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Research Article

ISOLATION AND SCREENING OF ANTAGONISTIC ACTINOMYCETES FROM MANGROVE SOIL

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ABSTRACT

Objective: The objective of the present study was to isolate and screen the antagonistic actinomycetes from mangrove soil in Visakhapatnam.
Methods: A total of 30 actinomycetes isolates were isolated by serial dilution plate technique, of these 20 isolates showed activity in primary screening against test pathogens used in this study. The active isolates were morphologically discrete on the basis of spore mass, colour, formation of aerial and substrate mycelia, production of diffusible pigment and biochemical characterization. The active isolates were subjected to shake flask fermentation and the secondary metabolites were extracted with ethyl acetate and screened for their antimicrobial activities against selected bacterial and fungal pathogens by agar well diffusion method.
Results: Out of 20 isolates, 13 isolates exhibited both antibacterial and antifungal activity and 7 isolates showed only antibacterial activity and did not inhibit fungi used in this study. These isolates were identified as *Streptomyces* species basing on their morphological, physiological and biochemical characters.
Conclusion: The results of the present study evidenced that mangrove source may be beneficial for the discovery of novel antibiotics from actinomycetes.

Keywords: Actinomycetes, primary screening, secondary metabolites, *Streptomyces*, antimicrobial activities

INTRODUCTION

Mangroves are woody plant communities that develop at the edge of the land and sea in tropical and subtropical latitudes. The microorganisms in mangrove forest sediments are not only involved in decomposition and mineralisation of litter fall but also versatile producers of various enzymes and antibiotics [1]. The mangrove surroundings are powerful origin for the isolation of antibiotic producing actinomycetes [2]. The utilization of the wealth of mangrove microorganism resources has been expanded in many aspects including the screening of novel actinomycetes that could be a hidden source of natural products [3].

Actinomycetes are widely distributed group of microorganism in nature and have the capacity to synthesise many biologically active secondary metabolites [4]. The secondary metabolites like antibiotics, herbicides, pesticides, anti-parasitic and enzyme inhibitors are obtained from actinomycetes are of special interest due to its diverse biological activities like antibacterial, antifungal, antioxidant, antitumor and antiviral. Two-third of the commercially obtained antibiotics is isolated from actinomycetes, So far 23,000 bioactive secondary metabolites are produced by the microbes, of these 10,000 compounds are produced by actinomycetes. Among actinomycetes, 7600 are produced by *streptomyces* species, many of them are potent antibiotics. Screening and isolation of potent actinomycetes for novel antimicrobial secondary metabolites is achievement in recent years.

In the present study, an attempt was made to isolate and screen the actinomycetes from the mangrove soil of Visakhapatnam, which can be wealthy valuable resource of secondary metabolites.

Materials and Methods

Collection of soil sample

Soil samples were collected from 6-10cm depth from mangrove regions in Visakhapatnam, Andhra Pradesh, India. Samples were stored under aseptic conditions until further use.

Isolation of Actinomycetes

Actinomycetes were isolated by serial dilution plate technique using yeast extract malt extract glucose agar media. The media were supplemented with Rifampicin (5µg/ml) and Nystatin (25µg/ml) to inhibit unwanted bacterial and fungal contamination respectively

[5]. An aliquot of 0.1ml was spread on the above media and incubated for 1-2 weeks at 28° C. The actinomycetes colonies were identified by their chalky, firm and leathery texture [6]. These colonies were sub cultured and maintained at 4°C for further characterization.

Primary Screening

The isolated actinomycetes were screened for their antagonistic activity against pathogenic microorganisms by using primary screening. Primary screening was done by cross streak method [7] and the lead isolates were selected and studied further.

Extraction of Secondary metabolites

The pure isolates were fermented by production medium consisting of Glucose 1%, Soya bean meal 1%, NaCl 1%, and $CaCO_3$ 0.1% at 28° C for 96 hrs at 180 rpm on a rotary shaker [8]. After 96hrs the fermented broth was extracted twice with ethyl acetate and shaken vigorously for 1hr for complete extraction. The ethyl acetate phase that contains bioactive compound was separated from the aqueous phase and was evaporated to dryness under reduced vacuum 80°-90°C[9]. The concentrated organic residue obtained was used to determine the antimicrobial activity.

Secondary Screening

The secondary screening was done by agar well diffusion method [10]. The assay plates were seeded with *Staphylococcus aureus* (MTCC 3160), *Bacillus Subtilis* (MTCC 441), *Bacillus cereus* (MTCC 430), *Escherichia coli,* (MTCC 443), *Pseudomonas aeruginosa* (MTCC 424), *Proteus vulgaris* (MTCC 426) using nutrient agar media and potato dextrose agar media for *Saccharomyces cerevisiae* (MTCC 170), *Candida albicans* (MTCC 227), *Aspergillus niger* (MTCC 961) and *Aspergillus flavus* (MTCC 3396). The antimicrobial activities were observed after 24hrs of incubation at 37°C for bacteria and 48hrs of incubation for *A.niger* and *A.flavus*, 24 hrs of incubation for *C. albicans* and *S.cerevisiae* at 28°C for fungi and the zone of inhibition were expressed as diameter (mm).

Characterization of Actinomycetes cultures

Selected and potential actinomycetes strains were studied for morphological, cultural, physiological and biochemical characteristics [11]. The morphology of spore chain and spore bearing hyphae were identified using optical microscope at 1,000X magnification. The color of spore mass was examined under light microscope and estimated by color chart [12].

RESULTS AND DISCUSSION

The present study was carried out to isolate antagonistic actinomycetes from mangrove soil in Visakhapatnam. A total of 30 different actinomycetes strains was isolated by using yeast extract malt extract glucose media and subjected to primary screening. Out of 30 isolates, 20 isolates exhibited the notable activity against test microorganisms in primary screening, the remaining 10 isolates exhibit meagre activity. Further work carried by the leading 20 isolates, these were cultivated in fermentation liquid medium for 96hrs. After fermentation, the extracted secondary metabolite was concentrated by solvent extraction method these were carried for secondary screening.

Screening of actinomycetes for antibiotics

Isolation of an antibiotic from culture filtrate is mainly gritty by its chemical nature. Solvent extraction is commonly hired for the extraction of antibiotics from the culture filtrates. Organic solvents have been used for the extraction of antimicrobial compounds from actinomycetes [13]. All the twenty actinomycetes were screened for their antimicrobial activity against six species of bacteria and four species of fungal pathogens. Antagonistic activities of actinomycetes isolates were tested by adopting agar well diffusion method. Out of 20 isolates, 13 isolates exhibited both antibacterial and antifungal activity and 7 isolates showed only antibacterial activity. The antibacterial activities of the 20 isolates against test pathogens are tabulated in table 1. In case of antibacterial studies, the isolates KVR 01, KVR 02, KVR 03, KVR 05, KVR 06, KVR 07, KVR 08, KVR 09, KVR 10, KVR 11, KVR 12, KVR 13, KVR 20, exhibited potent antibacterial activity against test pathogens and the maximum inhibition zone 20-18 mm was observed. The remaining isolates KVR 04, KVR 14, KVR 15, KVR 16, KVR 17, KVR 18 and KVR 19 exhibited intermediary activity against the test pathogens with the maximum zone of inhibition was 16-14mm observed for these isolates. Most of the isolates exhibited maximum antibacterial activity against gram positive bacteria used in this study.

Table 1: Antimicrobial Activity of the 20 Putative Isolates

Isolate No.	Name of the Test Organism (Inhibition zone diameter in mm)					
	Gram negative bacteria			Gram positive bacteria		
	E.coli	*P.vulgaris*	*P.aeruginosa*	*B.subtilis*	*B.cereus*	*S.aureus*
KVR 01	16	14	14	14	18	16
KVR 02	12	11	13	11	19	19
KVR 03	18	12	12	17	19	18
KVR 04	16	14	14	12	12	14
KVR 05	18	14	14	18	20	12
KVR-06	14	14	12	17	14	18
KVR 07	18	15	14	14	14	16
KVR 08	14	12	12	12	16	18
KVR 09	12	18	10	14	14	12
KVR 10	16	14	16	18	18	16
KVR 11	18	18	10	12	14	12
KVR 12	18	12	14	14	19	10
KVR 13	16	14	14	18	14	16
KVR 14	12	15	14	12	14	12
KVR 15	11	11	12	13	12	14
KVR 16	16	15	16	14	13	14
KVR 17	10	10	12	11	12	11
KVR 18	16	12	13	15	14	16
KVR 19	14	18	16	15	15	14
KVR 20	12	14	14	16	15	18

In case of antifungal studies the isolates KVR 02, KVR 05, KVR 06, KVR 07, KVR 10, KVR 12, KVR 13, KVR 14, KVR 15, KVR 16, KVR 17, KVR 18 and KVR 20 showed worthy against *A.niger, A.flavus, C.albicans and S.cerevisiae* . Among all the 13 isolates the maximum zone of inhibition was observed 20-18mm for *S.cerevisiae* and 15-12mm for *A.niger*. The maximum zone of inhibition observed for *A.flavus* was 17-14mm and 12mm for *C.albicans*. Among all the 13 isolates exhibits potent activity for *S.cerevisiae* and worthy activity for *A.niger, A.flavus* and mild activity for *C.albicans.* The results are tabulated in table 2. Comparatively Sonashia et al [14] reported 30 actinomyctes strains isolated from various locations of soil samples in Goa, out of them 28 isolates exhibited broad spectrum of antimicrobial activity against test pathogens and also Sakthi Velayudham [15] reported 36 actinomycetes were isolated from forest soil exhibited broad spectrum of antimicrobial activities.

Characterisation of the isolates

The morphological and physiological characteristics of these 20 isolates were studied and identified them up to genus level and presented in Table 3. All the 20 actinomycetes were identified at a generic level based on their colony morphology the isolates belonged to the genus *Streptomyces. Streptomyces* represent a leading portion of the actinomycetes in mangrove soil. Among all the members of actinomycetes the *Streptomyces* species are the producers of the secondary metabolites with broad spectrum of antibacterial, antifungal, antibiotic, vitamins, enzymes, antiphrastic, antitumor, antiviral, insecticide, herbicide, immunomodulators, antithrombatic agents [16].

Table 2: Antifungal activity of the 13 putative isolates

Isolate No.	Name of the Test Organism (Inhibition zone diameter in mm) Fungi			
	A.niger	*A.flavus*	*C.albicans*	*S. cerevisiae*
KVR 02	15	13	12	14
KVR 05	16	17	12	18
KVR 06	15	16	12	20
KVR 07	15	16	12	18
KVR 10	15	18	10	20
KVR 12	15	14	12	16
KVR 13	12	10	12	18
KVR 14	10	12	8	15
KVR 15	14	15	10	16
KVR 16	14	12	9	15
KVR 17	14	15	10	16
KVR 18	15	16	12	18
KVR 20	14	15	12	17

Biochemical characterization of the isolates

Various biochemical tests executed for the identification of the potential isolates were Melanin reaction, H_2S production, Tyrosine reaction, Starch hydrolysis, Casein hydrolysis, Gelatin hydrolysis, Nitrate reduction, Methyl red, Voges-Proskauer, Citrate, Urease and Catalase. Most of the isolates are positive against Melanin reaction, Citrate, Urease and negative against Voges-Proskauer and the results are tabulated in table 4.

Mangroves develop alongside bays in tropical and subtropical regions where sea water and fresh water mix. Indeed, mangrove forests may become a helpful source for discovering novel actinomycetes. Most of the natural products were derived from marine microbes of these the actinomycetes as a separate lineage for producing novel bioactive compounds which have an antibiotic and antitumor properties. They are the main source of clinically important antibiotics, most of which are too complex to be synthesized by combinatorial chemistry. Thus, microbialnatural products still appear as the most promising sources for developing future antibiotics. However, the research on actinomycetes from Indian isthmus is very flimsy and there is no reports regarding isolation of Actinomycetes from mangrove soil in Visakhapatnam (India). Therefore, the soil samples were collected from mangrove area in Visakhapatnam and made an effort to isolate potential actinomycetes strains. These isolates produced an extracellular compound which inhibits the growth of microbial cells. In the way of our systematic screening for bioactive actinomycetes, 20 isolates exhibited broad spectrum of antimicrobial activity. The cultural, morphological, biochemical, physiological characteristics reveals that these isolates belong to the genus *Streptomyces.* Results of the present investigation indicated that the isolates having a wide range of antimicrobial activities against all test pathogens used in this study

Table 3: Morphological characteristics of the 20 putative isolates on yeast malt extract Agar

Name of the isolate	Spore bearing hyphae	Spore mass colour	Growth	Vegetative Mycelia colour	Aerial hyphae colour	Soluble pigment
KVR 01	Retinaculum apertum	Brown	Abundant	Light brown	Brown	Brown
KVR 02	spirales	Blue	Abundant	Creamy	Bluish green	Reddish brown
KVR 03	spirales	Light yellow	Good	Light yellow	yellow	Dark brown
KVR 04	spirales	Reddish Brown	Abundant	Light brown	Brown	Dark brown
KVR 05	Flexous	Dark green	Abundant	Green	Grey	Dark green
KVR 06	spirales	Grey	Good	Light brown	Grey	Brown
KVR 07	spirales	Orange Brown	Abundant	Brown	Brown	Reddish brown
KVR 08	spirales	Reddish Brown	Good	Light brown	Brown	Dark brown
KVR 09	spirales	Green	Abundant	White	Green	Green
KVR 10	Rectus	White	Good	White	Grey	Nil
KVR 11	Flexous	Brown	Abundant	Light brown	Brown	Brown
KVR 12	Retinaculum apertum	Brown	Abundant	Light brown	Brown	Reddish Brown
KVR 13	Monoverticillus	Black	Abundant	Light brown	Brown	Nil
KVR 14	Flexous	Green	Abundant	White	Green	green
KVR 15	spirales	grey	good	grey	brown	brown
KVR 16	Retinaculum apertum	red	good	brown	yellow	yellow
KVR 17	spirales	grey	good	yellow	yellow	Nil
KVR 18	spirales	grey	good	brown	brown	Nil
KVR 19	Retinaculum apertum	grey	good	yellow	yellow	Nil
KVR 20	spirales	white	good	red	brown	brown

CONCLUSION

The present study comprises isolation, screening and partial characterization of novel bioactive compounds with a diverse range of actinomycetes. Further studies on the characterization of the isolates, purification of the antibiotic substance and study of its biological activities like antitumor, antiviral etc., and elucidation of its production pathways are underway. It is expected that the current attempt of isolation, screening and partial characterisation on mangrove actinomycetes of the local area of Visakhapatnam will be useful for identification of new antibiotics effective against challenging pathogens.

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Table 4: Biochemical characteristics of the 20 putative isolates

Name of the isolate	Melanin reaction	H_2S production	Tyrosine reaction	Starch hydrolysis	Casein hydrolysis	Gelatin hydrolysis	Nitrate reduction	Methyl red	Voges-Proskauer	Citrate	Urease	Catalase
KVR 01	+	+	+	-	-	+	+	-	-	+	+	-
KVR 02	+	+	+	+	-	+	-	+	-	-	-	+
KVR 03	+	+	+	+	+	+	+	-	-	+	+	-
KVR 04	+	+	+	-	-	-	+	-	-	+	+	-
KVR 05	+	+	+	+	-	+	+	+	-	+	+	-
KVR 06	+	-	-	+	+	+	+	-	-	+	+	+
KVR 07	+	+	+	+	+	+	+	+	-	+	+	-
KVR 08	+	+	+	+	+	+	+	-	-	+	+	-
KVR 09	+	+	+	-	-	-	-	-	-	+	+	-
KVR 10	-	-	+	+	+	+	-	+	-	+	+	+
KVR 11	+	+	+	-	-	+	+	-	-	+	+	-
KVR 12	+	+	+	-	-	+	+	+	-	+	+	-
KVR 13	-	+	+	+	+	+	-	+	-	+	+	-
KVR 14	+	+	+	-	-	-	-	-	-	+	+	-

KVR 15	-	-	+	-	+	+	+	+	-	+	+	-
KVR 16	+	-	+	+	-	+	+	-	-	+	+	+
KVR 17	-	+	-	+	-	-	-	+	+	+	-	-
KVR 18	+	-	-	-	+	-	+	-	-	-	+	+
KVR 19	-	+	+	+	+	+	-	-	+	+	-	+
KVR 20	-	+	-	+	-	-	-	+	-	+	+	-

"+" indicates Positive, "-" indicates negative

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