SCREENING OF ENDOPHYTIC BACTERIA FROM LEAVES AND FRUIT OF AVOCADO AT PIN-TA-YA TOWNSHIP

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ABSTRACT

Plant-associated bacteria that live inside plant tissues without causing any damage to plants are defined as endophytic bacteria. The term endophyte (Gr. endon, within; phyton plant) was first coined in 1866 by De Bary. The isolation and screening of endophytic bacteria from and their ability to produce antimicrobial and enzyme were carried out. The two samples were collected from Pin- Ta-Ya Township. The results of the endophyte isolation in these experiments gave ten strains (5species from leaves and 5 species from fruit) from leaves and fruit by using nutrient medium. Among the bacterial isolates, A 5, 7, 9 and 10 bacteria showed the antiagrobacteria against Agrobacterium sp. which can cause tumor. Another five bacteria isolates showed the antimicrobial activity against four test organisms by using well diffusion method. Continually, morphology and biochemical characters of these ten strains were further studied for identification. Only two isolated strains revealed hydrogen sulphide test. SN- 4, 7, 9 and 10 showed positive results of nitrate test. Among than SN- 1, 2, 3 and 5 showed positive results for glucose fermentation test. Except SN- 2, it was showed positive results of citrate utilization. Isolated strains SN- 1, 3, 5 and 10 showed positive results of urease. All isolated bacteria were found positive effect on catalase, salt tolerant, casein hydrolysis and starch hydrolysis, the results indicated that they can produce specific enzyme. Continually, morphology and biochemical characters of these ten strains were further studied for identification.

INTRODUCTION

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 important advantages of medicinal plants for therapeutic uses in various ailments are their safety and also being inexpensive, effective, and their easy accessibility. These advantages of the medicinal plants forced the traditional medical experts for extensively used in their day to day practice.

Plants can be considered as a new source of microorganisms. The plant parts can be utilized in the isolation of bioactive compound producing microorganisms. This means that there is much possibility of finding new microorganisms from plant sources. Today, the

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antimicrobial metabolites are required to have potent activity and be safe to humans, animals and ecosystems. The pharmaceutical agents derived from natural sources are an important part of our therapeutic strategies since beginning due to their chemical diversity and various bioactivities against diseases.

Plant-associated bacteria that live inside plant tissues without causing any damage to plants are defined as endophytic bacteria.Endophytic bacteria living in plant tissues deprived of doing substantive harm or gain benefit other than residency (Kado, 1992**).** The term endophyte (Gr. endon, within; phyton, plant) was first coined in 1866 by 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 (Tan, 2001 and Gunatilaka, 2006) .

The microbes existing in plants are called endophytic microbes. These microbes spent part or their entire lifetime in the living tissue of the host plant without causing any harm (Petrini et al., 1992). Microbes such as fungi, yeast, and bacteria associated with the host plant assist in the metabolites with potent medicinal activity such as antitumor, antibacterial, and antifungal compounds as decomposing enzymes, and also plant growth hormone (Petrini et al., 1992; Strobel et al., 1996; Strobel, 2002).

Now we are familiar with the fact that endophytic microorganisms that reside in the tissues of living plants are promising, less explored and useful sources of novel natural products for exploitation in agriculture, medicine, and industry. The importance of endophytes had been demonstrated over a long period as a source of pharmaceutical bioactive compounds, as many of endophytes were exposed to produce novel bioactive metabolites such as antibacterial, antifungal, antiviral, antitumor, antioxidant, anti-inflammatory, immunosuppressive drugs, and many related compounds.

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 and are grouped into various categories, which include alkaloids, terpenoids, steroids, lactones, phenolic compounds, quinones, lignans, etc. Importantly, secondary metabolites produced by endophytes provide a variety of fitness enhancements and exert several beneficial effects on host plants, such as stimulation of plant growth (Sturz et al., 1997) nitrogen fixation (Kirchhorf et al., 1997; Reinhold-Hurek and Hurek, 1998) and induce resistance to drought, herbivorous, parasitism (Chen et al., 1995; Sturz and Matheson, 1996).

The purpose of this study was to evaluate several isolated bacteria from leaves and fruit of *Persea gratissima* Gaertn and to select on endophytic bacteria for further morphology, biochemical characteristic and antimicrobial activity. In this investigation, altogether ten endophytic bacteria are isolated into pure culture by using nutrient medium from leaves and fruit of sample. The isolated strains are subjected into the examination concerning bioactive substances with medicinal activity of isolated bacteria as antimicrobial activity. The report on the isolation of useful microbes from native plants in Myanmar is still rare.

MATERIALS AND METHODS

Collection of plant materials

For the isolation of endophytic bacteria, healthy leaves and fruit were collected from healthy wild and cultivated plants from Pin-Ta-Ya Township. Samples were placed in clean plastic bags, brought to the laboratory and used for further experimental purpose.

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Isolation procedure of endophytic bacteria strains (Atlas, 1993 and Phay, 1997)

The collected samples of leaves and fruits were washed in running water for 10 minutes. These leaves and fruits were rinsed with sterilized distilled water for 5 minutes. Then, they were sterilized by soaking in 75% alcohol for 2 minutes. After that these fruits were dried on sterilized paper. Then, the fruits were cut into about 1-2 cm pieces and they were placed on nutrient medium plates. The plates were incubated for one to two days at room temperature. The nutrient medium was used as stock medium.

Biochemical Characteristic of Isolated Endophytic Bacteria

The biochemical of isolates was conducted according to Bergey's manual of Determinative Bacteriology (Holt et al., 1994) and Moore et al., (1988). For each strain of the following tests including Gram staining, oxygen requirement, Hydrogen sulfide, salt to tolerant, nitrate reduction, citrate utilization, methyl red, Voges proskauer, Urea hydrolysis, Determination of carbohydrate, Starch hydrolysis .

Antimicrobial activity Estimation

The study of antimicrobial activity was performed by paper disc method described by Cruickshank. 1975 and using six test organisms (*Pseudomonas aeruginosa, Escherichia coli, Staphylococcus aureus, Sacchroromyces sp. and Agrobacterium tumefaciens*). The clear zones appeared around the agar well, indicated the presences of antimicrobial activity secreted by respective isolated strains..

Results

Collection of Sample

The sample are collected from Pin-Ta-Ya Township

Family - Lauraceae

Scientific Name - *Persea gratissima* Gaertn

Myanmar Name - Htaw-bat-thee

Figure 1. Collection of Leave and Fruit of *Persea gratissima* Gaertn from Pin-Ta-Ya township

Screening of Endophytic Bacteria

In the present works, altogether ten isolated strains were maintained into the pure culture and designated as SN- 1 to 10. All the isolated bacteria SN-1 to 10 were sub cultured on to the respective selected media to provide pure culture. They were then sub cultured on the agar slants for further investigation.

Figure 2. Isolation of Endophytic Bacteria from *Persea gratissima* Gaertn Leaves and Fruit

Table 1. Biochemical Characteristics of Isolated endophytic Bacteria Strains (SN-1 to 10)

Tests	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Cell morphology	cocci	cocci	cocci	cocci	cocci	cocci	bacilli	cocci	cocci	cocci
Gram strain	-	-	-	-	-	-	-	-	-	-
Catalase	+	+	+	+	+	+	+	+	+	+
Aerobic/Anaerobic	Aero	Aero	Aero	Aero	Aero	Aero	Aero	Aero	Aero	Aero
Glucose(acid)	+	+	+	-	+	-	-	-	-	-
NaCl 6.5%	+	+	+	+	+	+	+	+	+	+
NaCl 1.0%	+	+	+	+	+	+	+	+	+	+
Urease	+	-	+	-	+	-	-	-	-	+
Citrate	+	+	+	+	+	+	_	+	+	+
Caseinase	+	+	+	+	+	+	+	+	+	+
Hydrogen sulphide	+	-	-	-	-	+	-	-	-	-
Starch hydrolysis	+	+	+	+	+	+	+	+	+	+
Growth at 23˚C	+	+	+	+	+	+	+	+	+	+
Growth at 45˚C	+	+	+	+	+	+	+	+	+	+
Nitrate Test	-	-	-	+	-	-	+	-	+	+

+ = positive result - = negative result

Preliminary study on antimicrobial activity of isolated strains

In this study, it was observed that SN-1.5, 7, and 10 showed high potential in six antimicrobial activity test as shown in Table (2) and Figure (5)

Table 2. Antimicrobial activity of all isolated Isolated EndophyticBacteria Strains (SN-6 to 10)

Strain No.	***Agrobacterium tumefaciens***	***Escherichia coli***	***Pseudomonas aerugesinosa***	***Saccharomyces sp.***	***Staphylococcus aureus***
SN-1	_	10mm	18mm	17mm	17mm
SN-2	_	_	_	_	_
SN-3	_	_	_	_	_
SN-4	_	_	_	_	_
SN-5	16mm	10mm	15mm	16mm	18mm
SN-6	_	_	_	_	_
SN-7	15mm	25mm	1omm	18mm	_
SN-8	_	_	_	_	_
SN-9	18mm	_	_	_	_
SN-10	14mm	14mm	10mm	15mm	18mm

well = 6 mm

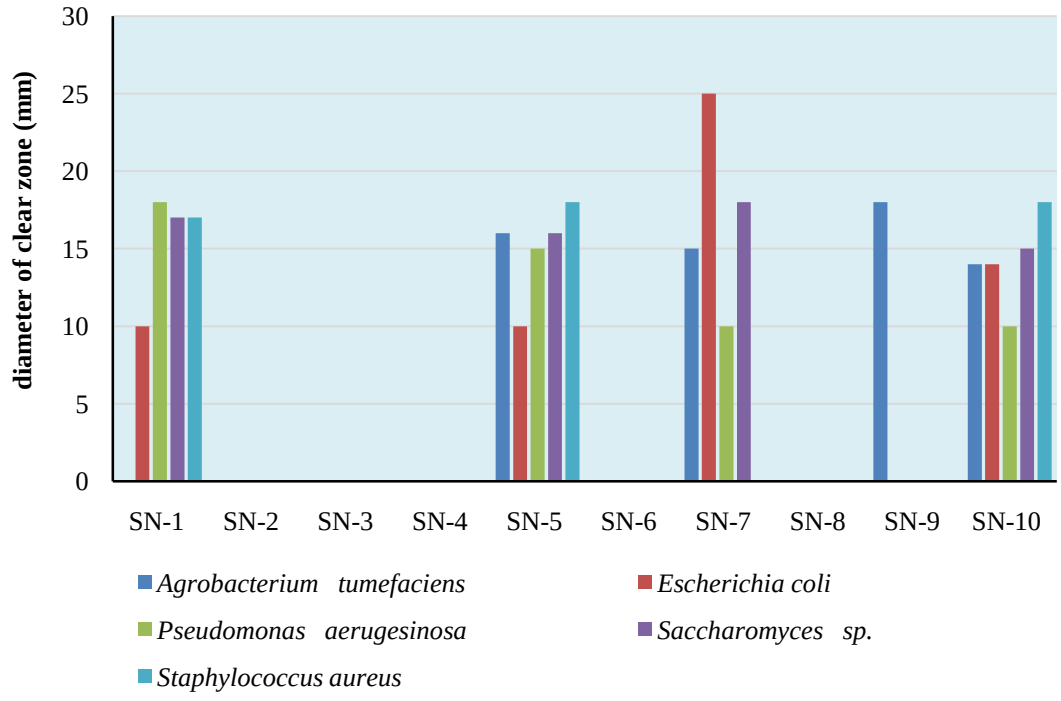
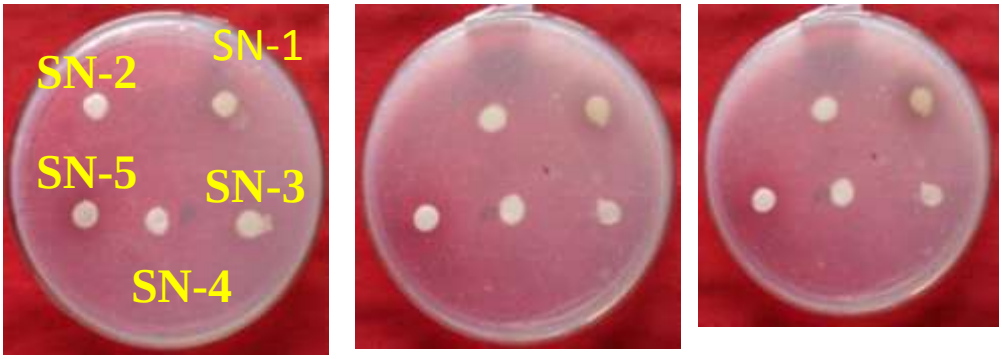
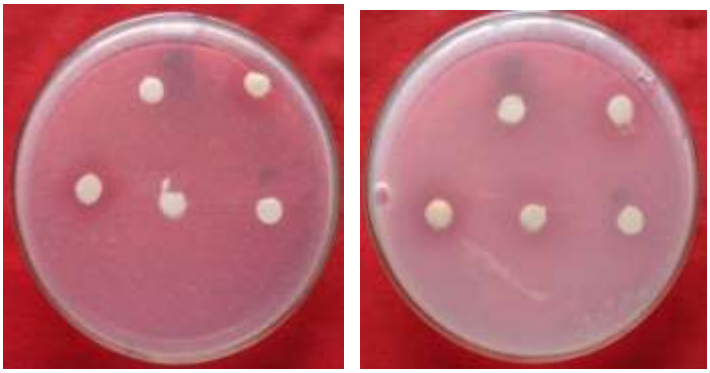


Figure 3. Antimicrobial activity of all isolated IsolatedEndophytic Bacteria Strains (SN-1 to 10)

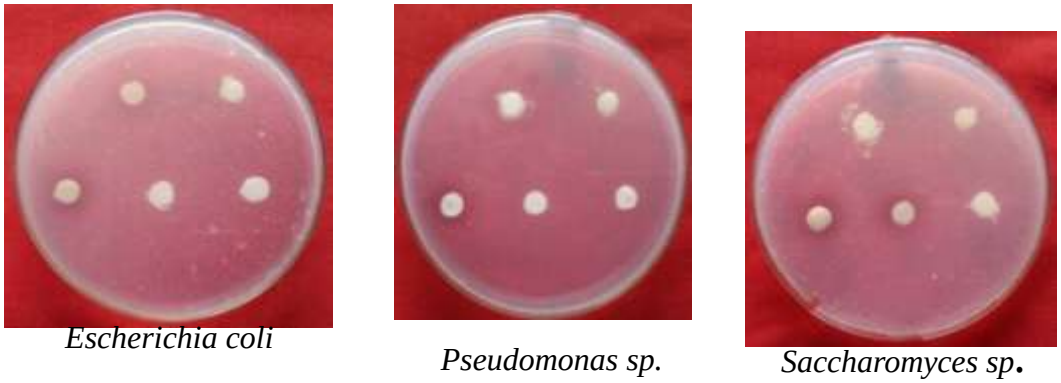


Pseudomonas sp. *Saccharomyces sp.*

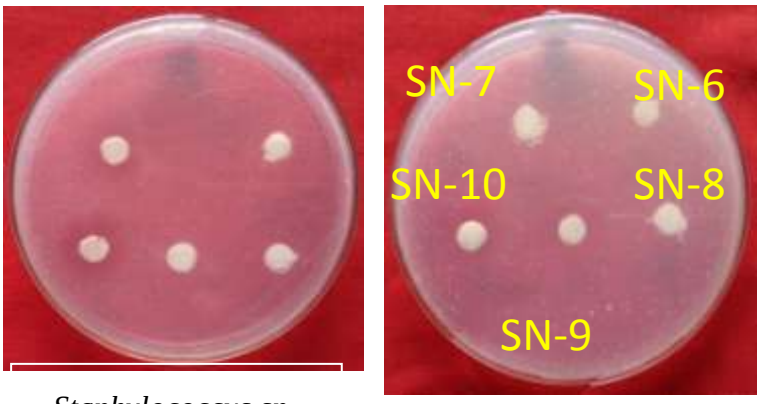


Saccharomyces sp. *Staphylococcus aureus*

Figure 4. Antimicrobial Activity of fermentation broth A 1 to 5Against FiveTest Organisms



Escherichia coli *Pseudomonas sp.* *Saccharomyces sp.*



Staphylococcus sp. *Agrobacterium tumefaciens*

Figure5 . Antimicrobial Activity of fermentation broth A 6 to 10Against Five Test Organisms

Discussion

An overall sample of 10 isolates of endophytic bacteria was recovered from leaves and fruit of (PerseagratissimaGaertn) thriving at Microbial Lab. at Dagon University. Endophytic bacteria showed creamy, slimy, soft, and sometimes mucoid, rough, dry colonies. In preliminary screening isolates were found Gram-negative .For biochemical and physiological tests, all isolates gave positive results for catalase and all showed positive results for starch hydrolysis .The result indicates that they can produce catalase and amylase enzyme. The amylase enzyme split starch into simple sugar.

Likewise, all isolates showed positive results for motility tests. SN- 1, 2, 3 and 5 showed positive results for glucose fermentation test and SN- 4, 6,7,8,9 and 10 not showed for glucose fermentation. In total 10 isolates showed aerobic and SN- 4, 7, 9 and 10 showed positive results and indicates that they can produce caseinase enzyme. Except SN-2, it was showed positive results of citrate utilization. As preliminarily screened for their antimicrobial properties in nutrient medium on against (Pseudomonas aeruginosa, Escherichia coli, Staphylococcus aureus, Sacchroromyces sp. and Agrobacterium tumefaciens) .SN - 1,5,7, 9 and 10 isolates exhibited some extent of inhibitory capacities (Figure 6 and 7).Distribution of important features of isolated bacterial endophytes is shown in Table (2). Out of two (SN- 1 and 6) gave positive results for Hydrogen sulphide. An overall sample of 10 isolates of endophytic bacteria was visible positive effect on milk hydrolysis.

Conclusion

In general, endophytic bacteria occur as lower population density than rhizospheric bacteria or bacterial pathogen. The surface of plants carries a wide range of microbial contaminants. To avoid this source of infection and for the isolation of endophytes, explants must be thoroughly surface-sterilized before inoculating them onto the nutrient medium. The optimum condition for the surface sterilization of the PerseagratissimaGaertnwas found as treating the leaves, and fruit with 70% alcohol for 1 minute followed by treatment of 3-5 times sterile rinse of the *Persea gratissima* Gaertn. These wide range of morphology and biochemical and characteristics of the endophytic bacterial isolates indicated that they are different bacterial species. Significant variation appeared in the types of indigenous bacteria isolated from different parts of host plant species.

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characteristics of the bacterial isolates entophytic indicated that they are different bacterial species. There appeared significant variation in the types of indigenous bacteria isolated from different parts of host plant species. 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 and are grouped into various categories, which include alkaloids, terpenoids, steroids, lactones, phenolic compounds, quinones, lignans (Tan and Zou 2003, Strobel and Daisy, 2003). Endophytes can be a promising source of bioactive compounds, and should be continuously isolated, characterized, and investigated for the discovery of lead bioactive compounds which can be employed in agriculture, medicine, and industries (Tiwari et al., 2013 and Sturz, 1997). Endophytes can be an alternate source of drugs which will help to conserve biodiversity and drug resistance.

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REFERENCES

Atlas, R.M., 1993. **Microbiology Fundamentals and Application**. Macmillan Publishing Co., a division of Macmillan, Inc.

Chen, Y.P., P.D. Rekha, A.B. Arun, F.T. Shen, W.A. Lai and C.C. Young, 2006**.Phosphate solubilizing bacteria from sub tropical soil and their tri calcium phosphate solubilizing abilities.Applied Soil Ecology**, 34: 33-41.

Cruickshank, R., J. P. Guguidand R. H. A. Swain**,** 1963. **Medical Microbiology** (11th ed.) The English Language Book Society and F.andS.Living stone Ltd., London.

Gunatilaka AA. 2006. **Natural products from plant-associated microorganisms: Distribution, structural diversity, bioactivity, and implications of their occurrence.** J Nat Prod; 69(3):509-26.

Kado CI. 1992. **Plant pathogenic bacteria.** In: Balows A, Truper HG, Dworkin M, Harder W, Schleifer KH, editors. The Prokaryotes.Vol. 1. New York, N.Y: Springer-Verlag; p. 659-74.

Kirchhorf G, Reis VM, Baldani JI, Eckert B, Dobereiner J, Hartmann A. 1997. **Occurrence, physiological and molecular analysis of endophyticdiazotrophic bacteria in gramineous energy plants. Plant Soil**; 194 : 45-55.

Reinhold-Hurek B, Hurek T. 1998. **Life in grasses: Diazotrophicendophytes**. Trends Microbiol; 6(4):139.

Petrini, O., T.N. Sieber, L. Toti and O. Viret, 1992.**Ecology, metabolitic production and substrate utilization in endophytic fungi.**Nat. Toxins, Vol.1, p.185-196.

Strobel G, Daisy B. 2003. **Bioprospecting for microbial endophytes and their natural products.**MicrobiolMolBiol Rev;67(4):491-502.

Strobel, GA., Hess W.M., Ford E., Sidhu R.S., Yang X. 1996. **Taxol from fungal endophytic and one issue of biodiversity**. J Indust Microbial 17:417-423.

Strobel, GA. 2002. **Microbial gifts from rain forest** : Symposium contribution. Can J Plant Pathol; 24:14-20.

Sturz AV, Christie BR, Matheson BG, Nowak J. 1997. **Biodiversity of endophytic bacteria which colonize red clover nodules, roots, stems and foliage and their influence on host growth.** BiolFertil Soil; 25(1):13-9.

Sturz AV, Matheson BG. 1996. **Populations of endophytic bacteria which influence host-resistance to Erwiniainduced bacterial soft rot in potato tubers.** Plant Soil; 184(2):265-71.

Tan RX, Zou WX. Endophytes: **A rich source of functional metabolites.** Nat Prod Rep 2001;18(4):448-59.

Tiwari R, Awasthi A, Mall M, Shukla AK, Srinivas KS, Syamasundar KV, et al. 2013. **Bacterial endophyte mediated enhancement of in plant a content of key terpenoidindole alkaloids and growth parameters of Catharanthusroseus**. Ind Crops Prod;43:306-10.