3. MATERIAL AND METHODS

3.1 STUDY AREA

3.1.1 Parangipettai

A coastal village, Parangipettai (Lat. 11°30’N; Long. 79°46’E), is one among the important commercial fish landing centres of southeast coast of India. It is situated in the northern bank of the mouth of Vellar River at a distance of 50 km from Puducherry. It is considered as a large trading centre because of its geographical position on Coramandel coast. It is endowed with three ecologically important biotopes, Vellar estuary, Killai back waters and Pitchavaram mangroves. Generally, gill nets and trawlers are operated in the near shore area for fishing. It is also one of the famous fish landing centre in Cuddalore district. The Annankoil landing centre in Parangipettai is located at the tip or ridges of Vellar estuary. About 402 non-mechanized boats are operated for fishing besides mechanized and indigenous crafts. There are 164 trawl nets, 5,471 drifts / gill nets, boats seines and bag nets, 213 hooks and lines, 65 shore seines and 483 other nets are used for fishing (Fig.1a).

3.1.2 Cuddalore

Cuddalore (Lat. 11°43’N; Long. 79°49’E) was the capital of the English possessions on the Coromandel Coast from the year 1748 to 1752. Cuddalore
district is located at south of Pondicherry on the coast of Bay of Bengal. Uppanar estuary is located at Cuddalore in southeast coast of India. Cuddalore is one of the dynamic centre of fishing in Cuddalore district with annual fish landing at about 2,000 tones. Cuddalore fishery plays a significant role in providing employment opportunities for 3,000 persons out of 12,855 living in nearby villages. The crafts used for fishing in Cuddalore are mostly indigenous. There are about 1,002 non-mechanized boats. Regarding the gears there are totally about 2,113 which includes 191 trawl nets, 1,151 drifts / gill nets, 52 boats seines and bag nets, 250 hooks and lines, 21 shore seines and 448 other nets (Fig.1b).

Due to its geographical position on the coromandel coast, Parangipettai and Cuddalore are the famous fish trade centres in TamilNadu. Thus, the marine edible fishes (Rastrelliger kanagurta, Lates calcarifer and Lutjanus fulviflammus) were collected from two stations in order to evaluate the degree of microbial contamination (Fig.2a, b).

3.2 SAMPLE COLLECTION

Marine edible fishes (Rastrelliger kanagurta, Lates calcarifer and Lutjanus fulviflammus) were collected from landing centres of Parangipettai and Cuddalore. The identified samples were placed individually in the pre-sterilized polythene bags, sealed and kept in a portable ice chest and transported to the laboratory for further bacteriological analysis. The following
fish species were chosen due to their worldwide availability in most tropical and subtropical waters throughout the year (Fig. 3. a, b, c).

3.2.1 *Rastrelliger kanagurta*

**Taxonomic Classification**

- **Kingdom**: Animalia
- **Phylum**: Chordata
- **Class**: Actinopterygii
- **Order**: Perciformes
- **Family**: Scrombidae
- **Genus**: *Rastrelliger*
- **Species**: *kanagurta*

**Common name**

Indian mackerel, Ayila meen in Tamil and Malayalam.

**Morphological features**

The Indian mackerel is moderately deep body, snout pointed and length of head distinctly greater than depth of body. Body bluish green with dark stripes or rows of dusky spots along upper half of the body, 2 dorsal fins, the
first being spiny and the second one rayed, 5-6 pairs of anal finlets, pelvic fin without spine, thin dark longitudinal bands on the upper part of the body, which may be golden on fresh specimens. There is also a black spot on the body near the lower margin of the pectoral fin. Dorsal fins are yellowish with black tips, while the caudal and pectoral fins are yellowish, others are dusky. It reaches the length of maximum 25 -35cm.

**Geographical Distribution**

The Indian mackerel *Rastrelliger kanagurta* is a pelagic fish, widely distributed in the tropical Indo-Pacific region. In India, *R. kanagurta* is distributed along east and west coast and in the Andaman and Nicobar Islands. Along the east coast, distribution and maximum abundance is recorded at about 70 - 100 m depth.

**Habitat**

The Indian mackerel is epipelagic, mostly found in shallow waters, coastal areas, harbours and deep lagoons and also in turbid waters rich in plankton. Juveniles feed on phytoplankton (i.e. diatoms) and small zooplankton such as cladocerans, ostracods, larval polychaetes etc. Hence, adult Indian mackerel prey primarily on Zooplankton such as larval shrimps and fish.
3.2.2 *Lates calcarifer*

**Taxonomic Classification**

- **Kingdom**: Animalia
- **Phylum**: Chordata
- **Class**: Pisces
- **Order**: Perciformes
- **Family**: Centropomidae
- **Genus**: *Lates*
- **Species**: *calcarifer*

Seabass has been placed under several families by various authors in the past (e.g., Family; Serranidae and Family; Latidae etc.). Centropomidae is the commonly accepted family name of this species and the recognized generic name is *Lates*. Grace Mathew (2004) has made known that above classification as accepted taxonomic classification of Seabass or giant perch.

**Common name**

Asian seabass, barra, barramundi perch, cock-up, giant perch, giant seaperch, palmer, silver barramundi, silver perch, white seabass, etc., Perca, Pseudolates, Holocentrus, Coins, Plectropoma, Latris and Pleotopomus are the other names given by various authors who collected the fish specimens from
different areas. Bloch (Schneider, 1801) stated that *Lates calcarifer* occurred in Japan Sea and named it as *Holocentrus calcarifer*.

**Morphological features**

Body is large, elongate and stout with pronounced concave dorsal profile in head and a prominent snout, concave dorsal profile becoming convex in front of dorsal fin. Mouth is large, slightly oblique, upper jaw reaching up to eye, teeth villiform, canine teeth are absent. Lower edge of pre-operculum is with strong spine, operculum with a small spine and with a serrated flap above original of lateral line.

Dorsal fin with 7 to 9 spines and 10 to 11 soft rays, a very deep notch almost dividing spiny from soft part of fin, pectoral fin short and rounded, several short, strong serrations above its base, dorsal and anal fins both have scaly sheath. Anal fin round, with three spines and 7–8 soft rays; caudal fin rounded. Scale large ctenoid (rough to touch). Eyes are bright pink, glowing at night. Two phases of colour occurs, either olive brown above with silver sides and belly in marine environment or golden brown in freshwater environment.

**Habitat**

It is a euryhaline and catadromous species, medium to large-sized bottom-living fishes of Seabass inhabit freshwater, brackish and marine habitats including streams, lakes, billabongs, estuaries and coastal waters. Due
to fast growth rate, good taste, flesh texture, high demand and high market value, it is considered as candidate species for culture in marine, brackish as well as fresh water environment (Maity et al., 2011).

**Geographic distribution**

Seabass between Longitude 50°E - 160°W and Latitude 24°N – 25°S is widely distributed in tropical and sub-tropical areas of the Western and Central Pacific and Indian Ocean. It occurs throughout the northern part of Asia, Southward to Queensland (Australia), westward to East Africa. Aquaculture of this species commenced in the 1970s in Thailand, and rapidly spread throughout of Southeast Asia. Caught mainly with bottom trawls, hand lines, bottom gillnets and traps, also esteemed as an exciting sport fish. It is freshly marketed.

**3.2.3 *Lutjanus fulviflammus***

**Taxonomic Classification**

- **Kingdom**: Animalia
- **Phylum**: Chordata
- **Class**: Actinopterygii
- **Order**: Perciformes
- **Family**: Lutjanidae
- **Genus**: *Lutjanus*
- **Species**: *fulviflammus*
Common name

Dory snapper, Black spot snapper and Black spot Sea perch.

Morphological features

Perch-like fishes, oblong in shape, moderately compressed, covered with moderate or small ctenoid scales (rough to touch). Lateral lines complete, straight or gently curved. Mouth is terminal, fairly large and protrusible. Jaw teeth are usually in few rows, conical and sharp, teeth usually present on vomer and palatines (roof of mouth). It grows up to 35cm in length, a series of six or seven horizontal yellow stripes runs on the side, mainly below the lateral line. A black spot is present near the lateral line on posterior part of the body.

Habitat

*Lutjanus fulviflammus* is one of the common species that occurs among snappers. Adults inhabit coral reefs, usually in schools in coastal reefs and in deep lagoons, juveniles are found in mangrove estuaries or in lower reaches of freshwater streams. It feeds mainly on fishes, shrimps, crabs and other crustaceans; it is an active carnivore, fishes and prawns forming the main constituent of the diet. Other organisms like crabs were also found in the stomach (Mohanraj and Prabhu, 2012). The diet of fish species showed clear spatial differences, which were dependent on size distributions.
Geographical distribution

Found in Red Sea, Tropical Indo – pacific, South to East London. Caught mainly by hand lines, traps, bottom set gill nets. Marketed in fresh condition. The snapper family represents an important fisheries resource in tropical and subtropical areas and as with many of our world fish stocks, most snapper fisheries are being harvested at or beyond their maximum sustainable yield. Because of their wide acceptance as an excellent food fish, high market price, and limited harvests from wild stocks, there is considerable interest in culturing a variety of snapper species (Stickney, 2000).

3.3 MICROBIOLOGICAL ANALYSIS OF BACTERIA

3.3.1 Sterilization of materials

All the glassware used were washed, dried and sterilized in hot air oven at a temperature of 160°C for 30 minutes. Culture media was also sterilized in an autoclave at a temperature of 121°C for 15 min. The wire loop was also sterilized using spirit lamp.
3.3.2 Media preparation

3.3.2.1 Zobell marine agar (2216)

Principle

Marine Agar 2216 was used for cultivating heterotrophic marine bacteria. Marine bacteria are present in nutrient sea water by the millions per ml and are essential to the life cycle of all marine flora and fauna. Peptone and yeast extract provide nitrogen, vitamins and minerals. The high salt content helps to simulate seawater. Numerous minerals are also included to duplicate the major mineral composition of sea water.

Composition: Ingredients Grams/Litre

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Grams/Litre</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptic digest of animal tissue</td>
<td>5.00</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>1.00</td>
</tr>
<tr>
<td>Ferric citrate</td>
<td>0.10</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>19.45</td>
</tr>
<tr>
<td>Sodium sulphate</td>
<td>3.24</td>
</tr>
<tr>
<td>Magnesium chloride</td>
<td>8.80</td>
</tr>
<tr>
<td>Calcium chloride</td>
<td>1.80</td>
</tr>
<tr>
<td>Potassium chloride</td>
<td>0.55</td>
</tr>
</tbody>
</table>
Potassium bromide 0.08
Sodium bicarbonate 0.16
Strontium chloride 0.034
Boric acid 0.022
Sodium silicate 0.004
Sodium fluoride 0.0024
Ammonium nitrate 0.0016
Disodium phosphate 0.008
Agar 15.00

Final pH (at 25°C) 7.6 ± 0.2

Procedure

The powder was suspended in 1 L of purified distilled water and was mixed thoroughly. Then it was heated with frequent agitation and boiled for 1 minute to dissolve the powder completely. Agar was melted by boiling in a water bath and pH was adjusted. Medium was then sterilized at 121°C for 15 minutes in an autoclave. After sterilization, it was cooled at 50°C. About 25-35 ml the medium was poured into sterile petriplates and the medium was set at ambient temperature.
3.3.2.2 Nutrient agar

Principle

Nutrient agar is a basic culture media which supplies the basic requirements of carbon, nitrogen and mineral source for growth, used for maintaining microorganism and cultivating fastidious organisms.

Materials required

Nutrient agar powder, conical flask, distilled water, pH meter, petridishes, measuring cylinder, Non-absorbent cotton etc.,

Preparation of medium

Peptone 1.0 gm
Beef extract 1.0 gm
Sodium chloride 0.5 gm
Agar 1.5 gm
Distilled water 100 ml

Procedure

23.0 grams of nutrient agar was weighed and added to 1.0 litre of distilled water in a 2.0 ml conical flask. Agar was melted by boiling in a water bath and pH was adjusted. Medium was sterilized at 121^0C for 15 minutes in an
autoclave. After sterilization, it was cooled at 50°C. In a sterile petriplates about 25-35 ml medium was poured and set at ambient temperature.

3.3.3 Sample Preparation

Surface of each fish samples was washed for one minute, individually with distilled water to remove excess external bacteria. Under sterile conditions, the fishes were dissected and separated into skin (1cm²), gill (1gm) and gut (1gm) was taken for analysis of bacteria. The skin, gill and gut regions of cut samples taken from edible fishes were crushed into small pieces in a sterile mortar and pestle with 10 ml sterile water and poured in the sterile test tubes.

3.3.3.1 Preparation of serial dilution

Principle

When one of the culture or sample is mixed with one part of diluents, the result will be 1: 2 dilutions. One part of the culture or sample is placed in nine parts of diluents; the result will be 1:10 dilution. When one part 1:10 dilution transferred to 9ml of the diluents the result will be an additional one to ten dilutions. This is because the final dilution is a multiple of all dilution. To determine final dilutions multiply all dilution factors.
1/10 \times 1/10 = 1/100, 1/100 \times 1/10 = 1/1000, 1/1000 \times 1/10 = 1/10000,

etc. A single dilution was calculated as,

\text{Dilution} = \text{Volume of sample / total volume of the sample and diluents.}

\textbf{Materials required}

9ml of water blanks in sterile test tubes, pipettes, cotton, nutrient agar, petriplates, marker etc.

\textbf{Procedure}

Nine millilitres of sterile water was poured aseptically into five tubes each and 1 ml of the original crushed fish sample was added to the first test tube and mixed thoroughly. Another 1 ml was taken from the first tube and added to the second test tube and mixed well. From the second test tube, another 1 ml was taken and introduced into the third test tube and mixed very well. This procedure is continued until the fifth test tube. The crushed sample was diluted from $10^{-1}$ to $10^{-5}$ for each fish sample.

\textbf{3.3.3.2 Spread plate method}

\textbf{Principle}

With the help of spread plate technique the isolation and enumeration of microorganisms from samples with lower population of bacteria and other
microorganisms are used. This method is called spread because L-Rod or cotton swab is used to spread the sample.

Materials required

Test tubes with 9ml of diluents, Cotton, Autoclave, Water bath, Zobell marine agar 2216, Nutrient agar or other agar media, Petriplates, L-Rod, Micropipettes, Spirit, etc.

Procedure

Prepared Zobell marine agar or nutrient agar medium was sterilized at 121°C and poured into the air dried petriplates under sterile conditions and allowed to set agar in petriplates. Samples were diluted up to 1:100000 (10^{-5}). From 10^{-2} dilution 0.1ml of sample was placed in the centre of an agar medium using sterile pipette, similar procedure was followed for the 10^{-3} and 10^{-4} dilutions. The glass spreader (L-Rod) was dipped into a beaker of ethanol/spirit, flamed ethanol soaked spreader on Bunsen burner was allowed to cool. The sample was spreaded evenly over the agar surface and plates were incubated at appropriate temperature 37 °C for 24 hours. The growth of the microorganisms was observed and recorded for colony enumeration and isolation.
3.3.3.3 Streak plate - pure culture technique

Principle

It is a good method for microbial purification. This method is called streak because it creates lines on the surface of the medium like streaking. Inoculation loop was used to transfer inoculums from the sample of streaking and is called looping out technique. Different types of streaking techniques are available; they are T-streak, Quadrant streak, simple streak and continuous streak. These techniques are named based on the type of line of streak drawn on the surface of the medium.

Materials required

Sample of mixed culture, Cotton, Autoclave, Hot air oven, Water bath, Nutrient agar or any one of the type of medium, Petriplates, Pipettes, Spirit, Inoculation loop etc.

Procedure

Nutrient agar was prepared and poured into the sterile petriplates which was allowed to solidify. The inoculation loop was sterilized using flaming technique. The microbial mixture was transferred from a tube to the edge of an agar plate with an inoculation loop and the petriplates was incubated at 37°C
for 24 hours. Purified individual colonies were noted and Individual colonies were subjected to identification.

3.4 QUANTITATIVE ANALYSIS OF BACTERIA

Triplicates were made for each dilution, after incubation the total number of colony forming units per gram (CFU g⁻¹) was determined.

3.4.1 Calculation of bacteria counts

Countable plates showing 1 to 32 colonies was selected and counted. The mean colony count on the Zobell marine agar plates of each given dilution was used to estimate the total viable count for the samples in colony forming units per gram (CFU g⁻¹) as,

\[ \text{Number of colonies } \times \text{dilution.} \]

3.4.2 Calculation of mean colony forming unit per gram (CFU g⁻¹)

The mean colony forming unit per gram (CFU g⁻¹) denoted by (x) was calculated as \( \frac{\Sigma fx}{\Sigma f} \), where \( \Sigma fx \) is the sum of the products of number of colonies and the colony forming unit per gram; while \( \Sigma f \) is the summation of the number of colonies.
3.4.3 Statistical analysis

The data for number of isolated bacterial genera were subjected to analysis of variance (ANOVA). One way ANOVA was performed using Graphpad Prism 6 version 6.03 for windows, Graphpad software, Sand-diego, Californea, USA www.graphpad.com.

3.5 QUALITATIVE ANALYSIS

Identification of microorganisms of the various purified bacterial isolates were examined for morphological and biochemical tests. They are used as a bias for identifying the isolates according to Bergey’s manual of Systematic Bacteriology (Holt, 1984).

3.5.1 Morphological analysis

3.5.1.1 Gram staining

Principle

The Gram stain, named after Dr. Christian gram, is a differential staining technique that differentiates bacteria into two groups: gram-positive and gram-negative. The procedure is based on the ability of microorganisms to retain the colour of the stains used during the grams staining reaction. Gram-positive bacteria retain the primary stain crystal violet and are not decolorized
by alcohol. In case of Gram-negative bacteria, they are decolorized by alcohol, losing the primary stain, purple during the decolorization step and retain the counter stain (saffranine) which imparts a pink colour to the decolorized gram-negative organisms.

Gram positive bacteria stains dark purple as it retains the primary stain called Crystal Violet in the cell wall. Gram-positive bacteria have a thick mesh-like cell wall made of peptidoglycan (50-90% of cell wall). The thick peptidoglycan layer of Gram-positive organisms allows these organisms to retain the crystal violet-iodine complex and stain purple. In case of Gram-negative bacteria, they have an additional outer membrane which contains lipid and which is separated from the cell wall by means of periplasmic space followed by a thin layer of peptidoglycan (10% of the cell wall). The gram negative bacteria losses the crystal violet-iodine complex during decolourization with the alcohol and retains the counter stain Saffranine, thus appearing reddish/pink.

**Materials required**

- Bunsen burner, Microscope, Inoculation loop, Slide, Water bottle, Crystal violet solution, Gram’s iodine solution, Ethanol 95%, Decolorizer and Saffranine solution.
Grams iodine

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iodine crystal</td>
<td>1.0 gm</td>
</tr>
<tr>
<td>Potassium iodide</td>
<td>2.0 gm</td>
</tr>
<tr>
<td>Distilled water</td>
<td>300 ml</td>
</tr>
</tbody>
</table>

The solution was made up to 300 ml.

Saffranine (Counter stain) 1%

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saffranine</td>
<td>1 gm</td>
</tr>
<tr>
<td>Distilled water</td>
<td>100 ml</td>
</tr>
</tbody>
</table>

Crystal violet stain

Ammonium Oxalate Crystal Violet (Hucker’s)

Solution A

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crystal violet</td>
<td>2 gm</td>
</tr>
<tr>
<td>Ethyl alcohol</td>
<td>20 ml</td>
</tr>
<tr>
<td>Distilled water</td>
<td>80 ml</td>
</tr>
</tbody>
</table>

Solution B

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ammonium oxalate</td>
<td>0.8 g</td>
</tr>
</tbody>
</table>

The solution A and B were mixed.
Destaining solution

Destaining solution was prepared by mixing 95 ml of ethanol in 5.0 ml of distilled water.

Procedure

A loopful of 18 – 24 hours bacterial culture was taken and was spread on a clean glass slide heat fixed and brought to room temperature. About 5 drops of crystal violet stain was added over the fixed culture, stained for 60 second and gently rinsed for the excess stain with a stream of water from plastic wash bottle. Again 5 drops of the iodine solution was added on the smear, enough to cover the fixed culture and stained for 30 seconds, excess iodine solution was rinsed with wash bottle water, the excess water was shaked from the surface.

A few drops of decolorizer (95% Ethanol) solution was added, so the solution trickles down the slide. It was rinsed with water after 5 seconds and was blot dried. And counterstained with 5 drops of saffranine stain for 20-30 seconds. The saffranine solution was washed off with water. The excess water was removed and blot dried alternatively, the slide was air-dried. Slide was then observed under low and high power objectives of the oil immersion (compound) microscope.
3.5.1.2 Motility test

Principle

Some microorganisms are capable of moving through the medium under their own power, others are non-motile. Bacteria uses flagella to move towards food source, move towards light and to move away from toxic chemicals. Some organisms are motile at room temperature (30\(^0\)C) but non-motile at body temperature (37\(^0\)C). There are different methods available to detect motility of bacteria.

Hanging drop method is a direct and best method to detect the bacteria. The name hanging drop is because a drop of culture is hanged on a concavity of slides on cover slip. In liquid suspension false motility can also be observed. It is essential to differentiate between actual motility and Brownian movement, a vibratory movement of the cells because of their bombardment by water molecules in the suspension.

Materials required:

24 hours broth culture of bacterial isolates, Bunsen burner, Inoculation loop, Depression slide, cover slip, microscope, petroleum jelly, tooth pick etc.
**Procedure**

Petroleum jelly/Vaseline was applied around the concavity of the depression slide as ring. Loopful of the culture was placed in the centre of the clean cover slip using aseptic technique. The depression slide was placed in the concave surface facing down, over the cover slip so that the depression covers the drop of the culture. The slide was pressed gently to form a seal between the slide and cover slip. The slide was turned quickly right side up so that the drop continues to adhere to the inner surface of the cover slip. It was observed under oil-immersion microscope for active motility of motile cells or non motile stable cells.

**3.5.2 Biochemical Analysis**

**a. Indole test**

**Principle**

The indole test screens the ability of an organism to degrade the amino acid tryptophan and produce indole. It is used as part of the IMViC procedures, a series of tests designed to distinguish among members of the family *Enterobacteriaceae*. Tryptophan an amino acid undergoes deamination and hydrolysis by bacteria that express tryptophanase enzyme.

\[
\text{Tryptophan} + \text{water} = \text{indole} + \text{pyruvic acid} + \text{ammonia}.\]
The chief requirement for culturing an organism prior to performing the indole test is that the medium should contain a sufficient quantity of tryptophan and the capacity to degrade it. The detection of indole, a by-product of tryptophan metabolism, relies upon the chemical reaction between indole and Kovac’s reagent (p-dimethyl amino benzaldehyde /DMAB) under acidic conditions to produce the red dye rosindole.

**Materials required**

Tryptone broth or peptone broth, Kovac’s reagent, Bacterial culture.

**Preparation of tryptone broth**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tryptone</td>
<td>1.0 gm</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>0.5 gm</td>
</tr>
<tr>
<td>Distilled water</td>
<td>100 ml</td>
</tr>
<tr>
<td>pH</td>
<td>7.4</td>
</tr>
</tbody>
</table>

The ingredients were dissolved and distributed in 2ml quantities in 12 × 100 mm test tubes and autoclaved at 121 °C for 15 minutes.

**Preparation of kovac’s reagent**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Paradimethyl aminobenzaldehyde</td>
<td>5.0 gm</td>
</tr>
<tr>
<td>Amyl alcohol</td>
<td>75.0 ml</td>
</tr>
<tr>
<td>Conc.HCL</td>
<td>250 ml</td>
</tr>
</tbody>
</table>
To the aldehydes dissolved in alcohol acid was slowly added and stored in brown bottles.

**Procedure**

Prepared tryptophan or peptone broth water medium was sterilized at 121°C for 15 minutes and inoculated with the test organism and incubated at 37°C for 24 hours. 0.5 ml or 1 ml of the Kovac’s reagent was added and gently agitated. The upper layer of liquid red to yellow or other colour changes was recorded.

b. **Methyl red test**

**Principle**

Methyl red is a pH indicator ranges between 6 (yellow) and 4.4 (red). The pH at which methyl red detects acid is considerably lower than the pH of other indicators used in the bacteriological culture media. Thus to produce a colour change, the test organisms must produce large quantities of acid from the carbohydrates substrate being used. Methyl red test is a quantitative test for the detection of mixed acid production (lactic, formic, acetic and pyruvic acids) from glucose through mixed acid fermentation pathway.
Materials required

MR-VP broth, Methyl red pH indicator, Inoculation loop, Cotton etc,

Preparation of MR/VP broth

Peptone 7.0 gm
Glucose 5.0 gm
Dipotassium phosphate 5.0 gm
Distilled water 5.0 gm
Final pH 6.9

Above substances was dissolved and distributed in 5ml quantities in
15 × 125 mm test tubes and autoclaved at 121 °C for 15 minutes.

Preparation of MR reagent

Methyl red 0.1 gm
Ethyl alcohol 300 ml
Distilled water 200 ml

Methyl red was dissolved in alcohol and distilled water and stored in
brown bottle at 4°C.
Procedure

Prepared MR-VP broth was taken in a test tube and sterilized at appropriate temperature. A test organism was inoculated using sterile technique and incubated at $37^\circ$C for 24-48 hours. At the end of the incubation, 5 drops of methyl red indicator was added directly to the medium, gently shaked and colour changes were observed.

c. Voges proskauer test

Principle

Voges and Proskauer are the two microbiologists who observed red colour reaction produced by appropriate culture media after the treatment of potassium hydroxide. It was later discovered that the active product formed after bacterial metabolism is acetyl methyl carbinol (acetoin) a neutral reacting end product. It is a product of butylenes glycol pathway.

Materials required

MR-VP Broth, Barritt’s Reagent A, Barritt’s Reagent B, Bacterial culture, Inoculation loop, Cotton, etc.
**Procedure**

Prepared MR-VP broth was taken in a test tube and sterilized at 121\(^{0}\)C for 10 minutes. The test organism was inoculated and incubated at 37\(^{0}\)C for 24-28 hours. 0.5 ml of Barritt’s reagent A and 0.5 ml of Barritt’s reagent B was added and gently mixed for 30 sec to 1 min and allowed to stand for 30 min, positive and negative results were recorded.

d. **Citrate utilisation test**

**Principle**

This test detects the ability of an organism to utilize citrate as the sole source of carbon and energy. Bacteria was inoculated in a medium containing sodium citrate and a pH indicator bromothymol blue. The medium also contains inorganic ammonium salts, which is utilized as sole source of nitrogen. Utilization of citrate involves the enzyme citrase, which breaks down citrate to oxaloacetate and acetate. Oxaloacetate is further broken down to pyruvate and CO\(_2\). Production of Na\(_2\)CO\(_3\) as well as NH\(_3\) from utilization of sodium citrate and ammonium salt respectively results in alkaline pH which gives a colour to the medium as green to blue.
Materials required

Simmon’s Citrate agar, Bacterial culture, Inoculation loop, Test tubes, Conical flask, Cotton plug, etc.,

Simmon’s Citrate agar Composition - Ingredients gm / litre

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount (gm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Magnesium sulphate</td>
<td>0.2</td>
</tr>
<tr>
<td>Ammonium dihydrogen phosphate</td>
<td>1.0</td>
</tr>
<tr>
<td>Dipotassium phosphate</td>
<td>1.0</td>
</tr>
<tr>
<td>Sodium citrate</td>
<td>2.0</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>5.0</td>
</tr>
<tr>
<td>Bromothymol blue</td>
<td>0.08</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000 ml</td>
</tr>
<tr>
<td>Final pH (at 25°C)</td>
<td>6.8 ± 0.2</td>
</tr>
<tr>
<td>Agar</td>
<td>15.0</td>
</tr>
</tbody>
</table>

Procedure

Simmons citrate medium was prepared and poured into the test tubes. The medium was sterilized at 121°C for 15 minutes. A well-isolated colony was picked up from the surface of a primary isolation and inoculated as a single
streak on the slant surface of the citrate agar tube and incubated at 37°C for 24 hours and colour changes were noted.

e. Catalase test

Principle

Catalase is an enzyme that decomposes hydrogen peroxide into oxygen and water. Hydrogen peroxide forms one of the oxidative end products of aerobic carbohydrate metabolism. Catalase converts hydrogen peroxide into water and oxygen. When small portion of the colony is introduced into hydrogen peroxide rapid evolution of bubbles occurs.

Materials required

3% Hydrogen peroxide, clean glass slide, Dropper, Bacteriological Loop, Bacterial culture in nutrient broth or agar, Cotton loop and wooden stick.

Procedure

With a loop or applicator stick a well-isolated colony from the 24 hours bacterial culture was transferred into a clean glass slide and 1-2 drops of 3% Hydrogen peroxide was added to the bacterial cells and observed for bubble formation.
f. Oxidase test

Principle

The cytochromes are iron containing haemoproteins that acts as last links in the chain of aerobic respiration by transferring electron to oxygen. The cytochrome oxidase test uses certain reagent dyes, such as p-phenylenediamine dihydrochloride, that substitute for oxygen as artificial electron acceptor. In the reduced state the dye is colourless, however, in the presence of cytochrome oxidase and atmospheric oxygen, p-phenylenediammine is oxidized, forming indophenol blue. The cytochrome system is found in aerobic or micro aerophilic and facultative anaerobic organisms. Oxidase test is important for identifying organisms based on oxygen utilization.

Materials required

Oxidase discs, Bacterial culture, Inoculation loop, Slide, etc.

Procedure

24 hours pure bacterial culture was taken and inoculated in a tube containing 5-10ml of nutrient broth and was incubated at 37°C for 12-24 hours. In a clean microscopic slide oxidase disc was taken, one or two drops of bacterial culture was placed on the disc. Within 10 seconds colour changes was observed in the disc.
g. Nitrate test

Principle

Nitrate is one of the nitrogen sources. Nitrate acts as an electron acceptor in anaerobic respiration. The presence of nitrites in the medium is detected by the addition of $\alpha$-naphthylamine and sulfanilic acid, with the formation of a red diazonium dye, P sulfobenzene-azo- $\alpha$-naphthylamine which gives red colour for the positive reaction.

Materials required

Nitrate broth, Nitrate reagent A, Nitrate reagent B, test tube, cotton, inoculation loop, conical flask, incubator etc.

Preparation of Nitrate broth

Beef extractor 0.3 gm  
Peptone 0.5 gm  
Potassium nitrate 0.1 gm  
Distilled water 100 ml  

The ingredients are dissolved and distributed in 5ml quantities in 15X125 mm tubes and autoclaved for 15mins at 121°C.
Reagent A

Alpha naphthalamine  0.5 gm
Acetic acid (5n) 30%  100 ml

Reagent B

Sulfanilic acid  0.8 gm
Acetic acid (5n) 30%  100 ml

The reagents are stored in brown bottles.

Procedure

Nitrate broth was prepared and sterilized, inoculated the medium with test organisms and incubated at 37°C for 24 hours. At the end of the incubation, 1ml of nitrate reagent A and nitrate reagent B was added to the test organisms, observed for the colour change.

h. Coagulase test

Principle

The enzyme coagulase is produced by the few Staphylococcus species a key feature of pathogenic Staphylococcus. It produces an enzyme coagulase which activates prothrombin to clot the rabbit plasma which differentiates S. aureus from other members in the genus.
Materials required

Rabbit plasma with EDTA, anti coagulant, saline, glass slides, test tubes, glass rod/platinum loop/plastic loop etc.

Procedure

0.5 ml of Rabbit plasma (diluted 1:5 with saline) was added to a test tube. Approximately 5 drops (250 µl) of overnight broth culture or small amount of the colony growth was added to the diluted plasma in the test tube and the test tube was incubated at 37°C for 4 hours. Clot formation was observed by gently tilting and shaking the tubes. If there is no clot formation, again the tubes were incubated at room temperature, after 18 hours results are recorded.

i. Esculin hydrolysis (bile esculin test)

Principle

Esculin is a glycosidic coumerin derivative (6-beta-glucoside-7-hyroxy coumerin) is linked together by an ester bond through oxygen. Hydrolysis of the esculin in the medium results in the formation of glucose and a compound called esculin. Esculatein, in turn, reacts with ferric ions to forms a black diffusible complex.

Esculin --------→ glucose + esculin------→ black complex.
**Materials required**

Bile esculin agar medium, Inoculation loop, Petriplates, Test tubes, Incubator, etc.

**Preparation of bile esculin agar**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brain Heart Infusion agar</td>
<td>4gm</td>
</tr>
<tr>
<td>Esculin</td>
<td>0.1gm</td>
</tr>
<tr>
<td>Ferric citrate</td>
<td>0.0 gm</td>
</tr>
<tr>
<td>Distilled water</td>
<td>100 ml</td>
</tr>
</tbody>
</table>

**Procedure**

Sterile bile esculin agar slant was prepared and autoclaved at 121°C for 15 minutes and was allowed to set in a slanting position. 2 or 3 morphologically similar colonies were inoculated in a zig-zag motion on slant and quadrant streak on the plate. The tubes/plates were incubated at 37°C for 24 hours in an ambient incubator. Colour changes were noted.

**j. Triple sugar iron agar test (H₂S production)**

**Principle**

Some bacteria liberate sulphur from sulphur containing amino acids or other sulphur containing compounds. The sulphur is used as finally hydrogen
acceptor during anaerobic respiration leading to the formation of hydrogen sulphide. When the following conditions are present, hydrogen sulphide can be detected by the test system.

**Materials required**

Test tube, conical flask, Inoculation loop, Cotton plug, TSI agar, etc.

**Preparation of Triple sugar iron**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beef extract</td>
<td>0.3 gm</td>
</tr>
<tr>
<td>Yeast extracts</td>
<td>0.3 gm</td>
</tr>
<tr>
<td>Peptone</td>
<td>2.0 gm</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>0.5 gm</td>
</tr>
<tr>
<td>Glucose</td>
<td>0.1 gm</td>
</tr>
<tr>
<td>Lactose</td>
<td>1.0 gm</td>
</tr>
<tr>
<td>Sucrose</td>
<td>1.0 gm</td>
</tr>
<tr>
<td>Ferrous ammonium sulphate</td>
<td>0.2 gm</td>
</tr>
<tr>
<td>Sodium thiosulphate</td>
<td>2.5 gm</td>
</tr>
<tr>
<td>Phenol red</td>
<td>2.5 gm</td>
</tr>
<tr>
<td>Distilled water</td>
<td>100 ml</td>
</tr>
<tr>
<td>Final pH</td>
<td>7.4</td>
</tr>
</tbody>
</table>
All the ingredients were dissolved, kept at appropriate pH. The agar was dissolved by boiling and the pH was checked again and distributed in 3-4ml quantities in 12x100mm test tubes to autoclave for 15 minutes at 121°C. The test tubes were allowed to set to obtain 1 inch butt.

**Procedure**

TSI agar medium was prepared and sterilized, a slant was formed with enough amount of butt (slant and butt must be in equal length). Single colony was picked up and inoculated by stabbing down the centre of agar butt carefully. The inoculating needle was withdrawn carefully then the surface of the slant was streaked and the tubes were incubated at 37°C. The results were noted only after 18-24 hour.

**k. Urease test**

**Principle**

The enzyme urease is possessed by the bacterium which hydrolyses urea and releases ammonia and carbon dioxide. Ammonia reacts in solution to form ammonium carbonate, which is alkaline leading to the increase in the pH. Phenol red which is incorporated in the medium changes its colour from yellow to red in alkaline pH.
**Materials required**

Agar, inoculation loop, petriplates, bacterial culture, Christensen’s agar medium, etc.

**Christensen’s agar medium preparation**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptone</td>
<td>0.1 gm</td>
</tr>
<tr>
<td>Glucose</td>
<td>0.1 gm</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>0.5 gm</td>
</tr>
<tr>
<td>Monopotassium</td>
<td>0.2 gm</td>
</tr>
<tr>
<td>Phenol red (1.2%)</td>
<td>1.0 ml</td>
</tr>
<tr>
<td>Agar</td>
<td>2.0 gm</td>
</tr>
<tr>
<td>pH</td>
<td>6.8</td>
</tr>
</tbody>
</table>

**Procedure**

Christensen’s agar medium slant was inoculated with a drop of 4-6 hour growth of bacterium on broth and incubated at 37° C for 24 hours or longer to observe the colour changes.

**3.5.3 Molecular Characterization of Bacteria**

The strains (*Micrococcus sedentarius* and *Paenibacillus alvei*) was obtained from the fish samples are subjected to PCR (Polymerase chain reaction) analysis.
3.5.3.1 Isolation of genomic DNA

Principle

In prokaryotes, DNA is double stranded and circular and is found throughout the cytoplasm. The cell membrane must be disrupted to release the DNA into the extraction buffer. SDS is used to disrupt the cell membrane. DNA can be protected from endogenous nucleases by chelating Mg\(^{2+}\) ions using EDTA. Mg\(^{2+}\) ion is considered as a necessary cofactor for most nucleases. Proteinase K enzyme degrades the proteins in the disrupted cell soup. Phenol: chloroform is used to precipitate and separate proteins from DNA. Chloroform is also a protein denaturant, which stabilizes the rather unstable boundary between the aqueous phase and a pure phenol layer. The denatured protein forms a layer at the inter phase between the aqueous organic phases which is formed after centrifugation. DNA released from the disrupted cells is precipitated by cold ethanol or iso-propanol.

Materials required

TE buffer (10mM trisHCl pH 8.0, 1mM EDTA pH 8.0). (Mix 1ml of 1M Tris HCl pH8.0 and 0.2 ml of 0.5M EDTA pH 8.0, made it up to 100ml with distilled water).
10% SDS, 20mg/ml – Proteinase K, Phenol: Chloroform(24:1), 95% ice cold Ethanol, 70% Ethanol, 3M Sodium acetate pH 5.2, Overnight culture (grown in LB broth)

**Procedure**

1.5ml of bacterial culture was transferred to a micro centrifuge tube and spun at 10000 rpm for 2 mins at 4° C. The supernatant was decanted and tube was drained well onto a tissue paper. The pellet was re-suspended in 467μl of TE buffer by repeated pipetting. 30μl of 10% SDS and 3μl of 20mg/ml of Proteinase K was added to the sample and incubated for 1 hr at 37° C. Equal volumes of Phenol: Chloroform (24:1) was added and mixed gently by inverting the tubes until the phase was completely mixed. The tubes were spun at 12,000 rpm for 10 mins at 4° C. The upper aqueous layer was transferred to a new tube and an equal volume of Chloroform was added. The samples were mixed by gently inverting the tubes and spun at 12,000 rpm for 10 mins at 4° C. The upper aqueous phase was transferred to a new tube and 1/10th volume of 3M sodium acetate was added. Double the volume of 95% ice cold ethanol was added and mixed by inversion until the DNA was precipitated. The tube was spun for 10 min at 12,000 rpm at 4° C and the supernatant was discarded. The pellet was washed with 0.2 ml of 70% ethanol and tube was spun as before. 70% ethanol was discarded and the pellet was air dried. The DNA was then suspended in TE buffer and run on 0.8% Agarose gel.
3.5.3.2 Electrophoresis on agarose gel

**Principle**

For the majority of DNA samples, electrophoretic separation is carried out in agarose gels. This is because DNA molecules and their fragments are considerably larger than proteins; therefore larger size agarose gels are required. Under an electric field, any given fragment of DNA should move towards the anode with the same mobility. This is due to the charge per unit length owing to the phosphate groups. Separation on agarose gels is achieved because of resistance to their movement caused by the gel matrix. Thus the largest molecules will have difficulty moving, whereas the smallest molecules will be relatively unhindered. Consequently the mobility of DNA molecules during gel electrophoresis will depend on size. Gel concentrations must be chosen based on the molecules to be separated such as for plasmid molecules – 1%; genomic DNA – 0.8% and RNA – 1.5%, mitochondrial DNA – 0.8% and amplified samples at 1.5% was use.

**Reagents**

1. TAE buffer (stock solution 50X).

   Tris base – 242g

   Glacial acetic acid – 57.1ml

   EDTA 0.5M

   Working concentration 1X
2. Gel Loading Buffer
   - Bromophenol blue
   - Xylene cynol
   - Sucrose

3. Agarose – 0.8%

4. Ethidium bromide 20mg/ml

**Procedure**

Agarose was weighed and transferred to a conical flask. 50 ml of 1X TAE was added and Agarose was melted to a clear solution by heating. It was allowed to cool until it reached bearable temperature. 2.5µl of ethidium bromide stock solution was added. A gel casting tray was placed in a levelling table and the melted agarose was poured. After the gel solidified, the comb was taken out carefully. The casted gel was placed in an electrophoresis tank and 1X TAE buffer was added until the gel was completely submerged. DNA sample was mixed with the gel loading buffer and loaded into the well. The samples were then electrophoresed at 50V until the gel loading buffer reached 2/3rd of the gel. This gel was then viewed under UV Trans-illuminator.
3.5.3.2 Quantitative and qualitative determination of DNA by spectrophotomeric method.

Principle

A solution of nucleic acids strongly absorbs UV with an absorbance maximum of 260nm and proteins at 280nm which is linearly related with the concentration of DNA and RNA and the amount of contamination in the solution in the solution. The intense absorption is primarily due to the presence of aromatic rings in the purine, pyrimidine. The concentration of nucleic acid in a solution can be calculated if one knows the value of $A_{260}$ of the solution. A solution of double-stranded DNA at a concentration of 50ug/ml in a 1cm quartz cuvette will give $A_{260}$ reading of 1. A solution of single-stranded DNA/RNA that has $A_{260}$ of 1 in a cuvette with a 1cm path length has a concentration of 40ug/ml. Proteins are usually the major contaminants in nucleic acids extract and these have absorption maximum at 280nm. The ratio of absorbance at 260 and 280nm provides a clear idea about the extent of contamination in the preparation. A ratio between 1.7 and 1.9 is indicative of fairly pure DNA preparation. But values less than 1.8 signify the presence of proteins as impurities. The values greater than 1.8 signify the presence of organic solvent in the DNA preparations. A ratio of 1.8 determines the pure DNA preparation.
Procedure

The spectrophotometer and the UV lamp were switched on. The wavelength was set at 260nm and 280nm. The instrument is set at zero absorbance with T.E buffer or sterile water as blank. 5 or 7ul of the sample is taken in a quartz cuvette and made up to 3ml with TE buffer or sterile water. Absorbance of the solution with the sample was read.

The concentration of DNA in the sample was calculated using the given formula: Concentration of dsDNA = $A_{260} \times 50\mu g \times \text{dilution factor}$.

**PURITY OF THE DNA**

$A_{260}: A_{280}$ ratio = $A_{260} / A_{280}$

- = 1.8: pure DNA
- = 1.7 – 1.9; fairly pure DNA (acceptable ratio for PCR)
- = less than 1.8; presence of proteins.
- = greater than 1.8; presence of organic solvent.

**3.5.3.3 Polymerase Chain Reaction (PCR)**

**Principle**

PCR is an *invitro* method of enzymatic synthesis of specific DNA sequence developed by Karymuller in 1988. It is a very simple and inexpensive technology for characterizing, analyzing, synthesizing, a specific DNA or RNA
from virtually, any living organism, plant, animal, virus or bacteria. It exploits the natural function of polymerase present in all living things to copy genetic material or to perform molecular photocopy. PCR consists of three steps:

- **Denaturation:** During this step, the two strands melts, open to form single stranded DNA and all enzymatic reaction stoops. This is generally carried out at 92 to 96°C.

- **Annealing:** Annealing of primer to each original strand for new strand synthesis is carried out between 40–60°C.

- **Extension:** The polymerase adds dNTPs complementary to the template strand at the 3’end of the primers. Since both the stands are copied in the PCR there is an exponential increase in the number of copies of the required gene.

These 3 steps are repeated for about 20 to 30 times in an automated thermal cycler, which heats and cools the reaction mixture in the tube in a very short time. This result in exponential increase accumulation of the specific DNA fragments.

**Procedure**

100mg of DNA was used for molecular identification of respective sample. The PCR reaction is performed for 20µl. PCR reaction was performed
for 16s rRNA gene. The PCR tubes were placed in thermo-cycler and the reaction was carried inside the thermo-cycler.

### REACTION SET UP FOR 16s rRNA GENE AMPLIFICATION

<table>
<thead>
<tr>
<th>COMPONENTS</th>
<th>STOCK CONCENTRATION</th>
<th>FINAL CONCENTRATION</th>
<th>VOLUME FOR 20 µl SETUP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Milli Q water</td>
<td></td>
<td></td>
<td>9.6µl</td>
</tr>
<tr>
<td>dNTP mix</td>
<td>2mM</td>
<td>0.2mM</td>
<td>2 µl</td>
</tr>
<tr>
<td>MgCl2</td>
<td>25Mm</td>
<td>1.5Mm</td>
<td>1.2µl</td>
</tr>
<tr>
<td>Taq buffer</td>
<td>10X</td>
<td>1X</td>
<td>2 µl</td>
</tr>
<tr>
<td>FORWARD PRIMER</td>
<td>3µm</td>
<td>0.3µm</td>
<td>2 µl</td>
</tr>
<tr>
<td>REVERSE PRIMER</td>
<td>3µM</td>
<td>0.3µM</td>
<td>2 µl</td>
</tr>
<tr>
<td>DNA Template</td>
<td>100ng/µl</td>
<td>100ng</td>
<td>1 µl</td>
</tr>
<tr>
<td>Taq DNA polymerase</td>
<td>5U/µl</td>
<td>1U</td>
<td>0.2 µl</td>
</tr>
</tbody>
</table>

**PCR reaction conditions**

- **Initial denaturation**: 94°C – 3min
- **Denaturation**: 94°C – 1min
- **Annealing**: 50°C – 1min
- **Extension**: 72°C – 1min 20sec
- **Final extension**: 72°C – 7min
Hold : 4°C
Total number of cycles : 40

3.6 DETERMINATION OF ANTIBIOTIC SUSCEPTIBILITY

Antibiotic susceptibility of the isolated bacterial cultures *Paenibacillus alvei* and *Micrococcus sedentarius* from the samples were tested by the antibiotic disc diffusion method (Bauer *et al.*, 1996).

**Principle**

The purpose of the Kirby-Bauer disk diffusion susceptibility test is to determine the sensitivity or resistance of pathogenic aerobic and facultative anaerobic bacteria to various antimicrobial compounds in order to assist a physician in selecting treatment options for his or her patients. The pathogenic organism is grown on Mueller-Hinton agar in the presence of various antimicrobial impregnated filter paper disks. The presence or absence of growth around the disks is an indirect measure of the ability of that compound to inhibit that organism.

**Materials and methods**

Sterile saline in 2ml tubes, 0.5 Mc Farland standard, 18-24hrs pure culture, Muller Hinton agar, Sterile Swabs, Caliper or Ruler, Antibiotic disks,
Antibiotic disks dispenser, Inoculating needle, 35°C to 37°C non-CO₂ incubator, Bunsen burner, Alcohol pads or isopropyl alcohol in a tube.

**Preparation of Muller Hinton Agar (MHA)**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beef, infusion from</td>
<td>300gm</td>
</tr>
<tr>
<td>Casein acid hydrolysate</td>
<td>17.5gm</td>
</tr>
<tr>
<td>Starch</td>
<td>1.5gm</td>
</tr>
<tr>
<td>Agar</td>
<td>17.0gm</td>
</tr>
<tr>
<td>Final pH (at 25°C)</td>
<td>7.3±0.1</td>
</tr>
</tbody>
</table>

38 grams was suspended in 1000 ml distilled water, boiled to dissolve the medium completely. It was sterilized at 15lbs pressure (121°C) for 15 minutes and mixed well before pouring into petriplates. Then it was poured to a uniform thickness of approximately 4mm (25ml per 9cm plates) and the agar was allowed to set at ambient temperature and the plates were stored at 4°C and used within a week.

**Preparation of McFarland standard (0.5)**

0.5 ml of 0.048 M BaCl₂ was added to 99.5 ml of 0.18 M H₂SO₄ with constant stirring. OD was ensured to fall within the range of 0.08 - 0.1 at 625 nm. It was stored in dark.
Procedure

Commercially available five antibiotic discs (Hi media labs) viz., Ampicillin 10μg, Methicillin 5μg, Rifampicin 5μg, Oxacillin 5μg, Amikacin 30μg were used. The petriplates containing 20 ml of Muller- Hinton agar were selected with 24 hours fresh culture of isolates. The response of the organism to antibiotic discs was determined by spreading 1x 10⁶ cells per ml broth (Micrococcus sedentarius (N3) and Penaebacillus alvei (N12) on MHA (Merck Pvt. Ltd.) plates. By making use of template drawn commercial antibiotics disc were dispensed with the help of a disc dispenser on the solidified Muller- Hinton agar and incubated for 37⁰C for 24- 48 hours was observed.

3.7 CURING OF PLASMIDS USING ACRIDINE ORANGE

(Pahwa et al., 2012)

Principle

Plasmids are extrachromosomal pieces of double-stranded circular DNA which have the capability to replicate independently of the host chromosome; many species of bacteria isolated from diverse habitats are known to contain plasmid DNA. Some plasmids are stable and can be maintained through successive generations by being partitioned to each daughter cell during cell division. This allows each cell to receive at least one plasmid copy. Some plasmids undergo spontaneous segregation and deletion. However, the majority
are extremely stable, and require the use of curing agents or other procedures (elevated growth temperature, thymine starvation), to increase the frequency of spontaneous segregation.

**Materials and methods**

Laminar air flow, Petri plate, Bunsen burner, Test tube rack, Loop, Micropipette, Incubator, Shaker incubator, Swabs, Penicillin discs bottle, Ciprofloxacin discs bottle, Forceps, Marker pen, etc.,

**Reagents required**

Acridine orange, Muller-Hinton Agar, LB medium.

**Lutriant Broth medium**

Dissolve the Yeast extract: 5 g, Tryptone: 10 g, NaCl: 10 g in 950 ml in deionized water and the pH of the medium was adjusted to 7.0 using 1N NaOH and made upto 1 litre then autoclaved for 20 minutes at 15psi and was allowed to cool at 55°C. It was then stored at room temperature.

**Procedure**

Overnight grown culture was inoculated into LB (pH 7.6) to give 10^5 to 10^8 cells per ml. LB broth (10 ml) containing final concentration of 100 μg/ml of acridine orange were incubated with log phase culture of *Paenibacillus alvei*
(N12) strains to give $10^5$ cells per ml. A control flask lacking acridine orange was also included. All flasks were incubated at 37°C at 200 rpm for 18-24hrs. Culture treated with acridine orange was analyzed for antibiotic susceptibility by following Kirby-Bauer disk diffusion method.

Antibiotic resistance patterns of the strains before and after curing of plasmids were compared. This was performed to determine the incidence of plasmids responsible for resistance mechanism of bacterial strains by adopting the method of Sambrook and Russel (2001).

3.8 PLASMID DNA ISOLATION BY ALKALINE LYSIS METHOD (Brinboim and Doly, 1979).

**Principle**

The principle of the method is selective alkaline denaturation of high molecular weight chromosomal DNA while covalently closed circular DNA remains double-stranded. Large and small plasmid DNAs have been extracted with this method (Birnboim and Doly, 1979). Plasmid DNA is a segment of DNA that is independent of chromosomes, but nevertheless capable of reproduction. Its strands are capable of recombining or annealing following heating or denaturation and it is this property on which alkaline lysis depends.
Materials required

Overnight grown culture, (Ampicillin – final conc.100µg/ml), Plasmid containing cultures (+ve control), Absolute alcohol, 70% ethanol, Centrifuge, Micropipettes, TE Buffer.

Alkaline lysis buffer I:

50 mM glucose
25 mM Tris-HCl (pH 8.0)
mM EDTA (pH 8.0)

Alkaline lysis buffer II:

0.4 N NaOH
2% (w/v) SDS

Solution II is prepared fresh and used at room temperature

Alkaline lysis buffer III:

5 M Potassium acetate 60.0 ml
Glacial acetic acid 11.5 ml
Millipore water 28.5 ml
**Procedure**

A single bacterial colony was transferred into 2 ml of growth medium containing the appropriate antibiotic in a loosely capped 15-ml tube. The culture was incubated for 24-48 hours at 28 ºC with vigorous shaking. The 1.5 ml of the culture was transferred into an Eppendorf tube. It was centrifuged at 12,000 g for 30 seconds at 4 ºC in a Sigma 1-14 micro centrifuge. The supernatant was drawn off and bacterial pellets obtained.

The bacterial pellet was resuspended in 100 μl ice-cold Solution I containing lysozyme (2.5 mg/ml), freshly prepared from crystalline lysozyme. The bacterial pellet was completely dispersed by vortexing and incubated in 37ºC incubator for 30 minutes for complete lysis. At the end of this time, the 200 μl of freshly prepared Solution II was added and mixed by inverting the tubes rapidly 5 times after closing the tube tightly. The tube was stored on ice. The 150 μl of ice-cold Solution III was added and tubes were vortexed gently in an inverted position for 10 seconds to disperse Solution III through the viscous bacterial lysate. The tubes were stored on ice for 3-5 minutes. After centrifugation at 12,000 g for 5 minutes at 4 ºC, the supernatants were transferred to fresh Eppendorf tubes. An equal volume of phenol:chloroform:isoamyl alcohol was added to the supernatant. It was mixed by vortexing and centrifuged at 12,000 g for 2 minutes at 4 ºC. The upper phase was transferred to a fresh Eppendorf tube. The dsDNA were precipitated with 2 volumes of
ethanol at room temperature. It was mixed by vortexing. The mixture was allowed to stand for 2 minutes at room temperature and centrifuged at 12,000 g for 5 minutes at 4 ºC. The supernatant was removed. The tube was stood in an inverted position on a paper towel to allow all of the fluid to drain away. The pellet of dsDNA was rinsed with 1 ml of 70% ethanol at 4 ºC. Again the supernatant was removed. The pellet of nucleic acid was allowed to dry in the air for 10 minutes. In the last stage, the nucleic acids were redissolved in 50 μl of TE (ph 8.0) containing DNase free pancreatic RNase (20 μg/ml). The DNA was vortexed briefly and stored at -20 ºC.

Pancreatic RNase (RNase A) at a concentration of 10 mg/ml was dissolved in 10 mM Tris.Cl (pH 7.5), 15 mM NaCl. It was heated to 100 ºC for 15 minutes and allowed to cool slowly to room temperature. Then it was dispensed into aliquots and stored at -20 ºC.

3.8.1 Agarose Gel Electrophoresis

Electrophoresis of plasmid DNA was performed 0.9% agarose gel run at 80 V for 90 minutes.

Preparation of an Agarose Gel

Agarose gel was prepared by adding 0.9 g to 100 ml of TAE (Tris-Acetate-EDTA) buffer. The slurry in the Erlenmeyer flask was heated in a microwave oven until all of the grains of agarose dissolved.
The solution was cooled to approximately 60 °C and ethidium bromide was added from a stock solution of 10 mg/ml in distilled water to a final concentration of 0.5 μg/ml and the solution was mixed thoroughly.

The warm agarose solution was poured into the mold. The gel was allowed to solidify completely for approximately 20-40 minutes at room temperature.

The electrophoresis tank was filled with approximately 1200 ml of TAE buffer. The comb was removed from the gel and the gel in the plastic tray was mounted in the tank so that the slots of the gel faced towards the negative pole-cathode. TAE buffer was added to the tank until the gel was covered to a depth of about 1mm.

The 10 μl of plasmid DNA sample was mixed with 2 μl of gel-loading buffer by sucking in and out of a micropipette. The mixture was slowly loaded into the wells of the submerged gel. The power supply was set to the constant voltage (80 volts). The gel was run for 90 minutes until the bromophenol blue dye-front have migrated to the bottom of the gel and then examined under UV light.
Molecular weight calculation for genomic DNA

\[ y = mx + b \quad \text{Where,} \]

\[ y = \log \text{MW} \]

\[ m = \text{the slope (1.743)} \]

\[ x = \text{Rf (of unknown DNA)} \]

\[ b = \text{the y-intercept (2.788)} \]

\[ R_f \text{Calculation} = \frac{\text{Migration distance of DNA}}{\text{Migration distance of dye front.}} \]

3.9 BIOINFORMATICS ANALYSIS

Phylogenetic or evolutionary tree was constructed based on 16s rRNA sequences showing the relationships of the isolates using a Portable software packages Molecular Evolutionary Genetics Analysis (MEGA) version 5 (Tamura, 2011). Multiple sequence alignment, distances and clustering with Neighbor - joining method were performed.