Mutagenesis of Indigenous *Streptococcus equisimilis* isolates for Enhanced Production of Streptokinase

By

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DECLARATION

I hereby declare that the contents of the thesis “Mutagenesis of Indigenous Streptococcus equisimilis isolates for Enhanced Production of Streptokinase” is product of my own research and no part has been copied from any published source (except the references, standard mathematical or statistical models/ equations/ formulate/ protocols etc). I further declare that this work has not been submitted for award of any other diploma/degree. The University may take action if the information provided is found inaccurate at any stage.

__________________________

Gull-e-Faran

2001-ag-409
DEDICATED

To

The HOLY PROPHET MUHAMMAD (PBUH)

Who is the source of inspiration and knowledge for whole Universe.
Acknowledgements

All praises for Almighty ALLAH Subhana-wa-Ta’ala, the beneficent, the most merciful, lord of the Day of Judgment. Thee (alone) we worship, and thee (alone) we ask for help. Show us the straight path. The path of those whom thou hast favored, Not (the path) of those who thane wrath nor of those who go astray (Ameen).

Countless Darood wa Salaam be upon Holy Prophet Hazrat Muhammad (S.A.W), the fountain of knowledge who guided his Ummah to seek knowledge from cradle to grave. He is the greatest and dignified reformer of the universe. He illuminated the whole world with knowledge, wisdom, human rights and social equality.

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GULL-E-FARAN
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ABSTRACT

Acute myocardial infarction and ischemic stroke are caused by thrombosis or the obstruction of blood vessels with clots and this is the leading causes of death. The single handling accessible is the use of thrombolytic agent to liquefy the blood lump. Hyper production of streptokinase was carried out in this research work. Beta hemolytic *Streptococcus equisimilis* was isolated from indigenous sources and then mutagenesis of this isolate was carried out by means of chemicals as well as radiations. To seek the optimum activity, different kinetic and thermodynamic parameters like pH, temperature, $K_m$, $V_{max}$, molecular weight, melting temperature, half-life, enthalpy and entropy etc. were applied on the purified enzyme. UV irradiated strain resulted in 335 U/mL activity with 1116.66 U/mg specific activity, 0.30 mg/mL protein, 41.92 fold purification and 69.79% recovery whereas Sodium azide derived mutant resulted in 400 U/mL activity with 2000 U/mg specific activity, 0.20 mg/mL protein, 71.94 fold purification and 64.51% recovery of the finally purified enzyme. Gamma irradiated strain exhibited 300 U/mL activity with 1428.57 U/mg specific activity, 0.21 mg/mL protein, 59.52 fold purification and 75.94% recovery whereas ethidium bromide derived mutant showed 365 U/mL activity with 1659.09 U/mg specific activity, 0.22 mg/mL protein, 66.52 fold purification and 85.08% recovery. Optimum pH and temperature of the finally purified enzyme was 7 and 45°C. Enthalpy of denaturation ($\Delta H^*$) of streptokinase at 45°C was 43.67 kJ/mole. The Energy of thermal denaturation $\Delta G^*$ was 101.14 kJ/mole and entropy of inactivation $\Delta S^*$ was -197.32 kJ/mole at 45°C. The negative value of $\Delta S^*$ indicated that streptokinase was thermodynamically stable. $K_m$ and $V_{max}$ values of streptokinase were 26.31 mM and 50 MS$^{-1}$. Streptokinase produced from sodium azide derived mutant exhibited activity within the pH range of 6 to 8 while it presented its best performance at pH 7. Thermal stability between 45°C to 80°C was shown by the streptokinase along with half-life of 244 minutes while less stability was shown at 80°C along with 45 minutes of half-life and 40.41 kJ/mole as enthalpy of denaturation ($\Delta H^*$).
Chapter # 1

INTRODUCTION

Streptokinase is a fast-acting drug, which is basically used in hospitals to liquefy the fibrin of blood clots. It is an enzyme which is produced by Streptococcus bacteria and it is a treatment for clots specifically found in the arteries of the lungs and heart. During kidney dialysis, shunts are blocked by formation of clots and these clots are effectively liquefied by streptokinase. So streptokinase is utmost effective in dissolving these clots. Newly-formed clots are mostly treated by inserting catheter into an artery and the clot get dissolved by releasing streptokinase at the site of the clot via this catheter. Amount of damage to heart muscle can be reduced by administrating streptokinase at early stages of heart attach to break the clots. If treatment with streptokinase is not closely supervised, it can result in extreme bleeding. In combination with another enzyme known as streptodornase, streptokinase is used to treat ulcers and wounds. For this purpose, this combination is applied locally (Malke et al., 1985).

Extracellular metallo-enzyme streptokinase, produced by beta-haemolytic Streptococci is being used as cheap and effective clot-liquifing remedy in several cases of pulmonary embolism and myocardial infarction. Streptokinase works by activating plasminogen through cleavage to produce plasmin and this novel drug belongs to a class of drugs which is known as fibrinolytics. Organisms including earthworms, bacteria and snakes contain fibrinolytic enzymes naturally, and so these enzymes are identified and discovered successfully from these sources. Among bacterial and fungal strains; Streptococcus equisimillis, Aeromonas hydrophila, Streptococcus pyogenes, Bacillus amyloquefacens, Mucor sp., Armillari amellea and Fusarium oxysporum contain fibrinolytic enzymes (Jiansha et al., 2003). Various foods such as Tofyou, Korean chugkook, Japanese Natto, edible honey mushroom and Jay soy sauce contain clot dissolving enzymes. Finally characterization and physiochemical properties were studied out after Purification of these fibrinolytic enzymes from these sources (Delvin, 1986).

These novel fibrinolytic enzymes are very effective for thrombolytic therapy. For nutraceutical applications and food enrichment, such enzymes can also be proficiently used along with the advantage to treat heart diseases. So in this way use of such enzymes can
efficiently avert cardiovascular diseases (Yoshinori et al., 2005). In the Western world, cardiovascular diseases are most vital cause of death (Viles et al., 2004). Tissue plasminogen activator (tpA), streptokinase and urokinase are the common clot dissolving agents being used for the medical treatment of these cardiovascular diseases (Hermentin et al., 2005). Hemostasis is body’s complex process obtained by balance between bleeding and blood clot formation. Fibrin clots are not dissolved in case of disturbed hemostasis so in such cases thromolytic agents are used; examples are urokinase, streptokinase and tissue plasminogen activators (tpA). For the treatment of thrombosis, these all have been widely used. Adverse side effects can be result of usage of these enzymes along with the high cost and thermolability of these enzymes (Chitte and Day, 2000).

For treatment of thromboembolic blockages and coronary thrombosis, streptokinase is in effective and efficient use (Banerjee et al., 2004; Endrogan et al., 2006). High affinity equimolar complex is formed with plasminogen, when streptokinase is used and that is the basic difference between the working of streptokinase, tissue type plasminogen activator and urokinase (Kim et al., 2000). In the development of acute transmural myocardial infarction, coronary thrombosis is the final pathway. Thrombolytic therapy with streptokinase is advantageous for the cure of such patients. Fibrinolytic system is indirectly activated by streptokinase by formation of plasminogen complex. Plasminogen is transformed to plasmin by the help of plasminogen activator, later the fibrin clots are degraded by this plasmin (Hermandez and Marrero, 2005).

Fibrin can be dissolved by a substance known as streptokinase which was found to be naturally produced by Streptococci as discovered by Tillet in 1933. In the human circulation this material streptokinase was shown relatively influential, to dissolve clots nearly 20 years ago (Johnson and McCarty, 1959). Gifford and Feinstein (1969) demonstrated that great caution is required regarding the use of anticoagulant therapy in myocardial infarction and the position of streptokinase is validated by extensive trials of irreprehensible experimental design. Correspondingly, results with streptokinase have been unsatisfactory in occlusion of peripheral arteries. Surgery is expected to be more victorious and more consistent in case of acute arterial obstruction (Persson et al., 1973) and again the results of treatment of chronically occluded vessels with streptokinase showed generally unsatisfactory results (Verstraete et al., 1971). So for treating patients in whom arterial obstruction is incomplete, streptokinase has only a small
part to play. Occlusion of the retinal artery may achieve better vision from thrombolysis in few patients but appropriately controlled studies have not been carried out. Treatment with streptokinase may result in a serious complication like hemorrhage, but it may be difficult and massive to control a convincing cause for limitation of use of streptokinase to circumstances where advantage has been obviously recognized. At the instant these are few but further developments, such as combining streptokinase with plasminogen may advance the results, but it seems more likely that streptokinase will remain a helpful drug for use in only a few cautiously chosen patients (Kakkar et al., 1975).

Healthy hemostatic system can suppress the blocking of circulatory system by the formation of blood clots. Development of plaque can occur due to the amassing of blood components, fat, cholesterol and cells in the interior walls of the blood vessels. Blood flow is restricted due to the clogging of these plagues and this failure of blood supply to the brain results in pulmonary embolism or stroke including deep vein thrombosis and to heart leads to myocardial infarction. Myocardial infarction is affecting nearly 8 million people globally and in India about 2.5 billions of people exist, these estimates are provided by World Health Organization. In India, deaths due to myocardial infarction falls nearly 25 million in a year and this value is going on increasing. Thus, a medical intrusion is necessary with the use of fibrinolytic agents like streptokinase (SK) (Collen et al., 1988, Collen, 1990; Francis and Marder, 1991; Banerjee et al., 2004). Plasminogen is activated to an enzyme plasmin with the help of streptokinase which is an extracellular protein. For cure of myocardial infarction, SK is currently used in clinical medicine. Group C Streptococcus species, S. equisimilis (Lancefield classification) are reported to produce maximum amount of streptokinase (Banerjee et al., 2004). Minor quantities of streptokinase are also produced from Group A and Group G Streptococcus species. Normal microbial flora in people suffering from sore throat contain Group A Streptococci naturally.

By using nuclear magnetic resonance (NMR) and circular dichronism (CD) techniques, thermal stability of different domains of streptokinase was demonstrated. These techniques indicate that domains are stretched out at diverse temperature is independent of presence of rest of domains have not effected the thermal stability of domain B and isolation from rest of chain has increased the thermal stability of domain C (Conejero-Lara et al., 1996). Streptokinase interacts with plasminogen all the way through numerous domains. Two binding
sites of streptokinase are there in which one site (C-terminal domain) is dependable for substrate detection and commencement (Kim et al., 1996). In binding of substrate, there is role of binding of second site (Asp41-His48 region) (Kim et al., 2000). Alpha domain of streptokinase is involved in activation of plasminogen and β-domain is responsible for development of complex between streptokinase and plasminogen so both domains play important role in streptokinase functioning (Robinson et al., 2000). Human plasmin unites with crystal structure of streptokinase by catalytic unit. Plasminogen activation and substrate recognition is done by the help of amino-terminal domain and carboxyl-terminal domain of streptokinase (Wang et al., 1998). Blood clots are dissolved by plasmin which is produced from plasminogen activators. Normally Plasminogen activator activates the plasminogen in the absence of fibrin which results in reduction of its competence as drug. Activation of plasminogen can take place through both fibrin dependent and fibrin independent mechanisms by streptokinase. Mutant streptokinase deficient in NH₂-terminal is not capable to stimulate plasminogen by fibrin independent method (Reed et al., 1998). Anti-streptokinase antibodies are formed as a result of anaphylactic response caused by streptokinase administration into circulation and this can lead to death also (Jennings, 1996). Half life of streptokinase is reduced in circulation by a cascade in which plasminogen is activated to plasmin by streptokinase and then plasmin cause breakdown of streptokinase. Tissue plasminogen activator shows half life of 5 minutes in circulation but streptokinase is active in circulation for longer time so tissue plasminogen activator is not suggested for competent treatment (Wu et al., 1998). Non-glycosylated streptokinase can be complexed with polyethylene glycol to extend the half life of streptokinase (Koide et al., 1982). Due to presence of neutralizing antibodies, inactivation of streptokinase was seen in blood of Streptococcal infected patients and this fact was revealed by early studies on immunogenicity. In infected patients, measurable level of antibodies against streptokinase is present (Ojalvo et al., 1999). Diverse Immunogenicity is exhibited by various domains of streptokinase (Reed et al., 1993).

Urokinase and streptokinase both activates circulatory plasminogen and clot-bound plasminogen but urokinase is not antigenic. Streptokinase stay drug of choice because the use of other activators is occasionally linked with blood loss (Rouf et al., 1996). Best bacterial plasminogen activators which are in wide use include streptokinase and staphylokinase (SAK) which is formed by Staphylococcus specie (Okada et al., 2001).
plasminogen activator is acylated plasminogen–streptokinase activator complex (APSAC) which contains streptokinase complied and complexed with an acetylated active site of human plasminogen (Marder et al., 1983). In circulation, the Acylated plasminogen–streptokinase activator complex has efficient half life as compared to streptokinase (Smith et al., 1981). By means of differential scanning calorimetric technique, investigation of effect of heat or thermal denaturation of streptokinase has been done. Unfolding of diverse domains of streptokinase is dependent on different conditions including sample concentration, pH and heat capacity (Azuaga et al., 2002).

Rigorous consequences including death can occur due to the maturity of thrombus (vascular obstruction) in the circulatory system. In a healthy homeostatic system, formation of blood lumps in regular blood flow is concealed but this homeostatic system reacts extensively to turn away blood loss in the incident of vascular damage. Stroke, thrombosis in deep veins, embolism of pulmonary system and acute myocardial infarction are the results of unbalanced homeostasis. In conditions like imbalance of natural homeostasis and formation of blood clots, intravenous injection of clot lysing or fibrinolytic agent is required. Tissue type plasminogen activator (tPA) and urokinase are included among such clot dissolving and thrombolytic agents (Banerjee et al., 2004). Extracellular enzyme (EC 3.4.99.22) streptokinase is produced by β-haemolytic Streptococci and it facilitates the blood clot lyses by activation of plasminogen. Circulatory plasminogen is activated indirectly by this single polypeptide enzyme and exerts its fibrinolytic action. Jackson and tang (1982) performed the absolute amino acid sequencing of streptokinase. Clinical handling of acute myocardial infarction and coronary blockage include use of streptokinase as therapeutic agent (Banerjee et al., 2004).

Streptokinase exhibit molar mass of 47 kDa along with composition of 414 amino acid residues (Malke and Ferretti, 1984). Isoelectric pH of streptokinase is 4.7 where as it gives best activity at 7.5 pH (Brockway and Castellino, 1974). Formation of fibrin clots can be inhibited by the use of anticoagulants and this treatment was graded best in case of thromboembolic vascular ailment in early days. After the initial use of this treatment, new approach was carried out which was the use of modification of plasminogen to plasmin and then its use for the fibrin clot lysis in vivo. Variety of clot dissolving agents are available for clinical use including anisylated purified streptokinase activator complex (APSAC), streptokinase, reteplase (r-PA), pro-urokinase and urokinase. Function of majority of fibrinolytic agents is based on the
principle of conversion of plasminogen to plasmin, this results in breakdown of fibrin network and ultimately clot lysis (Balaraman and Prabakaran, 2007). Streptokinase is produced naturally in microorganisms such as S. uberis, S. equisimilis and S. faecalis (Banerjee et al., 2004). In the previous few decades, employment of SK in various fields has elevated greatly so the demands for screening of newer streptokinase producing organisms have also increased. Due to increasing potential of SK application, mutually quantitative development and qualitative perfection is also required. Generally too low quantities of SK are formed by wild strains, so various quantitative enhancement strategies are required for the excessive production of the enzyme. These include strain improvement and medium optimization etc (Zia et al., 2013).

For treatment of venous thrombosis and pulmonary embolism, thrombolytic therapy with urokinase and streptokinase is gaining growing acceptance and presently acute myocardial infarction is being successfully treated by this enzyme and it is also being reevaluated (Goldhaber et al., 1984). Thrombolytic therapy may result in hemorrhage so it can be said that bleeding is the main side effect of streptokinase therapy. Mixture of lysis of fibrin in wounds and of a systemic hemostatic fault that occurs as an outcome of plasmin-mediated proteolysis of plasma coagulation factors result in bleeding. Hypofibrinogenemia is the systemic hemostatic fault which is represented as an increase in fibrinogen-split products (which causes defective fibrin polymerization) and reduction in the levels of factors V and VIII and probably by a fault in platelet function (Collen and Verstraete, 1991; Adelman, 1983). Extrinsic tissue plasminogen activator (t-PA) is a new plasminogen activator developed in recent years which can produce fibrinolysis without inducing a generalized hemostatic defect so it has potential advantages over urokinase and streptokinase. For medical uses, this new fibrinolytic product can be obtained from human melanoma cell line and it is a serine protease produced by recombinant DNA technology (Rijken and Collen, 1981; Pennica et al., 1983). On the fibrin surface; plasminogen is transformed to plasmin by t-PA which binds to fibrin with a better affinity than either streptokinase or urokinase. Thrombolysis without an important general coagulation defect was resulted after the infusion of melanoma cell-derived t-PA in animals and in humans (Korniger et al., 1982). Studies of DNA-recombinant t-PA have confirmed these findings recently. Induction of fibrinolysis without inducing a plasma proteolytic state is the ability of t-PA which confirm its probable advantages over urokinase
and streptokinase as a thrombolytic agent. Following relative contributions to bleeding associated with therapeutic thrombolysis are important: (1) local fibrinolysis in hemostatic plugs and (2) the plasmin-induced generalized coagulation defect. Relative contributions of these mechanisms to hemorrhage caused by thrombolytic therapy has not been defined yet (Gold et al., 1983).

In the developing countries there is increasing affluence of death from acute myocardial infarction and coronary heart disease so mainly with primary percutaneous coronary intervention and thrombolysis, there is need of increases in treatment. World Health Organization has taken priority of tackling coronary artery disease as first strategy (Longstaff et al., 2005). For treating acute myocardial infarction, principal clot dissolving agents include streptokinase which is in current use. Many countries are producing streptokinase worldwide and around 400,000-500,000 patients are treated with this fibrinolytic remedy every year. Different countries produce various streptokinase preparations. Streptokinase-plasminogen complex is made due to the interface of streptokinase and the protein plasminogen, systemic lytic state is created by this streptokinase-plasminogen complex which causes thrombolysis (Couto et al., 2004).

In the short term, deep venous thrombosis (DVT) is used for treating serious pulmonary embolism and in the long term same treatment can be used to manage the disease. Before the complete destruction of valves of the vein, removal of the whole thrombus is necessary for quick return of the venous system. Surgical thrombectomy and medical thrombolysis are the best ways used for the removal of thrombi. Further thrombosis can be prevented by conventional anticoagulant therapy and risk of pulmonary embolism is also reduced but occluded vein is not recanalized by this therapy. Thrombolysis has turn out to be the treatment of choice, as all thrombi present in the venous system are strongly effected by thrombolytic agents. Therefore, venous thrombi in the pulmonary artery and of the lower limb may be resolute at the same time. Application of thrombolytic agents gained renewed interest and wider approach due to the progress and evaluation of novel agents and the inspiring current confirmation of advantage in patients with cardiovascular disease. Expected benefit of thrombolysis has to be considered before treatment is instigated because it also has risks and limitations (Fletcher et al., 1962).
Patients can be benefited from thrombolysis but can also develop severe haemorrhage so it is contest for the clinician to recognize this difference dependably. Endogenous fibrinolytic system is activated by the induction of a fibrinolytic drug like streptokinase. Fibrin contained within the thrombus is affected by plasmin action which is potential property of all currently available fibrinolytic agents. Two reaction systems are exaggerated by induction of plasminogen activator: the fluid phase (blood) and the solid phase (thrombus). Degree of fibrinogenolysis is not dependent on efficacious breakage of the blood clot by fibrin dilapidation, however, rethrombosis and a hypocoagulable state is not permitted during treatment by thrombolytic agents. Three generations of plasminogen activators are available. Urokinase and streptokinase fall in first generation and are in medical use from last many decades. Second generation agents comprise acylated plasminogen-streptokinase activator complex, single-chain urokinase and recombinant tissue-type plasminogen activator. These all are currently under clinical study. High affinity for fibrin is shown by second generation drugs which outcomes in a small or vague hypocoagulable and lytic phase. Purification of streptokinase is done from streptococcal bacterial filtrate and this is utmost extensively used agent for break down of blood clots in deep vein thrombosis (Sikri and Bardia, 2007).

Streptokinase is deactivated by antibodies which are produced throughout drug induction and continue to be at high concentration for some months so this shows that streptokinase is extremely antigenic. Period of treatment is restricted to one week. Risks of allergic reactions increase due to elevated level of SK antibodies induced by previous streptococcal infections so fibrinolysis by using SK becomes unsuccessful. Before starting thrombolysis with SK, Prednisolone should thus be given. Streptokinase itself is inactive but catalyses the breakdown of the remaining plasminogen to plasmin only after making an intricate with plasminogen. Plasmin concentration is thus contrariwise proportional to the dosage of SK. SK has low specificity for fibrin and high affinity for plasminogen (Marder, 1979).

Two enzymatic actions are self-evident in mixtures of streptokinase and human plasminogen at least. Activator, an enzyme that quickly converts bovine plasminogen into plasmin, is the first and Plasmin, the major fibrinolytic protein of the blood is second. There is complex formation between streptokinase and human plasminogen or plasmin and it is understood for these complexes to be an activator (Ling et al., 1965). To respond under
controlled environment of molecular proportion, time and temperature, streptokinase and plasminogen were purified and radioactively labeled in present studies. Polyacrylamide-gel discontinuous electrophoresis was used to supervise the results of the reaction, bovine plasminogen activator activity, radioactivity and gel staining was used for detection of proteins. Human plasminogen and streptokinase were found to form a molecular complex with activator activity as confirmed by previous studies. But with the reaction conditions, the composition of the complex varies. When complex is exposed to mercaptoethanol, one major portion was released. Activator activity and components of both streptokinase and plasminogen are enclosed within this portion (estimated mol.wt. 70000) (Muramatu et al., 1969).

Variety of *Streptococcal* beta hemolytic strains produce streptokinase as a collection of extracellular proteins and it is a 414 amino acids containing plasminogen activator with a molecular mass of 47 kDa. High affinity equimolar complex is created by streptokinase with a plasminogen where as direct proteolysis is performed by tissue-type plasminogen activator and urokinase (Kim et al., 2000). Plasminogen is transformed to plasmin by this resulting complex; fibrin in the blood clot is degraded by this active protease (Wu et al., 1998). From past 30 years, this enzyme has been in use as a thrombolytic agent (Wong et al., 1994) and it is now broadly used for treating acute myocardial infarction, including coronary thrombosis as a thrombolytic agent (Kim et al., 2000). Major motives for examining of recombinant DNA technology means for this significant enzyme are its pathogenicity and small production yields from natural host. Fed-batch approach is the ideal technique for increasing the level of heterologous recombinant protein, which is comparative to both explicit cellular product yield and cell density. For this fed batch fermentation, *Escherichia coli* are the most frequently used host for heterologous protein production (Lee, 1996). To rise the output of a culture by raising the cell concentration, Fed-batch cultivation is a straight forward and efficient method. For recombinant protein production by *Escherichia coli*, Fed-batch cultures have been broadly used (Franz et al., 2005). For rising number of cells in recombinant cultures, numerous nurturing strategies have been used such as linear or exponential feed (Pal et al., 2001) and feedback control based feeding procedure so as to retain the pH or dissolved oxygen constant during the pre-induction phase (De Mare et al., 2005; Jeong et al., 2004).
Streptokinase cause human blot clot lysis, a bacterial protein first discovered by Tillet and Garner (Tillet and Garner, 1933) who then elaborated the medical significance of this enzyme. Streptokinase is an effective activator of plasminogen as well as an enzyme along with being the inactive precursor of plasmin also. These all facts are demonstrated by later studies (Schick and Castellino, 1974; Hoffman et al., 1991). Blood clots containing fibrin complex are solubilized by incomplete proteolysis by plasmin, which is dynamic thrombolysis constituent of the blood system (Rodriguez et al., 1995; Hermentin et al., 2005). During the secretion course of streptokinase from *Streptococcus equisimilis*, a fragment consisting of 26 amino acids is sliced which later on directs the secretion of streptokinase into the exterior medium. Growth of Beta-hemolytic *Streptococcus* in continuous culture was studied in which pH was set as a limiting factor (Ogburn et al., 1958). By adding buffer, the pH of the medium was controlled. Along with production of streptokinase, synthesis of some extra cellular antigens cells was studied. Effect of both tryptophan and glucose on growth of *Streptococcus faecalis* strain in continuous cultures was studied by Rosenberger and Elsden (1960). Restrictive factor should be an energy source, to get highest bacterial production per unit energy source as indicated by these conclusions.

From the culture media of numerous *Streptococci* strains, streptokinase is extracted which is later purified by numerous methods have been reported. Exceedingly refined streptokinase has been obtained by using DEAE-cellulose in amalgamation with other purification procedures in some cases (Blatt et al., 1964; De Renzo et al., 1967; Dillon and Wannamaker, 1965). Combination of more than one purification step has been used for streptokinase purification, moreover some new chromatographic processes are in wide application for streptokinase purification (Castellino et al., 1976; Taylor and Botts, 1968). For single step purification of streptokinase, affinity chromatography on immobilized Di-Isopropyl phosphate (DIP) - plasmin was used as reported by Castellino et al. (1976). Alteration of plasminogen by urokinase into plasmin and the plasmin protease’s reticence of action by diisopropyl fluorophosphates are involved in this process. Plasminogen as a ligand was used in an affinity chromatography (Jeong et al., 1993). By using glutathione as the ligand, production of fusion recombinant streptokinase is carried out which was later purified in a single step affinity chromatography (Nejadmoghaddam et al., 2007).
MI cause cardiac arrest in patients which is effectively treated with streptokinase during CRP as studied by Aliyev et al. (2005). Cardiocirculatory arrest is mainly caused by acute myocardial infarction and this results in intravascular thrombosis in 70 patients of cardiac arrest (Bedell and Fulton, 1986; Spaulding et al., 1997; Silvast, 1999). In 80% of non-survivors, reason of quick death is coronary artery disease. Survival in massive pulmonary embolism (PE) and acute myocardial infarction (AMI) has improved by thrombolytic treatment (Diehl et al., 1992; Grijseels et al., 1995). CPR was deliberated as a constrain for thrombolysis (Mueller et al., 1987; Gunnar et al., 1990; Curzen et al., 1998) because hemorrhagic complications can be induced by CPR procedures in AMI patients. On the other hand this assumption is not supported by any trial, certain scientific basis and serious study. Advantageous possessions of thrombolysis succeeding non-traumatic out of hospital CA with developed persistence and nervous outcome is demonstrated by current evidence (Van Campen et al., 1994; Voipio et al., 2001; Schreiber et al., 2002).

Fibrin formation and disseminated intravascular clotting is triggered by intravascular stimulation of blood thickening without adequate initiation of internal fibrinolysis which is associated with reperfusion (Bottiger et al., 1995; Gando et al., 1997). Vital organ dysfunction and outcome is determined by microcirculation impaired by formation of micro thrombi. Additional widespread enhancement in flow of microcirculatory together with the cerebral perfusion along with myocardium and lungs in myocardial infarction or pulmonary embolism is the result of thrombolysis by streptokinase (Fischer and Hossmann, 1995; Fisher et al., 1996). In fibrinolytic treatment of people with cardiac detention, streptokinase can be striking substitute to rt-PA by its applied production and induction in emergency setting and small expense.

**Aims and Objectives:**

- Isolation of streptokinase producing bacteria (*Streptococcus equisimilis*) from indigenous sources.
- Mutagenesis (radiation and chemicals) for strain improvement, to get enhanced streptokinase production.
- Isolation, purification and characterization of streptokinase by various techniques.
Chapter # 2

REVIEW OF LITERATURE

All over the world, a frightening condition is caused by stroke, deep vein thrombosis coronary artery disease and atherosclerosis. So much havoc is played by these diseases among the people that 12 million people expire due to cardiovascular problems every year and 80 million people are effected from heart diseases (Nagao et al., 2007). The health professionals and scientists have diverted interest on this condition and have been employed out to discover the therapy to deal this mortal disease after realizing the rigorousness of this health problem (Hina et al., 2010).

2.1. Streptokinase

A variety of strains of beta-hemolytic streptococci produce the extracellular enzyme streptokinase (EC 3.4.99.22). Circulatory plasminogen is activated by the fibrinolytic effect of this single-chain polypeptide enzyme. Jackson and Tang (1982) worked on complete amino acid order of streptokinase. This fibrinolytic enzyme is made up of 414 amino acid residues having molar mass of 47 kDa (Malke and Ferretti, 1984). Isoelectric pH of streptokinase is 4.7 and it works powerfully at pH of approximately 7.5 (El-Mongy and Taha, 2012). Conjugated carbohydrates, cysteine, lipids, cystine and phosphorous are not present in streptokinase. De Renzo et al. (1967) and Taylor and Botts (1968) reported other chemical and physical data on streptokinase. Considerable difference in structure is found in streptokinases because various groups of streptococci can produce this enzyme (Malke, 1993).

2.1.1. Structure of streptokinase and its mechanism of action

Various groups of streptococci produce streptokinases which acquire a substantial level of heterogeneity. Utmost of the accessible essential information on streptokinase is provided by biophysical techniques like distinction scanning calorimetry (DSC), nuclear magnetic resonance (NMR) spectroscopy, Fourier transform infrared (FT–IR) spectroscopy, circular dichroism (Beldarrain et al., 2001).

Important information is obtained by studies on fragments of streptokinase (Kim et al., 1996). Streptokinase consists of numerous structural domains (i.e., a-, h- and g-domains) with diverse linked functional properties. The protein is collection of two dissimilar domains as
recommended by Scanning calorimetric analysis (Welfle et al., 1992). Short plasminogen stimulation capability of the 60–414 amino acid residue domain of the protein is complemented by the N-terminal domain (i.e., residues 1–59) (Nihalani et al., 1998). Human plasmin light chain make complex with streptokinase and exhibit a crystal structure which is practically studied freshly (Wang et al., 1998). Beta domain of streptokinase also makes same crystal structure and has been worked out similarly (Wang et al., 1999). Azuaga et al. (2002) studied the thermal denaturation of streptokinase and Conejero-Lara et al. (1996) studied the temperature solidity of particular domains of the enzyme. The method of plasminogen activation by streptokinase has been the emphasis of widespread study (Wu et al., 2001; Wakeham et al., 2002; Sundram et al., 2003; Zhai et al., 2003); nonetheless, methods of plasminogen stimulation by streptokinase are still being explicated (Boxrud and Bock, 2000; Boxrud et al., 2001).

Plasminogen is activated both by fibrin independent and fibrin-dependent mechanisms of streptokinase (Reed et al., 1998). Interaction of plasminogen and streptokinase is however through numerous domains. Recognition of two self-regulating plasminogen compulsory sites of streptokinase was done by Nihalani and Sahni, (1995). In plasminogen substrate detection and stimulation, the C-terminal domain of streptokinase is involved (Kim et al., 1996; Kim et al., 2002; Zhai et al., 2003). In binding to the substrate plasminogen, the Asp41–His48 region of streptokinase is important similarly (Kim et al., 2000). Wakeham et al. (2002) studied plasminogen activation in reference to the part of contiguous region, i.e., residues 48–59. For plasminogen stimulation, the twisted region of the streptokinase g-domain is called to be necessary (Wu et al., 2001). For activating the plasminogen, streptokinase–plasminogen complex is involved which is formed by the h domain of streptokinase (Robinson et al., 2000). To activate conformational activation of plasminogen, streptokinase binds to plasminogen by the help of the lysine binding site (Boxrud and Bock, 2000; Boxrud et al., 2001).

To make best use of catalytic turnover, plasminogen-streptokinase activator complex intermingles with plasminogen by elongated protein–protein interfaces (Sundram et al., 2003). In streptokinase, the principal 59 amino acid residues appear to exhibit numerous practical roles (Shi et al., 1994; Young et al., 1995). Streptokinase has an unbalanced secondary structure in the nonexistence of these N-terminal residues. Fibrinolytic activity of the residual streptokinase portion is greatly reduced by the loss of residues 1–59 (i.e., residues 60–414)
Species-specific plasminogen stimulation is verified by utilizing isolates produced from pathogenic group C streptococci (McCoy et al., 1991).

### 2.1.2. Producing microorganisms

Billroth (1874) first recognized streptokinase producing streptococci in exudates of contaminated wounds. Later, similar microorganisms were found in the blood of scarlet fever patients. Founded on the diverse kinds of hemolytic reactions the variants produced on blood agar plates, Streptococcus sp. is categorized into gamma, alpha and beta variants. To additional segregation of the beta-hemolytic streptococci into groups A to O, Lancefield used serologic distinctions (Lancefield, 1933). Beta hemolytic streptococci belonging from Lancefield groups A, C and G are the best producers of streptokinase. Widely used organism for producing streptokinase is group C strain *Streptococcus equisimilis* H46A (ATCC 12449) which is secluded from a human source in 1945. Most active streptokinase is obtained from the strain H46A as it is chosen from more than a hundred fibrinolytic isolates. In its growth requirements, *S. equisimilis* H46A is less fastidious and does not produce erythrogenic toxins as the most of group A strains (Christensen, 1945). High levels of streptokinase can be obtaines by growing H46A isolate on semi synthetic medium (Christensen, 1945; Feldman, 1974). In various other microorganisms, gene used for streptokinase expression is mainly obtained from H46A (Malke et al., 1985; Hagenson et al., 1989; Wong et al., 1994).

### 2.1.3. Streptokinase fermentation

Commonly rich and complex growth media (augmented with numerous nutrients factors) is required by Group A hemolytic streptococci (Bernheimer et al., 1942). Bernheimer et al. (1942) originally developed an alteration in growth medium and it was used by Christensen (1945) for production of streptokinase from *S. equisimilis* H46A. The composition of medium included riboflavin, tryptophan, phosphate salts, biotin, glucose, nucleic acid (thiamine, uracil and adenine) peptone and glutamine. In the initial medium used by Bernheimer et al. (1942), the glutamine content was only 25%. Devoid of unnecessary production of acid, best growth was allowed by low glucose concentration at inoculation. For high density proliferation of cells, addition of glucose was done afterward the early overnight incubation. At several temperatures, dilution rates, pH in the growth medium with extra
glucose, a research work with constant cultures of a group C beta-hemolytic Streptococcus strain H46 evaluated the streptokinase production and biomass (Holmstrom, 1965).

At pH 7, there is no effect of glucose concentration and dilution rate on production of streptokinase and biomass; however, with increasing dilution rate, there was increase in streptokinase and biomass production. As compared to batch culture, there was two fold increase in SK production by continuous culture. In a study, an enriched tryptose broth medium was used for the production of streptokinase from various bacterial isolates. Out of 19 isolates, 18 strains of group E *streptococci* (SGE) were found to produce streptokinase (Ellis and Armstrong, 1971). This medium was composed of sodium chloride, tryptose, glucose, adenine, uracil, salts, vitamins, glutamine and tryptophan. For streptokinase production, Feldman (1974) used a medium composed of KH$_2$PO$_4$, cerelose, KHCO$_3$ at pH 7.0.

Substantial enhancement was seen in the explicit output of the recombinant protein when fedbatch cultivation was used instead of batch fermentations of IPTG-induced *E. Coli* (Yazdani and Mukherjee, 1998). Modeling of streptokinase fermentation and its kinetic analysis were studied out by Stuebner *et al.* (1991).

### 2.2. Production and purification of Streptokinase

Christensen (1949) reported method for the preparation of extra enormously purified streptokinase jointly with the consequence of temperature and pH on changeable inactivation. It was noted that an obvious damage of activity arises over a moderately narrow acid pH range (Christensen, 1945). In the sequence of studies on the properties and purification of streptokinase and more newly, that the damage can be totally overturned when solutions deactivated by this method are made marginally alkaline. The portent happens with crude concentrates as well as with incompletely purified streptokinase preparations.

De-Ranzo (1967) described procedures for the preparation of extremely purified streptokinase by chromatography on diethylaminoethyl cellulose and by column electrophoresis in a sucrose density gradient. On the basis of ultra centrifugal and gel electrophoresis analyses and constancy of specific activity, preparations chromatographed at least twice on diethylaminoethyl cellulose are shown to be essentially monodisperse. The molecular weight determined by equilibrium sedimentation was found to be 47,600 which are in good agreement with a previous finding from the same laboratory. A lowering of the
sedimentation coefficient without significantly changing the molecular weight was seen when streptokinase was treated at pH 7.5 in 0.1 M phosphate buffer with 5 M guanidine-hydrochloride or 8 M urea. This resulted in production of cystine and cysteine which were absent on amino acid analysis, and the molecule is therefore assumed to be a solo polypeptide chain with no subunits. The isoelectric point of streptokinase is about pH 4.7. The amino acid composition is consistent with the formula Aspee-Thrao-Serzr-Glu46-PTo20’-G1yzl-A1a~~-Val*--Met3-Ue22Leu40-Tyr20-Phe1 Lys3-HisS-Arg21-Try1 for molecular weight 47,754. Approximately 60 aspartic and glutamic residues are imitated per molecule of protein. The most highly purified preparations are devoid of carbohydrate and phosphorus and are inactive with basic amino acid esters, naphthyl esters, and acetyl tyrosine ethyl ester as substrates.

A methodology for persistent development of hemolytic *Streptococcus*, strain H 64 was suggested by Holmstrom (1968). Streptokinase production was studied at different temperature, pH and dilution rates in a complex medium supplemented with surplus glucose. With respect to increasing dilution rate, With respect to glucose, yield of streptokinase and cells amplified when dilution rate was increased in the sequence of 0.1 to 5.0 hr at pH 7.0 yield constant. Streptokinase production is directly affected by cell concentration and growth rate. Large scale production of streptokinase strongly depends on mode of fermentation. By employing batch cultures, higher concentrations of streptokinase are produced as compared to other modes of fermentation. While by using continuous production method, 2.3 times higher streptokinase production was obtained in comparison to batch cultures, so continuous production method is graded superior for large scale production of Sk.

Van De Rijn and Kessler (1980) formulated a new chemically distinct growth medium for the streptococci belonging from group A. This novel medium was superior over formerly defined media as: (i) development rate (i.e., replication cycles) of strains remained equivalent to the growth rates in complex media; (ii) In the new defined medium, every strain exhibited growth to a advanced culture density than in composite media; (iii) Without any previous adaptation procedure, transferral from multifaceted media with minor inocula quantity was conceivable ; and (iv) production of virulence elements (i.e., hyaluronic acid and M protein production) and extracellular enzymes during development in this novel medium was analogous to that in composite media.
Function of streptokinase in mammalian clot lysis system was studied by Caballero et al. (1999). Secretion of streptokinase was reported from non-human isolates of group C *Streptococci* including *S. zooepidemicus* and *S. equisimilis*. Diverse mammalian plasminogens openly bind with favored activity to this streptokinase. Segregation of streptokinase genes was done from *S. equisimilis* and then these genes were cloned in *Escherichia coli*. After sequencing and expression of these genes in *E-coli*, the comparison of activity of produced streptokinase was done on porcine and equine plasminogen, insufficient practical and organizational resemblances were revealed between the streptokinase produced by the human and swinish isolates. As streptokinase and plasminogen interacts in the fluid phase, labelled immobilized recombinant streptokinase reacted and stimulated the plasminogen in similar mode. Equine, porcine and human plasminogens are chopped at the same extremely preserved site, when streptokinase-plasminogen interaction was cleaved as shown by the investigation of these cleaved products. Altered streptokinase (Sk*) is produced after site particular cleavage of streptokinase. This study revealed that non-human *S. equisimilis* isolates produce a variety of plasminogen activator proteins which include streptokinase also.

Schroeder et al. (1999) studied that streptokinase demonstrate favored activation of plasminogens as this activation is dependent on source of the isolates. These group C *Streptococci* isolates were isolated from human and horse. For the analysis of this conclusion and for additional assessment, sequence of *Streptococcus equisimilis* isolates was isolated from horses and humans. For the assessment of potential of acquiring surface bound plasmin like activity, these *Streptococcal* strains were allowed to grow in the existence of equine and human plasma. Chromosomal DNA from equine Streptococcal isolates absolutely responded with skc (eq) probe and that of human *Streptococcal* strains responded differently with a skc (hu) probe in a separate pattern as shown by the results of Southern blotting. From each group, eight isolates were engaged. When isolates from human were grown in the presence of human plasma, important enzymatic activity was shown. In case of equine isolates, same response was shown which presented activity only in the existence of equine plasma. In case of activated plasminogen, streptokinase and fibrinogen, there is a straight association among the host protein and bacterial necessities as shown by this study. This shows that why limited
and specific number of mammalian hosts are prone to diseases caused by certain *Streptococcal* strains as explained by such definite association.

Banerjee *et al.* (2004) concluded that due to malfunction of hemostatic system and formation of blood clots in circulatory system, rigorous diseases like stroke and myocardial infarction can result. In such pathological progress of blood clot, medical interference with thrombolytic agents such as urokinase, tissue plasminogen activator and streptokinase is necessary. This study includes the aspects like the structure and structure–function associations, mode of action of streptokinase, recovery and purification of this protein from crude broths and recombinant streptokinase production from microbes.

By means of fed-batch culture approach, various recombinant proteins were produced from *Escherichia coli* as studied by Goyal *et al.* (2009). For investigating the effect of compound nitrogen sources, pH managing agents and dissolved oxygen concentration (DOC) on intracellular expression of streptokinase and cell enlargement, recombinant *E-Coli* BL21 (DE3) was used. In fed-batch culture, boost in DOC from 30% to 50% significantly affected the expression of SK (from 188 to 720 mg L\(^{-1}\)) where as in batch culture; raise in DOC did not affect SK expression. By supplementing the growth medium with yeast extract and tryptone, SK expression can be further enhanced. For pH control, liquid ammonia should be replaced with NaOH which always fallout in enhanced SK production without distressing production of cells. By process extended from flask to industrial bioreactor level, fed-batch mode of fermentation attained higher testified concentration of SK.

Babashamsi *et al.* (2009) studied that certain strains of beta hemolytic streptococci produce streptokinase as an extracellular protein. Fibrin clot is degraded by enzyme’s explicit lysine binding site is plasmin which is shaped from plasminogen after activation by streptokinase so consequently it is in use as a medication in thrombolytic therapy. The rate of bacterial growth and streptokinase production was studied in circumstance with pH maintenance and addition of surplus glucose to growth media. Salt precipitation was used to end first round extraction of this enzyme product of the bacterial culture the and then affinity chromatography on plasminogen substituted sepharose-4B was used for further purification in a state that the plasminogen active site was sheltered from streptokinase persuaded stimulation. Biological activity of this bacterial enzyme was performed in a precise streptokinase analysis.
along with the confirmation of its purity by SDS-PAGE. As compared with the batch culture, the level of streptokinase production enhanced double times in the fed–batch culture as shown by the results while at the same time 95% increase in the yield was obtained by limitation of the purification of streptokinase to a solitary step of the chromatography.

Karimi et al. (2011) studied that for the cure of acute myocardial infarction various intravenous thrombolytic agent are available out of which streptokinase is in wide clinical usage. Which is usually produced from cultures of H46A strain of *Streptococcus equisimilis*. Present study was intended with the purpose of the streptokinase production from H46A strain and use of biochemical process for its refinement. Under the consequence of variations in some factors of fermentation, the rate of streptokinase production was evaluated. Moreover, for the purification of streptokinase from the crude extract, a chemical reduction process was engaged due to the definite structure of streptokinase. The group C H46A streptococcus strain was developed in a bioreactor. By using glucose as carbon source in a best possible temperature, the proper pH was accustomed with NaOH. Techniques like filtration and ultra filtration were used for sterilization and concentration of the supernatant obtained from the crude product. The pH of the filtrate was attuned, cooled, and precipitated by methanol. Dithiothreitol (DTT) was used for the reduction of Protein solution. After the settling down of impurities by aldrithiol-2, the supernatant was analysed for its natural activity which showed the presence of streptokinase. Impurities were efficiently removed from streptokinase by reduction process and as compared with the batch culture, in the fed–batch culture the rate of streptokinase production improved over two times. Due to a boost in the production rate of logarithmic phase and decrease in lag phase period, SK production was improved. Chemical reduction method is simple to perform and it gives high yield of streptokinase along with the benefit over other approaches of purification which result in undesirable damages of streptokinase.

Felsia et al. (2011) studied that for the treatment of acute myocardial infarction, recombinant tissue plasminogen activator (tPA) is in common use but streptokinase is also much valuable and it is definitely more cost-effective. Uncertainty is being articulated about the constant feasibility of streptokinase therapy, in vision of the comparatively current accessibility of the competing recombinant tPA. It remains a fundamental and inexpensive treatment particularly in the world’s poorer healthcare systems, regardless of continuation of this research on streptokinase. Use of *Streptococcus pyogenes* species for SK production was
focused in this study and fractional purification of streptokinase by ammonium sulfate precipitation, dialysis and column chromatography was done. Lowry’s process was used for the quantification of streptokinase and SDS-PAGE was used for determination of its molecular weight and electrophoretic mobility.

Dubey et al. (2011) verified that in near future bacterial sources will be effectual as it is so apparent to get SK from bacterial sources. *Bacillus subtilis*, haemolytic *Streptococci* and urine sample were used for the separation of new clot lysing enzymes like nattokinase, urokinase and streptokinase. To purify these fibrinolytic enzymes, supernatants of haemolytic Streptococci, recombinant *E.coli* and *Bacillus subtilis* were processed. Thermophilic, hydrophilic, and strong clot dissolving activity was shown by culture broth of these organisms. Nattokinase, Urokinase and Streptokinase showed most favorable activity at 37-55°C, 55°C and 27-37°C respectively. These three fibrinolytic enzymes showed optimum activity at pH 9, 7 and 9 respectively. Sodium dodecyl sulfate polyacrylamide gel electrophoresis was performed for the estimation of molecular weight of these thrombolytic enzymes which were about 28 kDa, 47 kDa and 34 kDa respectively. Streptokinase, Urokinase and Nattokinase showed 467.73 U, 785.73 U and 576.73 U of caseinolytic activity respectively, when fibrin plate method was applied, fibrinolytic activity was 10 U, 5 U and 15 U, correspondingly.

Zia et al. (2013) stated that distinctive clot dissolving enzyme streptokinase is produced by numerous species of *Streptococci*, this enzyme works by binding and activating the plasminogen. For production of streptokinase, *Streptococcus mutans* was chosen and enzyme was produced by using molasses, corn steep liquor, sugarcane bagass and rice polishing as substrates of liquid state fermentation. Substrates were used in a concentration range of 0.1-0.8%. Maximum fibrinolytic activity was obtained at 0.5% molasses and rice polishing, 0.4% of sugarcane bagass and 0.3% level of corn steep liquor. Rice polishing, Corn steep liquor, sugarcane bagass and molasses showed 5.16, 5.08, 4.75, 5.5 units of clot lysis correspondingly. For Streptokinase assay, fibrin clot lysis method was used.

Shilpi et al. (2013) studied that numerous species of *streptococci* produce streptokinase which is a novel fibrinolytic protein. In the treatment of thromboembolic disorders where it dissolves a blood clot by the activation of plasminogen to plasmin, streptokinase can be used as a therapeutic agent. For the separation of haemolytic organisms, specimens from infected
throat can be an excellent source. When 34 throat swabs were collected from patients with acute tonsillitis, 43 bacterial isolates confirmed β-haemolysis. Among these β - haemolytic organisms, 11 isolates were streptococci. An important criterion of this work was screening the isolates for their effectiveness to produce streptokinase. Isolate SK-6 confirmed the maximum streptokinase activity as based on the results of blood clot dissolving assay and radial caseinolysis assay. This isolate (SK-6) was recognized as *Streptococcus equisimilis* when subjected to morphological and biochemical categorization based on Bergey’s criteria. This isolate may also be utilized for the large scale production of streptokinase as indicated by the thrombolytic prospective of this particular isolate.

### 2.3. Role of Streptokinase

Agnelli *et al.* (1985) acknowledged that thrombolysis without convincing plasma proteolytic state can be produced by tissue-type plasminogen activator (t-PA) so it is graded as auspicious fibrinolytic agent. It is indeterminate if this hypothetically significant aspect reduces t-PA as low clot dissolving than other plasminogen activators. Rabbits were used for analyzing the thrombolytic and hemorrhagic properties of t-PA and streptokinase in this research work. Only 28 + 6% thrombolysis was produced by 8000 U/kg/hr streptokinase over 4 hr and 4000 U/kg/hr streptokinase over 4 hr got unsuccessful to yield significant fibrinolysis. Plasmin interceded plasma proteolytic state is connected with both streptokinase regimens and both treatments formed a substantial surge in blood loss that was obvious within 15 min of commencement the mixture and was advanced over the 4 hr of drug induction. In distinction, 35 + 6% thrombolysis was resulted by 7500 U/kg/hr dose of t-PA, but even after the 4 hr of infusion, an imperative rise in hemorrhage or plasmin-mediated plasma proteolytic state was not attained. t-PA in a dose of 15,000 U/kg/r produced but was connected. While on the other hand, substantial bleeding and plasmin-mediated proteolytic state was attained by using t-PA in a dose of 15,000 U/kg/hr which resulted in 85 + 4% thrombolysis. Therefore, more effectual thrombolysis is attained by using streptokinase as this resulted in less hemorrhage than t-PA at same doses rate. Plasmin-mediated proteolytic state is linked with bleeding by the use of both fibrinolytic agents.

Into a clot dissolving protease plasmin (Pm), the function of bacterial enzyme streptokinase (SK) on the stimulation of human plasminogen (Pg) was demonstrated by Zhai
et al. (2003). From C-terminal domain of SK, only 40 residues show mobility when SK form composite with the catalytic domain of plasmin. Constructed and characterization of three C-terminal truncation mutants was done and this helped in for improved understating of the functions of SK in activation of plasminogen. SK residues 1-386, 1-401 and 1-378 were found in these mutants respectively. In case of plasminogen activation and amidolytic activity, a gradual reduction was seen on analysis. There was decreased compulsory affinity toward plasminogen, as more deletion of the C-terminus occur. For plasminogen-activation ability assessment, full dimension SK comparison was done by these three C-terminus truncation mutants. Almost 80% reduction in plasminogen activation ability was shown by the shortest constructed mutant SK (1-378). To the plasminogen catalytic domain, 30% rise in detachment constant was seen. The veracity of the SK C-terminal peptide is vigorous for the effective and proper role of SK as suggested by this study.

Most β-haemolytic streptococci from group A, C and G exude a 47-kDa protein streptokinase as verified by Couto et al. (2004). A dynamic complex able of converting other plasminogen molecules to plasmin is produced by the interaction of this isolated protein with human protein plasminogen. Assessment of five commercial streptokinase preparations accessible in Brazil was done. Tests of hydrolysis of the plasmin-specific substrate S-2251™ and euglobulin clot pattern was performed in vitro. A 96-well microtiter plate was used for euglobulin lysis time determination. In separate wells, human thrombin (10 IU/ml) and streptokinase were placed primarily. Addition of plasma euglobulin initiated the clot formation and then turbidity was calculated at 340 nm every 30 s. Activation of plasmin was analysed by using the plasmin-specific substrate S-2251™ in the second assay. Strongest thrombolytic activity in the euglobulin lysis analysis was exposed by Streptase™ so it was used as the reference formulation. By comparing with the reference formulation, about 50% activity was showed by Unitinase™ and Solustrep™ formulations which were the weakest. Considerable differences were observed amongst all tested streptokinase which all uniformly activated the plasminogen. Maximum activity was shown by Streptonase™ (94.7 ± 4.6 units) and Streptase™ (75.7 ± 5.0 units) in relation of total S-2251™ activity per vial while weakest performance was revealed by Strek™ (32.9 ± 3.3 units) and Unitinase™ (31.0 ± 2.4 units). Intermediary activity was presented by Solustrep™ (53.3 ± 2.7 units). For the amount of 47-kDa protein, SDS-PAGE densitometry results correlated with the variations between the
diverse formulations for both chromogenic substrate hydrolysis and euglobulin lysis test. Commercially accessible clinical formulatins of streptokinase differ appreciably in their activity (in vitro) as revealed by this data. It has to be investigated that whether these differences have experimental implications or not.

2.4. Uses as a therapeutic agent

Mi et al. (2005) evaluated that whether in the individuals after renal transplantation, the appliance of thrombolytic streptokinase fallout in the production of anti-streptokinase antibodies. It was concluded that streptokinase is being used for preflushing in non-heart beating donors (NHBDs). An ELISA was used to verify antibodies in the sera of patients. After kidney transplantation, serum were collected at 1 and 6 months. For conducting the trials, two groups of volunteers were chosen each containing 18 volunteers. Among both groups of the research trial, no any obvious difference was noted. These research groups included the thrombolytic-treated NHBDs kindey recievers and volunteers receiving NHBD kidneys devoid of such treatment.

Sikri and Bardia (2007) studied that in developing countries, streptokinase is still fundamental drug being used for treating cardiovascular diseases including acute myocardial infarction regardless of the wide application of the tissue plasminogen activator in advanced countries. William Smith Tillett laid the base for the use of streptokinase as a fibrinolytic agent in 1933 and same work was followed by his student Sol Sherry for many years and streptokinase emerged as an unexpected innovation. At the outset this drug was clinically applied in combating tuberculosis meningitis, fibrinous pleural exudates and hem thorax. Focus of treatment was distorted from palliation to “cure” in 1958 by oher scientists and Sherry when streptokinase was used for the treatment of patients with acute myocardial infarction. Contradictory results were produced in initial trials that used streptokinase infusion. Rentrop and colleagues initiated a pioneering approach of intracoronary streptokinase infusion in 1979. Reperfusion rates ranging from 70% to 90% were achieved afterwards larger trials of intracoronary infusion. Gruppo Italiano per la Sperimentazione Della Streptochinasi nell’Infarto Miocardico (GISSI) trial fulfilled the need for a thoroughly planned and methodically executed randomized multicenter trial in 1986, which not only recognized a standard method for its application in patients suffering from acute myocardial infarction but
also validated streptokinase as a successful beneficial mode. Presently in developing nations, streptokinase is still vital for the treatment of acute myocardial infarction in spite of the extensive use of tissue plasminogen activator in advanced nations.

Salarifar et al. (2009) sought to assess the effectiveness and security of Streptase (Aventis Behring GmbH, Marburg, Germany) and Heberkinasa (Heberbiotec, Havana, Cuba) which are diverse commercial types of streptokinase accessible in Iran. Thrombolysis is required by the patients suffering from acute myocardial infarction, a randomized double-blind clinical trial was conducted to contrast the two streptokinase preparations, i.e. Streptase (STP) and Heberkinasa (HBK) for the treatment of such patients. Assessment of thrombolysis achievement was done clinically or angiographically. After 30 days of thrombolysis, clinical follow-up was done. Patients were indiscriminately allocated to HBK (n=119) and STP (n=102) groups with a mean age of 56.9±10.8 years (males: 88.2%). Between the two groups, baseline demographic and clinical distinctiveness were similar and were not suggestively diverse in terms of pain-to-needle intervals and door-to-needle. There was no important dissimilarity between the rates of complications of the groups as given (42% [STP] vs 44.1% [HBK]). After thrombolysis, in the first 24 hours (9%) and in the first 72 hours (38.8%) angiography was made for 158 (71.5 %) patients. No major differentiation was found in lesion/patient ratio and lesion morphology of the two groups (1.67[STP] vs 1.87[HBK]). Similarity was found between two groups with reverence to patency ratio of angiography (67.5% [HBK] vs. 67.6% [STP]). Concerning impediments and medical outcome of both streptokinase regimes, the study groups were also similar. Heberkinasa is as effectual and as secure as a regular streptokinase, named as Streptase, in a scientific background as demonstrated by the study.

2.5. Mutagenesis for hyper production of streptokinase

Hemolytic mutants were produced when ATCC 19615 *Streptococcus pyogenes* and wild Lancefield strain SS-95 were treated with N-methyl-N'-nitro-N-nitrosoguanidine by Owens et al. (1978). Disease causing potential of these mutants retained for mice when reinsulated form dead animals. Lack of streptolysin S is seen in these mutants like naturally stirring non-hemolytic lowery strain. The level of nicotinamide adenine dinucleotidase, streptolysin, deoxyribonuclease and hyaiuronidase were same in wild strain.
Malke and Ferreti (1984) used *Escherichia coli* for cloning genomic DNA from *Streptococcus equisimilis* strain H46A by using an in vitro wrapping system and the bacteriophage lambda substitute vector L47. For screening of recombinants transporting the streptokinase gene (skc), an overlay technique of casein/plasminogen was applied. Isolation and physical characterization of total 10 independent clones containing skc was done along with the fact that the gene was present with a rate of recurrence of 1 in 836 recombinants. To sub clone skc in plasmid vector of *E. coli*, one recombinant clone was used. In three diverse *E. coli* host strains, use of recombinant plasmid strongly influenced streptokinase production. The plasmid used here is Plasmid pMF2. In either vector, expression of skc was self-governing of its alignment representing as its self promoter was existing and efficient in *E. coli*. Nevertheless, expression in pBR322 was much effectual in one alignment than in the other, reminiscent of that one or both of the bla gene promoters subsidized to skc expression. Numerous positions of proof, counting proof attained by the immune diffusion method, recognized the individuality of *E. coli* streptokinase. Analysing osmotic shock fluids, cell-free culture supernatant fluids and sonicates of osmotically shocked cells for streptokinase activity exposed the substance to be present in all three major positions, representing that *E. coli* cells were proficient of discharging considerable quantities of streptokinase into the growth medium.

Consequence of a diversity of mutagens on *Streptococcus lactis* was studied by Lautier et al. (1988). Mutagenic and lethal effects of methyl methanesulphonate (MMS), UV irradiation and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) and to a slight degree ethyl methanesulphonate (EMS) were studied on these three strains. These strains of *S. lactis* were comparatively less vulnerable to EMS, whereas all three strains showed much better reactivity to UV irradiation, MNNG and MMS as shown by lethality studies. A mutant Lac" exposed extra resistance in comparison to wild type after UV irradiation. Loss of 37-Md plasmid of this Lac" mutant results in this resistance. After using EMS, MMS and MNNG as mutagens, these all strains were effectively mutated by EMS for the inherited indicator assayed. A boost in the transformation rate was perceived in case of other two mutagens.

The outcome of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine on *Streptococcus lactis* strain was studied by Auffray et al. (1989). Decrease in the mutagenic and cytotoxic effects of this strain is caused by elevated concentration of MNNG and this study revealed that low levels of
this agent cannot perform this function. There is no competent inducible error-free repair system in after alkylation harm in *S. Lactis* as shown by this study.

Effectiveness of streptokinase as a competent blood clot-dissolving agent is restricted by its short in vivo half-life as assured by Wu *et al.* (1998). Minor intermediates of streptokinase are shaped after processing by plasmin during the clot-dissolving method. Lys59 and Lys386 are two of the chief processing sites. Engineering of two varieties of streptokinase was carried out with either one of the residue of lysine distorted to glutamine and a third type with both mutations. Protease-deficient *Bacillus subtilis* WB600 served as host and produced these mutant streptokinase proteins (muteins) by discharge. Contingent on the type of the mutation, the purified muteins showed diverse degrees of resistance to plasmin and also reserved equivalent kinetics parameters in plasminogen activation. In the radial caseinolysis assay, improved plasminogen activation activity with time was shown by muteins having double mutations and the half lives got prolonged 21-fold when *in vitro* assay was done by using a 1:1 molar ratio with plasminogen. Inactivation of streptokinase is the result of plasmin mediated processing and is not compulsory for the conversation of streptokinase to its functional state as indicated by this study. To create engineered enzymee with a longer in vitro purposeful half-life, obstruction of the N-terminal cleavage site is vital and without distressing their activity, Plasmin-resistant types of streptokinase can be produced.

Seclusion of β-haemolytic *Streptococci* (with blood lysis activity) from diverse biomass and blood samples was done by Abdelghani *et al.* (2005). This study was conducted by collecting 103 samples in total and out of these collected ones, 47 strains exhibited streptokinase (SK) production as confirmed by enzyme analysis. Submerged fermentation was used in this work for the SK production from selected isolates. T₃ was preferred as a novel option of *S.equisimilis* for mutagenesis as its showed admirable SK production. N-methyl-N’-nitro-N-nitroso guanidine (NTG) and UV treatment was used for mutagenesis of this T₃ strain. For SK production, first mutation was induced by using different means and later organisms were selected for further use for the enzyme production. Yield of the Selected UV mutant AUV10 showed120% more production in comparison to the wild strain (T3). While the selected NTG mutant NUV7 resulted in 146% higher yield production in contrast to the wild (T3) strain. For hyper production of SK from T3 strain of *S. equisimilis*, NTG and UV irradiation can be used as proficient mutagens as revealed by this study.
Wild strains were secluded and subjected to strain enhancement by employing mutagenesis (random UV irradiation) to monitor hyper production of streptokinase by Madhuri et al. (2011). From throat infected patients, total of 15 throat samples were unruffled and the presence of *Streptococcal species* was indicated by 3 samples out of these total 15 ones. To check for the production of streptokinase, analysis for determination of streptokinase activity was done for all strains, and *S. equisimilis* exhibited maximum activity. For strain improvement, UV irradiation was used and then activity of streptokinase activity of strains was analyzed. UVM6 (mutated *S. equisimilis*) showed highest activity as observed after enzyme assay. Improved streptokinase activity was given by mutated strains in association to the wild strain.

Lactic acid bacteria (LAB) were secluded from fresh sugar cane juice by Sobrun et al. (2012). Numerous isolates exhibited a plain zone and growth on deMan-Rogosa-Sharpe (MRS) agar supplemented with sodium azide, bromocresol purple and sucrose as found in this work. For further investigations, nevertheless, only 17 isolates which produced large yellow areas were chosen. According to their morphological and biochemical distinctiveness, these isolates were further recognized. It was found that 10 of these isolates were homofermenters. For mutagenesis using physical (UV-B) mutagens and chemical (Ethidium bromide) followed by biochemical characterization was done on one of these 10 isolates. For their capability to produce lactic acid, out of total 112 isolated mutants 9 homo fermentative isolates were further investigated. Lactic acid was produced as the sole fermentation product by all mutant isolates as confirmed by 1H-NMR spectroscopy.

### 2.6. Cloning for streptokinase production

Swab sample (collected at the Dra. Ângela Castro- Dep. de Microbiologia da Universidade Federal do Rio de Janeiro) was used for the inoculation of *Streptococcus dysgalactiae* sub sp. *equisimilis* of the group C and G by Lunardi et al. (2009). *Skc* and *skg* were augmented by PCR using explicit primers containing *NdeI* e *BamHI* limitation sites were used for the magnification of Streptokinase genes by PCR. These amplicon were cloned into pCR-Blunt cloning vector (Invitrogen) and the by using *NdeI* e *BamHI* restriction enzymes, *skc* and *skg* genes were subcloned into pET30a (+) expression vector (Novagen). These plasmids were nominated as pET30a (+)-skc and pET30a (+)-skg. pET30a(+)skc and pET30a(+)skg constructions were used respectively for *E. coli* BL21(DE3) and Rosetta(DE3)
cells and cultured at 37°C (pET30a(+)-skc) and 30°C (pET30a(+)-skg) in liquid LB medium during 6 hours (protein expression induced with 0.4mM IPTG). After sonication of the cells, SDS-PAGE electrophoresis was used for protein analysis. In the soluble and insoluble fraction, expression of streptokinase (SKC and SKG) was observed. A three-step protocol consisting of two anion exchange and one gel filtration chromatography columns using a FPLC system was used for the refinement of the recombinant protein. Consistent SK concentration was measured according to Bradford et al. (1976). Method of Pratap and Dikshit, (1998) using chromozyme PL (Boehringer Mannheim, Germany) as a synthetic substrate was used for SK activity assay. Bioreactor BIOSTAT B plus (Sartorius Stedium) was used to grow the recombinant streptokinases (SKC and SKG) after the determination of the purification protocol.

Lunardi et al. (2010) used a swab sample for obtaining *Streptococcus dysgalactiae* subsp. *equisimilis* of the group C (from Dra. Ângela Castro - Departamento de Microbiologia da Universidade Federal do Rio de Janeiro). Explicit primers containing *N*del e *B*am*H*I restraint sites were used for the magnification of Streptokinase gene by PCR. pCR-Blunt cloning vector (Invitrogen) was used for the cloning of this amplicon and the pET30a(+) expression vector (Novagen) using *N*del e *B*am*H*I restriction enzymes was used for the sub cloning of *ske* gene. This plasmid was nominated as pET30a (+)-skc. pET30a(+)-skc construction was applied for alteration of *E. coli* BL21(DE3) cells which were then cultivated at 30°C in LB medium during 6 hours (protein expression induced with 0.4 mM IPTG). 2L working volume stirred bioreactor (Biostat B plus, Sartorius Stedium, Germany) filled initially with 1L of medium was used for moving out both Batch and fed-batch cultivations. Two Ruston turbines with six flat-blades along with pH controllers, temperature, and aeration and agitation factors were used to endow the bioreactor. Two without feedback control (linear and exponential) and two with feedback control (DO-stat and pH-stat) fed-batch strategy cultivations were experienced along with diverse fed-batch media. Various initiation times were studied, after defining the better fed-batch media and strategy. SDS-PAGE electrophoresis was used in all experiments for protein analysis. Densitometry analysis (GS-700 Bio-Rad) and Bradford et al. (1976) method was used to measure concentration of SK. GE Pharmacia Äkta Purifier was used to conduct the purification experiments. Method of Pratap et al. (2000) using chromozyme PL (Roche) as a synthetic substrate was used to assay
streptokinase activity. In LB culture medium at 30°C for E. coli BL21 (DE3) strain, best expression of the SKC was obtained. A feeding medium containing MgSO₄ (25g/L) and glucose (400g/L) was used as fed-batch cultivation defined media. When compared to shaker cultivation, the maximal biomass production and protein expression were achieved using linear feeding strategy at 12h of orientation, increasing the biomass and cell output of approximately 8 fold and 7 fold, correspondingly.

2.7. Optimization of media for SK production

Isolation of streptokinase from β haemolytic streptococci, Streptococcus pyogenes was done by Patel et al. (2011). Batch fermentation was accepted out for culturing of these streptococci. U.V irradiation was used to treat these strains and to measure enzyme concentration; Lowry’s method for protein estimation was used. To examine the effect of diverse factors like development period of strain, volume of the inoculums and corn steep liquor on the enzyme production were studied and interpreted by regression analysis. To evaluate fibrinolytic activity of streptokinase, various biological activities including radial caseinolytic activity and fibrin lysis action were done. MI (45.05 mg/ml) was chosen as an excellent batch as it given almost five folds advanced production than wild strain (9.68 mg/ml). Lot F6 (296.67 mg/ml) was selected to be best among factorial experiment, so it was used for pilot scale streptokinase production as it demonstrates almost six times boost in yield. According to regression analysis, inoculum volume showed negative considerable effect on production where as incubation period and corn steep liquor are the factors which showed encouraging important effect on enzyme production.

Optimization of Streptokinase Production was demonstrated by El-Mongy and Taha, (2012). Enzyme production by Streptococcus pyogenes and Streptococcus equisimilis was monitored during the growth period by growing them on different media. pH of the culture media was accustomed after every 12 hours of incubation during growth and enzyme production levels were amplified significantly by this. Enzyme production was considerably improved when these microbes are grown on Strep-base medium. When glucose was used as carbon source, S. pyogenes and S. equisimilis showed most excellent growth while unsuitable carbon sources included mannitol and sorbitol. When Strep-base medium was used to grow microbes for production of streptokinase, chief source of organic nitrogen used were casein
and yeast extract. When 1.5% (w/v) casein and 1.5% (w/v) tryptone was used for *S. pyogenes* and *S. equisimilis* respectively, maximum levels of the Streptokinase were obtained. Methods used for streptokinase assay integrated common casein digestion method as well as the highly sensitive chromozym substrate digestion technique. For the assessment of various assay methods of streptokinase, protamine-sensitive electrode was employed for electrochemical analysis. Results presented by other methods were in correlation to the electrochemical method. These all assay methods are substitute and dependable for streptokinase detection during microbial growth. A faster and less exclusive procedure for streptokinase determination was provided by this and chiefly when enzyme was required to be analyzed in turbid media.

### 2.8. Characterization of Streptokinase

Brockway and Castellino (1974) performed contrast between the locally produced streptokinase and streptokinase purified from the human plasminogen complex (altered streptokinase). Native streptokinase showed 44,000 and altered streptokinase possessed 36,000 molecular weight. Native streptokinase showed lysine at carboxyl-terminal and an isoleucine residue at amino-terminal where as altered streptokinase showed lysine at carboxyl-terminal and an amino-terminal serine residue. For native streptokinase the amino terminal amino acid sequence was NH₂-Ile-Ala-Gly-Pro-Glu-Trp-Leu-Leu-Asp-Arg-Pro-Ser while for altered streptokinase sequence was NH₂-Ser-Lys-Pro-Phe-Ala-X-Asp-Ser-Gly-Ala-Met-Ser. Results of this study proved that loss of peptide at an amino terminal with a collective molecular mass of nearly 8000 take place upon composite development with human plasminogen or plasmin. Spherical dichroism and optical rotator dispersion analysis recommended differences in the conformations of altered and native streptokinase. In direct plasminogen activation assays, this fastidious type of transformed and native streptokinase perform in an equivalent manner and the streptokinase part of the activator complex most likely does not contain plasminogen activator active site in it.

1H n.m.r. spectroscopy was used for streptokinase investigation by Teuten *et al.* (1993). For this molecule of molecular mass 47 kDa, one- and two-dimensional well-resolved spectra have been obtained. A range of pKa values, between 5.6 and 8.2 has been shown by titration of all nine histidine residues edifying a range of environments for these residues in the protein structure. Reversible modification by diethylpyrocarbonate was given by at least eight histidine
residues. In chemically induced unfolding studies performed by thermal and chemical means, only one is adequately bare to be spontaneous towards photo-excited dye. The protein consists of at least three domains which have self-determining constancy, and that the protein can exist in a number of incompletely folded states as evidence is accessible here for numerous different unfolding transitions. It has been revealed that the N-terminal region of the molecule is widely extended, although other regions of the protein persist in original crumpled forms as for one of these that formed in 2 M guanidine hydrochloride.

Assured hemolytic *Streptococci* produce streptokinases which are proteins with plasminogen activator activity as studied by Nowicki *et al.* (1994). Equine streptokinases (ESKs) are produced from equine streptococcal isolates that bound both human and equine plasminogen but only voluntarily activated equine plasminogen. To cleanse a representative ESK produced by *Streptococcus equisimilis* strain 87-542-W, this property was exploited. A molecule with approximately 49,000 KDa molecular weight having two isoforms was secluded by using affinity chromatography with human plasminogen. Well characterized streptokinases (HSKs) that efficiently activate human plasminogen were subsequently compared with this ESK. In the highly conserved antigenic properties, peptide maps and amino-terminal amino acid sequence, variations in streptokinases were identified and DNA hybridization studies supported these differences. The family of proteins identified as streptokinase has much greater variety than formerly valued as indicated by the results.

For the expression of the pro fibrinolytic protein under the effect of a *tac* promoter, streptokinase (STK) gene of *Streptococcus equisimilis* was cloned in an expression vector of *Escherichia coli* by Avilan *et al.* (1997). Recuperation of all the recombinant STK to the periplasmic space was done after mild lysozyme digestion of induced cells. DEAE Sepharose chromatography followed by phenyl-agarose chromatography was performed on this periplasmic fraction. When a linear gradient was used, vigorous proteins eluted between 0 to 4.5% ammonium sulfate. Three major STK derivatives of 45 kDa, 47.5 kDa and 32 kDa were detected by western blot analysis by using a polyclonal antibody. As revealed by azymographic assay, when assayed with a chromogen-coupled substrate the 32-kDa protein created complex with human plasminogen but did not demonstrate Glu-plasminogen activator activity whereas a Km = 0.70 μM and kcat = 0.82 s⁻¹ was shown by 45-kDa protein. Perhaps consequential from fractional degradation during the export pathway or the purification steps, these proteins...
are recognized remains of STK as recommended by results. As revealed by peptide sequencing, the 47.5-kDa band corresponded to the native STK.

Microbial enzymes including one-domain (α) staphylokinases and three-domain (α, β, γ) streptokinases can stimulate mammalian protease plasminogen as studied by Johnsen et al. (2000). Proteolytic action of plasminogen of the host which eases microbial migration of the host organism is initiated by the resulting complexes of plasmin (ogen) cofactors. Kinetic method of the stimulation of plasminogen interceded by an innovative α and β domain streptokinase obtained from Streptococcus uberis (SkU) with specificity toward bovine plasminogen was investigated. Two steps occurred in the interaction between SkU and plasminogen: (1) quick association of the proteins and (2) gentle conversion to the active complex SkU−PgA. Plasmin is formed by the complex SkU−PgA from plasminogen with the given factors: Km ≤ 1.5 μM and kcat = 0.55 s1. The capability of proteolytic remains of SkU to stimulate plasminogen was studied. Only two C-terminal segments were shown to be active (97−261 and 123−261), which together comprise the β-domain (126−261). Useful resemblance to the staphylokinase was exhibited by the plasmin complexed with instigated plasminogen but not with plasminogen. In complex with plasmin, the blend protein His6−SkU (i.e., SkU with a small N-terminal tag) acted completely as well. These explanations validate that (1) the N-terminal α-domain, including a native N-terminus, was obligatory for “virgin” activation of the allied plasminogen in the SkU−PgA complex and (2) the C-terminal β-domain of SkU is imperative for gratitude of the substrate in the SkU−PgA complex.

Elevated expression systems assist recombinant protein refinement which makes better amounts of streptokinase protein as addition bodies as evaluated by Babu et al. (2008). The conditions are optimized to attain big amounts of streptokinase in the type of addition forms as the congregation of functional streptokinase is poisonous for the host cells. Yield and the solubility of wholesome protein are greatly reliant on a variety of blends of non-ionic and ionic and detergents, salts with solubilizing agents and chemical additives proceeded by remodeling of denatured protein into functional state. Careful complementary steps were required to extend under diverse well-ordered circumstances as subsequent remodeling step and denaturation is required for the drawing out of the purified streptokinase from inclusion forms. At this time the refined wreckages of remolded proteins were selected to indicate the circumstances that produce the vigorous streptokinase having instinctive conformation. By these methods, the
determined explicit enzyme activity of the streptokinase was attained. A purity of 99% was shown by RP-HPLC in the refolded recombinant streptokinase study. There are least collections in the dynamic streptokinase protein and the proportion of recovery is approximately 99% as shown by bulk segregation chromatography summary.

In this research work, hyper production of streptokinase was carried out by performing mutagenesis of wild *Streptococcus equisimilis* strain. In previous conducted studies, production of streptokinase was carried out from wild strains of group C hemolytic *Streptococci* but in present work mutagenesis was performed by using different physical and chemical means. Hyper production of streptokinase was attained by employing such techniques along with media optimization. This hyper production will help out to meet the demands of this clinically important enzyme by using cheap sources. There is great demand for reduction in production cost of this marvelous enzyme all over the world so that poor patients can also get advantage of treatment by this enzyme but this demand cannot be fulfilled without media optimization and mutagenesis of the bacterial strains. To meet the emerging elevated demands of this valuable enzyme, hyper production and advanced purification strategies are required. Present research work is totally based on mutagenesis, production and purification of streptokinase which resulted in valuable production of the enzyme along with the production of four mutant strains of *Streptococcus equisimilis*. This research work will help the poor people of this region in regard of easy availability of this marvelous enzyme. For the treatment of cardiovascular ailments.
Chapter # 3

MATERIALS AND METHODS

3.1. Chemicals and enzymes

All the enzymes and chemical reagents used in this research work were of analytical grade and this whole research work was carried out in Enzyme biotechnology laboratory (EBL), Department of Biochemistry, University of Agriculture, Faisalabad, Pakistan.

3.2. Microorganism

3.2.1. Isolation of beta hemolytic Streptococcal strain

The test organism used in this research work was *Streptococcus equisimilis* which was isolated from various indigenous blood and biomass sources including samples from patients suffering from sore throat, scarlet fever and acute tonsillitis. For the isolation of beta hemolytic *Streptococcus equisimilis*, patients were selected from Shafee medical Centre, Jinnah colony, Faisalabad. Total 10 samples including blood and throat swab samples were collected from patients having acute tonsillitis, sore throat and scarlet fever. Human and sheep blood agar plates were used for culturing of these samples. Direct streaking method was used and then these agar plates were incubated for 24 to 48 hours respectively at 37°C. Beta hemolytic *Streptococci* were distinguished from rest of other organisms on the basis of clear zones of hemolysis. Total 100 mL blood base agar was prepared and cooled, then 5 - 7% v/v defibrinated sheep blood and 5% v/v defibrinated human blood was added and then this media was poured into the petriplates. Simple streaking was done on the blood agar plates with throat swabs and blood samples from patients along with the control culture streaked by the same manner. All petriplates with test and control cultures were incubated at 37°C. After completion of stipulated time period, the colonies exhibiting clear zones of hemolysis were picked up and sub cultured into blood agar slants and again incubated. For the isolation of *Streptococcus equisimilis* strain, the samples which gave positive results for beta-hemolysis were subjected to Gram staining, morphological and biochemical characterization (Abdelghani *et al*., 2005; Shilpi *et al*., 2013).

Isolated strain was examined morphologically and taxonomically by Laboratory
Manual on Fundamental Principles of Bacteriology (Salle, 1948), Medical Microbiology (Cruickshank, 1968), Mackie and McCartney Practical Medical Microbiology (Collee et al., 1969), Bergey’s Manual of Determinative Bacteriology, (1974); Bergey’s Manual of Systematic Bacteriology, (1992) and District Laboratory Manual Practice in Tropical Countries (Monica, 2000). Pure culture of *Streptococcus equisimilis* (ATCC-H46A) was used as control for isolation of the right organism from indigenous blood and biomass samples. This pure culture was obtained from Department of Clinical Medicine and Surgery, Faculty of Veterinary Sciences, University of Agriculture, Faisalabad, Pakistan. Following biochemical tests were performed for the isolation of beta hemolytic *Streptococcus equisimilis* from blood and biomass samples.

### 3.2.1.1. Trehalose test:

#### 3.2.1.1.1. Principle

This test is basically used to check that whether the test organism can ferment the carbohydrate trehalose as a carbon source or not. Due to the production of acids as end products of trehalose fermentation, the pH of the medium drops. A changes Color of the pH indicator in the medium changes which indicate acid production. Numerous media are available for this purpose but phenol red trehalose broth is most commonly used. This is basically nutrient broth medium containing 0.5-1.0% trehalose. Phenol red is used as pH indicator which is red at neutral pH but changes to yellow at pH less than 6.8. When the pH is greater than 8.4, color change may be shown as magenta or hot pink.

#### 3.2.1.1.2. Procedure

Phenol red trehalose broth was used as medium and taken in caped tubes. Inoculation loop was sterilized on Bunsen burner. Caps from test tubes containing phenol red trehalose broth were removed and mouths of test tubes were heated on flame to avoid any contamination. Inoculum was picked aseptically with the help of loop from the culture plate of sample and immediately transferred into the fresh, sterile medium in tubes. Inoculating loop was re-heated again for sterilization. Same procedure was adopted for all remaining sample petri plates. Then inoculated tubes were placed in incubator at 35-37 °C for 24 hours.
3.2.1.3. Determination of test results

After completion of incubation time, incubated tubes were taken out from the incubator. Results were observed with the facts that if the trehalose was fermented, the culture will have changed to yellow in the presence of acids (indicating a positive test) or if the trehalose was not fermented, magenta or hot pink color was there due to the presence of bases/alkali (indicating a negative test). Results were recorded and cultures in tubes were disposed of to avoid any contamination. So by performing trehalose test, microorganisms were classified as trehalose negative and trehalose positive isolates (Abdelghani et al., 2005; Shilpi et al., 2013).

3.2.1.2. Haemolysis

Sample cultures obtained from indigenous sources were also analyzed to detect their capability of hemolysis. For this purpose sheep blood agar plates were used. Culture from all sample petri plates was aseptically transferred to blood agar plates separately. Then these blood agar petriplates were incubated at 37°C for 24 hours in incubator. After completion of stipulated time span, plates were taken out and keenly observed for hemolysis with naked eye and microscopically. Two types of hemolysis was observed as some petri plates showed complete clear zones known as beta hemolysis whereas few agar plates exhibited greenish incomplete discoloration named as alpha haemolysis (Abdelghani et al., 2005; Shilpi et al., 2013).

3.2.1.3. Gram staining

Most bacteria can be differentiated by the Gram staining reactions due to difference in their cell wall structure and classified as Gram positive bacteria having cell wall with large amount of peptidoglycan and Gram negative bacteria as they have less amount of peptidoglycan in their cell wall. Gram negative bacteria have lipopolysaccharide containing a compound known as lipid A or endotoxin. The organisms that retain purple color with crystal violet and are not decolorized by acetone iodine are called Gram positive bacteria. The organisms that drop their color of crystal violet after being treated with acetone iodine are called Gram negative bacteria (Abdelghani et al., 2005; Shilpi et al., 2013).
3.2.1.3.1. Procedure

Smears of all specimen cultures were fixed on glass slides either by heating or by alcohol fixation. Fixed smears were covered with crystal violet stain for 30 seconds and then washed off with water. Then Gram iodine was applied on smears for 30 seconds and washed off with water. De-colorization of smears was done by pouring acetone iodine (5 seconds) and rapid washing was done, then slides were covered with carbol fuschin for 30 seconds and it was also washed off with water. Slide were allowed to air dry and were observed under oil immersion lens. Results were noted as whether Gram positive or Gram negative along with the morphology of bacteria, whether cocci, bacilli and their arrangement. Use of old iodine should be avoided as old iodine is a poor mordant. By performing gram staining, isolates were classified into Gram positive and Gram negative categories (Abdelghani et al., 2005; Madhuri et al., 2011).

3.2.1.4. IMViC test

IMViC is an abbreviation that stands for four different tests including Indole test, Methyl red test, Voges-Proskauer test and Citrate utilization test. To obtain the results of these four tests, three test tubes were used for each test sample. Tryptone broth was used for indole test, MR-VP broth was used for methyl red and Voges Proskauer test and citrate was used for citrate utilization test. IMViC tests are employed for the identification/differentation of different bacteria (Abdelghani et al., 2005; Felsia et al., 2011; Madhuri et al., 2011).

3.2.1.4.1. Procedure for performing IMViC tests

Sample cultures were grown for 24 to 48 hours at 37°C and the respective tests were performed as followed:

3.2.1.4.2. Indole test

3.2.1.4.3. Principle

Indole is a compound formed after spiltting of amino acid tryptophan. So indole test is used to determine the ability of an organism to to form this compound. Tryptophanase cause hydrolysis of tryptophan which results in production of three possible end products, one of which is indole. Indole production is detected by Kovac’s or Ehrlich’s reagent which contains 4 (p)-dimethylamino benzaldehyde, this reacts with indole to produce a red colored compound.
Tryptophan broth is used to perform this test. Result was read after addition of Kovac’s reagent. The positive result was indicated by the red layer at the top of the tube after the addition of Kovács reagent whereas negative result was indicated by the lack of color change at the top of the tube after the addition of Kovács reagent. Indole test is a commonly used biochemical test which helps to differentiate *Enterobacteriaceae* and other genera.

### 3.2.1.4.4. Procedure

A conventional tube method requiring overnight incubation was used in this research work, which can identify even weak indole producing organisms. Isolated colonies of the sample cultures were emulsified in tryptophan broth separately for each sample and then incubated at 37°C for 24-28 hours in ambient air. 0.5 ml of Kovac’s reagent was added to the broth culture. Production of Pink colored ring after addition of appropriate reagent showed the positive result whereas no color change even after the addition of appropriate reagent was noted as negative. Most strains of *E.coli*, *P. vulgaris*, *M. morganii* and *Providenica* are indole positive. Indole test can also aid in species differentiation.

### 3.2.1.4.5. Methyl red test and Voges-Proskauer test

Both these tests were done in methyl red–Voges-Proskauer (MR-VP) broth, but the reagents added were different. Positive methyl red test was indicated by the development of red color after the addition of methyl red reagent whereas a negative methyl red test was indicated by no color change after the addition of methyl red reagent. Voges-Proskauer (VP) test was detected negative by lack of color change after the addition of Barritt’s A and Barritt’s B reagents. A positive Voges-Proskauer test was indicated by the development of red-brown color after the addition of Barritt’s A and Barritt’s B reagents.

### 3.2.1.4.6. Procedure

One tube of MR-VP broth was inoculated with fresh (18 to 24 hours) culture of the test sample. Transfer A light inoculum from an isolated colony was transferred and resuspended in the 5 ml MR-VP broth tube. As use of a heavy inoculum may result in aberrant results. Then all tubes containing inoculated broths of sample cultures were incubated at 37°C for 48 hours. Then 2.5 ml of culture was transferred into a new sterile culture tube and 5 drops of the methyl red reagent were added and results were noted (Holt, 1994; Abdelghani *et al.*, 2005).
3.2.1.4.7. Citrate utilization test:

Citrate utilization test was performed on Simmons citrate agar. Negative citrate utilization test was indicated by the lack of growth and color change in the tube whereas a positive citrate result was indicated by growth and a blue color change.

3.2.1.4.8. Principle

Citrate utilization test is commonly employed as part of a group of tests, the IMViC (Indole, Methyl Red, VP and Citrate) tests that distinguish between members of the Enterobacteriaceae family based on their metabolic by-products. Citrate utilization test is used to determine the ability of bacteria to utilize sodium citrate as its only carbon source and inorganic (NH$_4$H$_2$PO$_4$) is the sole nitrogen source. When an organic acid such as citrate (Remember Krebs cycle) is used as a carbon and energy source, alkaline carbonates and bicarbonates are produced ultimately. In addition, ammonium hydroxide is produced when the ammonium salts in the medium are used as the sole nitrogen source. The color change of the indicator is due to alkali production by the test organism as it grows on the medium. Growth usually results in the bromothymol blue indicator, turning from green to blue. The bromothymol blue pH indicator is a deep forest green at neutral pH. With an increase in medium pH to above 7.6, bromothymol blue changes to blue.

3.2.1.4.9. Procedure

Simmons citrate agar slants was inoculated lightly by touching the tip of a needle to a 18 to 24 hours old sample culture and then citrate agar slants were incubate at 35°C to 37°C for 18 to 24 hours. Some organisms may require up to 7 days of incubation due to their limited rate of growth on citrate medium. Development of blue color was observed which denotes alkalinization. In case of citrate positive organisms, growth was visible on the slant surface and the medium was an intense Prussian blue. The alkaline carbonates and bicarbonates produced as by-products of citrate catabolism raised the pH of the medium to above 7.6, causing the bromothymol blue to change from the original green color to blue. On the other hand for citrate negative samples, trace or no growth was visible. No color change occurred; the medium was remained deep forest green color of the un-inoculated agar. Only bacteria that can utilize citrate as the sole carbon and energy source will be able to grow on the Simmons
citrate medium, thus a citrate-negative test culture was virtually indistinguishable from an uninoculated slant.

3.2.1.5. Catalase test

Catalase is an enzyme, which is produced by microorganisms that live in oxygenated environments to neutralize toxic forms of oxygen metabolites; \( \text{H}_2\text{O}_2 \). The catalase enzyme neutralizes the bactericidal effects of hydrogen peroxide and protects them. Anaerobes generally lack the catalase enzyme (Abdelghani et al., 2005; Madhuri et al., 2011). Catalase mediates the breakdown of hydrogen peroxide \( \text{H}_2\text{O}_2 \) into oxygen and water. To find out if a particular bacterial isolate is able to produce catalase enzyme, small inoculums of bacterial isolate is mixed into hydrogen peroxide solution (3%) and the rapid elaboration of oxygen bubbles occurs. The lack of catalase is evident by a lack of or weak bubble production. Catalase-positive bacteria include strict aerobes as well as facultative anaerobes. They all have the ability to respire using oxygen as a terminal electron acceptor.

Catalase-negative bacteria may be anaerobes, or they may be facultative anaerobes that only ferment and do not respire using oxygen as a terminal electron acceptor (ie. Streptococci). The catalase test is primarily used to distinguish among Gram-positive cocci: Member of the genus *Staphylococcus* are catalase-positive, and members of the genera *Streptococcus* and *Enterococcus* are catalase-negative. Catalase test is used to differentiate aero-tolerant strains of *Clostridium*, which are catalase negative, from *Bacillus* species, which are positive. Semi quantitative catalase test is used for the identification of *Mycobacterium tuberculosis*. Catalase test can be used as an aid to the identification of *Enterobacteriaceae*. Members of *Enterobacteriaceae* family are catalase positive.

3.2.1.5.1. Procedure

Small amount of sample culture was transferred to a surface of clean, dry glass slide by using a loop or sterile wooden stick. A drop of 3% \( \text{H}_2\text{O}_2 \) was placed on to the slide and mixed. Rapid evolution of oxygen (within 5-10 sec.) as evidenced by bubbling was noted as positive result whereas a negative result was no bubbles or only a few scattered bubbles. All glass slides were disposed of after performing the complete procedure. Use of a metal loop or needle with \( \text{H}_2\text{O}_2 \) was avoided as it give a false positive result and degrade the metal. Care was taken in
using sample cultures from a blood agar plate, avoiding scraping up any of the blood agar as blood cells are catalase positive and any contaminating agar could give a false positive.

3.3. Selection of strain

The stock culture of the strain *Streptococcus equisimilis* used in the present study was maintained on Blood agar medium and the Todd Hewitt infusion Broth (Abdelghani *et al.*, 2005; Zia *et al.*, 2013). Pure culture was grown on THB medium for 24 hr. The composition of THB medium is presented in Table 3.1. After 24 hr, colonies were picked aseptically, transferred to the blood agar medium and incubated for 24 hr at 37 ºC. Composition of blood agar medium is given in Table 3.2. After 24 hr of the growth of the microbe on blood agar medium, the discrete colonies with clear hydrolysis zones were picked up, transferred into blood agar stabs and incubated for 24 hr at 37 ºC to obtain pure colonies. The microorganism, which formed clear zones after incubation (Felsia *et al.*, 2011), was selected, and subjected to strain improvement techniques along with extracellular streptokinase production by liquid state fermentation.

3.4. Strain improvement Techniques

The inoculum of *Streptococcus equisimilis* was prepared in phosphate buffer (pH 7), using 250mL Erlenmeyer flasks with working volume of 50mL in rotatory shaker operating at 120 rpm for 24hrs and temperature was adjusted at 37ºC (Abdelghani *et al.*, 2005; Carapito *et al.*, 2006).

3.4.1. Physical Mutagenesis

UV and gamma irradiation (Abdelghani *et al.*, 2005; Gromada and Fiedurek, 1997) were used to induce mutagenesis in *Streptococcus equisimilis* for hyperproduction of streptokinase. There are numerous advantages of ionizing radiations like gamma irradiation over chemical mutagens. Depending on the dosage time of radiation, such ionizing radiations infiltrate into tissues and cause the synthesis of various compounds. Some significant reducing and oxidizing species and molecular products like OH and H$_2$O$_2$ are formed respectively by the effect of gamma rays and water (Parker and Darbey, 1995). Chemicals like EMS have been reported having less mutagenic efficiency as compared to gamma rays (Dhulgande *et al.*, 2011). Kinase enzymes are indirect plasminogen activators (e.g. streptokinase) having no proteolytic role and
Table 3.1. Composition of Todd Hewitt Broth (THB) for culturing of *Streptococcus equisimilis*

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Quantity (g L⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beef extract</td>
<td>10</td>
</tr>
<tr>
<td>Tryptone</td>
<td>20</td>
</tr>
<tr>
<td>Dextrose</td>
<td>2</td>
</tr>
<tr>
<td>Sodium bicarbonate</td>
<td>2</td>
</tr>
<tr>
<td>NaCl</td>
<td>2</td>
</tr>
<tr>
<td>Disodium Phosphate Anhydride</td>
<td>0.4</td>
</tr>
</tbody>
</table>

pH 7.8, Temperature 30 ºC

The above mentioned contents were autoclaved at 121ºC for 15 minutes at 15 lbs, then medium was cooled and then it was used to culture *Streptococcus equisimilis*.

Table 3.2. Composition of blood agar medium for culturing of *Streptococcus equisimilis*

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Quantity (500mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infusion from beef heart</td>
<td>50 g</td>
</tr>
<tr>
<td>Tryptose</td>
<td>10 g</td>
</tr>
<tr>
<td>NaCl</td>
<td>5 g</td>
</tr>
<tr>
<td>Agar</td>
<td>15 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>500 ml</td>
</tr>
<tr>
<td>pH</td>
<td>7.4</td>
</tr>
</tbody>
</table>

The above mentioned contents were autoclaved at 121ºC for 15 minutes at 15 lbs, then medium was cooled to 45-50 ºC and aseptically added 50 mL of sterile defibrinated blood and mixed thoroughly and then poured into petriplates.
are chiefly produced by bacteria (Banerjee et al., 2004). To search for the mutants having better potential for streptokinase production, parent strain was subjected to mutagenesis by gamma irradiation.

3.4.1.1. Mutagenesis using gamma irradiation

*Streptococcus equisimilis* cell suspension (1x10^7 mL^-1) in a 24 hours phosphate buffer was transferred in 10 mL McCartney vials. 7 mL of cell suspension was transferred in each vial sealed with parafilm. These vials were exposed to gamma irradiation to Co\(^{60}\) source at Nuclear Institute of Agriculture and Biology (NIAB), Faisalabad, Pakistan.

For mutagenesis of *Streptococcus equisimilis* cell suspension, seven different doses of gamma radiation were selected. Which were 20, 40, 60, 80, 100, 120 and 140 K Rad. As dose depends on time i.e 1.25 K Rad hour\(^{-1}\) so 40 K Rad sample was collected on first day whereas 120 K Rad sample was collected on 4\(^{th}\) day (Weigel and Baggenstoss, 2012).

120 K Rad dose was selected as optimized dose for mutagenesis, as it gave 3log kill curve. It means that dose at which 1000 cells mL\(^{-1}\) of the cell suspension was killed due to the exposure of gamma radiation. As the log of 1000= 3, so it is called as 3 log kill curve. The kill/survival curve was prepared and time of exposure giving 3log kill was selected for mutagenesis of the *Streptococcus equisimilis* (Weigel and Baggenstoss, 2012).

3.4.1.2. Mutagenesis using UV lamp:

UV lamp (TUP 40w lamp which has about 90% of its radiation at 2540-2550 A\(^{0}\)) was used for the mutation of *Streptococcus equisimilis* cells (1x 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 52% 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 (Abdelghani et al., 2005; Sobrun et al., 2012).

3.4.2. Chemical mutagenesis

Sodium azide (Weigel and Baggenstoss, 2012; Eskil et al., 1998) and ethidium
bromoide (Sobrun et al., 2012) were used to induce mutagenesis in Streptococcus equisimilis for hyperproduction of streptokinase.

3.4.2.1. Mutagenesis using sodium azide

To prepare the stock solution, 0.0325 mg of sodium azide was dissolved in 10mL inoculum containing Streptococcus equisimilis and was placed in water bath at 37°C. Different time intervals were selected for chemical mutagenesis. After intervals of 30, 60, 90, 120, 150, 180, 210, 240 and 270 minutes, 1mL sample was withdrawn and centrifuged thrice for 15 minutes at 1000 rpm to remove the mutagen from cell suspension (Johnson et al., 1999). A dose (240 minutes) was selected as best dose by giving 82% killing rate.

3.4.2.2. Mutagenesis using ethidium bromide

A stock solution of 0.5 mg mL⁻¹ ethidium bromide was prepared and 1mL of this solution was added to 9 mL of inoculum medium containing cells of Streptococcus equisimilis (1 x 10⁷ cells mL⁻¹). After specific time intervals of 30, 60, 90, 120, 150, 180, 210, 240 and 270 minutes of incubation, 1mL sample was taken and it was centrifuged three times at 10,000 rpm for 15 minutes. A dose (240 minutes) producing 76% killing rate was selected as best dose.

3.4.3. Selection of Mutant

Following procedures/steps were adopted in the in the process to select the specific mutant having the ability to hyper produce streptokinase.

3.4.3.1. Selection by colony restrictor:

In order to restrict the formation of bacterial colonies, triton X-100 (1- 2% V/V) was used as colony restrictor (reference). Finally on the basis of results, triton X-100 was used in PDA medium obtaining the best results.

3.4.3.2. Selection of 3 log kill mutant dose by kill curve:

After treating inoculum with four diverse mutagens, mutated cells suspension was serially diluted 100 folds (each mutagen treated) to give approximately 30 or less colonies per plate. In a dark room, PDA media containing 1% triton X-100 as colony restrictor was inoculated by 0.1 mL. As control, non-irradiated inocculum was also plated. Strict aseptic
conditions were maintained in a laminar air flow to carry out all processes. Aluminum foil was used to cover the plates, placed in an incubator, set at 37°C for 2-4 days or till colony formation (different in each case). More than 1000 colonies were screened for selection of each mutant (each mutagen treated) and few mutants were isolated on PDA plates to study their enzyme activities. The best mutant was selected from a number of variants (Abdelghani et al., 2005; Madhuri et al., 2011).

3.5. Calculation of colony forming units (C.F.U. mL⁻¹)

The colony forming units were calculated as follows:

\[
\text{C.F.U mL}^{-1} = \frac{\text{Number of colonies on agar plate}}{\text{Amount plated (0.1)}} \times \frac{1}{\text{Dilution factor}}
\]

3.6. Screening procedures

3.6.1. Plate screening method

The basal medium used for selection of mutant was nutrient agar supplemented with 10% blood and 1% triton X-100 as colony restrictor. After 1-2 days of incubation at 37°C, the size of clearing zones was determined. The colonies showing bigger zone were further sub-cultured. A few colonies were obtained showing large clearance zones than wild type (El-Mongy and Taha, 2012).

3.7. Identification of mutant

For the identification of specific mutant of *Streptococcus equisimilis* following two tests were performed:

3.7.1. Enzyme diffusion zone test:

Screenings of fibrinolytic enzymes was done using nutrient agar medium containing 2% casein and 2 ml human serum. Streptokinase positive strain was identified on agar plate containing defibrinized blood. If streptokinase is formed, then haemolysis will be there giving rise to clear zones (Dubey et al., 2011).
3.7.2. Analytical test

The larger zone producing strains were scratched, dissolved and homogenized in buffer, filtered and then the reaction for enzyme activity by enzyme assay was determined quantitatively (Dubey et al., 2011; Felsia et al., 2011).

3.8. Production of streptokinase

3.8.1. Preparation of Inoculum

The selected strain was examined accurately by cultivation in blood agar medium and the pH of the medium was maintained at 7 before sterilization. Inoculum (50ml) was prepared in 250 ml flask in phosphate buffer (Abdelghani et al., 2005). The composition of phosphate buffer is given in table 3.3. The phosphate buffer was autoclaved for 15 min at 121°C and 15 lbs pressure. Then a loop full culture of *Streptococcus equisimilis* was transferred aseptically into the flask and incubated on an orbital shaker (120 rpm) for 24 hrs at 37°C (Carapito et al., 2006; Dubey et al., 2011).

3.8.2. Production of Enzyme in liquid state fermentation

In liquid state fermentation, abundantly available agricultural waste corn steep liquor was used as a substrate and other pre-optimized conditions were followed in order to analyze the streptokinase activity. The temperature was adjusted at 37°C and pH at 7 for 24 hrs of incubation on orbital shaker at 120 rpm. Abundantly available agricultural waste, corn steep liquor (CSL) 5% (w/v) was used as an economical substrate along with glucose 5%, yeast extract 5%, KH₂PO₄ 0.05 %, K₂HPO₄ 0.05 and MgSO₄.7H₂O 0.04%, NaHCO₃ 0.1%, CH₃COONa.3H₂O 0.1%, FeSO₄. 7H₂O 0.04%, MnCl₂. 4H₂O 0.02% and CaCO₃ 0.05% to achieve higher streptokinase yield by (LSF). These contents were autoclaved for 15 min at 121°C and 15 lbs pressure. Then 5% inoculum was added aseptically in each flask (triplicate was used) for incubation in shaker at 37°C and 120 rpm for 24 hrs (Baewald et al., 1975; Felsia et al., 2011).

3.8.3. Optimization of conditions for streptokinase production

Various parameters were optimized with desired strain, as to obtain the maximum yield of streptokinase. The parameters were; substrate concentration (1,2,3,4,5,6,7,8,9,10 %), fermentation period (12, 24, 36, 48, 60, 72 hrs.) pH (4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5, 9, 9.5,
10), temperature (27, 37, 45, 52, 60°C), glucose (1, 2, 3, 4, 5, 6, 7, 8, 9 and 10%), yeast extract (1, 2, 3, 4 and 5%), KH$_2$PO$_4$ (0.01%, 0.02%, 0.03%, 0.04%, 0.05%, 0.06% and 0.07%), K$_2$HPO$_4$ (0.05, 0.25, 0.5, 1 and 1.5%), MgSO$_4$.7H$_2$O (0.02, 0.04, 0.06, 0.08 and 0.1), NaHCO$_3$ (0.1, 0.15, 0.2, 0.25, 0.45), CaCO$_3$ (0.001, 0.002, 0.003, 0.004, 0.005, 0.006 and 0.007%), CH$_3$COONa.3H$_2$O (0.05, 0.1, 0.15, 0.2, 0.25), FeSO$_4$. 7H$_2$O (0.02, 0.04, 0.06, 0.08, 0.1), MnCl$_2$. 4H$_2$O (0.02, 0.04, 0.06, 0.08, 0.1) and inoculum size (2.5, 5, 7.5 and 10%). After each step of optimization of media for maximum production of streptokinase, it was compared with non-optimized conditions and hence showed highly significant results at optimized conditions (Patel et al., 2011; El-Mongy and Taha, 2012).

Table 3.3. Composition of Phosphate Buffer

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Quantity (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>K$_2$HPO$_4$ (salt)</td>
<td>16.2</td>
</tr>
<tr>
<td>KH$_2$PO$_4$ (acid)</td>
<td>9.79</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000ml</td>
</tr>
</tbody>
</table>

pH of the medium was maintained at 7 and the buffer was autoclaved for 15 min at 121°C and 15 lbs.

3.8.4. Harvesting of Sample

Enzyme was harvested from liquid state fermentation by centrifugation at 10,000 rpm for 20 min at 0°C and then it was filtered. After filtration residue was stored at 20°C and supernatant was assayed for enzyme activity (Dubey et al., 2011).

3.9. Enzyme assay

For the determination of enzyme activity, method of Parsad et al. (2006) was followed with slight modification. In present work, the streptokinase activity was determined by using the above mentioned method. One unit (IU) of the enzyme activity was defined as the Amount
of streptokinase causing the lysis of standard fibrin clot at pH 7.4 temperature 37°C within 90 min is termed as one unit (IU) of enzyme.

3.9.1 Development of an in vitro model to study clot lysis activity of indigenously produced streptokinase

In a pre-washed sterile microcentrifuge tubes, whole blood from healthy individuals was allowed to clot then serum was removed and clot was weighed (Parsad et al., 2006). The commercially available lyophylized streptokinase vial (1500000, 750000, 250000, 500 IU) each in 5 ml phosphate buffered saline (PBS) was added and mixed properly. These suspensions were used as stock from which appropriate dilutions were made to observe the thromolytic activity and used as a standard and compared the activity of indigenously produced streptokinase.

3.9.1.1. Procedure

Venous blood drawn from healthy volunteers was transferred to pre-weighed different sterile microcentrifuge tube (500µL) and incubated at 37°C for 60 minutes. After clot formation, serum was completely removed and each tube having clot was again weighed to determine the clot weight (Wt. of clot = Wt. of clot containing tube – Wt. of tube alone). Each tube was properly labelled and 200 µL of streptokinase along with various dilutions was added to the tubes. Water was added in one of the tubes containing clot as control. All the tubes were then incubated at 37°C for 90 minutes and observed for clot lysis. After incubation, fluid obtained was removed and tubes were again weighed to observe difference in weight after clot disruption. Difference obtained in weight taken before and after clot lysis was expressed as percentage of clot lysis. Test was repeated thrice with all the dilutions of the streptokinase in blood samples of different healthy volunteers.

3.10. Estimation of protein contents

Biuret method (Gornall et al., 1949; Slater, 1986) was applied for the estimation of protein contents.
3.10.1 Preparation of Biuret Reagent:

Both 12g of sodium potassium tartrate tetrahydrate and 3g of copper sulfate pentahydrate were dissolved in distilled water (500ml). 10% NaOH solution was added in 300ml of this solution and then distilled water was added to a final concentration of 1000ml.

3.10.2 Procedure:

For preparation of standard curve, bovine serum albumin was used with concentrations of 0.1, 0.2, 0.3, 0.4 and 0.5 mg/ml. One ml volume of standard dilution was mixed with 1ml of Biuret reagent and incubated for 15 minutes at 37°C. Optical density was recorded on spectrophotometer at 540nm and then standard curve was plotted between concentrations (mg/mL) and absorbance. Same procedure was repeated for the test solution and then protein contents were calculated.

3.11. Streptokinase Purification

Purification of crude extracellular streptokinase was carried out by ammonium sulfate precipitation, ion exchange chromatography and gel permeation chromatography (Carapito et al., 2006; Babashamsi et al., 2009; Dubey et al., 2011).

3.11.1 Ammonium sulfate precipitation technique

Crude enzyme was subjected to ammonium sulfate precipitation technique as first step of purification (Katherine et al., 2011).

3.11.1.1. Salting in of other proteins

Crude enzyme extract was saturated at 40% level by adding 28g of solid ammonium sulfate salt in 100ml of enzyme extract. Centrifugation was done at 10,000 rpm at 4°C for 15 min after placing this extract at 4°C for 4hrs. Sediments and supernatant are separated from each other.

3.11.1.2. Salting out of streptokinase

The supernatant of above step was saturated by adding 14 g salt and 60% ammonium sulfate saturation level was maintained. This extract was centrifuged at 10,000 rpm for 15 min, after 4hrs of incubation at 4°C. The supernatant was separated from the sediment, and then sediment was re-dissolved in minimum amount of distilled water.
3.11.1.3. Desalting of streptokinase

Against continuous stirring distilled water, this re-dissolved sample was dialyzed in the dialysis bag for 6 hrs. Enzyme assay was performed for all the fractions i.e. supernatant, sediment and desalted samples.

3.11.2 Purification by ion exchange chromatography

After ammonium sulfate precipitation, the enzyme was further purified by ion exchange chromatography (Carapito et al., 2006; Felsia et al., 2011).

3.11.2.1. Preparation of 0.5 N HCl

For preparation of 0.5 N HCl, 41.5mL of HCl (37%) was added to distilled water and volume was made upto 1000mL.

3.11.2.2. Preparation 0.5 N NaOH

For preparation of 0.5 N NaOH, 20g of NaOH was dissolved in 1000 mL distilled water.

3.11.2.3. Ion Exchange Column Preparation

Method of Ken and Isabelle (2012) was used for the preparation of DEAE (Diethyl amino ethyl) cellulose column. For preparation of slurry, the resin was gradually added to 0.1 M phosphate buffer (pH 7) and was heated at 95°C in water bath for 5 hrs. This whole process was carried out with the care that the slurry should not dry. To fill the outlet tube, buffer was passed through the column and slurry was poured into this column of 2x25 cm specifications. Column was left for 24hrs on levelled place for its stabilization. The buffer was removed from the column after opening the outlet tube and closed when just small amount of buffer was left on top of the column.

3.11.2.4. Washing the column with base

Washing of column was done by 50 mL of 0.5M NaOH, which was allowed to flow throughout the column. Distilled water was added to column after the complete removal of base and free flow of water was carried out until the pH of eluent was setteled at 7.
3.11.2.5. Washing the column with acid

After washing the column with base, 50 mL of 0.5 N HCl was poured on the column. It was allowed to flow throughout the column and then distilled water was passed until the eluent pH was settled at 7.

3.11.2.6. Equilibration of column

After washing with base and acid, equilibration of column was done with phosphate buffer (pH 7). It was achieved by washing the column continuously with buffer, so that the pH of eluent should be the same as that of buffer.

3.11.2.7. Application of sample

The buffer already present at the surface of column was allowed to flow by opening the outlet tube until a small amount of buffer was persisted at the top of the column. The desalted enzyme sample (1.5 mL) was poured on the surface of column by using micropipette. As sample penetrated into column bed, the outlet tube was opened carried out with Phosphate buffer (0.1 M, pH 7) was used for elution of sample. The drop rate of eluted sample was kept constant and 100 fractions of 2 mL each were collected. All these fractions were subjected to protein estimation and enzyme assay.

3.11.3 Gel filtration chromatography:

Method of Sukhacheva et al. (2004) and Erickson (2009) was adopted for the preparation of sephadex G-150 (Pharmacia) column.

3.11.3.1. Swelling of the resin

For preparation of gel filtration column, 1g dry sephadex G-150 was dissolved into 15 mL of phosphate buffer. It was heated in a water bath for 3 hour at 95°C (without drying the slurry).

3.11.3.2. Filling of column

For leveling of the column, it was placed vertically on stable stand. To fill the empty outlet tube, distilled water was added to column. To completely fill the column, the slurry was poured. When column was left undisturbed, distinct layers of water and gel appeared after some hours.
3.11.3.3. Application of sample

Distilled water present in column was removed by opening the outlet tube until a small layer on the top of column. Sample having maximum activity (obtained after ion exchange chromatography) was applied on gel filtration column and outlet was kept opened. The sample was allowed to penetrate in packed column. Elution was carried out by 0.1 M phosphate buffer pH 7 at a constant drop rate. A total 100 fractions of 2ml each were collected which were then subjected to enzyme assay and protein estimation.

3.11.3.4. 1 M phosphate Buffer preparation:

For preparation of 1 M phosphate Buffer, 9.79g KH$_2$PO$_4$ (acid) and 16.2g K$_2$HPO$_4$ (salt) were dissolved in 1000ml distilled water. pH of buffer was adjusted to 7 and molarity was kept 1. The pH of the buffer was adjusted at 7 with the help of 0.1 N HCl and 0.1 N NaOH.

3.11.4. Sodium Dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) is a technique for separating proteins based on their ability to move within an electrical current, which is a function of the length of their polypeptide chains or of their molecular weight. This is achieved by adding SDS detergent to remove secondary and tertiary protein structures and to maintain the proteins as polypeptide chains. The SDS coats the proteins, mostly proportional to their molecular weight, and confers the same negative electrical charge across all proteins in the sample (Sambrook et al., 1989). To analyze the homogeneity and purity of the enzyme, Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE; 10%) of different SK preparations was done as described by Hermentin et al. (2005), Reza et al. (2007) and Couto et al. (2004). To detect the small quantity of protein ranging from 1ng to 1 mg, silver staining was done. Polyacrylamide gel electrophoresis (PAGE) was used for separating proteins ranging in size from 5 to 2,000 kDa due to the uniform pore size provided by the polyacrylamide gel. By controlling the concentrations of acrylamide and bis-acrylamide powder used in creating a gel, Pore size of gel is controlled.

3.11.4.1. Stock solutions

1. 30% (w/v) acrylamide + 0.8% (w/v) bis-acrylamide
2. 1.5 M Tris/HCl pH 8.8 + 0.3 % (w/v) SDS
3. 0.5 M Tris/HCl pH 6.8 + 0.4 % (w/v) SDS

3.11.4.2. Preparation of resolving gel

For preparation of resolving gel, the following reagents were mixed together in a 250 mL Buckner flask and degassed by a vacuum pump for 5 minutes.

1. Stock solution-1 = 13.3 mL
2. Stock solution-2 = 10.0 mL
3. Distilled water = 16.7 mL

After degassing, the following reagents were added to initiate polymerization.

4. 2% (w/v) aqueous ammonium = 133 μL
   Persulfate (APS) freshly prepared
5. TEMED = 27 μL

By pouring the above mentioned mixture into gel apparatus which was assembled by sandwiching 2 spacers between two glass plates (10 cm x 8 cm x 1.5 cm), the resolving gels were prepared. To get even surface, 1-butanol was layered on the top of gel after polymerization. Then top of the gel surface was washed many times with distilled water after removal of 1-butanol.

3.11.4.3. Preparation of stacking gel

To concentrate the protein samples into a sharp band before their entry into the resolving gel is the main function of stacking gel. The pour size of stacking gel was large than the resolving gel. By using standard procedure of Laemmli, (1970) and Sambrook et al. (1989), 4% acrylamide stacking gel was prepared for streptokinase. Following reagents were mixed together in a 250 mL Buckner flask and degassed for 5 min.

1. Stock solution-1 = 1.5 mL
2. Stock solution-3 = 2.5 mL
3. Distilled water = 6.0 mL
4. 2% (w/v) aqueous ammonium= 100 µL

Persulfate (APS) freshly prepared

5. TEMED = 10 µL

The stacking gel mixture was then poured on the top of polymerized resolving gel. The stacking gel was allowed to polymerize after immediate insertion of comb (well maker).

3.11.4.4. Sample buffer

The following reagents were mixed to prepare sample buffer.

1. 0.75 M Tris/HCl buffer pH 6.5 = 200 µL
2. Distilled H2O = 6.3 mL
3. Glycerol = 2.5 mL
4. B-mercaptoethanol = 5% (v/v)
5. 10% w/v aqueous SDS = 1 mL
6. Bromophenol blue = 2.5 mg

On heating in the presence of SDS, non-covalently attached sub-units of proteins dissociated into monomers. If any S-S bridges between the subunits is present, B-mercaptoethanol was mixed to break them.

3.11.4.5. Stock electrode buffer

To prepare stock electrode buffer, following reagents were mixed and this stock solution was diluted 10 fold in distilled water just before use.

1. Tris base = 30 g
2. Glycine = 144g
3. SDS = 10g
4. Distilled water = 1L

3.11.4.6. Preparation of streptokinase for SDS-PAGE

Streptokinase (10 mg mL⁻¹) was mixed in SDS sample buffer (4X) and boiled for 4min.
3.11.4.7. Preparation of protein markers ladder for SDS-PAGE

For SDS-PAGE, protein markers ladder was used as standard. The ladder consisted of 4 bands ranging from 24-66 KD. The protein ladder was added in gel loading buffer (50% glycerol, 2% SDS, 30 mM NaCl, 1 mM NaN₃, 62.5 mM Tris/HCl pH 7, 0.01% bromophenol blue and 50 mM DTT) and applied directly to SDS-polyacrylamide gel after slight warming.

3.11.4.8. Running of PAGE

SDS gel was run at a constant voltage of 100 volts. The PAGE was stopped when tracking dye reached at the bottom of gel.

3.11.4.9. Protein staining procedure of SDS-Polyacrylamide gels:

To remove SDS, the gel was treated with 20% (v/v) isopropyl alcohol in 50 mM sodium acetate buffer (pH 5) and then washed thrice for 15 min. To remove isopropyl alcohol, the gel was immersed in 50 mM sodium acetate buffer (pH 5). Coomassie brilliant blue was used for final staining of polyacrylamide gels (Hermentin et al., 2005). For this purpose 0.025% coomassie blue was dissolved in 10% acetic acid for at least 2 hours. Then destained with 10% acetic acid for 2 hours (Couto et al., 2005). Protein bands were clearly visible after 10 minutes when gel was dipped in stain. After staining, gel was washed with distilled water to improve the visualization of bands.

3.12. Kinetic and Thermodynamic parameters:

To check the maximum activity of streptokinase, different kinetic and thermodynamic parameters were performed (Carapito et al., 2006; Dubey et al., 2011).

3.12.1. Optimum pH:

The enzyme activity of purified streptokinase derived from parent and mutant Streptococcus equisimilis was studied at different pH ranging from 4-8 to seek the optimum pH (Dubey et al., 2011).

3.12.2. Optimum Temperature

The purified parent and hyper produced streptokinase was assayed at different temperatures (20°C to 80°C) at optimized pH of 7. The activity of enzyme was checked by enzyme assay method (El-Mongy and Taha, 2012).
3.12.3. Substrate concentration:

Enzyme activity of purified streptokinase was checked at different substrate concentration such as 0.80, 1.00, 1.20, 1.40, 1.60, 1.80, 2.00, 2.20 and 2.40 µg of fibrin. The streptokinase activity was calculated by enzyme assay method (Yazdani and Mukherjee, 1998).

3.12.4. Activation energy (\(E_a\))

Activation energy was determined by assaying Streptokinase at various temperatures ranging from 20-70°C. The data was plotted according to Arrhenius as described by Rajoka et al. (2006).

3.12.5. Determination of kinetic constants of Michaelis-Menten Equation:

The Michaelis-Menten kinetic constants (\(V_{\text{max}}\) and \(K_m\)), catalytic constant (\(K_{\text{cat}}\)) and specificity constant (\(K_{\text{cat}}/ K_m\)) were determined by using blood as substrate. Streptokinase activity was determined in each fibrinogen (0.48-1.19 µg mL\(^{-1}\)) concentration keeping enzyme concentration (0.022 µ mole) constant. The values of \(V_{\text{max}}\) and \(K_m\) were calculated by Lineweaver – Burk plot (David et al., 2002).

3.12.6. Irreversible thermal denaturation

Irreversible thermal denaturation of SK was determined by incubating enzyme in phosphate buffer (pH 7) at different range of temperatures (40-80°C). Time course aliquots were withdrawn, cooled in ice for 30 minutes and then assayed for enzyme activity at 37 °C. This procedure was repeated at five different temperatures (40, 45, 50, 55, 60, 65, 70, 75 and 80 °C). The data was fitted to first order plots and analyzed.

3.12.7. Activation Energy of thermal denaturation

The first order rate constants for irreversible thermal denaturation (\(K_d\)) of SK were determined and Arrhenius plot was applied to determine the activation energy for denaturation (\(E_a\)).

3.12.8. Thermodynamics of irreversible thermal inactivation

The thermodynamic parameters for thermostability were calculated by rearranging the Erying’s absolute rate. Equation derived from the transition state theory.

\[
K_d = (K_B/h) \ e ^ {(-\Delta H^*/RT)} \ e ^ {(-\Delta S^*/R)} \ldots...\ldots\ldots(1)
\]
Where,

\[ h = \text{Plank's constant} = 6.63 \times 10^{-34} \text{ Js} \]

\[ K_B = \text{Boltzman constant (R/N)} = 1.38 \times 10^{-23} \text{ JK}^{-1} \]

\[ R = \text{Gas constant} = 8.314 \text{ JK}^{-1} \text{ mol}^{-1} \]

\[ N = \text{Avogadro’s No} = 6.02 \times 10^{23} \]

\[ T = \text{Absolute temperature} \]

\[ \Delta H^* = E_a - RT \]  

(2)

Where,

\[ \Delta H^* = \text{Enthalpy of activation of denaturation} \]

\[ E_a = \text{Activation energy for denaturation} \]

\[ \Delta G^* = -RT \ln (K_d, h/K_B, T) \]  

(3)

Where, \( \Delta G^* = \text{free energy of activation of denaturation} \)

\[ \Delta S^* = (\Delta H^* - \Delta G^*) / T \]  

(4)

Where, \( \Delta S^* = \text{Entropy of activation of denaturation} \)

3.13. Statistical Analysis:

Data obtained was analyzed by using two factor completely randomized design for assessment of means and standard error of means (Steel et al., 1997). Graphs of all recorded data were made on MS Excel and Slide Write Software.
Chapter # 4

RESULTS AND DISCUSSION

Streptokinase is one of the most important enzymes produced by certain Streptococci strains. This enzyme is used as a treatment for stroke and myocardial infarction. Decreasing the production costs of this enzyme is a strong demand for many users all over the world as it will make this effective treatment for such dangerous diseases in the reach of poor patients. Mutagenesis for hyper production of SK and optimization of the production process is the first step toward decreasing the production costs. In this work I isolated the beta haemolytic bacterial strain S. equisimilis, and used it for streptokinase production through liquid state fermentation. The isolated strain was grown and cultured on Todd Hewit Broth and blood agar medium.

Bacterial protein streptokinase is in wide use for the treatment of cardiovascular diseases and is graded as former method of treatment. In clinical practice, the local streptokinase is more applied and useful for cheap and rate effective treatment of thrombosis. As compared to recombinant process, mutagenesis by means of chemical and physical agents is more effective way to produce streptokinase as this result in production of safe and active streptokinase. Via bacterial fermentation, enormous amounts of streptokinase can be produced economically as on the other hand most of the active part separates out and decreases the enzyme activity in case of recombinant process. The objective of present research was to induce mutagenesis in indigenous strain of Streptococcus equisimilis by using physical and chemical agents and then to produce streptokinase by these physically and chemically mutant derived Streptococcus equisimilis strains. Finally partial purification of SK was carried out by ammonium sulfate precipitation method and additionaly purified by ion exchange chromatography and gel filtration chromatography. Different kinetic and thermodynamic parameters were applied to check the maximum activity of enzyme.

4.1. Microorganism

Streptococcus equisimilis is facultative anaerobic and Gram positive coccus shaped and bacteria. Clarke, 1924 first described the term Streptococci. Microscopic representation of Streptococcus is shown in Fig 4.1.
Several organisms produce streptokinase, but *Streptococcus faecalis* was used for industrial level streptokinase production. Circulation to heart muscle is blocked due to development of blood clots in case of acute myocardial infarction. For cure of acute myocardial infarction, numerous blood clot dissolving agents including tissue plasminogen activator and streptokinase are in use whereas uPA (urokinase plasminogen activator) belongs to other fibrinolytic agents group. In treating myocardial infarction, streptokinase is more effective than tissue plasminogen activator. Streptokinase was the treatment of choice for therapy of thrombosis as recommended by proportional clinical trials (Mucklow, 1995; Gillis and Goa, 1996).

Billroth in 1874 first discovered *Streptococci* in samples taken from infected lesions and later they discovered the Streptokinase production by same organisms. Later on same microorganisms were found in blood of scarlet fever patients. These *Streptococcal* variants are grouped into beta, alpha, and gamma variants as classified on diverse kinds of haemolytic responses showed on blood agar media plates. To further discriminate the Beta haemolytic Streptococci into groups A to O, in 1933, lancefield used serological grouping (Lancefield, 1933). Alpha haemolytic *Streptococci* of Lancefield groups G, C and A. Organisms from Group C are chosen for streptokinase production as they donot produce erthrogenic toxins so most of the streptokinases are obtained from these organisms. Ovoid cells set in pairs or chains are *Streptococci*. *Streptococci* are Gram positive, non-sporing, and catalase negative organisms which sometimes show motility. Some have need of addition of CO₂ for growth, most of them grow in air but all are facultative anaerobes. Some species are pathogenic; some are saprophytic along with the fact that many Streptococcal species are parasitic to animals or man.

Generally group a *Streptococci* are deliberated to be nutritionally meticulous bacteria. Early condiments which were thought to be required for growth of these bacteria involve serum, whole blood and other tissue fluids. Later it was testified that for growth in a casein hydrolysate basal medium, *Streptococcus equisimilis* necessitate a factor consequently designated as strepogenin. Along with making of lactic acid, all Streptococcal species flop to decrease nitrate by using glucose. *Streptococcus equisimilis* H46A is less demanding in its development necessities as equated to other strains. Multifaceted and rich media enhanced with a variety of nutritional factors is mandatory for growth of Group A haemolytic *Streptococci* (Bernheimer *et al.*, 1942; Stuebner *et al.*, 1991).
4.1.1. Isolation and screening of beta haemolytic *Streptococci* with potential of Streptokinase production

Isolation of *Streptococci* from specimens of pathological material is ordinarily not difficult. An enriched medium is required and for this purpose blood agar is medium of choice for both alpha and beta haemolytic *Streptococci*. Most specimens such as throat swabs and pus may be streaked directly on blood agar for isolation of haemolytic *Streptococci*. The colonial morphology is typical in the case of beta hemolytic *Streptococci* (Bergan *et al*., 1978). These beta haemolytic *Streptococci* are responsible for an acute infection of the throat commonly known as septic or streptococcus sore throat. So for the isolation of *Streptococcus equisimilis* strain in the present research work, samples were taken from the patients suffering from sore throat, scarlet fever and acute tonsillitis. For the isolation of haemolytic *Streptococci*, specimens from infected throat can be an excellent source (Abdelghani *et al*., 2005). Some *Streptococcal* strains showed alpha haemolysis on blood agar plates which was depicted as green discoloration zones while on other haem beta haemolysis was shown as formation of clear zones on blood agar (Collier *et al*., 1998). Proper identification and characterization of microorganisms is a very important because it expands the scope for exploitation of industrially important products. To establish the novelty or otherwise of the present isolates with those of reported in the literature, various morphological, cultural and biochemical characteristics of the isolated organisms were compared with the descriptions of the numerous *Streptococcus* species cited in the literature. The literature survey includes Bergey's Manual of Systematic Bacteriology (1992), Bergey's Manual of Determinative Bacteriology (1974), Mackie and McCartney Practical Medical Microbiology (Collee *et al*., 1969), District Laboratory Practice in Tropical Countries (Monica, 2000), Medical Microbiology (Cruickshank, 1968) and Laboratory Manual on Fundamental Principles of Bacteriology (Salle, 1948) Biological and Microbiological abstracts and all other relevant journals (Abdelghani *et al*., 2005).

The selected isolate was identified based on its morphological and biochemical characteristics. The morphological characterization involved culturing the isolate on nutrient agar plates for studying the appearance of the colonies following which Gram’s staining was performed. The biochemical characterization of the isolate was based on the results of indole test, methyl red test, Voges Proskauer test, citrate utilization test, trehalose test, haemolysis and catalase test (Shilpi *et al*., 2013). The biochemical, morphological and metabolic
characteristics of all isolates were compared with that of the reference culture. The reference cultures used in this research study was: *Streptococcus equisimilis* ATCC H46A strain, a group C *Streptococci* procured from Department of Clinical Medicine and Surgery, Faculty of Veterinary Sciences, University of Agriculture, Faisalabad, Pakistan. The physiological and biochemical properties, sugar fermentation tests of all samples along with reference culture is are shown in Table 4.1a. A detailed survey of the literature indicated that our isolates EBL-6 and EBL-10 belongs to group C *Streptococci* (Figure 4.6a, 4.6b and 4.6c). The data indicated that our isolates are closure to *S. equisimilis* as strict comparison with reference culture was adopted in aspects of morphology and biochemical tests. A detailed comparison of our isolate was made to establish its novelty or otherwise. However, some significant qualitative and quantitative differences could be noticed. Our isolates EBL-6 and EBL-10 differed from the reference culture in the following aspects: Good growth was observed for our isolates EBL-6 and EBL-10 on sodium azide and crystal violet blood agar media while moderate growth was observed for the reference culture on the same media. Reference culture produced acid from lactose and ribose while our isolate did not produce. In view of these significant differences, it is proper to consider our isolates EBL-6 and EBL-10 as new variant of *S. equisimilis*. The thrombolytic potential of these particular isolates indicated that they may also be utilized for the large scale production of streptokinase.

In present study, total of 10 samples were taken from various indigenous blood and biomass sources. Out of these 10 isolates, six isolated exhibited alpha- hemolysis while four isolates showed beta- hemolysis on blood agar plates in comparison to the reference culture. Alpha- haemolysis gave greenish zone around the colonies, while in beta- hemolysis clear zones were observed. In beta- hemolysis, other than *Streptococcus* spp, *Staphylococcus* spp. May also be present (Madhuri et al., 2011). When Gram staining was carried out, *Streptococci* gave Gram positive result with long chains of cocci, while *Staphylococci* gave Gram positive result with clusters of cocci. To further distinguish between *Streptococcus* and *Staphylococcus* species, the samples were inoculated on mannitol salt agar, only *Staphylococcus* species grows on mannitol salt agar as it is a selective media for *Staphylococcus*. Catalase test was also performed, only *Staphylococcus* spp. gave effervescence (positive result). All the positive results indicated that they were *Staphylococci* spp.
4.2. Strain improvement

Screening for novel streptokinase producing organisms is encouraged due to the growing potential of SK application in various fields of biomedical sciences. Extension in both quantifiable improvement and qualitative perfection is the emerging demand of the growing surge for the use of SK in numerous fields in the past few years. For the overproduction of the enzyme, quantitative enhancement requires optimization of medium and strain improvement as the wild strains produce generally too small amounts of the enzyme. Widespread use of mutation and selection is accredited to the enormous achievement examples of strain development in industry. To improve SK productivity, an attempt was made in the present work to subject the streptokinase producing strain *Streptococcus equisimilis* to strain improvement by random mutagenesis (Chemical and physical mutagens) (Madhuri *et al*., 2011; Weigel and Baggenstoss, 2012).

In the profitable development of microbial fermentation processes, industrial strain enhancement plays a dominant role. Physical and chemical mutagens induce mutations which
occur more commonly in some sites than probable and they are also non-randomly dispersed within a categorization instigating mutagen specific configurations of mutations. In latest new processes such as balanced selection and genetic engineering have activated to make a substantial involvement to this action but selection and mutagenesis termed as “random screening” is still a cheap technique and for dependable short term strain advancement is normally the optional technique (Rowlands, 1984). While numerous microorganisms have been testified to produce streptokinase, however, *Streptococcus equisimilis* is the main organisms being used for production of SK at industrial level. Screening, enhancement and assessment of novel streptokinase hyper producing strains is very significant in refining the competence and economics of the industrial scale procedure. Numerous attempts have been tried to advance streptokinase production from *Streptococcus equisimilis* by selection of strain by using haphazard/classical mutagenesis and screening practices, optimizing of fermentation parameters and genetic engineering (Abdelghani *et al.*, 2005).

In these studies the tenacity of mutagenesis was the selection of those *Streptococcus equisimilis* colonies which can result in hyper production of streptokinase enzyme. Kind of mutagen and dose rate/ concentration are the main factors in optimization of mutagenic procedures. In demand to get the determined occurrence of required wild variety amongst the strains to be selected, mutagen specificity can be considered and mutagenesis could be improved or focused. The physiological growth of isolated wild *Streptococcus equisimilis* strain is depicted in Fig 4.1. The industrial scale production of streptokinase from various sources have been in use from last many years but *Streptococcus equisimilis* is the most frequently used organism used for this purpose (Muhammad *et al.*, 2009). Strain enhancement for the wild strain was carried out by mutagenesis and choice. The parent organism was exposed to physical (UV irradiation and gamma irradiation) and chemical (Sodium azide and ethidium bromide) mutagens.

4.2.1. **Kill Curve Determination**

Gamma rays, very powerful, high energy beam and to defend them, considerable breadth of heavy metals as lead, steel or concrete is required. Primarily a kill curve was organized by employing gamma irradiation as a mutagen. For this purpose, numerous dosages of gamma rays were used to persuade mutation in *Streptococcus equisimilis*. A dose of 120 k Rad produced 88.50% killing (1x10⁴ CFU mL) as 3 log kill of the bacterial cells (Fig 4.3) UV
irradiation, carried out in a dark room, was used to increase the streptokinase activity of *Streptococcus equisimilis*. In order to optimize the treatment i.e. different doses of UV were compared evaluating cell survival and the frequency of positive and negative mutations (Zia et al., 2010). UV irradiation produced 87% killing (1.8 x 10³ CFU mL⁻¹) at 210 minutes of exposure where it produced 3 log kill as optimum dose and the detailed findings are arranged in Fig 4.4.

Higher doses of irradiation reduced the occurrence of positive mutation and the sum of viable colonies as defined by Abdelghani et al. (2005). To attain an immediate mutant, extraordinary dose of mutagen is compulsory. For this purpose, 0.326 mg of sodium azide was dissolved in 10 mL fresh inoculum. After 240 minutes dose rate produced 87% killing (Fig 4.5). This presented, revelation of cell suspension to sodium azide gave 3 log kill and this was selected as the best mutant with the capability to produce large amounts of streptokinase (Weigel and Baggenstoss, 2012; Eskil et al., 1998). A mutant strain with the capability to produce large quantities of streptokinase can be obtained by using a strong mutagen like ethidium bromide as it is reported to be an efficient and powerful mutagen. For this purpose 0.5 mg mL⁻¹ ethidium bromide solution was used for mutagenesis and 240 minutes exposure time was selected as the best dose as this resulted in 77% killing and 233% survival as shown in Fig 4.6. These results are in agreement with Sobrun et al. (2012).

4.2.2. Assessment and selection of mutant

After performing mutagenesis procedure, suspensions were serially diluted and 0.1 mL amount was plated on each PDA media plate. Colonies on each plate were restricted to 40 or less than this.

4.2.3. Colony restriction

By using colony restrictor, colonies were observed very visibly for their assortment. Triton X-100 was used to confine the bacterial colonies to minor size on selection medium (Sun et al., 2008). For colony restriction, the use of triton X-100 was found to be best as the colony size got restricted and small in size. This selection of colonies became the base of all further studies. Rodriguez et al. (2002) worked on the use 0.1% (V/V) triton X-100 to restrict radial colony growth, for streptokinase production. Carapito et al. (2006) used triton X-100 as a colony restrictor for the isolation of *Streptococcus equisimilis* mutants.
Fig. 4.2 Kill curve for the selection of optimum mutant dose of gamma irradiation for *S. equisimilis*

Fig. 4.3 Kill curve for the selection of optimum mutant dose of UV irradiation for *S. equisimilis*
Fig. 4.4 Kill curve for the selection of optimum mutant dose of sodium azide for *S. equisimilis*

Fig. 4.5 Kill curve for the selection of optimum mutant dose of ethidium bromide for *S. equisimilis*
4.2.4. Screening of mutant

4.2.4.1. Plate screening method

Nutrient agar supplemented with 10% blood was used for selection of mutant and 1% Triton X-100 was also added to this basal medium as colony restrictor. After incubating the plates for 1-2 days at 37 °C, the dimension of clearance zones was determined. Further sub-culturing of colonies presenting bigger clear zones was done. Outsized clearance zones were exhibited by few colonies in comparison to the parent strain and these large clearance zone producing colonies were selected for further use (Dubey et al., 2011; El-Mongy and Taha, 2012).

4.2.5. Mutant identification

Following two tests were implemented for the identification of specific mutant of *Streptococcus equisimilis*:

4.2.5.1. Enzyme diffusion zone test:

Screenings of streptokinase enzyme was done by using nutrient agar medium having 2 ml human serum and 2% casein. Streptokinase producing organism was recognized on blood agar plate containing defibrinized blood. If streptokinase is formed, then hemolysis will be there giving rise to clear zones (Dubey et al., 2011).

4.2.5.2. Analytical test

Strains producing the bigger clearance zone were dented, liquefied and normalized in phosphate buffer (pH 7), filtered and then enzyme assay was performed for the determination of the enzyme activity quantitatively. Reliang upon these trials, it was recommended that *Streptococcus equisimilis* EBL-SA-240, *Streptococcus equisimilis* EBL-UV-210, *Streptococcus equisimilis* EBL-G-120 and *Streptococcus equisimilis* EBL-EB-240 would be potential mutants for the maximum production of streptokinase (Table 4.1).

4.3. Production of streptokinase:

4.3.1 Microorganism production:

Phylum *Firmicutes* contain the lactic acid bacteria group which comprises genus of spherical Gram-positive bacteria including *Streptococcus*. Division of cells ensued in a sole alliance in these bacteria and named as *Streptococcus*. *Streptococcus* was classified according to their blood lysing qualities (Patterson, 1996). Alpha-hemolytic species caused iron oxidation which is present in hemoglobin molecules in erythrocytes provided green color on blood agar.
Complete heamolysis of erythrocytes is caused by Beta-hemolytic species. This was indicated by clear areas around the bacterial colonies.

Alpha-haemolytic species are called non-hemolytic as they do not cause hemolysis. Beta-hemolytic *Streptococci* were further classified with the help of Lancefield serotyping, which was described by the difference in bacterial cell wall composition in respect of specific carbohydrates (Facklam, 2002) There were 20 defined serotypes, entitled as Lancefield groups A to V (not including I and J). *Streptococcus pyogenes* was cultivated on blood agar at the neutral pH. Centrifugation of the culture was done for 25 min and enzyme purification was carried out by employing ammonium sulfate precipitation, dialysis and column chromatography (Felsia *et al*., 2011). *Streptococcus equisimilis* was cultured on nutrient agar at neutral pH and incubated for 24 hrs at 37°C.
Fig 4.6a. Beta-hemolytic *Streptococcus equisimilis* on blood agar medium

Fig 4.6b. Isolated beta-hemolytic *Streptococcus equisimilis* on blood agar medium
Fig 4.6c. Isolated beta-hemolytic *Streptococcus equisimilis* on blood agar medium

Table 4.1. Selected hyper producing mutants of *Streptococcus equisimilis* after screening and selection

<table>
<thead>
<tr>
<th>Sample</th>
<th>SK activity (U/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Streptococcus equisimilis</em> EBL-G-120</td>
<td>223.38±1.00</td>
</tr>
<tr>
<td><em>Streptococcus equisimilis</em> EBL-UV-210</td>
<td>201.78±3.18</td>
</tr>
<tr>
<td><em>Streptococcus equisimilis</em> EBL-SA-240</td>
<td>288.04±0.78</td>
</tr>
<tr>
<td><em>Streptococcus equisimilis</em> EBL-EB-240</td>
<td>230.61±4.33</td>
</tr>
<tr>
<td><em>Streptococcus equisimilis</em> (Wild)</td>
<td>197.02 ± 0.90</td>
</tr>
</tbody>
</table>
Fig. 4.7 Colony restriction with triton X-100 for *S. equisimilis* on PDA media (Gamma irradiation 120 KRad dose)

Fig. 4.8 Colony restriction with triton X-100 for *S. equisimilis* on PDA media (UV irradiation 210 minutes dose)
Fig. 4.9 Colony restriction with triton X-100 for *S. equisimilis* on PDA media (Sodium azide 240 minutes dose)

Fig. 4.10 Colony restriction with triton X-100 for *S. equisimilis* on PDA media (Ethidium bromide 240 minutes dose)
Table 4.1a. Results of biochemical tests for the isolation of *Streptococcus equisimilis* from different blood and biomass sources

* Voges proskauer Test.

<table>
<thead>
<tr>
<th>Sample Number</th>
<th>Disease</th>
<th>Source</th>
<th>Trehalose test</th>
<th>Hemolysis</th>
<th>Gram staining</th>
<th>Catalase test</th>
<th>*V.P Test</th>
<th>Methyl red test</th>
<th>Indole test</th>
<th>Citrate utilization test</th>
</tr>
</thead>
<tbody>
<tr>
<td>EBL- 1</td>
<td>Sore throat</td>
<td>Throat swab</td>
<td>+ ve</td>
<td>Beta</td>
<td>+ ve</td>
<td>- ve</td>
<td>+ ve</td>
<td>+ ve</td>
<td>Variable</td>
<td></td>
</tr>
<tr>
<td>EBL- 2</td>
<td>Sore throat</td>
<td>Throat swab</td>
<td>-ve</td>
<td>Alpha</td>
<td>- ve</td>
<td>+ ve</td>
<td>+ ve</td>
<td>-ve</td>
<td>+ve</td>
<td>-ve</td>
</tr>
<tr>
<td>EBL- 3</td>
<td>Acute tonsillitis</td>
<td>Human throat</td>
<td>-ve</td>
<td>Alpha</td>
<td>- ve</td>
<td>+ ve</td>
<td>+ ve</td>
<td>Variable</td>
<td>+ve</td>
<td></td>
</tr>
<tr>
<td>EBL- 4</td>
<td>Scarlet fever</td>
<td>Sheep blood</td>
<td>-ve</td>
<td>Alpha</td>
<td>- ve</td>
<td>-ve</td>
<td>-ve</td>
<td>-ve</td>
<td>+ve</td>
<td>-ve</td>
</tr>
<tr>
<td>EBL- 5</td>
<td>Scarlet fever</td>
<td>Sheep blood</td>
<td>-ve</td>
<td>Alpha</td>
<td>+ ve</td>
<td>+ ve</td>
<td>+ ve</td>
<td>+ ve</td>
<td>+ve</td>
<td>+ve</td>
</tr>
<tr>
<td>EBL- 6</td>
<td>Sore throat</td>
<td>Throat swab</td>
<td>+ ve</td>
<td>Beta</td>
<td>+ ve</td>
<td>- ve</td>
<td>- ve</td>
<td>-ve</td>
<td>-ve</td>
<td>-ve</td>
</tr>
<tr>
<td>EBL- 7</td>
<td>Scarlet fever</td>
<td>Human throat</td>
<td>+ ve</td>
<td>Beta</td>
<td>+ ve</td>
<td>+ ve</td>
<td>+ ve</td>
<td>-ve</td>
<td>+ve</td>
<td>-ve</td>
</tr>
<tr>
<td>EBL- 8</td>
<td>Acute tonsillitis</td>
<td>Human throat</td>
<td>-ve</td>
<td>Alpha</td>
<td>- ve</td>
<td>- ve</td>
<td>+ ve</td>
<td>Variable</td>
<td>+ve</td>
<td>+ve</td>
</tr>
<tr>
<td>EBL- 9</td>
<td>Sore throat</td>
<td>Human throat</td>
<td>-ve</td>
<td>Alpha</td>
<td>+ ve</td>
<td>- ve</td>
<td>+ve</td>
<td>+ve</td>
<td>-ve</td>
<td>-ve</td>
</tr>
<tr>
<td>EBL- 10</td>
<td>Acute tonsillitis</td>
<td>Throat swab</td>
<td>+ ve</td>
<td>Beta</td>
<td>+ ve</td>
<td>- ve</td>
<td>- ve</td>
<td>-ve</td>
<td>-ve</td>
<td>-ve</td>
</tr>
<tr>
<td>Control H46A</td>
<td>-</td>
<td>-</td>
<td>+ ve</td>
<td>Beta</td>
<td>-ve</td>
<td>-ve</td>
<td>-ve</td>
<td>-ve</td>
<td>-ve</td>
<td>-ve</td>
</tr>
</tbody>
</table>
4.3.2. Enzyme Production

4.3.2.1. Streptokinase production of in shake flask

The isolates, EBL- 6 and EBL- 10 were used for production of streptokinase. This research work defines the production of Streptokinase from beta *haemolytic streptococcus equisimilis*, its purification and characterization. So its production, purification and characterization were the main objectives of this research work. Streptokinase is now the emerging product of interest for its production from bacterial sources and its use as a powerful thrombosis agent. For thrombosis therapy, intravenous administration of Urokinase and Streptokinase has been extensively in use. To treat saphenous vein thrombosis in animals, enteric-coated Urokinase and streptokinase capsule were given to normal and experimental dogs and this latest study is called fibrinolytic treatment by oral drug induction. Intravenous administration improved fibrinolytic activity whereas oral drug induction did not indicate any strong thrombolytic effect as reported by Sumi et al (1990). All Streptokinases sequenced to date namely are formerly sequestered from streptococci which had caused infection to human host. These strains include group G *Streptococcus sp. Strain G19909* and group C *S equisimilis H46*. Significant resemblance to one another have been shown by these streptokinase. More than 85% homology at the amino acid level has been shown functionally and structurally by these enzymes. Whereas only 29.4% amino acid uniqueness was found in streptokinase produced by a *streptococcus* isolated from human hosts as described by Wang et al. (1998).

To define the influence of medium composition on streptokinase, a sequence of preliminary experiments in shake flask culture were performed. In 250 mL Erlenmeyer flasks, shake flask experiments (in triplicate) were conceded out. On a rotator shaker, cultivation was carried out at 37°C and 120 rpm with 100 mL working volume. For this purpose, formerly defined media was used with minor alterations (Bewald et al., 1975; Abdelghani et al., 2005). For the production of streptokinase enzyme, shake flask fermentation mode was used to culture *Streptococcus equisimilis* which is β-hemolytic streptococcus strain belonging to Group C. Corn steep liquor was used as substrate in shake flasks in a concentration of 5% CSL in 100 mL of fermentation medium and then these flasks were inoculated for production of streptokinase. All over the world, it is resilient demand of many users to decrease the production expenses of this enzyme as this will help the poor patients to treat such hazardous
ailments by this effective enzyme. First step toward decreasing the production costs is the optimization of the production process. In order to get the improved production of streptokinase, numerous parameters were optimized as given below.

### 4.3.2.2. Effect of substrate

Screening of numerous materials from agriculture wastes (agro-industrial) for product synthesis and microbial growth is involved for the assortment of an appropriate substrate for fermentation process so it is the most perilous factor (Banerjee et al., 2004). For fermentation, corn steep liquor (CSL) is a low price and cheap substrate (Kunamneni et al., 2007) and a nutritious source containing carbohydrates, vitamins and minerals. In current study corn steep liquor (CSL) was employed as substrate for culturing of *Streptococcus equisimilis* and for production of streptokinase by the same organism. Fermentation medium containing 1% to 10% substrate was imperiled to fermentation for 24hr at pH 7.4, 5% inoculum size and 37°C temperature. The findings given in figure 4.11 show the streptokinase activity in a medium containing 1% to 10% CSL concentration from wild and UV, sodium azide, gamma and ethidium bromide mutated *Streptococcus equisimilis* respectively. The results revealed that 5%, 4%, 4%, 5% and 5% CSL concentration supported the maximum production of streptokinase (198.49, 204.83, 289.21, 225.26 and 233.81 U mL⁻¹) from wild, UV, Sodium azide, Gamma and ethidium bromide mutated *Streptococcus equisimilis* respectively. The ANOVA (Table 4.2) exposed that the maximum production of streptokinase was supported by 4 and 5% substrate concentration, which differed significantly (p > 0.05) from other concentrations in SK production.

Most of the research conducted on streptokinase production resulted in same results as obtained from present study. For streptokinase production, 5% of corn steep liquor in fermentation medium was graded to be the superlative by Feldman (1974) for the optimization of substrate. Instead of casein hydrolyzate in growth medium of the strain *Streptococcus equisimilis*, use of corn steep liquor as substrate resulted in enhanced streptokinase yield. Banerjee *et al.* (2004) also studied the effect of CSL on SK yield. Best concentration of CSL used in fermentation medium was 5% which resulted in hyper production of streptokinase. So CSL at 5% concentration was used as the substrate for the production of streptokinase in succeeding trials. CSL is a widely used substrate for *Streptococcus equisimilis* for the
production of streptokinase as reported by Kunamneni et al. (2007). Maximum production of streptokinase was achieved by using 8\% CSL in fermentation medium as reported by Dubey et al. (2011). Patel et al. (2011) obtained maximum streptokinase production from UV irradiated Streptococcus equisimilis strain by using 8\% to 12\% CSL concentration in fermentation medium. Where as in the present study only 4 and 5\% CSL concentration resulted in hyper production of streptokinase from UV irradiated mutant strain of Streptococcus equisimilis.

4.3.2.3. Effect of fermentation period

Streptokinase production was carried out from wild Streptococcus equisimilis strain in liquid state fermentation for 12, 24, 36, 48, 60 and 72 hour with 5\% CSL concentration. Same fermentation period range was used to produce streptokinase from all four mutants i.e. UV, Sodium azide, Gamma and ethidium bromide mutated Streptococcus equisimilis at 4\%, 4\%, 5 and 5\% CSL concentration. Wild and all four mutants showed maximum streptokinase production (203.08, 237.58, 316.91, 247.01 and 250.57 U mL\(^{-1}\)) at 24, 24, 24, 36 and 24 hrs fermentation period respectively. With increase in fermentation time, enzyme production got declined. The results (Table 4.3) showed that 24 and 36 hour fermentation periods are statistically non-significant (p ˃ 0.05) in enzyme production from ethidium bromide and gamma mutants of Streptococcus equisimilis. Fermentation period of 24 hr is ideal for practical routine studies (Fig 4.12). Maximum production of streptokinase was achieved after 24 hr fermentation period as reported by Abdelghani et al. (2005) and Baewald et al. (1975).

The ANOVA showed that 24 and 36 hr fermentation periods are statistically non-significant (P >0.05) in enzyme production. Dubey et al. (2011) obtained maximum yield of streptokinase after 75 hrs of fermentation period. Patel et al. (2011) obtained maximum streptokinase production from UV irradiated Streptococcus equisimilis strain by using 48-72 hour fermentation time. Where as in the present study only 24 and 36 hours fermentation period resulted in hyper production of streptokinase. Findings of this research study are also in strong correlation with the study done by Madhuri et al. (2011), who obtained maximum streptokinase yield at 24 hours fermentation time. El-Mongy and Taha (2012) obtained maximum streptokinase yield (91.6 U mL\(^{-1}\)) after 72 hours of fermentation period where as in present study maximum production was carried out at 24 and 36 hours.
4.3.2.4. Effect of pH

To get highest enzyme yield, pH of medium was optimized after assortment of the appropriate fermentation period and substrate concentration. The maximum streptokinase production (225.71, 268.95, 335, 249.71 and 272.81 U mL⁻¹) was obtained at pH 7.5, 7.5, 7, 7.5 and 7 from wild, UV, Sodium azide, Gamma and ethidium bromide derived mutants (Fig 4.13). Statistical analysis through ANOVA also strongly valued the results of present study (Table 4.4). For metabolic activities and growth of microorganisms, optimal pH is very significant. Changes in pH greatly affect the metabolic activities of microorganisms so higher and lower pH effected the streptokinase production from *Streptococcus equisimilis* as compared to optimum one. Results of this research work are in accord with the conclusions of Abdelghani *et al.* (2005) who obtained optimal SK yield at pH 7.5 from *Streptococcus equisimilis*. These findings also showed good correlation with the findings of Nemirovick-Danchenko *et al.* (1985) and Hyun *et al.* (1997) that 6.8-7.5 was optimal pH for the synthesis of streptokinase by the bacterial strain. Davies *et al.* (1965) used pH 7.5 and attained the highest biosynthesis of streptokinase. To produce streptokinase from mutant *Streptococcus equisimilis*, Holmstrom (1965) subjected the medium to pH 7 and used the fed batch process for this purpose. Highest streptokinase yield from *Streptococcus equisimilis* was achieved at pH 7 by Dubey *et al.* (2011) and results are in great correspondence with the results of present study. pH is a restraining factor as revealed by the studies on the growth of β-hemolytic *Streptococci*. Concerning to pH sensitivity of streptokinase, this study supports the findings of Dubey *et al.* (2011). Comparatively high yields of streptokinase can be produced by instantaneous regulation of proper glucose feeding, inoculation rate and pH as shown by the present study. (Optimum pH of 7 is flawless for best streptokinase production and cell growth). Some portion of this product is dishonored at the same time, because of great sensitivity of streptokinase to temperature alteration and pH. Advanced SK yield can be attained by conditions that increase the growth rate of logarithmic phase and decrease lag phase period. Three times escalation in the rate of product can be attained by adjustment of pH maintenance with concentrated NaOH, glucose feeding and culture period (Karimi *et al.*, 2011). Patel *et al.* (2011) obtained maximum streptokinase production from UV irradiated *Streptococcus equisimilis* strain at pH 7. Results are in good correlation with the present study in which pH 7 and 7.5 resulted in hyper production of streptokinase. Madhuri *et al.* (2011) worked on
mutagenesis of *Streptococcus equisimilis* with UV irradiation for hyper production of SK. Results were in great correlation with the present study as over production of enzyme was attained at pH 7. El-Mongy and Taha (2012) obtained maximum streptokinase yield (91.6 U mL$^{-1}$) at pH 7.5 which is in good correlation with the results of current study as maximum production was attained at pH 7.5.

### 4.3.2.5. Effect of temperature

Highest streptokinase yield (287.64, 352.55, 263.89 and 289.37 U mL$^{-1}$) from UV, Sodium azide, Gamma and ethidium bromide derived *Streptococcus equisimilis* mutants was observed when medium was incubated at 37, 45, 37 and 37°C correspondingly in divergence with the wild which showed 242.38 U mL$^{-1}$ of streptokinase at 37°C (Fig 4.14). Incubation temperature affected the yield of enzyme as depicted by the results presented in Table 4.5. The bio processing temperature shoots up due to the enormous quantities of metabolic heat production. Metabolism of nitrogen source and substrate involve various enzymes which are deactivated at higher temperature and results in reduced production. These findings are in good association with the research work of other scientists. Improved secretion and synthesis of streptokinase into the extracellular medium was observed at 37°C as reported by Lee *et al.* (1997a). Highest yield of streptokinase was described by Feldman (1974) at 34°C while effect of the fermentation temperature on streptokinase yield from *Streptococcus equisimilis* was studied by Ozegowski *et al.* (1983), who studied the temperature range from 28-43°C.

Biomass production and cultivation temperature both are significant factors on which production of streptokinase mainly depends. In the present study on the, it was observed that, the enzyme production from *Streptococcus equisimilis* increased up to maximum level at 37°C as perceived in present study. However, at 37°C there was statistically non- significant effect of temperature on streptokinase production from UV irradiated and ethidium bromide mutant (p> 0.05). Abdelghani *et al.* (2005) and Dubey *et al.* (2011) got maximal streptokinase yield from *Streptococcus equisimilis* at 37°C which is in strong correlation with the findings of current study. Hyper production of SK was obtained from mutated strain of *Streptococcus equisimilis* at 37°C as that of in present study. Patel *et al.* (2011) obtained maximum streptokinase production from UV irradiated *Streptococcus equisimilis* strain at 37°C. Results are in good correlation with the present study in which hyper production of streptokinase was
Fig 4.11. Streptokinase production from wild and mutated *Streptococcus equisimilis* strains at different substrate concentrations
Table 4.2. Analysis of variance table for effect of substrate concentration on streptokinase production from wild and mutated strains of *Streptococcus equisimilis*

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>Degrees of freedom</th>
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<th>Mean squares</th>
<th>F-value</th>
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</tr>
<tr>
<td>Treatment</td>
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<td>11985.6</td>
<td>1188.98**</td>
<td>**</td>
</tr>
<tr>
<td>Substrate x Treatment</td>
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<td>50870</td>
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<td>**</td>
</tr>
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</tr>
</tbody>
</table>

** = Highly significant (P<0.01)
Fig 4.12. Streptokinase production from wild and mutated *Streptococcus equisimilis* strains at different fermentation times.
Table: 4.3. Analysis of variance table for effect of fermentation time on streptokinase production from wild and mutated strains of *Streptococcus equisimilis*

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>Degrees of freedom</th>
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<th>Mean squares</th>
<th>F-value</th>
</tr>
</thead>
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<td>Time</td>
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<td>49916.9</td>
<td>1924.75**</td>
</tr>
<tr>
<td>Treatment</td>
<td>4</td>
<td>73554</td>
<td>18388.4</td>
<td>709.04**</td>
</tr>
<tr>
<td>Time x Treatment</td>
<td>20</td>
<td>32418</td>
<td>1620.9</td>
<td>62.50**</td>
</tr>
<tr>
<td>Error</td>
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<td>1556</td>
<td>25.9</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>89</td>
<td>357112</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

** = Highly significant (P<0.01)
Fig 4.13. Streptokinase production from wild and mutated *Streptococcus equisimilis* strains at different pH
### Table: 4.4. Analysis of variance table for effect of pH on production of streptokinase from wild and mutant strains of *Streptococcus equisimilis*

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>Degrees of freedom</th>
<th>Sum of squares</th>
<th>Mean squares</th>
<th>F-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH level</td>
<td>12</td>
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<td>53799.2</td>
<td>53799.2</td>
</tr>
<tr>
<td>Treatment</td>
<td>4</td>
<td>175287</td>
<td>43821.6</td>
<td>12980.50**</td>
</tr>
<tr>
<td>pH level x Treatment</td>
<td>48</td>
<td>129012</td>
<td>2687.8</td>
<td>796.15**</td>
</tr>
<tr>
<td>Error</td>
<td>130</td>
<td>439</td>
<td>3.4</td>
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</tr>
<tr>
<td>Total</td>
<td>194</td>
<td>950328</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

** = Highly significant (P<0.01)
obtained at 37°C. Results obtained by El-Mongy and Taha (2012) are in strong correlation with the present study as maximum streptokinase yield (91.6 U mL⁻¹) was obtained at 37°C.

4.3.2.6. Effect of glucose

The energy source should be the restraining factor to acquire utmost enzyme production per unit energy source as revealed by the study done by Rosenberger and Elsden (1960). Glucose was used as carbon source in medium so glucose was used for medium supplementation and fermentation was proceeded under optimal conditions. Use of 2, 4, 2 and 3% glucose to growth medium resulted in maximal yield of streptokinase (300.52, 365.41, 280.50 and 295.19 U mL⁻¹) from UV, Sodium azide, Gamma and ethidium bromide derived mutants of *Streptococcus equisimilis* in contrast with the wild which showed 250.43 U mL⁻¹ of streptokinase activity at 5% glucose concentration (Fig 4.15, Table 4.6). However, 3%, 4% and 5% glucose concentrations differed significantly (P > 0.05). These concentrations also differed significantly from other concentrations of glucose used in streptokinase production. Appropriate glucose concentration was used by Nemirovic-Danchenko *et al.* (1985) to increase the yield of streptokinase from *Streptococcus equisimilis*. Maximum yield of streptokinase was obtained by using 5% glucose as studied by Ellis and Armstrong (1971). Maximal streptokinase yield was obtained by using 3.9% glucose concentration by Holmstorm (1965). While growth became sufficient without unnecessary production of acid when low glucose concentration in the enzyme production medium was used as reported by Bernheimer *et al.* (1942). The streptokinase production was enhanced, when restrictive quantities of all other nutrients were there along with surplus amount of glucose (Rosenberger and Elsden, 1960). Patel *et al.* (2011) worked on mutagenesis of *Streptococcus equisimilis* and obtained hyper production of streptokinase by using 7% glucose in fermentation medium whereas in the present study maximum SK yield was obtained from mutant strains of *Streptococcus equisimilis* by using only 2, 3 and 4% glucose in the fermentation medium. In present research work wild strain produced maximum enzyme level at 5% glucose level. Madhuri *et al.* (2011) obtained maximum streptokinase yield from mutant *Streptococcus equisimilis* strain.
by using 5% glucose in fermentation medium and same results were obtained by El-Mongy and Taha (2012) who also obtained maximal SK level at 5% glucose level. Results of present study are obtained by using smaller quantity of glucose in comparison to these studies.

4.3.2.7. Effect of yeast extract

On enzyme production, significant effect of yeast extract concentration in the growth medium was observed. Optimal streptokinase production (324.83, 381.09, 285.15 and 301.38 U mL\(^{-1}\)) from UV, Sodium azide, Gamma and ethidium bromide derived mutants of *Streptococcus equisimilis* was obtained at 3%, 2%, 2% and 3% concentration of yeast extract respectively in comparison with 266.13 U mL\(^{-1}\) activity showed by wild strain at 3% yeast extract concentration (Fig 4.16). As compared to other concentrations, highest streptokinase yield was obtained at 2 and 3% concentration of yeast extract in the present study (P< 0.01) (Table 4.7). Highest yield of streptokinase was obtained by using 3% yeast extract as reported by Narciandi *et al.* (1996). Yazdani and Mukherjee (2002) obtained maximal yield of streptokinase by using 5% yeast extract. Great quantities of streptokinase were obtained by using 7% yeast extract in fermentation medium (Hyun *et al.*., 1997). In present study, big quantities of streptokinase were produced at 2 and 3% level of yeast extract which is significantly higher than the previous production carried out by above said researchers at 5 or 7% level of yeast extract.

4.3.2.8. Effect of KH\(_2\)PO\(_4\)

With above determined optimum conditions, effect of different concentrations of KH\(_2\)PO\(_4\) (0.01, 0.02, 0.03, 0.04, 0.05, 0.06 and 0.07%) was studied on enzyme production. The results in Table 4.8 indicated that the maximum streptokinase production (273.59, 333.64, 400.34, 299.20 and 325.55 U mL\(^{-1}\)) was obtained from wild and UV, sodium azide, gamma, ethidium bromide derived mutants of *Streptococcus equisimilis* at 0.05, 0.04, 0.04, 0.06, and 0.04% concentration of KH\(_2\)PO\(_4\) respectively (Fig 4.17). Enzyme activity was decreased by further increase in the concentration of KH\(_2\)PO\(_4\). Abdelghani *et al.* (2005) got highest streptokinase yield by using 0.25%
KH$_2$PO$_4$ in the fermentation medium. Dubey et al. (2011) used 7% level of KH$_2$PO$_4$ for streptokinase production and obtained 467.73 U of enzyme where as in present study, by using only 0.04% of KH$_2$PO$_4$ in fermentation medium resulted in 400.34 U of streptokinase from sodium azide mutant of Streptococcus equisimilis. Patel et al. (2011) got maximal streptokinase yield from mutant strain of Streptococcus equisimilis by using 0.33% KH$_2$PO$_4$ in production medium whereas in the present study hyper production of same enzyme was attained by using only 0.04, 0.05 and 0.06% of KH$_2$PO$_4$ in the medium. Maximal streptokinase production from mutant Streptococcus equisimilis was obtained by using 0.25% KH$_2$PO$_4$ in the production medium whereas in the present study maximal streptokinase yield was attained by using only 0.04, 0.05 and 0.06% KH$_2$PO$_4$ in the fermentation medium of the mutant Streptococcus equisimilis strain (Madhuri et al., 2011).

4.3.2.9. Effect of K$_2$HPO$_4$

Frequency of fermentation and streptokinase production was boosted by using dipotassium hydrogen phosphate. Table 4.9 represents the results concerning the effect of K$_2$HPO$_4$ on the streptokinase production. Maximal enzyme production (280.95, 342.87, 418.31, 312.87 and 331.71 U mL$^{-1}$) was obtained from wild and UV, Sodium azide, Gamma, ethidium bromide derived mutated Streptococcus equisimilis by adding 0.25, 0.05, 0.05, 0.05 and 0.05% K$_2$HPO$_4$ to the fermentation medium respectively (Fig 4.18). ANOVA showed that 0.05% concentration of K$_2$HPO$_4$ was expressively different from all other concentrations tested ($P>0.05$). Production of streptokinase was induced by using 0.05% concentration of K$_2$HPO$_4$ as studied by Nemirovicc-Danchenko et al. (1985). Dubey et al. (2011) used 0.33% K$_2$HPO$_4$ in production medium for SK production from Streptococcus equisimilis strain and obtained 467.73 U of streptokinase. Whereas on the other hand in the present study by using only 0.05% level of K$_2$HPO$_4$ in production medium resulted in 418.31 U of streptokinase activity from sodium azide mutant of Streptococcus equisimilis. Results of present study are also in accord of the work done by Abdelghani et al. (2005), who obtained highest SK yield from wild Streptococcus equisimilis strain by using 0.25% of K$_2$HPO$_4$ in fermentation medium.
El-Mongy and Taha (2012) worked on the production of streptokinase from *Streptococcus equisimilis* by using 0.25% K$_2$HPO$_4$ in the fermentation medium of the organism and these results are in strong correlation with the findings of current study regarding the SK production from wild strain in which 0.25% K$_2$HPO$_4$ was used in the fermentation medium. In present study maximal streptokinase yield was obtained from mutated strain by using only 0.05% K$_2$HPO$_4$ in the fermentation medium which is quite better in comparison to the study done by El-Mongy and Taha (2012) in which 0.25% concentration was used for the production of same enzyme. Production of streptokinase from 19 strains of *Streptococci* was analyzed by Ellis and Armstrong (1971) in a study in which they used an enriched tryptose broth medium. This medium comprised of KH$_2$PO$_4$, sodium chloride, uracil, tryptose, glucose, adenine, glutamine, tryptophan, vitamins and salts. A medium containing corn steep liquor, cerelose, KH$_2$PO$_4$ with pH 7.0, was used by Feldman (1974) for producing streptokinase.
Fig 4.14. Streptokinase production from wild and mutated *Streptococcus equisimilis* strains at different temperatures.
Table 4.5. Analysis of variance table for effect of Temperature on streptokinase production from wild and mutant strains of *Streptococcus equisimilis*

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>Degrees of freedom</th>
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<td>Temperature</td>
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<td>2952.03**</td>
</tr>
<tr>
<td>Treatment</td>
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<td>41204</td>
<td>10300.9</td>
<td>419.77**</td>
</tr>
<tr>
<td>Temperature x Treatment</td>
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<td>95125</td>
<td>5945.3</td>
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</tr>
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<td>Error</td>
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<tr>
<td>Total</td>
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<td>427323</td>
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</table>

** = Highly significant (P<0.01)
Fig 4.15. Streptokinase production from wild and mutated *Streptococcus equisimilis* strains at different glucose concentrations
Table 4.6. Analysis of variance table for effect of glucose concentration on streptokinase production from wild and mutant strains of *Streptococcus equisimilis*

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<tr>
<th>Source of variation</th>
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<td>2088.48**</td>
</tr>
<tr>
<td>Glucose x Treatment</td>
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<td>Total</td>
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** = Highly significant (P<0.01)
Fig 4.16. Streptokinase production from wild and mutated *Streptococcus equisimilis* strains at different yeast extract concentrations
Table 4.7: Analysis of variance table for effect of Yeast extract concentration on streptokinase production from wild and mutant strains of *Streptococcus equisimilis*

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<tr>
<th>Source of variation</th>
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** = Highly significant (P<0.01)
4.3.2.10. Effect of MgSO₄·7H₂O

In the growth medium containing optimum concentrations of substrate (5%, 4%, 4%, 5% and 5%), yeast extract (3%, 3%, 2%, 2% and 3%), glucose (5%, 2, 4, 2 and 3%), K₂HPO₄ (0.05, 0.04, 0.04, 0.06, and 0.04%) and KH₂PO₄ (0.25, 0.05, 0.05, 0.05 and 0.05%), with growing organism at 37°C, 37°C, 45°C, 37°C and 37°C for 24, 24, 24, 36 and 24 hr and at pH (7.5, 7.5, 7, 7.5 and 7), five different concentrations (0.02, 0.04, 0.06, 0.08 and 0.1%) of MgSO₄·7H₂O was tested for optimal streptokinase production (296.65, 364.35, 436.43, 322.66 and 340.43 U mL⁻¹) from wild, UV, Sodium azide, Gamma and ethidium bromide derived mutated Streptococcus equisimilis respectively (Fig 4.19). Maximum activity of streptokinase was obtained from above said strains at 0.06, 0.04, 0.04, 0.06 and 0.04% MgSO₄·7H₂O concentration respectively as shown in Table 4.10. Maximal yield of streptokinase was obtained by using 0.04% MgSO₄·7H₂O (Narciaidi et al., 1996; Yazdani and Mukherjee, 2002). The statistical analysis of data also proved these results (P< 0.01). Abdelghani et al. (2005) used 0.04% of MgSO₄·7H₂O and obtained maximal yield of streptokinase and these results are in strong correlation with the present study in which highest SK yield (436.43 U mL⁻¹) was obtained by using 0.04% of MgSO₄·7H₂O in production medium. Madhuri et al. (2011) worked on the production of streptokinase from mutant Streptococcus equisimilis strain. They used 0.04% MgSO₄·7H₂O in the production medium and attained maximal SK yield and these results are in strong correlation with the results of present study in which by using 0.04% MgSO₄·7H₂O in the fermentation medium resulted in maximum SK production.

4.3.2.11. Effect of NaHCO₃

For optimal streptokinase production, effect of five diverse concentrations of NaHCO₃ (0.1, 0.15, 0.2, 0.25, 0.45%) was studied along with optimal yeast extract, glucose, substrate, KH₂PO₄, K₂HPO₄ and MgSO₄·7H₂O concentrations in the fermentation medium of wild, UV, Sodium azide, Gamma and ethidium bromide derived mutated Streptococcus equisimilis. Then organism was grown at optimum temperature, time and pH. The activity of streptokinase was maximized from wild, UV, Sodium azide, Gamma and ethidium bromide derived mutated Streptococcus equisimilis with 0.2, 0.15, 0.15, 0.15 and 0.15% NaHCO₃ in fermentation medium respectively ((Fig 4.20, Table 4.11). The ANOVA showed that 0.1% and 0.15% concentrations of NaHCO₃ differed significantly (P >0.05) for production of streptokinase. Though the maximum
yield (457.01 U) of SK in this research study was attained with 0.15% level of NaHCO$_3$ which is in strong correlation with the work done by Abdelghani et al. (2005) who obtained maximum streptokinase yield (681 U) by using 0.15% NaHCO$_3$. Patel et al. (2011) obtained maximum streptokinase production by using 0.2% NaHCO$_3$ in production medium and these results are in good correlation with the work done in present study in which by using 0.2% NaHCO$_3$, maximum SK was produced by wild _Streptococcus equisimilis_. Whereas in the present research study, mutated strains showed highest streptokinase yield by using 0.15% NaHCO$_3$ in the fermentation medium. For the highest production of streptokinase from mutant _Streptococcus equisimilis_, 0.15% NaHCO$_3$ was used in the production medium (Madhuri et al., 2011). These results are also in strong correlation with the findings of present study.

**4.3.2.12. Effect of CH$_3$COONa. 3H$_2$O**

Influence of five levels of CH$_3$COONa.3H$_2$O (0.05%, 0.1%, 0.15%, 0.2 and 0.25%) was checked for production of enzyme along with above selected optimal levels of various factors (Fig 4.21). The findings designated that maximal enzyme (322.66, 400.67, 465.33, 350.55 and 360.04 U mL$^{-1}$) production from wild, UV, Sodium azide, Gamma and ethidium bromide derived mutants was obtained at 0.15, 0.1, 0.1, 0.1 and 0.15% CH$_3$COONa.3H$_2$O concentration level respectively. The ANOVA test revealed that in SK production, 0.05%, 0.1% and 0.15% of CH$_3$COONa.3H$_2$O were statistically different (Table 4.12). When the medium contained 0.1% CH$_3$COONa.3H$_2$O, this resulted in maximum streptokinase production as demonstrated by Baewald et al. (1975) and Abdelghani et al. (2005). Madhuri et al. (2011) worked on production of streptokinase from UV irradiated _Streptococcus equisimilis_ strain and obtained maximal yield by using 0.1% CH$_3$COONa.3H$_2$O and these results are in strong correlation with the findings of present study in which hyper production of attained from UV irradiated mutant by utilizing 0.1% CH$_3$COONa.3H$_2$O in fermentation medium.

**4.3.2.13. Effect of FeSO$_4$.7H$_2$O**

FeSO$_4$.7H$_2$O was used to enhance the rate of fermentation and streptokinase production. The results regarding the effect of FeSO$_4$.7H$_2$O on enzyme production from wild UV, Sodium azide, Gamma and ethidium bromide derived mutants are presented in Table 4.13. The results showed that addition of 0.06, 0.04%, 0.04%, 0.04 and 0.04% FeSO$_4$.7H$_2$O to the to the fermentation medium respectively resulted in maximum production of streptokinase (331.70, 431.20, 486.40, 331.70, 431.20, 486.40,
367.20 and 376.30 U mL\(^{-1}\) respectively). The ANOVA showed that SK production at 0.04 and 0.06% level of FeSO\(_4\).7H\(_2\)O was significantly different from other concentrations (P >0.05). These concentrations of FeSO\(_4\).7H\(_2\)O in fermentation media resulted in maximum enzyme production from wild, UV, Sodium azide, Gamma and ethidium bromide derived mutants respectively. Results also revealed that 0.06%, 0.02% and 0.04% levels of FeSO\(_4\).7H\(_2\)O were similarly effective for streptokinase yield (P< 0.01). Streptokinase production was reduced by further addition of FeSO\(_4\).7H\(_2\)O. Synthesis of streptokinase was induced by 0.06% concentration of FeSO\(_4\).7H\(_2\)O as demonstrated by Yazdani and Mukherjee et al. (2002). Baewald et al. (1975) and Abdelghani et al. (2005) obtained maximal streptokinase yield when 0.002% level of FeSO\(_4\).7H\(_2\)O was used in production medium. These findings are not in accord with the results of present study. Hyper production of streptokinase was carried out by growing UV mutant of *Streptococcus equisimilis* in growth medium containing 0.04% FeSO\(_4\).7H\(_2\)O (Madhuri et al., 2011) and the findings are in strong correlation with the findings of present study in which maximal production was attained by using 0.04% FeSO\(_4\).7H\(_2\)O in the production medium.
Fig 4.17. Streptokinase production from wild and mutated *Streptococcus equisimilis* strains at different KH$_2$PO$_4$ concentrations
Table 4.8.  Analysis of variance table for effect of KH$_2$PO$_4$ concentration on streptokinase production from wild and mutant strains of *Streptococcus equisimilis*

<table>
<thead>
<tr>
<th>Source of variation</th>
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<th>Mean squares</th>
<th>F-value</th>
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</tr>
<tr>
<td>Treatment</td>
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<td>Total</td>
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<td></td>
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</tbody>
</table>

** = Highly significant (P<0.01)
Fig 4.18. Streptokinase production from wild and mutated *Streptococcus equisimilis* strains at different $K_2HPO_4$ concentrations.
Table 4.9. Analysis of variance table for effect of K$_2$HPO$_4$ concentration on streptokinase production from wild and mutant strains of *Streptococcus equisimilis*

<table>
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<th>F-value</th>
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<td>Treatment</td>
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<td>Concentration x Treatment</td>
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<td>Error</td>
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<td>Total</td>
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** = Highly significant (P<0.01)
Streptokinase production from wild and mutated *Streptococcus equisimilis* strains at different MgSO$_4$.7H$_2$O concentrations
Table 4.10. Analysis of variance table for effect of MgSO₄·7H₂O concentration on production of streptokinase from wild and mutant strains of *Streptococcus equisimilis*

<table>
<thead>
<tr>
<th>Source of variation</th>
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<th>Mean squares</th>
<th>F-value</th>
</tr>
</thead>
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<td>Concentration</td>
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<td>203583</td>
<td>50895.8</td>
<td>1612.99**</td>
</tr>
<tr>
<td>Treatment</td>
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<td>Concentration x Treatment</td>
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<td>Total</td>
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</tbody>
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** = Highly significant (P<0.01)
4.3.2.14. Effect of MnCl$_2$.4H$_2$O

With the above determined optimum conditions, five diverse levels of MnCl$_2$.4H$_2$O (0.02, 0.04, 0.06, 0.08 and 0.1%) were verified for optimum synthesis of streptokinase in the fermentation medium. The production of streptokinase from wild, UV, sodium azide, gamma and ethidium bromide derived mutants increased significantly (P > 0.05) by addition of 0.04, 0.02%, 0.02%, 0.04 and 0.02% MnCl$_2$.4H$_2$O respectively to production medium as compared to other four concentrations (Table 4.14). Nemirovich-Danchenko et al., (1985) used 0.02% concentration of MnCl$_2$.4H2O under similar conditions to enhance the streptokinase production. Highest streptokinase yield was obtained by using 0.02% level of MnCl$_2$.4H$_2$O in production medium as reported by Abdelghani et al. (2005). Hyper production of streptokinase was carried out by Madhuri et al. (2011) by using 0.02% MnCl$_2$.4H$_2$O in the production medium and the results are in strong correlation with the findings of present study in which maximal SK production was carried out from UV mutant of *Streptococcus equisimilis* by using 0.02% MnCl$_2$.4H$_2$O in fermentation medium.

4.3.2.15. Effect of inoculums size

Effect of four different inoculums sizes (2.5, 5, 7.5 and 10 mL) in the growth medium was studied for optimal streptokinase production along with the above determined optimal levels of various parameters. The streptokinase production from wild UV, Sodium azide, Gamma and ethidium bromide derived mutants increased significantly (P>0.05) by addition of 5% inoculum to the fermentation medium as compared to other three concentrations (Table 4.15). Abdelghani et al, (2005) used 10% inoculum of beta haemolytic *Streptococci* for streptokinase production. On the other hand Elmongy and Taha (2012) used 1% inoculum of *Streptococcus equisimilis* in fermentation media for streptokinase production. Madhuri et al. (2011) used 8-15% inoculum size and attained hyper production of streptokinase from *Streptococcus equisimilis* whereas in the present research work maximal streptokinase production was carried out by the same strain by using only 5% inoculum size.

4.3.2.16. Effect of CaCO$_3$

Seven different concentrations of CaCO$_3$ (0.001, 0.002, 0.003, 0.004, 0.005, 0.006 and 0.007%) were tested for maximum production of streptokinase in the growth medium with the above determined optimum conditions. The streptokinase production (367.72, 461.92, 583.87, 104
393.19 and 426.93 U mL⁻¹) from wild UV, Sodium azide, Gamma and ethidium bromide derived mutants increased significantly (P>0.05) by addition of 0.005%, 0.004%, 0.004, 0.005 and 0.004% of CaCO₃ to the fermentation medium as compared to other six concentrations ((Fig 4.25 and Table 4.16). When the medium contained 0.004% CaCO₃, this resulted in maximum streptokinase production as demonstrated by Baewald et al. (1975). Elmongy and Taha (2012) also used 0.005% CaCO₃ in fermentation media for streptokinase production from Streptococcus equisimilis.
Fig 4.20. Streptokinase production from wild and mutated Streptococcus equisimilis strains at different NaHCO$_3$ concentrations
Table 4.11. Analysis of variance table for effect of NaHCO$_3$ concentration on streptokinase production from wild and mutant strains of *Streptococcus equisimilis*

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>Degrees of freedom</th>
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<th>Mean squares</th>
<th>F-value</th>
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<td>Treatment</td>
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<td>699.42**</td>
</tr>
<tr>
<td>Concentration x Treatment</td>
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<td>89463</td>
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<td>189.00**</td>
</tr>
<tr>
<td>Error</td>
<td>50</td>
<td>1479</td>
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<td></td>
</tr>
<tr>
<td>Total</td>
<td>74</td>
<td>476574</td>
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<td></td>
</tr>
</tbody>
</table>

** = Highly significant (P<0.01)
Fig 4.21. Streptokinase production from wild and mutated *Streptococcus equisimilis* strains at different CH$_3$COONa.3H$_2$O concentrations
Table 4.12. Analysis of variance table for effect of CH₃COONa.3H₂O concentration on production of streptokinase from wild and mutant strains of *Streptococcus equisimilis*

<table>
<thead>
<tr>
<th>Source of variation</th>
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<th>Sum of squares</th>
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<td>733.24**</td>
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<td>Error</td>
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<td>Total</td>
<td>74</td>
<td>483984</td>
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** = Highly significant (P<0.01)
Fig 4.22. Streptokinase production from wild and mutated *Streptococcus equisimilis* strains at different FeSO$_4$.7H$_2$O concentrations
Table 4.13: Analysis of variance table for effect of FeSO$_4$.7H$_2$O concentration on streptokinase production from wild and mutant strains of *Streptococcus equisimilis*

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>Degrees of freedom</th>
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<td>476838</td>
<td>119209</td>
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<td>Treatment</td>
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<td>58699</td>
<td>14675</td>
<td>473.53**</td>
</tr>
<tr>
<td>Concentration x Treatment</td>
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<td>113887</td>
<td>7118</td>
<td>229.69**</td>
</tr>
<tr>
<td>Error</td>
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<tr>
<td>Total</td>
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<td>650973</td>
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<td></td>
</tr>
</tbody>
</table>

** = Highly significant (P<0.01)
4.4. Purification of streptokinase

Three steps were used for purification of streptokinase from crude extract. Crude enzyme was imperiled to ammonium sulfate precipitation, ion exchange chromatography and gel permeation for its final isolation and purification. Ammonium sulfate precipitation of crude enzyme was performed and then dialyzed against distilled water. Ion exchange column was used for further purification of streptokinase and for this purpose dialyzed sample of streptokinase was conceded through the column. Buffer containing 0.01 \( M \) Tris-HCl, 1 M NaCL, (pH= 8.0) was used to wash the column (Babashamsi et al., 2009).

4.4.1. Partial purification by ammonium sulfate precipitation:

Solubility of different proteins in salt solution is the basics of ammonium sulfate precipitation which was followed by increased protein-protein interaction. Because of its high solubility, ammonium sulfate precipitation is one of the most usually used agent for salting out of protein which allowed the recovery of solutions with high ionic strength (Voet and Voet, 1990). Under optimized conditions, enzyme was produced from physical and chemical derived mutants of \textit{Streptococcus equisimilis} and then it was subjected to ammonium sulfate precipitation. Crude enzyme produced from wild, UV, Sodium azide, Gamma and ethidium bromide derived mutants exhibited 370, 480, 620, 395 and 429 U mL\(^{-1}\) of enzyme activity. The specific activity of the crude enzyme was 13.45, 26.64, 27.80, 24 and 24.94 U mg\(^{-1}\) from wild, UV, Sodium azide, Gamma and ethidium bromide derived mutants respectively. Protein contents in the crude enzyme obtained from wild, UV, Sodium azide, Gamma and ethidium bromide derived mutants were 27.5, 18.02, 22.3, 16.5 and 17.2 mg mL\(^{-1}\).

Forty percent supernatant from wild, UV, Sodium azide, Gamma and ethidium bromide derived mutants showed the activity of 349, 410, 495, 362 and 374 U mL\(^{-1}\) with 19.07, 25.12, 24.14, 25.67 and 26.15 U mg\(^{-1}\) specific activity. Protein contents were 18.3, 16.32, 20.5, 14.1 and 14.3 mg mL\(^{-1}\) while 40% sediments of wild, UV, Sodium azide, Gamma and ethidium bromide derived mutants of \textit{Streptococcus equisimilis} showed the activity of 327, 350, 451, 320 and 325 U mL\(^{-1}\) respectively, which indicated the partial purification of enzyme. Specific activity was 21.09, 22.72, 25.05, 25.6 and 30.95 U mg\(^{-1}\) respectively. Protein contents were 15.5, 15.4, 18, 12.5 and 10.5 U mg\(^{-1}\) in streptokinase produced from wild, UV, Sodium azide, Gamma and ethidium bromide derived mutants of \textit{Streptococcus equisimilis}. Sixty percent supernatant from wild, UV,
Sodium azide, Gamma and ethidium bromide derived mutants showed the activity of 315, 300, 399, 278 and 289 U mL\(^{-1}\) while sediments of wild, UV, sodium azide, gamma and ethidium bromide derived mutants of *Streptococcus equisimilis* showed the activity of 322, 402, 476, 300 and 350 U mL\(^{-1}\) respectively. Specific activity was 22.82, 26.78, 26.6, 31.95 and 33.21 U mg\(^{-1}\) from 60% supernatants of wild, UV, sodium azide, gamma and ethidium bromide derived mutants of *Streptococcus equisimilis*. Following findings are in accordance with the results of Felsia *et al.* 2011.

After desalting process, the enzymatic activity from wild, UV, Sodium azide, Gamma and ethidium bromide derived mutants increased up to 328, 456, 520, 381 and 415 U mL\(^{-1}\) with specific activities of 28.52, 90.83, 71.72, 77.75 and 66.93 U mg\(^{-1}\) respectively. Enzyme produced from wild and mutant strains attained 2.12, 3.41, 2.58, 3.24 and 2.68 fold purification and 88.65, 95, 83.87, 96.45 and 96.73% recovery as compared to crude one after desalting process. These results are in great correlation with Babu *et al.* 2008 who attained 90% recovery after desalting process. Desired protein can be selectively precipitated by the addition of right amount of ammonium sulphate, while the other proteins persist to be dissolved. Removal of the contaminating salts existing in sediments is called desalting and this results in improvement of activity of enzyme (Felsia *et al.*, 2011; Zia *et al.*, 2001). Streptokinase was produced from *Streptococcus pyogenes* and then subjected to ammonium sulfate precipitation. The activity of crude enzyme was 806.7 IU/mg, protein contents were 1.21mg. The recovery of streptokinase after ammonium sulfate precipitation was 100%. After dialysis enzyme activity was 7600 IU/mg, protein contents were 0.77mg and recovery was 94.2% (Felsia *et al.*, 2011). The results of the present study (Table 4.17, 4.18, 4.19 and 4.20) strongly correlate with the results of Tomar (1968), who attained 2 to 3 times enhancement in specific activity after 40% (NH\(_4\))\(_2\)SO\(_4\) precipitation technique.

### 4.4.2. Ion Exchange Chromatography:

Streptokinase produced from the fermentation cultures of numerous streptococcal strains was purified and for this purpose several procedures have been described for this purification (Karimi *et al.*, 2011). Extremely purified product has been attained by applying DEAE-cellulose in amalgamation with other purification processes. DEAE-cellulose is the most frequently used cellulosic anion exchanger (Voet and Voet, 1990). Various other ion exchange resins i.e. carboxyl methyl cellulose, DEAE and sephadex etc. are likewise in use. But ion exchange cellulose is
preferred as the elevated density and fine particles size of the micro granular cellulose end in extra dense adsorbent bed, which gives improves resolution (Jakoby, 1971). For ion exchange chromatography desalted enzyme was exposed to DEAE-cellulose column. Adsorption of proteins on ion exchange resins includes chiefly the establishment of numerous ionic bonds between accessible groups of opposed charge on the adsorbent and charged groups on the proteins. Differential elution of the adsorbent proteins is the basics for chromatographic separation and by using a variation of techniques based either upon the change of pH or upon the application of agents proficient of competing with the adsorbed protein for the charged sites on the adsorbent (Jakoby, 1971). DEAE-cellulose was often used as cellulosic anion exchanger. The ion exchange capability of a resin is a measureable dimension of its ability to take up exchangeable ions. This property and exchange competence reveals the convenience of the inorganic groups to the exchanging ions.

So, it was observed that 51th fraction of UV mutant derived streptokinase showed 277.77 U mg\(^{-1}\) specific activity, 83.33 % recovery of the enzyme with 10.43 fold purification. In case of sodium azide mutant derived enzyme, 54th fraction of ion exchange showed 467.96 U mg\(^{-1}\) specific activity with 16.83 fold purification and 77.74 % recovery of the enzyme. On the other hand 316.26 U mg\(^{-1}\), 12.68 fold purification and 90.67% recovery was attained by ethidium bromide derived streptokinase. In comparison to wild and other two mutants, 50th fraction of gamma irradiated mutant derived SK resulted in 270.45 U mg\(^{-1}\) specific activity, 90.37 % recovery of the enzyme respectively with 11.27 fold purification. Among wild and mutant derived streptokinase, enzyme produced by EBL-SA240 mutated strain showed highest activity 482 U mL\(^{-1}\) along with maximum fold purification (16.83). These results strongly succeed the finding of Dubey et al. (2011) and Avilen et al. (1997) who applied DEAE-cellulose chromatography and attained 4.5 fold purified active enzyme.

These findings also correlate and supersede the work done by De Renzo et al. (1967) who used DEAE-cellulose column chromatography for the purification of relatively crude commercial preparation of streptokinase and obtained a 5-6 fold increase in purity. Perez et al., (1998) purified recombinant streptokinase and found 99% purity and Specific activity of 454 IU/mg. Felsia et al. (2011) produced streptokinase and after ion exchange chromatography estimated the enzyme activity of 6600 IU/mg, protein contents were 0.45mg and recovery of streptokinase was 81.8%. After using DEAE-cellulose chromatography, Avilen et al. (1997) acquired 4.5 fold purified active
enzyme. Crude streptokinase was purified by Tomar (1968) and 10 fold pure enzyme was obtained after employing ion exchange chromatography. The dialyzed sample was used for a DEAE-Sepharose balanced with phosphate buffer and bound protein was eluted by applying 1.0M NaCl at the drift speed of 2 ml/min. The activity of enzyme was 31.1 units and protein contents were 18.7mg (Hua et al., 2008). Several schemes have been described for recovery and purification of streptokinase either from the commercially available crude preparations or the fermentation broths of various Streptococci (Perez et al., 1998). De Renzo et al. (1967) purified streptokinase from a relatively crude commercial preparation by using ion exchange chromatography. Column chromatography on DEAE-cellulose was followed by column electrophoresis in sucrose density gradients to obtain a five- to six fold increase in purity. Repeated chromatography was necessary to remove the last detectable traces of impurities. In a similar procedure, starting from crude Varidase, Taylor and Botts (1968) attained a final specific activity of 100,000 units of streptokinase per mg of protein. This required a combination of ion exchange (DEAE-Sephadex A-50) and gel permeation (Sephadex G-100) chromatography.
Fig 4.23. Streptokinase production from wild and mutated *Streptococcus equisimilis* strains at different MnCl$_2$. 4H$_2$O concentrations.
Table 4.14.  Analysis of variance table for effect of MnCl$_2$. 4H$_2$O concentration on streptokinase production from wild and mutant strains of *Streptococcus equisimilis*

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>Degrees of freedom</th>
<th>Sum of squares</th>
<th>Mean squares</th>
<th>F-value</th>
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</thead>
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<td>Concentration</td>
<td>4</td>
<td>387410</td>
<td>96852.6</td>
<td>2271.22**</td>
</tr>
<tr>
<td>Treatment</td>
<td>4</td>
<td>35615</td>
<td>8903.8</td>
<td>208.80**</td>
</tr>
<tr>
<td>Concentration x Treatment</td>
<td>16</td>
<td>133929</td>
<td>8370.6</td>
<td>196.29**</td>
</tr>
<tr>
<td>Error</td>
<td>50</td>
<td>2132</td>
<td>42.6</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>74</td>
<td>559087</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

** = Highly significant (P<0.01)
Fig 4.24. Streptokinase production from wild and mutated *Streptococcus equisimilis* strains at different inoculum size.
Table 4.15. Analysis of variance table for effect of Inoculum size on production of streptokinase from wild and mutant strains of *Streptococcus equisimilis*

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>Degrees of freedom</th>
<th>Sum of squares</th>
<th>Mean squares</th>
<th>F-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inoculum size</td>
<td>3</td>
<td>187585</td>
<td>62528.5</td>
<td>1420.39**</td>
</tr>
<tr>
<td>Treatment</td>
<td>4</td>
<td>128051</td>
<td>32012.8</td>
<td>727.20**</td>
</tr>
<tr>
<td>Inoculum size x Treatment</td>
<td>12</td>
<td>15918</td>
<td>1326.5</td>
<td>30.13**</td>
</tr>
<tr>
<td>Error</td>
<td>40</td>
<td>1761</td>
<td>44.0</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>59</td>
<td>333316</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

** = Highly significant (P<0.01)
Fig 4.25. Streptokinase production from wild and mutated *Streptococcus equisimilis* strains at different CaCO₃ concentrations
### Table 4.16. Analysis of variance table for effect of CaCO$_3$ concentration on streptokinase production from wild and mutant *Streptococcus equisimilis*

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>Degrees of freedom</th>
<th>Sum of squares</th>
<th>Mean squares</th>
<th>F-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentration</td>
<td>6</td>
<td>366202</td>
<td>61033.7</td>
<td>1435.25**</td>
</tr>
<tr>
<td>Treatment</td>
<td>4</td>
<td>309057</td>
<td>77264.3</td>
<td>1816.92**</td>
</tr>
<tr>
<td>Concentration x Treatment</td>
<td>24</td>
<td>69643</td>
<td>2901.8</td>
<td>68.24**</td>
</tr>
<tr>
<td>Error</td>
<td>70</td>
<td>2977</td>
<td>42.5</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>104</td>
<td>747879</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

** = Highly significant (P<0.01)
4.4.3. Gel filtration chromatography:

Streptokinase can be isolated from the fermentation broths of various streptococci. For purification and recovery of streptokinase, numerous schemes have been described and same techniques can be used for the purification and recovery from the commercially accessible crude preparations (Banerjee et al., 2004). For gel filtration permeation chromatography, sephadex G-150 is used, a polymer containing cross linking with selected sized pores. In sephadex columns, bigger proteins travel quicker as compared to smaller ones as straighter route is taken by bigger molecules through the column. Through the column, more tortuous path is followed by the smaller proteins as when they enter the pores their speed of movement get reduced (Kelly and Reddy, 1986).

According to the differences in molecular size, molecules are separated by using gel filtration chromatography. By permeating the sample by a bed of absorbent and uncharged gel units, separation was achieved. Due to the economy, rapidity and simplicity of the method, gel filtration is widely accepted. Highly reproducible results are obtained by using gel filtration, whenever sufficient molecular size differences exist among sample substances. Large sample volume is easily accomplished along with 100% solute recoveries. Labile substances are effectively purified by using gel filtration as it is an enormously moderate technique that infrequently results in denaturation (Jakoby, 1971). The 53th fraction of UV, 51th of sodium azide, 55th of Gamma and 51th fraction of streptokinase produced from ethidium bromide derived mutants recovered from ion exchange chromatography was used for Sephadex G-150 column for gel filtration (size exclusion chromatography). After gel permeation it was perceived that enzyme produced from EBL-UV 210 mutant attained 1116.66 U mg⁻¹ specific activity with 41.92 fold purification and 69.79 % recovery. In comparison, enzyme produced from EBL-EB 240 strain showed 1659.09 U mg⁻¹ specific activity with 66.52 fold purification and 85.08 % recovery. Results are presented in table 4.17, 4.18, 4.19 and 4.20.

After gel filtration 55th fraction of enzyme produced from EBL-G 120 strain resulted in 1428.57 U mg⁻¹, 59.52 times fold purification and 75.94% recovery whereas 51th fraction of SK produced from sodium azide mutated strain exhibited 2000 U mg⁻¹ specific activity with 71.94 times fold purification and 64.51% recovery respectively. These results supersede the findings of Taylor and boots (1968) who obtained final specific activity of 1000 U mg⁻¹ after gel filtration chromatography. Recombinant SK produced by fermentation in E.coli K12, was purified by Perez
et al., (1998) with recovery yield of 49% and 99% purity. Zhang et al., (1999) purified streptokinase by gel filtration and found 95.7% purity and specific activity was $1 \times 10^5$ IU/mg. The gel column used by Zhang was Q-sepharose and sepharose G-10. Babu 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 sephadex 75 gel filtration column (1.0cm × 30cm) equilibrated with buffer and flow rate was 0.5ml/min. The activity of enzyme was 5.6 units, protein contents were 0.5mg and specific activity was 11.2units/mg. Babashamsi et al. (2009) attained 95% recovery after gel filtration chromatography. By using gel permeation (Sephadex G-100) chromatography and ion exchange (DEAE-Sephadex A-50) in combination, Einarsson et al. (1979) purified streptokinase and obtained 5-6 times increase in fold purification. Rodriguez et al. (1994) used the method in which a crude precipitate of streptokinase was obtained by using ammonium sulfate fractionation. Which was then dissolved and applied on gel permeation chromatography. DEAE-Sepharose column chromatography was used for further purification of this treated SK fraction. This resulted in 30% decrease in specific activity of streptokinase but in present study by using Sephadex G-150, there was drastic increase in specific activity of the enzyme. Patel et al. (2011) mutated the wild Streptococcus equisimilis strain with UV irradiation and got hyper production of streptokinase. Final purification was done by using gel permeation chromatography. In comparison to present work they obtained 9.68 mg mL$^{-1}$ protein and 296.67 U mg$^{-1}$ specific activity of the final purified enzyme. Whereas in the present research work, 1116.66 and 2000 U mg$^{-1}$ of specific activity were obtained after the gel filtration chromatography of SK produced from UV and sodium azide mutated Streptococcus equisimilis.
Figure: 4.26. Ammonium sulphate precipitation of streptokinase produced from wild Streptococcus equisimilis strain
Figure: 4.27. Ammonium sulphate precipitation of streptokinase produced from UV mutated *Streptococcus equisimilis* strain
4.4.4. Sodium Dodecyl sulfate polyacrylamide gel electrophoresis

When the purified enzyme was treated with mercaptoethanol while performing SDS-PAGE (10%), this established single band with a movement conforming to a molecular weight of 45 KDa. The enzyme consists of two matching subunits as demonstrated by this fact (Fig 4.3). The molecular mass of enzyme was in accord with that articulated by Reza et al. (2007) and Hermentin et al. (2005), while Ko et al. (1995) obtained 47 KDa molecular mass of purified streptokinase. SDS PAGE was performed with 12% resolving gel and 4% stacking gel to find out the molecular weight of enzyme streptokinase from streptococcus equisimilis. Staining was done by silver stain and destaining solution was 5% acetic acid. SDS-PAGE was done by using 25Mm tris buffer (pH 8.3) containing 0.1% (w/v) SDS as the mobile buffer to find the molecular weight 47K Da of streptokinase from Streptococcus Pyogenes (Felsia et al., 2011) Neger et al. (2008) performed SDS-PAGE with 100Mm tris buffer, 20% glycerol, 4% SDS, 2% β-mercaptoethanol and 0.2% bromophenol blue to solubulize the fusion protein. Coomassie brilliant blue was used to stain the gel. The figure depiction of SDS PAGE of streptokinase is presented in figure 4.44. In an SDS- PAGE analysis, the eluted product showed two bands of approximately 47 and 45 KDa.

4.5 Kinetic and Thermodynamic studies

Different kinetic parameters such as pH, temperature, substrate level and enzyme reactivation and inactivation were used to study the influence on the enzyme activity (Tueten et al., 1993; Babu et al., 2008; Rasul et al., 2011).

4.5.1. Optimum pH

The deviation in hydrogen ion concentration ($H^+$) is very important in case of functioning of enzymes. Substrate binding involves ionization of vital active site amino acid residues and eventually catalysis i.e. breakdown of substrate into products. Margin of active site may contain some ionisable residues, which are normally identified as non-essential residue. Modification of active site cleft may result in ionization of these residues and hence indirectly can influence the activity of enzyme (Niaz et al., 2004). Streptokinase was analysed at diverse pH extending from 4-8 to seek the optimum pH. Streptokinase from wild and sodium azide mutant showed optimum activity (293 and 416 U mL$^{-1}$) at pH 7 as indicated in Fig 4.32 and 4.33. Though,
it was perceived that pH range of 6-8 is best for good activity of streptokinase purified from *Streptococcus equisimilis*. Streptokinase activity increased up to pH 7 and then decreased. Altered streptokinase and native streptokinase showed different activity at diverse pH. At pH 12 streptokinase performance was reduced in comparison to the altered streptokinase.
Figure: 4.28. Ammonium sulphate precipitation of streptokinase produced from Sodium azide mutated *Streptococcus equisimilis* strain
Figure: 4.29. Ammonium sulphate precipitation of streptokinase produced from gamma irradiated *Streptococcus equisimilis* strain.
Streptokinase (Monica et al., 1979). Streptokinase showed decreased activity in acidic pH and activity enhanced in alkaline pH (Christensen 1947). Above stated results were favourably compared to those of Nemirovich-Danchenko et al. (1985) and Hyun et al. (1997) who studied that pH range of 6.8-7.5 is best for normal activity of streptokinase with optimal activity at pH 7.4. Davis et al. (1965) obtained maximum streptokinase activity at pH 7.5.

4.5.2. Optimum temperature

Wild and sodium azide mutated *Streptococcus equisimilis* resulting streptokinase was assessed at various temperatures ranging from 20-80°C at pH 7. The enzyme from wild strain showed maximal performance of 295 U mL⁻¹ at 40°C. It is clear from the Arrhenius plot that the streptokinase had a single conformation and idebility was seen after transition temperature. Dubey et al. (2011) obtained maximum activity of enzyme at 40°C. Streptokinase from sodium azide mutated strain showed maximum activity (416 U mL⁻¹) at 45°C. Several authors reported significant decrease in enzyme activity and maximum activity at a temperature range between 25-30°C (Wong et al., 2008). Streptokinase activity was calculated at 20, 25, 30, 35, 40, 45, 50, 55, 60 and 65°C. Maximum activity of streptokinase was found at 30°C (Hua et al., 2008).
Figure: 4.30. Ammonium sulphate precipitation of streptokinase produced from ethidium bromide mutated *Streptococcus equisimilis* strain
4.5.3. Determination of Michaelis-Menten constants

By spending diverse concentrations of fibrinogen from 0.48-1.19 µg mL⁻¹, the Michaelis-Menten kinetic constants (Vₘₐₓ, Kₘ, Kₜₐₜ, Kₜₐₜ/Kₘ) were calculated. By keeping enzyme concentration constant, Streptokinase activity was calculated on each fibrinogen level. Great affinity for fibrinogen was shown by streptokinase of wild *Streptococcus equisimilis* as it has small Km value for the substrate. This feature belongs to possessions of biotechnological relevance. In addition to its long term solidity in the pH range 6-8, this extraordinary substrate attraction and specificity confer the enzyme obtained from *Streptococcus equisimilis*, as an appropriate biocatalyst for medical uses. Puried SK from sodium azide mutated strain EBL-SA 240 exhibited 400 U mL⁻¹ while its Km value was 26.31 µ mol and Vₘₐₓ was 50 as determined from Lineweaver-Burk plot, with specificity constant (Kₜₐₜ / Kₘ) of 1762.43 s⁻¹ mM⁻¹.

4.5.4. Irreversible thermal denaturation

Melting temperature (Tₘₐₓ) is the temperature at the center of thermal unfolding transition of streptokinase and it was 60°C for the streptokinase produced from wild strain whereas melting temperature was 65°C for mutant produced streptokinase(Fig. 4.40 and 4.41.). Enzyme showed heat stability at 45°C with half-life of 244 min, whereas at 70°C stability was reduced and a half-life of 69 min was showed under same circumstances (Fig 4.42 and 4.43). The capability to attain stable enzymes is critical for their application as bio catalytic interactions (Jaenicke, 1991). From both commercial and scientific point of views, comprehensive clarification of the methods accountable for maintenance and undermining of enzymes particularly at raised temperatures is of distinct significance (Kristjansson and Kinsella, 1991). Attention in thermostable enzymes is mostly owed to the point that almost all industrial enzyme procedures are performed under uncertain/uncharacteristic physical environments, like elevated temperature, high pressure and high pH. Temperature is one of the utmost significant environmental aspects, supervising the life and biological activities of microorganisms and is possibly the greatest enhanced physically mutable in chemical reactions (Ward and Moo-Young, 1988). In industrial practices, enzymes acquired from heat resistant organisms offer some major compensations such as reduction of the amount of enzyme required is seen when the rate of reaction is elevated.
Table 4.17: Purification summary of streptokinase produced from wild and UV mutated *S. equisimilis*:

<table>
<thead>
<tr>
<th>Purification Stage</th>
<th>Activity (U/ml)</th>
<th>Protein (mg/ml)</th>
<th>Specific activity (U/mg)</th>
<th>Fold Purification</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wild</td>
<td>Mutant</td>
<td>Wild</td>
<td>Mutant</td>
<td>Wild</td>
</tr>
<tr>
<td>Crude</td>
<td>370</td>
<td>480</td>
<td>27.5</td>
<td>18.02</td>
<td>13.45</td>
</tr>
<tr>
<td>(NH₄)₂SO₄ desalted</td>
<td>328</td>
<td>456</td>
<td>11.5</td>
<td>5.02</td>
<td>28.52</td>
</tr>
<tr>
<td>DEAE-cellulose</td>
<td>300</td>
<td>400</td>
<td>6.4</td>
<td>1.44</td>
<td>46.87</td>
</tr>
<tr>
<td>Sephadex G-150</td>
<td>290</td>
<td>335</td>
<td>3.1</td>
<td>0.30</td>
<td>93.54</td>
</tr>
</tbody>
</table>
Table 4.18: Results for purification of streptokinase by Ammonium sulfate precipitation, dialysis and column chromatography from Gamma mutated *Streptococcus equisimilis*

<table>
<thead>
<tr>
<th>Purification Steps</th>
<th>Enzyme Activity U/mL</th>
<th>protein mg/mL</th>
<th>Specific Activity U/mg</th>
<th>Fold Purification</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>W</em></td>
<td><em>M</em></td>
<td>W</td>
<td>M</td>
<td>W</td>
</tr>
<tr>
<td>Crude Extract</td>
<td>370</td>
<td>395</td>
<td>27.5</td>
<td>16.5</td>
<td>13.45</td>
</tr>
<tr>
<td>Dialysis</td>
<td>328</td>
<td>381</td>
<td>11.5</td>
<td>4.9</td>
<td>28.52</td>
</tr>
<tr>
<td>Column Chromatography (Ion exchange)</td>
<td>300</td>
<td>357</td>
<td>6.4</td>
<td>1.32</td>
<td>46.9</td>
</tr>
<tr>
<td>Gel filtration</td>
<td>290</td>
<td>300</td>
<td>3.1</td>
<td>0.21</td>
<td>93.54</td>
</tr>
</tbody>
</table>

*Where W represents Wild strain and M represents Mutant strain.*
4.5.5. Thermodynamics of irreversible thermal inactivation

The potential of enzyme to defend in contradiction of thermal unfolding by heat in the absence of substrate is represented as thermostability and thermophilicity is the capability of enzymes to perform at elevated temperatures in the substrate presence (Georis et al., 2000). Enthalpy of denaturation (ΔH*) of the streptokinase derived from mutant Strep tococcus equisimilis was calculated to be 2.60 kJ/mol at 40°C. It was shown that free energy of thermal denaturation was calculated as 43.67 kJ/mol at 45°C. It was shown that ΔH* increased with increase in temperature. The value of free energy of thermal denaturation ΔG* exhibited virtually continuous tendency with temperature rise. When entropy of inactivation (ΔS*) was intended at each temperature, negative values were obtained which indicated the thermodynamically stable state of SK. Streptokinase exposed a ΔS* value of and -197.32 J mol⁻¹k⁻¹ at 45°C. The results are shown in Table 4.21.

Different experiments permitted the unfolding procedures of explicit streptokinase domains to be supervised and the absolute stabilities and inter domain interfaces to be categorized. Results demonstrated that streptokinase can be present in a number of incompletely unmolded forms, in which distinct domains of the protein behaved as sole cooperative units. In the first thermal transition, B domain unfolds compliantly at approximately 46°C and its steadiness is mainly sovereign of the occurrence of the other domains. The elevated temperature change in complete streptokinase corresponds to the unfolding of both domains A and C. Expressive thermal stability of domain C improved by its separation from the remaining chain. By contrast, cleavage of the Phe 63-Ala 64 peptide bond within domain A causes thermal destabilization of this domain. The two resulting domain portions (A1 and A2) adopted unstructured conformations when separated. A1 bound with high affinity to all fragments that contained the A2 portion (Conejero-Lara et al., 1996).

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, biotechnology 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 Strep tococcus equisimilis by using physical and chemical means. 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. Streptokinase isolated from chemically mutated \textit{Streptococcus equisimilis} is heat stable and can be applied for clinical presentations and use. The negative entropy of deactivation detected for streptokinase recommended that there was insignificant disorderness. A great value of free energy of thermal denaturation at 80°C designated that the streptokinase showed the opposition against the thermal unmolding at elevated temperatures.

To conclude, this is the first attempt to hyper produce this valuable enzyme by mutagenesis of indigenous bacterial strain and then employing those mutants to fermentation for enzyme production. The SK was hyper produced by conducting the mutagenesis of \textit{Streptococcus equisimilis} (Local strain). The crude extract was obtained having specific activity of 370 U mg$^{-1}$ and 100% recovery was obtained. On further purification SK with 2000 U mg$^{-1}$ of specific activity and 64.51 % recovery was obtained. The kinetic properties of this hyper produced SK were also assessed. Referring to technological relevance, SK of wild \textit{Streptococcus equisimilis} showed improved affinity for fibrinogen due to its low K$_{m}$ value for the substrate. Mutagenesis of \textit{Streptococcus equisimilis} for enhanced production of SK is one of the marvelous works ever done in Pakistan. Its hyper production will be more helpful in health centers for the treatment of cardiovascular diseases and thrombosis. The kinetic properties of this mutated SK were also studied for the first time; this increased the novelty and reliability of this research project. Mutagenesis is best technique for strain development and in present study this resulted in improved streptokinase activity. In future this could be of great value as this study resulted in acquiring high yielding mutant strains of these isolates which can be applied for large scale production of streptokinase. This study resulted in production of 4 different mutants of \textit{S. equisimilis} which all resulted in hyper production of SK. Among these four mutants, \textit{S. equisimilis} EBL-SA 240 was selected as best mutant on the basis of less nutrient requirements n more SK production with improved thermo stability.
Table 4.19: Results for purification of streptokinase by Ammonium sulfate precipitation, dialysis and column chromatography from Ethidium bromide mutated *Streptococcus equisimilis*

<table>
<thead>
<tr>
<th>Purification Stage</th>
<th>Activity (U/ml)</th>
<th>Protein (mg/ml)</th>
<th>Specific activity (U/mg)</th>
<th>Fold Purification</th>
<th>%Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude</td>
<td>* W 370</td>
<td>*M 429</td>
<td>W 27.5 M 17.2</td>
<td>W 1 M 1</td>
<td>W 100 M 100</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(NH$_4$)$_2$SO$_4$ desalted</td>
<td>328 11.5</td>
<td>415 6.2</td>
<td>28.52 M 66.93</td>
<td>2.12 M 2.68</td>
<td>88.64 M 96.73</td>
</tr>
<tr>
<td>DEAE-cellulose</td>
<td>300 6.4</td>
<td>389 1.23</td>
<td>46.87 M 316.26</td>
<td>3.48 M 12.68</td>
<td>81.08 M 90.67</td>
</tr>
<tr>
<td>Sephadex G-150</td>
<td>290 3.1</td>
<td>365 0.22</td>
<td>93.54 M 1659.09</td>
<td>6.96 M 66.52</td>
<td>78.37 M 85.08</td>
</tr>
</tbody>
</table>

*Where W represents Wild strain and M represents Mutant strain.*
**Table 4.20:** Results for purification of streptokinase by Ammonium sulfate precipitation, dialysis and column chromatography from Sodium azide mutated *Streptococcus equisimilis*  

<table>
<thead>
<tr>
<th>Purification Stage</th>
<th>Activity (U/ml)</th>
<th>Protein (mg/ml)</th>
<th>Specific activity (U/mg)</th>
<th>Fold Purification</th>
<th>%Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>W</em></td>
<td>*M</td>
<td>*W</td>
<td>*M</td>
<td>*W</td>
</tr>
<tr>
<td>Crude</td>
<td>370</td>
<td>620</td>
<td>27.5</td>
<td>22.3</td>
<td>13.45</td>
</tr>
<tr>
<td>(NH₄)₂SO₄ desalted</td>
<td>328</td>
<td>520</td>
<td>11.5</td>
<td>7.25</td>
<td>28.52</td>
</tr>
<tr>
<td>DEAE-cellulose</td>
<td>300</td>
<td>482</td>
<td>6.4</td>
<td>1.03</td>
<td>46.87</td>
</tr>
<tr>
<td>Sephadex G-150</td>
<td>290</td>
<td>400</td>
<td>3.1</td>
<td>0.20</td>
<td>93.54</td>
</tr>
</tbody>
</table>

*Where W represents Wild strain and M represents Mutant strain.*
Table 4.21: Kinetic and thermodynamic parameters for irreversible thermal inactivation of streptokinase

<table>
<thead>
<tr>
<th>Temperature (K)</th>
<th>$K_d$ (min)</th>
<th>$t_{1/2}$ (min)</th>
<th>$\Delta H^*$ (KJ mol$^{-1}$)</th>
<th>$\Delta G^*$ (KJ mol$^{-1}$)</th>
<th>$\Delta S^*$ (J mol$^{-1}$ k$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>313</td>
<td>4.7</td>
<td>171</td>
<td>43.28</td>
<td>97.77</td>
<td>-189.88</td>
</tr>
<tr>
<td>318</td>
<td>3.9</td>
<td>244</td>
<td>43.67</td>
<td>101.26</td>
<td>-197.32</td>
</tr>
<tr>
<td>323</td>
<td>6.10</td>
<td>163</td>
<td>43.84</td>
<td>101.32</td>
<td>-195.12</td>
</tr>
<tr>
<td>328</td>
<td>5.76</td>
<td>159</td>
<td>43.78</td>
<td>103.02</td>
<td>-195.67</td>
</tr>
<tr>
<td>333</td>
<td>9.97</td>
<td>93</td>
<td>43.70</td>
<td>100.11</td>
<td>-191.34</td>
</tr>
<tr>
<td>338</td>
<td>10.4</td>
<td>87</td>
<td>43.64</td>
<td>102.34</td>
<td>-194.01</td>
</tr>
<tr>
<td>343</td>
<td>12.6</td>
<td>69</td>
<td>43.51</td>
<td>103.66</td>
<td>-195.38</td>
</tr>
<tr>
<td>348</td>
<td>13.8</td>
<td>61</td>
<td>43.48</td>
<td>104.48</td>
<td>-196.70</td>
</tr>
<tr>
<td>353</td>
<td>18</td>
<td>45</td>
<td>43.41</td>
<td>105.28</td>
<td>-196.93</td>
</tr>
</tbody>
</table>
Fig 4.32. Effect of pH on enzyme activity from wild *Streptococcus equisimilis*

Fig 4.33. Effect of pH on enzyme activity from sodium azide derived mutant *Streptococcus equisimilis*
Fig 4.34. Effect of temperature on enzyme activity from wild *Streptococcus equisimilis*

Fig 4.35. Effect of temperature on enzyme activity from sodium azide mutant *Streptococcus equisimilis*
Figure 4.36. Arrhenius plot for activation energy requirement of streptokinase produced from wild strain for lysis of fibrin clot

Figure 4.37. Arrhenius plot for activation energy requirement of streptokinase produced from Sodium azide mutated strain for lysis of fibrin clot
Figure 4.38. Lineweaver Burk plot for calculation of Km and Vmax of SK produced from wild *Streptococcus equisimilis*

Figure 4.39. Lineweaver Burk plot for calculation of Km and Vmax of SK produced from sodium azide mutated *Streptococcus equisimilis*
Figure 4.40. Melting temperature of Streptokinase produced from wild *Streptococcus equisimilis*

Figure 4.41. Melting temperature of Streptokinase produced from sodium azide mutated *Streptococcus equisimilis*
Figure: 4.42. Irreversible thermal denaturation of streptokinase produced from wild *Streptococcus equisimilis*

Figure: 4.43. Irreversible thermal denaturation of streptokinase produced from sodium azide mutated *Streptococcus equisimilis*
Figure: 4.44. Evaluation of streptokinase purification by SDS-PAGE after ion exchange and gel filtration chromatography

Right to left:

1- Molecular marker
2, 3, 4, 5, 6. Purified and dialyzed sample
Chapter # 5

SUMMARY

The development of blood clot in the circulatory system as a result of failed hemostasis leads to serious events like stroke and myocardial infarction. This requires clinical intervention of thrombolytic agents like Urokinase, streptokinase and Tissue type plasminogen activator (tPA). Among these fibrinolytic agents SK has attracted great attention because it is cost effective. An important plasminogen activator known as streptokinase is naturally produced by most of the group C, A and G Streptococci which are sequestered from human hosts. This streptokinase mainly plays its role in the transformation of the plasma zymogen, plasminogen, to the serine protease plasmin. Blood clots are mainly made up of principal protein part known as fibrin. This fibrin in blood clots is consequently degraded by plasmin and this plasmin is produced by the action of 1:1 stoichiometric complex of streptokinase and human plasminogen on plasminogen. In treatment of coronary blockage and acute myocardial infarction, one of the best treatment is intravenous induction of streptokinase. Streptococcus equisimilis strain was isolated from indigenous blood and biomass sources. Isolated Streptococcus equisimilis was cultured on blood agar media and Todd Hewitt Broth (THB) and then store at 4°C. The central objective of this study was to subject this indigenous Streptococcus equisimilis strain to physical and chemical mutagens and then to produce streptokinase from these derived mutants in liquid state fermentation using corn steep liquor as substrate in medium. Mutagenesis was achieved by physical and chemical agents and four hyperproducing mutants were achieved in result of this research work, which were Streptococcus equisimilis EBL-G-120, EBL-UV-210, EBL-SA-240 and EBL-EB-240.

To achieve the hyper production of SK, optimization of the fermentation media was done in which numerous parameters were optimized after mutagenesis. It was observed that UV, Sodium azide, Gamma and ethidium bromide derived mutants showed maximum streptokinase production at 4%, 4 %, 5 and 5% substrate concentration. These all mutants showed maximal activity after 24hrs, 24hrs, 36 and 24hrs of fermentation period. Maximum Enzyme production was obtained at pH of 7.5, 7, 7.5 and 7 from UV, Sodium azide, Gamma and ethidium bromide derived mutants of Streptococcus equisimilis. Optimum temperature observed for best enzyme production was 37°C, 45, 37 and 37°C for UV, Sodium azide,
Gamma and ethidium bromide derived mutants. Best glucose concentration for maximum enzyme production from these mutants was 2%, 4, 2 and 3% respectively. UV, Sodium azide, Gamma and ethidium bromide derived mutants exhibited maximum streptokinase production at 3%, 2, 2 and 3% concentration of yeast extract. Finally UV irradiated strain resulted in 335 U/mL activity with 1116.66 U/mg specific activity, 0.30 mg/mL protein, 41.92 fold purification and 69.79% recovery whereas Sodium azide derived mutant resulted in 400 U/mL activity with 2000 U/mg specific activity, 0.20 mg/mL protein, 71.94 fold purification and 64.51% recovery of the finally purified enzyme. Gamma irradiated strain exhibited 300 U/mL activity with 1428.57 U/mg specific activity, 0.21 mg/mL protein, 59.52 fold purification and 75.94% recovery whereas ethidium bromide derived mutant showed 365 U/mL activity with 1659.09 U/mg specific activity, 0.22 mg/mL protein, 66.52 fold purification and 85.08% recovery.

Purified enzyme was further proceeded to check the effect of different parameters like substrate concentration, pH and temperature on activity of SK. Optimum pH and temperature of the finally purified enzyme was 7 and 45°C. The potential of streptokinase to defend in contradiction of thermal unfolding in the absence of substrate was called thermostability of streptokinase. Enthalpy of denaturation (ΔH*) of streptokinase at 45°C was 43.67 kJ/mole. The Energy of thermal denaturation ΔG* was 101.14 kJ/mole and entropy of inactivation ΔS* was -197.32 kJ/mole at 45°C. The negative value of ΔS* indicated that streptokinase was thermodynamically stable. K_m and V_max values of streptokinase were 26.31 mM and 50 MS⁻¹. Streptokinase produced from sodium azide derived mutant exhibited activity within the pH range of 6 to 8 while it presented its best performance at pH 7. Thermal stability between 45°C to 80°C was shown by the streptokinase along with half-life of 244 minutes while less stability was shown at 80°C along with 45 minutes of half-life and 43.41 kJ/mole as enthalpy of denaturation (ΔH*).


analysis, and characterization of streptokinase’s secreted by porcine and equine isolates of Streptococcus equisimilis. 67(12): 6478-6486.


Lunardi, J., L. A. Basso and D. S. Santos. 2009. Scale up of the Recombinant Streptokinase (Streptococcus dysgalactie subsp. equisimilis) Production in Bioreactor. Programa de Pós-Graduação em Biologia Celular e Molecular, Faculdade de Biociências, PUCRS.


