Chapter 4
Results
4. RESULTS

SCREENING OF METHICILLIN RESISTANT STAPHYLOCOCCUS AUREUS (MRSA)

Totally 92 wound samples were collected from the major sites of coastal area such as Cuddalore, Pondicherry, Kanya kumari, Gulf of Mannar and Chennai (East coast) Kerala-Alapuzha and Goa- Dona paula (West coast). Among the positive samples, 100 isolates of Methicillin Resistant *Staphylococcus aureus* (MRSA) were obtained (Plate: 1-A). The MRSA strains were confirmed by comparing the results with standard biochemical chart of *S. aureus* such as gram positive cocci in clusters as result of gram staining, indole negative, MR-VP positive, Citrate negative (Plate: 2), positive results were observed in case of Oxidase, Urease, Catalase, Nitrate reduction and gelatin liquefaction, whereas negative result were observed in case of motility test, starch hydrolysis, Lactose, glycerol, fructose, dextrose and maltose (carbohydrate) fermentation, acid production in TSI, oxidation fermentation, both Coagulase positive and negative strains were observed. Positive growth in *S. aureus* selective media such as golden yellow color colonies in MSA media, Yellow colony on Vogal Jonson agar media and Lipovitellin salt mannitol agar, black colonies on Baird parker media, mauve pigmented colonies on CHROM agar, pink colored colonies in MacConkey agar and MRSA was confirmed by positive growth in Oxacillin resistant screening agar base and MHA supplemented with 6 μg/ml oxacillin and 4% NaCl.

EPIDEMIOLOGICAL STUDY OF MRSA

In the fishermen community, Compared to that of females (22%), males (78%) were more prone to the infection of MRSA, based on gender (Fig: 1). Based on age groups adults were more prone to infection compared to that of children’s and old peoples. Maximum number
of 14 peoples got affected in the age group of 20-24 years (Table: 2), which is controversy in the terrestrial region with the prevalence among old peoples.

**ANTIBIOTIC SUSCEPTIBILITY ASSAY BY KIRBY-BAUER METHOD**

Since antibiotic susceptibility assay was considered as the best method and it was followed method of Kirby Bauer. It was performed for 100 MRSA strains against 24 antibiotics such as aminoglycosides, β-lactams, non-β-lactams and fluoroquinolones groups was analysed, based on its results (Plate: 3-A). After the incubation period of MHA plates, the diameter of the zone was measured and compared with the standard chart to determine the antibiogram pattern of MRSA (Table: 3). The resistance (R), intermediate (I) and susceptibility (S) of MRSA were observed and interpreted as per the National Committee for Clinical Laboratory Standards (NCCLS) chart (Gururaj Rao and Kelmani Chandrakanth, 2008).

**RESISTANT PATTERN OF MRSA**

Only one strain of MRSA showed resistant against all antibiotics used in the study except Vancomycin. 1 to 23 strain showed Pattern # 1, 1 to 22 strain showed Pattern # 2, 1 to 21 strain showed Pattern # 3, 4 to 20 strain showed Pattern # 4 to 7, 2 to 19 strain showed Pattern # 8, 9, 10 to 18 strain showed Pattern # 10 to 19, 12 to 17 strain showed Pattern # 20 to 31, 8 to 16 strain showed Pattern # 32 to 39, 5 to 15 strain showed Pattern # 40 to 44, 6 to 14 strain showed Pattern # 45 to 50, 14 to 13 strain showed Pattern # 51 to 62, 8 to 12 strain showed Pattern # 63 to 69, 7 to 11 strain showed Pattern # 70 to 76, 7 to 10 strain showed Pattern # 77 to 83, 5 to 9 strain showed Pattern # 84 to 88, 6 to 8 strain showed Pattern # 89 to 94, 1 to 7 strain showed Pattern # 95, 2 to 6 strain showed Pattern # 96, 97. Thus there were 97 different types of patterns among 100 isolates (those isolates with same pattern were from different samples) were observed (Table: 4).

Among 100 isolates, 72 MRSA isolates showed, more than 50% of resistance and 31 MRSA isolates showed more than 70% resistance, whereas only two isolate showed more than
90% resistance (Table: 8). MRSA isolate FM 68 possess maximum resistance of 95.83% (Table: 6) next to MRSA isolate FM 54 possessed maximum resistance of 91.66%. Among 24 antibiotics tested various pattern of resistance which persists among the isolates, isolated from different geographical regions of coastal area were observed (Table: 4 and Figure: 3) and vice versa to isolate to that of antibiotic. Variation of time had great influence in the production of antimicrobial metabolites which is directly proportional to each other.

**MIC BY AGAR DILUTION METHOD**

The lowest dose of the antibiotics, which is effective against the organism, can be estimated by HiComb test or agar dilution method, in which dosage level variation were examined by different strains. The MIC concentration of MRSA isolate showing greater than 70% resistance was first calculated using agar dilution method (Table: 10) in which growth was observed using the antibiotics Ciprofloxacin (Cf) -10 µg/10 ml, Rifampicin (R) -1000 µg/10 ml, Chloramphenicol (C) -1000 µg/10 ml, Tetracycline (T) -100 µg/10 ml, and Streptomycin (S) -100 µg/10 ml in range of 5 ml and 2.5 ml/100 ml stock (Plate: 3-B). The MIC concentration of MRSA ranged between 2-20 µg/ml. The MBC concentration of MRSA ranged between 0.5-15 µg/ml.

Comparative study of Multiple Antibiotic Resistance (MAR) index for isolates and antibiotics were calculated. In case of resistant isolates the maximum MAR index of 0.9583 was obtained for isolate FM 68 and the minimum value of 0.2500 was obtained for isolate FM 52. In case of resistant antibiotics, the maximum MAR index of 0.0370 was obtained for ampicillin and the minimum value of 0.0087 was obtained for gentamicin (Table: 7). The discovery and the development of new antibiotics are still a high priority in biomedical research.

**DNA EXTRACTION FROM MRSA**

In case of chromosomal DNA isolation, since the lysozyme becomes resistant, to most of the strains of *S. aureus*, it cannot be used to lysis the cell wall of those bacteria, only
lysostaphin can be used to lysis the cell wall. Few strains posses plasmids due to multidrug resistant of MRSA mostly lies on chromosomal DNA with a molecular weight of 23 kb plasmid DNA. Most of the MDR-MRSA does not bear the plasmids, since most of the antimicrobial resistance lies in the chromosomal DNA (Plate: 13).

ENZYMATIC ANALYSIS OF MARINE BACTERIA

Totally 27 stains were isolated from water and sediment samples of 20 stations, in the present work. The isolated marine bacterium includes Bacillus sp., and Vibrio sp from marine water, Pseudomonas sp., and Staphylococcus sp., from marine sediment, Serratia sp., from rat fish C. batrachus (Plate: 4). More bacterial load was observed in case of Kanyakumari region. Amount of Lipase (Plate: 6) was estimated by plate assay method (Table: 11) and titration method (Table: 13 and Figure: 4) in which few strains of Marine isolates showed Lipase production. Majority of strains are negative for Amylase (Plate: 7) production and positive for DNase (Plate: 8) and Gelatinase (Plate: 9) and negative for Alginase (Plate: 11) production. Variation of Esculin (Plate: 10) production was observed. But extending the incubation time results in DNase and Gelatinase production in case of few strains. The nature of enzymatic activity varies among different strains in case of marine bacteria (Table: 12). The extracellular enzymes were tested against MRSA and the enzyme, from the rat fish isolate seems to be more effective compared to other isolates. The enzymatic studies in marine bacteria were not preceded due to its less action on MRSA tested for antimicrobial activity.

The highest value of lipase by titration method was 55.99 units/ml/min, produced by MRSA isolate FM 3. Zymographic study shows that lipase bears activity against MRSA. But compared to that of sponge protein, marine bacterial enzymes were not much more effective.

Acetone had high stimulatory effect on the lipase activity. Tween 80 when used as the principal carbon sources produced maximum lipase. Yeast extract was the best nitrogen source for lipase production. Drop in enzyme activity was directly proportional to the concentration of
oleic acid used. Optimal assay temperature for lipase activity was found between 25 and 35°C. Marine bacteria shows drastic increase in its growth from pH 14.

Since the marine bacterial isolate from different marine source shows less activity against MRSA, different types of marine organism were isolated and characterized for enzyme production. Among which sponge was observed to posses efficient antimicrobial activity against MRSA.

**ANTIMICROBIAL ACTIVITY OF SPIRASTRELLA INCONSTANS SOLVENTS AGAINST MRSA**

The morphology of sponge was identified by the scientist of Zoological Survey of India (ZSI) as *Spirastrella inconstans* based on morphology and characters (Plate: 5). Some of MRSA isolates showed 60% resistance, those isolates were tested for this study against six solvents (Chloroform, Dichloromethane, Methanol, Ethanol, Petroleum ether and Acetone) of *Spirastrella inconstans*. Among this Chloroform showed much effective and Petroleum ether and acetone extract did not show efficient activity and optimum level of action was observed in case of other solvent extracts such as dichloromethane, methanol and ethanol (Table: 14). The strain numbers FM 4, FM 72, FM 84 and FM 88 showed maximum inhibition of 30 mm diameter against chloroform extract of *Spirastrella inconstans*, strain number FM 49 and FM 60 showed maximum inhibition of 18 mm diameter against Dichloromethane extract, strain number FM 68 showed maximum inhibition of 20 mm diameter against methanol extract, strain number FM 22 showed maximum inhibition of 22 mm diameter against ethanol extract compared to other MRSA strains (Plate: 12), strain number FM 20 and FM 74 showed maximum inhibition of 16 mm diameter against petroleum ether extract, strain number FM 84 showed maximum inhibition of 17 mm diameter against Acetone extract of *Spirastrella inconstans*.

**EXTRACTION OF PROTEIN FROM SPIRASTRELLA INCONSTANS**

Crude protein obtained from dialysis was further purified by Sephadex G-50 Column Chromatography by collecting about 75 fractions in 20% (Figure: 5), 40% (Figure: 6), 60%
(Figure: 7) and 80% (Figure: 8) saturation level. The fourth fraction of 40% and 60% bioactive compounds was indicated by dark coloration of those fractions obtained. The amounts of protein were estimated by UV-Vis Spectrophotometer (Plate: 14) in which absorbance and Transmittance was calculated (Table: 15).

If the absorbance value at 280 nm by using UV-Vis Spectrophotometer is 1 or 0.9 absorbance means it’s supposed to be protein. Lowry’s method was used to estimate the amount of protein content in the sample (Table: 16). From the results obtained, the fractions bearing the maximum protein content were taken into account for further characterization, such as fraction No. 3 and 4(40% saturation), 4(60% saturation), and 5(80% saturation) with absorbance value of greater than one.

**PRELIMINARY IDENTIFICATION OF BIOACTIVE COMPOUND FROM *SPIRASTRELLA INCONSTANS***

The fractions were collected by column chromatography and the nature of compound was detected by TLC, which showed the spots indicating the presence of amino acid content in that fractions. Those fractions bearing same compound among 300 fraction content each with 3 ml were combined in to single fractions. Based on the measurement of R_f value calculated the compound would be glutamic acid.

**SDS-PAGE**

Purified proteins were analyzed by SDS-PAGE. Among seventy five fractions collected in each percentage of saturation level, fourth fraction of 40% and 60% saturation level showed the protein content. The bands obtained from fraction number 4 and 79 were compared with the low molecular weight marker, thus indicating that the protein of *Spirastrella inconstans* extract showing 3,000 Da (Plate: 15) equal to that of insulin. As insulin can be used for the treatment of wound infection from olden days.
COMPOUND ESTIMATION IN *SPIRASTRELLA INCONSTANS*

The MRSA strain among fishermen community caused due to fishing, needs bioactive compound for the treatment, since almost all antibiotics developed resistance to the strains. Further purification was carried out by IR, UV and HPLC.

IR Spectrum was recorded on a NICOLET 360 FT-IR spectrophotometer (Figure: 9). Absorption value obtained by UV spectrum using TECHCOMP 8500 spectrophotometer was 1.7141 (Figure: 10). Wave length obtained by HPLC analysis was 254 nm (Figure: 11).

**SCANNING ELECTRON MICROSCOPE (SEM)**

At X 850 illumination shows 20 µm between the compounds, X 270 illumination 50 µm, at 2,300 X illumination 10 µm, at 6,000 X illumination 2 µm, at 2,700 illumination 5 µm and the compound was conformed as glutamic acid (Figure: 13).

**Carbon skeletal structure of Glutamic acid, C₅H₉NO₄ (2-aminopentadioic acid or 2-amino glutaric acid)**

![Carbon skeletal structure of Glutamic acid](image)

Thus the bioactive compound was identified as Glutamic acid by structural elucidation using ^1^H and ^1^3^C NMR (Figure: 12) from the marine source against dread full wound caused by MRSA among fishermans’ community.