Current Trends in Biotechnology and Pharmacy
Vol. 11 (4) 389-395 October 2017, ISSN 0973-8916 (Print), 2230-7303 (Online)

Production, Characterization and Evaluation of thrombolytic activity of Staphylokinase of *Staphylococcus hominis*

C P Chandrappa[1*], Megha Singh[1], N Chandrasekar[2] and M Govindappa[3]
[1]Department of Biotechnology, Shridevi Institute of Engineering and Technology, Sira Road, Tumkur- 572106, Karnataka, India
[2]Department of Chemistry, Shridevi Institute of Engineering and Technology, Sira Road, Tumkur-572106, Karnataka, India
[3]Department of Biotechnology, Dayananda Sagar college of Engineering, Bangaluru, India
*For Correspondence - chandrappacp@gmail.com

Abstract

Staphylokinase isolated from *Staphylococcus hominis* has 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. Finally the thrombolytic activity of Staphylokinase was evaluated by Radial Caseinolytic assay and Heated Plasma agar assay. It was suggested that 0.18 of enzyme is sufficient to dissolve the blood clot.

Key words: Staphylokinase, *Staphylococcus hominis*, SDS-PAGE, Caseinolytic, blood clot.

Introduction

Thrombolytic disorders have emerged to be one of the main causes of human mortality worldwide (1). A blood clot (thrombus) developed in the circulatory system can cause vascular blockage leading to life threatening consequences. A healthy homeostatic system suppresses the development of such blood clots in normal circulation. However, reacts extensively during vascular injury to prevent blood loss (2). The failure of the system to produce the bodily clot lysine such as tissue plasminogen activator (t-PA) and Urokinase, leads to stroke, pulmonary embolism, deep vein thrombosis and acute myocardial pathologies. The clinical intervention to cure these disorders is carried out by the external administration of thrombolytic agents (3).

Staphylokinase is a bacterially derived protein that has been used effectively as a plasminogen activator (4). Staphylokinase could be relatively inexpensive when compared to that of other thrombolytic agents and scaled up into large amounts for industrial production (5). Apparently, the only limitation with this thrombolysis is its bacterial origin that could raise undesired immune responses. Considering a thrombolytic agent for industrial scale development, the present work is undertaken to isolate the mature Staphylokinase from a new source.

Staphylococcus hominis is a coagulate-negative member of the bacterial genus of

Staphylococcus, consisting of Gram-positive, spherical cells in pairs or tetrads. *S. hominis* tends to colonize in areas with numerous porcine glands, such as axillae and the pubic region.

Materials and Methods

***Sample preparation*:** Staphylokinase producing *Staphylococcus* spp was isolated from curd and was mixed with the mixture of dall and fermented curd and it was allowed for incubation for 24 hours.

***Bacterial isolation and sub culturing*:** Serially diluted fermented sample were inoculated by spread plate method over nutrient agar and allowed for incubation 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 characterized and ensured the purity. 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** (6):* The isolated samples were analyzed for Staphylokinase production by heated plasma agar plate assay.

***Identification*:** The following tests were carried out to identify the bacteria. Gram staining, spore staining, motility, haemolysis test, antibiotic sensitivity test, Oxidase test, Catalase test, carbohydrate fermentation test and MRVP test.

Molecular screening by 16s rDNA sequencing: By 16s rRNA sequencing, the bacterial isolates were identified. The genomic DNA was extracted from pure culture and amplified by Polymerase Chain Reaction (PCR) by using *Taq* DNA polymerase, the universal primers forward (i.e. 5'-AGAGTTTGATCMTGGCTCAG) and reverse (i.e. 5'- AAGGAGGTGWTCCARCC).

***Culture Conditions*:** Nutrient broth and solid medium were used for propagation of *Staphylococcal* strains. 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 (7). One ml of uniformly prepared suspension of *staphylococcus hominis* was inoculated and incubated at 35°C and 150 rpm in an orbital shaker. Cells were removed at 48th hour by centrifugation and supernatant was collected.

Enzyme assay and characterization

***Enzyme assay*:** Fibrinolytic activity of crude enzyme was determined by heated plasma agar assay (8), Radial caseinolytic assay: It includes Caseinolytic Agar [5] and Skim Milk Agar.

***Enzyme purification*:** Collected supernatant was added to 3 volumes of acetone which was allowed to stand at 4°C for 1 day. Later the mixture was centrifuged at 10,000rpm for 15 min and the resultant precipitate was purified by ion exchange column chromatography (DEAE Cellulose) followed by Sodium Dodecyl Sulfate Polyacrylamide Gel electrophoresis (SDS-PAGE) with 20% gradient polyacrylamide gel and 4% stacking gel at 4°C.

***Ammonium salt precipitation for purification of enzyme** (7):* The Staphylokinase enzyme was also purified by ammonium sulfate saturation. The fraction of the protein was precipitated with 85% ammonium sulfate. Ammonium sulfate was found to enact the fibrinolytic movement after dialysis. Staphylokinase enzyme was partially purified by using anion exchange column chromatography (DEAE Cellulose, MERK) and affinity chromatography (8).

Protein Estimation: 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. HPLC was also carried out to check the purity of the enzyme.

***Estimation of protein by Lowry's Method** (9):* The relative activity and quantitative estimation of

fibrinolytic enzymes were estimated by Lowry's method spectrophotometrically at 600 nm. BSA was used as standard.

Modified Holmstorm Method *(10):* This is one of the important methods to test the thrombolytic activity of the enzymes. In this method we used the purified enzyme. 1ml of human blood sample was taken in eppendroff tube and allowed the blood to clot. After the blood clot completely, purified enzyme were added at a concentration of 10 to 100 µl. 10, 20, 40, 60, 80, 100 of purified enzyme were added. After that we have observed the time taken by the enzyme to liquefy the clots. Then we have calculated Enzyme units utilized (units/ml of clotted blood).

Results and Discussions

Bacterial isolation and Sub Culturing**:** The three isolates were named as MS1, MS2 and MS3 and sub cultured on nutrient agar and blood agar to screen for production of staphylokinase. Colonies on the plate, Gram staining and pure culture on nutrient agar and blood agar plates are shown in figure 1-4.

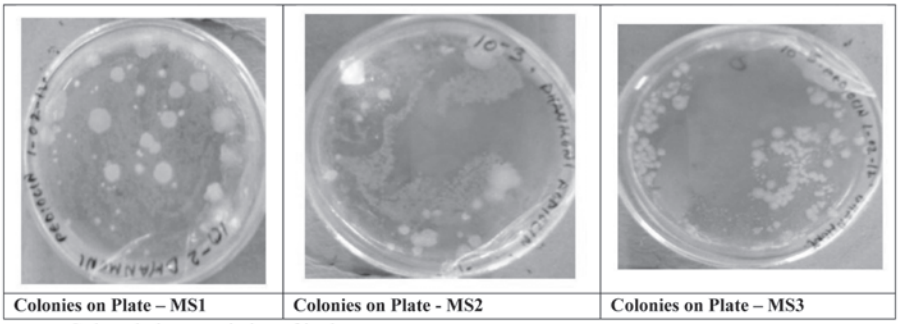

Fig. 1. Cultural characteristics of isolates

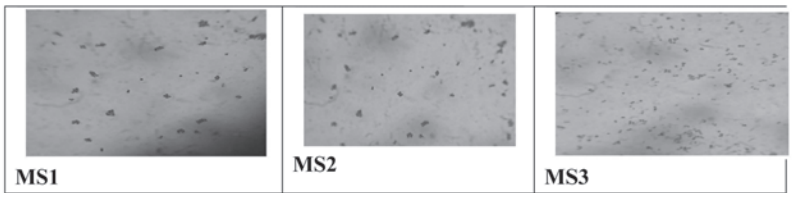

Fig. 2. Gram's staining

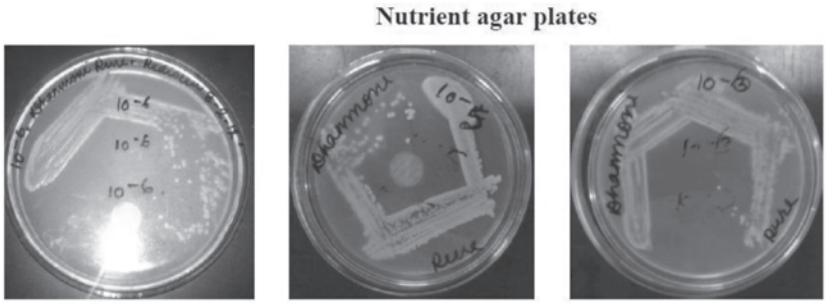

Fig. 3. Pure culture of MS1, MS2 and MS3 from left to right

Screening of Staphylokinase Producing *Staphylococcus* spp*:* The halo zones around the colonies indicate the positive result for this test. Out of the three strains isolated two have shown positive outcome.

Haemolysis test: MS3 was found to be non hemolytic remaining two were hemolytic (Fig. 4).

Further exploration and confirmation of thrombolytic activity of Staphylokinase, casein hydrolytic and skim milk agar assays were carried out and out of three strains, two strains showed positive results (fig 5 & 6). These two positive isolates were further experimented.

***Molecular screening by 16s rDNA Sequencing*:** Outcome of DNA sequencing of two

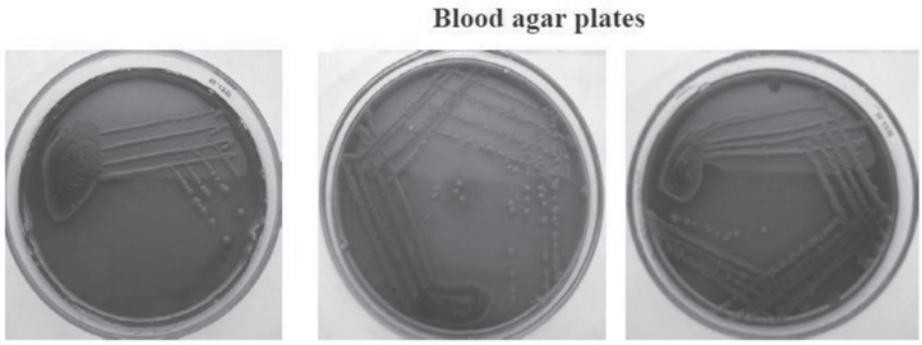

Fig. 4. Pure culture of MS1, MS2 and MS3 from left to right

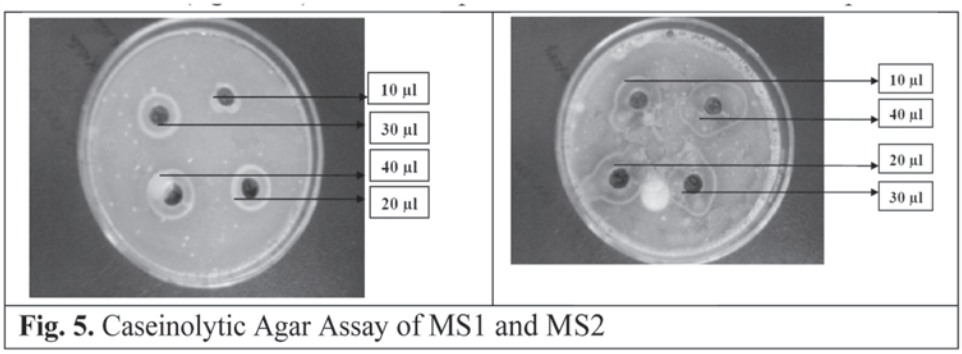

Fig. 5. Caseinolytic Agar Assay of MS1 and MS2

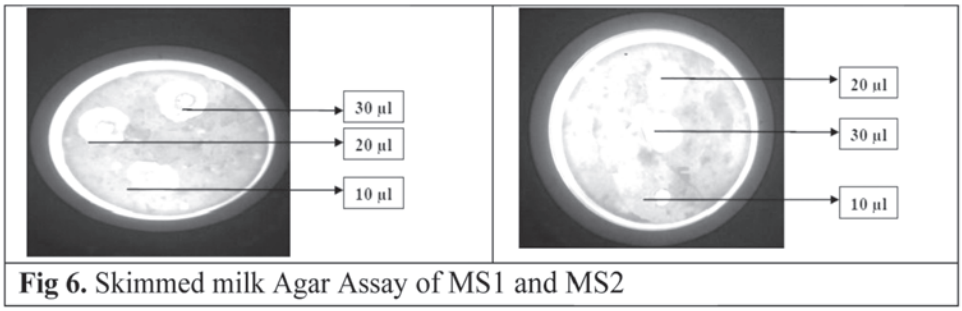

Fig 6. Skimmed milk Agar Assay of MS1 and MS2

isolates MS1 and MS2 confirmed that the identified organism as *Staphylococcus hominis* and further compared with NCBI gene library and determined closest homologous.

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 extracts (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 in an orbital shaker. After 2 days of fermentation, cells were removed by centrifugation.

Enzyme assay and characterization *:* Fibrinolytic activity was determined by the following methods. i) Heated plasma agar assay, ii) Radial caseinolytic assay: It included a) Caseinolytic Agar and 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. After incubation, halo zones around the wells were observed over night which indicated the positive result for this test. Diameter of the halo around the well was measured to check the functional activity of the proteins fig. 7.

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 was collected in fractions of 1 ml. UV-VIS spectroscopic reading was taken to know the protein concentration.

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. 8).

HPLC**:** The purity of the Staphylokinase was determined by HPLC and the retention time of the enzyme was 5.41 (Fig. 9) (12).

Modified Holmstrorm Method (13) : The enzyme was added at a concentration of 10 to 100µl and

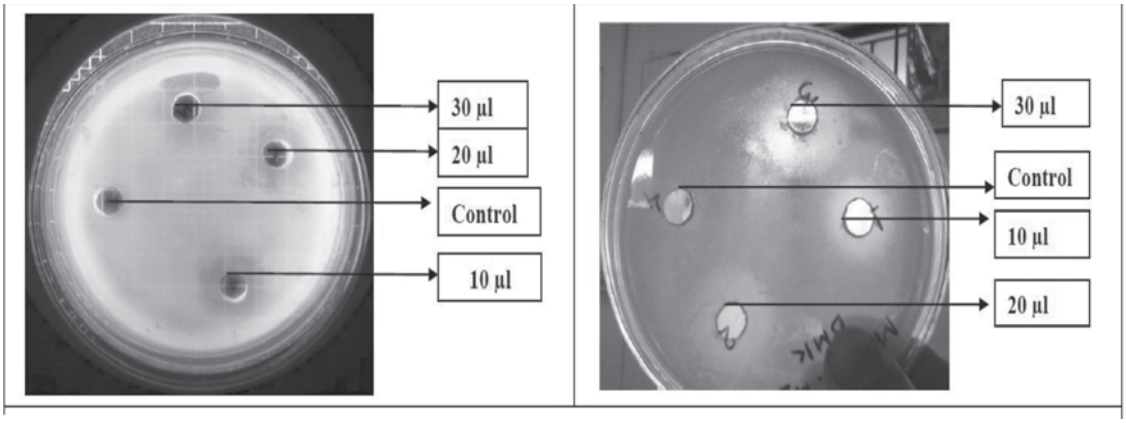

Fig. 7. Heated Plasma Agar Assay of MS1 and MS2

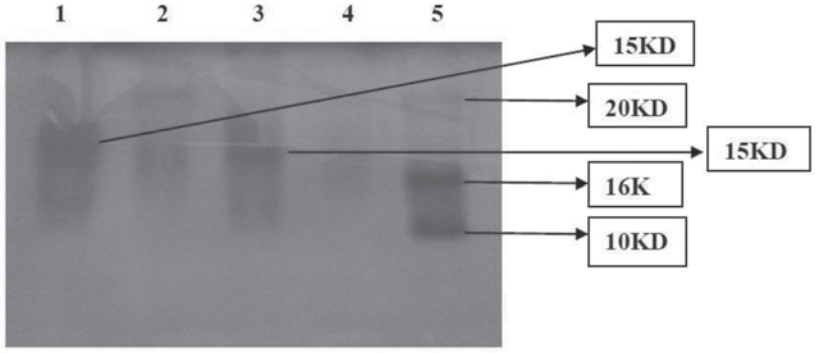

Fig. 8. SDS-PAGE of protein fractions collected from chromatography
1. Fraction 1, **2.** Fraction 2, **3.** Fraction 3, **4.** Fraction 4 and **5.** Protein marker

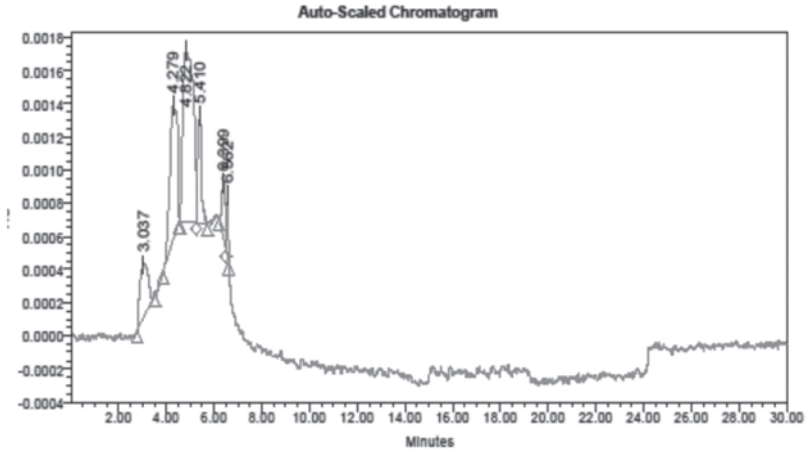

Fig. 9. HPLC of Staphylokinase extracted from Staphylococcus hominis

the enzyme concentration 10, 20, 40, 80, 100 showed positive results to liquefy the clots. The time taken by the enzyme to liquefy the clots was 20 to 30 minutes. Dubey *et al.* (14) announced that 55 µl of protein created by β-haemolytic streptococci is required to break down or lyse the coagulation. However here we have better outcomes similarly that of 10 µl of catalyst somewhat lysed the coagulations (14). But in between 40 to 60 µl the clot was lysed completely by the enzyme (Fig. 10).

Conclusion

Previously Dubey *et al.* (14) reported that 55ìl of enzyme was required for complete lysis of blood clot. But with reference to our study, some better results compared to that. The results of modified Holmstrorm method concluded that at very low concentration like 10, 20 and 40 µl of enzyme or 0.18, 0.36, 0.72 unit of enzyme could

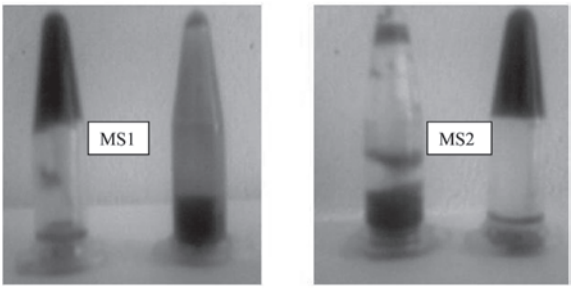

Fig. 10. Blood liquefied by staphylokinase produced by *Staphylococcus hominis (*MS1 and MS2)

lyse the clots. 10 µl of enzyme can partially lyse the clot, but in between 40 to 60 µl we observed complete lysis of the blood clot.

Acknowledgement

We express our sincere gratitude to Dr M R Hulinaykar, Honorable Managing Trustee, Sri Shridevi Charitable Trust, Tumakuru, Karnataka, India and Dr Gurulingappa M Patil, Principal, Shridevi Institute of Engineering and Technology, Sira Road, Tumkur, Karnataka, India for their support, encouragement and extending all the necessary facilities in the college for the successful completion of this project.

References

1. Allender, S., Scarborough, P., Peto,V. and Rayner, M. (2008). European Cardiovascular disease statistics.
2. MacFarlane, R D. (1977). Hemostasis-Introduction. *British Medical Bulletin.* 33: 183-94.
3. Collen, D. and Lijnen, H. R. (1996). Staphylokinase, a fibrin-specific plasminogen activator with therapeutic potential, Blood. 84: 680-686.
4. Wan, M., Wang, Y., Rabideau, S., Moreadith., R, Schrimsher., J. and Conn. G. (2002). An enzyme-linked immunosor-bent assay for host cell protein contaminants in recombinant PEGylated staphylokinase mutant SY161, Journal of Pharm Biomed Analysis. 28:5:953-63.
5. Mohana, S.V., Subathra Devi, C., Akata, S., Madhumanti, M., Sudeshna, D., Suganthi, V. and Selvarajan E. (2013). Screening for Staphylokinase producing *Staphylococcus* spp. from bovine milk sample, International Journal of Pharmacy and Pharmaceutical Sciences. 5:2. 601-604.
6. Pulicherla, K.K., Gadupudi, G.S., Rekha, V.P.B., Seetharam, K., Anmol, K. and Sambasiva Rao, K.R.S. (2011). 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. 30:35-42.
7. Tarek, A.E. (2017). Production of staphylokinase from locally isolated lysogenic *Staphylococcus aureus*, The Pharmaceutical and Chemical Journal, 4(1):25-31.
8. Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J. (1951). Protein measurement with the Folin phenol reagent, Journal of Biological Chemistry. 193: 265-275.
9. Viles-Gonzalez, J.F., Fuster. V. and Badimon, J.J. (2004). Atherothrombosis: A widespread disease with unpredictable and life-threatening consequences, European Heart Journal. 25: 1197-1207.
10. Kondo, I. and Fujise, K. (1977). Serotype B staphylococcal bacteriophage singly converting staphylokinase, Infection and Immunity. 18: 266-272
11. Mohana, S.V., Subathra Devi, C., Dhanmoni, K., Vaishnavi, B., Jemimah, N.S. and Kaustuvmani, P. (2015). Production and purification of staphylokinase from *Staphylococcus hominis* MSD1isolated from Kadi: A traditional Indian fermented food, International Journal of Pharm Tech Research. 8:6: 265-272.
12. Yoshinori, M., Ada, H.K.W. and Jiang B. (2005). Fibrinolytic enzymes in Asian traditional fermented foods, Food Research International. 38: 243-250.
13. Dubey., R, Kumar., J, Agrawala., D, Char., T. and Pusp., P. (2011). Isolation, production, purification, assay and characterization of fibrinolytic enzymes (Nattokinase, Streptokinase and Urokinase) from bacterial sources, African Journal of Biotechnology. 10:8: 1408-1420.