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ORIGINAL ARTICLE

In Vitro Susceptibility of *C. albicans* and *C. neoformens* to Potential Metabolites from *Streptomycetes*

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Abstract Forty two *Streptomycetes* isolates from soils of Kodachadri region in Western ghats were recovered by soil dilution technique. Cross streak method was followed for primary screening of antifungal activity. Positive isolates were subjected to secondary screening by cold extraction of fermentation broth in butanol solvent. Six isolates exhibited broad spectrum antifungal activity against all the tested yeast pathogens like *Candida albicans*, *Candida lipolytica, Cryptococcus neoformens* and *Saccharomyces cerevisiae*. One isolate showed excellent antifungal activity against all test organisms with maximum zone of inhibition 60 mm each incase of *C. neoformens* and *C. albicans.* Partial characterization of antifungal metabolite by TLC resulted in a purple spot with an R_f value 0.50. The UV absorption spectra at 218 nm indicated possible chemical nature of the active metabolite as polyene group and purity was assessed by analytical HPLC.

Keywords Antifungal activity · Cross streak · Secondary screening · HPLC · UV absorption

Introduction

The need for new, safe and effective antifungal antibiotics is a major challenge to the Pharmaceutical industry today, especially with the increased opportunistic infections in immunocompromised host and also lack of non toxic antifungal antibiotics [1]. Actinomycetes are one of the most attractive sources of antibiotics, of all the antibiotics 66% are produced by Actinomycetes [2–4]. Antibiotics predominate in therapeutic and commercial importance. One of the modern approaches is isolation and screening of Actinomycetes from relatively unknown areas. In this meaning Western ghats are of significant interest, as they are proven as eminent systems enriched with unraveled biological diversity. Aim of the present study was screening of antifungal metabolites from Actinomycetes against human yeast pathogens.

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Materials and Methods

Sampling

72 soil samples were collected (from May 2005 to October 2006) from Kodachadri forest areas of Western Ghats. Samples were collected from 20 cm depth into sterile polythene bags, air dried at room temperature and stored under aseptic condition until processing [5].

Isolation of Actinomycetes

Isolation and enumeration of actinomycetes was performed by soil dilution plate technique [6]. One gram of dried soil was serially diluted (up to 10^{-5} dilutions). Different aqueous solutions from 10^{-4} and 10^{-5} of the suspension were pour inoculated on plates containing selective media like Starch casein agar [7], Modified albumin agar, Actinomycetes isolation agar and Chitin agar [8, 9]. The incubation was carried out at 30 ± 2°C for 7–10 days [10, 11].

Characterization of Actinomycetes

Morphological observations of selected isolates were made with light microscope by using method of Shirling and

Gottlieb [12]. The isolates were identified up to genus level as described in Bergy's manual and by cover slip method. In cover slip method spore suspension of the actinomycetes were placed on the starch casein agar blocks covered with sterile cover slips and incubated in moist chamber for 2–3 days [13]. Biochemical characterization was done by performing starch hydrolysis, gelatin hydrolysis, casein hydrolysis, sugar fermentation and H_2S production [2, 14].

Bioassay

Pathogenic yeasts tested for in vitro antifungal activity are *C. albicans NCIM-3100, C. lipolytica NCIM-3472, C. neoformens NCIM-3541 and S. cerevisiae NCIM-3095* procured from National Collection of Industrial Microorganisms Pune, India.

The promising isolates identified in the present study were subjected to primary screening by cross streak method [15]. Actinomycetes were swab inoculated on half of the Petri plates containing Sabouraud's dextrose agar and incubated at 30°C for 72 h. Effectivity of the isolates was assessed by growth inhibition of pathogenic yeasts [16].

Isolates possessing antifungal activity were subjected to secondary screening by inoculating the culture to starch casein broth and incubated at 30°C for 8–10 days. After the 10th day the broth was centrifuged at 10,000 rpm for 20 min to separate mycelial biomass. For extraction of antibiotic, the supernatant was mixed with Butanol solvent, in 1:1 proportion (v/v), the solvent supernatant mixture was agitated for 45 min in homogenizer; the solvent was then separated by using separating funnel. Obtained extracts were assayed for antifungal activity by agar well diffusion method [16] using respective solvents as control.

Separation of Antibiotic

The solvent was evaporated by subjecting the crude extract to hot air at 40°C in an oven for 96 h. The concentrated residue obtained was dissolved in sterile water following which crude antibiotic obtained was subjected for purification [17]. The crude antibiotic was tested for its components by using precoated TLC plates using Ethanol:Chloroform:Water (40:40:20) [17] and Butanol:Acetic acid:water solvent systems [5]. Chromatograms were developed in Iodine chamber [17] and sprayed with Ninhydrin to know the possible chemical nature of the active component.

The UV–Visible absorption spectra of the bioactive component in solvent extracts were determined with a SHIMADZU UV-2550 spectrophotometer at 200–400 nm to determine the $\lambda_{maximum}$ of the band [6, 18].

High Performance Liquid Chromatography (HPLC)

The purity of the bioactive component from KSRO 04 was tested using HPLC analysis by the method employed by Chakravarthi et al. [19] with minor modifications. HPLC (Shimadzu) separation was performed using a C18-column (250 × 4.6 mm) at a flow rate of 1 ml/min and pressure 142 kgf by injecting 20 µl of sample. The mobile phase used was methanol:water (70:30, v/v). The absorbance was measured at 203 nm.

Results

Isolation of Actinomycetes

Forty two isolates were recovered from Kodachadri soils, among which six showed broad spectrum antifungal activity against test organisms.

Characterization of Actinomycetes

Six promising isolates were characterized by morphological and biochemical methods (Table 1). Microscopic characterization of these isolates by Cover slip method revealed them to be of the genus *Streptomyces*. Isolate KSRO4 showed straight arrangement of twelve to sixteen spores (Fig. 1).

Table 1 Biochemical activities of *Streptomycetes* isolates

Isolate no	KSRO1	KSRO2	KSRO3	KSRO4	KSRO5	KSRO6
Starch hydrolysis	+	−	+	+	+	+
Gelatin hydrolysis	+	−	−	−	+	[a]
Casein hydrolysis	+	−	−	−	−	[a]
Sugar fermentation						
S	−	+	−	−	−	[a]
M	−	+	−	−	−	[a]
G	+	+	−	−	−	[a]
H_2S production	−	−	+	+	−	[a]

[a] Results not clear

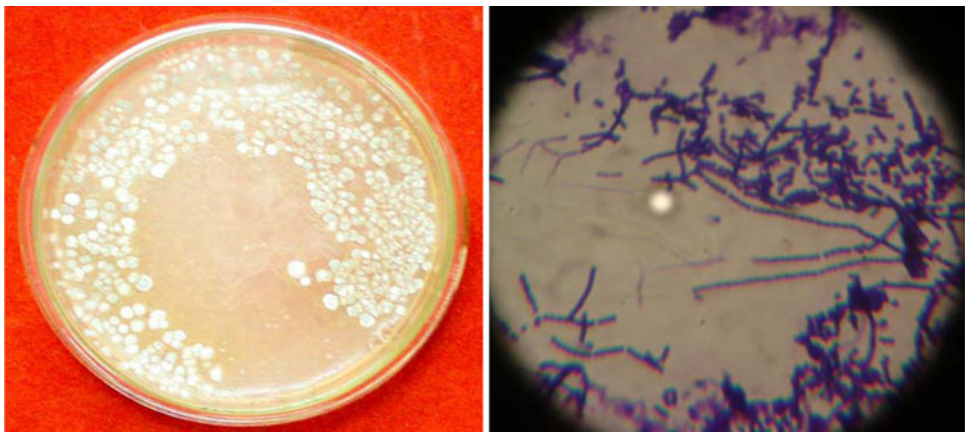


Fig. 1 Morphology and microscopic view of isolate KSRO4 (magnification 2000×)

Table 2 Antifungal activities of *Streptomycetes* isolates

Sl no	Isolate no	*C. albicans*	*C. neoformens*	*C. lipolytica*	*S. cerevisiae*
1	$KSRO_1$	18	10	–	20
2	$KSRO_2$	10	16	–	–
3	$KSRO_3$	50	20	–	80
4	$KSRO_4$	60	60	50	40
5	$KSRO_5$	11	–	30	–
6	$KSRO_6$	10	–	80	–

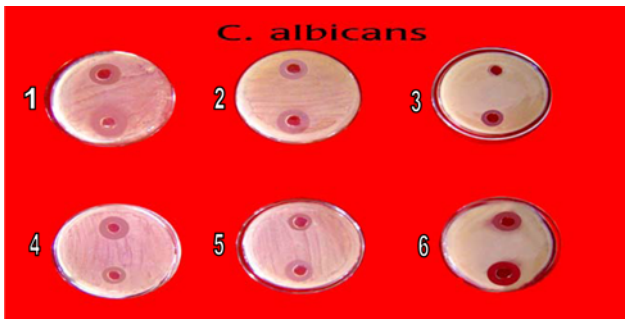


Fig. 2 Antifungal activity of *Streptomyces* isolates against *C. albicans* by well in agar method (*1*) KSRO-01, (*2*) KSRO-02, (*3*) KSRO-03, (*4*) KSRO-04, (*5*) KSRO-06, (*6*) KSRO-05

Bioassay

In primary screening 6 of the 42 isolates showed antagonistic activity against test fungi. Solvent extracts of culture filtrate tested in secondary screening showed inhibition zone varying from 10 mm to 60 mm by well in agar method. Two isolates showed potent antifungal activity against tested fungi (Table 2). All the six isolates inhibited *C. albicans* (Fig. 2), *C. neoformens* was inhibited by 4 isolates (Fig. 4) and three were effective on *S. cerevisiae* and *C. lipolytica* (Figs. 3 and 5). One isolate showed excellent antifungal activity in all tested fungi (Isolate KSRO4). The results are represented in bar diagram as follows.

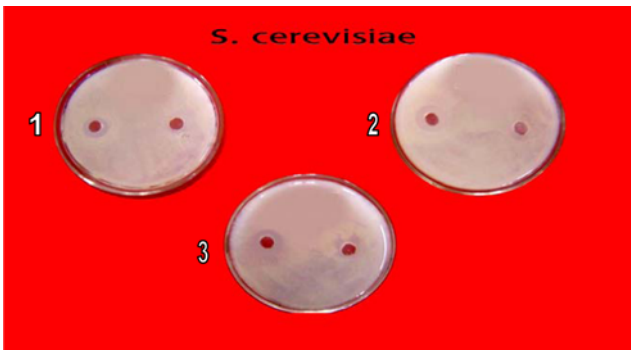


Fig. 3 Antifungal activity of *Streptomyces* isolates against *S. cerevisiae* by well in agar method (*1*) KSRO-01, (*2*) KSRO-04, (*3*) KSRO-03

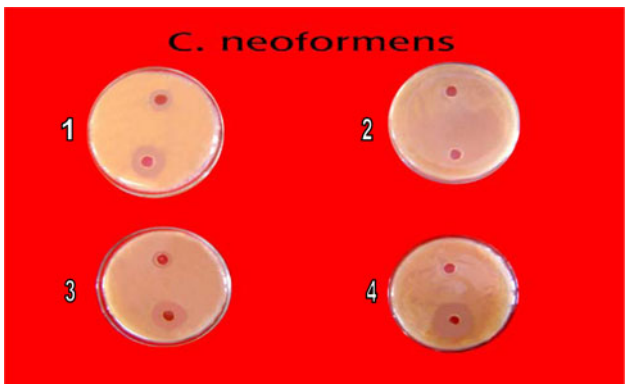
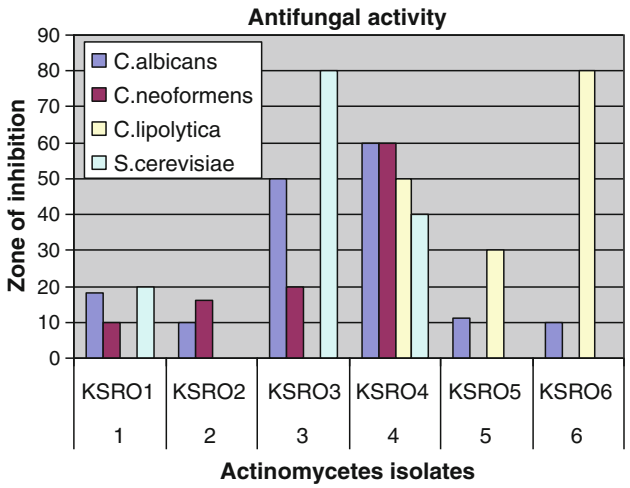


Fig. 4 Antifungal activity of *Streptomyces* isolates against *C. neoformens* by well in agar method (*1*) KSRO-01, (*2*) KSRO-02, (*3*) KSRO-04, (*4*) KSRO-03



Partial Characterization of Antibiotic

Concentrated solvent extract of isolate four subjected to TLC using solvent system [Butanol:Acetic acid:Water] showed spot having R_f value 0.50. The chromatogram developed in Iodine chamber, showed purple band indicating unsaturated fatty acid nature of active components (Fig. 6).

UV absorption range of the isolate KSRO-04 was 218 nm suggesting polyene nature of the component [5]. HPLC analysis was performed to check purity of extract obtained. Solvent system was standardized with different ratio of Methanol and Water. The solvent extract of isolate KSRO-04 revealed a prominent peak along with short peaks revealing traces of additional compounds which are indicated by additional peaks in the graph (Fig. 7).

Discussion

The present study emphasized on screening of actinomycetes from Western Ghats an unexplored biodiversity hot spot and

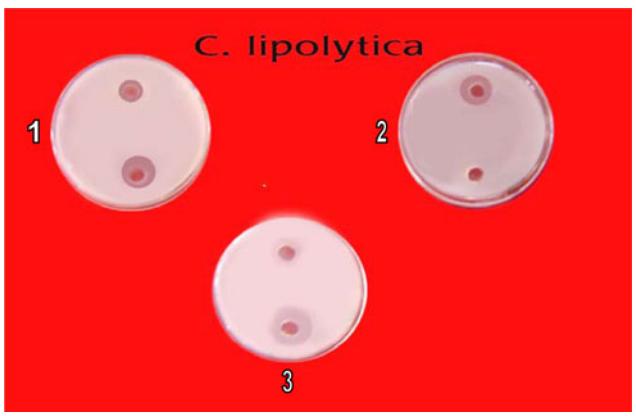


Fig. 5 Antifungal activity of *Streptomyces* isolates against *C. lipolytica* by well in agar method (*1*) KSRO-06, (*2*) KSRO-04, (*3*) KSRO-05

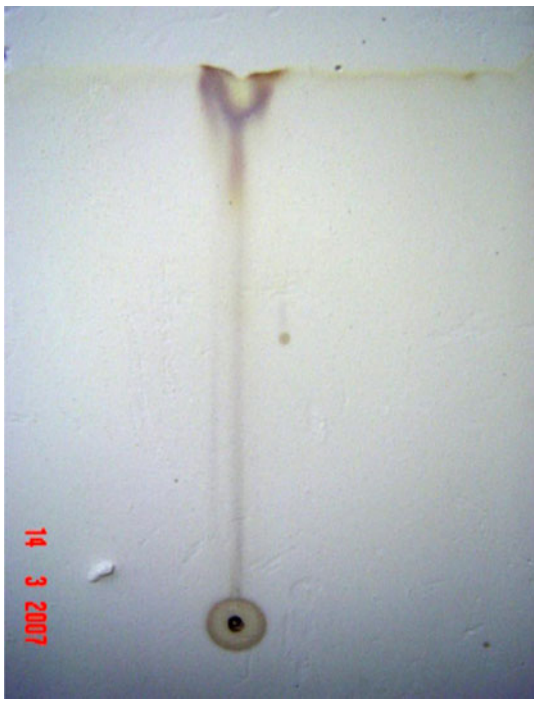


Fig. 6 Thin layer chromatogram of isolate no KSRO-04

partial characterization of antifungal metabolite effective against opportunistic yeast pathogens causing infections in immunocompromised hosts [20, 21]. Characterization of the antifungal metabolite indicated polyene nature of the active principle, similar activities have been earlier conducted and polyene nature has been confirmed by Sahin et al. [5]. Characterization of a non polyene antifungal antibiotic from *Streptomycetes* effective against yeast pathogen like *C. albicans* has been carried out by Augustine et al. [2]. The present investigation in comparison with other works focused on characterization of the antifungal metabolite, as in majority of the earlier studies *Candida albicans* has only been considered as a test pathogen but characterization of the metabolite has not been carried out. In this regard the metabolite is of high importance as polyene group of non toxic antifungal metabolites like Nystastin and Amphotericin B are very less in number.

Conclusion

With the above obtained results we conclude that polyene group of antibiotics are well suited for the control of opportunistic yeast pathogens and Western Ghats soils are a promising source for new and potent actinomycetes.

Future Prospect

In the past few decades emergence of new and resistant pathogens has increased the requirement of effective

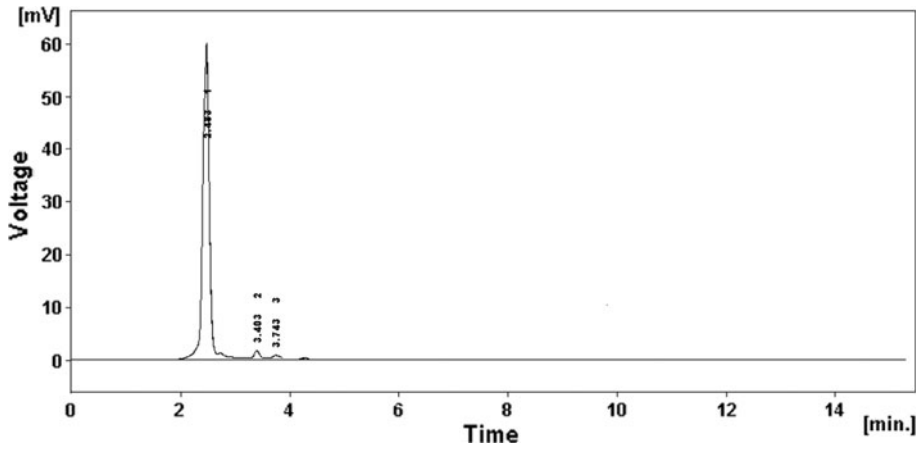


Fig. 7 High performance liquid chromatography (HPLC) analysis of the solvent extract of KSRO-04 isolate

antibiotics. In this regards, further investigations such as the taxonomic categorization of isolates by 16S rDNA analysis, electron microscopic studies, identification of purified active components by NMR and IR techniques and metabolites have to be subjected for assessment of their toxicological profiles, as a mandatory process suggested by all regulatory authorities are to be under taken.

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