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## Research Article

# Isolation investigation of Antifungal activity of Soil *Actinomycetes*

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**Abstract:** Infection caused by dermatophytic fungi in man and animals is common throughout the world. Dermatophytoses poses a serious concern to the economically poor population of India. Fungi cause both superficial and internal mycoses. The mycoses, though normally not lethal, is unpleasant and difficult to cure and cause considerable economic loss. Majority of superficial infections are caused by a closely related group of keratinophilic fungi called dermatophytes, which cause ringworm infection or *tinea* infection. Present study is aimed to develop an antibiotic (*Streptomyces* species) against dermatophytic fungi. A total of 25 soil samples were used for the isolation of soil microorganisms. They were subjected to primary screening by cross streak plate assay method against dermatophytic fungi. Then they were subjected to secondary screening by cross streak plate assay method to further test the capabilities of primarily screened organisms. Finally 5 isolates were selected for further study on the basis of (a) broad spectrum activity and (b) larger zone of inhibition in comparison to others. The antimicrobial substances were extracted with ethyl acetate from isolate- inoculated Kuster's broth fermented for 7 days at 28ºC by solvent extraction method. Minimum inhibitory concentration (MIC) was determined.
**Keywords:** Dermatophytic fungi, Streptomyces, MIC, Microsporum, tinea infection.

## INTRODUCTION

In the last two decades, a number of antifungal agents have been successfully developed for the topical treatment of superficial dermatomycosis. Most of these agents were reported to exhibit excellent therapeutic efficacy in experimental dermatophytic animals as well as in patients with dermatophytosis, so that mycological cure can be achieved [1].

Among the different types of drugs available in the market, antifungal antibiotics are limited but are a significant group of drugs that play an important role in the control of mycotic diseases. The need for new, safe and more effective antifungal is a major challenge to the pharmaceutical industry today, especially with the increase in opportunistic infections in the immuno-compromised host. The history of new drug discovery shows that novel skeletons from natural sources [2] in majority of cases. This involves the screening of microorganisms and plant extracts [3]. The search for new, safer, broad-spectrum antifungal antibiotics with greater potency has been progressing slowly [4].

Actinomycetes have been recognized as the potential producers of metabolites such as antibiotics, growth promoting substances for plants and animals, immunomodifiers, enzyme inhibitors and many other compounds of use to man. They have provided about two-thirds (more than 4000) of naturally occurring antibiotics including many of those important in medicine, such as aminoglycosides, anthracyclines, chloramphenicol, β-lactams, macrolide, tetracyclines etc [5].

## METHODS

### Collection of soil samples

Soil samples were collected from various places in Coimbatore district (Tamilnadu). The 25 areas from where samples were collected are 2 from Sulur, 3 from Parambi kulam, 4 from Kanuvai, 2 from Somaiyanur, 3 from Anaikatty, 2 from Maruthamalai hills, 3 from Siruvani, 4 from Kovaipudur, 2 and from Vadavalli. The samples were taken from a depth of 20cms depth [3].

### Isolation of actinomycetes

Actinomycetes were isolated on Kuster's agar medium by serial dilution method [6]. All cultures were purified by streak plate technique and confirmed by colony morphology and screened for their antifungal activity [7].

### Isolation of *Microsporum* species

Isolation of dermatophytes was routinely performed in this laboratory from infected skin, nail and hair samples of human patients. The sample was inoculated on Sabouraud's Dextrose Agar medium [8] and cultured at 26ºC for up to 4 weeks. The

identification of the dermatophyte isolates obtained were achieved by conventional microscopic method and confirmed by the Dermatology Department of PSG Institute of Medical Science and Research, Coimbatore, Tamilnadu, India.

**Screening of actinomycetes**

The screening method consists of two steps; primary screening and secondary screening.

In primary screening the antimycotic activity of pure isolates were determined by cross streak method on Sabouraud's dextrose agar medium [8]. The test organism used were, *Microsporum* species.

Secondary screening was performed by cross streak plate assay method against the test organism, *Microsporum* species.

**Phenotypic and biochemical characterization of actinomycetes**

The potent actinomycetes selected from screening were characterized by morphological and biochemical methods. Morphological methods consisted of macroscopic and microscopic methods. The microscopic characterization was done by cover slip culture methods [9]. The mycelium structure, colour and arrangement of conidiospore and arthrospore on the mycelium were observed through the oil immersion (1000 X). The observed structure was compared with Bergey's Manual of Determinative Bacteriology, ninth edition [10], and the organism was identified and characterized. The biochemical characterization performed were, acid production, NaCl resistance, temperature tolerance, and different carbon utilization, growth in alkali pH and growth in acid pH.

**Fermentation, extraction and antimycotic activity**

Selected streptomycetes were grown in submerged culture in 250ml flasks containing 50ml of liquid medium (NaCl 0.8g, $NH_4Cl$ 1.0g, KCl 0.1g, $KH_2PO_4$ 0.1g, $MgSO_4.7H_2O$ 0.2 g, $CaCl_2.2H_2O$ 0.04g, glucose 2.0g, yeast extract 3.0g and distilled water (pH 7.3) [3] . The flasks were inoculated with 1 ml of active *Streptomyces* culture and incubated at 28ºC for 120 h with shaking at 105t/min [3]. The broth was centrifuged at 10,000 rpm for 20min to separate the mycelial biomass.

Different solvents were used and tested for the extraction of the antibiotic from the culture supernatant. The solvents used were n-butanol, n-hexane, ethyl acetate, petroleum ether, chloroform, benzene and xylene that were used to determine the ideal solvent for extraction of the antibiotic from the culture supernatant. The solvent was added to the supernatant in 1:1 proportion. Solvent – supernatant mixture was agitated for 45min with homogenizer. The solvent was separated from the broth by a separating funnel. It was then centrifuged at 5000 rpm for 15min to remove traces of fermentation broth. All extracts were assayed for their antimycotic activity using respective solvents as control by cross streak assay method [6]. The solvent was evaporated to dryness in water bath at 80ºC - 90ºC and the residue obtained. The dark brown gummy substance obtained was dissolved in ethanol and concentrated. Silica gel plates, 10cm x 20cm x1mm thickness were prepared. They were activated at 150ºC for half an hour. Ten microliters of the ethyl acetate fractions and reference antibiotics were applied on the plates and the chromatogram was developed using ethanol: water: chloroform (40:40:20) as solvent system. The TLC plates were exposed to iodine vapour chamber or UV chamber to develop the antibiotic, if any. The fractions having same Rf value were mixed together and the solvent evaporated at 40ºC in a vacuum oven. These fractions were tested for their antimycotic activity by using the cross streak assay method. The fractions showing antifungal activity were again purified by Thin Layer Chromatography technique. The brown coloured powder obtained was stored in an ampoule at 4ºC [11].

The Rf values were found out by the formula,

$$\text{Relative Front} = \frac{\text{Distance travelled by the compound}}{\text{Distance travelled by the solvent}}$$

**Minimum inhibitory concentration**

The minimum inhibitory concentration of the antimycotic compound was determined by using the method of cross streak plate assay [7].

**RESULTS**

Out of thirty species of actinomycetes obtained from twenty-five different soil samples, five strains (PR01, PR05, KA07, SU09, AN10) produced the secondary metabolites that have antimycotic activity. The isolates PR01, AN10 exhibited strong antimycotic activity against the *Microsporum* (dermatophytic fungi) when grown on Kuster's agar media indicating that the secondary metabolite was produced in optimum amount on Kuster's agar medium. The highest activity was exhibited against *Microsporum* species was *Streptomyces* strain PR01 (30mm) followed by the strain AN10 (28mm). In contrast, strain SU09 showed the lowest inhibition zone (10mm) against *Microsporum* species. Biochemical characteristic showed that PR01 grew well at 45°C. None of the organisms grew well at a low temperature of 4°C and at a high temperature of 60°C. All the isolates were acid producers. Interestingly, all the isolates grew well at pH 6 and 8. Except PR01, none of the isolates grew well at pH 2 (Table 2). All the five isolates showed growth upto 0.25% salt tolerance (NaCl) and PR01 showed maximum salt tolerance upto 2.0% (Table 2).

A qualitative study of carbon utilization showed their ability to utilize citric acid, tartarate and oxalate (Table 2). Ethyl acetate and n-butanol extract of the culture

supernatant inhibited the growth of *Microsporum* species (Table 3)

The fraction collected by Thin Layer Chromatography (TLC) technique was checked for their antifungal activity. The active fraction PR01 and AN10 had an Rf value 0.73. The pure powder thus obtained was stored at 4°C.

**Table- 1: Antimycotic activity of actinomycetes against *Microsporum* species**

| Isolates | Inhibition zone diameter (mm) against *Microsporum* |
|---|---|
| SU09 | + (10mm) |
| SU11 | - |
| PR01 | + (30mm) |
| PR02 | - |
| PR03 | - |
| PR05 | + (16mm) |
| KA06 | - |
| KA07 | + (19mm) |
| KA08 | - |
| KA12 | - |
| SO14 | - |
| SO15 | - |
| SO13 | - |
| SO26 | - |
| AN16 | - |
| AN17 | - |
| AN18 | - |
| MA21 | - |
| MA19 | - |
| MA20 | - |
| SI04 | - |
| SI22 | - |
| VA25 | - |
| VA24 | - |
| KO23 | - |
| AN10 | + (25mm) |
| SI30 | - |
| KO29 | - |
| VA28 | - |
| AN27 | - |

+ indicates production of antimycotic compound against *Microsporum* species,
- indicates absence of antimycotic compound against *Microsporum* species.

## DISCUSSION

Twenty-five soil samples were collected from Coimbatore (Tamilnadu) and its surroundings, 30 isolates of actinomycetes were obtained. Five isolates showed inhibitory effect against *Microsporum* species (dermatophytic fungi). The report reveals that fungal infections have been gaining prime importance because of the morbidity of hospitalized patients [7]. Although synthetic drugs contribute to a major proportion of the antifungals used, natural antifungals have their own place in the antimycotic market [11].

Biochemical characteristics among the five selected isolates indicated that they were closely related to *Streptomyces* spp. These results agreed with earlier report [12]. The antimycotic compound from PR01, PR05, KA07, SU09, and AN10 were extracted from the supernatant using ethyl acetate solvent. Most of the

antifungal antibiotics are extracted using ethyl acetate [13].

The minimum inhibitory concentration (MIC) (Table 1) for the antimicrobial compound extracted from *Streptomyces* species, PR01 showed maximum (30mm) diameter zone inhibition and SU09 showed minimum (10mm) inhibition zone against *Microsporum* species. This results was agreement with earlier reports [3].

According to the TLC separation, the five extracts yielded components and its Rf value were similar to the commercially available antifungal compounds. This may mean that the same compounds are responsible for antimycotic activity of those isolates. In addition, the compounds on the TLC were fluoresced under UV radiation.

In conclusion, the findings of the present study showed that naturally occurring actinomycetes have a great potential to produce metabolite against dermatophytes enabling the discovery of new antibiotics and hence merit future studies.

**Table- 2: Biochemical characteristics of five active selected isolates**

| S. No | Characteristics | Isolates | | | | |
|---|---|---|---|---|---|---|
| | | **PR01** | **AN10** | **PR05** | **KA07** | **SU09** |
| 01 | Growth at | | | | | |
| | 45°C | + | - | - | - | - |
| | 4°C | - | - | - | - | - |
| | 60°C | - | - | - | - | - |
| 02 | Acid production in Kuster's broth | + | + | + | + | - |
| 03 | Salt tolerance (NaCl) | | | | | |
| | 0.25% | + | + | + | + | + |
| | 0.5% | + | + | - | - | - |
| | 1.0% | + | - | - | - | - |
| | 2.0% | - | - | - | - | - |
| 04 | pH tolerance | | | | | |
| | 2 | + | - | - | - | - |
| | 4 | + | + | + | - | + |
| | 6 | + | + | + | + | + |
| | 8 | + | + | + | + | + |
| 05 | Carbon utilization | | | | | |
| | Citric acid | + | + | + | + | + |
| | Tartrate | + | + | + | + | + |
| | oxalate | + | + | + | + | + |

+ indicates positive, - indicates negative.

**Table-3: Different solvents used for extraction of antimycotic compound from *Streptomyces* species**

| Solvent used for extraction of antimycotic compound | Isolates | | | | |
|---|---|---|---|---|---|
| | **PR01** | **AN10** | **PR05** | **KA07** | **SU09** |
| n – hexane | - | - | - | - | - |
| Petroleum ether | - | - | - | - | - |
| n – butanol | + | + | + | + | + |
| Chloroform | - | - | - | - | - |
| Benzene | - | - | - | - | - |
| Xylene | - | - | - | - | - |
| Ethyl acetate | + | + | + | + | + |

+ indicates positive to compound extraction,
- indicates negative to compound extraction.

**REFERENCES**

1. Gupte M, Kulkarni P, Ganguli BN; Antifungal Antibiotics. Appl. Microbiol. Biotechno.2002; 58(1): 46-57.
2. Bevan P, Ryder H, Shaw I; Identifying small-molecule lead compounds: The screening approach to drug discovery; Trends Biotechnol. 1995; 13(3): 115-121.

3. Itoyama T, Uchida K, Yamaguchi H; Therapeutic effects of omoconazole nitrate on guinea – pigs experimentally infected with Trichophyton mentagrophytes. J. Antimicrob. Chemother.1997; 39(6): 825–827.
4. Uchida K, Tanaka T, Yamaguchi H; Achievement of Complete Mycological Cure by Topical Antifungal Agent NND – 502 in Guinea Pig Model of Tinea Pedis. MIcrobiol. Immunol.2003; 47(2): 143-146.
5. Shadomy S; Preclinical evaluation of antifungal agents; in recent trends in the discovery, development and evaluation of antifungal agents. New Jersy: Prous Sciences, 1987; 8-14.
6. Sahin N, Ugur A; Investigation of the Antimicrobial activity of some Streptomyces isolates. Turk. J. Biol.2003; 27(2): 79-84.
7. Hwang BK, Ahn SJ, Moon SS; Production, purification and antifungal activity of the antibiotic nucleoside, tubercidine, produced by Streptomyces violaceoniger. Can. J. Bot, 1994:72(4): 480-485.
8. Takahashi H; The Second Study Group of Liranaftate Cream. Clinical evaluation of 2% liranaftate cream in treatment of dermatophytosis: Open trial (Phase III study) of 2% liranaftate cream. Jpn. Pharmacol. Ther.193; 21:509 – 522.
9. Uchida K, Matsuzaka A, Yamaguchi H; Therapeutic effect of amorolfine on experimental dermatophytosis. Jpn. J. Antibiot.1991; 44(9):1020 – 1031.
10. Bergey's Manual of Determinative Bacteriology. Actinomycetales, 9th edition, 2000;
11. Oskay AM, Üsame T, Cem A; Antibacterial activity of some actinomycetes isolated from farming soils of Turkey. African journal of Biotechnology,2005; 3(9): 441-446.
12. Beck-Sague CM, Jarvis WR; Secular trends in the Epidemiology of nosocomial fungal infections in the United States, J. Infect. Dis,1990;167:1247-1251.
13. Williams ST, Goodfellow M, Alderson G. In: Bergey's Manual of Systematic Bacteriology.1989; 4: 2452-2492.