Chapter - III
Materials and Methods
3. MATERIALS & METHODS

3.1. Collection of seaweeds

Since the aim of present study was concerned with isolation and characterization of bacteria associated with chosen species of some seaweeds found along the coast of Thondi, Ramanathapuram District, Tamilnadu, India, a portion of healthy, matured and fresh seaweeds was collected from the coast of Thondi (Lat: 9º 44’ 10” N and Long: 79º 00’ 45” E, Palk Bay) (Figure.1 & 2), Ramanathapuram district, Tamilnadu, India. The collected samples were transferred in to clean screw cap plastic bottles which contain sterilized sea water and the same were immediately brought to the laboratory of department of Zoology, Raja Doraisingam Government Arts College, Sivagangai for further study. The seaweeds were identified with the help of CMFRI bulletin No.41. And then, the collected seaweeds were rinsed thrice with sterilized seawater to remove external particles and epiphytes.

List of Seaweed species collected for the present study

The following seaweeds were collected from the coast of Thondi, Ramanathapuram district, Tamilnadu, India for the present study: Red seaweeds; 1. Jania rubens (Linnaeus) Lamouroux and 2. Gracilaria corticata (J.Agardh), Brown seaweeds; 1. Dictyota dichotoma (Hudson) Lamouroux, and 2. Chnoosphora implexa (Herring) J.Agardh and Green seaweeds; 1. Enteromorpha intestinalis (Linnaeus) Nees and 2. Caulerpa racemosa (Forsskal) J.Agardh (Figure.3).

3.2. Isolation of endophytic bacteria associated with collected seaweeds

Seaweed was thoroughly washed thrice with sterile sea water. One gram of seaweed sample was homogenized in sterilized glass mortar and pestle and the extract was made up to 10ml by adding sterilized sea water. Then, the extract was centrifuged
at 6000 rpm for 15 minutes. The supernatant was taken and made up to 10 ml by using sterile sea water and serially diluted with the sterile sea water up to 10^6 dilutions. Each dilution was spread on the labelled petriplates containing 15 ml of autoclaved marine agar medium (ZoBell medium). The plates having the supernatant dilutions were kept for incubation at 37°C for 24 hrs or until the colonies were observed (Nakanishi and Nishijima, 1996). Pure bacterial colonies were obtained through repeated streaking and single bacterial colony was isolated. After 24 hrs at 37°C, different colonial morphologies of bacteria were chosen and purified by successive re-streaking. The pure bacterial cultures obtained were maintained on Nutrient Agar slants.

### 3.3. Isolation of epiphytic bacteria associated with collected seaweeds

Seaweed was thoroughly washed thrice with sterile sea water to remove the epiphytes. A sterile cotton swab was used to rub the seaweed surface and the removed bacteria present in the cotton swab were inoculated on marine agar and marine broth (Himedia) isolation plates. The inoculated plates were incubated for 24 hrs at 37°C and until colonies were observed. After 24 hrs at 37°C, different colonial morphologies of bacteria were chosen and purified by successive re-streaking. The pure bacterial cultures obtained were maintained on Nutrient Agar slants (Takizawa et al., 1993; Ravel et al., 1998).

### Preparation of culture media

The general culture media described below were prepared using the routine methods and used in appropriate experiments:
Composition of Marine Agar Medium (ZoBell Marine Agar-Himedia, Mumbai, India.)

1. Peptic digest of animal tissue - 5.000g
2. Yeast extract - 1.000g
3. Ferric Citrate - 0.100g
4. Sodium Chloride - 19.450g
5. Magnesium Chloride - 8.80g
6. Sodium Sulphate - 3.240g
7. Calcium Chloride - 1.800g
8. Potassium Chloride - 0.550g
9. Sodium bicarbonate - 0.160g
10. Potassium bromide - 0.080g
11. Strontium Chloride - 0.034g
12. Boric acid - 0.022g
13. Sodium silicate - 0.004g
14. Sodium fluorate - 0.0024g
15. Ammonium nitrate - 0.0016g
16. Disodium phosphate - 0.0080g
17. Agar - 15g
18. pH - 7.6 ± 0.2

Preparation of the medium

55.25 grams of ZoBell Marine Agar were suspended in 1000 ml of distilled water and boiled to dissolve the medium completely. Then, it was sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes.
**Nutrient Agar Medium (NA Medium)**

1. Peptone    -0.5g
2. Beef extract   -0.3g
3. Yeast extract   -0.3g
4. Sodium chloride   -0.5g
5. Agar   -20.g
6. pH  -7
7. Distilled water  -100ml

**Preparation of the medium**

Nutrient agar medium was prepared according to the manufacturing company. It was used for general experiments, cultivation and activation of bacterial isolates when it is necessary (MacFaddin, 2000).

**3.4. Cultural characterization of bacterial colony**

After the incubation period of inoculated petriplates, the bacterial colonies cultured in the plates were observed for the following characterizations: Size (The size of the colonies cultured on the inoculated petriplates such as pinpoint, small, moderate and large were observed), Pigmentation (Colour of the colonies was observed), Form (The shape of the colony was observed; the shapes were described as followed; Circular: Unbroken peripheral edge. Irregular: Indented peripheral edge. Rhizoid: Root like, spreading growth), Margin (The appearance of outer edge of the colony was observed. The margin was described as follows; Entire: Sharply defined, even; Lobate: Marked indentations; Undulate: Wavy indentations; Serrate: Tooth like appearance; Filamentous: Thread like spreading edge) and Elevation (The elevation of the growth of the colony was observed. The degree to which colony growth is raised on the agar surface is described as follows; Flat: elevation not discernible; Raised: slightly elevated; Convex: Dome-shaped elevation; Umbonate: Raised with elevated convex central region).
3.5. Microscopical characterization

1. Motility Test

Motility was performed by the hanging drop method. With a cotton swab, a ring of petroleum jelly was applied around the concavity of the depression slide. Using sterile technique, a loopful of the culture was placed in the centre of a clean cover slip. The depression slide was placed, with the concave surface facing down, over the cover slip so that the depression covers the drop of culture. The slide was gently pressed to form a seal between the slide and the cover slip. The slide was quickly turned right side up so that the drop continues to adhere to the inner surface of the cover slip. The drop of culture was first focused under the low power of objective (10X) and then observed under high power objective (40X).

2. Gram staining

Bacterial smear was prepared on a clean micro slide. The smear was primary stained with crystal violet (Hucker’s) for 1 minute. The smear was gently washed with tap water. The smear was gently flooded with the Gram’s iodine mordant for 1 minute. Then, the smear was gently washed with tap water and decolourized with 95% ethyl alcohol. The smear was gently washed with tap water. Finally the smear was counter stained with safranin for 45 seconds. Then, the smear was washed with tap water. The smear was blot dried with bibulous paper and examined under oil immersion.

3. Shape

After over the Gram’s staining, the smear was observed under microscope (40x) for the shape of the bacteria. The shape was observed for short rod, straight rod, slightly curved, ovoid, rod chains or Cocci.
Reagents used for Gram staining

Crystal violet (Primary stain)

Solution A

- Crystal violet (96%) dye content - 2g
- Ethanol - 20ml

Solution B

- Ammonium oxalate - 0.8g
- Distilled water - 80ml

Gram’s iodine (Mordant)

- Iodine - 1.0g
- Potassium iodide - 2.0g
- Distilled water - 300ml

Ethyl alcohol (Decolourizer)

- Ethyl alcohol (100%) - 95%
- Distilled water - 5ml

Safranin (counter stain)

- 2.5% of safranin in 95% ethyl alcohol - 10ml
- Distilled water - 1000ml

3.6. Biochemical studies

Biochemical characterizations such as Catalase test, ONPG test, Lysine Utilization test, Ornithine Utilization test, Urease, Phenyl alanine Deamination test, Nitrate reduction test, H₂S production test, Citrate test, Voges-Proskauer’s test, Methyl red test, Indole test, Malonate test, Esculin Hydrolysis test, Arabinose test, Xylose test, Adonitol test, Rhamnose test, cellobiose test, Melibiose test, Saccharose test, Raffinose test, Trehalose test, Glucose test, Lactose test and Oxidase test.
Biochemical Identification Kit

Commercially available systems reduce the need for preparing a variety of test media and reagents and the time required for interpretation of results, thereby making the identification of various bacterial species more plausible in the routine laboratory. Himedia Rapid Biochemical Identification kit, *Enterobacteriaceae* Identification Kit [KB003 Hi25®] was used in the present study as KB003 is the comprehensive test system used for identification of gram negative *Enterobacteriaceae* species. Himedia provides a range of Biochemical Identification test kit (KB001 to KB012) involving single step procedure of inoculation which leads to final identification of test organism being studied. Each Biochemical Identification test kit is a standardized colorimetric identification system utilizing conventional biochemical tests and carbohydrate utilization tests. The tests are based on the principle of pH change and substrate utilization. On incubation organisms undergo metabolic changes which are indicated by a colour change in the media that is either interpreted visually or after addition of a reagent.

**Preparation of the reagents and solutions**

**Catalase reagent**

This reagent was used at a concentration (3%) using H₂O₂ in Distilled water and stored in a dark container. It was used for identification of Catalase producing bacteria (Forbes *et al.*, 2007).

**Readymade reagents (Himedia /India)**

These reagents were brought with the rapid identification system kits:

1. NaOH (40%): Sodium hydroxide was used in alkaline phosphatase production test to detect the ability of organism to produce sufficient phosphatase enzyme.
2. TDA reagent: It was used in phenylalanine deamination test to detect phenylalanine deamination activity by bacteria.

3. Nitrate reduction reagent: It was used to detect nitrate reduction. This reagent was composed of two reagents, A and B as follows: Reagent A: sulphanilic acid. Reagent B: N-dimethyl-naphthylamine.

4. Vogus-Proskauer’s reagent: It was used in Voges-Proskauer’s test to detect acetoin production. This reagent was composed of two reagents, Baritt reagent A and Baritt reagent B.

5. Methyl red reagent: It was used in methyl red test to detect acid production.

6. Kovac's reagent: It was used in indole test to detect deamination of tryptophan.

7. Oxidase disc: It was used in oxidase test to detect oxidase positive bacteria.

**Muller- Hinton agar medium**

- Beef infusion from - 300g
- Casein acid hydrolysate - 17.50g
- Starch - 1.50g
- Agar - 17.00g
- Distilled water - 1000ml
- pH - 7.3 ± 0.1

Muller- Hinton agar was prepared according to the manufacturing company. It was used in anti-bacterial activity testing (Mac Faddin, 2000).

**Brain Heart Infusion (BHI) broth**

Brain heart infusion (BHI) broth was prepared according to the manufacturing company. It was used in biochemical testing.
Brain Heart Infusion (BHI) broth–glycerol medium

This medium was prepared by mixing 5 ml of glycerol with 95 ml of BHI broth (sterilized by autoclave). It was used for preservation of bacterial isolates as stock for long time (Forbes et al., 2007).

Biochemical Characterization

Preparation of Inoculum

Single well isolated colony was picked up and inoculated in 5 ml Brain Heart Infusion broth and incubated at 37°C for 4-6 hours until the inoculum turbidity is ≥ 0.10D at 620nm. Kit was opened aseptically and sealing tape was peeled off. Each well was inoculated with 50 µl of the above inoculums by surface inoculation method and kept for incubation at 35- 37°C for 18-24 hours. At the end of the incubation period, a series of reagents were added in designated wells as per manufacturer’s specifications to carry out different biochemical tests.(Collee, J.G., et al., 1996, Macfaddin, J.F., 2000, Murray, P.R., et al., 1999).

For the ultimate characterization of isolated strains the following biochemical tests were performed that were being discussed as below:

1. Catalase test

A few drops of 3% hydrogen peroxide (H₂O₂) were added over the colonies of fresh culture of isolated bacteria. Culture tubes were observed for the formation of nascent oxygen in the form of air bubbles. This indicated the positive for Catalase production.

2. ONPG test

O-nitrophenyl-β-D-galactopyranoside had been fixed in the well of readymade biochemical test kit. This well was inoculated with 50 µl of the above inoculums by surface inoculation method and kept for incubation at 35- 37°C for 18-24 hours. In the
presence of β-galactosidase, ONPG was cleaved into galactose and O-nitrophenol, a yellow compound. At the end of the incubation period, development of yellow colour was noted. Development of a yellow colour indicated positive result.

3. Lysine Utilization test

The medium of this test contained Bromocresol purple as pH indicator. When carbohydrate present in the medium was utilized, pH was lowered due to acid production changing the colour of medium to yellow. The acid produced stimulated decarboxylase enzyme. The formation of lysine (positive reaction) due to this reaction increased the pH of the medium, changing the colour of the indicator from olive green to purple. Negative reaction was indicated by development of yellow colour.

4. Ornithine Utilization test

The medium of this test also contained Bromocresol purple as pH indicator. When carbohydrate present in the medium was utilized, pH was lowered due to acid production changing the colour of medium to yellow. The acid produced stimulated decarboxylase enzyme. The formation of Ornithine (positive reaction) due to this reaction increased the pH of the medium, changing the colour of the indicator from olive green to purple. Negative reaction was indicated by development of yellow colour.

5. Urease test

This test detected the ability of an organism to ammonia by the action of enzyme urease. The medium contained phenol red indicator so that the medium turned pink under alkaline conditions. No change in colour indicated negative reaction.
6. Phenyl alanine Deamination test

This test detected the ability of an organism to oxidatively deaminate phenylalanine with production of phenylpyruvic acid which reacted with ferric salts to give a green colour. On addition of TDA reagent (R036) to the medium after incubation, appearance of green colour indicated a positive reaction. No change in colour indicated negative reaction. The results were interpreted within 5 minutes after addition of reagent as the green colour fades quickly.

7. Nitrate reduction test

Gram-negative bacilli vary in their ability to reduce nitrates. Members of Enterobacteriaceae characteristically reduce nitrate to nitrite which reacts with sulphanilic acid (R015) and N, N-dimethyl-1-napthylamine reagent (R009) to produce pinkish red colour. Appearance of pinkish red colour indicated the positive reaction. Negative reaction was indicated by colourless appearance.

8. H₂S production test

All members of Enterobacteriaceae are capable of producing various amounts of H₂S. Microorganisms are capable of enzymatically liberating sulphur-containing amino acids or inorganic sulphur compounds. The hydrogen sulphide released reacted with ferric ions or lead acetate to yield ferrous sulphide or lead sulphide, which were insoluble black precipitates. Blackening of medium indicated positive reaction.

9. Citrate test

The medium contained sodium citrate as sole source of carbon, Bromothymol blue as indicator and inorganic ammonium salts. The organism that was capable of utilizing citrate as its sole source of carbon also utilized the ammonium salts as source of nitrogen. Ammonium salts were broken down to ammonia (NH₃) with resulting alkalinity. The indicator therefore turned blue from yellowish green indicating alkaline condition.
10. Voges-Proskauer’s test

Some organisms had the ability to produce a neutral end product acetyl methyl carbinol (acetoin) from glucose utilization. This could be detected by addition of 1-2 drops of Barritt Reagent A (R029) and 1-2 drops of Barritt Reagent B (R030). A positive result was indicated by pinkish red colour within 2-5 minutes. No change in colour indicated negative result.

11. Methyl red test

The methyl red test was based on the use of a pH indicator, methyl red to determine the hydrogen ion concentration (pH) when an organism utilized glucose. On addition of one drop of methyl red indicator (I007) at the end of the period of incubation, the colour changed to distinct red. It indicated positive test whereas if the reagent turned yellow or yellowish orange, it indicated negative result.

12. Indole test

This test demonstrated the ability of certain bacteria to decompose the amino acid tryptophane to indole which accumulated in the medium. On addition of Kovac’s Indole reagent (R008), development of reddish pink colour within 10 seconds indicated positive result.

13. Malonate test

Malonate test medium contained Bromothymol blue as indicator. Sodium malonate was the carbon source and ammonium sulphate was the nitrogen source. Organism, which was able to utilize malonate, released sodium hydroxide. The resulting alkaline conditions caused by the indicator to change from light green to blue. Malonate -negative organisms did not cause any change in the colour of the medium.
14. Esculin Hydrolysis test

Esculin was substituted by glucoside that could be hydrolysed by certain bacteria to yield glucose and esculetin. The latter combined with ferric ions in the medium to form a black coloured complex.

15. Arabinose test

Specific carbohydrate (Arabinose) had been added to basal medium which contained phenol red as indicator. On fermentation of Arabinose, acid was liberated which lowered down the pH of the medium and this change of colour was indicated by pH indicator dye. Positive test was indicated by colour change to yellow due to acid reaction. No change in colour or red/pink colour indicated negative reaction.

16. Xylose test

Specific carbohydrate (Xylose) had been added to basal medium which contained phenol red as indicator. On fermentation of Xylose, acid was liberated which lowered down the pH of the medium and this change of colour was indicated by pH indicator dye. Positive test was indicated by colour change to yellow due to acid reaction. No change in colour or red/pink colour indicated negative reaction.

17. Adonitol test

Specific carbohydrate (Adonitol) had been added to basal medium which contained phenol red as indicator. On fermentation of Adonitol, acid was liberated which lowered down the pH of the medium and this change of colour was indicated by pH indicator dye. Positive test was indicated by colour change to yellow due to acid reaction. No change in colour or red/pink colour indicated negative reaction.

18. Rhamnose test

Specific carbohydrate (Rhamnose) had been added to basal medium which contained phenol red as indicator. On fermentation of Rhamnose, acid was liberated
which lowered down the pH of the medium and this change of colour was indicated by pH indicator dye. Positive test was indicated by colour change to yellow due to acid reaction. No change in colour or red/pink colour indicated negative reaction.

19. **Cellobiose test**

Specific carbohydrate (Cellobiose) had been added to basal medium which contained phenol red as indicator. On fermentation of Cellobiose, acid was liberated which lowered down the pH of the medium and this change of colour was indicated by pH indicator dye. Positive test was indicated by colour change to yellow due to acid reaction. No change in colour or red/pink colour indicated negative reaction.

20. **Melibiose test**

Specific carbohydrate (Melibiose) had been added to basal medium which contained phenol red as indicator. On fermentation of Melibiose, acid was liberated which lowered down the pH of the medium and this change of colour was indicated by pH indicator dye. Positive test was indicated by colour change to yellow due to acid reaction. No change in colour or red/pink colour indicated negative reaction.

21. **Saccharose test**

Specific carbohydrate (Saccharose) had been added to basal medium which contained phenol red as indicator. On fermentation of Saccharose, acid was liberated which lowered down the pH of the medium and this change of colour was indicated by pH indicator dye. Positive test was indicated by colour change to yellow due to acid reaction. No change in colour or red/pink colour indicated negative reaction.

22. **Raffinose test**

Specific carbohydrate (Raffinose) had been added to basal medium which contained phenol red as indicator. On fermentation of Raffinose, acid was liberated which lowered down the pH of the medium and this change of colour was indicated
by pH indicator dye. Positive test was indicated by colour change to yellow due to acid reaction. No change in colour or red/pink colour indicated negative reaction.

23. Trehalose test

Specific carbohydrate (Trehalose) had been added to basal medium which contained phenol red as indicator. On fermentation of Trehalose, acid was liberated which lowered down the pH of the medium and this change of colour was indicated by pH indicator dye. Positive test was indicated by colour change to yellow due to acid reaction. No change in colour or red/pink colour indicated negative reaction.

24. Glucose test

Specific carbohydrate (Glucose) had been added to basal medium which contained phenol red as indicator. On fermentation of Glucose, acid was liberated which lowered down the pH of the medium and this change of colour was indicated by pH indicator dye. Positive test was indicated by colour change to yellow due to acid reaction. No change in colour or red/pink colour indicated negative reaction.

25. Lactose test

Specific carbohydrate (Lactose) had been added to basal medium which contained phenol red as indicator. On fermentation of Lactose, acid was liberated which lowered down the pH of the medium and this change of colour was indicated by pH indicator dye. Positive test was indicated by colour change to yellow due to acid reaction. No change in colour or red/pink colour indicated negative reaction.

26. Oxidase test

Oxidase test was performed on organism to be tested to differentiate *Enterobacteriaceae* from other gram negative rods using the Oxidase disc (tetramethyl paraphenylene diamino dihydrochloride) provided with the kit. The test was performed using oxidase disc (DD018) provided with the kit. A well isolated colony
was picked up and rubbed on a single oxidase disc. Positive reaction was indicated by
development of deep purple colour within 10 seconds. Colour in 10-60 seconds
indicated a delayed positive reaction. Colour development after 60 seconds or no
change in colour indicated a negative reaction. The result was recorded.

3.7. Antimicrobial activity test

Principle

Antimicrobial activity testing of bacterial and fungal isolates is a common and
important technique in most clinical laboratories. The results of these tests are used
for selection of the most appropriate antimicrobial agent(s) for treatment against the
infectious organisms. Till the 1950s, laboratories were lacking in the methodologies
and equipments for the accurate determination of in vitro responses of organisms to
antimicrobial agents. Bauer et al., began the development of standardized methods for
antimicrobial activity testing, using disc diffusion system. However the activity
results may not always correlate with the patient's response to therapy. The response
of an infected patient to antimicrobial agent(s) is a complex interrelationship of host
responses, drug dynamics and microbial activity. Antimicrobial activity tests are
either quantitative or qualitative. Disc diffusion test is a qualitative test method. The
National Committee for Clinical Laboratory Standards (NCCLS), now known as
Clinical Laboratory Standards Institute (CLSI) has published comprehensive
documents regarding the disc diffusion systems. The agar disc diffusion test is the
most convenient and widely used method for routine antimicrobial activity testing. In
subsequent and current practice, antimicrobial impregnated paper discs are applied
onto the agar surface. Based on the Bauer-Kirby Method (1966), standardized
reference procedures for the disc systems were published by WHO and FDA and are
periodically updated by the CLSI (formerly NCCLS).
3.7.1. Antifungal activity Test

For antifungal activity test, three fungi such as Fusarium oxysporum, Rhizoctonia solani and Pyricularia oryzae were selected. These fungal strains were obtained from Tamilnadu Agricultural University, Coimbatore. The bacterial isolates were tested against these three fungi for antifungal activity.

Preparation of Inoculum

1. Inoculum was prepared by picking five distinct fungal colonies of approximately 1mm from 24 hours old culture grown on Sabouraud Dextrose Agar (M063) and incubated at 35 ± 2º C. Colonies were suspended in 5ml of sterile 0.85% saline.

2. Inoculum of bacterial isolates of present study were also prepared by picking five distinct colonies of approximately 1mm from 24 hours old culture grown on Sabouraud Dextrose Agar (M063) and incubated at 35 ± 2º C. Colonies were suspended in 5ml of sterile 0.85% saline.

3. The resulting suspensions of both were adjusted the turbidity to yield 1 x 10⁶ - 5 x 10⁶ cells /ml (i.e. 0.5 McFarland standards).

Test Procedure

1. Plates with Muller Hinton Agar + 2% Glucose were prepared. The medium in the plates was sterile and had a depth of about 4 mm.

2. A sterile non-toxic cotton swab on a wooden applicator was dipped into the standardized inoculum of selected fungus and rotated the soaked swab firmly against the upper inside wall of the tube to express excess fluid. The entire agar surface of the plate was streaked with the swab three times, turning the plate at 60º angle between each streaking. The inoculum was allowed to dry for 5 - 15 minutes with lid in place.
3. The empty sterile discs were dipped into the standardized inoculum of bacterial isolates of present study and applied using aseptic technique. The discs were deposited in the centre at least 24 mm apart.

4. The plates were inverted and placed in the incubator at 35 ± 2°C within 15 minutes after the discs were applied.

5. Each plate was examined after 20 - 24 hours of incubation. When insufficient growth is observed after 24 hours incubation, the plates were read after 48 hrs of incubation. The zone of inhibition was measured in mm (diameter).

3.7.2. Antibacterial activity test

For antibacterial activity test, three pathogenic bacteria of poultry such as *Eicheria coli*, *Pasteurella multocida* and *Salmonella pullorum* and three pathogenic bacteria of cattle such as *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Eicheria coli*, were selected. These strains were obtained from Veterinary College, Namakkal, Tamilnadu, India. The bacterial isolates were tested against these six pathogenic bacteria for their antibacterial activity.

**Preparation of Inoculum**

1. Inoculum was prepared by picking five distinct colonies of selected pathogenic bacteria of poultry as well as cattle separately of approximately 1mm from 24 hours old culture grown on Sabouraud Dextrose Agar (M063) and incubated at 35 ± 2°C. Colonies were suspended in 5ml of sterile 0.85% saline.

2. Inoculum of bacterial isolates of present study were also prepared by picking five distinct colonies of approximately 1mm from 24 hours old culture grown on Sabouraud Dextrose Agar (M063) and incubated at 35 ± 2°C. Colonies were suspended in 5ml of sterile 0.85% saline.

3. The resulting suspensions of both were adjusted the turbidity to yield 1 x 10⁶ - 5 x 10⁶ cells /ml (i.e. 0.5 McFarland standards).
Test Procedure

1. The Plates with Muller Hinton Agar + 2% Glucose were prepared. The medium in the plates was sterile and had a depth of about 4 mm.

2. A sterile non-toxic cotton swab on a wooden applicator was dipped into the standardized inoculum of selected bacterium of poultry as well as cattle and rotated the soaked swab firmly against the upper inside wall of the tube to express excess fluid. The entire agar surface of the plate was streaked with the swab three times, turning the plate at 60° angle between each streaking. The inoculum was allowed to dry for 5 - 15 minutes with lid in place.

3. The empty sterile discs were dipped into the standardized inoculums of bacterial isolates of present study and applied using aseptic technique. The discs were deposited in the centre at least 24 mm apart.

4. The plates were inverted and placed in the incubator at 35 ± 2°C within 15 minutes after the discs were applied.

5. Each plate was examined after 20 - 24 hours of incubation. When insufficient growth is observed after 24 hours incubation, the plates were read after 48 hrs of incubation. The zone of inhibition was measured in mm (diameter).

3.8. Antibiotic susceptibility test

For testing the antibiotic susceptibility of the bacterial isolates of present study, five antibiotics such as Amikacin-30mcg, Gentamycin-10mcg, Cefoperozone-75mcg, Cefpodoxime-10mcg and Ceftazidine-30mcg (HiMedia, Mumbai-India) were used. The bacterial isolates of present study were tested against these five antibiotics for antibiotic susceptibility test.
**Test procedure**

1. The plates with Mueller-Hinton Agar (M173/M1084) were prepared for rapidly growing aerobic organisms as per Bauer- Kirby Method (1966). The medium in the plates was sterile and had a depth of about 4 mm.

2. Four or five colonies of bacterial isolates of present study were inoculated with a wire loop to 5ml of Tryptone Soya Broth (M011) and incubated at 35-37°C for 2-8 hours until light to moderate turbidity developed. The inoculum turbidity was compared with that of standard 0.5 McFarland (prepared by mixing 0.5ml of 1.175% barium chloride and 99.5 ml of 0.36N sulphuric acid).

3. A sterile non-toxic cotton swab on a wooden applicator was dipped into the standardized inoculum and rotated the soaked swab firmly against the upper inside wall of the tube to express excess fluid. The entire agar surface of the plate was streaked with the swab three times, turning the plate at 60° angle between each streaking. The inoculum was allowed to dry for 5-15 minutes with lid in place.

4. The discs were applied aseptically using sterile forceps.

5. The discs were deposited with centres at least 24 mm apart.

6. The plates were incubated immediately at 35 ± 2°C and examined after 16-18 hours or longer, whenever necessary.

7. The zones showing complete inhibition were measured and the diameters of the zones to the nearest millimeter were recorded.
3.9. Molecular characterization

3.9.1. Preparation of Genomic DNA and PCR amplification of the 16s rRNA gene

The genomic DNA was isolated from the twelve isolates according to the procedure described by Marmur et al., (1961) and the small subunit of rRNA gene was amplified using the two primers 16S1 (5’-GAGTTTGATCCTGGCTCA-3’) & 16S2 (5’-CGGCTACCTTGGTACGACTT-3’), which are complementary to the conserved regions at the 5’ - and 3’ ends of the 16S rRNA gene corresponding to positions 9-27 and 1477-1498 of the Escherichia coli 16S rRNA gene (Lane, 1991).

3.9.2. 16s rRNA gene sequencing

The purified PCR product, approximately 1.5Kb in length, was sequenced using the primers 16S1 (5’-GAGTTTGATCCTGGCTCA-3’) and 16S2 (5’-CGGCTACCTTGGTACGACTT-3’), the same which were already used for PCR amplification. Sequencing of the purified PCR product was carried out using 5 pmol of a given sequencing primer and 8 µl ready-reaction mix from either the Big Dye Terminator sequencing kit (Perkin Elmer) or the Thermo Sequenase Dye Terminator cycle sequencing kit (Amersham) in a total volume of 20 µl. Cycle sequencing was carried out in a GeneAmp PCR machine (9600 ; Perkin Elmer). The thermal sequence consisted of 30 cycles as follows: 96 °C for 10 s, 50 °C for 5 s and 60 °C for 4 min. After the PCR, the products were precipitated using 2 µl sodium acetate (3 M, pH 4±6) and 125 µl ethanol and stored on ice for 10 min. The pellet was recovered by centrifugation at 15,000 rpm for 20 min. at 4 °C, washed with 70 % ethanol, dried under vacuum and dissolved in 4 µl loading buffer [formamide : 25 mM EDTA (4 : 1)]. The samples were denatured for 2 min. at 90 °C before being loaded on to the sequencing gel (6 % bis-acrylamide gel). Two microlitre aliquots
from each sample were loaded per gel lane and the gel was run for 10 hrs on a DNA sequence (ABI Prism model 377 version 2.1.1). The obtained sequences were involved for Blast analysis for the closest match and the same were submitted in NCBI GenBank for getting accession numbers.

3.9.3. Phylogenetic analysis

The 16S rRNA sequences of all isolates were aligned with reference sequences from the NCBI database, using the multiple sequence alignment program CLUSTAL W. The aligned sequences were then manually checked for gaps. The pair wise evolutionary distances for the above aligned sequences were computed using the MEGA 5 program. To obtain the confidence values for the rRNA sequence-based genetic affiliations, the original sequence data set was resembled 500 times using SEQBOOT and subjected to bootstrap (%) analysis. The multiple distance matrices thus obtained were used to construct phylogenetic tree showing the relationships between bacterial isolates, using various distance matrix-based clustering algorithms [FITCH, the unweighted pair group method with averages (UPGMA), KITCH and the neighbour-joining (NJ) method] (G.S.N. Reddy et al., 2000).