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MOLECULAR IDENTIFICATION AND EVALUATION OF STREPTOKINASE ACTIVITY AMONG *STREPTOCOCCUS PYOGENES* IN RELATION TO STREPTOKINASE GENOTYPE ISOLATED FROM TONSILLITIS PATIENTS

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November 2022

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ABSTRACT

MOLECULAR IDENTIFICATION AND EVALUATION OF STREPTOKINASE ACTIVITY AMONG *STREPTOCOCCUS PYOGENES* IN RELATION TO STREPTOKINASE GENOTYPE ISOLATED FROM TONSILLITIS PATIENTS

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Master of Science in Biology

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About 100 throat swab samples were taken from a patient suffering from tonsillitis, and 60 were obtained through biochemical tests. Of these, 40 were identified as hemolytic, and 6 were identified as S. pyogenic. The ability of these six strains to produce streptokinase was evaluated. Among the six strains, the one with the most variable production degrees was *S. pyogenes* S30. Its activity was 0.35 U/mg. *S. pyogenes* S30 was selected to determine the optimal conditions for streptokinase production. The maximum amount of the enzyme was obtained after the strain was given a base medium containing glucose of 0.5%, a yeast extract of 1.5%, and an inoculum with a size of 10.4. The optimal conditions for streptokinase production were established by precipitation with a 45% saturation of ammonium sulfate. The resulting enzyme was then purified using a combination of ion exchange, gel filtration, and DEAE-Cellulose. After the last step, the enzyme's specific activity was increased to 54 U/mg. *S. pyogenes* was then analyzed using a PCR method to determine the presence of the *pyogenes* specific primer. The results indicated that the six production strains exhibited 100% positive bands.

2022, 55 pages

Keywords: PCR, Tonsillitis, Streptokinase, *S. pyogenes*, Bacterial isolates

ÖZET

BADEMCİK HASTALARINDAN İZOLE EDİLEN STREPTOKİNAZ GENOTİPİNE İLİŞKİN STREPTOKOKKUS PYOGENLERİ ARASINDAKİ STREPTOKİNAZ AKTİVİTESİNİN MOLEKÜLER TANIMI VE DEĞERLENDİRİLMESİ

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Bademcik iltihabı olan bir hastadan yaklaşık 100 boğaz sürüntü örneği alındı ve bunlardan 60 tanesi biyokimyasal testlerle elde edildi. Bu 60 kişiden 40'ı hemolitik, altısı da S.piyojenik olarak tanımLandı. Bu altı suşun streptokinaz üretme yeteneği değerlendirildi. Altı suş arasında en değişken üretim derecesine sahip olanı S.pyogens S30 idi. Aktivitesi 0,35 U/mg idi. *S.pyogens* S30, streptokinaz üretimi için optimal koşulları belirlemek üzere seçildi. Enzimin maksimum miktarı, suşa %0,5 glukoz, %1,5 maya özütü ve 10,4 büyüklüğünde bir inokulum içeren bir baz ortamı verildikten sonra elde edildi. Streptokinaz üretimi için optimal koşullar, çöktürme ile belirlendi. %45 amonyum sülfat doygunluğu. Elde edilen enzim daha sonra iyon değişimi, jel filtrasyonu ve DEAE-Selüloz kombinasyonu kullanılarak saflaştırıldı. Son adımdan sonra enzimin spesifik aktivitesi 54 U/mg'ye yükseltildi. Daha sonra, S.pyogens *pyogenes*'e özgü primerin varlığını belirlemek için bir PCR yöntemi kullanılarak analiz edildi. Sonuçlar, altı üretim suşunun %100 pozitif bantlar sergilediğini gösterdi.

2022, 55 sayfa

Anahtar Kelimeler: PCR, Tonsillit, Streptokinase, *S.pyogens*, Bakteriyel izolatlar

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LIST OF SYMBOLS

%	Percentage
±	Plus-minus
°C	°C
g	Gram
m^2	Square meters
mg	Milligram
mL	Milliliters
ng	Nanogram
nm	Nanometer
µg	Microgram
µL	Microliter

LIST OF ABBREVIATIONS

DNase	Deoxyribonuclease
GAS	Gastric acid syndrome
pH	Potential hydrogen
SLS	Sodium lauryl sulfate

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1. INTRODUCTION

One of the most common Gram-positive bacteria is the *streptococcus* (*Streptococcus pyogenes*) that is known to colonize the skin and throat and is responsible for various non-suppurative sequelae. Its members have developed various strategies to evade the host's defenses (Bisno 1995).

Most of bacterial pharyngitis is caused by skin and throat strains of group A *streptococcus* that induce impetigo and scarlet fever. The notion of unique skin and throat strains is the product of decades of research (Wannamaker 1972) has found that different types of group A *streptococcus* can cause throat infections.

Streptokinase, a 47 kDa extracellular protein made up of 414 amino acids, is produced by some strains of *Streptococci*. It is a plasminogen activator and is involved in dissolving blood clots. Blood clots are often treated with streptokinase (Dubey *et al.* 2011) have found that SK has a high-affinity bond with plasminogen, which is different from tissue-type plasminogens.

In average circulation, a healthy and normal hemostatic system prevents the formation of blood clots. However, failure to maintain this system could lead to death in some instances such as acute myocardial infarction or pulmonary embolism. A hemostatic system can achieve an optimal balance of blood flow and hemorrhage. Although a blood clot may not be lysed, it can still form. This is because the formation of a blood clot can occur in an imbalanced situation. In order to prevent this, anti-coagulants are used to treat thrombosis (Balaraman and Prabakaran 2007). The studies have demonstrated that streptokinase may help treat acute myocardial infarction in human beings and animals (Banerjee *et al.* 2004).

The related studies have focused on finding more strains from bacteria that are capable of producing *Streptococcus* pyogenes. This led to an increase in the amount of streptokinase, which was made possible by:

- Identification and isolation of pyogenes from tonsillitis.

- Streptokinase production involves several processes, which should be examined in detail.

- Purification of the most efficient strain.

- Purification and molecular assay for streptokinase production.

2. LITERATURE REVIEW

2.1 *Streptococcus Pyogenes*

- Eubacteria are a kingdom.
- Firmicutes are a phylum.
- Cocci are a kind of fungus.
- The Streptococcaceae are a family of Lactobacillales.
- Streptococcus is the genus.
- *S. pyogenes* is the scientific name for this species (Facklam 2002).

Streptococcus pyogenes is a Gram-positive anaerobic bacterium that does not generate spores, is non-motile, and does not produce toxins. Since its members are divided along a plane, they have varying cell sizes (0.6 to 1.0μm) and temperatures. They can grow at varying temperatures, ranging from 22 to 40 °C (Faddin 2000).

These bacteria can cause various infections such as pharyngitis and scarlet fever. They can also invade the body's deeper tissues and cause various immune-mediated post-streptococcal conditions such as acute rheumatic fever (Todar 2011).

Streptococcus pyogenes is a human pathogen that belongs to the group A family of bacteria Streptococcus. It can cause serious diseases (Tan *et al.* 2014).

There were more than 600.000 cases of pharyngitis and over 111 million cases of pyoderma in the United States between 2005 and 2007, according to a comprehensive

study investigating prevalence and incidence statistics of GAS. This data set is an impressive global record about the mortalities and morbidities caused by this bacterium (Bisno *et al.* 200, Ralph and Carapetis, 2013). After the person is infected with gastric acid syndrome (GAS), the patient's body enters the cavity of the mouth, skin, and wounds. The most common colonization sites are the gastrointestinal tract and the oropharynx (Tan *et al.* 2014).

According to Cunningham, GAS usually causes superficial and self-healing wounds and gastrointestinal symptoms such as pharyngitis and pyoderma in healthy individuals. But people with diabetes or weakened immune systems, for example, can get serious infections that can kill them. Patients with necrotizing fasciitis or toxic shock syndrome must receive an antibiotic treatment because of high mortality and morbidity rates. Secondary autoimmune disorders such as rheumatoid arthritis and glomerulonephritis have been associated with these infections.

2.1.1 Classification of *streptococci*

Streptococci are a heterogeneous group of bacteria. Even though they can be involved in different groups, Brooks and his colleagues have found that the main factors in their classification have been known for a long time (Brooks *et al.* 2007).

The morphology and the reaction between the bacteria on blood agar are the first factors used to classify *Streptococci* (Brown 2005). Brown explained what beta, gamma, and alpha mean and how they describe the various phenomena that take place when blood is put on agar (Davies, 1990). Rebecca Lancefield, a bacteriologist from the University of California, studied the serological characteristics of various cell wall group-specific substances and capsular antigens (Greenwood *et al.* 2002).

According to their serological features, Lancefield recommended in 1933 that bacteria be divided into three groups: A, B, and C (Brown 2005). The bacteria have an ability to recognize their antigens Rhamnose-acetyl glucosamine (A), Rhamnose-galactosamine

(B), and Rhamnose-glucoside (C). *Streptococci* are identified by their rhamnose-acetyl glucosamine polysaccharide (Brook *et al.* 2007).

The classification system still relies on serological typing and hemolysis tests to determine the resistance to various chemical factors and physical properties. Brooks *et al.* (2007) also states that different biochemical tests are used to determine the presence of enzymes.

2.1.2 Pathogenesis

Adherence and colonization

The most critical step in colonizing the body is to attach a group of *streptococci* to a dermal or pharyngeal cell (Baddour *et al.* 1990). Even though gastric acid cannot stick to the host's epithelial lining, it may be eliminated by several means including salivary and mucous processes (Courtney *et al.* 2002). Before bacteria can compete with the body's bacteria for tissue locations, they must first establish a foothold in the skin by attaching to and colonizing it. Understanding the factors that influence the development of resistance to these infections is vital for developing effective vaccines and antibiotics (Hasty *et al.* 1992). In the light of these reviews, the mechanisms by which adherence is initiated are described. After the first contact with the mucosa, a second event happens to make the tissue-specific site (Hasty *et al.* 1992).

Multiple adhesions could make a strain more adherent and virulent. However, these characteristics may be able to direct colonization at this location, even though their effects on forming a particular tissue-specific site are still unclear (Courtney *et al.* 1999). *Streptococci* are thought to be more easily transported from the surface or mucosa into deeper tissues if particular adhesion mechanisms are present. Wang *et al.* (1997) found that these mechanisms could activate inflammatory responses and cause formation of cytokines.

Intracellular invasion

Recent studies have suggested that group A *streptococcus* can also enter the human body's cells. These bacteria can invade human tissues somehow similar to Salmonella and other pathogens (Penta *et al.* 1996). Multiple labs have validated the initial findings, which have attracted much attention. The high frequency of invasion is caused by M protein (Cue *et al.* 1998). Both the SFbI and M1 protein are known to be invasive agents that can be used by the body's cells to absorb the extracellular matrix. It has been theorized that the internalization of group A *streptococci* could cause the disease. The internalization of group A *streptococci* may facilitate the growth of deeper tissues in the body. In their study, Penta *et al.* (1994) revealed that this process could be associated with the protein's low level of virulence.

There is a possibility that both of the bacterial invasion ideas are valid, depending on their features and whether or not they enter through the skin or throat. In addition, the host's capacity to keep the pathogen under control via the utilization of host cells may play a role in the strategy's effectiveness (Schrager *et al.* 1996).

2.1.3 Virulence factors of *S. Pyogenes*

The factors that can affect its spread and adhesion of *S. pyogenes* are divided into two groups (Hook *et al.* 1960).

1-Somatic virulence factors.

2-Surface exposed factors.

Somatic virulence factors

They included the capsule, M protein, and adhesions (Bisno *et al.* 2003**)**.

The Capsule

The genes hasA, hasB, and hasC are responsible for the hyaluronic acid in the capsule. It is helpful for various cellular functions such as killing and colonization. The capsule may alter the cytoskeleton in host cells by attaching to receptors on those cells, which aid in cellular invasion (Mitchell 2003, Wessels and Bronze 1994).

M Protein

The two main components of the M-protein are a pair of protein molecules arranged in a coiled-coil structure. The C-terminal domain is conserved, while the N-terminal portion varies. This structure is located inside the cell membrane.

The M-protein is a significant surface antigen involved in development of various diseases, such as GAS. This protein regulates the immune system's reaction to tissue invasion and externalization and can also prevent the phagocytosis of polymorphonuclear leukocytes (Bisno *et al.* 2003). The bacteria also have other adhesion candidates that can promote the attachment of host cells. The LTA, which is attached to the bacterial surface, is one of them. The protein F, which is a fibronectin-binding protein, can also help maintain the binding of fibronectin to the mucosal surfaces of the host and internalize respiratory epithelial cells (Penta *et al.* 1994, Bisno *et al.* 2003). Laminin, CD44, galactose, and fructose are other receptors implicated in fibronectin's attachment to the gas surface (Musser and Leo 2005).

Extracellular virulence factors

Streptolysin S: An enzyme known as Streptolysin S, thermolabile, is responsible for hemolysis in blood agar. As stated by Sierig *et al.* (2003), sodium lauryl sulfate is one of the most potent cytotoxins (SLS). It can be used to treat various diseases and conditions. It has a broad spectrum of activity and can be used in various cellular structures (Bisno *et al.* 2003, Nizet, 2002). The genetic region responsible for this

substance's production is the sag operon. When it is in contact with specific cells, such as macrophages, it can cause lysis and transmembrane pores and promote resistance to the killing of phagocytes (Kwinn and Nizet, 2007).

Streptolysin O: An amino acid in the Streptolysin O protein that can bind to cholesterol in human cells is oxygen-labile, immunogenic, and cytolytic. This cell can be caused by the vast transmembrane holes created by this process. As stated by Sierig *et al.* (2003), these cells, macrophages, leukocytes, and erythrocytes are also possible targets of SLO in addition to cells (Palmer *et al.* 1998). In a model of epithelial cells, the presence of SLO prevents bacteria from lysosomal localization (Kwinn and Nizet, 2007). In addition, the presence of SPN in the host cell environment can decrease the cell energy stores and increase the level of cytotoxicity. The cytolysin-mediated translocation mediates this process (Meehl and Caparon, 2004).

Streptococcal **chemokine protease**

A *Streptococcal* chemokine protease prevents the movement of neutrophils from the infected area (Hidalgo-carass *et al.* 2006). The serine protease C enzyme removes the chemokine interleukin-8 generated by *Saccharomyces pyogenes* by inhibiting the chemokine from the infection site. However, it is not required to remove the chemokine C5a in order to prevent bacteria from spreading (Hidalgo-carass *et al.* 2004).

Superantigens

The superantigens produced by the bacteria *Streptococcus pyogenes* are responsible for the development of various symptoms such as scarlet fever (Patterson 1996).

Hyaluronidase

Saccharomyces pyogenes and other pyogenic *streptococci* utilize an enzyme known as a hyaluronidase to degrade the hyaluronic acid, a vital component of connective tissues. This can help spread infections along the fascial planes.

This enzyme is also an opacity factor, as it can appear in growth media combined with a serum. It is produced by strains commonly associated with skin infections (Mark *et al.* 2009).

Deoxyribonuclease (DNase)

The four DNases produced by *Saccharomyces pyogenes* are A, B, C, and D. The most common form is DNase B, which hydrolyzes nucleic acids. This enzyme may also play a role in spreading factors by liquefying the exudates of body tissues (Buchanan *et al.* 2006).

Streptokinase

Different strains of the hemolytic *streptococci* generate streptokinase (Babashamsi et al. 2009). Hemolytic strains without erythrogenic toxins provide most of the medicines used to dissolve clots (Hagenson *et al.* 1989, Wang *et al.* 1994). Identification of streptokinase depends on the enzyme's ability to activate the plasminogen and hydrolyze an indicator substrate. The hydrolysis may be affected by several circumstances, including the presence of casein, fibrin clot, and other proteins (e.g., lysine methyl ester, lysine ethyl ester, L- arginine methyl ester). The SK enzyme has been proved to be effective in detection of plasminogen activator activity (Pratap *et al.* 2000, Mundada *et al.* 2003). Compared to other similar enzymes, its half-life is rather longer, and it is well-known that it has a strong affinity for circulating plasminogen (Banerjee *et al.* 2004). The enzyme's action was initially discovered in 1933. In the 1940s, it was put into use in therapeutic studies. The next year, other tissue-type or urokinase-type activators do not form a high-affinity complex with plasminogen, but SK is an exception to this rule. Treatment of acute and chronic blood disorders is now most

commonly used (Dubey *et al.* 2011). Boersma *et al.* (2003) state that it is now the best medication for treating different blood problems.

In average circulation, the formation of blood clots is prevented by a healthy and normal hemostatic system. This system can help stop blood clots from forming, which can lead to stroke, pulmonary embolism, and acute myocardial infarction, which are all life-threatening conditions.

Previously, the treatment of thrombosis was mainly based on the use of anti-coagulants to prevent the formation of blood clots. However, the enzyme's activity can also lyse fibrin deposits in an unbalanced situation (Balaraman and Prabakaran 2007). Several clinical trials have revealed that the use of streptokinase as a treatment for acute myocardial infarction is cost-effective and valuable for human beings and animals (Banerjee *et al.* 2004).

Streptokinase structure

Simply put, this single-chain polypeptide with molecular weight of 47 kDa only does not contain any lipid or carbohydrate components. Because of its affordable price, it is commonly utilized to treat ailments in underdeveloped countries (Welfle *et al.* 1992). An enzyme complex is activated by the activation of the plasminogen gamma domain. The plasminogen's activation results from this complex (Banerjee *et al.* 2004). It has been revealed that proteins' N-terminal domains (residues 1–59) activate plasminogen. This mechanism is different from the 60– 414 amino acid residue domain (Nihalani *et al.* 1998).

The stability of the enzyme's secondary structure is an issue. When the residual fragment is not filled with N-terminal amino acids, it has a diminished activity, as well (Young *et al.* 1995). It has a long half-life cycle, similar to other plasminogen activators (Banerjee *et al.* 2004). The crystal structure of human plasmin's catalytic domain gets complexes with streptokinase, as shown in Figure 2.1.

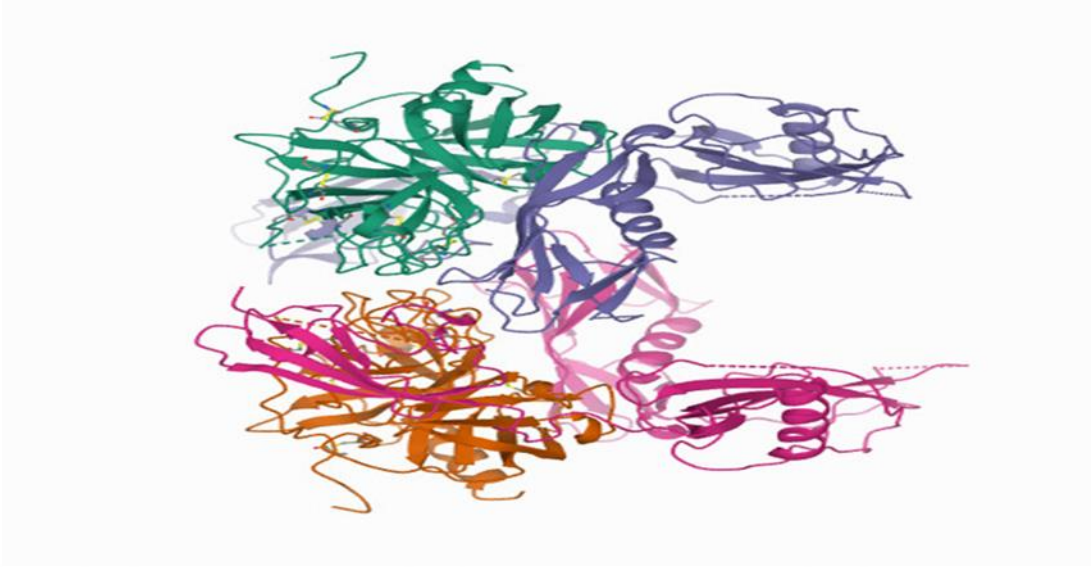

Figure 2.1 The three-dimensional structure of the plasmin catalytic domain in relation to streptokinase (Wang *et al.* 1998).

Streptokinase's action mechanism

Activation of plasminogen by streptokinase is well-documented since it binds to many protein domains (Reed *et al.* 1998). Streptokinase has a variety of domains that interact with plasminogen in a complex way. At least two distinct binding sites for plasminogen have been identified (Nihalani and Sahni 1995). The enzyme's C-terminal domain carries out the activation and recognition of plasminogens (Kim *et al.* 2002). The His48 area is most likely to have a role in plasminogen binding (Kim *et al.* 2000). Residues 48–59 are explored with plasminogen activation (Wakeham *et al.* 2002). The streptokinase is thought to be critical for plasminogen activation (Wu *et al.* 2001). The domain is considered crucial in regulating this process (Robinson *et al.* 2000). The extension of the plasminogen binding site contains the Streptokinase binding site, which stimulates the protein activation (Boxrud *et al.* 2001). The plasminogen activator complex interacts with plasminogen to enhance the catalytic turnover by streptokinase (Sundram *et al.* 2003). In the formation of Streptokinase, the complex's first 59 amino acid residues have several functions (Shi *et al.* 1994, Young *et al.* 1995). This enzyme's secondary structure and the loss of residues 1–59 may become unstable if the N-

terminal residues are missing. Plasminogen activation was shown in a study using 56 pathogenic isolates (Coy *et al.* 1991).

Types of Streptokinase

1. Streptokinase: also known as Streptase, it is a type of enzyme produced by a Group C Beta Hemolytic bacterium. The German company, Aventis Behring, developed this product for treatment of fibrinolytic activity.

2. Heberkinase: In Cuba, heberkinase is a type of Streptokinase. Currently, it is considered to be a safe treatment, as laboratory tests have proven that it can effectively treat patients who suffer from acute heart failure. These tests have revealed that the drug can stimulate the production of antibodies, which can then be used to treat the disease (Lierena *et al.* 2004).

Activity of streptokinase

The enzyme's activity was examined in 1933 by Tillet and Garner for the first time. It was discovered that it could activate the plasminogen activator. This was an analysis factor for blood clot coagulation. This activity is produced by strains of the bacteria known as groups A, C, and G. According to the Lancefield classification, this group has several breakage interfaces (Kulisek *et al.* 1989, Nejadmoghaddam *et al.* 2007).

Streptokinase hydrolyzes plasmin to produce a particular plasmin-specific tripeptide. This method can be used to detect the presence of this enzyme in the sample. A new enzyme-linked immunosorbent assay has been developed to detect the presence of this enzyme in the bloodstream (Shemanova *et al.* 1995, Leonardi *et al.* 1983). The test's sensitivity is 10 times greater than that of the other methods. It only requires a small amount of blood serum to perform the test. Plasma cross-linked degradation products are mainly measured using this technique (Eisenberg *et al.* 1990).

Thrombolysis or fibrinolysis

The protein fibrin is then applied to the blood cells to form a blood clot. These enzymes are responsible for breaking up this clot by coagulating it. Their roles can explain their presence in different blood courses in blood clot development. One example is the plasmin enzyme, which is produced by a protein. The process control solution for formation of blood clot is then prepared using inhibitors of plasminogen activators. These include the fast-reacting tPA and uPA and inhibitors of the other protein types, such as macroglobulin- 2 and antiplasmin. One type of plasminogen that Streptokinase represents does not exist naturally in the human body. This protein can function with the complex composition of the plasminogen activator to produce a different type of enzymes called as plasmin (Banerjee *et al.* 2004)

Clinical uses of streptokinase

Treatment for a wide range of illnesses, including cancer, depends on this enzyme:

1. Coronary thrombosis.

2. Acute heart failure.

3. Acute myocardial infarction.

4. An embolism in the pulmonary artery.

5. A blood clot.

6. Stroke (Banerjee *et al.* 2004, Dubey *et al.* 2011).

For over half a century, Tillet has been using various drugs such as Deoxyribonuclease and Streptokinase to treat patients with Empyema disease. Some materials in the pleura

may degenerate due to the administration of these medications, resulting in the ejection of these materials (Zhiwen *et al.* 2011).

At one point, 44 patients were treated with Streptokinase. These patients were placed under surveillance and the enzyme successfully acted as a mediator of Fibrinolysis. It can also be used to treat other conditions where treatment of fibrinolytic agents is not suitable (Simpson *et al.* 2000).

Optimum conditions of streptokinase production

The live bacterial cells nourish themselves and develop in the microbial media. It contains the energy needed to perform its vital functions.

Various environmental parameters, including temperature and pH conditions, the length of incubation time, and media components, might affect enzyme synthesis from these media. These conditions affect media production, although there may be purification procedures (Aunstrup 1979). The optimum conditions for enzyme generation and proliferation have been investigated in various studies.

These conditions include:

The media type

Complex and rich media are often used to promote the growth of the Group A hemolytic *streptococci*. A modified medium is also used to produce the streptokinase enzyme. This mixture comprises various nutrients such as phosphate salts, peptone, thiamine, and riboflavin (Bernheimer *et al.* 1942). In the original medium, 25% of the solution allowed growth to begin without excessive acid generation. After the overnight incubation, additional glucose was added to the solution to increase the concentration. Cells grew with a high density. The other 75% was lost due to the low glucose concentration. This medium is commonly used for producing streptokinase (Suh *et al.*

1984). The activity of the bacteria increased during exponential growth. The maximum activity of streptokinase was detected during this period. The chemical-defined medium, commonly used for the growth of group A *streptococci*, does not require the use of inoculum. This allows the bacteria to grow in a relatively small amount of media and has the potential to double its activity (McCoy *et al.* 1991).

Temperature

The difference in temperature between the solution and the active enzyme affects the total state of the enzyme and its reaction speed. Enzyme performance is adversely affected by heat in terms of its structure and function. For instance, the temperature difference between the solution and the active enzyme can affect the protein concentration in the solution and its persistence (Fullbrook 1983).

Potential Hydrogen (pH)

The lack of control over the pH level of the medium can lead to the development of harmful bacteria that can affect the production and growth of biomass. The sodium hydroxide concentration should be added to the medium to maintain its pH level at neutral-7. The rapid growth phase and the acclimatization lag phase are characterized by developing a new batch culture in *streptococcus* bacteria. Compared to the continuous culture, the rapid growth and acclimatization lag phases are highly productive. The change in the pH level affects the properties of the enzymes, such as their charge and distribution, as well. Molecules in the practice site move around randomLy when polyionic polymers are present.

Most enzymes are less effective in hydrogen and perform best in the middle pH range. However, they are also affected by the effects of hydrogen stored in different numbers. This phenomenon causes a loss of efficiency and prevents the enzyme from functioning properly. The pH 7 is the ideal level for Streptokinase's stability (Illanes *et al.* 2008).

Purification of streptokinase

The process of protein isolation aims to remove a particular protein from a biological or bio-product material since living systems only produce these materials. It also aims to separate the different types of proteins from one another. The process is complicated since different types of proteins have similar properties. In an ideal situation, one can remove all the contaminating proteins without losing interest in the other one so that the quantity of protein would be reduced, but its activity would not change (Clive 2002). Streptokinase was isolated in 1968 by Tomar using ammonium sulfate as a solvent. The activity of the resultant solution increased by two to three times. A gradient elution process was then used to remove the dialyzed solution. Ultrafiltration was the primary means through which the activity reached its climax. Finally, highly pure streptokinase was isolated from a commercial Kabikinase product, the Swede (Kabi Vitram). In order to distinguish between the dissolved and eluted streptokinase solutions, the ammonium sulfate fractionation procedure was followed by gel permeation chromatography. The eluted streptokinase solution was then treated with column chromatography. Other chromatographic procedures such as combining multiple steps were also used to purify the bacteria. The procedure was then performed to distinguish the eluted and dissolved streptokinase solutions using a single-step purification method. The immobilized di-Isopropyl phosphate prevented the plasmin protease from functioning (Jeong *et al.* 1993).

Pharyngitis

Pharyngitis is referred to the inflammation of the pharynx's submucosal structures and mucous membranes. *Streptococcus pyogenes* is responsible for around 30% of cases. Pharyngitis in children may be caused by streptococcus, which accounts for 20% to 30% of cases (Slusarcick and Caig 2000).

The most common cause of childhood pharyngitis is *Streptococcus pyogenes*. It affects children aged between 5 and 15 years. The symptoms of this infection include fever,

throat pain, nausea, and vomiting. These symptoms usually occur during the winter and early spring (Wannamaker 1972).

Red fever is a common cause of pharyngitis that can be triggered by the bacteria *Streptococcus pyogenes*. It is characterized by a fine erythematous rash on the face and sometimes desquamate during convalescence. Exudative pharyngitis is rare in children under 3 years old and characterized by generalized adenopathy, coryza, and excoriated nares. Usually, the fever and throat pain can be alleviated within 3 to 5 days (Denny *et al.* 1953)..

3. MATERIALS AND METHODS

3.1 Materials

In order to complete the present study, we used various devices and materials - the countries of manufacturing and producers of which will be cited later. These included an autoclave, a distillator, a compound light microscope, and an electrophoresis unit -- all to ensure that the results are as accurate as possible. Other helpful equipment included an electrophoresis unit, a distillation device, and an electrophoresis constant power unit, all of which are made in Germany. Acetic acid, Agar/India, and other biological and chemical materials were also present. This product contained British a-naphthol (Sigma), Indian beef extract, Bovine serum albumin (BDH), Indian casein, Coomassie brilliant blue (G250) and British DEAE-Cellulose Carbon dioxide (CO2), Glucose (GL), Hydrogen peroxide (HP), Hydrochloride (HC), KH2po4, Lactose, Mannitol, Peptone, Sephadex G-200/Sweden, Skim milk and Sodium chloride/UK. Sodium chloride/UK and Sodium hydroxide are also included in this product.

3.1.1 Media used in the present study

The following media used in the study were sterilized according to the manufacturers' instructions. They were then autoclaved at 121 °C for 15 minutes (Table 3.1).

Table 3.1 Media used

Medium before preparation	Country of product
Nutrient agar	China
Nutrient broth	India
Simon citrate agar	India
Transport medium swabs	China
Brain heart infusion broth	India
Todd-Hewitt broth	China
Blood agar	India

Prepared media: The media were thoroughly prepared and used according to the references of the manufacturing company. They were then autoclaved at 121 ºC for 15 minutes. They were then placed in sterilized dishes and incubated for 22 hours.

Blood agar: According to Collee et al.'s method, 5% of human blood was added into a warm agar base to prepare a blood agar that can be used to isolate and identify Streptococcus. The mixture was kept at 4 °C until it would be used for isolation and identification of *Streptococcus spp.* (Collee *et al.* 1996).

Skim milk agar: Principle and interpretation; Skimmed milk agar is used and prepared in studies to prove coagulation and hydrolysis of casein protein. This medium is recommended by APHA (2) (which is a specialist) for the cultivation and enumeration of microorganisms (including bacteria) in the dairy industry. The addition of skim milk to any medium rich in nutrients creates favorable conditions for the growth of organisms in milk or its derivatives. As a result, the number of bacteria to be isolated is more than the number of live organisms isolated on a standard medium. Bacterial lysis and culture for the analysis of casein protein to form soluble nitrogenous compounds are indicated as a clear and precise area surrounding the colonies (Kiran *et al.* 2017).

Strep-base medium (56921- M): This medium was composed of the following conditions.

Quality Level	200
Sterility	non-sterile
Form	powder
Shelf life	limited shelf life, expiry date on the label
Composition	agar, 15.0 g/L

Buffer, 2.5 g/L	chromogenic mixture, 2.54 g/L
Protein hydrolysate,	17.5 g/L
Selective agents,	0.110 g/L
Manufacturer/trade name	NutriSelect® Plus
Final pH	7.3±0.2 (25 °C)
Application(s)	clinical testing, veterinary microbiology
Storage temp.	2-8°C
Suitability	*Streptococcus* spp.

3.1.2 Reagents and dyes

Catalase (CAT) Activity reagent (Cat.No.:E-BC-K031-S): Ammonium molybdate may immediately halt the catalase (CAT) process that decomposes H_2O_2. As a result, a yellowish complex is formed when ammonium molybdate interacts with the remaining H_2O_2. The yellowish complex may be formed at 405 nm to estimate CAT activity (Table 3.2).

Table 3.2 Performance characteristics

Synonyms	**CAT**
Sample type	Serum, plasma, cells, tissue
Sensitivity	0.27 U/mL
Detection range	0.27-155.4 U/mL
Detection method	Colorimetric method
Assay type	Enzyme Activity
Assay time	70 min
Precision	Average inter-assay CV: 5.1%Average intra-assay CV: 3.1%
Other instruments	Micropipettor, Vortex mixer, Incubator or Water-bath
Other reagents	Normal saline (0.9% NaCl) or PBS (0.01 M, pH 7.4)
Storage	2-8℃
Valid period	6 months

The following tests were carried out according to the instructions of the manufacturing company:

1. Oxidase test reagent (70439- Sigma-Aldrich)
2. Kovac's reagent
3. Methyl red Reagent
4. Gram stain solution
5. Buffers and Solutions
6. Streptokinase assay
7. Tris-HCl (0.1 M, pH 8.0)
8. Tris-HCl buffer (0.1M) and pH 8.0
9. Bovine Serum Albumin (BSA)

These materials used in the study were prepared according to the instructions of the manufacturer.

Tris-HCl buffer (0.0211 M, pH 7.899): Tris-HCl was dissolved(0.314 g) in 90 mL of distilled water, the pH was adjusted to 8, and then the volume was completed to 100 mL by distilled water.
Casein 1% : Casein was prepared by dissolving 1 g of it in 90 mL of Tris-HCl buffer 0.1 M. The pH was adjusted to 8.0 and then heated to 80 ºC, until casein dissolved, The final volume of 100 mL was obtained by using a Tris-HCl buffer.

Trichloroacetic acid 5%: 5 g of trichloroacetic acid was dissolved in 100 mL of distilled water. Using methanol, 0.25 g of Coomassie Brilliant Blue R-250 was dissolved in 90 mL of H2O (1:1), producing 10 mL of glacial acetic acid. The solution was then filtered via Whatman filter No. 1 to eliminate undesirable particles. The temperature for preservation was room temperature.

3.1.3 Streptokinase purification solution

Streptokinase, an extracellular protein enzyme generated by bacteria, is isolated from hemolytic and beta-hemolytic *Streptococcus* strains. Streptokinase was isolated by affinity chromatography on sepharose-4B variant plasminogen if the active site of plasminogen was shielded from streptokinase-induced activation of streptokinase. SDS-PAGE validated the purity of streptokinase and a streptokinase-specific test showed its biological activity.

3.2 Methods

The main objective of the study was to identify and isolate bacterial strains that can be used to produce streptokinase. This step was carried out through the collection of specimens, isolation, and the optimum conditions for the streptokinase production.

3.2.1 Specimen collection

From 1/10/2021 to 1/12/2021, pharyngeal swabs were taken from children (2-14 years old) with pharyngitis and 75-100 samples were collected in Baghdad Governorate / Medical City Hospitals. The swab specimens were transferred aseptically to the laboratory under cooling conditions.

3.2.2 Isolation of *Streptococcus pyogenes*

Group A beta *streptococci* are often isolated on blood agar and then utilized to analyze and isolate the enzyme Streptokinase. The following are possible outcomes of an infection with *Streptococcus pyogenes*:

- The colonies are small, difficult to distinguish, white to gray, and about 1 mm in diameter.

- Each colony is surrounded by a zone of beta-hemolysis with a diameter of 2-3 mm.

One of the nodal hemolytic peptide forms is streptolysin S. *Hemorrhaging* may be observed more clearly if the agar is stabbed multiple times with Streptolysin O. Under the water's surface, particular creatures create colonies that thrive without access to oxygen. A more prominent beta-hemolysis zone may be noticed because both streptolysin S and streptolysin O are active in the stabbed region.

As seen by the area of inhibition surrounding the TaxoA disc, a paper disc with low quantities of bacitracin, only the group A beta *streptococci* are susceptible to the antibiotic. Bacitracin is resistant to other groups of Streptococcus, and periodical inhibition is absent in these strains.

3.2.3 Identification of *Streptococcus pyogenes*

On agar medium enriched with blood, *streptococci* thrive. An external supply of catalase improves the development of *streptococci* by detecting hemolysis and allowing the following identification processes to proceed. Cultivating *S. pyogenes* on selective Gram-positive agar media containing phenylethyl alcohol or Columbia agar containing colistin and nalidixic acid is possible. Most streptococcal strains prefer 35°C to 37°C under 5% CO2 or anaerobic conditions for optimal incubation. The viridans group streptococci benefit significantly from these circumstances; however, *S. pyogenes* may not be able to thrive under these conditions.

Biochemical tests: Isolates of suspected *Streptococcus pyogenes* were submitted to the biochemical assays described by Murray *et al.* (2003) and Table 3.3 shows the test results:

Table 3.3 Biochemical tests of Strep. *pyogenes*

Characteristics	**Results (*Streptococcus pyogenes*)**
CAMP	Negative (-ve)
Capsule	Capsulated
Catalase	Negative (-ve)
Gram Staining	Positive (+ve)
Hemolysis	Beta
Motility	Non-Motile
OF (Oxidative-Fermentative)	Fac. Anaerobes
PYR	Positive (+ve)
Shape	Cocci
Spore	Non-Sporing
Urease	Negative (-ve)
VP (Voges Proskauer)	Negative (-ve)
6.5% NaCl Growth	Negative (-ve)

Identification of bacteria by VITEK 2 system: In the present study, the VITEK 2 device using a growth-based technique was employed to obtain the results. This type of technique allows the identification of many different types of bacteria.

Streptococcus pyogenes isolates: In this study, the isolated bacterial isolates were preserved according to the scientific methods recommended by the companies.

Short–term storage (few weeks): Overnight incubation at 37 °C was used to keep bacteria alive for a few weeks after being inoculated on nutrient agar plates. It was wrapped and kept at 4 °C so that they were ready.

Medium-term storage (1 to 4 months): The isolates were inoculated in small bottles containing 4 to 7 mL of sterile nutrient agar and placed in a candle-jar incubated at 37°C for 24 hours to maintain their stab culture. The bottles were then tightly packed and stored at 4 °C .

Long-time storage: Inoculated with a bacterial isolate, a test tube containing 12 mL of sterile brain-heart broth was placed in a candle jar for 24 hours. After the initial incubation, the mixture was stirred with sterile glycerol (20%) by the vortex**.**

3.2.4 Screening of S. *pyogenes*

The agar medium was then used to prepare the wells. The wells were incubated at 37 °C for 24 hours, adding 23 µl of streptokinase crude enzyme. The positive result was recorded when the presence of the enzyme formed a clear zone around the well in the agar plate.

Quantitative screening of Streptokinase: The *Streptococcus pyogenes* were grown in the (23 mL) brain heart-based culture at 37 C until the turbidity level reached 580 nm. The supernatant was centrifuged for 25 minutes at 9.000 rpm. The supernatant was then filtered through a 0.45m (millipore) filter. Upon its collection, it was labeled as a crude enzyme.

Streptokinase activity assay: the Streptokinase Activity Assay Kit (Colorimetric) (ab273313) was used to measure the effect of bulk culture-based Streptokinase on

plasminogen activation in serum and to make comparisons between the two. The test is straightforward and only requires three simple procedures to complete. It may detect streptokinase activity within less than 60 minutes.

Determination of protein concentration (Grimsley and Pace 2003): The bovine serum albumin curve used in the present study was calculated and completed using different concentrations of BSA in the stock solution. The process was carried out as follows: 2.5 mL (measured by automatic pipette) of blue brilliant G-250 dye was added, mixed, and left to dry for two minutes at 25 °C. Color spectrometry was used to measure the absorbance at 280 nm. The blank was made by diluting 0.5 mL of 0.02 M Tris-HCl solution with 2.5 mL of dye reagent. When plotting BSA uptake with albumin, the standard curve was utilized Table 3.4. 0.1 mL of the test sample (isolate bacteria) was mixed with 0.4 mL of 0.02 M Tris-HCl and 2.5 mL of brilliant blue G-250, and the absorbance at 280 nm was measured after it was kept at 25 °C for 2 minutes.

Table 3.4 Surface area and body concentrations used in preparing the standard protein curve

BSA	**Tris-HCl Buffer**	**Protein (µg/mL) concentration**	**Final Volume**
0.0	100.0	0.0	0.1
20.0	80.0	20.0	0.1
40.0	60.0	40.0	0.1
60.0	40.0	60.0	0.1
80.0	20.0	80.0	0.1
100.0	0.0	100.0	0.1

Estimating specific activity of Streptokinase: The following equation was used to estimate streptokinase's specific activity (Longstaff and Whitton 2004).

Specific activity (unit/mg) = Enzyme activity (unit/mL)/ Protein concentration (mg/mL).

3.2.5 Conditions required for streptokinase production

One mL of a new culture was added to 99 mL of the production medium to identify the optimum conditions for synthesizing streptokinase. Afterwards, the bacteria were incubated in a shaker incubator at 100 rpm at 37ºC for 24 hours, and the protein concentration and activity were estimated.

Optimum pH of the medium: The pH level of the medium used to produce the bacteria was then adjusted to different values for 24 hours. The results of the experiments were then applied in the following steps to determine the optimum pH for the production of streptokinase.

Optimum incubation of streptokinase and temperature: To determine the optimum (perfectly) temperature for the production of streptokinase, the isolate was inoculated under various incubation conditions. These temperature values ranged from 20 to 40 °C. At the next step, the optimum temperature was set.

3.2.6 Streptokinase purification method

The Streptokinase enzyme produced by isolated S. *pyogenes* was wholly cleaned and identified using different purification techniques as mentioned below:

- Enzyme production and preparation of cell-free filtrate,
- Ammonium sulfate precipitation,
- Dialysis of the crude enzyme,
- Purification by ion-exchange chromatography (DEAE-Cellulose preparation).

- Purification by Gel filtration chromatography.

3.2.7 Molecular assay

Isolation of genomic DNA

- The samples of DNA taken from bacterial cells

- Use of the Wizard Genomic DNA Purification Kit (supplied by Promega USA) to purify DNA.

In order to carry out the processes, the following actions had to be taken:

Pellet Cells: The supernatant was discarded after the culture overnight was centrifuged for 2 minutes at 13.000 rpm. Pipette: 600 µl of the Nuclei Lysis Solution was mixed gently for 5 minutes of incubation at 80°C. The mixture was left to cool to ambient temperature. Three milliliters of RNase Solution was mixed with 3 milliliters of water and incubated at 37°C for 15 minutes.

Precipitation of Proteins: Protein Precipitation Solution was added and mixed with a vortex to precipitate proteins. For six minutes, the mixture was incubated on ice and then centrifuged for four minutes at 11.000 rpm.

DNA Precipitation and Rehydration: The supernatant was transferred to a new 600-microliter isopropanol tube at room temperature. Then, it was combined to obtain DNA. The supernatant was decanted from the centrifuged tubes 1 minute after centrifugation at 13.000 rpm. 580 microliter of water was mixed with 70 percent ethanol and poured into a bottle with an 11.000 rpm centrifuge and a 3-minute run time. After removing the ethanol, the pellet was left to air dry for 8–13 minutes. For 1 hour at 65°C or overnight at 4°C, the DNA pellet was rehydrated in 100 l of Rehydration Solution.

The DNA purity and concentration (determination): Measurement or determination of the DNA A NanoDrop Spectrophotometer was used to determine the DNA's purity and concentration. The concentration and purity of DNA may be determined using a nanodrop spectrophotometer.

Detection of Streptokinase gene (*ska* gene) by PCR: The sequence of the ska gene was obtained from the NCBI database. The resulting primers were then designed to identify *Streptococcus* species that carry the ska gene.

Primers: The software was used to create a primer designed to target a specific sequence of the ska gene using NCBI's Primer Blast software. Table 3.5 shows the primers' sequence.

Primers were dissolved in nuclease-free water, yielding a primary concentration of 100 uM. The stock was diluted with 90 l of nuclease-free water, resulting in a final volume of 10 l. The experiment was completed at 10 M, the ultimate solution concentration (Ramirez-Peña *et al.* 2010, Derbise *et al.* 2004).

Table 3.5 *Priming of the ska gene*

Primer	Sequence	m.w(bp)
ska-F	ATGAAAAATTAC TTATCTATTGGAGTG	1020
ska-R	TTTGTCTTTAGGGTTATCA GGT	

Screening procedure: Several trials were also carried out to improve the PCR conditions and prepare the sequence-specific primers. The components and program of the mixture are presented below or the mixture contains Green Taq Master (12.5), Ska-F (1.0), Ska-R (1.0), D.W (8.5), a DNA sample (2.0), and final volume (25.0 l). Table 3.6 shows PCR program for the combination A.

Table 3.6 The PCR program for the combination A

No.	Steps	Time	Temperature C°	No. Of cycles
1	Initial Denaturation	5 minutes	95	1 cycle
2	Denaturation	45 seconds	95	
	Annealing	45 seconds	62	35 cycles
	Extension	60 seconds	72	
5	Extension	7 minutes	72	1 cycle
6	Holding		4	

Agarose gel electrophoresis. Agarose gel electrophoresis was used to validate the integrity and presence of DNA extracted using PCR, which is the test designed for this study. DNA ladder marker and Ethidium bromide (10 mg/mL) were added to the TBE buffer.

Preparation of agarose: In a beaker filled with 100 mL of 1X TBE, 1 g (for 1 percent) of agarose was added. The water bath was used to bring the solution to a boil. When the agarose was completely dissolved, the Ethidium Bromide (10 mg/mL) was added. The agarose was well mixed and bubbles were avoided before allowing the solution to cool to 50-60°C.

Placing the agarose gel horizontally: Once the agarose solution had let for 30 minutes, it was put onto a gel tray. A 1X TBE-Electrophoresis buffer was used in the tray to prevent the gel from being harmed. As a result, the buffer was allowed to sit in the tray until it was around 3mm above the gel's surface.

DNA loading: Each DNA sample was injected with 2 l of loading dye from the orange-blue and the samples were only added to the individual wells. There was no need to transfer the PCR products into the wells. One hour of 7v/cm electrical electricity was used. The DNA molecule moves from Cathode to (+) Anode poles,. A gel imaging system was used to see the stained bands in the gel.

3.2.8 Streptokinase dissolving the blood clot

4mL blood of a healthy individual was drawn and collected in Eppendorf tubes. The procedure for the formation of a clot was performed at room temperature for about 45 minutes. Following the clot formation, the serum (100 μL) was taken out and placed in three Eppendorf tubes. The mixture was then coagulated at 37 °C within one hour. After the incubation period, the blood clot was dissolved in the clotted blood. In the control distilled water (100 μL) was then added to the blood clot (1mL) (Banerjee *et al.* 2004).

4. RESULTS AND DISCUSSION

4.1 Isolation of Streptococcus

The samples were collected from patients who had tonsillitis. Out of the 100 medical samples, 60 were bacterial strains. However, only 40 were able to produce β-hemolysis. The formation of whole hemolysis quarters is usually attributed to *S. pyogenes* (Nizet 2002).The presence of these bacteria was confirmed through their Gram staining and characteristic microscopically. The study's results indicated that the bacterial strains suspected of being S. pyogenes were gram-positive. They were also frequently arranged in chains, as stated by (Ahmed *et al.* 2015).

4.2 Biochemical Characteristics of bacterial strains of Streptococcus pyogenes

Various biochemical tests were used to examine the properties of bacterial isolates that were thought to be subjected to S. *pyogenes* tests. The sensitivity of the bacteria was confirmed by the presence of large inhibition zones on blood agar (Cunningham 2000).

The results of this study (Table 4.1) showed that all 6 isolates did not produce catalase and oxidase enzymes, which were the targeted enzymes in the present study, as well as negative for the methyl red, citrate, Fujis-Proskauer and indole tests. This test is frequently associated with *Streptococcus pyogenes,* as stated in Bergey's Guide to Specific Bacteriology.

Table 4.1 Biochemical assays for *Streptococcus pyogenes*

Biochemical assays	Isolate					
	S 5	S 9	S 15	S 25	S 30	S 37
Growth under anaerobic condition	Positive	Positive	Positive	Positive	Positive	Positive
β-hemolysis	Positive	Positive	Positive		Positive	Positive
Catalase	Negative	Negative	Negative	Negative	Negative	Negative
Oxidase	Negative	Negative	Negative	Negative	Negative	Negative
Indole	Negative	Negative	Negative	Negative	Negative	Negative
Methyl red	Negative	Negative	Negative	Negative	Negative	Negative
Voges Proskauer	Negative	Negative	Negative	Negative	Negative	Negative
Citrate	Negative	Negative	Negative	Negative	Negative	Negative
Glucose fermentatio	Positive	Positive	Positive	Positive	Positive	Positive
Lactose fermentatio	Positive	Positive	Positive	Positive	Positive	Positive
Maltose fermentatio	Positive	Positive	Positive	Positive	Positive	Positive
Mannitol fermentati	Positive	Positive	Positive	Positive	Positive	Positive

4.2.1 Semi-quantitative screening

Analysis and evaluation of the efficiency of certain strains of *S. pyogenes* on streptokinase production were carried out as follows: the radial caseinolytic is a classical method of enzyme determination. The bacterial cultures were put in a single-day incubation chamber and centrifuged for 25 minutes at 100 rpm to carry out this process. A 0.45 m cellulose acetate filter was then used to remove the cell-free supernatant. The enzyme's purest form was then regarded as filtrates (Babashmasi *et al.* 2009). Afterwards, the supernatants were incubated at 37 °C for 24 hours on milk agar plates (Himedia, India). Following incubation, the diameter of the casein hydrolysis regions was measured to the nearest millimeter width (Figure 4.1). In the present study, the hydrolysis capacity of a specific isolate was calculated by taking the excellent diameter from the casein hydrolysis zone (Ahmed *et al.* 2015). Among the six isolates, the isolate S30 showed the highest streptokinase activity (Table 4.2).

Figure 4.1 Biological activity of streptokinase

Table 4.2 Caseinolytic analysis of bacterial isolates

Streptokinase Producers	Zone of radial caseinolysis (mm)
S 5	10
S 9	8
S 15	12
S 20	10
S 30	13
S 37	9

The results of this study indicated that the S30 isolate was selected to improve the production of streptokinase.

4.2.2 Quantitative screening for producing isolates

The bacterial cultures were centrifuged at 1200 rpm for 24 minutes after incubating for one day or 24 hours. The cell-free supernatant was then filtered using a 0.45 m cellulose acetate filter. The capacity of specific strains to produce streptokinase is associated with the variations in the genes responsible for its production (Caballero *et al.* 1999) (Table 4.3).

Table 4.3 The activity of the streptokinase enzyme

The presence of *S. pyogenes* regulates the activity of the streptokinase enzyme in cultur after 24 hours of incubation at 37 °C under an aerobic condition	
Isolate name	**Specific activity(U/mg)**
S 5	0.32
S 9	0.29
S 15	0.37
S 20	0.32
S 30	0.42
S 37	0.30

4.3 Optimum Conditions for Streptokinase Production

The effects of cultural and nutritional factors on the production of streptokinase were investigated in order to identify the optimal conditions for the S. pyogenes S30 as follows:

4.3.1 Optimum pH medium

In order to investigate the effects of the initial mean pH on the production of streptokinase, the intermediate production of S. pyogenes was modified to a range of pH values from four to ten in the study. Figure 4.2 shows the results of the study. The maximum amount of streptokinase production was obtained when the pH value was adjusted to seven. This led the enzyme-specific activity to increase to 3.1 U/mg.

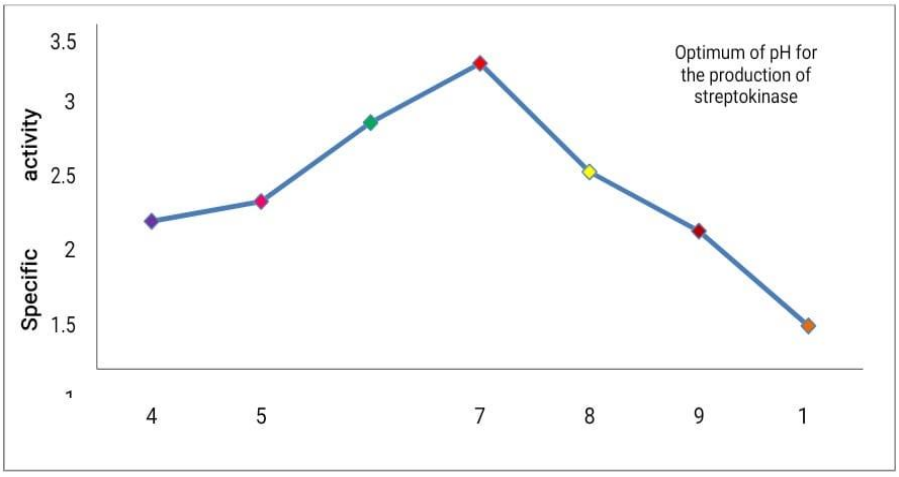

Figure 4.2 Optimum of pH for the streptokinase production

After a 24-hour incubation at 37 °C in a shaker incubator at 100 rpm, the optimum pH for generating streptokinase by the S. pyogenes isolate S30 was determined. Streptokinase production was most efficient when the pH of the production medium was 7 (Carron *et al.* 2009, Duby *et al.* 2011), in this study. The reduction in the production of streptokinase in the way of life medium below pH 8 is also attributed to the effect of the various factors that affect the microorganism metabolism and the balance of the bio-molecules in the medium (Park *et al.* 2018). Several structural changes occur when hydrogen ions are reduced or concentration increases in an environment. The enzymes get generated due to competition between H+ and/or OH components (Tortora *et al.* 2004). The pH level affects the production process of enzymes by varying its effect on the availability of nutrients and the formation of the substrate. It also affects the stability of the produced enzyme (Bull and Bushnel 1976).

4.3.2 Optimum incubation temperature

Streptokinase production was optimized at temperatures ranging from 20 to 25 °C, 30 to 35 °C, and 40 °C. Figure 4.3 also shows that the streptokinase synthesis was concentrated when the microorganism was cultured in the medium at 35 °C. The exact reproduction of streptokinase (3.1 U/mg) was achieved under these circumstances. Streptokinase production might be affected by temperature fluctuations throughout the incubation phase (Arshad *et al.* 2018).

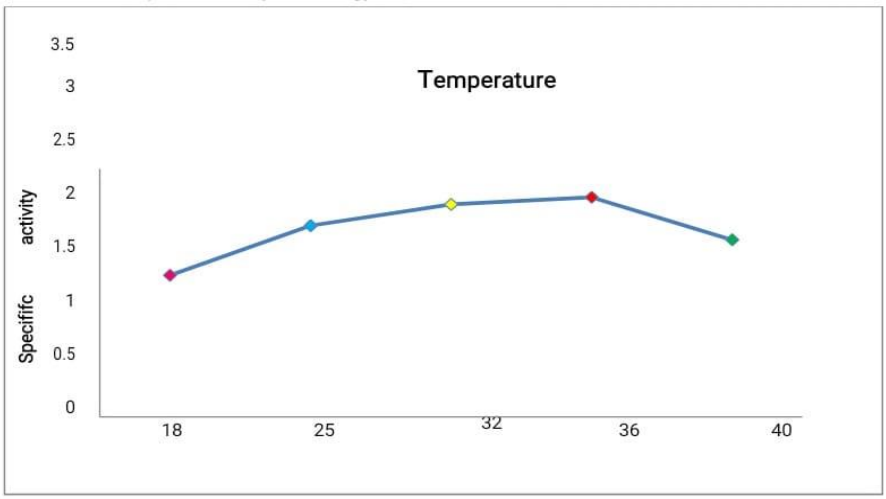

Figure 4.3 High-Quality Streptokinase in S. *pyogenes* S30

S. pyogenes' S30 isolate produced high- quality streptokinase at 37 °C after 24 hours in a shaker incubator at 100 rpm. According to (Bhardwaj *et al.* 2013), the acceptable temperature at which the production of streptokinase began was 75 °C. A temperature below or above this level will significantly decrease the reaction rate. This could be caused by the degradation of the enzyme's three-dimensional structure. This could be also associated with the denaturation of the protein. This process involves the breaking of hydrogen bonds and non-covalent bonds. Temperature can affect the growth of microorganisms by altering the conditions that allow them to grow. These include the amount of oxygen they can absorb, their reaction velocity, and their kinetic energy; all of which may affect Streptokinase production.

4.4 Purification of Streptokinase

S. pyogenes S30 is very useful in producing streptokinase when the most demanding or favorable conditions are present. In order to perform the purification, four steps were taken to obtain and purify the enzyme in addition to the characterization investigation. The four steps of purification are below:

4.4.1 Ammonium sulfate precipitation

One of the most common techniques used in the purification of enzymes is their concentration. This process depends on the ammonium sulfate salts produced during the precipitation. It is also an expensive method to produce a protein extract (Grodzki and Berenstein 2010). The precipitation of ammonium sulfate was carried out utilizing various saturation rates in this study (ranging between 30 percent and 80 percent). It was done by extracting the streptokinase produced by S. pyogenes S 30 below the ideal conditions. Table 4.4 shows the results indicating that the enzyme's activity was 12U/mg when the ammonium sulfate solution's concentration was 45%.

Because of its inexpensive cost and outstanding solubility, it is commonly employed in producing enzymes. The enzyme's stability and pH are not affected by ammonium sulfate salts, in contrast to other organic solvents. Precipitates in the crude enzyme supernatants, with 30 to 80 percent (NH4)2SO4 saturations, were compared to keep the fibrinolytic enzyme as much as possible according to the activity test. The results showed that $(NH_4)2SO_4$ was

collected in a 30-60 percent $(NH_4)2SO_4$ saturation (Wang *et al.* 2009). After the ammonium sulfate precipitation, the solution was dissolved in a small quantity of Tris-HCL (0.02 M) buffer at pH8.

4.4.2 Ion exchange chromatography

Negatively charged streptokinase generated by S. pyogenes S30 was removed using ion-exchange chromatography. This method was performed by washing a single column with a dilute solution of Tris-HCl buffer.

S. pyogenes S30 streptokinase negative (-) was removed using this method. After precipitation with ammonium sulfate, a DEAE-cellulose column was used to purify negative (-) streptokinase. Then, they were rinsed with an equivalent amount of M Tris-HCl buffer (5%) at (pH8). A NaCl concentration gradient was used to extract the negatively charged proteins (ranging from 0.2 to 0.5 M). Streptokinase activity was determined by analyzing the two protein peaks produced after the rinsing procedure. The results revealed that the removed protein fractions (from 19 to (31) contained most of the activity (36 units/mL.) The specific activity of the enzyme was measured to be 18 units/mg. Ion alternate chromatography is an exemplary technique for separating various and purification protein components. It is very easy to perform and depends on the protein's binding to the ion exchange. DEAE-cellulose exchanger was used to purify Streptokinase enzyme from Bacillus licheniformis (TRIA 2016). Gel filtration chromatography (GFCt) was utilized to purify the streptokinase method further after it was purified using ion-change chromatography (ICC). Costa *et al.* (2014) mentioned that streptokinase unique recorded (46.87 U/mg) and purification fold 3.48 via the use of DEAE-cellulose.

4.4.3 Gel filtration chromatography

The gel filtration method was used to remove the impurities from the streptokinase produced using isolated S. pyogenes S 30. After purification, the ion trade purification step was performed. The resulting product was accumulated from the Sephadex G-200 column with a separate limit of 5000 to 600000 Dalton. This allows for larger separation capacities with an excessive degree of purification. It is characterized by its quick running and high healing

properties. It can be used for a long time due to its simple preparation and balance properties (Shamsi and Khan 2016). Tris HCL buffer, pH 8.0, was previously used for equilibration (Figure 4.4).

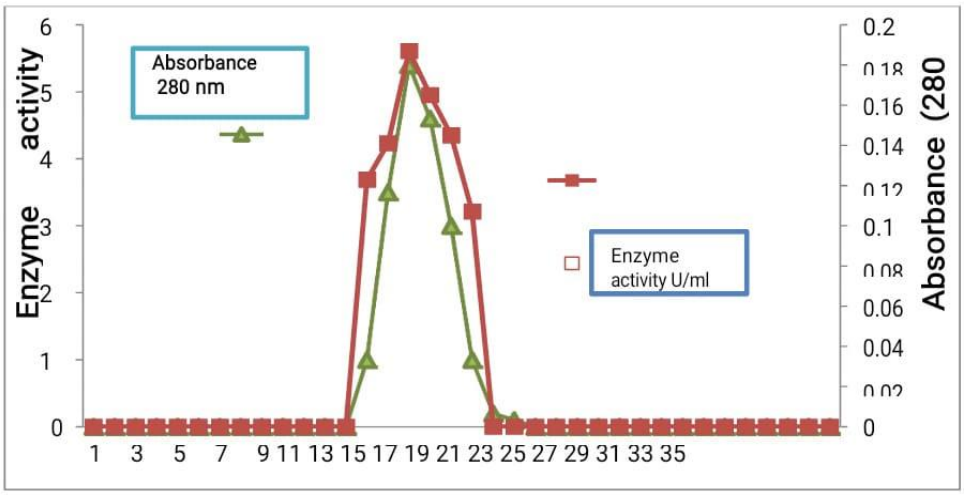

Figure 4.4 Gel filtration chromatography using Sephadex G -200 column

Figure 4.4 shows the peak of streptokinase uptake. After rinsing with Tris-HCl buffer, the activity reached its peak, observed in the present study. The activity and specific activity of streptokinase enzyme, as well as protein concentration, were then determined. Streptokinase's specific activity increased with purification factor 6 (Table 44). In a study, S. pyogenes streptokinase was purified using Sephadex G-150, the third purification step after the Precipitation of ammonium and DEAE-cellulose. The enzyme was then produced with a specific activity of 93.54 units/mg (6.96-fold purification) (Babu *et al.* 2008).

Table 4.4 Purification steps for Streptokinase produced by locally isolates

Purification ste	Volume (mL)	Enzyme activity (U/mL)	Protein concentration (mg/mL)	Specific activity (U/mg)	Total activity (U)	Purification (folds)	Yield (%)
Crude enzyme	80	18	3	6	1440	1	100
Ammonium sulfate Precipitation 45	20.0	33.42	2.806	12.02	672.56	2.01	46.6(
DEAE-cellulos	15	36	2	18	540	3	37.5
Sephadx G-20(	7	54	1.5	36	378	6	26.2

4.5 Molecular Assay

4.5.1 Isolation of genomic DNA

All of the samples used in the Genomic DNA extraction were successful. Gel electrophoresis was used to verify DNA integrity, whereas Nanodrop spectrophotometer was used to verify DNA purity. Agarose gel was applied to fine bands, the attention of which varied from 70 to 110 ng/l. The purity also ranged between 1.75 and 1.90.

4.5.2 Detection of streptokinase gene by the polymerase chain reaction

Streptokinase is a protein secreted by *streptococcus* and binds to plasminogen. As early as 1933, the protein gene, known as streptokinase, is located in A strains. These strains are polymorphic and have nine different genes (Simbolo *et al.* 2013, Johnston *et al.* 1992).

Streptokinase is an activator of plasminogen that can be produced by various groups of bacteria, including G *streptococci*, C *streptococci*, and A *streptococci*. This enzyme helps streptococcal virulence to develop by producing plasmin which can lead to bacterial unfolding from the first area of infection by inflicting fibrinolysis and the degradation of the basement membrane and extracellular matrix aspects (Kuusela *et al.* 1992, Lizano *et al.* 2005) Streptokinase can trigger an infection by stimulating the activation of the complement system (Nitzsche *et al.* 2015).

The DNA sequence of a fragment of ska was compared with that of the *Streptococcus* species. The lines and nucleotides of the sample were verified to be 95-100% match with the sequence identity of the sample. Salvà-Serra *et al.* (2020) stated that the prognosis of patients infected with *S. pyogenes* depended on the serological test.

After a preliminary infection, the result of the life cycle is useless. However, a positive S. *pyogenes* antibody can be used to determine if a bacterial infection has occurred.

In order to overcome this problem, it is recommended to use a specific primer for the production and detection of *Streptococcus pyogenes*. The results of PCR amplification for 6 production samples are impressive (100%). While no band was detected with other samples, the results of this procedure were still remarkable (Figure 4.5).

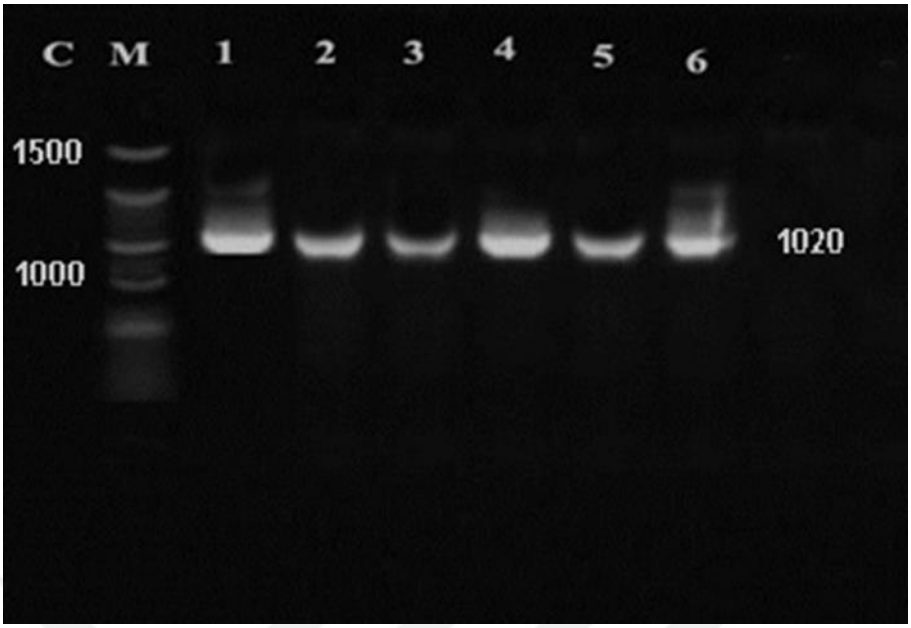

Figure 4.5 Agarose gel electrophoresis-PCR amplification products of streptokinase gene from six *S. pyogenes* (agarose gel 1.5 %, 60 (volt, 2x hours), Line M : DNA marker (100-1500bp)

4.6 Streptokinase Dissolving Blood Clot

100 μL of the blood was added into three Eppendorf tubes and was incubated at 37 °C for 1 hour.

Results illustrated in Figure 4.6 indicated that the streptokinase completely dissolved the blood clot in tube B while the streptokinase partly dissolved it in tubes C and D. This result was then compatible with the result of Hwang *et al.* (2007). The researcher carried out the degradation process by spreading red blood cells in the solution containing the bacterial fibrinolytic enzyme after 60 minutes.

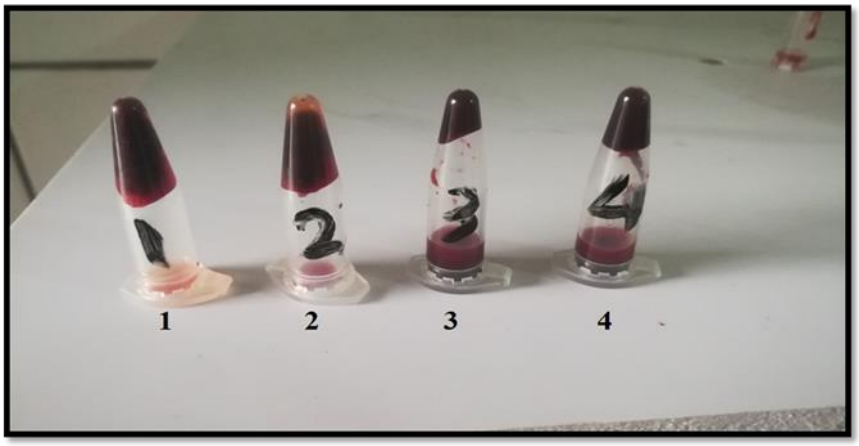

Figure 4.6 Activity of streptokinase in dissolving the blood clots

A: only normal saline + blood clot as control.

B: Purified streptokinase (100 µL) + Blood clot 1 mL.

C: Purified streptokinase (75 µL)

D: Purified streptokinase (50 µL)

+ Blood clot 1 mL.

+ Blood clot 1 mL.

5. CONCLUSIONS AND RECOMMENDATION

The presence of *Streptococcus* allowed researchers to isolate *Streptococcus pyogenes* and produce streptokinase from children with tonsillitis. A high percentage of streptokinase production was found in S. *pyogenes* isolates using a specific primer. In this study, conditions were optimum for producing and extracting streptokinase enzyme from S. pyogenes. The isolate producing S30 for streptokinase was obtained after addition of nodal medium (pH7) containing glucose of 0.05, yeast extract of 1.5%, and an inoculum with size of 1 x 104. The incubation period was 24 hours at 36°C. The incubator was run at 100 rpm for 48/hours. In the same study, the streptokinase enzyme was produced and obtained from S. pyogenes S 30 bacteria and was purified. Ammonium sulfate precipitation, ion-exchange chromatography (DEAE-Cellulose), and gel filtering (Sephadex G200) were used to purify the product. When incubated at 37°C for one hour, the purified streptokinase (100 l) dissolved a blood clot sample.

REFERENCES

Ahmed, Y. T., Zbar, N. S. and Al-Shaibani, A. B. 2015. Detection of Hyaluronidase and Streptokinase Virulence Factors from Streptococcus pyogenes. Al-Nahrain Journal of Science., 18(1): 136-140.

Arshad, A., Zia, M. A., Asgher, M., Joyia, F. A. and Arif, M. 2018. Enhanced production of streptokinase from Streptococcus agalactiae EBL-31 by response surface methodology. Pakistan Journal of Pharmaceutical Sciences., 3: 1.

Aunstrup, K. 1979. Production of Microbial enzymes. Microbial technology., 1: 282-309.

Babashamsi, B. M., Razavian, M. H. and Nejadmoghaddam, M. R. 2009. Production and purification of streptokinase by protected affinity chromatography. Avicenna J Med Biotechnol., 1: 47-51.

Babu, P. C., Srinivas, V. K., Mohan, V. K. and Krishna, E. 2008. Renaturation, purification and characterization of streptokinase expressed as inclusion body in recombinant E. coli. Journal of Chromatography B., 861(2): 218-226.

Baddour, L. M., Lowrance, J. H. and Simpson, W. A. 1990. The role of fibronectin binding in the rat model of experimental endocarditis caused by Streptococcus sanguis. The Journal of clinical investigation., 86(1): 7-13.

Balaraman, K. and Prabakaran, G. 2007. Production and purification of a fibrinolytic enzyme (thrombinase) from Bacillus sphaericus. Indian Journal of Medical Research., 126(5): 459.

Banerjee, A., Chisti, Y. and Banerjee, U. C. 2004. Streptokinase a clinically useful thrombolytic agent. Biotechnology advances., 22(4): 287-307.

Bernheimer, A. W., Gillman, W., Hottle, G. A. and Pappenheimer Jr, A. M. 1942. An improved medium for the cultivation of hemolytic Streptococcus. Journal of Bacteriology., 43(4): 495-498.

Bhardwaj, S., Angayarkanni, J., Bhattacharya, S., Das, A. and Palaniswamy, M. 2013. Isolation, screening and characterization of β-haemolytic Streptococci with potential of streptokinase production. Int Res J Biol Sci., 2: 63-66.

Bisno, A. L. 1995. Molecular aspects of bacterial colonization. Infection Control and Hospital Epidemiology, 16(11): 648-657.

Bisno, A. L., Brito, M. O. and Collins, C. M. 2003. Molecular basis of group A Streptococcal virulence. The Lancet Infectious Diseases, 3(4): 191-200.

Bisno, A. L., Rubin, F. A., Cleary, P. P. and Dale, J. B. 2005. Prospects for a group A Streptococcal vaccine: rationale, feasibility, and obstacles report of a National Institute of Allergy and Infectious Diseases workshop. Clinical Infectious Diseases , 41(8): 1150-1156.

Boersma, E., Mercado, N., Poldermans, D., Gardien, M., Vos, J. and Simoons, M. L. 2003. Acute myocardial infarction. The Lancet., 361(9360): 847-858.

Boxrud, P. D., Verhamme, I. M., Fay, W. P. and Bock, P. E. 2001. Streptokinase triggers conformational activation of plasminogen through specific interactions of the amino-terminal sequence and stabilizes the active zymogen conformation. Journal of Biological Chemistry., 276(28): 26084-26089.

Brooks, G. F., Butel , J. S., Carroll, K. C. and Morse, S. A. 2007. Medical Microbiology. 24th ed. McGraw Hill. London., 13: 233-248.

Brown, A. E. 2005. Microbiological Applications. 9th ed. McGrawHill. New York., 2: 83-433.

Buchanan, J. T., Simpson, A. J., Aziz, R. K., Liu, G. Y., Kristian, S. A., Kotb, M., and Nizet, V. 2006. DNase expression allows the pathogen group A Streptococcus to escape killing in neutrophil extracellular traps. Current Biology., 16(4): 396-400.

Bull, A. T., and Bushell, M. E. (1976). Environmental control of fungal growth. Filamentous fungi., 5: 6-9.

Caballero, A. R., Lottenberg, R. and Johnston, K. H. 1999. Cloning, expression, sequence analysis, and characterization of streptokinases secreted by porcine and equine isolates of Streptococcus equisimilis. Infection and Immunity., 67(12): 6478-6486.

Carron, J. L., Keefe, G. P., McKenna, S. L., Dohoo, I. R. and Poole, D. E. 2009. Evaluation of the University of Minnesota Tri-plate and 3M Petrifilm for the isolation of Staphylococcus aureus and Streptococcus species from clinically mastitic milk samples. Journal of dairy science., 92(10): 5326-5333.

Clive, D. 2002. A guide to protein isolation. Kluer Academic.

Collee, J. G., Miles, R. S. and Watt, B. 1996. Tests for identification of Bacteria. Mackie and McCartney practical Medical Microbiology., 14: 131-49.

Costa, H. B., Fernandes, P. M., Romão, W. and Ventura, J. A. 2014. A new procedure based on column chromatography to purify bromelain by ion exchange plus gel filtration chromatographies. Industrial Crops and Products., 59: 163-168.

Courtney, H. S., Dale, J. B. and Hasty, D. L. (2000). Strategies for preventing group A Streptococcal adhesion and infection. In Handbook of Bacterial Adhesion, pp. 553-579, Humana Press, Totowa, NJ.

Courtney, H. S., Hasty, D. L. and Dale, J. B. 2002. Molecular mechanisms of adhesion, colonization, and invasion of group A Streptococci. Annals of Internal Medicine., 34(2): 77–87.

Coy, H. E., Broder, C. C. and Lottenberg, R. 1991. Streptokinases produced by pathogenic group C Streptococci demonstrate species-specific plasminogen activation. Journal of Infectious Diseases., 164(3): 515-521.

Cue, D., Dombek, P. E., Lam, H. and Cleary, P. P. 1998. Streptococcus pyogenes serotype M1 encodes multiple pathways for entry into human epithelial cells. Infection and Immunity., 66(10): 4593-4601.

Cunningham, M. W. 2000. Pathogenesis of group A Streptococcal infections. Clinical Microbiology Reviews., 13: 470–511.

Davies, D. R. 1990. The structure and function of the aspartic proteinases. Annu. Rev. Biophys. Biophys. Chem., 19: 189-215.

Denny, F.W., Wannamaker, L.W. and Hahn, E. O. 1953. Comparative effects of penicillin, aureomycin and terramycin on Streptococcal tonsillitis and pharyngitis. Pediatrics., 11: 7-13.

Derbise, A., Song, Y. P., Parikh, S., Fischetti, V. A. and Pancholi, V. 2004. Role of the C-terminal lysine residues of Streptococcal surface enolase in Glu-and Lys-plasminogen-binding activities of group A Streptococci. Infection and Immunity., 72(1): 94-105.

Dubey, R., Kumar, J., Agrawala, D., Char, T. and Pusp, P. 2011. Isolation, production, purification, assay and characterization of fibrinolytic enzymes (Nattokinase, Streptokinase and Urokinase) from Bacterial sources. African Journal of Biotechnology., 10(8): 1408-1420.

Eisenberg, P. R., Jaffe, A. S., Stump, D. C., Collen, D. and Bovill, E. G. 1990. Validity of Enzyme-linked Immunosorbent assays of cross-linked fibrin degradation products as a measure of clot lysis. Circulation., 82(4): 1159-1168.

Facklam, R. 2002. What happened to the Streptococci: overview of taxonomic and nomenclature changes. Clinical Microbiology reviews., 15(4): 613-630.

Faddin, J. F. 2000. Individual Biochemical tests. Biochemical tests for identification of medical bacteria., 3: 27-439.

Greenwood, D. Slack, R. B. and Peutherer, J. 2002. Medical Microbiology. 16th ed.Churchill Livingstone, New York., 9: 175-188.

Grimsley, G. R. and Pace, C. N. 2003. Spectrophotometric determination of protein concentration. Current protocols in protein science., 33(1): 3-1.

Grodzki, A. C. and Berenstein, E. (2010). Antibody purification: ammonium sulfate fractionation or gel filtration. In Immunocytochemical methods and Protocols, pp. 15-26, Humana Press.

Hagenson, M. J., Holden, K. A., Parker, K. A., Wood, P. J., Cruze, J. A., Fuke, M. and Stroman, D. W. 1989. Expression of streptokinase in Pichia pastoris yeast. Enzyme and Microbial Technology., 11(10): 650-656.

Hasty, D. L., Ofek, I., Courtney, H. S. and Doyle, R. J. 1992. Multiple adhesins of Streptococci. Infection and immunity., 60(6): 2147-2152.

Hidalgo-Grass, C., Dan-Goor, M., Maly, A., Eran, Y., Kwinn, L. A., Nizet, V. and Hanski, E. 2004. Effect of a bacterial pheromone peptide on host chemokine degradation in group A Streptococcal necrotising soft-tissue infections. The Lancet., 363(9410): 696-703.

Hidalgo-Grass, C., Mishalian, I., Dan-Goor, M., Belotserkovsky, I., Eran, Y., Nizet, V. and Hanski, E. (2006). A Streptococcal protease that degrades CXC chemokines and impairs bacterial clearance from infected tissues. The EMBO journal., 25(19): 4628-4637.

Hook, E. W., Wagner, R. R. and Lancefield, R. C. 1960. An epizootic in Swiss mice caused by a group A Streptococcus, newly designated type 50. American Journal of Hygiene., 72: 111-119.

Hwang, K. J., Choi, K. H., Kim, M. J., Park, C. S. and Cha, J. H. 2007. Purification and characterization of a new fibrinolytic enzyme of Bacillus licheniformis KJ-31,

isolated from Korean traditional Jeot-gal. Journal of Microbiology and Biotechnology., 17(9): 1469-1476.

Illanes, A., Altamirano, C. and Wilson, L. (2008). Homogeneous Enzyme kinetics. In Enzyme Biocatalysis, pp. 107-153, Springer, Dordrecht.

Jeong, I. K., Young, E. L. and Jeung, S. J. 1993. Myung. B. Purification of streptokinase by affinity chromatography using human plasminogen. Korean Biochem J., 26(2): 172-175.

Kim, D. M., Lee, S. J., Kim, I. C., Kim, S. T., and Byun, S. M. 2000. Asp41-His48 region of streptokinase is important in binding to a substrate plasminogen. Thrombosis research., 99(1): 93-98.

Kiran, S., Swarnkar, M. K., Mayilraj, S., Tewari, R. and Gulati, A. 2017. Paenibacillus ihbetae sp. nov., a cold-adapted Antimicrobial producing Bacterium isolated from high altitude Suraj Tal Lake in the Indian trans-Himalayas. Systematic and applied Microbiology., 40(7): 430-439.

Kulisek, E. S., Holm, S. E. and Johnston, K. H. 1989. A chromogenic assay for the detection of plasmin generated by plasminogen activator immobilized on nitrocellulose using a para-nitroanilide synthetic peptide substrate. Analytical biochemistry., 177(1): 78-84.

Kuusela, P., Ullberg, M., Saksela, O. and Kronvall, G. 1992. Tissue-type plasminogen activator-mediated activation of plasminogen on the surface of group A, C, and G Streptococci. Infection and Immunity., 60(1): 196-201.

Kwinn, L. A. and Nizet, V. 2007. How group A Streptococcus circumvents host phagocyte defenses. Future Microbiol., 2: 75-84.

Lefebre, M. D. and Valvano, M. A. 2001. In vitro resistance of Burkholderia cepacia complex isolates to reactive oxygen species in relation to catalase and superoxide dismutase production. Microbiology., 147(1): 97-109.

Leonardi, M. S., Gazzara, D., Fava, C., Foca, A. and Mastroeni, P. 1983. Enzyme-linked immunosorbent assay (ELISA) for streptokinase Antibodies. Diagnostic Immunology., 1(2): 64-67.

Lierena, L., Quiros, J., Sainz, B., Valdes, J., Zorio, B., Villanueva, L., Filgueiras, C., Cabrera, F., Echarte, J., Perezdeltodo, J., Guerrero, I., Lopez, L., Garcia, E., Nadal, B., Betancourt, B., Diaz-Rojo, G., Jarcia, A., Light, R., Nguyen, T. and

Mulligan, M. 2000. The in vitro efficacy of Varidase versus streptokinase or urokinase for liquefying thick purulent exudative material from loculated empyema . Lung., 178: 13-18.

Longstaff, C. and Whitton, C. M. 2004. A proposed reference method for plasminogen activators that enables calculation of enzyme activities in SI units. Journal of Thrombosis and Haemostasis., 2(8): 1416-1421.

Mark, E. H., Morgan, J. H. and Anna, J. R. 2009. Genotypic and Phenotypic Assessment of Hyaluronidase among Type Strains of a Select Group of Staphylococcal Species, International Journal of Microbiology., 8: 34-38.

Meehl, M. A. and Caparon, M. G. 2004. Specificity of streptolysin O in cytolysin-mediated translocation. Molecular Microbiology., 52(6): 1665-1676.

Mundada, L. V., Prorok, M., DeFord, M. E., Figuera, M., Castellino, F. J. and Fay, W. P. 2003. Structure-function analysis of the streptokinase amino terminus (residues 1–59). Journal of Biological Chemistry., 278(27): 24421-24427.

Musser, J. M. and DeLeo, F. R. 2005. Toward a genome-wide systems Biology analysis of host-pathogen interactions in group A Streptococcus. The American Journal of pathology., 167(6): 1461-1472.

Nejadmoghaddam MR, Modarresi MH, Baba shamsi M, Chamankhah M .(2007). Cloning and over expression of active recombinant fusion streptokinase: a new approach to facilitate purification. Pak J Biol Sci. 10(13):2146–2151.

Nihalani, D. and Sahni, G. 1995. Streptokinase contains two independent plasminogen-binding sites. Biochemical and Biophysical research communications., 217(3): 1245-1254.

Nihalani, D., Kumarm, R., Rajagopal, K. and Sahni, G. 1998. Role of the amino-terminal region of streptokinase in the generation of a fully functional plasminogen activator complex probed with synthetic peptides. Protein Science., 7(3): 637-648.

Nitzsche, R., Rosenheinrich, M., Kreikemeyer, B. and Oehmcke-Hecht, S. 2015. Streptococcus pyogenes triggers activation of the human contact system by streptokinase. Infection and Immunity., 83(8): 3035-3042.

Nizet, V. 2002. Streptococcal β-hemolysins: genetics and role in disease pathogenesis. Trends in microbiology., 10(12): 575-580.

Palmer, M., Vulicevic, I., Saweljew, P., Valeva, A., Kehoe, M. and Bhakdi, S. 1998. Streptolysin O: a proposed model of allosteric interaction between a pore-forming protein and its target lipid bilayer. Biochemistry., 37(8): 2378-2383.

Park, S. Y., Yang, D., Ha, S. H. and Lee, S. Y. 2018. Metabolic engineering of Microorganisms for the production of natural compounds. Advanced Biosystems., 2(1): 1700190.

Penta, D., Rubens, C., Chi, E. and Cleary, P. P. 1994. Group A streptococci efficiently invade human respiratory epithelial cells. Proceedings of the national academy of sciences., 91(25): 12115-12119.

Penta, D., Zhang, X. P. and Cleary, P. P. 1994. Streptococcus pyogenes type IIa IgG Fc receptor expression is co-ordinately regulated with M protein and Streptococcal C5a peptidase. Molecular Microbiology., 12(6): 873-879.

Pratap, J., Rajamohan, G. and Dikshit, K. L. 2000. Characteristics of glycosylated streptokinase secreted from Pichia pastoris: enhanced resistance of SK to proteolysis by glycosylation. Applied Microbiology and Biotechnology., 53(4): 469-475.

Ralph, A. P. and Carapetis, J. R. 2012. Group a Streptococcal diseases and their global burden. Host-pathogen interactions in Streptococcal diseases., 1: 27.

Ramirez-Peña, E., Treviño, J., Liu, Z., Perez, N. and Sumby, P. 2010. The group A Streptococcus small regulatory RNA FasX enhances streptokinase activity by increasing the stability of the ska mRNA transcript. Molecular Microbiology., 78(6): 1332-1347.

Reed, G. L., Liu, L., Houng, A. K., Matsueda, L. H. and Lizbeth, H. (1998, October). Mechanisms of fibrin-independent and fibrin-dependent plasminogen activation by streptokinase. In Circulation., 98(17): 41.

Robinson, B. R., Liu, L., Houng, A. K., Sazanova, I. Y., and Reed, G. L. 2000. The streptokinase beta domain plays a critical role in activator complex formation and substrate docking. In Circulation., 102(18): 103.

Salton, M. R., Kim, K. S. and Baron, S. (1996). Medical Microbiology. University of Texas Medical Branch at Galveston: Galveston, TX., 1: 9-6.

Salvà-Serra, F., Jaén-Luchoro, D., Jakobsson, H. E., Gonzales-Siles, L., Karlsson, R., Busquets, A. and Moore, E. R. 2020. First complete genome sequences of

Streptococcus pyogenes NCTC 8198T and CCUG 4207T, the type strain of the type species of the genus Streptococcus: 100% match in length and sequence identity between PacBio solo and Illumina plus Oxford Nanopore hybrid assemblies. Bio. Rxiv., 03(10): 985267

Schrager, H. M., Rheinwald, J. G. and Wessels, M. R. 1996. Hyaluronic acid capsule and the role of Streptococcal entry into keratinocytes in invasive skin infection. The Journal of Clinical investigation., 98(9): 1954-1958.

SHAMSI, Y. and KHAN, S. 2016. Review on Chiraita (Swertia chirata Bush. Ham.)–A Unani Herbal Medicine. Ind. J. Unani Med., 9(1): 30-37.

Shao, Y., Forster, S. C., Tsaliki, E., Vervier, K., Strang, A., Simpson, N. and Lawley, T. D. 2019. Stunted Microbiota and opportunistic pathogen colonization in caesarean-section birth. Nature., 574(7776): 117-121.

Shemanova, F., Postnikova, T. M. and Rostov, G. 1995. Enzyme Immunoassay of antistreptokinase. Klin Lab.\Diagn., 2: 26-9.

Shi, G. Y., Chang, B. I., Chen, S. M., Wu, D. H. and Wu, H. L. 1994. Function of streptokinase fragments in plasminogen activation. Biochemical Journal., 304(1): 235-241.

Sierig, G., Cywes, C., Wessels, M. R. and Ashbaugh, C. D. 2003. Cytotoxic effects of streptolysin O and streptolysin S enhance the virulence of poorly encapsulated group A Streptococci. Infection and Immunity., 71(1): 446-455.

Simbolo, M., Gottardi, M., Corbo, V., Fassan, M., Mafficini, A., Malpeli, G. and Scarpa, A. 2013. DNA qualification workflow for next generation sequencing of Histopathological samples. PloS One., 8(6): e62692.

Simpson, G., Roomes, D. and Heron, M. 2000. Effects of streptokinase and deoxyribonuclease on viscosity of human surgical and empyema pus. Chest., 117(6): 1728-1733.

Slusarcick, A. L. and McCaig, L. F. 2000. National Hospital Ambulatory Medical Care Survey: 1998 outpatient department summary. Advance Data., (317): 1-23.

Suh, H., Kim, K. H., Kim, S. S. and Han, M. H. 1984. Culture conditions of Streptococcus sp. for streptokinase production. Microbiology and Biotechnology Letters., 12(3): 231-231.

Sundram, V., Nanda, J. S., Rajagopal, K., Dhar, J., Chaudhary, A. and Sahni, G. 2003. Domain truncation studies reveal that the streptokinase-plasmin activator complex utilizes long range protein-protein interactions with macromolecular substrate to maximize catalytic turnover. Journal of Biological Chemistry., 278(33): 30569-30577.

Tan, L. K., Eccersley, L. R. and Sriskandan, S. 2014. Current views of haemolytic Streptococcal pathogenesis. Current Opinion in Infectious Diseases., 27(2): 155-164.

Todar, K. (2011). Bacterial resistance to antibiotics. Todar's online textbook of bacteriology., 4: 3.

Tortora, G. J., Funke, B. R. and Case, C. L. 2004. Microbial diseases of the digestive system. Microbiology an Introduction., 3: 712-713.

TRIAL, A. 2016. Industrial Enzymes: Recovery and Purification Challenges. Agro-Industrial Wastes as Feedstock for Enzyme Production: Apply and Exploit the Emerging and Valuable Use Options of Waste Biomass., 9: 5.

Wakeham, N., Terzyan, S., Zhai, P., Loy, J. A., Tang, J. and Zhang, X. C. 2002. Effects of deletion of streptokinase residues 48–59 on plasminogen activation. Protein engineering., 15(9): 753-761

Wang SL, Chao CH, Liang TW, Chen CC. (2009) Purification and characterization of protease and chitinase from Bacillus cereus TKU006 and conversion of marine wastes by these enzymes. Mar Biotechnol.

Wang SL, Ye RQ, Nathoo S. (1994). Engineering and production of streptokinase in Bacillus subtilis expression – secretion system. Appl Environ Microbiol 60:517 – 23.

Wang, B., Ruiz, N., Pentland, A. and Caparon, M. 1997. Keratinocyte proinflammatory responses to adherent and nonadherent group A Streptococci. Infection and immunity., 65(6): 2119-2126.

Wannamaker, L.W. (1972) Perplexity and precision in the diagnosis of streptococcal pharyngitis. Am. J. Dis. Child.;124:352–358.

Welfle, K., Pfeil, W., Misselwitz, R., Welfle, H. and Gerlach, D. 1992. Conformational properties of streptokinase—differential scanning calorimetric investigations. International Journal of Biological macromolecules., 14(1): 19-22.

Wessels, M. R. and Bronze, M. S. 1994. Critical role of the group A streptococcal capsule in pharyngeal colonization and infection in mice. Proceedings of the National Academy of Sciences., 91(25): 12238-12242.

Wu, D. H., Shi, G. Y., Chuang, W. J., Hsu, J. M., Young, K. C., Chang, C. W. and Wu, H. L. 2001. Coiled coil region of streptokinase γ-domain is essential for plasminogen activation. Journal of Biological Chemistry., 276(18): 15025-15033.

Young, K. C., Shi, G. Y., Chang, Y. F., Chang, B. I., Chang, L. C., Lai, M. D. and Wu, H. L. 1995. Interaction of streptokinase and plasminogen: studied with truncated streptokinase peptides. Journal of Biological Chemistry., 270(49): 29601-29606.

Zhiwen, Z., Hawthorne, M. L., Guo, Y., Drake, W., Bilaceroglu, S., Misra, H. L. and Light, R. W. 2006. Tissue plasminogen activator combined with human recombinant deoxyribonuclease is effective therapy for empyema in a rabbit model. Chest., 129(6): 1577-1583.

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