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RESEARCH ARTICLE

Strain improvement, optimization and purification studies for enhanced production of streptokinase from *Streptococcus uberis* TNA-M1

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BACKGROUND: Screening of isolates for their potency to produce streptokinase was an important criterion of this research. The current study emphasizes the strain improvement, optimization and purification studies for enhanced production of streptokinase from *Streptococcus uberis* TNA-M1 isolated from bovine milk.
METHODS: The study was carried out on samples collected from milk sample. Primary screening and characterization is used as an excellent source for the isolation of β-hemolytic organisms. Strain improvement was done by both physical & chemical mutagenesis. The enzyme activity was checked by clot lysis assay and confirmed by fibrin plate method. The partially purified and crude enzyme were analysed by high-performance liquid chromatography. Molecular weight & enzyme purity was checked by SDS –PAGE, further confirmed by fibrin zymography.
RESULTS: Out of the 3 isolated strains, only one isolate expressed β-haemolysis with streptokinase (SK) activity. Based on the results of radial caseinolytic assay and blood clot dissolving assay, isolate TNA-M1 demonstrated the highest streptokinase activity. Based on morphological , biochemical and molecular characterization, it was identified as *Streptococcus uberis* and the strain was named as *Streptococcus uberis* TNA-M1. The results indicated that ultra-violet (UV) and ethyl methane sulfonate (EMS) were effective mutagenic agents for strain improvement of *Streptococcus uberis* TNA-M1 and enhanced SK productivity. HPLC analysis was performed in order to confirm the presence of streptokinase with the similar retention time (0.875 min) with its standard (0.854) min. SDS-PAGE of the enzyme showed protein band of approximately 47 kDa and confirmed by fibrin zymography. It exhibited fibrinolytic activity, which was more potent than other fibrinolytic enzymes. Glucose and peptone were recorded to be the optimum carbon and nitrogen sources respectively.
CONCLUSION: Thus this study presents its novelty by highlighting the potential of *Streptococcus uberis* TNA-M1 as a significant source for the production of fibrinolytic enzymes.

Keywords Streptokinase, *Streptococcus uberis*, clot busters, mutagenesis, optimization

Introduction

Thrombotic diseases are responsible for heavy toll in death and disability worldwide. These are the most common diseases in the United States and in almost all western industrialized countries. Each year cardiovascular disease (CVD) causes 4.3 million deaths in Europe while in United States 2.5 million deaths (Kumar et al., 2011). A blood clot (thrombus) developed in the circulatory system can cause vascular blockage leading to serious consequences including death. A healthy homeostatic system suppresses the development of blood clots in normal circulation, but reacts extensively in the event of vascular injury to prevent blood loss. Outcomes of a failed homeostasis include stroke, pulmonary embolism, deep vein thrombosis and acute myocardial infarction. Pathologies involving a failure of homeostasis and the development of clot require clinical intervention consisting of intravenous administration of thrombolytic agents Streptokinase is one such agents (Taleb et al., 2005). The clinical importance of streptokinase was first noted by Tillet and Garner (Tillett et al.,1933), who

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discovered that this bacterial protein caused the lysis of human blood clots. It was later found that streptokinase is not an enzyme but rather a potent activator of plasminogen, the inactive precursor of plasmin (Schick and Castellino,1974). Plasmin is the active fibrinolytic component of the circulatory system, solubilizing the fibrin network in blood clots through limited proteolysis (Rodríguez et al,1995). Streptokinase (SK) is an extracellular protein produced by many strains of β- hemolytic streptococci (Tillett and Garner, 1933) which is currently used in clinical medicine as a therapeutic agent in the treatment of thromboembolic blockages, including coronary thrombosis (Banerjee et al., 2004). The enzymes should be produced at a lesser cost, must be reproducible and reusable. The production cost around 30 to 40 per cent for producing enzymes depends on the composition of production medium. By the introduction of mutant strains which have the high capacity of production of enzymes the cost can be further reduced. Mutation is currently used as a basic tool to improve the efficacy of the microorganism producing streptokinase. Strain improvement could be done by mutating the microorganism which produces the enzyme by exposing the organism to physical mutagens like UV rays and chemical mutagens like N-Methyl-N'-nitro-N-nitrosoguanine (NTG), Ethidium Bromide (EtBr) and Ethyl Methane Sulfonate (EMS) (Parekh et al., 2004). Hence the study is focused on strain improvement, optimization and purification studies for enhanced production of streptokinase from *Streptococcus uberis* TNA-M1 isolated from bovine milk.

Materials and methods

Isolation of β-hemolytic streptococci

Isolation of the samples was performed by the serial dilution plate technique (Uversky and Fink, 2004). β-hemolytic streptococci were isolated from the bovine milk sample. Serial dilutions were done with 1 mL of milk sample in 9 mL of sterilized distilled water.The 100μL of each dilution was plated on blood agar medium and incubated for 24 h. The isolates with clear zone of hemolysis around the colonies were purified through repeated streaking on fresh agar plates and maintained on the Brain Heart Infusion Agar (BHIA) until further use.

Morphological and biochemical characterization

Morphological and biochemical characterization was done according to Bergey's Manual of Systematic Bacteriology (Grimont and Grimont, 1984).

Molecular characterization

Total genomic DNA was isolated using the phenol chloroform method (Nathan et al., 2004). PCR amplification of 16S r-DNA was carried out using the primers FC27 (5′_ to 3′ _AGAGTTTGATCCTGGCTCAG) and RC1492 (5′_to3′ _TACGGCTACCTTGTTACGACTT). 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 program of the National Centre for Biotechnology Information (NCBI) and the sequence data was submitted to the GenBank database under the accession number (KT783532).

Phylogenetic analysis

The acquired sequences was used for a gene homology search, with the 16S r-DNA sequences available in the public databases from BLAST and identified to the generic level. Using the CLUSTAL-X Multiple Sequence Alignment Program (Strasburg, France), the 16S r-DNA sequences of the strains were aligned with sequences of related organisms obtained from GenBank and a phylogenetic tree was constructed via the neighbor-joining method using the EvolView program (Huangkai et al. 2012). To validate the reproducibility of the branching pattern, a bootstrap analysis was performed.

Ultravoilet (UV) Irradiation

Ultraviolet (UV) light is electromagnetic radiation with a wavelength shorter than that of visible light, but longer than X-rays .The bacterial cell suspension of wild strains (1×10^6) was prepared in phosphate buffer, pH 7.2 and 10 mL was transferred aseptically into sterile flat bottomed petridishes of 100 mm diameter. The exposure to UV light was carried out in laminar air flow chamber fitted with germicidal lamp that has about 90% of its radiation at 2540-2550Å. The exposure was carriedout at a distance of 20 cm away from the center of the germicidal lamp. 1 mL of the treated bacterial cell suspension was transferred test tubes covered with a black paper at different intervals of time (0, 10, 20, 30, 40, 50, 60 and 70 min) and kept in the refrigerator overnight, to avoid photo reactivation. 100 μL of the irradiated bacterial suspension was plated onto Pike Streptococcal agar and incubated for 24 h at 37°C, the number of colonies was counted to determine survival rates after exposure to UV irradiation. (Hyun et al., 1997)

Chemical mutagenesis

The potential streptokinase producer from UV irradiation was further processed to chemical mutagenesis with Ethyl methane sulphonate (EMS) & N-methyl-Ń-nitro-N-nitroso-guanidine (NTG) which have high capability of inducing the mutations. The selected UV irradiated isolate was incubated in Todd Hewitt medium at 37°C for 12 h and was harvested at logarithmic phase by centrifugation at 14000 r/min for 10 min at 4°C and washed twice with phosphate buffer (0.1M) pH

7.2. UV irradiated potent isolate was incubated with different concentration of EMS and NTG (0, 5, 10, 15, 20, 25, 30, 35, 40, 45 and 50 µg/mL was added into the cell suspension. After incubation for 1 h at 37°C with rotation speed at 100 rpm in shaker incubator, the cells were centrifuged and washed immediately with buffer. The treated cells were transferred into Pike Streptococcal agar plates. The plates were incubated at 37°C for 24 h. (Ibrahim and O'Sullivan, 2000)

Production of streptokinase

The mutants were selected from Pike Streptococcal agar plates after mutagenesis and maintained in Todd Hewitt medium. The SK production was carried out in 50 mL Erlenmeyer flasks containing 25 mL of the production medium (Baewald et al., 1975). The mutant strains of *Streptococcus uberis*TNA-M1 was incubated at 37°C for 12 h with agitation of 150–200 rpm. The streptokinase activity was measured by the addition of 50µL of culture supernatant in the punched wells (1.5mm) on the casein plasminogen overlay agarose medium which was incubated for 12 h at 37°C. The maximum activity was determined by the zone of hydrolysis around the punched wells. The mutants, showing higher streptokinase activity were again mutated by same method described above and this procedure was repeated twice to get the mutants with maximum streptokinase activity. The improved mutated strains were then sub cultured on Pike Streptococcal agar plates further to get pure colonies of these mutants.

Enzyme purification

Ammonium sulfate (salt) precipitation was performed to carry out protein extraction. Different concentrations of salt (20%-80%) were used to facilitate and optimize maximum protein extraction. The pellet obtained was dissolved using PBS buffer, and stored in 4°C. The supernatant was used for further extraction processes with increasing salt concentration.

Dialysis

The partially purified protein sample was added to the membrane from one end (having highest protein concentration from ammonium sulfate precipitation) and the other end was closed with tight knots. The tube containing protein was inserted into 25 mM Tris HCL buffer. The sample was left at 4°C for 6 h in a magnetic stirrer.

Gel filtration chromatography

The resulting dialyzed protein sample was further purified using gel filtration with Sephadex G-100 column (5 mL), previously equilibrated with 10mM Sodium Phosphate Buffer (pH 7.0). Proteins were eluted in the same buffer at 30 mL/h. The obtained protein fractions were subjected to casein hydrolysis and quantities using Lowry's Method in order to find the protein fractions with high specific activies. A chromatogram of absorbance at 560nm Vs fraction number was plotted using the readings obtained from casein hydrolysis of the various fractions. The active enzyme fractions with high specific activities were then pooled and further subjected to enzyme assay to compare the activity of crude and purified enzyme. (Feldman, 1974)

Casein hydrolysis for crude, partial and purified enzyme

Casein broth method was adopted to check for the proteolytic activity of crude enzyme before extraction and further purification steps. Casein broth was prepared and the crude enzyme-casein solution was subjected to UV-Vis spectroscopy and the absorbance values were recorded at 560 nm. Casein broth was prepared using the following reagents. Spectrophotometer was standardized using a blank solution. The tyrosine concentration in crude enzyme solution was estimated using standard tyrosine graph. The Lowry method was applied to determine protein concentration in the crude enzyme sample before extraction and purification, collected with BSA as the reference (Lowry et al., 1951)

Clot lysis assay

Varying volumes (20-100µL) of purified enzyme sample of interest were tested for clot lysis activity in venous blood taken from healthy volunteers with distilled water as negative control. The tubes with clot were incubated at 37°C for 90 min and observed for clot lysis. The difference in weight taken before and after clot lysis was expressed as percentage of clot lysis. (Holmstrom et al., 1965) Streptokinase from β. hemolytic *Streptococci* (sigma), 10000 KU was used as positive control in the assay. The positive control diluted to 100 U/ mL was used for the blood clot lysis assay.

Fibrin plate assay of purified enzyme

Fibrinolytic activity of the purified protease enzyme was further confirmed by using fibrin plate method (Aradhye and Chavan, 2015). Fibrin plates were prepared by adding the solution composed of 4.5 mg/mL fibrinogen in 10 mM Tris–HCl buffer (pH 7.0), 1.2% agarose and 0.45 U/ mL thrombin into the petri plate. The solution in the plate was left for 30 min at room temperature allowing it to form fibrin clot. 10µL of the purified enzyme was carefully loaded onto the punctured circular well (3.5mm in diameter) on the plate and then the plate was incubated at 37°C for 24 h. Fibrinolytic activity was estimated by measuring the diameter of the lytic circle around the well. The activity was expressed in the unit

of lysed area per gram of the protein (mm^2/g) Plasmin from human plasma was used as the positive control.

HPLC

The partially purified and crude enzyme were analyzed by high-performance liquid chromatography (HPLC). Proteins were eluted with 70% (v/v) acetonitrile as the mobile phase at the flow rate of 1.0 mL $h^{-1}$and detected at 280 nm with C18 column (3.0 mm × 300 mm). The retention times for the peaks of the partially purified samples were obtained.

Determination of molecular weight using SDS-PAGE

Wild and the mutant purified protein sample obtained from gel filtration were loaded and compared with BSA as standard protein marker to determine the molecular weight of the purified protein (Lamelli, 1970).

Fibrin zymography

Fibrin zymogram was performed by simple modification of the method (Chung et al., 2011).Purified streptokinase enzyme was loaded in 10% SDS-polyacrylamide gel containing 0.012% of bovine fibrinogen, 10U of thrombin and 0.1% of bovine plasminogen. Further SDS was removed by washing twice for 20 min with 2.5% Triton X-100. The gel was washed and incubated in a mixture of solution containing 50mM TrisHCl (pH-8), 150mM NaCl, 10mM $CaCl_2$ at 37°C for 48h. The gel was stained for 20 min in 0.5% Coomassie brilliant blue in glacial acetic acid isopropanol-distilled water (1:3:6). Washing with distilled water revealed clear area in the shape of bands, lysis of fibrin was visualized, on a blue background. (Chung et al., 2011)

3 Results

Among the 15 bacterial colonies isolated from milk samples 3 isolates were selected based on its colony morphology (yellow colonies, smooth, convex, regular, round colony) and the isolates were found to be gram positive cocci in chains. *Streptococcus* sp. was differentiated from other microorganisms by using differential growth media like pike streptococcus agar (Fig. 1). The isolate TNA-M1 utilizing exhibiting halo zone formation around the colonies on blood agar medium confirmed the presence of β-hemolytic *Streptococcus* sp. The observations were concordant with the reports showing that characteristic feature. The partial hemolytic zone found on the blood agar plate showed the confirmation of the γ-hemolytic *Streptococcus*. The observations were concordant with the reports showing that characteristic feature. The biochemical characteristics of the isolate TNA-M1 were found to exhibit gram positive characteristics. Based on the morphological and biochemical characterization, the TNA-MI strain was identified as *Streptococcus* sp. The 16S rRNA sequencing was exported to the database and checked for homologous alignment. Based on the alignment results, the TNA-M1 strain was confirmed as *Streptococcus* which showed 99% similarity with other *Streptococcus uberis*. The partial 16S rRNA sequences were deposited in Gen Bank under the accession number KT783532 (Fig. 2). A phylogenetic tree was constructed for the TNA-M1 strain with bootstrap values. The phylogenetic tree based on 16S rRNA sequences showed that the isolate occupies a distinct phylogenetic position within the *Streptococcus* family. Based on the molecular taxonomy and phylogeny, the strain was identified as *Streptococcus uberis* and designated as *Streptococcus uberis* TNA-M1.

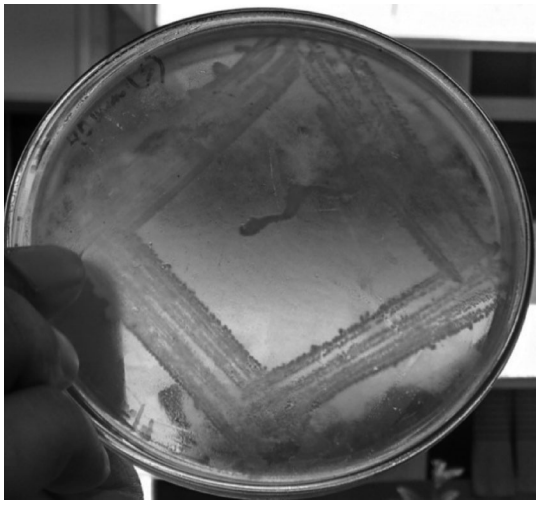

Figure 1 *Streptococcus uberis* TNA-M1.

Survival rate of parent strain *Streptococcus uberis* TNA-M1 was found to be inversely proportional to the exposure time. Minimal survival rate of 44.1% was observed after exposure to120 min of UV-irradiation at 254 nm (Fig. 3). The mutant colonies from UV60 and UV90 showed maximum zone of hydrolysis for streptokinase by radial caseinolytic plate assay. The colonies observed on the pike streptococcal agar plates after treating the cells with the physical and chemical mutagens were further inoculated on the skim milk agar plates to determine their proteolytic activity and the mutants having prominent zone of clearance on skim milk agar plates were selected for further experiments. The *Streptococcus uberis* mutant strain after 60 min exposure was found to give the highest zone of clearance (Fig. 4 A). The mutant strain treated with different concentrations of chemical mutagens EMS (Ethyl methane Sulfonate) and N-Methyl-N'-nitro-N-nitrosoguanine (NTG) respectively were screened on the skim milk agar plates and the mutants having a prominent zone of clearance on the skim milk agar plates were selected for further experimental research. The *Streptococcus uberis* mutant with the EMS concentration of 15µg/ mL was found to give the highest zone of clearance (Fig. 4 B).

Streptokinase activity was confirmed by casein hydrolytic assay. The diameter of zone of hydrolysis around the culture

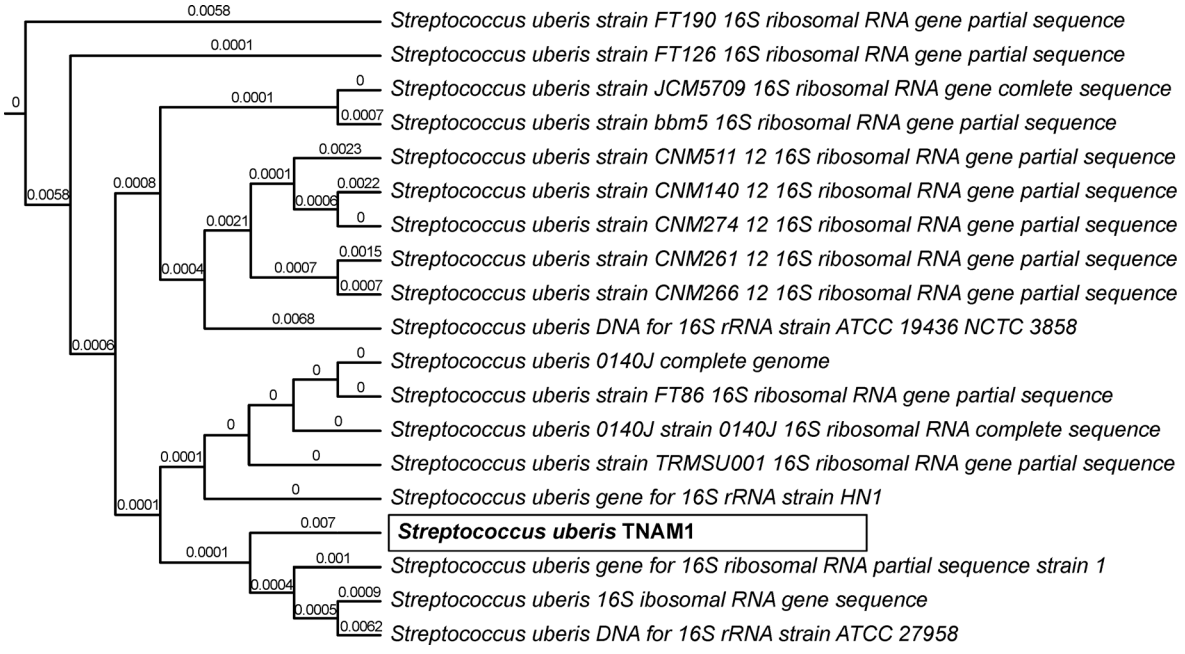

Figure 2 Phylogenetic tree of *Streptococcus uberis* TNA-M1

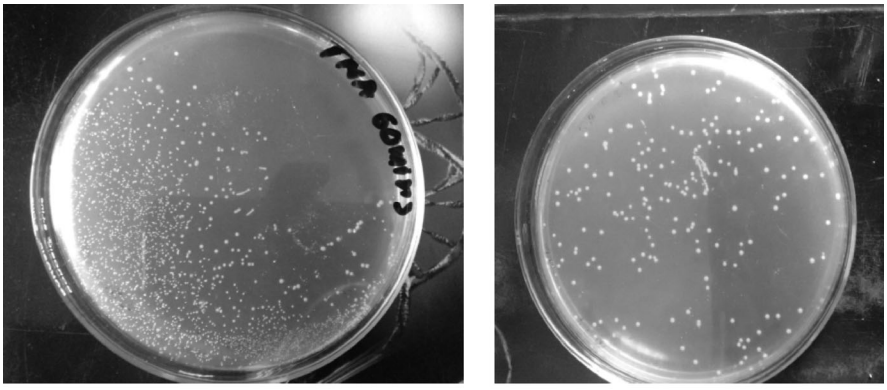

Figure 3 Mutated colonies after UV irradiation for 60 min(A); Mutated colonies after treatment with chemical mutagen-EMS (15 μg/mL)(B).

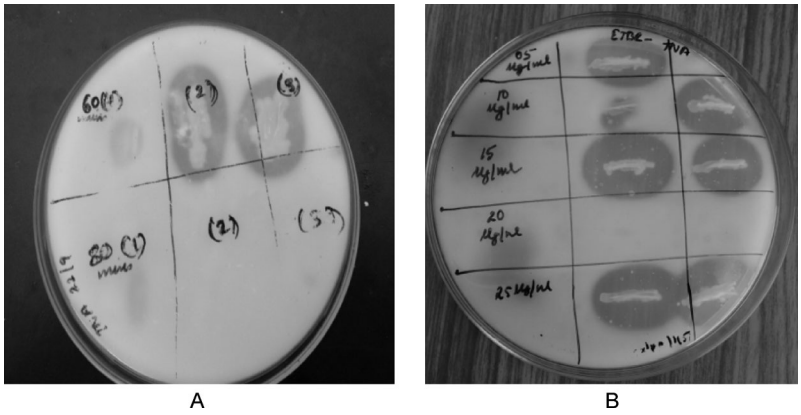

Figure 4 Zone of lysis (physical mutagen) (chemical mutagen).

supernatant were clearly observed and measured. The maximum zone of clearance of 5mm was observed for the isolate (Fig. 5). Blood clot lysis activity was observed visually at different concentrations from 50 to 200 μL of purified streptokinase. The enzyme volume of 200 μL was able to liquefy 79% of the clot within 6 h of incubation at

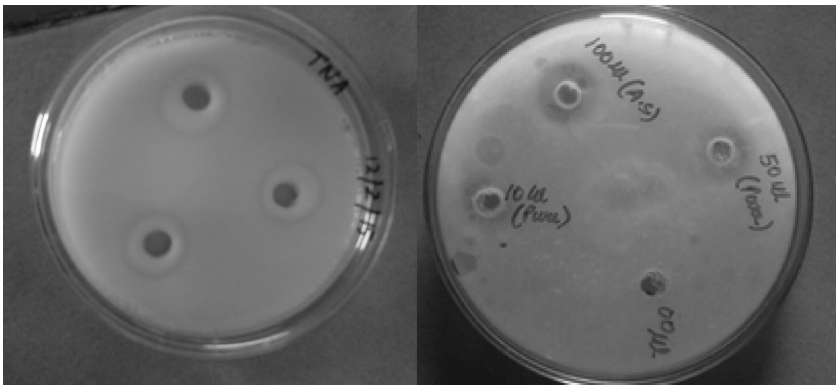

Figure 5 Zone of hydrolysis by the crude and partially purified Streptokinase on Casein Plasminogen agarose overlay and on fibrin plate.

room temperature. The blood clot lysis was also performed that to confirm the Streptokinase activity on blood clot (Fig. 6). Based on the results it was found that the enzyme could lyse natural clot as well as synthetic clot of fibrinogen, plasmin and thrombin. The release of RBC was 100% with purified Streptokinase at the 30th minute of incubation, while control clot indicated no release of RBC. The enzymatic specificity of streptokinase enzyme were analyzed by fibrin zymography. Hence the plasmin-like fibrinolytic enzyme were confirmed in nanogram quantity (Fig. 7A) In SDS-PAGE precipitated protein, partially purified enzyme and purified enzyme were electrophoresed. The band around 47kDa confirms the presence of purified streptokinase (Fig. 7B). The retention time of the partially purified Streptokinase was 0.854 min. Gel Filtration was carried out and the fractions and the fractions were analyzed for protein content and streptokinase activity. The fractions eluted from 18 to 22 shows maximum activity and protein content which was pooled together and analyzed for purity Fig. 8 Streptokinase purified by using Sephadex G-100 column.

The potent isolate *Streptococcus uberis* TNA-M1 in the production medium showed maximum enzyme productivity

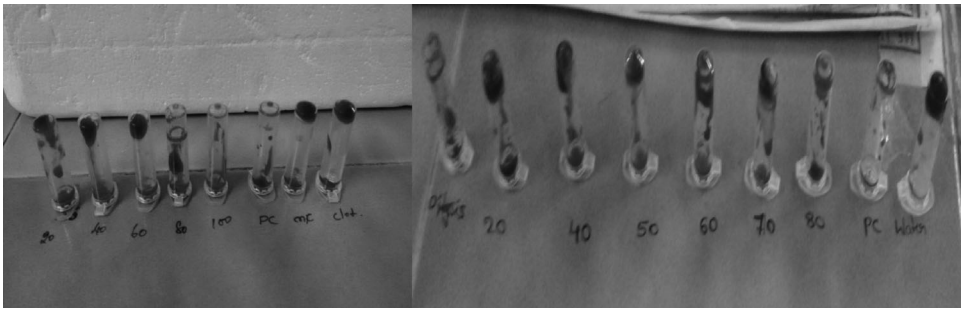

Figure 6 Blood clot lysis of purified Streptokinase from wild and mutant strain.

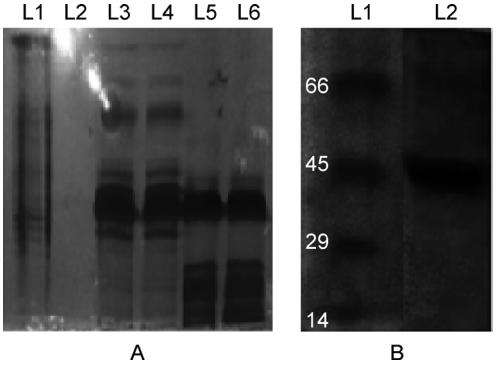

Figure 7 (A) Zymogram of crude, ammonium sulfate, dialysed and purified (B) SDS- PAGE. A: L1- Marker; L2- empty well; L3- Crude; L4- Ammonium sulfate; L5- Dialysed; L6- Purified. B: L1- Molecular marker; L2- Purified enzyme.

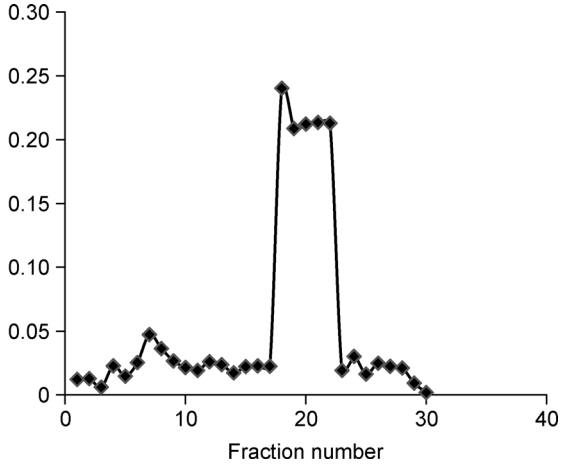

Figure 8 Gel Filtration on Sephadex G100 column eluted with 0.020 TrisHcl buffer shows the fractions from 18 to 22 shows maximum streptokinase activity.

with maximum protein content. Different parameters, pH and temperature affect various biological processes and play important roles in the optimization process. However, after a certain optimum percentage, the addition of further inoculum negatively affects fermentation by decreasing the dilution rate (Fig. 9). Based on the obtained results, the optimum carbon source for the enzyme productivity by *Streptococcus uberis* TNA-M1 mutant strain was found to be glucose (1024 U/mL) and peptone as nitrogen source (1135 U/mL). Furthermore, *Streptococcus uberis* grown on shrimp shell powder supplemented medium, showed better activity than the strain grown in medium with pH 7.5 (1167 U/mL) and 8th h of incubation (1353U/mL). The inoculum concentration 1.5% was more favorable than the generally employed concentrations of up to 3%. The protein content and enzyme activity was found extremely high with the inoculum amount of 1.5% (431 U/mL). The production rate gradually increased with the increase in inoculum percentage. The retention time and purity was analyzed and confirmed by HPLC with standard streptokinase as reference (Fig. 10). The retention time peak was found as 0.854 min closer to 0.875 min.

Discussion

Streptokinase and are the best investigated fibrinolytic proteins of microbial origin, but there are others.A medium containing corn steep liquor, cerelose, KH_2PO_4 and $KHCO_3$, pH 7.0, was used by Feldman (1974) for producing

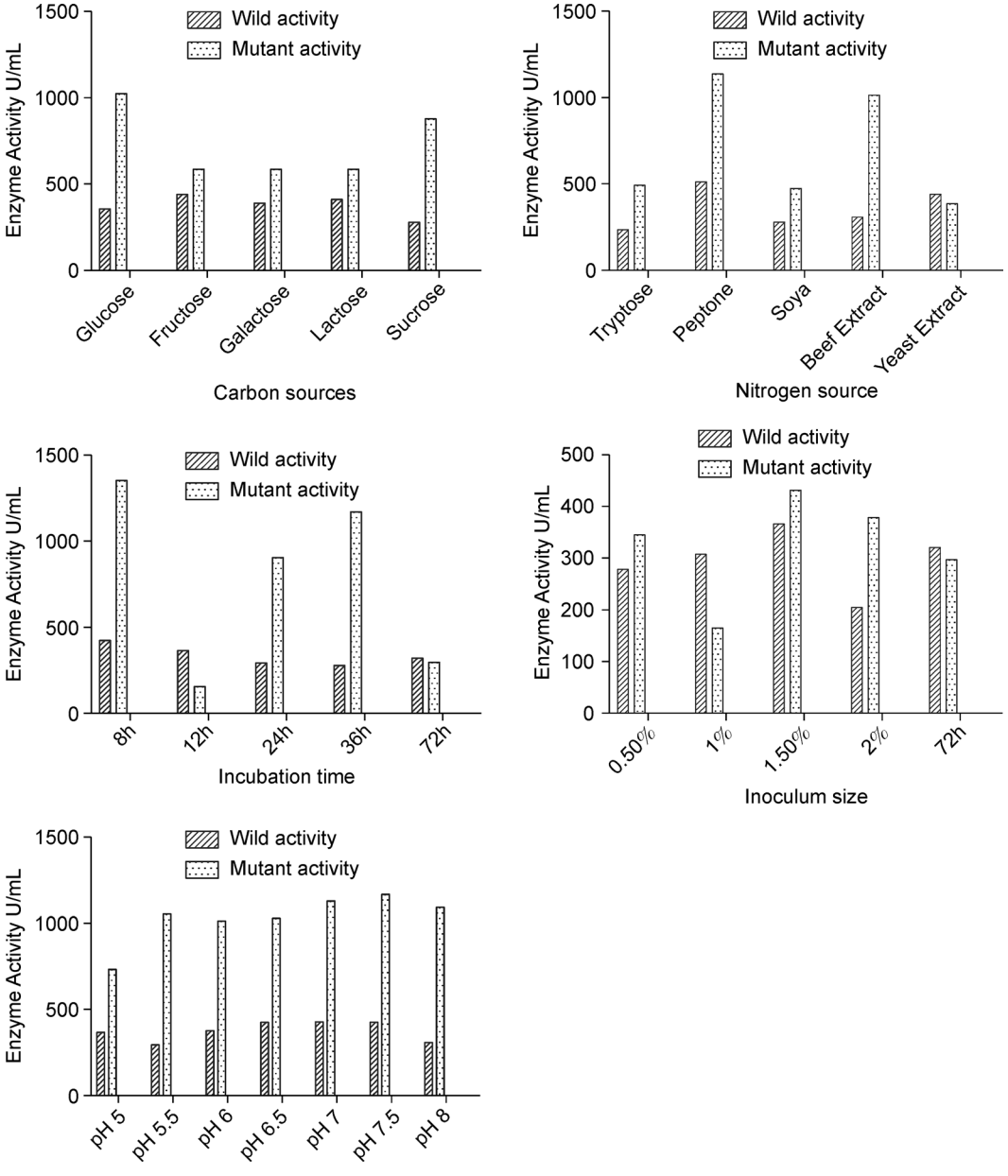

Figure 9 Optimisation studies of *Streptococcus uberis* TNA-M1

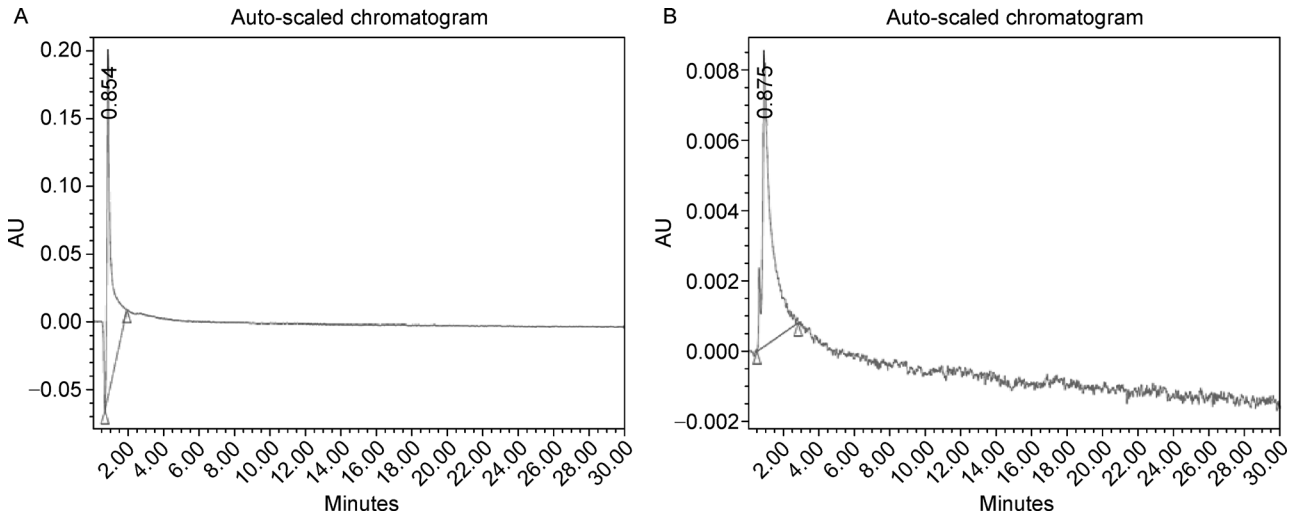

Figure 10 (A) and (B) shows HPLC profile of standard and purified Streptokinase from *Streptococcus uberis* TNA-M1.

streptokinase. Use of corn steep liquor instead of casein hydrolyzate enhanced streptokinase yield in cultures of the strain H46A. Baewald et al. (1975) used a simple and inexpensive medium to obtain high yields of streptokinase from *S. equisimilis.* The medium contained yeast autolyzate or corn steep liquor as the nitrogen source, glucose, and various salts. High titers of streptokinase were attained at 28°C, pH 7.2–7.4, within 24 h in agitated cultures. Relatively recently, work has focused on elucidating the fermentation conditions for producing streptokinase from mutants of the wild-type streptococci and other genetically engineered microorganisms. In contrast, the production of streptokinase was maximized when glucose was in excess and the other nutrients were present in limiting amounts. The mutant strain showed highest activity in the medium glucose and sucrose as carbon sources and peptone and beef extract as nitrogen sources. The mutant was cultured at pH 6.8–7.2, 35–38°C, in broth aerated at 0.1–1.0 vvm. The titer of streptokinase exceeded (8500 $U \times mL^{-1}$). In 1949, Christensen devised the first quantitative method for determining streptokinase (Christensen and Macleod, 1945). The fibrin plate method originally introduced for determining proteolytic activity in blood has been widely used for measuring fibrinolysis. The method has been criticized for various shortcomings and numerous attempts have been made to improve it. In the fibrin plate method, a zone of lysis produced on a fibrin film in a petri dish is measured and related to the concentration of the fibrinolytic protein,either from the commercially available crude preparations or the fermentation broths of various *streptococci*, purified streptokinase from a relatively crude commercial preparation (Varidase; Lederle Laboratories, American Cyanamid, USA). Column chromatography on DEAE-cellulose was followed by column electrophoresis in sucrose density gradients to obtain a five- to 6-fold increase in purity. Precipitation of streptokinase with 40%–50% ammonium sulfate resulted in a two- to three-fold increase in specific activity. The precipitate was recovered by centrifugation and dialyzed against 25mM Tris HCL . The dialyzed solution was further purified by gradient elution from a DEAE-cellulose chromatography column. The major peak of eluted activity was concentrated 10-fold by ultrafiltration.

Conclusion

Native streptokinase is useful for cost-effective thrombolytic therapy in clinical practice. Large quantities of streptokinase can be produced inexpensively via bacterial fermentation. Strain improvement is achieved by inducing genetic mutation by the physical and chemical basis. Thus the developed strain with better productivity is desirable as it is cost effective. Thus future investigation on cloning of the SK gene for mass production of streptokinase could be initiated with this study. Further modification of the desired functional trait would promote the production of potent strain with increased specific enzyme activity.

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Compliance with ethics guidelines

The authors declare that they have no conflict of interest. This article does not contain any studies with human or animal subjects performed by the any of the authors.

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