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Isolation, Screening and Characterization of β-Haemolytic Streptococci with Potential of Streptokinase Production

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Abstract

Streptokinase is a novel fibrinolytic protein produced by several species of streptococci. As a therapeutic, streptokinase can be used in the treatment of thromboembolic disorders where it dissolves a blood clot by the activation of plasminogen to plasmin. Specimens from infected throat can be an excellent source for the isolation of haemolytic organisms. From the 34 throat swabs collected from patients with acute tonsillitis, 43 bacterial isolates demonstrated β-haemolysis. Among these β-haemolytic organisms, 11 isolates were streptococci. Screening the isolates for their potency to produce streptokinase was an important criterion of this paper. Based on the results of radial caseinolysis assay and blood clot dissolving assay, isolate SK-6 demonstrated the highest streptokinase activity. When subjected to morphological and biochemical characterization based on Bergey's criteria, isolate SK-6 was identified as Streptococcus equisimilis. The thrombolytic potential of this particular isolate indicated that it may also be utilized for the large scale production of streptokinase.

Keywords: *Streptococcus equisimilis*, streptokinase, tonsillitis, caseinolysis, haemolysis.

Introduction

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[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[2].

An effective therapy against thrombus can be the intravenous administration of thrombolytic agents, usually a plasminogen activator that activates the inactive plasminogen to plasmin which in turn degrades fibrin to soluble products and thus establish normal blood flow[3,4]. Extensively investigated sources of thrombolytic agents include snakes, earthworms, bacteria, actinomycetes and fungi[5]. The plasminogen activators from these sources are mainly categorized into two types: non fibrin specific plasminogen activators such as streptokinase, urokinase and fibrin specific plasminogen activators such as tissue-type plasminogen activators[6].

Streptokinase (E.C.3.4.99.22) is an extracellular, single chain, non-enzymatic, monomeric protein, consisting 440 amino acids, including a 26-amino acid *n*-terminal signal peptide which is cleaved during secretion to yield the mature 414 amino acid protein residues of 47 kDa molecular weight[7,8]. Streptokinase is produced by many strains of β-haemolytic streptococci, isolated naturally from upper respiratory tract. Streptokinase produced by different groups of streptococci differs considerably in structure[9,10].

The objectives of the present study were isolation of β-haemolytic streptococci from patients with throat infections, screening and selection of a suitable isolate for streptokinase production and identification of the isolate based on its morphology and biochemical characteristics.

Material and Methods

Isolation of β-haemolytic streptococci: A total of 34 throat swabs of the biomass were collected from patients suffering from acute tonsillitis. Before processing the sample, the swabs were immersed in sterile physiological saline for at least 15 min under aseptic condition. The samples were subjected to serial dilution and pour plated on commercially available sheep blood agar medium (HiMedia Pvt. Ltd, Mumbai). Incubation of the plates was carried at 37°C for 24 h[1]. 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 (BHI) agar (HiMedia Pvt. Ltd, Mumbai) until further use.

Production and recovery of streptokinase: The pure culture colonies showing clear zone of haemolysis on blood agar plates were inoculated in 10 ml of mineral salt medium (MSM) (g/L: KH_2PO_4, 0.42; K_2HPO_4, 0.375; $(NH_4)_2SO_4$, 0.244; NaCl, 0.015;

$CaCl_2$. $2H_2O$, 0.015; $MgSO_4.7H_2O$, 0.05; and $FeCl_3.6H_2O$, 0.054; pH 7±0.1) 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* for 30 min. The cell free supernatants were filtered through 0.45 µm cellulose acetate filter and the filtrates were considered as crude enzyme[11].

Screening of streptokinase producing haemolytic streptococci: Radial caseinolysis assay: The cell free supernatants were loaded on to the commercially available skimmed milk agar plates (HiMedia Pvt. Ltd, Mumbai) and incubated at 37°C for 12 h. Following incubation, the diameters of the zone of caseinolysis were measured to the nearest millimeter[12,13]. Actual count of hydrolytic ability of respective isolate was determined by subtracting the diameter of the well from the zone of caseinolysis. The isolate showing the highest zone of caseinolysis was selected for further studies.

Blood clot dissolving assay: Sterile empty microcentrifuge tubes were taken, labelled suitably and their weights determined (W_1). Sheep blood was freshly collected. 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 (W_2). To determine the clot weight, W_1 was subtracted from W_2. 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 (W_3) to observe the difference in clot weight. Percentage of clot lysis was calculated using the following equation[14]:

$$\text{Percentage lysis} = 100-\{[(W_3- W_1)/(W_2- W_1)]*100\}$$

Identification: The characteristics of the selected isolate of our study were compared with that of the reference culture *Streptococcus pyogens* MTCC 1923 obtained from Microbial Type Culture Collection, Chandigarh, India. The selected isolate was identified based on its morphological and biochemical characteristics. The morphological characterization involved culturing the isolate on nutrient agar plates for studying the appearance of the colonies following which gram's staining and motility test were performed.

The biochemical characterization of the isolate was based on the results of indole test, methyl red test, Voges Proskauer test and citrate utilization test. Growth on MacConkey agar and Bile Esculin agar were checked. Growth in the presence of 6.5% NaCl, 40% bile, growth at pH 9.6, 10°C and 45°C, under aerobic and anerobic conditions, growth was determined. The isolate was tested for its ability to produce acid from inulin, lactose, salicin, sucrose, maltose and glucose. Arginine, esculin, gelatin, starch and casein hydrolysis were also carried out. Bergey's Manual of Determinative Bacteriology (9th Edition) was used as a reference to identify the isolate[15].

Results and Discussion

Streptokinase was the first thrombolytic drug to be introduced for the treatment of acute myocardial infarction[16]. 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 Medicines[17].

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[1].

From the 34 throat swabs collected from patients suffering from acute tonsillitis, 43 isolates demonstrated clear zone of haemolysis on the blood agar plates. Gram staining results revealed that of these 43 isolates having haemolytic capabilities, 11 were gram positive cocci in short chains, while rest were either gram positive cocci in clusters or in pairs. Earlier, Doss *et al.*[18] found that among a total of 15 throat samples collected, both α-haemolysis (greenish zone around the colonies) and β-haemolysis (clear zone around the colonies) were observed. Out of these throat samples, 10 showed α-haemolysis and 5 samples showed β-haemolysis. In β-haemolysis, other than *Streptococcus* sp, *Staphylococcus* sp. was also present. Thus it is evident that as compared to the organisms causing partial breakdown of the blood cells, the organisms causing complete breakdown were slightly lesser in number in such infected biological samples.

The screening of microorganisms for the production of useful products continues to be an important aspect of biotechnology. Although advances in instrumentation, genetics and microbial physiology are having an impact, screening programs are still primarily based on classical techniques of enrichment and mutagenesis[19]. These 11 streptococcal isolates were selected for further evaluation of their streptokinase activities based on the results of radial caseinolysis assay and blood clot dissolving assay. Among the 11 isolates, isolate SK-6 demonstrated the highest streptokinase activity as outlined in table-1.

Proper identification and characterization of microorganisms is very important because it expands the scope for utilization of potent isolates for the production of industrially important products. SK-6 was subsequently subjected to morphological and biochemical characterization and later identified as *Streptococcus equisimilis*. The significant results of the characterization of the isolate have been clearly presented in table-2.

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

SK producers	Zone of radial caseinolysis (mm)	Percentage of clot lysis
S. pyogens MTCC 1923	17	38.73
SK-2	11	23.90
SK-3	10	19.83
SK-5	13	27.22
SK-6	15	36.00
SK-9	12	30.17
SK-13	13	28.07
SK-17	9	18.03
SK-26	11	23.87
SK-29	13	27.83
SK-31	9	17.95
SK-38	11	22.97

Table-2
Morphological and biochemical characterization of isolate SK-6 and reference strain

Biochemical Tests	Reactions	
	SK-6 Isolate	*S. pyogens* MTCC 1923
Growth under aerobic condition	+	+
Growth under anaerobic condition	+	+
Growth on MacConkey agar	-	-
Bacitracin sensitivity	R	S
Growth on Bile Esculin agar	-	-
Growth at 10°C	-	-
Growth at 45°C	-	-
Growth at pH 9.6	-	-
Growth with 6.5% NaCl	-	-
Growth with 40% bile (Oxgall)	-	-
α-haemolysis on blood agar	-	-
β-haemolysis on blood agar	+	+
Hydrolysis of arginine	+	+
Hydrolysis of esculin	-	-
Gelatin hydrolysis	-	+
Starch hydrolysis	-	-
Casein hydrolysis	+	+
Acid formation		
Inulin	-	-
Lactose	+	+
Salicin	-	+
Sucrose	+	+
Maltose	+	+
Glucose	+	+
Ribose	+	-
Indole test	-	-
Methyl red test	-	-
Voges Proskauer test	-	-
Citrate utilization test	-	-

Keys: +, positive; -, negative; R, resistant to bacitracin; S, susceptibile to bacitracin

Lancefield classification differentiates the β-haemolytic streptococci into groups A to O[20]. Most of the streptokinases are obtained from β-haemolytic streptococci of human and animal origin and belong to the Lancefield groups C, G and also *S. pyogenes*[21]. The maximum amount of streptokinase is produced by Group C streptococcus species i.e. *S. equisimilis*[22]. The fact that *skc* and *skg* alleles of *S. equisimilis* are closely related to the *ska* subcluster 2a of *S. pyogenes* (that is strongly associated with throat isolates of *S. pyogenes*), may possibly contribute to the throat tropism of *S. equisimilis*[23].

S. equisimilis are preferred for producing streptokinase as they lack erythrogenic toxins, are less fastidious in its growth requirements than the majority of group A strains and can be grown on semisynthetic media to secrete large quantities of streptokinase[22,24].

Conclusion

Results from our study have shown that throat samples contain β-haemolytic bacteria capable of producing streptokinase. The screening for a potent streptokinase producer revealed that the bacterial isolate SK-6, later identified as *S. equisimilis* produced the highest streptokinase activity as compared to the other isolates. Taking into account the current finding, SK-6 isolate of *S. equisimilis* can be exploited industrially for the production of streptokinase. But in order to enhance the level of secretion of this thrombolytic metabolite by the isolate, it is necessary to adopt strategies for the optimization of the media and cultural conditions.

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