CHAPTER 3
MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 Chemicals Used
All the chemicals used in the present study including Bromophenol blue, EDTA, PIPES buffer (Hi-Media Pvt. Ltd. India); 2, 4-dinitrophenylhydrazine, ACC, Basic fuchsin, Ethidium bromide, HDTMA (Sigma Aldrich); Aniline, Molybdenum oxide, Sodium thiosulphate (RANKEM); PCR buffer, Tetrazolium salt (ROCHE); Bromothymol blue, Kovac’s reagent, Sulfanilic acid, Tetramethyl-para-phenylenediamine dihydrochloride (BDH) and Primers 16SF Universal 5’-AGAGTTTGTCCTGGCTCAG-3’ and 16SR Universal 3’-ACGGCTACCTTGTTACGACCT-5’, Taq-DNA polymerase (Merck bioscience) and Napthol solution, Potassium dichromate, Sodium citrate, Starch (CDH) etc. were of AR/GR grade.

3.1.2 Instruments used
Various instruments used during the present investigation include: Autoclave, Incubator, Orbital Shaking cum BOD Incubator, (NSW, India); Electronic Balance (Afcoset, India); Hot plate, Inoculation Loop, Magnetic stirrer (KFW Scientific Industries); Shaker, Vortex shaker (Perfit, India); ABI 3730xl Genetic Analyzer (Applied Biosystems); Bunsen burner (R. K. Singh & Sons); Deep freezer (Blue Star); Digital photo colourimeter (Countronics); Electrophoretic-unit (Genei); Laminar Air Flow (Rescholar Equipment); Laminar Air Flow (Double R. Optics & Scientific Works); Micropipette (Rankem); Microscope (Olympus); Microwave Oven (Onida); Hot air oven, pH meter (Popular, India); Refrigerator (LG); Research Centrifuge (Remi); Sonicator (Hielscher, Germany); UV-VIS Spectrophotometer Double beam with PC (Systronics); Thermocycler (Lark); UV-transilluminator (National Analytical Corporation, India).

3.1.3 Cultures Used
All the cultures used in the present investigation were isolated from soil samples. The culture of fungus *Fusarium oxysporum* MTCC 3656 was obtained from Institute of Microbial Technology (IMTECH), Chandigarh, India.
3.2 METHODS
A systematic study was undertaken to isolate native strains of rhizobacteria from *Ocimum* sp. of different localities of Delhi, Haridwar and Kurukshetra. Details of the various methods/protocols followed for conducting the experiments are as follows:

3.2.1 Plant growth promoting rhizospheric bacteria from *Ocimum* rhizosphere
3.2.1.1 Collection of Soil Sample
Soil samples were collected from the rhizospheric soil *Ocimum* sp. plants from different localities of Kurukshetra, Delhi and Haridwar. The intact soil of the roots was collected carefully in sterile plastic bags and stored at 4 °C in the laboratory till their further use.

**Kurukshetra**, the *dharamkshetra*, district of Haryana state in India extends from latitude 29°-52’ to 30°-12’ and longitude 76°-26’ to 77°-04’. Saraswati, Markanda and Ghaggar are the main rivers of the district. The climate of Kurukshetra is very hot in summer and very cold in winter. It has maximum temperature of 45 °C in summer and minimum temperature of 3 °C in winter.

**Delhi**, the capital city of India, spreads over an area of 1483 km². The latitudinal and longitudinal location of Delhi are 23.38 °N and 77.13 °E. Delhi experiences tropical steppe type of climate and hence its seasons are marked with extreme temperatures. Temperature reaches almost 45 °C in the summer months and winters are quite cold.

**Haridwar** (also known as *Mayapuri, Kapila, Mokshadwar, and Gangadwar* and finds mention in various ancient Hindu epics) district in Uttarakhand state of India spreads over an area of 2360 km². It extends from Shivalik Hills in the North and Ganges in the South. Temperature ranges from 25 °C to 44 °C during summers and between -2 °C to 24 °C during winters.

**Table 4. Collection sites for soil samples**

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Place</th>
<th>No. of Soil Samples collected</th>
<th>Isolates obtained</th>
</tr>
</thead>
<tbody>
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<td>1</td>
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<td>30</td>
</tr>
<tr>
<td>2</td>
<td>Delhi</td>
<td>12</td>
<td>69</td>
</tr>
<tr>
<td>3</td>
<td>Haridwar</td>
<td>10</td>
<td>167</td>
</tr>
</tbody>
</table>
3.2.1.2 Isolation of Plant Growth Promoting Rhizobacteria
About 10 g of rhizospheric soil was taken into a 250 ml of conical flask and 90 ml of sterile distilled water was added to it. The flask was shaken for 10 min on a rotary shaker. One ml of suspension was added to 10 ml vial and serial dilution technique was performed up to $10^{-7}$ dilution. About 0.1 ml of suspension was spread on the plates of respective media (Nutrient agar medium, King’s B medium, Pikovaskaya medium, Ashby medium and YEMA medium). Plates were incubated at 30 °C for 3 days to observe the colonies of bacteria. Bacterial colonies were further streaked on the respective media plates to get their pure cultures (Ashrafuzzaman et al., 2009).

3.2.1.3 Morphological and biochemical characterization
All the isolates obtained were morphologically and biochemically characterized. Different colony characteristics such as shape, colour, size, etc. were noted. Also, various biochemical tests such as indole test, catalase test, oxidase test, citrate utilization, gelatin liquefaction, etc. were performed.

3.2.2 Screening of the PGPR for various desirable properties

3.2.2.1 Ammonia Production
Bacterial isolates were tested for the production of ammonia in peptone water. Isolates were inoculated in 10 ml peptone water and incubated for 48 to 72 h at 36 °C. After incubation, 0.5 ml Nessler’s reagent was added in each tube. Development of brown to yellow colour indicated ammonia production (Cappuccino and Sherman, 2010).

3.2.2.2 Phosphate Solubilization
The plates were prepared with Pikovaskaya’s medium. All the isolates were tested for their ability to solubilize phosphate using Pikovaskaya’s medium. The culture was inoculated on the Pikovskaya medium plates and incubated at temperature 28 °C for 7 days. Formation of clear zone around bacterial growth was taken as a positive test (Pikovaskaya et al., 1948).

3.2.2.3 Hydrogen Cyanide Production
Isolates were tested for their ability to produce hydrogen cyanide (Lorck, 1948). The culture was streaked on nutrient media plates amended with 4.4 g glycine/L. The
Whatman filter paper No. 1 was soaked in a solution of Sodium carbonate (2%, v/v) in 0.5% picric acid. The filter paper was placed on top of the plate and parafilm was used for sealing the plates. The plates were incubated at 36 °C for 4 days. Following incubation, filter paper was observed for the colour change. Brownish colour indicated good production of HCN and reddish brown indicated strong HCN production.

3.2.2.4 Siderophore Production
The production of siderophore by the isolated bacterial cultures was tested by plate assay. The tertiary complex formed by bacterial isolates was assessed by the method of Schwyn and Neilands (1987). Deferration was the first step which included removal of the iron that could contaminate the glassware. Due to the good ion exchange property of glass, it could easily get contaminated with iron. Hence it was necessary to remove the contaminating iron. This was done by soaking the glassware in 2 N HCl solution, followed by washing with distilled water. About 1 L CAS medium was prepared by mixing 60.5 mg dehydrated Chromo Azurol S in 50 ml water to 10 ml iron solution (1 mM FeCl$_3$.6H$_2$O in 10 mM HCl). This mixture was then slowly added to 40 ml aqueous solution of 72.9 mg hexadecyl trimethyl ammonium bromide and the mixture was stirred continuously. To prepare agar medium, 30.2 g PIPES was added to 18 g agar (pH 6.8). All the contents are then autoclaved. After cooling, CAS dye was added slowly along the glass wall. Poured media in the plates and allowed to solidify. The bacterial culture was streaked on CAS plates and incubated at 28 ºC for 7 days. Plates were observed for the formation of yellow to orange coloured clear zone around the bacterial growth.

3.2.2.4.1 Nature of siderophore
The nature of siderophore produced by the isolate was determined by following tests:

(i) **Hydroxamate nature** (tetrazolium salt test)
This was done by adding tetrazolium salt to siderophore sample under alkaline conditions. Development of deep red colour indicated positive test (Snow, 1954).

(ii) **Carboxylate nature** (Vogel’s chemical test)
Phenolphthalein was added to the sample of siderophore and observed for the disappearance of pink colour (Vogel, 1987).
3.2.2.5 Antifungal Activity
The culture of fungus *Fusarium oxysporum* was spot-inoculated on the centre of the previously plated sterilized modified PDA medium. The bacterial isolate was streaked onto the opposite side of the petri plate. The plates were incubated at 28 °C for 7 days. Following incubation, plates were observed for the inhibition of the fungal growth and results were recorded (Tiwari *et al.*, 2010). The inhibition level was calculated by subtracting the distance (mm) of fungal growth in the direction of an bacterial isolate from the fungal radius. The percent inhibition was calculated as follows:

\[
\text{% inhibition} = \frac{(R - r)}{R} \times 100
\]

where ‘r’ is radial growth of the fungal colony opposite the bacterial growth and,

R is the radial growth of the fungus in the control plate.

3.2.2.6 IAA Production
IAA production of indole acetic acid by the bacterial isolates was assessed colorimetrically (Okon *et al.*, 1977). Isolates were grown in Luria-Bertani (LB) medium amended with 100 mg/L tryptophan and incubated at 28 °C for 7 days at 250 rpm. After incubation, cultures were centrifuged and supernatants collected. IAA was estimated colorimetrically by mixing 2 ml supernatant with 4 ml of Salkowski reagent (1 ml of 0.5M FeCl₃ in 50 ml of 35% HClO₄) and kept for 30 min. Further, it was observed for the development of pink colour. The absorbance was taken at 535 nm.

3.2.2.7 ACC Deaminase Activity
The isolates were checked for ACC-deaminase activity qualitatively by using salt minimal medium (Dworkin and Foster, 1958). The activity of the enzyme was assayed quantitatively by the modified methods of Honma and Shimomura (1978) and Penrose and Glick (2003). The amount of α-ketobutyrate produced on cleavage of ACC by the enzyme was measured by comparing the absorbance of sample at 540 nm with the α-ketobutyrate standard curve. The 100 mM α-ketobutyrate stock solution was prepared in 0.1 M Tris-HCl (pH 8.5) and diluted to prepare 10 mM solution for generating standard concentration curve. About 200 μl of known α-ketobutyrate concentration was mixed with 300 μl of 2, 4- dinitro-phenylhydrazine reagent (0.2% 2, 4-dinitrophenylhydrazine in 2 M HCl) and incubated at 30 °C for 30 min. This denaturizes α-ketobutyrate as a
phenylhydrazone. Added 2 ml of 2 M NaOH to this solution and absorbance was measured at 540 nm after development of colour of phenylhydrazone.

For measuring ACC-deaminase activity in bacterial extracts, 1 ml of 0.1 M Tris-HCl, pH 7.6, was used for suspending the bacterial cell pellets, centrifuged at 13,500 rpm for 5 min and removed the supernatant. Suspended pellet in 600 μl of 0.1 M Tris-HCl (pH 8.5) and added 300 μl of toluene. Vortexed the contents for 30 seconds and assayed for ACC-deaminase activity. About 200 μl toluenized cells were mixed with 20 μl of 0.5 M ACC, vortexed and incubated at 30 °C for 15 min. Added 1 ml of 0.56 M HCl to the mixture, vortexed and centrifuged for 5 min at 13,500 rpm. About 1 ml supernatant was taken and mixed with 800 μl of 0.56 M HCl and vortexed. To this solution, added 300 μl of the 2, 4- dinitro- phenylhydrazine reagent (0.2 % 2, 4-dinitrophenylhydrazine in 2 M HCl) and incubated at 30 °C for 30 min. Added 2 ml of 2 M NaOH, and the absorbance was measured at 540 nm. Enzyme activity was expressed as μ mol α-ketobutarate /mg protein/h.

3.2.2.7.1 Protein concentrations determination: The protein concentration of the toluenized cells was determined according to the method of Lowry (1951). About 26.5 μl of toluenized cell sample was diluted with 173.5 μl of 0.1 M Tris-HCl (pH 8.0). The contents were boiled with 200 μl of 0.1 N NaOH for 10 min. Cooled the solution and added 200 μl of Lowry’s reagent. Recorded the absorbance at 660 nm. The standard curve for protein concentration was determined using Bovine serum albumin (BSA).

3.2.2.8 Heavy Metal Tolerance

The selected bacterial strains were tested for their resistance to heavy metals by agar dilution method (Cervantes et al., 1986). Freshly prepared nutrient agar plates amended with various soluble heavy metal salts namely Ni (nickel II sulfate; NiSO₄), Hg (mercuric II chloride; HgCl₂), Co (Cobalt II nitrate; CO(NO₃)₂), Cd (Cadmium chloride; CdCl₂), Cu (copper II sulphate; CuSO₄), Pb (lead acetate; Pb(C₂H₃O₂)₂), Zn (Zinc sulphate; ZnSO₄) and Cr (Potassium dichromate; K₂Cr₂O₇) at concentrations 25 μg/ml, 100 μg/ml and 400 μg/ml were inoculated with overnight grown cultures. Heavy metal tolerance was indicated by appearance of bacterial growth after incubating the plates at 35 °C for 24 - 48 h.
3.2.3 Statistical Analyses

Statistical analysis of all tests was carried out using SPSS 16.0 design. The tests were performed in triplicate. Data was reported as mean ± standard deviation (SD) and was analyzed with standard error at 0.5% significance.

3.2.4 Optimization of cultural conditions for improvement of properties exhibited by PGPR

3.2.4.1 Effect of temperature on growth

The bacterial isolates were streaked on nutrient media plates and incubated at 10 °C, 20 °C, 28 °C, 37 °C and 45 °C. Plates were observed for the bacterial growth after 3 days of incubation.

3.2.4.2 Growth of isolates with different pH conditions

The bacterial isolates were inoculated in the media adjusted with the respective pH i.e. 4, 5, 6, 6.5, 7, 7.5 8, 9 and incubated at 35 °C for 3 days. Plates were observed for the bacterial growth.

3.2.4.3 Growth of isolates with different carbon sources

The bacterial isolates were inoculated in the media of different carbon sources i.e. glucose, fructose, sucrose, lactose, maltose, starch, mannitol, citrate, succinate and incubated at 35 °C for 3 days. Plates were observed for the bacterial growth.

3.2.5 Characterization of PGPR Isolates

3.2.5.1 Morphological Characterization of selected isolates

Selected isolates were examined for the colony morphology. Different morphological characteristics such as colony size, colour, shape, margin, pigmentation, elevation etc., were recorded for the chosen PGPR isolates.

3.2.5.2 Phenotypic Characterization

3.2.5.2.1 Gram staining

Thin smear of the bacterial isolate was made on clean glass slide, air dried and heat fixed the smear. Covered the smear with crystal violet for 1 min. and washed with tap water. Gently flooded the smear with Gram’s iodine solution for 1 min. and washed with tap
water. Decolorized the slide with 95% ethyl alcohol. Washed the slide with tap water and counter stained with Safranin for 45 seconds. Gently washed the slide with tap water, blot dried and air dried. Examined under oil-immersion (Cappuccino and Sherman, 2010).

3.2.5.2.2 Endospore staining
Thin smear of the bacterial isolate was made on clean glass slide, air dried and heat fixed the smear. Flooded the smear with malachite green and placed on top of a beaker of water sitting on a warm hot-plate, heated the slide to steaming. Steamed for 2 to 3 min. and washed with tap water. Counter-stained with Safranin for 30 seconds. Washed with tap water, blot dried and examined under oil-immersion objective (Cappuccino and Sherman, 2010).

3.2.5.2.3 Flagella staining
Cleaned slides by boiling in chromic acid solution. Preparation of smear: Added 2 drops of distilled water to the culture and incubated for 20 min. Took a drop of suspension, added on a slide kept in slanting position and air dried the smear. The slide was treated with Loeffler’s mordant and heated till steam appears approximately 3 min. Gently washed the slide by tap water. Further slide was treated with Carbol fuchsin stain and heated till steam appears. Finally slide was tap washed with water following which slide was air dried and observed under oil immersion (Cappuccino and Sherman, 2014).

3.2.5.2.4 Motility test
Inoculated SIM agar deep tubes with the isolate by means of stab inoculation and incubated the tubes at 37 °C for 48 h. Observed the tubes for the presence of turbidity (Cappuccino and Sherman, 2010).

3.2.5.3 Biochemical Characterization
The biochemical characterization of selected PGPR isolates was done according to the methods outlined by Cappuccino and Sherman (2010). The media composition for the biochemical tests are listed in Appendix. The tests conducted are as follows:
3.2.5.3.1 Catalase activity
About few drops of H$_2$O$_2$ (3%) was added to the cultures and observed for the effervescence.

3.2.5.3.2 Gelatin liquefaction
The isolates were inoculated to the pre-sterilized nutrient gelatin deep tubes and incubated at 28 °C for 24 h. Following incubation, tubes were kept at 4 °C in a refrigerator for 30 min. Liquefaction in the gelatin deep tubes indicated positive test.

3.2.5.3.3 IMViC tests
3.2.5.3.3.1 Indole production
Pre-sterilized tryptone broth tubes were inoculated with the bacterial isolates and incubated at 28 °C for 48 h. After incubation, 10 drops of Kovac’s reagent were added to each tube. Tubes were observed for the appearance of cherry red color. This indicated positive test for production of indole.

3.2.5.3.3.2 MRVP production
Pre-sterilized MRVP broth tubes were inoculated with the bacterial isolates and incubated at 28 °C for 24-48 h. After incubation, added methyl red indicator to one tube for MR test and VP reagents to the second tube for VP test. Appearance of red to yellow colour indicated positive methyl red (MR) test and development of crimson to ruby pink colour was taken as positive VP test.

3.2.5.3.3.3 Citrate utilization test
Pre-sterilized Simmon’s citrate agar slants were inoculated with the isolates and incubated at 28 °C for 48 h. Change in colour from green to blue indicated positive test for citrate production.

3.2.5.3.4 Hydrogen sulfide production
Pre-sterilized TSI agar tubes were inoculated with the isolates and incubated at 28 °C for 48 h. Blackening of the culture medium indicated positive test for H$_2$S production.
3.2.5.3.5 Oxidase test
The filter paper was soaked in 1% tetra-methyl-p-phenylenediamine dihydrochloride solution and the bacterial culture was rubbed on this filter paper. Development of dark purple colour indicated positive oxidase test.

3.2.5.3.6 Carbohydrate fermentation test
Pre-sterilized TSI slants were inoculated with the isolates and incubated at 35 °C for 48 h. After incubation, tubes were observed for change in color. The tubes were observed for changes in colour. Alkaline slant (red) and acid butt (yellow) indicated glucose fermentation; acid slant (yellow) and acid butt (yellow) indicated lactose and/or sucrose fermentation; alkaline slant (red) and acid butt (red) or no change in colour indicated negative test for carbohydrate fermentation.

3.2.5.3.7 Nitrate reduction
Pre-sterilized nitrate broth tubes were inoculated with the isolates and incubated at 30 °C. Following incubation, added nitrite reagent A (Sulfanilic acid) and reagent B (Naphthylamine) to the tubes and observed for appearance of red colour. If colour does not change, added a pinch of powdered zinc. Development of pink colour indicated negative test for nitrate reduction.

3.2.5.4 Molecular Characterization
3.2.5.4.1 Genomic DNA Extraction (Biopure™ Kits)
About 1 ml of bacterial culture was taken in centrifuge tubes and centrifuged for 5 min. at 10,000 rpm. About 100 μl of Buffer-A was added to the pellet. The samples were incubated at 37 °C for 30 min. Then, 20 μl of Buffer- B1 and 10 μl of Buffer -B2 were added and incubated at 65 °C for 30 min. following which, 250 μl of Buffer -B3 was added and vortexed at low speed for 30 seconds. Again centrifuged at 10,000 rpm for 10 min. The clear supernatants were pipetted out without disturbing the pellets into the Spin columns. To each of the Spin columns, 250 μl of Buffer -PC was added and centrifuged at 12,000 rpm for 1 min. The flow through was collected in the collection tube and discarded. About 250 μl of Buffer-C was added in the column and centrifuged at 12,000 rpm for 1 min. The flow through collected in collection tube was discarded and the columns were air dried. After this, 50 μl of Buffer -D was added and centrifuged at
10,000 rpm for 1 min. The flow through containing the genomic DNA was collected and preserved at 4 °C.

3.2.5.4.2 Agarose Gel Electrophoresis
About 40 ml of 0.8% Agarose gel was prepared by dissolving 0.32 g of Agarose in 40 ml of TAE buffer. The solution was kept in microwave oven at power level 800 W for 2 min. for proper dissolving and to get a clear transparent solution. The agarose solution was allowed to cool at room temperature and 5 μl of Ethidium Bromide was dissolved. The Gel casting tray, Chamber and combs were wiped and cleaned with 70% Ethanol. The boundaries of the tray were sealed with cello tape carefully. The Agarose gel was poured into the tray, comb was placed properly and the gel was allowed to solidify for about 20-30 min. After solidification, the comb and tape were removed carefully. The loading samples were prepared by mixing 10 μl of the extracted DNA and 5 μl of loading dye. The samples were loaded in the corresponding wells made by removing the comb. The gel was allowed to run for 1 h at 100 volts. The DNA bands were observed under U.V. Transilluminator.

3.2.5.4.3 Amplification of 16S rDNA gene
The primers used for the amplification of 16s rDNA gene were 16SF Universal 5’-AGAGTTTGA TCCTGGCTCAG-3’ and 16SR Universal 3’-ACGGCTACCTTG TTACGA CTT-5’.

**PCR reaction:**

<table>
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<th>Profile</th>
<th>Step</th>
<th>Temperature</th>
<th>Duration (min)</th>
<th>No. of cycles</th>
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<td>6</td>
<td>Hold temperature</td>
<td>4 °C</td>
<td>Forever</td>
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</tr>
</tbody>
</table>
3.2.5.4.3.1 Materials required
Genomic DNA/Template DNA = 2 μl (sample); Two primers *i.e.* forward primer = 2 μl and Reverse primer = 2 μl; PCR buffer = 4 μl; DNTPs = 4 μl; Taq-DNA polymerase enzyme = 0.2 μl; MgCl₂ = 2 μl; Nuclease free water for making volume upto 25 μl.

Protocol:

- **Pre-denaturation:** 94 °C for 5 min. (tightly coiled complementary double helical strands of DNA get unwinded and other small ions or particles attached to DNA are degraded/removed).
- **Denaturation:** 94 °C for 1 min. (two strands of the genomic DNA get denatured in which the Hydrogen bonds between them is broken down exposing the two separate DNA templates).
- **Annealing:** 55 °C for 1 min. (wherein the forward primer and reverse primer will bind to the complementary sequences present on both strands of template DNA. These primers help in the synthesis of the new strands by using DNA-polymerase enzyme).
- **Extension/Elongation:** 72 °C for 2 min. In this reaction a new strand of complementary strands is synthesized by Taq- DNA polymerase enzyme by utilizing dNTPs present in the sample. The steps of Denaturation, Annealing and Extension are run for 30 cycles to yield enough amplicons that can be subjected for sequencing.
- **Final Extension:** 72 °C for 10 min (step proof reading takes place wherein any mis match/mis pairing are repaired).
- **Soak temperature/preservation temperature:** set at 4 °C (due to which the amplicons remain safe at an optimum temperature of 4 °C until next use).
- The amplicons were run on 1% agarose gel electrophoresis as a qualitative check. The amplicons were purified by washing with 1 M sodium acetate and 70% of ethanol and subjected for sequencing.
3.2.5.4.4 Separation of amplified products by agarose gel electrophoresis

**Materials Required:** Electrophoretic unit: Gel casting trough, gel combs, power-pack and UV Transilluminator; Agarose (1.2%); Bromophenol blue; Ethidium bromide (0.5 μg/ml); 50 x TAE (stock): Tris – free base (60.5 g), Glacial acetic acid (14.25 ml), 0.5 M EDTA (25 ml) and make up the volume to 250 ml (pH 8.0); Working solution (1 x TAE): 20 ml of 50 x TAE was made upto 1000 ml by using distilled water.

**Protocol:**
About 1 g of agarose was weighed and added to a 250 ml conical flask containing 100 ml of 1 x TAE buffer. The agarose was melted by heating the solution in a micro oven and the solution was stirred to ensure even mixing and complete dissolution of agarose. The solution was then cooled to about 50 ºC. Two to three drops of ethidium bromide (0.5 μg/ml) was added. The solution was mixed and poured into the gel casting plat form after inserting the comb in the trough. While pouring sufficient care was taken for not allowing the air bubbles to trap in the gel. The gel was allowed to solidify and the comb was removed after placing the solidified gel into the electrophoretic apparatus containing sufficient buffer (1 x TAE) so as to cover the wells completely. The amplified products (20 μl) to be analysed were carefully loaded into the sample wells, after adding bromophenol blue with the help of micropipette. Electrophoresis was carried out at 60 volts until the tracking dye migrated to the end of the gel. The DNA bands stained with Ethidium bromide were viewed under UV-transilluminator and photographed for documentation.

3.2.5.4.5 Sequencing
Forward and reverse DNA sequencing reaction of PCR amplicons was carried out on ABI 3730xl Genetic Analyzer by Sanger’s Method to obtain the sequence.

3.2.5.4.6 Similarity Search by BLAST
The obtained sequences were subjected to similarity search by BLAST with the nrdatabase of NCBI to find out the source names. The obtained sequence was input in the 1st box of the BLAST work page and BLAST was run at default parameters. The subject microbe whose sequence shows maximum identity with the input sequence is considered to be the source microbe of the input sequence.
3.2.5.4.7 Phylogenetic Analysis by CLUSTAL W

For this 10-11 sequences that were most identical to the query sequence were retrieved from BLAST in Fasta format and submitted on Workbench. Multiple Sequence Alignment was performed on Clustal W to generate the rooted tree as dendrogram. The organisms on the same branch are considered to be closely related while those on the farther branches are supposed to be distinctly related in evolution.