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STUDIES ONANTICANDIDAL ACTIVITY OF THE ACTINOMYCETES ISOLATED FROM COIMBATORE REGION OF TAMILNADU

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ABSTRACT

A novel actinomycetes strain designated KMA15, producing anticandidal substances was isolated from different locations in Coimbatore has been studied. Morphological and biochemical studies indicated that strain KMA15 belonged to the genus *Streptomyces.* A total of 35 actinomycetes were subjected to agar disc method against *Candida albicans.* It was observed that 12 isolates were active against *Candida albicans.* The anticandidal substances were extracted by solvent extraction method with ethanol from isolate inoculated in yeast malt extract broth fermented for 7 days 28^0C. Fungicidal activity was determined by observing clear zone, the actinomycetes strain KMA15 possessed great fungicidal activity for *Candida albicans* by showing the 22mm of zone of inhibition.Minimum inhibitory concentration of the solvent extract was determined as 1.562 mg/ml against *Candida albicans*. The antifungal substance active against the *Candida albicans* were purified using column chromatography. The nature and further biological activity of the active principle are under investigation.

KEYWORDS: **Soil, Actinomycetes, Candida albicans, Anticandidal activity, Streptomyces.**

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INTRODUCTION

Since the early 1960s *Candida albicans* has emerged from being an infrequent pathogen to become one of the commonest agents of nosocomial infection [1]. Among the different types of drugs prevailing in the market, antifungal antibiotics are a very few but vital group of drugs whereas, it has an important role in the control of mycotic diseases. *Candida albicans* being the most common etiological agent of fungal bloodstream infections. Systemic *Candida* infections are associated with a high mortality rate (38%) and prolonged hospital stay. Globally, 2.5% and 9% of the *Candida* species isolates analyzed were resistant to fluconazole and itraconazole, respectively [2].Because of the serious problems in fungal disease control a serious search is needed to identify novel antibiotics. The need for new, safe and more effective antifungal agents are a major challenge to the pharmaceutical industry today [3]. *Streptomyces* is the largest antibiotic producing genus, producing both antibacterial and antifungal and also a wide range of other bioactive compounds such as immunosuppressant [4]. *Streptomycetes* are among the richest sources for antibiotics. Several *Streptomyces* sp producing antibiotics are used in human and veterinary medicine, agriculture and fishing industry. *Candida albicans* causes infection (candidiasis or thrush) in humans and other animals. Candida species are becoming nearly as common a cause of hospital-acquired infection as the more familiar bacterial pathogens. By far the commonest species to cause clinical infection is *C. albicans*[5]. In the present study we described the isolation of new actinomycetes strain from Coimbatore soil having antifungal activity against *Candida albicans* and its identification by conventional method as well as the optimal condition for the antifungal compound production.

MATERIALS AND METHODS

Soil sampling and isolation of antibiotic producing actinomycetes

Actinomycetes used in this study were isolated from the soil of Coimbatore region. Soil from different places of Coimbatore region were brought to the laboratory in aseptic condition. Actinomycetes from the soil had been isolated by spread plate technique on starch casein agar after serial dilution in distilled water. After 48 hours of incubation at 30˚C, actinomycete colonies appearing on the starch casein agar were transferred to the yeast malt extract agar for colony maintenance [6]. The antifungal activity of the pure actinomycete isolates were determined on PDA against *Candida albicans*. Each actinomycetes strain was streaked in the centre of PDA followed by incubation for 7 days at 28^0C. The 7mm agar disc of the *Candida albicans* were placed in the side of the inoculated actinomycetes. After incubation for 5 to 7 days at 28^0 C, the inhibition zone of *Candida albicans* growth was measured. Selected active actinomycetes which showed anticandidial activity were used for further *invitro* bioassay[7].

Invitro bioassay of anticandidial activity

The culture filtrates of the selected antagonistic actinomycetes tested for *invitro* anticandidal activity by paper disc method[8]. Selected actinomycetes were inoculated yeast malt extract broth and incubated in rotary shaking incubator for 7 days 28^0C. The supernatant of the culture broth was evaluated by paper disc method against *Candida albicans*. From this *invitro* bioassay the best strain which showed high anticandidal activity was selected for the further production.

Selection of suitable broth medium and correct culture conditions

In favour of optimum production of antibiotic, the selected organism was batch fermented in the different fermentation media viz yeast extract and malt extract glucose broth (ISP_2), inorganic salts- starch broth (ISP_4), tryptone yeast extract and glucose broth, Krasilnikov's synthetic broth (SMK) and starch nitrate broth. After incubation at 28^0C for 7 days in shaker at 200 rpm, anticandidal activity was measured for each culture supernatant. After achieving the better anticandidal activity,

effect of various carbon and nitrogen sources at the concentration of 1%and temperatures ranges from 25, 28, 31, 34, 37 and 40^{0}C on the antibiotic production were also investigated in the same culture conditions described above to improve the maximum production [9]. Culture conditions were found to affect antifungal metabolite production by *Streptomyces rochei* AK 39[10].

Isolation of anticandidal substances
Extraction and purification of anticandidal compounds

The supernatant from batch fermentation was taken for further extraction. The solvent, ethanol was evaporated by subjecting the supernatantto rotating the flash evaporator at 40^{0}C (50 rpm) under vacuum. The dried residues obtained were dissolved in ethanol. The solvent extracts were purified by column chromatography. Fifty fractions were collected, and the anticandidal activity of each fraction was measured by paper disc method[11].

Determination of shelf life of active compound

To measure the stability of the active solvent extract in soluble state, 5mg/ml samples were prepared in distilled water and placed in small vials. These samples were kept at room temperature and tested using Agar diffusion method for anticandidial activity at 14 days intervals as long as the activity persisted[12].

Thermal stability of the anticandidal compound

To determine the effect of temperature on stability of the antibiotic, 1 ml of supernatant was harvested from broth culture and added to sterile screw cap ampoules and treated at 4^{0}C in refrigerator for 24 h, at 25, 37, 56, 70 and 90^{0}C in a water bath for 30 min and at 120^{0}C in autoclave for 15 min [13]. Finally, the residual anticandidial activity of different temperature treated samples were determined by measuring the inhibition zone.

Determination of minimum inhibitory concentration

To measure the MIC values, two-fold dilutions of 50, 25, 12.5, 6.25, 3.125, 1.562 and 0.781 mg/ml of the crude extract were prepared in ethanol solvent and assayed by well diffusion method[14].

Detection of fungicidal and fungistatic activity

Small blocks of Actinomycetes (1 mm ^{3}) against *Candida albicans* was transferred to fresh PDA plates and incubated for 7 days at 26-28^{0}C. During incubation, growth or lack of fungus growth was investigated both visually and microscopically. Rejuvenation of growth was indicative of fungistatic and lack of growth represented fungicidal properties of the antagonist[12].

Taxonomic grouping of active actinomycetes isolates

Morphological observations were made with a light microscope [15]. The morphology of spore bearing, hyphae with entire spore chain and structure of spore chain with the actinomycete morphologies were observed by using cover slip method and compared as described by Bergey's manual[16]. Carbon utilization was determined on plates containing ISP basal medium 9 to which carbon sources were added to a final concentration of 1.0%. The plates were incubated at 27˚C and growth was read after 15 days using glucose as positive control. The ability to utilize nitrogen sources was determined in a basal medium containing different nitrogen sources. Results were observed after 15 days.

RESULTS AND DISCUSSION

Isolation of antagonistic Actinomycetes and invitro bioassay of anticandidal activity

35 actinomycete isolates were obtained from 3 different soil samples collected in Coimbatore. By performing agar disc method, 12 actinomycetes produced antibiotic against *Candida albicans* were selected for further *invitro* bioassay. To determine the anticandidial activity of the selected 12 actinomycetes by paper disc method, each strain was batch fermented in the yeast malt extract broth. The crude extract was bio assayed against *C.albicans.* Among all the tested actinomycetes, the strain KMA15 showed greater anticandidal activity. This

active strain KMA15 has been selected for the further production of anticandidal substances (Table.1).The above mentioned results were in agreement with those obtained by many investigators, they found that the antimicrobial activities of *Streptomyces* spp. were increased [14, 17]. The present study results were parallel with the results of Devi *et al* (2006) and singh*et al* (2006) towards the activity of actinomycetes against pathogenic fungi[18,19].

Table 1
Invitro bioassay of antagonistic actinomycetes

S.No	Strain	Zone of inhibition(mm)
1	KMA01	9
2	KMA08	18
3	KMA13	12
4	KMA15	22
5	KMA16	13
6	KMA20	11
7	KMA21	14
8	KMA22	15
9	KMA23	7
10	KMA24	9
11	KMA29	12
12	KMA30	11

Selection of suitable broth medium and correct culture conditions

Different broth media, carbon and nitrogen sources, temperatures were tested for the best production of active compounds. It was found that, Krasilnikov's synthetic broth (SMK), glucose and corn meal at concentration of 1% each as carbon and nitrogen sources respectively for 120 hr at 28^0 C in orbital incubator with shaking at 200 rpm were the most suitable for antibiotic formation. In the optimal corn meal concentration, the zone of inhibition was measured as 22 mm. Results obtained match with what was reported by chattopadhyay and sen 1997[20]. Shahat *et al* 2011 concluded, local isolate of *Streptomyces* could produce anticandidial metabolite on different media composition and carbon, nitrogen sources[21].

Extraction and purification of anticandidial compounds

Extraction process was carried out using ethanolic solvent at the level 1:1 (v/v). The organic phase was concentrated to dryness under vacuum using a rotary evaporator at 45 ^{0} C. The purification of the anticandidial compound was carried out using silica gel column chromatography. The anticandidal activity of each fraction was tested by paper disk method against *Candida albicans.* The most active fractions against the *Candida albicans* ranged from 22mm to 35mm. The purification of the compound was confirmed by TLC. The active fraction showed a single spot with an RF value 0.78. In the elution system the best anticandidial activity was observed in 40:60.

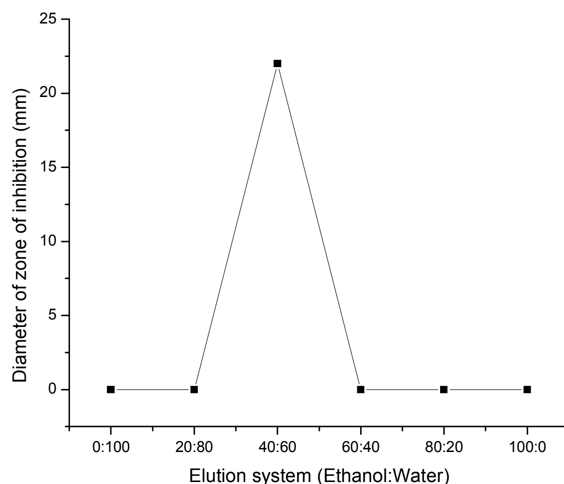

Figure 1
column chromatogram of the solvent extract from Streptomyces strain KMA15.

Guangying *et al*., (2005) used chloroform and ethanol 95:5 (v/v) as an elution solvent(11). Atta *et al*., (1999) purified bioactive substance through column chromatography packed with silica gel was eluted with a mixture of chloroform and methanol 8:2 (v/v), revealed that the most active fractions against the fungal pathogen ranges from 23 to 30[22]. Ethanol was used as the extraction solvent in this study to that are safer in handling as compared to other organic solvents, such as methanol and acetone.

Determination of shelf life of active compound

Antibiotics from the strain KMA15 stored at 4^0C and retained their activity for the entire testing. The extract retained its anticandidial activity even 12 months of storage. In the present study the room temperature does not affect the activity of the antibiotic. Antibiotic from *Streptomyces* KMA15 stored at 4^0C and retained their activity for the entire one year duration of testing. Bonjar *et al*., (2005) reported that at 30^0C and 40^0C the activity of strain 101 at 10mg/ ml against *V.dahliae* showed 18 mm zone of inhibition[23].

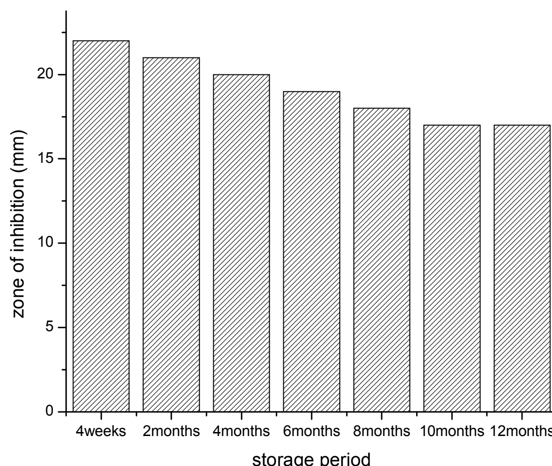

Figure 2
Shelf life of Antibiotic from actinomycetes strain KMA15

Thermal stability of the anticandidal compound

The antibiotic was stable at different temperatures, however the antibiotic lost its activity completely after autoclaving at 121° C for 15 min. Muiru *et al.,* (2007) reported from his study that none of the antibiotic was stable at 90^{0}C and above. Antibiotics from culture filtrates 28P and CS35 were sparingly stable at 80^{0}C. High temperatures were found to destabilize the antibiotics and this has an important implication on the storage of the antibiotics from culture filtrates[24].

Determination of minimum inhibitory concentration

In well-diffusion method MIC of the solvent extract was determined as 1.562 mg/ml against *Candida albicans.* Ebrahimi Zarandi *et al.*,(2009)*,* in well diffusion method MIC of the solvent extract was determined as 3.125 mg/ml against *M.oryzae*[12]. . The isolates of rare actinobacteria obtained from the samples of limestone quarry appear to be very prominent with potential antimicrobial features (25).

Detection of fungistatic and fungicidal activity

Fungistatic activity was determined by observing the positive subculture of the blocks of the clear zone on the PDA. The actinomycetes strain KMA15 possessed fungistatic activity for *Candida albicans.* These results may suggest that this active compound could be used for the valuable pharmaceuticals as it has a strong activity against *Candida albicans* .

Taxonomic grouping of active actinomycetes isolates

On the basis of the morphological and biochemical property, the strain KMA15 was classified in the genus *Streptomyces*[26]. The scanning electron shows the spores were numerous very fine and oval with smooth membranes (Fig.3). The colour of the colony is gray and no diffusible pigment. The colour of the aerial mycelium is white. It varied depending on the type of used media. The strain hydrolysed starch and liquefied gelatin. It utilized Glucose, Mannitol, Xylose, it could not utilized galatose. As nitrogen sources it utilized nitrates (Table.2).

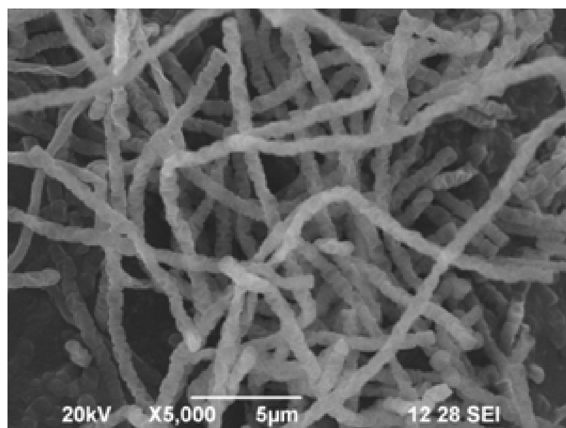

Figure 3
The scanning electron micrograph of the spore chains of KMA15

Table 2
Morphological and Physiological characteristics of the active actinomycetes isolates

Characteristics	Results
Spore mass	Gray
Spore surface	Smooth
Color of aerial mycelium	White
Sugar Pattern	Not detected
Diffusion pigment	Not produced
L-Valine	+
L-Phenylalanine	+
L-Histidine	+
L-Lysine	+
L-Tyrosine	+
Glucose	+
Mannitol	+
Xylose	+
Galatose	-
Nitrates	+
Starch hrdrolysis	+
Liquefied Gelatin	+

+ positive - negative

The characterization of *Streptromyces* sp mainly based on aerial mycelia the shape and ornamentation of spore surface. Further physiological characters such as temperature, utilization of sugar and reduction of nitrates where also considered to identify the genus *Streptomyces*[27]. .

CONCLUSION

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 immune compromised host and drug resistant pathogens. The result obtained in the present investigation indicated that KMA15 demonstrated the obvious inhibitory effect against *Candida albicans*. The potential of the compound in the control of *Candida albicans* should also be further evaluated.

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