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# Isolation and identification of endophytic bacteria associated with leaves of *Tecomella Undulata* collected from Jodhpur, Rajasthan

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**Abstract**
Endophytic bacteria are those which are associated with internal parts of plants and by releasing various factors, they help in growth of plants. In the present study, we isolated and identified endophytic bacteria from leaves of *Tecomella undulata* collected from Jodhpur, Rajasthan. The isolated bacterial strains were identified by morphological and biochemical basis. Out of total 20 bacterial strains, 17 stains were found to be gram positive while 3 were Gram negative. All Gram-negative strains were cocci and Gram-positive strains were rod shaped. All were found to be catalase and lipase positive, 9 were amylase positive, 10 were MR positive, 10 were indole positive, 9 were VP positive, 13 were gelatinase positive, 18 were urease positive, 9 were citrate positive, 16 were motile, 17 were protease positive, and 13 were $H_2S$ production positive. 2 IAA producing strains were identified by molecular sequencing as *Bacillus cereus* and their accession number for NCBI are SUB13994283 OR838723 and was SUB13994283 Seq3 OR838725.

**Keywords:** Endophytic bacteria, *Tecomella undulata*, leaves, molecular sequencing etc

## Introduction

Endophytes are endosymbiont bacteria or fungi that occupy all or a portion of their life cycle inside healthy plant tissues without leaving behind any apparent indications of their existence. Endophytic bacteria benefit plants in a number of ways, including by promoting growth and increasing disease resistance through producing antibiotics. Endophytes produce valuable pharmaceutical substances of biotechnological interest as well as peculiar secondary metabolites of plant value while under stress.

Interactions between plants and endophytes improve plant health and are crucial for low-input, high-productivity, and sustainable agriculture (Mapelli *et al*., 2013, Aroca *et al*., 2013, Veneklaas *et al.,* 2012) [2, 8, 11]. Any plant studied over the past many decades potentially have endophytes (Hardoim *et al*., 2015, Giauque *et al*., 2019) [3, 5]. Numerous endophytic traits have been documented, including their widespread distribution, prolonged presence in plants, non-pathogenic nature, capacity to increase the ability of their plant hosts to withstand biotic and abiotic stress (Rodriguez *et al.,* 2009) [9], increase access to soil nutrients, and increase plant yield (White *et al*., 2019; Kharwar, 2022) [6, 13].

Numerous plants have endophytic bacteria, which have been isolated from stems, leaves, flowers, leaves, fruits, and seeds. They don't cause any disease symptoms, unlike phytopathogenic bacteria, and they can even help plants flourish. Through both direct and indirect processes, plant growth-promoting bacterial endophytes (PGPBE's) aid in the growth of plants. The creation of phytohormones including indole acetic acid (IAA), cytokinin, zinc (Zn), and phosphorus (P) solubilization, along with an increase in the amount of assimilable nitrogen made available to the host through biological nitrogen fixation (BNF), directly promote plant growth. Bacterial endophytes produce secondary metabolites, such as siderophores, antibiotics, hydrogen cyanide (HCN), and enzymes like 1-Aminocyclopropane-1-carboxylate (ACC) deaminase, cellulase, and protease that are crucial for granting tolerance to biotic and abiotic stressors. These secondary metabolites also operate as a source of hydrogen cyanide (HCN). The bacteria were isolated from whole plant rice samples and tested for IAA generation, phosphate solubilization, and nitrogenase activity. As iron accessibility to plants is typically low, siderophores produced by bacteria may encourage plant growth directly by supplying iron to plants. As a result, organic chelators produced by bacteria will aid in iron absorption or benefit plants indirectly by restricting the availability of iron to pathogens, thereby inhibiting the growth of pathogens. This finally strengthens the plant immune system and guards it against infection by plant diseases (Verma *et al*., 2022) [12].

In the present study, endophytic bacteria were isolated from leaves of *Tecomella undulata* and those were identified using different techniques.

### Field survey and collection of seeds

A sample of leaves of *Tecomella undulata* was done from Jodhpur, Rajasthan state. Approximately, 250 g leaves were collected from sampling site. Collected leaves were subjected to isolation of associated pathogenic bacterial strains.

### Isolation and purification of the pathogen

The collected healthy leaves were surface sterilized by immersion in 90% (v/v) ethanol for one minute followed by 1% (v/v) NaOCl for 10 min and then washed six times with sterile distilled water. Sterilized leaves were pleated on nutrient agar (NA) medium and incubated for 24-48 h at 30°C. The colonies that appeared under the leaves were picked up and diluted by dilution series and subsequently streaked on TZC agar plates**.**

Colonies showing similar characteristics were confirmed by plating on various semi selective media, and by other biochemical and molecular studies described below:

**Identification by morphological characteristics**

**Gram's staining:** To study the morphological features of bacterial isolates, a smear from its suspension was prepared on glass slide; air dried and stained by Hucker's modified Gram's staining method**.** The airdried stained slides were examined under compound microscope under high power and oil immersion objectives.

**Identification using physiological/ biochemical methods:** On the basis of morphological and cultural characteristics of colonies, the isolates were differentiated and subjected to other biochemical tests:

**Catalase test:** The catalase enzyme presence was assessed on pre-screened isolates by introducing small amounts of colonies onto individual glass slides. Hydrogen peroxide was subsequently introduced into the cells via a Pasteur pipet.

**Amylase test:** Starch agar [(percent w/v) peptic digest of animal tissue, 0.5; yeast extract, 0.15; beef extract, 0.15; soluble starch, 0.2; NaCl, 0.5; agar, 1.5] was used to do this examination. The culture plates were filled with iodine solution after 2-4 days of proper development.

**MR-VP:** All of these experiments were carried out in MR-VP broth (0.5 percent $K_2HPO_4$; 0.5 percent dextrose; 0.7 percent buffered peptone; with a pH of 6.9). The deposition of acid caused by the oxidative processing of glucose reduces the pH levels. A favorable MR test suggests a low pH as a result of acid output. MR process was done by incorporating some drops of methyl red after 2-4 days of development on MR-VP broth (0.1 g methyl red in 300 ml of 95 percent ethanol, volume made up to 500 ml with distilled water).

**Indole:** The development of indole was identified by applying Kovac's reagent and examining the forming of a red circle. New isolates were inoculated in tryptone broth with 0.2 ml Kovac's reagent (conc. HCl, 25 ml; amyl alcohol, 75 ml; p-dimethyl amino benzaldehyde, 5g) and thoroughly combined.

**Gelatinase:** The pre-selected isolates were introduced into 10 mL of Nutrient Gelatin (NG) solution, which consisted of 13 g/L of dehydrated Nutrient Broth and 120 g/L of gelatin, using a stab inoculation method. A negative control was prepared with uninoculated NG. The tubes were placed in an environment with a constant temperature of around 20 degrees Celsius for a duration of 48 hours. After incubation, the tubes were placed in the freezer for a duration of 15 minutes to allow them to harden.

**Urease:** Inoculated cultures were in urea broth containing (% w/v) - $KH_2PO_4$, 0.91; yeast extract, 0.01; phenol red, 0.001; urea, 2; $K_2HPO_4$, 0.95; filter sterilised. Within 4-5 days of developing, extreme change in color of the culture medium was observed.

**Citrate utilization:** Simmon's Citrate Agar [(percent w/v) $Na_4H_2PO_4$, 0.1; agar, 1.5; $MgSO_4.7H_2O$, 0.02; Sodium citrate, 0.2; $K_2HPO_4$, 0.1; Bromothymol blue, 0.008; NaCl, 0.5; and the pH-6.8] was used to verify citrate use. Plates were incubated and observed for the change in color of the medium from greenish to yellow.

**Motility:** The Triphenyl Tetrazolium Chloride (TTC) approach was used to assess motility. In test tubes, a nutrient agar medium containing 0.02 percent TTC was packed, and collected strains of bacteria were hooked and incubated for a whole day at 37 °C.

**Protease:** The isolates were initially screened by conducting a proteolytic activity experiment on Milk Agar plates. The composition of the agar plates included 3 g L-1 yeast extract, 5 g L-1 peptone, 12 g L-1 agar, and 100 mL L-1 sterile UHT non-fat milk. The plates were injected with bacteria and then incubated for a duration of 24 hours.

**$H_2S$ production:** Development of the strains on Kligler's iron agar slants was used to search for $H_2S$ output. The medium contains (percent w/v) - beef extract 0.3; ferrous sulphate 0.02; peptic digest of animal tissue 1.5; phenol red 0.0024; peptone 0.5; yeast extract 0.3; dextrose 0.1; lactose 1; agar 1.5, $Na_2S_2O_3$, 0.03; NaCl 05, and the pH remains 7.4.

**Molecular characterization**

The soil samples were subjected to 16S rRNA sequencing to molecularly characterize the isolated bacteria. To achieve this objective, genomic DNA was extracted from the bacterial cells and then underwent PCR amplification of the 16S r-gene. Subsequently, the sequencing of the amplified gene was done at Barcode Bioscienecs, Bangalore. The obtained sequences were uploaded to NCBI to get accession numbers.

**Results**

A total of 20 bacteria were isolated from the collected root of the plant. Their morphological and biochemical characteristics are being described in table 1.

Out of total 20 bacterial strains, 17 stains were found to be gram positive while 3 were Gram negative. All Gram-negative strains were cocci and Gram-positive strains were rod shaped. All were found to be catalase and lipase positive, 9 were amylase positive, 10 were MR positive, 10 were indole positive, 9 were VP positive, 13 were gelatinase positive, 18 were urease positive, 9 were citrate positive, 16 were motile, 17 were protease positive, and 13 were $H_2S$ production positive.

**Table 1:** Morphological and biochemical characterization of the isolated endophytes.

| Strain code | Gram staining | shape | catalase | amyalse | MR | lipase | Indole | VP | gelatinase | Urease | Citrate | Motility | protease | $H_2S$ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | + | Rod | + | + | - | + | + | + | - | + | + | + | + | - |
| 2 | - | cocci | + | - | - | + | - | - | - | + | - | + | + | + |
| 3 | + | Rod | + | + | - | + | + | + | - | + | + | + | + | - |
| 4 | + | Rod | + | + | - | + | + | + | + | + | + | + | + | - |
| 5 | + | Rod | + | - | + | + | - | - | + | + | - | + | + | + |
| 6 | - | cocci | + | - | + | + | - | - | + | + | - | - | - | + |
| 7 | + | Rod | + | + | - | + | + | + | + | + | + | + | + | - |
| 8 | + | Rod | + | + | - | + | + | + | - | + | + | + | + | - |
| 9 | + | Rod | + | + | - | + | + | + | - | + | + | + | + | - |
| 10 | + | Rod | + | - | + | + | - | - | + | + | - | - | - | + |
| 11 | + | Rod | + | - | + | + | - | - | + | + | - | + | - | + |
| 12 | + | Rod | + | + | - | + | + | + | + | + | + | + | + | + |
| 13 | + | Cocci | + | - | + | + | - | - | - | + | - | - | + | + |
| 14 | + | Rod | + | - | + | + | - | - | + | + | - | + | + | + |
| 15 | + | Rod | + | + | - | + | + | + | - | - | + | + | + | + |
| 16 | + | Rod | + | - | + | + | - | - | + | - | - | + | + | - |
| 17 | + | Rod | + | + | - | + | + | + | + | + | + | + | + | + |
| 18 | - | Cocci | + | - | + | - | - | - | + | + | - | - | + | + |
| 19 | + | Rod | + | - | + | + | + | - | + | + | - | + | + | + |
| 20 | + | Rod | + | - | + | + | - | - | + | + | - | + | + | + |

**Molecular determination of the isolated endophytes**

Two indole positive strains 1 (S1) and 3 (S3) were selected for molecular sequencing. Those were identified as *Bacillus cereus*. Both sequences were submitted to NCBI. The obtained accession number for S1 was SUB13994283 OR838723 and for S3 was SUB13994283 Seq3 OR838725 The details of sequence code and phylogenetic tree are given below-

**Sequence of S1**

| | |
|---|---|
| 1 | tcggagagtt tgatcctggc tcaggacgaa cgctggcggc gtgcctaata catgcaagtc |
| 61 | gagcggacag aagggagctt gctcccggat gttagcggcg gacgggtgag taacacgtgg |
| 121 | gtaacctgcc tgtaagactg ggataactcc gggaaaccgg agctaatacc ggatagttcc |
| 181 | ttgaaccgca tggttcaagg atgaaagacg gtttcggctg tcacttacag atggacccgc |
| 241 | ggcgcattag ctagttggtg gggtaatggc tcaccaaggc gacgatgcgt agccgacctg |
| 301 | agagggtgat cggccacact gggactgaga cacggcccag actcctacgg gaggcagcag |
| 361 | tagggaatct tccgcaatgg acgaaagtct gacggagcaa cgccgcgtga gtgatgaagg |
| 421 | ttttcggatc gtaaagctct gttgttaggg aagaacaagt gcgagagtaa ctgctcgcac |
| 481 | cttgacggta cctaaccaga aagccacggc taactacgtg ccagcagccg cggtaatacg |
| 541 | taggtggcaa gcgttgtccg gaattattgg gcgtaaaggg ctcgcaggcg gtttcttaag |
| 601 | tctgatgtga aagcccccgg ctcaaccggg gagggtcatt ggaaactggg aaacttgagt |
| 661 | gcagaagagg agagtggaat tccacgtgta gcggtgaaat gcgtagagat gtggaggaac |
| 721 | accagtggcg aaggcgactc tctggtctgt aactgacgct gaggagcgaa agcgtgggga |
| 781 | gcgaacagga ttagataccc tggtagtcca cgccgtaaac gatgagtgct aagtgttagg |
| 841 | gggtttccgc cccttagtgc tgcagctaac gcattaagca ctccgcctgg ggagtacggt |
| 901 | cgcaagactg aaactcaaag gaattgacgg gggcccgcac aagcggtgga gcatgtggtt |
| 961 | taattcgaag caacgcgaag aaccttacca ggtcttgaca tcctctgaca accctagaga |
| 1021 | tagggctttc ccttcgggga cagagtgaca ggtggtgcat ggttgtcgtc agctcgtgtc |
| 1081 | gtgagatgtt gggttaagtc ccgcaacgag cgcaaccctt gatcttagtt gccagcattc |
| 1141 | agttgggcac tctaaggtga ctgccggtga caaaccggag gaaggtgggg atgacgtcaa |
| 1201 | atcatcatgc cccttatgac ctgggctaca cacgtgctac aatggacaga acaaagggct |
| 1261 | gcaagaccgc aaggtttagc caatcccata aatctgttct cagttcggat cgcagtctgc |
| 1321 | aactcgactg cgtgaagctg gaatcgctag taatcgcgga tcagcatgcc gcggtgaata |
| 1381 | cgttcccggg ccttgtacac accgcccgtc acaccacgag agtttgcaac acccgaagtc |
| 1441 | ggtgaggtaa cctttatgga gccagccgcc gaaggtgggg cagatgattg gggtgaagtc |
| 1501 | gtaacaaggt agccgtatcg gaaggtgcgg ctggatcacc tccttt |

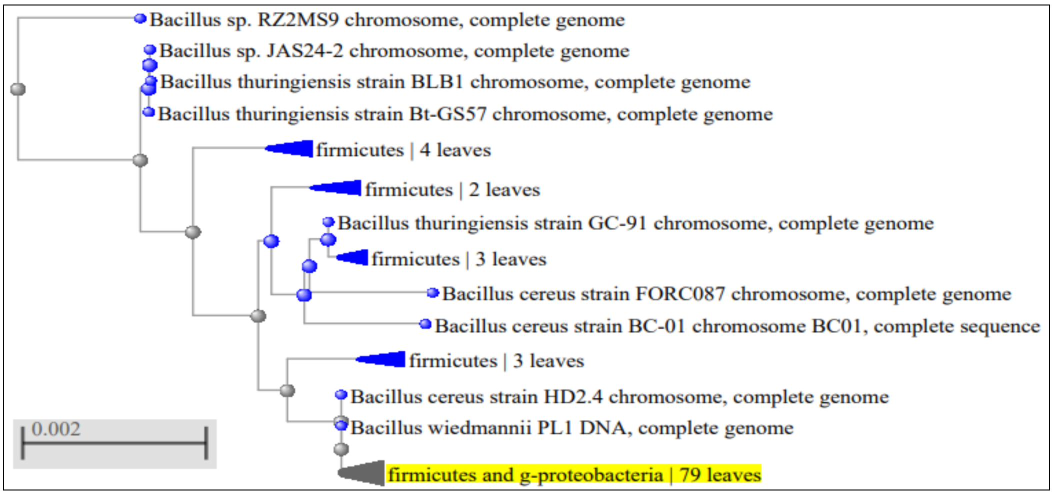


**Fig 1:** Phylogenetic tree of isolated endophyte S1

## Sequence of S3

| | |
|---|---|
| 1 | atgcaagtcg agcgaatgga ttaagagctt gctcttatga agttagcggc ggacgggtga |
| 61 | gtaacacgtg ggtaacctgc ccataagact gggataactc cgggaaaccg gggctaatac |
| 121 | cggataacat tttgaaccgc atggttcgaa attgaaaggc ggcttcggct gtcacttatg |
| 181 | gatggacccg cgtcgcatta gctagttggt gaggtaacgg ctcaccaagg caacgatgcg |
| 241 | tagccgacct gagagggtga tcggccacac tgggactgag acacggccca gactcctacg |
| 301 | ggaggcagca gtagggaatc ttccgcaatg gacgaaagtc tgacggagca acgccgcgtg |
| 361 | agtgatgaag gctttcgggt cgtaaaactc tgttgttagg gaagaacaag tgctagttga |
| 421 | ataagctggc accttgacgg tacctaacca gaaagccacg gctaactacg tgccagcagc |
| 481 | cgcggtaata cgtaggtggc aagcgttatc cggaattatt gggcgtaaag cgcgcgcagg |
| 541 | tggtttctta agtctgatgt gaaagcccac ggctcaaccg tggagggtca ttggaaactg |
| 601 | ggagacttga gtgcagaaga ggaaagtgga attccatgtg tagcggtgaa atgcgtagag |
| 661 | atatggagga acaccagtgg cgaaggcgac tttctggtct gtaactgaca ctgaggcgcg |
| 721 | aaagcgtggg gagcaaacag gattagatac cctggtagtc cacgccgtaa acgatgagtg |
| 781 | ctaagtgtta gagggtttcc gccctttagt gctgaagtta acgcattaag cactccgcct |
| 841 | ggggagtacg gccgcaaggc tgaaactcaa aggaattgac gggggcccgc acaagcggtg |
| 901 | gagcatgtgg tttaattcga agcaacgcga agaaccttac caggtcttga catcctctga |
| 961 | aaaccctaga gatagggctt ctccttcggg agcagagtga caggtggtgc atggttgtcg |
| 1021 | tcagctcgtg tcgtgagatg ttgggttaag tcccgcaacg agcgcaaccc ttgatcttag |
| 1081 | ttgccatcat taagttgggc actctaaggt gactgccggt gacaaaccgg aggaaggtgg |
| 1141 | ggatgacgtc aaatcatcat gccccttatg acctgggcta cacacgtgct acaatggacg |
| 1201 | gtacaaagag ctgcaagacc gcgaggtgga gctaatctca taaaaccgtt ctcagttcgg |
| 1261 | attgtaggct gcaactcgcc tacatgaagc tggaatcgct agtaatcgcg gatcagcatg |
| 1321 | ccgcggtgaa tacgttcccg ggccttgtac acaccgcccg tcacaccacg agagtttgta |
| 1381 | acacccgaag tcggtggggt aacctttttg gagccagccg cctaaggtgg gacagatgat |

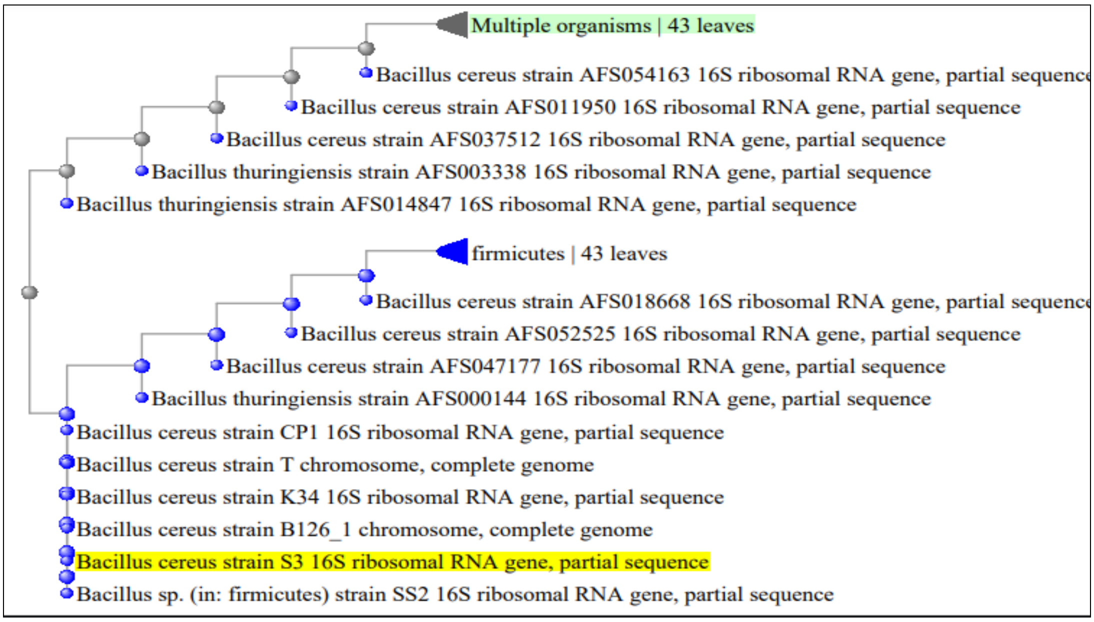


**Fig 2:** Phylogenetic tree of the isolated endophyte S3.

## Discussion

Plant growth-promoting bacteria (PGPB) are typically found colonising the rhizosphere (termed rhizobacteria) or the plant's intercellular spaces, where they are referred to as endophytic bacteria. Since almost all plant species belong to one of these categories, the PGPB has significant ecological and biotechnological significance. Numerous growth-promoting processes have been discovered. According to Ludwig and Kamilova (2009), the major genera of PGPB are *Azotobacter, Azospirillum, Acetobacter, Pseudomonas, Bacillus, Gluconacetobacter, Herbaspirillum*, and *Burkholderia.* Several commercial formulations for use as inoculants for commercial crops are available from these genera.

IAA is the most significant phytohormone that directly stimulates the growth of microorganisms and plants, in fact. Endophytic bacteria with the capacity to produce IAA can promote root growth and root length, increase the root surface area and enable the plant to absorb more nutrients from the soil (Souza *et al*., 2015) [10].

In the current study, 20 endophytic bacteria associated with root of *Tecomella undulata* have been isolated, 2 of which were identified as *Bacillus cereus* which also showed production of indole acetic acid by a biochemical test. Production of Indole acetic acid from bacteria indicated its plant growth promoting behaviours. Those bacteria were also identified as PGRP in the study of Goes *et al.,* (2012) [4] and Akinsanya *et al.* (2015) [1].

## Conclusion

For the results of the present study, it can be concluded that in the collected root of *Tecomella undulata*, different kinds of endophytic bacteria are associated which have plant growth promoting activity and help plant to grow in different environmental conditions.

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