

# ISOLATION AND ANTIMICROBIAL ACTIVITY OF RHAMNOLIPID (BIOSURFACTANT) FROM OIL-CONTAMINATED SOIL SAMPLE USING HUMIC-ACID SALTS-VITAMIN AGAR

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## Abstract

In this study, actinomycetes were isolated from the soil sample contaminated with petroleum oil in Naval Dockyard near Visakhapatnam. The isolate was identified as *Streptomyces coelicoflavus* comprising 16S rDNA sequence. Ten isolates of soil actinomycetes were initially screened in humic acid-salts vitamin agar plates. Soil organic matter is generally rich in humic acid and fluvic acid. Humic acid is an important ingredient for the extraction of biosurfactant producing actinomycetes. In the present study, humic acid substances used for screening of biosurfactant producing Actinomycetes were extracted by using soil organic matter. Humic substances were extracted by treating this organic soil with alkalis like dilute sodium hydroxide. The ten isolates were further grown in Kim's medium, containing olive oil as the sole source of carbon, for extracellular biosurfactant activity by oil spreading method. Out of these ten, only three isolates namely, NDYS-1, NDYS-3, NDYS-4, showed promising biosurfactant activity compared to the standard, sodium laryl sulphate. These three promising isolates were further screened for their biosurfactant activity by using Para film-M method, Emulsification index and Phenol H<sub>2</sub>SO<sub>4</sub>. Out of these three, only NDYS-4 showed maximum biosurfactant activity for the above tests. The result showed that the extracellular surface active agent produced by the isolate NDYS-4 was Rhamnolipid in nature, and was further confirmed by orcinol assay taking L-rhamnose as standard. Then crude extract obtained from this isolate was used in contact bioautography for antibacterial activity. It was observed that as the antibacterial activity increased, the biosurfactant activity also increased and vice versa.

**Keywords:** Humic acid, Fluvic acid, Organic matter, Biosurfactant, Bioautography

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## 1. INTRODUCTION

Humic substances are considered as the most important constituents of soils. These are formed by the decomposition of plant and animal residues by microorganisms. The humic substances play an important role in increasing biosurfactant productivity and soil fertility. In organic soils, this humic substance forms a large part of the total reserve of organic matter up to 80 - 90 % [1, 2].

Biosurfactants are a chemically diverse group of surface-active molecules produced by various groups of microorganisms that may belong to yeast, bacteria, filamentous fungi, plants and animals [3, 4]. Surfactants manufactured by the chemical industry pose environmental problems because of their toxicity and resistance to

degradation [5]. Currently, there is interest in replacing these chemical surfactants with biological surfactants [5, 6].

Biosurfactants have potential advantages over chemical surfactants, because of their low toxicity, high biodegradability, high selectivity and specific activity, and availability of its raw material where biosurfactant can be produced from low-cost raw materials, which are available in large quantity [5-8].

Biosurfactants are categorized mainly by their chemical composition and their microbial origin [9]. In general, biosurfactants are classified into lipopeptides, lipoproteins, glycolipids, polymeric fattyacids, phospholipids, neutral lipids and particulate biosurfactants [10].

Glycolipid biosurfactants are the most promising, owing to their high productivity from renewable resources and versatile biochemical properties. Among the glycolipids, the best known rhamnolipids are trehalolipids and sophorolipids [11].

Rhamnolipids have several potential industrial and environmental applications including the production of fine chemicals, the characterization of surfaces and surface coatings and as additives [12]. They can be efficiently used in handling industrial emulsions, biodegradation and detoxification of industrial effluents, control of oil spills, and in bioremediation of contaminated soils [13-15].

Pharmaceutically, antibiotic resistance is a serious and widespread problem i.e., hydrophobic antibiotics are those cells which do not take them up. Rhamnolipids are one of the main biosurfactants, tested for the uptake of hydrophobic antibiotics in-vivo to enhance their activity [16, 17].

The objective of this study was to isolate soil actinomycetes from oil contaminated soil in naval dock yard to screen for extracellular biosurfactant activity and their identification methods.

## 2. MATERIALS AND METHODS

### 2.1 Isolation of Biosurfactant Producing Actinomycetes

#### 2.1.1 Selection of Soil Samples

The soil sample was collected in sterile plastic bags from Naval Dockyard in Visakhapatnam. Soil mixed with petrochemicals was taken for screening of biosurfactant producing microorganisms.

#### 2.1.2 Selective Isolation of Biosurfactant Producing Actinomycetes

Collected soil sample was air dried at room temperature for 1 week, then was treated at 55 °C in a hot air oven for 3h, and then was stored at room temperature in sterile bags. The sample was labelled as NDYS. About one gram of soil sample was weighed and transferred to 50ml of sterile water, and then was placed on rotary shaker at 120 rpm for 30min. After 30min, serial dilutions were made from these solutions up to 10<sup>-10</sup> order by taking 1ml and diluting it to 10ml with distilled water.

Humic acid supplemented actinomycetes selective medium was used for specially obtaining biosurfactant producing actinomycetes [18]. 100µl aliquots of the appropriate dilution were applied to Humic acid-Salts-Vitamin agar plates which the pH was adjusted to 7.0.

These plates were supplemented with Rifampicin (50µg/ml) and cycloheximide (50µg/ml) and were incubated at 28°C for 7 days for the growth of actinomycetes colonies. Humic acid-salts-vitamin agar medium was used to isolate specifically the biosurfactant producing actinomycetes [19].

After 7 days of incubation, actinomycetes colonies were preliminarily selected based on colony morphology and small portion of colony streaked on Bennet's agar medium with the help of sterile inoculating loop. These strains were preserved in refrigerator at 4°C for further identification studies.

### 2.1.3 Laboratory Synthesis of Humic Acid

50g of soil sample was taken in a conical flask, and was washed with 50 ml of 0.1M HCl. Humic acid from the soil sample was extracted by adding 100 ml of 0.5M NaOH and keeping this flask on an orbital shaker at 120 rpm for 2 h. Subsequently, the extracted solution was filtered and was adjusted to pH 1 by concentrated 6M HCl. As a result of the pH adjustment, a solid precipitate representing the crude humic acid fraction was produced, while the crude fulvic fraction remained in the filtrate. The obtained crude humic acid was dissolved in 0.5M NaOH and was reprecipitated by acidification using 6M HCl. The solid precipitate was redissolved in 0.5M NaOH to obtain the humic acid solution. This solution was dried in the hot air oven to convert it into humic acid crystals. These crystals were used in the preparation of Humic acid-salts-vitamin agar medium [20].

### 2.2 Screening for Biosurfactant Activity

Isolates were sub-cultured from the working stocks, and were incubated at 28 °C for 7 days. After incubation, the microbial growth from each slant was suspended in 2ml of sterile distilled water, and was transferred in to 50ml of Kim's medium in a 250ml Erlenmeyer flask [21]. This flask was then incubated on a rotary shaker for 7 days at 28 °C. After incubation, the content of the flask was centrifuged at 7000 rpm for 20min, and was filtered. This cell-free supernatant was used for experimentation in the following preliminary tests for identifying the presence of biosurfactant.

#### 2.2.1 Oil Spreading Method

10µl of crude oil was added to the surface of 40ml distilled water in a petri dish to form a thin layer of oil. Then 10µl of culture supernatant were gently placed on the centre of the oil layer. In few seconds, a clear zone was formed on this oil layer, which indicated the presence of biosurfactant in the supernatant. The diameter of this clearing zone on the oil layer correlates to the effectiveness of surfactant activity [22].

#### 2.2.2 Para Film-M test

One drop of Bromophenol blue indicator was added to 2ml of cell-free supernatant. 10µl of this sample was carefully placed like a drop on parafilm-M with a micropipette. The shape of

this drop on the surface was inspected after 1min. Sodium lauryl sulfate and phosphate buffer (pH 7.0) were used as positive and negative controls respectively. If the drop becomes flat, it indicates the presence of biosurfactant. If it remains in dome shape, it indicates the absence of biosurfactant [23].

### 2.2.3 Emulsification Index

Emulsifying capacity of the biosurfactant was evaluated by emulsification index (E24). The E24 of the culture sample was determined by adding 6ml of kerosene, 4ml of water and 1ml of the cell-free broth in a test tube, and was 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 [24, 25].

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

### 2.2.4 Phenol-Sulphuric Acid Method

To 1ml of cell free supernatant, 1ml of 5% phenol was added. To this mixture, 2-5ml of concentrated sulphuric acid was added drop by drop, until characteristic colour was developed. Development of orange colour indicated the presence of glycolipids [26].

## 2.3 Conformation Tests for Rhamnolipid

### 2.3.1 Orcinol Assay

400µL of cell-free supernatant was taken and its pH was adjusted to 2 by adding 2N HCl to separate the Rhamnolipid. To this, 750µl of diethyl ether was added and mixed thoroughly to extract Rhamnolipid in to an organic layer. This organic layer was collected. Solvent addition and extraction were repeated twice. Ether fractions were pooled and dried by evaporation. 400µl of pH8 phosphate buffer was added to the remaining precipitate (this equals to 1x dilution). 2.7ml of orcinol was added to 300µl of sample.

300µl of phosphate buffer, taken as blank, was also treated with 2.7ml of orcinol reagent. Standard solutions of L-Rhamnose between 50-250µg/ml were prepared with phosphate buffer, and were treated with orcinol reagent. Samples, standards and blank were boiled for 20 min, and test solutions were left in dark for 35min to cool down to room temperature. Then, optical density was measured at 421nm. The rhamnolipid concentrations were calculated from standard curves prepared with L-rhamnose and were expressed as rhamnose equivalents [27, 28].

## 2.4 Time Course of Biosurfactant Production

The time course of biosurfactant production was followed in batch cultures at optimum conditions. This experiment was designed for 10 days starting from the log phase to stationary phase under submerged culture conditions [29]. The resultant cell-free supernatant was removed by filtration followed by cold centrifugation at 10,000 rpm at 4 °C for 20 min. The supernatant was analyzed for biosurfactant production by orcinol assay.

## 2.5 Extraction of Crude Biosurfactant

Biosurfactant was recovered from the filtrate by acid precipitation method [30]. To 5ml of cell free supernatant, 2N HCl was added to adjust its pH to 2, then was placed in refrigerator at 4°C for overnight so that Rhamnolipid is precipitated. Ethyl acetate was added to the filtrate in the ratio of 1:1 (v/v) and was shaken vigorously for 1 hour for complete extraction. The ethyl acetate phase that contains biosurfactant was separated from the aqueous phase.

It was evaporated to dryness and the residue was washed twice with ethyl acetate, later dried. Thus obtained compound was dissolved in phosphate buffer pH8 and used to determine antimicrobial activity and to perform bioautography for the screened isolate NDYS-4.

### 2.5.1 Bioautography

The crude extract was loaded on to pre-coated Thin Layer Chromatographic (TLC) plates with the capillary tubes and eluted using 50% ethyl acetate, 50% hexane as mobile solvent system. The developed plates were dried under a stream of fast moving air for an hour to remove traces of solvent on the plates. Bacterial (*S.aureus*, *E.coli*) suspension of 24h cultures grown on nutrient agar slants were freshly prepared in sterile distilled water. Then plating was done using inoculated nutrient agar.

After solidification, the air-dried TLC plates were placed onto the media with the help of sterilized forceps. These plates were kept in refrigerator for one hour for diffusion. After marking, TLC plates were removed. This whole process was carried out aseptically in a Laminar flow cabinet. Thereafter, these plates were incubated overnight at 37°C for bacterial growth. A control was also kept using solvent alone. The inhibition zone diameters were measured and noted [31].

## 2.6 Identification of the Strain NDYS-4

The different characteristics of the new isolate or unidentified organism were determined in adequate detail, for its identification and classification. The major characteristics which were determined are as follows:

### 2.6.1 Macroscopic Examination

The isolates were grown on 20ml of Benett's agar medium by pouring on a petri plate, and were incubated at 28°C for 7 days, and were visually observed for the evaluation of colony morphology, such as colony size, shape, surface texture, arrangement of cell masses and margin.

### 2.6.2 Microscopic Examination

The presence of spores, structure of hyphae, features of aerial and substrate mycelium were determined by microscopic observation. The smear of isolate was prepared by adding 0.5ml of culture suspension to thin smear of YEME medium on a sterile Petri plate. The culture was spread gently over the solidified medium and was incubated at 28°C for 7 days. Morphological examination was then carried out at 100X magnification using Transmission Electron Microscope [32].

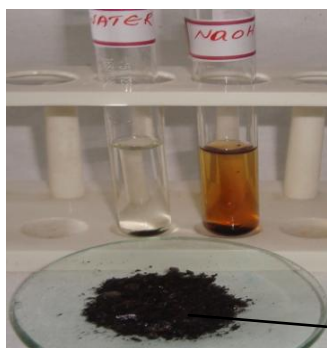
### 2.6.3 Culture Characteristics

The growth characteristics on different media, nutrients, physical conditions required for growth, were examined. Proper identification and characterization of microorganisms is very important because it expands the scope for exploitation of industrially important products. The isolate NDYS-4 was further identified by 16S r-DNA sequencing, biochemical and culture characterizations [33].

## 3. RESULTS AND DISCUSSION

### 3.1 Extraction of Humic Acid

As per modified Essington method, black coloured shiny crystals were extracted from organic soil. Humic acid is soluble in sodium hydroxide and insoluble in water. The extracted crystals have passed this test, as shown in **Fig-1**, and were conformed as humic acid crystals. These humic acid crystals were used for screening of biosurfactant producing actinomycetes.



**Fig-1:** Identification test for humic acid and extracted humic acid crystals.

### 3.2 Isolation and Screening of Biosurfactant

#### Producing Actinomycetes

A total of 10 strains of actinomycetes were isolated from oil contaminated naval dockyard soil named NDYS. All these isolates were initially screened for extracellular biosurfactant production grown on Kim's medium containing olive oil as sole source of carbon [21]. Out of these 10, only three isolates of NDYS, namely, NDYS-1, NDYS-3 and NDYS-4, had shown positive results for Oil spreading method [22]. As per the results observed for these three isolates, the drop displaced zone for NDYS-4 had shown maximum displaced area with a diameter of 8.5 cm, indicating a good biosurfactant production. Again, these three isolates were tested for biosurfactant production by parafilm-M test, where a flat drop was shown by NDYS-4 taking sodium laryl sulphate as positive control and distilled water as negative control [23].

The biosurfactant activity of NDYS-1, NDYS-3 and NDYS-4, were evaluated by Emulsification index. The emulsification of these isolates were detected from the first day of incubation period, had shown that the highest of emulsion was formed at 72h by NDYS-4, compared with the standard sodium laryl sulphate [34]. In order to identify the type of biosurfactant, phenol-H<sub>2</sub>SO<sub>4</sub> test was performed with cell-free supernatant of NDYS. According to the method described by Dubois M *et al*, NDYS-4 isolate had shown orange colour when phenol and sulphuric acid were added. The orange colour indicated that the biosurfactant obtained was glycolipid in nature [26]. These results are shown in **Table-1**.

### 3.3 Conformation Assay for Rhamnolipid

In the present study, Orcinol assay was performed for the conformation of Rhamnolipid. According to Chandrasekher *et al*, this test was conducted, and only NDYS-4 isolate had shown Rhamnolipid production [27]. This had confirmed that NDYS-4 isolate is a potential producer of glycolipids. The types of glycolipid were found to be Rhamnolipid in nature from the results shown in **Table-1**. Therefore, this isolate NDYS-4 was selected for quantification of Rhamnolipid by taking L-Rhamnase as standard.

**Table-1:** Detection of biosurfactant producing actinomycetes

S. No	Test	Control	NDYS-1	NDYS-3	NDYS-4	SLS
1	Drop spreading test	-	2.5cm	4cm	8.5cm	10cm
2	Parafilm-M test	-	+	+	+++	+++
3	Emulsification index	-	20.21%	23.47%	52.43%	57.31%
4	Phenol H <sub>2</sub> SO <sub>4</sub> test	-	-	-	+	-

(1) Diameter of zone of oil displaced in 'cm' (2) (+) spreading of drop, (-): dome shape  
 (3) Emulsification index in '%' (4) Phenol-H<sub>2</sub>SO<sub>4</sub> test (+): orange colour to yellowish orange

### 3.4 Bioautography

By performing the bioautography for cell-free supernatant of NDYS-4, the bioactive compound was found to be antibiotic. Significant inhibition zones were observed against *Escherichia coli*, as shown in **Fig-2**.

### 3.5 Antimicrobial Activity

The isolates of actinomycetes were tested for microbial sensitivity against Gram positive and Gram negative bacterial strains by Cup plate method. The biosurfactant isolated from NDYS-4 by acid precipitation method, had shown significant activity against *E.coli* and *S.aureus*, and significant zones of inhibition were observed [31].



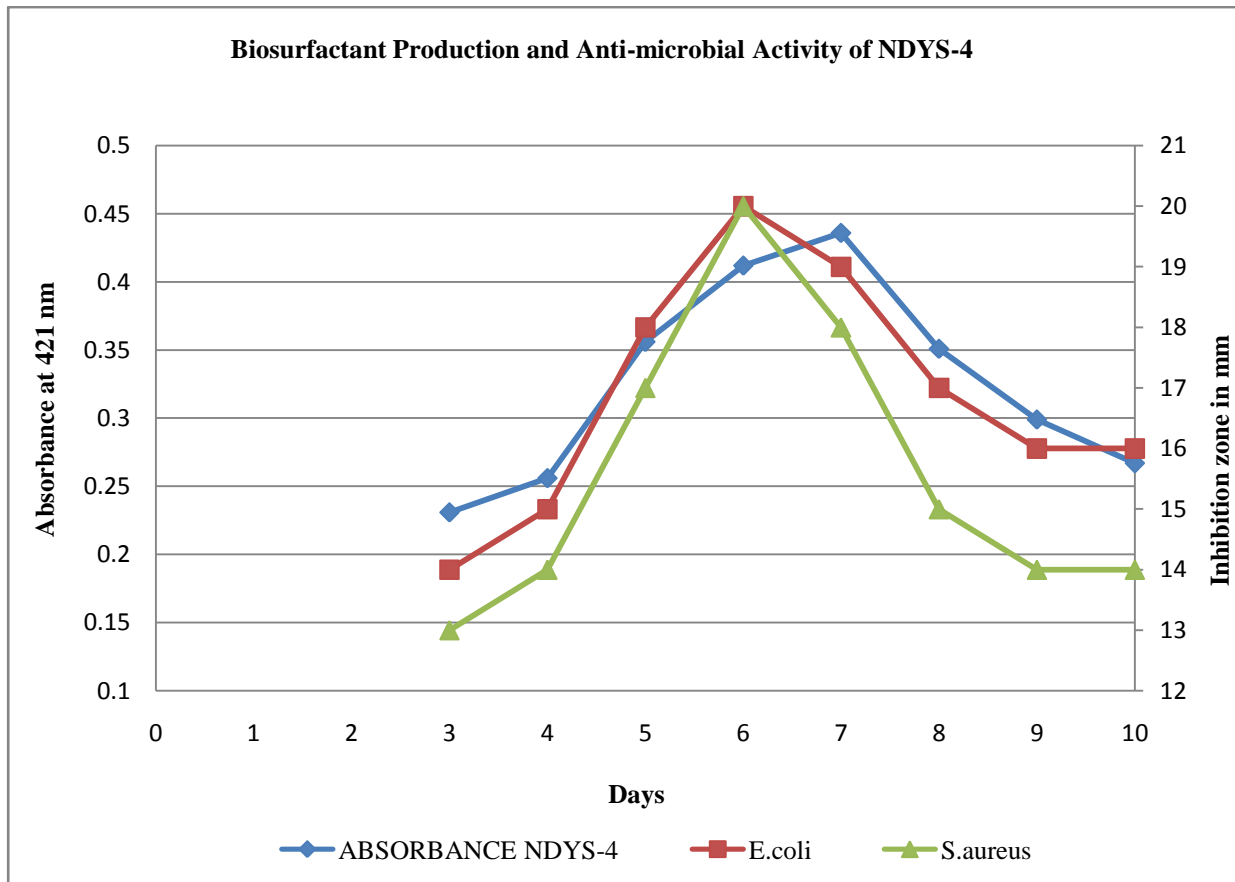
**Fig-2** Bioautography of NDYS-4 against bacterial test organism

### 3.6 Time Course of Biosurfactant Production and Antimicrobial Activity

The biosurfactant 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 observed on 7<sup>th</sup> day.

The decrease in antimicrobial activity after 8<sup>th</sup> day of incubation had shown that the biosurfactant biosynthesis had stopped, and was probably due to the production of secondary metabolites. This indicated that the biosurfactant production had occurred predominantly in the exponential growth phase, suggesting that the biosurfactant produced was primary metabolite as shown in **Fig.3** [29].



**Fig-3** Biosurfactant Production and Antimicrobial Activity of NDYS-4

### 3.7 Characterization of Strain NDYS-4

The strain NDYS-4 had shown promising growth in Kim's medium using olive oil as sole source of carbon at temperature range of 28 °C for 7 days. Outer surface of colonies were perfectly round initially, but later developed thin wavy mycelium. The colour of the aerial mycelium observed was white and the colour of the substrate mycelium was pink. By studying the morphology and 16S rDNA sequencing (Fig-4), the isolated strain was found to be *Streptomyces coelicoflavus*.

The screened isolate of NDYS-4 was sent to IMTECH, Chandigarh, INDIA, for the analysis of the Phylogenetic tree and nomenclature of the strain, as shown in Fig-4. In this figure, the numbers at the nodes indicate the levels of bootstrap support (%), based on neighbour-joining analysis of 1000 resample datasets, and values more than 50% only are given. IMTECH accession numbers are given in parentheses.

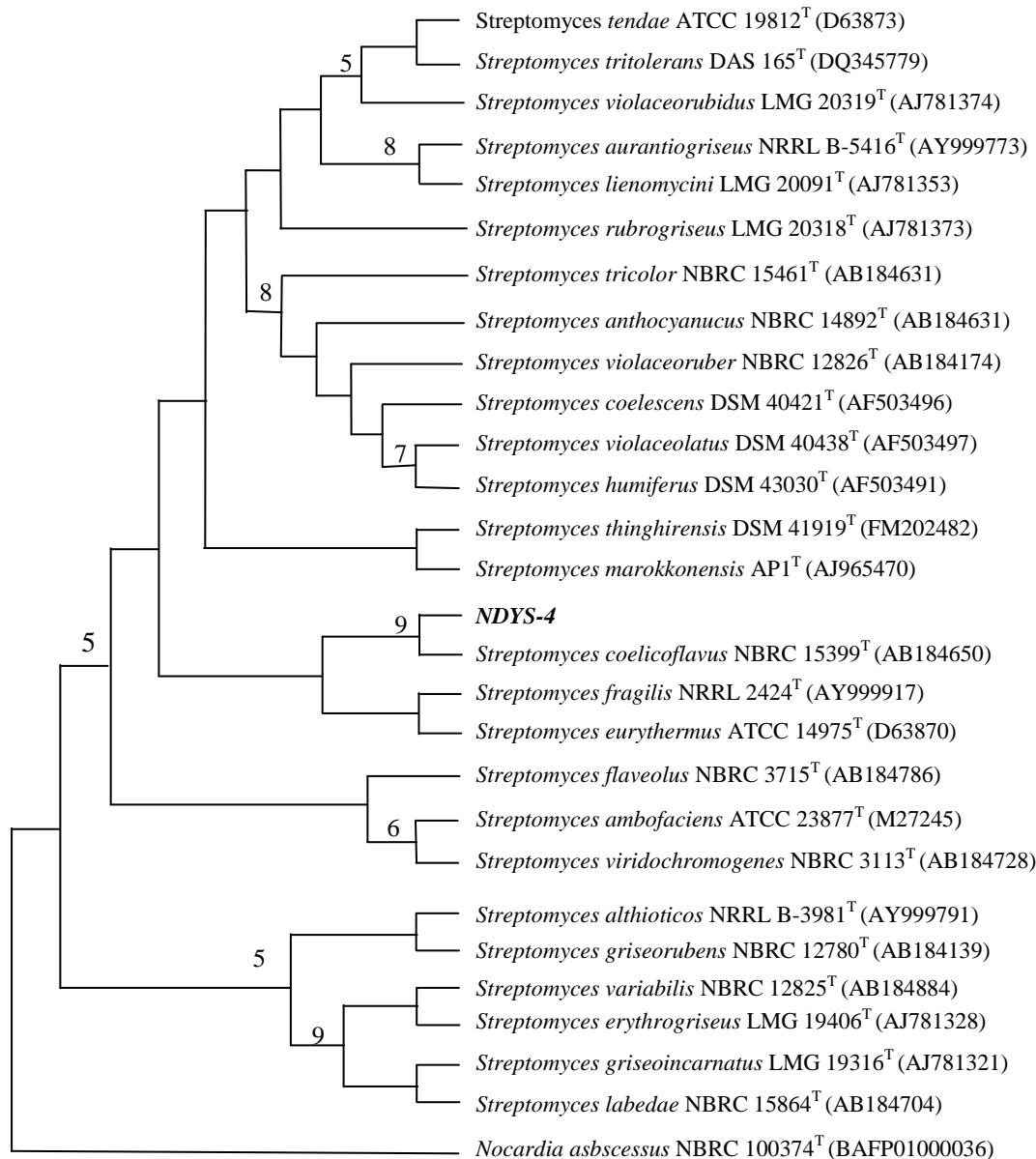
## 4. CONCLUSIONS

In the present study, *Streptomyces coelicoflavus* isolated from oil contaminated Naval dockyard soil, had shown significant biosurfactant activity. The importance of this biosurfactant for

industrial use was proven by its physical properties like emulsification. The functional characterization of this isolated biosurfactant had indicated that the biosurfactant produced was Rhamnolipid in nature, which belongs to glycolipids class. The isolated biosurfactant had shown significant antibacterial activity.

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**Fig-4:** Neighbour-joining Phylogenetic Tree of strain NDYS-4 made by IMTECH, Chandigarh, India.

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