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Enzymatic activity of endophytic bacteria from *Tamarindus indica* leaves

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Abstract
Endophytic microorganisms are to be found in virtually every plant on earth. These organisms reside in the living tissues of the host plant and do so in a variety of relationships, ranging from symbiotic to slightly pathogenic. The bacterial isolates that showed broad spectrum activity on all the five test organisms were selected for further characterization. Morphological characteristics; the colony shape, color, elevation, texture and the bacteria Gram type were observed. Biochemical activities of microorganisms were studied for the purpose of identification as well as classification. The endophytes of medicinal plants participate in biochemical pathways and produce novel bioactive compounds. (Strobel and Daisy, 2003). The aim of the study was to identify the various morphological characterization of endophytic bacteria from leaves of *Tamarindus indica.* The preliminary identification of the endophytic bacterial isolates was done based on various morphological features of isolated endophytic bacteria from leaves of *Tamarindus indica.*

Keywords: Endophtic bacteria, *Tamarindus indica*, bacterial isolates, biochemical pathways

Introduction
Endophytes are microbes that colonized inner healthy plant tissues and established for all or part of their life cycle without causing any disease symptoms (Willson, 1995).
Almost all plants on this planet are believed to have association with endophytic microbes but only few of the plant species have ever been completely studied for endophyte. The most frequently encountered endophytes are fungi, and it seems that other microbial forms certainly exist in plants as endophytes, but there is meagre information on their occurrence. El-Deeb *et al*. (2013) [4] had conducted a study on endophytic bacteria from *Plectranthus tenuiflorus* medicinal plant in Saudi Arabia desert and studied their antimicrobial activities. Endophytic bacteria were isolated from root, stem and leaves of *Plectranthus tenuiflorus* plant. Among 28 endophytic bacterial isolates from different organs of *Paenibacillus tenuiflorus* plant, 8 isolates were identified by partial sequencing of their 16S rRNA gene.
The isolated endophytic bacteria were *Bacillus sp*., *Bacillus megaterium*, *Bacillus pumilus*, *Bacillus licheniformis*, *Micrococculuteus*, *Paenibacillus* sp, *Pseudomonas* sp., and *Acinetobacter calcoaceticus*. Most isolates that exhibited extracellular enzymatic activity were belong to the genus *Bacillus*. Furthermore, *Bacill us sp*. (HE613660) exhibited the stronger activities in extracellular enzymes such as *amylase, esterase, lipase, protease, pectinase, xylanase and cellulase* than other strains.

Material and methods
Location and place of work
The proposed work was conducted in the Department of Veterinary Pharmacology and Toxicology, College of Veterinary Science and Animal Husbandry, N.D.V.S.U., Jabalpur (M.P.).

Collection of leaves
Fresh mature leaves of of *Tamarindus indica* were procured from, three different places of Jabalpur *viz*. Jawaharlal Nehru Krishi ViswaVidyalaya (J.N.K.V.V), Tropical Forest Research (T.F.R.I.), State Forest Research Institute (S.F.R.I), Jabalpur. Samples were immediately brought to laboratory and were used within 24 hrs and finally processed for isolation of endophytic bacteria.

Sterilization of leaves

The sterilization of leaves and isolation of endophytic bacteria from the leaves was done according to Mahajan *et al.* (2014) [7], with some modifications. The leaves were excised with autoclaved scalpel and forceps in laminar air flow.

Plate 1: Sterilization of leaves and isolation of endophytic bacteria from *Tamarindus indica*

Sterility check: To confirm that the surface of leaves were effectively sterilized, 1 ml of the sterile distilled water that was used in final rinse of surface sterilization procedures was plated on to nutrient agar media and incubated at 37°C for 24 hrs. Then bacterial growths were observed.

Preparation and sterilization of media

King's B (KB) media (HiMedia), Mueller Hinton media (HiMedia), Blood agar media (HiMedia) and BHI broth (HiMedia) were prepared by adding agar into the distilled water. Hot plate was used for the proper mixing of media and autoclaved at 121°C for 15-20 minutes at 15 lbs.

Inoculation of leaves and isolation of endophytic bacteria

The media were poured into different autoclaved small petri plates and leaves of the plant were embedded in small petri plates. These plates were then incubated at 37°C for 24 hrs. Characterization of the bacteria was done according to its morphology and by Gram's staining. After that a single colony was transferred into BHI broth and incubated at 37°C for 24 hours.

Morphological characterization

Form, elevation, margin, surface, opacity and chromogenesis of isolated endophytic bacterial colonies were noted

Table 1: Growth of *E*ndophytic bacteria from leaves of *Tamarindus indica* on King's B media

S. No.	Isolate No.	Form	Elevation	Margin	Surface	Opacity	Chromo-genesis
1	JN-1a	Irregular	Flat	Undulated	Smooth	Opaque	Absent
2	JN-1b	Irregular	Flat	Undulated	Smooth	Opaque	Absent
3	JN-1c	Circular	Flat	Undulated	Smooth	Opaque	Absent
4	JN-1d	Irregular	Raised	Entire	Smooth	Opaque	Absent
5	JN-1e	Irregular	Flat	Undulated	Smooth	Glistning	Absent
6	TF-2a	Irregular	Flat	Undulated	Smooth	Opaque	Absent
7	TF2b	Circular	Raised	Entire	Smooth	Opaque	Absent
8	TF-2c	Irregular	Flat	Undulated	Smooth	Opaque	Absent
9	TF-2d	Irregular	Flat	Undulated	Smooth	Opaque	Absent
10	TF-2e	Circular	Flat	Entire	Smooth	Opaque	Absent
11	SF-3a	Irregular	Raised	Undulated	Smooth	Opaque	Absent
12	SF-3b	Circular	Flat	Undulated	Smooth	Opaque	Absent
13	SF-3c	Irregular	Flat	Undulated	Smooth	Opaque	Absent
14	SF-3d	Circular	Raised	Entire	Smooth	Glistening	Absent
15	SF-3e	Irregular	Flat	Undulated	Smooth	Opaque	Absent

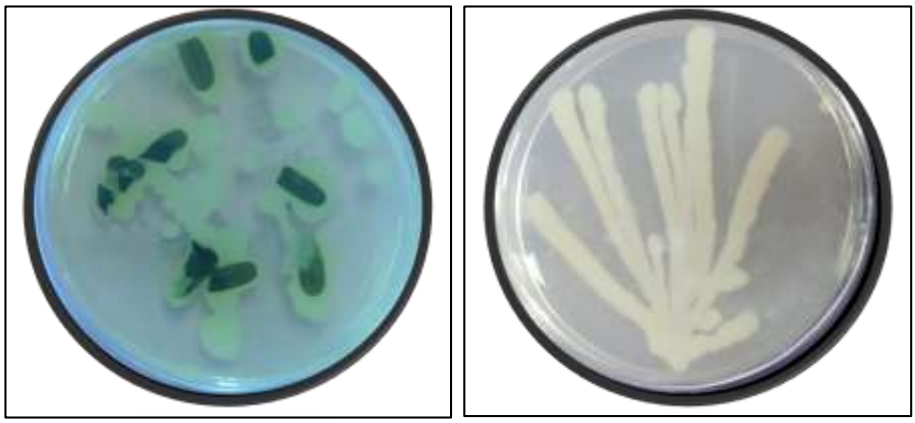

Plate 2: Growth of *E*ndophytic bacteria from leaves of *Tamarindus indica* on King's B media

Purification of endophytic bacteria

For purification of endophytic bacteria, sub culturing was mainly done by streaking a loop full of BHI broth on the fresh pre solidified blood agar plates and then incubated at 37°C for 24 hrs.

After incubation the colony was transferred into BHI broth and then incubated at 37°C for 24 hrs and purity was checked by Gram's staining and stored for further work.

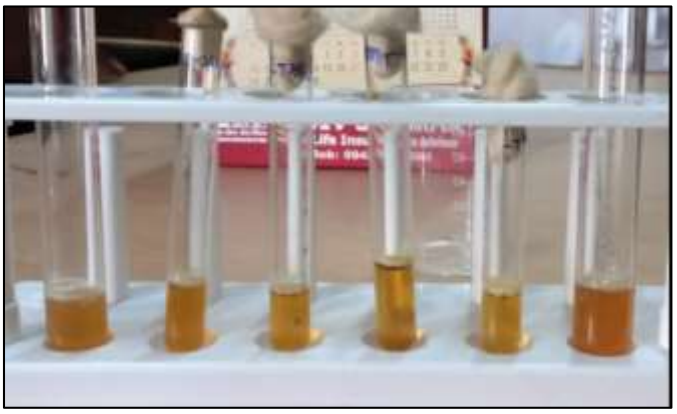

Plate 3: Growth of *E*ndophytic bacteria from leaves of *Tamarindus indica* on BHI

Growth characteristic of isolated endophytic bacteria from *Tamarindus indica* showed characteristics as 33.33 per cent isolates with turbidity and 80 per cent isolates with pellicle formation. Sediment formation was seen in 26.67 per cent isolates and 53.33 per cent isolate showed ring formation.

Table 2: Growth of endophytic bacteria isolated from leaves of *Tamarindus indica* in BHI broth

S.No	Isolate No.	Turbidity	Pellicle	Sediment	Ring formation
1	JN-1a	Absent	Present	Absent	Present
2	JN-1b	Absent	Present	Absent	Absent
3	JN-1c	Present	Present	Present	Absent
4	JN-1d	Absent	Present	Absent	Present
5	JN-1e	Absent	Present	Absent	Present
6	TF-2a	Present	Present	Absent	Present
7	TF-2b	Absent	Absent	Present	Present
8	TF-2c	Absent	Present	Absent	Absent
9	TF-2d	Absent	Absent	Absent	Absent
10	TF-2e	Present	Present	Absent	Present
11	SF-3a	Absent	Present	Absent	Present
12	SF-3b	Present	Absent	Present	Absent
13	SF-3c	Present	Present	Absent	Present
14	SF-3d	Absent	Present	Absent	Absent
15	SF-3e	Absent	Present	Present	Absent

Plate 4: Growth of Endophytic bacteria from leaves of *Tamarindus indica* on 5 per cent sheep blood agar

Gram's staining: The smear was prepared and fixed. Gently flooded the smear with Gram's iodine and allowed to stand for 1 minute. Tilted the slide gently and rinsed with distilled water. Decolorized with 95 percent ethyl alcohol and tilted the slide gently and applied alcohol drop by drop to 10 seconds. Immediately rinsed with distilled water gently flooded with safranin to counter stain and allowed to stand for 1 minute. Tilted the slide gently and rinsed with distilled water, dried the slide. Viewed the smear under 100X microscope.

Table 3: Gram's staining of Endophytic bacterial isolated from leaves *Tamarindus indica*

S. No.	Isolate No.	Gram's staining	Shape	Types of bacteria
1	JN-1a	Positive	Rod	01
2	JN-1b	Positive	Rod	01
3	JN-1c	Positive	Rod	01
4	JN-1d	Positive	Rod	01
5	JN-1e	Positive	Rod	01
6	TF-2a	Positive	Rod	01
7	TF-2b	Positive	Rod	01
8	TF-2c	Positive	Rod	01
9	TF-2d	Positive	Rod	01
10	TF-2e	Positive	Rod	01
11	SF-3a	Positive	Rod	01
12	SF-3b	Positive	Rod	01
13	SF-3c	Positive	Rod	01
14	SF-3d	Positive	Rod	01
15	SF-3e	Positive	Rod	01

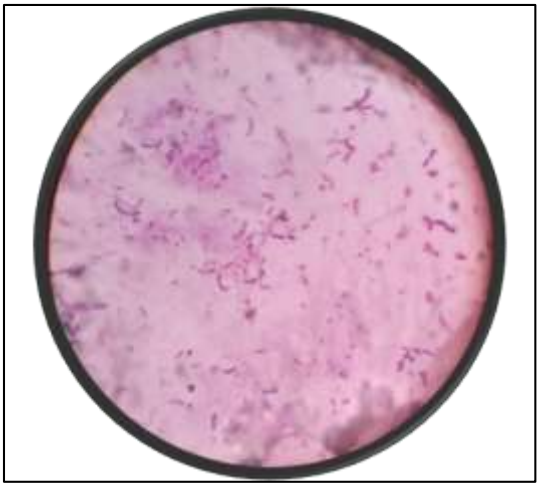

Plate 5: Gram's staining of Endophytic bacterial isolated from leaves *Tamarindus indica*

Biochemical characterization: The endophytic bacteria isolated from *Tamarindus indica* had shown positive reaction to Catalase test, Coagulase test, Voges- Proskauer (VP), ortho nitrophenyl β-galactoside (ONPG), Urease, Arginine utilization and Sugar fermentation tests were done.

Plate 6: VP (1), ONPG (2), Urease (3), Arginine utilization (4) and Sugar fermentation tests (5, 6, 7) of *E*ndophy bacteria isolated from leave *Tamarindus indica*

Table 4: Biochemical tests of endophytic bacteria

S. No	Isolate No.	Catalase test	Coagulase test	VP test	ONPG test	Urease test	Arginine utilization test
1	JN-1a	Positive	Negative	Positive	Positive	Positive	Negative
2	JN-1b	Positive	Negative	Positive	Positive	Positive	Negative
3	JN-1c	Positive	Negative	Positive	Positive	Positive	Negative
4	JN-1d	Positive	Negative	Positive	Positive	Positive	Negative
5	JN-1e	Positive	Negative	Positive	Positive	Positive	Negative
6	TF-2a	Positive	Negative	Positive	Positive	Positive	Negative
7	TF-2b	Positive	Negative	Positive	Positive	Positive	Negative
8	TF-2c	Positive	Negative	Positive	Positive	Positive	Negative
9	TF-2d	Positive	Negative	Positive	Positive	Positive	Negative
10	TF-2e	Positive	Negative	Positive	Positive	Positive	Negative
11	SF-3a	Positive	Negative	Positive	Positive	Positive	Negative
12	SF-3b	Positive	Negative	Positive	Positive	Positive	Negative
13	SF-3c	Positive	Negative	Positive	Positive	Positive	Negative
14	SF-3d	Positive	Negative	Positive	Positive	Positive	Negative
15	SF-3e	Positive	Negative	Positive	Positive	Positive	Negative

Plate 7: Coagulase test reaction of isolated *E*ndophytic bacteria from leaves of *Tamarindus indica*

Plate 8: Coagulase test reaction of isolated Endophytic bacteria from leaves of *Tamarindus indica*

Enzymatic activity test

The agar diffusion method was used to detect extracellular hydrolytic enzyme activity. The isolates were grown on different indicator media including cellulase activity indicator medium, amylase activity indicator medium and protease activity indicator medium. Bacterial cultures were incubated at 30°C for 48 h. clearing zones in the medium indicated positive enzymatic activity, were recorded (El-Deeb *et al*., 2013) [4].

Plate 9: Enzymatic activity test from leaves of *Tamarindus indica*

Table 5: Enzymatic activity test reaction of endophytic bacteria isolated from leaves of *Tamarindus indica*

S.No	Isolate No.	Cellulase activity	Amylase activity	Protease activity
1	JN-1a	Negative	Negative	Negative
2	JN-1b	Negative	Negative	Negative
3	JN-1c	Negative	Negative	Negative
4	JN-1d	Negative	Negative	Negative
5	JN-1e	Negative	Negative	Negative
6	TF-2a	Negative	Negative	Negative
7	TF-2b	Negative	Negative	Negative
8	TF-2c	Negative	Negative	Negative
9	TF-2d	Negative	Negative	Negative
10	TF-2e	Negative	Negative	Negative
11	SF-3a	Negative	Negative	Negative
12	SF-3b	Negative	Negative	Negative
13	SF-3c	Negative	Negative	Negative
14	SF-3d	Negative	Negative	Negative
15	SF-3e	Negative	Negative	Negative

Result and Discussion

The microscopic examination of endophytic bacterial isolates had shown that all endophytic bacterial isolates from *Tamarindus indica* were gram positive rods and only one type of bacteria was present. The biochemical characterization of endophytic bacterial isolates showed positive reaction to catalase, Vogas proskaur's (VP) Orthro-Nitrophenyl-β –galactoside (ONPG), urease and negative reaction to coagulase, arginine utilization test. The endophytic bacteria isolates were evaluated for the presence of active hydrolytic. The endophytic bacteria isolated from *Tamarindus indica* were evaluated for the presence of active hydrolytic enzymes including cellulase, amylase and protease. No cellulolytic, amylolytic and proteolytic activity was observed as there was no clearing of zones in agar plates observed for the endophytic bacterial isolates of *Tamarindus indica.* Khanam and Chandra (2015) [6] had isolated endophytic bacteria from *Beta vulgaris* having morphological characteristics of umbonate elevation, entire margin and red coloured colonies. Soman (2018) [12] had isolated endophytic bacteria from leaves of *Acacia nilotica* and the colonies were circular in shape with raised elevation while endophytic bacteria isolated from the leaves of *Acacia catechu* were irregular in shape, flat elevation on petri plate, entire colony margins, and the surface of the growth was smooth, opaque and white in colour. The endophytic bacteria isolated from the leaves of *Acacia auriculiformis* were irregular in shape, flat elevation on petri plate, undulated colony margin; the surface of the growth was equally smooth and dull, opaque and white in colour. Baghat *et al*. (2014) [1], who found that the 90 per cent of isolated endophytic bacteria from *Capparis sinaica* were gram positive in nature. Prasad and Dagar (2014) [9] had isolated endophytes from Avacado and Black grapes which were gram positive rods. The coagulase negative reaction suggests that isolates were nonpathogenic in nature. The present findings are very near to the work of Khanam and Chandra (2015) [6]. The endophytic bacteria isolated from *Tamarindus indica* were evaluated for the presence of active hydrolytic enzymes including cellulase, amylase and protease. No cellulolytic, amylolytic and proteolytic activity was observed as there was no clearing of zones in agar plates observed for the endophytic bacterial isolates of *Tamarindus indica* (Table 05; Plate 09). The present findings are very near to the work of Soman (2018) [12] where she found that endophytic bacteria isolated from different varieties of babool leaves did not show enzymatic activity reaction (Cellulase activity, amylase activity and protease activity). Khanam and Chandra (2015) [6] conducted a study in which the isolates from the dye yielding plant *Beta vulgaris* did not show any enzymatic activity reaction. Kewat (2019) [5] found that endophytic bacteria isolated from *Moringa oleifera* and *Cymbopogon citratus* did not show any enzymatic activity reaction. However, El-Deeb *et al.* (2013) [4] found that endophytic bacteria isolated from *Plectranthus tenuiflorus* had exhibited extracellular enzymatic activity.

Conclusion

In the present study, five leaves samples of *Tamarindus indica* each from J.NK.V.V. T.F.R.I. and S.F.R.I. were taken. The leaves were sterilized using 0.1 per cent sodium hypochlorite, 0.01 per cent bavistin, 0.05 per cent streptomycin sulphate, 70 per cent ethanol and incubated into King's B media and then again sub cultured into sheep blood agar and then transferred into BHI broth. All the endophytic bacterial isolates from leaves of *Tamarindus indica* were non-haemolytic in nature when grown on 5 per cent sheep blood agar. Growth characteristic of isolated endophytic bacteria from *Tamarindus indica* in BHI broth showed as 33.33 per cent isolates with turbidity and 80 per cent isolates with pellicle formation. Sediment formation was seen in 26.67 per cent isolates and 53.33 per cent isolate showed ring formation. The microscopic examination of endophytic bacterial isolates had shown that all endophytic bacterial isolates from *Tamarindus indica* were gram positive rods and only one type of bacteria was present. The biochemical characterization of endophytic bacterial isolates showed positive reaction to catalase, Vogas proskaur's (VP) Orthro-Nitrophenyl-β –galactoside (ONPG), urease and negative reaction to coagulase, arginine utilization test. The endophytic bacteria isolates were evaluated for the presence of active hydrolytic. Enzymes including cellulase, amylase and protease. No cellulolytic, amylolytic and proteolytic activity was observed as there was no clearing of zones in agar plates observed for the endophytic bacterial isolates of *Tamarindus indica.*

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