

Isolation and characterization of antagonistic actinobacteria from mangrove soil

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Abstract

The aim of the present study was to isolate and screen actinobacteria having antagonistic activities against pathogenic microorganisms. A total of twenty actinobacteria strains were isolated from the mangrove sediment. Of these four active isolates were identified as *Streptomyces* species by means of morphological, physiological, biochemical and cultural characteristics. These 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. The results showed that among the active isolates, four isolates (BC 01, BC 02, BC 03 and BC 04) showed promising activities against the selected test pathogens. These four extracted isolates were analyzed for UV Spectrophotometric and HPLC. Spectral data of the extracted compound revealed its antimicrobial nature. The UV spectrum of the methanol extracts for the active isolates showed absorbance peaks ranging between 207-223 nm. Two to three bioactive regions were detected on the HPLC. The results indicate that *Streptomyces* strains isolated from mangrove sediment produce potential antibacterial, antifungal and broad spectrum antibiotic compounds.

Key words: Actinobacteria, *Streptomyces*, Mangrove, antimicrobial, UV Spectrophotometric, HPLC.

Introduction

Mangrove ecosystem has a saline environment rich with organic matter due to microbial enzymatic and metabolic activities. Several

Mangrove ecosystems are well known potent areas for distribution and occurrence of microbes (Gupta et al. 2007 and Xu et al. 2009). Mangroves inhabit intertidal zones and can tolerate a wide range of salinities (Liang et al. 2008). They are regarded as highly productive ecosystems and abode to unexplored microbial diversity including actinobacteria (Hong et al. 2009).

Marine environment contains a wide range of distinct microorganisms that are lack in terrestrial environment. Though some reports were available on antibiotic and enzyme production by marine actinobacteria, marine environment is still a prospective source for new actinobacteria, which can yield novel bioactive compounds and industrially important enzymes (Sharma and Pant 2001). Actinobacteria are the most economically and biotechnologically worthy prokaryotes and are present in various ecological habitats such as soil, fresh water, back water, lake, compost, sewage and marine environment. They are considered to be highly valuable and hold a prominent position as targets in screening programs due to their diversity and their proven ability to produce novel secondary metabolites including antibiotics and other therapeutic compounds of pharmaceutical importance (Berdy 2005). Approximately, two-thirds of the thousands of naturally occurring antibiotics have been isolated from actinobacteria (Takaizawa et al. 1993) and majority from *Streptomyces* hence, these natural products is an extraordinary source for lead structures in the development of new drugs (Goodfellow et al. 1989). Several reports were available on marine actinobacteria, but only a few reports are available pertaining to actinobacteria from mangroves.

In India, only 41 species of actinobacteria belonging to eight different genera have been isolated and studied for its potential role (Sivakumar et al. 2007). *Streptomyces* were the major isolates in most of marine samples collected in the south east coastal region of India (Sahu et al. 2006). Marine *Streptomyces* are widely distributed in biological sources such as fishes, molluscs, sponges, seaweeds, mangroves, besides seawater and sediments. Studies were aimed mainly on isolation, identification and maintenance of actinobacteria in different culture media but only few studies alone were reported to evaluate the antagonistic potential of marine actinobacteria, hence the present study aims at the isolation and characterization of biologically diverse strains of actinobacteria from mangrove soil for the production of bioactive secondary metabolites which were identified by HPLC and UV –Visible

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absorption spectrophotometry with the ultimate objective of discovering novel bioactive compounds.

Materials and methods

Collection of soil sample

Soil sample were collected from 10-15 cm depth from mangrove regions in local area of Visakhapatnam. Samples were taken in a zipped polythene bags and were carried to the laboratory under aseptic conditions for further studies.

Isolation of Actinobacteria

Actinobacteria were isolated by serial dilution plate technique using Glucose yeast extract malt extract agar media, Starch casein agar media and Actinomycetes isolated media. The media is supplemented with Rifampicin 5µg/ml and Fluconazole 25µg/ml to inhibit unwanted bacterial and fungal contamination respectively. The sediment sample was diluted with sterilized distilled water and an aliquot of 0.1ml was spread on the media. After incubation for 2-4 weeks at 28° C, the actinobacterial colonies developed on the plates were identified by their chalky, firm and leathery texture of the colonies. They were sub cultured and maintained in starch casein agar slants for further characterization.

Characterization of Actinobacteria cultures

The selected potential actinobacteria strains were studied for morphological, cultural, physiological and biochemical characteristics (Shirling & Gottlieb 1966). The morphology of spore chain and spore bearing hyphae were identified using optical microscope at 1,000 X magnification. The color of spore mass was examined under light microscope and estimated by color chart (Pridham 1965).

Screening of the actinobacteria for antagonistic activity

The isolated actinobacteria were screened for the antagonistic activity using primary and secondary screening. Primary screening was done by cross streak method (Lemos et al. 1985) and the secondary screening was done by agar well diffusion method (Audrey 2007) against selected bacteria and fungi and the lead isolates were selected and studied further.

Fermentation

Based on the results of primary screening, 12 putative isolates were selected for the fermentation and assessment of antibiotic production. The isolates were inoculated in 250 ml Erlenmeyer flasks containing production medium. The production medium consists of Glucose 1%, Soya bean meal 1%, NaCl 1%, and CaCO₃ 0.1%. The inoculated cultures in the production medium were incubated at 28° C for 96 hrs at 180 rpm on a rotary shaker (Siva kumar et al. 2011).

Extraction of crude secondary metabolites

After fermentation the fermented broth was centrifuged at 4,000 rpm for 10 min and the filtrate was separated. The supernatant was extracted twice with ethyl acetate and shaken vigorously for 1 hr for complete extraction. The ethyl acetate phase that contains bioactive compound was separated from the aqueous phase. It was evaporated to dryness under reduced vacuum 80°-90°C and the residue obtained

was used to determine the antimicrobial activity (Westley et al. 1979).

Determination of Antimicrobial activities

The antimicrobial activities were determined by agar well diffusion method (Audrey 2007). 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 24 hrs of incubation at 37° C for bacteria and 48° C for fungi and the zone of inhibition were expressed as diameter (mm).

UV-Visible Spectrophotometer and HPLC Analysis of Crude Extracts

The dried ethyl acetate extracts of the four isolates (BC 01, BC 02, BC03 and BC 04) were analyzed by HPLC and UV/VIS spectrophotometer. The samples were mixed with HPLC grade methanol and filtered using 0.22µm Millipore membrane filter and were analyzed for UV spectra. The absorption spectrum of four active extract were determined in the UV region (200-400nm) by UV/VIS spectrophotometer (UV-1800-Shimadzu) by using UV Probe software. Analytical HPLC was carried out with Waters Spherisorb 5µm ODS2 4.6 X 250 mm analytical cartridge (C-18 column) on a Waters 515 pump; isocratic Reverse phase system with a 2998 photodiode array detector at 210nm and the range given was 190-600 nm. The flow rate was 1.0ml/min, and additional UV detector was measured at 254 nm by using Empower 2 software. The mobile phases Methanol and water were (HPLC grade) used in the ratio of 80:20. The samples were run for 10 min and the retention time was noted. The elution time was compared with the standards and the bioactive compounds were assumed as described by Mohan & Vijayakumar (2008).

Results and discussion

The filamentous bacteria, especially *Streptomyces* are commonly found in all most all habitats. In the present study, totally 20 different actinobacterial strains were isolated from the mangrove soil collected from the local area.

Screening of actinobacteria for antibiotics

Biological activity testing of fermentation products from the actinobacteria revealed to have antagonistic activities against pathogenic bacteria and fungi. Out of 20 isolates, 12 isolates showed the significant activity against test microorganisms during the preliminary screening. These 12 isolates were cultivated in specific fermentation liquid medium for 96hrs. After fermentation, the antimicrobial compounds were purified from the filtrate by solvent extraction method. These isolates were then subjected to secondary screening, only 8 isolates were found to active against test organisms while the remaining 4 isolates exhibited very poor activity. Of these 8 isolates the four isolates BC 01, BC 02, BC 03, and BC 04 exhibited broad spectrum of antimicrobial activities and the results are shown in Fig: 1, 2 and 3. The isolate BC 01, exhibits the highest activity against *P.vulgaris* whereas moderate activity was observed against *E. coli*, *B. Subtilis* *S. aureus* *P. aeruginosa* and *B. cereus*. The isolates BC 02, BC 03 and BC 04 exhibited

highest activity against *P. vulgaris*, *E. coli*, *B. Subtilis* and exhibited moderate activity against *S. aureus*, *P. aeruginosa*, and *B. cereus*. In case of the antifungal studies, the four isolates BC 01, BC 02, BC 03, and BC 04 exhibited highest activity against *A. niger*. BC 01, BC 02 and BC 04 showed moderate activity against *C. albicans*, *A. flavus*, and *S. cerevisiae* but BC 03, showed less activity. The present findings highlight the importance of further investigation towards the goal of obtaining the antimicrobial activities. The present study results were parallel with the results of Devi et al. (2006) and Singh et al. (2006) towards the antagonistic activity of marine actinomycetes against the pathogenic bacteria and fungi.

Characterization of the isolates

Among all the active isolates, only four isolates BC 01, BC 02, BC 03, and BC 04 exhibited significant antimicrobial activities against the human pathogens used in this study. The morphological, physiological and biochemical characteristics of these isolates were studied, to identify them up to genus level (Table I). Based on the results obtained, the active isolates were identified as genus *Streptomyces*. Exploitation of terrestrial actinobacteria over many years estimated 95% rediscovery rate of known compounds but *Streptomyces* from the estuarine ecosystem were halophilic nature and were adapted to the rhizosphere soil, will produce novel antibiotics. It is evident that mangrove rhizosphere habitats are an abundant novel source of actinobacteria for discovering natural products originated from terrestrial environment (Fenical et al. 1999). Mangroves are a poorly studied ecosystem for the isolation of bioactive compounds, there is an essential need for the development of new approaches for isolation and subsequent description of potent actinobacteria. The present results were found to correlate with the earlier reports which showed that actinobacteria are isolated from rhizosphere soil of mangroves and all of them showed antibacterial and antifungal properties against human pathogens (Rajesh Kannan. 2011).

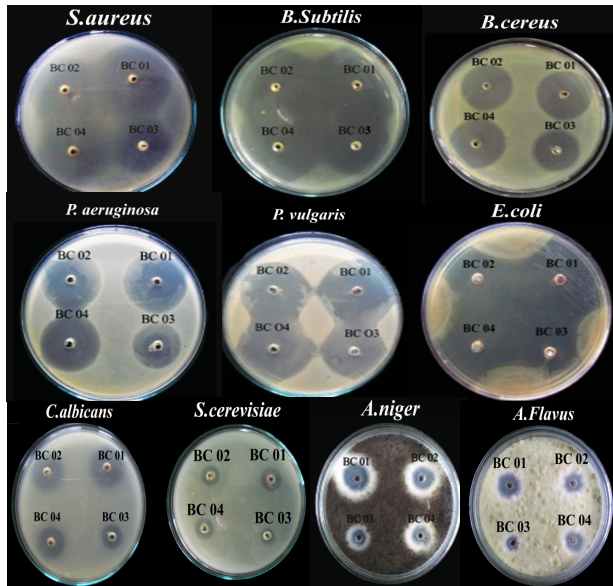


Fig 1: Antimicrobial activities of the four putative isolates

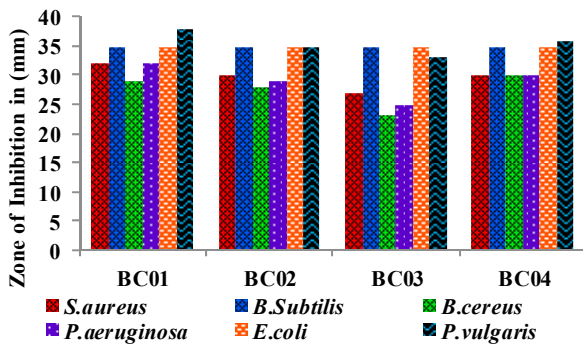


Fig 2: Antibacterial Activities of the four putative isolates

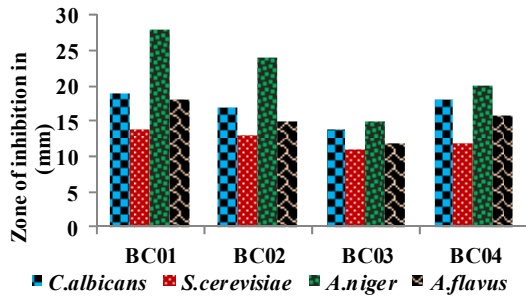


Fig 3: Antifungal Activities of the four putative isolates

Table 1: Morphological, Biochemical and Cultural characteristics of the four Putative isolates

Name of the test	BC 01	BC 02	BC 03	BC 04
Substrate Mycelia	White	Pale green	Creamy	Green
Aerial Mycelia	Gray	Black	Grey	Dark green
Pigmentation	Nil	Bluish black	Black diffusible pigment	Nil
Spore bearing aerial hyphae	Flexuous	Filamentous	Monoverticillus	Biverticillus
Growth	Good	Good	Good	Poor
Indole production	+	+	+	+
Methyl red	+	+	+	-
Voges proskauer	+	-	+	+
Citrate utilization	+	-	+	-
Melanin Formation	+	-	+	+
Gelatin Hydrolysis	+	+	+	+
Tyrosine Reaction	+	-	+	+
H ₂ S Production	+	+	+	+
Casein Hydrolysis	+	-	+	+
Starch Hydrolysis	+	+	+	+
Nitrate Reduction	+	-	+	+
Urease	-	-	-	-
catalase	+	+	+	+
Gelatin	+	+	+	+

+ indicates positive, - indicates negative.

The UV absorption spectrum of the antimicrobial agents recorded a maximum absorption at 207 nm for BC 01, 227 nm for BC 02, 220 nm for BC 03 and 223nm for BC 04. The UV spectral data of the four isolates showed the maximum absorbance peaks in the wavelength range of 207-223 nm may be an indicator for the presence of polyene compounds in the culture extracts (Swaadoun et al. 1999). The absorbance peaks are displayed in Fig. 4. The

analysis of UV absorbance shows a differential pattern for each isolate and it might indicate that these isolates produce different bioactive compounds involved in the inhibition of bacteria and fungi.

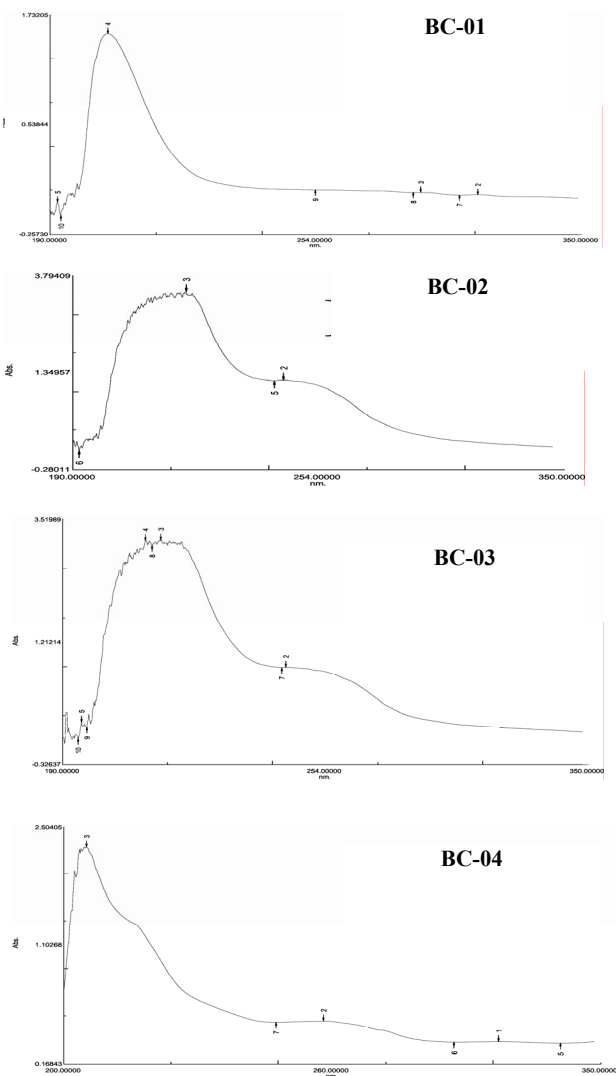


Fig 4: UV Spectra's of the four putative isolates

HPLC is being routinely used for the analytical estimation of various antibiotics. In the present study, HPLC profile of the antimicrobial compounds BC 01, BC 02, BC 03 and BC04 was performed by Spherisorb analytical cartridge (C-18) up to 10 min at 210 nm. The absorption peaks were showed in Fig: 5. the antimicrobial compound of BC 01 showed absorption peaks at RT (Retention time in min) 1.514, 1.941, 2.036, 3.146, 3.247, and 3.412. The major peaks showed at 1.941 and 2.036 min were the peaks representing the antimicrobial compounds of BC 01. The antimicrobial compound of BC 02 showed absorption peaks at RT 2.040, 2.944, 5.477 and 6.748 min. and the major peaks which had the antimicrobial activity were identified to be at 2.944, 2.040 and 6.748 min which represent the bioactive compounds. The antimicrobial compound of the BC 03 showed absorption peaks at RT 2.115 and 3.027 min. the major peak showed at 2.115 min was the peak representing the antimicrobial component. Similarly, the

antimicrobial compound of BC 04 showed absorption peaks at RT 2.054, 3.012 and 4.487 min and the major peak which had antimicrobial agent were identified to be at 2.054 min.

All the standard antibiotics were purchased from Hi Media (Mumbai). The reference standards were prepared by 1mg/ml concentration with a HPLC grade methanol and filtered through a 0.22 μ Millipore filter paper before injection into HPLC. The reference standard antibiotics used for the HPLC analysis are streptomycin, rifampicin, tetracycline, kanamycin and nystatin. All the standard antibiotics were detected at 210nm. All the four isolates showed the RT within the range of the above standard antibiotics. So the antimicrobial compounds are assumed as antibiotics with good significant value. The results from antimicrobial studies, spectral and HPLC analysis revealed that these strains were effective producers of antibacterial and antifungal compounds.

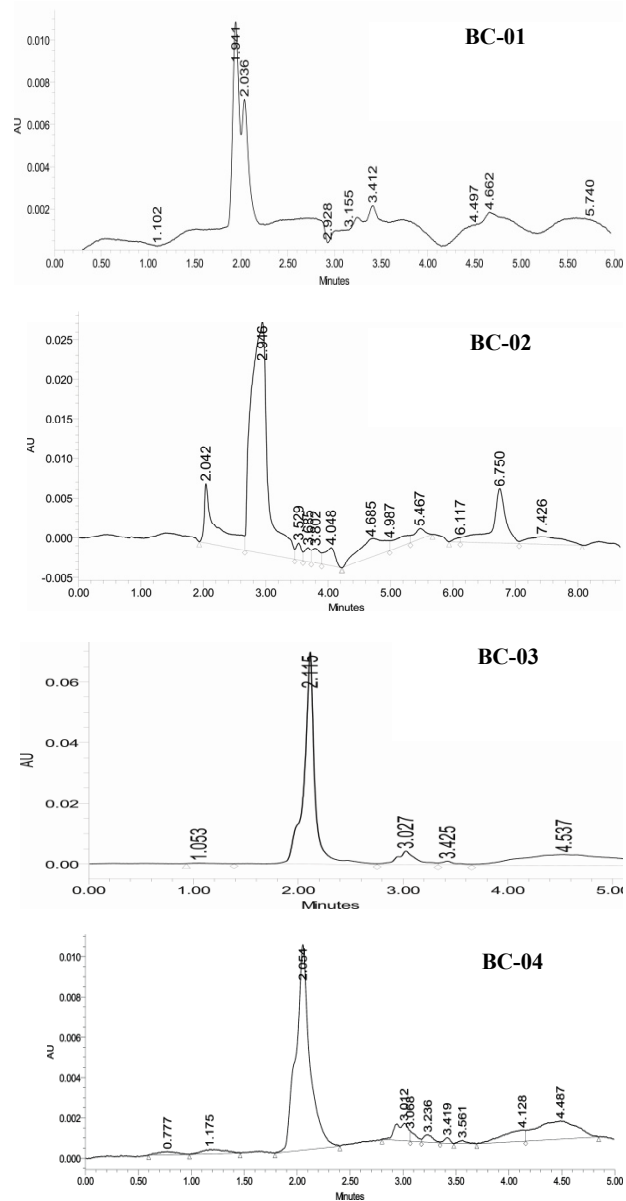


Fig 5: HPLC analysis of the four putative isolates

Conclusion

The present study was an attempt to identify and pick out the useful strains of *Streptomyces* from the mangrove area that display antimicrobial activity against a variety of microbial pathogens intrinsically and shows the importance of further investigation towards the goal of obtaining novel antimicrobial agents from this untapped habitat like mangroves, which appears to be an unexplored area in this environment and rich in biodiversity. Further studies on the characterization of the isolates, purification of the antibiotic substance and its anticancer activity and elucidation of its production pathways are underway. It is expected that the current attempt of isolation, characterization and the study on mangrove actinobacteria of the local area of Visakhapatnam will be useful for identification of new antibiotics effective against challenging pathogens.

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