www.sphinxsai.com

International Journal of ChemTech Research
CODEN (USA): IJCRGG ISSN : 0974-4290
Vol.6, No.8, pp 4007-4014, September 2014

ICSET-2014 [6th – 7th May 2014]
International Conference on Science, Engineering and Technology

Production of fibrinolytic Staphylokinase from UV Mutated Staphylococcus aureus VITSDVM7

V. Mohanasrinivasan*, C.Subathra Devi, Monidipa Banerjee, Jannatul Firdous Siddiqui, Pragya Lakshmi, Jemimah Naine S, Vaishnavi.B

School of Bio Sciences and Technology, VIT University, Vellore

***Corres.author: v.mohan@vit.ac.in**

Abstract : Staphylokinase is an extracellular protein produced by strains of *Staphylococcus aureus* which digest fibrin clots. Hence it is widely used as thrombolytic agent due to its low cost as compared to several other thrombolytic agents. The current work has been attempted to characterize the *Staphylococcus aureus* SPK-7 producing staphylokinase. The strain was subjected to morphological, biochemical and molecular characterization. 16Sr DNA sequencing of the isolate revealed close affiliation with *Staphylococcus* genera. BLAST search analysis of the sequence showed maximum identity with *Staphylococcus aureus* CMV201 and *Staphylococcus aureus* SV4 (99% similarity). Hence the strain SPK-7 was coined as *Staphylococcus aureus* VITSDVM7. The preliminary screening was done by casein hydrolysis method followed by plasmolytic activity and showed the maximum zone of inhibition of 15 and 17mm respectively. Random mutagenesis was carried out for the enhanced production of staphylokinase by varying time intervals. The purification of staphylokinase from *Staphylococcus aureus* VITSDVM7 was carried out by ammonium sulphate precipitation, dialysis and gel filtration chromatography resulted in specific activity of 780 , 1053, 1105, 1152 U/mg respectively. Eventually there was a gradual increase with corresponding purification fold of 1, 1.4, 1.4, and 1.5. The clot busting activity was found with 50 % lysis. Hence the mutated strain produced staphylokinase enzyme which can be considered as a good thrombolytic agent.
Keywords: Staphylokinase, mutagenesis, clot busters, thrombolytic agent**.**

Introduction

Cardiovascular diseases have become the leading cause of death in the western world[1]. Novel fibrinolytic enzymes derived from microbial source which are useful for thrombolytic therapy that is currently used in managing and preventing cardiovascular diseases[2]. So far fibrinolytic enzymes have been identified from snakes, earthworms, and bacteria: *Streptococcus pyogenes, Aeromonas hydrophila,Serratia* E15, *B. natto, Bacillus amyloliquefacens, Staphylococcus aureus,* Actinomycetes and fungi: *Fusariums sp*; *Mucor* sp, *Armillaria mellea*[3]. Thrombolysis is the breakdown of blood clots which works by stimulating secondary fibrinolysis which normally activates plasmin. Many blood clot-dissolving agents, such as streptokinase, urokinase, tissue plasminogen activator (t-PA) have been used in treatments for cardiovascular diseases. *S.aureus* can produce protease enzyme encoded by the bacteriophage, called staphylokinase. It is a protein of 15.5KDa and consisting of 136 amino acids and a single chain without disulfide bridges. Its 3D structure reveals that it consists of a α helix (central) and a five- strand β sheets and the connecting loop[4]. The in vitro

studies on human plasma have shown fibrin specific clot- lysis by recombinant staphylokinase which is also proven in animal models of thrombosis and in acute myocardial infection patients[5]. Mutagenesis plays a very crucial role in human lives. Mutation is sometimes advantageous for survival purpose and for the evolution also. In recent years advanced procedures, mutagenesis have begun to make a significant role since it is still cost effective procedure, and reliable. Hence the current study was focused on the enhanced staphylokinase production by UV mutated *Staphylococcus aureus.*

Materials and Methods

Isolation and characterisation

The strain SPK-7 isolated from milk sample was streaked on Mannitol salt agar medium. Simultaneously, Cultural, morphological and biochemical characterisation was done according to Bergey's Manual of Systematic Bacteriology.[6]

Molecular characterization

Total genomic DNA was isolated using the phenol Chloroform method. PCR amplification of 16SrDNA was carried out using the primers FC27 (5_to3_ AGAGTTTGATCCTGGCTCAG) and RC1492 (5_to3_ TACGGCTACCTTGTTACGACTT) [7].The PCR product was detected by agarose gel electrophoresis. Sequencing was performed using big dye terminator cycle sequencing kit (Applied Bio Systems, USA). The sequence was subjected to homology search using BLAST programme of the National Centre for Biotechnology Information (NCBI) and the sequence data has been submitted to the Gene Bank database under the accession number (KJ725089). The acquired sequences were used for a gene homology search, with the 16S rDNA sequences available in the public databases from BLAST and were identified to the generic level. Using the CLUSTAL-W Multiple sequence alignment program (Strasburg, France), the 16SrDNA sequences of the strains were aligned with sequences of related organisms obtained from Gene Bank and a phylogenetic tree was constructed by neighbour-joining method using the Evol View program[8]. To validate the reproducibility of the branching pattern, a bootstrap analysis was performed.

UV mutagenesis

Mutagenesis of the wild type strain SPK-7 was carried out using different exposure times and radiation intensities following the method[9, 10]. The percentage of survival rate was measured after every exposure time and the selected mutants were used for further studies[11].

Production of staphylokinase enzyme

The staphylokinase was produced using satoh's medium. After sterilization the mutant strain was inoculated in the production media at 30ºC for 24 h. The broth was kept for centrifugation for 10,000 rpm at 4°C for 10 min. Supernatant was collected and refrigerated for further experiment[12].

Casein hydrolysis assay

Casein hydrolysis assay was performed to check the proteolytic activity. Casein agar was prepared and sterilized. The wild type and the mutant strain were streaked on two different plates. Further to study the zone of hydrolysis the crude enzyme from mutant strain was added into casein plate and incubated for 24 h at 37 °C[13].

Heated –plasma agar assay

Plasmolytic activity of the extracted enzyme was determined by the heated Plasma Agar assay[14]. Hydrolysis is expressed as diameter of clear zone in mm. The mutant SPK-7 showing prominent zones of clearance were further processed.

Enzyme precipitation

Ammonium sulphate was added to the mutant culture supernatant in small amount with continuous stirring in order to achieve 60-70% saturation. The precipitates were dissolved in 50mM phosphate buffer (pH 7) and subjected for dialysis over night against the same buffer. The resultant dialysates were considered as partially purified and used for further studies.

Gel filtration chromatography

The dialysate (1mL) solution was loaded onto a gel filtration chromatography using Sephadex G-50 column (5 by 30 cm) pre-equilibrated with 50 mM sodium phosphate buffer solution (pH 7.0). The column was washed out to remove the unadsorbed materials with the same eluting buffer at a flow rate of 1mL/1min. The fractions eluted were assayed for the enzyme activity and the fractions were combined and concentrated.

Protein estimation by Lowry's method

The total protein content of the crude enzyme, precipitated and the dialysed samples were determined by Lowry's method [15].

Casein digestion

The activity of staphylokinase was determined by using casein digestion method[13].One unit of caseinolytic activity was determined as the amount of enzyme releasing 1µmole of tyrosine equivalent /min.

Clot lysis activity by holmstrom method

Holm strom method is an important method for checking the clot lytic activity of an enzyme. In this method clotted blood was taken in six separate eppendorf tubes. One tube was kept as the control i.e. without the addition of the enzyme. The weights of the clots were determined before the addition of the enzyme. Different volumes of 10, 20,30, 40,50, 60, 70, 80, 90 and 100µL of the purified enzyme samples were added into five tubes respectively. The tubes were incubated for 60 min at 37˚C. The tubes were then centrifuged at 10,000 rpm for 5 min in a cooling centrifuge. The supernatants were discarded and the clots were weighed again after the enzyme treatment. The percentage of clot lysis was determined using the following formula:

$$\frac{\text{Weight of the released clot}}{\text{Weight of the initial clot}} \times 100$$

The enzyme which tcompletely liquefies 1mL of clotted blood can be considered as 1 enzyme Unit[16].

Results

The *Staphylococcus* strain SPK-7 isolated from milk sample appeared to be yellow colored colonies on the nutrient agar and mannitol salt agar plate. Upon Gram staining it showed purple colored cocci with grape like appearance when observed under microscope as well the cultural, biochemical properties are depicted (Table, 1, Fig 1). The comparative degradation of wild type and mutant SPK-7 was observed by streaking on casein agar plate (Fig 2). The mutant SPK-7 colonies resulted after UV-mutagenesis and the numbers of survivals from each exposure are represented (Table 2). The minimum survival rate of 0.8 % was observed after exposure 20 min of exposure with UV irradiations at 254 nm (Fig 3). The wild type strain SPK-7 assessed for casein degradation showed moderate zone of inhibition of about 11mm. The enhancement for the productivity of the enzyme was followed by random UV mutagenesis of SPK-7 was found to exhibit prominent zone of clearance on casein agar plate with 15mm diameter. The heated plasma method relatively revealed more efficient zone of hydrolysis for the mutant SPK-7 of 17mm. (Fig 4). The identification of phylogenetic neighbours of 16S rDNA gene sequence were found similar with the members of the closely related genera *Staphylococcus* and shared closest relationship with the strains *Staphylococcus aureus* CMV201 and *Staphylococcus aureus* SV4 99% similarity. The strain SPK-7 was coined as *Staphylococcus aureus* VITSDVM7. The phylogenetic tree was constructed and the bootstrap analysis was performed to assess the confidence limits of the branching (Fig 5).

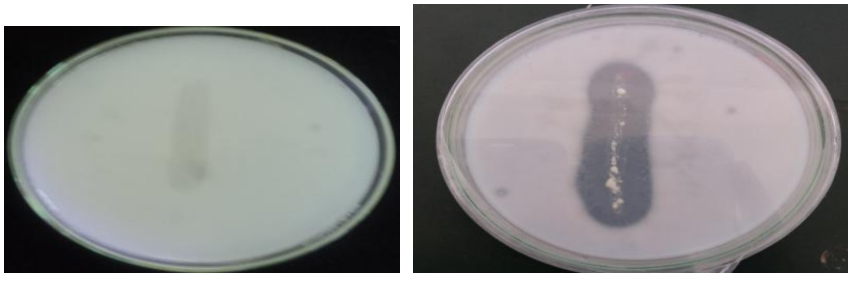
The purification of Staphylokinase from mutant *Staphylococcus aureus* VITSDVM7 was carried out by ammonium sulphate precipitation, dialysis and gel filtration chromatography which resulted in specific activity of 780, 1053, 1105, 1152 U/mg respectively.The purified staphylokinase showed its corresponding purification fold of 1.5 with a total yield of 12.8% (Table 3). The elution profile of Staphylokinase from mutant after gel filtration chromatography is shown (Fig 6). In order to determine the enzymatic activity, the extent of lysis was checked after the coagulation of blood. One unit of enzyme activity was defined as the minimum amount of enzyme at which the bloods clot lysis completely. The maximum clot lysis was shown to be 50% for 100 µL enzyme (Fig7,8)

Table 1. Cultural, morphological and biochemical characterization of *Staphylococcus aureus* VITSDVM7

	Characteristics	Result
Cultural characters	Colony morphology on nutrient agar	Small, round, regular, mucoid,creamy yellow, fast growing colonies
	Configuration	Circular
	Margin	entire
	Elevations	Convex
	Surface	Rough
	Density	Opaque
	Appearance	Shiny
	Pigments	Cream,Golden yellow
Microscopic characters	Gram staining	Gram positive rods
	Motility	Non motile
	Shape	Rods
	Size	Moderate
	Arrangement	Chains
Biochemical characters	VP	Negative
	Esculin hydrolysis	Positive
	PYR	Positive
	ONPG	Positive
	Arginine utilization	Positive
	Glucose	Positive
	Lactose	Positive
	Arabinose	Negative
	Sucrose	Positive
	Sorbitol	Negative
	Mannitol	Positive
	Raffinose	Negative

A B C

Fig 1 (A. Yellow colored colonies on nutreint agar ; B. Yellow coloured colonies on mannitol salt agar; C.Gram staining showing purple clusters)

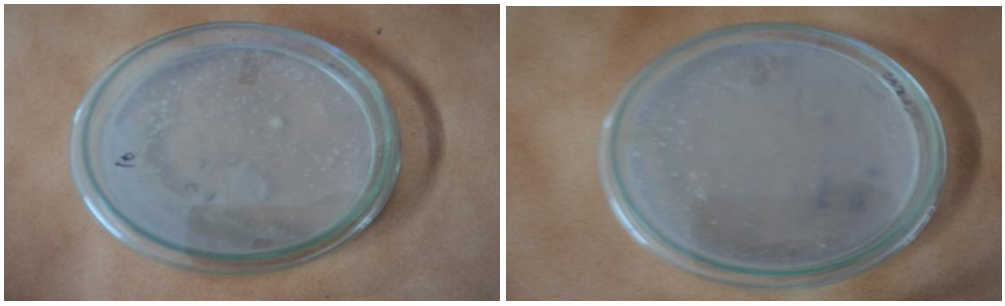


(A) (B)

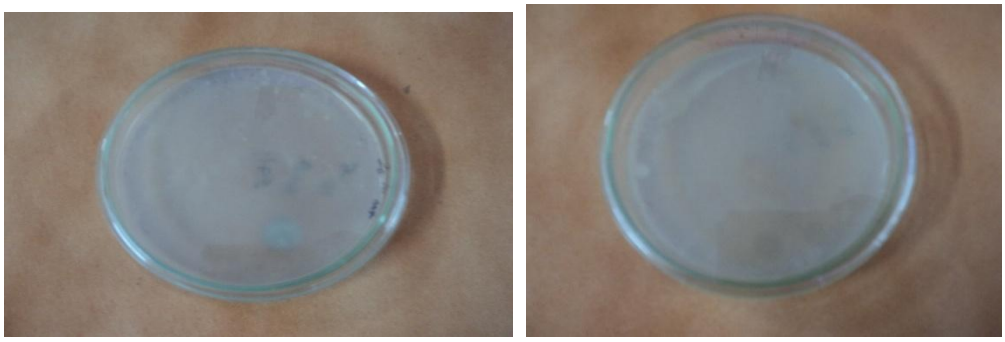
Fig:2 Caseinolytic activity (A) Wild type SPK-7, (B) Mutant SPK strain

Table: 2 Survival of *Staphylococcus aureus* VITSDVM7 UV exposure using different doses

Irradiation time (min)	Number of colonies germinated after UV exposure at 254nm	Survival rate%
0	125	100
5	69	55.2
10	52	41.6
15	37	29.6
20	12	9.6
25	1	0.8
30	0	0

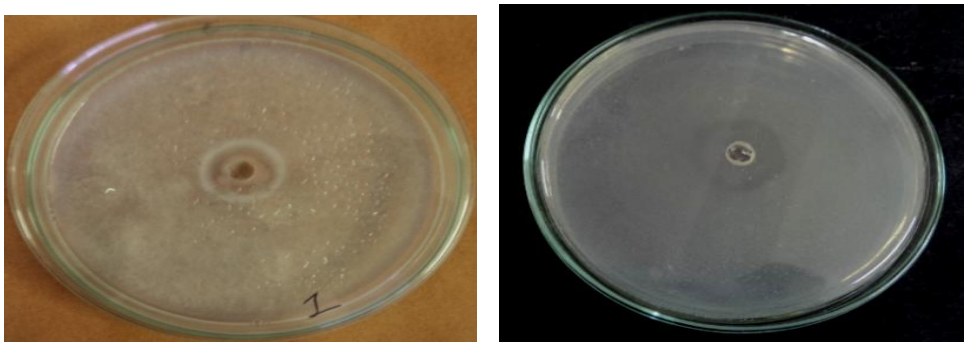


(A) (B)



(C) (D)

Fig: 3 Survival of mutants SPK-7(A-10min;B-15min;C-20min;D-25min)



(A) (B)

Fig:4 Mutant SPK-7 (A) Casein agar plate showing zone of hydrolysis of 15mm.(B) Heated Plasma agar plate showing 17mm zone of hydrolysis

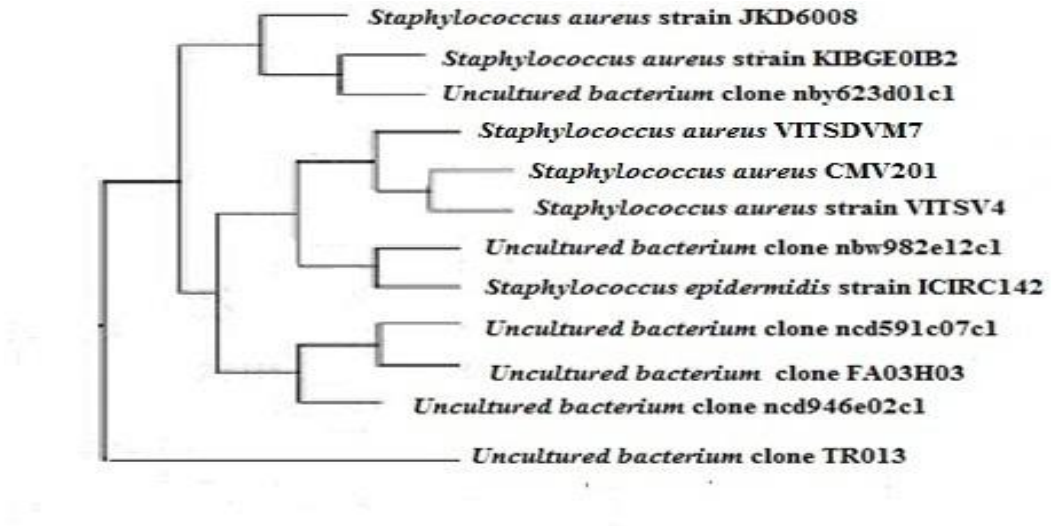


Fig: 5 Phylogenetic tree of the strain *Staphylococcus aureus* VITSDVM7

Table: 3 Purification of staphylokinase enzyme produced by *Staphylococcus aureus* VITSDVM7

Purification step	Total Volume	Total enzyme activity(U/mg)	Total Protein(mg)	Specific activity(U/mg)	Recovery (%)	Purification (fold)
Crude	50	46683	59.9	780	100	1
Ammonium sulphate precipitation	10	15249	14.5	1053	32.7	1.4
Dialysis	4.5	7547	6.8	1105	16.2	1.4
Sephadex G-100	3	5997	5.2	1152	12.8	1.5

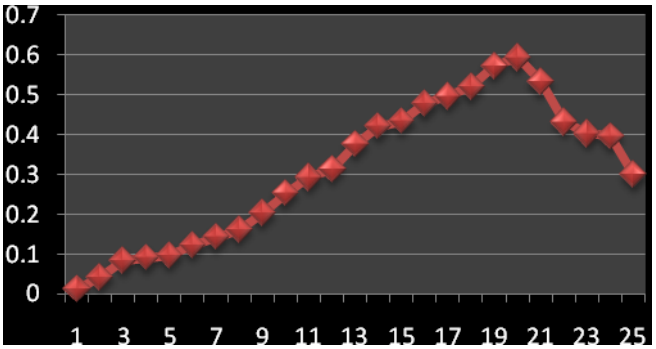


Fig: 6 The elution profile of a staphylokinase after Gel filtration chromatography

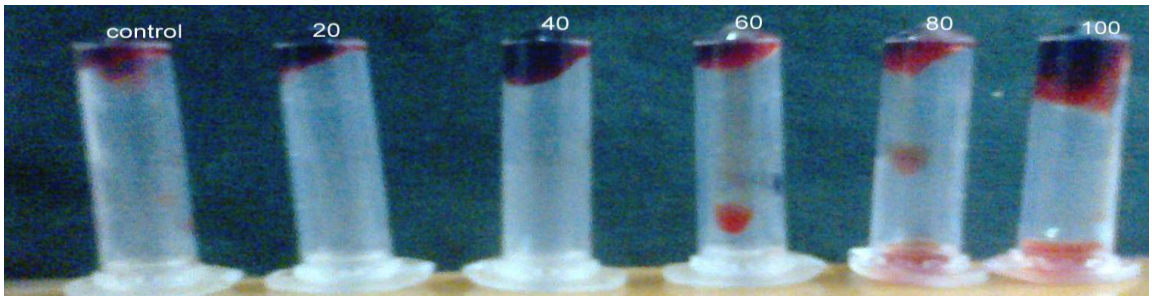


Fig: 7 Pictorial representation of clot lysis activity

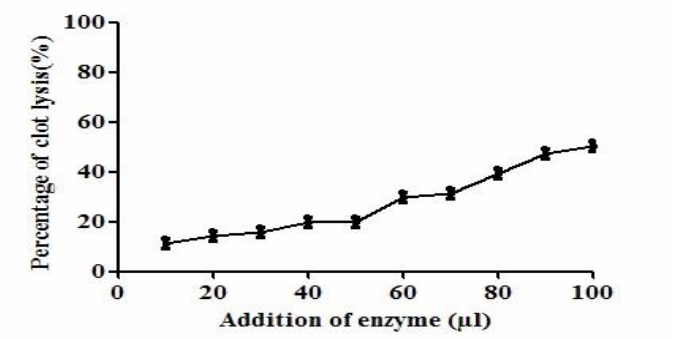


Fig 8 Percentage of clot lysis

Discussion

The accumulation of fibrin in blood vessels leads to cardiovascular diseases (CVD)[11]. The synthetic thrombolytic drugs used to lyse the clot have several drawbacks. Hence task of the study was to produce a cost effective thrombolytic enzyme from *Staphylococcus aureus* VITSDVM7. The ideal features of thrombolytic drugs from bacterial sources including cost effective and stability. Staphylokinase is a bacterial protein isolated from a culture medium which is produced by many strains of *Staphylococcus aureus*. They converts inactivated plasminogen to plasmin in active form[21, 22] which is well known to have profibrinolytic effects[23].In the present study the maximum production of staphylokinase in the culture medium was found with maximum enzyme productivity, which may be due to the variations occurred in genes and differences in their ability of repairing their DNA[17,18].The present study is supported by previous observations,where Staphylokinase, *from Staphylococcus aureus* isolated from local wound sample is reported to have a therapeutic function. The heated plasma agar plate test showed a very good clearance zones after overnight incubation[12, 20]. The clot lysis activity of thrombolytic drugs has been reported[19]. The present study is also supported by the literature study where, Staphylokinase precipitated from culture supernatants by adjusting the pH to3.3 with 10M – HCl[24, 25]. The concocurrent study on separation of staphylokinase was precipitated with 75 % $(NH4)_2S0_4$ and purified by chromatography on CM-cellulose column [26] plasminogen – Sepharose[27]. The in vitro fibrinolytic properties of staphylokinase were evaluated[28] Staphylokinase – neutralizing activity was detected by using a clot lysis assay [29]. Hence the present study proved with fibrinolytic potential of staphylokinase produced from mutated *Staphylococcus aureus* VITSDVM7

Conclusion

The successful development of various strains in the fermentation industry holds the promise of valuable enzymes isolated from microorganisms. In this present study, the purification and characterization of mutant staphylokinase enzyme with beneficial virtue on clot lysis. Also suggests UV irradiation as an effective mutagen for enhanced staphylokinase production from *Staphylococcus aureus* VITSDVM7. Hence this holds great value for the future which would enable one to obtain high yielding mutant strains for large scale production of staphylokinase.

Acknowledgement

We are greatly indebted to Vellore Institute of Technology for the constant encouragement, help and support for extending necessary facilities.

References

1. Viles-Gonzalez J.F.,Fuster V., Badimon J,J., Atherothrombosis., A widespread disease with unpredictable and life-threatening consequences, Eur. Heart J., 2004, 25, 1197-1207.
2. Yoshinori M., Ada H.K.W., Jiang B., Fibrinolytic enzymes in traditional fermented food, Food. Res., 2005, 38, 243-250.
3. Jian Sha C.L., Galindo V., Pancholi V.L., Popov Y., Zhao C.W.H., Chopra A.K., Differential expression of the enolase gene under in vivo versus in vitro growth conditions of *Aeromonas hydrophila*, Microbial pathogen.,2003, 34, 195-204.
4. Wagner P. L., Waldor M. K., Bacteriophage control of bacterial virulence, Infec Immun., 2002, 70, 3985–3993.

5. Collen D., Van de Werf F., Coronary Thrombolysis with Recombinant staphylokinase in patients with evolving myocardial infection, Circul., 1993, (87), 1850-53.
6. Grimont P.A.D., Grimont F., Bergey's Manual of systematic bacteriology. Balti more, Williams and Wilkins.1.,1984, 477 - 484.
7. Rainey F.A., Ward-Rainey N.L., Kroppenstedt R.M., Tackebrandt E., The genus *Norcardiopsis* repre - sents a phylogenetically coherent taxon and a distinct actinomycetes lineage: proposal of *Nocaridio psaceae* fam. Nov, Int. J. Syst. Bacteriol., 1996, 46, 1088-1092.
8. Huangkai Zhang., Shenghan Gao., Wei-Hua Chen., EvolView, an online tool for visualizing, annotating and managing phylogenetic trees. *Nucleic. Acids. Res.*, 2012, 40,569-572.
9. Justin C., Khodursky A., Peter B., Brown P.O., Hanawalt P.C. Comparative gene expression profiles following U.V exposure in wild type and SOS-deficient *Escherichia coli*. Genetics.,2001, 158, 41-64.
10. Hopwood D.A., Bibb M.J., chater K.F., Kieser T., Bruton C.J. Kieser H.M., Lydiate D.J., Smith C.P., ward J.M and Schrempt H., Gentic manipulation of streptomyces - A Laboratory manual, 1985, The John Innes Foundation, Norwick.
11. Wang C., Du M., Zheng D., Kong F., Zu G., Feng Y., Purification and characterization of nattokinase from *Bacillus subtilis* Natto B-12. J. Agric. Food .Chem., 2009, 57, 9722–29.
12. Mohana Srinivasan V., Subathra Devi C., Saha A., Mondal M., Dhar S., Suganthi, V., Selvarajan E., Screening for staphylokinase producing *Staphylococcus* spp. from bovine milk sample. Int. J. Pharm.Pharm. Sci., 2013, 5(2).
13. Mohanasrinivasan V., Subathra Devi C., Ritusree Biswas., Falguni Paul., Mohor Mitra., Selvarajan E., Suganthi V., *Enhanced production of nattokinase from UV mutated Bacillus sp*. Bangladesh J Pharmacol., 2013, 8, 110-115.
14. Dubey R., R. Dubey., Kumar J., Agrawala D., Char T., Pusp P., Isolation, production, purification, assay and characterization of fibrinolytic enzymes (Nattokinase, Streptokinase and Urokinase) from bacterial sources. African J BiotechnoL., 2011,10(8), 1408-1420.
15. Lowry O.H., Rosenbrough N. J., Farr A.L., Randall R.J., Protein measurement with Folin-Phenol reagent. J. Biol. Chem., 1951, 193, 265-275 .
16. Holmstrom B., Streptokinase assay on large agar diffusion plates. Act. Chem. Scand. 1965, 19, 1549-54.
17. Ben F.K., Microbial repair mechanisms after U.V treatment, Technical Backgrounds Article Berson UV-Technick., 2003, 1:1.
18. Uehra H.K., Yonder Y., Yamano K., Marvo B., Regulation of neutral Protease productivity in *Bacillus subtilis* transformation of high protease productivity, J Bacteriol.,1974, 33, 91-92.
19. Sweta Prasad., Rajpal S Kashyap., Jayant Y Deopujari., Hemant J Purohit., Girdhar M Taori., Hatim F Daginawala., Delvelopment of an *in vitro* model to study clot lysis activity of thrombolytic drugs, Thrombosis J., 2006, 4:14.
20. Pulicherla KK., Gadupudi G.S., Rekha V.P.B., Seetharam K., Anmol Kumar., 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, Int. J. Adv. Sci. Technol., 2011, 30, 35-42.
21. Gerheime B., Staphylococcal coagulation and fibrinolysis, Nat., 1948, 162,732.
22. Lack C.H., Staphylokinase: an activator of plasma protease, Nat., 1948, 161, 559.
23. Lewis J.H., Ferguson J.H., A proteolytic enzyme system of the blood, 111. Activation of dog serum profibrinolysin by staphylokinase. Am. J. Physiol., 1951, 166, 594 – 602.
24. Davidson F., Orma L., The activation of plasminogen by staphylokinase : comparison with streptokinase, Biochem. J., 1960, 76, 56 – 61.
25. Glanville Kitty L.A. A simple method of purifying staphylokinase, Biochem. J., 1963, 88, 11 – 14.
26. Jackson KW, Esmon N and Tang J: Streptokinase and staphylokinase, Methods Enzymol., 1981, 80, 387.
27. Kondo I, Itoh S and Takagi T. Purification of staphylokinase by affinity chromatography with human plasminogen, in Jeljaszewicz (ed): *Staphylococci* and Staphylococcal Infections, Zbl. Bakt. Suppl., 1981, 10, 343.
28. Sweet B., McNicol G.P ., Douglas A.S., In vitro studies of staphylokinase, Clin Sci., 1965, 29, 375.
29. Lewis JH, Kerber CW and Wilson JH. Effects of fibrinolytic agents and heparin on intravascular clot lysis, Am. J. Physiol., 1964, 207, 1044 – 48.

******* *******