

ENHANCED PRODUCTION OF STREPTOKINASE BY CHEMICAL MUTAGENESIS OF STREPTOCOCCUS SPECIES

*Submitted in partial fulfilment of the requirements for the award of Master of Science
in Medical Biotechnology and Clinical Research*

By

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BONAFIDE CERTIFICATE

This is to certify that this Project Report is the bonafide work of **Mr. JOHN ANTONY P (42101007)** who carried out Project entitled "**ENHANCED PRODUCTION OF STREPTOKINASE BY CHEMICAL MUTAGENESIS OF STREPTOCOCCUS SPECIES**" under the supervision of **Dr. A. Dayanandan, Professor (Research)** from September 2023 to April 2024.



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DECLARATION

I, **Mr. JOHN ANTONY P (42101007)** hereby declare that the Project Report entitled **"ENHANCED PRODUCTION OF STREPTOKINASE BY CHEMICAL MUTAGENESIS OF STREPTOCOCCUS SPECIES"** done by me under the guidance of **Dr. A. DAYANANDAN Professor (Research)** at the Centre for Molecular and Nanomedical Sciences, Sathyabama Institute of Science and Technology, Chennai, is submitted in partial fulfilment of the requirements for the award of Master of Science in Medical Biotechnology and Clinical Research.

DATE: 18/04/2024

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SIGNATURE OF THE CANDIDATE

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John Antony P

Abstract

An extracellular enzyme called streptokinase, which is obtained from different strains of β -hemolytic streptococci, is used as an intravenous thrombolytic treatment for diseases such deep vein thrombosis, pulmonary embolism, and acute myocardial infarction. With 414 amino acid residues and a molecular weight of 47 kDa, this single-chain polypeptide promotes fibrinolysis via activating circulatory plasminogen. Strict biosafety measures are necessary due to its immunogenic potential, which raises biosafety concerns during commercial manufacturing. The inability of streptokinase to produce plasminogen in the absence of fibrin and its fibrin-independent "catalytic switch" activation process limit the enzyme's efficacy. The synthesis of streptokinase is increased by chemically mutating *Streptococcus* species. The fibrin plate assay facilitates the detection of streptokinase activity, which helps with qualitative identification. The function of streptokinase in streptococcal pathogenicity. It also serves as a differential medium for the detection of hemolysis, which is the destruction of red blood cells brought on by cytolytic toxins produced by particular strains of the bacteria *Aerococcus*, *Bacillus*, *Streptococcus*, and *Staphylococcus*. Blood agar is an excellent medium for the cultivation of picky bacteria that do not flourish on broad media like Nutrient Agar and need particular nutrients. Approximately 5% of defibrinated mammalian blood (human, sheep, or horse) is added to the autoclaved basal media to create blood agar medium. High rates of morbidity and mortality are associated with ST-segment elevation myocardial infarction (STEMI), the most severe form of coronary artery disease.

Key Words: Streptokinase, *Streptococcus*, Chemical mutagenesis, Ethylmethanesulfonate.

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LIST OF ABBREVIATIONS

TPA	Tissue Plasminogen Activator
AMI	Acute Myocardial Infarction
CVD	Cardio Vascular Diseases
DVT	Deep Vein thrombosis
EMS	Ethyl Methanesulfonate
HBsAg	Hepatitis B Surface antigen
SK	Streptokinase

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CHAPTER-1

1. INTRODUCTION

1.1 Streptococcus

Streptococcus is a genus of spherical bacteria or gram-positive cocci (also known as cocci) that are members of the Streptococcaceae family, within the phylum Bacillota and the order Lactobacillales (lactic acid bacteria). The Streptococcus genus, *Streptococcus pyogenes* is a species of aerotolerant, Gram-positive bacteria. These extracellular bacteria are composed of non-sporing, non-motile cocci that have a tendency to form chains. Streptokinase is an extracellular protein secreted by various strains of streptococci and is used clinically as an intravenous thrombolytic agent for the treatment of acute myocardial infarction.

Streptokinase is a more economical treatment option for acute myocardial infarction than recombinant tissue plasminogen activator (tPA). It is equally effective in treating the condition. Because streptokinase has the potential to trigger immunogenic reactions in process workers, biosafety precautions must be given particular attention throughout commercial manufacturing. Streptococci are catalase-negative, nonmotile, gram-positive, nonspore-forming cocci that grow in chains or pairs. Cultures that are older could become less Gram-positive. While certain streptococci are obligate (strict) anaerobes, the majority are facultative anaerobes. Most need blood agar, or enriched media. The hyaluronic acid capsule of group A streptococci is present. The synthesis of streptokinase from *Streptococcus pyogenes* species and its partial purification using column chromatography, dialysis, and ammonium sulfate precipitation. Streptokinase is an extracellular protein secreted by various strains of streptococci and is used clinically as an intravenous thrombolytic agent for the treatment of acute myocardial infarction.

Streptokinase, an enzyme derived from numerous naturally occurring strains of β -haemolytic streptococci isolated from the upper respiratory tract, is used to break down the fibrin matrix of blood clots, particularly those in the heart and lung arteries. Streptokinase is a single-chain polypeptide with 414 amino acid

residues and a molecular weight of 47 kDa. Different strains of β -hemolytic Streptococci generate the extracellular enzyme streptokinase. By stimulating the circulatory plasminogen, the single polypeptide enzyme indirectly causes fibrinolysis. The pH of the protein is 4.7 when it is isoelectric, and it reaches its maximal activity at 7.5. Streptokinase is potentially immunogenic to process workers, hence biosafety concerns during commercial manufacturing need to be given extra attention. Blood clots within blood arteries are treated with these, although their effectiveness as a medication is reduced due to their inability to induce plasminogen in the absence of fibrin. It can be activated by a "catalytic switch" that contains the NH₂-terminal in both the presence and absence of fibrin. By a fibrin-independent mechanism, mutant SK missing NH₂-terminal is unable to activate plasminogen. In order to increase the production of the blood-clotting enzyme streptokinase, chemical agents are used to induce mutations in the bacterial genome. This process is known as chemical mutagenesis of Streptococcus species.

In order to treat myocardial infarction, pulmonary embolism, and deep vein thrombosis, streptokinase is administered therapeutically as a clot-dissolving agent. The fibrin plate assay is a frequently employed staining technique that enables the qualitative identification of streptokinase activity, to detect the existence of the streptokinase enzyme that is produced by Streptococcus species. By producing plasmin, streptokinase contributes to the pathogenicity of streptococcal bacteria. Plasmodium causes fibrinolysis, the breakdown of extracellular matrix, and the disruption of basement membrane components, which facilitates the spread of germs from the original site of infection. Additionally, by activating complement, streptokinase causes inflammation, which may contribute to post-infectious illnesses including glomerulonephritis. The previous few decades have seen an exponential expansion in the use of streptokinase in a variety of sectors, necessitating both qualitative and quantitative improvement.

1.2 Level of Classification for streptococcus

1. Domain: Prokaryotes
2. Kingdom: Bacteria
3. Phylum: Firmicutes

4. Class: Bacilli
5. Order: Lactobacillales
6. Family: Streptococcaceae
7. Genus: Streptococcus
8. Species: Streptococcus pyogenes

Streptococci divided into six group on the basis of their 16S rdna Sequences

- S. anginosus
- S. gallolyticus
- S. mitis
- S. mutans
- S. pyogenes and
- S. salivarius.

Numerous species of Streptococcus are known to cause illness in both humans and animals, while other species are crucial to the production of various fermented goods.

1.2.1 Streptococci infection divided into several groups

- Group A Streptococcus
- Group B Streptococcus
- Group C Streptococcus
- Group G Streptococcus

Group A streptococci: Group A strep, typically affects the skin and throat. In certain regions, people may carry GAS without exhibiting any symptoms. Though these bacteria seldom result in severe or even fatal illness, most group A strep infections cause rather minor illness.

There are several types in Group A Streptococcal infections that can range from mild infection like a soar throat, into severe or life threatening conditions.

I. Mild streptococcal infections

- **Cellulitis** - A tissue-deep infection that attacks subcutaneous tissues
- **Erysipelas** - An infection affecting the epidermis.
- **Impetigo** - Blisters and sores on your arms, legs, or in the area around your mouth and nose.
- **Sore throat** - Discomfort and pain in the throat.
- **Strep throat** - An infection of the tonsils and throat.

II. Severe streptococcal infections

- Infections of the bloodstream (bacteremia).
- An infection that results in a high temperature, sore throat, and rash (scarlet fever).
- Infected multiple organs (toxic shock syndrome).
- Rheumatic fever, or inflamed tissues in your heart and joints.
- Necrotizing fasciitis is a flesh-eating illness.

Group B streptococci: Group B streptococcus, generally referred to as group B strep or GBS, is a type of bacteria that can infect individuals of all ages, however it can be very serious in neonates, where it typically results in sepsis, pneumonia, and meningitis. Infections of the urinary system, skin, bloodstream, soft tissues, bones, and joints, as well as pneumonia, are the most frequent health problems in adults that result from GBS. According to the CDC, 1 in 20 non-pregnant adults who have an invasive strep B infection pass away on average.

Types of Infection Group B Streptococcal

- Bacteremia - A bloodstream infection
- Meningitis is an infection of the spinal cord and brain lining.
- Sepsis - The severe reaction of the body to an illness
- Pneumonia Illness of the lung
- Bone and joint infections
- Skin and soft tissue infections
- Urinary tract infections

Group C and G streptococci: Because the illnesses caused by group C and G streptococci are significantly less common than those caused by strep A and B,

much less is known about these bacteria. The most common hosts of Group C and G strep are horses and cattle, and contact with these animals or raw milk can transmit the illness to people. In addition to human skin, the bacteria can also reside in people's throats. This is especially true in sections of the skin that have been damaged by eczema or on mucous surfaces like the gut or vagina.

Types of Infections Group C and G streptococcal

GCS and GGS are common asymptomatic colonizers of the epidermis, gastrointestinal system, and female vaginal tract and are considered normal commensal flora of the human upper airway. In addition, they have been linked to pharyngitis, bacteremia, endocarditis, septic arthritis, osteomyelitis, puerperal infections, meningitis, and mono-and polymicrobial infections of the skin and soft tissues.

1.3 Production of streptokinase

Blood clots that have developed in the blood arteries can be removed with streptokinase. It is applied as soon as a heart attack occurs in order to increase patient survival. Blood clots in the legs (deep vein thrombosis) and lungs (pulmonary embolism) can also be treated with this medication. Streptokinase, an enzyme produced by Streptococcus bacteria, is used as a thrombolytic drug to break up fibrin blood clots, particularly those that have developed in the arteries leading to the heart and lungs. It is also used to remove clots that have formed in shunts during dialysis. Streptokinase was made by a variety of streptococci and bacteria that carried the genetic material from streptococcus of Lancefield groups A, C, and G. Agent thrombolytic is streptokinase. When patients receiving hemodialysis therapy, it is utilized as a medication to treat clots that have developed in the tubes by giving them medications for extended periods of time. Streptokinase research keeps improving the medication's effectiveness as a thrombolytic agent. In the therapy of cardiovascular disorders, streptokinase remains an important drug despite the presence of urokinase and tissue plasminogen activator. Because it is so affordable, streptokinase plays a significant role in the treatment of heart problems in underdeveloped nations. Using clot-dissolving or thrombolytic medications was the main aid in the treatment of cardiovascular diseases (CVD). Streptococci that generate β -haemolytic

produce SK, one of the main plasminogen activators. It is a protein, not an enzyme, and it forms the active complex with plasminogen to convert free plasminogen to plasmin that is used in proteolysis. SK administration's growing promise as a thrombolytic drug spurs the creation of quantitative augmentation techniques. For increased production of highly active SK, similar optimization of the medium composition and growth conditions is required.

Due to blood clot-induced blockage of blood circulation, thrombosis is most common in the aged population. For the treatment of conditions like stroke, acute myocardial infarction (AMI), deep vein thrombosis (DVT), and pulmonary embolism, a range of thrombolytic medicines with different pharmacologic and pharmacokinetic features are available. It has been established that the enzyme streptokinase is produced by β hemolytic *Streptococcus* belonging to Lancefield groups A, C, and G. Additionally, it was recently found that *S. agalactiae*, a member of Group B *Streptococcus*, produces SK. For the purpose of improving the production of highly active SK by EMS-induced mutations and maximizing the nutritional needs and culture conditions for the development of microorganisms, natively isolated *S. agalactiae* EBL-20 has been implemented. By utilizing a two-level Plackett-Burman (PB) architecture to screen carbon and nitrogen inputs, SK production was maximized. Traditionally, process variable optimization entails altering one variable at a time while keeping the others same. In practice, this strategy requires a lot of experiments to examine every potential combination of test variables. Response surface methodology (RSM) is an alternative that may be used to assess and comprehend how different process factors interact. RSM was effectively used to optimize bioprocess parameters. The synthesis of hepatitis B surface antigen (HBsAg) in *S. cerevisiae* using *P. pastoris*' GAP promoter has been previously described.

The SK expression made use of the same vector backbone. We have looked into the following in the current investigation

- (1) Using *P. pastoris*' GAP promoter to express SK intracellularly in *S. cerevisiae*.
- 2) Optimization of nutrients for SK production utilizing the aforementioned expression system using response surface methodology (RSM) and comparison between normal and baffled flasks.

(3) Qualitative SK detection by the Lyticase-nitroanilide test (LNA)

Using random mutagenesis, the amount of SK secreted by *Streptococcus equi* was increased. A silanizing agent that was placed around the SiO₂-MNPs and had a surface rich in 3-aminopropyltrimethoxysilane was used to encapsulate the tPA and SK A. In vitro thrombolysis testing was performed using fibrin-containing agarose plates and blood clot lysis tests. One method for streptokinase (SK) fermentation—the focus of this work—postulates the existence of both active and inactive cells, the latter of which develops from the former. Under such circumstances, the productivity and stability patterns are determined by the relative effects of noise on various cell types. Biologically active recombinant streptokinase was produced at high levels employing the *Pichia* cell recycling technique, which resulted in a volumetric product yield increase of up to 2.4fold at the shake flask level, reaching 3049.53 mg/L of rSK-His (PIR1).

1.4 Mutagenesis of streptococcus

Mutagenesis is the process by which an organism's genetic makeup is altered through the creation of a mutation. It could happen on its own in the wild or as a result of being around mutagens. Deoxyribonucleic acids (DNA) in an organism can alter through a process called mutagenesis, which can lead to a mutation in a gene. Mutations can lead to altered protein function and phenotypic alterations since they are heritable and permanent changes in genetic material. In cases where a specific target is prone to mutations, mutations might be site-specific. Exposure of wild strains of microorganisms that produce enzymes to physical or chemical stimuli is one of the random mutations. When it comes to preventing cancer, mutagenesis is a crucial phase in the carcinogenesis process that must be avoided. An rising body of research suggests that a wide variety of plants, phytochemicals, and the component formulations they contain can block the processes that lead to mutagenesis and cancer.

1.4.1 Types of Mutagenesis

- Chemical Mutagenesis
- Radiation Mutagenesis
- Site-Directed Mutageneis
- Transposon Mutagenesis

1.4.1.1. Chemical Mutagenesis

Chemical mutagenesis is the process of altering an organism's DNA by means of chemicals. One helpful method for quickly producing a large number of random mutations is chemical mutagenesis. It can, however, also result in a high frequency of non-specific mutations, which makes it challenging to pinpoint the precise mutations in charge of a given phenotype. Chemical mutagens can work through a number of different methods, including as:

- Frameshifting
- Base substitution and
- Breakage in DNA strands.

Random-and-extensive mutagenesis (REM) and site-directed mutagenesis (SDM) are the two main mutagenesis methods. A substantial portion of these techniques are carried out using polymerase chain reaction (PCR) and non-PCR.

1.4.1.2. Site – directed Mutageneis

Using the SDM approach, an amino acid can be changed in DNA by modifying it at a particular nucleotide position. The structure and function of proteins can be drastically altered by these substitution alterations. A DNA template containing the target gene, knowledge of the target gene's nucleotide sequence, and a short primer complementary to the target sequence, usually ranging from 20 to 30 base pairs, are required for this technique. The primer must be modified to contain a mismatched nucleotide, usually 1 to 3 base pairs, which will result in an amino acid change.

1.4.1.3. Random and Extensive method

Although there is less control over the final alterations, REM is a valuable method when multiple mutations are sought. Protein essential residues (such as EcoRV restriction endonuclease) have been mapped out with the aid of this approach.

1.5 Blood Agar

An enhanced medium called blood agar (BA) is used to cultivate bacteria or other microorganisms that are difficult to grow. Because these bacteria need a different, richer nutritional environment than other common bacteria, they are referred to as "fastidious" bacteria. Many pathogens, particularly those that are more challenging to grow, like *Neisseria* species, *Streptococcus pneumoniae*, and *Haemophilus influenzae*, can be grown on blood agar. Haemolytic bacteria must also be identified and distinguished, especially those belonging to the *Streptococcus* species. Additionally, it functions as a differential medium for the detection of hemolysis, or the breakdown of red blood cells caused by cytolytic toxins generated by specific strains of *Staphylococcus*, *Aerococcus*, *Bacillus*, and *Streptococcus* bacteria.

For the culture of fastidious bacteria that do not proliferate on general media such as Nutrient Agar and require specific nutrients, blood agar is a great medium. Blood agar medium is made by adding approximately 5% of defibrinated mammalian blood (human, sheep, or horse) to the autoclaved basal media. The most severe form of coronary artery disease, ST-segment elevation myocardial infarction (STEMI), is linked to high rates of morbidity and mortality. The majority of instances of STEMI are caused by a complete thrombotic blockage that results from an atherosclerotic plaque in an epicardial coronary artery. The best strategies to reduce myocardial ischaemia and infarct size and, consequently, the risk of heart failure and consequences following STMI are early detection and prompt reperfusion.

CHAPTER – 2

2. REVIEW OF LITERATURE

2.1 Streptococcus

The synthesis of streptokinase from *Streptococcus pyogenes* species and its partial purification using column chromatography, dialysis, and ammonium sulfate precipitation. Lowry's technique is used to quantify the enzyme, and SDS-PAGE is used to measure its molecular weight and electrophoretic mobility (X Freeda Felsia et al., 2011).

Better fibrinolytic activity can be produced by streptokinase through the use of trace elements, growth factors, co-factors, co-enzymes, inhibitors, activators, nitrogen and carbon sources, inoculum size, and ideal physical conditions, including temperature, pH, incubation time, and the right concentration of substrates, such as CSL, molasses, rice polishing, and sugarcane bagasse, to meet the needs of this enzyme (Hadia Naseem et al., 2021).

Streptokinase derivatives that are resistant to C-terminal degradation were created by region-specific combinatorial mutagenesis around the C-terminal processing sites. A derivative that exhibited a specific activity increase of 2.5 times was suggested to be a more effective thrombolytic drug (SL Wong et al., 1994).

According to the findings, UV and NTG were efficient mutagenesis agents for improving the *S. Equisimilis* T3 strain and increasing SK output. The high yielding mutant strain of *S. equisimilis* T3 isolate is thought to be commercially viable for large-scale industrial SK production (Thaer Taleb Abed Abdelghani et al., 2005).

Biological activity was observed in the partly purified SK. It was discovered that *Streptomyces lividans* is a useful host for the synthesis of a significant biopharmaceutical product with a global market (Elsa pimienta et al., 2007).

The capacity of an activator to directly convert plasminogens from guinea pigs and cows to plasmin can be used to characterize its activity. This conversion cannot be completed by plasmin, streptokinase, or plasminogen alone. The soy bean trypsin inhibitor does not inhibit the activator, and it can convert guinea pig plasminogen to plasmin in the casein guinea pig serum assay. Other characteristics of the activator include its capacity to cause lysis on unheated but not heated beef fibrin agar plates (F.B. Taylor Jr. et al., 1978).

The potential of this yeast rSK for thrombolytic therapy and large-scale manufacture was shown by its comparable in vitro thrombolytic activity to that of a commercial SK (Abdullah S.Assiri et al., 2014).

The toxicity of recombinant proteins to the expression host is still a major obstacle to their large-scale synthesis. In this investigation, the straightforward expedient of obtaining the product in the culture supernatant significantly increased the production of recombinant SK, which is harmful to its host. In high cell density fermentation, the maximum product accumulation in the culture supernatant was 4.25 g/L. the great yield and purity attained for this crucial macromolecule in medicine (Adivitiya et al., 2016).

According to current research, serum from immunized mice and people can identify recombinant streptokinase as an antigen. Thus, the epitopes of the natural version of this antigen and the recombinant SKa protein are identical. For the serologic diagnosis of *S. pyogenes* infections, recombinant SKa also appeared to be a promising antigen (Neda molaee et al., 2013).

The fibrin clot lysis method was utilized for the enzyme assay, and corn steep liquor (CSL) was added to the liquid state fermentation culture medium in quantities ranging from 0.1% to 0.8%. CSL was found to exhibit 44% blood lysis in liquid state fermentation culture media for the manufacture of streptokinase, with 0.3% being the optimal concentration among the tested concentrations (Abdul Ghaffar et al., 2015).

We are constantly searching for breeds that produce SK hyperproduction. Based on the available data, it was found that UV and EtBR were useful mutagens for improving the strain of *S. equisimilis*, with UV being the most efficient mutagen, resulting in 400% increased SK activity in mutant strain UV6, relative to natural type. Commercial exploitation of the mutant strain is possible (Naeem et al., 2018). In contrast to streptokinase, which has little to no impact and may only break down loculations in cases of empyema, DNase dramatically lowers pus viscosity. If these in vitro modifications result in therapeutic benefits, clinical research ought to be done. There may be further uses for the straightforward viscometer that was developed for these investigations (Graham Simpson MD et al., 2000).

The preparation and application of SiO₂-MNP-tPA-SK for the immobilization of tPA and SK was discovered during this investigation to have a high protein-loading efficiency and activity retention, which can mitigate the hemorrhagic adverse effects of the medication and enzyme. When compared to traditional treatment using free medicines and enzymes at the same dosage, magnetic targeted delivery of SiO₂-MNP-tPA-SK can effectively shorten the thrombolysis time (Ateke Tadayon et al., 2015).

The preparation and application of SiO₂-MNP-tPA-SK for the immobilization of tPA and SK was discovered during this investigation to have a high protein-loading efficiency and activity retention, which can mitigate the hemorrhagic adverse effects of the medication and enzyme. When compared to traditional treatment using free medicines and enzymes at the same dosage, magnetic targeted delivery of SiO₂-MNP-tPA-SK can effectively shorten the thrombolysis time (Wezenet Tewodros et al., 1995).

According to a sequence study of the transposon-hosting region, the lacZ fusion happened close to the 3'-end of a gene that codes for a homolog of an ATP-binding protein from a family of Gram-positive ABC transporters. These results show that environmental regulation of genes in *S. mutans* and maybe other therapeutically relevant streptococcal species can be identified using Tn917-lac mutagenesis (Dennis G. Cvitkovitch et al., 2000).

The digestive tract of mice was discovered to have *Streptococcus equinus* and other *Streptococcus* species that, when added to animals that had previously received antibacterial treatment, produced mutagenic activation of amoscanate. Similar to this, these strains produced a mutagenic product when incubated with amoscanate in vitro (C. J. Molineaux et al., 1981).

S. pneumoniae and *S. pyogenes* are two other common streptococcal pathogens, and while *S. sanguinis* is a significant disease in and of itself, the relative ease with which these discoveries may be applied to identify critical genes in these pathogens emphasizes the significance of this discovery. Furthermore, it appears from our analysis that most bacteria for whom an annotated genome sequence is available can have their important genes predicted using our methods with little extra work (Ping Xu et al., 2011).

The genetic mutations that set this strain apart from its original strain are unknown, and its genome has not yet been made public. In the experimental portion of this work, a whole-genome sequencing protocol for streptococcal hyaluronic acid manufacturers was established since this knowledge may be helpful in determining the origins of some of the unique manifestations of this strain (BcAdrián Baláži et al., 2022).

Strains of *S. mutans* lacking in Fpg, MutY, and Smn shown higher virulence in comparison to the parent strain in the *Galleria mellonella* pathogenicity model. According to our findings, *S. mutans* may be more virulent due to mutator phenotypes caused by the lack of BER enzyme (Kaisha Gonzalez et al., 2013).

In comparison to crude enzyme, 2.6 times less enzyme activity and 75.66 times more specific activity were observed after purifying procedures. Compared to crude extract, 38.09% more streptokinase was found in purified form. The increased activity per unit of the purified enzyme in this work is a step towards the cost-effective manufacture of streptokinase, which may have significant applications in the treatment of cardiovascular illnesses (Akbar et al., 2021).

It was discovered that ethyl methane sulfonate random mutagenesis was a more effective method of improving strain than UV irradiation. The mutant strain was discovered to be much more halophilic than the wild type, extremely

thermotolerant, and capable of growing at very high pH levels. Because of the very high KI value, the *S. venezuelae* mutant was thought to be extremely resistant to increased initial lactose concentrations (Balakrishnan Naveena et al., 2012).

The stability of streptokinase is largely dependent on its NH₂-terminal (Ile1-Lys59), and that inhibiting cleavage at Lys59 of streptokinase extends the half-life of streptokinase in complex with plasmin (Guey-Yueh Shi et al., 1998).

The formation of ATR, which depended on the culture's pH and the medium's initial phosphate concentration, had a significant impact on the rate and degree of streptokinase breakdown. The suppression of ATR caused by high pH and high initial phosphate concentration was observed to boost streptokinase productivity by at least 2.5 times and significantly reduce streptokinase degradation (Kalpana Sriraman et al., 2006).

According to the conclusion, current developments in structural and synthetic biology have improved SK, resulting in a longer half-life and lower antigenicity. These kinds of enzyme compositions seem like the ideal thrombolytic drugs (Aslam et al., 2020).

It is commonly recognized that streptokinase functions fibrinolytically and activates plasminogen. Through its interaction with plasminogen, streptokinase forms a "activator complex" that transforms plasminogen into the proteolytic enzyme. Plasmin is responsible for the breakdown of all plasma proteins, including fibrinogen and fibrin clots. Several techniques, such as batch processing and repeated batch processing, can be used to make them (Amul Sankha et al., 2023).

The thermodynamic stability of streptokinase was demonstrated by the negative value of ΔS^* . The streptokinase K_m and V_{max} values were 50 MS-1 and 26.31 mM, respectively. The pH range of 6 to 8 was where the sodium azide generated mutant streptokinase showed activity, with pH 7 being the optimal value (Faram et al., 2015).

We used CRISPR-Cas9 to successfully knockout the *SagD* gene in order to improve the stability of mRNA and increase the expression of streptokinase, which is inhibited by *SagA*. We observed a 13.58-fold increase in streptokinase transcript

level and a 1.48-fold increase in protein level in the mutant strain compared to wild type. We have successfully employed CRISPR-Cas9 to knock out the *SagD* gene in *S. equisimilis*, and the resultant modified strain of the bacteria can be exploited to overexpress streptokinase for therapeutic purposes (Armi M. Chaudhari et al., 2022).

After treating DEAE cellulose with $(\text{NH}_4)_2\text{SO}_4$ or hydroxylapatite, streptokinase was produced using ion-exchange chromatography. After processing, the finished product had 3-5% of the initial absorbance (280nm) and 22-40% of the initial activity. It was discovered that trypsin and streptokinase were very different proteins, and that streptokinase served as trypsin's substrate, shielding it from autolysis and DFP inhibition (Russell H tomar et al 1968).

According to the study, optimization studies may be a more cost-effective way to get SK for the treatment of heart patients. *S. agalactiae*, a newfound source of SK, was discovered to be just as appropriate as other β hemolytic *Streptococcus* species. The current study was designed to satisfy SK's growing needs in the near future. While native SK-based thrombolytic treatment is beneficial, its administration frequently causes allergic responses. Thus, it is imperative to give careful thought to the synthesis of genetically engineered SK that has a longer half-life in blood circulation and lower immunogenicity in the future (Arooj Arshad et al., 2018).

Numerous clinical trials conducted on patients suffering from acute myocardial infarction unequivocally demonstrate that intravenous infusion of streptokinase, as compared to placebo, is related with a decrease in both short- and long-term fatality. The current state of Sk production and its role as a clinically significant medication in the treatment of thrombotic diseases are discussed in this study. The history, the enzyme's sources, its mechanism of action, structural alterations that enhanced its functionality, and information on recombinant streptokinase's creation and present application are all covered (P. Suresh Kumar et al., 2012).

A 47.4 kDa protein and a smaller hydrolyzed protein (44 kDa) were discovered when the promoter for *sprT* [*Streptomyces griseus* trypsin (SGT)] was utilized in the host *S. lividans* TK24. This suggests that posttranslational hydrolysis had

occurred, as has been described in other expression systems. The experiment using casein/plasminogen showed that the plasmid construct with the SGT signal peptide produced more SK than the one with the SK signal peptide. The amount of SK that could be produced at its maximum rate in 0.25 units per milliliter of culture broth was determined; this was five times more than the amount that could be produced in the same host utilizing alternative expression methods that utilised the *ermE* and *tipA* promoters (Kim Hi-ran et al., 2012).

A reduction in the lag phase duration and an increase in the logarithmic phase growth rate are responsible for improvements in SK output. While the chemical reduction approach yields a high yield of streptokinase and is simple to use, purifying processes sometimes result in unacceptable losses of the enzyme (Z Karimi et al., 2011).

Several side effects were linked to streptokinase infusion. The most frequent of these was hypotension, while mild bleeding and allergic reactions were less frequent occurrences. Patients with diabetes, hypertension, dyslipidemia, and smoking had higher adverse events reported (Afifa Rahman et al., 2020).

According to our study, there are more men than women who come with an acute myocardial infarction. The two most prevalent risk factors, smoking and hypertension, are present in 44% and 41% of patients, respectively. Thirteen percent of the individuals had diabetes mellitus. The use of streptokinase results in a considerable ($p < 0.0001$) reduction in discomfort. ST elevation significantly resolves ($p < 0.0001$) after streptokinase is administered. Streptokinase can be employed as a first-time thrombolysis in our nation due to its excellent efficacy, greater availability, lower cost, and less risk of hemorrhagic consequences (N Swetha Ramya et al., 2018).

This treatment technique is more effective than thrombolytic therapy, as evidenced by a significant drop in QTD of patients with fatal arrhythmias in the primary PCI group and an overall reduction in QTD in that group. EF is a key marker of healthy heart function and can predict better heart function in the primary PCI group on its own (Abbas Valizadeh et al., 2020).

In diabetic smokers who have ST Elevation Myocardial Infarction (STEMI) and no other risk factors, the success rate of streptokinase is 52.4%. To enhance the treatment outcome of these instances, the management of cases with these problems must take this into account and the treatment techniques must be reviewed (Hasan Sohail et al., 2020).

Compared to patients without diabetes, those with diabetes have a significantly greater failure rate of ST-segment resolution 180 minutes after streptokinase infusion. Higher Killip class and lower ejection fraction are associated with this failure rate (Mohammad Masoomi et al., 2012).

In diabetics with acute STEMI, streptokinase treatment exhibited ST segment resolution at 90 minutes in 15.38% of cases (n = 26), whereas no ST segment resolution was seen in 84.62% of cases (n = 143). The study concludes that in diabetic people suffering from STEMI, thrombolytic treatment is ineffective. It is important to look into more recent approaches like primary angioplasty and peri-infarction metabolic control in diabetics in order to enhance outcomes (Muhammad Adeel Alam et al., 2019).

An overview of the general incidence of SK-induced hypotension has been provided by this study. It is important to evaluate the benefits and risks of inferior MI patients experiencing SK-induced hypotension. Safe treatment techniques can be put in place to guarantee that SK is safe for all STEMI patients (Adyani Md Redzuan et al., 2021).

The thrombolytic efficacy of streptokinase varies significantly between patients with ST-elevation myocardial infarction (STEMI) who are diabetic and those who are not. Variations in the success and failure rates of thrombolysis indicate how type 2 diabetes affects how acute myocardial infarction is treated. Compared to their non-diabetic counterparts, who have a better success rate (more than 70% ST resolution), diabetic STEMI patients had a higher failure rate (less than 30% ST resolution) following thrombolysis. This suggests that in patients with diabetes-related STEMI, achieving favorable thrombolytic results may be challenging. However, those without diabetes who have STEMI usually benefit more from

thrombolytic therapy. One of the suggested alternative therapy methods is PCI because diabetics have a higher (Dr Sreedhar Vasala et al., 2023).

In our cohort, the STEMI patients treated with streptokinase had a low death rate and an outstanding survival rate. Regression analysis revealed that the most important predictors of in-hospital death among STEMI patients were increasing age, blood potassium level, and right ventricular involvement. The survival of these patients was strongly correlated with higher systolic and diastolic blood pressure at presentation and >50% settlement of increased ST segment on ECG on the first post-admission day. Additionally, it was discovered that higher BMI, obesity, diabetes, hypertension, and inferior heart wall involvement by infarction all increased the risk of death in our patients (MUHAMMAD SHAHID et al., 2019).

CHAPTER – 3

AIM AND OBJECTIVE

3.1 Aim

To produce streptokinase from *Streptococcus* and enhanced by Chemical Mutagenesis.

3.2 Objective

- To collect the fertile soil sample and isolate bacteria
- To screen the production of extracellular streptokinase from isolated cultures.
- To enhance the production of streptokinase by chemical mutagenesis.
- To optimize and produce streptokinase.
- To purify the produced enzyme and increase its efficiency.
- To characterize the produced enzyme for its activity and stability.

CHAPTER- 4

MATERIALS AND METHODS

4.1 Materials

For this experiment, we used most of the chemicals and materials were purchased from Himedia and TCI chemicals.

4.2 Methods

4.2.1 Sample collection

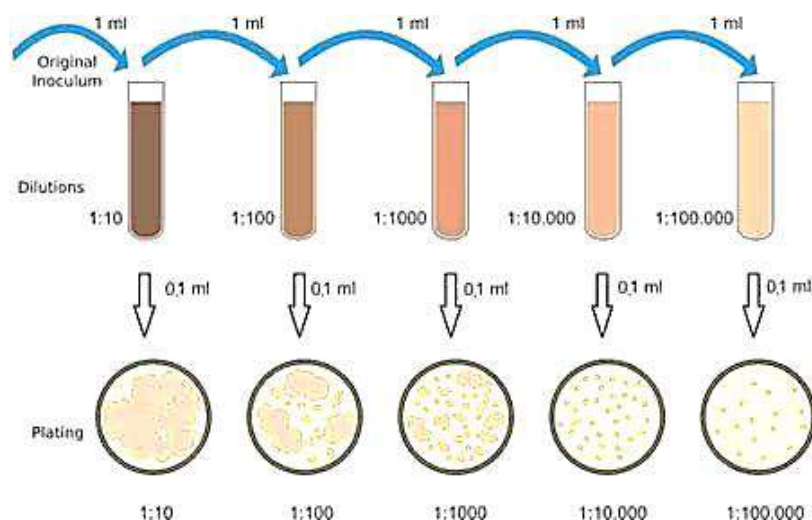
Soil sample was collected at Sathyabama university, Chennai. The soil sample was transported to the laboratory and stored at 4°C.

4.2.2 Isolation of bacteria from the soil sample by serial dilution

The collected soil sample was diluted in distilled water with the concentration 10^{-1} to 10^{-9} and it was prepared without any clumps.

Prepare the Nutrient agar and autoclave it.

Keep the isolated bacteria sample and media inside the laminar air flow. After the media get into warm temperature pour the media in the petri plates. Then streak the plates with the isolated bacteria, dry it for 15-20 minutes. Incubate it for 24 hours.



4.2.3 Isolation of streptococcus

To isolate the streptococcus, the sample were streaked on to the sheep blood agar plates and were incubated for 24- 48 hours.

For 100ml of autoclaved and cooled blood agar was prepared, then 5%-7% defibrinated sheep blood was added and then poured into the Petri plates.

Simple streaking was done on the plates with inoculation loop or throat swap.

Incubate the plates for 24 hours at the temperature 37°C.

Isolated colonies present in the clear zones.

4.3. Identification of streptococcus by gram staining method

- Once the isolated bacterial strain was purified, it was applied to a glass slide using heat fixing and then subjected to a series of staining procedures.
- First, stained with crystal violet for one minute and then rinsed with water.
- Then it washed with decolorizer that contains ethanol or acetone, again rinsed with water.
- Finally counterstained with safranin for 30 seconds and dry it for few minutes.
- Observe the slide under the microscope, wheather the bacteria is gram positive or gram negative.

4.4 Screening of streptokinase produced by streptococcus

- Isolated colonies were picked up from the blood agar plates and add in the 5ml of distilled water which was then added into the selective agar medium. Mix it well.
- Then poured the selective agar medium into the petriplates and streaked with inoculation loop. Then incubate for 24-48 hours.
- Observe the petriplates, wheather the streptokinase is present or not.

4.5 Streptokinase Assay

Clot lysis assay

First the fibrin plate was prepared in the following manner:

Two solutions ((a) and (b)) were prepared as follows:

- a) 200 mg of agarose (Merck, Germany) was dissolved in 20 mL phosphate buffer solution with pH of 7.2 and maintained at 56°C.
- b) 50 mg of human fibrinogen (Fibrinogen, Fraction 1, Type 1 from Human Plasma, Sigma-Aldrich, USA) was dissolved in 10 mL phosphate buffer solution (pH = 7.2).

Twenty hundred μL of 1 mg/mL human plasminogen (Human plasminogen, Hyphen Biomed, France) was added to solution (a) and 500 μL of thrombin (10 NIH unit/vial, from human plasma, Sigma-Aldrich, USA) to solution (b). Clot formation was initiated by mixing solutions (a) and (b) in a Petri dish. It was maintained at 4°C for 30 min. Dilutions, over the range 100-1000 IU/mL, were prepared from the international standard with Milli-Q water and loaded, as duplicate, onto fibrin plate and incubated for 8 h. The zones of lysis produced on the fibrin plates were measured and related to concentration to obtain the dose-response curve for streptokinase. The test samples were prepared by reconstitution of each vial with 1 mL of Milli-Q water. Using the dose-response curve, the streptokinase activity of the samples was determined from zones of lysis produced by appropriate dilutions of each sample loaded on fibrin plates.

Chromogenic substrate assay

The chromogenic method is an endpoint method using a chromogenic substrate (S-2251 (Val-leu-lys-p-nitroaniline.2HCl); Chromogenix, Milan, Italy), where Streptokinase converts plasminogen to plasmin in solution, and in the absence of fibrin. In this method, a given concentration of Streptokinase results in linear generation of active plasmin and accelerating hydrolysis of S-2251, hence resulting in the optical density (OD) of the solution increasing exponentially.

The total amount of product formed, and hence the OD, is proportional to the Streptokinase concentration. Substrate solution was prepared by adding 1 mL of 0.5 M Tris-HCl with pH of 7.4 at 37°C to 1 mL of 3 mM S-2251 (reconstituted in water) and 5 µL of 10% Tween 20 (Usb®,USA). This solution was maintained at 37°C and, immediately before use, 45 µL of plasminogen solution (1 mg/mL) were also added. Dilutions for the dose-response curve for Streptokinase were prepared in 10 mM Tris-HCl with pH of 7.4 containing 0.1 mM NaCl and 1mg/mL albumin, over the range 4.0, 2.0, 1.0, 0.5 IU/mL and maintained at 37°C in a microtiter plate. Plasminogen activation was initiated by mixing 60 µL of Streptokinase solution with 40µl of substrate solution. Concentrations of Streptokinase in the activation mixture were, therefore, 2.4, 1.2, 0.6, 0.3 IU/mL. The reaction was allowed to proceed for 20 min and then stopped by addition of 50 µL of 50% acetic acid and the OD was measured at 405 nm (9).

4.6 Chemical Mutagenesis:

Chemical Mutagenesis of Streptococcus by Ethyl Methanesulfonate(EMS):n

To induce mutation, Streptococcus was exposed to EMS for 0-200 minutes, with a 20-minute break in between. After preparing the wild Streptococcus EBL-20 spore suspension in tryptone broth, it was rinsed and suspended in 100 mM potassium phosphate buffer (pH 7.5). 12(L of EMS was added to a sterile test tube along with 1 mL of spore suspension aseptically [25]. Every 20 minutes, aliquots were taken, cleaned, and then placed onto nutrient agar plates following serial dilution. The plates were incubated for twenty-four hours at 37°C.

Screening of mutants:

Placing bacterial culture on nutrient agar plates enriched with triton X-100 allowed for the removal of the strong mutant bacterial colonies. On skimmed milk agar plates, the colonies cultured on triton X-100 underwent additional analysis. In order to do this, isolated colonies were allowed to develop on agarose plates that contained a 0.8% agarose, 200 μ L of human plasma, 10% skim milk, 150 mM NaCl, and 50 mM Tris-HCl (pH 8.0) combination [15]. The plates were incubated for 12–16 hours at 37°C. The clean zones surrounding the colonies that resulted from SK's proteolytic activity were measured in order to ascertain the activity of SK. The ability of the colonies with the highest zone of clearance to lyse clots was examined in order to make the final selection.

4.7 Optimization of culture condition for SK production:

Using a statistical method, the interaction between four variables—pH, temperature, incubation/fermentation period, and inoculum size—and the yield of SK was examined. For five levels of four separate variables, a central composite design (CCD) of response surface methodology (RSM) was used. A total of 30 ($= 2k + 2k + 6$) experimental runs were carried out, where k is the number of independent factors/variables. To determine the pure error, the design included sixteen factorial and eight axial experimental runs with six replicates at the center point. Every experiment was carried out three times. Using multiple regression and variance analysis, the second order polynomial coefficient for each equation term "X" was determined. Response surfaces were created using Contour plots and response surfaces were produced using Design-Expert 7.0.

4.8 Production of Streptokinase:

Colonies of the chosen mutant, *Streptococcus* EBL-32, that had been cultured on nutrient agar plates were selected, and they were injected into Todd Hewitt Broth over a 24-hour period at 37°C. Three separate liquid state fermentations were carried out in Erlenmeyer flasks using 50 mL of production media that had been previously tuned. One milliliter of the inoculum was added to the production media, and it was then incubated at 37°C for twenty-four hours to promote liquid state fermentation. Following the incubation period, the culture was centrifuged for 30

minutes at 10,000 g to extract the supernatant, which contained the crude enzyme. While RSM optimized the fermentation/cultivation conditions, single factor analysis was used to optimize the microorganism's nutritional needs

CHAPTER- 5

RESULTS AND DISCUSSION

5.1 Isolation of the bacteria growth

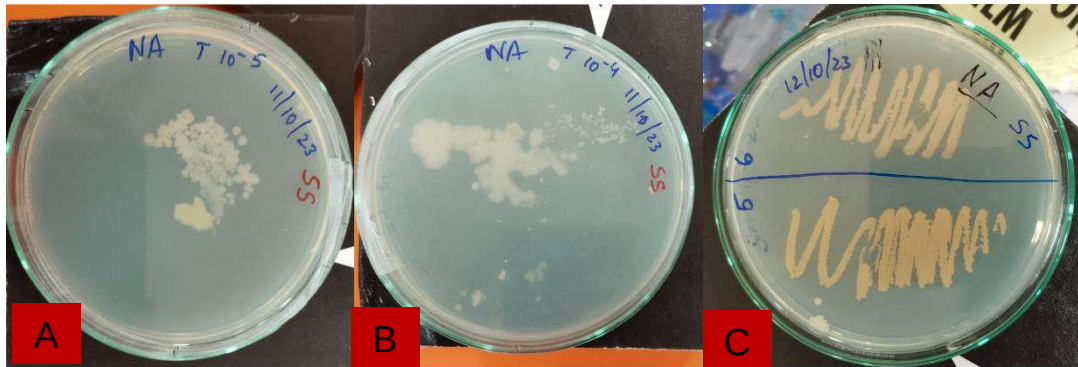


Fig:5.1 Growth of the bacteria is shown in different concentration

The bacterial colonies were isolated using bergey's manual into separate place. Based on screening results, the samples with good enzymatic activity were taken forward for the production.

5.2 Identification of streptococcus bacteria by Gram Staining

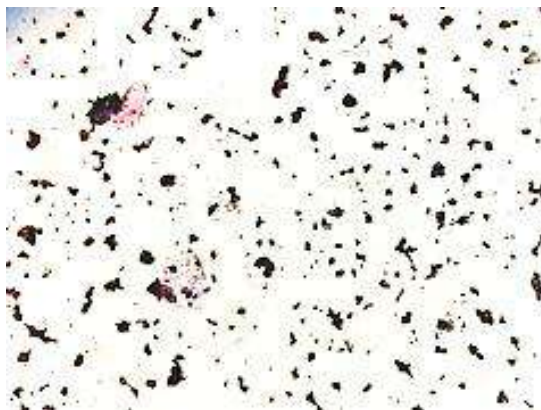


Fig:5.2 Gram positive-cocci with long chains

The most important characteristics are shown in the above picture. Streptococcus species gave gram positive with long chains of cocci.

5.2.1 Screening of streptokinase produce by streptococcus bacteria

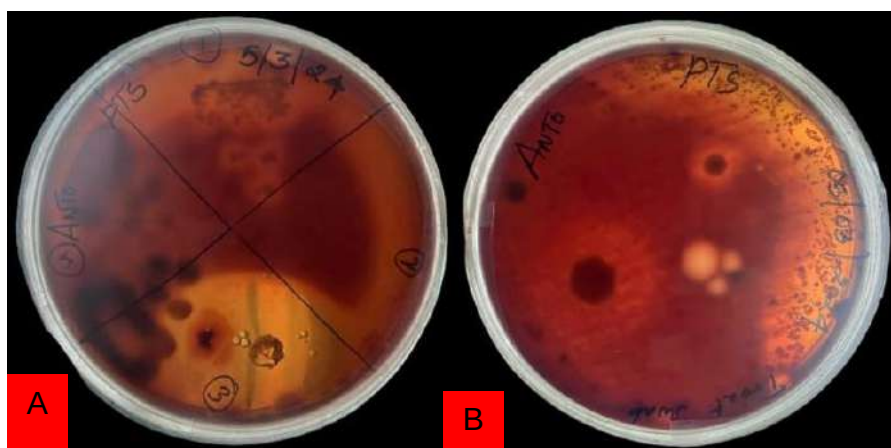


Fig:5.3 Haemolytic activity of mutated *Streptococcus sp.* in blood agar plate shows that the streptokinase production by bacteria

Streptokinase Activity in bacteria:

If the right dosage of streptokinase is given within a specific amount of time after a myocardial infarction, the prognosis improves significantly. As a result, the ability to accurately determine the potency values of streptokinase preparations is essential. The activity of two products (native vs. recombinant) was assessed in this investigation using two distinct approaches. Table 1 presents the average potency data for both samples using the two approaches, and Figure 1 shows this information diagrammatically. Using the Clot lysis and Chromogenic substrate assays, the activity of streptase was determined to be $101 \pm 4\%$ and $97 \pm 5\%$ of the label claim, respectively. These findings are between the permissible bounds of the potency standards specified by the European Pharmacopeia (90–111% of declared potency).

NativePAGE analysis of the two products was done in order to look into the cause of the varied results found in the current work when compared to the previously described study. The findings demonstrated that the native version of SK found in Streptase® migrated more slowly on the gel than the recombinant form found in Heberkinasa. This result implies that there are some structural differences between the two types of SK. This result was validated by the two proteins' N-

terminal sequencing. The recombinant protein has an extra methionine (Met), as shown by the sequencing of the first six amino acids.