CHAPTER-2
Screening of endophytic bacteria for synthesis of nanoparticles and antimicrobial activity

GRAPHICAL ABSTRACT
1. INTRODUCTION

a. Isolation of endophytic bacteria

Endophytic bacteria inhabit almost all variety of plant species from both monocotyledonous and dicotyledonous ranging from woody tree such as yew, oak, pear, to herbaceous crop plants such as sugar beet, maize, tomato and rice. The diversity of bacterial endophytes mainly depends on the type of plant species and geographical area. Surface sterilization protocol plays an important role to eliminate the epiphytic flora. Majority of the scientific literatures mainly use disinfectant such as sodium hypochlorite, formaldehyde, ethanol, calcium hypochlorite etc., (Schulz and Boyle, 2005; Zhang et al., 2006). In order to isolate bacterial endophytes use of antifungal agents such as cycloheximide and Bavistin are widely used in surface sterilization protocols to suppress the growth of fungal species (Zin et al., 2010; Baker et al., 2012c). One of the important aspects in isolation of potent bacterial endophytes relies on the selection of plant species which is further depend on the different criteria.

b. Selection of Plant Material

Plants with ethno botanical history form important criteria towards screening of endophytes which may lead to isolate endophyte with potent activity and few of the scientific reports suggests that plants growing in rich biodiversity lodge diverse group of endophytes with activity. Similarly plants growing in unique habitats such as harsh environment and plants growing in a disease area with being infected can yield potent endophytes (Strobel et al., 1999). Apart from selection of plant, type of plant tissues also influences the isolation of endophytes for instance young plant tissue is more suitable for isolation of endophytes than older tissues (Bacon and White, 2000).

c. Screening of endophytes for synthesis of nanoparticles and antimicrobial activity

Recent studies demonstrate that endophytes may play undeniable roles in reducing the metal salts due to their unique metabolic diversity. In general, microbes are reported to interact with other living organisms along with environmental elements such as metals and minerals causing alteration to their physical and chemical
properties. In most scenarios these changes cause inevitably impact on microbial growth, few succumb and most of them flourish by growing luxuriantly. Research has now begun to shed light on some of the phenomena unanticipated activities surrounding microbial communities are embracing their stability and the mechanisms that govern the progress and development in science by isolating the novel microorganisms from various biological niches. Endophytes are one such plethora which harbours unique niches in higher plants screening such plethora bearing unique diversity can reduce and synthesize nanoparticles. In majority of cases commonly encountered microbial genera lack their ability to reduce metal salts unless they have been isolated from the mining fields which become an ease for its use for synthesis of nanoparticles as they encounter metal debris or metal contamination.

Whereas to achieve the synthesis by exploiting the microbial plethora which have not encounter the metal salts becomes a challenging task during which various parameters come into play such as selecting the source for isolation, culturing the isolated microorganisms in an enriched media different from the normal media, acclimatization of these microorganisms to sustain and encounter the metal salts. During such paradigmatic process most of the microbial flora gets screened and only the microorganisms capable of growing survives. Such microorganisms are rapidly exploited as nanofactories for the synthesis of nanoparticles. With this general scientific knowledge the present study is executed to screen bacterial endophytes from plants in surge of their evaluation for nanoparticle synthesis and antimicrobial activity.

Endophytic system for nano-material biosynthesis has come in vogue in recent past as an effective alternative to chemical synthesis. The ability to synthesis nanoparticles rapidly with morphology control by eco-friendly biological methods is exciting and represents an important advance in making them viable alternatives to the more popular chemical methods. Especially, extracellular synthesis offers the advantage of obtaining significant quantities in relatively pure state and can easily be processed. These studies also exemplify the ability of microbes to tolerant metal and also supports the hypothesis of endophyte plays a major role in defence mechanism of its host by forming mutualistic relationship and prevent its host from toxic metals and harsh environments. Exploiting endophytic flora in synthesis of nanoparticles against
other biological entity can lead to significant advances. The enthusiasm fuelling progress is towards microbial plethora synthesising nanoparticles and their applications which in turn address key implications caused by chemical synthesis protocols (Baker and Satish, 2012b; Ali et al., 2013).

Endophytes can be screened for antimicrobial activity through various techniques which are grouped into preliminary screening techniques such as agar overlay and dual culture technique. Secondary techniques include disc diffusion, well diffusion and minimal inhibitory concentration (Hayakava et al., 2004). These screening techniques aid in separating activity bearing endophytes from other endophytes.

2. REVIEW OF LITERATURE

a. Isolation of endophytic bacteria with bioactivity

Three bacterial endophytes were isolated from the leaves of Gaultheria procumbens collected from the Forest Research Institute of Malaysia (FRIM). Which were identified as Pseudomonas resinovorans, Paenibacillus polymaxa and Acenitobacter calcoaceticus using amplified 16S rRNA gene sequence method. These putative bacterial endophytes contributed for the growth and development of Gaultheria procumbens (Bhore et al., 2010). Endophytic bacterium was isolated from Solanum nigrum L. which was characterized based on 16S rRNA to reveal its genera belonging to Bacillus sp. The isolated bacterium was evaluated for bioremediation of heavy metals such as Cu (II), Cd (II) and Pb (II) which resulted in the remediation at 10 mg/ml concentration of the metals, thus forming a multi-metal resistant isolate (Guo et al., 2010).

Endophytic bacteria were isolated from three arboreal species Carapa guianenses, Ceiba pentandra and Swietenia macrophylla. Isolated endophytes were identified through partial sequencing of the 16S rRNA encoding gene. The results were matched with the gene bank to reveal the majority of the isolates belonging to Bacillus species (Coelho et al., 2011). Endophytic bacteria were isolated from green gram Vigna radiate L. twenty five isolates obtained were evaluated for plant growth promotion. Four isolates showed significant response for plant growth promoting
effects on greenhouse plants. Based on genomic 16S rRNA analysis revealed 40% of the isolated bacteria belongs to the *Azotobacter* genus these strains were capable of production of IAA and solubilizing phosphate which suggested that endophytic bacteria for the use as biofertilizers (Aung et al., 2011).

*Catharanthes roseus* L. was screened for isolation of endophytic actinomycetes. Among the 38 strains obtained, 20 strain exhibited different morphologically characteristics which showed antimicrobial activity against human and phytopathogens (Kafur and Khan, 2011). Endophytic bacteria were isolated from *Cymbidium eburneum* for production of indole which resulted in isolation of twelve endophytic bacteria. Eight endophytes showed production of indole and phosphate solubilizing activity under greenhouse conditions (Faria et al., 2012).

According to the study of Pradeepa and Jennifer, (2013) endophytic bacteria were screened and isolated from the leaves of *Tabernaemontana divaricata* L. obtained isolates were designated with numbers Tde 1, Tde 2, Tde 3, Tde 4, Tde 5. The bacteria were screened for production of cytokinin and maximum activity was observed with Tde 1 which was further identified as *Alcaligenes faecalis* by 16S rRNA sequencing and phylogenetic tree was constructed by neighbour joining method. Screening of endophytic bacteria inhabiting mangrove species, *Rhizophora mangle* and *Avicennia nitida* located at Bertioga and Cananeia of Brazil resulted in isolation of *Bacillus* species as one of the most frequently isolated genus, comprising 42% of the species isolated from Cananeia and 28% of the species from Bertioga. Other genera such as *Pantoea*, *Curtobacterium* and *Enterobacter* were also isolated from these plants. The isolated bacterial endophytes were evaluated for enzyme production. According to the authors this was the first reported diversity analysis on endophytic bacteria obtained from the branches of mangrove trees (Castra et al., 2014).

Six endophytic bacteria were isolated from *Zea mays* L. Which belong to *Bacillus* and *Enterobacter* species to evaluate for nitrogen fixation and biocontrol against fungal pathogens. Among six isolates, four were positive for the nitrogen and two *Bacillus* strains exhibited antagonists against the pathogenic fungi *Fusarium verticillioides*, *Colletotrichum graminicola*, *Bipolaris maydis* and *Cercospora*
Culturable endophytic bacteria colonizing grapevine were isolated and characterized by molecular methods which resulted in three hundred and eighty one culturable endophytes. Obtained results showed 30% of the isolates belonged to the genus *Bacillus* and other isolates belong to *Staphylococcus*, *Microbacterium*, *Paenibacillus*, *Curtobacterium*, *Stenotrophomonas*, *Variovorax*, *Micrococcus* and *Agrococcus* respectively (Baldan *et al.*, 2014).

**b. Screening of endophytes for nanoparticles synthesis**

Scientific literature represents availability of few reports on the endophytes towards synthesizing nanoparticles. According to study conducted by Sankar *et al.*, (2003), use of geranium leaves (*Pelargonium graveolens*) and its endophytic fungus resulted in extracellular synthesis of gold nanoparticles. Sterilized geranium leaves and an endophytic fungus *Colletotrichum* sp. were separately challenged with chloroaurate ions. Rapid reduction of the metal ions was observed in both the cases resulting in the formation of stable gold nanoparticles of variable size. In Geranium leaves, the reducing and capping agents appear to be terpenoids while they are identified to be enzymes in the case of *Colletotrichum* sp. The TEM analysis revealed spherical shaped gold nanoparticles using the fungus, whereas leaves exhibited a variety of shapes that included rods, flat sheets and triangles. This report confirms monodispersity of nanoparticles with endophytic fungus compared to its host.

Similarly, *Amylomyces rouxii* KSU 09 an endophytic fungus synthesizes silver nanoparticle. The synthesized silver nanoparticles were characterized by determining the time dependent increase in surface plasmon resonance (SPR) through UV-Vis spectrophotometry, by X-ray diffraction (XRD), transmission electron microscopy (TEM) and atomic force microscopy (AFM). The broad spectrum antimicrobial activity of the silver nanoparticles was observed against Gram-negative and Gram-positive bacteria pathogenic fungi (Musarrat *et al.*, 2010).

The endophytic fungus *Aspergillus clavatus* isolated from sterilized stem tissues of *Azadirachta indica* was challenged with 1 mM gold chloroaurate solution resulted in triangular shaped gold nanoparticles along with some spherical as well as hexagonal shaped. It was also observed that the synthesis of gold nanotriangles are extracellular and showing a high aspect ratio. The study reported single-step green
protocol for the generation and stabilization of nontoxic gold nanotriangles. Nanoparticles were characterized using UV-Visible spectroscopic technique, FTIR, XRD, AFM and TEM (Verma et al., 2011).

An endophytic Pestalotia sp. isolated from healthy leaves of Syzygium cumini L. was evaluated for the extracellular synthesis of silver nanoparticles. Silver nanoparticles synthesized showed antibacterial activity against human pathogens. The study also confirmed the formation of spherical and polydispersed nanoparticles in the range of 10-40 nm having average size of 12.40 nm when endophyte was treated with silver nitrate (Raheman et al., 2012). According to the study conducted by Devi et al., (2012), an endophytic fungus Penicillium sp. isolated from the Centella asiatica resulted in synthesis of silver nanoparticles using the filtrate of cell mass of an endophyte. The SEM studies confirmed the formation of silver particles in the size of 100 nm, a clear indication of the formation of silver nanoparticles. The silver nanoparticle synthesized exhibited antimicrobial effect on various human pathogens.

3. MATERIALS AND METHODS

a. Plant selected for the present investigation

Selection of plant species was attributed based on their traditional scientific records and their earlier reports on bacterial endophytes. Healthy plant materials such as leaves, stem and bark were randomly collected from different sampling sites located within Karnataka, Southern India. Five medicinal plants viz., Annona squamosa L., Coffea arabica L., Tridax procumbens L., Euphorbia hirta L., Mimosa pudica L. were selected for the present study.

i. Annona squamosa L.

Annona squamosa L. belongs to family Annonaceae. It is a semi-deciduous tree with approximate 3-7 m in height with irregular branches bearing leaves which are more or less 6-17 x 3-6 cm in size with lanceolate or long lanceolate in shape (Figure 2.1a). Annona squamosa L. hails a reputed medicinal values based on the scientific literatures reported. Leaves extract of Annona squamosa L. is reported to have anti diabetic, anticancerous, antihyperthyroidism, antifertility, antitumor, antimutagenic, anthelmintic, scavenging, antidiabetic, licidical, hepatoprotective, antithyroid, antigenotoxic, antiplasmodial, molluscicidal and antimicrobial properties.
The ripened fruit of this plant is consumed as a healthy diet and unripe fruit powder is used as insecticides. *Annona squamosa* seeds powder is used in curing wounds. Overall the plant and its products hail significant therapeutic values (Vanitha *et al*., 2011; Yushau *et al*., 2011). Based on the earlier reports on bacterial endophytes and traditional value *Annona squamosa* L. was selected for screening of bacterial endophytes in the present investigation whose samples were collected from Manasagangotri campus; University of Mysore situated at Mysore lying between 11°30’ north to 12°50’ east and has an average altitude of 770 m (2,526 ft). It is situated in the southern region of the state of Karnataka, India, at the base of the Chamundi hills and spreads across an area of 128.42 km$^2$ (50 sq m).

![Figure 2.1(a): Annona squamosa L.](image)

**ii. Coffea arabica L.**

*Coffea arabica* L. is a small shrub which belongs to family Rubiacaeae. It grows up to average height of 2 to 4 m with characteristic horizontal branching bearing shiny with dark glossy evergreen leaves (Figure 2.1b). *Coffea arabica* L. is a tropical crop and the second most valuable international commodity in the world due to the fact that its seeds are been used since ancient times as one of the most popular beverages across the globe. The epidemiologic significance of the research on *Coffea arabica* L. has yielded its pharmaceutical importance with therapeutic properties such as decrease in plasma glucose levels, reduction of plasma homocysteine levels and improves the vaso reactivity in cardiovascular disease, antioxidant activity, antimicrobial activity (Bisht and Sisodia, 2010; Farah, 2011). Based on the earlier reports on bacterial endophytes and traditional value *Coffea arabica* L. was selected for screening of bacterial endophytes in the present
investigation whose samples were collected from the farm Coorg coffee estate located at outskirts of Madikeri which is situated in Kodagu, Karnataka, India. Its geographical coordinates are 12° 25'0'' North, 75° 44' 0'' East. It has a geographical area of 4,102 km\(^2\) (1,584 sq m). Madikeri features a tropical highland climate as it has an elevation of 1061 m (3484 ft).

![Coffea arabica L.](image)

**Figure 2.1(b): Coffea arabica L.**

### iii. *Euphorbia hirta* L.

*Euphorbia hirta* L. belongs to family Euphorbiaceae, it is a small ascending annual herb reaching up to 50 cm, with hairy stems and leaves. The flowers are small, numerous and crowded together in dense cymes about 1 cm in diameter (Figure 2.1c). *Euphorbia hirta* L. is reported to posses medicinal properties they have been used in the treatment of gastrointestinal disorders, posses antioxidant properties, anti-inflammatory, antimicrobial properties anticancer activity, nematicidal activity, bronchial and respiratory (Upadhyay *et al.*, 2010; Huang *et al.*, 2012). Based on the earlier reports on bacterial endophytes and traditional value *Euphorbia hirta* L. was selected for screening of bacterial endophytes in the present investigation whose samples were collected from Srirangapatna is historical rocky island formed by Cauvery River with area 13 km\(^2\) (5 sq mi), situated 15-20 km from Mysore, Karnataka, India. Geographical coordinates are 12.41° North 76.7° East. It has an average elevation of 679 m (2227 ft).
iv. *Mimosa pudica* L.

*Mimosa pudica* L. belongs to family Fabaceae, it is a short prickly plant with its branches growing close to ground. It grows up to a height of about 0.5 m and spreads up to 0.3 m. The stem is erect, slender, prickly and well branched. Leaves are bipinnate, fern like and pale green in colour with a tendency of closing when disturbed (Figure 2.1d). *Mimosa pudica* L. is widely used in medicine which can be traced down with various scientific literatures pertaining to ayurvedic medicine. It has been used in treating headache, alopecia, diarrhoea, dysentery, insomnia, arresting bleeding, skin diseases, hyperosteogeny and arthralgia.

The extract of the plant is reported to have highly significant biological activities such as anthelminthic activity, anti-hepatotoxic activity, antivenom activity, antiulcer activity and antimicrobial activity (Kaur *et al.*, 2011 and Varnika *et al.*, 2012). Based on the earlier reports on bacterial endophytes and traditional value *Mimosa pudica* L. was selected for screening of bacterial endophytes in the present investigation whose samples were collected from Srirangapatna is historical rocky island formed by Cauvery River with area 13 km² (5 sq mi), situated 15-20 km from Mysore, Karnataka, India. Geographical coordinates are 12.41° North 76.7° East. It has an average elevation of 679 m (2227 ft).
v. *Tridax procumbens* L.

*Tridax procumbens* L. is a procumbent herb with leaves size ranging from 3-6 x 1.5-3 cm whose stems are branched, creeping at the base and trailing above (Figure 2.1e). *Tridax procumbens* L. is a common medicinal herb used by ethnomedical practitioners in India. It is reported to bear significant therapeutic properties such as wound healing, anticoagulant, antifungal, insect repellent, antioxidant, antidiabetic, antiparasitic, antimicrobial, to cure diarrhea and dysentery (Kumar and Naidu, 2011). Based on the earlier reports on bacterial endophytes and traditional value *Tridax procumbens* L. was selected for screening of bacterial endophytes in the present investigation whose samples were collected from Chamundi hills reserve forest (12°15'34"-12°17'5" North to 76°39'63"-76°42'02" East) comprises an isolated range of hillocks ranging in altitude from 861-944 m above sea level, surrounding a plateau that is 1,024-1,057 m high popularly known as Chamundi hills, with a periphery of 11 km, situated south-east of Mysore city, Karnataka, India.
b. Sample processing

The materials were collected in a sterilized polythene bags and transported to laboratory. Collected plant materials were thoroughly washed under running tap water and then were immersed in double distilled water containing 50 µg/ml of cycloheximide for 60 minutes (Baker et al., 2012c).

c. Surface sterilization protocol

Plant materials were subjected to surface sterilization under aseptic condition and washed thoroughly with tap water followed by distilled water to remove adhering soil and debris. Later the surface sterilization was carried by sequential steps initially by immersing in 3.15% sodium hypochlorite for 120 seconds then followed by 70% ethanol for 60 seconds and dried using sterile blotter sheets for 30 seconds (Figure 2.2). In every step of the surface sterilization procedures the plant materials were washed in sterile double distilled water. To confirm that the surface disinfestations process was successful and to verify that no biological contamination from the surface sterilized plant segments, sterility checks were carried out for each sample to monitor the effectiveness by impressions and 0.1 ml from the final rinse was plated out on nutrient agar as control plate (Webster et al., 2001; Zin et al., 2010; Baker et al., 2012c). Colonies emerging from surface sterilized plant segments was subcultured and maintained with alpha-numeric code based on the source of isolation.

![Figure 2.2: Schematic representation of surface sterilization of plant material for isolation of endophytes](image-url)
d. Screening of endophytic bacteria for synthesis of nanoparticles

Endophytic bacteria were cultured in the media incorporated with metal salts such as silver nitrate and gold chloroaurate and incubated at 37°C until visible growth was observed. Further the colonies emerging from this enrich media was cultured in nutrient broth and incubated for 72 hours. The culture broth was centrifuged at 8000 rpm at 4°C for 20 minutes and supernatant was assessed for synthesis of nanoparticles by challenging 1 mM of silver nitrate for silver nanoparticles and gold chloroaurate for gold nanoparticles and incubated until colour change was observed. Samples were drawn periodically and monitored using UV-Visible spectrophotometry to confirm the synthesis of nanoparticles (Baker and Satish, 2012d).

e. Optimization parameters for the synthesis of nanoparticles

i. Temperature

The reaction mixture of the metal salts and the cell free supernatant of each isolates were incubated at different temperatures ranging from 30°C to 80°C and the synthesis of nanoparticles was monitored by drawing the samples periodically and analyzing it using UV-Visible spectrophotometry.

ii. Concentration of metal salts and supernatant

Effect of concentration of the metal salts was optimized by varying the different concentration of metal salts ranging from 1.0 to 2.5 mM and ratio of supernatant with metal salts was studied to determine the optimal ratio required for rapid synthesis. The reaction mixture was incubated at room temperature. The synthesis of nanoparticles was monitored by drawing the samples periodically and analyzing it using UV-Visible spectrophotometry.

iii. pH

Effect of pH influencing the nanoparticle synthesis was carried out by varying the pH of the reaction mixture from 6 to 9. The synthesis of nanoparticles was monitored by drawing the samples periodically and analyzing it using UV-Visible spectrophotometry.
f. Screening of endophytic bacteria for antimicrobial activity

Primary screening of antibacterial activity of endophytic bacteria isolated from surface sterilized plant segments was carried out by agar overlay assay based on the protocol described by Hayakava et al., (2004). In brief each endophytic isolate were point inoculated and incubated for 72 hours. The colonies were inactivated by adding 1-5 ml chloroform onto sterilized blotter sheets placed on the inverted upper lid and incubated for 20 minutes. The inactivated colonies were overlaid with 10 ml of 0.65% of sloppy soft nutrient agar seeded with test organisms viz., Staphylococcus aureus (MTCC 7443) and Escherichia coli (MTCC 7410). Plates were incubated at 37º C for 24 hours (Baker et al., 2012b). For antifungal activity dual culture technique assay was performed based on the protocol of Lind et al., (2005), in brief each isolate was individually streaked perpendicularly and was incubated at 37º C for 72 hours. Later the test fungal spore suspensions viz., Fusarium verticilloides, Aspergillus niger and Aspergillus flavus were seeded onto the media across the streaking and incubated at room temperature for 7 days. The antagonistic activity of the endophytic bacteria was measured with inhibition of test fungi.

g. Molecular characterization of selected endophytes

Bioactive endophytic bacterial strains were inoculated to sterile 3 mL of LB medium and incubated overnight at 37º C and 200 rpm for approximately 24 hours. 2 mL of the resulting bacterial suspension (OD at 0.6) was pelleted at 13,000 rpm for 3 minutes and the total cellular DNA was extracted using the CTAB method outlined in Wilson, (1990). Cell pellet (approximately 0.1 gram) resuspended completely with 600 µl TE buffer using sterile toothpick to ensure complete mix and resuspended. Approximately 10 µl (10 µg) lysozyme solution added to the cell suspension. Mixture was mixed thoroughly by inverting Eppendorf tube several times and incubated for 10-60 minutes at 37º C. 10µl of Proteinase K (10 mg/ml) and 30 µl SDS (20%). was added by mixing thoroughly and incubated at 37º C until the suspension becomes clear and viscous. 100 µl of NaCl (5 M) was added by mixing and incubated suspension at 65º C for 2 minutes. 100 µl CTAB solution was pre-heated at 65º C and mix thoroughly by incubating suspension at 65º C for 10 minutes.
Above suspension was extracted with an equal volume of chloroform/isoamyl alcohol (24:1) solution. Centrifuge (10,000×g, 5 minutes). Upper (aqueous) phase (Supernatant 1), containing the nucleic acids, was transferred into a separate 2.0 ml Eppendorf tube and 0.7 volumes of isopropanol was added to above aqueous phase to precipitate nucleic acids, mixed gently by inverting the tube several times DNA should appear as a white, viscous, precipitate. Centrifuge (12,000×g, 15 minutes) at room temperature to pellet genomic DNA and upper isopropanol was carefully removed without disturbing the pellet.

The pellet was washed with 500 µl ethanol (70%) by inverting the tube several times. Centrifuge at 12,000×g, 15 minutes at room temperature. Ethanol was carefully removed and rim of the tube was blot dried with a paper towel to remove of excess liquid and pellet was dried in a speed-vac. The above DNA pellet was re-suspended in 100 µl TE buffer and stored at -20° C. The quality of extracted genomic DNA was assessed using agarose gel electrophoresis by loading 10 µl of total DNA solution onto a 0.8% agarose gel containing ethidium bromide (0.5 µg/ml) and electrophoresed to separate DNA and visualized under a gel documentation system (Bio Rad) (Wilson, 1990).

h. Molecular taxonomy of endophytic bacteria

Extracted DNA was amplified using universal primers, Forward primer UPF 5′-AGTTTGATCCTGGCTCA-3′ and Reverse primer UPR as 5′-ACGGCTACCTTGTTACGACTT-3′. Polymerase chain reaction (PCR) amplification was carried out by preparing 50 µl PCR reaction mixture containing 4µl extracted DNA with 100 ng/ml concentration, 5.0 µl of 10× PCR buffer (Merck Genei, India), 5 µl of 2.0 mM dNTP mix, 2.0 µl of 0.2 µM of each primer and 2.0 µl Taq DNA polymerase (1 U) (Fermentas) and 30 µl sterile deionized water. PCR amplification was performed with an Eppendorf Master cycler Gradient (Eppendorf, Germany) using the following program: Initial denaturation at 95° C for 4 minutes, followed by 30 cycles of denaturation at 95° C for 1 minute, annealing temperature at 55° C for 1 minute and extension at 72° C for 1 minute with a final extension at 72° C for 10 minutes (Mirza et al., 2006). The PCR products were electrophoresed in 0.8% (w/v) agarose gel containing ethidium bromide (0.5µg/ml) and the amplicons were visualized under a gel documentation system (Bio Rad). The gel section with desired
band was carefully excised under UV light and subjected to extraction using a gel band purification kit (Qiagen) and sequenced bidirectional way in an ABI 3730 sequencer (Applied Biosystems, United States) using the UPF and UPR primers.

Sequences were processed using BioEdit (Hall, 1999) and subjected to BLAST search at NCBI (www.ncbi.nlm.nih.gov) to assign putative identity, designation of operational taxonomic units based on sequence similarity measures and phylogenetic inference. Partial nucleotide sequences were deposited in NCBI Gen Bank and authentic accession number were obtained. Phenogram was constructed with Clustalw (Thompson et al., 1997) software by grouping the isolates deposited at GenBank to reveal the relationships of identified strain with taxonomically similar bacteria by constructing the phylogenetic tree. Alignments were manually edited wherever necessary and a phylogenetic analysis was performed to assess phylogenetic affiliation.

4. RESULTS

a. Isolation of endophytic bacteria

Mining the selected medicinal plants towards haunt for potent endophytic bacteria claimed in successful isolation of 322 isolates upon screening five significant plants which have cited in table 2.1. During surface sterilization protocol addition of cycloheximide resulted in suppression of fungal endophytes. Sterility checks showed no growth which claimed the successful sterilization procedure. Emergence of endophytic bacteria was clearly observed from the surface sterilized plant material as shown in the figure 2.3. Each endophytic bacterium was designated alpha-numeric code based on their source of isolation. Each isolate was subcultured until further use (Figure 2.4) majority of the isolated bacteria were Gram-negative compared to Gram-positive.

Upon screening Annona squamosa L. fostered towards isolation of eighty four endophytic bacteria, among which forty one isolates were obtained from surface sterilized stem and forty three isolates from leaves. The obtained isolates were given accession number AS 01 to AS 84 until further use. Similarly from Coffea arabica L. eleven isolates were obtained from surface sterilized stem and twenty one from
surface sterilization leaves which were maintained with accession number CA 01 to CA 32.

**Table 2.1: Bacterial endophytes isolated from five medicinal plants**

<table>
<thead>
<tr>
<th>Plant species</th>
<th>Plant part used</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Stem</td>
<td>Leaves</td>
</tr>
<tr>
<td><em>Annona squamosa</em> L.</td>
<td>41</td>
<td>43</td>
</tr>
<tr>
<td><em>Coffea arabica</em> L.</td>
<td>11</td>
<td>21</td>
</tr>
<tr>
<td><em>Euphorbia hirta</em> L.</td>
<td>12</td>
<td>18</td>
</tr>
<tr>
<td><em>Mimosa pudica</em> L.</td>
<td>57</td>
<td>-</td>
</tr>
<tr>
<td><em>Tridax procumbens</em> L.</td>
<td>14</td>
<td>06</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>332</td>
<td></td>
</tr>
</tbody>
</table>

**Figure 2.3: Endophytic colonies emerging from plant segments**

*Mimosa pudica* L. revealed highest numbers of isolates with forty one endophytes, as this herb is very shy which folds itself upon touch, hence large segments of stems and roots were evaluated in isolation of potent endophytes among the total isolates of *Mimosa pudica* L. fifty seven isolates were obtained from stem and eighty four were obtained from roots. All the isolates were given accession number from MB 01 to MB 141 and were well maintained until further use. When
Euphorbia hirta L. was evaluated, forty six isolates were obtained among which twelve isolates were obtained from stem segments, eighteen isolates from leaves and sixteen isolates from root segments which were maintained with accession number from EH 01 to EH 46 until further use. Ultimately, medicinal plant Tridax procumbens upon evaluation resulted in isolation fourteen isolates from surface sterilized stem, six isolates from leaves and nine isolates from roots respectively. All the obtained isolates were provided with accession number from T 01 to T 29.

Figure 2.4: Pure culture of endophytic bacteria

b. Screening of endophytic bacteria for synthesis of nanoparticles

All the endophytic isolates were designated using a code consisting of alphanumerical characters based on the plant source (Table 2.1). Among the total three hundred and thirty two endophytic isolates obtained, forty one endophytic isolates were capable of growing in the media enriched with metal salts. These forty one endophytic bacteria showing positive results with primary screening were further evaluated for secondary screening for nanoparticles synthesis which resulted in isolation of only seven strains capable of synthesizing both gold and silver nanoparticles respectively (Figure 2.5) which was initially confirmed with change in colour from the supernatant colour to dark brown colour with respect to silver nanoparticles and pink or ruby red in for gold nanoparticles. This change in colour is due to the surface plasmon resonance of the nanoparticles. Further confirmation was achieved using UV-Visible spectrophotometer with peak occurring between 200 to
600 nm for silver nanoparticles and 300 to 800 nm for gold nanoparticles. Each isolate showed different pattern of peaks which was studied in order to observe the maximum absorption.

Figure 2.5: Screening of bacterial endophytes for synthesis of nanoparticles

i. UV- analysis of silver nanoparticles by endophytic bacteria

Silver nanoparticles was ascertain with supernatant of strain AS 41G which reduced silver nitrate to silver nanoparticles as shown in figure 2.6 with maximum absorption between 410 to 420 nm with a sharp and prominent peak and the synthesis was achieved within 12 hours of incubation time.

Figure 2.6: UV-Visible spectrum of silver nanoparticles mediated by Strain AS 41G
The increments of optical density between 420 to 450 nm as represented in below (Figure 2.7) confirm the nanoparticles synthesis by using strain CA 417. The obtained peak was sharp and prominent and synthesis was achieved within 12 hours of incubation time.

Figure 2.7: UV-Visible spectrum of silver nanoparticles mediated by Strain CA 417

Strain EH 419 assisted the silver nanoparticles synthesis as illustrated in the figure 2.8 displaying the peak between 150 to 350 nm which confirms the formation of nanoparticles. The peak was broad with low intensity and the synthesis of nanoparticles was achieved within 24 hours of incubation time.

Figure 2.8: UV-Visible spectrum of silver nanoparticles mediated by Strain EH 419
Silver nanoparticles synthesis was observed with strain MB 13 as represented in figure 2.9, with peak conferring between 350 to 650 nm and maximum absorption between 350 to 400 nm confirming the nanoparticles synthesis. The figure illustrates the peak with the presence of shoulder and the synthesis was completed with 36 hours of incubation time.

Figure 2.9: UV-Visible spectrum of silver nanoparticles mediated by Strain MB 13

Silver nanoparticles synthesis was achieved with strain MB 141 as shown in the (Figure 2.10) with broad peak and maximum absorption peak 200 to 250 nm. Synthesis of nanoparticles was completed within 30 hours of incubation time.

Figure 2.10: UV-Visible spectrum of silver nanoparticles mediated by Strain MB 141
The reduction of silver ions to nanoparticles was achieved with strain MB 149 as shown in the figure 2.11 which illustrate the maximum absorption between 350 to 450 nm confirming the nanoparticles synthesis. The synthesis of nanoparticles was achieved within 30 hours of incubation time.

**Figure 2.11: UV-Visible spectrum of silver nanoparticles mediated by Strain MB 149**

Synthesis of silver nanoparticles was achieved with strain TP 416 (Figure 2.12). The figure shows the maximum absorption between 350 to 400 nm confirming the nanoparticle formation. The peak appears to be like a small hump with very low intensity and the synthesis was achieved within 30 hours of incubation time.

**Figure 2.12: UV-Visible spectrum of silver nanoparticles mediated by Strain TP 416**
**ii. UV analysis of gold nanoparticles by endophytic bacteria**

Synthesis of gold nanoparticles was initially observed with change in colour of the reaction mixture from colour of the supernatant to pink or ruby red in colour based on the intensity of synthesis the colour varied among the different isolates. Results of the secondary screening from these isolates were as similar to that of silver nanoparticles but the absorption spectra varied with the emergence of peak between 400 to 800 nm.

The synthesis of gold nanoparticles mediated by strain AS 41G with maximum absorption at around 550 to 560 nm as shown in the figure 2.13. The obtained peak was prominent peak. Similar kind of observation was seen with the same isolate with respect to silver nanoparticles as mentioned earlier in the chapter and the synthesis was achieved within 10 hours of incubation time.

![UV-Visible spectrum of gold nanoparticles mediated by Strain AS 41G](image)

**Figure 2.13: UV-Visible spectrum of gold nanoparticles mediated by Strain AS 41G**

Bioreduction of gold chloroaurate into gold nanoparticles was achieved with strain CA 417 as shown in figure 2.14 with maximum absorption occurring between 500 to 600 nm, conferring the formation of gold nanoparticles. The obtained peak was sharp and prominent. The synthesis was achieved within 10 hours of incubation time.
Figure 2.14: UV-Visible spectrum of gold nanoparticles mediated by
Strain CA 417

Synthesis of gold nanoparticles using strain EH 419 as shown in figure 2.15 with maximum absorption between 500 to 550 nm which confirmed the formation of gold nanoparticles. The peak obtained was broad with low intensity and the synthesis of nanoparticles was achieved within 36 hours of incubation time.

Figure 2.15: UV-Visible spectrum of gold nanoparticles mediated by
Strain EH 419
Gold nanoparticles were synthesized using strain MB 13 as shown in (Figure 2.16) wherein maximum absorption was observed between 500 to 600 nm confirming the nanoparticles synthesis the obtained peak was of low intensity and the reaction was completed within 32 hours, further synthesis was not observed.

![Figure 2.16: UV-Visible spectrum of gold nanoparticles mediated by Strain MB 13](image)

Similarly, strain MB 141 mediated gold nanoparticles synthesis as shown in figure 2.17 with maximum absorption occurring between 500 to 600 nm, conferring the formation of gold nanoparticles. The obtained peak was sharp and prominent. The synthesis was achieved within 10 hours of incubation time.

![Figure 2.17: UV-Visible spectrum of gold nanoparticles mediated by Strain MB 141](image)
Gold nanoparticles was synthesized using strain MB 149 as shown in the figure 2.18 with the maximum absorption occurring at around 550 nm confirming the nanoparticles formation. The figure displayed small low intensity peak and synthesis of nanoparticles was achieved within 26 hours of incubation time.

![Figure 2.18: UV-Visible spectrum of gold nanoparticles mediated by Strain MB 149](image)

Gold nanoparticles were synthesized from strain TP 416 as shown in the figure 2.19 with maximum absorption between 450 to 600 nm confirming the formation of gold nanoparticles. The peak appears to be like broad hump and the synthesis was achieved within 30 hours of incubation time.

![Figure 2.19: UV-Visible spectrum of gold nanoparticles mediated by Strain TP 416](image)
c. Optimization parameters for synthesis of nanoparticles

The primary and secondary screening results claimed completion of nanoparticle synthesis with 48 to 50 hours of incubation which was the critical variable. Hence to minimize the time, further optimization study was carried out in order to achieve rapid synthesis of silver and gold nanoparticles. When different parameters such as concentration of metal salts, pH and temperature were varied resulted in reduction of time from hours to minutes. Each isolate had different time intervals as shown in the table 2.2.

Table 2.2: Incubation time required for selected endophytic bacteria for synthesis of nanoparticles

<table>
<thead>
<tr>
<th>Endophytic bacteria</th>
<th>Plant name</th>
<th>Incubation time</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Initiation Completion</td>
</tr>
<tr>
<td>AS 41 G</td>
<td><em>Annona squamosa</em> L.</td>
<td>7 minutes 18 to 20 minutes</td>
</tr>
<tr>
<td>CA 417</td>
<td><em>Coffea arabica</em> L.</td>
<td>9 minutes 20 to 25 minutes</td>
</tr>
<tr>
<td>EH 419</td>
<td><em>Euphorbia hirta</em> L.</td>
<td>15 minutes 30 to 40 minutes</td>
</tr>
<tr>
<td>MB 13</td>
<td><em>Mimosa pudica</em> L.</td>
<td>30 minutes 60 to 80 minutes</td>
</tr>
<tr>
<td>MB 141</td>
<td><em>Mimosa pudica</em> L.</td>
<td>9 minutes 20 to 30 minutes</td>
</tr>
<tr>
<td>MB 149</td>
<td><em>Mimosa pudica</em> L.</td>
<td>20 minutes 40 to 50 minutes</td>
</tr>
<tr>
<td>TP 416</td>
<td><em>Tridax procumbens</em> L.</td>
<td>29 minutes 50 to 60 minutes</td>
</tr>
</tbody>
</table>

Note: The initiation time refers to the time where the reaction mixture tends to change the color of the solution. Similarly the completion of the time refers to the time beyond which there was no further change in color of the reaction mixture.

Effect of variables on the reduction of metal salts and the formation of stable nanoparticles greatly varied and influenced with change in parameters (Table 2.3 a&b). In the present study it was observed that elevated temperature influenced the synthesis of nanoparticles with the temperature above 50° C was optimal for six endophytic bacteria except MB 13 which reduced the metal salts at 40° C. Similarly, four endophytic bacteria showed nanoparticles synthesis at pH ranging from 7-9 except MB 149 and MB 13 showed the reduction of metal salts at pH 5-7. The concentration of the metal salts resulted in maximum synthesis of nanoparticles both silver and gold was attributed at 1 mM concentration. When concentration of
metal salts versus supernatant was evaluated, it was observed that at ratio 7:3 (metal salts: supernatant) was optimal for synthesis of nanoparticles.

Table 2.3(a): Influence of different parameters on synthesis of nanoparticles from endophytic bacteria

<table>
<thead>
<tr>
<th>Endophytic Bacteria</th>
<th>Temperature</th>
<th>pH</th>
<th>Concentration of metal salts</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10-40°C</td>
<td>50-70°C</td>
<td>70-90°C</td>
</tr>
<tr>
<td>AS 41 G</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>CA 417</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>EH 419</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>MB 13</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>MB 141</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>MB 149</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>TP 416</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

Note: “+” indicates the synthesis of nanoparticles within 30 minutes. “-” indicates no synthesis occurred within 30 minutes of incubation time.

Table 2.3(b): Ratio of metal salts with supernatant secreted from endophytic bacteria

<table>
<thead>
<tr>
<th>Endophytic bacteria</th>
<th>Metal Salts: Supernatant Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>9:1</td>
</tr>
<tr>
<td>AS 41 G</td>
<td>-</td>
</tr>
<tr>
<td>CA 417</td>
<td>-</td>
</tr>
<tr>
<td>EH 419</td>
<td>-</td>
</tr>
<tr>
<td>MB 13</td>
<td>+</td>
</tr>
<tr>
<td>MB 141</td>
<td>-</td>
</tr>
<tr>
<td>MB 149</td>
<td>-</td>
</tr>
<tr>
<td>TP 416</td>
<td>-</td>
</tr>
</tbody>
</table>

Note: “+” indicates the synthesis of nanoparticles. “-” indicates no synthesis

Among the selected endophytic bacteria, AS 41 isolate was exemplified for describing the influence of variables on the synthesis of nanoparticles. The reason for highlighting this isolate is based on its rapid synthesis compared to other isolates.
When concentration of metal salt silver nitrate was varied, it was observed that synthesis was maximum at 1 mM (Figure 2.20) followed by 0.5 mM and there was no significant change with 1.5 mM and 2 mM. The result was analyzed based on the maximum absorption recorded at 410 nm using UV-Visible spectrophotometer.

![Graph showing concentration of metal salts influencing synthesis of silver nanoparticles](image1)

**Figure 2.20: Different concentration of metal salts influencing synthesis of silver nanoparticles**

When influence of pH on nanoparticle synthesis was studied resulted in maximum synthesis was observed at alkaline pH 8 followed by pH 9 and 7 whereas no synthesis was observed with pH 5 and 6 (Figure 2.21). This result indicated that the alkaline pH was influencing the nanoparticle synthesis based on the absorption recorded at 410 nm at UV-Visible spectrophotometer.

![Graph showing pH influencing synthesis of nanoparticles](image2)

**Figure 2.21: Different pH influencing synthesis of nanoparticles**
With the temperature of the reaction mixture ranging from 10 to 90°C showed maximum synthesis between 70 to 80°C followed by 50 to 60°C. Whereas, no synthesis was observed with temperature 10 to 20°C (Figure 2.22) this result indicated that elevated temperature influenced the synthesis of nanoparticles. All the absorption was recorded at 410 nm at UV-Visible spectrophotometer.

![Graph showing the relationship between temperature and optical density](image)

**Figure 2.22: Different temperature influencing synthesis of nanoparticles**

Based on the results obtained, three endophytic bacteria AS 41 G, CA 417 and MB 141 showed sharp, prominent peaks with UV-Visible spectra, also capable of synthesizing nanoparticles within 30 minutes of incubation time as shown in the table 2.2. Which were further selected for molecular characterization and large scale synthesis of nanoparticles.

d. **Screening of endophytes for antimicrobial activity**

The isolates obtained expressed different morphological characteristic, the preliminary screening of antimicrobial activity by dual culture method inhibited the growth of test fungal pathogen viz., *Fusarium verticilloides*, *Aspergillus niger* and *Aspergillus flavus* with perpendicular streak of isolate across the test pathogen (Figure 2.23). Agar overlay method for bacteria displayed clear zone in the lawn of test pathogenic bacteria laid over point inoculated endophytic isolate expressing the antimicrobial activity (Figure 2.23). Preliminary screening of endophytic bacteria for antimicrobial activity resulted in isolation of twelve endophytic bacteria among the total endophyte isolated but when these bioactive endophytic bacteria were cultured to
obtain crude extract only four endophytic bacteria were capable of showing activity. With two isolates showing activity against only Gram-positive pathogens and two isolates showed activity against all the test pathogens. Hence these two isolates bear a unique feature with its catabolic pathways and were capable of synthesizing nanoparticles and capable of secreting antimicrobial metabolites which were subjected to molecular characterization and selected for future experiments.

![Figure 2.23: Antimicrobial activity of bacterial endophytes against test pathogens by agar overlay and dual culture techniques](image)

**e. Molecular characterization of bioactive endophytes**

The molecular characterization of bioactive endophytic strains was carried out by using set of universal primers as described in materials and method. The molecular characterization results revealed that endophytic bacteria PCR amplification of 16S rRNA were able to produce an amplicon which is measured about ~1300-1500 base pairs (~1.3-1.5 kb) as depicted in figure 2.24. The obtained 16S rRNA gene sequence was matched at NCBI using BLAST tool to reveal its maximum similarity. Acquisition of AS 41G sequences was 99% homologous to gene sequence of *Pseudomonas veronii* strain CIP 104663. The strain CA 417 showed 99% homologous to gene sequence of *Pseudomonas fluorescens* strain BCPBMS. Whereas strain MB 141 showed 99% homologous to the *Aeurinibacillus migulanus* strain 2012 BaDB 21. All the homologous was carried out with the database available online at NCBI site. Based on the results acquired from molecular characterization, each
strain was designated with generic and species nomination, the sequences of all three strains were deposited at Genbank and received accession numbers as cited in the table 2.4.

![Figure 2.24: Agarose Gel showing genomic DNA of bioactive endophytic bacteria](image)

Table 2.4: Designated name and accession number for bioactive isolates showing positive for nanoparticles synthesis

<table>
<thead>
<tr>
<th>Isolate number</th>
<th>Designated name</th>
<th>Accession number</th>
</tr>
</thead>
<tbody>
<tr>
<td>AS 41G</td>
<td><em>Pseudomonas veronii</em> strain AS 41G</td>
<td>KC 480604</td>
</tr>
<tr>
<td>CA 417</td>
<td><em>Pseudomonas fluorescens</em> strain CA 417</td>
<td>KC 480603</td>
</tr>
<tr>
<td>MB 141</td>
<td><em>Aneurinibacillus migulanus</em> strain 141</td>
<td>KF 606762</td>
</tr>
</tbody>
</table>

f. Phylogenetic relation of bioactive endophytes with other bacteria

Phylogram was constructed to reveal the relationship of identified *Pseudomonas veronii* strain AS 41G with taxonomically similar bacteria based on the 16S rRNA gene sequences. The phylogenetic tree was constructed using ClustalW software by grouping the isolates deposited at GenBank as represented in figure 2.25.
Figure 2.25: Phylogram expressing the relationships of *Pseudomonas veronii* strain AS 41G to taxonomically similar bacteria based on the 16S rRNA gene sequences

Phylogram was constructed to reveal the relationships of identified *Pseudomonas fluorescens* strain CA 417 with taxonomically similar bacteria based on the 16S rRNA gene sequences. The phylogenetic tree was constructed with ClustalW software by grouping the isolates deposited at GenBank as represented in figure 2.26.

Figure 2.26: Phylogram expressing the relationships of *Pseudomonas fluorescens* strain CA 417 to taxonomically similar bacteria based on the 16S rRNA gene sequences

Phylogram was constructed to reveal the relationships of identified *Aneurinibacillus migulanus* strain 141 to taxonomically similar bacteria based on the 16S rRNA gene sequences. The phylogenetic tree was constructed with Clustalw software by grouping the isolates deposited at GenBank as represented in figure 2.27.
Figure 2.27: Phylogram expressing the relationships of *Aneurinibacillus migulanus* strain 141 to taxonomically similar bacteria based on the 16S rRNA gene sequences

- *Aneurinibacillus migulanus* strain NBRC 15520 (NR113764)
- *Aneurinibacillus migulanus* strain DSM 2895 (NR112214)
- *Aneurinibacillus migulanus* strain G1 JQ337949
- *Aneurinibacillus migulanus* strain 2012BaDB21 (JX04916)
- *Aneurinibacillus migulanus* strain 141* (KF606762)
- *Aneurinibacillus migulanus* strain B0270 (NR036799)
- *Aneurinibacillus migulanus* strain A72 (GU397386)
- *Aneurinibacillus sp.* AT8 (FJ821593)

**g.** GenBank submission of three bioactive endophytic bacteria at NCBI

**i.** *Pseudomonas veronii* strain AS 41G

![GenBank image](image-url)
ii. *Pseudomonas fluorescens* strain CA 417
iii. Aneurinibacillus migulanus strain 141

5. DISCUSSION

The results obtained in the present investigation attributed the assumption of endophytes being one of the rich sources of functional bioactive metabolites in nature. The results showed isolation of endophytic bacteria from viz., *Annona squamosa* L., *Coffea arabica* L., *Euphorbia hirta* L., *Mimosa pudica* L. and *Tridax procumbens* L. During surface sterilization protocol, use of 3.5% sodium hypochlorite resulted in elimination of surface microbial flora. Majority of earlier reports suggests that sodium hypochlorite is one of the widely used disinfectants. As the aim of the study is to isolate endophytic bacteria, antifungal agents such as cycloheximide and bavistin were employed this suppressed the fungal endophytes based on the previously reported literatures (Webster *et al*., 2001 and Zin *et al*., 2010).
Selection of plants for isolation of endophytic bacteria was carried out based on their availability and previous reports on bacterial endophytes. Very scanty reports were available on the selected plant species and majority of these reports were on fungal endophytes in the survey made as far.

According Lin et al., (2011), Xylarialean A45 was isolated from Annona squamosa L., which represented cytotoxic activity. Similarly another study reports the isolation of Xylaria A23 which was capable of secreting new class of Cytochalasin H2 compound with cytotoxic activity (Li et al., 2012). Further no reports were available on endophytic bacteria from Annona squamosa L. Which prompted us to select this plant and attempt was made in isolation of endophytic bacteria resulting in isolation of eighty four isolates with myriad morphological characteristics (Baker and Satish, 2013).

Earlier study on Coffea arabica L. revealed isolation of eighty seven endophytic bacteria which belong to nineteen different genera. The study also reports the isolation of higher Gram-negative (68%) bacteria compared to Gram-positive (Vega et al., 2005). These findings were in accordance with the results obtained in present investigation with higher number of Gram-negative bacteria compared to Gram-positive bacteria.

Similar study on Coffea arabica L. resulted in isolation of forty bacterial endophytes from surfaced sterilized leaves and branches. Among these isolates twenty three were tested for the biocontrol activity in coffee plants (Shiomi et al., 2006). These results correlate with the findings of present investigation with thirty two endophytic bacteria were isolated from surface sterilized stem and leaves segments. When Mimosa pudica L. was cited for earlier reports on endophytic bacteria, only Burkholderia sp. has been reported as endophyte from root nodules of Mimosa pudica L. (Pandey et al., 2005) no other reports on endophytes have been so far reported to best of our knowledge. Due to the scanty reports on this plant which made us the subject of interest in the present investigation resulting in isolation of one forty one endophytic bacteria from surface sterilized stem and roots (Baker and Satish, 2013c).

Endophytic bacteria were isolated from surface sterilized leaves and stems of Tridax procumbens L. resulting in isolation of fifty culturable endophytes belonging
to different genera, among which Bacillus species was more predominant compared to other bacterial species such as Cronobacter sakazakii, Enterobacter spp., Lysinibacillus sphaericus, Pantoea, Pseudomonas and Terribacillus saccharophilus (Praveena and Bhore, 2013). Similar findings were observed in the present investigation with twenty-nine culturable endophytes have been isolated from the Tridax procumbens.

However, only a single literature pertaining to Tridax procumbens in this case also factors such as seasonal and restriction to geographical area may account for scanty reports on endophytes. Moreover in most of the cases this plant has been recognized as a weed leading to less interest. When reports on Euphorbia hirta L. was surveyed, no reports were obtained to best of our knowledge. Even in this same reason may be implied such as seasonal, geographical area and most of the cases it might be considered as a weed as it is majorly habitats with the unwanted weeds. To best of our survey there were scanty reports available pertaining to endophytic bacteria towards synthesizing silver, gold and bimetallic nanoparticles. There are quite large numbers of reports on fungal endophytes mediates nanoparticles synthesis from different plant species synthesizing silver, gold and bimetallic nanoparticles (Shankar et al., 2003; Musarrat et al., 2010; Raheman et al., 2012; Devi and Joshi, 2014). This might be due to the fact that fungal endophytes have emerged as one of the rich source of metabolites bearing various biological activities hence the researchers are concentrating much on fungal endophytes for the synthesis of nanoparticles. It can be noteworthy here that endophytic bacteria inhabits in large and diverse communities compared to fungal endophytes. There are quite large numbers of reports on fungal endophytes synthesizing silver, gold and bimetallic nanoparticles (Shankar et al., 2003; Musarrat et al., 2010; Raheman et al., 2012; Devi and Joshi, 2014).
various biological activities hence the researchers are concentrating much on fungal endophytes for the synthesis of nanoparticles. In the present study an attempt has been made for the synthesis of nanoparticles using endophytic bacteria isolated from five different medicinal plants and the results are promising enough to report five endophytic bacteria towards synthesizing silver and gold nanoparticles.

To best of our knowledge and survey there are no reports as such available for primary screening of endophytes for the synthesis of nanoparticles. Hence an attempt was made for the first time to screen potent endophytic bacteria for nanoparticle synthesis by incorporating the metal salts into the media and incubated, which resulted in successful screening of endophytic bacteria for synthesis of silver and gold nanoparticles. In the present investigation much effort were devoted to achieve synthesis within shorter time intervals as compared to conventional methods.

During the secondary screening the cell free supernatant was evaluated for reduction of metal salts into nanoparticles. The experiments were designed and executed based on the earlier report (Prakash et al., 2010 and Qian et al., 2013). The reduction of ions into nanoparticles resulted in colour change which served as an initial tool for indicating synthesis of nanoparticles as the synthesis starts initiating the colour of the reaction mixtures tends to change its colour from the colour of supernatant to brown in colour for silver nanoparticles and pink or ruby red in colour for the gold nanoparticles. This is due to the surface plasmon resonance band of the nanoparticles as per the literatures reported (Kathiresan et al., 2009; Zonooz and Salouti, 2011 & Qian et al., 2013).

One such study documented the screening of *Lactobacillus* strains from milk products and their evaluation for synthesis of silver nanoparticles by using cell free supernatant during which 9 isolates were obtained and only one isolate was capable of showing positive result for synthesis of nanoparticles (Ranganath et al., 2012). These results justifies with findings of the present study with only seven isolates capable of synthesizing both silver and gold nanoparticles with secondary screening. These results clearly suggest that any microbe to synthesize nanoparticle depend on their physiological and biochemical mechanisms which is not present in all the organisms (Reith et al., 2007 and Benzerara et al., 2010).
In a recent study screening of isolates was carried out based on the nitrate reductase activity, wherein 12 plant growth promoting bacteria and 4 actinomycetes were isolated from Philippine soils sixteen and tested for the presence of nitrate reductase activity. This study is based on the fact that nitrate reductase enzymes are responsible in the bioreduction. The study was carried out for the synthesis of gold nanoparticles and the result obtained showed ten plant growth promoting bacteria could exhibit nitrate reductase activity after 5 days of incubation at 37°C whereas no activity was conferred with four tested actinomycetes (Fernando et al., 2013).

In most of the earlier reports of microbial route of nanoparticle synthesis, majority of the studies display prolong time for synthesis forming one of the major drawbacks. In the present study it was also witnessed that most of the isolates were capable of synthesizing nanoparticles after two to three days of incubation time which was not feasible. Hence the study focused and devoted to the isolates which could synthesize with shorter time interval. In order to reduce the time interval, different variables were optimized such as temperature, pH, concentration of the metal salts and concentration of supernatant.

These optimization studies revealed that the elevated temperature and alkaline pH influenced the nanoparticle synthesis this might be due to the fact that sufficient activating energy is obtained to reduce the metal salt, similarly dielectric constant is achieved resulting in high ionic strength which enhance the synthesis process. These results correlates with the earlier findings which reports elevated temperature at 100°C and alkaline pH 10 influenced the synthesis of silver nanoparticles by cell free supernatant of *Pseudomonas aeruginosa* and the study also reports the stabilization of silver nanoparticles by capping around the nanoparticles (Oza et al., 2012).

Screening of isolates bearing antimicrobial activity can be achieved with different screening protocols but majority of literatures report the use of agar over lay assay with point inoculation of isolates which screens the bioactive isolates. In the present investigation only 12 bacterial endophytes could show positive results against test organisms. The results obtained in the present investigation is in accordance with the findings of Ramazani et al., 2013 which reports the screening of only 8.7% of
Streptomyces genus isolates bearing activity among the total isolates screened for antimicrobial activity.

Similarly indigenous endophytic bacteria belonging to Pseudomonas, Bacillus, Enterobacter, Klebsiella, Acetobacter, Burkholderia, Rhizobium and Xanthomonas were isolated from soybean (Glycine max (L.) Merril.) by Dalal and Kulkarni, (2013). Endophytic bacteria were screened in vitro for the antagonistic activity against soil-borne fungal pathogens of soybean viz., Rhizoctonia solani, Fusarium oxysporum, Sclerotium rolfsii, Collectotruchum truncatum, Macrophomina phaseolina and Alternaria alternata. Five isolates JDB 3 (Pseudomonas sp.), JDB 5 (Pseudomonas sp.), JDB 9 (Bacillus sp.), JDB 11 (Bacillus sp.) and JDB 14 (Bacillus sp.) were found to considered as efficient antagonists against the above mentioned pathogens. These results correlated with preliminary screening outcome in which endophytic bacterial isolates belonging to genera Bacillus and Pseudomonas were exhibited antagonistic activity against Aspergillus and Fusarium species by dual culture technique.

The bioactive strains exhibiting synthesis of nanoparticles and antimicrobial activity were subjected to genotypic and phenotypic characterization. Identification can be carried out using different techniques and most of the techniques are biochemical based identification and characterization of bacteria which are time consuming and laborious. Since then advent of rapid molecular biology techniques have provided accurate identification of large number of bacteria within short span of time. Identification, classification and to perform taxonomic studies of prokaryotes especially bacteria involve gene sequence analysis of small subunit ribosomal RNA (16S rRNA). Genes of rRNA are transcribed from the ribosomal Operon as 30S rRNA precursor molecules and then cleaved by RNase III into 16S, 23S, and 5S rRNA molecules in bacteria. The ribosomal operon size, nucleotide sequences and secondary structures of the three rRNA genes are conserved within a bacterial species (Maidak et al., 1997).

Nine “hyper variable regions” within bacterial 16S rRNA genes demonstrate considerable sequence diversity among different bacterial species and can be used for species identification (Van de Peer et al., 1996). PCR amplification of target sequences using universal primers facilitated by hyper variable regions are flanked by
conserved stretches in most bacteria (Baker et al., 2003 and Munson et al., 2004). 16S rRNA hyper variable region sequences analyses from various earlier studies were identify a single bacterial species or differentiate among a limited number of different species or genera (Becker et al., 2004 and Maynard et al., 2005)

One of the major functions of 16S rRNA is binding with the Shine-Dalgarno sequences of mRNA during translation and thus the anti-SD sequence is one of the most significant and conserved features in 16S rRNA genes (Gunasekaran and Rajendhran, 2011). Since 16S rRNA is the most conserved of these three rRNAs, it has been proposed as an “evolutionary clock”, which has led to the reconstruction of the tree of life (Woese, 1987). For the past two decades, microbiologists have primarily relied on 16S rRNA gene sequences for the identification and classification of bacteria. The 16S rRNA sequence analysis is used in two major applications, Identification and classification of isolated pure cultures and Estimation of bacterial diversity in environmental samples without culturing through metagenomic approaches (Gunasekaran and Rajendhran, 2011).

Present molecular taxonomic studies on endophytic bacteria using PCR amplification of 16S rRNA were able to produce an amplicon which is measured about ~1300-1500 base pairs (~1.3-1.5 kb) as depicted in figure 2.24. This result was in consistence with the earlier reports by Minaxi and Saxena, (2010) for molecular characterization of rhizobacteria Pseudomonas aeruginosa RM-3 biocontrol agent against Macrophomina phaseolina and Dreschlera gramineae.

Similarly, the above molecular characterization results for Aneurinibacillus spp., which is reported in the present study is correlated with previous literature by Mandepudi et al., (2013).