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Research Article

ISOLATION, CHARACTERIZATION AND EXPLORATION OF THE BIOLOGICAL ACTIVITY OF ENDOPHYTIC BACTERIA FROM MEDICINAL PLANTS

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ABSTRACT

Bacterial endophytes are known to reside inside tissues of plants and can form a variety of different relationships including symbiotic, mutualistic, commensalistic and ammensalism. There has been increasing evidence, that endophytic bacteria can stimulus plant growth significantly by the production of phytohormones analogous to PGPR activity. In the present study, leaf explants of 6 medicinally important plants viz. *Abrus precatorius* (A_P), *Aegle marmelos* (A_M), *Aloe barbadensis* (A_B), *Annona squamosal* (A_S), *Azadirachta indica* (A_I) and *Oscimum sanctum* (O_S) were used for isolation and characterization of bacterial endophytes via morphological, biochemical examinations. Total 21 endophytes were isolated on Luria-Bertani (LB) and Nutrient agar isolation medium. Isolates were further screened for the enzymatic activity (amylase, protease, cellulase and lipase), and antifungal potential against plant fungal pathogen *Aspergillus niger*. Endophytic bacteria isolates from *Aegle marmelos*, *Aloe barbadensis*, and *Azadirachta indica* showed maximum amylase, protease, cellulase, and lipase activities in addition to antifungal activities. The screened endophytic isolates will be useful as a possible source of novel bioactive metabolites and potential biopesticides and biofertilizers.

Keywords: Endophytes, Medicinal Plants, PGPR.

1. INTRODUCTION

Herbal medicine is widely used in the traditional healthcare system such as Ayurvedic, Unani, Hekimi and other forms of folk treatments. Almost 80% of the rural population is dependent on medicinal plants for their primary health care. Now medicinal plants are gaining more importance in pharmaceutical industries for the preparation of new phytomedicines [1]. Antimicrobial screening is the first stage of antimicrobial drug research to ascertain the susceptibility of pathogenic microorganisms to any plant agent. Nowadays, multiple drug resistance has developed due to the indiscriminate use of commercial drugs commonly used in the treatment of infectious diseases [2]. In addition to this problem, antibiotics are sometimes associated with adverse effects on the host, including hypersensitivity, immune suppression and allergic reactions [3], there is a constant need for new and effective therapeutic agents [4]. Therefore, there is a need to develop alternative antimicrobial drugs for the treatment of infectious diseases from medicinal plants [5]. Medicinal plants represent a rich source of antimicrobial agents. Plants are used medicinally in different countries and are a source of many potent drugs. A wide range of medicinal plant parts is used for extract as raw drugs and they possess varied medicinal properties [6]. Medicinal plants are believed to be an important source of new chemical substances with potential therapeutic effects. The first definition of an endophyte, as "any organism that grows within plant tissues are termed as endophytes" [7]. Endophytes can live within plants without causing disease symptoms [8]. The endophytic communities have been divided into different subgroups, such as 'obligate' or 'facultative,' which are associated with all

types of plants [9].

In the present study, an attempt was made to isolate and characterize endophytic bacteria from the medicinal plants and determine their antibacterial and antifungal property.

2. MATERIAL AND METHODS

2.1. Isolation of bacterial endophytes

The leaves of green, healthy mature plants of *Abrus precatorius* (A_P), *Aegle marmelos* (A_M), *Aloe barbadensis* (A_B), *Annona squamosal* (A_S), *Azadirachta indica* (A_I) and *Oscimum sanctum* (O_S) were collected. The plants were selected based on their medicinal values. For the isolation of endophytic bacteria, fresh leaf samples were subjected to pretreatment wash in distilled water followed by a 2 min. wash in 70% methanol. The leaf samples were then washed in 2% sodium hypochlorite solution for 1 min. followed by sterile distilled water for 2 min. Leaves were crushed in sterile distilled water using mortar and pestle and plant extracts were prepared. About 1 ml of crushed samples were serially diluted and 0.1 ml spread onto Luria-Bertani (LB) medium. Plates were incubated at 37°C for 2-3 days. Cultural morphology such as colour, texture, consistency and size limited number of representative isolates were selected for further investigation.

2.2. Screening of endophytic bacteria for enzymatic activity

Isolated microbial endophytes were screened for different biological activities such as Amylolytic activity in 1% starch agar plates, Proteolytic activity in 1% (v/v) milk agar plates, Lipolytic activity in tributyrene (TBA) and calcium carbonate plates and Cellulolytic activity in cellulose-containing plates.

2.3. Plant growth promoting assays

2.3.1. Phosphate solubilization

Isolates were inoculated on Pikovaskaya's phosphate solubilizing agar [10]. Plates were incubated for three days at 28±2°C.

2.3.2. Zinc solubilization

Isolates were inoculated in the modified Pikovaskaya's agar containing 0.1 % insoluble zinc compounds such as ZnO and ZnS. Then the plates were incubated at 28°C for 24-48 hours.

2.3.3. IAA production

Isolates were inoculated in Luria Broth (LB) supplemented with tryptophan (0.01%) for 48 hours. Bacterial cultures were centrifuged at 5000 rpm for 10 minutes at 4°C to harvest the cells. 0.5 ml supernatant was taken into a fresh microcentrifuge tube and added 1 ml Salkowaski reagent.

2.4. Screening of endophytic bacteria for salt tolerance

Endophytic bacteria were inoculated onto a nutrient agar medium supplemented with different concentrations of NaCl (2, 5 and 10%). All the plates were incubated at 28°C for 5 days and bacterial growth was observed every 24 hours.

2.5. Biochemical tests

Isolates were characterized based on twelve biochemical properties like Sugar Utilization, Methyl Red Test, Voges-Proskauer Test, Citrate Utilization Test, Indole Production Test, Phenylalanine Deamination Test, Urea Hydrolysis Test, Nitrate Reduction Test, Ammonia Production Test, Catalase Test, Oxidase Test and Triple Sugar Iron (TSI) Agar Test. All the biochemical tests were performed as per standard reference methods [11].

2.6. *In vitro* antifungal study

Endophytic microbes that were isolated from different medicinal plants were further tested for antifungal activity against the common plant pathogenic fungus *Aspergillus niger*. The fungal growth was placed in the middle of the nutrient agar plate whereas the endophytic isolates were streaked on both sides at an equal distance of the fungal pathogen. The inhibition zone was measured after 5-7 days.

3. RESULTS AND DISCUSSION

3.1. Isolation of bacterial endophytes

Total 21 leaves bacterial endophytes were isolated on Luria-Bertani (LB) and Nutrient agar isolation medium. From six varieties of medicinal plant leaves their respective numbers of isolates are mentioned in table 1.

Table 1: The numbers of isolates with reference names

Leaves of different medicinal plants	No. of Isolates	Reference name
Rosary pea (*Abrus precatorius*)	2	Ap1,Ap2
Golden apple (*Aeglemarmelos*)	5	Am1,Am2, Am3,Am4, Am5
Aloe vera (*Aloe barbadensis*)	5	Ab1, Ab2, Ab3, Ab4, Ab5
Sugar apple (*Annona squamosal*)	3	As1,As2, As3
Neem (*Azadirachta indica*)	2	Ai1,Ai2
Tulsi (*Oscimum sanctum*)	4	Os1,Os2, Os3,Os4

Results indicate endophytes were isolated previously studied by this method. Kumar *et al.,* isolated a total of 14 fungi and 7 bacteria from leaves and stem of medicinal plants viz. *Azadirachta indica* and *Catharanthus roseus* [12]. 3 isolates were obtained from the leaf extract of *Calotropis procera* [13]. 94 bacteria were isolated from the leaf of the plant *Moringa oleifera* [14].

3.2. Morphological characterization of endophytic bacteria

The 21 isolates showed the diversified colonies among their size; shape such as round, irregular, punctiform; pigmentations produced by some of the colonies. Further microscopic observations results are shown in Table 2.

3.3. Screening of endophytic bacteria for enzymatic activity

Extracellular enzyme amylase was produced by leaf endophytes and a zone of hydrolysis was observed. From the total 21 isolates, 16 colonies were found active in producing amylase. The development of a clear zone surrounding the growth indicates the formation of paracasein due to hydrolysis of casein protein present in milk agar plate by the enzyme caseinase produced by the isolates. The formation of a clear zone due to solubilization of calcium carbonate indicates positive lipolytic activity due to the production of the enzyme lipase. Lipolytic activity was readily given by the isolates A_M5, A_I1, A_I2, A_P2, A_B1 and A_B2. Isolates were inoculated into the agar plate containing cellulose. Growth was observed in all the plates but only a few colonies were observed producing cellulolytic activity which was detected through the zone of hydrolysis of cellulose by the enzyme cellulase. Arora *et. al.*, obtained 50% positivity of their total isolates and the same results are obtained by us for this activity [15].

Table 2: Results of gram reaction and enzymatic screening

Isolates	Gram Reaction	Amylolytic activity	Proteolytic activity	Lipolytic activity	Cellulolytic activity
Ap1	Positive, Cocci in groups	+	-	-	+
Ap2	Negative, Long rods in chain	+	+	+	-
Am1	Negative, Short rods	+	+	-	-
Am2	Positive, Cocci in groups	+	-	-	+
Am3	Negative, Cocci in chain	+	-	-	+
Am4	Negative, Short rods	+	-	-	-
Am5	Positive, Long rods in chain	+	-	+	-
Ab1	Positive, Cocci in chain	+	+	+	+
Ab2	Negative, Short rods	+	-	+	-
Ab3	Negative, Short rods	+	-	-	+
Ab4	Negative, Cocci in groups	-	-	-	-
Ab5	Negative, Short rod in group	-	+	-	-
As1	Positive, Cocci in chain	-	-	-	-
As2	Positive, Cocci in groups	+	-	-	+
As3	Negative, Long rods in chain	+	-	-	-
Ai1	Negative, short rod singly, group	-	+	+	+
Ai2	Positive, rods in chains	-	-	+	+
Os1	Positive, rods in chain	+	-	-	-
Os2	Negative, Short rods in groups	+	+	-	-
Os3	Positive, rods in chain	+	-	-	+
Os4	Positive, Cocci in chain	+	-	-	+

3.4. Plant growth promoting assays

Among 21 isolates, 13 endophytic bacterial isolates shows phosphate solubilisation activity (Fig. 1). 52% of the total isolates were active against the zinc solubilisation effect (Fig. 1). For qualitative assessment of IAA, 9 out of 21 isolates displayed colour changed ranging from light pink to reddish, on the addition of Salkowski's reagent, indicating positive results for IAA production (Figure 1).The diameter of these zones was recorded and are mentioned in table 3. A method

described by Arora *et. al.,* for the inorganic phosphate solubilization, they have isolated only 10% colonies of their total 20 endophytic bacteria showing this activity [15]. Overnight grown single colonies were transferred aseptically by inoculating as spot on respective zinc medium plates [16].

3.5. Screening of endophytic bacteria for salt tolerance

All the 21 endophytic bacteria showed growth at 2% NaCl concentration just after 24 hours, late growth was observed at 5% NaCl and only a few isolates viz. O_S2, A_I2, A_M4 and A_P2 showed their growth in 10% NaCl concentration after 48 hours which was regularly checked every 24 hours. Thus the ability of the particular bacterial isolate to grow in high salinity can be essential for plant adaptation in a stressful environment and can elucidate the survival mechanism used by endophytes in natural environments with high salinity.

3.6. Biochemical tests

The utilization of Maltose, Lactose and Dextrose was studied using the fermentation broth with Durham's tube and the results are presented in Table 3. Out of 21 isolates, 8 isolates fermented maltose from which 2 isolates viz. A_B1 and A_B2 produced gas, 8 isolates fermented lactose, 6 isolates fermented dextrose.

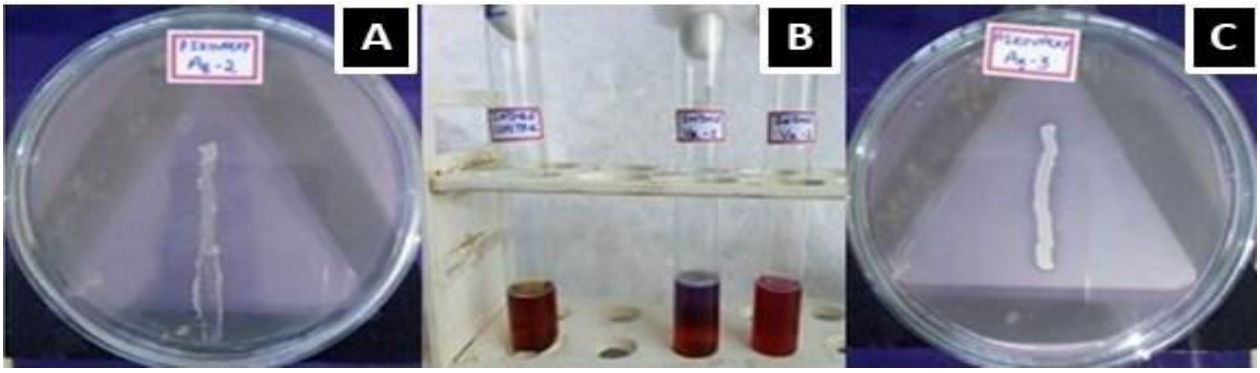

Fig. 1: A. Phosphate Solubilisation; B. Indole Production; C. Zinc Solubilisation Activity

Table 3: Carbohydrate utilization and plant growth promoting activity of bacterial endophytes

Isolates	Maltose	Lactose	Dextrose	Phosphate solubilization Zone (mm)	Zinc solubilization Zone (mm)	IAA production
Ap1	-	-	-	8.0	0.0	+
Ap2	-	+	-	4.0	7.0	-
Am1	+	+	+	0.0	12.0	+
Am2	-	-	-	5.0	10.0	-
Am3	-	-	-	10.0	0.0	-
Am4	-	-	-	3.0	0.0	+
Am5	+	+	+	0.0	6.0	-
Ab1	+ / G	+	-	0.0	0.0	+
Ab2	+ / G	-	+	12.0	6.0	+
Ab3	-	-	-	7.0	12.0	-
Ab4	-	-	-	8.0	18.0	+
Ab5	-	-	-	0.0	5.0	-
As1	-	+	-	8.0	0.0	+
As2	-	-	+	6.0	2.0	-
As3	-	+	-	0.0	7.0	+
Ai1	+	+	-	0.0	8.0	-
Ai2	+	-	+ / G	14.0	0.0	-
Os1	-	-	+	7.0	0.0	-
Os2	-	-	-	9.0	0.0	-
Os3	+	-	-	0.0	9.0	-
Os4	+	+	-	0.0	0.0	+

Most of the endophytic isolates were active for the biochemical and physiological characterization. Among the 6 selected medicinal plants, endophytic bacterial isolates from the plants' *Aloe barbadensis* (A_B1,A_B3), *Aegle marmelos* (A_M1,A_M2 A_M3,A_M4,A_M5), *Azardirchata Indica* (A_I1), *Annona squamosa* (A_S1,A_S3) and *Ocimum sanctum* (O_S2,O_S3,O_S4) gave the positive results for the methyl red test which conveys that they isolates are mixed acid fermenters. Endophytic bacterial isolates from the plants *Aegle marmelos* (A_M2,A_M4,A_M5), *Aloe barbadensis* (A_B4,A_B2), *Annona squamosa* (A_S2), *Ocimum sanctum* (O_S1), gave the Voges Proskauer test positive due to the presence of diacetyl function. Bacterial isolates from the plants *Abrus precatorius* (A_P1,A_P2), *Aloe barbadensis* (A_B1), *Annona squamosa* (A_S1,A_S2,A_S3) and *Ocimum sanctum* (O_S1,O_S2) were able to utilize the citrate in the medium, with the production of alkaline products indicating the Citrate utilization test positive. The indole production test was positively given by the isolates of the plants *Abrus precatorius* (A_P1,A_P2), *Aegle marmelos* (A_M1,A_M2,A_M3,A_M4), *Aloe barbadensis* (A_B1,A_B2,A_B3,A_B4,A_B5), *Azardirchata Indica* (A_I2), *Annona squamosa* (A_S2,A_S3) and *Ocimum sanctum* (O_S3, O_S4). Negative results were obtained for the Phenylalanine deamination test. Positive Urea hydrolysis tests were obtained from the isolates of the plants *Aegle marmelos* (A_M4), *Aloe barbadensis* (A_B1,A_B3), *Annona squamosa* (A_S1,A_S2,A_S3), *Azardirchata Indica* (A_I1), *Ocimum sanctum* (O_S2). Nitrate reduction test was obtained positive for most of the isolates and the change of red litmus to blue indicates positive ammonia production. The catalase test was positively obtained for almost all the isolates. Oxidase test was obtained positive for the isolates of the plants *Abrus precatorius* (A_P1), *Aegle marmelos* (A_M1), *Aloe barbadensis* (A_B2), *Annona squamosa* (A_S1,A_S2, A_S3) and *Ocimum sanctum* (O_S1). The triple sugar iron test was indicated positive by the isolates of the plants' *Aloe barbadensis* (A_B2,A_B3,A_B4,A_B5), *Azardirchata Indica* (A_I1) *Ocimum sanctum* (O_S3) (Table 4).

Table 4: Biochemical tests results of bacterial endophytes

Isolates	MR	VP	Citrate Utilization	Indole Production	Phenylalanyl Deamination	Urea Hydrolysis	Nitrate Reduction	Ammonia Production	Catalase Test	Oxidase Test	TSI
Ap1	-	-	+	+	-	-	-	-	+	+	-
Ap2	-	-	+	+	-	-	+	-	+	-	-
Am1	+	-	-	+	-	-	+	+	+	+	-
Am2	+	+	-	+	-	-	+	-	+	-	-
Am3	+	-	-	+	-	-	-	+	+	-	-
Am4	+	+	-	+	-	+	+	-	+	-	-
Am5	+	+	-	-	-	-	+	-	+	-	-
Ab1	+	-	+	+	-	+	+	-	+	-	-
Ab2	-	+	-	+	-	-	+	-	+	+	+
Ab3	+	-	-	+	-	+	+	-	+	-	+
Ab4	-	+	-	+	-	-	+	-	+	-	+
Ab5	-	-	-	+	-	-	-	-	+	-	+
As1	+	-	+	-	-	+	+	-	+	+	-
As2	-	+	+	+	-	+	+	-	+	+	-
As3	+	+	+	+	-	+	+	-	+	+	-
Ai1	+	-	-	-	-	+	+	+	+	-	+
Ai2	-	-	-	+	-	-	+	+	+	-	-
Os1	-	+	+	-	-	-	+	+	+	+	-
Os2	+	+	+	-	-	+	+	+	+	-	-
Os3	+	+	-	+	-	-	+	+	+	-	+
Os4	+	+	-	+	-	-	+	+	+	-	-

3.7. *In vitro* antifungal study

Endophytic culture A_P1, A_P2, A_B1, A_B2, A_B3, A_S3, A_I1, A_I2, O_S1, O_S2, O_S3 and O_S4, showed a remarkable control over *Aspergillus niger* (Table. 5). Jalgaonwala *et al.,* isolated 78 bacterial endophytes from the aerial parts and roots of eight medicinal plants [17]. Out of

seventy-eight isolates, ten inhibited the growth of the fungal pathogen *Aspergillus niger*. Similar to their observation, in our study, the endophytic bacterial strains isolated from the medicinal plants, *Abrus precatorius, Aloe barbadensis, Annona squamosa, Azadirachta indica* and *Oscimum sanctum* showed remarkable inhibitory zones against the fungal pathogen *Aspergillus niger*.

Table 5: Antifungal activity of endophytic isolates

Isolates	A_P1	A_P2	A_B1	A_B2	A_B3	A_S3	A_I1	O_S1	O_S2	O_S3	O_S4
Zone of Inhibition (mm)	15	17	08	13	14	09	11	13	10	12	14

4. CONCLUSION

The aim of studying endophytic bacteria is important not only for understanding their ecological role in their interaction with plants but also for their possible biotechnological applications. The methodology employed in this work was effective for the isolation of endophytes from the leaves of six medicinal plants. A total of 21 endophytic bacteria were isolated from selected plants out of which isolates of the plants *Aegle marmelose*, *Aloe barbadensis* and *Azadirachta indica* showed the largest biodiversity among the enzymatic, plant-growth-promoting and different biochemical activities. While some endophytic bacterial isolates showed major activity of hydrolytic enzymes and reasonable ability in controlling a pathogenic fungus. It has already become obvious that enormous potential for the organism, product, and utilitarian discovery in this field holds exciting promise. Therefore, it would be very interesting to further identify and analyse the main cause (Microbial Endophytes) behind these plants that make them so versatile.

Conflict of interest

All the authors involved in designing and execution of this work and given due recognition and have no conflicts in composing and submission of the article.

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