Chapter 3

Isolation And Identification Of Endophytes
3.1 Introduction

Plants provide a nutrient rich niche for the growth and development of various groups of microorganisms, especially bacteria. Mutualistic interactions between host plant and associated bacteria have emerged as a result of clear positive selection exerted on these associations (Garbeva et al., 2006; Lugtenberg and Kamilova, 2009). In recent years, researchers have begun to realize that plants may serve as a repository of untold numbers of organisms known as endophytes (Bacon and White, 2000; Strobel, 2002).

The number of reports on bacteria being isolated from inside healthy plant tissues considered as endophytes is fastly increasing (Carroll, 1988; Hallmann et al., 1997; Chanway, 1998; Azevedo et al., 2000; Recuenco and Vuurde, 2000; Kuc, 2001; Wakelin et al., 2004). The internal tissues of the plants provide a uniform and safe environment for the endophytes.

Most endophytes are capable of synthesizing bioactive compounds that may provide plants with a defense against pathogens, and some of these compounds have proven useful for novel drug discovery (Guo et al., 2008; Yan et al., 2011). They constitute a great reservoir of bacterial diversity with a remarkable biotechnological potential (Ryan et al., 2008). Bioprospecting endophytes thus offer tremendous promise to discover natural products with therapeutic value which have attracted increasing attention among microbiologists, ecologists, agronomists, and natural product chemists.

To date, many strains of endophytic bacteria have been reported such as Azorhizobium, Bacillus, Bradyrhizobium, Gluconacetobacter, Klebsiella, Burkholderia, Enterobacter, Pseudomonas, and Streptomyces (Menpara and Chanda, 2013). Both Gram positive and Gram negative bacterial endophytes have been isolated from several types of tissues from numerous plant species (Kobayashi and Palumbo, 2000). Several different bacterial endophyte species can be isolated from a single plant (Zinniel et al., 2002). Endophytic Gram negative bacteria, such as Pseudomonas sp., have been intensively investigated as biological control agents with regard to the production of antimicrobial metabolites (Nielsen et al., 2002).
Endophytic Gram positive bacteria, such as *Bacillus* sp., have been isolated from cotton (Reva et al., 2002), cucumber root (Mahaffee and Kloeppper, 1997), balloon flower root (Islam et al., 2010), and citrus plant (Lima et al., 2005). *Paenibacillus polymyxa* from Korean ginseng root have also been investigated (Jeon et al., 2003).

To date, estimates of the diversity of endophytic bacterial species have been largely based on culture techniques. Rangjaroen et al., (2014) reported the community structure of endophytic bacteria in different plant tissues of five highland rice landraces at different stages of growth. Also, there are many reports on the isolation and diversity of endophytic bacteria from various plant species, such as grapevine (Compant et al., 2011), *Panax notoginseng* (Ma et al., 2013), cucumber (Sun et al., 2013), *Ocimum sanctum* (Murugappan et al., 2013), and Rhizome of ginger (Jasim et al., 2014).

These findings encouraged us to investigate and explore the endophytic bacterial community of *Pongamia pinnata*. Although a few reports exist on the endophytic fungal assemblage of *P. pinnata* (Huang et al., 2010; Tiwari, 2013; Tiwari and Chittora, 2013), but to date, there has been no study of endophytic bacteria from leaf and stem.

This study is therefore the first to isolate and identify endophytic bacteria associated with healthy Karanj plants. In the present study, isolation of endophytic bacteria from asymptomatic leaf and stem of *P. pinnata* has been carried out. Later on microbiological examinations, biochemical characteristics and molecular approaches are used to identify the isolates.

### 3.2 Materials and Methods

#### 3.2.1 Collection of plant material

Samples of healthy and asymptomatic leaf and stem of *Pongamia pinnata* L. were collected from Rajkot, Gujarat, India in the months of July and August 2012. Samples were collected between 9:00 A.M. and 11:00 A.M, stored in polythene bags, brought to the laboratory and processed on the same day for isolation of endophytic
bacteria. The plant was compared with voucher specimen (Voucher Specimen No. PSN180) deposited at Department of Biosciences, Saurashtra University, Rajkot, Gujarat, India.

3.2.2 Surface sterilization of leaf and stem

The surface sterilization process and the isolation of the endophytic bacteria were performed based on the procedures described by Araujo et al., (2002) with some modifications. Leaf and stem were kept under running tap water for washing for 10 min to remove attached soil. Briefly, endophytic bacteria were isolated after removing epiphytes by surface sterilization by serially placing samples in 70% ethanol for 5 min, sodium hypochlorite solution (5% available Cl⁻) for 2.5 min, and 70% ethanol for 30 s and finally, washed five times with sterile distilled water. It is important to validate the effectiveness of surface sterilization process. It is the most confronting step and proves that the surface sterilization protocol was effective in removing epiphytic organisms and that the bacterial-isolated strains can be considered as endophytic organisms. It was checked by pressing the surface sterilized leaf and stem onto Nutrient Agar (NA) (HiMedia, India) medium plates and a 100µl sample of the 5th rinse water was plated onto the same medium and the plates were examined for growth after incubation at 28°C for 5 days. After incubation of 5 days non-sterile samples were discarded and only samples, for which no bacterial growth occurred in the sterility check, were considered in further analysis.

3.2.3 Isolation of endophytic bacteria

For the isolation of plant-associated bacteria, the surface sterilized leaf and stem were aseptically cut into pieces of approximately 5-8mm long with sterile surgical blade and were placed on Nutrient Agar (NA) medium plates and incubated at 28°C for 2 to 4 days to allow growth of endophytic bacteria from the edges of cut pieces.

In an additional experiment, pieces of leaf and stem were macerated in a sterile mortar and pestle with 5 mL of 12.5 mM potassium phosphate buffer (pH 7.0). Tissue extracts were then serially diluted in potassium phosphate buffer (pH 7.0) and
plated in triplicate to recover any bacterial endophytes present in the plant tissue. The plates were incubated at 28°C for 4 days. The population density of the endophytic bacteria in the tissue samples was determined and expressed as colony forming units (CFU) per gram of tissue (Fig. 3.1).

After incubation, a representative of each bacterium, recovered from each plant fragment and/or homogenized sample as evident from their colony morphology was transferred to fresh Nutrient agar medium plates to establish pure cultures of endophytic bacteria. The entire isolation experiment was repeated nearly ten times around the same time duration. The colonies obtained over and over again were the only ones which were considered as endophytes.

**Collection and Surface Sterilization of plant material**

*(leaf and stem)*

- Aseptically cut leaf and stem into 5-8mm pieces
- Place on Nutrient Agar (NA) medium plates
- Incubation at 28°C for 2 to 4 days
- Observe for the growth of endophytic bacteria from the edges of cut pieces.
- Macerate pieces of leaf and stem in a sterile mortar and pestle with 12.5 mM potassium phosphate buffer (pH 7.0)
- Serially dilute tissue extracts and plate in triplicate on NA
- Incubation at 28°C for 4 days
- Observe for the growth of endophytic colonies on NA plates

**Fig. 3.1:** A schematic representation of isolation procedure
3.2.4 Maintenance and preservation

The pure cultures were preserved on the Nutrient agar media and stored at 4°C. The cultures were subsequently transferred on fresh Nutrient agar media at 3 months intervals.

3.2.5 Characterization of the organisms

3.2.5.1 Colony characteristics

For the primary characterization, the pure culture of all the isolated bacteria was streaked on the Nutrient agar plate and their colony characteristics were observed.

3.2.5.2 Cell morphology and Gram reaction

For the differentiation on the basis of the cell morphology and cell arrangement, individual bacterium was studied for the Gram reaction, in activated culture in Nutrient broth.

3.2.5.3 Biochemical characterization

For further differentiation, the isolates were studied for biochemical and metabolic activities. The biochemical tests included production of catalase, oxidase, H₂S, ammonia, indole, hydrolysis of urea, reduction of nitrate and litmus; fermentation of the sugars such as glucose, fructose, sucrose, maltose, lactose and xylose. All the biochemical media and their test reagents were prepared as mentioned by Cappuccino and Sherman, (2004). The individual isolate was inoculated to the respective biochemical medium and incubated at 37°C for 24-48h and results were subsequently observed.
3.2.6 16S rRNA amplification and nucleotide sequencing

Colonies of five pure cultivated bacteria showing potential antimicrobial activity were chosen for identification. The colonies were suspended in 0.5 ml of sterilized saline and centrifuged at 10,000 rpm for 10 min. After removal of supernatant, the pellet was suspended in 0.5 ml of InstaGene Matrix (Bio-Rad, USA). It was incubated at 56°C for 30 min and then heated at 100°C for 10 min. After heating, supernatant was used for PCR.

Amplification of 16S rRNA gene was performed in 20 µl of PCR reaction solution containing 1 µl of template DNA. 27F (5′-AGAGTTTGATC(A/C)TGGCTCAG-3′) and 1492R (5′-GG(C/T)TACCTTGTACGACTT-3′) primers were used and then 35 amplification cycles at 94°C for 45 sec, 55°C for 60 sec, and 72°C for 60 sec were performed. About 1,400 bp of DNA fragments were amplified. Unincorporated PCR primers and dNTPs from PCR products were removed by using Montage PCR Clean up kit (Millipore).

The purified PCR products of approximately 1,400 bp were sequenced by using 518F (5′-CCAGCAGCGGCTATC-3′) and 800R (5′-TACCCCTTCTTAATCC-3′) primers. Sequencing was performed by using Big Dye terminator cycle sequencing kit (Applied BioSystems, USA). Sequencing products were resolved on an Applied Biosystems model 3730XL automated DNA sequencing system (Applied BioSystems, USA). This part of work was carried out at Macrogen Inc., Seoul, Korea.

3.2.7 Phylogenentic analysis of the 16 S rRNA sequences

The sequences obtained were compared with sequences within the NCBI database (http://www.ncbi.nlm.nih.gov/) using Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1990). Multiple sequence alignment was carried out using CLUSTALW (Tompson et al., 1994) and phylogenetic tree was constructed using MEGA 6 (Tamura et al., 2013) by Neighbour Joining method with 1,000 bootstrap replicates. The 16S rRNA gene sequence data were deposited in GenBank.
3.3 Results and Discussion

It has been always fascinating to study the microbial community occupying unique ecological niches. Hence new strategies have to be developed to isolate them. As lot of spotlight has been given to fungal endophytes, isolation of bacterial endophytes would add great value to make available the unexplored world of organisms.

3.3.1 Efficacy of sterilization

The surface sterilization protocol was a critical prerequisite for isolating plant endophytic bacteria. The result of effectiveness of the surface sterilization protocol was determined. The major key step to succeed in isolating and studying endophytes is to ensure the sterility of the plant surface (Hallmann et al., 1997). This study proved that the surface sterilization protocol was effective in removing epiphytic organisms and that the bacterial-isolated strains can be considered as endophytic organisms.

3.3.2 Isolation of endophytic bacteria

The number of colony-forming units per gram fresh weight (CFU) of culturable endophytic bacteria isolated from leaf and stem of *P. pinnata* plant were determined. The results revealed that CFU g⁻¹ fresh weight value in stem 1.2×10⁴ is lower than in leaves 8.4×10⁴. A total of 17 morphologically distinct bacterial strains, 10 (L1-L10) from leaf and 7 (S1-S7) from stem were isolated from *P. pinnata* which demonstrates that medicinal plants hosted a diverse selection of culturable bacteria.

Diverse endophytic bacterial consortia have been colonising plants (Rashid et al., 2012). Literature mining suggests that the endophytic realm is in dynamic interaction with their hosts. It is obvious that there might not be any spatial constraints and hence they might be able to live in harmony. CFU g⁻¹ fresh weight value in stem was lower than in leaves. Similar results were obtained in prior studies by El-Deeb et al., (2013). However, opposite results were obtained by Ma et al., (2013) where stem (5.2×10⁶ CFU g⁻¹) showed high CFU g⁻¹ fresh weight value than leaf (2.0×10⁶ CFU g⁻¹). In our opinion, the standardized surface sterilization process and different selective
isolation procedures with isolation media met nutritional requirements which increased the range of endophytes isolated.

3.3.3 Characterization of the organisms

3.3.3.1 Colony characterization

Although, the genetic data and molecular techniques are extensively being used for the identification and phylogenetic relatedness of organisms belonging to prokaryotes during the last many years, the traditional classification methods based on phenotypic, morphological and microbiological observation have its own importance in studying.

The isolated endophytic bacteria were primarily characterized on the basis of their colony characteristics, as described in Table 3.1. Some common characters are collectively showed by the majority of isolates such as, round and entire shape with opaque colony, smooth texture and creamish white pigmentation. Fig. 3.2 shows the colony characteristics of endophytic isolates.

On the basis of colony size, 80% of isolates were between 1-3mm in colony size while about 20% had large (4-5mm) colony size. With reference to colony shape, around 50% of the isolates had round shape, 40% had irregular shape and 10% of the isolates had elliptical shape. With respect to colony margin, majority of the isolates had irregular margins. With respect to elevation parameter majority of isolates (60%) were with flat elevation, whereas around 20% were with raised and slightly raised elevation. For, colony texture parameter, all isolates were found to be smooth in nature. There was not much diversity observed with reference to opacity parameter; and only 20% of bacteria were translucent and rest all were opaque in nature and none of the total collected pool of bacteria had transparent opacity.
Fig. 3.2: Colony characters of endophytic isolates

Table 3.1: Characterization of organisms on the basis of colony characteristics

<table>
<thead>
<tr>
<th>Isolates</th>
<th>Size (mm)</th>
<th>Shape</th>
<th>Margin</th>
<th>Elevation</th>
<th>Opacity</th>
<th>Texture</th>
<th>Pigmentation</th>
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<tbody>
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<td>S1</td>
<td>3</td>
<td>Round</td>
<td>Entire</td>
<td>Flat</td>
<td>Opaque</td>
<td>Smooth</td>
<td>Creamish white</td>
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<td>S2</td>
<td>4</td>
<td>Round</td>
<td>Irregular</td>
<td>Raised</td>
<td>Opaque</td>
<td>Smooth</td>
<td>Creamish white</td>
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<td>S3</td>
<td>3</td>
<td>Round</td>
<td>Entire</td>
<td>Slightly raised</td>
<td>Opaque</td>
<td>Smooth</td>
<td>Creamish white</td>
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<td>S4</td>
<td>3</td>
<td>Round</td>
<td>Entire</td>
<td>Flat</td>
<td>Opaque</td>
<td>Smooth</td>
<td>Creamish white</td>
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<tr>
<td>S5</td>
<td>1</td>
<td>Round</td>
<td>Irregular</td>
<td>Flat</td>
<td>Opaque</td>
<td>Smooth</td>
<td>Creamish white</td>
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<tr>
<td>S6</td>
<td>3</td>
<td>Elliptica</td>
<td>Irregular</td>
<td>Flat</td>
<td>Opaque</td>
<td>Smooth</td>
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<tr>
<td>S7</td>
<td>1</td>
<td>Elliptica</td>
<td>Irregular</td>
<td>Flat</td>
<td>Opaque</td>
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<td>L1</td>
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<td>2</td>
<td>Irregular</td>
<td>Irregular</td>
<td>Raised</td>
<td>Translucent</td>
<td>Smooth</td>
<td>White</td>
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<td>L5</td>
<td>3</td>
<td>Irregular</td>
<td>Irregular</td>
<td>Flat</td>
<td>Translucent</td>
<td>Smooth</td>
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<td>L6</td>
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<td>Irregular</td>
<td>Irregular</td>
<td>Flat</td>
<td>Opaque</td>
<td>Smooth</td>
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<td>L7</td>
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<td>Entire</td>
<td>Raised</td>
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<td>L8</td>
<td>3</td>
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<td>Slightly raised</td>
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<td>L9</td>
<td>3</td>
<td>Round</td>
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<td>Slightly raised</td>
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<td>Smooth</td>
<td>Creamish white</td>
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<td>L10</td>
<td>2</td>
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<td>Flat</td>
<td>Translucent</td>
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<td>Creamish white</td>
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</tbody>
</table>
Looking at the Gram reactions, Gram positive character was dominating over the Gram negative by a small margin (Fig. 3.3). On the basis of Gram’s reaction, 9 out of 17 isolates i.e., 53% were Gram positive in nature and rest 47% were Gram negative. In the literature, Gram positive endophytic bacteria appear to be widely described (Ceballos et al., 2012, Miller et al., 2012, Aranda et al., 2011, Islam et al., 2010, Lima et al., 2005). Our results of the isolates are in agreement with this.

With respect to cell arrangement; among all the isolates, approx. 23% of total isolates were in chains, 60% were singly and 17% were singly and in clusters. All the isolates were rod shaped (Fig. 3.3). Table 3.2 shows the characterization of organisms on the basis of Gram’s reaction.

Table 3.2: Characterization of organisms on the basis of Gram’s reaction

<table>
<thead>
<tr>
<th>Isolates</th>
<th>Gram reaction</th>
<th>Shape</th>
<th>Arrangement</th>
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<tbody>
<tr>
<td>S1</td>
<td>Positive</td>
<td>Rods</td>
<td>Singly</td>
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<td>S2</td>
<td>Positive</td>
<td>Rods</td>
<td>Chains</td>
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<td>S3</td>
<td>Positive</td>
<td>Rods</td>
<td>Chains</td>
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<td>S4</td>
<td>Negative</td>
<td>Rods</td>
<td>Singly</td>
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<tr>
<td>S5</td>
<td>Negative</td>
<td>Rods</td>
<td>Chains</td>
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<tr>
<td>S6</td>
<td>Negative</td>
<td>Rods</td>
<td>Singly and in clusters</td>
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</table>
3.3.3.2 Biochemical characterization

In the present day of increasing emphasis on the molecular tools, the metabolic and physiological status of the organisms is still important to diversify and differentiate organisms. The microorganisms have their own identifying biochemical characteristics. These biochemical fingerprints are the properties controlled by the cell’s vital molecules and they are responsible for the bioenergetics, biosynthesis and biodegradation.

With these objectives, the biochemical and metabolic activities of all the isolates were studied for the further differentiation and characterization. The detail outline for the biochemical reactions of all the isolates is depicted in Table 3.3, Figs. 3.4 and 3.5.
Table 3.3: Biochemical profile of endophytic organism

<table>
<thead>
<tr>
<th>Isolates</th>
<th>H₂S production</th>
<th>Nitrate reduction</th>
<th>Indole production</th>
<th>Methyl red</th>
<th>Voges proskauer</th>
<th>Citrate Utilization</th>
<th>Catalase</th>
<th>Oxidase</th>
<th>Urea hydrolysis</th>
<th>Starch hydrolysis</th>
<th>Gelatin hydrolysis</th>
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<td>-</td>
<td>-</td>
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<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>M</td>
</tr>
</tbody>
</table>
### Table 3.4: Triple Sugar Iron test of isolates

<table>
<thead>
<tr>
<th>Isolates</th>
<th>Slant</th>
<th>Butt</th>
<th>Gas production</th>
</tr>
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<tbody>
<tr>
<td>S1</td>
<td>Alkaline</td>
<td>Alkaline</td>
<td>Nil</td>
</tr>
<tr>
<td>S2</td>
<td>Acid</td>
<td>Acid</td>
<td>Nil</td>
</tr>
<tr>
<td>S3</td>
<td>Acid</td>
<td>Acid</td>
<td>Nil</td>
</tr>
<tr>
<td>S4</td>
<td>Alkaline</td>
<td>Alkaline</td>
<td>Nil</td>
</tr>
<tr>
<td>S5</td>
<td>Acid</td>
<td>Alkaline</td>
<td>Nil</td>
</tr>
<tr>
<td>S6</td>
<td>Alkaline</td>
<td>Acid</td>
<td>Nil</td>
</tr>
<tr>
<td>S7</td>
<td>Alkaline</td>
<td>Acid</td>
<td>Nil</td>
</tr>
<tr>
<td>L1</td>
<td>Acid</td>
<td>Acid</td>
<td>Nil</td>
</tr>
<tr>
<td>L2</td>
<td>Acid</td>
<td>Alkaline</td>
<td>Nil</td>
</tr>
<tr>
<td>L3</td>
<td>Alkaline</td>
<td>Acid</td>
<td>Nil</td>
</tr>
<tr>
<td>L4</td>
<td>Acid</td>
<td>Acid</td>
<td>Nil</td>
</tr>
<tr>
<td>L5</td>
<td>Acid</td>
<td>Acid</td>
<td>Nil</td>
</tr>
<tr>
<td>L6</td>
<td>Alkaline</td>
<td>Alkaline</td>
<td>Nil</td>
</tr>
<tr>
<td>L7</td>
<td>Acid</td>
<td>Acid</td>
<td>Nil</td>
</tr>
<tr>
<td>L8</td>
<td>Acid</td>
<td>Acid</td>
<td>Nil</td>
</tr>
<tr>
<td>L9</td>
<td>Acid</td>
<td>Acid</td>
<td>Nil</td>
</tr>
<tr>
<td>L10</td>
<td>Acid</td>
<td>Acid</td>
<td>Nil</td>
</tr>
</tbody>
</table>

### Table 3.5: Sugar utilization profile of isolates

<table>
<thead>
<tr>
<th>Isolates</th>
<th>Sugar Utilization</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Lactose</td>
</tr>
<tr>
<td>S1</td>
<td>-</td>
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<tr>
<td>S2</td>
<td>-</td>
</tr>
<tr>
<td>S3</td>
<td>A</td>
</tr>
<tr>
<td>S4</td>
<td>-</td>
</tr>
<tr>
<td>S5</td>
<td>-</td>
</tr>
<tr>
<td>S6</td>
<td>-</td>
</tr>
<tr>
<td>S7</td>
<td>-</td>
</tr>
<tr>
<td>L1</td>
<td>-</td>
</tr>
<tr>
<td>L2</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>H2S production, 0</td>
</tr>
<tr>
<td>---</td>
<td>------------------</td>
</tr>
<tr>
<td>L3</td>
<td>-</td>
</tr>
<tr>
<td>L4</td>
<td>-</td>
</tr>
<tr>
<td>L5</td>
<td>A</td>
</tr>
<tr>
<td>L6</td>
<td>A</td>
</tr>
<tr>
<td>L7</td>
<td>A</td>
</tr>
<tr>
<td>L8</td>
<td>-</td>
</tr>
<tr>
<td>L9</td>
<td>-</td>
</tr>
<tr>
<td>L10</td>
<td>-</td>
</tr>
</tbody>
</table>

**Fig. 3.4:** Overall profile of biochemical tests for total number of positive isolates
**Fig. 3.5:** Overall profile of biochemical tests for total number of positive isolates

**Fig. 3.6:** TSI profile of isolates
Among the isolated endophytic bacteria, none of the isolates showed $\text{H}_2\text{S}$ and indole production. This suggests lack of tryptophanase in these organisms. This observation was unlike as shown in the one of the reports where 40% of the isolates were indole positive (Pal et al., 2012). Approximately, 58% isolates were capable of nitrate reduction. Maximum organism were able to hydrolyse starch with 82%
whereas gelatin and casein hydrolysis was noticed to be 53%, while 35% of the total isolates were noticed to be hydrolyzing urea. This phenomenon of positive urease activity has also been observed in other study on endophytes (Mbai et al., 2013). Organisms were also able to utilize and generate diversified result with respect to other biochemical parameters. 29% of organisms were able to utilize citrate. Approximately 47% of the isolates were catalase positive suggesting that they are moderate aerobic in nature and only 11% were oxidase positive. Ikeda et al., (2013) and Allu et al., (2014) had found 100% of catalase positive isolates. Majority of the isolates were motile (76%). Nearly 64% of isolates were positive for methyl red whereas only 47% were positive for Voges proskauer test. With reference to TSI test, maximum alkaline reaction was observed in the slant and maximum acid reaction was produced in butt (Table 3.4, Fig. 3.6). Slant was shown to be of acidic nature by 11 isolates whereas butt by 12 isolates. None of the isolates showed gas production.

Ability of the organisms to metabolize different sugars for the bioenergetics purpose is one of the approaches to diversify the organisms. The extent of sugar utilization was highly varied among the isolates (Table 3.5, Fig. 3.7). None of the isolates were able to produce gas in Durham’s tube. Maximum isolates were able to ferment dextrose (glucose) extensively (100%) followed by sucrose (82%), fructose (70%), maltose (64%), galactose and mannitol (47%) and lactose (23%) (Fig. 3.8). Sugar utilization characters of our isolates are in correspondence with Singh et al., (2013) describing lactose, dextrose and galactose positive isolates. Goryluk et al., (2009) demonstrated 100 % isolates fermenting dextrose which is exactly in accordance with our results.

### 3.3.4 Phylogenetic identification

16S rRNA, as “living fossil” of bacteria, is selected principally for the classification and identification of bacteria (Petrosino et al., 2009). 16S rRNA gene sequencing is widely used as a reliable technique in bacterial taxonomy. Evidently, it is routinely used in the polyphasic approach, describing any bacterial species or higher taxa (Ludwig and Schleifer, 1999; Mora, 2005). In the present study, potential isolate showing promising results in antimicrobial screening; isolates designated as L3, L5, L8, S3 and S7 were identified on the basis of 16S rRNA gene homology. As
described in materials and method, approx. 1500bp rRNA genes was amplified by using forward and reverse primers. Using consensus primers, the ~1.5 kb 16S rDNA fragment was amplified using high-fidelity PCR polymerase. The PCR product was bi-directionally sequenced using the forward, reverse and an internal primer. Sequence data was aligned and analyzed for finding the closest homologs for the microbe by comparing gene sequence with reference strains. The sequences were submitted to NCBI, as *Bacillus cereus* L3 (GenBank Number, KJ572277), *Bacillus licheniformis* L5 (GeneBank Number, KJ572278), *Bacillus cereus* L8 (GenBank Number, KJ572279), *Bacillus megaterium* S3 (GenBank Number, KJ572280) and *Bacillus pumilus* S7 (GenBank Number, KJ572281) (Table 3.6). The results confirmed that all five isolates belonged to the genus *Bacillus*. They belonged to the Gram positive, Firmicutes, and low G+ C content bacteria. Phylogenetic tree was constructed with all five isolates and closely related *Bacillus* species (Fig. 3.9 (a), (b)). Prior studies on endophytic bacteria have demonstrated the predominance of the genus *Bacillus* (Melnik et al., 2008; Aranda et al., 2011; Ren et al., 2013; Ji et al., 2014).

### Table 3.6: 16S rRNA identification and characterization of five potential endophytic isolates

<table>
<thead>
<tr>
<th>Isolates</th>
<th>Nearest match with accession no.</th>
<th>Sequence length (base pairs)</th>
<th>Sequence identity (%)</th>
<th>Accession no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>L3</td>
<td><em>Bacillus cereus</em> (AB215099)</td>
<td>1481</td>
<td>99</td>
<td>KJ572277</td>
</tr>
<tr>
<td>L5</td>
<td><em>Bacillus licheniformis</em> (KF535146)</td>
<td>1476</td>
<td>99</td>
<td>KJ572278</td>
</tr>
<tr>
<td>L8</td>
<td><em>Bacillus cereus</em> (KJ722448)</td>
<td>1437</td>
<td>100</td>
<td>KJ572279</td>
</tr>
<tr>
<td>S3</td>
<td><em>Bacillus megaterium</em> (KJ461522)</td>
<td>1473</td>
<td>100</td>
<td>KJ572280</td>
</tr>
<tr>
<td>S7</td>
<td><em>Bacillus pumilus</em> (KF535137)</td>
<td>1476</td>
<td>99</td>
<td>KJ572281</td>
</tr>
</tbody>
</table>

L3, L5, L8- endophytic isolates from leaf; S3, S7- endophytic isolates from stem
3.3.4.1 16S r-RNA sequences of the identified bacteria in the FASTA format

>gi|636806231|gb|KJ572277.1| Bacillus cereus strain L-3 16S ribosomal RNA gene, partial sequence

CTCTGCTCAGGATGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGC
GAATGGATTAAAGAGCTTGCTCTTATGAAGTTAGCGGCGGACCGGAGTTGAGTA
ACACGTGGGTAAACCTGCCCAAGAAGACTGGGATAACTCCGGGAAACGGG
GCTAATACCCGATAAACATTGTGAAACCCTATGCTCGAATTTGAAAGCCCGG
CTTCGCCGTCATCTTATGGGACCCCGCTCGCATTAGCTTTGTTGAG
GTAACGGCTCAACAAAGGCAACGATCGTAGCCGACCTGAGAGGGTGATC
GGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGAGCACAG
TAGGGATCTCTCCGCAATGGGAAAGTCTGACGGAGCAACGCCTGAG
GTGATGGAAGGCTTCTCCTGCTGTAACAACTCTGTTGTATAGGGAAGAACAGT
GCTAGTGTGAATAGCTGGGACCTTTGCGGTACTCTAACCAGAAAGCCACGG
CTAAGTACGTGGCGACAGCCCGGTAATAAGTAGGGGAAAGGTTATCC
GGAATTATTGGGCGTAAAGCGCGCGGCAGGTTGGTCTTAAGCTGTGATGTG
AAAGCGGCACGGCTCAACCCGTTGGAGGCTACTGAAACTGGGAACTGGGAGACCTTGA
GTGCAAGAAGAAGAAATGGGAAATTTCCATGTGGTCAGGGGATACTGCTAG
GATATTGGAGGAACACCAGTGCCGAGGCAGTTTTTCTGTTCTGTAACCTGAC
ACTGAGCGCGAAAGCGTGGGGAGCAGAGATAGAAGGTTTCCGCCCTCTTA
TCCACCCGTAACAGATGAGTCTAAGTGTGTTAGGAGGCTTCCGCCCTTTTA
GTGCTGAAAGTTAACAGCTAAAGCCTCCGTCGCGGGAGATCGGCGACAAG
GCTGCAAAACTAACAGGAATTGACGGGGGCGCCAGCAACGCGGTGGGACATG
TGGTTTAAATTCGGAAGCAGGAAAGTCTTTACCACGGTCTGCTGCATCCTCT
GAAAAACCTTAGAGATAGGGCTTCTCCTCCTCCTGGAGACAGAGTGACAGGGTGT
GCTAGTTGTGTGCTCGTCAAGTGCTGTGCTGGAGATGTTGGTTAAAGTGCTCCGCAAC
GAGCGCAACCTTGGATCTTACTGTGCCATTACTTAAAGTTGGCGACTCTAAGGT
GACTGCGGTGACCAAACCCGGAAGAGGTGGGGAGTGACGTCAAACCATCA
TGCCCCCTATGACCTGGGCTACACACGCTGCTACAAATGGGACCGTGACAAGA
GCTGCAAGCCGCCGTTGGAGCTCTAATCTCATAAACCCTTGCTCAGTGGC
GATTTGAGGCTGCAACTCAGGCTTATCGAAGTTGCAAGCTGGATCGTAATC
GGATCAGCAGCCCGCCTGGATACTAGTTCCCAGGCGCCTGCTACACACCAGCC
GTCACACCACGAGATTTTGAACACCCGAAGTCGGTGGGTAACCTTTTT
GGAGCCAGCGCCCTAAGGTTGGGACAGATGTGAGG
>gi|636806235|gb|KJ572278.1| Bacillus licheniformis strain L-5 16S ribosomal RNA gene, partial sequence

AGGACGAACGCTGGCGGCGTGTCAAATACATGCAAGTCGAGCGGACCGA
CGGGAGTGCTGCCCTCTTAGGTTCAGCGGCGACGGGTGAGTAACACGGTGG
TAAACTGCTGTAAGACTGGGAATAATTCCGGGAAACCCGGGCTAATACCG
GATGCTTGATTTAGCCGCATGTGGTCAATCTGAAAGATTGGCTTATTAGC
CACCTACAGATGGAACCCGGCCGCGATTAGCTAGTGTGGTGAAGTACGGCT
CACCAAGGCGAGCTGCTTACGCGCCACCTGAGAGGGTGACTGAGATGGCA
GGACTGAGACACCGGCCCAGACTCCTACGGGAGCGACGTAGGAATGCA
TTCCCGCAATGGACGAAAGTCTGACGAGCAACCGCGGCGTGCAGTGATGGA
GTTTTTCGGATCGTTAAACTCTGTGTTAGGGAAGAAAGGATACCGTTCG
AATAGGGCCGACCTTGAGCGTACAATACCGAGAAAGCCACGGCGTAAACTAC
GTGCCAGCAGCCGCGGTATAACCTAGTGGCAGCAAGCCTTTGTCGGGAAATAT
TGCCGATCAAAGCCGGCGCCAGGGTTCTCTTATACTCGATGTGAAAAGC
CGCTCAACCGGGGAGGGTCATTGGAAAATGGAACCTGTTAGATGCA
GAGGAGATGGAAATCCACGTGTAGCGGTGAAAAATCGTAGAGATGTGGA
GGAACACCGTGGCAGAGCCAGACTCTGCTGTCTGAACGTACGCTAGGC
GCGAAAGCGTGGGAGCGCAACAGAGATAGATGATTAGTGGTAAGCTGC
CTACGCAGCTAGCTCAGCGTACGATGTTAGTGGTGAATTCGCC
GCAAACCCATTAAGCGACTCCGCCCTGGGGGAGTACGGTCGCAAGACTGAA
TCGAAGCAACCCGAAGAAACCTTCAGGTCAGCTCTCTAGCTGGACATC
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AGCCGGAGGCTAAGCCCAATCCACAAATCTGTCTACTCTCAGTGATCGCAA
GGTCAAAACCCGGAGAAGGTGGGAGTGACTGCAAAATATCAGATC
ATGACCTGGGCTACACAGTGTCAAATGGGGCACAAAGGAGGGGAGCGA
AGCCGGAGGCTAAGCCCAATCCACAAATCTGTCTACTCTCAGTGATCGCA
GTCGTCAGCTGTCGTCGTAAGTGGTGAAATACCTGTTGGTTAAATAGCTGC
GACAGCCGAGGCTGGCAGCGACTCAATGGGCAGAAAGAACGGCGA
AGCCGGAGGCTAAGCCCAATCCACAAATCTGTCTACTCTCAGTGATCGCA
GTCGTCAGCTGTCGTCGTAAGTGGTGAAATACCTGTTGGTTAAATAGCTGC
GACAGCCGAGGCTGGCAGCGACTCAATGGGCAGAAAGAACGGCGA
AGCCGGAGGCTAAGCCCAATCCACAAATCTGTCTACTCTCAGTGATCGCA
GTCGTCAGCTGTCGTCGTAAGTGGTGAAATACCTGTTGGTTAAATAGCTGC
GACAGCCGAGGCTGGCAGCGACTCAATGGGCAGAAAGAACGGCGA
AGCCGGAGGCTAAGCCCAATCCACAAATCTGTCTACTCTCAGTGATCGCA
>gi|636806239|gb|KJ572279.1| Bacillus cereus strain L-8 16S ribosomal RNA gene, partial sequence

CAGGATGAACGCTGGCGGCGTGCCTAATACATGCAAGTGAGCGAATGG
ATTAAGAGCTTGCTCTTATGAAGTGTACGCGGGACGGGTGAGTAACCG
GGTAACCTGCCCATAAGACTGGGATAACTCCGGGGAAACCGGGGCTAATA
CCGGATAACATTTTGAACCAGCTGTGCTGAAATTGAGGAAGCCCAGCCG
TGTCACCTATTGAGTGAACCCCGCTGCATATTAGCTCTTGGTGAGTAAC
GCTCACCAGGCAAAGATGCTGACCGACCTGGAGAGTCAACCG
ACTGGGACTGAGACACGCCCCAAGCTCTACGAGGAGCAACGATAGG
ATCTTTCCGCAATAGGAGAAATGTCTAGCGAGCAACGCCCAGCTGGAG
AAGGCTTTTCCGGGTCAAAACTCTGTGTTTAGGGAAGAACAGTGTGACT
TGAATAAAGCTGGCACCCTTGACGTTACCTAACCAGAAAGCACCAGGCTA
ACCGTGCCAGCAGCAGCGCTGTAATACGTAGGTGGAGGAAACGCTTTACCC
ATTTGGGTGAAGCGCGCAGAGTTGGTTTTTAAGTCTGATGTGAAAGCC
CAGGCTCAACCGTGAGGATCATGAAACTGGGAGACTTGAGTGCAGAG
AGAGAAAGTGGAAATTCCATGTGTTAGCGTGAATATCGTAGAGATATGG
AGGAAACCCAGTTGGCAAGCGACCTTCTGTGTAACCTGACACTGAGG
CGCGAAAGCTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGC
CGTAAACAGTGAAGTCTAAGTGTTAGGAGGTTCCTCCGCCCCCTTAGTCTG
AGTTAACGCAATAGCATTCCGCGCTTGGGGAGTACGGCGCAAGGCTTAA
CTCAAGGAATGGACCGGCACGCTAGTGAGCATGTTTCTTA
TTCAAGCAACCGGAAGACCTTTACCGGTCTTTGACATCTCTCTGACACCC
CTAGAGATAGGGCTCTCTCCTCGGAGCAGAGTACGGCAGGTGTTGCTAGTG
TGTCGTCAGCTCGTGTGAGATGTGTGGGTTTAGTCAAGTCGCCAACAGGC
AACCCTTGATCTTAAGTGGCATATGTAAGTGGCACTCTAAGGTCACCTG
CGGTGACAAACCGGAGGAAGTGGGGAGTACGCTGAAATCTCATCATGCCC
TATGACCTTGCGTACACACGTGCTACATGGACGTCAGAAAGAAGACTGCC
GACCGCGAGGTGGAGCTAATCTCAATAAACCGTTCTCAGTGCGATTGTA
GGCTGCAACTCGCCTACATGAAGCTGGAATCTCGTATAGTAATCAGGCGATAG
CATGGCGCGGTGAATACGGTCCCCGCTTGTACACACCCGCGGCACACAC
CAGGAGATTGTAACACCCGAAGTCGGGTAACCCCGGAGAAGTCGGGTAACCTTT
>gi|636806241|gb|KJ572280.1| Bacillus megaterium strain S-3 16S ribosomal RNA gene, partial sequence

GATGAACGCTGGCAGCTGCTAATACATGAAGTCGAGCGAACTGATTA
GAAGCTTGGTTCTATGACGTTAGCGGAGGAGGAGCTGAGTGATGAAGGGC
ACCTGCGCTGGAAGATGAGTAACTGCGAAGATGAGTGAAGGGC
ATTAGGATCTCTCTCCTGGAAGATGATTGAAAGATGGTTTCGGCTATCA
CTTACAGATGGGCCCCGGGTGCATAGCTAGCTGTTGAGTAGTTACCGCTCA
CCAAGGCAACAGATGCATAGCCTGACGGAGGAGGTGTAGATTACCGCTGA
GACTGAGACACGGCCAGACTCCTACGGAAGGAGCAGATGAGTGAACCGTGC
CGCAATGGGACGAAAGCTGACGGGAGCAGCAGCGGAGTGGCATGGAACG
TTCCGGGTCTGAAACTCCTGGTTAGGAAAGAGACATGCGAGTACCGTAC
TGCTCCTGACCTTGACGGTAGCTACAAACAGACAGAGGAGCTTACCGACT
CAGCAGCCTGCTAATACGGTGGGCAACGCTTATCCGGGAAATTATGGG
CGTAAAGCCCGCCGAGCCTTTCTTCTAAGTCTGATGTGAAGCGCCACG
TCAACCCGTGGAAGGTCATTGGAAGACTGATGAGGCGAAGAGA
AAAGCGGAAATTCACGCTGAGCGGAAAGCTGAGATGTTGAGGAA
CACAGTGGCAGCGCTGCTGCTGTAACCTGCTGAGCTAGGCTGAGGCGA
AGCGTAGGAGGAGCAAGAGTAGGATACCCAGTACCCTGTAGTATCCACGCG
ACGATGAGTCCTAAGTGTAGGAGTTTTCGCGTCCTTACTGCTGCTGACG
ACGCCTAAACAGCACTCCGCGCTGGGGAGTAGCGTCGAAGACTGAAACT
AGGAATTGACGGGGGAGCAGGAGCTGAGTGGGCTCTAAGGTGAAGCCG
AGCAACGGAGAACCTAGCGTATGTAACGTCGTTGTAGTGAAGGAGTTG
GATAGACGCTGGCTCGGGGACAGGAATGCTGACAGGTGTTGCTGACGG
CGTCAGCTGTCGTAAGAGTGTTGGGTAAAGTTAGCTGCGAAGCGAAC
ACCTGATCTTAGGCGAGGACTTTAGGATTGGAAGCATTGCTGAGGCG
TGCAAAACCCGGAGGAAGTTGGGAGTACGTAACATCATTAGCCTGACCT
GACCTGGGGCTACACATGCCTAATGGGATGTAACAAAGGCTGCAAGAC
CGCGAGGTCAAGGCAAATCCACAAAAACATTTCAGTCCGAGATTGTAGG
TGCAACTCGCCCTACATGAAGCTGGAATCGCTAGTAATCGCGGATCGCAT
GCCGGGTTGAAATACGTTCCGCGCCGCTTGTACACACGCGCCGCACAC
GAGAGTTGTAACACCGGAAGTCGGTGAGTAAACGTGAAGGAGCTAGCCG
CCTAAGGTTGGGACAGATGATTGGG

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>gi|636806244|gb|KJ572281.1| Bacillus pumilus strain S-7 16S ribosomal RNA gene, partial sequence

TCAGGACGCAACGCTGGCGCGTGCTAATACATGCAAGTCGAGCGA
GAAGGGAAGCTTGCTCCTCCCGGATGGTTAGCAGCGGCGGAGCTGATGAACTAC
GGATACTTGTTGAGATGCTAAGACTGGGATAACTCCGGGAAACCCGAGCTAATAC
CGGATAGTTCCCTGGACCCGATGTGTTCAAGGATGAAAGACGGTTTCGGCT
GTCACTTACAGATGGACCCGGCCGCACTTAGCTAGTTGGGTGGGAATGG
CTCACCAGGCCAGGCTGCTAGGAGGCGAGGGCCATGAGTGAGATG
GGTTTTCGGATCGAAAAGTCTGTTTGATAGGAAGAACATAGGCAGAGAT
AAGGCACGAGTGGGAAATTCACGTCGAGCGGATGGAATCGTAGAGATGGGA
GAACACCCGATGGGCAAGCGGACACTTCTCCTGGGTCTAGCTGAGCTGAGG
CGAAAGCCGGGGAGCGAAGGGTTAGAGATTAGATACCTGGTACGATCAGCCG
AAACGATGCTAACGTCTAGTTGGATGTTTGTTTCGCGCCCCTAGTGCTGAGC
TAACGCATTAGCAGTCCCTGGACGGGATACGTCGAAAGGAACTACTAGG
AAAGGAATTCGGGCGGCAAAGACGGCGGAGGTGACAAAGTGGTAGTTT
GAAGCAACGGAAGAACCTTACCAGGCTTTGACATCCAGGAGCAAGG
GAGATAGGCGCTTTCCCTGGGGACAGAGTGCAGACGATGTGGAATGG
CGTCAGCTGCTGCTGAGATGGTGTTGGTAGATAACCATCGCAGGAAAC
CCTTGATCTTTAGCAGCTTGGCCACTTCAGGTGTGAGCCCTATG
TGACAAACCGGAGGAAGGTGGGGATGACGTCAATATCATCAGCTG
GACCTGGGCTACACACGTCTACATGGACAGAAACAAAGGCGCTG
CCGCAAGGTTAGCAGCAATCCATATCTGTTTCAGTTGGAGATCGAGT
TGCAACTCAGCTGCTGAGATGGTGTTGGTAGAAAACTGCTTGG
GCGCGGTGAATAGCTGGGCTGATCGAGCTGACACCCGCCTACAC
GAGATTGGCAACACCCGGAAGTGGATGATCATCATTGTGGGAC
GCCGAGGCTGGGGCGAGATGATGGTGTGAGCCGACG

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Neighbour-joining tree of potential bacterial endophytes obtained from leaf and stem of *Pongamia pinnata* based on the 16S rRNA gene sequences using MEGA 6. Bootstrap values based on 1,000 replications were listed as percentages at the nodes. The evolutionary distances were computed using the Kimura 2-parameter method and are in the units of the number of base substitutions per site. Note: numbers in parentheses represent the sequences Accession Number in the GenBank.

**Fig. 3.9(a):** Phylogenetic tree, exhibiting the phylogenetic relations between the five endophytic bacterial isolates.
Bacillus licheniformis L-5 (KJ572278)
  Bacillus sp. MG26 (JN791327)
  Bacillus sp. WB21-21 (GU595371)
  Bacillus aryabhattai W44 (KC441850)
  Bacillus licheniformis SCD 126066 (JQ030985)
    Bacillus sonorense SML-6 (KC688290)
    Bacillus sp. LLVI-4 (GU573844)
      Bacillus sp. KT100 (KJ734000)
        Bacillus amyloliquifaciens subsp. plantarum Hk1-11 (JF899253)
        Bacillus subtilis W39 (GQ872187)
    Bacillus thuringiensis LMA (KJ628093)
      Bacterium SEHEN2 (EU723272)
        Bacillus sp. N3a (DQ376940)
          Bacillus thuringiensis CHMAB4 (KJ787129)
            Bacillus cereus EM1 (KJ612528)
              Bacillus sp. 240B1 (AF350926)
                Bacillus cereus L-8 (KJ572279)
                  Bacillus thuringiensis BAC1141 (HM355730)
                    Bacillus sp. CWP1-6-1 (HQ179147)
                      Bacillus cereus (AB592505)
    Bacillus megaterium BAC1141 (HM355730)
      Bacillus sp. 3452CO2 (KF600573)
        Bacillus aryabhatai CU2 (KJ855785)
          Bacillus megaterium S-3 (KJ572280)
            Bacillus megaterium 1-4 (JX122839)
              Bacillus sp. CSBb (AB552877)
                Bacillus sp. HB12161 (KJ423084)
                  Bacillus flexus (HE801970)
                    Bacillus sp. PNS-25 (JQ218455)
                      Bacterium Antarctica-14 (EF667987)
                        Bacillus flexus BMU6 (KC595872)
Neighbour-joining tree of potential bacterial endophytes obtained from leaf and stem of *Pongamia pinnata* and their closest relatives based on the 16S rRNA gene sequences using MEGA 6. Bootstrap values based on 1,000 replications were listed as percentages at the nodes. The evolutionary distances were computed using the Kimura 2-parameter method and are in the units of the number of base substitutions per site. Note: numbers in parentheses represent the sequences Accession Number in the GenBank.

**Fig. 3.9(b):** Phylogenetic tree, exhibiting the phylogenetic relations between the endophytic bacterial isolates with their nearest homologs
3.4 Conclusions

- 17 morphologically distinct bacterial strains were isolated from *P. pinnata*; 10 (L1-L10) from leaf and 7 (S1-S7) from stem.

- 80% of isolates were between 1-3mm in colony size while about 20% had large (4-5mm) colony size. 50% of the isolates had round shape, 40% had irregular shape and 10% of the isolates had elliptical shape.

- Some common characters were showed by the majority of isolates such as, round and entire shape with opaque colony, smooth texture and creamish white pigmentation.

- Gram positive character dominated over the Gram negative by a small margin. 53% of the isolates were Gram positive in nature and rest 47% were Gram negative.

- None of the isolates showed H₂S and indole production. 47% of the isolates were catalase positive suggesting that they are moderate aerobic in nature and only 11% were oxidase positive.

- 100% of the isolates were able to ferment glucose followed by sucrose (82%). None of them produced gas in Durham’s tube.

- The 16S rRNA sequencing revealed L3 as *Bacillus cereus* (GenBank Number, KJ572277), L5 as *Bacillus licheniformis* (GeneBank Number, KJ572278), L8 as *Bacillus cereus* (GenBank Number, KJ572279), S3 as *Bacillus megaterium* (GenBank Number, KJ572280) and S7 as *Bacillus pumilus* (GenBank Number, KJ572281).

- Thus all five isolates belonged to the genus *Bacillus* (Gram positive, Firmicutes, and low G+ C content bacteria).
3.5 References


