Chapter 3

Materials and Methods

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A hemolytic *Staphylococcus* strain isolated from soil sample around the Brahmaputra River of Assam, India, was selected for the present study. The study sites were situated in Tinsukia and Dibrugarh district. The bacterial strain was isolated from soil samples and pure cultures were maintained at the Biotechnology Department of CSIR-North East Institute of Science and Technology, Jorhat, Assam. It was identified based on gram staining, catalase test and growth on mannitol salt agar, coagulation test and 16S rDNA primers (Bharadwaz *et al.,* 2014). The present investigations were designed to perform purification of the different extracellular proteins isolated from studied bacteria, followed by the individual amino acid analysis of the different proteins fractions. Chemicals used in this study were of analytical grade. Isolation of bacterial strain was done as per the method of Aneja (2005). Isolates were maintained in nutrient agar slants.

3.1. Catalase test:

The enzyme catalase is produced by some bacterial strain to facilitate the cellular detoxification function. Catalase is useful to break down hydrogen peroxide (H_2O_2) into Water (H_2O) and Oxygen (O_2).When a loopful pure culture of the bacterial strain mixed with a drop of 3 % H_2O_2 on a clean glass slide, and then rapid formation of bubbles after few seconds indicates that the organism showing catalase positive reaction. Catalase test was performed as per standard protocol obtained from the URL- (http://www.austincc.edu/microbugz/handouts/Catalase%20and%20Oxidase%20Tests.pdf).

$$2H_2O_2 + \text{Catalase} \rightarrow 2H_2O + O_2$$

Materials:

- Pure culture of bacterial strain (maintained at CSIR-NEIST, Jorhat).

- 3 % Hydrogen peroxide.
- Clean Glass slide.
- Laminar air flow chamber.
- A sterile toothpick.

Procedure:

1) A loopful of the bacterial strain was placed on a clean microscope slide.
2) 2-3 drops of H_2O_2 was added onto the smear.
3) Mixed with a toothpick.
4) Rapid formation of air bubbles indicated positive result.

3.2. Bacterial growth on Manitol salt agar:

Manitol salt agar (MSA) was used as a selective medium for isolation of staphylococcal strain. MSA allows growth of only those bacteria which can able to tolerate high concentrations of sodium chloride and prevent the growth of other bacterium. It contains 7.5% sodium chloride. In the present study the bacterial growth on manitol salt agar media was observed following the method of Davis *et al.* (2006).

Materials:

- Manitol salt agar powder (HIMEDIA Chemicals, India).
- Pure culture of bacterial strain (maintained at CSIR-NEIST, Jorhat).
- Sterile Petri plates.
- Laminar air flow chamber.

The ingredients of Manitol salt agar (ready to use) are:

1) Enzymatic Digest of Casein ..... 5 g
2) Enzymatic Digest of Animal Tissue ..... 5 g
3) Beef Extract ..... 1 g
4) D-Mannitol ..... 10 g
5) Sodium Chloride ..... 75 g

6) Phenol Red..... 0.025 g

7) Agar..... 15 g

Final pH (25 ^{o}C) 7.4 ± 0.2.

3.3. Antibiotic sensitivity test:

Antibiotic sensitivity test was performed following the method as described elsewhere (www.microrao.com/micronotes/coagulase.pdf). There are several procedures through which one can find out the resistance of a particular bacterial strain towards antibiotics. In this case I choose attempted the qualitative method to find out the sensitivity of the isolated bacterial strain.

3.3.1. Qualitative Methods:

Qualitative methods are categorizing a bacterial isolate as sensitive, intermediate or resistant towards a particular antibiotic.

3.3.2. Disk diffusion test:

In disk diffusion method a particular bacterial isolate is allowed to spread on an agar plate. Then different types of paper disc containing specific concentration of an antibiotic are placed on it and along with the antibiotic disk the agar plate is incubated at 37^{o}C for overnight. Strains resistant to an antibiotic grow up to the margin of disk. A zone of inhibition will appear indicating the absence of the bacterial strain around the disk which means the isolate is susceptible to the specific antibiotic. If the strain is resistant to the antibiotic then the bacterial growth can be seen up to the margin of the disk. Then the diameter of the zone of inhibition will be measured to find out whether the strain is sensitive, intermediate or resistant.

Materials:

- Muller - Hinton agar powder (Tulip Diagnostics, India).
- Pure culture of bacterial strain (maintained at CSIR-NEIST, Jorhat) .

- Sterile Petri plates.
- Laminar air flow chamber.

The ingredients of Mullar- Hinton agar (ready to use) are:

1) Casein acid hydrolysate
2) Beef infusion
3) Starch-1.5
4) Agar-17.0

Final pH (25^{o}C) 7.3 ± 0.2

Procedure:

1) Suspend 38 gms of the Mullar – Hinton agar powder in 1000 mL of distilled water.
2) Mixed thoroughly.
3) Boiled with frequent agitation to dissolve the powder completely.
4) Sterilized by autoclaving at 121^{o}C for 15 minutes at 15 lbs pressure.
5) Mixed well before pouring.
6) Inoculated a loopful of pure culture to peptone water.
7) With the help of a sterile cotton swab it was then swabbed into Muller-Hinton agar plate.
8) Different antibiotic disc were putted on it.
9) Along with the antibiotic disc it was incubated for around 24 hrs at 37^{o}C.
10) Zone of inhibition was observed and measured.

3.4. Coagulase test:

Coagulation test with some modification was performed following the method as described elsewhere (www.microrao.com/micronotes/coagulase.pdf). The clumping factor is also known as bound coagulase. It cross-links both α and β chain of fibrinogen in plasma to form fibrin clot that deposits on the cell wall. As a result,

individual coccus sticks to each other and clumping is observed. Some strains of *staphylococcus* may not produce bound coagulase, and such strains must be identified by tube coagulase test.

Materials:

- Clean test glass slides.
- Marking pencil.
- Pure culture of bacterial strain (maintained at CSIR-NEIST, Jorhat).
- Laminar air flow chamber.
- Applicator stick.

Procedure:

1) Dense suspensions of *Staphylococci* was made from the pure culture are made on two ends of clean glass slide.
2) One end was labelled as ―test‖ and the other as ―control‖.
3) The control suspension serves to rule out false positivity due to auto-agglutination.
4) The test suspension was treated with a drop of citrated plasma.
5) Mixed thoroughly using a applicator stick.
6) Agglutination or clumping of cocci within 5-10 seconds was taken as positive.
7) Positive result was recorded in the present study.

3.5. Culturing of the bacterial strain:

The Bacterial strain was inoculated in soybean casein digest medium to get sufficient amount of cell free extract of the coagulase protein as per the method previously described by Ritsu Sonohara *et al*. (1995).

Materials:

- Fluid soybean casein digests powder (HIMEDIA Chemicals, India).
- Pure culture of bacterial strain (maintained at CSIR-NEIST, Jorhat) .

- 250 mL conical flasks.
- Laminar air flow chamber.

The ingredients of soybean casein digest agar media (ready to use) are:

1) Casein peptone- 17 g/L
2) Soybean peptone- 3 g/L
3) Sodium chloride- 5 g/L
4) Dibasic potassium phosphate- 2.5 g/L
5) Glucose- 2.5 g/L

Procedure:

1) Pure culture of the bacterial strain was maintained in fluid soybean casein digest agar slants at CSIR-NEIST, Jorhat.
1) 6 g of fluid soybean casein digest media powder was suspended in 200 mL distilled water and then taken in two conical flasks.
2) After the media was dissolved completely in distilled water, it was plugged tightly with cotton and sterilized by autoclaving at 15 lbs pressure at 121°C for 15 minutes.
3) A loopful of the bacterial strain was inoculated into fluid soybean casein digest medium from a pure culture of the bacterial strain inside a laminar air flow chamber under sterilized conditions.
4) The broth with the bacterial strain was kept in a shaker incubator at 200 rpm for around 48 hours at 28°C.

3.6. Estimation of total CFU:

Estimation of the number of colony forming unit (CFU) was done as per the method described by Wacup (1951).

Materials:

- Soybean casein digests medium powder (HIMEDIA Chemicals, India).

- Soybean casein digests agar powder (HIMEDIA Chemicals, India).
- Sterile distilled water.
- Sterile cotton.
- Glass marking pencil.
- Pure culture of bacterial strain (maintained at CSIR-NEIST, Jorhat) .
- 250 mL conical flasks.
- Laminar air flow chamber.
- Clean test tubes.

The ingredients of Fluid soybean casein digest medium (ready to use) are:

1) Pancreatic digest of casein ..... 17.00 g/L
2) Peptic digest of soybean ..... 3.00 g/L
3) Sodium chloride ..... 5.00 g/L
4) Potassium dihydrogen phosphate..... 2.50 g/L
5) Glucose monohydrate ..... 2.50 g/L

Final pH (25^0C) 7.3 ± 0.2

The ingredients of soybean casein digest agar media (ready to use) are:

1) Pancreatic digest of casein…… 15.00
2) Paptic digest of soyabean meal ……....5.00
3) Sodium chloride …… 5.00
4) Agar…… 15.00

Final pH (25 ^{o}C) 7.4 ± 0.1

Procedure:

1) 200 mL of sterilized broth was taken in a clean conical flask.
2) A loopful of pure culture of the bacteria was inoculated into it laminar air flow chamber.
3) 7 clean test tubes was taken and labelled as 1 to 7 on it.

4) 9 ml of sterilized double distilled water was taken into it.
5) 1 ml of inoculated broth was added to the first test tube and mixed using vortex.
6) 1ml of the solution from the first test tube was transferred to the second test tube and mixed using vortex.
7) The process was continued up to the 7^{th} test tube.

From the preparation 0.1 ml of the solution from 7^{th} tube was added into soybean casein digest medium and mixed thoroughly using vortex.

8) 5 numbers of sterile petri plates was taken.
9) 20 ml of the media with the strain was poured on it.
10) The plates were allowed to get soiled and sealed.
11) The sealed plates were then kept in an incubator for 72 hours to grow the strain properly.
12) Then the colonies were counted using a colony counter.

The total plate count was done using the formula described below.

Total plate count = total number of colonies in average x dilution factor x 10/ml of water.

3.7. Characterization of bacterial strain by Gram staining:

The Gram staining of the bacterial strain was performed to characterize the bacterial strain following the method of Hucker and Conn (1923). Kits and reagents procured from HiMedia Laboratories, India were used for this study.

Procedure:

1) A glass slide was cleaned with a mixture of ethanol and water in the ratio of 1:1.
2) A drop of sterile double distilled water was placed on the slide.
3) The inoculation loop was made sterile and a loopful of bacterial culture (24 hrs overnight culture is preferred) was placed on the slide.
4) A smear was made.
5) The smear was allowed to dry in air.

6) The air dried sample was fixed by passing over flame 3-4 times. The slide was allowed to cool.
7) The smear was completely covered with crystal violet stain for 1 min.
8) The stain was poured off and it was covered with Gram's iodine solution for 1 min.
9) The stain was drained off from the slide and washed with distilled water.
10) The slide was decolourised with alcohol and acetone (1:1) for 10 seconds till purple stain just stops coming out of the slide.
11) The slide was washed with distilled water till stain stopped coming out of the film.
12) The slide was then flooded i.e. decolourised with counter stain, safranine for 10 seconds. Then the slide was washed with distilled water.
13) Water was drained off and the slide was allowed to dry in air and then observed under phase contrast microscope.

3.8. Isolation of extracellular proteins from bacterial strain:

3.8.1. Acetone precipitation of proteins:

Procedure:

Generally protein sample absorbed with a number of contaminants that may interfere with downstream applications. There are several strategies which are available to eliminate these substances from protein samples. Using dialysis technique or by gel filtration technique the Small soluble substances may be removed from the mixture. There are some other strategies that exist for removing undesirable substances from the mixture which is by adding a suitable chemicals/compound that allow protein to precipitate. Protein samples can be separated by centrifugation and the supernatant that containing the interfering substance has been removed. The protein

pellet is then re-dissolved in suitable buffer compatible with the downstream application.

Acetone precipitation of the protein was carried out as per the method of Visith Thongboonkerd *et al.* (2002). The *staphylococcal* strain were taken along with the broth and centrifuged at 8000 rpm for 10 minutes at 4°C. The supernatant (protein sample) was collected in separate acetone compatible tubes and the pellet (media) was discarded.

Reagents and materials:

- Bacterial strain in Nutrient agar media
- Refrigerated Centrifuge machine
- Rotor
- Centrifuge tubes
- Acetone (cooled to -20°C)
- Acetone compatible tubes
- Refrigerated Centrifuge machine Rotor
- 0.02M phosphate buffer (pH 7)

Procedure:

1) Acetone was cooled to -20°C.
2) The supernatant (protein sample) collected from the bacterial strain, was kept in acetone compatible tubes.
3) 50% of chilled acetone (-20°C) was added to the supernatant in the tubes.
4) The tubes were vortexed and incubated at -20°C overnight.
5) The supernatant along with the acetone was centrifuged at 8000 rpm for 10 min at 4°C.
6) The supernatant was disposed off properly and care was taken not to dislodge the protein pellet. (Centrifugation repeated if pellets do not occur).

7) The acetone was allowed to evaporate from the uncapped tubes at room temperature for 30 minutes. (Over drying the pellet was prevented or else the pellet may not dissolve properly).
8) The pellet was completely dissolved in minimum amount of 0.02M phosphate buffer of pH 7.2. This is the required crude protein.

3.8.2. Ammonium sulphate precipitation and dialysis:

The salt induced precipitation is one of the common types of precipitation of protein. Ammonium sulphate is commonly used salt for the precipitation of proteins because of its solubility, it is less expensive, commonly available in highly pure level and it does not denature proteins. Protein contain some polar amino acids such as serine, glycine etc. Normally hydrophilic amino acids in native proteins are resides on the surface of the proteins and hydrophobic amino acids are buried. The interaction between the nearby oppositely charged groups is ion pairs or salt bridges. Earlier investigations have discovered the fact that in folded proteins there are four attractive ion pairs and one repulsive ion pair are present per one hundred amino acids. Water interacts with these surface amino acids as a powerful solvent and keeps them in solution. Though the solubility of protein depends upon several factors but it is varies according to the ionic strength and to the salt concentration of the solution.

Ammonium sulphate precipitation technique is used to get the cell free extract of coagulase protein was performed following the method as described by Green and Hughes (1955). The Crude protein was saturated with different concentrations of Ammonium sulphate. The precipitated protein obtained was dissolved in Phosphate buffer (0.02M, pH 7.2).

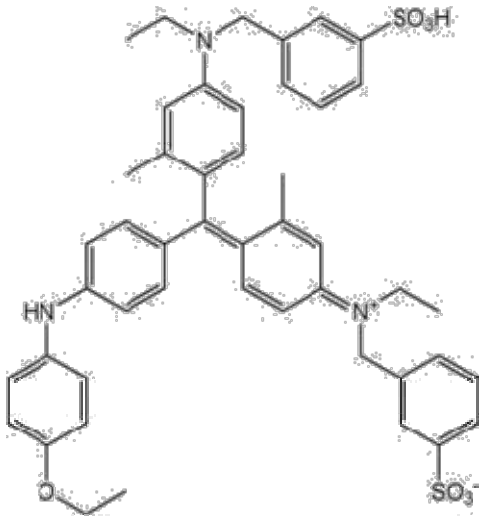
The protein samples collected after ammonium sulphate precipitation was dialyzed using dialysis membrane – 50 (Av. Flat width 24.26 mm, Av. Diameter 14.3 mm and the capacity approximately 1.61ml/cm, Himedia) against Phosphate buffer (0.02M, pH 7.2) to remove excessive salts and stored at 4°C.

3.9. Estimation of extracellular protein concentration:

The protein content in the crude extract was estimated by the method described by Bradford (1976).

Principle:

The Bradford assay is a protein determination method that involves the binding of Coomassie Brilliant Blue G-250 dye to proteins (Bradford, 1976). The dye exists in three forms: cationic (red), neutral (green), and anionic (blue) (Compton and Jones 1985). Under acidic conditions, the dye is predominantly in the doubly protonated red cationic form (Amax = 470 nm). However, when the dye binds to protein, it is converted to a stable unprotonated blue form (Amax = 595 nm) (Reisner *et al.* 1975, Fazekes de St. Groth *et al.* 1963, Sedmack and Grossberg 1977). It is this blue protein-dye form that is detected at 595 nm in the assay using a spectrophotometer or microplate reader. Work with synthetic polyamino acids indicates that Coomassie Brilliant Blue G-250 dye binds primarily to basic (especially arginine) and aromatic amino acid residues (Compton and Jones, 1985). Spector (1978) found that the extinction coefficient of a dye-albumin complex solution was constant over a 10-fold concentration range. Thus, Beer's law may be applied for accurate quantitation of protein by selecting an appropriate ratio of dye volume to sample concentration. Certain chemical-protein and chemical-dye interactions interfere with the assay. Interference from non-protein compounds is due to their ability to shift the equilibrium levels of the dye among the three colored species. Known sources of interference,such as some detergents, flavonoids, and basic protein buffers, stabilize the green neutral dye species by direct binding or by shifting the pH (Compton and Jones, 1985; Fanger, 1987). Nevertheless, many chemical reagents do not directly affect the development of dye colour when used in the standard protocol.



Materials:

- Spectrophotometer capable of measuring absorbance in the 595 nm region.
- Test tubes, 13 x100 mm
- 3 ml Disposable Plastic Cuvettes
- 1 ml Disposable Plastic Cuvettes
- Protein Standard (BSA) Solution, (2 mg/ml)
- Protein Standard (BSA) Solution, (1 mg/ml)
- Protein with Unknown concentration

Procedure:

1) The Bradford Reagent was brought to room temperature and gently mixed before use. Bradford assays are routinely performed at room temperature.
2) Three test tubes were taken labelled as blank (consists of buffer with no protein), a protein standard (known concentration of protein), test (an unknown sample).

3) 0.1 ml of the protein sample and 3 ml of the Bradford Reagent was added per tube.
4) A protein standard of appropriate concentrations in the same buffer as the unknown Samples was prepared. The standards were created by serially diluting either the 2 mg/mL or 1 mg/mL of BSA protein standard.
5) A dilution scheme was prepared to ensure the concentration is within the linear range of 0.1–1.4 mg/mL for the protein samples with unknown concentrations. Tube 6 was taken represents an unknown sample with a 2-fold dilution.

Tube No.	Sample(ml)	BSA protein standard (mg/mL)	Bradford reagent (mL)
1	0.1	0	3
2	0.1	0.25	3
3	0.1	0.5	3
4	0.1	1.0	3
5	0.1	1.4	3
6	0.1	Unknown	3

6) After adding 3 mL of Bradford Reagent was added to each tube, it was then vortexed gently for thorough mixing. The total liquid volume in each tube is 3.1 mL.
7) The samples are then incubated at room temperature for 5minutes.
8) The samples were then transfer into cuvettes.
9) Measured the absorbance at 595 nm.
10) A standard curve was created by plotting the 595 nm values (y-axis) versus their concentration in mg/mL (x- axis).
11) Determination of the protein concentration in the unknown sample was done using the standard curve.

3.10. Column chromatography:

The chromatographic separation of crude protein was done using Sephadex G-75. A column with a height of 0.25 m, diameter 0.02 m and area 1.5714 cm^2 was packed with Sephadex G-75 and equilibrated with 0.02 M phosphate buffer (pH 7.2). One ml of dialyzed protein was allowed to pass through the column until the sample was completely eluted out. 3 ml of fractions were collected separately (one ml in each tube) and Spectrometric readings were taken at 280 nm and the observations recorded and fractions were lyophilised for further tests. In the present study the method was obtain form http://web.uvic.ca/~berryde/techniques/column%20chrom.pdf.

3.11. Lyophilization:

The three protein fractions that were collected from column chromatography and the crude protein isolated from the staphylococcal strain were collected separately in round bottom flasks. Then these flasks were taken and lyophilized using a lyophilizer / freeze dryer at – 70oC till the samples were dried to powder form.

3.12. Blood Coagulating Activity (Bioassay):

Blood coagulation activity of the protein was done as per of Lee-White method.

1. Reagents and Materials
2. Clean glass slide
3. Fresh human blood
4. Five fractions of the lyophilized extracellular proteins of Bacterial strain.
5. Spatula
6. Ethanol
7. Marker pen
8. Stop watch

Briefly, saline water which was taken as control. Crude protein isolated by acetone and ammonium sulphate precipitation was taken in two different slides. Similarly a bit of lyophilised protein sample was placed on a third glass slide and a drop of fresh blood serum was added to each slide. After mixing the both substance coagulation time was recorded.

3.13. Sodium dodecyl sulphate- Polyacrylamide gel electrophoresis (SDS-PAGE):

Sodium dodecyl sulphate- polyacrylamide gel electrophoresis (SDS-PAGE.) technique was applied for determination of molecular weight as well as to cheek the purity of the protein isolated from the staphylococcal strain. After lyophilisation, protein fractions were dissolved in sample buffer and subjected to SDS-PAGE along with standard protein markers as described by Laemmle (1970).

Principle:

SDS is an anionic detergent which binds strongly and denatures proteins. The number of SDS molecules bound to a polypeptide chain is approximately half of the number of amino acid residues in that chain. The protein SDS complex carries net negative charges; hence more towards the anode and the separation is based on the size of the protein.

Sodium dodecyl sulfate (SDS or NaDS), sodium laurel sulphate, or sodium lauryl sulfate (SLS) is an organic compound with the formula $CH_3(CH_2)_{11}OSO_4Na$. It is an anionic surfactant used in many cleaning and hygiene products. The salt is of an organo sulfate consisting of a 12-carbon tail attached to a sulfate group, giving the material the amphiphilic properties required of a detergent. Being derived from inexpensive coconut and palm oils, it is a common component of many domestic cleaning products. Sodium coco-sulfates is essentially the same compound, but made from less purified coconut oil.

SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis, describes a technique widely used in biochemistry, forensics, genetics and molecular biology to separate proteins according to their electrophoretic mobility (a function of

the length of a polypeptide chain and its charge) and no other physical feature. SDS is an anionic detergent applied to protein sample to linearize proteins and to impart a negative charge to linearized proteins. In most proteins, the binding of SDS to the polypeptide chain imparts an even distribution of charge per unit mass, thereby resulting in a fractionation by approximate size during electrophoresis.

Reagents:

- **30% Acryl amide, and 0.8% Bisacrylamide (Himedia, India):**

30 gm of acrylamide and 0.8 grams of N, N methylene bisacrylamide were mixed and dissolved in a total volume of 100 mL distilled water. The solution was filtered through 0.45 μm whatman filter and stored in dark colored bottle.

- **1M Tris-Cl buffer (pH 8.8) (Himedia, India):**

12.114 gm of Tris-buffer was weighed and dissolved in 50 mL of distilled water. pH was adjusted to 8.8 with 1N HCl and made up to 100 mL with distilled water. It was filtered through 0.45 μm Whatman filter paper and stored in dark colored bottle at 40C.

- **1M Tris–Cl buffer (pH 6.8):**

12.114 gm of Tris–Cl was weighed and dissolved in 50 mL of distilled water. pH was adjusted to 6.8 with 1N HCl and made up to 100 mL with distilled water. It was filtered through 0.45 μm Whatman filter paper and stored in dark colored bottle at 4°C.

- **Ammonium per sulphate (10 % w/v) (Himedia, India):**

0.1 gm of ammonium persulfate were dissolved in 1.0 mL of distilled water (Freshly prepared).

- **10% SDS (w/v) (Himedia, India):**

10 grams of SDS was dissolved in 100 mL of distilled water, stored at room temperature.

- **Coomassie Blue Solution (Staining solution) (Sigma, USA):**

0.1 grams of Coomassie Brilliant Blue R-250 was dissolved in 40 mL methanol (G. S. Chemicals, India). To this 10 mL acetic acid and 40 mL distilled water was added.

➢ **Destaining Solution:**

40 L methanol and 10 mL acetic acid was mixed with 50 mL distilled water.

➢ **TEMED (Himedia, India)**:

N, N, N/, N/-tetramethylene diamine, the chain initiator

Gel contents:

Gel composition	10%	11%	12%
29.2% Acrylamide , 0.8% Bisacrylamide	6.67 ml	7.33 ml	8 ml
0.4% SDS, 1.5M Tris-Cl pH8.8	5 ml	5 ml	5 ml
Distilled water	8.33 ml	7.66 ml	7 ml

Stacking gel:

- Water - 6.3mL
- 30% Acryl amide - 1.7 mL
- 1.5 M Tris (pH 6.8) - 1.25 mL
- 10% SDS - 0.1 mL
- 10% APS - 0.1 mL
- TEMED - 0.01 mL

Loading dye:

- Tris buffer (pH 6.8) – 5 mL
- SDS – 0.5 g
- Glycerol – 2 mL
- B-marcaptoethanol – 0.25 mL
- Bromophenol blue – 1 mL

Make up the volume to 10 mL with distilled water.

Experimental Procedure:

3.13.1. Pouring the Separating Gel:

The gel plates were assembled using two clean glass plates and spacers and locked to the casting stand. The separating gel slurry was prepared using the reagents mentioned above. The mixture was gently swirled for mixing and poured in the chamber between the glass plates. Distilled water was layered on the top of the gel and left to polymerize for 30 minutes at room temperature.

3.13.2. Pouring the Stacking gel:

After polymerization, layer of water was poured off completely and stacking gel slurry was prepared using the reagents mentioned above. The slurry was mixed gently and poured on the top of the separating gel. A Teflon comb was inserted into the layer of stacking gel and it was allowed to polymerize for 45 minute at room temperature (approx. 30 min).

3.13.3 Preparation of samples for electrophoresis:

100 µL of crude sample was mixed with 50 µL loading dye in a 1.5 mL tube. Mixed well and then this sample is boiled in hot water bath for 2-3 minutes. The sample was then centrifuged at 3000 rpm for 3 minutes. The supernatant was collected.

3.13.4 Loading the samples to the well:

After polymerization of stacking gel, Teflon comb was removed gently and wells formed were filled with SDS electrophoretic buffer. To the buffer filled wells, 150 µL of the sample was loaded with micropipette. Similarly 100 µL marker protein was also loaded in one of the well.

3.13.5 Running the gel:

The power supply was connected to the cell and run at 15 mA of constant current until tracking dye (Bromophenol blue) enters the separating gel. Then the

current was increased to 30 mA. After the bromophenol blue dye reached the bottom of the separating gel, the power supply was disconnected.

3.13.6 Disassembling the gel and staining the gel:

The buffer from the upper buffer chamber was poured off and the plates were removed gently. The gel was exposed and was carefully immersed in staining solution overnight with uniform shaking on a shaker. The proteins absorb the Coomassie brilliant blue.

3.13.7 Destaining the gel:

The staining solution was poured off and gel was dipped in destaining solution with uniform gentle shaking. Dye not bound to protein was removed. The destaining solution was changed frequently until the background of the gel was colourless. Five blue colored bands were observed in the crude protein of the bacterial strain using the gel documentation system (transilluminator). The gel was observed and then photographed.

3.14. HPLC fractionation:

High pressure liquid chromatography (HPLC) technique was applied using high-pressure liquid chromatography (HPLC)apparatus (Waters 600 controller, Waters inline degasser Symmetry C-18 column 5µm with height and width 4.6 x 250mm, setup with WatersTM 600 Pump and Waters 2489 UV visible detector) to the lyophilized protein samples to cheek the purity of it and also for different individual amino acids was quantified from the protein fractions collected from column chromatography as per the method of Turnel *et al.* (1982).

Principle:

High-performance liquid chromatography (sometimes referred to as high-pressure liquid chromatography, HPLC) is a chromatographic technique used to separate a mixture of compounds in analytical chemistry and biochemistry with the

purpose of identifying, quantifying and purifying the individual components of the mixture. Some common examples are the separation and quantitation of performance enhancement drugs (e.g. steroids) in urine samples, or of vitamin D levels in serum.

Procedure:

HPLC typically utilizes different types of stationary phases (i.e. sorbents) contained in columns, a pump that moves the mobile phase and sample components through the column, and a detector capable of providing characteristic retention times for the sample components and area counts reflecting the amount of each analyte passing through the detector. The detector may also provide additional information related to the analyte, (i.e. UV/Vis spectroscopic data, if so equipped). Analyte retention time varies depending on the strength of its interactions with the stationary phase, the composition and flow rate of mobile phase used, and on the column dimensions. HPLC is a form of liquid chromatography that utilizes small size columns (typically 250 mm or shorter and 4.6 mm or smaller; packed with smaller particles), and higher mobile phase pressures compared to ordinary liquid chromatography.

With HPLC, a pump (rather than gravity) provides the higher pressure required to move the mobile phase and sample components through the densely packed column. The increased density arises from the use of smaller sorbent particles. Such particles are capable of providing better separation on columns of shorter length when compared to ordinary column chromatography.

For purification, the fraction obtained from Sephadex G-75 was subjected to HPLC and C-18 column was used for analysis. The flow rate was set up at 1 ml/min. Absorbance was taken at 280 nm. The 25µl protein sample was injected and run for 20 minutes to get separate the protein from the sample and prepared the chromatogram. The same protein was used to run with a standard protein sample to compare the molecule of the protein sample.

Materials:

- Extracellular protein isolated from the bacterial strain
- 6 N HCl containing 1% phenol.
- Distilled water
- Triethylamine
- PITC Whatmann nylon membrane filters
- Deproteinization solution (5ml of 6N HCl containing 1% phenol).

It was prepared by taking 50ml out of 12N HCl (Himedia) and adding it to 50ml of distilled water, and finally 1% of phenol.

Re-drying solution:

This was prepared by adding Ethanol, Water, and triethylamine together at ratio 2:2:1.

Derivatization solution:

Ethanol	:	Triethylamine	:	Water	:	PITC (Phenylisothiocyanate)
7	:	1	:	1	:	1

Sample Preparation:

Individual amino acids were quantified from the protein fractions isolated from the bacterial strain, collected using column chromatography. 400 µL of the protein fractions were taken separately in wide mouthed test tubes and 5 mL of 6 N hydrochloric acid containing 1% phenol were added to it. Then the mouths of all the test tubes were sealed. The protein samples along with hydrochloric acid and phenol were digested for about 24 hours in boiling water bath. After digestion, each sample was filtered using Whatmann-1 filter paper and 1.5 mL sample from the filtrate was taken and 300 µL of redrying solution was added to it. All the samples were vortexed and then boiled in boiling water bath for 2 hours. Then 60 µL of derivatizing solution

was added. It was mixed by vortexing and dried in hot water bath to remove traces of PITC. The samples were filtered using Whatmann nylon membrane filters (0.45 μm) of 25 mm. The filtrate was centrifuged at 6000 rpm for 5 min to precipitate any impurities if present. The supernatant is collected.

Experimental procedure:

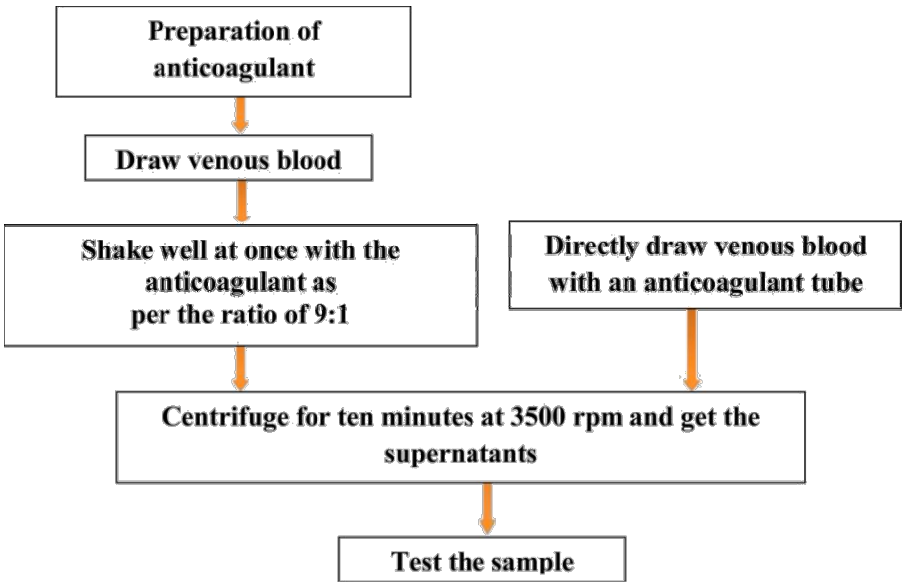
Eluent 1 and eluent 2 are used for free amino acid analysis. Symmetry® C-18 column 5 μm with height and width 4.6 × 250 mm was used. HPLC set up had WatersTM 600 Pump and Waters 2489 UV visible detector. 25 μL of protein sample was injected to the CI8 column. All the samples were run for 20 minutes and the graph was recorded. The absorbance was taken in 254 nm.

3.15. Coagulation assay of the purified protein sample:

Coagulation assays other then the slide coagulation test were performed to cheek the alteration of the other coagulation parameter of human plasma sample. The parameters including Prothrombin time (PT), activated partial thromboplastin time (APTT) , thrombin time (TT), fibrinogen (Fib) assay were considered for this present study. The blood samples were collected from the clinical centre of CSIR–NEIST Jorhat. "Coag 2051" coagulation analyzer from analytical technologist ltd. was used for this purpose. Reagents from "SUNBIO", INDIA were used for coagulation assay of the coagulase protein.

3.15.1. Sample preparation:

There are two methods for sample collection, as illustrated in the following flowchart



1) The blood sample was mixed thoroughly with the anticoagulant.
2) Centrifugation of the blood sample was performed as soon as possible at 3500 rpm for ten minutes.
3) Plasma was collected in a separated test tube.
4) The test tube with the plasma was plugged and sealed promptly to avoid increase of pH.
5) The coagulation assay was performed within 60 minutes of the collection of the sample.

3.15.2. Prothrombin time (PT) assay:

Materials:

- Prothrombin time (PT) assay kit
- Clean test tube
- Sterilized micotips
- Coagulation analyzer

Procedure:

1) Preheated the Prothrombin time reagents at 37^{O}C for ten minutes.
2) 20 µL of plasma added carefully at the bottom to a tube avoiding the air bubbles and preheated for two minutes.
3) The preheated samples then putted into the testing channel and aspirate 40 µL of the Prothrombin time reagent with the help of the online pipette.
4) After pressing the "OPTIC key" on the coagulation analyzer the screen displayed as "please add the reagent" command and then reagent was added(the pipette was pressed down thoroughly until a beep sound was heard then the instrument start measurement.
5) Approximately 80 sec later, the screen displayed the result.
6) Step 2-5 was repeated continuously to continue the readings.
7) Results were recorded.

3.15.3. Activated partial thromboplastin time (APTT) assay:

Materials:

- Activated partial thromboplastin time (APTT) assay kit
- Clean test tube
- Sterilized micotips
- Coagulation analyzer

Procedure:

1) The $CaCl_2$ solution of activated partial thromboplastin time (APTT) reagent kit was preheated at 37^{O}C for ten minutes.
2) 20 µL of plasma was added to 20 µL of the activated partial thromboplastin time reagent then mix them and preheated at 37^{O}C for three minutes.
3) The preheated mixture was placed into the testing channel and 20 µL of the 0.025 mol/L $CaCl_2$ solution (preheated) was aspirated with the help of the online pipette provided with the coagulation analyzer.

4) After pressing the "OPTIC" key on the coagulation analyzer screen display "please add the reagent" command in to CHX and then $CaCl_2$ solution was added into the tube until a beep sound was heard, and then the instrument starts measuring.
5) Approximately 100 seconds later, the screen displays the result.
6) Step 2-5 was repeated for continuous assay.
7) Results were recorded.

3.15.4. Thrombin time (TT) assay:

Materials:

- Thrombin time (TT) assay kit
- Clean test tube
- Sterilized micotips
- Coagulation analyzer

Procedure:

1) Add 30 µL of plasma was added to a tube avoiding air bubbles and preheated for two minutes.
2) The preheated tubes were then placed into the testing channel and aspirate 30 µL of the thrombin time reagent with the help of the online pipette.
3) After pressing the "OPTIC‖ key on the coagulation analyzer the screen displayed display "please add the reagent" command into the test channel, the thrombin time test reagent was added to the tube.
4) Then the instrument started measuring.
5) Approximately in 60 second latter, the screen displayed the test result.
6) Steps 2-5 were repeated for continuing the readings.

3.15.5. Fibrinogen assay (FIB):

Materials:

- Fibrinogen assay (FIB) assay kit
- Clean test tube
- Sterilized micotips
- Coagulation analyze

Procedure:

1) 40 µL of the diluted test plasma was put it into a tube, and then it was preheat for three minutes.
2) 20 µL of the fibrinogen assay reagent was aspirated with the help of the online pipette attached to the coagulation analyzer.
3) After pressing the "OPTIC‖ key on the coagulation analyzer the screen displayed "please add the reagent" command into the test channel and then the fibrinogen assay reagent was add into the tube.
4) Then the instrument started measuring.
5) Approximately 60 seconds later the screen displayed the result.
6) Steps 1-4 were repeated for continuing the readings.

3.16. Computational analysis of coagulase protein using bioinformatics tools:

3.16.1. Translation of nucleotide sequence to amino acid:

The sequence of coagulase in *Staphylococcus* strain was obtained from NCBI having accession number ―EU246837.1_4‖ of *Staphylococcus sp. Cobs2Tis23* 16S ribosomal RNA gene, partial sequence; in FASTA format and used the ―Transeq‖ tool (http://www.ebi.ac.uk/Tools/st/emboss_transeq/) for conversion to amino acid sequence. We have chosen all the six open reading frame and selected standard codon table during launching of the tool, keeping other parameters by default.

3.16.2. Preliminary characterization:

A few bioinformatic tools and database were used to search out some insight and prediction about the coagulase in the Staphylococcal strain. The preliminary characterization including the amino acid composition, molecular weight, theoretical pI, atomic composition, instability index, aliphatic index and grand average of hydropathicity (GRAVY) of the protein can be calculated through theoretical computation from an available amino acid sequence. In the present study, the primary characterization of staphylocoagulase was done using the expasy proteomics tools program at http://au.expasy.ch/tools.

3.16.3. Protein Theoretical Model Generation:

Template selection through PDB-BLAST:

The query sequence of coagulase from *Staphylococcus* strain was searched to find out the related protein structure to be used as a template(s) by the BLAST program [www.ncbi.nlm.nih.gov/BLAST] against Protein Data Bank database with default parameters.

As in our template search we found the templates having less than 35% identity, so we prefer the multiple template based model generation approach of MODELER 9v8 software. MODELLER implements comparative protein structure modelling by satisfaction of spatial restraints and can perform many additional tasks, including de novo modelling of loops in protein structures. The best templates found during PDB-BLAST were used as multiple templates.

3.16.4. Model Evaluation and Refinement:

There are different software tools and Meta servers are available for validation and evaluation of a generated model. Structural evaluations of the generated structure of the Staphylococcal coagulase were performed by using two programs called PROCHECK and ERRAT. The predicted model was submitted to the Meta server ―SAVES‖ (http://nihserver.mbi.ucla.edu/SAVES/) for structural evaluation.

Evaluation of a model is important to check the overall fold/structure, bond lengths and angles, errors over localized regions and other stereo-chemical parameters.

3.16.5. Protein Disorder region analysis and Toxicity prediction:

There are numerous insilico tools are available, which can predict the immunogenicity and toxicity of the peptides. Herein we used the ToxinPred tool, a unique in silico method useful in predicting toxicity of peptides/proteins developed by CSIR-IMT, Chandigarh. In addition, it will also be useful in designing least toxic peptides and discovering toxic regions within the proteins (Kapur *et al.,* 2012). Here, we have used the three modules of Designing Peptides, Batch Submission and Protein Scanning for toxicity analysis.

3.16.6. Binding Sites prediction:

The binding sites of the staphylococcal coagulase were predicted though Discovery Studio 4.0 Molecule Viewer which predicts the possible binding sites in receptor protein.

3.17.1. Isolation of genomic DNA from the bacterial strain:

Reagents:

- 24 hours fresh culture (broth) of bacterial strain
- Tris-EDTA buffer
- 10% (w/v) SDS
- 20 mg/mL proteinase-K
- 5 M NaCl
- CTAB/ NaCl solution
- Chloroform
- Isoamyl alcohol
- Buffered phenol
- Isopropanol
- 70% (w/v) ethanol

Experimental procedure:

1) 25 mL broth culture of bacterial strain was grown until saturated. 1.5 mL of each broth was taken separately in Eppendorff tubes and microfuged for 10 min at 10000 rpm. Pellet was formed. The pellet was resuspended in 657 µL TE buffer.
2) 30 µL of 10% SDS and 3 µL of 20 mg/mL proteinase K was added and mixed thoroughly (vortexed) and incubated for 1 hour at 37 °C.
3) 100 µL of 5 M NaCl was added and mixed thoroughly.
4) 80 µL of CTAB/ NaCl was added and mixed thoroughly and incubated for 10 min at 65°C.
5) 1 volume of chloroform: isoamyl alcohol in the ratio of 24: 1 was added and mixed thoroughly and microfuged for 4–5 min. Supernatant was transferred to a fresh tube.
6) 1 volume of phenol: chloroform: isoamyl alcohol in the ratio of 25: 24: 1 was added to the supernatant, extracted thoroughly and microfuged for 5 min. The supernatant was transferred to a fresh tube.
7) 0.6 volume of Isopropanol was added and mixed thoroughly until a stringy white precipitate was formed. The pellet was transferred to fresh tube containing 70% ethanol.
8) The pellet was microfuged for 5 min at room temperature and the pellet was dried. The pellet (DNA) was stored in refrigerator.

3.17.2. Preparation of agarose gel for agarose gel electrophoresis:

Method:

- 1% Agarose
- 100 mL of 10 X TBE Buffer (10 X TE buffer from HIMEDIA Chemicals is made 1 X)
- 4 µL Ethidium bromide

Procedure:

1) 0.5 grams of agarose (from HIMEDIA Chemicals) was weighed and dissolved in 50 mL of 1 X TBE buffer. It was dissolved well.
2) Add 4 µL ethidium bromide.
3) The solution was poured in the Agarose gel electrophoretic apparatus (Genei, Bangalore).
4) The comb was inserted.
5) The gel was allowed to solidify.
6) The DNA samples isolated from bacterial strain were dissolved in 20 µL of TE buffer. It was mixed well.
7) The DNA samples were dissolved in 5 µL loading dye.
8) After the agarose gel is solidified, the comb is slowly taken out.
9) The TBE buffer is poured into the apparatus.
10) Then the DNA samples from bacterial strain and PGPR was loaded onto the gel.
11) The power supply was connected to the apparatus. Voltage of 100 volts and current of 75 mA was applied.
12) After the loading dye reached the bottom of the gel, the power supply was disconnected.
13) Then the DNA samples were seen in the Gel documentation system (transilluminator).

3.17.3. PCR amplification and sequencing:

The 16S rDNA sequencing of bacterial strains was done at Bangalore Genei, Bangalore, India. Approximately 1.5 kb 16S rDNA fragment was amplified using consensus primers and Taq DNA polymerase. The PCR product was bi-directionally sequenced using the forward, reverse and an internal primer. The sequenced data were aligned and analysed for finding the closest homologue for the bacteria. Alignment was developed using a combination of NCBI GenBank and RDP database. PCR Samples containing final volume of the 25µl reaction mixture containing 2.5 µl of 10x

Taq buffer, 10 mM of dNTP mix, 10 pM of primer, 1 unit of Taq polymerase (MBI Fermentas, USA) and DNA were amplified by a thermal cycler (Thermal Cycler Model 2720, Applied Biosystems, USA). The PCR conditions consisted of 45 cycles at 94ºC for 1 min, 37ºC for 1 min, 72ºC for 2 min and 72ºC for 5 min. The PCR amplified product was resolved on 1% agarose gel (High EEO). The gel was visualized and analysed with a Gel documentation system (UVP system, Cambridge, UK).

3.17.4. Data analysis:

PCR amplified product data obtained were used to estimate the genetic similarity among different isolates on the basis of shared amplification products (Harris *et al.,* 2002). A dendogram was constructed by the Unweighted Pair Group Method with Arithmetic mean (UPGMA), (Nei and Li *et al.,* 1979***)*** to group individuals into discrete clusters. The phylogenetic tree was made in MEGA 3.1 software using the Neighbour Joining method. The gel was visualized by staining with ethidium bromide and photographed under UV light by a Gel Doc-It Digital Imaging System (UVP Ltd. UK).