International Journal of Biology Research
ISSN: 2455-6548
Impact Factor: RJIF 5.22
www.biologyjournal.in
Volume 3; Issue 1; January 2018; Page No. 213-216

Screening of potential streptokinase producing *Streptococcus* strain

Patil RC
Department of Microbiology, Bhavan's College, Andheri (West), Mumbai, Maharashtra, India

Abstract
Streptokinase is a very well known novel fibrinolytic protein. It is produced by several species of Streptococci and possesses therapeutic importance. Streptokinase can be dissolves a blood clot by the activation of plasminogen to plasmin. Soil is an excellent source for isolation of enzymes. In the present study a total of 50 soil samples were collected from nearby ENT hospital, 23 isolates were found as streptococci which were isolated on glucose azide medium and demonstrated for β-haemolysis on blood agar. Among them 11 *Streptococci* strains showed β-haemolysis and subjected to caseinolysis assay and blood clot dissolving assay as well as morphological and biochemical characterization based on Bergey's criteria. Highest streptokinase activity showed S9 isolates were subjected for the large scale production of streptokinase. The optimum temperature and pH of Streptokinase is 27-37°C and 9 respectively. The molecular weight of Streptokinase was obtained 47 kDa. 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.

Keywords: streptokinase, bacterial strain, caseinolysis assay, blood clot

Introduction
The extracellular enzyme streptokinase is produced by various strains of β-hemolytic streptococci. The enzyme is a single-chain polypeptide that exerts fibrinolytic action indirectly by activating the circulatory plasminogen. A considerable degree of heterogeneity exists among the streptokinases produced by different groups of streptococci. Biophysical techniques such a circular dichroism, nuclear magnetic resonance (NMR) spectroscopy, Fourier transform infrared (FT–IR) spectroscopy and differential scanning colorimetry (DSC) have provided most of the available structural information on streptokinase (Beldarrain *et al*., 2001) [3].
Streptokinase is not only an enzyme but also a potent activator of plasminogen, the inactive precursor of plasmin. Plasmin is the active fibrinolytic component of the circulatory system, solubilizing the fibrin network in blood clots through limited proteolysis. Streptokinase is used recently as therapeutic agent in the treatment of thromboembolic blockages, including coronary thrombosis (Dubey *et al*., 2011) [5].
Human physiology is so well articulated that in case of any haemorrhage, the healthy haemostatic system responds extensively by forming a blood clot or thrombus to prevent excessive blood loss. However, under normal condition, for an effective vascular functioning, the development of thrombus in circulation is usually suppressed (Abdelghani *et al*., 2005) [1]. In case of an uncontrolled haemostasis, development of a thrombus in the vascular system may result in vascular blockage, pulmonary embolism, deep vein thrombosis and acute myocardial infarction (AMI) including death (Hamid *et al*., 2011) [6]. Streptokinase is used 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) [2].
The objectives of the present study were isolation of β-haemolytic streptococci from soil samples collected from nearby ENT hospital, screening and selection of a suitable isolate for streptokinase production and identification of the isolate based on its morphology and biochemical characteristics.

Materials and methods
A total of 50 soil samples were collected from nearby ENT hospital, 23 isolates were found as *Streptococci* which were isolated on glucose azide medium and demonstrated for β-haemolysis on blood agar. Among them 11 *Streptococci* strains showed β-haemolysis and subjected to caseinolysis assay and blood clot dissolving assay as well as morphological and biochemical characterization based on Bergey's criteria.

Isolation and Identification of Isolates
For isolation of *Streptococci* strains, soil samples were collected from nearby ENT hospital and serial dilution plate technique was performed. In this method 1g of each soil sample was taken in 9 ml of sterilized distilled water in pre-sterilized test tube. Serial aqueous dilutions were prepared by transferring 1ml of the soil suspension into 9 ml of sterilized distilled water in sterilized test tubes. Different aqueous dilutions (10^{-6}) of the soil suspension were applied separately into sterilized Petri dishes containing sterilized Glucose azide medium and incubated for 24-48 hr at 37^0C. The selected isolate was identified by its morphological and biochemical characteristics based on Bergy's Manual of Determinative Bacteriology.

Preparation of blood agar medium and Isolation of β-haemolytic *Streptococci*

For the isolation of β-haemolytic *Streptococci* strain, preparation of blood agar was done by taking a Blood base agar (Hi-media) and autoclaved at 121^0C for 20 mins. After that medium was cooled, and then freshly collected sterile defibrinated blood was added and mixed uniformly, and poured in sterile Petri dishes. The medium was incubated for 24 hours to examine the sterility. All the samples were freshly inoculated 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 Brain Heart Infusion Agar (BHIA).

Production, Extraction, recovery and assay of streptokinase

Isolates showing clear zone of haemolysis on blood agar plates were maintained on BHIA. Potential strain was inoculated in mineral salt medium and incubated at 37^0C for 24 h. Upon overnight incubation, the individual cultures were centrifuged at 10,000 rpm 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) [11]. To the supernatant 2.5ml streptavidin solution and 0.1M tris HCl buffer was added and incubated at 37^0C for 20minutes. After incubation period reaction was stopped by adding 2ml of 1% TCA and incubated at 50^0C for 30minutes. The solution was centrifuged and the supernatant (1ml) was taken. To that 1ml of 2% sodium carbonate and 0.5% copper sulphate was added and incubated at room temperature for 10minutes. To the mixture 1ml folins phenol reagent was added, mixed well and incubated at room temperature for 30minutes in dark condition. The colored solution was read at 660nm in spectrophotometer.

Ammonium salt precipitation

The fibrinolytic enzymes were also purified by ammonium sulfate saturation. The protein fraction was precipited with 85% ammonium sulfate. Ammoniun sulfate was found to activate the fibrinolytic activity after dialysis. Fibrinolytic enzymes were partially purified by using anion exchange column chromatography (DEAE cellulose, MERK).

Effect of Temperature and pH on enzyme activity

The effect of temperature and pH on streptokinase on production was carried out using the following temperature values such as 20^0C, 30^0C, 40^0C and 50^0C whereas the effect of pH using pH values of 6, 7, 8, and 9 and the enzyme activity was measured.

Sodium Dodecyl Sulphate-Poly acrylamide Gel Electrophoresis (SDS-PAGE)

For the SDS-PAGE, resolving gel and stacking gel were prepared and poured into the gel apparatus and allowed to polymerize. After that sample buffer (β-merceptoethanol and bromophenol blue) was added into the sample that containing volume 1:5 and then this mixture was heated at 100^0C for 2 min. Then spinned down for few seconds and this mixture was loaded in the wells of acrylamide polymerized gel in gel apparatus. For the determination of molecular weight, the marker was also loaded in separate well and electrophoresis was carried out at 50V constant voltage. Process was kept up to the dye reaches about 1cm from the end of the gel. The gel was taken off with the help of spacer and stained with coomassie blue, and the protein bands were visualized by destaining with methanol-acetic acid solution.

Radial caseinolysis assay of streptokinase producing haemolytic *Streptococci*

For determination of caseinolysis activity, skimmed milk agar plates were prepared with help of standard protocol. The cell free supernatants were loaded on to the skimmed milk agar plates and incubated at 37^0C for 12 h. Following incubation, the diameters of the zone of caseinolysis were measured. The isolate showing the highest zone of caseinolysis was selected for further studies.

Blood clot dissolving assay

Blood clot dissolving assay was carried out by taking sterile empty microcentrifuge tubes and labeled and their weights determined as (W1). Sheep blood was freshly collected. In each microcentrifuge tube 500 µl of blood was taken and incubated at 37^0C for 45 min for clot formation. After clot formation, serum was completely removed by aspiration, without disturbing the clot. The weights of the microcentrifuge tubes with the clots were noted as (W2). To determine the clot weight, W1 was subtracted from W2. After that in each tube 500 µl of the respective cell free supernatants were added. For control test, pre-sterilized distilled water was added to one of the tubes containing clot. All the tubes were then incubated at 37^0C for 90 min and observed for clot lysis. Following incubation, the fluid on each tube was removed and tubes were again weighed as (W3) to observe the difference in clot weight. Percentage of clot lysis was calculated using the following equation (Shil *et al.,* 2013) [11].

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

Results

Isolation and Identification of Isolates

In the present study a total of 50 soil samples were collected from nearby ENT hospital, 23 isolates were found as *Streptococci* which were isolated on glucose azide medium and identified on the basis of morphological and biochemical characterization based on Bergey's criteria. Gram staining of these isolates also revealed that isolates were gram positive cocci in short chain. The organisms were shown growth under aerobic and anaerobic conditions, negative results on indol, methyl red, and citrate utilization tests while acid fermentation in glucose, lactose, sucrose, maltose, and ribose test.

Screening of isolates for β-Haemolysis on blood agar

Isolates demonstrated for β-haemolysis on blood agar. Isolates were compared with both α-haemolysis and β-haemolysis and α-haemolysis with greenish zone, indicates the reduction of the red blood cell hemoglobin to methemoglobin in the medium surrounding the colony whereas the β-haemolysis with clear zone indicates complete or true lysis of red blood

cells. On that basis, if the isolates showed a clear zone surrounds the colony were observed and considered as β-haemolysis producers. Among them 11 *Streptococci* strains showed β-haemolysis.

Caseinolysis of bacterial isolates

Potential strain was used 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. mutans* MTCC497. Among 11 isolates 6 isolates showed potential caseinolytic activity, isolate S9 revealed the highest activity of 19 mm. From that S9 strain considered as promising isolate for streptokinase production.

Blood clot dissolving pattern of bacterial isolates

Potential strains were used for blood clot dissolving assay. Percent clot lysis obtained from the calculating clot lysis percentage by equation (1) after treating the clots with streptokinase produced from isolates and that produced from *S. mutans* MTCC497. Maximum clot lysis was observed with isolate S9 of 67% whereas the percentage obtained from the standard *S. mutans* MTCC497 of 63%, confirming that isolate S9 is a very promising isolate for streptokinase production. The other samples S8 and S5 also showed higher activity of 56% and 53% respectively.

Table 1

SN	Isolates	β-haemolysis producer	Caseinolytic activity (mm)	Blood clot lysis (%)
1	S1	+	10	32
2	S2	+	10	34
3	S3	-	-	-
4	S4	+	12	34
5	S5	+	15	53
6	S6	-	-	-
7	S7	+	14	48
8	S8	+	16	56
9	S9	+	19	67
10	S10	-	-	-
11	S11	-	-	-
12	S12	+	14	47
13	S13	-	-	-
14	S14	-	-	-
15	S15	+	11	32
16	S16	-	-	-
17	S17	-	-	-
18	S18	-	-	-
19	S19	-	-	-
20	S20	-	-	-
21	S21	+	14	44
22	S22	-	-	
23	S23	+	10	38
24	*S. mutans* (MTCC 497)	+	16	63

Characterization of Streptokinase

Highest streptokinase activity showed by S9 isolates was subjected for the large scale production of streptokinase. Streptokinase enzyme activity was carried out using the following temperature values such as 20^0C, 30^0C, 40^0C and 50^0C whereas pH with using pH values of 6, 7, 8, and 9 and the enzyme activity was measured. The optimum temperature of Streptokinase activity was obtained at 27-37^0C is 128U/ml and at pH 9 is 127U/ml. The molecular weight of Streptokinase was obtained 47 kDa.

Discussion

Now a day the increasing potential of streptokinase application promoted us to screen for newer streptokinase producing organisms. Application of streptokinase in various fields exponentially increases in both qualitative improvement and quantitative enhancement (Mahboubi *et al.,* 2012) [7]. 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. In this study, samples were collected nearby ENT hospital and isolates were isolated and screened for β-hemolysis. Observation of α- haemolysis and β-hemolysis shows the difference, α- haemolysis 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 whereas the β-hemolysis will never include the brown or green discoloration of the cells in the surrounding medium, is defined as complete or true lysis of red blood cells. On prolonged incubation, many alpha hemolytic organisms will begin to appear clearer, but if the surrounding medium contains any shades of brown or green the haemolytic is still considered alpha (Rebbeca, 2005) [10].

Doss *et al.,* (2011) [4] 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) [5] found only 3 β-haemolytic *streptococci* with haemolytic activity isolated from different samples of blood and biomass from infected throat. 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. 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) [8, 9]. Maximum clot lysis was observed with isolate S9 of 67% that isolate S9 is a very promising isolate for streptokinase production. The other samples S8 and S5 also showed higher activity of 56% and 53% respectively. Highest streptokinase activity showed S9 isolates were subjected for the large scale production of streptokinase. The optimum temperature and pH of Streptokinase is 27-37°C and 9 respectively. The molecular weight of Streptokinase was obtained 47 kDa.

Conclusion

In the present study a total of 50 soil samples were collected from nearby ENT hospital, among them 11 *Streptococci* strains showed β-haemolysis and subjected to caseinolysis assay and blood clot dissolving assay. Highest streptokinase activity showed S9 of 67% blood clot lysis. Isolate S9 is a very promising isolate for streptokinase production. The optimum temperature and pH of Streptokinase is 27-37°C and 9 respectively. The molecular weight of Streptokinase was obtained 47 kDa. 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.

References

1. Abdelghani TTA, Kunamneni A, Ellaiah P. Isolation and mutagenesis of streptokinase producing bacteria, Am. J Immunol. 2005; 1(4):125-129.
2. Abdelmongy M, Taha T. In vitro detection and optimization of streptokinase production by two *streptococcal* strains in relatively low cost growth medium. Int. Res. J Microbiol. 2012; 3(4):153-163.
3. Beldarrain A, Lopez-Lacomba JL, Kutyshenko VP, Serrano R, Cortijo M. Multidomain structure of a recombinant streptokinase. A differential scanning calorimetry study. J Protein Chem. 2001; 20:9-17.
4. Doss HM, Manohar M, Singh NA, Mohanasrinivasan V, Devi CS. Studies on isolation, screening and strain improvement of streptokinase producing β- hemolytic streptococci, World J Sci. Technol. 2011; 1(3):7-11.
5. Dubey R, Kumar J, Agrawala D, Char T, Pusp P. Isolation, production, purification, assay and characterization of fibrinolytic enzymes (Nattokinase, Streptokinase and Urokinase) from bacterial sources, Afr. J Biotechnol. 2011; 10(8):1408-1420.
6. Hamid M, Rehman KU, Nejadmoqaddam MR. Investigation of fibrinolytic activity of locally produced streptokinase, Asian J Chem. 2011; 23(1):251-254.
7. Mahboubi A., Sadjady S.K., Abadi M.M.S., Azadi S. and Solaimanian R., (2012). Biological activity analysis of native and recombinant streptokinase using clot lysis and chromogenic substrate assay, Iranian J Pharma. Res. 2011; 11(4):1087-1093
8. Mundada LV, Prorok M, DeFord ME, Figuera M, Castellino FJ, Fay WP. Structure– function analysis of the streptokinase amino terminus (residues 1–59), J Biol. Chem. 2003; 278:24421-24427.
9. Pratap J, Rajamohan G, Dikshit KL. Characteristics of glycosylated streptokinase secreted from Pichia pastoris: enhanced resistance of SK to proteolysis by glycosylation. Appl. Microbiol. Biotechnol. 2000; 53:469 475.
10. Rebbeca B. Blood agar plates and haemolysis protocols. Am. Soc. Microbiol, 2005.
11. Shil B, Sourav B, Arjiti D, Palanisway M. Isolation, screening and characterization of beta haemolytic *Streptococci* with potential of streptokinase production. Int. J Bio sci. 2013; 2(4):63 66.