Screening Of Endophytic Actinomycetes Residing In *Eucalyptus globus* For Antimicrobial Activity Against Human Pathogenic Bacteria

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ABSTRACT

Among the three Endophytic actinomycetes genera isolated from surface sterilized leaves, segment of Bark and segment of stem of Eucalyptus globus, streptomyces showed antibacterial activity against E.coli, K.pneumoniae, P.vulgaris and S.marsesense.Micomonospora and Actinomycetes did not show any antibacterial activity against the tested pathogen. The culture filtrate of Streptomyces species and Micromonospora was also evaluated for cytotoxic activity using human blood cells .No distinct effect could be observed in both tested cases.

Key words: Endophytic actinomycetes, Eucalyptus globus, antimicrobial activity

INTRODUCTION

Endophytic microorganisms are those which inhabitat the internal part of plants, causing apparently no visible changes to their hosts. It has been found also that some endophytic microorganisms can produce valuable pharmaceutical substances of biotechnological interest (Strobel et al, 1996; Strobel and Long,1998).One of the most promising endophytic micro-organisms to be isolated would be an actinomycete, or specifically a streptomycets, sincethese organisms often produce antibiotics. Recently, one of the first antibiotic-producing endophytic streptomyctes was isolated from a medicinal plant (snake vine, Kennedia nigriscans) used by Aboriginal Australians to treat and dress bleeding wounds (Castillo et al., 2002). The snake vine consistently yieldeda yellowish-orange culture of Streptomyces sp. which was subsequently demonstrated to produce a unique family of functionalized peptide antibiotics, the munumbicins. The rationale to direct a study of the snake vine plant was related to the traditional ethno botanical knowledge of Aboriginal people. Otherstreptomycetes may be found as endophytes in other higher plants, and ethno botanical or other approaches could be used to findthem.

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In the present study, leaves , stem and bark segment that were evaluated for endophytic actinomycete and their antimicrobial activity against human pathogenic bacteria such as E.coli, K.pneumoniae,P.vulgarisand S.marsesense.Micomonospora and Actinomyces. Moreover , he culture supernatant containing the crude antibiotic substance was also evaluated.cytotoxic activity against human blood cells.

MATERIALS AND METHODS

The materials used and the methods followed in conduct of various experiments are detailed here.

a)Selections of plant materials

Leaves, segment of bark and segment of stem of Eucalyptus globules were collected from the University campus for the present Investigation.

b) Size of the explants

The fresh plant materials were taken into the polythene bag in the laboratory under aseptic condition. Using sterile scalpel, the samples were cut from the explants approximately 1cm long and 0.5 cm diameter

c) Culturing and maintenance of endophytic actinomycetes

Endophytic actinomycetes were isolated from surface sterilized samples by the following method. Samples were washed in running water to remove soil particles and sterilized by sequential immersion in 70% (v/v) ethanol for 5 min and sodium hypochlorite solution (0.9% available chlorine) for 20 min. The surface sterilized samples were then washed in sterile distilled water for three times in order to remove the excess

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surface sterilization agents. The samples were soaked in 10% $NaHCO_3$ solution for 10 min to retard the growth of endophytic fungi.

Starch casein agar medium was prepared according to the method described by Demain and Davies (1999). The sterilized plant samples were then plated on to the medium under aseptic condition. They were then incubated at 28°C for 20 days. At a regular interval of 2-8 days, the presences of endophytic actinomycetes colonies were monitored on the plate.

Pure culture was maintained and stored on the starch casein agar slants. Based on the Bergey's manual of systemic bacteriology, the Endophytic actinomycetes colonies on the slant were then identified

d)Isolation of actinomycetes from the cultured broth

Actinomycetes isolates were cultured in a liquid broth as follows. 100ml of seed medium was prepared at a pH of 7.0. A loop full of colony from the respective slant was taken and it was then inoculated into the conical flask containing the seed medium. The inoculated flasks were shaken on a rotary shaker (200 rpm) at 30ºC for 3 days. 3 ml of the seed culture was transferred into a 500ml conical flask containing the production medium. The inoculated flasks were shaken on a rotary shaker (200rpm) at 30ºC for 6 days. The broths containing the mycelia were centrifuged at 10000 rpm for 5 min. The resulting supernatant was extracted and transferred to a 100ml fresh conical flask. Double the volume of ethyl acetate was then added to the flask. It was incubated in a rotary shaker at 30ºC for 2 hours. The organic layer was collected; evaporated using flask evaporator. The obtained residues were used for doing further bioassay.

e) Antimicrobial assay

In order to evaluate the antibacterial activity of the actinomycetes present in the extract, the following five human pathogenic bacteria such as Pseudomonas aeruginosa, Staphylococcus aureus, E. coli, Proteus vulgarius and Serratia marcesencs were used. All those strains were maintained on nutrient agar slants at 4ºC.

Muller Hinton agar plates were prepared and the plates were swabbed individually with the bacterial strains. Wells were made by using the gel puncher. 20µl of extract was then added to the wells and it was incubated at 37ºC for 24 hours. Later, the zone of inhibition was evaluated.

f)Cytotoxicity assay against Human blood cells:

Using 10ml sterile syringe a peripheral was collected in sterile 15ml centrifuge tube containing 0.1 % of EDTA and the collected blood was centrifuged at 2500rpm for 5mts.The supernatant was discarded and the collected pellet was washed with sterile PBS. 0.1 ml of washed cell suspension was incubated with cell supernatants with different concentrations ranged from 0.1 to 1 % in 0.9ml of PBS. The mixture was incubated at 37æ% C for 5hrs and every 30mts blood cell count was using Hemocytometer and microscopic examination with Leishmann stain.

RESULTS AND DISCUSSION

Isolation of Actinomycetes from eucalyptus

A total of 114 Actinomycetes colonies (isolates) belong to three different genera such as Streptomyces, Micromonospora and Actinomyces were isolated on starch casein agar media. All the isolates were identified based on their growth on selection medium and morphological characteristics of the colonies. Among the three parts tested, leaf recorded maximum Actinomycetes growth of 75 isolates, followed by segment of stem with 31 isolates and least Actinomycetes growth were recorded from segment of bark with 8 isolates.

Time taken to grow on the media was also changed in different plant part tested. All the isolates from leaf started growth after 3 days and full growth was observed in 7 days. Actinomycetes from segment of stem started growth at the 5th day and complete growth was observed at 10th day whereas the segment of bark had taken 10 days to growth and complete growth was observed at the 20th days (Table-1).

Heena Rintalaetial (2003) isolates "Streptomycetes in indoor environment by PCR based detection and diversity". The streptomyces are group of environmental bacteria present in almost all kinds of environments. They are common in soil, but also found in sediments, compost and fodder, aquatic habitats. A PCR based detection method was applied and tested with soil and dust samples. The PCR primers were specific for streptomycetes. Streptomycetes were detected in 81% of the dust samples by PCR and mesophilic actinomycetes in 36% of the samples by culture.

Sahar El-shatoury (2006) isolated the endophytic Actinomycetes from selected Medicinal

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plants". The Endophytic Actinomycetes were isolated from surface sterilized living aerial parts of four medicinal plants. Artemisia herba alba, Echinops spinosus, Mentha longifolia and Ballota undulate. A total of 41 isolates were obtained belonging to different genera with a prevalence of Streptomyces. The highest number of isolates from Mentha longifolia . Artemia salina had antimicrobial activities against four bacterial strain, and six clinical bacterial cultures and two clinical fungal cultures.

Among the five different human pathogenic bacteria tested against the isolated Actinomycetes revealed that all the pathogens except Pseudomonas aeruginosa (Table-2) were susceptible to Streptomyces. A inhibitory zone was formed around the bacterial culture was 12.7mm followed by 10.1 mm in Ecoli and K.pneumonia tested plates.. The other two Actinomycetes isolates didn't inhibit the growth of any of the bacterial cultures.

Luiz Henrique Rose et al., (2003) studied on "Antimicrobial Activity of Brazilian Basidiomycetes". A total of 103 isolates of Basidiomycetes representing 84 species from different ecosystem were evaluated for their antifungal and antibacterial activity in a panel of pathogenic and non pathogenic microorganism .Crude extract from Tyromyces duacinus, Irpex lacteus etc ... presented significant activity against one or more of the target microorganism .Eight isolates were active only against bacteria while three inhibited exclusively the growth of fungi. Differences in the bioactivity of extracts obtained from isolates from the same species were observed. .(Table 3)

Cytotoxicity assay:

The cytotoxic assay using human blood cells reveals no distinct cytotoxicity could be observed in both tested actinomycetes culture supernatant. Moreover no lysis, structural changes and reduction was observed .In both tested time periods RBC count was found to be stable and 1.94, 1.92 X 10^6 cells were recorded at 30min and 1 hr.Similiarly micromonospora treated RBCS showed similar counts at tested time periods.Eventhough any distinct cytotoxicity could be observed clumping of RBC occurred at 1hr of incubation.

The identification, Characterization of these Bioactive compound responsible for this antimicrobial activity is now progress.This study will be useful to exploit the metabolite produced by wide range of Endophytic actinomycetes as a novel drugs against human pathogenic multidrug resistant bacteria.

Table:1 Growth pattern of endophytic Actinomycetes from different part of Eucalyptus

S.No.	Parts	No. of Isolates	Time taken to grow	
			Initial Growth	Fully Mature
1.	Leaf	75	3 ± 1	7
2.	Segment of stem	31	5 ± 0.1	10
3.	Segment of bark	8	10 ± 0.5	20

Table 2: Antibacterial activity of Actinomycetes isolate.

S.No.	Tested pathogen	Zone of Inhibition (mm)		
		Streptomyces	*Micromonospora*	*Actinomyces*
1.	*E.coli*	12.7	0.0	0.0
2.	*Klebsiella pneumoniae*	10.1	0.0	0.0
3.	*Pseudomonas aeruginosa*	0.0	0.0	0.0
4.	*Proteus vulgaris*	11.3	0.0	0.0
5.	*Serratia marcesense*	13.1	0.0	0.0

Changes in Human RBC count using cultured filtrates of tested Actinoycetes

S.No.	Tested Actinomycetes	RBC Count (10 6 Cells)		
		0.5	*1.0*	*1.5*
1.	*Streptomyces*	1.94	1.92	1,92
2.	*Micromonospora*	1.94	1.92	1.92
3	.Control	2.0	2.0	2.0

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