RESEARCH ARTICLE

Thrombolytic Activity of Purified Staphylokinase Produced from Clinical Isolates

Luma Saleem Hachim*, Khawlah Jebur Khalaf, Ikbal Khudhur Aljoofy

Department of Biology, College of Science, Mustansiriyah University, Baghdad, Iraq

Received: 09th September, 2020; Revised: 10th October, 2020; Accepted: 16th November, 2020; Available Online: 25th Decemeber, 2020

ABSTRACT

The present study is designed for the screening of staphylokinase producing *Staphylococcus aureus* which is isolated from different clinical sources. Mannitol salt agar and Blood agar were used to isolate the *Staphylococcus aureus*. On the other hand, 40(66.6 %) *Staphylococcus aureus* could develop production staphylokinase by casein hydrolysis assay and 45 (75%) by heated plasma agar assay. While Satoh's media is the best media for staphylokinase production, the period 24 hours was optimum incubation on the staphylokinase production. Using the casein digestion method was confirmed enzyme activity. Determined total protein content by the Bradford method. The first stage of purification of staphylokinase is ammonium sulphate precipitation in saturation 85% and dialysis, the second stage is ion-exchange chromatography and the end-stage is gel filtration chromatography. Increased effect thrombolytic activity for purified staphylokinase than of crude enzyme.

Keywords: *Staphylococcus aureus***,** Staphylokinase**,** Detaction of staphylokinase assay, purification of staphylokinase and thrombolytic activity.

International Journal of Drug Delivery Technology (2020); DOI: 10.25258/ijddt.10.4.11

How to cite this article: Hachim LS, Khalaf KJ, Khudhur AI. Thrombolytic Activity of Purified Staphylokinase Produced from Clinical Isolates. International Journal of Drug Delivery Technology. 2020;10(4):571-576.

Source of support: Nil.

Conflict of interest: None

INTRODUCTION

Staphylococcus aureus is one of the world's leading pathogens, causing a large range of infectious diseases.[1] *S. aureus* is a gram-positive, cocci-shaped bacteria that appear to be clustered in clusters characterized as "grape-like." These organisms can grow up to 10 % in salt on the media.[2] That promotes colonization of tissue, tissue damage, and distant diseases. To survive the host immune response attack and develop an infection, *S. aureus* presents a vast array of determinants of virulence, including extracellular toxins (e.g., enterotoxins, leukotoxins, and hemolysins), enzymes (e.g., staphylokinase, coagulases hyaluronidase, and proteases), and surface proteins (e.g., clumping factors, adhesions and *S. aureus* surface proteins).[3]

Staphylokinase (SAK) belongs to the family of staphylococcal proteins and is made up of 136 amino acids and approximately a 15 kDa molecule. *S. aureus* synthesizes SAK primarily in the late exponential phase of its growth, where it needs to invade the host tissues by dissolving blood clots formed at the site of injury, and scavenged all the fibrinogen from the system.[4] Staphylokinase was invented when SAK was found to have fibrinolytic activity, such as Streptokinase.[5] Thrombolytic disorders (Thrombosis) have emerged as one of the major causes of human mortality worldwide[6]. Thrombosis is an abnormal blood clot (thrombus) inside a blood vessel. The blood clots can detach from the vascular wall and travel in the bloodstream.[7]

METHODS

Isolation of Bacteria

Bacteria isolated as pure colonies on Mannitol salt agar and Blood agar after incubated at 37°C for 24 hours were inspected microscopically via the gram stain technique and symmetry tests, including cultural and morphological characteristics of all bacterial isolates.[8]

Detection of Staphylokinase Production

Casein Hydrolysis Assay: This medium was prepared according to Pulicherla *et al.* (2011). Casein Hydrolysis Assay agar was prepared by adding 2.8gms of skim milk powder and 2ml of human serum to nutrient agar medium. A well diffusion technique was used to detect the plasmolytic activity of the enzyme. These plates were inoculated by isolated *S. aureus* (100 µL of 1.5×10^8 cell/mL) for each strain in each well and incubated at 37°C for 24 hours to determine the hydrolysis. The positive result was recorded by creating a clear zone around well in Casein hydrolysis agar plate.[9]

*Author for Correspondence: lumaslem474@yahoo.com

Heated Plasma Agar Assay: This medium was prepared according to Shagufta *et al.* (2014). By adding pre- hated human plasma at 56°C for 20 minutes to nutrient agar medium in a percentage of 20% of the total volume, then media was mixed gently and poured into Petri plates and kept for solidification. A well diffusion was used to check the plasmolytic activity of the enzyme. These plates were inoculated by isolated *S. aureus* (100 µL of 1.5×10^8 cell/mL) for each strain in each well and incubate it at 37°C for 24 hours.[10]

The Media used for Staphylokinase Production

The effect of different culture medium used to estimate staphylokinase production by the most powerful Staphylococcus isolate (LKI-43) is listed in Table 1.

To determine staphylokinase production by LKI-43isolate was studied. This was performed by inoculating 5 mL of broth medium in test tubes with LKI-43(1.5×10^8 cell/ml)) incubated at 37°C overnight. These media were added individually to the flask, then inoculated each flask with LKI-43isolate at 30ºC in a rotary shaker at 100 rpm for 24 hours and in addition to estimate enzymatic activity.

Optimum Incubation Period

The effect of the incubation period on staphylokinase production by the most powerful *Staphylococcus* isolates LKI-43isolate was studied by incubating the production medium at 24, 48, and 72 hours.

Production and Extraction of Staphylokinase

The over producer *S.aureus* was grown in the staphylokinase production medium (Satoh's medium) and incubated at 30 ºC in a shaker incubator at 100 rpm for 24 hours.[11] After that, the whole broth was collected by centrifugation at 10,000 rpm for 10 minutes. at 4°C, the supernatant was filtered through a 0.22 µm Millipore filter, and it was collected and labeled as a crude enzyme.

Table 1: Different culture media for staphylokinase production

Culture medium
Nutrient broth
Brain Heart broth
Tryptic soy broth or Trypticase soy broth (TSB)
Nutrient broth 10 gm/L and 3 gm/L yeast extract
Brain Heart Infusion (BHI) Broth and 3 gm/L yeast extract
Tryptic soy broth and 3 gm/L yeast extract
10 gm/L Nutrient broth and NaCl
Brain Heart Infusion (BHI) Broth and 5 gm/L NaCl
Tryptic soy broth and 5 gm/L NaCl
10 gm/L Nutrient broth and 10 L glycerol
Brain Heart Infusion (BHI) Broth and 10 gm/L glycerol
Tryptic soy broth and10 gm/L glycerol
Satoh's medium(10 gm/L Nutrient broth, 3 gm/L yeast extract, 5 gm/L NaCL and 10 L glycerol
Brain Heart Infusion (BHI) Broth, 3 gm/L yeast extract , 5 gm/L NaCL and10 L glycerol
Tryptic soy broth or Trypticase soy broth (TSB), 3 gm/L yeast extract, 5 gm/L NaCl, and 10 L glycerol

Staphylokinase Activity Assay

Staphylokinase activity was determined using the casein digestion method according to Sutar *et al.* (1986), Vesterberagn and Westerberg (1972) as follows: Incubated 1.8 mL of 1% casein in a water bath at 50°C for 5 minutes and mixed with 0.1 mL of the enzyme solution, the solution was incubated at 37°C for 30 minutes. To stop the reaction, 3mL of 5% trichloroacetic acid (TCA) was added. The solution was centrifuged for 15 minutes at 6000 rpm. The control test was prepared by mixing 3 ml of 5% TCA to 1.8 mL of 1% casein solution and then adding 0.2 ml of enzymatic solution. The supernatant's absorbency was measured by spectrophotometer at 280 nm.[12,13]

The activity and Specific activity of the enzyme were calculated as;

Enzyme activity = (Absorbance at 280nm)/(0.01×30×0.2)

0.01: Constant, 30: Reaction time (min), 0.2: Enzyme volume (mL)

Specific activity (U/mg) = Enzyme activity/Protein concentration (mg/mL)

Protein Assay

Bradford (1976) calculated the total protein content using bovine serum albumin (BSA) as standard.[14]

Purification of Staphylokinase [6]

Ammonium Sulfate Precipitation and Dialysis of Crude Enzyme

The crude enzyme supernatant was fractionated between 40 to 85 % of saturation by precipitation with ammonium sulfate. Centrifuged at 10,000 rpm at 4°C for 30 minutes, the precipitate was then taken and dissolved in the amount of phosphate buffer saline (pH 7.0) and dialyzed at 4°C in the same buffer overnight.

Ion Exchange Chromatography

Thereafter, the dialyzed enzyme was added to the ion exchange chromatography column packed with DEAE-Cellulose equilibrated previously with 0.05M Tris-HCl buffer pH8 after that column was washed with an equal volume of the same buffer; while the proteins attached were slowly eluted with gradual concentrations of sodium chloride (0.1-0.9 M respectively).

Gel Filtration Chromatography

A volume of concentrated staphylokinase obtained from the ion exchange stage was loaded onto the gel filtration chromatography using Sephadex G-100, with 0.01 M Tris-HCl buffer pH8 suspended, degassed, and packed in a glass column (1.535 cm) and balanced with 0.01 M Tris-HCl buffer pH 8. Elution was done at a flow rate of 3 ml/fraction using the same equilibration buffer, and the column was washed out with the same eluting buffer to eliminate the unabsorbed materials. The absorbance was estimated at 280 nm for each fraction. staphylokinase activity was also determined for a fraction.

Determination of Molecular Weight

The method of gel filtration chromatography on a column Sephadex G-150 was followed to estimate the molecular

weight of the staphylokinase enzyme, using a standard protein (Alcohol dehydrogenase150 KDa, Bovine serum albumin66 KDa, Carbonic anhydrase29KDa, and lysozyme 14.4 KDa) by drawing the relationship between the logarithm of a standard protein molecular weight and the size of recovery/size of Void (Ve/Vo).

Determination of the Thrombolytic Activity of Staphylokinase

The Holmstrom method was used to assess the activity of the enzyme. In this procedure, clotted blood was taken in twenty-one separate eppendorf tubes each containing 1 mL of blood (Ten eppendorf tubes for the pure enzyme as well as for crud enzyme). When the blood clotted full, the weights of the clots were determined before the enzyme was added.[15]

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

One tube has been kept under control. Separate concentrations of 10, 20 ... 100 µL of purified enzyme samples were added to ten tubes, respectively. All the tubes were incubating at 37˚C for 60 minutes. Then centrifuged at 10,000 rpm for 5 min in a cooling centrifuge. The supernatants were discarded and the clots were reweighted after enzyme therapy. The percentage of clot lysis was calculated using the following formula:

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

An enzyme that fully liquefies 1 mL of clotted blood can be considered to be 1 enzyme unit.[7]

RESULTS AND DISCUSSION

In the present study, after culturing eighty-three clinical samples which isolated from different sources, samples were collected from the patients who attended to Baghdad-Iraq Hospitals (Al-Kindi Hospital and Ibn Al-Nafees). Sixty isolates of *S. aureus* were diagnosed according to biochemical tests and VITEK® 2 Compact system.

Detection of Staphylokinase Production

Casein hydrolysis assay

Casein hydrolysis is one of the oldest methods used specifically for the qualitative determination of the enzyme. Casein hydrolysis was defined by a clearance zone around the growth of colonies on casein agar plates [9].

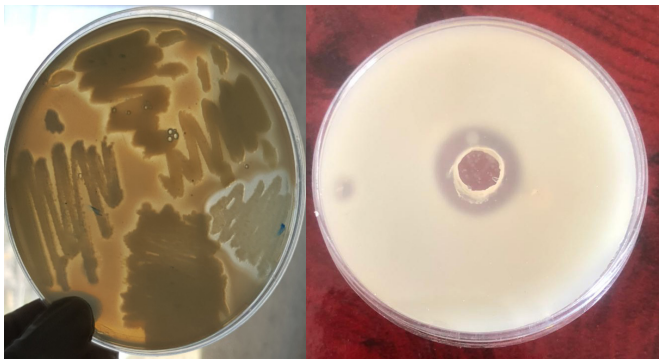

Figure 1: Casein hydrolysis assay for staphylokinase production.

The diameters of the caseinolysis zones were determined by the millimeter shown in Figure 1. Only 40(66.6 %) isolates showed clear halo zones around the colonies. A halo zone (11 to 20) mm was measured in 15 (25%) isolates, (21 to 25) mm in 12(20%) isolates, and 9 (15%) isolates were observed at zone (26-29) mm while the highest zone about 30 mm was observed 4 (6.6%) isolates.

Where the results appeared that 66.6% of isolates able to produce staphylokinase enzyme, the results agreed with the results 65% of researcher Al-Taai (2017)[16] and this agreed with the results of the study conducted by Devi *et al*. (2012)[17] who found 58% of isolates.

The casein has been used for many years as a substrate to study proteolytic enzymes (tryptase, streptokinase, staphylokinase, serum protease, etc.). It has been seen in various investigators as a plasmin substrate instead of fibrin and fibrinogen.[18] Staphylokinase is a bacterial protein isolated from a culture medium produced by many strains of *S. aureus* and transforms inactivated plasminogen into active plasmin known to have profibrinolytic effects.[7]

Heated Plasma Assay

The Heated Plasma method was used primarily for the qualitative determination of the enzyme activity, where clear fibrinolytic halos were observed around the well[10]. The hydrolysis of plasma was identified by a zone of clearance around the growth of colonies on plasma agar plates as shown in Figure 2.

The diameters of the clear zones were measured by millimeter, Only 45(75 %) isolates showed clear halo zones around the colonies. A halo zone (11 to 20) mm was measured in 15 (25%) isolates, (21 to 25) mm in 12 (20%) isolates, and in 12 (20%) isolates, a zone (26-29) mm was observed. The highest zone measuring about 30 mm was observed in 6 (10%) isolates.

The present result of staphylokinase producers was 75% were agreeing to the results of Wieckowska-Szkiel M. *et al.* (2007)[19] was 75.5%. While the results were to Van wamel *et al*. (2006),[20] was 76.6% isolates able to produce staphylokinase enzyme. The plasma has been used as a substrate for the study of proteolytic enzymes.[7]

The Best Medium for Staphylokinase Production

Fifteen different media were used to optimize the growth and staphylokinase production of *S. aureus* isolate. The enzyme activity was measured in the different media. The results in Tables 3-7 indicate that Satoh's medium was the highest value

Figure 2: Heated plasma assay for staphylokinase production

Table 2: The media were used for staphylokinase production

Medium	*Enzyme activity (U/ml)*
Nutrient broth	10
Brain Heart Infusion (BHI) Broth	6.6
Tryptic soy broth or Trypticase soy broth (TSB)	3.3
Nutrient broth and yeast extract	15
Brain Heart Infusion (BHI) Broth and yeast extract	13.3
Tryptic soy broth and yeast extract	8.3
Nutrient broth and NaCl	10
Brain Heart Infusion (BHI) Broth and NaCl	6.6
Tryptic soy broth and NaCl	3.3
Nutrient broth and glycerol	12.5
Brain Heart Infusion (BHI) Broth and glycerol	8.3
Tryptic soy broth and glycerol	6.6
Satoh's medium(nutrient, yeast extract, NaCl, and glycerol)	21.6
Brain Heart Infusion (BHI) broth, yeast extract, NaCl, and glycerol	18.3
Tryptic soy broth or Trypticase soy broth (TSB), yeast extract, NaCl, and glycerol	11.6

Table 3: Purification steps for staphylokinase produced by LKI-43isolate.

Purification step	*Volume (ml)*	*Enzyme activity (U/ml)*	*Protein concentration (mg/ml)*	*Specific activity (U/mg protein)*	*Total activity (U)*	*Purification (folds)*	*Yield (%)*
Crude enzyme	100	22	3.4	6.4	2200	1.0	100
Ammonium sulfate precipitation (85%)	30	40	3.5	11.4	1200	1.7	54.5
Dialysis	25	55	2.6	21.1	1375	3.2	62.5
DEAE-cellulose	21	88.3	1.5	58.8	1854.3	9.1	84.2
Sephadex G-100	24	76.6	0.3	255.3	1838.4	39.9	83.5

among the media were used for staphylokinase production and enzyme activity 21.6 U/mL. The present result of Satoh's medium was agreeing to the results of Mohanasrinivasan *et al.* (2015),[6] and Elbashiti (2017).[21]

The yeast extract is used as a nutritional resource in bacterial culture media. Yeast extract is a mixture of amino acids, peptides, water-soluble vitamins, and carbohydrates.[22] Added the glycerol to culture media as the carbon source.[23] On the other hand, added NaCl that shortening the latent period of *S. aureus* growth.[24]

Optimum Incubation Period

The effect of incubation periods on the Staphylokinase production was that enzyme production was in 24 hours of incubation and increased the activity of the enzyme but when the incubation period was increased until it reached 48 to 72 hours. there was a decrease in enzyme activity.

Enzyme activity reached the maximum 22 U/mL after 24 hours of incubation, and then it began to decrease, reaching 18.5 U/mL after 48 hours of incubation and 13.8 U/mL after 72 hours of incubation.

T The possible explanation for a decline in staphylokinase production beyond 24 hours. It may be due to the rapid depletion of nutrients in the medium; the Accumulation of excess acid in the media due to the use of sugar, and the developed oxygen tension.[25] In another study, it was found that incubation temperature affects microorganisms' growth and then streptokinase production.[26]

Production, Extraction, and Purification of Staphylokinase

The crude enzyme from the production medium (Satoh's medium) of *S. aureus*. The purification of staphylokinase was carried out by ammonium sulphate precipitation in concentration 85%, dialysis, Ion exchange chromatography, and gel filtration chromatography, which resulted in the specific activity of 6.4, 11.4, 21.1, 58.8, 255.5 U/mg, respectively. The purified staphylokinase showed its corresponding purification fold of 1.0 with a total yield of 39.9 in Table 3. The elution profile of staphylokinase following Ion exchange chromatography is shown in Figure 3. The elution profile of staphylokinase after gel filtration chromatography is shown in Figure 4.

Determination of Molecular Weight

The gel filtration determined the molecular weight based on the molecular weight of the logarithm and the volume/volume of the elution (Ve / Vo) measured (MW) of the staphylokinase found to be 14.5 KDa. Depending on the size of the separate molecules with their charge. It was possible that the different methods of estimation may be used.[27] Sephadex-G150 was used for estimation the molecular weight of purified staphylokinase from *S. aureus* illustrated in Figure 5.

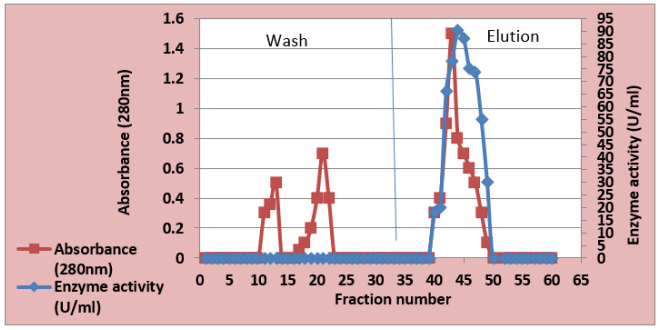

Figure 3: Wash and Elution profile of staphylokinase purified from LKI-43 isolate on DEAE- cellulose column

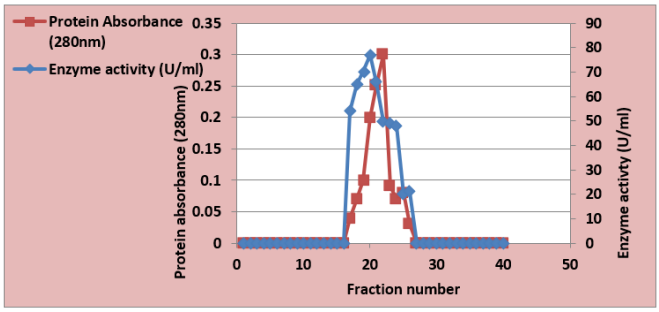

Figure 4: Gel filtration chromatography on Sephadex G-100

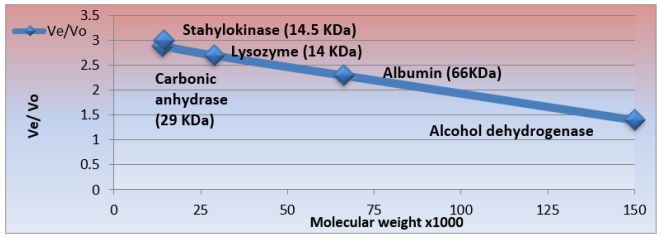

Figure 5: Standard curve to estimate the molecular weight of purified staphylokinase enzyme

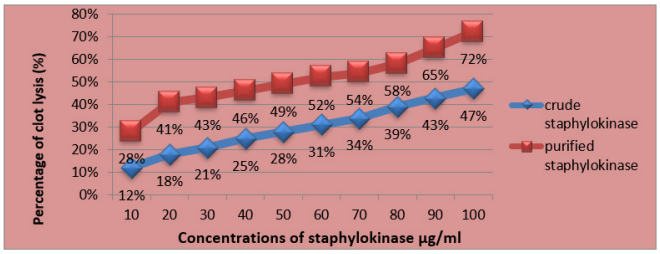

Figure 6: Percentage of clot lysis by crude and purified for staphylokinase produced by LKI-43 isolate.

The molecular weight of staphylokinase form *S. aureus* was found 14.5 KDa the result agrees with the reported by Bashiti (2017).[28]

Mohanasrinivasan *et al.* (2015)[6] showed that staphylokinase purified from *Staphylococcus hominis* had a molecular weight found approximately around 15 KDa. In another study (Mohanasrinivasan *et al.*, 2014)[7] mentioned the molecular weight of staphylokinase was 15.5KDa.

Thrombolytic activity for staphylokinase

Holmstrom method by Mohana *et al.* (2013)[15] confirmed the thrombolytic property of the enzymes, the clot lysis capability

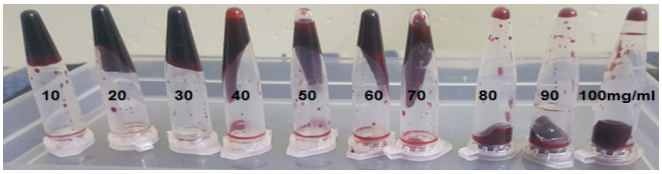

Figure 7: Effect of the crude enzyme on the dissolve blood clot.

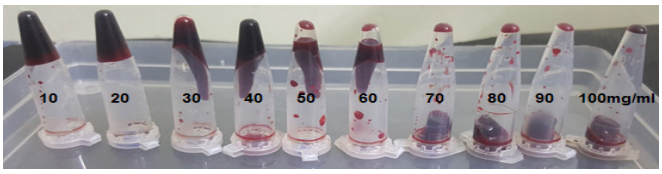

Figure 8: Effect of a purified enzyme on the dissolve blood clot.

of SAK was checked by In vitro clot lysis assay method. Results illustrated in Figure 6, in Figure 7, and Figure 8 showed an increased effect of the purified enzyme on dissolve blood clot than of crude enzymes. The maximum clot lysis by crude and purified was in concentration 100 µg / ml. The highest 72 % of clot lysis in 60 min for purified enzyme while for the crude enzyme was 47%. This result agrees with the results obtained by Prasad *et al.* (2006)[29] the highest 72% of clot lysis.

Srinivasan *et al.* (2013)[30] was seen showed that crude SAK from most of the isolated staphylococcus strains did not show clot lysis activity at lower concentrations. Where Mohanasrinivasan *et al.* (2014)[7] explained the maximum clot lysis was shown to be 50% for 100 µL/mL enzyme

CONCLUSION

The staphylokinase is similar to the streptokinase Produced by *Streptococcus pyogenes* except it is easy to provide the optimal conditions for staphylococcus growth *auras* with the best medium for staphylokinase production, Optimum incubation period, the purification, and Thrombolytic activity for staphylokinase that beneficial on clot lysis.

On the other hand, staphylokinase production from the non-clinical source could be useful for cost-effective therapeutic protein production and safer in clinical practice. Also, experiment thrombolytic activity is the need for staphylokinase that beneficial on clot lysis in vivo.

ACKNOWLEDGMENT

The authors are grateful to the Biology Department, College of Science, Mustansiriyah University, Baghdad, Iraq.

REFERENCE

1. Kwieciński J, Josefsson E, Mitchell J, Higgins J, Magnusson M, Foster T, Jin T, Bokarewa M. Activation of plasminogen by staphylokinase reduces the severity of Staphylococcus aureus systemic infection. The Journal of infectious diseases. 2010 Oct 1;202(7):1041-1049.
2. Taylor TA, Unakal CG. *Staphylococcus aureus*. In StatPearls. StatPearls Publishing PMID: 28722898. 2019.
3. Zecconi A, Scali F. Staphylococcus aureus virulence factors in evasion from innate immune defenses in human and animal diseases. Immunology letters. 2013 Feb 1;150(1-2):12-22.
4. Sumera, A., Chaudhury, P., Haque, S., Saha, P., Paul, D., and Jawed, A. Staphylokinase: A Potent Thrombolytic Agent. Agent

International Journal of Engineering Science Invention (IJESI). 2018;7(1):2319-6734.
5. Christensen L. R.Streptococcal fibrinolysis: A proteolytic reaction due to serum enzyme activated by streptococcal fibrinolysin. J. Gen. Physiol. 1945;28:363-383.
6. Mohanasrinivasan.V, Subathra Devi. C, Dhanmoni K.,Vaishnavi. B, Jemimah Naine.S., Kaustuvmani,P. Production and purification of staphylokinase from *Staphylococcus hominis* MSD1isolated from Kadi: A traditional Indian fermented food. International Journal of PharmTech.0974-4304 Vol.8, No.6, (2015),265-272.
7. Mohanasrinivasan, V., Devi, C. S., Banerjee, M., Siddiqui, J. F., Lakshmi, P., & Naine, J. Production of fibrinolytic Staphylokinase from UV Mutated *Staphylococcus aureus*. Vitsdvm. Int. J. ChemTech Res, 6,(2014),4007-4014.
8. Benson, H.J.Microbiological Applications: Laboratory Manual in General Microbiology.(2002), 8thed.McGraw-Hill Company, Boston, USA.
9. Pulicherla K.K., Gadupudi G.S., Rekha V.P.B., Seetharam K., Anmol K., Sambasiva Rao K.R.S. Isolation, Cloning, and Expression of Mature Staphylokinase from Lysogenic *Staphylococcus aureus* Collected from a Local Wound Sample in a Salt Inducible *E.coli* Expression Host. International Journal of Advanced Science and Technology,30:(2011),35-42.
10. Shagufta Naseer, B., Ravi, M., and Subhashchandra, M. G. Screening of staphylokinase producing *Staphylococcus aureus* from clinical samples. Int J Res Biol Sci, 4(2),(2014),46-48.
11. Tarek A. E. Production of Staphylokinase from Locally Isolated Lysogenic *Staphylococcus aureus*. The Pharmaceutical and Chemical Journal,4(1):(2017),.25-31
12. Vesterberagn K. Vesterberg O. Studies on staphylokinase, journal Med. Microbiol (1972): Vol.5.
13. Sutar II, Vartak HG, Srinivasan MC, Siva Raman H. Production of alkaline protease by immobilized mycelium of Conidiobolus. Enzyme Microb. Technol, 8: (1986),632-634.
14. Bradford, M. A rapid and sensitive method for the quantitation of microgram quantities of protein using the principle of protein-dye binding. Annl Biochem;72,(1976),248-254.
15. Mohana V. , Subathra C., Akata S., Madhumanti M., Sudeshna D., Suganthi V. and Selvarajan E.Screening for staphylokinase producing *staphylococcus* spp. From bovine milk sample. International Journal of Pharmacy and Pharmaceutical Sciences. (2013),Vol 5, Issue 2.
16. Al-Taai, H. R. R. Molecular detection of erm (A), mef (A) in *Staphylococcus* spp resistant to macrolide from different clinical infections. Diyala Journal of Agricultural Sciences,9(3 Special),(2017),1-15.
17. Devi, C. S., Sinha, D., Sharma, V., & Mohanasrinivasan, V. Screening for staphylokinase producing Staphylococcus spp. from different environmental samples. Asian j pharm Clin res,5(4),(2012),125-128.
18. Christensen, L. R. The action of proteolytic enzymes on casein proteins. Archives of biochemistry and biophysics,53(1),(1954), 128-137.
19. Więckowska-Szakiel, M. A. R. Z. E. N. A., Sadowska, B. E. A. T. A., & RÓżalska, B. A. R. A. Staphylokinase production by clinical *Staphylococcus aureus* strains. Polish Journal of Microbiology, 56(2),(2007),97-102.
20. van Wamel, W. J., Rooijakkers, S. H., Ruyken, M., van Kessel, K. P., & van Strijp, J. A. The innate immune modulators staphylococcal complement inhibitor and chemotaxis inhibitory protein of *Staphylococcus aureus* are located on β-hemolysin-converting bacteriophages. Journal of bacteriology, 188(4),(2006), 1310-1315.
21. Elbashiti, T. A. Production of staphylokinase from locally isolated lysogenic. *Staphylococcus aureus,* The Pharmaceutical and Chemical Journal,4(1), (2017),25-31.
22. Zarei O., Dastmalchi S., and Hamzeh-Mivehroud, M. A simple and rapid protocol for producing yeast extract from *Saccharomyces cerevisiae* suitable for preparing bacterial culture media. Iranian Journal of pharmaceutical research: IJPR,(2016),15(4), 907.
23. Küster, E., & Williams, S. T. Selection of media for isolation of *streptomycetes*. Nature, 202(4935), (1964),928-929.
24. Omotoyinbo, O. V., & Omotoyinbo, B. I. Effect of Varying NaCl Concentrations on the Growth Curve of *Escherichia coli* and *Staphylococcus aureus*. Cell Biol, (2016): 4, 31.
25. Ferreira, F. A., Souza, R. R., Bonelli, R. R., Américo, M. A., Fracalanzza, S. E. L., & Figueiredo, A. M. S. Comparison of in vitro and in vivo systems to study ica-independent Staphylococcus aureus biofilms. Journal of microbiological methods, 88(3),(2012),393-398.
26. Novak, E. K., and Philips. A. W. L-Glutamine as a substrate for l- asparaginase from Serratia marcescens. J. Bacteriology,117(2): (1974),593-600.
27. Seagel, I.H. Biochemical Calculations, 2nd ed. John and Sons. Inc. NewYork.(1976).
28. Bashiti, T. A. Production of Staphylokinase from Locally Isolated Lysogenic *Staphylococcus aureus*. Production of Staphylokinase from Locally Isolated Lysogenic *Staphylococcus aureus*, (2017): 4(1).
29. Prasad, S., Kashyap, R. S., Deopujari, J. Y., Purohit, H. J., Taori, G. M., & Daginawala, H. F. Development of an in vitro model to study clot lysis activity of thrombolytic drugs. Thrombosis Journal, (2006):4(1), 14.
30. Srinivasan, V. M., Devi, C. S., Saha, A., Mondal, M., DhaHAR, S., Suganthi, V., & Selvarajan, E. Screening for staphylokinase producing Staphylococcus spp. from bovine milk sample. International Journal of Pharmacy & Pharmaceutical Sciences, (2013):5(2), 601.