

Original Research Article

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Isolation, Biochemical and Molecular Characterization of Endophytic Bacteria from Tomato (*Lycopersicon esculentum* Mill.)

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ABSTRACT

Keywords

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In the present study, 24 isolates of endophytic bacteria were obtained from different plant tissues including root, stem and fresh leaves regions of tomato plants cv. Arka Vikas. The study characterizes morphological, biochemical and molecular characters of isolated bacterial endophytes. Among twenty-four isolates, nineteen isolates were found positive to HCN production, seventeen isolates to IAA production, and seven isolates to siderophore production. The isolates that were found to be highly effective were characterized on molecular basis by 16S rRNA gene sequencing and based on the sequencing, the endophytic isolates were identified as *Bacillus xiamenensis* (EBT8), *Bacillus aerius* (EBT14), *Bacillus stratosphericus* (EBT18) and *Bacillus safensis* (EBT22).

Introduction

Tomato (*Lycopersicon esculentum* Mill.) is one of the most important vegetable crops in India. It is a good source of vitamins, minerals, organic acids, essential amino acids and dietary fibers. The main tomato developing nations are the USA, a few European nations, China and Japan. In India, tomato is cultivated in an area of about 193.7 mha in the states Andhra Pradesh (27.93mt) followed by Madhya Pradesh (24.60mt), Karnataka (18.37 mt), Gujarat (14.25mt), Odisha (13.12mt), West Bengal (12.65 mt) and Telangana (8.91 mt). In Telangana,

tomato is cultivated in an area of 0.7 mha with an annual production of 19.37 million tons. The productivity is 28.1 MT/ha (INDIASTAT, 2017-18). The leading tomato growing districts in Telangana are Ranga Reddy, Medak, Khammam, Adilabad and Mahaboobnagar

The productivity of tomato is low due to several abiotic and biotic stresses. The pervasiveness of high humidity and warm temperature amid the growing season makes the crop vulnerable to infection by various biotic factors such as fungi, bacteria, virus and nematodes bringing about noteworthy

yield misfortunes. Fungicide application is the most widely used approach for the management of the disease. However, use of fungicides poses serious problems such as development of resistance in the pathogen, environmental pollution, accumulation of fungicide residues and reduction of beneficial microbe population. Hence, alternative techniques like use of plant extracts and biocontrol agents that cause little or no loss to the environment are to be taken into consideration. But most of the research focused on the use of bacteria as biocontrol agents has involved the use of rhizospheric bacteria and not much attention was given to endophytic bacteria.

Endophytic bacteria are those bacteria that colonize the inner tissues of healthy plants without causing symptoms of disease or detrimental effect on their host. Endophytic bacteria have been isolated from many different plants including trees, fodder crops, vegetables, fruits, cereal grains and other crops (O'Sullivan *et al.*, 1992). Endophytic bacteria are prokaryotes that colonize internal tissues of healthy plants without causing any disease symptoms. After they gain entry into the plant, they may be either localized at the point of entry or spread through the plant (Hallman *et al.*, 1997). Endophytes synthesize bioactive compounds like alkaloids, steroids, terpenoids, peptides, polyketones, flavonoids, quinols and phenols that stimulate plant growth and increase resistance to the plant pathogens (Rosenblueth *et al.*, 2006).

Use of endophytic bacteria can be considered as a new source of biocontrol agents in the plant disease management (Backman *et al.*, 2008), as they share the same ecological niche as that of plant pathogens, which makes them suitable for biocontrol (Ryan *et al.*, 2008).

The aim of present work was to isolate new bacterial endophytes, characterizing them

biochemically and for plant growth promotion.

Materials and Methods

Isolation of endophytic bacteria

Sample collection and isolation of endophytic bacteria

Healthy tomato plant samples were collected and separated into root, shoot and leaf portions. The plant portions were surface sterilized by sequential immersion in 70 per cent alcohol for 30 seconds and 5 per cent sodium hypochlorite for 15 minutes followed by three washes in sterile distilled water and ground using mortar and pestle (Feng *et al.*, 2013).

The ground sample was then serially diluted and spread on the Tryptic Soy Agar (TSA) plates. The plates were then incubated at $28 \pm 2^\circ \text{C}$ in the BOD incubator for 2-3 days (Padder *et al.*, 2017).

An aliquot of 0.1 ml was taken from the final rinse and plated on TSA to check the efficacy of surface sterilization. A total of 24 isolates obtained in this manner were maintained on TSA slants and stored at 4°C .

Biochemical characterization of endophytic bacteria

HCN production

The ability of the bacterial isolates to produce HCN was estimated by growing the bacterial isolates on TSA amended with 4.4 g/L of glycine (Bakker *et al.*, 1987). The indication for HCN production was recorded as a change in the colour of filter paper to brown and the isolates were scored based on intensity of the colour of filter paper.

Siderophore production

Chrome azurol S (CAS) assay was used to detect siderophores produced by endophytic bacteria. Siderophore production was tested on petri dishes contained CAS - agar. Pure isolates of endophytic bacteria were spotted on CAS agar plates and incubated at 28±2°C for 5 days in the dark.

The colonies with orange zones were considered as positive for siderophore production. The control plates of CAS - agar were incubated under the same conditions as described above and no color change in the CAS - blue agar was observed, after incubation period of 3-5 days.

Ammonia production

Ammonia production by the endophytic bacterial isolates was tested according to Cappuccino *et al.*, 1992. Based on the intensity of colour, the isolates were divided into four groups i.e., +, ++, +++,++++.

IAA production

Indole acetic acid production test was carried out by using the following method (Glickmann *et al.*, 1995). The bacterial cultures were grown in TS Broth amended with 0.1 % DL - tryptophan and incubated at 30±2°C at 180 RPM in the dark for 5 days in a shaker incubator. Two ml supernatant was taken and two drops of orthophosphoric acid and 4 ml of Salkowski reagent were added to the supernatant. The tubes were incubated at room temperature for 30 minutes. Based on the colour intensity, the isolated bacterial endophytes were divided into four groups i.e., -, +, ++, +++ and the scores assigned were 0, 1, 2 and 3 respectively. The quantitative analysis of IAA was performed by measuring the OD at 530nm in a spectrophotometer.

Molecular characterization of endophytic bacteria

The total genomic DNA of each isolate was extracted by following standard method (Chun *et al.*, 1995). The PCR amplification of the 16S rRNA gene of the selected strains was done by using forward and reverse primers including, 27F5'- AGAGTTTGATCCTGG CTCAG-3' and 1492R 5'-CGGTTACCTG TTACGACTT-3' primers. The reaction mixture was incubated in a thermal cycler (Eppendorf, Germany) and the conditions for PCR amplification were 94°C for 3 min for initial denaturation, followed by 35 cycles of 94°C for 30sec, 50°C for 30sec, 72°C for 90/60 sec and final extension at 72°C for 7 min and the products were sequenced. The BLAST search program (<http://www.ncbi.nlm.nih.gov/BLAST/Blast.cgi>) was used to compare the sequence homology of nucleotides.

Results and Discussion

Isolation of endophytic bacteria

Twenty four isolates of endophytic bacteria were isolated from tomato root, stem and leaf portions of tomato plants on the TSA medium. Colonies with different morphological characters on TSA were further characterized. The number of isolates and the source of their isolation are mentioned in Table 1.

Inuwa *et al.*, 2017 isolated sixteen endophytic bacteria were isolated from roots and leaves of lemon grass wherein the roots harbored higher populations of endophytic bacteria. Similarly, abundance of *Bacillus* in tomato plants was reported when eight endophytic bacteria from tomato plants out of which two were found to be Gram negative and the remaining six isolates were Gram positive (Amaesan *et al.*, 2012).

Morphological characterization

Colony morphology is commonly used to distinguish bacterial genotypes on plates (Saxer *et al.*, 2010). All the 24 isolates of endophytic bacteria were selected based on the different morphological characteristics. The morphological characters of all the isolates are shown in the Table 2.

Gram's staining

Out of 24 isolates tested, 15 isolates were gram positive bacilli, 5 were gram positive cocci, 3 were gram negative rod and only 1 was gram negative cocci. This indicated that majority (62.5%) of the bacteria observed in this study belong to gram positive bacilli followed by 20.5% of gram positive cocci. Gram negative cocci seemed to be most uncommon accounting to only 4.16% of the total isolates.

Biochemical characterization of endophytic bacterial isolates

Ammonia production

Another important feature of endophytic bacteria is the production of ammonia, which indirectly affects the growth in plants. Certain endophytic bacteria can provide nitrogen to the plants through biological nitrogen fixation, which is an important source of nitrogen input in Agriculture and represents a

promising substitute for chemical fertilizers (Puri *et al.*, 2018). Results of the Table 3 showed that all the 24 endophytic bacterial isolates tested for ammonia production gave positive reaction. The isolate EBT22 recorded very high (++++) ammonia production and 45.8% of the total isolates showed highest (+++) production of ammonia. The least production of ammonia was shown only by four isolates (EBT5, EBT11, EBT24 and EBT25) (Fig. 1).

HCN production

HCN is produced by many rhizobacteria and is known to play a major role in biocontrol of pathogens (Defago *et al.*, 1990). The ability of 24 endophytic bacterial isolates to produce HCN was determined by picric acid assay. Among the 24 isolates, eight isolates (EBT1, EBT2, EBT8, EBT11, EBT15, EBT16, EBT21 and EBT23) have shown highest (+++) production of HCN while Seven isolates (EBT4, EBT6, EBT7, EBT9, EBT14, EBT18 and EBT22) have shown least (+) production of HCN. Moderate (++) production of HCN was recorded by four isolates (EBT3, EBT13, EBT24 and EBT25) and only five isolates (EBT5, EBT10, EBT17, EBT19, EBT20) have not shown (-) HCN production (Table 3). Six endophytic bacteria isolated from corn roots were identified as *Bacillus* sp. and *Enterobacter* sp. by 16S rRNA gene sequencing.

Table.1 List of endophytic bacterial isolates and their source of isolation

S.No	Number of isolates	Isolate Name	Source of Isolation
1	8	EBT1, EBT5, EBT7, EBT 12, EBT15, EBT19, EBT23, EBT25	Stem
2	12	EBT2, EBT3, EBT6, EBT8, EBT9, EBT10, EBT13, EBT16, EBT18, EBT20, EBT21, EBT24	Root
3	4	EBT4, EBT14, EBT17, EBT22	Leaf

Table.2 Colony characteristics of different isolates of endophytic bacteria

Isolate	Shape	Pigmentation	Margins	Elevation	Appearance	Gram staining reaction	Shape under microscope
EBT 1	Circular	White	Entire	Raised	Shiny	Gram Positive	Rod
EBT 2	Irregular	White	Undulate	Raised	Dull	Gram Positive	Rod
EBT 3	Irregular	White	Undulate	Flat	Dull	Gram Positive	Rod
EBT 4	Circular	White	Undulate	Flat	Dull	Gram Positive	Rod
EBT 5	Irregular	White	Undulate	Flat	Shiny	Gram Positive	Rod
EBT 6	Irregular	White	Undulate	Flat	Dull	Gram Positive	Rod
EBT 7	Irregular	White	Undulate	Flat	Shiny	Gram negative	Rod
EBT 8	Circular	White	Entire	Flat	Dull	Gram positive	Rod
EBT 9	Wrinkled	White	Undulate	Flat	Dull	Gram Positive	Rod
EBT 10	Irregular	White	Undulate	Raised	Shiny	Gram Positive	Rod
EBT 11	Circular	White	Undulate	Flat	Dull	Gram Positive	Rod
EBT 13	Circular	White	Entire	Raised	Dull	Gram Positive	Rod
EBT 14	Circular	White	Undulate	Flat	Dull	Gram Positive	Rod
EBT 15	Circular	White	Entire	Flat	Shiny	Gram Positive	Cocci
EBT 16	Circular	White	Entire	Raised	Shiny	Gram negative	Rod
EBT 17	Circular	Cream	Entire	Raised	Shiny	Gram negative	Cocci
EBT 18	Irregular	Cream	Entire	Flat	Shiny	Gram positive	Rod
EBT 19	Irregular	White	Undulate	Flat	Dull	Gram Positive	Cocci
EBT 20	Circular	White	Entire	Flat	Shiny	Gram Positive	Cocci
EBT 21	Wrinkled	Yellow	Undulate	Raised	Shiny	Gram Positive	Cocci
EBT 22	Irregular	White	Entire	Flat	Dull	Gram Positive	Rod
EBT23	Irregular	Creamy yellow	Undulate	Flat	Shiny	Gram Positive	Rod
EBT 24	Circular	White	Entire	Flat	Shiny	Gram negative	Rod
EBT 25	Circular	White	Entire	Raised	Shiny	Gram Positive	Cocci

Fig.1 Screening of endophytic bacterial isolates for (a) ammonia production (b) HCN production- a) Weak production of ammonia ; b) Moderate production of ammonia; c) Strong production of ammonia; d)Very strong production of ammonia

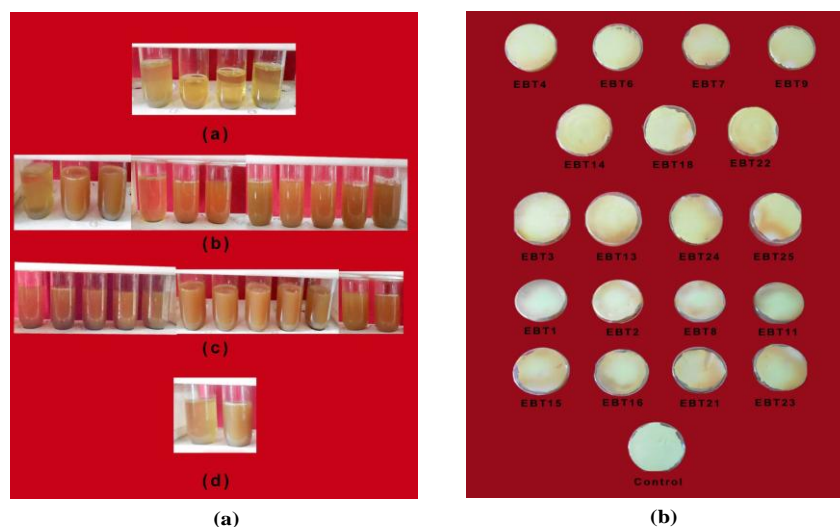


Table.3 Biochemical tests for characterization of endophytic bacterial isolates

Isolate	IAA production	Ammonia production	HCN production	Siderophore production
EBT 1	+	+++	+++	++
EBT 2	-	++	+++	-
EBT 3	+	++	++	-
EBT 4	-	++	+	-
EBT 5	-	+	-	-
EBT 6	-	+++	+	-
EBT 7	-	+++	+	-
EBT 8	++	++	+++	+
EBT 9	++	+++	+	-
EBT 10	++	+++	-	+
EBT 11	+++	+	+++	+
EBT 13	+++	++	++	-
EBT 14	+++	+++	+	+
EBT 15	+++	++	+++	-
EBT 16	+++	++	+++	-
EBT 17	+	+++	-	-
EBT 18	+++	++	+	++
EBT 19	++	+++	-	-
EBT 20	++	+++	-	++
EBT 21	-	+++	+++	-
EBT 22	+	++++	+	-
EBT 23	+	+++	+++	-
EBT 24	+	+	++	-
EBT 25	-	+	++	-

Table.4 Quantitative estimation of IAA produced by isolates of endophytic bacteria

S.No.	Isolate ID	IAA production with tryptophan $\mu\text{g/ml}$	IAA production without tryptophan $\mu\text{g/ml}$
1	EBT1	23.1 \pm 0.30	19.8 \pm 0.45
2	EBT3	25.2 \pm 0.40	14.5 \pm 0.40
3	EBT8	29.6 \pm 0.35	21.5 \pm 0.30
4	EBT9	25.7 \pm 0.35	16.1 \pm 0.35
5	EBT10	27.7 \pm 0.45	17.8 \pm 0.25
6	EBT11	29.5 \pm 0.20	19.7 \pm 0.32
7	EBT13	28.6 \pm 0.15	21.70 \pm 0.35
8	EBT14	30.7 \pm 0.41	16.0 \pm 0.50
9	EBT15	29.7 \pm 0.20	17.4 \pm 0.45
10	EBT16	27.5 \pm 0.35	18.88 \pm 0.36
11	EBT17	20.2 \pm 0.30	11.3 \pm 0.25
12	EBT18	40.2 \pm 0.10	19.2 \pm 0.10
13	EBT19	23.6 \pm 0.25	21.73 \pm 0.31
14	EBT20	23.4 \pm 0.26	19.64 \pm 0.23
15	EBT22	27.9 \pm 0.55	13.4 \pm 0.30
16	EBT23	16.4 \pm 0.25	15.3 \pm 0.20
17	EBT24	16.67 \pm 0.02	12.5 \pm 0.15

Fig.2 Screening of endophytic bacterial isolates for siderophore production

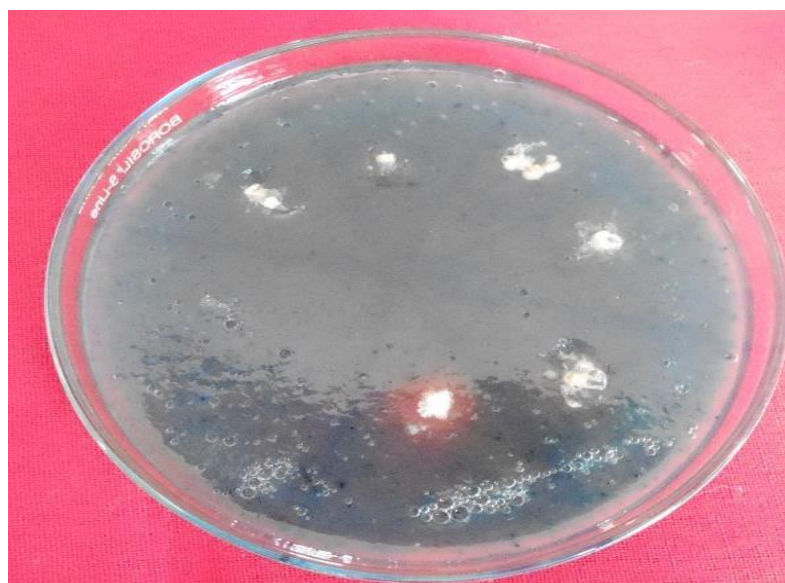
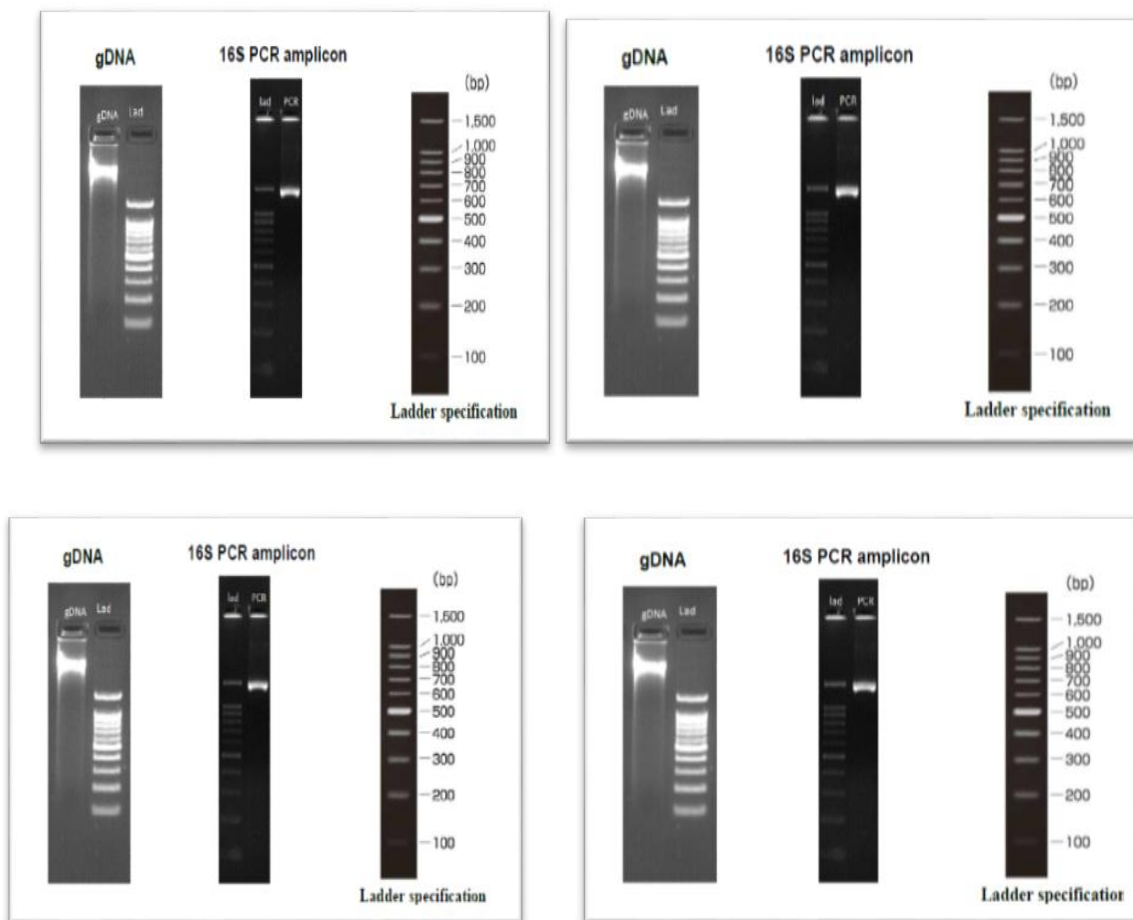


Table.5 Phylogenetic identity of 16S rRNA gene sequences after BLAST analysis

Isolate Name	Similarity percentage	Closest Gen Bank match	Strain identified	Accession No.
EBT8	99.27 %	<i>Bacillus xiamenensis</i> strain MCCC 1A00008 16S ribosomal RNA, partial sequence	<i>Bacillus xiamenensis</i>	MK881100
EBT14	99.07 %	<i>Bacillus aerius</i> strain 24K 16S ribosomal RNA, partial sequence	<i>Bacillus aerius</i>	MK881084
EBT18	94.09 %	<i>Bacillus stratosphericus</i> strain 41KF2a 16S ribosomal RNA, partial sequence	<i>Bacillus stratosphericus</i>	MK881075
EBT22	99.28 %	<i>Bacillus safensis</i> strain NBRC 100820 16S ribosomal RNA, partial sequence	<i>Bacillus safensis</i>	MK836054

Fig.3 Gel photographs of genomic DNA of isolates of endophytic bacteria [(a) EBT8; (b) EBT 14; (c) EBT 18; (d) EBT 22)



Four of these isolates were found to be positive for nitrogen fixation and the remaining two strains have shown outstanding production of IAA, siderophores and lytic enzymes but none of them have shown positive production of HCN (Szilagyi *et al.*, 2014). However, in the present study, except five isolates all the other isolates were positive for HCN production

Siderophore production

The siderophore production was found to become of the mechanisms to outcompete the pathogens (O'Sullivan *et al.*, 1992; Schippers *et al.*, 1987). Production of antimicrobial compounds is directly induced by siderophore producing ability of bacteria (Joseph *et al.*, 2012). Out of the 24 isolates tested for siderophore production, seven isolates have shown positive response which includes EBT1, EBT8, EBT10, EBT11, EBT14, EBT18 and EBT20 with highest production by the isolate EBT20 (Table 3). All other isolates were found to be negative for production of siderophore. Siderophore production by endophytic microorganisms facilitates in colonization of bacteria to the host tissue from rhizospheric zone (Loaces *et al.*, 2011) (Fig. 2).

IAA production

All the 24 isolates were tested for IAA production and 17 isolates exhibited significant amount of IAA production after 24 hours of incubation with tryptophan. The data (Table 3) indicated that seventeen (17) isolates of endophytic bacteria from the plant were able to produce IAA, the primary auxin in plant growth promotion by utilization of tryptophan. Several bacteria with the ability to anabolize IAA with the supplementation of L-tryptophan have been isolated from the plant surface (Patel *et al.*, 2012)]. Bacterial IAA enhances the development of root system and

thus resulting in high water and nutrient uptake (Patten *et al.*, 1996). Bacterial IAA producers (BIPs) have the potential to interfere with any of plant's physiological processes by input of IAA into the plant's auxin pool (Johan *et al.*, 2005).

Quantitative production of indole acetic acid

The quantity of IAA produced was determined by measuring the OD values at 530 nm. Among all the twenty four isolates tested, IAA production varied with and without tryptophan supplementation and results are presented in the (Table 4). In the presence of tryptophan, the highest IAA production was shown by the isolate EBT18 (40.2 µg/ml) followed by the isolate EBT 14 (30.7 µg/ml). The least production of IAA was recorded by the isolate EBT23 (16.4 µg/ml) followed by EBT24 @ 16.67 µg/ml in the presence of tryptophan. In the absence of tryptophan, the highest production of IAA was recorded by the isolates EBT19 and EBT13 that are on par with each other with respective values of 21.73 µg/ml and 21.70 µg/ml whereas the least production of IAA was observed in the isolate EBT17 followed by the isolate EBT24 that have shown IAA production of 11.3 µg/ml and 12.5 µg/ml respectively. Many bacteria have the ability to synthesize IAA either in the presence or absence of tryptophan but the microbes produce larger quantities of IAA in the presence of tryptophan (Normanly, 1997; Venis *et al.*, 1991).

Molecular characterization of endophytic bacteria

The four isolates which were found to be effective in plant growth promotion and per cent disease reduction under glass house conditions were characterized and identified based on 16S rRNA gene sequencing (Figure 3).

The 16S rRNA gene sequence of the isolates were compared with other bacterial sequence by BLAST (<http://www.ncbi.nlm.nih.gov/Blast/Blast.cgi>). The result was compared with the sequence of GenBank based on partial 16S rRNA to check the relationship and similarity with the endophytic isolates. The results showed similarity of EBT 8 at 99.27 per cent with *Bacillus xiamenensis*, similarity of EBT 14 at 99.07 per cent with *Bacillus aerius*, similarity of EBT18 with *Bacillus stratosphericus* at 94.09 per cent and similarity of EBT 22 at 99.28 per cent with *Bacillus safensis*. The details of the sequence data of all the five potential isolates are presented in the Table 5.

This research concluded that the endophytic bacteria from *Lycopersicon esculentum* Mill produced one or more different characteristics that have better potential than generally used commercial fungicides. They produced phytohormones like IAA, ammonia, HCN and siderophore, which are beneficial in plant growth promotion and disease management. The potential isolates of endophytic bacteria were further characterized at molecular level by 16S rRNA gene sequencing for identification. Based on the sequencing, the potential endophytic isolates were identified as *Bacillus* sp.

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