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A potential surface sterilization technique and culture media for the isolation of endophytic bacteria from *Acalypha indica* and its antibacterial activity

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Abstract
The objective of the current study was to optimize the isolation procedure of the endophytic bacteria from medicinal plant. Medicinal plants are the potential hosts of endophytic microorganisms like bacteria, fungi, actinomycetes etc. Endophytic bacteria live symbiotically within the plant and in turn helping the plant in number of ways like growth, protection to environmental conditions, and sustainability, in favour of the hosts. They produce a wide range of bioactive compounds that are economical importance to humans. The present investigations were undertaken to optimize the isolation and identification of bacterial endophytes in leaf tissue of *Acalypha indica* plant growing at Coimbatore, India. Studies on the optimization of growth of the isolates were performed by varying growth medium. The ability of bacterial isolates were tested for abtimicrobial activity assay. Future studies will determine the potential medium to isolate endophytic bacteria that can be further applied for various applications like biological control, growth promotion and enzyme production.

Keywords: *Endophytic bacteria, Acalypha indica,* bioactive compound, optimization

Introduction
Bacterial endophytes are a class of endosymbiotic microorganisms including bacteria, fungi, and actinomycetes widespread among intra- and intercellular plant tissues for all or part of their life cycle do not cause plant disease or any morphological changes. Endophytes have the ability to colonize internal plant tissues of healthy leaves, petioles, stems, twigs, bark, root, fruit, flower, and seeds without causing any apparent harm or pathogenic infection to their host plants. Endophytic bacteria have been shown to have several beneficial effects on their host plant, including growth promoting activity, modulation of plant metabolism and phytohormone signalling that leads to adaptation to environmental abiotic or biotic stress. Use of endophytic bacteria presents a special interest for development of agricultural applications that ensure improved crop performance under cold, draught or contaminated soil stress conditions or enhanced disease resistance (Inga Miliute *et al*., 2015) [7]. Recenty Jha *et al*., 2013 [8] reported that endophytic microorganisms resides inside the plant to for improve plant performance in integration with plant disease management systems. Many bioactive metabolites are originated from microbial organisms, bacteria are the heart important groups of eukaryotic organisms that have wide capacity to produce numerous metabolites with antimicrobial activities and possess potential application as drugs. Recent studies have reported hundreds of natural products including substance of alkaloids, terpenoids, flavonoids, steroids etc. Metabolites of endophytes have been reported to inhibit a number of microorganisms (Fisher *et al*., 1984; Gurney & Mantle, 1993) [3, 5]. It is estimated that there might be as many as one million different endophyte species, however, only a handful of them have been described (Petrini; 1991) [11], which means investigating the metabolites of endophytes can increase the chance of finding novel antimicrobial natural products. There are numerous new endophytic species may exist in medicinal plants, it follows that endophytic microorganisms are important components of microbial biodiversity (Zhang *et al*., 2009) [14]. *Acalypha indica* Linn, commonly known as Indian copper leaf is a medicinal plant from the tropical Africa and the Indian Ocean islands is widely used in traditional medicine to treat skin

parasites, scabies and other skin problems. In addition it is used to treat asthma, and also to clean the liver and kidneys. The root decoction of this plant is also taken against intestinal worms and stomach-ache. *A. indica* plant may host useful microbial community that might be potentially has several biological and ecological role in their environment. However, information about the biological and ecological role of endophytic bacteria of *A. indica* plant is still unknown. Therefore, the aim of this study was to optimize the isolation and identification of bacterial endophytes survived inside the leaves of *A. indica* plant, to determine the antimicrobial. So far very little information is known about the biology bacterial endophytes; subsequently, isolation and characterization of bacterial endophytes that colonize different plant species of various habitats and ecosystem is potentially useful.

Materials and Methods

Plant sampling and study area

The plants of *A. indica* were collected at the flowering stage from SNMV college campus in Coimbatore, (11°1′6″N 76°58′21″E) Tamil nadu India. The plant materials were carefully placed in sterile polyethylene bags and brought to the laboratory in portable cool chambers (4°C). The green, healthy mature leaves were collected for isolating endophytic bacteria. Plant picture is shown in Plate 1.

Plate 1: *Acalypha indica*

Isolation of Endophytic Microorganism

Surface sterilization method

In order to get rid of exophytic bacteria and other dirt substances on the surface of the sample will be done by surface sterilization. It is usually accomplished by treatment of plant tissues with oxidant or general sterilizing agent for a period, followed by a sterile rinse (Hallmann, 2001) [6]. A new novel method of surface sterilization of medicinal plants was designed effectively to achieve the selective isolation of endophytic microorganisms from medicinal plants. Two methods were considered as base to design and formulate new protocols of surface sterilization. The collected plants were briefly washed under running tap water to remove the soil debris and further subjected to simple and new surface sterilization by subsequent soaking them in series of solutions as follows: sterile distilled water for 1 min, 2% of Sodium hypochlorite containing 0.1% of Tween 20 solution for 3 minutes, ethanol 70% for 1 min, and finally washed in sterile distilled water twice for 3 times and the excess moisture was blotted with a sterile filter paper. The last washing water was plated onto bacterial, fungal, and actinomycetales culture media of Nutrient agar, Czapek Dox agar, and Starch nitrate agar, respectively. The success of surface sterilization method was confirmed by the absence of any microbial growth onto the cultural media from the plating of last washing water. Surface sterilization was also performed by using the conventional method i.e. by immersing the leaf tissues in 70% ethanol for 5 minutes and then plating them in the culture media (Zin *et al*., 2007) [15].

Culture media for endophytic bacteria

The choice of growth medium is crucial as it directly affects the number and the type of endophytic bacteria that can be isolated from plant tissues. The sterilized plant leaves were cut by surgical blade into 0.5 cm segments. The sterilized leaves segments were macerated in sterile saline solution by sterile homogenizer. After 20 minutes one ml of sterilized crushed samples was serially diluted till 10^{-5} and 0.1 ml was spread onto Nutrient agar (A), TSA agar (B) and LB (C) agar media and incubated at room temperature. Regular observations were done from the 12 hours onwards for a period of 3-4 days for bacterial growth and monitored every day to check the growth of endophytic colonies from the crushed leaf tissues. The colonies were sub cultured into the fresh respective medium and stored at 4 ºC for further study.

Evaluation of Antimicrobial Activity

The pure culture of endophytic bacterial culture was used for antimicrobial activities by using well or cup agar diffusion method. Well agar was prepared by scooping out the MHA media with a sterile cork borer (6mm in diameter), the multidrug resistant human pathogens *Staphylococcus aureus* and *Pseudomonas aeruginosa* were swapped on LB agar. All the clinical pathogens were obtained from TNAU, Coimbatore, Tamilnadu. The wells were filled with 50 µl of the pure culture and the plates were incubated at 35±1ºC for 24 hours. After incubation the zone of inhibition was recorded and compared with control.

Results

Efficiency of surface sterilization methods on the growth and contamination of endophytic bacteria

Isolation of endophytes from inner parts of plant material is a significant step for pure culture isolation. Based on this rationale various innovative pretreatment methods have been explored. Better understanding of sterilization concepts has provided the basis for an explosion in number of chemical compounds which can be experimented for surface sterilization (Justin & Christopher, 2003; Cao *et al*., 2004) [9, 2].

In conventional method, (Zin *et al*., 2007) [15] there was no growth of endophytic bacteria in the 12 hours, but contamination was increased with prolonged incubation period. In new method, more number of bacterial colonies was observed from the 24 hours with maximum number of endophytic bacteria and no contamination.

Table 1: Efficiency of surface sterilization methods on growth and contamination of endophytic bacteria on NB, LB and TSA.

Methods	12 hours						24 hours						36 hours					
	NB		LB		TSA		NB		LB		TSA		NB		LB		TSA	
	G	C	G	C	G	C	G	C	G	C	G	C	G	C	G	C	G	C
Conventional method	--	--	+	--	--	--	+	--	++	--	--	+	+	++	++	++	+	++
New method	--	--	+	--	--	--	+	--	+++	--	+	--	++	+	+++	--	+	--

G-Growth, C-Contamination, NB-Nutrient Broth, LB- Luria-Bertani medium, TSA-Tryptic Soy Agar medium, +++ Highest growth, + + Moderate growth, + Low growth, -- No growth.

Efficiency of culture media on the growth of endophytic bacteria

The different medias like NB, LB and TSA broth are used during endophyte isolation strongly influence the number and diversity of cultivable bacteria. The samples from crushed leaf tissues of *Acalypha indica*ts were placed on three different media. More growth of endophytic bacteria was obtained in LB agar with almost negligible amount contamination. Whereas nutrient agar showed moderate growth with very less contamination. Maximum contamination negligible amount of growth of endophytic bacteria was observed TSA agar. Overall highest number of endophytic bacterial isolates were obtained from LB media followed by NB and TSA agar media.

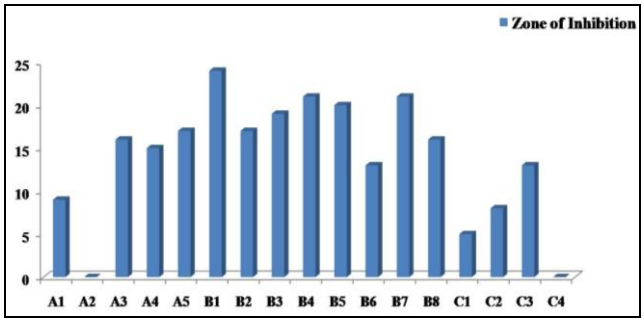
Isolation and screening of bacterial Endophytes

The pure culture of endophytic bacteria was used for antimicrobial activities against the multidrug resistant human pathogens *staphylococcus aureus*, and *Pseudomonas* by the well diffusion method. A total of 19 isolates from NB agar (A), 28 isolates from LB agar (B) and 14 isolates from TSA agar (C) respectively (Table 2). The bacterial crude extract was used for well diffusion method. Out of 19 isolates from NB agar 5 showed inhibition against resistant *S. aureus and Pseudomonas.* Out of 28 isolates from LB agar 8 isolates showed inhibition against resistant *S. aureus and Pseudomonas.* Out of 14 isolates from TSA agar only 4 isolates showed inhibition against resistant *S. aureus* and *Pseudomonas.* The zone of inhibition was obtained in methicillin resistance *S. aureus* plate. The zone of inhibition range was 2 to 24 mm (Graph 1).

Table 2: Observation of Antimicrobial Activity and measurement of Zone of Inhibition from the isolated Endophytic bacteria

Bacterial Code	Zone of Inhibition
A1	09 mm
A2	Negative
A3	16 mm
A4	15 mm
A5	17 mm
B1	24 mm
B2	17 mm
B3	19 mm
B4	21 mm
B5	20 mm
B6	13 mm
B7	21 mm
B8	16 mm
C1	05 mm
C2	08 mm
C3	13 mm
C4	Negative

A: NB agar B: LB agar C: TSA agar



Graph 1: Graphical view of measurement of zone of inhibition from the isolated Endophytic bacteria

Discussion

In the present study surface sterilization and culture media were optimized to obtain endophytic bacteria from a medicinal plant *A. indica*. To our knowledge, this is the first comprehensive report concerning the endophytic bacteria from *A. indica*. It can be considered that the new method employed in this study were productive and resulted in the successful isolation of numerous endophytic bacteria and that the isolates obtained can be considered as true endophyte. And paper also provides reports on simple and powerful method of surface sterilization of plant tissues for isolation of endophytic microorganisms. Surface sterilization of plant tissue for isolation of endophytic microorganisms using conventional method showed that surface sterilization with ethanol 70% was not effective to eliminate microorganisms on the plant surface. Ethanol 70% does not possess sporadically activity and is not efficient in eliminating spore forming bacteria.

To isolate true endophytes it is important to select a suitable sterilization method, and several factors need to be considered, one of which is safety, and this means avoiding antibiotics or potentially poisonous chemicals (Webster *et al*., 2003) [12]. Previously George (1993) [4] suggested that for the most effective disinfection of plant material is using of hypochlorite solution at pH 6-7. In this study low concentration sodium hypochlorite (0.1%) is found to be more effective disinfectant in eliminating plant surface microorganisms. The sodium hypochlorite showed a very low contamination of bacteria and fungus because sodium hypochlorite (NaOCl) is very effective as disinfectant agent against many bacteria as previously described (Zin *et al*., 2007) [15]. Hypochlorite (OCl^-) as a strong oxidant can denature by aggregating essential proteins of bacteria as previously described (Winter *et al*., 2008). Our results are in agreement with earlier studies on attempts using various sterilization methods (Nurul *et al*., 2012) [10]. In summary, combination of Sodium hypochlorite and Tween 20 was found to be very effective in eliminating epiphytic microorganisms.

Another part of this is to find the most suitable medium for the isolation of endophytic. Our studies suggested that using LB agar media is a good platform for isolating endophytic bacteria from the plant tissues. In future, isolation of endophytic bacteria from various medicinal plants will be a promising source of producing new antibiotic.

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