ISSN: 0975-8585

Research Journal of Pharmaceutical, Biological and Chemical Sciences

Production and Partial Purification of Staphylokinase from *Staphylococcus hominis.*

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ABSTRACT

Staphylokinase (SAK) is an amino acid enzyme activates plasminogen to form plasmin, which digest fibrin clots. This disrupts the fibrin meshwork which can often form to keep an infection localized. Staphylokinase was isolated from Staphylococcus hominis having therapeutic function to dissolve the blood clots. Staphylococcus hominis was isolated from curd. Staphylococcus spp was confirmed by morphological, biochemical and molecular techniques such as 16s rDNA sequencing. Satoh's medium was used for the production Staphylokinase. Cells were separated from culture broth by centrifugation and the supernatant fluid was added to 3 volume of acetone. After centrifugation of the mixture, the resultant precipitate was purified by ion exchange column chromatography (DEAE Cellulose). Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was done by using 10 to 20% gradient polyacrylamide gel and a 4% stacking gel at 4°C. The purity of the Staphylokinase was determined by SDS-PAGE and HPLC. The proteolytic and the plasmolytic activity of the enzyme produced by the isolated staphylococcus hominis was screened by Casein hydrolysis assay and heated plasma agar assay respectively.

Keywords: Staphylokinase, fibrin clots, *Staphylococcus hominis,* Satoh's medium, SDS-PAGE.

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ISSN: 0975-8585

INTRODUCTION

Staphylokinase is a bacterial plasminogen activator protein which is currently in clinical testing for treatment of myocardial infarction and peripheral thrombosis. We have recently completed a structural determination of the wild type protein and are currently investigating structural and functional properties of a series of staphylokinase mutants with a view to engineering this protein to improve clinical properties.

Staphylokinase (SAK) forms a bimolecular complex with human plasmin (ogen) and changes its substrate specificity by exposing new exosites that enhances accession of substrate plasminogen (PG) to the plasmin (Pm) active site. Staphylokinase (SAK), a 16kDa profibrinolytic protein from the *Staphylococcus aureu*s, has been demonstrated to induce highly fibrin-specific thrombolysis in both human plasma and in limited clinical trials [1]. Recent studies on the thrombolytic potential of recombinant SAK in achieving early perfusion in myocardial infarction and in the dissolution of platelet-rich clot [2,3] have clearly established its immense utility in clinical medicine as a thrombolytic agent and suggested that it can be developed as a potent clot-dissolving agent. Unlike some other plasminogen (PG) activators, e.g. tissue PG activator and urokinase, SAK has no proteolytic properties by itself, but acts by forming a 1:1 stoichiometric complex with plasmin (Pm), which, in turn, activates other molecules. In this bimolecular complex, SAK acts as a cofactor and provides substrate binding exosites for docking and enhanced presentation of the PG `substrate' to the Pm active site [4].

Fibrinolytic enzymes were identified and studied among many organisms including snakes, earthworms, and bacteria: *Streptococcus pyogenes, Aeromonas hydrophila, Serratia* E15, *B.natto*, *Bacillus amyloliquefacens*, Actinomycetes and fungi: *Fusarium oxysporum*; *Mucor* sp, *Armillaria mellea* [5]. These novel Fibrinolytic enzymes derived from traditional Asian foods are useful for thrombolytic therapy. They will provide an adjunct to the costly Fibrinolytic enzymes that are currently used in managing heart disease, since large quantities of enzyme can be conveniently and efficiently produced. In addition, these enzymes have significant potential for food fortification and nutraceutical applications, such that their use could effectively prevent cardiovascular diseases [6]. Accounts of cardiovascular diseases have become the leading cause of death in the Western world [7]. Many blood clot-dissolving agents, such as urokinase, streptokinase, and tissue plasminogen activator (t-PA), have been utilized in clinical treatments for cardiovascular diseases. Hemostasis is a complex process obtained through an optimal balance between bleeding and blood clot formation. Screenings of fibrinolytic enzymes was done using nutrient agar medium containing 2% casein and 2 ml human serum. The relative activity and quantitative estimation of fibrinolytic enzymes were estimated by Lowry's method spectrophotometrically at 560 nm; L-arginine, casein, BSA, mixture of amino acids and phenylthiohydant ions (PTHs) was used as standard [8].

Staphylokinase, a 163 amino acid bacterial protein from *Staphylococcus aureus* is reported to have a therapeutic function and used as a potential alternative for the available clot dissolving drugs and some countries proved to be a potent alternative over the available clot dissolving drugs. Thrombolytic disorders have emerged to be one of the main causes of human mortality worldwide [9].

The main reason for selecting the enzyme is because Staphylokinase could be relatively inexpensive when compared to other thrombolytic agents and an alternative cure against cardial blood clot. Therefore, the objectives of this study were to isolate and screen Staphylokinase (SAK) producing staphylococcal sp from curds sample, to produce and purify SAK and to determine its dissolving capability.

MATERIALS AND METHODS

Sample preparation

The curd was used to isolate Staphylokinase producing *staphylococcus spp.* Sample was prepared by mixing daal with fermented curd. The mixed sample was kept for overnight incubation.

Bacterial isolation and sub culturing

Fermented sample were serially diluted and plated in nutrient agar plate. Spread plate method was followed here to grow the organism. After plating the plates were incubated at 35°c for 24 hours. Each single strain was isolated by repeated streaking on nutrient agar medium and blood agar medium. The individual colonies were observed. The colonies were checked for their purity by microscopy. Three bacteria were isolated and isolates were maintained on nutrient agar and blood agar plates.

Screening of Staphylokinase producing *Staphylococcus spp*.

Screening by heated plasma agar plate [10].

The isolated samples were analyzed for Staphylokinase production by heated plasma agar plate assay. Here 15 ml of nutrient agar medium was prepared, and boiled at 100°C in water bath. 5 ml of heated plasma was added in the nutrient agar medium. Then mixed the heated plasma with the NA medium and poured in the plates and allowed to solidify. After the media got solidified, inoculate the bacterial culture by zigzag streaking. Incubate the plates at 37°c for overnight. The halo zones around the colonies were observed.

Identification

The isolated bacteria were identified by following microbiological and biochemical tests such as Gram staining, spore staining, motility, haemolysis test, antibiotic sensitivity test, Oxidase test, Catalase test, carbohydrate fermentation test, MRVP TEST. The above tests were performed to confirm the strain.

Molecular screening by 16s rDNA sequencing

The bacterial isolates were identified by 16s rRNA sequencing. The bacterial isolates were identified by 16s rDNA sequencing. The genomic DNA was extracted from pure culture and using *Taq* polymerase, the ~1.5 kb 16S rDNA fragment was amplified. The amplified PCR product was bi-directionally sequenced using the forward and reverse primers. The universal primers forward (5'-AGAGTTTGATCMTGGCTCAG) and reverse (5'-AAGGAGGTGWTCCARCC) were used to amplify the 16S rDNA. The amplified data was aligned with NCBI gene library and closest homologous was determined.

Culture Conditions

Nutrient broth and solid medium were used for propagation of *Staphylococcal* strains. The isolated samples were streaked on blood agar medium (Peptone: 5g/L; Beef Extract: 3g/L; NaCl : 5 g/L; Blood: *5ml*/100ml; Agar: 1.5% and pH: 7.0) to find the non haemolytic organism. The selective medium, Mannitol salt agar medium (Beef extract: 1g/L; Peptone: 10g/L; NaCl: 75g/L; Mannitol: 10 g/L; Phenol red: 0.025; Agar: 1.5% and pH: 7.0) served for the screening of *Staphylococcus hominis* strains. In the present study, blood agar medium (contains a base similar to nutrient agar) was added with 5% of human blood cells and used for discriminating the collected microbes.

Enzyme production

Staphylococcus hominis was grown on Satoh's medium [9]. It consists of 1% nutrient broth (Difco Laboratories, Detroit, Mich.), 0.3% yeast extract (Difco), 0.5% sodium chloride, and 1% glycerol with pH adjusted to 7.4.. The pH was adjusted to 7.4 with 2 M acetic acid and 2 M NaOH. Medium was sterilized by autoclaving at 121°C for 35 min and cooled to room temperature. One ml of uniformly prepared suspension of *staphylococcus hominis* was used as an inoculum; incubated at 35°C and 150 rpm (Rotation per minute) in an orbital shaker. After 2 days of fermentation, cells were removed by centrifugation.

Enzyme assay and characterization

Enzyme assay

Fibrinolytic activity was determined by heated plasma agar assay [11], Radial caseinolytic assay: It includes Caseinolytic Agar [10] and Skim Milk Agar.

Enzyme purification

Cells were separated from culture broth by centrifugation (8,000 × g, 15 min) and the supernatant fluid was added to 3 volume of acetone. The mixture of supernatant and acetone was allowed to stand at 4°C for 1 day. After centrifugation (10,000 × g, 15 min) of the mixture, the resultant precipitate was purified by ion exchange column chromatography (DEAE Cellulose). Sodium dodecyl sulfate polyacrylamide gelelectrophoresis (SDS-PAGE) was done by using 10 to 20% gradient polyacrylamide gel and a 4% stacking gel at 4°C.

Ammonium salt precipitation for purification of enzyme [11].

This is also one of the important methods for purification of enzymes. Ammonium salts, PBS, dialysis membrane are used here.

The fibrinolytic enzymes were also purified by ammonium sulphate saturation. The protein fraction was precipitated with 40%, 50%, 60% and 70% ammonium sulphate. Ammonium sulphate was found to activate the fibrinolytic activity after dialysis. Fibrinolytic enzymes were partially purified by using ion exchange column chromatography (DEAE Cellulose, MERK), and affinity chromatography and the 5 ml of total sample were collected in different tubes UV spectroscopy reading was taken to determine the concentration of enzyme.

Purification by Chromatographic Techniques

The staphylokinase product of the bacterial culture was preliminary extracted by salt precipitation and then purified by ion exchange chromatography and affinity chromatography.

Ion exchange chromatography

Chromatography is a technique used to separate organic compounds on the basis of their charge, size, shape and their solubility. This procedure involves a mobile phase and a stationary phase. In ion exchange chromatography separation is based on charge of the molecule. Proteins contain many ionisable groups on the side chains of their amino acids as well as their amino and carboxyl- termini. DEAE cellulose is a weak anion exchanger; it will bind to the opposite charge of the protein of interest. Ion exchange chromatography technique demonstrates the purification and estimates the protein concentration.

Affinity chromatography [12]

Affinity chromatography techniques are used to purify HRP enzyme from crude sample. Affinity ligand (Concanavalin A-Agarose) is covalently coupled to cross- linked agarose by divinyl sulfone activation method. The crude sample (enzyme) binds with the ligand, when the sample is passed through the column. The crude sample (enzyme) binds with the ligand, when the sample is passed through the column. The unbound proteins are removed completely by washing the column. The crude sample is eluted with elution buffer by changing the pH. Since Con A requires MN^{++} and Ca^{++} ions for carbohydrate binding, buffers should either include these metal ions or the gel should be equilibrated with these ions, immediately prior to use. This is particularly important if the sample may contain traces of chelating agent or if the resin had been treated under acidic condition.

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Protein Estimation

SDS-PAGE

The purity of staphylokinase was confirmed by SDS-PAGE. Protein expressions were analyzed by running on 15% SDS-PAGE. 40 ml of separating gel and 10 ml of stacking gel was prepared to run 15% SDS-PAGE. Low molecular protein marker was used here.

Estimation of protein by Lowry's Method [13]

The relative activity and quantitative estimation of fibrinolytic enzymes were estimated by Lowry's method spectrophotometrically at 600 nm. BSA was used as standard.

Protein Estimation

SDS-PAGE

Protein expressions were analyzed by running on 15% SDS-PAGE and a very clear 15.5 KD and 15 KD protein band was identified against a low molecular weight protein marker.

RESULTS AND DISCUSSION

Bacterial isolation and Sub Culturing

Fermented sample was taken and serially diluted and plated in nutrient agar plates. After 24 hours of incubation colonies were observed in the plates. Different colonies were observed in the plates and initial identification was done by gram staining method. Six different colonies were taken for gram staining. Out of six isolates three isolates were found to be gram positive and three were gram negative. Gram positive isolates were taken for further experiment. The three isolates were named as MS1, MS2 and MS3.

The three isolates were selected and pure culturing of those colonies was done in nutrient agar plates and blood agar plates. Colonies on the plate, gram staining and pure culture plates are shown below from figure no. 1.

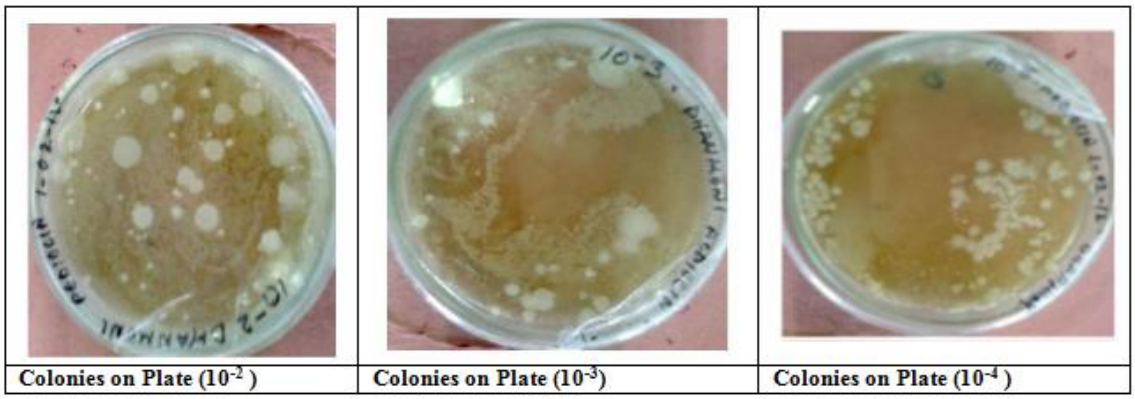

Fig 1: Cultural characteristics of *Staphylococcus hominis*

Gram's staining

The colony morphology was examined and they were identified as cocci and Gram staining results revealed that the bacterial species to be a Gram positive. Cocci cells were found in singly, pairs and tetrads (fig 2).

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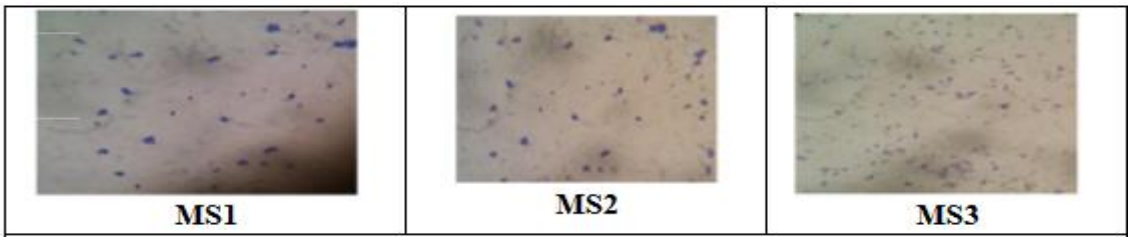

Fig 2: Gram's staining

Pure culture on nutrient agar and blood agar plates

Sub culturing or pure culturing of the isolated strain was done in nutrient agar and blood agar plates. Single individual colonies were developed in the pure culture plates and it is represented in fig 3 and 4.

Fig 3: Pure culture of MS1, MS2 and MS3 from left to right

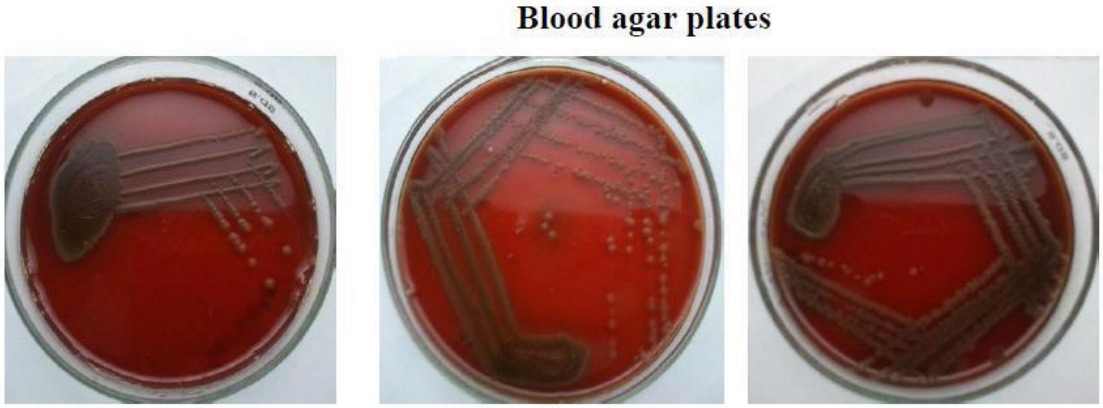

Fig 4: Pure culture of MS1, MS2 and MS3 from left to right

Screening of Staphylokinase Producing *Staphylococcus spp.*

Screening by heated Plasma Agar Plate

The isolated samples were analyzed for Staphylokinase production by heated plasma agar plate method this test was performed to confirm that whether the isolated strain has the ability to produce thrombolytic enzyme or not. The plates were incubated at 37°c for overnight. The halo zones around the colonies were observed which indicates the positive result for this test. Out of the three isolated strains which we used for this test two of the strains showed positive result.

The heated plasma agar plate is reported to be the more specific and accurate method for determining the Staphylokinase activity. The observation revealed that when staphylokinase producing strains

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were inoculated overnight at 37ºC onto the heated plasma agar plate, there is a formation of clear fibrinolytic halos. To further explore and confirm the thrombolytic activity of Staphylokinase, casein hydrolytic assay (one of the specific tests for detecting the thrombolytic activity of Staphylokinase) and skim milk agar assay was carried out. The zones of clearance were clearly observed on the plates after an overnight incubation. This test involves cleavage of casein analogous to fibrin in the clot atmosphere when activated by plasma milieu. Out of three strains two of the strains showed positive result for this screening method for production of staphylokinase. The positive strains which showed the positive results are MS1 and MS2. These two positive isolates were taken for further experiment.

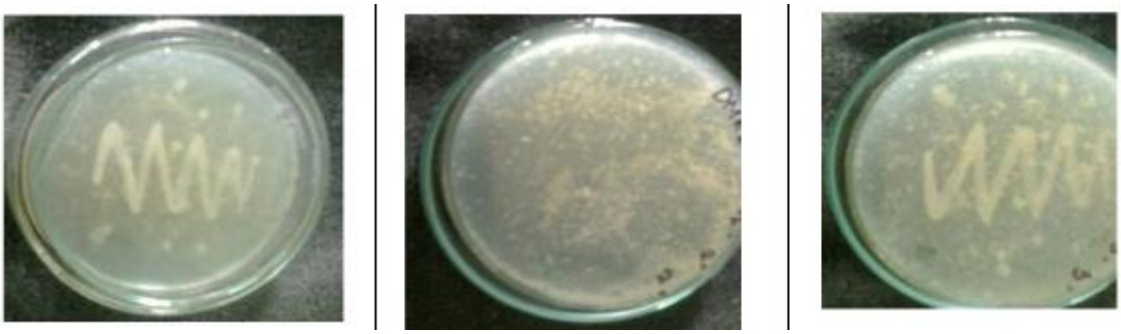

Fig 5: Pure culture of MS1, MS2 and MS3 on heated plasma from left to right

Haemolysis test

The isolated strains MS1, MS2, MS3 was found to be non hemolytic after doing the haemolysis test in blood agar medium (fig 6).

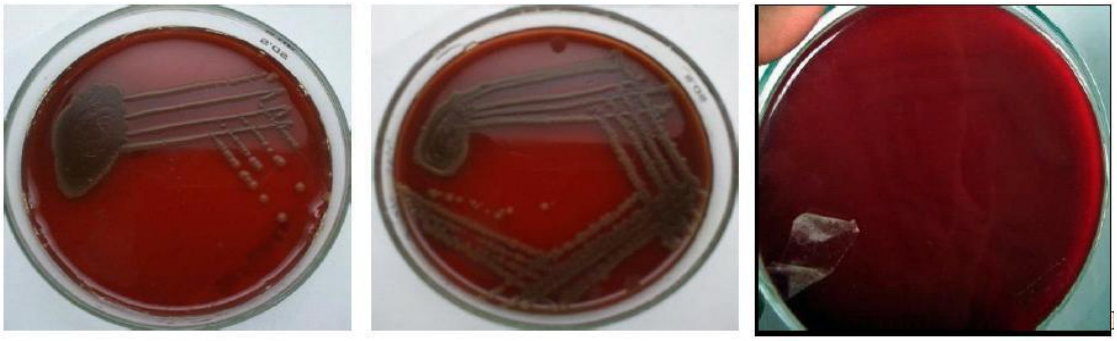

Fig 6: Pure culture of MS1, MS2 and MS3 on heated plasma from left to right

Molecular screening by 16s rDNA sequencing

The bacterial isolates were identified by 16s rDNA sequencing. After sequencing the two isolates MS1 and MS2 were identified as *Staphylococcus hominis. The* genomic DNA was extracted from pure culture and using *Taq* polymerase, the ~1.5 kb 16S rDNA fragment was amplified. The universal primers forward 5'-AGAGTTTGATCMTGGCTCAG and reverse 5'- AAGGAGGTGWTCCARCC were used to amplify the 16S rDNA. The amplified data was aligned with NCBI gene library and closest homologous was determined.

Enzyme Production [11]

Satoh's medium was prepared for the production of enzyme. it was prepared by mixing of 1% nutrient broth (Difco Laboratories, Detroit, Mich.), 0.3% yeast extract (Difco), 0.5% sodium chloride, and 1% glycerol with pH adjusted to 7.4.

Staphylococcus hominis was grown on Satoh's medium. The pH was adjusted to 7.4 with 2 M acetic acid and 2 M NaOH. Medium was sterilized by autoclaving at 121°C for 35 min and cooled to room

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temperature. One ml of uniformly prepared suspension of *staphylococcus hominis* was used as an inoculum; incubated at 35°C and 150 rpm in an orbital shaker. After 2 days of fermentation, cells were removed by centrifugation.

Enzyme assay and characterization

Enzyme assay

Fibrinolytic activity was determined by following methods. They are, i) Heated plasma agar assay

ii) Radial caseinolytic assay: It includes
a) Caseinolytic Agar
b) Skim Milk Agar

Heated Plasma agar assay [11]

The isolated samples were analyzed for Staphylokinase production by heated plasma agar assay method. 10, 20, 40µl of enzyme sample was added into the wells and the plates were incubated at 37°C for overnight. After incubation halo zones around the wells were observed, this indicated the positive result for this test. Diameter of the halo around the well was measured to check the functional activity of the proteins. The diameter of the zones was measure and the data is presented in table 1and fig 7.

Table No. 1: Heated Plasma Agar Plate Assay of MS1 and MS2

Isolates	Enzyme concentration in µl	Diameter of zones in mm
MS1	Control	0.0
	10	1.0
	20	1.1
	40	1.3
MS2	Control	0.0
	10	1.0
	20	1.4
	40	1.8

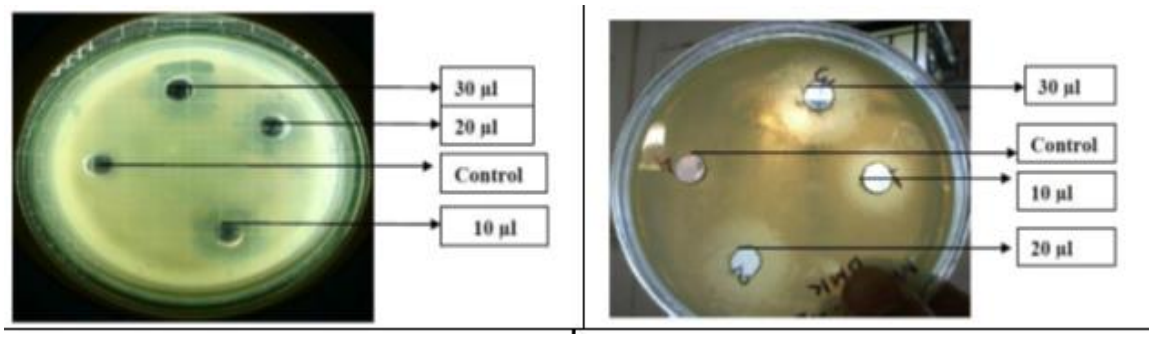

Fig 7: Heated Plasma Agar Assay of MS1 and MS2

Radial Caseinolytic Assay

Caseinolytic Agar Assay

Radial caseinolytic assay was performed to test the activity of the enzyme. To explore and confirm the thrombolytic activity of Staphylokinase, casein hydrolytic assay (one of the specific tests for detecting the thrombolytic activity of Staphylokinase) was carried out. The diameter of the zones was given below in table 2 and fig 8.

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Table 2: Caseinolytic Agar Assay of MS1 and MS2

Isolates	Enzyme concentration in µl	Diameter of zones in mm
MS1	10	0.2
	20	0.6
	30	0.7
	40	0.8
MS2	10	0.7
	20	1.0
	30	0.9
	40	1.4

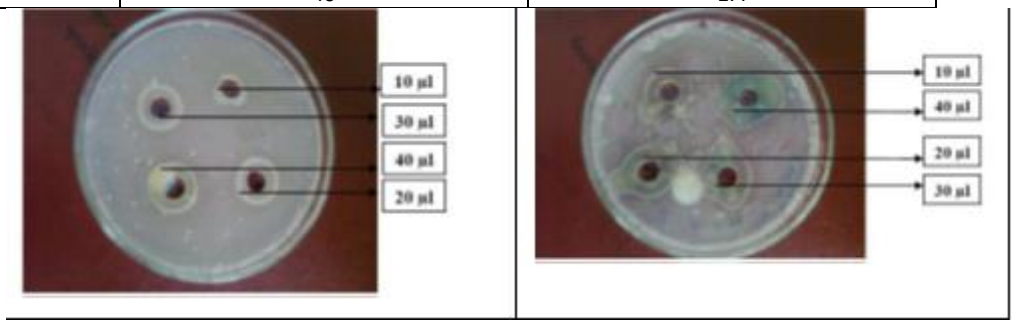

Fig 8: Caseinolytic Agar Assay of MS1 and MS2

Skim Milk Agar Assay

Skim milk agar assay was done to detect the thrombolytic activity of the enzyme. This is one of the methods to detect the thrombolytic activity of enzyme. After incubation the halo zones around the wells were observed and the diameter of the zones was measured. The diameter of the zones is given below in table 3 and fig 9.

Table 3: Skim Milk Agar Assay of MS1 and MS2

Isolates	Enzyme concentration in µl	Diameter of zones in mm
MS1	10	0.4
	20	0.5
	30	0.6
MS2	10	0.0
	20	0.8
	30	0.6

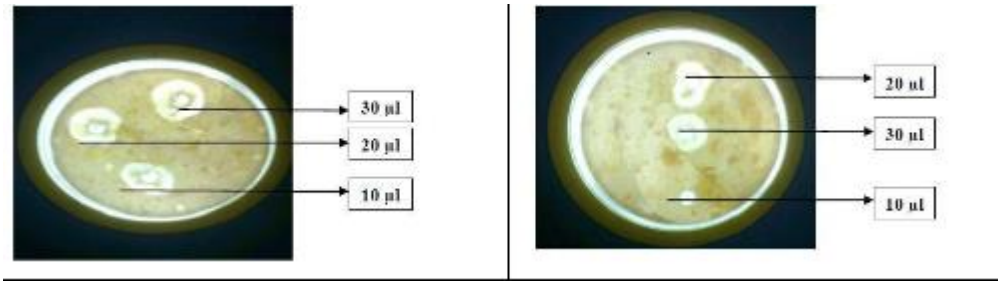

Fig 9: Caseinolytic Agar Assay of MS1 and MS2

Enzyme purification

Ammonium Sulphate Precipitation Method

The enzyme staphylokinase was purified by ammonium sulphate precipitation method by using dialysis membrane. Ion exchange chromatography and affinity chromatography were used to purify the enzyme. After purification by these two chromatographic techniques sample were collected in fractions of 1 ml. UV-VIS spectroscopic reading was taken to know the protein concentration.

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Protein Estimation

SDS-PAGE

Protein expressions were analyzed by running on 15% SDS-PAGE and a very clear 15.5 KD and 15 KD protein band was identified against a low molecular weight protein marker (fig 10).

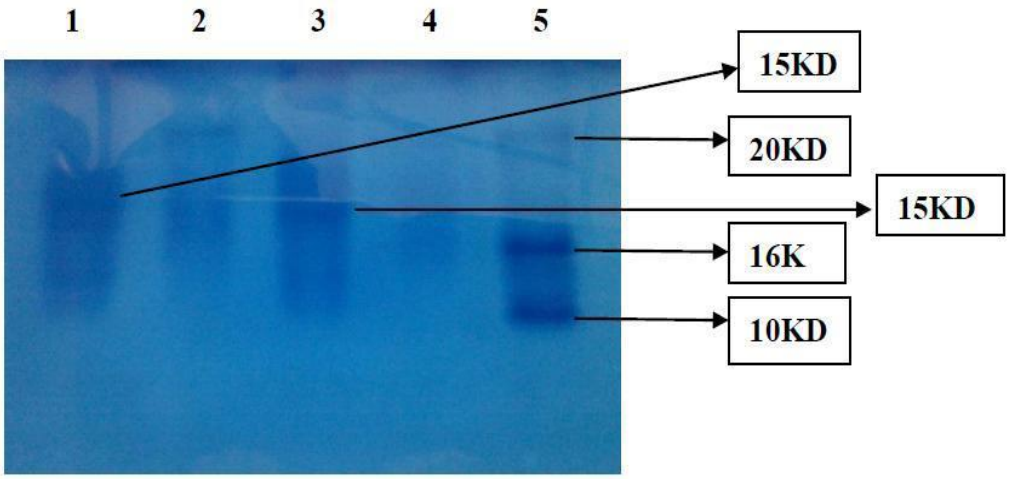

Fig 10: SDS-PAGE of protein fractions collected from chromatography
1. Fraction 1, **2.** Fraction 2, **3.** Fraction 3, **4.** Fraction 4 and **5.** Protein marker

CONCLUSION

In the present study staphylokinase was successfully isolated from a bacterial source. The bacterial spp. *Staphylococcus hominis* was normally found in human and animal skin, but here we have found a new source to isolate the organism from a fermented source. The productions, purification, assay, optimization and characterization of fibrinolytic enzyme staphylokinase were done here in this study.

ACKNOWLEDGEMENT

We express gratitude toward Dr M R Hulinaykar, Managing Trustee, Sri Shridevi Charitable Trust, Dr Gurulingappa M Patil, Principal, SIET, Tumakuru, India for providing all facilities.

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