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Distribution of Endophtyic Actinomycetes of Three Medicinal Plants and Evaluation of Their Antibacterial Potencies

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Abstract: This study was under taken to find actinomycetes diversity within some medicinal plants and focused on their antibacterial potentials. Presently endophytic microorganisms are less explored microbial habitat and most interesting source of novel antimicrobial agent. Three medicinal plants; *Vinca rosea*, *Cinnamomum* sp. and *Solanum sisymbrifolium* were selected for actinomycetes isolation. Surface sterilized stems were subjected to various media for endophyte isolation. Total 14 actinomycetes were isolated in this study in which 57 % belonged to genera *Streptomyces*, 7 % each of *Nocardia*, *Streptoverticillium* and *Micromonospora*, 15 % *Streptosporangium* and 1 isolate remained unidentified. Isolates were identified by microscopic (SEM), morphological, physiological and molecular characterization. All isolates were evaluated for antibacterial property against 5 Gram negative and 2 Gram positive human pathogenic bacteria. Study showed that endophytic isolate *Streptomyces thermovilaceus* NT1, *S. rochei* CH1 and isolate *Streptomyces* sp. BT5 were active against most of the test pathogens where no isolate except NT1 was active against *P. aeruginosa*. Based on above results it can be concluded that endophytic actinomycetes are an advanced source of potential antimicrobial agent for future medicine.

Key words: Endophytic, *Vinca rosea*, *Cinnamomum* sp. *Solanum sisymbrifolium,* Actinomycetes, Antibacterial.

Introduction

Actinomycetes are Gram positive, filamentous, high G+C content bacteria mainly reside in soil. This group of prokaryote is a high value industrial agent as it has the capability to produce a large number of antibiotics and other bioactive secondary metabolites [5]. But multiplication in the number of drug-resistant pathogens and the throttled success of strategies like combinatorial chemistry in providing new compounds turns us in finding some new strategies for microbial isolation. Endophytes are comparatively less investigated ecosystem yet to be explored. These microorganisms live symptomless inside plant's internal tissue and they play beneficial role in many respect including protection form pathogens by producing some secondary metabolites to their host in most cases [18]. This symbiotic association leads this group of microbes to provide better information in drug discovery because of probability for genetic recombination with host during coevolution [3]. This phenomenon again draws importance to plants with ethnobotanical properties to be more potent sources of endophytes producing active natural products than other plants [14].

There are many endophtyic actinomycetes already have been isolated as endophyte to various medicinal plants that produce significant

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bioactive compound and some of them with new chemical structure. *Vinca rosea*, *Cinnamomum sp* and *Solanum sisymbrifolium* are very well known for their medicinal values [8,10,11]. This study aimed to screen endophytic actinomycetes from these Indian medicinal plants and to evaluate their *in vitro* potential against selected human bacterial pathogens.

Materials and methods

Plant collection

Healthy stems of locally available medicinal plants (*Vinca rosea*, *Cinnamomum* sp. and *Solanum sisymbrifolium*) were collected from various places of Paschim Medinipur, West Bengal, India. Samples were collected in zip locked bag and immediately stored in 4°C till endophyte isolation.

Isolation of endophytic actinomycetes

Endophyte was isolated after proper surface sterilization of plant stems following standard method [21] with slight modifications. Stems of each plant were cut at about 1 cm length and washed well under running tap water to remove dusts. After rinsing with sterile water the samples were surface sterilized using sodium hypochlorite and 70% ethanol as surface sterilizing agent and then flamed after dipping in 90 % ethanol for 10 to 15 s to remove all epiphytic microorganisms. Barks were pealed, stems were thin sectioned to increase surface area of endophyte emergence and plated on water agar media, actinomycetes isolation agar media, glycerol-asparagine agar media (ISP5), and yeast extract malt extract agar media (ISP2) supplemented with actidione (50 µg/ml) after soaking in $NaHCO_3$ for 2 min. Incubation was done for at least 15 days at 28 ±2°C [7]. Endophytes were immediately made in pure culture when they came out and preserved in 30 % glycerol at -20°C.

Microscopic characterization of endophytes

All isolated actinomycetes strains were grown on media where they were isolated and sterile coverslips were placed at 45°C at edge of colony. Isolates were allowed to grow further a week and coverslips were then observed under compound microscope after Gram staining. Colonies were further scrapped and washed for three times with phosphate buffer saline (pH - 7.2). After addition of 0.25 % gluteraldehyde (in Na-phosphate, pH 7.2), cells were dehydrated in graded (30 to 100 %) ethanol and dried up to critical point after which attached to the stub. The sputter was coated with gold and used for SEM (Model- S530 HITACHI, JAPAN and Vega© TESCAN, Czech Republic). Cellular and spore morphology of each isolates were examined under compound and electron microscope.

Physiological and biochemical characterization

Aerial mycelium, spore mass color, substrate mycelium and pigmentations were recorded after growing the isolates in various media following the standard procedures [12,19]. Routine enzyme productions by isolates were checked for amylase, protease and cellulase by growing on specific media. Optimum growth temperature and pH was also determined after growing it in wide range of temperature and pH conditions [4].

Test microorganisms

In this study bacterial pathogens; *Aeromonas cavie* (ATCC 15468), *Vibrio parahemolyticus* ATCC 1782, *Pseudomonas aeruginosa* (ATCC 9027), *Proteus vulgaris* (ATCC 12453), *Shigella flexnerii* (ATCC 12022), *Bacillus subtilis* (ATCC 11774), *Bacillus cereus* (ATCC 14579) were used. All the pathogens were pure cultured in Muller Hinton Agar plate (MHA) and maintained in same broth with 30 % glycerol at -20°C.

Production of secondary metabolites

Isolates were grown on agar media and 1cm^2 actinomycetes grown agar blocks were transferred to individual conical flask containing 50 ml formulated secondary metabolite production media [bacto tryptone- 0.5 %, yeast extract- 0.2 %, dextrose- 1 %, K_2HPO_4- 0.2 %, NaCl- 0.2 %, $MgSO_4$, $7H_2O$- 0.05 %]. Production of antibacterial metabolites from isolated endophytes was carried out at 28 ±2°C for 12 days with 200 rpm orbital shaking.

Antibacterial assay by agar diffusion method

Antibacterial properties against selected pathogens of isolated strains were determined by

agar diffusion technique on MHA plates. Pathogens were grown in MH broth for 24 h and 100 µl was seeded on individual plates on which wells were made of 5 mm diameter. Each actinomycetes fermented broth was taken in separate microcentrifuge tube and centrifuged at 12,000 rpm for 15 min. Cell free 100 µl culture supernatant was loaded on each well of petriplate where sterile culture broth was treated as control. All plates were placed at 4°C for 30 min for better diffusion and then placed in incubator at 35°C for 24 h. Zone of inhibition if produced by endophyte culture broth was recorded. Each experiment was carried in triplicate and mean, standard deviation and standard error of each value was calculated by standard statistical methods.

Results

A total of 14 endophytic actinomycetes were isolated from surface sterilized stems in this study between which 6 strains were isolated from *Vinca rosea*, 3 from *Cinnamomum* sp. and 5 actinomycetes were isolated from *Solanum sisymbrifolium* **(**Table 1). All the strains produced various cultural characteristic on different ISP media. Their morphology, physiology and biochemical characters were summarized in table 2. It was found that *Streptomyces* were predominant (57 %) among isolated endophytic actinomycetes where as *Nocardia*, *Streptoverticillium*, *Streptosporansium*, *Micromonospora* were also obtained (Figure 1). Two of the isolates were identified up to species level and characterized as *Streptomyces thermovilaceous* NT1 (GenBank accession number KJ486841.1) and *Streptomyces rochei* CH1 (GenBank accession number KJ486840.1). They were isolated respectively from *Vinca rosea* and *Cinnamimum* sp. Colony morphology, cellular morphology with spore orientations of few isolates were presented in figure 2 and 3 respectively. Isolated actinomycetes *S. thermoviolaceus* NT1 was able to grow at 40°C though it was isolated at 28°C.

Culture filtrates of about 79 % of isolated endophytic actinomycetes were able to produce antimicrobial metabolite against test pathogens. *S. thermoviolaceus* NT1 was active against all test pathogens whereas *Streptomyces rochei* CH1 and *Streptomyces* sp. BT5 were unable to inhibit *P. aeruginosa* only (Figure 4). Rests of

Table 1. Isolated endophytes, their source plant and antimicrobial producing ability

Plant Name	Code of isolates	Endophyte isolates	Antimicrobial potential
Vinca rosea	NT 1	*Streptomyces thermoviolaceous* NT1	++
	NT 2	*Streptomyces* sp	+
	NT 3	*Streptomyces* sp	-
	NT 4	*Nocardia*	+
	NT 5	*Streptosporangium* sp	+
	NT 6	*Streptoverticillium* sp	+
Cinnamomum sp.	CH 1	*Streptomyces rochei*	++
	CH 2	*Micromonospora* sp	+
	CH 4	*Streptomyces* sp.	+
Solanum sisymbrifolium	BT 1	Unidentified	-
	BT 2	*Streptomyces* sp	+
	BT 3	*Streptomyces* sp.	+
	BT 4	*Streptosporangium* sp	-
	BT 5	*Streptomyces* sp	++

+: active against at least 2 of pathogens
++: active against most of the pathogens
-: active against none of test pathogens

Table 2. Summary of characterizations of some of the endophytic isolates

Characters	Code Names					
	NT1	**NT4 (ISP5)**	**CH1 (ISP5)**	**CH2 (ISP2)**	**BT4**	**BT5**
Colony morphology	Elevated, rough surface, dry, brownish to white colony.	Fine edge, chalky, dry irregular foldings, white to grey colony.	Elevated, wrinkled surface, dry, irregular folding white colony.	Elevated with irregular folding, dry, white to orange colony.	Dry, smooth surface, Whitish to grey center.	Smooth surface with irregular folding, dry. White to brownish.
Cell morphology	Highly branched filamentous, 0.2 to 0.8 µm diameter.	Bbranched filamentous 0.8 to1 µm diameter, whorle forming.	Highly branched mycelium.	Bbranched mycelium 0.8 to 1 µm diameter	Highly branched mycelium of 0.8 to 1 µm diameter.	Extensive branched mycelia of 1 to 1.2 µm diameter.
Spore morphology	3- 5 spores at filament tip. Cylindrical, smooth surface about 0.8 µm diameter.	Smooth, spiny spores at along with mycelia.	3-8 spores at tip of the filaments, Oval, smooth and hairy surfaceabout 1 to1.2 µm diameter.	Single spores on mycelium. Spores are spherical to oval, smooth.	Spherical sporangia 2.5 to 3 µm diameter. oval, smooth.	2-3 spores on mycelium. Spores are spherical to
Gram character	Gram positive, aerobic.	Gram positive, aerobic.	Gram positive, aerobic.	Gram positive, aerobic.	Gram positive, aerobic.	Gram positive, aerobic.
Aerobic growth	Grow well	Grow well	Grow well	Grow well	Grow well	Grow well
Extra cellular enzymes	Cellulase, amylase, protease (+) protease (-)	Cellulase, amylase, (+) protease (-)	Cellulase, amylase, (+) protease (+)	Cellulase, amylase, protease (+)	Cellulase (+), amylase (-), Protease (-)	Cellulase, amylase, (+)

table 2. (continued).

Characters	**Code Names**					
	NT1	**NT4 (ISP5)**	**CH1 (ISP5)**	**CH2 (ISP2)**	**BT4**	**BT5**
Growth temperature						
20°C	-	-	-	-	-	+
28°C	++	++ (*)	++ (*)	++	++	++ (*)
35°C	++ (*)	+	+	+	+	-
40°C	+	-	-	-	-	-
Growth pH						
5	-	-	-	-	-	+
7	+	++ (*)	+	++ (*)	++(*)	++ (*)
8	++ (*)	++	++(*)	++	+	+
10	+	-	-	+	-	+

* indicates optimum value, optimizations were determined of triplicate study
-: no growth
+: moderate

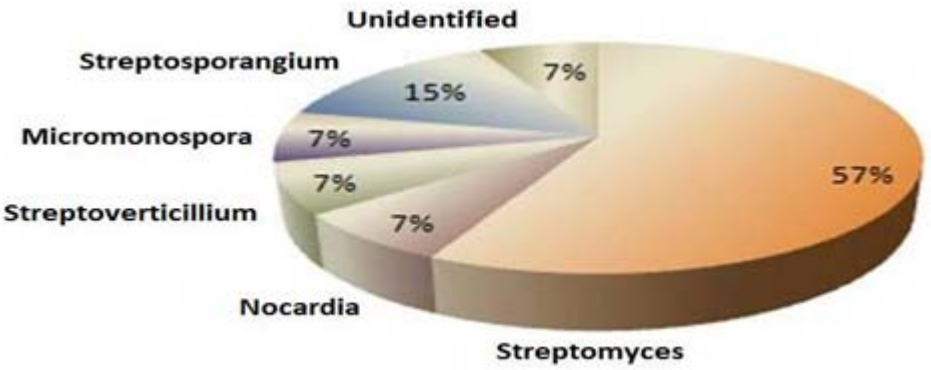

Fig 1. Distribution of various genera of endophytic actinomycetes among total isolates

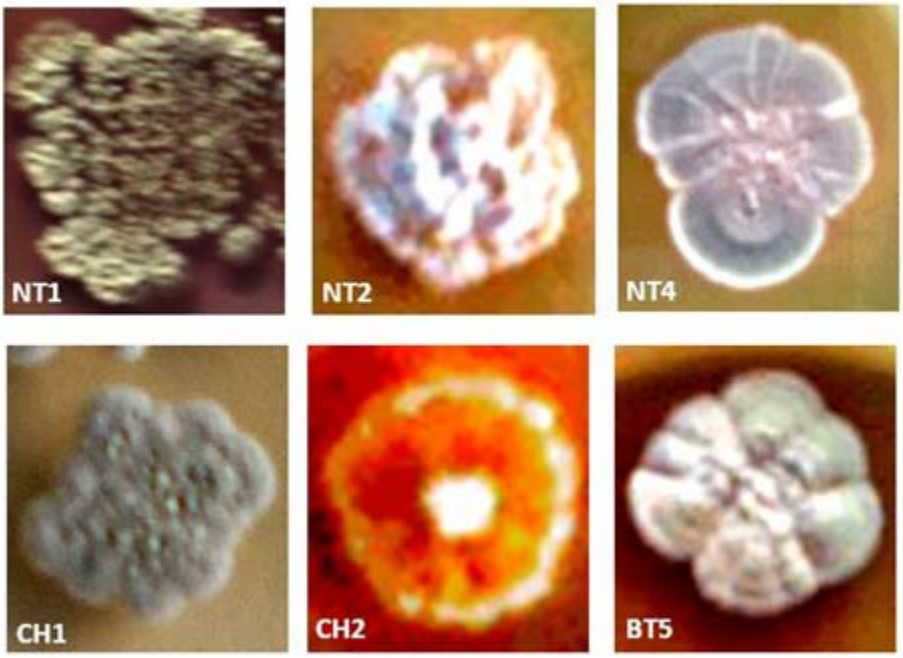

Fig 2. Colony morphology of various actinomycete isolates (from upper left isolates are NT1: *S. thermoviolaceus* NT1, NT2: *Streptomyces* sp., NT4: *Nocardia* sp., and from lower left isolates are CH1: *S. rochei* CH1, CH2: *Micromonospora* sp. and BT5: *Streptomyces* sp. respectively)

the 79 % isolates were active to at least 2 of the experimental pathogens (data not shown). In this study endophytic *Streptomyces* sp. BT1, was isolated from *Solanum sisymbrifolium* remain unidentified that need identification through molecular characterization. A total of three (21 %) isolates were found to be inactive against test pathogens.

Discussion

Currently research has started on isolation of endophytic actinomycetes but proper documentation of distribution of endophytic actinomycetes in Indian medicinal plants is yet unavailable. In addition yet it is unclear about role of those actinomycetes to medicinal properties of these plants. 5 different genus was obtained within 14 isolates suggest strong diversity among isolates. Some of isolated endophytes produce amylase, cellulase and protease to some extent in this study that may be for sustaining life and obtaining nutrient from internal plant tissues [15]. However during study on endophtyic actinomycetes from *Aquilaria crassna*, 10 actinomycetes were found and

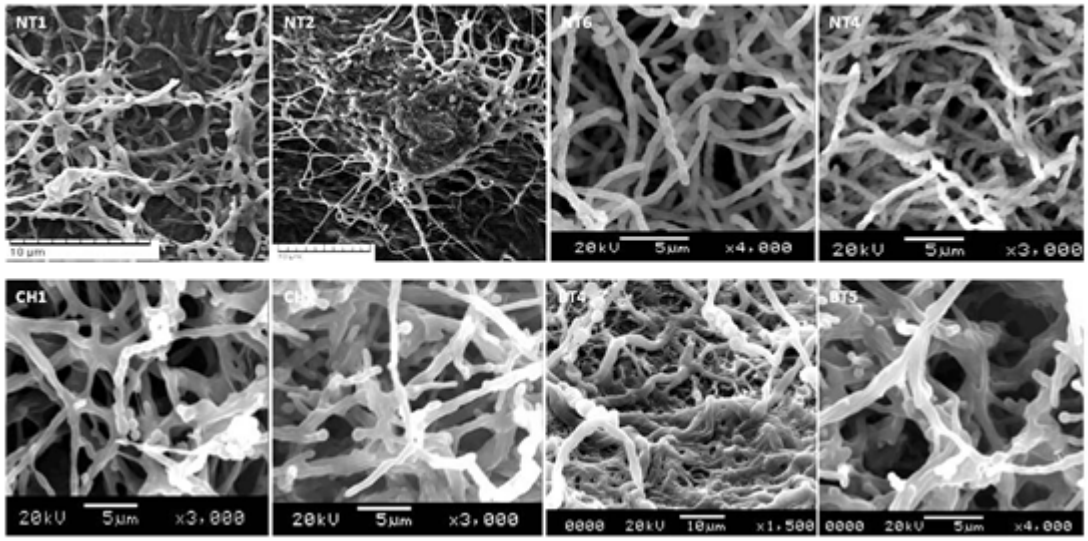

Fig 3. Scanning electron microscopic view of some antimicrobial producing endophytic actinomycetes (from upper left isolates are NT1: *S. thermoviolaceus* NT1, NT2: *Streptomyces* sp., NT6: *Streptoverticillium* sp., NT4: *Nocardia* sp. and from lower left CH1: *S. rochei* CH1, CH2: *Micromonospora* sp, BT4: *Streptosporangium* sp. and BT5: *Streptomyces* sp.)

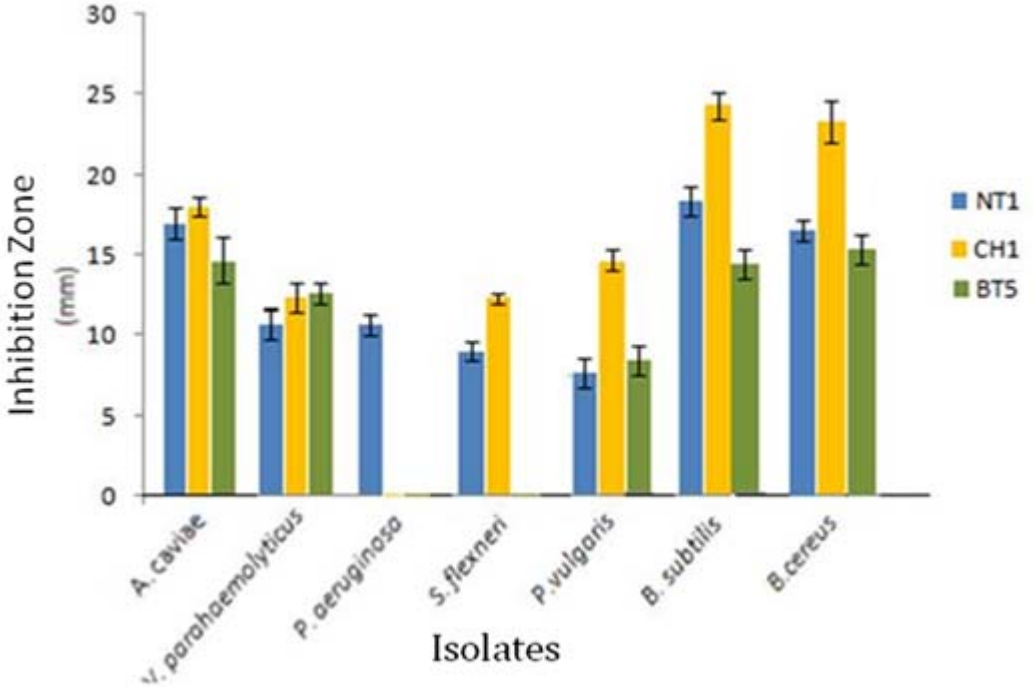

Fig 4. Anti bacterial potency of three selected actinomycetes against few human pathogens

isolates belonged to members of the genera *Streptomyces*, *Nonomuraea, Actinomadura*, *Pseudonocardia*, *Nocardia* and 2 were unidentified [9]. It is well known that diversity of isolated actinomycetes is largely dependent on the isolation method [2], we have tried here variety of media for maximize the isolate numbers and by suppressing emergence of undesired group of endophyte by using antifungal antibiotic and $NaHCO_3$ that reduce fungal growth.). In comparison to present result, 53 actinomycete were isolated from leaves and roots of maize (*Zea mays* L.) among which

43.4n% showed antimicrobial activity [1].

Recent studies have highlighted the bioactive importance of endophytic actinomycetes, including bio-control of fungal plant pathogens [16], production of plant growth regulators [6] and production of enzymes [13,17]. Most of the endophytic *Streptomyces* so far isolated are good anti fungal producer but fewer reports are found where they are lethal to bacteria [21].

All the plants selected in this study are well known for their medicinal properties, so isolation of residing actinomycetes from their internal tissues have tremendous scope for the screening of novel bioactive compounds. Based on the results of this study it can be concluded that endophytic diversity study of medicinal plants of India may offer some new species of actinomycetes with great potential for the synthesis of bioactive compounds.

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