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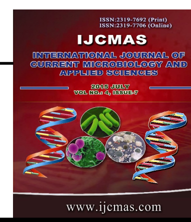

Original Research Article

A Potential New Isolate for Streptokinase Production

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A B S T R A C T

Keywords

Streptokinase, β- haemolysis, *Streptococcus pyogenes*, Caseinolysis

Screening of isolates for their potency to produce streptokinase was an important criterion of this research. The study was carried out on 60 throat swabs collected from patients with acute tonsillitis. Primary screening and characterization of the specimens from the infected throat can be an excellent source for the isolation of haemolytic organisms. Three bacterial isolates (5%) demonstrated β- haemolysis. Based on the results of radial caseinolysis assay and blood clot dissolving assay, isolate SK-2 demonstrated the highest streptokinase activity. When subjected to morphological and biochemical characterization based on Bergey's criteria, it was identified as *Streptococcus pyogenes*. The thrombolytic potential of this particular isolate indicated that it could extract a promising streptokinase with potent activity also it may be utilized for large scale production of streptokinase.

Introduction

Thrombotic diseases are responsible for heavy toll in death and disability worldwide. These are the most common diseases in the United States and in almost all western industrialized countries. Each year cardiovascular disease (CVD) causes 4.3 million deaths in Europe while in United States 2.5 million deaths (Kumar *et al.,* 2010).

A blood clot (thrombus) developed in the circulatory system can cause vascular blockage leading to serious consequences including death. A healthy homeostatic system suppresses the development of blood clots in normal circulation, but reacts extensively in the event of vascular injury to prevent blood loss. Outcomes of a failed homeostasis include stroke, pulmonary embolism, deep vein thrombosis and acute myocardial infarction. Pathologies involving a failure of homeostasis and the development of clot require clinical intervention consisting of intravenous administration of thrombolytic agents Streptokinase is one such agents (Taleb *et al.,* 2005).

Streptokinase (SK), the oldest and best-known plasminogen activator, is produced

by various strains of β-hemolytic *Streptococci*. Its fibrinolytic activity was first described in 1933. Streptokinase found its initial clinical application in combating fibrinous pleural exudates, hemothorax and tuberculous meningitis. Streptokinase is udes in hospital to dissolve the fibrin of blood clots, especially those in the arteries of heart and lungs it is also used on the clots formed in shunts during kidney dialysis (Abdelmongy and Taha, 2012). Streptokinase was first used in patients with acute myocardial infarction and this changed the focus of treatment. Earlier streptokinase produced contradictory results until the GISSI (Gruppo Italiano per la Sperimentazione della Streptochinasi nell'Infarto Miocardico) trial in 1986, which validated streptokinase as an effective therapy (Kumar *et al.,* 2010).

Streptokinase is an extra cellular protein, extracted from certain strains of beta hemolytic *streptococci*. It is a non-protease plasminogen activator that activates plasminogen to plasmin, the enzyme that degrades fibrin clot through its specific lysine binding site; it is used therefore as a drug in thrombolytic therapy. Streptokinase is currently used in clinical medicine as a therapeutic agent in the treatment of thromboembolic blockages, including coronary thrombosis (Dubey *et al.,* 2011).

The objective of this study was to isolate β-hemolytic *streptococci* species form Sudanese patients suffering acute tonsillitis, with potential for streptokinase production.

Materials and methods

A total of 60 throat swabs of the biomass were collected using sterile swab from patients suffering from acute tonsillitis in Khartoum Hospital ENT Department, and were given number prefixed with SK Then transferred aseptically to the Lab during Jan–April, 2015.

Isolation of microorganisms

Isolation of the samples was performed by the serial dilution plate technique (Uversky and Fink, 2004). In this technique; 1g of each soil sample was taken in 9 ml of sterilized distill water in pre- sterilized test tube. Serial aqueous dilutions were prepared by transferring 1ml of the soil suspension into 9 ml of sterilized distill water in sterilized test tubes. Different aqueous dilutions (10^{-7}) of the soil suspension were applied separately into sterilized Petri dishes containing sterilized nutrient agar and incubated for 24 hr at 37□C.

Preparation of blood agar medium

Commercially available blood agar medium was used. 42 g of the medium was suspended in one liter of purified water. Then heated with frequent agitation until the medium was completely dissolved and autoclaved at 121 °C for 15 minutes. After the medium was cooled, 10 ml of freshly collected sterile defibrinated blood was added, cooled and poured on Petri dishes. The medium was incubated for 24 hours to examine the microbial growth and hemolytic reactions.

Isolation of B-haemolytic *Streptococci*

All the samples were freshly poured on the previously prepared blood agar medium and anaerobically incubated for 24 hours. The isolates with clear zone of haemolysis around the colonies were purified through repeated streaking on fresh agar plates and maintained on the on the Brain Heart Infusion Agar (BHIA) until further use (Shil *et al.,* 2013).

Extraction and recovery of crude streptokinase

The pure culture colonies showing clear zone of haemolysis on blood agar plates were inoculated in 10 ml of mineral salt medium and incubated at 37°C for 24 h. Following the development of turbidity, 1 ml of these individual cultures were transferred to 49 ml of mineral salt medium and incubated as previously mentioned. Upon overnight incubation, the individual cultures were centrifuged at 10,000 *g* using cold centrifuge for 30 min. The cell free supernatants were filtered through 0.45 µm cellulose acetate filter and the filtrates were considered as crude streptokinase (Shil *et al.,* 2013).

Screening of streptokinase producing haemolytic *Streptococci*

Radial caseinolysis

Preparation of skimmed milk agar plates

51.5 grams of skimmed milk agar, was suspended in 1 liter of distilled water and heated to boiling until completely dissolved. Sterilized by autoclaving, mixed well and poured into sterile Petri dishes. After cooling, the plates were incubated for 24 hours to examine microbial contamination. The cell free supernatants were loaded on the pores that made on the plates and incubated for 24 hours.

Blood clot dissolving assay

Sterile empty microcentrifuge tubes were taken, labelled suitably and their weights determined (W1). Human blood was freshly collected. A 500 µl of blood was transferred into each microcentrifuge tube and incubated at 37°C for 45 min. After clot formation, serum was completely removed by aspiration, without disturbing the clot. The weights of the microcentrifuge tubes with the clots were noted (W2). To determine the clot weight, W1 was subtracted from W2. A 500 µl of the respective cell free supernatants were added to the respective tubes. Pre-sterilized distilled water was added to one of the tubes containing clot and this served as control. All the tubes were then incubated at 37°C for 90 min and observed for clot lysis. Following incubation, the fluid on each tube was removed and tubes were again weighed (W3) to observe the difference in clot weight. Percentage of clot lysis was calculated using the following equation (Shil *et al.,* 2013).

Percentage lysis = 100-{[(W3- W1)/(W2-W1)]*100}50 Equ (1)

Blood clot lysis on slide

Three clean slides were labeled suitably, clotted blood was fix on each slide, then 200 µl of the crude enzyme was added to the clot on the slide and observed for clot lysis activity.

Identification

The selected isolate was identified based on its morphological and biochemical characteristics based on Bergy's Manual of Determinative Bacteriology. (Reference) that involved cultural microscopic and biochemical characteristics.

Bacitricin sensitivity

Bacitricin Susceptibility Test Discs are used for the identification and differentiation of Group A *Streptococci* (especially *S. pyogenes*) from other β --haemolytic *Streptococci*. Bacitricin susceptibility test discs are filter paper discs impregnated with 0.04 units of Bacitricin. Bacitricin discs can save considerable time, labour and materials

if used as a screening test before serological grouping.

Results and Discussion

Isolation and screening of the samples

Haemolysis on blood agar

Among a total of 60 throat samples collected, both α - haemolysis with greenish zone, indicates the reduction of the red blood cell hemoglobin to methemoglobin in the medium surrounding the colony. While β -haemolysis with clear zone indicates complete or true lysis of red blood cells. A clear zone, approaching the color and transparency of the base medium, surrounds the colony were observed

Out of these throat samples, three isolates (5%) showed β -haemolysis according to figure 1, and 7 (11.6%) isolates showed α - haemolysis according to figure 2, the rest of the samples (83.4%) showed (γ) haemolysis according to figure 3 indicates the lack of haemolysis.

Caseinolysis of bacterial isolates

Table 1 shows caseinolytic activity of the crude streptokinase after the cell-free fluid was used to fill the pre-made wells in the skim agar medium. The activity illustrated by measuring the diameter of zone of inhibition and compared to the reference strain *S. pyogenes* MTCC1923.

On e unit of enzyme activity was defined as the amount of enzyme that produce a clear zone of 1 mm at pH of 7 and 37 $^{\circ}$C for 24 hr. All the samples give high activity ranged from 10 to 13 mm, while sample SK2 revealed the highest activity of 13 mm. From table 1 we could conclude that Sk 2 is a promising isolate for streptokinase production as it obtained activity near to *S. pyogenes* obtained from microbial type culture collection1923 (MTCC1923).

Blood clot dissolving pattern of bacterial isolates

As shown in table 1, percent clot lysis obtained from the calculating clot lysis percentage by equation (1) after treating the clots with streptokinase produced from our isolates and that produced from *S. pyogenes* MTCC 1923. Maximum clot lysis was observed with isolate SK2 of 38.2% same as the percentage obtained from the standard *S. pyogenes* MTCC 1923, confirming that isolate SK2 is a very promising isolate for streptokinase production. The other samples SK1 and SK3 also recorded higher activity of 37.1% and 36.4% respectively.

Blood clot dissolving activity on slides

A clear visual representation of clot lysis is shown in figure 4 after 200 µl of the crude streptokinase produced by SK1, SK2 and SK3 were added to the clot on slide.

Characterization of producing microorganism

Among the 3 isolates, isolate SK-2 demonstrated the highest streptokinase activity and larger zone of inhibition as outlined in table 1.

SK-2 was subjected to morphological and biochemical characterization according to Bergy's criteria. The organism shown negative results on indol, methyl red, and citrate utilization tests while positive result on gram staining and glucose test, and later identified as *Streptococcus pyogenes*. The significant results of the characterization of the isolate have been clearly presented in table 2. Streptokinase was the first thrombolytic drug to be introduced for the treatment of acute myocardial infarction.

Being a leading fibrinolytic agent and finding its usage in the treatment of thromboembolic conditions, streptokinase is now been included in the World Health Organization (WHO) Model List of Essential Medicine The increasing potential of streptokinase application promoted us to screen for newer streptokinase producing organisms. Also the exponential increase in the application of streptokinase in various fields in the last few decades demands extension in both qualitative improvement and quantitative enhancement (Mahboubi *et al.,* 2012).

In the present study, most of the clinical samples were isolated from the throat of human beings. The reason behind this strategy was the fact that the respiratory tract is being used by most βHS as a port of entry in to the body of their hosts (Kermati *et al.,* 2012).

Alpha haemolysis (α) is the reduction of the red blood cell hemoglobin to methemoglobin in the medium surrounding the colony. This causes a green or brown discoloration in the medium. The color can be equated with "bruising" the cells. Microscopic inspection of alpha-hemolyzed red blood cells shows that the cell membrane is intact, so it is not, in fact, true lysis. Beta haemolysis will never include the brown or green discoloration of the cells in the surrounding medium. On prolonged incubation, many alpha hemolytic organisms will begin to appear more clear, but if the surrounding medium contains any shades of brown or green the "haemolytic" is still considered "alpha (Rebbeca, 2005).

Beta haemolysis (β) is defined as complete or true lysis of red blood cells. A clear zone, approaching the color and transparency of the base medium, surrounds the colony. Many species of bacteria produce toxic by-products that are capable of destroying red blood cells (Rebbeca, 2005). Gamma haemolysis (γ) is somewhat self-contradictory. Gamma indicates the lack of haemolysis. There should be no reaction in the surrounding medium.

The principal factor responsible for β-haemolysis in GAS is SLS, an oxygen-stable cytolysin that is not immunogenic in the course of natural infection. According to Thaer *et al.* (2005) the streptokinase yield of the best mutant strain in their work indicated an increase of 120 % as compared to the wild strains. Hyun *et al.* (1997) produced copious quantities of streptokinase using a mutant Streptococcus spp. Doss *et al.* (2011) found that among a total of 15 throat samples collected, both alpha haemolysis and beta haemolysis were observed. Out of these throat samples, 10 showed alpha haemolysis and 5 samples showed beta haemolysis. In beta haemolysis, other than *Streptococcus* sp, *Staphylococcus* sp. was also present. Also Dubey *et al.* (2011) found only 3 β-haemolytic *streptococci* with haemolytic activity isolated from different samples of blood and biomass from infected throat.

Table.1 Caseinolysis and blood clot dissolving pattern of bacterial isolates

Streptokinase producers	Zone of caseinolysis in mm	Percentage of clot lysis
S. pyogenes MTCC 1923	17	38.73%
Sk1	11	37.1%
Sk2	13	38.2%
Sk3	10	36.4%

Table.2 Morphological and biochemical characterization of isolateSK-2 and reference strain

Biochemical test	SK2	*S. pyogenes MTCC1923*
Growth under aerobic condition	+	+
Growth under anaerobic condition	+	+
Growth on MacConkey agar	-	-
Bacitracin sensitivity	+	+
Alpha haemolysis on blood agar	-	-
Beta haemolysis on blood agar	+	+
Casein hydrolysis	+	+
Indol test	-	-
Methyl red test	-	-
Citrate utilization test	-	-
Gram staining	+	+
glucose	+	+

Keys: +, positive; -, negative; S, susceptible to bacitricin

Figure.1 β- haemolysis on blood agar

Figure.2 α- haemolysis on blood agar

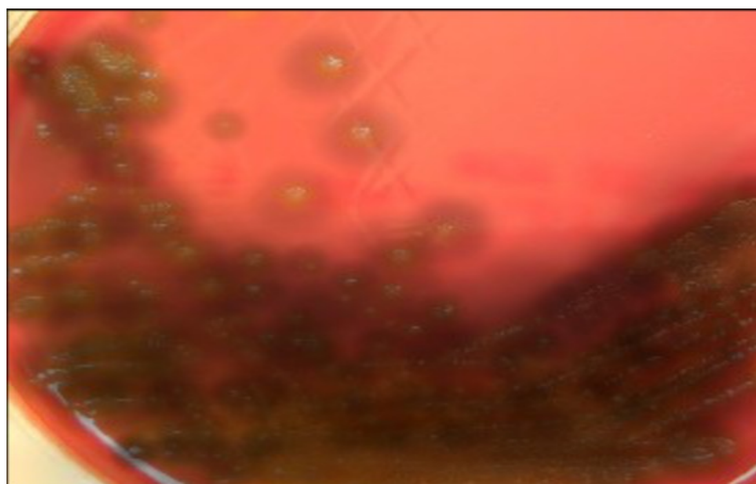


Figure.3 γ -haemolysis on blood agar

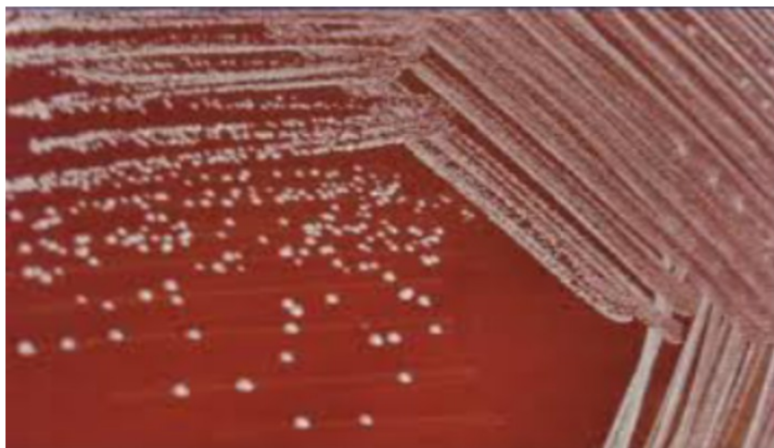
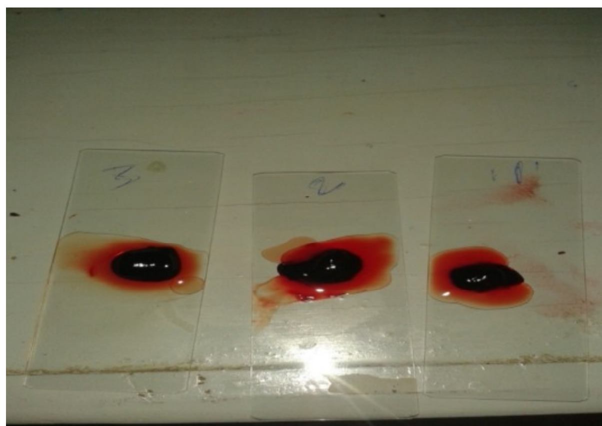


Figure.4 Streptokinase clot dissolving activity on slide



Streptokinase assay depends on its ability to activate plasminogen to plasmin which in turn, hydrolyzes an indicator substrate and the extent of hydrolysis over a given period of time is related back to the concentration of streptokinase. In our study after treating the clots with streptokinase produced from our isolates, maximum clot lysis was observed with isolate SK2 of 38.2% within 20 min. confirming that isolate SK2 is a very promising isolate for streptokinase production. Substrates for plasmin may include the fibrin clot, casein, other proteins, and various synthetic esters which have been used successfully for the sensitive detection of the enzyme activity (Pratap *et al.,* 2000; Mundada *et al.,* 2003). In the Saksela (1981) study two methods were used: Radial caseinolysis method of agarose gel containing both casein and plasminogen is commonly used for simple detection of the enzyme. The method is simple but it lacks accuracy and it is time consuming. This method is based on the determination of the liberated tyrosine from digested casein after plasminogen activation (Mounter and Shipley, 1957).

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References

Abdelmongy, M., Taha, T. 2012. In vitro detection and optimization of streptokinase production by two *streptococcal* strains in relatively low cost growth medium. *Int. Res. J. Microbiol.,* 3(4): 153–163.

Doss, H.M., Madhuri, Manohar, Neha, Atul Singh, Mohanasrinivasan, V., Subathra Devi, C. 2011 Studies on isolation, screening and strain improvement of streptokinase producing B- hemolytic *Streptococci*. *World J. Sci. Technol.,* 1(3): 07–11

Dubey, R., Kumar, J., Agrawala, D., Char, T., Pusp, P. 2011. Isolation, production, purification, assay and characterization of fibrinolytic enzymes: Nattokinase, Urokinase and Streptokinase from bacterial sources. *Afr. J. Biotechnol.,* 10(8): 1408–1420.

Hyun, H.H., Lee, Y.B., Song, K.H., Jeon, J.Y., Lee, H.H. 1997. Strain improvement for enhanced production of streptokinase and streptodornase in *Streptococcus spp*. *J. Microbiol. Biotechnol.,* 7: 101–6.

Kermati, M., Roohvand, F., Mehdi, M., Reza, M., Sedat, M. 2012. Screening, cloning and expression of active streptokinase from an Iranian isolate of *S. equisimilis* group C in *E. coli*. *Iran. J. Basic Med. Sci.,* 16(4): 620–627.

Kumar, A., Pulicherla, K., Seetha, K., Sambasira, K. 2010. Evolutionary trend of thrombolytics. *Int. J. Bio-sci. Bio-technol.,* 2(4): 51–68.

Mahboubi, A., Kazem, S., Mirzaei, M., Azadi, S., Solaimanian, R. 2012. Biolologiacal activity analysis of native and recombinant streptokinase using clot lysis chromogenic substrate assay. *Iran. J. Pharm. Res.,* 11(4): 1087–1093.

Mounter, L.A., Shipley, B.A. 1957. The inhibition of plasmin by toxic phosphorus compounds. *J. Biol. Chem.,* 226: 855–861.

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

Pratap, J., Rajamohan, G., Dikshit, K.L. 2000. Characteri stics of glycosylated streptokinase secreted from Pichia pastoris: enhanced resistance of SK to proteolysis by glycosylation. *Appl. Microbiol. Biotechnol.,* 53: 469–475.

Rebbeca, B. 2005. Blood agar plates and haemolysis protocols. *Am. Soc. Microbiol,*

Saksela, O. 1981. Radial caseinolysis in agarose: a simple method for detection of plasminogen activators in the presence of inhibitory substances and serum. *Anal. Biochem.,* 111: 276–282

Shil, B., Sourav, B., Arjiti, D., Palanisway, M. 2013. Isolation, screening and characterization of beta haemolytic *Streptococci* with potential of streptokinase production. *Int. J. Bio sci.,* 2(4): 63–66.

Taleb, T., Kunameeni, A., Ellaiah, P. 2005. Isolation and mutagenesis of streptokinase producing bacteria. *Am. J. Immun.,* 1(4): 125–129.

Thaer, Taleb, Abed Abdelghani, K., Adinarayana, E. Poluri, 2005. Isolation and mutagenesis of streptokinase producing bacteria. *Am. J. Immunol.,* 1(4): 125–12.

Uversky, V.N., Fink, A.L. 2004. Conformational constraints for amyloid fibrillation: The importance of being unfolded. *Biochem. Biophys. Acta,* 1698: 131–153.