



IMPROVED STREPTOKINASE PRODUCTION; UV IRRADIATION OF STREPTOCOCCUS EQUISIMILIS

Gull-e-Faran¹, Muhammad Anjum Zia², Muhammad Shahid³, Sajid Abdullah⁴

1. Enzyme Biotechnology Laboratory
Department of Biochemistry,
University of Agriculture,
Faisalabad, Pakistan.
2. Enzyme Biotechnology Laboratory
Department of Biochemistry,
University of Agriculture,
Faisalabad, Pakistan.
3. Department of Biochemistry,
University of Agriculture,
Faisalabad, Pakistan.
4. Department of Zoology, Wildlife
and Fisheries,
University of Agriculture,
Faisalabad- 38040, Pakistan

Correspondence Address:

Gull-e-Faran
Enzyme Biotechnology Laboratory
Department of Biochemistry,
University of Agriculture, Faisalabad,
Pakistan.
gull_uaf@yahoo.com

Article received on:

02/02/2015

Accepted for publication:

09/02/2015

Received after proof reading:

06/05/2015

ABSTRACT... Several strains of beta-hemolytic Streptococci produce streptokinase enzyme that can bind and activate human plasminogen to plasmin. Streptokinase degrades the fibrin lump by its explicit lysine joining site and so it is applied as a remedy in thrombolytic therapy. The purpose of the study was to subject wild strain of Streptococcus equisimilis to strain development technique, using random mutagenesis by UV irradiation for enhanced production of streptokinase. **Objective:** To evaluate the hyper production of streptokinase after mutagenesis of wild Streptococcus equisimilis by means of UV irradiation. **Study Design:** Randomized study. **Period:** 2012-2014. **Setting:** Enzyme Biotechnology Laboratory, Department of Biochemistry, University of Agriculture, Faisalabad-Pakistan. **Materials and Methods:** UV lamp (TUP 40w lamp which has about 90% of its radiation at 2540-2550 Å) was used for the mutation of Streptococcus equisimilis cells (1×10^7 cells mL⁻¹) for enhanced production of streptokinase. 10 mL fresh inoculum was transferred to sterile petri plates, which were exposed to UV light for 30, 60, 90, 120, 150, 180, 210, 240 and 270 minutes. The exposure was carried out at distance of 20cm from the centre of lamp. A dose producing 87% killing was selected as optimum dose, after preparing kill curve. The kill/ survival curve was prepared and time of exposure giving (210 minutes) 3 log kill was selected for mutation of the Streptococcus equisimilis for hyper production of streptokinase enzyme. **Results:** Enzyme assay was performed for both wild and mutant strains. Dose of 210 minutes was selected as best dose which was followed by the selection using triton X-100. Finally the selected strain S. equisimilis EBL-UV-210 showed 480 U mL⁻¹ of streptokinase activity in quantitative blood clot liquefaction test, which is quite higher than wild strain (370 U mL⁻¹). This maximum yield of streptokinase was obtained after 24h, at CSL 4%, pH 7.5, 37°C, KH₂PO₄ 0.04%, K₂HPO₄ 0.05%, MgSO₄·7H₂O 0.04%, NaHCO₃ 0.15%, CaCO₃ 0.004%, CH₃COONa·3H₂O 0.10%, FeSO₄·7H₂O 0.04%, MnCl₂·4H₂O 0.02%, glucose 2%, yeast extract 3% and 5% inoculum size in liquid state fermentation. **Conclusions:** Results showed that mutated strain gave enhanced streptokinase activity in comparison to the wild strain. Our current study focused on streptokinase production from this UV mutated streptococcus equisimilis species and purification of this enzyme by ammonium sulfate precipitation, Ion exchange and gel filtration chromatography. The activity of streptokinase was determined by using quantitative blood clot liquefaction method.

Key words: Streptococci, streptokinase, UV- mutagenesis, ammonium sulfate precipitation.

Article Citation: Gull-e-Faran, Zia MA, Shahid M, Abdullah S. Improved streptokinase production; uv irradiation of streptococcus equisimilis. Professional Med J 2015;22(5):656-663.

Novelty Statement:

Streptokinase, a powerful therapeutic agent is in clinical use from last many decades for the treatment of cardiovascular blockage. As myocardial infarction is becoming major health problem worldwide now days with no proper treatment available other than surgery and removal of blood clots. Discovery of streptokinase was a great mile stone in medical science and microbiology. This discovery has provoked new hope for mankind regarding human health, cure

and treatment of myocardial infarction. This study was conducted for improved production of streptokinase from mutagenesis of Streptococcus equisimilis by UV irradiation. This resulted in surplus production of streptokinase with efficient clot dissolving activity. This hyper produced enzyme was purified and can be marketed at cheap rates to the poor people of Pakistan for treatment of this life threatening illness. In short such valuable production of streptokinase can serve mankind for saving lives.

INTRODUCTION

Martin (1982)¹ reported that a wide variety of beta-haemolytic Streptococci produce streptokinase as their extracellular enzyme. Stoichiometric 1:1 complex is formed by the interaction of streptokinase and plasminogen activator, which then converts plasminogen to plasmin. The fibrin matrix of blood clots is then dissolved by this plasmin. From past 30 years streptokinase is in wide use as a clot dissolving agent.² Mohammad et al. (2009) observed that with the help of plasminogen activator (non-protease), plasminogen is activated to plasmin which then lyses the fibrin lump and consequently applied as a remedy for thrombolytic action.

Abdelghani et al. (2005)³ worked on isolation of 103 β -haemolytic Streptococci (with haemolytic activity). These Streptococci were secluded from various sources of biomass and blood from infected throat, out of which 47 samples showed streptokinase (SK) activity. For strain improvement studies, T3 was chosen as best strain as it was recognized as a new modification of Streptococcus equisimilis and given perfect SK production in liquid state fermentation. UV and N-methyl-N'-nitro-N-nitroso guanidine (NTG) were used for the mutagenesis of this selected strain. Seclusion of mutants was done and finally SK activity of these mutants was described. Best UV mutant AUV10 showed significantly higher yield of SK as compared to the wild strain (T3). While apparently higher yield of SK was also shown by best NTG mutant NUV7 as compared to wild strain (T3). For strain enhancement of *S. equisimilis*, UV and NTG were proved as efficient mutagenic agents for improved SK productivity.

Molecular mass of streptokinase is 47 kDa made up of 414 amino acids. Tissue type plasminogen activator and urokinase act directly by causing proteolysis whereas on other hand streptokinase act by forming high affinity equimolar complex with plasminogen.⁴ Circulating plasminogen is then transformed to plasmin by this complex, later on fibrin in blood clot is then lysed by this efficient proteolytic enzyme.⁵ Such thrombolytic agents are in broad application in the cure of myocardial

infarction.⁴

Madhuri et al. (2011)⁶ conducted a study to isolate wild strain of Streptococcus equisimilis and its random mutagenesis by UV irradiation for strain improvement. After conducting mutagenesis, screening was done to evaluate improved streptokinase production. Throat samples were collected from patients suffering from throat infection and out of these samples presence of Streptococcal species was confirmed in 3 samples. Streptokinase assay was performed for the wild as well as mutant strains, to evaluate the streptokinase production and highest production was given away by *S. equisimilis*. For strain improvement UV irradiation was applied on wild strains and finally streptokinase activity was evaluated. Highest activity was showed by UVM6 (mutated *S. equisimilis*), it was concluded that mutated strains exhibit enhanced streptokinase production as compared to the parent strains.

For the treatment of myocardial infarction, streptokinase is being widely used as thrombolytic remedy from last 40 years.⁷ Severe medical problems can be caused by high or low doses of such drugs so maintenance of dose rate is much important in case of such drugs.⁸ For the purification of streptokinase, DEAE-cellulose ion exchange chromatography is in wide use which results in highly purified product. So such methods of production and purification introduce new ways in the application of such proteins with improved biological activity.^{9,10,11} In the purification of streptokinase numerous chromatographic techniques like affinity chromatography and gel filtration chromatography are also in wide use.^{4,12} Group C streptococcus species (Lancefield classification), *S. equisimilis* are reported as best producers of streptokinase as they give highest yields of streptokinase.¹⁰ Lesser quantities of streptokinase are also being produced by Group A and Group G streptococcus species, people afflicted from sore throat contain Group A streptococci as their normal microbial flora.

In the previous few decades, application of SK in various disciplines has increased exponentially

which elevated the demands for the promotion and screening for newer streptokinase producing organisms. Due to increasing potential of SK application, quantitative development and qualitative perfection is also required equally. Generally too low amounts of streptokinase are produced from wild strains, so various quantitative enhancement strategies are required for the over production of the enzyme. These include strain improvement and medium optimization etc. The amazing achievement of strain enhancement in industry is generally accredited to the broad appliance of selection and mutation. In the current work, an effort was made for strain improvement by UV irradiation to improve streptokinase production in comparison of the wild strains which produce low amounts of streptokinase naturally.

MATERIALS AND METHODS

Organism

In this research work, test organism *Streptococcus equisimilis* (Fig 1) was isolated from various indigenous blood and biomass sources including samples from patients suffering from sore throat, scarlet fever and acute tonsillitis. Test cultures were grown and preserved on blood agar medium.

Strain improvement studies

The wild strain was inoculated into nutrient agar containing 10% v/v defibrinated sheep blood at 45°C and incubated at 37°C for 24 hours. After growth on blood agar medium, isolated colonies were obtained and suspended in phosphate buffer (pH 7) for the preparation of inoculum. 1ml of the suspension was poured into the vials and exposed to UV light for 0, 30, 60, 90, 120, 150, 180, 210, 240 and 270 minutes. These plates were kept 20cm away from the lamp. After the exposure to UV light, to evade photo reactivation the suspension was stored in dark overnight. It was then diluted in succession by adding phosphate buffer and finally plated on PDA medium.¹³ Then plates were incubated at 37°C for 24 hours and the numbers of colonies in each plate were counted. Each colony was supposed to be produced from a single cell. Total 15 colonies were selected from the plates presenting less than 1% survival rate and then

screened for streptokinase activity. Then best UV mutant strain was chosen from these selectants which was then used for further studies.^{13,14}

Screening of mutated streptococcus equisimilis

After performing UV mutagenesis, kill curve was made by using exposure time and log natural of colony forming units (CFU). 210 minutes exposure time was selected as best dose (Fig 2). This best selected mutant was named as *S. equisimilis* EBL-UV-210 (Fig 1).

Production of streptokinase

Isolated colonies were singled out from PDA plate and added in 25ml sterilized phosphate buffer (pH 7.0) and placed in orbital shaker at 120rpm at 37°C for 24 hours for inoculum preparation. 5ml of this inoculum was then added to 100ml of the production medium¹⁵ composed of Glucose 2%, Yeast Extract 2%, KH_2PO_4 0.05%, CaCO_3 0.005% and CSL 0.5% at pH 7 and flasks were incubated at 37°C for 24 hours in an orbital shaker at 120 rpm. Corn steep liquor (CSL) was used as substrate. Along with mutant *S. equisimilis* EBL-UV-210, wild strain was also used for production and purification of streptokinase for comparison. Different parameters like substrate concentration, fermentation time, pH, Temperature, concentration of yeast extract, KH_2PO_4 , CaCO_3 , glucose and inoculums size were optimized.^{16,17}

Sample harvesting

After 24 hours incubation, the biomass was harvested by filtration and centrifuged at 10,000 rpm for 20 min at 0°C. After centrifugation, supernatant (crude enzyme) was assayed for enzyme activity and residue was stored at -20°C.⁹

Quantitative blood clot liquefaction assay

First of all whole blood from healthy individuals was collected and then sterile empty micro centrifuge tubes were taken, weighted and labelled suitably. Initial weight was termed as (W1). Into each micro centrifuge tube, 0.5 mL blood was taken and incubated at 37°C for 45 min. Serum was totally removed by aspiration, after clot formation without disquieting the clot. Now weight of tubes

with clots was designated as (W2). Clot weights were calculated by subtracting W1 from W2. To all respective tubes, 0.5 mL cell free supernatants were added. For control same procedure was adopted but Pre-sterilized distilled water was added instead of crude enzyme. Clot lysis was checked in all tubes after incubating them at 37°C for 90 min. To examine the difference in clot weight, the fluid on each tube was separated and tubes were weighed again after incubation (W3). By using the following equation, clot lysis percentage was calculated.¹⁸

$$\text{Percentage lysis} = 100 - \left\{ \frac{(W3 - W1)}{(W2 - W1)} \right\} * 100$$

One unit of streptokinase is an amount of enzyme converting 1 micro-mole of substrate in one minute. Streptokinase assay is reliant upon the activation of plasminogen to plasmin that hydrolyzes the substrate in a defined period which is linked back to concentration of streptokinase.

Streptokinase purification

Extracellular streptokinase was purified by ammonium sulfate precipitation, ion exchange and gel filtration chromatography. Ammonium sulfate precipitation was done on crude enzyme.¹⁹ The fraction was precipitated by 40% to 60% ammonium sulfate saturation level. A column of DEAE (Diethyl amino ethyl) cellulose was prepared.²⁰ The resin was steadily added to 0.1 M phosphate buffer (pH 7) until slurry was prepared and then it was heated in water bath at 95°C for 5 hrs, avoiding drying the slurry. The column was washed by continuous passage of buffer which gradually filled the outlet. Finally the slurry was poured into column. Then it was placed on leveled surface for 24 hrs. Outlet was opened for the removal of buffer from the column. 25ml of 0.5M NaOH was used to wash the column, which was drawn out from the column later on. To attain neutral pH, distilled water was added to column until the pH of eluent was adjusted at 7. Then a column of sephadex G-150 (Pharmacia) was prepared by the following method.²¹ In 8 ml of distilled water, 1g dry sephadex G-150 was dissolved which was then heated in a water bath for 3 hrs at 95°C (without drying slurry). The

column was positioned vertically on stable stand. To fill the empty outlet tube, distilled water was flowed through the column. For complete filling of the column, slurry was poured into it. For setting of distinct layers of gel and water, column was left uninterrupted. Leaving a small layer on the top of column, all distilled water was removed by opening the outlet tube. The sample having the highest activity (obtained after ion exchange chromatography) was applied on it and outlet was opened to collect fractions. The sample was allowed to make a way into the packed column. 0.1 M phosphate buffer (pH 7) was used to carry out the elution. Total 100 fractions of 2ml each were collected which were then subjected to enzyme assay and protein estimation.²²

Statistical analysis

Data obtained was analyzed by two factor completely randomized design for assessment of means and standard error of means.²³

RESULTS AND DISCUSSION

Hemolytic *Streptococcus equisimilis* was used for enhanced production of streptokinase enzyme. It is an important blood clot dissolving agent and act as tissue plasminogen activator. By using corn steep liquor as substrate, streptokinase was produced by submerged state fermentation at pH

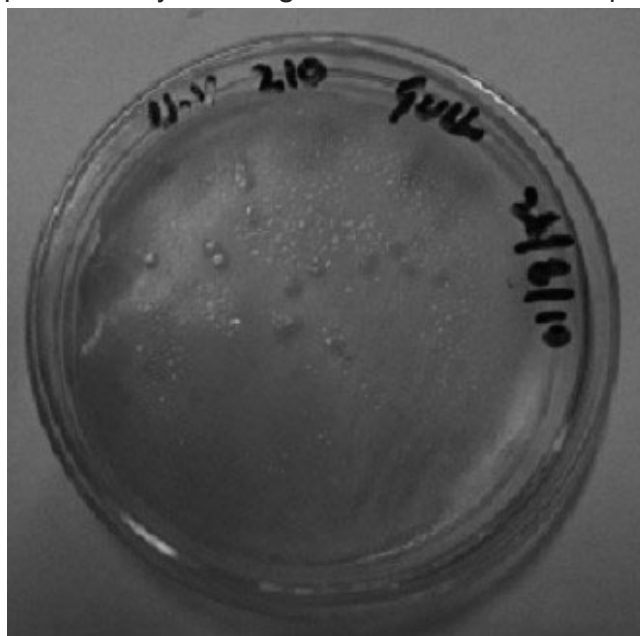


Fig-1: UV mutated *S. equisimilis* EBL-UV210

7.0.⁶ Precipitated proteins, purified proteins and crude extracts were estimated by using biuret method of protein estimation.¹¹

For strain improvement, UV irradiation was used to subject the wild strain. Fermentation was carried out for control, wild and mutant strains simultaneously. Results obtained showed significant effects of UV irradiation on streptokinase production. Dubey⁹ et al. (2011) stated that *Streptococcus* species was grown by using 8% CSL and it exhibited 55U mL⁻¹ of streptokinase, but in present study by using only 0.4% CSL concentration, fibrinolytic activity was increased up to 204.83 U mL⁻¹ which shows the significant effect of UV mutagenesis for hyper production of streptokinase.

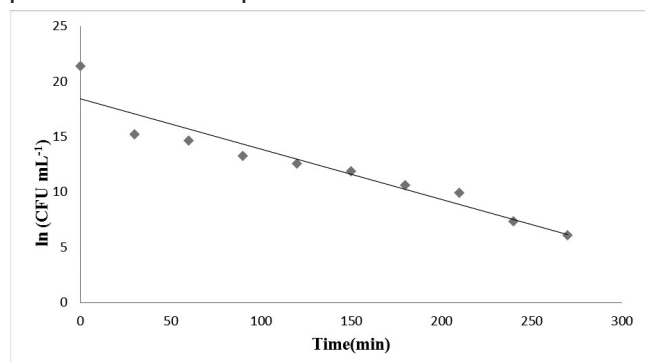


Fig 2: Effect of UV-irradiation on *S. equisimilis* to formulate the kill curve.

Crude enzyme exhibited 370 U mL⁻¹ and 480 U mL⁻¹ of enzyme activity for mutant and wild strain respectively. Ammonium sulfate precipitation was based on desolation of various proteins in salt solution. This was followed by improved protein-protein interaction. Ammonium sulfate precipitation was used for enzyme purification because of its high solubility; it was used for salting out of proteins from the solution with

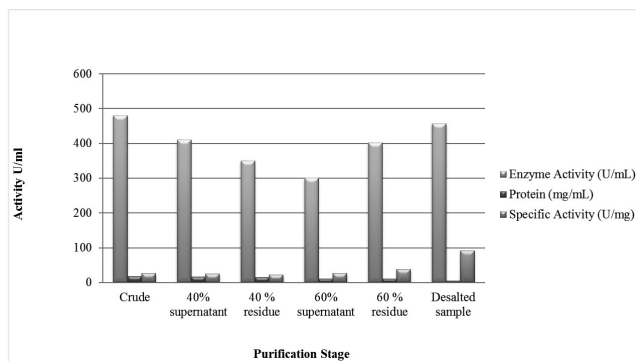


Fig 3: Ammonium sulfate precipitation of SK produced from UV mutated *S. equisimilis*.

high ionic strength.²⁴ After ammonium sulfate precipitation wild strain showed 328 U mL⁻¹ and mutant strain showed 456 U mL⁻¹ of streptokinase. Streptokinase was produced from *Streptococcus pyogenes* and then subjected to ammonium sulfate precipitation. The activity of crude enzyme was 6.7 IU/mg, protein contents were 1.21mg. The recovery of streptokinase after ammonium sulfate precipitation was 100%. After dialysis enzyme activity was 76 IU/mg, protein contents were 0.77mg and recovery was 44.2%.²⁵ After dialysis enzyme was subjected to Ion exchange chromatography which resulted in 300 UmL⁻¹ and 400 UmL⁻¹ of streptokinase for wild and mutant strain respectively. The dialyzed sample was applied to a DEAE-Sepharose column balanced with phosphate buffer and bound protein was eluted by applying 1.0M NaCl in the buffer. The activity of enzyme was 31.1 units and protein contents were 18.7mg.²⁶ In present study gel filtration resulted in 290 UmL⁻¹ and 335 UmL⁻¹ of streptokinase from wild and mutant strains respectively. The percentage recovery was 78.37 and 69.79% after gel filtration chromatography for wild and mutant strain respectively (Table-I).

Purification Stage	Activity (U/ml)		Protein (mg/ml)		Specific activity (U/mg)		Fold Purification		% age Recovery	
	Wild	Mutant	Wild	Mutant	Wild	Mutant	Wild	Mutant	Wild	Mutant
Crude	370	480	27.5	18.02	13.45	26.64	1	1	100	100
(NH ₄) ₂ SO ₄ desalted	328	456	11.5	5.02	28.52	90.83	2.12	3.41	88.65	95
DEAE-cellulose	300	400	6.4	1.44	46.87	277.77	3.48	10.43	81.08	83.33
Sephadex G-150	290	335	3.1	0.30	93.54	1116.66	6.96	41.92	78.37	69.79

Table-I. Purification summary of streptokinase produced from wild and UV mutated *S. equisimilis*:

Protein in crude enzyme was 27.5 and 18.02 mg mL⁻¹ for wild and mutant strain respectively whereas concentration of protein in partial purified enzyme was 11.5 and 5.02 mg mL⁻¹ respectively. Streptokinase assay was performed for all samples (wild and mutant strains) by quantitative blood clot liquefaction method. The results showed that maximum amount of streptokinase was produced by the *S. equisimilis* EBL-UV210. Madhuri⁶ et al. (2011) reported that highest enzyme activity was given by UVM6 (mutated *S. equisimilis*). In comparison to the wild strain, mutated strains gave enhance streptokinase activity. Enzyme assay was based on the actuality that least quantity of enzyme solution was necessary to dissolve the fibrin coagulate. From the results, it can be concluded that 210 minutes of exposure time to the organism was the most effective UV irradiation dose in producing favorable mutation as compared to other doses (Fig.3). It was observed that mutated strain *S. equisimilis* EBL-UV210 (mutated *S. equisimilis*) produce extra amount of streptokinase in comparison to the wild strains. According to Madhuri⁶ et al. (2011) it can be concluded that 6 minutes of exposure time to the mutagen was the most effective dose in producing favorable mutation as compared to 2,4,8 and 10 minutes. It was observed that mutated strain UVM 6 (mutated *S. equisimilis*) produce more amount of streptokinase in comparison to the wild strains. According to Abdelghani³ et al. (2005) the streptokinase yield of the best mutant strain in their work indicated an increase of 120 % as compared to the wild strains. Thus keeping this as the reference, the results showed that the enzyme units produced by mutant strain were 480 U mL⁻¹ and for wild strain units were 370 U mL⁻¹. Hyun et²⁷ al. (1997) demonstrated that by using a mutant *Streptococcus* spp, plentiful quantities of streptokinase can be obtained. Zhang²⁸ et al., (1999) purified streptokinase by gel filtration and found 95.7% purity and specific activity was 1×10⁵ IU/mg. The gel column used by Zhang was Q-sepharose and sepharose G-10. Babu²⁹ et al. (2008) analyzed recombinant streptokinase by HPLC and indicated purity of 99% and gel filtration indicated minimal accumulation of active streptokinase and renaturation was 99%.

When enzyme was purified with superdex 75 gel filtration column (1.0cm × 30cm) equilibrated with buffer and flow rate was 0.5ml/min. In the current study, it was seen that only the best UV mutant strain has produced three fold more yield of streptokinase as compared to the wild strain after UV mutagenesis.

CONCLUSION

UV irradiation is an efficient mutagenic agent as evident from results so its best technique for strain development and this resulted in improved streptokinase activity. In future this could be of great value and can be used to acquire high yielding mutant strains of these isolates which can be applied for large scale production of streptokinase. Streptokinase production should also be checked by using various substrates other than CSL.

Copyright© 09 Feb, 2015.

ACKNOWLEDGEMENT

The authors are thankful to HEC for providing financial support through HEC-Indigenous PhD fellowship to G.F to carry out this work. This research work was carried out at EBL, UAF Pakistan under the supervision of M.A.Z.

Copyright© 09 Feb, 2015.

REFERENCES

1. Martin M (1982). **Streptokinase in chronic arterial disease**. CRC Press Inc, Boca Raton, Fla.
2. Mohammad B, Hossein M, Mohammad R (2009). **Production and purification of streptokinase by protected affinity chromatography**. Avicen.J Med Biotechnol 1: 47-51.
3. Abdelghani, T. T. A., A. Kunamneni and P. Ellaiah. 2005. **Isolation and mutagenesis of streptokinase producing bacteria**. Am. J. Immunol., 1: 125-129.
4. Kim DM, Lee SJ, Kim IC, Kim ST, Byun, SM (2000). **Asp14-His48 region of streptokinase is important in binding to substrate plasminogen**. Thromb Res 99: 93-98.
5. Wu XC, Ye R, Duan Y Wong, SL (1998). **Engineering of Plasmin-Resistant Forms of Streptokinase and Their Production in *Bacillus subtilis*: Streptokinase with Longer Functional Half-Life**. Appl J Envir Microbiol 64: 824-9.

6. Madhuri DH, Manohar M, Singh NA, Mohanasrinivasan V, Devi CS (2011). **Studies on isolation, screening and strain improvement of streptokinase producing β -hemolytic Streptococci.** World J Sci Technol 1: 7-11.
7. De- Renzo EC, Siiteri PK, Hutchings BL, Bell PH (1967). **Preparation and certain properties of highly purified streptokinase.** J Biol Chem 242: 533-542.
8. Hermentin PT, Cuesta-Linker J, Weisse KH, Schmidt M, Knorst M, Thimme SM (2005). **Comparative analysis of the activity and content of different streptokinase preparations.** Eur Heart J 26: 933- 940.
9. Dubey R, Kumar J, Agrawala D, Char T, Pusp P (2011). **Isolation, production, purification, assay and characterization of fibrinolytic enzymes (Nattokinase, Streptokinase and Urokinase) from bacterial sources.** Afr J Biotechnol 10: 1408-1420.
10. Feldman L. J (1974). **Streptokinase manufacture in German.** German pat. 23, 540; 19.
11. Gornall AG, Bardwill CJ, David MM (1949). **Determination of serum proteins by means of biuret reagent.** J Biol Chem 17: 751-766.
12. Jackoby WB (1971). **Enzyme purification and related techniques** - Methods in Enzymology. Vol. XXII. Academic press, USA.
13. Mazhar M, Rehman K, Ali A, Zia MA, Akhtar MS (2012). **Gamma rays mutagenesis of Aspergillus niger for hyperproduction of mutarotase.** Cand J Appl Sci 3(2):362-368.
14. Zia MA, Rasul S, Iftikhar T (2012). **Effect of gamma irradiation on Aspergillus niger for enhanced production of glucose oxidase.** Pak J Bot 44(5): 1575-158.
15. Baewald G, Mayer G, Heikel R, Volzke KD, Roehlig R, Decker KL. (1975). **Fermentative production of Streptococcus metabolites, especially streptokinase.** German pat. 1, 110; 96.
16. Patel VP, Patel KS, Patel RM (2011). **Isolation and optimization of streptokinase production by batch fermentation.** Intern J Pharm and Biolog Arch 2: 1488-1492.
17. Zia MA, Faisal R, Abbas RZ, Faran G, Saleemi MK, Khan JA (2013). **Comparison of streptokinase activity from Streptococcus mutans using different substrates.** Pak Vet J 33(1): 77-79.
18. Prasad S, Kashyap R, Deopujari JY, Purohit HJ, Taori GM, Daginawala HF (2006). **Development of an in vitro model to study clot lysis activity of thrombolytic drugs.** Thromb J; 4 -14.
19. Shilpi B, Angayarkanni J, Bhattacharya S, Das A, Palaniswamy M (2013). **Isolation, Screening and Characterization of beta Haemolytic Streptococci with Potential of Streptokinase Production.** Int Res J Biological Sci 2: 63-66.
20. Ken C, Usher, Isabelle HB (2012). **Web based applet is a learning tool that simulates ion exchange chromatography purification of overexpressed proteins from E.coli cells lysate.** J Chem Educ 89: 555-556.
21. Erickson, HP (2009). **Size and Shape of Protein Molecules at the Nanometer Level Determined by Sedimentation, Gel Filtration, and Electron Microscopy.** Biolog Proc Online 11: 32-51.
22. Karimi Z, Babashamsi M, Asgarani E, Niakan M, Salimi A (2011). **Fermentation, fractionation and purification of streptokinase by chemical reduction method.** Iran J Microbiol 3: 2-46.
23. Steel RGD, Torrie JH, Dickey DA (1997). **Principles and Procedures of Statistics: A Biomaterial Approach. 3rd ed.** McGraw Hill Book Co. Inc., New York, USA. 336-352.
24. Voet D, Voet JG, Pratt CW (1990). **Fundamentals of biochemistry.** John wiley and sons, UA.pp. 687-689.
25. Felsia FX, Kummar RV, Kalpana S (2011). **Production and partial purification of streptokinase from Streptococcus pyogenes.** J Biochem Tech 3: 289-291.
26. Hua Y, Jiang B, Mu W (2008). **Purification and Characterization of a Novel Fibrinolytic Enzyme from Bacillus sp. nov. SK006 Isolated from an Asian Traditional Fermented Shrimp Paste.** J Agric Food Chem 56: 1451-1457.
27. Hyun HH, Lee YB, Song KH, Jeon JY, Lee HH (1997). **Strain improvement for enhanced production of streptokinase and streptodornase in Streptococcus sp.** J Microbiol Biotechnol 7: 101-6.
28. Zhang WX, Sun T, NiHuang X, Liu X, XiangGu D, Tang ZQ (1999). **Recombinant streptokinase production by fed-batch cultivation of Escherichia coli.** Enzy Microbial Technol 24: 647-65.
29. Babu, C.V. P., V. K. Srinivas, V.K. Mohan, E. Krishna. 2008. **Renaturation, purification and characterization of streptokinase expressed as inclusion body in recombinant E. coli.** J Chromatograp 861: 218-226.



“Discipline is the bridge between goals and accomplishment.”

Jim Rohn



AUTHORSHIP AND CONTRIBUTION DECLARATION

Sr. #	Author-s Full Name	Contribution to the paper	Author=s Signature
1	Gull-e-Faran	Ph. D student prepare research paper after completion of his Ph. D research project.	
2	Muhammad Anjum Zia	Supervisor under his supervision and guidance reasearcher completed his research work and research article.	
3	Muhammad Shahid	He provided technical assistance in designing reaserch project and research article.	
4	Sajid Abdullah	He provided the valuable suggestion for the improvement of research work and research article.	