lipids) were performed with cell-free supernatant of PLS. Phenol-H<sub>2</sub>SO<sub>4</sub> test was conducted as per the method described by Dubois M et al, in which, PLS-1 isolate had shown orange colour when phenol and concentrated sulphuric acid were added.<sup>16</sup> The results for this test are shown in Figure 3(a). Biuret reagent should turn to violet

or pink ring due to the reaction of peptide bond proteins. However, a negative result was obtained in this study, i.e. colour did not change to violet.<sup>17</sup> For phosphate test also, yellow colour precipitate was not observed in this study.<sup>18</sup> These results were shown in Table 2.

**Conformation tests for rhamnolipid**

In the present study, CTAB/methylene blue agar, a semi-quantitative assay was done for the detection of anionic bio-surfactants or extracellular glycolipids. PLS-1 has shown positive result forming dark blue colour zones around the cups.<sup>15</sup> Orcinol assay is a quantitative assay for the conformation of rhamnolipid. This test was conducted as per Chandrashekar et al, and only PLS-1 isolate has shown Rhamnolipid production.<sup>20</sup>

The results for Orcinol assay and methylene blue agar plate method are shown in Figure 3(b), (c). It was confirmed that PLS-1 isolate is a potential producer of

glycolipids. The type of glycolipids was found to be Rhamnolipid in nature from the results shown in Table 3. Therefore, this isolate was selected for the quantification of Rhamnolipid by taking L-Rhamnose as standard.

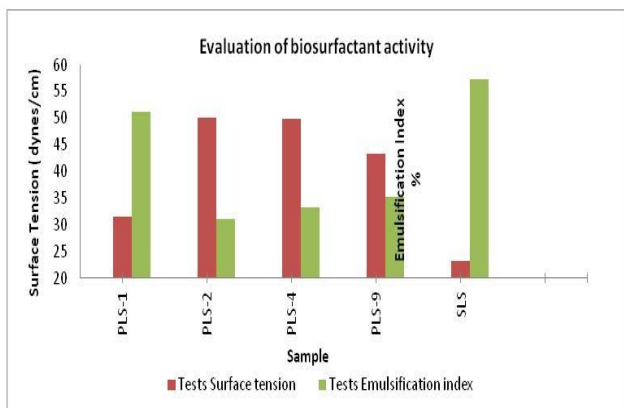
**Table 1:** Detection of bio-surfactant producing actinomycetes

Test	Control	PLS-1	PLS-2	PLS-4	PLS-9
Oil displacement test	-	7.5cm	0.5cm	1cm	4cm
Parafilm-M test	-	++	-	-	-
Lipase test	-	++	-	-	-

1. Diameter of oil displaced zone. 2.(+) spreading of drop, (-):dome shape. 3.(+) clear zone around the streak,(-): no zone



**Figure 1:** Preliminary Identification tests for Biosurfactant Producing Actinomycetes

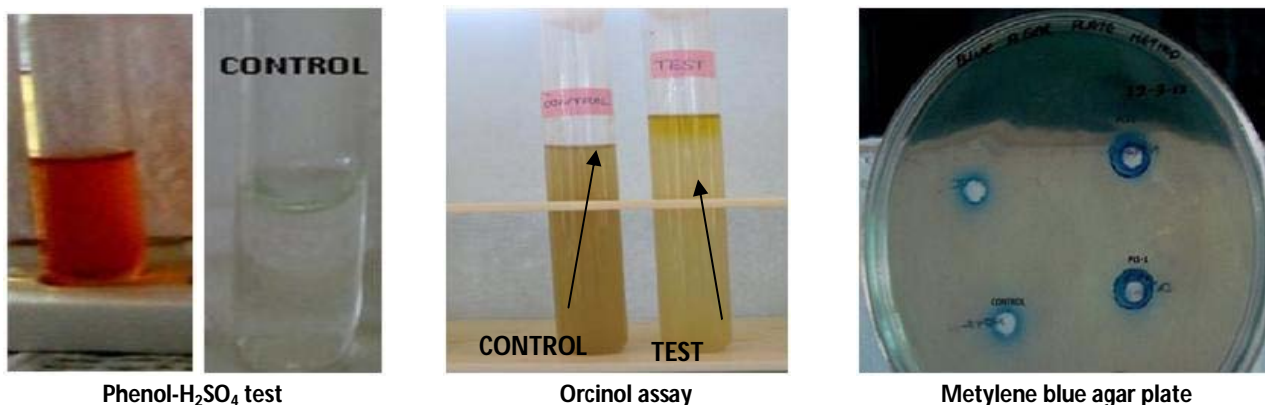


**Figure 2:** Evaluation of bio-surfactant activity

**Table 2:** Detection of type of bio-surfactant produced by isolates

Name of the isolate	Tests		
	Phenol-H <sub>2</sub> SO <sub>4</sub> test	Biuret test	Phosphate test
PLS-1	+	-	-
PLS-2	-	-	-
PLS-4	-	-	-
PLS-9	-	-	-

Phenol-H<sub>2</sub>SO<sub>4</sub> test (+): orange colour to yellowish orange. (-): yellow, Biuret test (+): violet or pink ring (-): blue, Phosphate test (+): yellow precipitate, (-): colour less

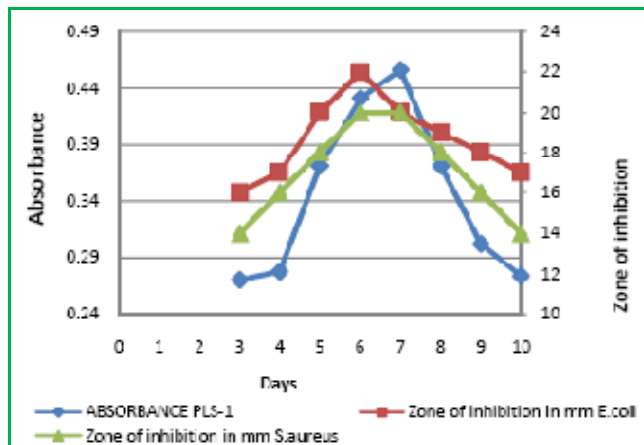


**Figure 3:** Specific Identification tests for type of Biosurfactant

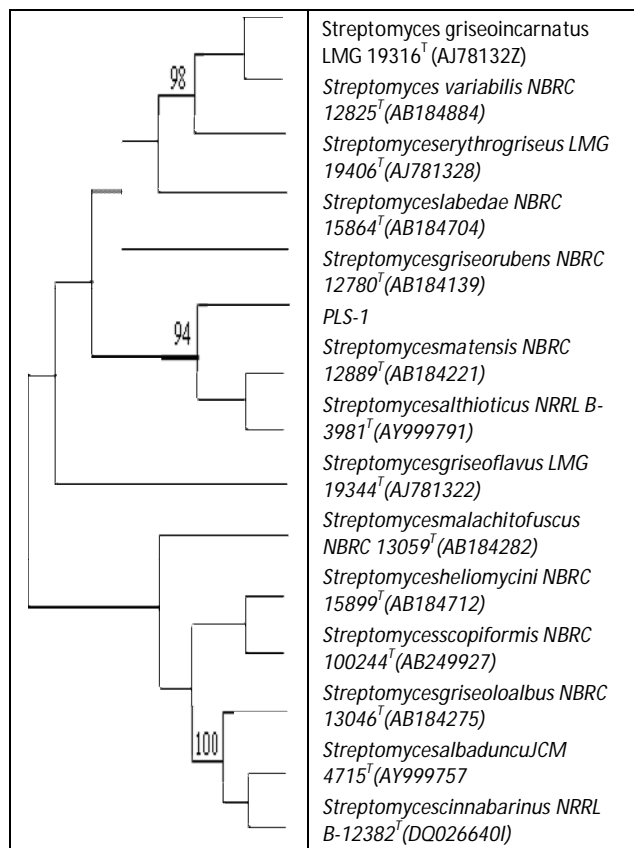
**Table 3:** Identification tests for rhamnolipid producing actinomycetes

Isolates	Tests	
	Methylene blue agar plate method	Orcinol assay
PLS-1	+	+
PLS-2	-	-
PLS-4	-	-
PLS-9	-	-
CONTROL	-	-

(+): yellowish orange colour; (-): dark blue colour halos



**Figure 4:** Bio-surfactant Production and Antimicrobial Activity of PLS-1



**Figure 5:** Neighbour-joining Phylogenetic Tree of strain PLS-1 made by IMTECH.

Numbers at nodes indicate levels of bootstrap support (%) based on neighbour-joining analysis of 1000 resample datasets; only values more than 50% are given. IMTECH accession numbers of given in parentheses.

**Antimicrobial Activity**

The isolates of actinomycetes were tested for microbial sensitivity against Gram positive and Gram negative bacterial strains by cup plate method. Bio-surfactant isolated from PLS-1, by acid precipitation method showed antimicrobial activity against *E.coli* and *S.aureus*, significant zones of inhibition were observed.<sup>22</sup>

**Time course of bio-surfactant production and Antimicrobial activity**

The bio-surfactant production was dependent on growth of culture in the fermentation medium at about 3<sup>rd</sup> day of growth surfactant concentration started to increase, reaching its maximum after about 7<sup>th</sup> day. As antimicrobial activity depends on the concentration of rhamnolipid maximum activity was exhibited on 7<sup>th</sup> day. The decrease in antimicrobial activity after 8<sup>th</sup> day of incubation showed that bio-surfactant biosynthesis stopped and is probably due to the production of secondary metabolites. This indicates that bio-surfactant production occurred predominantly in exponential growth phase, suggesting that bio-surfactant is produced as primary metabolite from the results shown in Figure 4.<sup>27</sup>

**Characterization of Strain PLS-1**

The strain PLS-1 showed good growth in Kim’s medium using olive oil as sole source of carbon at temperature range 30±2°C in 7 days. Outer surface of colonies was perfectly round initially, but later they developed thin wavy mycelium. The aerial and substrate mycelia mass colour is white. By morphology and 16S rDNA sequencing Figure 5, the isolated strain was found to be *Streptomyces matensis*.<sup>24</sup>

**CONCLUSION**

In the present study *Streptomyces matensis* PLS-1, isolated from poultry soil showed significant bio-surfactant activity. The importance of this bio-surfactant for industrial use is shown by their physical properties like high emulsification index and reduced surface tension. The functional characterization of isolated bio-surfactant indicated that the bio-surfactant produced was rhamnolipid, which belongs to glycolipids class. The isolated bio-surfactant showed 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 alternative to chemical surfactants in industries and for the potential use of the compound as an alternative to antibacterial agents.

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