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Screening of actinomycetes for antifungal metabolites production from kodachadri soils

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ABSTRACT

42 Actinomycetes isolates were recovered from soils of Kodachadri by soil dilution technique. Cross streak method was followed for primary screening of antifungal activity. Positive isolates subjected to secondary screening from fermentation broth of isolates extracted in butanol solvent. Six isolates characterized as Streptomycetes species showed broad spectrum antifungal activity against *C.albicans*, *C.neoformens*, *S.cerevisiae*, *Fusarium and Colletotrichum spp.* One isolate showed excellent antifungal activity against all test organisms with maximum zone of inhibition 60mm (*C.neoformens*) 50mm (*C.albicans*) and 40mm (*Fusarium spp*). Partial characterization of antifungal metabolite by TLC revealed active component as unsaturated fatty acid by a purple spot with an Rf value 0.50. The active metabolite exhibited UV absorption at 218 nm indicating possible chemical nature of the component as polyene group and purity was assessed by analytical HPLC.

KEYWORDS

Antifungal activity;
Cross streak;
Secondary screening;
HPLC;
UV absorption.

INTRODUCTION

The history of new drug discovery process shows that novel skeletons have,in the majority of cases come from natural sources[4]. This involves screening of microorganisms and plant extracts[21]. Actinomycetes are one of the most attractive sources of antibiotics, of all the antibiotics 66% are produced by Actinomycetes. Antibiotics predominate in therapeutic and commercial importance[18,20]. 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

The need for new, safe and effective antifungal antibiotics is a major challenge to the Pharmaceutical industry today, especially with the increase opportunistic infections in immunocompromised host and also lack of non toxic antifungal antibiotics. Aim of the present study was isolation of Actinomycetes producers of antifungal substances from kodachadri soils.

MATERIALS AND METHODS

Sampling procedures

72 soil samples were collected (from May 2005 to October 2006) from forest areas of Western Ghats.

Samples are collected from 20 cm depth into sterile polythene bags, air dried at room temperature and stored under aseptic condition until processing[16].

Isolation of actinomycetes

Isolation and enumeration of actinomycetes was performed by soil dilution plate technique[12]. One gram of dried soil was serially diluted up to 10^{-4} different aqueous solutions from $10^{-4}$and 10^{-5} of the suspension were applied onto plates containing selective media like Starch casein agar, Modified albumin agar, Actinomycetes isolation agar and Chitin agar[9,10]. The cultivation was carried out at 30 ±2 for 7 to 10 days[4,8].

Characterization of actinomycetes

Morphological observations of selected isolates were made with light microscope by using method of Shirling and Gottlieb[22]. The isolates identified up to genus level as described in Bergy's manual and by cover slip method[3] where spore suspension of the actinomycetes was placed on the blocks of starch casein agar covered with sterile cover slips. Incubated in moist chamber for 2-3 days, biochemical characterization was done performing starch hydrolysis, gelatin hydrolysis, casein hydrolysis, sugar fermentation and H_2S production[14,15].

Bioassay

Pathogenic fungi tested in-vitro for antifungal activity includes *C.albicans*, *C.lipolytica*,*C.neoformens* and *S.cerevisiae* collected from National Collection of Industrial Microorganisms Pune, India. Plant pathogens like *Fusariumspp* and *colletotrichumspp* (Rot pathogens) were isolated from infected pods of Vanilla *in vitro*.

The promising isolates identified in the present study were subjected to primary screening by cross streak method[7] actinomycetes were swab inoculated in half of the Petri plates containing potato dextrose agar and incubated for two to three days at 30^0C. For 72 hours. The effectivity was assessed in terms of inhibition of growth of pathogenic fungi.

Isolates possessing antifungal activity were subjected to secondary screening by inoculating the culture to starch casein broth and incubated at 30^0C for 8-10 days. The broth was centrifuged at 10,000 rpm for 20 min to separate mycelial biomass. For extraction of antibiotic the supernatant was mixed with solvent butanol, in 1:1 proportion (V/V). The solvent supernatant mixture was agitated for 45 min in homogenizer the solvent was separated by separating funnel. Remaining extracts were assayed for antifungal activity by agar well diffusion method[1] using respective solvents as control.

Separation of antibiotic

The solvent was evaporated by subjecting the crude extract to hot air at 40^0C to50^0C in oven for 96 hours. The residue obtained, dissolved in sterile water and concentrated, following which crude antibiotic obtained was subjected to purification[2].

The crude antibiotic was tested for number of number of components by using precoated TLC plates using Ethanol: Chloroform: Water (40:40:20)[14] Butanol: Acetic acid: water solvent system[16]. Chromatogram was developed in Iodine chamber[14] and dyed with Ninhydrin to arrive at possible chemical nature of active component.

UV/Visible absorption

The UV-Visible absorption spectra of the bioactive component in solvent extracts were determined with a SHIMADZU UV-2550 spectrophotometer at 200-400nm to determine the $\lambda_{maximum}$ of the band[17] (Ilic et al., 2005; Wu et al., 2007).

High performance liquid chromatography (HPLC)

The purity of the bioactive compound was tested using HPLC. The crude solvent extracts of isolate S7 was subjected to HPLC analysis by the method employed by Chakravarthi et al. (2008) with minor modifications. The isolate was grown in 500 ml of Starch casein broth in flask at 30±2^{0}C in stationary cultures. After 11 days, culture was filtered, filtrate was centrifuged and the supernatant was extracted with an equal volume of n-butanol. The organic phase was taken to dryness. The residue was dissolved in 1 ml of sterile distilled water. HPLC (Shimadzu) separation was performed using a C18-column (250×4.6 mm) at a flow rate of 1 ml/min and Pressure 142kgf. A 20µl amount of sample was injected. The mobile phase used was methanol: water (70:30, v/v). The absorbance was monitored at 203 nm.

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TABLE 1 : Biochemical activities

Isolate no		Kss1	Kss2	Kss3	Kss4	Kss5	Kss6
Starch hydrolysis		+	-	+	+	+	*
Gelatin hydrolysis		+	-	-	-	+	*
Casein hydrolysis		+	-	-	-	-	*
Sugar fermentation	S	-	-	-	-	-	*
	M	-	-	-	-	-	*
	G	-	+	-	-	-	*
H_2S Production		-	-	+	-	-	*

*Results not clear

TABLE 2: Antifungal activities of active isolates

		Zone of inhibition in mm					
Sl. no.	Isolate no	*C.albicans*	*C.neoformens*	*C.lipolytica*	*S.cerevisiae*	*Fusarium sp*	*Colletotrichum sp*
1	Kss_1	80	100	30	90	50	-
2	Kss_2	100	-	-	90	80	80
3	Kss_3	60	160	40	80	-	-
4	Kss_4	150	100	50	40	140	60
5	Kss_5	110	100	110	-	70	140
6	Kss_6	100	160	80	20	-	-

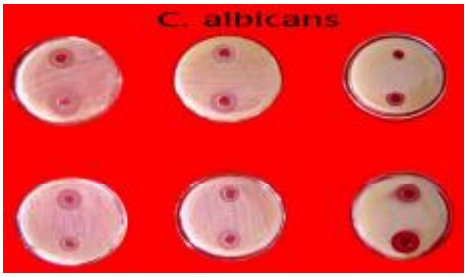


Figure 1: 1. kss4 2. kss1 3. kss3 4. kss2 5. kss6 6. kss5

Figure 2 : 1. kss3 2. kss4 3. kss1

RESULTS

Isolation of actinomycetes

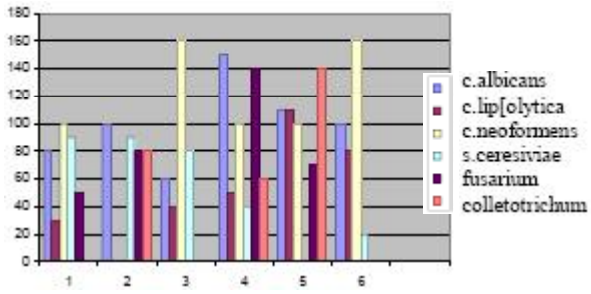
42 isolates were recovered from Kodachadrisoils, among which six showed broad spectrum antifungal activity against test organisms.

Characterization of actinomycetes

Promising 6 isolates were characterized by morphological and biochemical methods. (TABLE 1). Microscopic characterization of 6 isolates by Cover slip method revealed them to be belonging to the genus *Streptomycetes.*

Bioassay

In primary screening 6 of the 42 isolates showed antagonistic activity against test fungi (TABLE 2). Solvent extracts of culture filtrate tested in secondary screening showed inhibition zone varying from 10mm to 160mm by well in agar method. Two isolates showed potent antifungal activity against tested fungi (TABLE 2). All the 6 isolates inhibited *C.albicans*, (figure 1) *C.neoformens* was inhibited by 4 isolates (figure 3) and 3 were effective on *C.lipolytica* and *S. cerevisiae* (figures 2 and 4) .One isolate showed excellent antifungal activity in all tested fungi. (Isolate no kss 4). The results can be represented in bar diagram as follows.



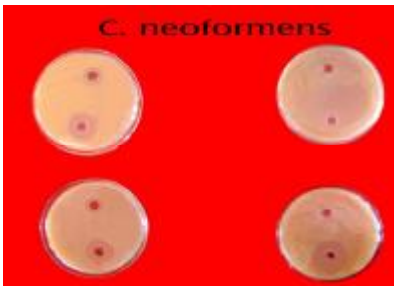


Figure 3 : 1. kss1 2. kss2 3. kss4 4.kss3

Partial characterization of antibiotic

Concentrated solvent extract of isolate4 subjected to TLC using solvent system (Butanol: Acetic acid: Water) showed spot having R_f value 0.50 When the chromatogram was developed in Iodine chamber,

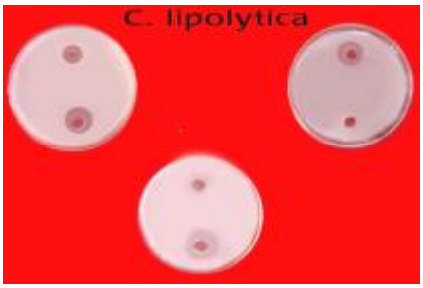


Figure 4 : 1. kss6 2. kss4 3. kss5

Figure 5 : Thin layer chromatogram of isolate no kss4

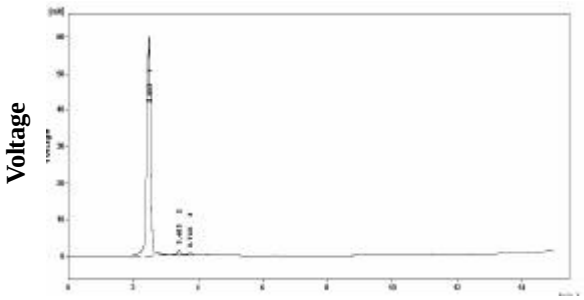


Figure 6 : HPLC analysis of isolate kss4

showed purple band indicating unsaturated nature of active components (figure 5).

UV absorption range of the isolate kss4 was 218 nm suggesting polyene nature of the component.

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 kss4 revealed a prominent peak along with short peaks revealing traces of additional compounds which are indicated by additional peaks in the graph (Figure 6).

DISCUSSION

The diversity of terrestrial actinomycetes has been of extraordinary significance in several areas of medicine. Characterization of antifungal metabolite from actinomycetes has been studied by Augustine etal. Exploitation of antagonistic potential of actinomycetes in biological control of plant pathogens has given fruitful results[1]. Broad spectrum antimicrobial activity is also exhibited by some of the actinomycetes by inhibiting Yeasts and filamentous fungi[8]. The present study also emphasized on screening of actinomycetes which were effective on human fungal pathogens, like candida and cryptococcus may be a new source of antifungal antibiotic. Potential of actinomycetes in biological controle was also studied here by testing against rot pathogens of Vanilla like *Fusarium* and*colletotrichum.*

Overall the present study was successful in isolation of potent actinomycetes isolates, their preliminary taxonomic identification and partial characterization of the active principle.

So further investigations are under progress to confirm the taxonomic categorization of these isolates and purify the active components so as to explore the antibiotics produced by these isolates.

CONCLUSION

The present Research was promising research for diverse actinomycetes from an unraveled soil source like kodachadri in Wsetern ghats.

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