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BIOPROSPECTING OF ENDOPHYTIC BACTERIA ISOLATED FROM *FICUS HISPIDA* PLANT

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ABSTRACT
Endophytes are endosymbionts residing in the internal tissues of host plants without causing any apparent damage or harm to the host. The need for novel chemical compounds to treat human diseases is ever increasing. The rapid development of drug-resistant microbes, the discovery of new cases of life-threatening infections, and the constant recurrence of diseases have pushed for advances in the field of drug discovery. The study analyses the bioprospecting of endophytic bacteria from *Ficus hispida* plant leaves. In this study totally 16 morphologically different endophytic bacteria were isolated and screened for bioactivity, enzymatic activity, dye degradation and plant growth promoting substances. Four strains showed broad spectrum antimicrobial activity against test pathogens. In enzyme screening activity all the 16 strains produce any one of the enzyme. In degradation studies the maximum of 11 isolates degrade crystal violet, 9 isolates degrade malachite green, 7 strains degrade methylene blue and 5 strains degrade phenol. Four plant growth substances (acetoin, phosphatase, ammonia, indole acetic acid) are produced by endophytic bacteria isolates. These studies also studied for antibiotic susceptibility and salt tolerance of endophytic bacterial isolates for presence of antibiotic resistant genes and for withstand salt tolerance of isolated strains. This study demonstrates the occurrence of culturable bacterial endophytes in the plant under study. The presence of bacterial endophytes in the leaf tissues has uncovered a new arena which remains largely unexploited in the bioprospecting point of view. The present study concludes that endophytic bacteria isolated from *Ficus hispida* plant were potential for pharmaceutical environmental application.

KEYWORDS: Endophytes, Bioactivity, Enzyme, Degradation and Plant growth substance.

INTRODUCTION
Endophytes are microorganisms (bacteria or fungi or actinomycetes) that dwell within robust plant tissues by having a symbiotic association. Recent molecular studies on endophytic bacterial diversity have revealed a large richness of species. Endophytes promote plant growth and yield, suppress pathogens, may help to remove contaminants, solubilize phosphate, or contribute assailable nitrogen to plants. Plants and animals normally associate with diverse microorganisms.[1] Plants constitute vast and diverse niches for endophytic organisms. Endophytic bacteria have been isolated from a large diversity of plants as reviewed.[2] Endophytic bacteria in a single plant host are not restricted to a single species but comprise several genera and species. Microbial endophytes are known to produce a wide range of bioactive compounds which may actually contribute to host plant health such as phyto-hormones or by increasing resistance against the plant pathogens and parasites.[1]

The study of endophytic bacteria has evoked great interest due to their beneficial properties like fixing nitrogen, producing auxins and protecting the host plants from plant pathogens. Endophytic bacteria have been isolated from many plants like maize, rice, wheat, sugarcane, marigold, coffee, potatoes, tomatoes, lettuce, fodder plants like alfalfa, sorghum, and trees like yew and pine.

The plants with ethnobotanical importance are the ones which are more likely to harbour endophytes capable of producing pharmaceutically important bioactive compounds. It is highly possible that various unique bioactive compounds associated with the host tree are in reality being produced by the residing endophytes. Thus

the isolated endophytes could be cultured and used for commercial production of the bioactive compounds.[3]

Considering that only a few endophytes have been studied, they constitute a poorly investigated group of microorganisms with abundant potential as a source of biologically important and chemically novel compounds with significant pharmaceutical and agricultural applications.[4]

Plants are one of the most vital sources of medicines. Currently, large numbers of drugs in use are derived from plants. Medicinal plants are the chief source of secondary metabolites used as drugs and essential oils of therapeutic importance. The term endophyte (Gr. endon, within; phyton, plant) was first coined in 1866 by De Bary. An endophyte can be defined as a microorganism such as fungi or bacteria that expends either the complete or part of its lifecycle within the healthy tissues of a living plant, typically causing no symptoms of disease.[5]

Keeping in view the importance of the four selected ethno medicinal plants, their pharmaceutical applications and biological activity, further study on purification, optimization, and structure elucidation of bioactive compound produced by the best isolates that exhibit wide spectrum activity and the identification of the potential isolate is under progress. The need for novel chemical compounds to treat human diseases is ever increasing. The rapid development of drug-resistant microbes, the discovery of new cases of life-threatening infections, and the constant recurrence of diseases have pushed for advances in the field of drug discovery.

Endophytes refer to the microorganisms (mostly fungi and bacteria) colonising the intercellular and intracellular regions of healthy plant tissues at a particular time, whose presence is unobtrusive and asymptomatic. Most plant species that have been previously studied host at least one endophytic microbe with plants growing in unique environmental settings generally hosting novel endophytic microorganisms.[6] Endophytes are a potential source of novel bioactive compounds. Nonetheless, fine screening, purification, and identification methods are required to target active compounds since each microorganism may contain a large pool of compounds with only few being bioactive.

MATERIALS AND METHODS

Pretreatment of leaf sample

For the isolation of endophytic bacteria fresh leaf samples of *Ficus hispida* plant was collected from Ninnaikarai Village, Kattangulathur, Tamilnadu. The leaf samples were collected in sterile plastic bags and transported aseptically to the laboratory. For the pretreatment of leaf samples and for isolation of endophytic bacteria, the method described by Sun[7] *et al.*, was adopted. Fresh leaf samples were washed in running tap water, followed by 20min wash in 70% ethanol. Then the leaf sample was washed on 2% sodium hypochlorite for 1 min. Finally leafs were washed in distilled water for minutes and dried.

Isolation and characterization of endophytic bacteria

After pretreatment, plant leaves were crushed with sterile distilled water using mortar and pestle. About 1 ml of crushed sample was serially diluted and 0.1ml of aliquot from 10^2 to 10^5 dilutions were taken and spread onto nutrient agar medium using sterile glass L-Rod. Plating was done in duplicates and all the plates were incubated at room temperature for 48 hours. From the total isolates, based on the morphology characters such as colour, texture, consistency, size, and shape limited number of colonies were selected from the plates for further investigations. All the selected isolates were subcultured in nutrient agar slants and preserved in refrigerator at 4°C. Phenotypic characters such as Grams reaction, endospore staining, capsule staining, motility, catalase, oxidase activity of all the isolates were performed by adopting standard procedures.[8]

Screening of endophytic bacteria for antimicrobial activity

Antimicrobial substances from bacterial isolates were produced by submerged fermentation using fermentation medium[9] (peptone 1%, glucose 1%, potassium dihydrogen phosphate 0.1%, magnesium sulphate 0.05%, pH 7). For the preparation of 18hrs inoculum, Nutrient broth was prepared and all the bacterial isolates were inoculated and incubated at 28ºC. For the production of antimicrobial compound, 10% inoculum were transferred into fermentation medium and incubated in rotary shaker at 28°C for 120 hours. Cell free supernatant was prepared by centrifuging fermentation medium for 10,000 rpm for 10minutes. Antimicrobial activity of culture supernatant was tested by agar well diffusion method using Muller Hinton Agar medium. Test bacterial strains used in this study include *Staphylococcus aureus, Bacillus sp., Klebsiella, Pseudomanas sp., E. coli* from Centre for DDD, Sathyabama University, Chennai.

Screening of endophytic bacteria for extracellular enzymatic activity

All the bacterial isolates were screened for various enzymes such as Amylase, Protease, lipase, invertase, pectinase, agarase, glutaminase and urease by plate Method. All the isolates were spot inoculated on respective enzyme screening media and incubated at 28ºC for 5-7days.[8]

Enzyme	Media	Method of detection	Reagents used
Amylase	Starch Agar	Zone of clearance	1% iodine
Protease	Skim Milk agar	Zone of clearance	-
Lipase	Tween 80 agar	Opaque zone	1% cuso4
Invertase	Minimal agar +1% sucrose	Growth	-
Pectinase	Minimal agar+1% pectin	Growth	-
Urease	Christensonºs urea agar	Pink colour	-
Agarase	Artificial agar medium	Zone of clearance	1% Iodine
Glutaminase	Nutrient agar+1%glutamine	Pink color	-
Asparginase	Nutrient agar + 1% aspargine	Pink colour	-

Screening for biodegradation activities

Dye degradation: All the bacterial isolates were tested for dye degradation using different dyes such as Crystal violet, Saffranin and Malachite green by Plate method. All the bacteria were spot inoculated into screening medium supplemented with Crystal violet (0.01%), Malachite green (0.01%), Saffranin (0.01%) in respective plate and incubated at 28ºC for 5 days. Clear zone around the bacterial spot indicates dye degradation.[10]

Phenol degradation: All the bacterial isolates were screened for phenol degradation. The isolates were spot inoculated on mineral agar medium supplemented with phenol (0.05%) as sole carbon source. The plates were incubated at 28ºc for 5 days. Presence of growth on the mineral agar medium indicates phenol degradation.[11]

Screening for plant growth promoting substances

Acetoin production: The Voges-Proskauer test was used as a qualitative method for the detection of acetoin, a precursor for 2,3 butanediol. All the bacterial isolates were inoculated on each 2ml of MR-VP broth and incubated at 28ºC for 5 days. After incubation, to the 1ml bacterial culture 3ml of freshly prepared 5% α-Napthol in absolute ethanol and 1ml of 40% KOH were added and the mixture was stirred vigorously. The formation of a red colour is indicative of the presence of acetoin.[12]

Ammonia Production: For the screening of ammonia production, all the bacterial isolates were inoculated into each 5ml of sterile peptone broth and incubated at 28ºC for 48 hours. After incubation, about 0.5ml of Nessler's reagent was added into all the tubes. The production of ammonia is indicated by the appearance of pink colour.

Phosphate solubilization: All the isolates were screened for phosphate solubilization by the method described.[12] All the bacterial isolates were spot inoculated on Pikovoskaya's agar plates and incubated at 28ºC for 2-3days. After incubation the plates were observed for clear halo formation around the bacterial growth for every 24hours.

Nitrogen fixing bacteria: Nitrogen fixing activity of bacterial isolates were studied by inoculating them into Jensen medium and incubating them into 5 days for 28ºC. Presence of growth on Jensen medium indicates nitrogen fixation.[9]

Indole acetic acid (IAA): For the screening of IAA production, all the bacterial isolates were inoculated into each 5ml of sterile nutrient broth supplemented with 3mg/ml of L-tryptophan and incubated at 28ºC for 5 days. After incubation, cell free supernatant was obtained by centrifuging at 10,000rpm for 10min and to that a drop of orthophosphoric acid and 2ml of Solwaski's reagent was added and kept for 20min at room temperature. The development of pink colour indicates the production of IAA.[8]

Screening of endophytic bacteria for antibiotic susceptibility

Antibiotic susceptibility pattern for bacterial isolates were determined by Kirby-Bauer disc diffusion method.[13] 18 hrs broth cultures of bacterial isolates was prepared in nutrient agar and adjusted to 0.5 McFarland standards. All the cultures were inoculated into Muller Hinton Agar plates using sterile cotton swab. Standard antibiotic disc streptomycin (10µg), vancomycin(30µg), trimethoprin(5µg), bacitracin(10µg), were placed on nutrient agar plates and incubated at 37°C for 24 hours. After incubation antibiotic susceptibility pattern was determined by measuring zone of inhibition.

Screening of endophytic bacteria for salt tolerance

Endophytic bacteria were inoculated onto nutrient agar medium supplemented with different concentrations on NaCl (0-10%). All the plates were incubated at 28ºC for five days and bacterial growth was observed for every 24 hours.

RESULTS

Isolation and Characterization of endophytic bacteria

Nutrient agar plates inoculated with *Ficus hispida* plant leaves sample showed morphologically differenct bacterial colonies. Totally 28 colonies were recovered from sample, of which 16 isolates were selected for further studies.

Of 16 isolates selected 5 were pigmented and 11 isolates were non-pigmented. Regarding morphology characteristics 3 strains were Gram positive cocci, 5 stains were Gram positive rod, 8 strains were Gram negative rods. Out of 16 isolates 4 strains showed endospore positive, possibly genus *Bacillus*.

Antimicrobial activity

Regarding antimicrobial activity, 10 (62.5%) isolates showed inhibitions against any one of test strains. Out of 16 isolates, 4(25%) showed broad spectrum activity, 4 (25%) showed against only for Gram positive bacteria, 2(12.5%) showed activity only for Gram negative bacteria.

Enzyme activity

Enzymatic activities of endophytic bacterial shows majority of isolates exhibit positive activity for protease. Maximum number of strain showed positive activity for Protease (62.5%), Amylase (43.75%), Glutaminase (31.25%), Urease (43.5%), Agarase (18.75%), Pectinase (75.5%), Lipase(37.5%), Invertase (31.5%). Number of bacteria showing different enzymatic activities are given in fig 1.

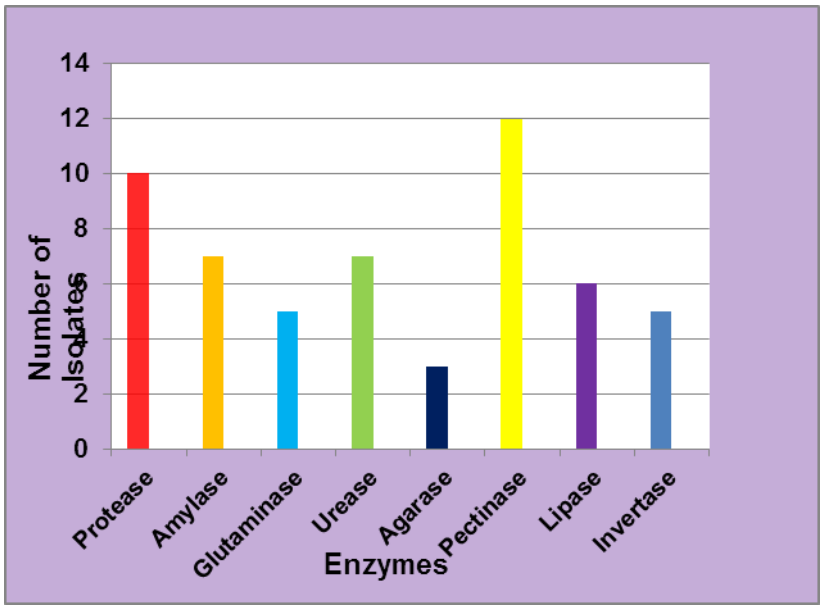

Figure 1: Screening of extracellular enzymes of endophytic isolates.

Biodegrading activity of Bacteria

Out of 16 endophytic isolates 7 strains showed degradation against Methylene blue, 11 strains showed crystal violet degradation, 9 strains showed malachite green degradation and 5 strains showed phenol degradation (Fig 2).

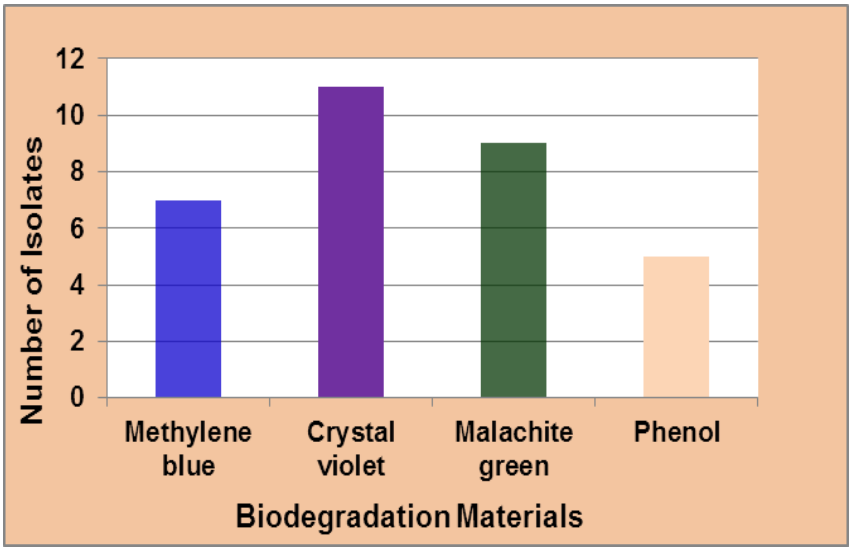

Figure 2: Biodegradation activity of endophytic isolates.

Plant growth promoting substances activity of endophytic isolates

Plant growth promoting substances of bacterial isolates reveals positive results for plant growth promoting substances. Of 16 isolates, 5(31.25%) strains exhibited indole acetic acid, 6(31.5%) isolates showed positive for ammonia production, 4 (25%) isolates showed acetoin production, 6(31.5%) showed positive for phosphate solubilization (Fig 3).

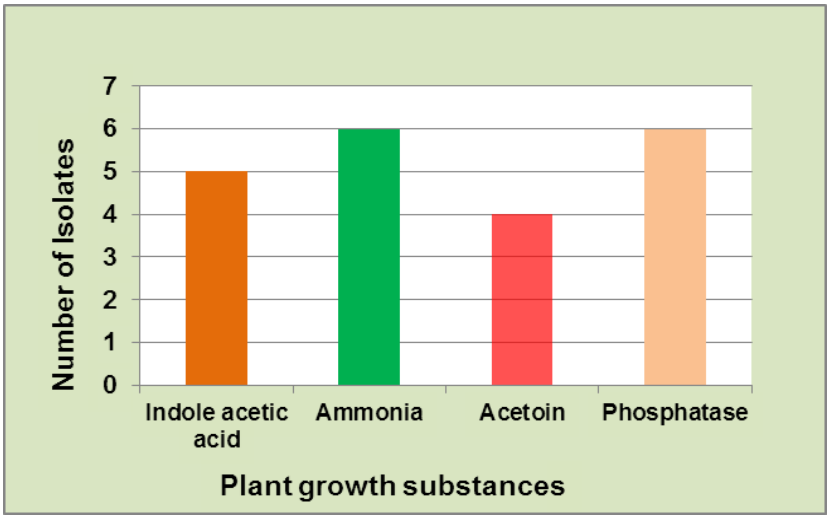

Figure 3: Screening activity of plant growth substances by endophytic isolates.

Screening of endophytic bacteria for antibiotic susceptibility

Of the isolates studied for antibiotic susceptibility, 87.5% of endophytic bacteria was susceptible to streptomycin, 62.5% and 75% of endophytic isolates was susceptible to bacitracin and trimethoprin. About 81.25% of isolates were sensitive to vancomycin.

Effect of Sodium Chloride on the growth of bacteria

All the 16 isolates showed good growth at 0% to 5% NaCl concentration, 5 isolates showed good growth upto 6% NaCl concentration and 3 isolates showed growth at 7% NaCl concentration. The growth rate decreased when the NaCl concentration increased.

DISCUSSION

In the present study *Ficus hispida* plant leaves yielded total 28 colonies. Several reports have been reported on endophytic bacteria and fungi in different plant leaves. However there is no or less report on endophytic bacteria from *Ficus hispida* plant leaves. In general endophytic bacteria occur at low population than rhizospheric area. Based on the visible morphological difference 16 isolates were selected from 24 isolates.[14]

Endophytes are the chemical synthesizers within plant.[8] Many of them are capable of synthesizing bioactive compounds that can be used by plants for defense against pathogens and some of these compounds have been proven useful for drug discovery. Most of the natural products discovered from endophytes are antibiotics, anticanceragents, antivirals, antidiabetic agents and other bioactive compounds having different functional roles.[15] In this present study, culture filtrate of 10 isolates showed antimicrobial activity whereas 4 strains showed broad spectrum activity. Detection of active compounds from these isolates and further studies are needed for their proper utilization.

Next to antibiotics, enzymes are the most important products. In the present study, while screening the endophytic bacterial isolates for 8 different extracellular enzymes, 10 isolates showed protease, 12 isolates produce pectinase, 5 isolates produce glutaminase and invertase, 7 isolates produce amylase and urease, 6 isolates produce lipase and 3 isolates produce agarase enzymes. Maria[16] *et al.*, have also reported enzymatic activity of mangrove-derived endophytic fungi. The results of the present study showed the production of several enzymes by endophyte bacteria which might play role in mangrove litter degradation.

Pandey[17] *et al.*, reported that endophytic bacteria residing inside the plant have been promoting the growth of plant directly or indirectly through production of phytohormones, plant nutritional status and biocontrol of host plant diseases. The present study also showed four plant growth promoting activities like acetoin (4 isolates), indole acetic acid (5 isolates), ammonia(6 isolates) and phosphatase (6 isolates) produced by endophytic bacteria isolated from *Ficus hispida*.

Endophytes are recently reported in the degradation of environmental toxins. The bacteria also degrading recalcitrant compounds from contaminated soils. The improvement of phytoremediation of water soluble and colatile organic pollutants by endophytic bacteria was reported by Barac et al.[18] In this present study out of 16 isolates 7 endophytic bacterial isolates degrade methylene blue, 11 isolates degrade crystal violet, 9 isolated showed degradation of malachite green and 5 strains showed degradation of phenol. Further the dye degrading endophytes isolated in this study will be a potential candidate for dye degrading enzymes.

Antibiotic resistant genes, in addition to clinical pathogens, are also present in environmental isolates which are horizontally transferred to other microorganisms. In the present study, susceptibility pattern of antibiotic for endophytic isolates were studied using different antibiotics. Most of the isolates were susceptible for antibiotics particularly for streptomycin, vancomycin and bactiracin, trimethoprin respectively.

In this present study, salt tolerance was studied for all endophytic isolates. Out of 16 isolates 3 isolates showed upto 7.5% of salt tolerance and 5 isolates were tolerated upto 6% of salt where as other isolates were showed 0 to 5% salt tolerance. Kamalraj[19] *et al.*, reported the effect of NaCl on endophytic fungal assemblage in the leaves of mangrove plant.

The plants have the great potential but are under-exploited source for endophytic bacteria. In this present study endophytic bacteria isolated from *Ficus hispida* plant produce substances of antimicrobial compounds, enzymes, plant growth harmones and also biodegrading activities. The commercial utility of this potential biochemical's need further study.

CONCLUSION

This study demonstrates the occurrence of culturable bacterial endophytes in the plant under study. The presence of bacterial endophytes in the leaf tissues has uncovered a new arena which remains largely unexploited. The endophytic isolates also showed production of some compounds which if further identified can be used for future probable applications. Many such medicinal plants associated with endophytes are an untapped source of novel bioactive compounds which could have significant industrial, pharmaceutical and agricultural applications.

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