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Influence of different sterilizing methods on isolation endophytic bacteria from *Rauvolfia serpentina*

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Abstract
The plant micro-ecosystem has been associated to improved plant productivity, and bacterial endophytes are one of the major constituents of this ecosystem. Endophytic bacteria reside intercellularly within the plant tissues without causing any damage to host plant. Therefore, these bacteria are considered more advantageous for plant survival as protection from environmental stress and microbial competition. Population density of endophytic bacteria is less as compared to epiphytes or rhizospheric bacteria hence isolation of true bacterial endophytes without any contamination is a major challenge. In the current study different surface sterilization methods were conducted to isolate true endophytic bacteria from the roots of *Rauvolfia serpentina*. Ethanol, sodium hypochlorite, tween 20 and mercuric chloride at various concentrations and duration were employed to optimize the surface sterilization for the isolation of endophytes from *Rauvolfia serpentina*. Further morphological and biochemical characterization of isolates were carried out. Future studies will determine the potent endophytic bacteria that can be applied for various applications like growth promotion, biological control, and enzyme production.

Keywords: plant tissue, endophytic bacteria, surface sterilization, contamination

Introduction
The broadly accepted definition of endophytes that was given by Bacon and White [1] is microbes that colonize intra and intercellular plant tissues without having any negative effects to the host plant. As endophytes reside within plant tissues, they are exposed to a more specific and stable habitat than rhizospheric bacteria [2]. In 1926 Perotti [3] found endophytic growth at a specific stage in the life of bacteria, described as an advanced stage of infection and having a strong mutualistic relationship with the plant. Since then, endophytes have been characterized as microbes that could be isolated from surface-sterilized plant tissue [4]. Despite this fact presence of endophytes in plants is variable and, occasionally transient [5]. As endophytic bacteria are closer to plant than rhizospheric and phyllosphere bacteria, they have a direct and intense effect on the growth and development of plant [6]. In recent years, several studies demonstrated the effectiveness of endophytic bacteria in agricultural applications by protecting plants from a series of abiotic stresses including low temperature [7, 8] drought [9], and salinity [10]. Endophytes are also an important source of pharmaceutical bioactive metabolites such as antitumor, antioxidant, antibacterial, antifungal, antiviral, anti-inflammatory, immunosuppressive drugs, and many related compounds. Endophytes are also known for the production of different natural products and to exhibit a wide range of biological activity and are classified into numerous categories, which include steroids, terpenoids, phenolic compounds, lactones alkaloids quinines, lignans, etc [11].
The isolation procedure is mandatory and critical step while working with endophytes. Hypothetically, the sterilizing agent used, during isolation should be strong enough to remove any microbe present on the root surface at the same time sensitive enough to recover endophytic bacteria [12]. Though, it is challenging to achieve because in time the agent may invade the plant tissue and can be lethal for some endophytic bacteria. Therefore, the selection of isolation media and surface sterilization are important to maximize the successful isolation of endophytes, thus, enhancing the possibility to discover new endophytes [13].
Medicinal plants are an important resource of isolating endophytic bacteria, which can induce secondary metabolite of very important value. There are reports on numerous new endophytic species may exist in medicinal plants, it follows that endophytes are important components of microbial biodiversity [14]. *Rauwolfia serpentina* was commonly known as Sarpagandha, belonging to family Apocynaceae. *R. serpentina* has been used for the treatment of skin cancer, eczema [15].

Reportedly large number of alkaloids (1.7% to 3.0 %) is present in the roots of Sarpagandha [16].
The present study was carried out to show the effectiveness of surface sterilization methods to obtain pure bacterial endophytes from the roots of medicinal plant *Rauvolfia serpentina*.

Material and Methods

Sample collection of plant materials

Mature healthy medicinal plants of *Rauvolfia serpentina* were collected from Patanjali herbal garden site nursery, Haridwar, (29° 58' N, 78° 13' E Elevation: 298m.). Samples were placed in clean plastic bags, brought to the laboratory and used for further experimental purpose.

Isolation of endophytic bacteria

Surface sterilization: The root samples were washed under running tap water to remove the soil debris and were carefully excised and subjected to three different surface sterilization methods. The method I samples were immersed in 70% ethanol for 5 min [17]. Method II samples were surface sterilized with 70 % ethanol for 3 min, 0.5 % sodium hypochlorite for 3 min and 70 % ethanol for 30s [18]. Method III samples were washed with 0.1% mercuric chloride, then washed with 70% ethanol for 1 min and finally rinsed with 3% Sodium hypochlorite for -5 min [19]. Method IV samples were washed with Tween 20 (1 drop in 200ml sterile distilled water) and then immersed in 3% sodium hypochlorite (10 min in a shaking incubator at 120 rpm) later washed with 70% ethanol for 1 min [20]. In all methods root samples were washed with sterile distilled water after each step. Each method was performed in triplicate.

Sterility check: In each step of the surface sterilization process the root samples were washed in sterile distilled water. To monitor the effectiveness of the surface sterilization process, a sterility check was carried out for each sample. For this final rinse was plated on nutrient agar as control, incubated at 28ºC for 48 hrs and checked for possible microbial growth. Root sample was used if no growth appears [21].

Endophytic bacteria isolation, purification and preservation: For this purpose the surface-sterilized root segments were cut into a small disc of about 1cm pieces. Then placed on the plates containing medium and incubated at 28°C for 48 h [22].

Morphological and Biochemical Characterization of the Isolated Endophytic Bacteria

All selected Endophytic bacteria will be identified through differential staining; their morphological and biochemical properties will be evaluated as described in Bergey's Manual of Systematic Bacteriology [23].

Result

The efficiency of surface sterilization methods on the growth and contamination of endophytic bacteria

In order to obtain pure endophytes from inner root tissues *of Rauvolfia serpentina*, epiphytic microorganism and other contamination must be eliminated, through surface sterilization method. For this root samples were treated individually and by the different combination of a chemical disinfectant. Method I (70% ethanol) that was initially selected was not found an effective individually as a high percentage of contamination was observed along with the growth of endophytes. Whereas in method II, III, IV, root samples were treated with different combination and duration of ethanol, sodium hypochlorite, and mercuric chloride to achieve a satisfactory result. In method III, mercuric chloride was effective in eliminating contamination but survival percentage of bacterial endophytes decreased. Therefore, only method IV (3% sodium hypochlorite and 70% ethanol for 10 minutes) was found effective for surface sterilization of *Rauvolfia serpentina* root tissues, with high percentage survival and no contamination. The results of the optimization process for the surface sterilization are shown in the Fig.1.

Morphological and Biochemical Characterization of the Isolated Endophytic Bacteria

A total of 16 different bacterial isolates were selected on the basis of colony morphology and colors. (Fig. 2 and 3). Nearly 37% of isolates were gram-negative rods, 12% were gram-positive cocci and rest were gram-positive rods. Morphology and biochemical characteristics, isolates showed characteristics of genera *Bacillus*, *Pseudomonas*, *Micrococcus*, *Serratia*, and *Actinobacteria.* Details of the preliminary characterization are shown in Table 1.

Discussion

In the current study, surface sterilization method was optimized to obtain maximum bacterial endophytes from medicinal plant Rauvolfia *serpentina*. To best of our knowledge, this is the first comprehensive report concerning the endophytic bacteria from *Rauvolfia serpentina*. The population density of endophytic bacteria is less as compared to epiphytes or rhizospheric bacteria. Hence to avoid contamination or infection isolation of bacterial endophytes, root samples must be thoroughly surface sterilized before inoculating them into the solid agar medium. In this study, simple and powerful method of surface sterilization was employed for isolation of bacterial endophytes from the roots tissue. Surface sterilization of *Rauvolfia serpentina* medicinal plants for isolation of bacterial endophytes using ethanol 70% was found not effective to eliminate microorganisms present on the plant surface. Thus it was estimated that 70% ethanol was not efficient in eliminating epiphytic bacteria. Use of mercuric chloride for the surface sterilization of *Rauvolfia serpentina* was not found effective because mercuric chloride was found to be a good decontaminating agent, but the survival percentage of endophyte decreased. In this study, high concentration of sodium hypochlorite (3%) is found to be more effective than low concentration (0.5%) disinfectant in eliminating plant surface microorganisms. Another objective of the study was a preliminary characterization of isolates. Endophytic bacteria showed a wide range of morphological and biochemical characteristics indicating that they are different bacterial species.

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Table 1: Morphological and Biochemical Characterization of the Isolated Endophytic Bacteria

Strain	Gram Staining	Shape	Colour	Urease	Starch	Indole	MR	VP	Citrate	H_2S	Catalase
RS 1	+ive	Rods	White	-	+	-	-	+	-	-	+
RS 2	+ive	Rods	Colorless	-	-	-	-	-	+	-	+
RS 3	+ive	Rods	Creamish	+	+	+	+	-	-	+	+
RS 4	+ive	Rods	White	-	+	-	-	-	-	-	+
RS 5	-ive	Rods	Colorless	-	+	-	-	-	+	-	+
RS 6	-ive	Rods	Yellowish Green	-	+	-	-	-	-	-	+
RS 7	-ive	Rods	White	+	-	-	+	-	-	+	+
RS 8	+ive	Cocci	Creamish	-	+	-	-	-	+	-	+
RS 9	+ive	Rods	Milky white	-	+	-	-	-	-	+	+
RS 10	+ive	Rods	Milky White	-	-	+	-	-	+	+	+
RS 11	+ive	Rods	White	-	+	-	-	+	-	-	+
RS 12	-ive	Rods	Light green	+	-	-	-	-	+	-	+
RS 13	+ive	Cocci	White	+	-	+	-	-	-	+	+
RS 14	+ive	Rods	White	-	+	-	-	-	-	-	+
RS 15	-ive	Rods	Orange	+	-	-	-	-	-	+	+
RS16	-ive	Rods	Green	-	+	-	-	-	-	-	+

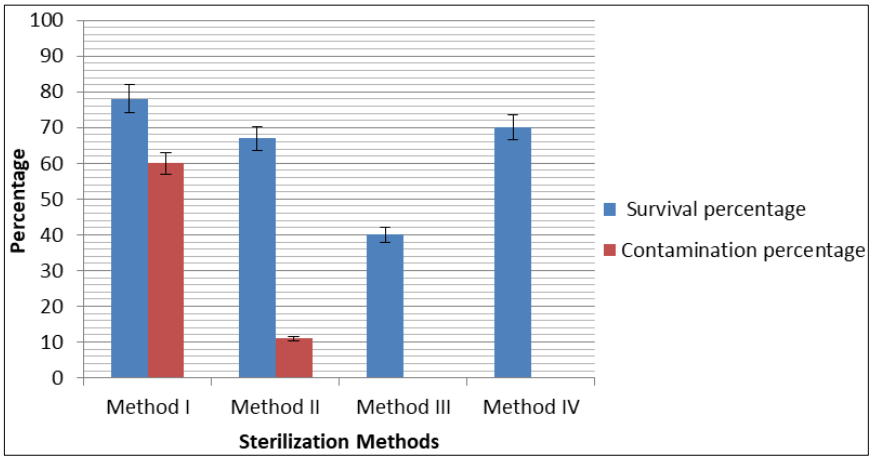

Fig 1: Optimum condition for the surface sterilization of *Rauvolfia serpentina*

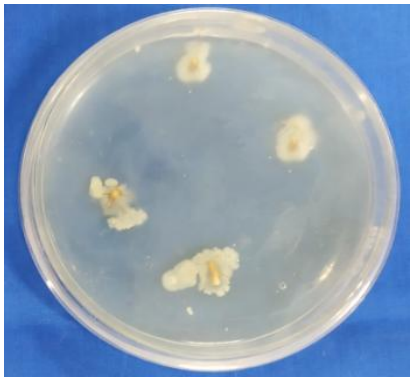

Fig 2: Isolation of endophytes from roots of *Rauvolfia serpentina*

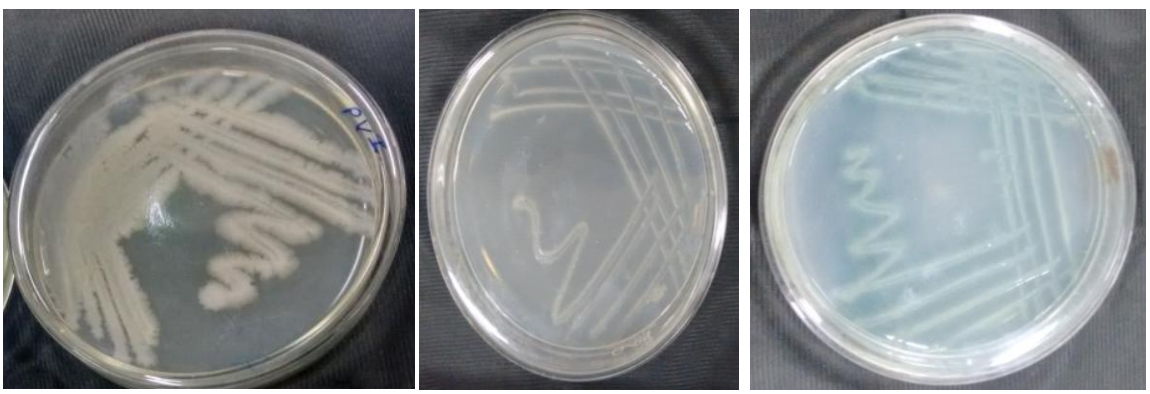

Fig 3: Purified culture of bacterial endophytes isolated from roots of *Rauvolfia serpentina*

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