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Diversity of Actinobacteria in Bamboo Ecosystems and its Antibiotic Activity Against MRSA

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Abstract: The major global health burden is rising of drug resistance. New screening approaches, exploration of unscreened ecosystem are continuously required to combat antibiotic-resistant concern. Bamboo which is the one of the important medicinal value plants and the impact of bamboo properties might influence the crucial activity of soil microbes. Rhizosphere microorganisms provide an excellent resource for the isolation and identification of therapeutic important products. Among them, actinobacteria are an important source in antibiotic development. Based on this view, in the present investigation, an effort was made to screen different ecosystem which is a unique unscreened and a diverse bamboo ecosystem for the isolation of potent antibiotic producing actinobacteria. Actinobacteria isolates were obtained from rhizospheres of bamboo in Megamalai forest, Western Ghats of Tamil Nadu, India. The physico-chemical properties of rhizosphere soil sample were analysed. Isolates were selected based on the different morphological characteristics. Totally 32 actinobacteria species, including rare M*icromonospora* and S*treptosporangium* were isolated. Based on the morphological characteristics, 22strains were screened for antibacterial activity against methicillin-resistant *Staphylococcus aureus* (MRSA).Among that, five actinobacterial strains BS 6, BS 9, BS 11, BS 12 and BS 19 have possessed zone of inhibition of 15mm, 14mm, 16 mm, 17 mm and 30 mm respectively. The screening results highlighted that bamboo environment represented a rich reservoir for isolation of actinobacteria, which are potential sources for discovery of prime antibiotics.

Keywords: Actinobacteria, bamboo, antibiotic, MRSA, antibacterial.

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Introduction

Infective pathogens ESKAPE (*Enterococcus, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa,* and *Escherichia coli*) are the major cause of infections worldwide. Antibiotic resistance of ESKAPE organisms is commonly related to higher morbidity, mortality and also affects economic growth [1]. Antibiotic resistance is one of the leading global health burdens. Hence, attention towards the ESKAPE organisms can help us to overcome the burden of antibiotic resistance. New screening approaches, exploration of unscreened ecosystem are continuously required to combat antibiotic-resistant concern.

Bamboo (*Bambusa vulgaris*) vegetation has the potential to sequester carbon from the atmosphere. Bamboo minimizes carbon dioxide in the atmosphere and generates 35% more oxygen than an equivalent stand of trees. Bamboo's another specific character is medicinal value. Each and every part of the bamboo such as leaves, shoot and root has potential for novel nutritional and medicinal source. Several varieties of the species possess significant antioxidant property; they can be used in the treatment of different diseases such as antitumor, anti-inflammatory, etc. Moreover the common secondary metabolites, extracts of bamboo contain biologically active polysaccharides and peptides. The combination of polyphenols with macromolecules may lead to potential biological activities like anti-microbial, anti-aging, anti-free radical, anti-fatigue [2], [3].

Bamboo which is one of the important medicinal value plants and the impact of bamboo properties might influence the crucial activity of soil microbes. The rhizosphere becomes a desirable niche for microbial communities to proliferate. One teaspoon of bare or tilled or bare soil contains more microorganisms than there are people on earth, i.e. the rhizosphere soil can have 10^{10}-10^{12} cells per gram. Due to the nutrient availability, microbial growth is highest in the rhizosphere compared to the bulk soil; however, many microbes are competing for these nutrients, some more successfully than others. The plant rhizosphere-microbe relationships that have received the most attention include those of a variety of research. Rhizosphere microorganisms provide an excellent source for the isolation and identification of important therapeutic products [4, [5]. Among them, actinobacteria are an important source in antibiotic development. Actinobacteria are well-known producers of several novel medicinally useful compounds. Actinobacteria are widely distributed in terrestrial and also in aquatic ecosystem. Actinobacteria are one of the most efficient groups of secondary metabolite producers and are very important from an industrial point of view. Among its various genera, *Streptomyces, Saccharopolyspora, Amycolatopsis, Micromonospora* and *Actinoplanes* are the major producers of commercially important biomolecules. Around 33,500 bioactive secondary metabolites are produced by microorganisms, among that 13,700 are produced by actinobacteria, representing 40 % of all bioactive microbial metabolites discovered. The actinobacteria are potential producers of antibiotics and other therapeutically useful compounds. The bioactive secondary metabolites produced by actinobacteria include antibiotics, antitumor agents, immunosuppressive agents and enzymes [6],[7].The genus *Streptomyces* was first introduced and discovered streptomycin from *Streptomyces griseus* by Waksman and Henrici in 1943. Continuous efforts of the scientific

community have been put in to explore the potential of these actinobacteria and their genus now contains over 860 species and subspecies isolated from various ecosystems [6]. Consequently, there is a higher possibility to isolate novel compounds from actinobacteria from bamboo rhizosphere.

Material and Methods

Study area

Megamalai also known as high wavy mountains, (Latitude:9.64612° N, Longitude: 77.40134° E) is located at an altitude of 1,500 m above the sea level in the Western Ghats in Theni zone in Tamil Nadu. The Western Ghats, a chain of mountain ranges (~1600km in length) that runs parallel to the west coast from the river Tapti (Gujarat) in the north to Kanyakumari (Tamil Nadu) in the south. It was declared as one of the 34 biodiversity hotspots of the world. The landscape is endowed with an array of vegetation types varying from wet (evergreen) forests on the western side to dry (thorn forests) in the eastern side due to wide altitude gradient (220–2000 m above sea level) and varied rainfall pattern (wind ward and leeward zones)[8]. For present investigation, we have selected three bamboo tree area sites for sample collection.

Collection of soil samples

All the three samples (MS1-MS3) were collected from the rhizosphere region of bamboo trees during May 2016.Rhizophere soil samples were collected at a depth of 15 to20 cm from the surface after removing the top layer.Samples were collected into sterilized zip lock covers using a spade whichwas thoroughly cleaned and disinfected during sampling so as to prevent cross-contamination. The soil samples texture morphology was recorded. Temperature was determined in situ with using mercury bulb thermometer. Gravimetric method used to determine the soil moisture content. Soil samples pH were analysed potentiometrically using pH meter.

Isolation and enumeration of actinobacteria

Isolation and enumeration of actinobacteria were processed by standard serial dilution plate technique. The soil samples were pre-treated at room temperature. One gram of soil was mixed into 99ml of sterile distilled water and agitated vigorously. Aqueous dilutions, 10^{-2} to 10^{-4} of the suspensions were prepared and aliquot of 0.1 ml from each dilution was taken and spread on starch casein agar (SCA)supplemented with cycloheximide (100µg/ml) and nalidixic acid (20µg/ml) to inhibit the growth of fungi and bacteria respectively. The plates were incubated at 28^{o}C for 10-15 days. After the incubation period, the actinobacterial colonies were counted and load of the actinobacterial colony was expressed as number of colony forming unit (cfu). After the enumeration of actinobacteria, morphologically different colonies of actinobacteria were streaked on yeast extract malt extract agar (YEME/ISP2) plates and incubated at 28^{o}C for 7-10 days to obtain pure colonies [9]. The isolated cultures were maintained by slant culture and preserved by glycerol stock for further studies.

Cultural characterization

The isolated cultures were observed by macroscopic and microscopic view. They were tentatively identified based on Bergey's Manual of Systematic Bacteriology. All isolated 32 actinobacterial cultures were inoculated into the YEME agar medium and incubated for 10 days at 28^0C. Cultural characteristics were observed and recorded including colony morphology, growth, consistency, aerial mass colour, presence of reverse side pigment and soluble pigment production.

Micro-morphological characterization

Micro-morphological characteristics were screened by adopting slide culture technique [9]. About 2ml of YEME agar medium inoculated with actinobacteria were poured as a thin layer over the surface of sterile microscopic slides. The slides were kept in sterile petriplates and incubated at 28^0C for 10 days. During incubation, the slides were observed under light microscope at 40X magnification. The characteristics like presence of aerial mycelium, substrate mycelium, mycelial fragmentation and spore chain morphology were observed and recorded as streptomycetes and non-streptomycetes / rare actinomycetes.

Screening of actinobacteria for antibacterial activity

Cross streak method

Preliminary screening for antibacterial activity was done by cross streak method. Twenty two actinobacteria strains were screened for antibacterial activity against clinical isolates such as methicillin-resistant *Staphylococcus aureus* (MRSA), *Bacillus subtilis, Escherichia coli, Klebsiella pneumoniae* and *Pseudomonas aeruginosa*. The isolates were cross streaked on Modified Nutrient Glucose Agar (MNGA) medium and incubated at room temperature for 5–7 days. After observing a good growth of actinobacterial cultures, overnight cultures of pathogens were streaked at the right angle of actinobacterial cultures. Plates were incubated at 37°C for 24hrs and the zone of inhibition was recorded. MNGA plates without actinobacteria isolates but streaked with the same stock of pathogens were used as control [9].

Agar plug diffusion method

Agar plug diffusion method is frequently used to highlight the antagonism between microorganisms. Mueller Hinton agar (MHA) plates were inoculated with overnight cultures of pathogens. Agar plugs of 5 mm in diameters of all isolated actinobacterial strains were placed on the plates and incubated for 37°C for 24 hrs. After the incubation, the plates were observed for the zone of inhibition and results were recorded [10].

Results

Soil analysis

The soil on the low hills is shallow, stony and dry. The soil is a thin layer of black peaty earth over yellow clay in the shola forests occurring at higher elevations. The top soils that are exposed

to high climatic range of temperature, winds and grazing become prone to erosion in rains of even moderate intensity. The Megamalai forest has red loamy soil. The rhizosphere soil sample is reddish brown with loamy texture with temperature range from 24-26^{o}C. The pH of the samples was observed in the range of6.9-7.4.

Isolation and enumeration of actinobacteria

The total actinobacteria population in the rhizosphere soil ranged from 6 x $10^4$to 7.5 x 10^4 cfu gm^{-1}. Totally thirty two actinobacteria strains were isolated based on the morphological difference. The stock of the isolates were prepared by using glycerol stock and also maintained by slant culture for further use.

Cultural characterization

All the thirty two isolated actinobacetrial cultures were studied for the cultural characteristics like colony morphology, growth, consistency, aerial mass color, presence of reverse side pigment and soluble pigment production and all the morphological pattern of actinobacterial strains were listed out in table 1. Among the 32actinobacterial strains, good growth was observed in 78.12% of strains and powdery consistency of 84.37% strains whereas 15.63% strains are exhibited in leathery consistency on ISP2 agar plates. Five different variety of aerial mass colour were observed such as white, gray, green, orange and yellow. Only 37.5% of strains were produced soluble pigment. Among 32 actinobacterial isolates, 22 isolates were finally selected based on their diverse cultural, morphological characteristics and its pigment production for further screening studies. Among that, fifteen strains were suspected as *Streptomycetes,* three were *Micromonospora,* two were S*treptosporangium* and another two were rare actinobacteria.

Table 1. Growth and morphological pattern of actinobacterial strains

Characteristics	**Appearance**	**No. of Isolates (%)**
Growth	Good	25 (78.12%)
	Moderate	7 (21.88%)
Consistency	Powdery	27 (84.37%)
	Leathery	5 (15.63%)
Aerial Mass colour	White	15 (46.87%)
	Gray	12 (37.5%)
	Green	2 (6.25%)
	Orange	1 (3.13%)
	Yellow	2 (6.25%)
Reverse side pigment		20 (62.5%)
Soluble pigment		12 (37.5%)
Micromorphology	Aerial and substrate mycelium	30(93.75%)
	Substrate mycelium alone	2 (6.25%)

Spore chain morphology	Rectus Flexibile (RF)	15 (46.87%)
	Retinaculum Apertum (RA)	6 (18.75%)
	Spirals (S)	2 (6.25%)
	Others	9 (28.13%)

Antibacterial activity

Out of twenty two strains, fourteen actinobacterial strains showed good antagonistic activity in cross streak method. Among that, seven actinobacterial strains (BS 2, BS 6, BS 9, BS 11, BS 12, BS 16 and BS 19) showed prominent activity against MRSA and seven actinobacterial strains(BS 4, BS 7, BS 8, BS 14, BS 17, BS 21 and BS 22)exhibited notable activity against gram negative bacteria such as *E.coli, K.pneumoniae* and *P.aeruginosa* (Table 2).

Table 2. Antibacterial activity of actinobacteria by cross streak method

S.No.	Strain No.	Test pathogens				
		MRSA	*B. subtilis*	*E. coli*	*K.pneumoniae*	*P. aeruginosa*
1.	BS 1	-	-	-	-	-
2.	BS 2	+	+	-	-	-
3.	BS 3	-	-	-	-	-
4.	BS 4	-	-	+	+	
5.	BS 5	-	-	-	-	-
6.	BS 6	+	-	-	-	-
7.	BS 7	-	-	+	-	-
8.	BS 8	-	-	-	-	+
9.	BS 9	+	+	-	-	-
10.	BS 10	-	-	-	-	-
11.	BS 11	+	+	-	-	-
12.	BS 12	+	-	-	-	-
13.	BS 13	-	-	-	-	-
14.	BS14	-	-	+	-	-
15.	BS 15	-	-	-	-	-
16.	BS 16	+	+	-	-	-

17.	BS 17	-	-	-	-	+
18.	BS 18	-	-	-	-	-
19.	BS 19	+	+	-	-	-
20.	BS 20	-	-	-	-	-
21.	BS 21	-	-	+	+	-
22.	BS 22	-	-	+	-	-

Based on the cross streak assay results, fourteen actinobacterial strains were selected and used to carry out for agar plug diffusion study. Among that BS 6, BS 9, BS 11, BS 12 and BS 19 showed prominent activity against test pathogens (Table 3). BS 19 possessed 30 mm zone of inhibition against MRSA (Figure 1). It clearly indicated that, BS 19 has novel bioactive secondary metabolite producing ability.

Table 3. Antibacterial activity of actinobacteria by agar plug diffusion method

S.No.	Strain No.	Test pathogens - zone of inhibition (diameter in mm)				
		MRSA	*B. subtilis*	*E. coli*	*K.pneumoniae*	*P. aeruginosa*
1.	BS 2	10	11	-	-	-
2.	BS 4	-	-	13	12	
3.	BS 6	15	-	-	-	-
4.	BS 7	-	-	12	-	-
5.	BS 8	-	-	-	-	11
6.	BS 9	14	12	-	-	-
7.	BS 11	16	15	-	-	-
8.	BS 12	17	-	-	-	-
9.	BS14	-	-	12	-	-
10.	BS 16	12	14	-	-	-
11.	BS 17	-	-	-	-	12
12.	BS 19	30	14	-	-	-
13.	BS 21	-	-	14	13	-
14.	BS 22	-	-	17	-	-

Figure 1. Antibacterial activity of actinobacteria by agar plug diffusion method

Active trend approach study on potential culture BS 19 may lead to obtain prime compound with antibiotic activity against multidrug resistant pathogens. The morphology of potential strain BS 19 is yellow to orange colonies with sticky aerial mycelia. The aerial mycelium and substrate mycelium were present. The soluble pigments were observed in yellow colour, but there is no melanoid pigment was observed. The potential strain BS 19 was tentatively identified as *Streptomyces* sp. based on the culture morphology.

Discussion

Bamboo is one of the perennial plants in medicinal field, they found all over the world. Recently, research has increased all the scientific community and evidence show the vast potential of bamboo as a therapeutic plant which is used in various medical practices. The pharmacological studies on bamboo such as antimicrobial, antioxidant, anticancer, anti-ulcer, anti-fertility properties etc., were exhibited critically line evidence of its potential [2-4].[11]. Hence, the present study focuses on the bamboo rhizosphere. Although the potential of Bamboo may vary, the more perspective view is required for critically assured outputs. The isolation and enumeration of actinobacteria from rhizosphere soil sample demonstrated that starch casein agar results with different colonies and good growth. The addition of cycloheximide and nalidixic acid to the medium enhanced the isolation of actinobacterial culture. Based on the observation, the SC agar with cycloheximide and nalidixic acid are effective for the isolation of actinobacterial culture from rhizosphere soils. It is generally known that pre-treatment and selective media are needed to avoid isolates of unwanted fast growing gram negative bacteria and fungi [9],[10].

A large body of literature describes the potential uses of plant-associated microbes as agents for stimulating plant growth and managing soil and plant health. Among that, bamboo plants have significant characteristics [11]. Additionally, many rhizosphere isolates make bioactive natural

compounds that are the potential source of novel products for use in medicine, agriculture, and industry such as new antibiotics, chemotherapeutic agents, and agrochemicals that are highly effective with low toxicity. The population diversity of actinobacteria is abundant in the rhizhophere of bamboo plant. Actinobacteria were the second most abundant group from the rhizospheric isolates of moso bamboo plants [12].[13]. Few antibiotics have been reported from the novel species. For example Aculeximycin, an antibiotic produced from *Streptosporangium albidum*[12]. *Streptomyces gramineus*sp.nov. has reported for antibacterial activity against plant-pathogenic bacteria. The secondary metabolites contain major fatty acids and isoprenoids squinones [13]. The isolates from bamboo (Bambuseae) rhizosphere soil, produces antibiotics and secondary metabolites against a broad range of bacterial and fungal pathogens. It shows the highest degree of similarity with *Streptomyces albosporeus* subsp. *labilomyceticus* [14]. It is interesting to note that novel species of the genus Streptomyces, *Streptomyces bambusae,* was reported for antifungal and antibacterial activities, isolated from bamboo [15].Keeping with this line, bamboo actinobacteria are the potential source for secondary metabolites and along with the CO_2 sequestration capability.

Conclusion

WHO estimates of global antibiotic resistance strains like *Escherichia coli, Klebsiella pneumoniae*, and *Staphylococcus aureus* are the three main agents of greatest concern, associated with both hospital- and community acquired infections. Colistin is the last antibiotic available in the world for infections when the strongest antibiotics fail to treat. The frequency increases the health burden worldwide, so need for novel antibiotics were increasing to fight against multidrug resistant bacterial pathogens. In this present study, BS 19 possessed 30mm zone of inhibition against MRSA in agar plug diffusion method. Thus, this actinobacterial strain may be a good source for potential drug against MRSA. The significant properties of Bamboo not only important in health concern, but also possesses environmental amenable too. Hence, the actinobacterial cultures which were isolated from rhizosphere of Bamboo may also be aimed for its CO_2sequestration properties.

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References

1. Mulani, M.S., Kamble, E.E., Kumkar, S.N., Tawre, M.S., and Pardesi, K.R, 2019, "Emerging Strategies to Combat ESKAPE Pathogens in the Era of Antimicrobial Resistance: A Review." Front Microbiol. 10 (539), pp.1-24.
2. Lee, H.J., 2013,"Biodiversity and Phylogenetic Analysis of Streptomyces Collected from Bamboo Forest Soil", Korean J. Microbiol.,46(3), pp.262-269.
3. Hao, Y., Chen, H., Wei, Y.M., and Li, Y.M., 2016, "The influence of climate change on CO2 (carbon dioxide) emissions: an empirical estimation based on Chinese provincial panel data", J. Clean. Prod., 131, pp. 667-677.
4. Petra, M., David, C., and Zed, R., 2011,"Rhizosphere interactions between microorganisms and plants govern iron and phosphorus acquisition along the root axis – model and research methods", Soil Biol. Biochem., 43(5), pp.883-894.
5. Amir, H. A., Richard, A.W., Pubudu, P.H., and Christer,J. 2017, "Rhizosphere engineering: Enhancing sustainable plant ecosystem productivity", Rhizosphere,3(2),pp.233-243.
6. Bérdy,J., 2012,"Thoughts and facts about antibiotics: Where we are now and where we are heading", J. Antibiot., 65, pp.385-395.
7. Su, H., Shao, H., Zhang, K., Li, G. 2016,"Antibacterial metabolites from the actinomycete *Streptomyces sp.* P294."J Microbiol. 54(2), pp.131–135.
8. Bhupathy, S., and Babu, S., 2013, "Meghamalai Landscape: A Biodiversity Hotspot",J. threat.Taxa, 5(15), pp. 4939–4944.
9. Balagurunathan, R., and Subramanian, A., 2001, "Antagonistic *Streptomycetes* from marine sediments", Advances in Biosciences, 20(2), pp.71– 76.
10. Balouiri, M., Sadiki, M., and Ibnsouda, S.K., 2016,"Methods for in vitro evaluating antimicrobial activity:A review", J. Pharm. Anal., 6,pp.71–79.
11. Han, J., Xia, D., Li, L., Sun, L., Yang, K., and Zhang, L., 2009, "Diversity of Culturable Bacteria Isolated from Root Domains of Moso Bamboo (Phyllostachysedulis)", MicrobEcol ., 58,pp.363-373.
12. Ikemoto, T., Matsunaga, H., Konishi, K., Okazaki, T., Enokita, R., and Torikata, A., 1983,"Aculeximycin, a new antibiotic from *Streptosporangium albidum*. I. Taxonomy of producing organism and fermentation", J Antibiot (Tokyo)., 36 (9),pp.1093-1096.
13. Lee, H.J., Han, S.I., and Whang, K.S. 2012, "*Streptomyces gramineus* sp. nov., an antibiotic-producing actinobacterium isolated from bamboo (Sasa borealis) rhizosphere soil", Int J Syst Evol Microbiol., 62, pp.856-859.
14. Su, H., Shao, H., Zhang, K., Li, G. 2016,"Antibacterial metabolites from the actinomycete *Streptomyces sp.* P294."J Microbiol.54 (2), pp.131–135.
15. Nguyen, T.M., and Kim, J., 2015,"*Streptomyces bambusae* sp. nov., showing antifungal and antibacterial activities, isolated from bamboo (Bambuseae) rhizosphere soil using a modified culture method", CurrMicrobiol., 71(6), pp.658-68.