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Actinomycetes of Western Ghats Karnataka, India

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ABSTRACT

Western Ghats in Karnataka are one of the 25 biodiversity hotspots in world. These forest soils were studied for Actinomycetes diversity, by screening for antibiotics, enzymes and pigment production. 27 isolates were subjected to *in-vitro* bioassay by agar - well diffusion method. The results revealed the broad spectrum antimicrobial potential of these isolates against pathogenic microorganisms like *Salmonella typhi, Staphylococcus aureus, Pseudomonas fluorescence, Escherichia coli, Proteus vulgaris, Bacillus subtilis, Klebsiella pneumoniae, Streptococcus spp, Vibrio cholerae, Cryptococcus neoformens, Candida albicans, Candida lipolytica and Sacchromyces cerevesiae.* Characterization of metabolites by TLC (Rf value 0.29 to 0.93) and purity assessment by HPLC was done. UV absorption studies of the active metabolites showed varied absorption peaks (203nm to 345nm) indicating diversity of antibiotics.

KEYWORDS

Western Ghats;
Actinomycetes;
Antibiotic;
UV absorption;
HPLC.

INTRODUCTION

Actinomycetes are ubiquitous in soils. Actinomycetes are physiologically diverse bacteria as evidenced by their production of antibiotics, enzymes and pigments. Actinomycetes are also responsible for biodeterioration and biodegradation processes in nature. These versatile capabilities have promted biologists to screen these organisms from different geographical regions of the world like Antartica, Mount Everest, Forests and Marine habitats[17].

The Western Ghats constitute the beautiful array of mountains along the western coast of India. About sixty percent of the Western Ghats are located in the state of Karnataka. These hills cover 60,000 km² and the average elevation is around 1,200 meters. The area is one of the world's twenty five "Biodiversity Hotspots" and has over 5000 species of flowering plants, 139 mammal species, 508 bird species and 179 amphibian species. At least 325 globally threatened species occur in the Western Ghats. Microbial Diversity of the soils of Western Ghats in Karnataka has not been attempted, so the present study concentrated on the exploration of actinomycetes from these soils.

MATERIALS AND METHODS

Sampling areas

The soil samples were collected from Kodachadri, Nagara, Hosanagara, Ripponpet, Agumbe, Tirthahalli and Chikmagalore. These areas comprise Western Ghats in Karanataka

Collection of soil samples

The soil samples were randomly collected from a depth of 15 cm in sampling bags from different areas[5,6]. The samples were kept in aseptic condition until they were subjected to plating.

Isolation of actinomycetes

The air dried soil samples were subjected to serial dilution up to 10^{-5}[20]. The diluted samples were plated on various media like Starch Casein Agar (SCA), Chitin Agar (CA), Kenknight and Munaier's Medium (KMM), Actinomycetes Isolation Agar (AIA), Arginine Glycerol Salt Agar (AGS) and Yeast Extract-Malt Extract Agar (YEMEA) for isolation of actinomycetes. The inoculated plates were incubated at 30±2°C for up to 14 days in aerobic condition. The colonies obtained on plates were purified by streak plate technique and confirmed as Actinomycetes by microscopy, staining and biochemical reactions[2].

Identification of actinomycetes

Cover slip technique was performed to study the spore chain arrangement and morphology. A thin block of Starch Casein Agar was placed on sterile slide, inoculated with the pure culture and sterile cover slip was placed on it. The slide was incubated in moist chamber for 3-4 days under aseptic condition. After incubation, the growth on cover slip was fixed in 5% aqueous tannic acid for 10-15 minutes, washed in distilled water, stained in 0.2% aqueous Crystal violet for 3 minutes and observed under oil immersion objective using 20X eye piece[3,12,21].

Antibiotic assay

The actinomycetes isolates were cultivated for 5 days using Starch Casein Agar plates at 28°C[20]. 500 ml Erlenmeyer flasks containing 250 ml of starch casein liquid medium were inoculated with the isolates. The inoculated flasks were kept on a rotary shaker at 28-30°C for 9 days. The broth was then centrifuged at 10,000 rpm for 20 minutes, the supernatant collected was extracted with solvents like butanol and hexane[1,20]

Bioassay was done by well in agar method[15] was followed to assess the antibiotic potential. The plates were first inoculated with the test organisms by swab culture method. Then the well was bored in the media using sterile cork borer to make well of 8 mm in diameter. About 100-200µl of extract was dispensed into the wells. The test organisms used were gram positive *Staphylococcus aureus, Bacillus subtilis and Streptococcus sp,* gram negative *Pseudomonas fluorescence, Escherichia coli, Proteus vulgaris, Salmonella typhi and Klebsiella pneumoniae,* yeast species *Cryptococcus neoformens, Candida albicans, Candida lipolytica and Saccharomyces cerevesiae*.

Metabolite characterization

Characterization of crude antibiotics was carried out by,

A) TLC

The n-butanol and n-hexane solvent extracts containing bioactive components were concentrated and fractionated using Thin layer chromatography (TLC) on a 12 X 22 cm silica gel plate and developed with chloroform-ethanol-water (2:4:4) and n-butanol-acetic acid-water (4:1:2) solvent systems[20]. Detection was done in three ways. The TLC plates were exposed to iodine vapors, sprayed with vanillin and ninhydrin separately to develop the antibiotic, if any Wu *et al.* (2007).

B) UV absorption spectra

The UV-Visible absorption spectra of the crude antibiotics were determined in (SHIMADZU UV-2550) spectrophotometer at 200-400nm to determine the λ_{max} of the metabolite, to deduce the probable nature of the metabolite[8,10].

C) HPLC

The purity of the crude antibiotics was tested using analytical HPLC (SHIMADZU). The residue was dissolved in 1 ml of sterile distilled water. Separation was performed using a C18-column (250 x 4.6 mm) at a flow rate of 1 ml/min and Pressure 142kgf. A 20 μl amount of sample was injected. Methanol: water (70:30, v/v) was used as mobile phase. The absorbance was monitored at 203, 330 and 360 nm[4].

RESULTS

Isolation of actinomycetes

It was found that SCA (30%) and AIA (30%) were the ideal media for isolation of maximum number of actinomycete isolates from the soil samples followed by AGS (18%), KMM (11%) and CA (11%). (TABLE: 01). Most of the isolates were identified as *Streptomyces* species as noticed with straight, open loops, closed spirals and hook ap-

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pearance of spores. Straight arrangement of spores was observed in case of 11 actinomycete isolates, three isolates showed open loop pattern and ten isolates showed open or closed spiral arrangement spores (TABLE 1).

Antibiotic assay

The solvent extracts of broth culture filtrates when subjected to secondary screening by Well diffusion method, exhibited significant inhibitory effects. Among the tested bacterial pathogens, *S. typhi* was found to be more inhibited followed by *P. vulgaris*, *K. pneumoniae*, *B. subtilis*. *Streptococcus* sp and *E. coli* were least affected target bacteria (TABLE 2).

Antifungal activity was done by testing against 6

TABLE 1 : Colony characteristics of actinomycete isolates

Isolate	Medium used	Pigmentation	Colony morphology	Spore formation	Spore arrangement	Tentative genera
S1	AGS	-	White, cottony with grey sporulation	+	Straight	*Streptomyces*
S2	SCA	Yellow	Creamish	+	Straight	*Streptomyces*
S3	AGS	-	Grey colored, leathery	+	Open loops	*Streptomyces*
S4	AGS	-	Ash colored, powdery appearance	+	Straight	*Streptomyces*
S5	SCA	-	Light yellow, mealy	+	Closed spirals	*Streptomyces*
S6	SCA	-	Dark grey colored	+	Flexous	*Streptomyces*
S7	SCA	Dark red	Bright white, leathery, red pigmentation	+	Primitive spirals	*Streptomyces*
S8	SCA	Red	Bright white, leathery, light red pigmentation	+	Primitive spirals	*Streptomyces*
S9	SCA	Light yellow	Creamy, tough colony, light yellow pigmentation	+	Straight	*Streptomyces*
S10	KMM	Brown	Dull white, with greenish black back	+	Short chain of spores	*Streptomycts*
S11	SCA	-	White, leathery	-	Bacilli like spores	*Nocardia*
S12	SCA	-	Creamy, mealy	+	Straight	*Streptomyces*
S13	AIA	-	White, leathery	+	Open spiral	*Streptomyces*
S14	AGS	-	Grey	+	Straight, long chain of spores	*Streptomyces*
S15	AGS	-	Creamy, yellow	+	hook	*Streptomyces*
S16	CA	-	Grayish White, ery	-	Fragmented mycelia	*Nocardia*
S17	KMM	Brown	Dark grey, powdery	+	Short chain of spores	*Streptomyces*
S18	AIA	-	Light white powdery	+	Two open loops	*Streptomyces*
S19	CA	-	Light green, powdery	+	Long chain of spores	*Streptomyces*
S20	AIA	Brown	Light green wlth yellow margin	+	Closed spirals	*Streptomyces*
S21	AIA	Yellow	Light yellow;leathery	+	Long chain of spores	*Streptomyces*
S22	KMM	-	Brown with white centre	+	Long chain of spores	*Streptomyces*
S23	AIA	-	Creamy with light green powdery	+	Two spores	*Microbispora*
S24	AIA	-	White leathery	+	Straight	*Streptomyces*
S25	CA	-	Light green	–	Fragmented mycelium	*Nocardia*
S26	AIA	Dark green	Light grey with dark green	+	Open loop	*Streptomyces*
S27	AIA	-	Light green,powdery	+	Closed spirals	*Streptomyces*

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TABLE 2 : Antibacterial activity of actinomycetes in secondary screening

Sl. No.	Isolate No.	Zone of inhibition in mm							
		B. subtilis	*P. aeruginosa*	*S. typhi*	*S. aureus*	*P. vulgaris*	*K. pneumoniae*	*E. coli*	*Streptococcus sp*
01	S_1	36	-	-	30	40	36	-	-
02	S_2	36	-	24	14	12	-	-	-
03	S_3	-	-	24	-	14	-	-	-
04	S_4	-	16	16	-	10	-	-	-
05	S_5	-	-	24	-	-	16	-	-
06	S_6	-	-	28	20	22	-	-	16
07	S_7	10	16	16	14	10	14	-	-
08	S_8	08	14	08	14	12	10	-	-
09	S_9	-	-	-	-	12	22	-	-
10	S_{10}	14	08	14	-	24	18	-	-
11	S_{11}	-	-	-	-	-	-	-	-
12	S_{12}	10	16	12	-	-	36	-	-
13	S_{13}	-	08	08	-	-	-	-	-
14	S_{14}	-	-	-	-	-	-	-	-
15	S_{15}	16	10	-	08	-	26		
16	S_{16}	16	18	16	-	14	18	-	-
17	S_{17}	12	-	12	-	-	18	-	-
18	S_{18}	-	-	12	14	12	12	-	-
19	S_{19}	-	12	12	12	10	10	-	-
20	S_{20}	36	08	12	36	16	28	-	-
21	S_{21}	16	16	32	30	32	28	-	-
22	S_{22}	26	10	20	36	20	18	-	-
23	S_{23}	08	-	-	08	-	16	-	-
24	S_{24}	12	16	18	12	10	-	-	-
25	S_{25}	36	22	16	16	30	16	-	-
26	S_{26}	-	-	-	-	-	26	20	14
27	S_{27}	12	26	16	-	10	-	16	-
28	Std (Cef)	26	26	26	40	30	30	26	20

fungi. 17 actinomycete isolates inhibited *C. albicans* followed by *C. lipolytica* and *S. cerevisiae* by 11 isolates. 10 isolates were effective against *C.neoformans*. 8 isolates were effective against *Collectotrichum* sp and *Fusarium* sp was the least affected among all the tested fungi. (TABLE 3).

Metabolite characterization

A. Thin layer chromatography

The thin layer chromatograms of various isolates were developed with ninhydrin detection agent showed red spots with Rf values from 0.29 to 0.69. Isolate S6 showed a purple band with Rf value 0.93 in iodine vapour chamber. About 7 isolates exhibited Rf values from 0.50 to 0.59 while isolates S3, S4, S5, S12 and S16 showed Rf values from 0.60 to 0.69 (TABLE 4).

B. UV spectral analysis

The isolates exhibited absorption peaks ranging from 203nm to 345nm. The isolates S1, S10, S13, S16, S22, S24, S25 and S26 showed absorption maxima at 280nm. About 13 isolates exhibited absorption at 290nm and 300nm in 6 isolates. Five isolates namely S3, S5, S11, S24 and S25 showed three absorption peaks ranging between 280nm and 340nm, probably suggesting the presence of mixture of bioactive components in the solvent extracts. Isolate S16 exhibited 4 absorption peaks ranging from 280nm to 295nm (Figure 1).

C. HPLC

HPLC analysis was performed to check purity of

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TABLE 3 : Antifungal activity of actinomycetes in secondary screening

Sl. No.	Isolate No.	Zone of inhibition in mm					
		C. albicans	*C. lipolytica*	*C. neoformans*	*S. cerevisiae*	*Fusarium sp*	*Colletotrichum sp*
01	S_1	-	08	16	14	-	-
02	S_2	14	08	14	16	-	-
03	S_3	12	10	-	08	-	-
04	S_4	12	-	-	10	22	22
05	S_5	14	-	-	-	-	-
06	S_6	18	08	10	14	34	18
07	S_7	22	14	26	12	-	-
08	S_8	12	14	26	-	-	-
09	S_9	12	12	10	-	-	-
10	S_{10}	-	-	-	-	-	-
11	S_{11}	08	16	-	-	-	-
12	S_{12}	14	08	-	-	-	-
13	S_{13}	12	-	-	14	-	-
14	S_{14}	-	-	-	-	-	-
15	S_{15}	-	-	-	-	-	-
16	S_{16}	14	-	16	12	-	-
17	S_{17}	-	-	-	-	-	-
18	S_{18}	20	-	-	16	12	26
19	S_{19}	26	-	-	-	18	22
20	S_{20}	16	26	-	10	18	22
21	S_{21}	22	38	22	16	22	12
22	S_{22}	20	34	10	20	26	16
23	S_{23}	-	-	-	-	-	-
24	S_{24}	10	22	30	14	10	20
25	S_{25}	12	-	24	10	16	-
26	S_{26}	-	-	-	-	-	-
27	S_{27}	-	-	-	-	-	-
28	Std (Flu)	-	30	-	-	-	-

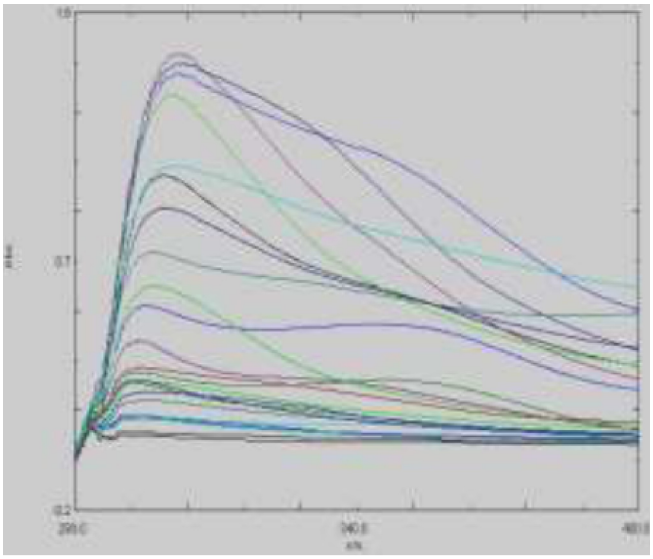

Figure 1 : UV spectral analysis

extract obtained. The solvent extract of isolate S7, S2 and S26 revealed a prominent peak along with short peaks revealing traces of additional compounds.

DISCUSSION

In the present study, forest soils of Shivamoga and Chikmagalore districts were screened for the diverse and potent actinomycete isolates, as forest soils and uncultivated soils are proven to be rich in actinomycetes population. It has been reported in earlier studies by[18,17], that the carbon and nitrogen sources greatly affect the actinomycete growth and metabolite production. In this study also, carbon sources like starch and glycerol have influenced such enhanced activity since most of the ac-

TABLE 4 : Thin layer chromatography

Isolate	Solvent system Butanol:Acetic acid:Water		Solvent system Chloroform:Ethanol:Water	
	Detection agent	Rf value	Detection agent	Rf value
S1		-		-
S2		0.58-		-
S3		0.60		-
S4		0.60		-
S5		0.61		-
S6		-		0.93
S7		0.79		-
S8		0.58		-
S9		0.50		-
S10		-		-
S12		0.55		-
S13		0.69		-
S15	Ninhydrin	0.50	Iodine chamber	-
S16		0.53		-
S17		0.53		-
S118		0.67		-
S119		0.52		-
S20		-		-
S21		0.58		-
S22		-		-
S23		0.57		-
S24		0.57		
S25		-		-
S26		Diffused		-
S27		-		-

tinomycete isolates were recovered from Starch Casein Agar and Actinomycete Isolation Agar. In this study, the tentative genera of actinomycetes was assigned based on the morphological features like color of aerial mycelium, substrate mycelium, soluble pigment produced, and characteristic spore arrangement by most convenient, simple cover slip technique. The *Streptomyces* isolates showed varied pattern of spore arrangements like open loops, hooks, spirals, closed spirals, straight and flexous indicating diversity of *Streptomyces* in Western Ghats soils. Similar studies were also conducted by[14,19], to identify the actinomycete populations in different geographical locations. In the present study, *in vitro* antimicrobial activity of n-butanol solvent extracts of 27 actinomycete isolates was tested by Well diffusion method against Gram positive bacteria, Gram negative bacteria, molds and yeasts. The zone of inhibition was found to be varying between 8mm to 36mm in bacteria. Gram negative bacteria namely *S.typhi, P.vulgaris* and *K.pneumoniae* were inhibited more than *E. coli*. In case of gram positive bacteria, *B. subtilis* was inhibited by more number of isolates followed by *S. aureus*. In total, the n-butanol solvent was found to be suitable solvent for extracting antibacterial principles. More number of isolates inhibited opportunistic pathogens like *C. albicans* and *C. neoformans* indicating the significance of these isolates in biomedical field where availability of effective antifungal drugs is very less. The present investigation recovered isolates with promising antimicrobial activity especially antibacterial activity (81% of isolates) followed by antifungal activity (59% of isolates).

Most of the isolates exhibited UV absorption at 280-290nm while few isolates exhibited absorption above 300nm also. The identical absorption pattern in most of the isolates could be due to the similarity in antibiotics. Studies reveal that antibiotics namely Thienamycin, (beta lactam), YL704 (macrolide), Tylosin (macrolide) exhibited absorption between 280 to 290nm[9,11,13].

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