Journal of
Plant Pathology & Microbiology
ISSN: 2157-7471

Waheeda and Shyam, J Plant Pathol Microbiol 2017, 8:2
DOI: 10.4172/2157-7471.1000399

Research Article Open Access

Formulation of Novel Surface Sterilization Method and Culture Media for the Isolation of Endophytic Actinomycetes from Medicinal Plants and its Antibacterial Activity

Waheeda K and Shyam KV*

Department of Biotechnology and Microbiology Karnataka University, Dharwad, India

Abstract

Endophytic actinomycetes from medicinal plants were rarely reported as compared to those from soil and marine sources. The present results correlate the presence of endophytic actinomycetes in medicinal plants by isolating them from three medicinal plants i.e. *Ocimum basillicum, Withania somnifera* and *Rauvolfia tetraphylla*. In the present work surface sterilization method and media was standardized and 32 endophytic actinomycetes were isolated from three medicinal plants. We assessed the efficiency of four different surface sterilization methods and four medium for isolation of endophytic actinomycetes. The method with calcium hypochlorite, sodium hydrogen carbonate and sodium azide (New method) used was more effective in eliminating epiphytic microorganisms. Among four tested media starch casein agar (SCA) found to be best media for the isolation of endophytic actinomycetes. We succeeded in obtaining fast and luxurious growth of pure culture of endophytic actinomycetes on modified ISP-4 (M) (inorganic salt solution agar) in comparison with standard ISP-4(S). Preliminary antibacterial assay of all isolates were tested by confrontation test. The secondary screening of selected isolates were tested by disc diffusion test by using ethyl acetate extract which showed broad spectrum antibacterial activity against test human pathogens. Based on the morphological and phenotypic characters 12 isolates were identified as *Streptomyces* spp. Among 12 isolates A3 as a representative was characterized by SEM and identified by 16SrRNA analysis as *Streptomyces flavoviridis* A3WK, it showed significant antibacterial activity against test human pathogen. This is the first report of successful isolation of endophytic actinomycetes from the said medicinal plants, for using newly formulated surface sterilization method and new comparative study on ISP-4 medium.

Keywords: Endophytic actinomycetes; Surface sterilization; Isolation media; 16SrRNA; ISP-4; Antibacterial activity

Introduction

The inclusive and widely accepted definition of endophyte that was given by Bacon and White [1] is microbes that colonize living internal tissues of plants without causing any immediate, overt negative effects. Plants constitute vast and diverse niches for endophytic organisms, and closer biological associations may have developed between these organisms and their respective hosts than for epiphytes of soil related organisms [2] Plant associated bacteria, both endophytic and rhizospheric, are known to have beneficial effects on the host plant [3]. Rhizospheric bacteria are known to be more easily cultivable than comparable endophytic species [4]. Endophytes reside within plant tissues and are therefore exposed to a more specific and stable habitat than rhizospheric bacteria.

Among endophytes, a distinction can be made between obligate endophytes [5] and facultative endophytes [6]. Obligate endophytes are expected to be the hardest to cultivate, since they require more specific conditions and therefore the main focus of this manuscript. The beneficial interaction of endophytic actinomycetes with plants is considered as an important area of research [7].

Medicinal plants are the important resource of isolating endophytic actinomycetes, which can induce secondary metabolite of very important value. The importance of medicinal plants in treatment of various types of ailment is also undebatable as noted in Ayurveda and other traditional medicine practices. Though several types of plants are covered with actinomycetes, it is known that not all plants contain endophytic actinomycetes [2,8]. The symbiotic existences of endophytic actinomycetes in medicinal plants and their role in deciding the medicinal value of plants due to the production of various bioactive compounds is worth probing. Relevance of screening medicinal plants is an important approach to explore and exploit new endophytes [9].

Although collection of plants is relatively inexpensive and easy, little is known about the endophytic actinobacterial distribution and their abundance compared to soil actinomycetes, and the vast majority of endophytic actinobacteria remain unexplored and unknown. Possibility of isolating more endophytic actinobacterial strains, especially rare actinobacteria and new species is highly challenging. To solve this problem, new isolation strategies and media must be introduced. Four cultivation principles and methods merit particular attention: 1) ensuring complete release of endophytes from inner tissues of plant samples; 2) cells enrichment; 3) designing media that simulates host plant environment; 4) extended incubation times [10].

Various methods have been used by researchers for isolation of endophytic actinomycetes. Takahashi and omura [11] emphasized that the diversity of actinomycetes depends mainly on the methods of isolation. The most frequently employed method for their detection and enumeration involves isolation from effective surface sterilized host plant tissue. Isolation of endophytic actinomycetes depends on various factors, which include host plant species, age, type of tissues, sampling seasons, surface sterilants, selective media and culture conditions [12,13].

***Corresponding author:** Shyam K Vootla, Professor, Department of Biotechnology and Microbiology, Karnataka University, Dharwad, 580001, India, Tel: +919880322743; E-mail: vootlashyam@kud.ac.in

Received February 07, 2017; **Accepted** February 24, 2017; **Published** February 27, 2017

Citation: Waheeda K, Shyam KV (2017) Formulation of Novel Surface Sterilization Method and Culture Media for the Isolation of Endophytic Actinomycetes from Medicinal Plants and its Antibacterial Activity. J Plant Pathol Microbiol 8: 399. doi: 10.4172/2157-7471.1000399

In spite of a growing and robust literature on endophytes, cultivation-dependent techniques still strongly determine the number of actinomycetes present in plant tissues. The literature suggests that only 0.001% to 1% of the endophytes present in plant tissues are cultivable [14,15].

The preliminary task in isolating endophytic actinomycetes is fool proof sterilization methods to thoroughly clean the epiphytic microbes to ensure the isolation of only endophytic actinomycetes [16]. However; the isolation procedure is a critical and important step in working with endophytes. Diversity of isolated actinomycetes largely depends on isolation methods [11].

The specificity of the isolation method influences the number of endophytic actinomycetes isolated in medicinal plants [17,18]. Endophytic actinomycetes unlike the conventional microbes in culture do not show growth immediately. Typically, a specific media composition and constituents are required for the isolation of distinct endophytes [19]. Previously unstudied groups of soil bacteria were isolated by using different types of media [19-21].

The research on endophytic actinomycetes is relatively less because surface sterilization of the samples a very important step, if the sterilization time is too long, the plant cell would be damaged and hamper endophyte isolation [22]. Therefore, the selection of isolation media and surface sterilization are important to maximize the successful isolation of endophytic actinomycetes, thus, enhancing the possibility to discover new endophytes [23].

Three medicinal plants have been chosen for the present study i.e. *Ocimum basillicum, Withania somnifera and Rauvolfia tetraphylla*. Thai or sweet basil is a common name for the culinary herb *O. basillicum* belongs to family *Lamiaceae*. It is considered to be antibacterial, antifungal, antispasmodic, carminative, diaphoretic, digestive and expectorant [24]. *W. somnifera* known commonly as ashwagandha belongs to family Solanaceae, the root and berry are used for arthritis, anxiety, tumors, tuberculosis, asthama, insomnia, leukoderma, backache [25]. *R. tetraphylla* is a plant in the *Apocyanaceace* family is commonly known as the devil pepper or sarpagandha, roots are sedative, tonic and febrifuge [26].

This is the first report of the isolation of endophytic actinomycetes and till now, there are no reports on endophytic actinomycetes from *O. basillicum*, *W. somnifera* and *R. tetraphylla*, inspite of the fact that these are significant medicinal plants and have huge importance in pharmacology. The present study was carried out to show the effectiveness of surface sterilization methods and standardization of most suitable growth and purified media. This work is a part of larger study which relates the presence of endophytic actinomycetes in medicinal plants and gives the distinctive features to medicinal value of the plants.

Materials and Methods

Selection and collection of medicinal plants

Important regional medicinal plants *O. basillicum* (OB), *W. somnifera* (WS) and *R. tetraphylla* (RT) were selected for the isolation of endophytic actinomycetes. Plants were sampled over growing seasons (May to February). All the three experimental medicinal plants were sampled from botanical garden of Karnatak University, Dharwad. At each sampling events, a total of 10 plants were dug out carefully to ensure that maximal amount of roots material were collected. Two batches of five plants were collected at interval of 10 min. Each sample was collected in a separate sterilized bag and brought to the laboratory and processed within 24 h as per the standard procedure prescribed by Justin [27].

Surface sterilization methods

Surface sterilization is the first and obligatory step for endophyte isolation in order to kill all the surface microbes. It is usually accomplished by treatment of plant tissues with oxidant or general sterilizing agent for a period, followed by a sterile rinse [28,12].

The plant samples were thoroughly washed under tap water to remove the soil debris from the leaves, stem, and roots. The inner tissue and the outer tissue of leaves, stem, and root of the three plants were carefully excised and subjected to three different conventional surface sterilization methods. A new novel method of surface sterilization of medicinal plants was designed effectively to achieve the selective isolation of endophytic actinomycetes from medicinal plants. Three conventional methods were considered as base to design and formulate new protocols of surface sterilization.

Conventional method I [29] samples were immersed in 70% ethanol for 5 min. Conventional method II [23] Samples were immersed in a solution of cyclohexamide (50 µg/ml) for 4 h followed by a 15 min wash in 3.15% of sodium hypochlorite and stored in refrigerator (4°C) overnight. Conventional method III [30] sample were immersed in 70% ethanol for 5 min followed by 0.9% NaOCl for 20 min followed by 10 min wash in 10% sodium hydrogen carbonate. New Method which includes 10 min wash in 3.15% calcium hypochlorite, followed by a 15 min wash in 10% sodium hydrogen carbonate and 2 min wash in 1% sodium azide. In all the four methods samples were rinsed with sterile distilled water after each step. Each method was performed in triplicate and in control plant tissues were placed on media without surface sterilization.

Culture media for endophytic actinomycetes

The choice of growth medium is crucial as it directly affects the number and the type of endophytic actinomycetes that can be isolated from plant tissues. Starch casein Agar (SCA) [19], Yeast extract malt extract Agar (YEMA) [31]. Actinomycetes isolation Agar (AIA) [32], Humic acid vitamin B Agar (HV) [33] are currently used for the isolation of endophytic actinomycetes. All media are supplemented with cyclohexamide 50 µg/ml and nystatin 50 µg/ml to suppress the gram negative bacterial and fungal growth.

Isolation and purification of endophytic actinomycetes

The plant tissue blocks of three medicinal plants were carefully excised into small tissues. Each representative tissue of each plant was placed on SCA, YEMA, AIA, and HV. The inoculated sample was incubated for 3-4 weeks at 30°C. Colonies with typical characteristics of actinomycetes was sub cultured and purified on ISP-4 Agar media. Pure cultures of obtained isolates were purified by repeated streaking on modified media of ISP-4 Agar medium. A comparative study was done on standard and modified ISP-4(M) Agar medium. The new modified composition of ISP-4 medium was achieved by modifying the composition of inorganic salts in ISP-4 medium i.e. $ZnSO_4$, $FeSO_4$ and $MnCl_2$. The efficacy of surface sterilization method, resulting from lack of microbial growth, was authenticated by rinsing the samples with sterile distilled water and shaken for 30 sec at final step of each procedure. Then 10 µl of washing water was cultured on the NA and kept overnight at 37°C as a positive control to eliminate the surface contaminating epiphytic microorganisms as described by [8].

Preliminary identification of actinomycetes

Selected colonies were picked from plates and purified on SCA and

Methods	First Week								Second Week								Third Week								Fourth Week							
	SCA		AIA		YEMA		HV		SCA		AIA		YEMA		HV		SCA		AIA		YEMA		HV		SCA		AIA		YEMA		HV	
	G	C	G	C	G	C	G	C	G	C	G	C	G	C	G	C	G	C	G	C	G	C	G	C	G	C	G	C	G	C	G	C
Method I	-	-	-	+	+	-	-	-	-	+	-	++	+	-	+	+	-	++	-	+++	+	-	+	++	-	+++	-	+++	+	+++	++	+++
Method II	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	+	-	-	-	-	-	+	-	++	-	-
Method III	-	+	-	+	-	+	-	+	-	++	+	++	-	++	-	++	-	+++	+	+++	-	+++	-	+++	-	+++	+	+++	-	+++	-	+++
Method IV	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	-	-	+	+++	-	+	++	-	-	++	+

G-Growth, C- Contamination, SCA-Starch casein agar, AIA-Actinomycetes isolation agar, YEMA-Yeast extract malt extract agar, HV- Humic acid vitamin B agar, +++ - Highest growth, ++ - Moderate, + -Low growth, - No growth.

Table 1: Efficiency of surface sterilization methods on growth and contamination of endophytic Actinomycetes on SCA, AIA, YEMA and HV.

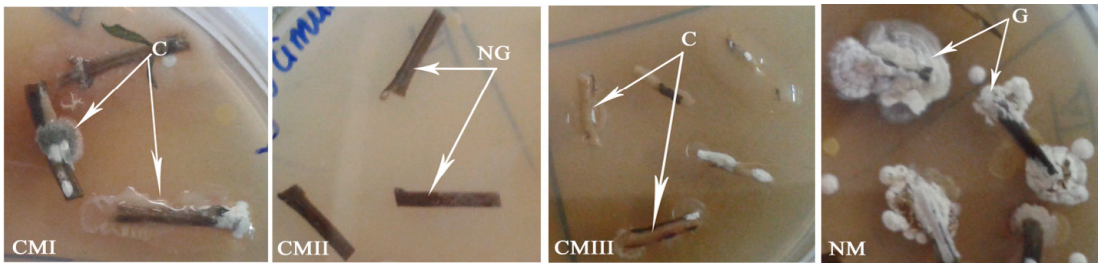

Figure 1: Efficiency of surface sterilization method on the growth and contamination of endophytic actinomycetes on Starch casein agar (SCA). Conventional method (CM), new method (NM), Arrow mark indicating Growth (G) and contamination (C).

ISP-4 media (International Streptomyces Project). The isolates were tentatively identified and grouped according to their morphological and cultural characteristics, including the properties of colonies on the plate; the presences of aerial mycelium and substrate mycelium; distinctive reverse colony color; and diffusible pigments, sporophore and spore chain morphology, were used as identification characters [34,35]. These phenotypic properties allowed them to be segregated into distinct groups. Further, observation visualization of both morphological and microscopic characteristics using light microscopy, acid-fast and Gram stain properties were also performed.

Primary screening for antibacterial activity

The potentiality of active endophytic actinomycetes isolates was determined by the cross-streak method slightly modified method of [36]. Isolates were suspended in sterile distilled water and inoculated on the surface of nutrient agar. After 72 h of incubation at 28°C, test human pathogens were inoculated in perpendicular directions of the endophytic isolates and incubated at 28°C for 48 h [37,38]. The potentiality of isolates is determined by producing antibiotics which is expressed by inhibition zones between the test isolates. The inhibition zone was observed after 48h of inoculation. Plates with the same medium without inoculation of endophytic actinomycetes but with simultaneous streaking of test human pathogens were maintained as control.

Fermentation and extraction

The most potent strains were grown on ISP-4 (International Streptomyces Project-4) as a production media. The isolate was inoculated in ISP-4 broth (Inorganic salt solution agar; soluble starch 10 g/500 ml, K_2HPO_4 1.0 g $MgSO_4.7H_2O$ 1.0 g, NaCl 1.0 g, $(NH_4)_2SO_4$ 2. 0 g, $CaCO_3$ 2.0 g, Distilled water 500 ml, Trace salts solution 1 ml) and incubated at 28°C in a shaker (180 rpm) for 7 days to allow optimum production of bioactive compound. The culture broth of endophytic actinomycetes was filtered to remove mycelium and extracted with ethyl acetate and was concentrated with a rotatory evaporator at room temperature [39].

Secondary screening

The antimicrobial susceptibility of the potent endophytic actinomycetes extracts were tested against Gram positive bacteria *Corneybacterium diphtheria* (ATCC75415) *and Streptococcus faecalis* ATCC47077, *Gram* negative bacteria *E. coli* ATCC25922, *Klebsiella pneumoniae* ATCC10031, *Salmonella typhi* ATCC700931. Test organisms were incubated in lysogeny broth (LB) for 20h at 37°C until the stationery phase was reached. Antimicrobial activity of endophytic actinomycetes extract was evaluated by Disc diffusion paper-disc method with slight modification [40]. Discs impregnated with an ethyl acetate extracts, dried in laminar air flow and placed over the agar surface of plates freshly inoculated with test organisms. Suspensions of test organism were adjusted to 10^6 cfu/ml. The plates were kept at room temperature for 2 h to allow the diffusion of extracts and then incubated at 37°C for 24 h. The antimicrobial activity was measured by the diameter of the inhibition zone.

16SrRNA gene sequencing

Based on the potentiality of the endophytic actinomycetes one of the highly potent isolate as a representative was characterized by 16SrRNA and Scanning Electron Microscopy (SEM).

Results

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

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 [27,41-43].

In conventional method, I [29] there was no growth of actinomycetes in the first week, but contamination was increased with prolonged incubation period. And in second and third week along with contamination there was growth of actinomycetes on YEMA and HV media. But this was not considered as true endophytes because true endophytic actinomycete starts appearing from the fourth week as shown in Table 1. In conventional method II [23] even after the fourth

week there was no growth of actinomycetes on SCA, AIA, YEMA and HV shown in Table 1. In conventional method III [30] starting from the first week there was more contamination along with the growth of

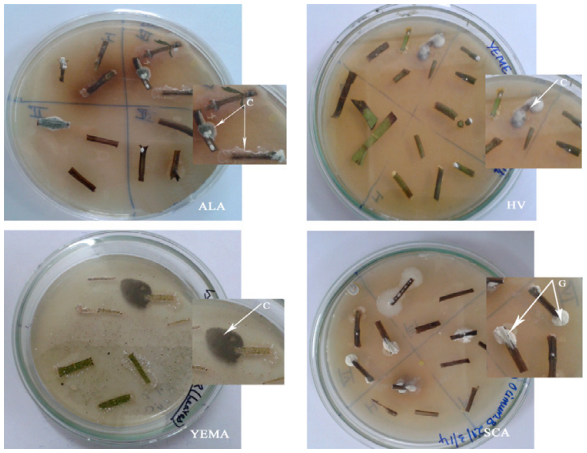

Figure 2: Efficiency of culture media on the growth and contamination of endophytic actinomycetes on new method IV. AIA-Actinomycetes isolation Agar, HV-Humic acid vitamin B agar, YEMA-Yeast extract malt extract agar, SCA-Starch casein agar. C-Contamination, G-Growth of endophytic actinomycetes.

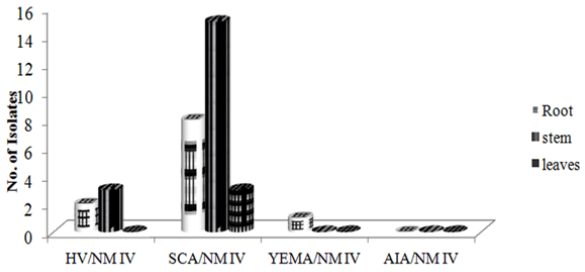

Figure 3: Efficiency of new method (NM) on four different media and three part of plants.

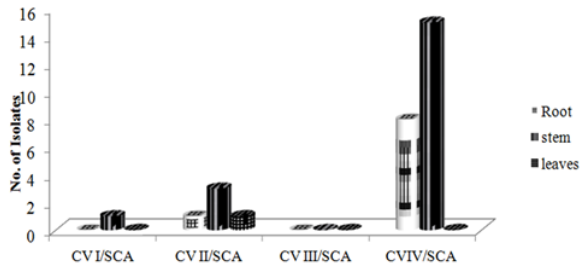

Figure 4: Efficiency of starch casein agar on four different surface sterilization methods in root, stem and leaves.

Figure 5: Growth and pigmentation of pure culture of endophytic actinomycetes on A- No growth on standard ISP-4 (S) and B- luxuriously growth on modified ISP-4 (M).

Isolates	ISP-4(S)		ISP-4(M)	
	Growth	Pigmentation	Growth	Pigmentation
A1	-	-	+++	+++
A2	-	-	+++	+++
A3	-	-	+++	+++
A5	-	-	+++	+++
A6	-	-	+++	+++
A7	-	-	+++	+++
B1	-	-	++	++
B2	-	-	++	++
B3	-	-	+++	+++
C1	-	-	+++	+++
C2	-	-	+++	+++
D1	+++	+++	-	-
E1	-	-	+++	+++

*ISP-4(S) - Standard ISP-4, IS-4(M) - Modified ISP-4, (-) No growth and pigmentation, (+++) highest growth, Moderate growth.

Table 2: Growth and pigmentation pattern of pure cultures of endophytic actinomycetes on standardISP-4(S) and modified ISP-4 (M).

actinomycetes on AIA and YEMA media in comparison with that of SCA and AIA shown in Table 1 and Figure 1. In new method, more number of actinomycetes colonies was recovered from the fourth week with no contamination on SCA followed by HV. Maximum numbers of endophytic actinomycetes were recovered from stem. But there was contamination on AIA and YEMA media shown in Table 1 and Figure 1. This is the first attempt to isolate endophytic actinomycetes by using calcium hypochlorite, sodium hydrogen carbonate and sodium azide and proved most efficient method for the isolation of endophytic actinomycetes.

Efficiency of culture media on the growth and contamination of endophytic actinomycetes

The different media that are used during endophyte isolation strongly influence the number and diversity of cultivable actinomycetes [44]. The samples from root stem and leaves of three plants were placed on four different media. More growth of endophytic actinomycetes was obtained in method IV on SCA media with very less contamination whereas method II shows no growth with very less contamination on YEMA and AIA. Where as in method III more contamination with less growth on YEMA media. And method I shows maximum contamination negligible amount of growth of endophytic actinomycetes on AIA as shown in Figure 2.

Isolation and purification of endophytic actinomycetes

A specific protocol for isolation of actinomycetes from a given plant is important, as isolation represents the most crucial step during the process of obtaining pure cultures, sampling strategy, tissue typing and culture conditions, surface sterilization and selective media all influences the detection and enumeration of endophytes [45].

Thirty-two endophytic actinomycetes isolates were successfully obtained from the selected plant samples of the three medicinal plants i.e. *O. basillicum*, *W. somnifera* and *R. tetraphylla.* Amongst the obtained isolates highest number of actinomycetes was isolated from stem followed by root and least number of isolates was obtained from leaves shown in Figure 3. According to Qin et al. [46] the maximum endophytic actinobacteria have been recovered from root followed by stems and least in leaves. The results obtained by [47] suggested that the different location within the plants also differ in the diversity of actinomycetes flora.

Highest number of endophytic actinomycetes isolates was obtained

from SCA media followed by HV and YEMA. Figure 4 shows the least number of endophytic actinomycetes isolates obtained from AIA. In this study the most suitable medium for the isolation of endophytic actinomycetes is SCA. After successful isolation, the obtained isolates were purified on modified ISP-4 media (M). According to Shirling and Gottlieb [32], ISP medium is the best medium for the growth of actinomycetes [31]. In this work, all the isolates showed luxurious growth with more pigmentation on modified ISP-4 (M) medium comparing with that of standard ISP-4(S). The main difference between Standard(S) and modified(M) ISP-4 medium is increased concentration of Zinc sulphate ($ZnSO_4$), Manganese chloride ($MnCl_2$) and ferrous sulphate ($FeSO_4$) from 0.100 g to 0.150 g. One of the purified representative isolate of endophytic actinomycetes on Standard(S) and modified (M) ISP-4 medium is shown in Figure 5 and Table 2.

Preliminary identification of endophytic actinomycetes

The endophytic actinomycetes isolates were picked and purified on ISP-4 and identified based on morphological, phenotypical criteria features morphology of substrate and aerial hypae, spores and pigment production and colony characters [48] 32 isolates were identified as endophytic actinomycetes and among these 13 were identified as *Streptomyces* as shown in Table 3. Thirteen isolates were characterized by MALDI- TOF MS and SEM (Results are not shown). To confirm the reliability of morphological identification, one among the selected bioactive endophytic actinomycetes strain A3 were subjected to *16SrRNA* gene sequence analysis characterized by SEM in Figures 6 and 7). The A3 isolate subjected to *16SrRNA* gene sequence revealed the endophytes as *Streptomyces flavoviridis* A3WK. The percentage of *16SrRNA* gene sequence similarities of these isolate to the closest type strain of NCBI database are presented in Figure 8. The nucleotide sequences of *S. flavoviridis* A3WK obtained in this study was deposited in Gene Bank with accession number KP260508.

Sl. No	Aerial Mycelium	Substrate mycelium	Microscopic features	Soluble pigment
A1	Pinkish white	Blackish Gray	Gram positive Straight Smooth	Pinkish orange
A2	Grayish White	Grayish Black	Gram positive Rectus Warty	White Grayish Black
A3	White	Yellowish	Gram positive Spiral Smooth	Yellow
A5	White	Blackish	Gram positive Straight Smooth	Fully Black
A6	White Grayish	Grayish Brown	Gram positive Flexuous Warty	Light Brown
A7	Whitish Yellow	Light Yellow	Gram positive Rectus Smooth	Light Yellow
B2	White	Greyish White	Gram positive Flexuous Not Determined	Whitish gray
B3	White	Blackish White	Gram positive Flexuous Smooth	Black
C1	Whitish Yellow	Yellowish	Gram positive Flexuous Smooth	Yellow
C2	Whitish Yellow	Grayish Yellow	Gram positive Rectus Smooth	Pale Yellow
D1	White Brown	Dark Brown	Gram Positive Marty Spiny	Dark Brown
E1	Grey White	Gray	Gram positive Flexuous Smooth	Grayish Brown

Table 3: Phenotypic characterization of isolated endophytic Actinomycetes isolates on SCA.

Figure 6: Colony morphology of *Streptomyces flavoviridis*A3WK on ISP-4 after 8days.

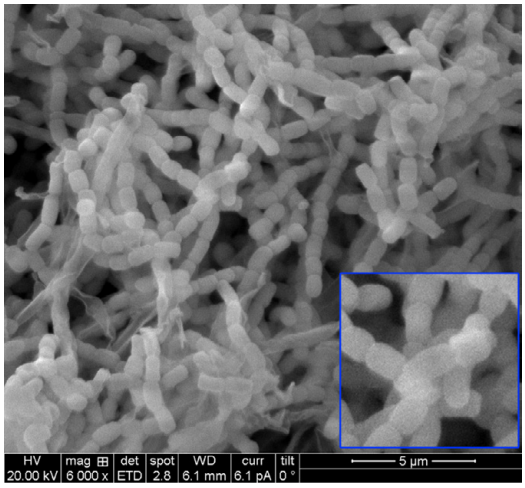

Figure 7: Scanning electron microscopy of *Streptomyces flavoviridis* A3WK Showing barrel shaped spore formation

Primary screening

In this study, 12 isolates were subjected to primary screening. Among 12 isolates few were active against all the tested Gram positive and gram negative bacteria which confirm their broad spectrum of activity. Among 12 isolates A3 showed highest activity against Gram positive and gram negative bacteria (Figure 9).

Antibacterial susceptibility test by Disc diffusion assay

Based on the primary screening for antibacterial activity by confrontation test, phenotypic characterization and spore structure we have selected 10 isolates for antibacterial susceptibility test by disc diffusion testing with their ethyl acetate extract. Among 10 isolates A3 isolate showed robust antibacterial activity in comparison with other isolates (Table 4).

Discussion

In the present study surface sterilization and culture media were optimized to obtain endophytic actinomycetes from three medicinal plants. To our knowledge, this is the first comprehensive report concerning the endophytic actinomycetes from *O. basillicum*, *W. somnifera* and *R. tetraphylla*. It can be considered that the new method

employed in this study were productive and resulted in the successful isolation of numerous endophytic actinomycetes and that the isolates obtained can be considered as true endophyte. And this is the first report on surface sterilization of plant tissues using the combination of calcium hypochlorite, sodium hydrogen carbonate and sodium azide for isolation of endophytic actinomycetes.

Surface sterilization of plant tissue for isolation of endophytic actinomycetes using Method I 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. While in method II which includes the sample immersion in cyclohexamide followed by sodium hypochlorite showed very less and slow growth with contamination. As cyclohexamide being antifungal and sodium hypochlorite being effective disinfectant agent against many bacteria, however, some fungus and bacteria survived might be due to resistance towards cyclohexamide and sodium hypochlorite (OCl^-) [49].

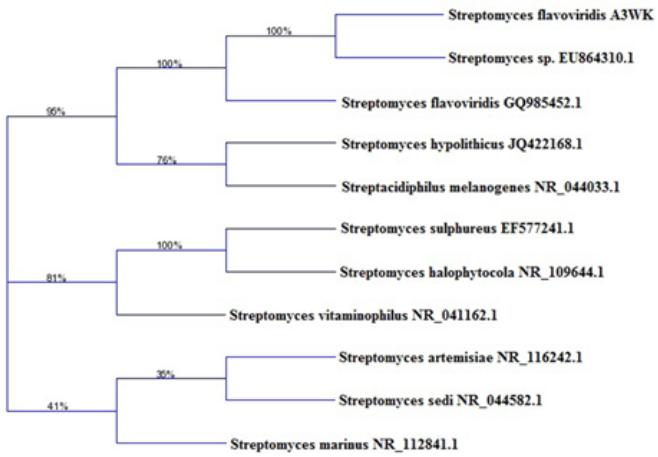

Figure 8: Phylogenetic tree constructed by Neighbor-joining method based on *16SrRNA* gene sequencing showing the phylogenetically closest species of *Streptomyces flavoviridis* A3WK.

Figure 9: Preliminary screening for antibacterial activity of endophytic actinomycetes against human pathogens by confrontation test.

Sl. No	Isolates	Zone of inhibition				
		Corney-bacterium diphtheria	*Salmonella parathypi*	*Strepto-coccus faecalis*	*E. coli*	*Aerobacter aeromonas*
1	A2	++	+	+	+	+
2	**A3**	**+++**	**+++**	**++**	**+++**	**++**
3	B3	+++	++	+	+	++
4	D1	++	++	++	++	+
5	B2	+	++	+	+	+
6	C2	_	_	+	+	+
7	C1	_	_	+	+	_
8	A5	+	+	+	_	+
9	A6	+	+	+	+	+
10	A7	+	_	_	+	_

(-) no inhibition, (+) inhibition zone >5 mm, (++) inhibition zone >10 mm, (+++) inhibition zone >15 mm

Table 4: Secondary Screening for Anti-bacterial activity by disc diffusion assay.

The bactericidal action of hypochlorite solution (Bleach) is due to both hypochlorus acid (HOCl) and the NaOCl (OCl^-) ion with the former being more active so that the disinfecting efficiency of chlorine is best in slightly acid hypochlorite solution [50,51]. It is suggested that for the most effective disinfection of plant material, hypochlorite solution at pH 6-7 is effective [50]. The results of Nurul hazwani et al. [52] shows that treatment with 5.25% sodium hypochlorite as the active ingredient even at high concentration was ineffective to control fungal and bacterial contamination in the explants. The use of antibiotics *in vitro* is not very effective in eliminating microorganisms and these compounds can be phytotoxic [53].

For designing a suitable sterilization method, several factors need to be considered, one of which is safety, and this means avoiding antibiotics or potentially poisonous chemicals such as mercuric chloride [23]. In method III which resulted more contamination and the poor performance of this method can be attributed to 70% ethanol and sodium hydrogen carbonate were not sufficient to eliminate surface microorganisms, however sodium hydrogen carbonate is only effective in eliminating endophytic fungi [30].

New method in which calcium hypochlorite in combination with sodium hydrogen carbonate and sodium azide was more effective in eliminating epiphytic microorganisms. This is the first attempt to isolate endophytic actinomycetes using calcium hypochlorite for surface sterilization of plant tissues. For chlorine solution to be a good disinfectant it must meet the chlorine demand. The chlorine demand is the amount of free available chlorine, or (FAC)often called HOCl, hence its activity is more before a FAC residual is reached [54]. If the chlorine demand is not met complete disinfection may not be achieved. The solution with the NaOCl (OCl^-) ion is a very poor disinfectant which is far less effective as a disinfectant than HOCl. In this study calcium hypochlorite is found to be more effective disinfectant in eliminating plant surface microorganisms [40]. Our results are in agreement with earlier studies on attempts using various sterilization methods [55,52].

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 [29]. Hypochlorite (OCl^-) as a strong oxidant can denature by aggregating essential proteins of bacteria as previously described [56]. The effectiveness of bleach (1.4% NaOCl solution for 1 min) as sterilization agent for plant tissue is reported [57]. In our study NaOCl proved to be very poor sterilizing agent. Interestingly calcium hypochlorite in combination with sodium hydrogen carbonate and sodium azide was more effective in eliminating epiphytic microorganisms [58]. The efficacy of calcium hypochlorite in comparison with sodium hypochlorite might be due to its pH 7, at this pH hypochlorous acid is produced and it is considered as the most germicidal of all chlorine compounds between 80 to 120 times stronger than the OCl^- ion. Most fresh HOCl solutions have a pH between 7 to 8 [59,53]. However, all (fresh and old) NaOCl solutions, ("chlorine bleach") unless buffered by an acid, have a pH 10.25+ producing no HOCl. These solutions produce only the OCl^- ion, a very poor

disinfectant which is far less effective as a disinfectant than HOCl. Hence, during our study we paid attention to the pH issue which resulted in very less contamination proving the effectiveness of calcium hypochlorite at pH 7 [58].

Use of sodium azide as a biocide, is commonly used to inhibit microbial growth during experimental studies [60]. The effort of scientists in realizing effective sterilization methods for various purposes is well documented [52].

In summary, a three-step sterilization new method was found to be very effective in eliminating epiphytic microorganisms using calcium hypochlorite, sodium hydrogen carbonate and sodium azide yielded 98% of endophytic actinomycetes from the three medicinal plants.

In this study the most suitable medium for the isolation of endophytic actinomycetes was SCA. Contrastingly none of the previous studies confirmed good media for actinomycetes colonies using SCA media. Studies using AIA and YEMA which were not ideal during our studies (Results not shown), but these media were successfully used in earlier studies [29,61,62]. Our studies using SCA media is a good platform for isolating endophytic actinomycetes from the various plant tissues of medicinal plants as evident from our results.

After isolation the endophytic actinomycetes colonies emerged from the tissues on media were purified on ISP-4 media [63]. When these colonies were purified on ISP-4, the growth was slow and with no pigmentation on the media. However, our initial studies showed slow growth and no pigmentation with in stipulated time. A study was done by using different concentration of mineral salts or inorganic salt or trace salt i.e. by comparing standard ISP-4(S) and modified ISP-4(M). Generally, in standard ISP-4(S) 0.1% of trace salt solution ($FeSO_4$, $MnCl_2$, and $ZnSO_4$) are prescribed for isolation of actinomycetes which yielded less growth of colonies with no pigmentation.

Hence in modified ISP-4(M) step wise increase in salt solution was adopted and to our surprise 0.150 g of inorganic salt yielded good colony growth and pigmentation. These results might be due to the fact that endophytic actinomycetes need higher concentration of trace salts for growth, which inside the plants is available through plant system. Another reason for luxuriant growth might be due to the habitat of endophytic actinomycetes which are present abundantly in intercellular space where the inorganic salts are abundant [64,65].

These endophytic actinomycetes when grown on ISP4 media require the same quantity of these trace salt as in the intercellular spaces of the medicinal plants for their luxurious growth. Results of Okazaki [18] emphasized that adding a certain amount of plant extracts into the isolation medium is effective. Although the choice of a suitable growth medium is crucial during isolation, a comparative study of ISP-4 media has not been published. The inorganic salts obviously affect both the number and diversity of endophytes that can be isolated from a specific plant tissue and it may also determine the ultimate cultivability of endophytic actinomycetes.

The isolates were identified based on morphological criteria, including characteristics of colonies on plate, morphology of substrate and aerial hyphae, morphology of spores and pigment production [48]. Based on morphological and phenotypic characterization 12 isolates were identified. Hence morphology is still an important characteristic for the description of taxa and it is not adequate in itself to differentiate between many genera. In fact, it was the only characteristic which was used in many earlier descriptions, particularly of *Streptomyces* species in the first few editions of Bergey's Manual (2012). Our results showed that the highest percentage of bioactive strains found was genus *Streptomyces* which is consistent with other reports [46,66]. *Streptomyces* spp. is dominant over the rest of taxa in endophytic colonization [36]. Our findings indicate that bioactivity of endophytic actinomycetes against tested Gram negative bacteria is more than the Gram-positive bacteria.

In order to isolate endophytic actinomycetes, microbes present on the plant surface have to be fully eliminated first. So, requirement for surface sterilization protocol are that all microbes present on plants surface are killed but also that these procedures have as small as possible negative effect on the endophytic actinomycetes. It means that the procedure should be sensitive enough to recover maximum endophytic actinomycetes and strong enough to eliminate the epiphytes present on the surface of explants. In conclusion, we developed an efficient new method for isolation of endophytic actinomycetes, comparison and modification of ISP-4(S) and ISP-4(M) to obtain pure cultures. To our knowledge, this is the first method developed and first report to isolate endophytic actinomycetes from the said medicinal plants and endophytic actinomycetes are promising source of producing new antibiotic.

Acknowledgement

Author Waheeda Khanam grateful to University Grants Commission New Delhi, for providing Research Fellowship for PhD through University of Potential Excellence (UPE) Scheme (No. KU/Sch/UGC-UPE/2013-14/109) during the year Feb 2013 to Feb 2016.

Conflicts of Interest

The authors declare that they have no conflict of interest

References

1. Bacon CW, White JF (2000) Microbial endophytes. CRC Press, Boca Raton, Florida, USA.
2. Strobel GA, Daisy B (2003) Bioprospecting for microbial endophyte and their natural products. Microbiol Mol Biol Rev 67: 491-502.
3. Taule C, Mareque C, Barlocco C, Hackembruch F, Reis VM, et al. (2012) The contribution of nitrogen fixation to sugarcane (*Saccharum officinarum* L.), and the identification and characterization of part of the associated diazotrophic bacterial community. Plant Soil 356: 35-49.
4. Bafana A (2012) Diversity and metabolic potential of culturable root-associated bacteria from Origanum vulgare in sub-Himalayan region. World J Microbiol Biotechnol 29: 63-74.
5. Croes S, Weyens N, Janssen J, Vercampt H, Colpaert JV, et al. (2013) Bacterial communities associated with *Brassica napus* L. grown on trace element-contaminated and non-contaminated fields: A genotypic and phenotypic comparison. Microb Biotechnol 6: 371-384.
6. Kamnev AA, Tugarova AV, Antonyuk LP, Tarantilis PA, Polissiou MG, et al. (2005) Effects of heavy metals on plant-associated rhizobacteria: Comparison of endophytic and non-endophytic strains of *Azospirillum brasilense*. J Trace Elem Med Biol 19: 91-95.
7. Patrycja G, Magdalena W, Gauravi A, Dnyaneshwar R, Hanna D, et al. (2015) Endophytic actinobacteria of medicinal plants: Diversity and bioactivity. Antonie Van Leeuwenhoek 108: 267-289.
8. Machavariani NG, Ivankova TD, Sineva ON, Terekhova LP (2014) Isolation of endophytic actinomycetes from medicinal plants of the Moscow region, Russia. World Appl Sci J 30: 1599-1604.
9. Zhang J, Wang JD, Liu CX, Yuan JH, Wang XJ, et al. (2014) A new prenylated indole derivative from endophytic actinobacteria Streptomyces sp. neau-D50. Nat Prod Res 28: 431-437.
10. Suryanarayanan TS, Thirunavukkarasu N, Govindarajulu MB, Sasse F, Jansen R, et al. (2009) Fungal endophytes and bioprospecting. Fungal Biol Rev 23: 9-19.
11. Takahashi Y, Omura S (2003) Isolation of new actinomycetes strains for the screening of new bioactive compounds. J Gen Appl Microbiol 49: 141-54.
12. Hallmann J (2001) Plant interactions with endophytic bacteria. In: Jeger

MJ, Spence N (Eds). Biotic interactions in plant pathogen association. CAB International, Wallingford, Oxfordshire, UK. 87-119 p.

13. Gaiero JR, McCall CA, Thompson KA, Day NJ, Best AS, et al. (2013) Inside the root microbiome: Bacterial root endophytes and plant growth promotion. Am J Bot 100: 1738-1750.
14. Torsvik V, Øvreås L (2002) Microbial diversity and function in soil: From genes to ecosystems. Curr Opin Microbiol 5: 240-245.
15. Alain K, Querellou J (2009) Cultivating the uncultured limits, advances and future challenges. Extremophiles 13: 583-594.
16. Jacobs PT, Wang JH, GorhamRA, Roberts CG (1998) Disinfection, sterilization and antiseptics in health care. Polyscience Publishers, New York.
17. Okazaki T, Takahashi K, Kizuka M, Enokita R (1995) Studies on actinomycetes isolated from plant leaves. Annual Report of the Sankyo Research Laboratory 47: 97-106.
18. Okazaki T (2003) Studies on actinomycetes isolated from plant leaves. In: Kurtboke I, editor. Selective isolation of rare actinomycetes. National Library of Australia, Canberra, Australia. 102-12 p.
19. Kuster E, Williams ST (1964) Selection of media for isolation of streptomycetes. Nature 202: 928-929.
20. Janssen PH, Yates PM, Grinton BE, Taylor PM, Sait M (2002) Improved culturability of soil bacteria and isolation in pure culture of novel members of the divisions acidobacteria, actinobacteria, proteobacteria and verrucomicrobia. Appl Environ Microbiol 68: 2391-2396.
21. Sait MP, Hugenholtz P, Janssen H (2002) Cultivation of globally distributed soil bacteria from phylogenetic lineages previously only detected in cultivation-independent surveys. Environ Microbiol 4: 654-666.
22. Xu Z, Gao D, Song X, Xu Y (2012) A review of endophyte and its use and function. Adv Biomed Eng Rev 8: 124-130.
23. Webster S, Mitchell SA, Ahmed MH (2003) A novel surface sterilization method for reducing microbial contamination of field grown medicinal explants intended for *in vitro* culture. 17th SRC conference, Biotechnology Centre, Jamaica.
24. VaughanJ G, Geissler C (1997) The new Oxford book of food plant. Oxford University Press, Oxford, UK.
25. James ES, Mario RM, Winthrop BP, Roberto FV, Zhigang H (1999) Basil: A source of aroma compounds and a popular culinary and ornamental herb. In: Janick J (Ed). Perspectives on new crops and new uses. ASHS Press, Alexandria, Egypt. 499-505 p.
26. Adel ET, El-Awady AM, Attia AO, Dessoky DSE (2012) *In vitro* multiplication of the important medicinal plant, harmal (*Rhazya stricta* Decne). J Med Plants Res 6: 3586-3590.
27. Justin TC, Christopher MMF (2003) Isolation and identification of actinobacteria from surface-sterilized wheat roots. Appl Environ Microbiol 69: 5603-5608.
28. Sturz AV (1995) The role of endophytic bacteria during seed piece decay and potato tuberization. Plant Soil 175: 257-263.
29. Zin NM, Sarmin NI, Ghadin N, Basri DF, Sidik NM, et al. (2007) Bioactive endophytic Streptomycetes from the Malay Peninsula. FEMS Microbiol Lett 274:83-88.
30. Madhurama G, Sonam D, Urmil PG, Ravindra NK (2014) Diversity and biopotential of endophytic actinomycetes from three medicinal plants in India. Afr J Microbiol Res 8: 184-191.
31. Shirling EB, Gottlieb D (1966) Methods for characterization of *Streptomyces* species. Int J Syst Bacteriol Evol 16: 313-340.
32. Ajit KP, Vineet KM, Ratul S, Vijai KG, Bhim PS (2015) Isolation, abundance and phylogenetic affiliation of endophytic actinomycetes associated with medicinal plants and screening for their *in vitro* antimicrobial biosynthetic potential. Front Microbiol 7: 273.
33. Hayakawa M (1990) Selective isolation methods and distribution of soil actinomycetes. Actinomycetologica 4: 103-112.
34. Ruan JS, Liu ZH, Liang LN, Yang DC (1990) Study and application of actinomycetes. Bios-Scientific publisher limited, Beijing, China. 1-18 p.
35. Yan XC (1992) Classification and identification of actinomycetes. Bios Scientific publisher, Beijing, China. 623-656 p.
36. Yashavantha HCR, Devaraju R, Sreedharamurthy S (2015) Antimicrobial properties of endophytic Actinomycetes isolated from *Combretum latifolium* Blume, a medicinal shrub from Western Ghats of India. Front Biol 10: 528-536.
37. Highley TL, Ricard J (1988) Antagonism of *Trichoderma* spp. and *Gliocladium* virens against wood decay fungi. Mater Org 23: 157-169.
38. Bensultana A, Ouhdouch Y, Hassani L, Mezrioui N, Rafouk L (2010) Isolation and characterization of wastewater sand filter actinomycetes. World J Microbiol Biotechnol 26: 481-487.
39. Lei W, Peng Q, Xiu-Feng L, Shuai Z, Zhi-Gang Z, et al. (2016) Comparative analysis of chemical constituents, antimicrobial and antioxidant activities of ethyl acetate extracts of Polygonum cuspidatum and its endophytic actinomycete, *Streptomyces* sp. As0916. Chin J Nat Med 14: 0117-0123.
40. Lin W, Huang T, Cornell JA, Lin C, Wei C (1996) Bactericidal activity of aqueous chlorine and chlorine dioxide solutions in a fish model system. J Food Sci 61:1030-1034.
41. Rutala WA, David JW (1995) APIC guidelines for selection and use of disinfectants. Am J Infect Control 23: 313-342.
42. Cao L, Qiu Z, You J, Tan H, Zhou S (2004) Isolation and characterization of endophytic Streptomyces strains from surface-sterilized tomato (*Lycopersicon esculentum*) roots. Lett Appl Microbiol 39: 425-430.
43. Sardi P, Saracchi M, Quaroni S, Petrolini B, Borgonovi GE, et al. (1992) Isolation of endophytic Streptomyces strains from surface-sterilized roots. Appl Environ Microbiol 58: 2691-2693.
44. Eevers N, Gielen M, Sánchez-López A, Jaspers S, White JC, et al. (2015) Optimization of isolation and cultivation of bacterial endophytes through addition of plant extract to nutrient media. Microb Biotechnol 8: 707-715.
45. Zhang HW, Song YC, Tan RX (2006) Biology and chemistry of endophytes. Nat Prod Rep 23: 753-771.
46. Qin S, Li J, Chen HH, Zhao GZ, Zhu WY, et al. (2009) Isolation, diversity, and antimicrobial activity of rare actinobacteria from medicinal plants of tropical rain forests in Xishuangbanna, China. Appl Environ Microbiol 75: 6176-6186.
47. Nimnoi P, Pongsilp N, Lumyong S (2010) Endophytic actinomycetes isolated from *Aquilaria crassna* Pierre ex Lec and screening of plant growth promoters production. World J Microbiol Biotechnol 26: 193-203.
48. Goodfellow M, Cross T (1984) Classification. In the biology of the Actinomycetes. Academic Press, London.
49. Ascenzi JM (1996) A handbook of disinfectants and antiseptics. CRC Press, Boca Raton, Florida, US.
50. George EF (1993) Plant propagation by tissue culture, part 1. The technology (2ndedn), exegetics, westbury, UK.
51. Mc.donnell G, Russell AD (1999) Antiseptics and disinfectants: Activity, action, and resistance. Clin Microbiol Rev 12: 147-179.
52. Nurul HD, Shashita J, Rozi M (2012) Methods paper: An improved surface sterilization technique for introducing leaf, nodal and seed explants of *Aquilaria malaccensis* from field source into tissue culture. AsPac J Mol Biol Biotechnol 20: 55-58.
53. Priscila GM, Angela FJ, Letícia Célia de LN, Patricia M, Thereza CVP (2009) Choice of sterilizing/disinfecting agent determination of the Decimal Reduction Time (D-Value). Braz J Pharma Sci. 45(4): 701-708.
54. Bloomfield SF, Miller EA (1989) A comparison of hypochlorite and phenolic disinfectants for disinfection of clean and soiled surfaces and blood spillages. J Hospl Infect 13: 231-239.
55. Çölgeçen H, Koca U, Toker G (2011) Influence of different sterilization methods on callus initiation and production of pigmented callus in *Arnebia densiflora* Ledeb. Turk J Biol 35: 513-520.
56. Winter J, Ilbert M, Graf PC, Ozcelik D, Jakob U (2008) Bleach activates a redox regulated chaperone by oxidative protein unfolding. Cell 135: 691-701.
57. Okudera Y, Ito M (2009) Production of agar wood fragrant constituents in Aquilariacalli and cell suspension cultures. Plant Biotech 26: 307-315.
58. Hawkins CL, Pattison DI, Davies MJ (2003) Hypochlorite-induced oxidation of amino acids, peptides and proteins. Amino Acids 25: 259-274.
59. Dukan S, Dadon S, Smulski DR, Belkin S (1996) Hypochlorous acid activates the heat shock and soxRS systems of Escherichia coli. Appl Environ Microbiol 62: 4003-4008.

60. Benny C, Kerenstomler S (2006) Interaction of sodium azide with triazine herbicides: Effect on sorption of soil. Chemosphere 65: 352-357.

61. Surang C, Suphatra H, Akkaraphol S, Pattra C, Watanalai P (2013) Endophytic Actinomycetes: A novel source of potential acyl homoserine lactone degrading enzymes. Bio Med Res Int 8.

62. Kanchanadevi D, Hemashenpagam H, Sandhya R (2013) Characterization and plant growth promoter production of endophytic actinomycetes isolated from agricultural crops. Int J Adv Pharma Sci 4: 808-827.

63. Thongchai T, Chunhua L, Yuemao S, Saisamorn L (2005) Secondary metabolites from endophytic *Streptomyces aureofaciens* CMUAc130 and their antifungal activity. Microbiology 151: 1691-1695.

64. Jesus MB, Ben JJL (2014) Biotechnology application of bacterial endophytes. Current Biotechnol 3:60-75.

65. Dhanya NN, Padmavathy S (2014) Impact of endophytic microorganisms on plants, environment and humans. Scientific world J 1- 11.

66. Li J, Zhao GZ, Huang HY, Qin S, Zhu WY, et al. (2012) Isolation and characterization of culturable endophytic actinobacteria associated with *Artemisia annua* L. Antonie van Leeuwenhoek 101: 515-527.