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Research Article

Screening and Molecular characterization of Staphylokinase producing *Staphylococcus spp* isolated from Bovine milk

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ABSTRACT

The study was carried out on 50 samples collected from different regions in Khartoum State. Mannitol Salt Agar and Blood Agar were used to isolate the microorganism. Casein Hydrolytic Assay and Heated Plasma Agar Assay were performed to screen staphylokinase producing *Staphylococcus spp*. Molecular detection was done to confirm the presence of stapylokinase gen and identified as *staphylococcus aurus*. Satoh's media was used as the production media for the enzyme production. Based on the result of Casein Hydrolytic Assay and Heated Plasma Agar Assay, four isolates (9A), (11B), (25B) and (21C) showed positive results of *Staphylococcus spp,*. The clot lysis capability of stapylokinase was checked *in vitro* using Clot Lysis Assay Method. Isolate (25B) showed highest clot lysis of 91% in 90 min. while samples (9A), (11B) and (21C) gave clot lysis of 75%, 72% and 75% repectively. It was observed that the crude staphylokinase extracted from *Staphylococcus sp* showed thrombolytic activity at concentrations of 80-100 µl using Holmstrom method. Partial purification of the enzyme showed maximum activity at concentrations of 10 µl. The thrombolytic potential of this particular isolate indicated that it may be a promising and utilized for the large scale production.

Key words: Haemolytic Activity. Fibrinolytic Activity, *Staphylococcus aurus and* Staphylokinase

INTRODUCTION

Staphylokinase is a 136 amino acid protein produced by certain strains of *Staphylococcus aureus*. It belongs to fibrin-specific plasminogen activator.

.As myocardial infarction is becoming major health problem worldwide now days with no proper treatment available other than surgery and removal of blood clots.

Streptokinase is a powerful therapeutic agent in clinical use for the treatment of cardiovascular blockage. Research in thrombolyis leads to a new hope for mankind regarding human health, cure and treatment of myocardial infarction or commonly known as heart attack due to blood clot[1]. The formation of a clot in the blood vessels might cause acute myocardial infarction which will results in obstructing the blood flow to heart tissue, as cardiovascular disease is the single largest cause of death worldwide and is commonly associated with myocardial infarction[2] . Thrombolytic therapy of acute myocardial infarction is based on the premise that coronary artery thrombosis is its proximate cause rupture of a theromatous plaque leads to occlusive thrombosis that produces myocardial ischemia and cell necrosis, leading to loss of ventricular function and possibly death[3]. One approach to the treatment of established thrombosis consists of pharmacological dissolution of the blood clot by intravenous infusion of plasminogen activators that activate the fibrinolytic system. According to the WHO, 17.3million deaths in 2008 were attributable to cardiovascular disease, with 7.3 million (42% of all cardiovascular deaths) being due the result of a

myocardial infarction[4]. In 2009, approximately 1 of 6 people in the United States died of coronary heart disease. These diseases are mainly due to the accumulation of fibrin in blood vessels. The use of blood clot dissolving agents is one of the well-established methods in treating patients with acute myocardial infarction. Fibrinolytic enzyme such as urokinase, streptokinase and nattokinase, staphylokinase are agents that dissolve fibrin clots[3], were identified and studied among many organisms including snakes, earthworms, fungi , bacteria and a variety of foods, and have been purified and their physiochemical properties have been characterized[5]. Thrombolytic therapy with intravenous infusion of plasminogen activators has become an established treatment for patients with acute myocardial infarction. The most frequently used thrombolytic agents are streptokinase and recombinant tissue-type plasminog n activator. Staphylokinase e is an *Staphylococcal* extracellular protein and it is an immunogenic, inexpensive and aids in converting plasminogen to plasmin which is a major factor in blood clotting[4,6]. Hemolytic Streptococcus was used for enhanced production of streptokinase enzyme. It is an important blood clot dissolving agent and act as tissue plasminogen activator[7]. The main purpose for selecting the enzyme staphylokinase because it could be relatively inexpensive when compared to other thrombolytic agents. Although *staphylococcus aureus* is one of the most important etiologic agents in mastitis of cows, goats, and sheep[8], it produces a wide range of pathogenicity and virulence factors like staphylokinase, hyaluronidases, coagulases and haemolysins[9] . In spite of this fact, there is no local production of the cardio vascular therapeutic. This study was conducted with aim to extract the Staphylokinase from *Staphylococcus spp*. isolated from bovine milk samples. Thrombolytic therapy has become a conventional treatment for myocardial infarction but currently clinically prescribed thrombolytic drugs have such problems as delayed action and other side effects like bleeding, reocclusion etc. The current research work of isolation and screening of staphylokinase producing *Staphylococcus spp*. involves the traditional methods which are simple, rapid and can be easily carried out, which help in making the production process of staphylokinase cost effective. Economically the isolation, screening and production of staphylokinase are reliable.

MATERIAL AND METHOD

This study was conducted at the Central Lab. Sudan. All the materials used during the study were of high quality and purity.

Sample collection

A total of 50 bovine milk samples were collected from different cow farms and brought aseptically to the Lab. Serial dilution and cultured on nutrient agar medium.

Isolation of Microorganism

Isolation of the microorganism was done using selective and differential media: Mannitol Salt Agar (MSA). Blood Agar media is also used to check the haemolytic and acid producing properties. The selected strains were sub -cultured and preserved at 4°C for further testing[10].

Enzyme screening using qualitative Method

Enzyme produced from the isolated *Staphylococcus spp* was screened by Casein Hydrolysis Assay and Heated Plasma Agar Assay to check the proteolytic and the plasmolytic activity of the enzymes[11].

Casein Hydrolysis Assay

Casein Hydrolysis Assay was prepared by mixing non-fat dry milk (casein), serum and nutrient agar. The serum was prepared by collecting 5 ml of blood and the blood was allowed to clot for 5 hrs. The yellow colour fraction was used as serum. Well diffusion plate technique was used to check the caseinolytic activity of the enzyme[7].

Morphological and Molecular Characterization

Morphological and biochemical Identification of the selected microorganism is determined according to the directions given by the Bergey's Manual of Systematic Bacteriology[12,13]. and involved cultural microscopic and biochemical characteristics

Molecular Characterization

DNA extraction

DNA was extracted from *staphylococcus aureus* using boiling[14]. In brief, one loop-ful of bacterial colonies was picked and suspended in 500 µl TE buffer in 1.5 ml tube and vortexed. Then 5µl of proteinase K and 5 µl of lysozyme enzyme were added respectively. 100 µl of lysis buffer was added for each samples and incubated at 37°C for 30 min., then the samples were boiling at 100°C for 1 hr. The samples were centrifuged at 12,000 rpm for 5 min. supernatant were collected and stored at -20 °C till used for further study.

Polymerase chain reaction (PCR)

The PCR was performed by processing the extracted DNA from *staphylococcus aureus* with primers that are specific for the stapylokinase gene. The primers used consisted of forward primer and reverse primer

(5'- CGCGGATCCTCAAGTTCATTCGAC-3')
(5'-GAATCTAGACCCAAGCTTTTTCCTTTCTAT AACAAC-3').

The reaction was performed in 25µl volume using Solis Bio dyne master mix. The volume included: 5µl master mix, 1µl forward primer, 1µl of reverse primer, 3µl of extracted DNA and 15µl of distilled water. The DNA was amplified in thermo-cycling conditions using PCR machine Techno (Japan) as follows: initial denaturation at 95˚C for 5 min, followed by 35 cycles of denaturation at 94˚C for 1 min, annealing at 51˚C for,1 min and extension at 72˚C for 1 min, with a final extension 72˚C for 10 min. 5µl of the amplified product was subjected to direct analysis by gel electrophoresis in 2% Agarose, the gel was prepared by adding 0.7 g of Agarose to 75 ml 5X Tris Borate EDTA buffer. The product was visualized by staining with 0.15% Ethidium bromide using UV gel documentation system INGeNius. The expected size of staphylokinase gene amplicon was 400 bp.

Enzyme Extraction

Extraction of Staphylokinase from *Staphylococcus spp* was carried out by culturing on Satoh's medium containing 10 g/L nutrient broth, 3 g/L yeast extract, 5g/L NaCl, and 10 ml/L glycerol, at 30°C, at 100 rpm for 24 hr. The pH of the medium was adjusted to 6.8 before sterilization. The isolated culture was then inoculated into the production medium. The cells were then harvested by centrifugation at 10000 rpm, at 4 ˚C for 10 min.

Heated Plasma Agar Assay

Heated Plasma Agar Assay method is one of the important methods to determine the activity of the enzyme. Crude enzyme was prepared by centrifugation at 10000 rpm for 25 min. 15ml of nutrient agar medium was prepared. Plasma was prepared by collecting of 10 ml of blood. Anticoagulant (EDTA) was added and blood was centrifuged at 13000 rpm for 10 min. The supernatant served as plasma. The plasma was then heated at 56 °C for 20 min. Then it was mixed with nutrient agar and plated. Well diffusion technique was used to check the plasmolytic activity of the enzyme[7].

Partial purification of Enzyme

Partial purification of the Enzyme was achieved using ammonium sulphate precipitation method: 50 ml of the supernatant was taken and ammonium sulphate was added slowly at concentration of 30 %, 50% and 70% saturation level, magnetic stirrer at 4 °C, after 1 hr the samples were centrifuged at 8000 rpm for 30 min, pellets collected were resuspended with 1X phosphate buffer solution (PBS) of pH 7.0 at 1:1 pellets to buffer ratio[7].

Enzyme activity by Modified Holmstrom method

The thrombolytic activity of the enzyme was checked using Modified Holmstrorm method. This is one of the most important methods to test the thrombolytic activity of an enzyme. In this method both crude and ammonium sulphate precipitated samples were used. 1 ml of human blood were taken in Eppendorf tubes and allowed to clot. After the blood clotted completely, enzyme was added at a concentration of 10 -100 µl. The minimum concentration of the enzyme which completely liquefies 1ml of clotted blood is considered as 1 enzyme unit[15].

***In vitro* clot lysis study**

Venous blood was drawn from healthy volunteer and was transferred to four reweighed sterile Eppendoff tube (500 µl/ tube) and incubated at 37°C for 45 min. till clot formation. As soon as clot formation is observed, serum was completely removed. Weight of individual tube having clot was again weighed for determination of the clot weight.

Clot weight = weight of clot containing tube – weight of tube alone.

All the eppendoff tubes containing clot was properly labelled. 100 µl of streptokinase and distilled water were added at 3:4 concentrations ratio (streptokinase isolated *Staphylococcus aureus* strains and distilled water). All the tubes were then incubated at 37 ^{0}C for 90 min. and observed for clot lysis. After incubation, fluid obtained was removed and tubes were again weighed to observe the difference in weight after clot lysis. Difference obtained in weight taken before and after clot lysis was expressed as percentage of clot lysis. Streptokinase and water were used as a positive and negative control respectively[16].

% clot lysis = (Weight of the lysed clot / Weight of clot before lysis) × 100

Blood clot lysis on slide

Eights clean slides labelled suitably, clotted blood was fixed on each slide, and then 100 µl of Semi purified enzyme was added to the clot on the slide and observed for clot lysis activity.

Blood clot lysis on tube

Eights clean tubes labelled suitably, clotted blood was poured on each tube, and then 100 µl of semi purified enzyme was added to the clot on the tube and observed for clot lysis activity at different time interval[17].

Time course of enzyme production

The colonies that showed large zone on blood agar medium were used of inoculum preparation .A volume of 200 ml of brain heart infusion taken in a 250 ml Erlenmeyer flask was inoculated with a loop-ful of cells from 24 hr. old slant and kept at 37˚C in rotary shaker after 18 hr. of incubation 10 ml of this broth culture was used as inoculums. 10% inoculum was added to 100 ml of production medium into 250 ml cultures then incubated at 30˚C and 100 rpm on rotary shaker incubator for 26 hr. Sample were removed periodically every 6 hr. and cell growths as well as staphylokinase activity were determined[18].

RESULTS AND DISCUSSION

In the present study a total of 50 presumptive bovine milk samples were recovered from different cow farms in Khartoum State. Samples were transferred aseptically using falcon tubes to the lab. and gave a prefixed number.

Isolation of the microorganism

Mannitol Salt Agar

Among a total 50 milk sample collected, 30 out of 50 sample (60%) showed complete fermentation of mannitol, according to Figure (1) as the colour of the media was changed from pink to yellow, the colonies were able to grow on MSA, confirmed the presence of *Staphylococcus spp*. All samples that show complete fermentation from the mannitol salt agar were taken and streaked on the nutrient agar plates and the pure cultures were maintained**.** Study on isolation of 103 β-haemolytic *Streptococci* with haemolytic activity,their *Streptococci* were secluded from various sources of biomass and blood from infected throat[19]. Other results[20] stated that several strains of beta-hemolytic Streptococci produce streptokinase enzyme that can bind and activate human plasminogen to plasmin. Our result is in accordance with the earlier observation which shows that the isolates from a local wound formed due to lysogenic. *Staphylococcus aureus* are used for economic production of staphylokinase[7]. Large quantities of Staphylokinase can be produced inexpensively by bacteria[21].

Screening for staphylokinase activity

Blood haemolysis

Colonies developed on the blood agar plates, 9out of 30 sample (30%) showed β-hemolysis (clear zone around the colonies) indicates complete or true lysis of red blood cells, clear zone approaching the color and transparency of the base media , surrounds the colony were observed according to (Figure 2) and the rest of samples did not show haemolysis. Eight isolated strains produce staphylokinase after incubation, showed distinct halo zones indicating proteolytic and plasmolytic activity[22].

Microsocpic and Molecular characterization

These microorganisms were subjected to morphological and biochemical characterization according to Berge's criteria and all the selected isolates showed positive results for grams staining, catalase, co-agulase, MR, VP tests, and anaerobic growth and negative results for citrate utilisation test. The isolates were identified and it could be of *Staphylococcus spp.*

PCR amplification of stapylokinase gene

One primer was used to amplify region in stapylokinase gene, thus the DNA fragments were obtained, amplification produced DNA amplicon of about 400 bp on 2% agarose gel.Ten samples were successfully amplified and included in this study as shown in (Figure 3).

Screening for staphylokinase activity using Casein Hydrolytic Assay

In Casein Hydrolytic Assay, samples (9A) produced 29 mm halo zone which is found to be the largest zone, whereas sample (21C) produced halo zones of 10 mm. But samples (11B) and (25B) did not give any halo zones (Figure 4). Study on *Staphylococcusspp* from wound pus showed maximum halo zone of 30 mm[7,23]. This test involves cleavage of casein analogues to fibrin in the clot atmosphere when activated by the added plasma. Another results revealed that among 20 isolates, four isolates showed β-hemolytic activity and only one strain showed efficient hydrolysis in the casein hydrolytic assay[24].

Heated Plasma Assay

After incubation at 37º C onto the heated plasma agar plate, there is a formation of clear fibrinolytic halos. Samples (7C), (11B) and (25B) produced halo zones of 10 mm,12 mm and 8 mm respectively, Sample (11B) gave the best zone when compare to other samples (Figure 5). One study stated that out of 12 isolates only one potent strain showed maximum hydrolysis of diameter 18 mm on heated plasma plate assay[22].

Partial purification of Staphylokinase

Ammonium Sulphate precipitation is one of the most commonly used methods for protein purification from a solution. In solution, proteins form hydrogen bonds with water molecules through their exposed polar ionic groups. When high concentration of small,

highly charged ions such as ammonium sulphate are added, these groups compare with the proteins to bind to the water solubility resulting in precipitation

Determination of enzyme activity by Modified Holmstrom method

Holmstrom method confirmed the thrombolytic property of the isolated enzymes. Table (1) showed that crude enzyme of sample (11B) showed thrombolytic property only at 100 µl concentration whereas precipitated enzyme showed the activity from concentrations of 70-100 µl. Precipitated (11B) showed thrombolytic activity of 80µl onwards. Whereas precipitated samples (21C) and (25B) showed the clot lysis activity at lower concentration of 10µl onwards. So, the least concentration that gives the complete lysis of 1 ml of clotted blood is 10 µl enzyme units for sample (21C) and (25B) only.

Clot lysis study

In vitro thrombolysis study was done on the selected stapylokinase producing *S. aureus* isolates. Addition of 100 µl of sterile distilled water after 90 min. of incubation at 37°C to the negative control shows negligible clot lysis (4.0 %). To the positive control when 100 µl of streptokinase (30,000 I.U.) was added 86% of clot lysis was observed. The 4 semi purified samples (9A), (11B), (21C) and (25B) showed 75 %, 72%, 75% and 91% clot lysis respectively. One study stated that a strain *S. equisimilis EBL*- showed 480 U mL^{-1} of streptokinase activity in quantitative blood clot liquefaction test[22], which is quite higher than wild strain of 370 U mL^{-1}.

Blood clot dissolving activity on Slide and Tube

A clear, visual representation of clot lysis is shown in (Figure 6 and 7) after 200 µl of the semi purifide staphylokinase produced by sample (25B) were added to the clot on slide and tube then observed for 45 min. One works reported that 0.12 ml of streptokinase liquefied 1ml of clotted blood in 18 h[25], whereas in our work only 0.01 ml of staphylokinase extracted from sample (25B). Now a days researchs focusing on a thrombolytic therapy with blood clot specific, efficiently, in a shorter duration time and with minimum side effects.

Time course of Enzyme production

The enzyme activity and bacterial growth kinetic are depicted on Figure (8). Fibrinolytic enzyme production from bovine milk sample at different time period (0 - 30 hr.). It shows that the growth profile during 30 hr cultivation. Maximum growth of the bacterium was obtained within 24-30 hr of cultivation. The activity of the enzyme reached maximum within 5 hr after inoculation beyond 5 hr of growth no increase in enzyme activity was recorded. The two profiles were similar and show that the fermentation kinetics of streptokinase production by *Staphylococcus spp.* might be classified as growth associated.

CONCLUSION

In the present study 50 bovine milk samples were brought from different location around Khartoum State screened for staphylokinase production. Nine positive isolate on the basis of the growth characteristics on MSA and BA plate and were subjected to heated plasma and casein hydrolysis study. Morphological and molecular characterization were done to confirm the presence of stapylokinase gen and identified as *staphylococcus aurus*. Four strains out of the nine isolate (8%), samples (9A), (11B), (25B) and (21C) exhibit proteolytic and fibrinolytic activity, and they revealed their ability to *in vitro* clot lysis of 86% and they also shows 75 %, 72%, 75%, 91% clot lysis respectively

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Table 1
The clot busting ability of the crude and precipitated samples using Modified Holmstrom Method

Enzyme conc / Samples	10µL	20 µL	30µL	40 µL	50 µL	60 µL	70 µL	80 µL	90 µL	100 µL
Crude 9A	-	-	-	-	-	-	-	-	-	|+
Precipitate 9A	-	-	-	-	-	-	+	+	+	+
Crude 11B	-	-	-	-	-	-	-	+	+	+
Precipitate 11B	-	-	-	-	-	-	-	+	+	
Crude 21C	-	-	-	-	-	-	-	+	+	+
Precipitate 21C	+	+	+	+	+	+	+	+	+	+
Crude 25B	-	-	-	-	+	+	+	+	+	+
Precipitate 25B	+	+	+	+	+	+	+	+	+	+

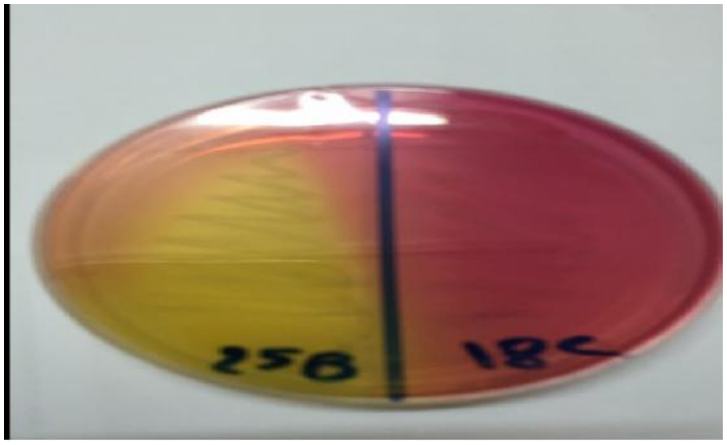
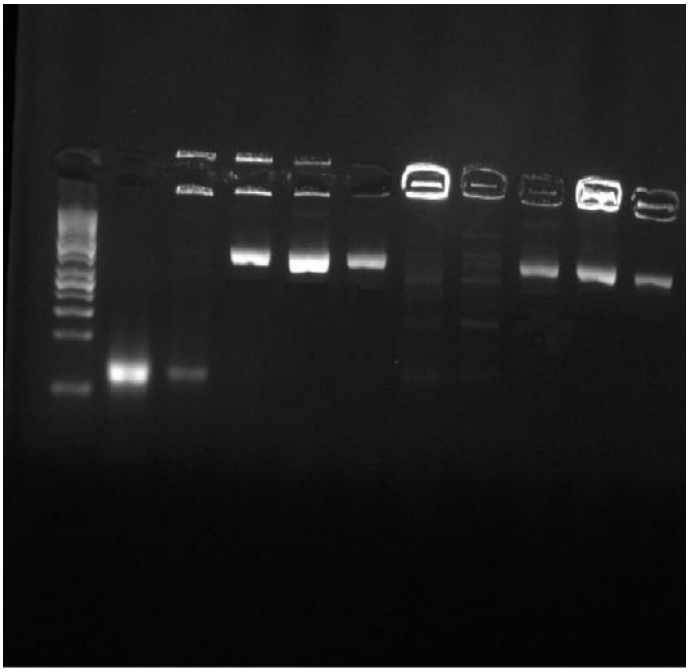


Figure 1
Mannitol Fermentation

Figure2
Blood Haemolysis on Blood Agar medium



M 1 2 3 4 5 6 7 8 9 10

Figure 3
Agarose gel of the PCR products of stapylokinase gene. Fragment size is in 400bp DNA marker (50bp)
M: DNA marker
1and 2: Negative control
3, 4, 5, 6, 7, 8, 9 and 10: Samples

Figure 4
Zone of inhibition on casein hydrolysis Assay

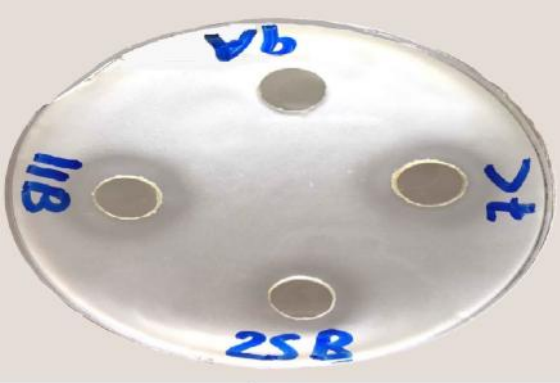


Figure 5
Zone of inhibition on heated plasma agar

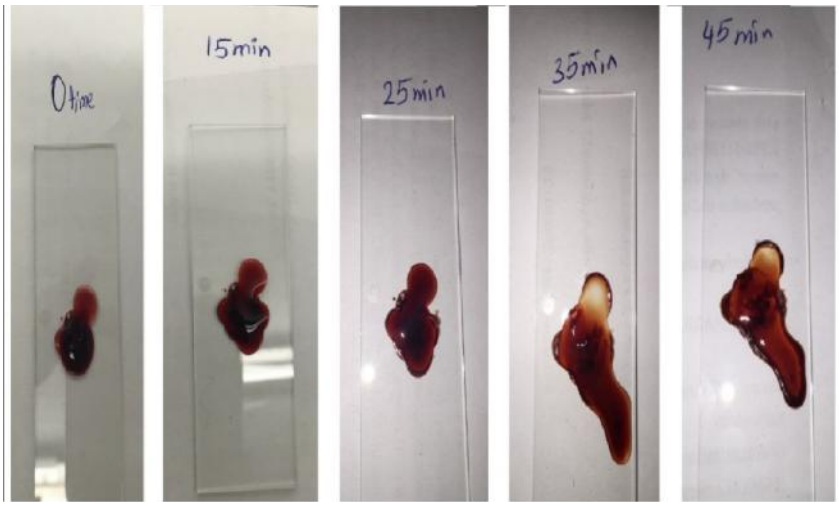
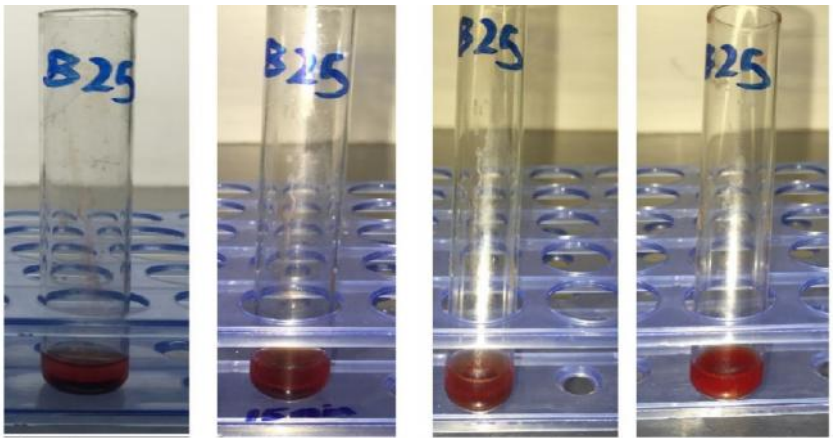


Figure 6
Clot dissolving activity on slides from 0-45 min



0 min. 15 min. 35 min 45min

Figure 7
Clot dissolving activity on tube from 0- 45 min

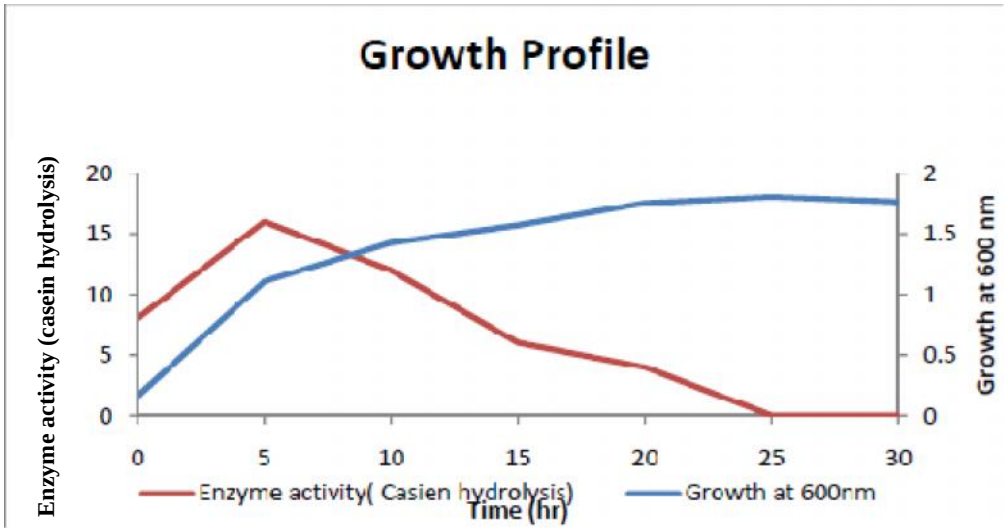


Figure 8
Staphylokinase activity profile during the growth of *Staphylococcus Spp*

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