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*Full Length Research Paper*

# Screening of Actinomycete strains for the production of antifungal metabolites

A. Kavitha[1], M. Vijayalakshmi[1]*, P. Sudhakar[2] and G. Narasimha[3]

[1]Department of Botany and Microbiology, Acharya Nagarjuna University, Guntur – 522 510, A. P., India.
[2]Centre for Biotechnology, Acharya Nagarjuna University, Guntur – 522 510, A. P., India.
[3]Department of Virology, Sri Venkateswara University, Tirupati, A.P., India.

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**4 different actinomycete strains (A1, A2, A3 and A4) were isolated from the laterite soil samples of Guntur region. Growth pattern and antifungal profiles of the strains were evaluated against the test fungi such as *Aspergillus flavus*, *Aspergillus niger*, *Candida albicans* and *Fusarium oxysporum*. Based on the cultural, morphological and physiological characteristics, the strains A1, A2 and A4 were identified as the species of *Streptomyces* while the strain A3 was assigned to *Nocardia*. Among the 4 tested strains, *Streptomyces* sp. A1 showed strong antifungal activity which may provide a potent source for antifungal metabolites.**

**Key words:** Actinomycetes, *Streptomyces*, *Nocardia*, antimicrobial activity.

## INTRODUCTION

Soil microorganisms provide an excellent resource for the isolation and identification of therapeutically important products. Among them, Actinomycetes are an important group of filamentous, gram-positive bacteria producing antibiotics of agricultural and medicinal importance. *Streptomyces* spp. rank first and cover around 80% of total antibiotics (Berdy, 2005). Though there are number of antibiotics, the need to search for new and efficient antibiotic. Producing strains keeps rising due to the emergence of drug resistant pathogens (Wise, 2008). Particularly, the incidences of infections by opportunistic fungi are increasing, especially in patients whose immune systems are compromised by AIDS, cancer, diabetes, age and other causes. Many antifungal compounds have been identified, but safe and effective antifungal drugs have not yet been developed because of the high degree of similarity between fungi and mammalian cells (Berdy, 1989).

Therefore, amphotericin B, which was developed many years ago, is still widely used in treatment for deep-seated mycoses despite its serious side effects (Gallis et al., 1990). Azole group antifungal agents, miconazole, ketoconazole, fluconazole, and itraconazole are also used clinically. But these medicines have nephrotoxicity and hepatotoxicity and cause vomiting and impotence (Fukai et al., 2003). This has consequently resulted in a strong demand for potent drugs that have least side effects and the search for the new antifungal metabolites remains as a challenging task. Our ongoing search for new antifungal metabolites led to the isolation of 4 actinomycete strains from the laterite soils of Guntur region and the present study deals with the cultural, morphological, physiological and antifungal profiles of these 4 strains.

*Corresponding author. E-mail: muvvavl@yahoo.co.in. Tel.: 0863 – 2293189 Ext. 167 (O); 0863 – 2293378.

## MATERIAL AND METHODS

### Isolation

Soil samples collected from the laterite soils of Guntur region were pretreated with calcium carbonate and dried in hot air oven at 45°C for 1 h in order to reduce the incidence of bacteria and molds (El-Nakeeb and Lechevalier, 1963). Soil dilution plate technique was employed to isolate the actinomycete strains on different media such as asparagine-glycerol-salts, asparagine-glucose and starch casein salts agar media with pH adjusted to 7.2 and the plates were incubated at 30°C for 10 days. The actinomycete strains predominant on different media were picked out, purified and preserved on yeast extract-malt extract-dextrose (YMD) agar medium at 4°C (Williams and Cross, 1971).

**Cultural, physiological and biochemical characteristics**

Cultural characteristics such as color of aerial mycelium, color of substrate mycelium and pigmentation of the selected actinomycete strains were recorded on YMD agar medium (Shirling and Gottlieb, 1966). Micromorphology of the actinomycete strains were examined by slide culture method (Williams and Cross, 1971). The utilization pattern of carbon sources by the strains was carried out according to the methods of Gottlieb (1961) since it can be used as an aid for species determination (Pridham and Gottlieb, 1948). The ability of the strains to produce different enzymes was examined by using standard methods (Holding and Collee, 1971). Tolerance of the strains to NaCl concentration was also evaluated (Tresner et al., 1968). The actinomycete strains were tested for their ability to produce $H_2S$, indole and melanin pigments (Holding and Collee, 1971). In addition, the sensitivity of the strains to different antibiotics was determined by paper disc method (Cappuccino and Sherman, 2004).

**Production of antifungal metabolites**

The secondary metabolites produced by the actinomycete strains were extracted by the method of Ellaiah et al. (2005). Pure culture of the strains was transferred aseptically and individually into the seed medium (YMD broth). After 24 h of incubation, the seed culture at a rate of 10% was inoculated into the production medium of the same composition. The fermentation was carried out at 28 ± 2°C for 1 week under agitation at 250 rpm. At every 24 h interval, the flasks were harvested and the biomass was separated from the broth. The dry weight of the biomass was recorded and expressed in mg/100 ml. The culture filtrates were extracted twice with ethyl acetate and the pooled solvent extracts were evaporated to dryness under vacuum to yield a crude residue. For the extraction of secondary metabolites, similar protocol was followed for all the 4 strains. The residue was then dissolved in dimethyl sulphoxide (DMSO) and the extracts thus obtained were used for antifungal activity against the test fungi such as *Aspergillus flavus*, *A. niger*, *Candida albicans* and *Fusarium oxysporum* by using agar well diffusion method (Cappuccino and Sherman, 2004). Czapek-Dox agar medium was used for culturing the test fungi. The solvent extracts dissolved in DMSO at a concentration of 1000 µg/ml were added to each well using DMSO as a negative control. The plates were incubated at 37°C for 48 - 72 h and the diameter of the inhibition zones of the test fungi around each well was measured.

## RESULTS AND DISCUSSION

Our continuous search for new antifungal metabolites from the laterite soil samples of Guntur region led to the isolation of 4 predominant actinomycete strains *viz*., A1 and A3 on asparagine-glycerol-salts, A2 on asparagine-glucose and A4 on starch casein salts agar media by using the soil dilution plate technique. Cultural, physiological and biochemical characteristics of the 4 strains are recorded in Table 1. On YMD agar medium, the color of the aerial mycelium was light grey for A1, white for A2 and A3 and green for A4 while the color of substrate mycelium appeared brown for A1 and A4, light yellow for A2 and hyaline for A3. Light brown pigmentation was observed for A4 and the others are non-pigmented. Rizk et al. (2007) reported grey and white color series of Actinomycetes as the dominant forms in the soil as compared to yellow, red, violet and green ones. Different color series of Actinomycetes were recorded in soil (Ndonde and Semu, 2000) as well as in marine environments (Remya and Vijayakumar, 2008).

Sporophore morphology of the strains grown on YMD for 5 days showed rectus-flexibilis pattern in A2 and retinaculum apertum type in A1 and A4. Based on these characters, the three strains A1, A2 and A4 were assigned to the family of Streptomycetaceae and the genus *Streptomyces* (Pridham et al., 1958; Williams et al., 1983). The strain A3 exhibited well-developed vegetative hyphae with irregular branches penetrating the agar and bearing white aerial sparse hyphae. At a late stage of their growth, the filaments fragment into rod-shaped elements characteristic of the family Nocardiae and the genus *Nocardia* (Hoshino et al., 2004).

Kampfer et al. (1991) suggested the physiological tests as indispensable tools for classification and identification of actinomycetes. Strain A2 had the ability to secrete enzymes such as amylase, asparaginase, catalase, cellulase, nitrate reductase, pectinase, phosphatase and urease. Strains A1 and A4 also exhibited the production of all the enzymes tested except pectinase and phosphatase for A1 and asparaginase, pectinase and phosphatase for A4. Strain A3 could produce only nitrate reductase and urease enzymes. All the strains are unable to produce lipase, DNAse and RNAse. Carbohydrate utilization test plays a prominent role in the taxonomic characterization of actinomycete strains (Pridham and Gottlieb, 1948). Strains A1 and A4 efficiently utilized the carbon sources such as fructose, glucose, mannitol, sucrose and xylose. Maltose, rhamnose and sucrose were assimilated by the strain A3 as the good sources of carbon and energy while arabinose, fructose, inositol, rhamnose, glucose and xylose were well utilized by the strain A2.

In addition to cultural and physiological tests, biochemical characteristics of the 4 strains are recorded. The strains A1 and A2 exhibited positive response to citrate utilization, melanoid pigmentation on tyrosine agar, indole and $H_2S$ production while all these properties were found to be negative for the strain A3. Strain A4 showed only indole production and melanoid pigmentation on tyrosine agar. Except A4, all the strains displayed tolerance to crystal violet and none of them showed resistance to phenol (0.1%). Tolerance of the strains to NaCl concentration also serves as an important character for species identification. All the strains exhibited salt tolerance up to 7% except for the strain A4 which could tolerate only up to 5%. Hence, the strains could be placed in intermediate salt tolerance group as suggested by Tresner et al. (1968). The susceptibility of the 4 strains towards various antibiotics differed from one strain to another. The strains A1, A2, A3 and A4 exhibited sensitivity to all the antibiotics tested except ampicillin and tetracycline for A2 and streptomycin for A4. Goodfellow and Orchard (1974) reported the antibiotic sensitivity of some Nocardioform bacteria as one of the valuable criteria for their taxonomic differentiation. Shirling and Gottlieb (1966) also stated

**Table1.** Cultural, physiological and biochemical characteristics of the 4 actinomycete strains (A1, A2, A3 and A4).

| Characteristics | A1 | A2 | A3 | A4 |
|---|---|---|---|---|
| **Cultural characters of the strains on YMD medium** | | | | |
| Color of the aerial mycelium | Light grey | White | White | Green |
| Color of the substrate mycelium | Brown | Pale yellow | hyaline | Brown |
| Pigmentation | Nil | Nil | Nil | Light brown |
| Spore morphology | Rectinaculum -apertum | Rectus flexibilis | Fragmentation of hyphae into rods | Rectinaculum-apertum |
| Physiological characters | | | | |
| Amylase | + | + | - | + |
| Asparaginase | + | + | - | - |
| Catalase | + | + | + | + |
| Cellulase | + | + | - | + |
| Chitinase | + | + | - | - |
| DNAse | - | - | - | - |
| Lipase | - | - | - | - |
| Nitrate reductase | + | + | + | + |
| Pectinase | - | + | - | - |
| Phosphatase | - | + | - | - |
| Protease | + | + | - | + |
| RNAse | - | - | - | - |
| Urease | + | + | + | + |
| Utilization of carbon sources | | | | |
| Arabinose | Moderate | Good | Moderate | Moderate |
| Fructose | Good | Good | Moderate | Good |
| Galactose | Moderate | Moderate | Moderate | Moderate |
| Glucose | Good | Good | Moderate | Good |
| Glycerol | Good | Moderate | Poor | Moderate |
| Inositol | Moderate | Good | Moderate | Moderate |
| Lactose | Moderate | Moderate | Moderate | Moderate |
| Maltose | Moderate | Moderate | Good | Moderate |
| Mannitol | Good | Moderate | Moderate | Good |
| Sorbitol | Moderate | Moderate | Moderate | Moderate |
| Sucrose | Good | Moderate | Good | Good |
| Xylose | Good | Good | Moderate | Good |
| Biochemical characters | | | | |
| Indole production | + | + | - | + |
| Citrate utilization | + | + | - | - |
| $H_2S$ production | + | + | - | - |
| Melanin pigments | + | + | - | + |
| Tolerance to | | | | |
| Crystal Violet | + | + | + | - |
| Phenol (0.1%) | - | - | - | - |
| NaCl concentration | Up to 7% | Up to 7% | Up to 7% | Up to 5% |
| Susceptibility to antibiotics (µg/disc)* | | | | |
| Ampicillin (10) | S | R | S | S |
| Amikacin (30) | S | S | S | S |

**Table 1** Contd.

| | | | | |
|---|---|---|---|---|
| Nalidixic acid (30) | S | S | S | S |
| Rifampicin (5) | S | S | S | S |
| Streptomycin (10) | S | S | S | R |
| Tetracycline (30) | S | R | S | S |

+, Positive result; -, negative result; *, concentration of antibiotics; R, Resistant; S, Sensitive

**Table 2.** Antifungal spectra of the actinomycete strains A1, A2, A3 and A4.

| Age of the culture (h) | A1* | | | | A2 | | | | A3 | | | | A4 | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | CA** | AF | AN | FO | CA | AF | AN | FO | CA | AF | AN | FO | CA | AF | AN | FO |
| 24 | 6*** | - | - | 2 | - | - | - | - | 3 | - | - | - | 4 | - | 2 | 2 |
| 48 | 14 | 2 | 3 | 4 | 2 | - | - | - | 4 | - | - | - | 10 | 3 | 4 | 4.5 |
| 72 | 15 | 3 | 7 | 5 | 5 | - | 2 | - | 6 | 2 | 5 | 3 | 11 | 5 | 5 | 6 |
| 96 | 19 | 6 | 10 | 10 | 6 | 2 | 5 | 3 | 9 | 3 | 6 | 4 | 14 | 6 | 8 | 9 |
| 120 | 15 | 4 | 6 | 8 | 9 | 4 | 7 | 5 | 10 | 5 | 9 | 6 | 17 | 9 | 11 | 10 |
| 144 | 9 | 2 | 3 | 3 | 6 | 2 | 4 | 3 | 7 | 2 | 4 | 3 | 9 | 2 | 3 | 5 |
| 168 | - | - | - | - | - | - | - | - | 5 | - | 2 | - | 3 | - | - | - |

*A1 - *Streptomyces* sp., A2 - *Streptomyces* sp., A3 - *Nocardia* sp., A4 - *Streptomyces* sp.
** Test fungi: CA - *Candida albicans*, AF - *Aspergillus flavus*, AN - *A. niger*, FO - *Fusarium oxysporum*
***Diameter of the inhibition zone (mm)

that the actinomycetes strains tested for resistance to different antibiotics could be useful as a taxonomic aid.

## Growth pattern and antifungal profiles of the 4 Actinomycete strains

All the four strains A1, A2, A3 and A4 entered log phase after 24 h of incubation and continued up to 72 h in the same phase whereas the strains A3 and A4 exhibited a very slight increase in their growth up to 120 h. The length of the stationary phase for the strains A2, A3 and A4 ranged from 72 - 120 h while the strain A1 showed an extended stationary phase up to 144 h of incubation (Figure 1).

The metabolites collected from 24 hold culture of the *Streptomyces* sp. (A1) exhibited antifungal activity against *C. albicans* and *F. oxysporum* while they had no effect on other test fungi such as *A. niger* and *A. flavus* (Table 2). A gradual rise in the antifungal spectrum was observed with increasing age of the culture up to 4 days. Thereafter, a subsequent decline in its antifungal activity was noticed. Of all the test fungi, *C. albicans* was highly sensitive to the metabolites of the 4-day old culture of the strain.

Results regarding the antifungal spectrum of the ethyl acetate extracts of the *Streptomyces* sp. A2 and *Nocardia* sp.A3 are recorded in Table 2. The crude extracts obtained from 24 h old culture of the strains A2 and A3 had no activity on all the test fungi except for *C. albicans* by the strain A3. The metabolites of the strains A2 and A3 showed antifungal spectrum from 48 h onwards and the activity of the metabolites was maximum on 5$^{th}$ day in which *C. albicans* and *A. niger* exhibited high sensitivity.

The ethyl acetate extracts collected from 24 h old culture of the *Streptomyces* sp. A4 showed antifungal activity on the test fungi including *C. albicans*, *A. niger* and *F. oxysporum*. The strain A4 showed an extended trend of antifungal spectrum with the advancing age of the cultures up to 5 days respectively. The metabolites produced by the strain A4 was highly inhibitory to *C. albicans* followed by *A. niger*, *F. oxysporum* and *A. flavus* (Table 2).

The crude extracts collected from the culture filtrates of the 4 strains exhibited good antifungal activity especially on *C. albicans*. Strain A1 showed strong antifungal activity on the test fungi such as *A. flavus*, *A. niger*, *C. albicans* and *F. oxysporum* when compared to other tested strains A2, A3 and A4.

Antimicrobial agents have been widely used in several fields viz., human therapy, veterinary, phytopathology, food industries and treatment of leather and wood. Many of these antibiotics are produced by microorganisms and actinomycetes are likely the most important ones. Antifungal metabolites have been reported from the genus *Streptomyces* (Igarashi et al., 2005; Sontag et al., 2006) and a few from *Nocardia* (Mikami, 2007). Unfortunately, the indiscriminate use of antibiotics had predictably resulted in the emergence of drug resistant pathogens. Fungal infections often pose threat to immunosuppressive and healthy individuals. For example, *Candida* species were pathogenic to immunosuppressive as well as healthy people (http://en.wikipedia.org/wiki/*Candida_albicans*).

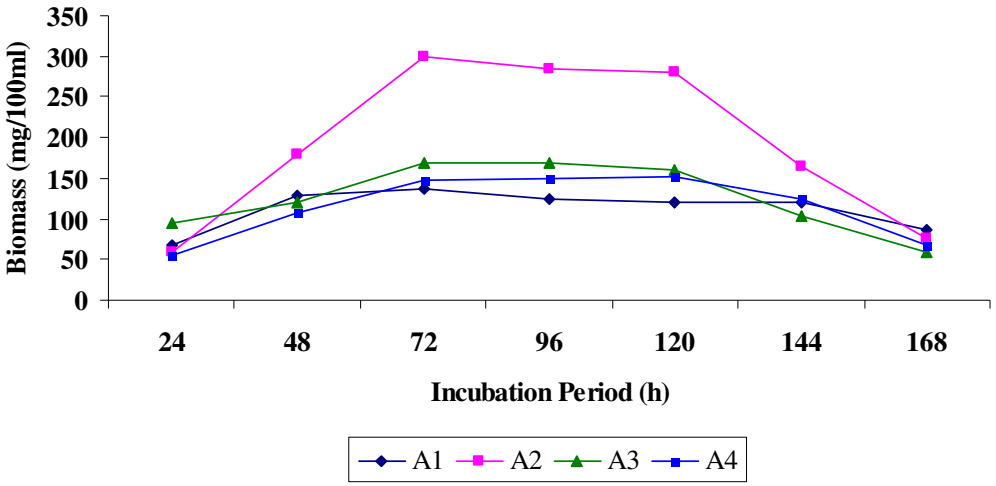


**Figure 1.** Growth patterns of the 4 actinomycete strains (A1, A2, A3 and A4) on yeast extract-malt extract-dextrose broth.

Other limitations for the treatment of fungal infections include resistance to antifungal metabolites, toxicity, drug interactions and expense (Scorzoni et al., 2007). Therefore, the field of antifungal therapy is currently undergoing accerlated changes and new antifungal agents with novel mechanisms of action will likely to enter clinical practice.

In the present study, five-day old cultures of *Streptomyces* spp. (A2 and A4) and *Nocardia* sp. (A3) produced maximum yields of antifungal metabolites while that of *Streptomyces* sp. (A1) showed high antifungal activity on 4$^{th}$ day. Secondary metabolites extracted from 5-day old cultures of *Streptomyces* sp. CDRIL-312 (Harindran et al., 1999), *Streptomyces* spp. (Kathiresan et al., 2005) and *S. purpueofuscus* (Anupama et al., 2007) showed good antifungal activity against filamentous fungi while the metabolites collected from four-day old culture of *S. griseus* also showed good antifungal activity (Otani et al., 1988). The genera *Streptomyces* and *Nocardia* are well known for antifungal metabolites (Berdy, 2005; Mikami, 2007). As the strains selected in the present study showed good antifungal activity, further studies regarding the extraction and purification of antifungal metabolites are in progress. It is hoped that the 4 strains may provide the most promising array of pharmacologically new antifungal compounds which will improve our armamentarium for prevention and treatment of life threatening fungal infections in human life.

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