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# ISOLATION, CHARACTERIZATION AND ANTIBACTERIAL ACTIVITY OF ENDOPHYTIC BACTERIA FROM *ADHATHODA BEDDOMEI*

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## ABSTRACT

*Adhathoda beddomei* is used traditionally to treat various diseases. The endophytic bacteria present in the adhathoda may enhance its medicinal property.The current study concentrates to screen the endophytic bacteria from leaves of *adhathoda beddomei*. The surface sterilized leaves of the plant were used for the isolation of the endophytic bacteria. The isolates were identified using polymerase chain reaction using 16s ribosomal DNA (rDNA) method. Based on the similarity correlated with bacterial species and submitted to gen bank.From the obtained entophytic bacterial isolates only the pure culture was chosen for further analysis. The isolated bacteria were identified as enterobacter. The screened and isolated bacteria were concluded as enterbacter.

**KEY WORDS:** ***adhathoda beddomei*; ribosomal DNA; enterobacter; polymerase chain reaction; gen bank**

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# INTRODUCTION

Bacteria are common inhabitants of both the surface and internal tissues of most plants. An endophyte is a bacterial or fungal organism which spends the whole or part of its life-cycle by colonizing inter and / or intracellularly inside the healthy tissues of the host plant typically causing no symptoms of disease [1, 2]. Endophytic bacteria colonize the internal tissues of their host plants and can form a range of relationships including symbiotic, mutualistic, commensalistic and trophobiotic. Recently endophytic bacteria have gained attention due to their interesting features related to plant growth and health. Some of the bacteria are known to increase nutrient availability, produce growth hormones, convey stress tolerance, induce systemic resistance, or deter plant pathogens [3, 4].

# MATERIALS AND METHODS

Sample collection: The fresh leaves of the plant were collected from the Siddha Medicinal Plants Garden, Mettur in December 2013 in sterile plastic bags and transported to lab and processed with in 4hrs.

***Isolation of endophytic bacteria from leaves***

The endophytic bacteria were isolated according to the [5, 6] with minor modifications. Fresh and healthy leaves were thoroughly washed under tap water and transferred to a laminar air flow chamber and immersed into 70% ethanol for 2-3 minutes and then into sodium hypochlorite(0.5%) for 5-10 minutes. Finally leaf samples were exposed to 70% ethanol for 30 seconds and then washed thrice with autoclaved distilled water. Samples were blot dried on sterile towels and cut aseptically into small sections before plating[7]. The leaf pieces were aseptically inoculated on petridishes containing nutrient agar and Luria Bertani (LB) Agar. The nutrient agar plates were incubated at $30^0$C for 24 hours while the LB agar plates were incubated at $30^0$C for 18-20 hours in darkness and observed for growth of bacterial colonies surrounding the leaf sections.

***Biochemical and Molecular Characterization of bacterial isolate***

The tests conducted were gram staining, cell morphology, motility, catalase activity and different biochemical tests like indole, methyl red voges-proskauers, citrate utilization tests were performed[8] and observed for results. Further molecular characterization was done using 16s rRNA gene sequencing polymerase chain reaction using specific universal primers, purification of the sample and sequencing with 500bp data using ABI 3500 XL Genetic Analyzer. The pure DNA sequence obtained was redefined as BLAST analysed at NCBI web site http://www.ncbi.nlm.nih.gov to determine the similarity of the sequence with already reported sequences in gen bank. The phylogenetic tree was produced using BLAST pair wise alignments. The nucleotide sequence data obtained was submitted to gen bank and accession numbers were obtained.

***Antibiotic sensitivity assay***

Antibiotic sensitivity test was performed based on the standard procedure with two different antibiotics gentamycin 10mcg and rifampicin 5mcg. Extraction of crude chloroform extracts from endophytic bacterial fermentation broth The pure culture obtained were subcultured on LB agar plates for 24 hrs then subcultured on to LB broth in a 1L Erlenmyer flask and incubated in shaker incubator at $37^o$c for 48hrs. After incubation the culture broth was subjected to sonication for 1hr to break the cells and chloroform was used for extraction in separating funnel by shaking vigorously for 30mins. The mixture was allowed to settle until two distinct layers are appeared, the upper solvent layer and lower aqueous layer. The upper solvent layer was separated and evaporated in rotary evaporator and the crude extract was obtained. This crude extract was used for further experimental purpose.

***Antibacterial assay***

The crude chloroform extracts of the endophytic bacteria were tested for their antibacterial activity

against. Antibacterial activity of bacterial extract was tested by an agar well diffusion method using Mueller- Hinton agar medium. Test bacterial strains used in this test include human pathogens like *Staphylococcus aureus* and *Escherichia coli.* The test cultures were swabbed on the surface of the solidified media and allowed to dry for 10 minutes. DMSO was used as the negative control. The antibiotic amoxicillin (0.2mg/ml) was used as the positive control. The plates were incubated for 24 hours at $37^0$C. Zone of inhibition was recorded in mm.

## RESULTS AND DISCUSSION

Screening for endophytic bacteria revealed the presence of bacteria was characterized as gram negative rods and motile in nature. The biochemical tests revealed that their biochemical nature as catalase- positive, indole -negative, methyl red- negative, vogues proskeur- positive and citrate -positive.

**Table 1**
***Biochemical test of the bacterial isolate***

| Bacterial isolate | Catalase | Indole | Methyl-red | Voges-Proskauer | Citrate |
|---|---|---|---|---|---|
| EB1 | Positive | Negative | Negative | Positive | Positive |

***Identification of endophytic bacteria***

Further identification of the endophytic bacterial isolate EB1 was done by 16S rRNA analysis. A homology search was done for the nucleotide sequence by insilico method with the help of BLAST (Basic Local Alignment Search Tool). Also a microscopic observation revealed that the isolated bacteria are gram negative rods and arranged in chains they are also motile in nature. Based on the BLAST and 16S rRNA analysis phylogenetic tree was constructed. From this the genetic sequence was correlated with enterobacter the accession number given as KJ680325.

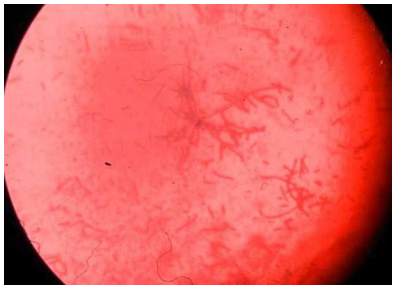

**Figure 1**
***Gram staining***

GAGTGGCGGAACGGGTTGAAGTAATGTCTGGGAAACTGCCTGATGGAGGGGG
AGAACTACTGGGAAACGGTACTAATACCGCATAACGCGCAAGACCAAAGAGGG
GGACCTTCGGGCCTCTTGCCATCAGATGTGCCCAGATGGGATTAGCTAGGTAG
GTGGGGTAACGGCTCACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGATGAC
CAGCCACACTGGAACTGAGACACGGTCCAGACTCCTTACGGGAGGCAGCAGTG
GGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTATGAA
GAAGGCCTTCGGGTTGTAAAGTAACTTTCAGCGGGGAGGAAGGTGTTGTGGTT
AATAACCACAGCAATTGACGTTACCCGCAGAAGAAGCACCGGCTAACTCCGTG
CCAGCAGCCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTCTGTCAA
GTCGGATGTGAAATCCCCGGGGCTCAACCTGGGAACTGCATTCGAAACTGGCAGGCTAGAGTCTTGTAGAGGGGGGTAGA
ATTCCAGGGTGTAGCGGTGAAATGCGTAGAGATCCTGGAGGAATACCGGTGGCGAAAGCGGCCCCCTGGACAAAGACTGA
CGCTCAGTGCGAAAGCGGTGGGGAGCAAACAGGATTAGATACCCTTGGGTAGTCCACGCCGTAAACGATGTCGATTTGGG
AGGTTGTGCCCTT.

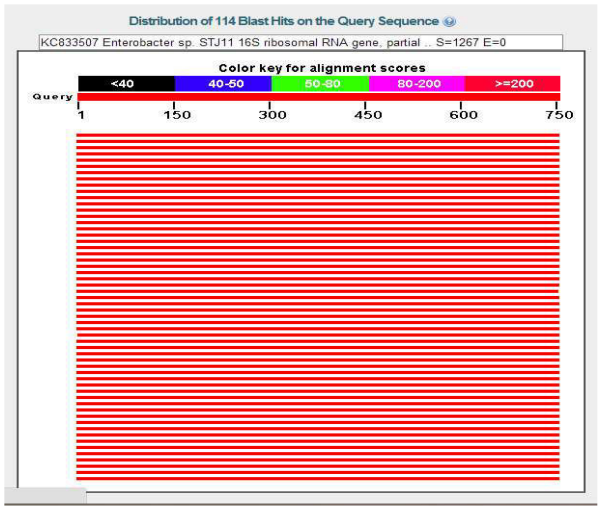


**Figure 2**
***Distribution of Blast hits on the 16S rRNA sequence of the endophytic bacteria***

| | | | | | | |
|---|---|---|---|---|---|---|
| Enterobacter cloacae strain USC-803 16S ribosomal RNA gene, partial sequence | 1267 | 1267 | 100% | 0.0 | 97% | KC934172.1 |
| Enterobacter sp. STJ11 16S ribosomal RNA gene, partial sequence | 1267 | 1267 | 100% | 0.0 | 97% | KC833507.1 |
| Enterobacter sp. DL5.1 16S ribosomal RNA gene, partial sequence | 1267 | 1267 | 100% | 0.0 | 97% | JQ912517.1 |
| Enterobacter sp. S11 partial 16S rRNA gene, isolate S11 | 1267 | 1267 | 100% | 0.0 | 97% | HF572842.1 |
| Enterobacter cloacae strain J6N 16S ribosomal RNA gene, partial sequence | 1267 | 1267 | 100% | 0.0 | 97% | JX490077.1 |
| Enterobacter cloacae subsp. dissolvens SDM, complete genome | 1267 | 10143 | 100% | 0.0 | 97% | CP003678.1 |
| Enterobacter sp. NCCP-287 gene for 16S rRNA, partial sequence | 1267 | 1267 | 100% | 0.0 | 97% | AB641896.1 |
| Enterobacter sp. NCCP-280 gene for 16S rRNA, partial sequence | 1267 | 1267 | 100% | 0.0 | 97% | AB641889.1 |
| Enterobacter cloacae strain FR 16S ribosomal RNA gene, partial sequence >gb\|KC492095.1\| Enterobacter cloacae strain V8 16S ribosomal RNA gene, partial seque | 1267 | 1267 | 100% | 0.0 | 97% | JF894166.1 |
| Enterobacter cloacae strain SF2 16S ribosomal RNA gene, partial sequence | 1267 | 1267 | 100% | 0.0 | 97% | JF489149.1 |
| Enterobacter sp. EMB19 16S ribosomal RNA gene, partial sequence | 1267 | 1267 | 100% | 0.0 | 97% | JF281095.1 |
| Salmonella sp. enrichment culture clone CL107 16S ribosomal RNA gene, partial sequence | 1267 | 1267 | 100% | 0.0 | 97% | HQ686272.1 |
| Enterobacter cloacae strain C111 16S ribosomal RNA gene, partial sequence | 1267 | 1267 | 100% | 0.0 | 97% | HQ407286.1 |
| Enterobacter cloacae strain EB89 16S ribosomal RNA gene, partial sequence | 1267 | 1267 | 100% | 0.0 | 97% | FJ194527.1 |
| Endophytic bacterium CK04 16S ribosomal RNA gene, partial sequence | 1267 | 1267 | 100% | 0.0 | 97% | FJ205665.1 |
| Enterobacter sp. 5CoNi43 partial 16S rRNA gene, isolate 5CoNi43 | 1267 | 1267 | 100% | 0.0 | 97% | AM231085.1 |
| Uncultured bacterium clone MW1_8F_h09 16S ribosomal RNA gene, partial sequence | 1266 | 1266 | 100% | 0.0 | 97% | KC712639.1 |
| Enterobacter cloacae strain STPF 6 16S ribosomal RNA gene, partial sequence | 1266 | 1266 | 100% | 0.0 | 97% | JF723553.1 |
| Uncultured Enterobacter sp. clone B24h45 16S ribosomal RNA gene, partial sequence | 1264 | 1264 | 100% | 0.0 | 97% | KF680982.1 |
| Uncultured Enterobacter sp. clone B24h50 16S ribosomal RNA gene, partial sequence | 1264 | 1264 | 100% | 0.0 | 97% | KF680981.1 |
| Enterobacter cloacae strain A27CK 16S ribosomal RNA gene, partial sequence | 1264 | 1264 | 100% | 0.0 | 97% | KF220490.1 |
| Uncultured bacterium clone A0-11 16S ribosomal RNA gene, partial sequence | 1264 | 1264 | 99% | 0.0 | 97% | JF766389.1 |
| Enterobacter cloacae strain M21 16S ribosomal RNA gene, partial sequence | 1264 | 1264 | 100% | 0.0 | 97% | JX017254.1 |
| Enterobacter cloacae strain JNVU TF1 16S ribosomal RNA gene, partial sequence | 1264 | 1264 | 100% | 0.0 | 97% | JN228093.1 |

**Figure 3**
***Sequence producing significant alignments with the 16S rRNA sequence of the endophytic bacteria***

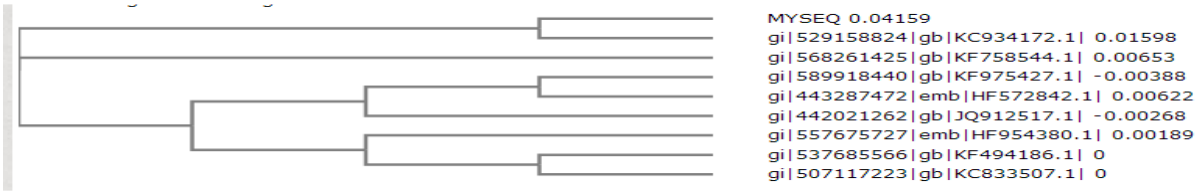


**Figure 4**
***Phylogenetic tree showing the relationship between the 16S rRNA sequence of the endophytic bacteria and its possible homologs***

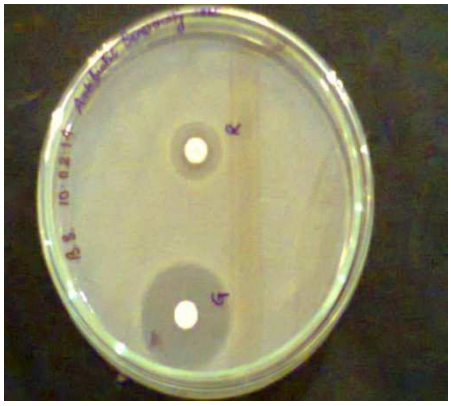

**Figure 5**
***antibiotic sensitivity test***

***Antibacterial activity***

Endophytic bacterial extracts did not show any antibacterial activity against both gram positive and gram negative bacteria (fig 6). However, one of the previous reports suggests that extract of endophytic bacteria *Bacillus amyloliquifaciens* obtained from diethyl ether and chloroform were effective in the inhibition of *S. aureus.* It had the bacteristatic activity while the crude extract obtained from diethyl ether and ethyl acetate could inhibit the growth of *E.coli* and *B. cereus,* giving bactericidal activity [9]. In spite of this antibacterial activity of endophytic fungi isolated from adhathoda beddomei was also proved by prabavathy et al [10].

**A** **B**

**Figure 6**
***Antibacterial assay of endophytic bacterial extract***

***A. Against E coli.***
***B. B. Against Staphylococcus aureus***

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