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Effect of chemical mutagens on expression of therapeutic protein-streptokinase in wild strain *Streptococcus equinus* VIT_VB2

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Streptokinase breaks down the clot in myocardial infarction, affecting three million people globally. The current study, enhanced the production of industrially important fibrinolytic enzyme, streptokinase (SK), this can be used to reduce death rate due to myocardial infarction. The ultra-violet (UV) mutated strain, UVSE6 of *S. equinus* VIT_VB2 showed maximum substrate specific-SK activity (864±0.6 IU mL^{-1}) and partial clot lysis (79%). Hence, the mutant strain UVSE6 was further enhanced by chemical mutagenesis. The improved mutant strain EMS1 after chemical mutagenesis showed maximum SK activity (1004.5±0.7 IU mL^{-1}) and partial clot lysis activity (89%), significantly higher than wild strain. The amidolytic activity of purified SK from mutant strain EMS1 of *S. equinus* VIT_VB2 was found to be 8253 ± 1.6 IU. The molecular weight of SK was determined as 47 kDa by SDS-PAGE and purity of SK was confirmed by HPLC (retention time: 2.82 min). Presence of *SK* gene isolated from EMS1 mutant strain *S. equinus* VIT_VB2 was confirmed using molecular gene sequencing (1200 bp). Structural analysis reveals 3.7% of the amino acid residues in outlier region in the wild type model increase in the mutant. The variation of amino acid in the sequences is observed in RAMPAGE analysis.

Keywords: Amidolytic activity, Clot busters, Ethyl methyl sulphonate (EMS), N-methyl- N′-nitro- N-nitroso guanidine (NTG), RAMPAGE analysis

More than 620 million people are living in the world due to heart related issues[1]. In India, about 14 million people suffer from myocardial infarction every year, and around 2.5 to 3 million patients are treated by thrombolytic drugs instead of catheterization[2]. Thrombolytic drugs like t-PA, urokinase and SK are being commercialized worldwide in the present decade to reduce thrombotic disorders. The substandard and inaccessibility of thrombolytic drugs are the foremost concern in the developing countries. Hence, the side effects of thrombolytic drugs have appended new disputes and certain criteria to manufacture effective drug formulations[3]. Potential fibrinolytic proteases were reported from several sources such as microorganisms, earthworms, snake venom, mushrooms and fermented foods. Among the clot lysing drugs, SK has become one of the primary life-saving drugs for treatment of thrombosis disorders[3]. Economically, the manufacturing of this clot buster drug with high-cost equipment, materials and techniques has led to an increase in the drug cost. The existing clot buster drugs cost between 15,000 and 40,000 INR. The new drug developed by CSIR-IMTECH (Council of Scientific & Industrial Research - Institute of Microbial Technology) costs around 3000 INR this drop in price increases the availability and affordable access to life-saving medication[4]. The involvement of strain improvement techniques introduces improved strains of microorganisms which can reduce the cost of process flow with increased productivity and it may also possess some specialized additional desirable characteristics than wild type strains[4].

The application of strain improvement techniques together with recombinant technology has been exploited industrially to improve the rate of enzyme production[5]. The use of classical *in vitro* mutagenesis with physical and chemical mutagens has developed numerous variants from wild strains[5]. Therefore, short term and reliable strain improvement can minimize the use of tissue plasminogen activator (t-PA) and urokinase (u-PA) exhibiting adverse effects like hemorrhage and anaphylaxis. The incorporation of *SK* gene modification and recombinant *SK* gene expression shows reduced immunogenicity. Consequently, constant efforts have been

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concentrated in the pursuit of more secure and less cost fibrinolytic drugs from different sources[6].

The current study emphasizes the enhanced production of SK from *S. equinus* VIT_VB2 by physical mutagen using UV irradiation followed by chemical mutagen using ethyl methyl sulphonate (EMS) and N-methyl-N′-nitro-N-nitrosoguanidine (NTG) at different concentrations. The contemporary work was the first to report *in vitro* mutagenesis of *S.equinus* VIT_VB2 isolated from bovine milk[7]. The study reports the activity and yield of SK as well as isolation of *SK* gene from the mutant strain. Thus, the findings show a considerable impact on clot buster enzymes with pharmaceutical and medical significance.

Materials and Methods

Microorganism

Streptococcus equinus VIT_VB2 [Accession no: JX406835], isolated from bovine milk was maintained on Pike Streptococcal agar[7]. All the experiments were repeated thrice to confirm the constant production of SK.

Strain improvement

Ultraviolet (UV) irradiation is an electromagnetic spectrum of light with wavelength less than visible light and higher than X-rays. The bacterial strain *S. equinus* VIT_VB2 ($1x10^6$) was maintained in 0.1 M phosphate buffer (pH 7.2). About 10 mL of the culture was transferred aseptically into sterile flat-bottomed petri dish (width of 100 mm) and was exposed to UV irradiation at a distance of 20 cm away from the UV lamp (254-255 nm). At regular intervals of every 10 min (0, 10, 20, 30, 40, 50, 60 and 70 min), 1 mL of the bacterial culture was transferred to sterile test tubes wrapped with dark paper and incubated in a dark chamber overnight to prevent photo-reactivation. 100µL of the irradiated bacterial culture (serially diluted up to 10^{-8}) was spread plated on Pike Streptococcal agar. After 24h incubation at 37°C, the mutation survival rate of the strain was determined[8]. The chemical mutagenesis of *S. equinus* VIT_VB2 was primarily tested to select the mutagens on Pike Streptococcal agar medium using a sterile paper disc with 10 µg mL^{-1} of silver nitrate ($AgNO_3$), ciprofloxacin, EMS, NTG, negative control (water, 10µL) as well as a disc impregnated on a blank area. The potential streptokinase producer from UV irradiation was further treated with EMS and NTG as chemical mutagenic agent which has high rate of induced mutations. The selected UV irradiated isolate was incubated in Todd Hewitt broth (THB) medium at 37°C for 12h. The microbial pellet was harvested by centrifugation at 12000 rpm for 10 min at 4°C and washed twice with 100 mM phosphate buffer (pH 7.2). Different concentrations of EMS and NTG (0, 5, 10, 15, 20, 25, 30, 35, 40, 45 and 50 µg/mL) was added to the cell suspension of the physically mutated (UV irradiated) potent isolate and incubated for 60 min at 37°C at 100 rpm. After incubation the pellet was harvested by centrifugation and washed immediately with 100 mM phosphate buffer. The treated cells were inoculated on Pike Streptococcal agar plates and incubated at 37°C for 24h. Further, the survival rate of the EMS and NTG treated UV irradiated mutant isolate was determined[9-11].

Screening and selection of mutants

The mutant colonies were selected from Pike Streptococcal agar plates after mutagenesis and maintained in THB medium. The mutant strains of *S.equinus* VIT_VB2 was inoculated in 25 mL of production medium and incubated at 37˚C for 12h, 150 rpm[10]. The culture supernatant of the production medium was considered crude enzyme source. 50 µL of crude enzyme was added in the punched wells (1.5 mm) on the casein plasminogen overlay agarose medium and incubated at 37°C for 12h. The SK activity was determined by the zone of hydrolysis around the punched wells[11]. The mutants, showing maximum zone of hydrolysis were again mutated twice with the same mutagen to enhance the SK activity. The improved mutated strains were then sub-cultured on Pike Streptococcal agar plates[9].

Purification gel filtration chromatography

The crude enzyme of the mutant strain *S.equinus* VIT_VB2 was collected and precipitated by ammonium sulfate between 40-90 % of saturation[12]. The dialyzed protein solution (2 mL) was then applied onto Sephadex G-100 column (width of 1.5 cm with 30 cm gel bed height) equilibrated with 20 mM Tris–HCl, pH 7.2. The fractions were collected with 1 mL/min flow rate and analyzed for protein content at 280nm[13].

Molecular weight and purity analysis of SK

The purified protein was analyzed for molecular weight by 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) with standard marker (29-97 kDa)[14]. The SK purity was determined by High Performance Liquid

Chromatography (HPLC) comprised with Waters 2487 separation module with dual-wavelength absorbance detector, a binary pump of Waters 1525 and equipped with a C18 column (250 x 4.6 mm, 5 µm) (Waters Corporation, Milford, MA, USA). Acetonitrile and water in the ratio of 1:1 was used as mobile phase[15].

Partial clot lysis assay

The SK activity of the mutant *Streptococcus equinus* VIT_VB2 was determined by partial clot lysis activity of the culture supernatants in terms of percentage. The clot lysis percentage was determined by measuring the release of RBCs in the fluid at 542 nm and calculated with reference to the calibrated curve of RBCs[16,17].

Chromogenic assay

The *in vitro* activation of plasminogen of SK was analyzed using chromogenic substrate[4]. The amidolytic activity was based on the release of the p-nitroanilide (extinction coefficient -8,800 $M^{-1}cm^{-1}$) from the substrate H-D-Valyl-L-Leucyl-L-Lysine-p-nitroaniline dihydrochloride. The SK and control samples consisted of 20 µL 100 mM Tris–HCl (pH 7.4), 30 µL 0.6 mM substrate and 100 µL 0.1 IU plasminogen which was carried out in 96-well plates. Absorbance of the wells was measured at 405 nm and the intensity of hydrolysis was determined. The crude and purified SK activity was evaluated using calibrated graph using standard SK (Sigma, Mumbai). One unit of SK activity was expressed as the amount of enzyme which converts 1 µM of substrate/ min/mL[18]. (reaction volume-150 µL).

***In vitro* blood clot lysis assay**

The blood clot lysis activity of SK was analyzed by Holmstrom method with some modifications[19].

DNA extraction and sequencing

The potent producer of streptokinase, EMS1 was characterized by extracting the total genomic DNA[20]. The extracted DNA was amplified using the following specific primer for *SK* gene with forward and reverse primers were Skc-F 5' CGCGGATCCATTGCTGGACCTGAG 3' and Skc-R, 5'GCTGGATCCTTATTTGTCGTTAGGGTTATC 3'.The amplification reaction contained 1µg of *S.equinus* VIT_VB2 (UVSE6-EMS1) DNA and 3µL of each oligo primers (5µM), 2.5µL of 10× PCR buffer, 12.8 µL double distilled water, 1.5 µL 25mM $MgCl_2$, 1 µL 5mM dNTPs and 0.2 µL 5 U Taq DNA polymerase in a final concentration of 25 µL. The polymerase chain reaction was performed with Mastercycler gradient (Eppendorf). The DNA was denatured at 95°C for 5 min and subjected to 29 cycles of PCR which included denaturation at 94°C for 30 sec, amplification at 50°C for 30 sec, polymerisation at 72°C for 45 sec and final extension of single cycle at 72°C for 7 min[21]. The PCR product was sequenced for the confirmation of streptokinase gene. The sequence was compared with the database search using BLAST form National Center for Biotechnology Information (NCBI) database[22]. The generation of phylogenetic tree was done by distance matrix analysis using the neighborhood joining method[23].

The predicted 3D structure of the protein was structurally aligned using SWISS MODEL https://swissmodel.expasy.org/interactive. The wild type and mutant gene sequence 3D alignment revealed amino acid changes. The outputs of a structural alignment were superposition of the atomic coordinate sets and a minimal root mean square deviation (RMSD) between the structures validated. The Ramachandran plots were also depicted for each model by Rampage at http://mordred.bioc.cam.ac.uk/~rapper/rampage.php.
The RMSD of two aligned structures indicated their divergence from one another. The differences in relative orientation of the domains between two structures to be aligned are artificially inflated the RMSD[24].

Statistical analysis

All the experimental methods in the study were conducted in triplicates and the results are represented as mean ± standard deviation (S.D). A statistical evaluation of SK production (Sigmaplot) and survival curve analysis using a Kaplan-Meier plot (GraphPad Prism 8) was performed accordingly.

Results

Physical mutagenesis

The survival rate of *S. equinus* VIT _VB2 was found to be 3% after 60 min of exposure to UV. The survival curves were analyzed by Kaplan-Meier plot (GraphPad Prism 8), which linearly decreased with the increase in time of UV irradiation (Fig. 1A, Table 1). From UV mutagenesis, 8 mutants were selected on the basis of different morphology and activity on casein plasminogen agarose overlay medium. Out of the 8 mutants, only one mutant isolate, UVSE6, showed maximum hydrolysis (14.6±0.6mm) (Fig. 1C) and 79% of RBCs were

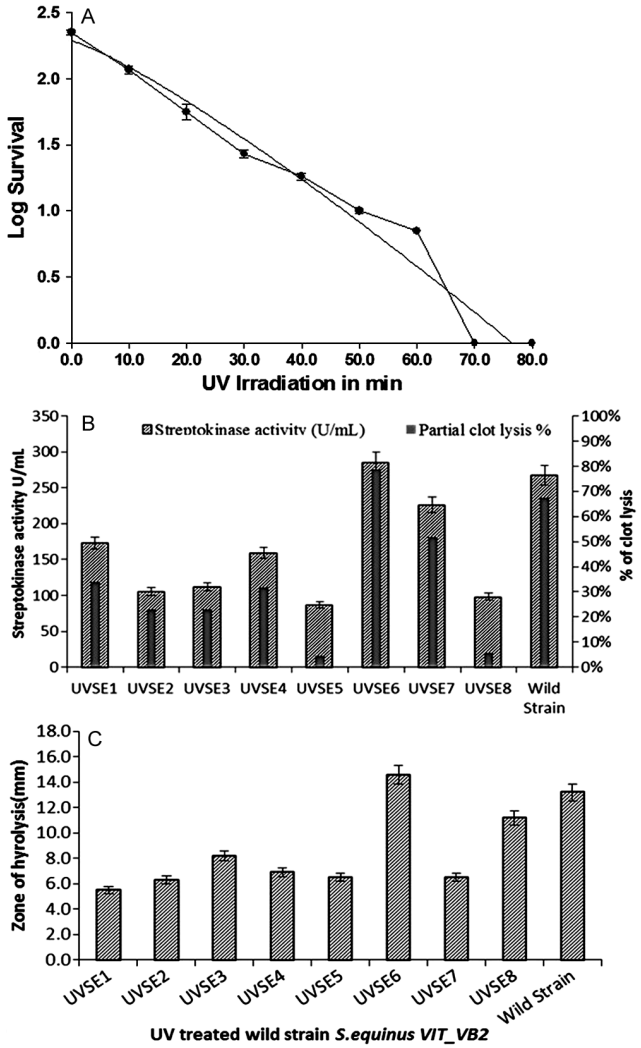

Fig. 1 — (A) UV survival curve of *S. equinus* VIT _VB2 (B) Streptokinase activity of UV mutated *S.equinus* VIT_VB2 (C) Zone of hydrolysis by UV mutated *S. equinus* VIT_VB2.

Table 1 — Amidolytic activity of wild and mutant strains of *S. equinus* VIT_VB2

Mutants	Streptokinase activity (IU mL^{-1})
UVSE6	864±0.6
EMS1	1004.5±0.7
NTG1	979.2±0.4
NTG3	964.5±0.6
NTG8	989.5±0.6
Purified streptokinase	8253 ± 1.6
S.equinus VIT_VB2 (Wild strain)	414.5±0.55

released from the clot which was comparatively higher than the SK activity from wild strain (68%). UVSE6 mutant strain showed maximum SK activity of 285.2±0.5 IU mL^{-1} than the wild strain 267.4±0.5 IU mL^{-1} by casein digestion method (Fig. 1B). The plasmin substrate specificity in terms of amidolytic activity was found to 864.0±0.6 IU mL^{-1}

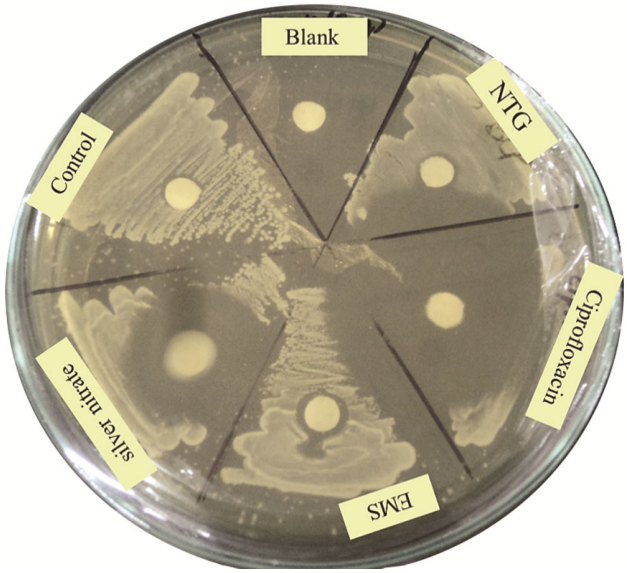

Fig. 2 — Selection of mutagen – 10µg of EMS , NTG, ciprofoxacin, silver nitrate solutions and negative control with water.

which was two-fold higher than that from the wild strain. The productivity was increased than the parental strain from 267.4±0.5 IU mL^{-1} to 285.2±0.5 IU mL^{-1}.

Chemical mutagenesis

The UV mutant UVSE6, potential producer of SK, was screened for chemical mutagenesis (EMS, NTG, silver nitrate, ciprofloxacin). The disc with silver nitrate and ciprofloxacin showed complete growth retardation when compared with EMS and NTG. The control disc with water was surrounded by colonies, and the blank area was negative control. Therefore, EMS and NTG were selected for chemical mutagenesis (Fig. 2). The survival rate of EMS treated with varying concentration from 5-50 µg/mL for 60 min on UV mutant UVSE6 was found to be 3.7%. The log survival curve (Kaplan-Meier plot) gradually decreased from 2.0 to 0.57 (Fig. 3, Table 2). The plates showing 3.7% survival rate was selected for the chemically induced mutants. Similarly, the survival rate of NTG treated with varying concentration from 5-50 µg/mL for 60 min on UV mutant UVSE6 was found to be 11.1%. The log survival curve (Kaplan-Meier plot) expressed a gradual decrease from 2.0 to 0.0 with the increase in the NTG concentration (Fig. 4).

Screening for mutants

Out of 12 mutants selected from EMS mutagenesis, EMS1 showed maximum hydrolysis (16±0.7 mm) and 89% clot lysis activity which was comparatively

higher than the SK from UVSE6 exhibiting at 79% of RBCs release from the clot. The maximum SK and amidolytic activity of EMS1 mutant strain was found to be 321.5±0.8 IU mL^{-1} and 1004.5±0.7 IU mL^{-1} respectively (Fig. 3B & C, Table 1). Similarly, out of the 12 selected mutants, NTG1, NTG3 and NTG8 showed maximum hydrolysis of 18.7±0.4 mm, 16±0.7 mm and 18.2±0.5 mm respectively and the percentage of partial clot lysis with maximum release

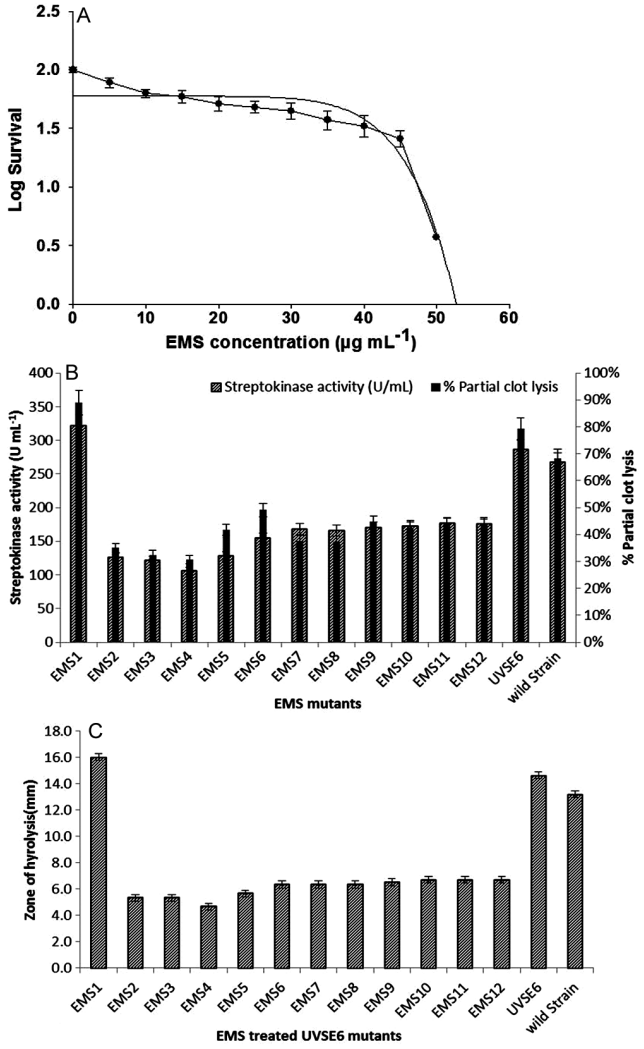

Fig. 3 — (A) EMS treated survival curve of UVSE6 of *S. equinus* VIT _VB2 (B) Streptokinase activity and partial clot lysis of EMS mutants of UVSE6 *S. equinus* VIT_VB2 (C) Zone of hydrolysis by EMS mutants of UVSE6 *S. equinus* VIT_VB2.

was 94%, 89% and 91% which is comparatively higher than the streptokinase from UVSE6 exhibiting a 79% RBC release from the clot. The streptokinase activity of the mutant NTG1, NTG3 and NTG8 showed 389.2±0.75 IU mL^{-1}, 319.3±0.5 IU mL^{-1} and378 ±0.54 IU mL^{-1} (Fig. 4B & C). The amidolytic activity was found to be 979.2±0.4 IU mL^{-1}, 964.5±0.6 IU mL^{-1} and 989.5±0.6 IU mL^{-1}

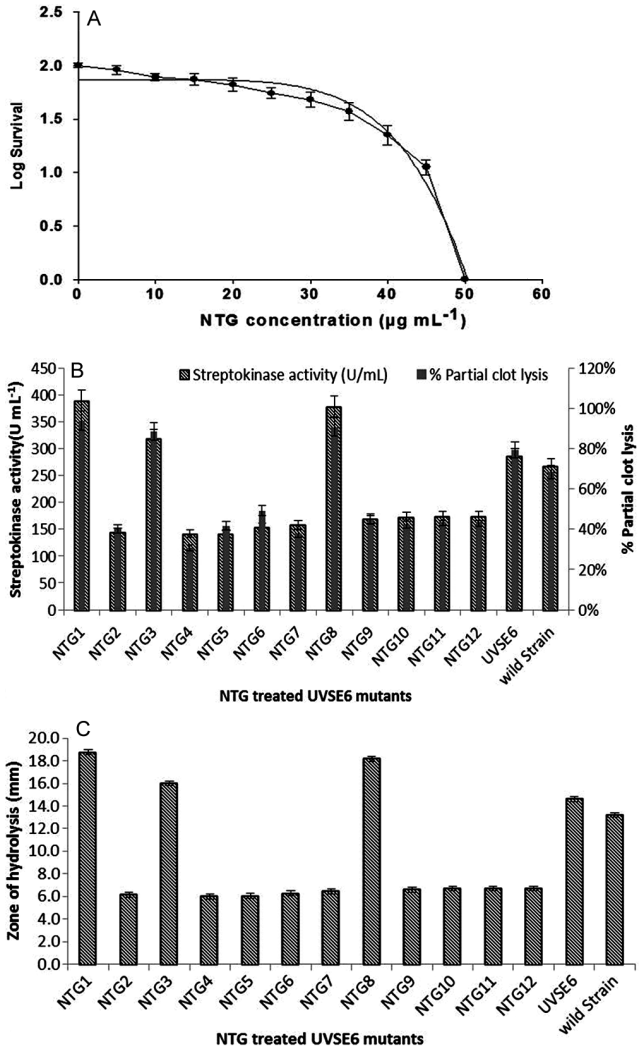

Fig. 4 — (A) NTG treated survival curve of UVSE6 of *S.equinus* VIT _VB2 (B) Streptokinase activity and partial clot lysis of NTG treated mutants of UVSE6 *S. equinus* VIT_VB2 (C) Zone of hyrolysis by NTG treated mutants of UVSE6 *S. equinus* VIT_VB2.

Table 2 — Purification of streptokinase

Purification steps	Relative activity (IU mL^{-1})	Total Activity (IU)	Total protein Content (mg)	Specific Activity (IU mg^{-1})	Fold Purified	% Yield
Crude enzyme	341.46	34146.0	95	359.4	1	100
Ammonium sulfate (%) precipitate	918.52	8266.7	9.3	888.9	2.47	24.2
Dialysis	1232.40	7394.4	5.1	1449.9	4.03	21.7
Gel filtration chromatography	1675.90	6703.6	0.8	8215.2	22.86	19.6

respectively (Table 1). The wild and mutant strains expressed variation in the cultural morphology (Fig. 5). The SK activity was observed by the zone of hydrolysis and liquefaction in casein plasminogen assay (Fig. 6).

***In vitro* blood clot lysis activity**

The lysis of blood clot was observed using 50 µL of crude SK from the selected mutant strains EMS1, NTG1 and NTG8. The SK lysed the blood clot completely within 1h of incubation at 28°C with standard streptokinase as positive control (10,000 IU mL^{-1}, Sigma Aldrich) and distilled water as negative control (Fig. 7)

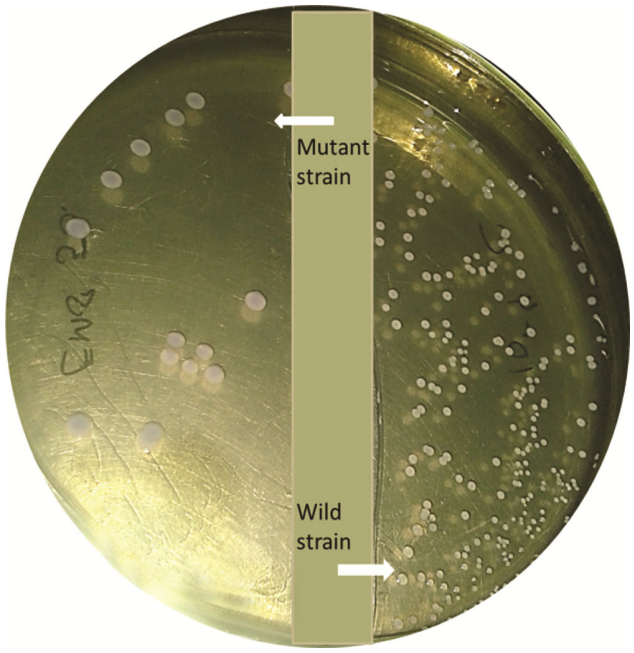

Fig. 5 — Mutant and wild strain of *S. equinus* VIT_VB2 on Pike Streptococcal agar plate.

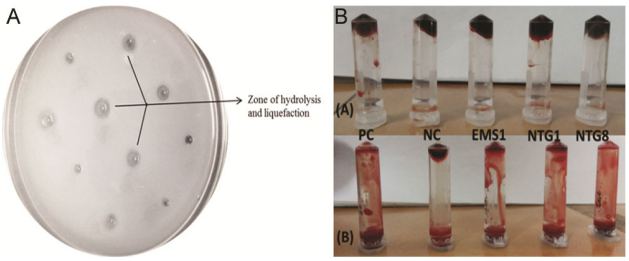

Fig. 6 — (A) Zone of hydrolysis and liquefaction of culture supernatants on casein plasminogen overlay agarose (pH 7.5) (B) *In vitro* blood clot lysis assay PC-postive control (1000U/mL standard SK); NC- ngative control (water); EMS1- extracted enzyme from EMS1 mutant strain; NTG1- extracted enzyme from NTG1 mutant strain; NTG8 - extracted enzyme from NTG8 mutant strain (a) before addition (b) after addition (50µL)

Assay of purified streptokinase

The total SK activity from *S.equinus* VIT_VB2 mutant EMS1 was found to be 34146 IU and the total protein content was 95 mg (Table 2). The purification of SK was fractionated and analyzed from Sephadex G-100 column (Fig. 8). A set of active fractions from 15 to 25 with maximum SK activity was pooled together which expressed 22.86 fold purity and 19.6 % yield (Table 2). The specific activity of purified SK from *S.equinus* VIT_VB2 mutant EMS1 was found to be 8215.2 IU mg^{-1}. The molecular weight of SK was confirmed by the presence of a band of around 47 kDa in SDS-PAGE (Fig. 8). The HPLC analysis confirms the purity of SK with the retention time of 2.8 min which was similar to standard SK (Fig. 10). The plasmin substrate specificity for the purified streptokinase from the double mutated strain UVSE6-EMS1 of *S.equinus* VIT_VB2 was found to be 8253±1.6 IU mL^{-1}.

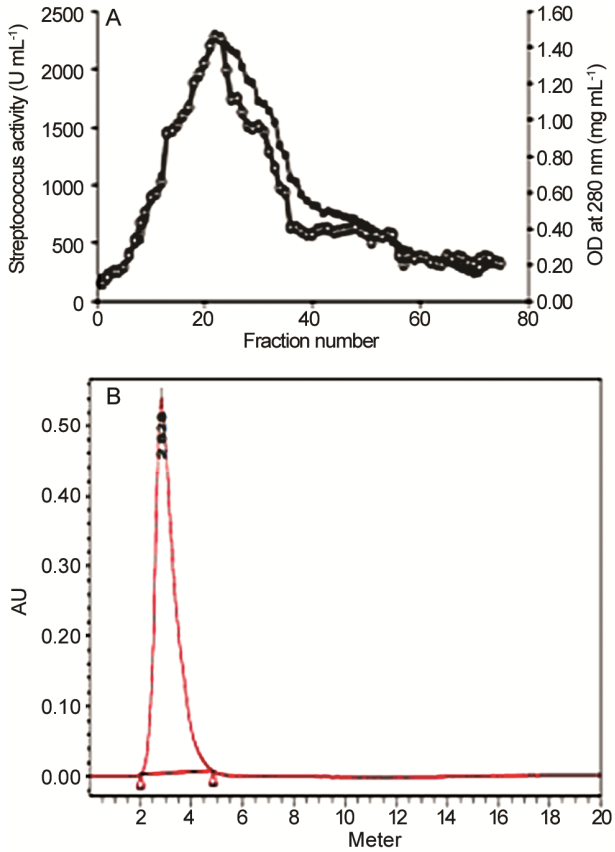

Fig. 7 — (A) Gel filtration chromatography using Sephadex G-100 – SK eluted with 0.020 M Tris–HCl buffer, pH 7.6. Symbols ○--○ OD at 280 nm (mg mL^{-1}), ■--■Streptokinase activity (U mL^{-1}) (B) HPLC analysis of purified streptokinase from mutant potent EMS1 strain of *S. equinus* VIT_VB2.

SK gene characterization

The gene sequence of streptokinase extracted from mutated strain UVSE6-EMS1 of *S. equinus* VIT_VB2 showed 99% similarity with the streptokinase gene from *Streptococcus dysgalactiae subsp. equisimilis* strain GGS_S88 of 1200 bp. The phylogenetic tree was constructed based on Evolview software with bootstrap values ≥50% (Fig. 9).

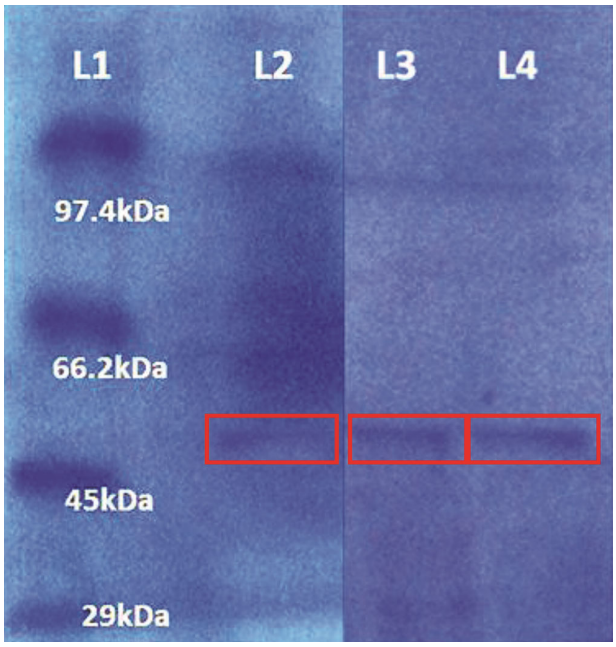

Fig. 8 — 12% SDS-PAGE analysis L1 (Lane 1) - Protein marker; L2 (Lane 2) - 60 % ammonium sulfate precipitate; L3 (Lane 3) - partially purified SK; L4 (Lane 4) - purified SK from Sephadex G-100 gel filtration column chromatography.

Model refinement

The Ramachandran plots of the wild type and mutant models were depicted and compared after refinement (Fig. 10). In wild type SK sequence model, the percent residues in favoured region were

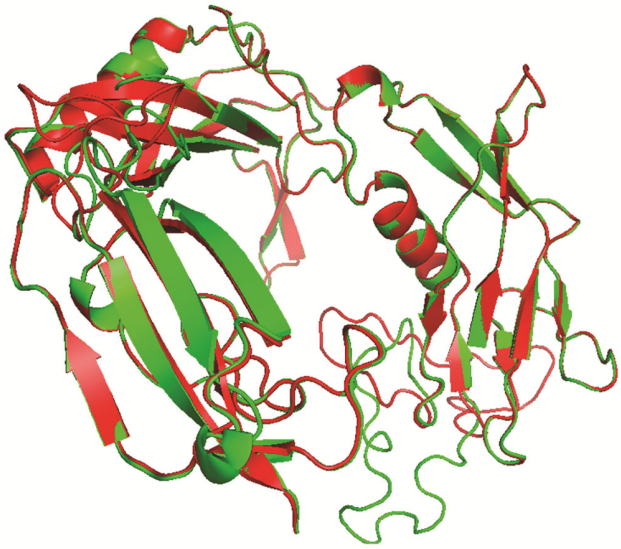

Fig. 10 — 3D modeling alignment between wild type (red) and mutant (green) SK sequence.

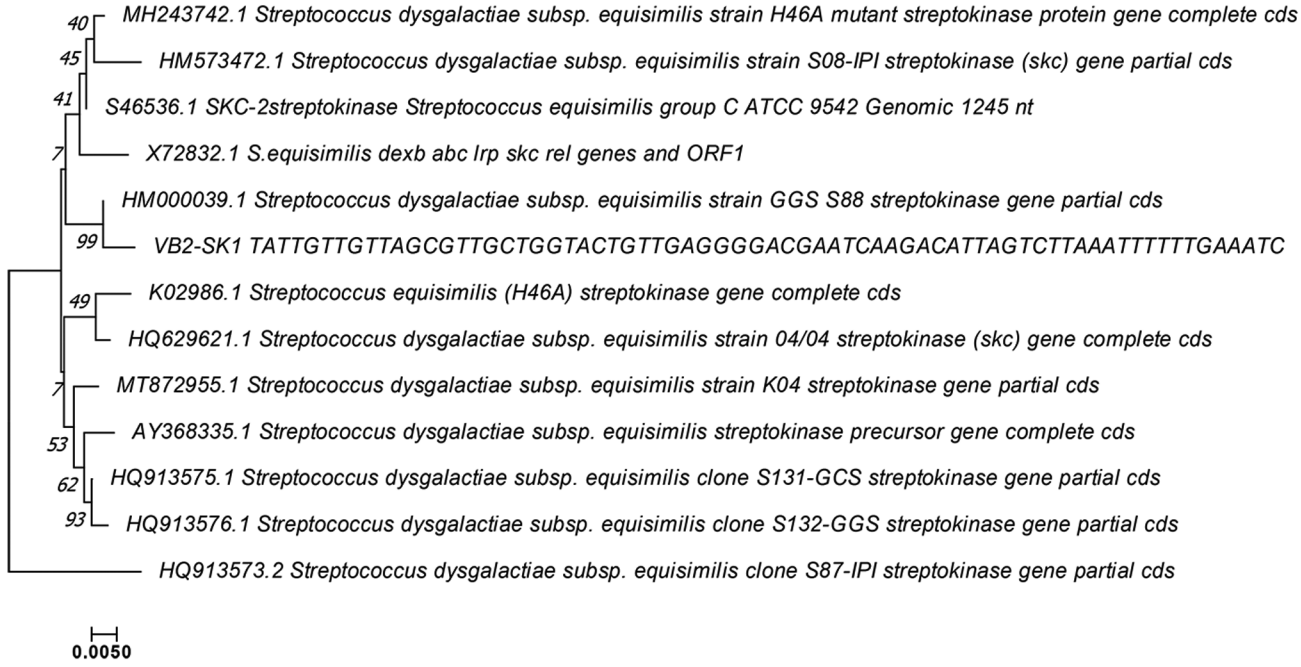

Fig. 9 — Phylogenetic relationship with streptokinase gene from mutant potent EMS1 strain of *S. equinus* VIT_VB2 with other streptokinase gene sequences.

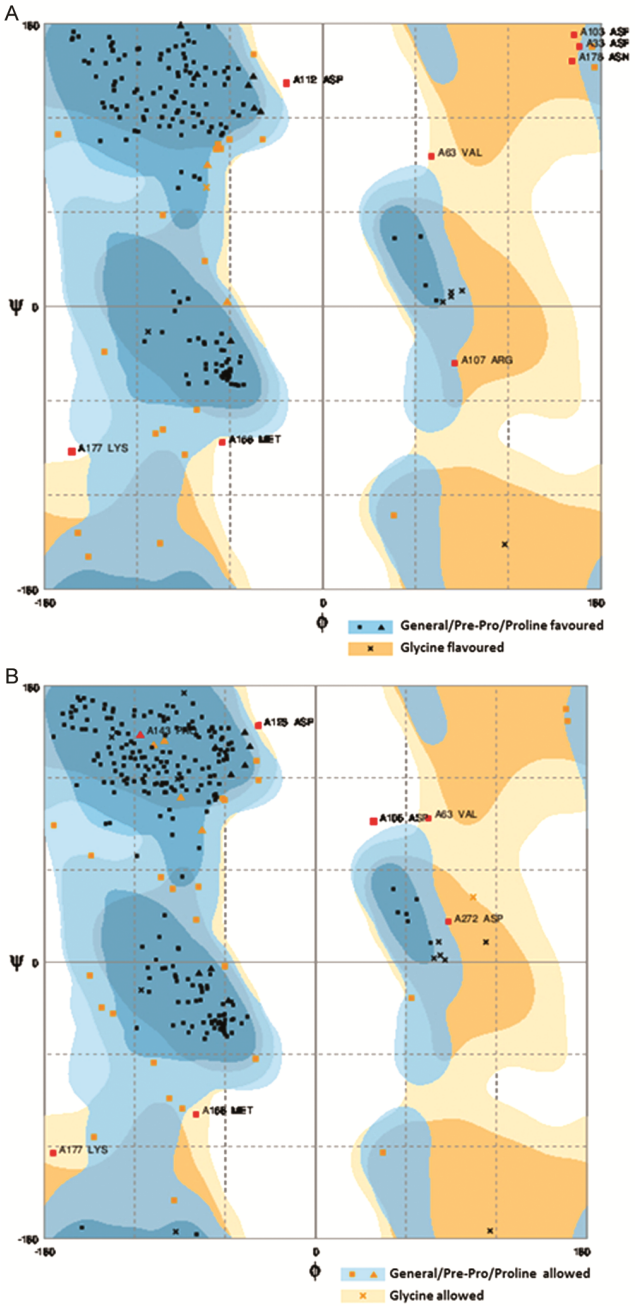

Fig. 11 — Ramachandran Plot for wild type and mutant SK sequence.

84.1% while 87.8% in the mutant SK sequence model 3.7% of the amino acid residues in outlier region in the wild type model increase in the mutant. The Ramachandran plot (Fig. 11) indicated that some amino acids in the best predicted structure were located at outlier region.

Structures alignments

Based on SWISS model structure alignments no significant discrepancy was seen between the wild type and the mutant SK sequence (Fig. 11). RMSD between wild type and mutant was calculated as 0.195, which was <2.0.

Discussion

Strain improvement plays a vital role in the development of microbial fermentation process[25]. Usually, the wild strains produce limited quantities of desired enzymes that are useful for commercial application[26]. Therefore, adoption of simple selection techniques has evolved in the substantial increase in industrial application[27]. A large number of variants are identified by this random mutagenesis, which depends on the screening of improved and efficient strain with a considerable increase in yield[28]. Previous report has been stated that the absence of 42 amino acids at the C terminal region due to the mutagenesis expressed less immunogenicity than wild SK[29]. Thus, the introduction of SK variants has enhanced plasminogen activation of SK with decreased immunogenicity, which includes complexing and replacing of normal amino acids[30-32]. The enhanced stability was reported in some of the variants with few modifications like replacement of Lys 59 with glutamic acid residue[33,34]. The present study involves the use of UV as a physical mutagen. Isolate UVSE6 was found to be potent producer of SK when exposed to chemical mutagenesis. The activity of SK from UV mutant and wild strain was found to be merely equal but was not stable. This instability may be because of the UV irradiations due to which it might have undergone back mutations[35]. Previous studies have used the chemical mutagens to enhance the production and to increase the stability[36]. In the current study, it was observed that the chemical mutagenesis has an impact on the survival of the UVSE6 mutant and its SK activity. Both EMS and NTG mutagen play a significant role by enhancing SK production. The use of EMS mutagen resulted in only one potent strain, EMS1 which showed maximum SK activity. Similarly, with NTG mutagen, NTG1, NTG3 and NTG8 expressed maximum SK activity among other strains. The selection of potent mutant strain for further studies was based on the determination of maximum SK activity with chromogenic substrate. Therefore, SK from EMS1 showed maximum plasminogen activation and selected further pilot scale production of SK. Previous studies have used UV, NTG and other physical and chemical mutagens to increase the production of SK from different *Streptococcus spp.*[12,37]. This was the

first study to report the successful mutation using UV and EMS and stable enhanced production of SK from *S. equinus*. *Streptococcus uberis* TNA-M1 and *Streptococcus dysgalactiae subsp. equisimilis* SK-6 are previously reported SK producing organisms[35,38.] EMS1 isolate in the current study was observed to produce higher quantify of SK when compared to *Streptococcus dysgalactiae subsp. equisimilis* SK-6[35,38]. Enhanced production of streptokinase from the standard strain *Streptococcus Sp.ATCC 12449* was performed and found to be evident in the isolation of hyperproductive mutant UB111 using NTG as the mutagen. The estimated streptokinase activity was 820 U/mL upon mutagenesis[39]. The study explains that mutagens can enhance the enzyme production by conventional method. The activity of crude enzyme has increased 2.5-fold from EMS1 mutant than the wild strain *S. equinus* VIT_VB2 (13463 IU and 51.5 mg)[7]. The purified SK from the mutant strain expressed 1.2 times increased specific activity than SK from *S. equinus* VIT_VB2 (6585 IU mg^{-1}) which has been already reported.

In comparison with previous studies, the current study achieved the largest production of SK from *S. equinus*[40]. The retention time of the mutant SK from HPLC analysis was corresponding to the retention time (2.14 min) of standard SK[7]. The *SK* gene was confirmed by DNA sequencing with specific primers amplification reaction. This gene has maximum similarity with other streptokinase gene sequence from other strains of *Streptococcus* sp. The integrated wild type and mutant SK sequence on structure analysis and modelling substantiates that effect of mutagens has improved its quality. 3D models are constructed to visualize deviation among the mutant and wild-type protein models. The RMSD measures the difference between Cα atom positions between two proteins. The smaller the deviation is, the more spatially equivalent the two proteins are. Ideally, it should be 0.0 for two same proteins, but measurement errors and other variations cause deviation. Biological functions of proteins are performed in interaction with other molecules[41]. The RAMPAGE score provides an estimate of the absolute quality of a model by comparing the same protein before and after mutation.

The growing prospective of streptokinase as a thrombolytic drug elicits the expansion of reckonable augmentation approaches. In the current study, *S. equinus* VIT_VB2 isolated from bovine milk has been used for the greater production of very active streptokinase by UV, EMS and NTG induced mutations. Earlier quoted research reports are obtainable representing the improved production of streptokinase by persuading mutation in wild Streptococcal strains by physical or chemical mutagens. One of the preceding research results confirmed that streptokinase yield from UV and NTG mutated *S. equismilis* was 120% and 146% more than the wild strain[34]. In another study, a 3 fold improvement in production of streptokinase was reported from UV mutated S. *equisimilis*[42]. SK activity attained from EMS mutated strain *S.agalactiae* was reported to be 1.6 fold greater as related to the activity reported by wild strain[36]. Nevertheless, not much data has been reported on the usage of mutant *S. equinus* for enhanced production of streptokinase.

Conclusions

The increased potential applications of SK have emphasized the need to enhance the production of SK from *S. equinus* VIT_VB2. Through physical mutation, the current study achieved to develop a mutant strain UVSE6 with an enhanced production of SK than the wild strain. The mutant strain UVSE6, further mutated with chemical mutagens (EMS and NTG) enhanced the production of SK. The results of the present study provide evidence for the significant impact of UV irradiation, EMS and NTG on strain improvement. This holds great value for future studies. The 47 kDa SK from EMS1 was purified using Sephadex G-100 column and the purity was confirmed by HPLC. This facilitated to obtain a high yielding mutant strains for industrial production of SK in large scale application. *Streptococcus dysgalactiae subsp. equisimilis* strain GGS_S88 was genomically similar to the UVSE6-EMS1 of *S. equinus* VIT_VB2. *In silico* analysis confirmed that in the secondary structure, alpha helix dominated, followed by a random coil, extended strand and beta turns. It was also evident that the effect of mutagen affects the variation in amino acid residues, resulting in higher activity than the wild type SK. The forthcoming research work focuses on the genetic expression of the SK from potent, mutant EMS1 strain of *S.equinus* VIT_VB2.

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Conflict of interests

The authors declare that they have no competing interests

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