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ISOLATION OF ENDOPHYTIC ACTINOMYCETES FROM MEDICINAL PLANTS AND ITS MUTATIONAL EFFECT IN BIOCONTROL ACTIVITY

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ABSTRACT

In the present study, the endophytic actinomycetes were collected from three medicinal plants *Azadiracta indica, Ocimum sanctum* and *Phyllanthus amarus*. Endophytic actinomycetes were isolated using different media like Starch casein agar, Starch casein nitrate agar, Actinomycetes isolation agar and Soyabean agar, while it showed more colonies in Starch casein agar. The endophytic actinomycetes were stained and biochemical tests were performed. Antimicrobial compound was purified from the filtrate by ethanol extraction method. Antagonistic activities of endophytic actinomycetes isolates were tested against bacterial pathogens (*Staphylococcus aureus, Streptococcus pyogenes, Klebsiella pneumoniae* and *Pseudomonas aeruginosa)* and the fungi *Rhizopus*. For the selected isolates antibiotic resistance was checked using various antibiotic discs like Amoxycillin, Penicillin, Rifampicin and Ampicillin. The strains which showed efficient antibacterial activity were selected to study the effect of mutation by physical and chemical method. In this study, UV mutated endophytic actinomycetes increase antibiotic production than non-mutated endophytic Actinomycetes, whereas in chemical mutation it does not increase the antibiotic production.

INTRODUCTION: India is rich in all three levels of biodiversity, as species diversity, genetic diversity and habitat diversity [1]. Today, there is a renewed interest in traditional medicine and an increasing demand for more drugs from plant sources. The use of medicinal plants by man for the treatment of diseases has been in practice for a very long time.

Screening of compounds obtained from plants for their pharmacological property has resulted in the isolation of innumerable therapeutic agents. In fact, many of the current drugs mimic naturally occurring molecules or have structures that are fully or in part derived from natural motifs [2, 3].

It is believed that the whole plant has more effective healing properties than its isolated constituents. Any part of the plant may contain active components [4].

Azadiracta indica is widely used in traditional system of medicine for centuries now. Each part of *A. indica* is used in medicines and thus commercially exploitable. It is also considered to be a natural source for medicines and industrial products. The bark, seeds, leaves, fruit, extracts and oils of the neem tree contain pharmacological constituents which offer some impressive therapeutic qualities, like antimicrobial [5], anti-pyretic and anti-inflammatory [6], anti-tumour [7], anti-helminthic activities [8].

Ocimum sanctum is found growing naturally in moist soil nearly all over the globe. In ayurvedic system of medicine*, Ocimum sanctum* has been employed for the treatment of diseases like cancer, leprosy [9], hepatic disease, paralysis [10], urinary stone track disease, depression and other nervous disorder [11] and diabetes.

Phyllanthus amarus widely distributed throughout the trophics and subtrophics in sandy regions as a weed in cultivated and waste lands. The powdered leaves of *P.amarus* were given in the form of capsules to the patients with chronic viral hepatitis B. Due to its antiseptic, coolant, febrifugal, stomachic, astringent and diuretic properties of this plant it is very much utilized in traditional medicine [12].

Endophytic microorganisms are microbes that colonized inside plants tissues with symptomless to their hosts. The most frequently isolated endophytes are fungi, however, both gram positive and negative bacteria can be found as endophytes [13]. The endophytic actinomycetes which are associated with plants also play important role in protection their host from phytopathogenic invasions [14]. Several endophytic actinomycetes act as plant growth promoter by producing of phytohormone, indole-3- acetic acid (IAA) or iron chelating molecules, siderophores *in vitro* [15].

In modern agriculture, application of agrochemicals is still an invaluable and effective method to control plant diseases. However, since use of agrochemicals has been fallen into disfavour because of environmental pollution and detrimental effects on a variety of non-target organisms, potential use of microbe-basic bio-control agents as replacements or supplements for agrochemicals has increased in agricultural importance. The success of bio-control largely depends on basic knowledge about the selected antagonist.

The present study was undertaken with a view to test the potential of mutated and non-mutated endophytic actinomycetes and as biocontrol agents which were isolated from surface sterilized leaves of medicinal plants (*Azadiracta indica, Ocmum sanctum, Phyllanthus amarus*) against various human pathogenic bacteria like *Staphylococcus aureus, Streptococcus pyogenes, Klebsiella pneumoniae* and *Pseudomonas aeruginosa.*

MATERIALS AND METHODS:

Sample collection: The medicinal plant samples like neem (*Azadiracta indica*), thulsi (*Ocimum sanctum*) and *Phyllanthus amarus* were collected for the isolation of endophytic actinomycetes.

Isolation of endophytic actinomycetes from plant samples: The plant materials were first dried for about two days in room temperature and separated into root and leaf. Then it was immersed in 70% ethanol for surface sterilization followed by crushing them with phosphate buffer in mortar and pistil. The crushed leaves were plated on starch casein agar, Starch casein nitrate agar, Actinomycetes isolation agar and soya bean agar [16] plates containing cycloheximide and nystatin (each at concentration of 50µg/ml of medium) and incubated at 28^0C for 7 days.

Colonies on the plates were examined using a microscope and picked on the basis of morphological features and colours of pigmentation and biochemical tests were performed for the identification of endophytic actinomycetes.

Agar Well Diffusion Method:

1. **Antibacterial activity:** The antibacterial activity was tested against ethanol extract of endophytic actinomycetes obtained from *Azadiracta indica, Ocimum sanctum* and *Phyllanthus amarus*. The inoculation of microorganism was prepared from bacterial culture [17]. About 15-20 ml of Muller-Hinton agar medium was poured in the sterilized petridish and allowed for solidifying. One drop of bacterial cultures like *Staphylococcus aureus*, *Streptococcus pyogenes*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* was inoculated by swabbing with sterile swab over the medium.

   Wells of 6nm in diameter and about 2cm apart punctured in the culture medium using sterile cork borers. Different concentrations (25, 50, 75 and 100µl) of the ethanol extracts were added to the wells and 75µl of ethanol was added to the centre well as control. The plates were incubated in air at 37^oC for 24 hours. Antibacterial activities were evaluated by measuring inhibition zone in diameters.

2. **Antifungal activity:** The antifungal activity was tested against ethanol extract of endophytic actinomycetes obtained from *Azadiracta indica, Ocimum sanctum* and *Phyllanthus amarus*. The inoculation of microorganism was prepared from the fungal culture. About 15-20 ml of Saborauds dextrose agar medium was poured in the sterilized petridish and allowed for solidifying. One drop of the *Rhizopus* culture was inoculated by swabbing with sterile over the medium. Wells of 6nm in diameter and about 2cm apart punctured in the culture medium using sterile cork borers. Different concentrations (25, 50, 75 and 100µl) of the ethanol extracts were added to the wells and 75µl of ethanol was added to the centre well as control. The plates were incubated at 37^{o}C for 24 hours. Antifungal activities were evaluated by measuring inhibition zone diameters.

3. **Antibiotic sensitivity test:** The isolated endophytic actinomycete strains were subjected to antibiotic sensitivity test. Muller Hinton agar was prepared and the strains were swabbed over the plate. Antibiotic discs like Penicillin, Ampicillin, Rifampicin and Amoxycillin were placed on the plate and it was gently pressed. The plates were incubated at 37^{o}C for 24 hrs and observed for inhibition zone diameters.

Effect of mutation of Antimicrobial activity: The strains which showed efficient antimicrobial activity were further selected to study the effect of mutation.

1. **Physical mutation:**

a. **UV Irradiation:** The selected strains were cultured in the tubes containing 9 ml Yeast Extract Malt Extract Broth. The tubes were inoculated with one loopful of the strain and incubated in a rotatory shaker at 30^{o}C for 96 hours. After incubation, the tubes were removed from the shaker and 3 ml of each culture was exposed to UV irradiation at a distance of 30 cm for 180 sec. One ml of the exposed cultures was transferred to 9 ml of Glycerol Starch Broth Medium and the tubes were incubated for 96 hours on a shaker at 30^{o}C. After incubation, the tubes were removed from the shaker and the broth was centrifuged at 2000rpm for 20 min and the supernatant was used to examine the post mutation effect on the strains for their antibacterial activity [18].

b. **Chemical mutation:** The selected strains were cultured in the tubes containing 9 ml Yeast Extract Malt Extract Broth. The tubes were inoculated with one loopful of the strain and incubated in a rotatory shaker at 30^{o}C for 96 hours. The culture broth was centrifuged at 3000 rpm for 10 min and the pellets were collected. The pellets were suspended with 2ml of TRIS buffer (pH 7.2) and 50 µg/ml of NN Dimethyl Diphenylene diamine sulphate was added to the test tubes. Then the test tubes were incubated at 30^{o}C for 30 minutes.

After incubation 1 ml of treated culture was added to 9 ml of GS fermentation medium (10g glucose, 10g soya bean meal, 1g $CaCO_3$ and 1 l H_2O, pH 7.5) and the tubes with culture was incubated for 96 hours on a shaker at 30^{o}C. The tubes were removed from the shaker and the broth was centrifuged at 2000rpm for 20 min. The supernatant was used to examine the post mutational effect on the strains for their antibacterial activity.

RESULTS AND DISCUSSION: The present study was undertaken in three medicinal plants namely Neem (*Azadiracta indica*), Thulsi (*Ocimum sanctum*), Phyllanthus (*Phyllanthus amarus*). The cultural characterization of endophytic actinomycete isolates were studied by using different media.

Starch casein agar has been recognized as a successful media for the better recovery of endophytic actinomycete compared with soyabean agar, actinomycetes isolation agar, starch casein nitrate agar, since these media showed growth along with fungi and unicellular bacteria. Hence, the maximum population was recorded in starch casein agar in Neem than thulsi and phyllanthus (**Plate 1, Fig. 1**).

PLATE 1: COLONY MORPHOLOGY IN *A. INDICA*

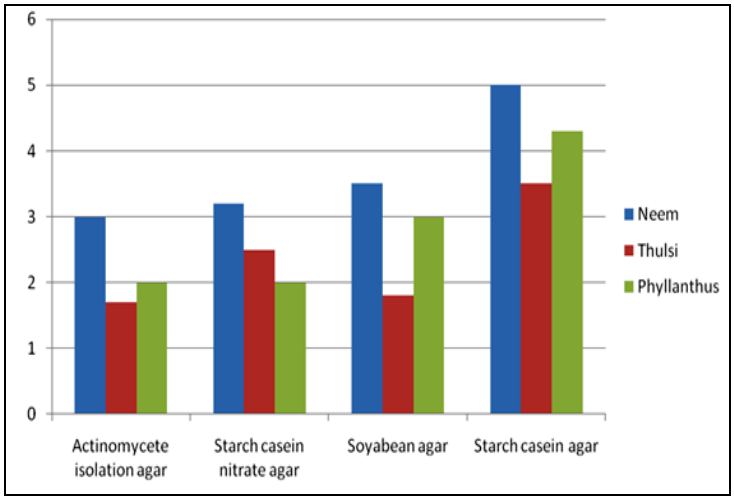

FIG. 1: CULTURAL CHARACTERIZATION OF ENDOPHYTIC ACTINOMYCETES IN THREE MEDICINAL PLANTS ON DIFFERENT MEDIA

The isolates were identified according to the morphological criteria including characteristic colony on plates, morphology of spores on pigment production. After 1-4 weeks of incubation in Starch casein agar, Starch casein nitrate agar, Actinomycetes isolation agar and Soyabean agar, the endophytic colonies were observed. The isolates were characterized based on the biochemical tests- Indole, Methyl red, Voges proskauer, Citrate utilization, Catalase and Oxidase.

The results on morphological, physiological and biochemical activity revealed that all isolated strains obtained from three medicinal plants belonging to *Streptomyces* sp. All the strains were found to be gram positive rod and showed branched mycelium in their morphology similar to fungal characterization (**Table 1**).

TABLE 1: BIOCHEMICAL CHARACTERIZATION OF ENDOPHYTIC ACTINOMYMCETES

S.No	Biochemical characterization	Strain isolated from *A.indica*	Strain isolated from *O. sanctum*	Strain isolated from *P. amarus*
1	Gram staining	Gram positive rod	Gram positive rod	Gram positive rod
2	Type of spore	Long chain spore	Spiral chain spore	Long chain spore
3	Indole	-ve	-ve	-ve
4	Methyl red	+ve	+ve	+ve
5	*Voges proskauer*	-ve	-ve	-ve
6	Citrate utilization	+ve	+ve	+ve
7	Catalase	+ve	+ve	+ve
8	Oxidase	+ve	+ve	+ve

Antagonistic activity:

1. **Antibacterial activity:** antibacterial activity of Endophytic actinomycetes obtained from *Azadiracta indica, Ocimum sanctum and Phyllanthus amarus* against all the pathogenic microbes was positive except *Phyllanthus*. When compared with three isolates, the *Azadiracta indica* isolate have a promising effect on antibacterial activity against the various pathogens like *Staphylococcus aureus, Streptococcus pyogenes, Pseudomonas aeruginosa* and *Klebsiella pneumoniae.* In that, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* shows a maximum zone of inhibition [2.5cm] whereas in *Ocimum sanctum*, the endophytic actinomycete isolates showed the inhibition only against *Staphylococcus aureus* [2.3cm] [**Plate 2, Table 2**].

TABLE 2: EFFECT OF ETHANOL EXTRACT ON ENDOPHYTIC ACTINOMYCETES IN MEDICINAL PLANTS AGAINST PATHOGENIC BACTERIA

Endophytic actinomycetes from medicinal plant	*Zone of inhibition against pathogenic bacteria in cm*															
	S. aureus				*S. pyogenes*				*P. aeruginosa*				*K. pneumoniae*			
	25 µl	50 µl	75 µl	100 µl	25 µl	50 µl	75 µl	100 µl	25 µl	50 µl	75 µl	100 µl	25 µl	50 µl	75 µl	100 µl
Strain 1 (A. *indica)*	1.3	1.5	1.7	2	1.8	2	2	2.2	2	2	2.2	2.5	1.8	2	2.2	2.5
Strain 2 (*O. sanctum)*	1.2	1.5	2	2.3	-	-	-	-	-	-	-	-	-	-	-	-
Strain 3 (*P. amarus)*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

No Zone

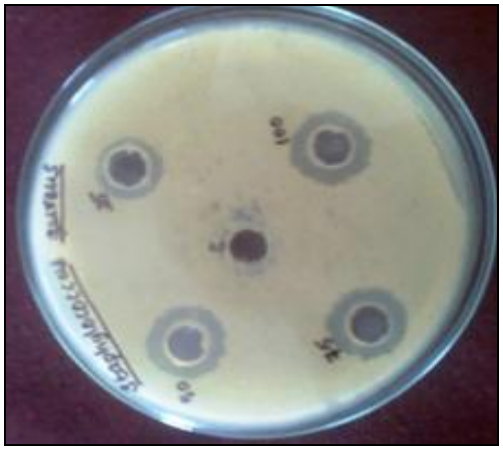

PLATE 2: ANTIBACTERIAL ACTIVITY OF *A. INDICA* AGAINST *S. AUREUS*

2. **Antifungal activity:** The endophytic actinomycetes isolates obtained from the three medicinal plants showed positive result, when tested against *Rhizopus* at different concentrations [25, 50, 75 and 100µl]. The maximum zone of inhibition was obtained in *Azadiracta indica* extract [2.8cm]. [**Plate 3, Table 3**].

TABLE 3: EFFECT OF ETHANOL EXTRACT ON ENDOPHYTIC ACTINOMYCETES IN medicinal plants against *Rhizopus*

S. No	Endophytic actinomycetes from medicinal plant	Rhizopus (Zone of inhibition in cm)			
		25µl	50 µl	75µl	100µl
1	Strain1 (*A. indica*)	2.5	2.6	2.7	2.8
2	Strain2 (*O. sanctum*)	1.2	1.3	1.5	2
3	Strain3 (*P. amarus*)	1	1.3	1.5	2

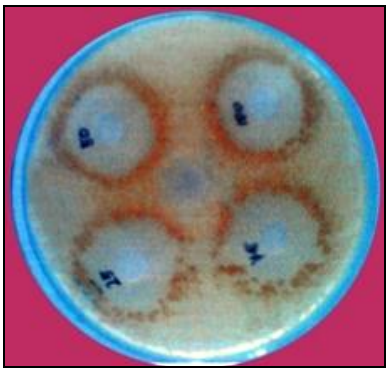

PLATE 3: ANTIFUNGAL ACTIVITY OF *A. INDICA*

3. **Antibiotic resistance:** The endophytic actinomycetes obtained from the three medicinal plants were tested for antibiotic resistance. The antibiotics used were Penicillin, Ampicillin, Rifampicin and Amoxycillin. In *A. indica* extract there was a complete inhibition of tested antibiotics. Whereas for penicillin disc, the *Phyllanthus amarus* extract showed 2.3cm zone of inhibition [**Plate 4, Table 4**].

TABLE 4: ANTIBIOTIC RESISTANCE OF MEDICINAL PLANTS

Endophytic actinomycetes from medicinal plant	Zone of inhibition in cm			
	Penicillin	Rifampin	Ampicillin	Amoxycillin
A. indica	-	-	-	-
O. sanctum	1.2	2	1.5	-
P. amarus	2.3	2.2	2	1.2

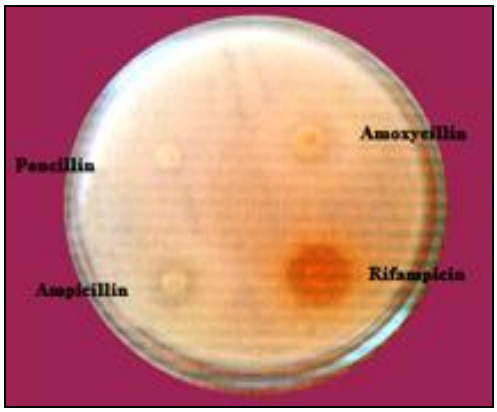

PLATE 4: ANTIBIOTIC RESISTANCE OF *OCIMUM SANCTUM*

Effect of mutation on Antibacterial Activity:

1. **Physical method:** The strain which showed efficient antibacterial activity was further selected to study the effect of mutation on their antibiotic production.

TABLE 5: EFFECT OF MUTATION ON ANTIBACTERIAL ACTIVITY (PHYSICAL METHOD)

Pathogenic organism	Zone of inhibition in cm		
	Mutated	Non mutated	Control
S. aureus	2.2	1.8	1
S. pyogenes	-	-	-
K. pneumoniae	1.8	1.7	1.3
P. aeruginosa	-	-	-

1. No zone of inhibition

The mutated strain was checked for their antibacterial activity against bacterial pathogens such as *Staphylococcus aureus, Streptococcus pyogenes, Klebsiella pneumoniae* and *Pseudomonas aeruginosa*. But the inhibition zone was observed only against *Staphylococcus aureus* and *Klebsiella pneumoniae* [**Plate 5, Table 5**].

PLATE 5: EFFECT OF PHYSICAL MUTATION IN *S. AUREUS*

2. **Chemical method:** The mutated strain showed more zone of inhibition when compared to non-mutated and control. The chemically mutated strain was checked for its antibacterial activity against *Staphylococcus aureus, Streptococcus pyogenes, Klebsiella pneumoniae* and *Pseudomonas aeruginosa*. But the strain did not show any inhibitory effect against any of the pathogen [**Plate 6, Table 6**].

TABLE 6: EFFECT OF MUTATION ON ANTIBACTERIAL ACTIVITY (CHEMICAL MUTATION)

Pathogenic organism	Zone of inhibition in cm		
	Mutated	Non mutated	Control
S. aureus	-	1.8	1
S. pyogenes	-	1.5	1.6
K. pneumoniae	-	1.7	1.3
P. aeruginosa	-	1.2	1

3. No zone of inhibition

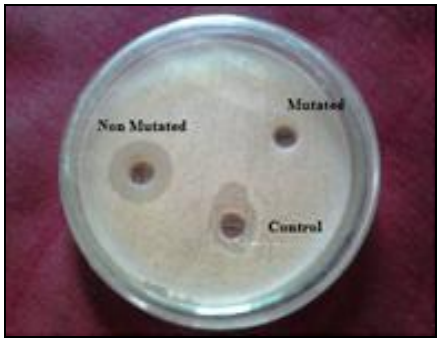

PLATE 6: EFFECT OF CHEMICAL MUTATION IN *K. PNEUMONIAE*

Endophytic actinomycetes are now considered as an existing novel source for obtaining new active bio compounds and have been reported from several hosts such as tomato, banana, wheat and maize with promising antimicrobial activity against pathogenic strains [19, 20, 21, 22]. Our present study suggests that endophytic actinomycetes offer promise for the discovery of novel natural products with pharmaceutical and agricultural potential.

CONCLUSION: We report that the *Streptomyces* sp after mutated by UV increase antibiotic production, whereas chemically treated strain showed decrease in production. This is due to the fact that the mutation of the active gene of this strain, responsible for the production of antibiotics might have been partially inactivated. This could indicate that bacterial species is more resistant to the antimicrobial substances produced by the actinomycetes.

It is assumed that actinomycetes that colonise internal tissues of medicinal plants are highly adapted to such habitat and have potential use as a source of bioactive agents.

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