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Research Article

# ISOLATION AND SCREENING OF ANTIBIOTIC PRODUCING ACTINOMYCETES FROM GARDEN SOIL OF SATHYABAMA UNIVERSITY, CHENNAI

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**ABSTRACT**

**Objectives:** To isolate and screen actinomycetes with antibacterial and antifungal activity from garden soil samples of Sathyabama University, Chennai.

**Methods:** Six soil samples were collected, serially diluted and plated on starch casein agar supplemented with nalidixic acid and cyclohexamide for inhibition of bacteria and fungi, respectively. Primary screening of actinomycetes isolates was done by following cross streak method against test organisms. Submerged fermentation was followed for the production of crude antibiotics. Agar well diffusion method was done to determine the antimicrobial activity of the crude extract. The minimum inhibitory concentrations (MIC) also quantified for the crude extract by microtiter plate assay. The promising isolate was characterized by conventional methods.

**Results:** On primary screening, 13 out of 22 actinomycete isolates (59%) showed potential antimicrobial activity against one or more test bacteria and/or fungus. The isolate BN8 shows antagonistic activity against all the tested bacteria and fungi, isolates BN5 and BN16 were active against only bacteria not fungi, and isolate BN2 was active against all tested fungi. The zone of inhibition was measured by using the crude extracts of all the four isolates. The crude extract produced by isolate BN8 showed zone on inhibition against all the tested bacterium in 100 µg/ml against *Pseudomonas aeruginosa* (22 mm), *Klebsiella pneumonia* (25 mm), *Bacillus cereus* (20 mm), *Staphylococcus aureus* (22 mm), *Escherichia coli* (15 mm), *Aspergillus flavus* (14 mm), *Aspergillus niger* (20 mm), *Aspergillus fumigatus* (10 mm), respectively. The crude extracts of isolates BN2, BN5, and BN16 were not exhibited any zone of inhibition against the test microbes on agar well diffusion assay. The MIC of the crude extract against *P. aeruginosa* (50 µg/ml), *S. aureus* (25 µg/ml), *K. pneumonia* (25 µg/ml) and *B. cereus* (25 µg/ml) *and E. coli* was 12.5 µg/ml. The MIC of 12.5 µg/ml was observed for the crude extract against *A. flavus*, 25 µg/ml against *A. niger*, and 50 µg/ml against *A. fumigatus,* respectively.

**Conclusion:** This groundwork concluded that the soil samples of Sathyabama University have actinomycetes with metabolites inhibits bacterial and fungal pathogens. One of the promising isolates was identified which active against the test bacteria and fungi. The crude metabolite exhibits a very large zone of inhibition when compared to the control antibiotic. The type of antimicrobial agents produced by the isolate and the phylogenic relationship of the isolate has to be investigated as well.

**Keywords:** Actinomycetes, Isolation, Screening, Antimicrobial activity, Cross streak method.

## INTRODUCTION

It is indisputable that new antibiotics are urgently needed to halt and reverse the relentless spread of antibiotic resistant pathogens which cause life-threatening infections and risk undermining the viability of health care systems [1]. The screening of microbial natural products continues to represent an important route to the discovery of valuable chemicals, for the development of new therapeutic agents and for evaluates of the potential of lesser known and/or new bacterial taxa are of increasing interest [2]. It has been estimated that roughly two-third of the thousands of naturally occurring antibiotics have been isolated from actinomycetes [3].

Actinomycetes are a prolific source of structurally diverse secondary metabolites; many of these possess pharmaceutically relevant biological activities [4]. Around 23,000 bioactive secondary metabolites from microorganisms have been reported, and over 10,000 of these are from actinomycetes, constitute 45% of all bioactive microbial metabolites. So, the searching for novel actinomycete constitutes an essential component of natural product-based drug discovery is appreciably in recent years.

According to the World Health Organization, over-prescription and improper use of antibiotics have led to the resistance of many pathogens. Clinically-important bacteria, such as *Staphylococcus aureus*, are becoming resistant to commonly used antibiotics. Now-a-day, new resistant strains are emerging more quickly while the rate of discovery of new antibiotics is slowing down. Because of this, many scientists have focused on screening programs of microorganisms, primarily of actinomycetes, for their production of antibiotics [5].

Antibiotic producing actinomycetes have not been well-investigated [6]. The majority of these microorganisms in the soil that are potential drug sources remain unfruitful, and for that reason inaccessible for novel antibiotic discovery. This study is expected to become an important component in the production of new natural bioactive products. The study was undertaken to isolate and screen garden soil derived actinomycetes for antibiotic activity.

## METHODS

### Sample collection and isolation of actinomycetes

A total of six soil samples were collected aseptically from the rhizosphere of six different fig plants in the Garden of Sathyabama University, Chennai. The collected samples were transferred to the Microbiology laboratory, Department of Biotechnology, Sathyabama University, Chennai, where the entire work was carried out. In the laboratory, the samples were air dried for 3 days. After the 3 days of pre-treatment, all the samples were pooled together and used for isolation of actinomycetes. 1 g of soil sample was added to a test tube containing 9 ml of sterile water and mixed well. From this, 1 ml of aliquot was again transferred and mixed with another 9 ml of sterile water to make $10^{-2}$ dilution factor. Similarly, dilutions up to $10^{-5}$ were made using serial dilution technique. A volume of 1 ml of suspension from $10^{-1}$ to $10^{-5}$ serially diluted tubes were taken and spread evenly with sterile L-shaped glass rod over the surface of sterile starch casein agar plates

aseptically using spread plate technique. Nalidixic acid 20 µg/ml and cyclohexamide 25 µg/ml were added in the media to inhibit bacterial and fungal. All the plates were incubated at 30°C for 7-14 days. The plates were often observed for the growth of actinomycetes. The based on colony morphology, the different colonies, were chosen, isolated and maintained in International *Streptomyces* Project (ISP-2) agar slants at 4°C for subsequent studies.

**Bacterial and fungal strains**

Test bacterial strains such as *S. aureus* National Collection of Industrial Microorganisms (NCIM) 5021, *Pseudomonas aeruginosa* NCIM 2242, *Escherichia coli* NCIM 2065, *Klebsiella pneumonia* NCIM 2957, *Bacillus cereus* NCIM 2063 and fungal test strains, such as *Aspergillus niger* NCIM 620, *Aspergillus flavus* NCIM 1028, *Aspergillus fumigatus* NCIM 902, were obtained from the NCIM, National Chemical Laboratory, Pune, India.

**Primary screening**

All isolated actinomycetes were screened for their antimicrobial activity. The test bacteria used for primary screening were *E. coli*, *S. aureus*, *P. aeruginosa*, *K. pneumonia, and B. cereus*. Antifungal activity of actinomycetes was determined using *A. niger*, *A. flavus, A. fumigatus* the test organisms. Activities were assessed using modified nutrient agar (India) for bacteria and for fungi. Each plate was streaked with each isolate at the center of a plate and incubated at 37°C for 7 days. Then, fresh subcultured test organisms were streaked perpendicular to the actinomycete isolate [7,8], and the plates were incubated for 24 hrs at 37°C for bacteria and 48 hrs at 28°C for fungi. After incubation, the suppressed growth of the test organisms on the streaked line indicates the isolate were an antibiotic producer, and the full growth of the test organisms on the streaked line indicates the isolate were negative for antibiotic production.

**Fermentation and extraction of crude extracts**

Based on the zone of inhibition in primary screening, isolates (designated as BN2, BN5, BN8, and BN16) that have potential antimicrobial activity were selected for secondary screening. The crude bioactive compound was produced by submerged fermentation followed by solvent extraction, and then the antimicrobial activity of the crude extracts was assessed following agar well diffusion methods [9].

The promising cultures of actinomycete isolates were grown in ISP-2 broth (500 ml) at 37°C for 7 days. To concentrate the antimicrobial metabolite produced from BN2, BN5, BN8, and BN 16 isolates, first the cell mass was separated by centrifugation at 4000 rpm followed by 50% of ethyl acetate (250 ml) was added in fermented broth cultures and shaken vigorously for 20 minutes and kept stand to get a clear solvent phase and aqueous phase. The clear solvent phase was separated and allowed to evaporation to get concentrated crude extract.

**Secondary screening**

*Agar well diffusion assay*

The ethyl acetate crude extract obtained from the selected isolates were dissolved in 5% dimethyl sulfoxide (DMSO) (1 mg/ml) and used as a stock solution to determine the antimicrobial activity against test fungi and bacteria using 1% DMSO as a control. The wells (6 mm diameter) were cut using a sterile corl porer on Muller Hinton agar. 24 hrs young culture of *E. coli, S. aureus, P. aeruginosa, K. pneumonia, and B. cereus*. Anti-fungal activity of actinomycetes was determined using *A. niger*, *A. flavus, A. fumigatus* were swabbed with sterilized cotton swab on the surface of prepared Muller Hinton agar for bacteria and fungi. 100 µl of the dissolved crude extract was loaded onto each well and left for 30 minutes until the metabolite was diffused. Then the plates were incubated for 24 hrs at 37°C for bacterial and 48 hrs at 28°C for fungi. After incubation, the zone of inhibitions was measured and recorded. The assay was conducted in duplicate, and mean values are reported (Table 1).

**Table 1: Agar well diffusion assay of ethyl acetate extract of potential isolates against test organisms**

| Crude extract and standard antibiotics | Zone of inhibition (mm) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | PA | KP | BC | SA | EC | AF | AFU | AN |
| BN2 | 0±0 | 0±0 | 0±0 | 0±0 | 0±0 | 0±0 | 0±0 | 0±0 |
| BN5 | 0±0 | 0±0 | 0±0 | 0±0 | 0±0 | 0±0 | 0±0 | 0±0 |
| BN8 | 22±1 | 25±2 | 20±1 | 22±1 | 15±1 | 14±1 | 10±1 | 20±2 |
| BN16 | 0±0 | 0±0 | 0±0 | 0±0 | 0±0 | 0±0 | 0±0 | 0±0 |
| AB solution | 20±2 | 22±1 | 15±1 | 20±1 | 18±1 | 30±1 | 10±1 | 10±2 |
| 1% DMSO | 0±0 | 0±0 | 0±0 | 0±0 | 0±0 | 0±0 | 0±0 | 0±0 |

AB solution: Antibiotic antimyctotic solution containing amphotericin B (25 µg), 10,000 units of penicillin, 10 mg streptomycin×100 liquid.
PA: *Pseudomonas aeruginosa*, KP: *Klebsiella pneumonia*, BC: *Bacillus cereus*, SA: *Staphylococcus aureus*, EC: *Escherichia coli*, AF: *Aspergillus flavus*, AFU: *Aspergillus fumigatus*, AN: *Aspergillus niger*, DMSO: Dimethyl sulfoxide

**Determination of minimum inhibitory concentrations (MIC) for the crude extract**

*Microplate assay*

The MIC values, which represent the lowest concentration that completely inhibits the growth of microorganisms, were determined by following micro well dilution method [10]. The sterile 96-well microplate was used for the assay (0.5 ml volume, Fisher Scientific). 2 mg of crude extract was dissolved in small amount of 2 ml of DMSO. Stock solutions before dilution were no more than 5% DMSO so that final concentrations in the microwells were typically <1% DMSO and solvent controls were run at these concentrations. Samples were diluted to twice the desired initial test concentration with nutrient broth for bacteria and Sabouraud's dextrose agar (SDA) broth for fungi. All wells, except the first, were filled with respective broth (50 µl). Test sample (100 µl) was added to the first well, and serial two-fold dilutions were made down to the desired minimum concentration. 1-day-old cultures of bacteria grown in nutrient broth and fungi were in the SDA broth until the turbidity was equal to a 0.5 McFarland Standard. The plates were inoculated with the bacterial and fungal suspension (5 µl per well) and incubated at 37°C overnight. After that, 40 µl of 3-(4, 5-dimethyl-thiazol-2-yl)- 2,5-diphenyl-tetrazolium bromide (MTT) at a final concentration 0.5 mg/ml freshly prepared in water was added to each well and incubated for 30 minutes. The change to dark purple indicated that the bacteria were biologically active. The MIC was equal to the concentration of crude added in the well, where no change of color of MTT was observed. The MIC assay was done in triplicate.

*Characterization of active isolate*

The most potent isolate BN8 was characterized by conventional methods includes morphological, biochemical, and physiological studies was carried out as described in the ISP [11].

**RESULTS**

**Sample collection and isolation of actinomycete**

A total of 22 morphologically different actinomycetes were isolated from six soil samples collected from the rhizosphere of six fig plants from the Garden of Sathyabama University, Chennai. All the 22 isolates were sub-cultured on ISP-2 media, and the cultural characters of all the isolates were recorded (Table 2) and were preserved on ISP-2 slants for further usage.

**Primary screening**

All isolates were screened for antimicrobial activity by cross streak method against five bacteria *S. aureus, K. pneumonia, E. coli, B. cereus, P. aeruginosa* and three fungi *A. niger*, *A. flavus, A. fumigatus.* The isolate designated as BN8 was active by suppressed the growth of all the tested bacteria and fungi; the isolate BN2 was active against only the three fungi tested. The isolate BN5 and BN16 were active

against all the tested bacteria, not fungi. The isolates BN6, BN12, BN14, BN19, BN20, and BN21 were suppressing the growth of two bacteria. The isolates BN1, BN9, and BN18 were active against one bacterium.

**Mass cultivation and crude extract preparation**

Based on the primary screening results the isolate BN8, BN2, BN5, and BN16 were selected for mass cultivation. The whole cultured broth (500 ml) of the selected actinomycetes isolates were extracted with ethyl acetate and the extract was evaporated to dryness. The crude extracts of isolates BN8, BN2 were yellow oily residue and for the isolate BN5, BN16 were brown.

**Secondary screening by agar well diffusion method**

The antimicrobial activity of the crude extract against microorganisms was examined, and their potency was qualitatively assessed by the presence or absence of inhibition zones and zone diameter (mm) by agar well diffusion method.

On agar well diffusion assay the crude extract of isolate BN8 showed zone of inhibition against all the tested bacterium in 100 µg/ml against *P. aeruginosa* (22 mm), *K. pneumonia* (25 mm), *B. cereus* (20 mm), *S. aureus* (22 mm), *E. coli* (15 mm), *A. flavus* (14 mm), *A. niger* (20 mm), *A. fumigatus* (10 mm), respectively. The crude extract of isolates BN2, BN5, and BN16 were not exhibited any significant microbial activity.

**Determination of MIC for the crude extract**

*Microplate assay*

The antimicrobial activities of the crude extract against microorganisms examined in the present study and their potency were quantitatively assessed by the MIC values. This crude extract displayed varied antibacterial and antifungal activities across the studied pathogens. The change to dark purple after the addition of MTT indicated that the bacteria were biologically active. The MIC was determined based on the change of color of MTT in the well.

The crude extract inhibited the growth of both bacterial and fungal strains with an MIC value of 50 µg/ml against *P. aeruginosa*, 25 µg/ml against *S. aureus, K. pneumonia,* and *B. cereus.* The MIC value of the crude extract against the bacterium *E. coli* was 12.5 µg/ml. The MIC of 12.5 µg/ml was observed in the crude extract against *A. flavus,* 25 µg/ml against *A. niger,* and 50 µg/ml against *A. fumigatus,* respectively.

**Conventional characterization of active isolate**

*Cultural characteristics*

The color of the aerial mycelium was observed on the 14th day of incubation. The aerial mycelium of the selected test strains was white, pale pink, white, pale yellow, pale pink, and white in ISP-1, ISP-2, ISP-5, ISP-6, starch casein agar, and actinomycetes isolation agar, respectively, and are represented in Table 3.

*Pigment production*

Selected isolates were grown in ISP-1, ISP-7 media to determine the melanoid pigment production. The selected isolate does not produce melanoid pigment on ISP-1 and ISP-7 (Table 3).

**Physiological and biochemical characters of the selected actinomycetes**

The active isolate was utilized lactose, D-xylose, L-maltose, sucrose, D-fructose, glycerol, and D-glucose (Table 4). To understand the native nature of the marine actinomycetes, sodium chloride tolerance test was carried out. Different concentrations of sodium chloride (0%, 2%, 5%, 7%, 10%, and 20%) solution were added to the starch casein agar medium. The presence or absence of growth was recorded on 7th day onward. The tested isolate was growing well at 7% of NaCl, and there is no growth above 7% of NaCl. This indicates that the isolate can tolerate only up to 7% of NaCl.

**Spore chain morphology**

The spore chain morphology of the selected isolates was observed under light microscopy and scanning electron microscopy and it is shown in Fig. 1. The spore chain of selected isolate was observed as flexuous, flattened, and smooth.

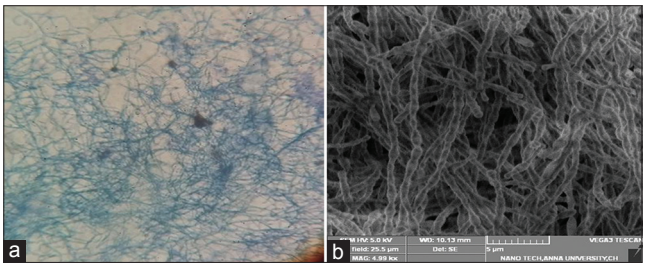


**Fig. 1: Spore chain morphology of the potent isolate BN8, (a) Light microscopic view (×40), (b) scanning electron microscopy image**

**Table 2: Cultural characters of actinomycetes isolates**

| S. No. | Isolate code | Colony morphology | Reverse side pigment | Soluble pigment | Melanoid pigment | Nature of growth |
|---|---|---|---|---|---|---|
| 1 | BN1 | Pinkish white | Orange | Red | - | +++ |
| 2 | BN2 | Pale yellow | Brown | - | - | +++ |
| 3 | BN3 | Pale yellow | Yellow | - | - | +++ |
| 4 | BN4 | Pink | Brown | Brown | - | +++ |
| 5 | BN5 | White | Black | Brown | + | +++ |
| 6 | BN6 | White | Yellow | - | - | +++ |
| 7 | BN7 | White | Pale yellow | - | + | ++ |
| 8 | BN8 | Pale pink | Orange | Pink | + | +++ |
| 9 | BN9 | Pale green | White | - | - | ++ |
| 10 | BN10 | White | Black | - | - | +++ |
| 11 | BN11 | White | White | - | - | ++ |
| 12 | BN12 | White | Brown | Dark brown | - | +++ |
| 13 | BN13 | White | Orange | - | - | +++ |
| 14 | BN14 | Gray | Light gray | Gray | - | ++ |
| 15 | BN15 | Light gray | White | - | - | +++ |
| 16 | BN16 | Gray | Orange | - | + | +++ |
| 17 | BN17 | Light gray | Pale yellow | Yellow | - | +++ |
| 18 | BN18 | Dark gray | Gray | Gray | - | +++ |
| 19 | BN19 | White | Pale yellow | - | - | +++ |
| 20 | BN20 | Gray | Black | - | - | ++ |
| 21 | BN21 | White | Nil | - | + | ++ |
| 22 | BN22 | Pinkish white | Pale yellow | - | - | +++ |

BN: Designation for each isolated actinomycetes

**Table 3: Cultural characteristics of selected isolate BN8**

| S. No | Medium used | Aerial mass color | Reverse side pigment | Soluble color | Melanoid pigment | Nature of growth |
|---|---|---|---|---|---|---|
| 1 | ISP-1 | White | Pale yellow | – | – | Good |
| 2 | ISP-2 | Pale pink | Orange | Pink | Brown | Good |
| 3 | ISP-5 | White | White | – | – | Slow |
| 4 | ISP-6 | Pale yellow | Orange | – | – | Slow |
| 5 | SIP-7 | – | – | – | – | No |
| 6 | SCA | Pale pink | Brown | – | – | Good |
| 7 | AIA | White | Olive green | – | – | Good |

–: Negative. ISP: International *Streptomyces* project, SCA: Starch casein agar, AIA: Actinomycetes isolation agar

**Table 4: Physiological and biochemical characters of the selected actinomycetes BN8**

| Characteristics | Isolate BN8 |
|---|---|
| Utilization of carbon sources | |
| Lactose | + |
| D-xylose | + |
| L-maltose | + |
| Sucrose | + |
| D-Fructose | + |
| Glycerol | + |
| D-glucose | + |
| NaCl (%) | |
| 0 | + |
| 2 | + |
| 5 | ++ |
| 7 | +++ |
| 10 | – |
| 20 | – |

## DISCUSSION

Antibiotics are the primary bioactive compounds for the cure of infectious diseases. Since the emergence of multidrug resistant pathogens, there are crucial challenges for successful healing of infectious diseases. Due to the burden for the high frequency of multidrug resistant pathogens in our surroundings, there has been increasing interest for searching effective antibiotics from soil actinomycetes in diversified ecological niches [12].

In the present study, soil samples were taken from the rhizosphere of fig plants in isolation of actinomycetes. For efficient isolation of actinomycetes from environmental samples requires an understanding of the potential soil sample areas and the environmental factors favors its growth. Previous studies showed that selection of different potential areas such as rhizosphere soil samples was an important activity in isolation of different types of potent antibiotic producing soil actinomycetes [13]. The present study of primary screening using single streak methods indicated that, 13 (59%) out of 22 actinomycete isolates showed potential antimicrobial activity against one or more test bacteria and/or fungus. This result (59%) is higher than 21.88% and equal to 59.09% from previous reports [12,14].

The presence of clear inhibition zones around the wells on the inoculated plates is a signal of the antimicrobial activities of antibiotics extracted from actinomycetes against test organisms. Kesavan and Selvam, [15] reported 5-25 mm inhibition zone of crude extracts against selected test organisms. For the present study, a range of inhibition zone was recorded for the crude extract of one isolate against test organisms were 10-20 mm. The inhibition zone of crude extracts from four selected isolates against test microorganisms ranged from 0 to 25 mm which was found to be good when compared to Yücel and Yamaç's results [16]. The results of the present study were interesting and encouraging because the crude extracts from the isolates may have promising antibiotics.

According to the present result, the used standard antibiotic antimyctotic solution containing amphotericin B (25 µg), 10,000 units of penicillin, 10 mg streptomycin ×100 exhibits a range of inhibition zone of against test microorganisms from 10±2 to 30±1 mm. The crude extracts had greater inhibition zone against five pathogenic microbes when compared to the zone of antibiotic solutions from the isolates. Therefore, the further purification process is significant to get pure antibiotic substance for the application of treatment of different pathogenic microorganisms. In the present study, the effect of the crude extracts of promising isolate BN8 has shown higher antimicrobial activity.

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