WORLD JOURNAL OF PHARMACY AND PHARMACEUTICAL SCIENCES
SJIF Impact Factor 7.421
Volume 8, Issue 2, 738-745 Research Article ISSN 2278 – 4357

OPTIMIZATION AND ISOLATION OF VARIOUS ENDOPHYTIC BACTERIA FROM MEDICINAL PLANTS

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Article Received on
23 Nov. 2018,

Revised on 13 Dec. 2018,
Accepted on 02 Jan. 2019

DOI: 10.20959/wjpps20192-13058

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ABSTRACT

Endophytes are plant-associated microorganisms that live within the living tissues of their host plants. These endophytes represent a promising source of biologically active metabolites useful for pharmaceutical and agricultural applications. The present study investigates the diversity of endophytic bacteria and antibiotic susceptibility of those bacteria. A total of 15 endophytic bacteria were isolated from *Withania somnifera and Premna japonica*. The antibiotic susceptibility test was conducted for four antibiotics, viz. Ampicillin, Alkem, Tetracin and Doxy-T. The susceptibility of all the endophytic bacteria isolated, was determined by disc diffusion method. Some of these bacteria showed sensitivity to antibiotics.

KEYWORDS: Endophytic bacteria, *Withania somnifera, Premna japonica,* Antibiotic susceptibility.

INTRODUCTION

Medicinal plants are the chief source of secondary metabolites used as drugs. As a result of this, the traditional experts have been using them extensively in their day to day practice. Endophytic bacteria live symbiotically with plants. While growing inside the plants, the endophytes show no visible symptoms of infection and disease (Kobayashi and Palumbo, 2000). Endophytes are known to enhance the quality and growth of plants (Patle et al, 2018). The study of endophytes and their association with plants have, therefore, gained immense scientific and commercial interest (Gupta et al, 2015). Close association of plants and endophytes has been demonstrated over a long period as source of pharmaceutical bioactive compounds (William R Strohl, 2000). Many endophytes were exposed to produce novel bioactive metabolites such as antibacterial, antifungal, antiviral, antitumor, antioxidant, anti-

inflammatory, immunosuppressive drugs, and many related compounds (Gouda et al., 2016). Endophytes are well known for the production of various classes of natural products and have been reported to exhibit a broad range of biological activity. (Stierle and Stierle, 2015). They are grouped into various categories, which include alkaloids, terpenoids, steroids, lactones, phenolic compounds, quinones, lignans, etc.

Molecules derived from natural products, particularly those products by plant and microbes have an excellent record of providing novel chemical structural compounds for development of new pharmaceutical products. Endophytic bacteria are found in stems, leaves, roots, seeds, fruits, ovules, tubers, and as well inside legume nodules (De Bary, 1866; Kobayashi and Palumbo, 2000). In general, bacterial populations are in decreasing order from roots, stems and leaves. In most plants, generally roots have greater numbers of endophytes compared to above ground tissues. Bacterial endophytes have been isolated from leaves, stems, roots, flowers, seeds, and fruits of various plant species (Karmakar and Pramanik, 2011). The isolation procedure is an important and critical step while working with endophytic bacteria and fungi. It must be sensitive enough to recover endophytic microorganisms, but at the same time it should be strong enough to eliminate epiphytes from the plant surface. *Withania somnifera* is a perennial shrub from the Solanaceae or Nightshade family. The herbal root extract has been traditionally used as a tonic and as a sedative but recent research shows that the leaf extract contains Withanolides which have been found to have regenerative properties on brain-cell synapses in mice and in human cell lines in laboratory studies (Ven Murthy, 2010). *Withania Somnifera* grows abundantly in India (especially Madhya Pradesh), Pakistan, Bangladesh, Sri Lanka and parts of Northern Africa. The main chemical constituents are alkaloids and steroidal lactones (Kuruyanagi, 1999). These include tropine and cuscohygrine. The leaves contain the steroidal lactones and withanolides (McInroy and Kloepper, 1994).

Premna japonica is a small tree/shrub in the family Lamiaceae. It bears flowers and fruits between May and November. During flowering season, it attracts a large number of butterflies and bees.The plant is extensively used in traditional medicine in Maharashtra. The present studies were therefore undertaken to investigate and identify endophytic bacteria present in the tissues of leaves and stems of *Withania Somnifera* and *Premna japonica.* Studies were also performed on biochemical characterization of the endophytes and their ability to interact with different antibiotics.

MATERIALS AND METHODS

Sample collection

For the isolation of endophytic bacteria, healthy leaves and stems of *Withania somnifera and Premna japonica* plant were collected from Ratnagiri.

Pre-treatment

The leaves and stems of each plant were washed separately under the tap water to remove soil particles and the surface epiphytes which are present on the surface of plants.

Surface sterilization

All the samples of both the plants were separately subjected to a following surface sterilization procedure. Fresh leaves and stems of the plant were washed under running tap water. These were then washed with sodium hypochlorite containing 0.1% tween 20 for 10 sec. followed by five washes of sterile distilled water. The leaves and stems were further washed with 70% ethanol for a minute and subsequently given three washes of distilled water. They were then dried at room temperature.

Media for isolating endophytic bacteria

Nutrient agar media were used for the isolation of endophytic bacteria. The nutrient agar did not contain any component which could suppress the growth of endophytic fungi. Therefore, the media used for the isolation of endophytic bacteria was supplemented with an antifungal agent.

Isolation, purification, and subculture of endophytic bacteria

After sterilization of the plant material, the surface of the stems was removed using a sterile scalpel in the laminar air flow cabinet under aseptic condition. The leaves were cut into pieces and each piece was placed on nutrient agar medium-supplemented with antifungal agents. The plates with plant tissues were sealed using parafilm tape and incubated at 28±2°C for the growth of bacterial endophytes. The observation was made for 48 hours. After 24 hrs, morphologically different bacterial colonies were selected from the cultures. The colonies are repeatedly streaked in order to achieve pure bacterial isolates. All the selected isolates were sub-cultured in nutrient agar slants and finally, the purified endophytes were stored at 4°C for further use.

Preliminary characterization of endophytic bacteria

Phenotypic characteristics such as microscopic features, Gram reaction, endospore staining, motility, catalase, and oxidase activity of all the isolates were determined using standard procedures.

Effect of sodium chloride on growth of endophytic bacteria

To study the effect of sodium chloride on the growth of endophytic bacteria, the nutrient agar medium was supplemented with different concentrations of NaCl and the endophytic bacterial isolates were inoculated into it. All the plates were incubated at 27°C for 3-4 days and observed for every 24 hrs.

Antibiotic susceptibility pattern of endophytic bacteria

Antibiotic susceptibility pattern of endophytic bacterial isolates was determined by disc diffusion method. All the cultures were inoculated into nutrient agar plates using sterile swab. Antibiotics viz, Ampicillin (50mg), Alkem (50mg), Tetracin (25mg), Doxy-T (10mg) were placed on nutrient agar plates and incubated at 30°C for 24 hours.After incubation, the antibiotic susceptibility pattern was determined by measuring the zone of inhibition.

RESULTS

Isolation of endophytic bacteria

Nutrient agar media was used for isolation of endophytic bacteria, which showed different bacterial colonies. Totally 15 bacterial colonies were recovered from the sample of leaves and stems (Table 1). All isolates were selected for further investigation.

Table 1: Cultural characterization of endophytic bacteria.

Sr.no.	Culture	Colour	Size	Margin	Consistency	Texture
1.	WSL1	colourless	small	circular	creamy	raised
2.	WSL2	colourless	small	regular	creamy	raised
3.	WSL3	colourless	small	regular	creamy	flat
4.	WSL4	colourless	small	regular	creamy	raised
5.	WSS1	colourless	small	irregular	creamy	raised
6.	WSS2	colourless	small	irregular	creamy	raised
7.	WSS3	colourless	small	irregular	creamy	raised
8.	PJL1	colourless	large	irregular	creamy	raised
9.	PJL2	colourless	small	circular	creamy	raised
10.	PJL3	colourless	small	irregular	creamy	raised
11.	PJL4	colourless	large	irregular	creamy	raised
12.	PJS1	colourless	small	circular	creamy	raised
13.	PJS2	colourless	small	regular	creamy	raised
14.	PJS3	colourless	small	irregular	creamy	flat
15	PJS4	colourless	large	irregular	creamy	raised

Note: Codes regarding origin WSL: *Withania somnifera leaf,* WSS: *Withania somnifera stem,* PJL: *Premna japonica leaf,* PJS: *Premna japonica stem* & individual isolate is indicated as 1, 2, 3 and 4.

Preliminary characterization of endophytic bacteria

All the 15 endophytic bacterial isolates were non-pigmented. Out of 15 isolates, 8 Gram positive cocci, 5 Gram negative bacilli and 2 Gram positive bacilli were observed. A total of 9 isolates showed positive results for endospore staining, possibly belonging to genus *Bacillus*. For biochemical and physiological tests, 11 isolates gave positive result for catalase, 5 showed positive results for oxidase test and 6 isolates showed positive results for motility test. The results of all the isolates are listed in Table 2.

Table 2: Microscopic characterization of endophytes.

Sr.no.	Culture	Gram staining	Motility	Catalase	Oxidase	Spore staining
1.	WSL1	G+ve,cocci	-	-	+	+
2.	WSL2	G-ve,bacilli	+	+	-	+
3.	WSL3	G-ve,bacilli	+	+	-	+
4.	WSL4	G+ve,bacilli	+	-	-	+
5.	WSS1	G+ve,cocci	-	-	+	-
6.	WSS2	G+ve,cocci	-	+	+	-
7.	WSS3	G+ve,cocci	-	+	-	-
8.	PJL1	G+ve,cocci	-	-	-	+
9.	PJL2	G+ve,cocci	-	+	-	-
10.	PJL3	G+ve,cocci	-	+	+	-
11.	PJL4	G-ve,bacilli	+	+	+	+
12.	PJS1	G+vecocci	-	+	-	-
13.	PJS2	G-ve,bacilli	-	+	-	+
14.	PJS3	G+ve,bacilli	+	+	-	+
15	PJS4	G-ve,bacilli	+	+	-	+

"+" = positive, "-" = negative

Antibiotic susceptibility pattern of endophytic bacteria

Results of the Antibiotic susceptibility test of the isolated endophytic bacteria are given in Table 3. Out of 15 isolates, 4 isolates were sensitive to Ampicillin, 8 isolates were sensitive to Alkem, 12 isolates were sensitive to Tetracin and 5 isolates were sensitive to Doxy-T.

Table 3: Antibiotic susceptibility pattern of endophytic bacteria: (Zone size in mm).

Sr.no.	Culture	Ampicillin	Alkem	Tetracin	Doxy-T
1.	WSL1	S (25)	S (22)	S (24)	S (26)
2.	WSL2	S (27)	S (22)	S (21)	S (26)
3.	WSL3	R (0)	S (21)	S (24)	S (22)
4.	WSL4	R (10)	S (25)	S (27)	S (24)
5.	WSS1	R (0)	S (20)	S (20)	I (18)
6.	WSS2	R (0)	R (0)	R (11)	R (0)
7.	WSS3	R (0)	R (10)	R (11)	R (0)
8.	PJL1	R (0)	S (22)	S (26)	I (14)
9.	PJL2	R (0)	S (25)	S (24)	I (10)
10.	PJL3	R (0)	R (0)	R (12)	R (0)
11.	PJL4	R (0)	R (21)	S (16)	I (18)
12.	PJS1	R (0)	S (23)	S (17)	S (26)
13.	PJS2	S (23)	R (10)	S (27)	R (0)
14.	PJS3	S (29)	R (0)	S (19)	R (0)
15.	PJS4	R (0)	S (25)	S (25)	R (0)
Total		4	8	12	5

R- Resistant, S- Sensitive, I- Intermediate

Effect of sodium chloride on the growth of endophytic bacteria

All the 15 isolates showed good growth at 0% to 12.5% NaCl concentration. No adverse effect of sodium chloride was seen in any isolates till 12.5%. In fact, the growth of the isolates was enhanced with an increase in the concentration of NaCl provided, and there was no contamination observed.

DISCUSSION

Endophytic bacteria, which exist in the inner tissues of living plants, have attracted increasing attention among taxonomists, ecologists, agronomists, chemists and evolutionary biologists. Numerous studies have indicated that these prolific actinobacteria are capable of producing an impressive array of secondary metabolites. The metabolites play important role in a wide variety of biological activity, viz. antibiotics, antitumor and anti-infection agents, plant growth promoters and enzymes, etc. They sometimes, promote growth and enhance the ability of the host plant to withstand the environmental stresses. These microorganisms may represent an underexplored reservoir of novel species, which can be exploited in pharmaceuticals, agriculture and industry.

In the present study, the plants of *Withania somnifera* and *Premna japonica* were collected from Ratnagiri, Maharashtra. Selective pre-treatment is a prerequisite for the isolation of endophytic bacteria. A total of 15 bacterial strains were recovered from stems and leaves of

the selected plants. The wide range of biochemical and physiological characteristics of the endophytic bacterial isolates indicated that they are different bacterial species. Antibiotic susceptibility pattern of endophytic bacteria were studied using 4 different antibiotics. Most of the isolates (4-12) are sensitive to Ampicillin, Alkem, Tetracin, Doxy-T, but some are resistant to all antibiotics. This study also showed the tolerance of endophytic bacteria to sodium chloride solution 12.5% concentration. Growth rate of the endophytic bacteria increased with the increase of sodium chloride concentration.

CONCLUSION

Based on results, it was concluded that the selected medicinal plants contain different types of endophytic bacteria and most of the endophytic bacterial isolates are Gram-positive, catalase positive and also spore forming. This study is the first of its kind to report the endophytic bacteria from *Withania somnifera* and *Premna japonica* in Maharashtra. Keeping in view the importance of the selected ethanomedicinal plants, this study is the stepping stone for exploring the role of the bacterial endophytes in the metabolism of these plants.

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