Chapter 4

Results and Discussion

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4.1. Identification and characterization of the bacterial strain:

The haemolytic *Staphylococcal* strain was identified using various biochemical processes such as gram staining method, growth observed in Mannitol salt agar plate, catalase activity, coagulation test and 16S rDNA primers .Isolates were maintained in nutrient agar slants (Himedia, India) as shown in **Figure I.**

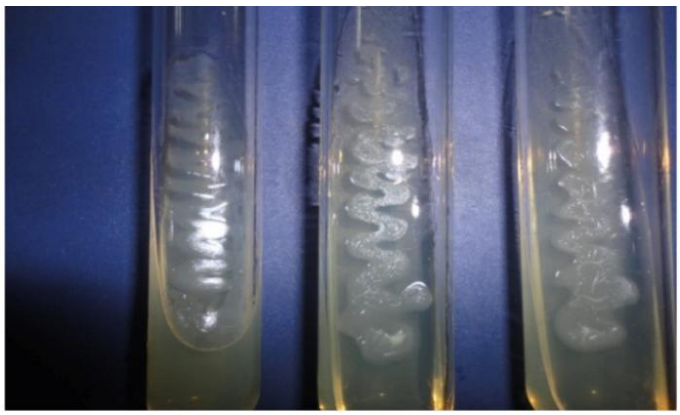


Figure I: *Staphylococcal* strain preserved in nutrient agar slant.

4.1.1. Catalase Test:

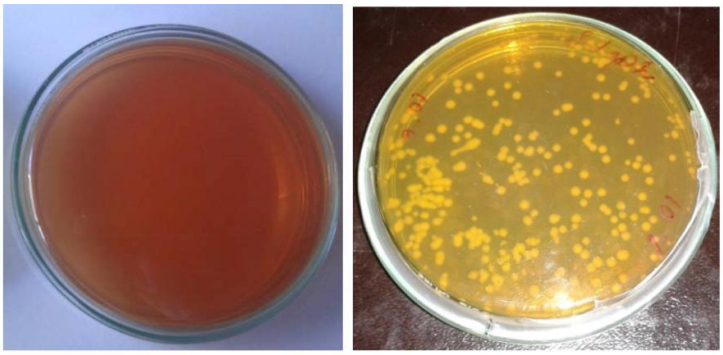
The traditional method for detection of catalase activity in bacteria grown on agar plates is the observation of effervescence after flushing colonies on the plate with dilute hydrogen peroxide solution. The bacterial strain was subjected to catalase test during the prelimanary chraterization.The formation of bubbles of oxygen after few seconds was observed when a loopful pure cuture was mixed with a drop of 3 % hydrogen peroxide (H_2O_2) on a clean glass slide. In the present study the strain showed catalase positive reaction as represented in **Figure II.**

-VE + VE

Figure II: Catalase test showing positive reaction.

4.1.2. Bacterial growth on Manitol salt agar:

Selective media allows the growth of certain type of organisms, while inhibiting the growth of other organisms. This selectivity is achieved in several ways. For example, organisms that have the ability to utilize a given sugar are screened easily by making that particular sugar the only carbon source in the medium for the growth of the microorganism.Similarly, the present bacterial strain was innoculated on Mannitol salt agar (MSA) plates and incubated at 37^{O}C for 24 to 48 hours for growth and change in the colour of the medium was observed.A significant effect on the growth patterns of the strain and change of colour of the medium from red to yellow indicated the the presence of a Staphylococci species.The result is shown in **Figure III.**



(a) (b)

Figure III (a): Mannitol Salt Agar Media before inoculating the bacterial strain. (b) Staphylococcal strain grown in mannitol salt agar culture media (48 -72 hours after inoculation)

4.1.3. Antibiotic sensitivity test:

Antibiotic sensitivity was performed to cheek the susceptibility of bacteria to certain antibiotics. Antibiotic sensitivity test (AST) was done to determine the antibiotics that is useful in treating bacterial infection in vivo. Several antibiotic dice were used for the purpose as shown in **Figure IV**.

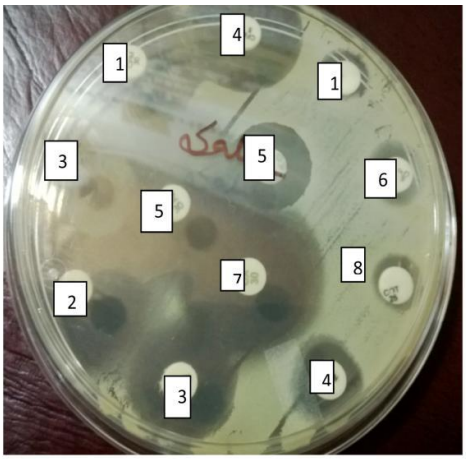


Figure IV: Antibiotic sensitivity test of the bacterial strain on Muller-Hinton agar; (1) Ampicillin (Sensitive), (2) Chloramphenicol (Sensitive), (3) Erythromycin (Sensitive), (4) Gentamycin (Sensitive), (5) Methicillin (Sensitive), (6) Tetracycline (Sensitive), (7) Co- trimoxazole (Sensitive), (8) Ciprofloxacin (Sensitive)

Normally bacteria show two kinds of resistance towards antibiotics, firstly intrinsic resistance that means the species showed resistance to an antibiotic before its introduction and secondly acquired resistance that means the species was originally susceptible to a specific antibiotic, but later it became resistant to it. Many bacterial strains can acquire resistance towards antibiotic either by mutation or by exchange of genetic material among same species or closely related species. This sudden acquisition of resistance towards antibiotics brings many difficulties in treating infections during treatment of an infection caused by the bacterial strain. The Present study was aimed to demonstrate the sensitivity towards a different kind of antibiotic that routinely used.

4.1.4. Coagulase test:

The coagulase test is an in vitro method for direct detection of coagulase positive Staphylococcal strains.Coagulase test was used to identify and differentiate the strain from coagulase negative *Staphylococci.* In the present study the staphylococcal strain was found to be coagulase positive on coagulation test. The bacterial strain was tested for the coagulation reaction as per the method described earlier. The suspected staphylococcal strain was found to be coagulase positive in a slide coagulation test as shown in **Figure V**.

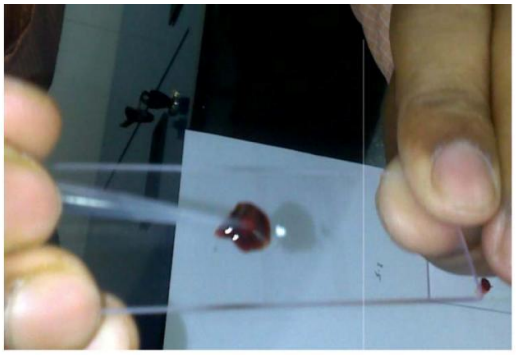


Figure V: Slide coagulation test of the bacterial strain performed against human blood

4.1.5. Culturing of the bacterial strain:

The bacterial strain was cultured in fluid soybean casein digest medium for getting the sufficient amount of the cell free extract from the strain.

4.1.6. Estimation of total CFU:

The coagulase activity of the bacterial strain was obtained when the bacteria was allowed to grow in fluid soybean casein digest medium with rich aeration at 37°C for 48 hours for getting the sufficient amount of the cell free extract from the strain. Then an attempt was made to get an idea about the total number of CFU as described in the previous chapter. After the assay the results are as follows. The colony forming unit harvested after the growth in the agar plates was 63.6 x 105 cfu /ml of distilled water. It could be concluded the bacterial strain produced maximum coagulase protein when grown in soybean casein digest medium.

4.1.7. Characterization of bacterial strain by Gram staining:

Further characterization of the *Staphylococcous* strain was done by subjecting to gram stain and observing under phage contrast microscope, as shown in **Figure VI**. The study showed the predominant strain as gram- stain positive that lives in colony with its characteristic of grape - like clusters appearance and forming cocci with non spore forming bacterium that was observed under Phase contrast microscope.

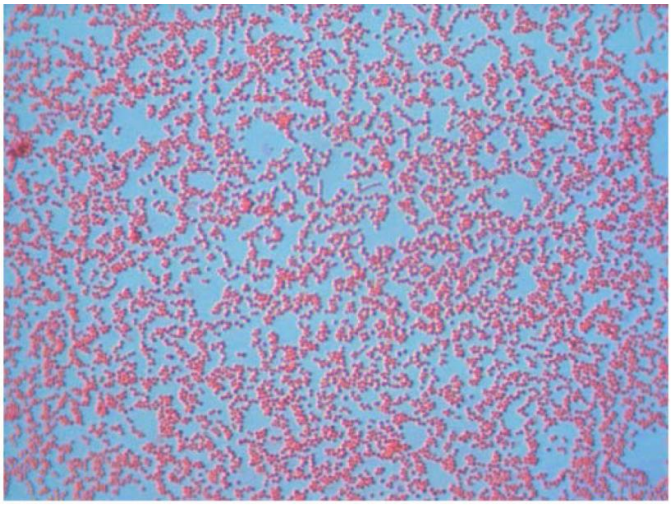


Figure VI: Gram staining of the bacterial strain seen under phase contrast microscope (100X)

4.2.1. Isolation of extracellular proteins from bacterial strain:

Proteins can be purified by a variety of methods based on their unique physical and chemical properties such as size, solubility, charge, hydrophobicity and binding affinity. The present study focuses on ammonium sulfate precipitation as a convenient first step in coagulase purification in that, it allows the concentration of the starting material and the precipitation of the desired protein. The principle of ammonium sulfate precipitation lies in "salting out" proteins from the solution. The proteins are prevented to form hydrogen bonds with water and the salt facilitates their interaction with each other forming aggregates that afterward precipitate out of solution. Another precipitating technique known as accetone precipitation was also performed simultaneously to compare the protein abundance.The cultured media with

the bacterial strain were treated with two different precipitating agents that found to be more suitable for the present study are acetone and ammonium sulphate.The samples were collected and filtered through filter paper to separate the trace of media from the solution.It was then subjected to bioassy with human blood sample to check the clotting activity of the cell free extracts.The results of the bioassy of acetone precipitate residue are shown in **Figure VII** and ammonium sulphate precipitated residue are shown in **Figure VIII**. It was subjected to dialysis to remove the excess amount of salts and other contaminants to get a more purified protein.

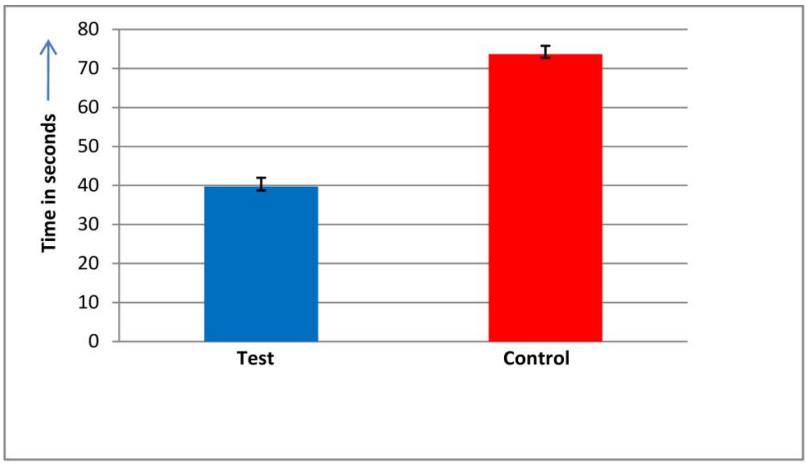


Figure VII: Bioassay of the Crude protein with human blood samples obtained by acetone precipitation. Crude protein of acetone precipitation was taken as test and whole blood with the buffer was considered as control.

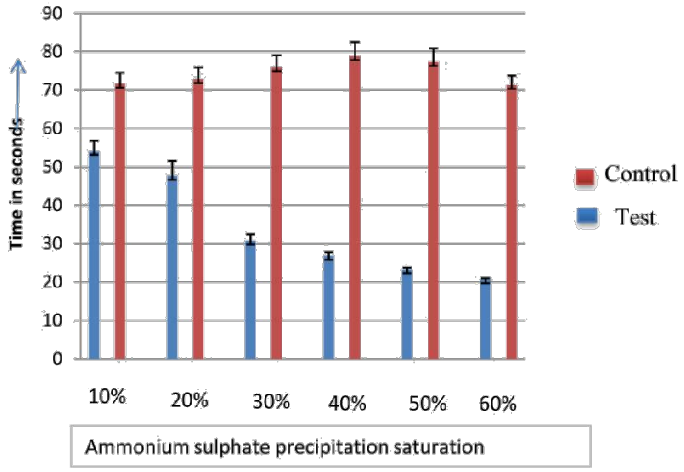


Figure VIII: Bioassay of the Crude protein with human blood samples obtained by ammonium sulphate precipitation.

4.2.2. Dialysis and Lyophilization:

The dialyzed protein sample obtained was subjected to column chromatography, and eluted three protein fractions from the Sephadex G-75 column dissolved in 0.02 M phosphate buffer. All the three fractions which had a higher absorbance at 280nm were collected and lyophilised to get more concentrated sample.

4.2.3. Estimation of extracellular protein concentration:

The cell free extract of the bacterial strain was further tested for estimation of the total protein.The total protein of the cell free extract was measured several times during different phases of the present study.As shown in the **Table III.**

Table III: Protein content (gm%) during different phases of purification

Steps of purification	Protein in gm%
Crude Protein extract	0.6
Ammonium sulphate precipitation (60%)	2.7
Dialysed sample	1.8
Different fraction collected after filtration: Fraction 1. Fraction 2. Fraction 3.	 0.8 1.6 1.3
Lyophilised Sample	1.4

4.2.4. Blood Coagulating Activity (Bioassay):

The extracellular proteins isolated from *Staphylococcus* strain have high coagulation activity. The coagulation time of the human blood was found to be 30 seconds when the blood was treated with the third protein fraction of column chromatography. Control blood takes a long time to coagulate. As shown in **Figure IX**.

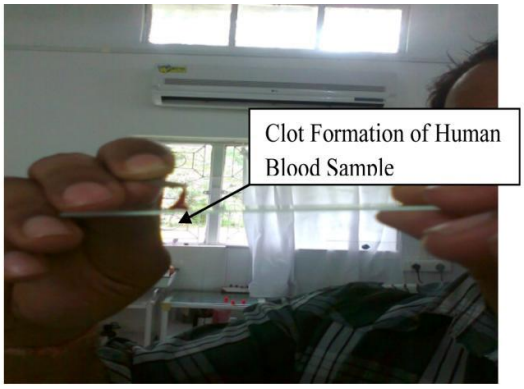


Figure IX: Slide Coagulation test of the lyophilised fraction of the coagulase protein extracted from the bacterial strain performed against human whole blood

During the present study an attempt was made to check whether the coagulase protein has any role in the homology of coagulation cascade or not. We tried the extracellular protein for routine coagulation assay parameters like prothrombin time, thrombin time, activated partial thromboplastin time, and fibrinogen time with human blood sample. The findings are listed in **Figure X- XIII.**

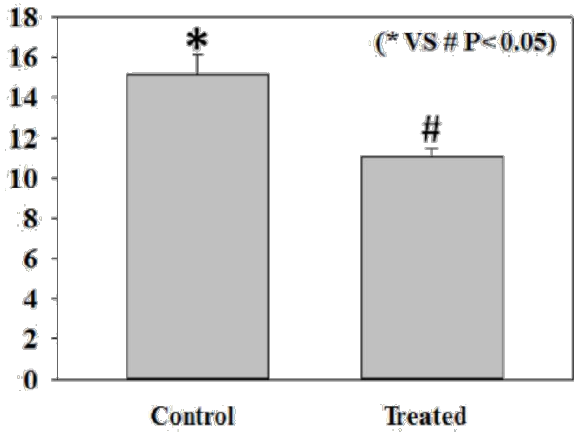


Figure X: Changes in the prothrombin time after the treatment with the lyophilised protein

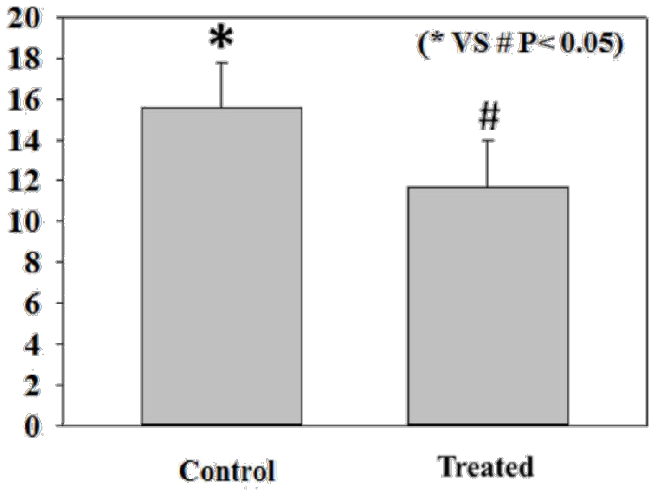


Figure XI: Changes in the thrombin time after the treatment with the lyophilised protein

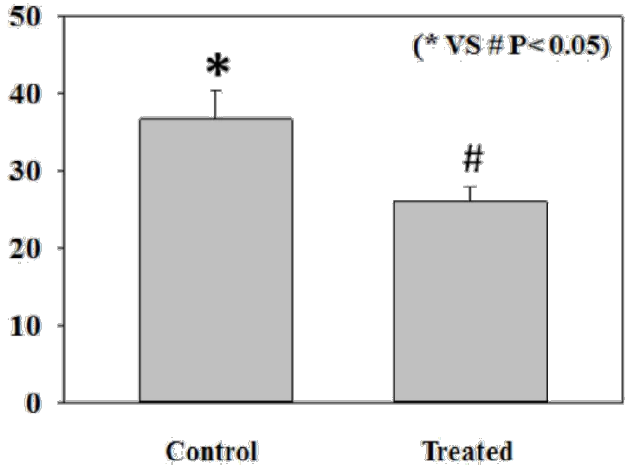


Figure XII: Changes in the activated partial thromboplastin time after the treatment with the lyophilised protein

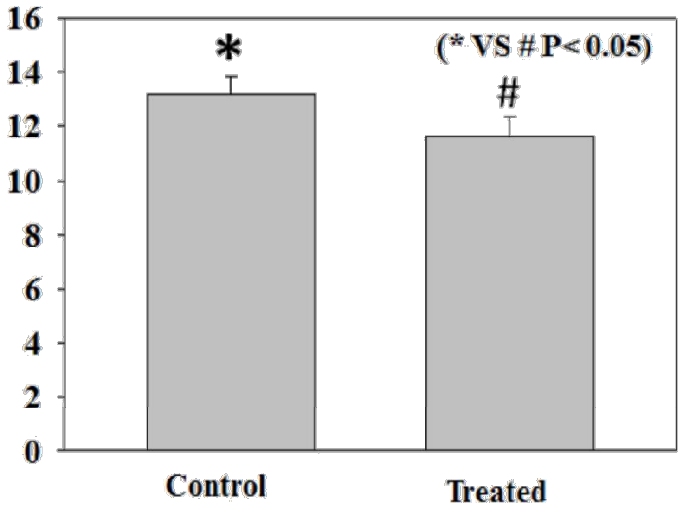


Figure XIII: Changes in the fibrinogen time after the treatment with the lyophilised protein

4.2.5. Stability of the coagulase protein in different temperature:

The coagulase protein was kept at different temperature such as 2^{o}C, 4^{o}C, 6^{o}C, -4^{o}C,-20^{o}C, and -86^{o}C to find out the stability. Preliminary observations showed that either crude extract (10% w/v) or the ammonium sulphate precipitated protein fraction

(precipitation was done in phosphate buffer with a stabilizing agent (e.g. glycerol)) can be kept at 4^{o}C to 8^{o}C for a period of up to one month. Sample kept in -20^{o}C can be stored for several months without losing functional properties.

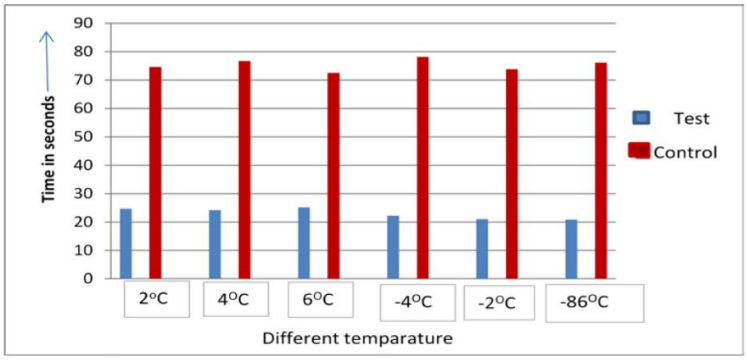


Figure XIV: Stability of coagulase protein at different temperature. 60% ammonium sulphate saturated coagulase protein sample was taken as test and human whole blood sample with buffer was taken as control.

4.2.6. SDS-PAGE for determination of molecular weight:

By SDS-PAGE, the *Staphylococcus* bacterial proteins got separated based on their molecular sizes. In this study the molecular weights of the individual extracellular protein fraction were found to be 14kDa, 33 kDa, and 64kDa respectively. In this study, these three fragments have been isolated and biochemically characterized **Figure XV.**

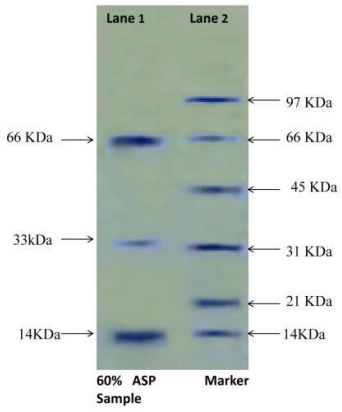


Figure XV: SDS PAGE of Staphylocoagulase Protein

Lane 1- Protein sample, Lane 2-Marker

4.2.7. HPLC fractionation:

The HPLC technique was used for analysis and quantification of individual amino acids of the three protein fractions purified from the crude sample of *Staphylococcus Sp*. The prominent amino acids found in the fractions were Aspartic acid, Glutamic acid and Proline in addition to other amino acids**.** The amino acid composition individual protein fractions were done by comparing the peaks of each amino acid with the peaks of the 21 standard amino acids **Figure XVI** in the standard graph with respect to the retention time. Quantification of the individual amino acids was done by comparing the area of the peak of each amino acid against the area of the standard peak of glycine. As shown in **Table IV-VI.**

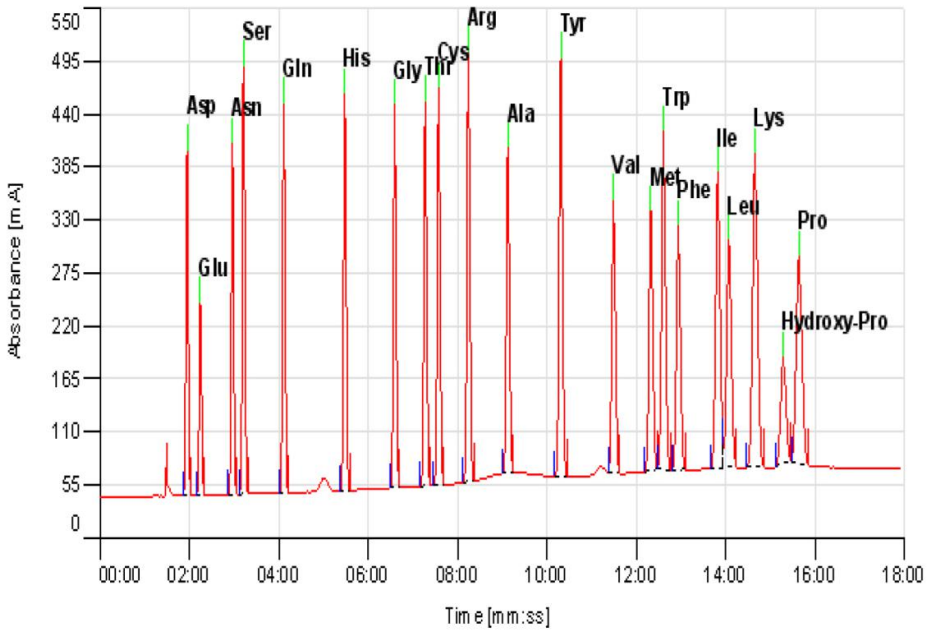


Figure XVI: Standard graph of individual amino acids (shown in peaks) showing the retention time (min) against absorbance.

The 66 kDa fragment shows a high affinity for human prothrombinin**.** The active complex of the 66 kDa fragment with human whole blood samples exhibited clotting activities when mixed a small amount of coagulase protein with a drop of

blood sample. The lowest time for this fragment was recorded as 21 seconds. The amino acids composition of the 66 kda fraction was shown in figure **Figure XVII.**

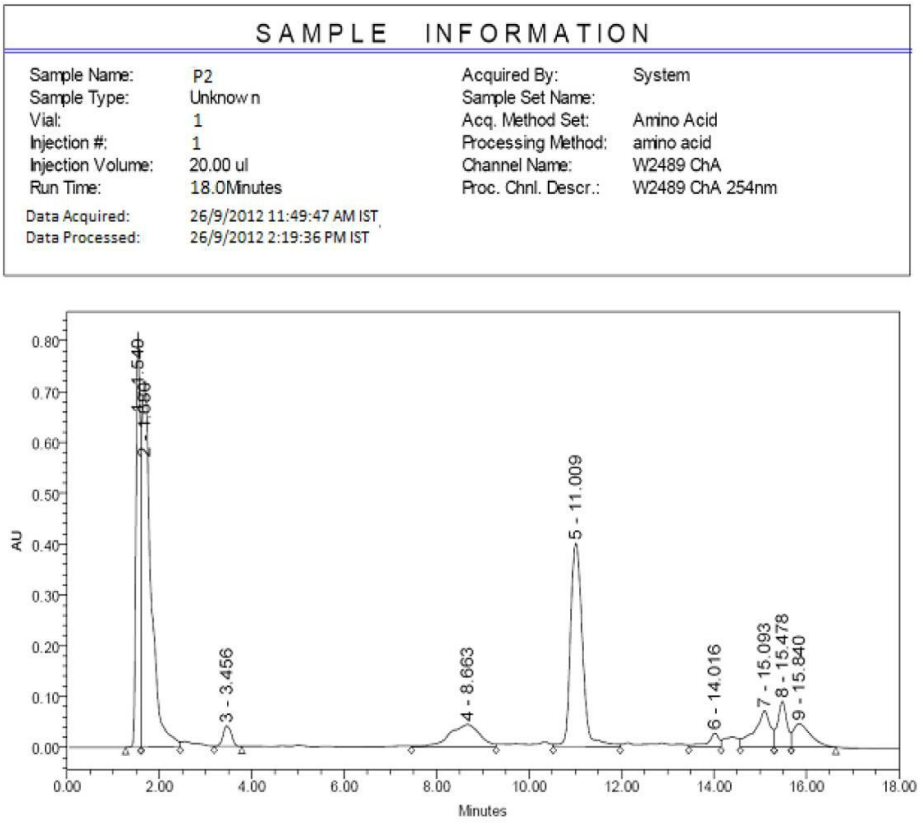


Figure XVII: Chromatogram showing amino acids of coagulase protein fraction having molecular weights of 66 kDa using HPLC.

	PEAK NAME	RT	AREA	%AREA	HEIGHT	UNIT
1	1	1.540	5200780	17.41	819084	<ppm
2	2	1.680	10333239	34.59	721195	<ppm
3	3	3.456	507740	1.70	40208	<ppm
4	4	8.663	1994580	6.68	43526	<ppm
5	5	11.009	7242561	24.24	401174	<ppm
6	6	14.016	463888	1.55	27249	<ppm
7	7	15.093	1689447	5.66	71403	<ppm
8	8	15.478	1239494	4.15	89875	<ppm
9	9	15.840	1200965	4.02	45862	<ppm

Table IV. Composition and quantification of the amino acids present in protein fraction.

Peak no.	Amino acid	Quantity of amino acid (µg)
1	Aspartic acid	117.745
2	Glutamic acid	233.944
3	Serine	11.495
4	Arginine	45.157
5	Valine	163.97
6	Isoleucine	10.502
7	Lysine	38.249
8	Hydroxyproline	28.062
9	Proline	27.189

The 33 kDa fragment, which is a major product, also showed cogulation properties with human whole blood sample. The lowest time for this fragment was recorded as 23 seconds. The amino acids composition of the 33 kDa fraction was shown in **Figure XVIII.**

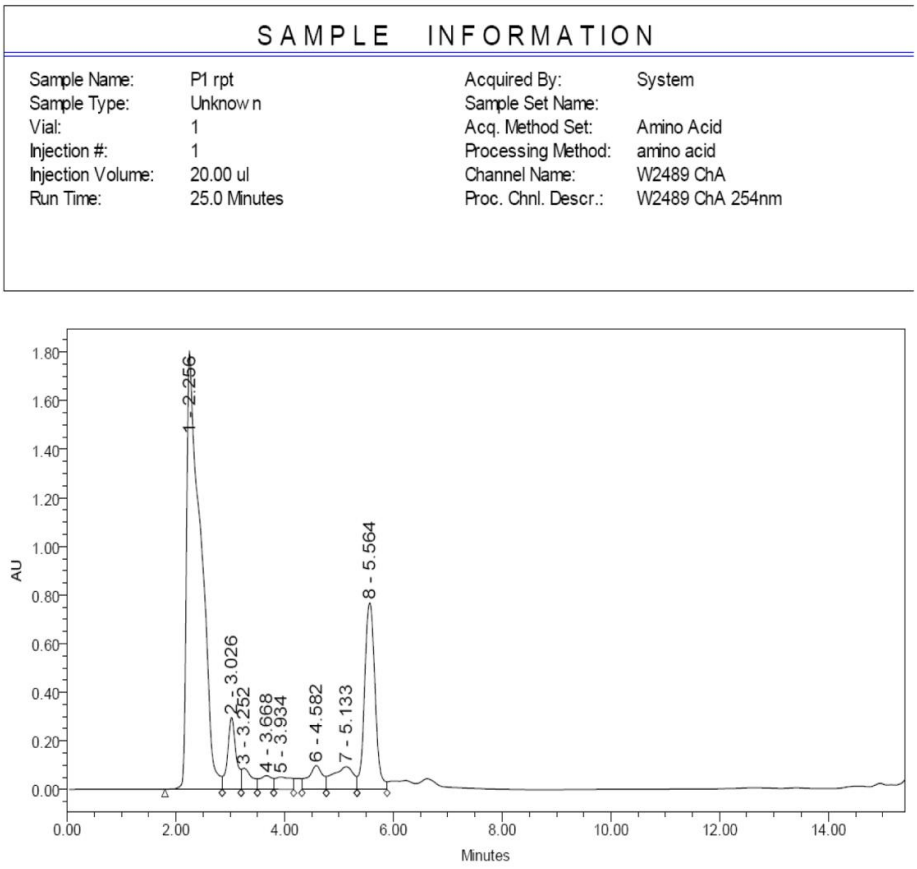


Figure XVIII: Chromatogram showing amino acids of coagulase protein fraction having molecular weights of 33 kDa using HPLC.

	PEAK NAME	RT	AREA	%AREA	HEIGHT	UNIT
1	1	2.256	32252857	60.79	1807651	<ppm
2	2	3.026	3217474	6.06	296366	<ppm
3	3	3.252	1155027	2.18	86134	<ppm
4	4	3.668	887362	1.67	56377	<ppm
5	5	3.934	1037508	1.96	50775	<ppm
6	6	4.582	1784433	3.36	97891	<ppm
7	7	5.133	2438371	4.60	93528	<ppm
8	8	5.564	10282931	19.38	768331	<ppm

Table V. Composition and quantification of the amino acids present in protein fraction.

Peak no.	Amino acid	Quantity of amino acid (µg)
1	Aspartic acid	730.204
2	Glutamic acid	72.843
3	Asparagine	26.149
4	Serene	20.089
6	Glutamine	40.399
8	Histidine	232.805

The 14 kDa fragment retains the ability to bind to clot human blood sample to some extant but fails to induce any activation of prothrombin as the lowest coagulation time for this fragment was recorded as 46 seconds. The amino acids composition of the 14 kDa fraction was shown in **Figure XIX.**

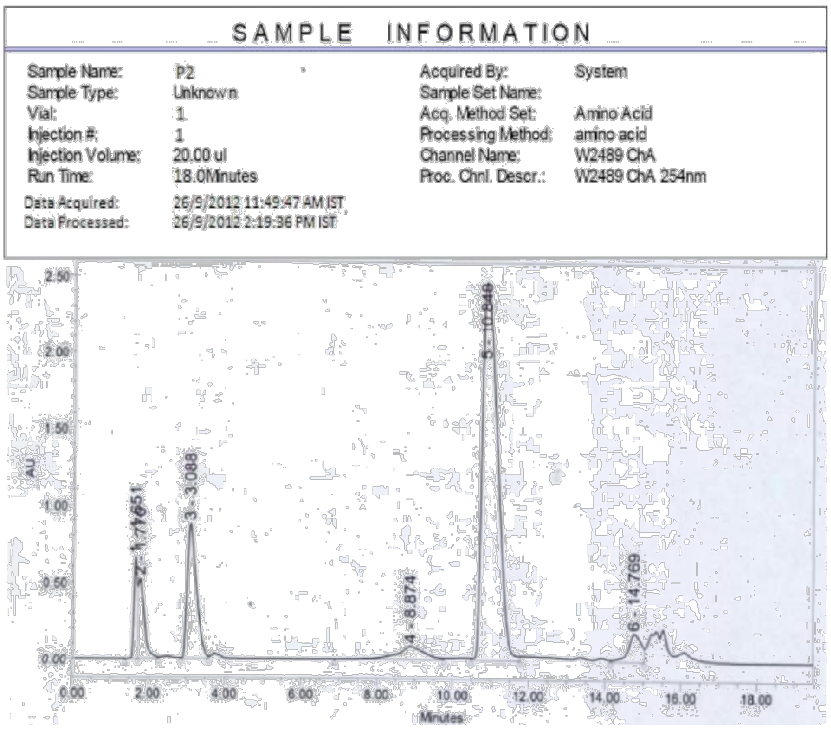


Figure XIX: Chromatogram showing amino acids of coagulase protein fraction having molecular weights of 14 kDa using HPLC.

Sl no.	Peak Name	RT	Area	% Area	Height	Unit
1	1	1.651	4369796	4.25	661782	<ppm
2	2	1.770	5751447	5.60	519943	<ppm
3	3	3.088	14661595	14.28	878752	<ppm
4	4	8.874	4649076	4.53	90423	<ppm
5	5	10.848	68749046	66.94	2467147	<ppm
6	6	14.769	4520091	4.40	188322	<ppm

Table VI. Composition and quantification of the amino acids present in protein fraction.

Peak no.	Amino acid	Quantity of amino acid (µg)
1	Aspartic acid	117.745
2	Glutamic acid	233.944
3	Serine	11.495
4	Arginine	45.157
5	Valine	163.97
6	Isoleucine	10.502

4.3. Computational analysis of coagulase protein using bioinformatics tools:

4.3.1. Translation of nucleotide sequence to amino acid:

The nucleotide sequence from NCBI database having the accession number ―EU246837.1_4‖ of *Staphylococcus* sp. *Cobs2Tis23* 16S ribosomal RNA gene, partial sequence; is converted to its corresponded amino acid sequence by using the ―Transeq‖ tool *(http://www.ebi.ac.uk/ Tools/st /emboss_transeq/)* under EMBOSS.

4.3.2. Preliminary characterization:

The various physiochemical properties of a targeted protein can be deduced from a protein sequence using bioinformatic tools. We used the ProtParam tools *(http://www.expasy.org/tools/protparam.html)* to compute.

4.3.3. Protein Theoretical Model Generation:

4.3.3. I. Template selection through PDB-BLAST:

The 3D structure of a protein is required to understand its function as it can make many complex shapes and each shape can have different functions. Preliminary characterization can help to understand the nature, activities and other properties of the targeted protein. In the present study, the assumption of the structure of staphylocoagulase isolated from the bacterial strain was generated using Homology modeling since no experimental structure is available for this protein as per literature.

The screening of the templates is the process of finding out the best homologous sequences that are highly similar to the target protein sequence and which have an experimental 3D structure. Herein, we used the PDB-BLAST where searching on template done by multiple sequence alignment of those sequences that have structures in protein data bank. During BLAST, we kept the parameters by default and searched templates in organisms excluding *Homo sapiens*.

During NCBI PDB BLAST of the sequence, three templates as 1BXC (Chain A, Xylose Isomerase from Thermus Caldophilus [Thermus caldophilus]), 1N5Y (Chain L, Hiv-1 Reverse Transcriptase Crosslinked to Post- Translocation Aztmp-Terminated Dna (Complex P) [Mus musculus]) and 1SZJ of (Chain G, Structure of Holo-Glyceraldehyde-3-Phosphate-Dehydrogenase from Palinurus Versicolor) were selected having identity and E value around 32%, 26%, 30% and 0.067, 3.3, 9.2 respectively and chosen for the model building. In the present study we have used MODELER multiple template based method. The generated model was submitted to PDBSum database having id ―r631‖as shown in **Figure XX**.

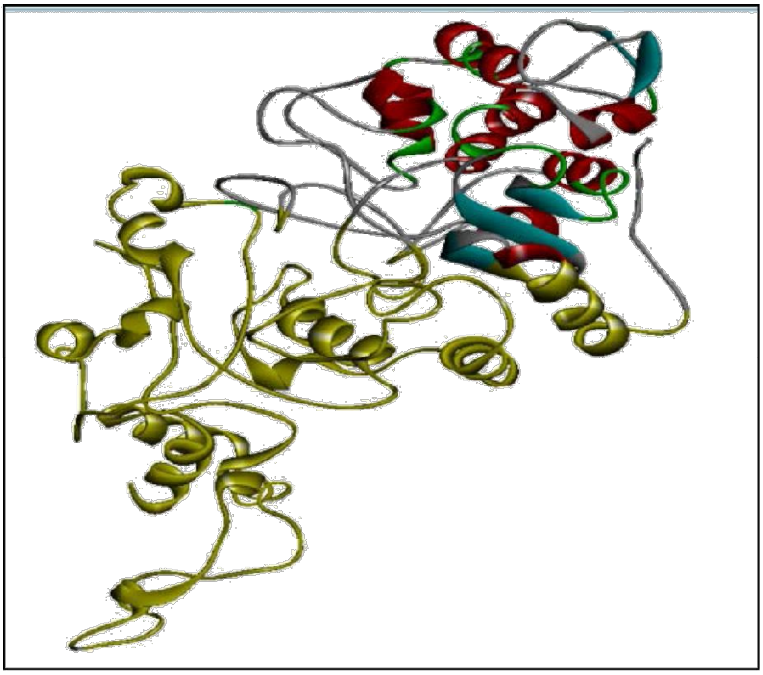


Figure XX: Protein model generated through MODELER 9v8 (MODELS view through Discovery Studio 4.0 molecule viewer).

4.3.3. II. Model Evaluation and Refinement:

The model assessment of a protein is the process of finding out the best theoretical model among the generated models based on their Model quality, Error values and Ramachandran plot analysis. In Ramachandran analysis (**Figure XXI**), a good distribution of 460 amino acid residues of the generated model of staphylocoagulase was found where about 95.1% residues are found to be in most favored and additionally allowed region, and only 2.5% residues are in generously allowed region (**Table VII).** During model assessment the stereo chemical and geometrical parameter of the generated model was evaluated using different online and offline bioinformatics tools and meta servers. The QMEAN, DDFIRE & ERRAT values for the generated structure are recorded as 0.097 -734.35 kj/mol and 26.386, respectively (**Table VIII)**.

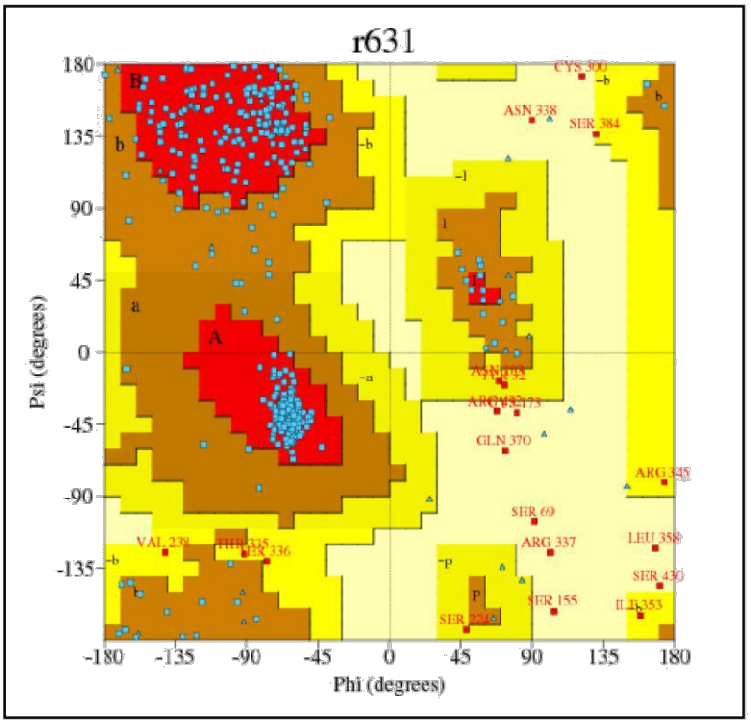


Figure XXI: Ramachandran plot of the generated model of the coagulase protein

Table VII: Ramachandran Plot analysis of the generated model of the coagulase protein

Most favoured regions [A,B,L]	**79.4%**
Additional allowed regions [a,b,l,p]	**15.7%**
Generously allowed regions [~a,~b,~l,~p]	**2.3%**
Disallowed regions [XX]	**2.6%***
Non-glycine and non-proline residues	**100.0%**

Table VIII: Assessment of stereo chemical and geometrical parameter of the generated model through PROCHECK and ERRAT

Method	Model	QMEAN	DDFIRE	ERRAT
MODELER	Moddeler_model.pdb	0.097	-734.35	26.386

4.3.4. Protein Disorder region analysis and Toxicity prediction:

There are a large number of proteins having a globular structure and static in nature, but some protein that have regions which are natively disordered. These regions are flexible, dynamic and can be partially or fully soluble. The primary

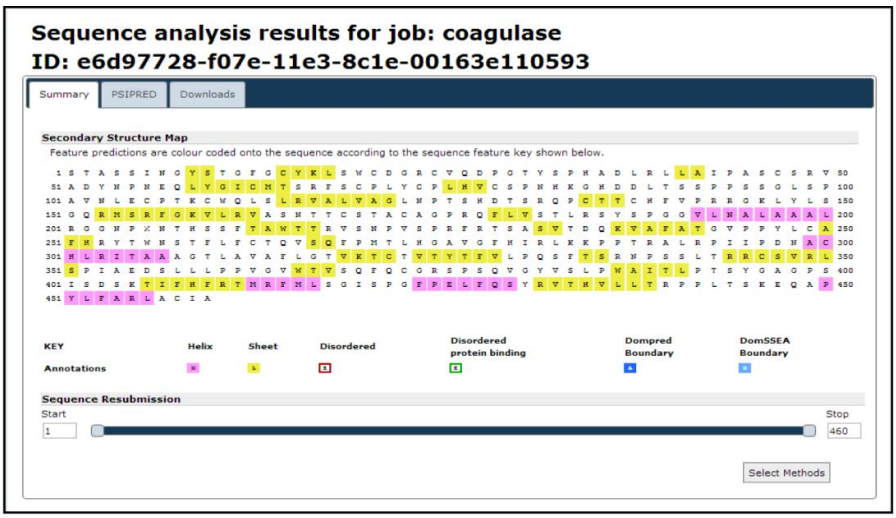


Figure XXII: Disorder Prediction through "DISOPRED"for protein disordered region detection

function of such region appears to be molecular recognition of protein and nucleic acids. Low sequence complexity, compositional bias to aromatic and hydrophilic residues and high flexibility are often appears as main characteristics. The prediction of native disordered regions of protein is very important because it may have some contribution in regulatory processes such as transcription and cytokinesis. The present study did not show any disordered region for the given sequence as shown in **Figure XXII**.

Though Peptides have numerous advantages over small molecules such as high biological activity, high specificity, however the toxicity, immunogenicity and stability are becoming major concerns. From the present *insilico* toxicity prediction studies, the concerned protein did not show any mutation or disorder region and found to be non toxic as can be revealed from **Figure XXIII**.

Mutation Position	SVM score	Prediction	Hydrophobicity	Hydropathicity	Hydrophilicity	Charge
No Mutation	-0.80	Non-Toxin	-0.21	-0.45	0.10	49.50

Figure XXIII: Results of ToxinPrediction for Toxicity of the coagulase protein

4.3.5. Binding Sites prediction:

The binding sites are smaller pockets on the tertiary structure where ligands bind. It is a place of chemical specificity and affinity on a protein that binds or forms chemical bonds with other molecules and ions or protein ligands. The affinity of the binding of a protein and a ligand is a chemically attractive force between the protein and a ligand. The best three binding sites as we have found in our study are shown in **Figure XXIV** with values as in **Table IX**. The dominant amino acid residues surrounding the binding sites are as follows.

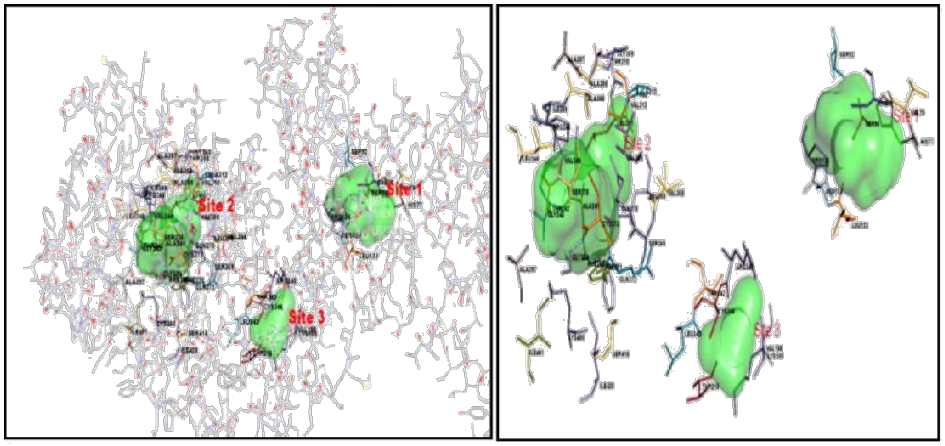


Figure XXIV: Active sites of generated protein model with residues view through Discovery Studio 4.0

Cavity 1:Lue 123, Asn 124,Pro 125,Ser 92,Ser 96,Cys 79,Val 78,His 77.

Cavity 2:Leu 311,Val 380,Ala 312,Val 313,Ser 378,Ala 241,Thr242,Gly 243,Lev 248,Pro 245,Val 244,Phe 371,Gly.

Cavity 3: Lys 159, Val 160, Thr 343, Arg 345, Leu 342, Cys 346, Thr 216.

Table IX: Protein Binding Sites with XYZ direction and respective volumes.

Protein Binding Sites	Directions			Volume (Å)
	X	Y	Z	
Site 1	24.153	-13.459	-12.97	42.375
Site 2	18.153	-13.209	-43.97	103.625
Site 3	32.153	-11.209	-30.22	6.875

5.1. PCR amplification and sequencing:

The 16S rDNA sequencing of bacterial strains was done at Bangalore Genei, India. The genomic DNA of the bacterial strain was run in 1% Agarose gel. The gel along with DNA bands is represented in **Figure XXV**

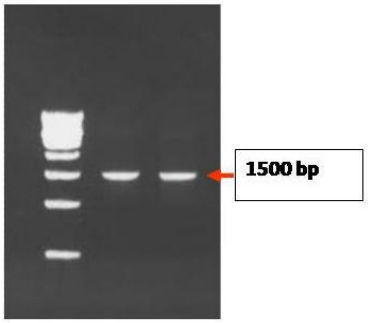


Figure XXV: the PCR amplified product in agarose gel (1%) using bacteria specific primers.

Based on nucleotide homology and phylogenetic analysis the studied bacterial strain was identified as: *Staphylococcus sp.Cobs2Tis23* (EU246837); isolated from Staphylococcus *NEIST Basab -1* strain r- 43 (b) soil sample. The phylogenetic tree was made using MEGA 3.1 software and the Neighbour Joining method (**Figure XXVI**). The gel was observed by staining with ethidium bromide and the photograph was taken under UV light by a Gel Doc-It Digital Imaging System (UVP Ltd. UK).

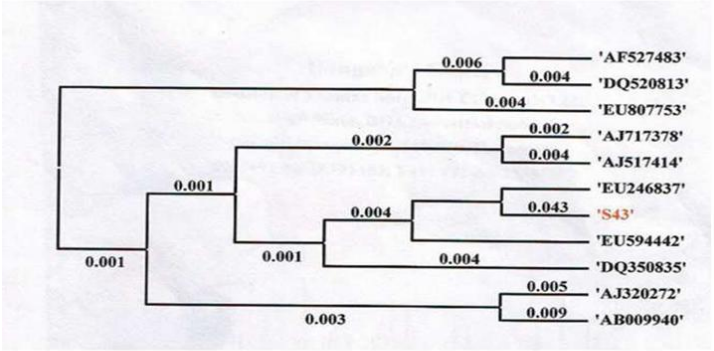


Figure XXVI :Phylogenetic tree of *Staphylococcus NEIST basab -1(S 43)* in MEGA 3.1 software using neighbour joining method