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***Corresponding authors**

Baseer Ahmad
E-mail
dr.baseerahmadkhan@gmail.com

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#Note

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Optimization of Metal Ions in Sugarcane Bagasse Fermenting Medium for the Production of Streptokinase by *Streptococcus equisimilis*

Rida Tanveer^{1#}, Huma Gulzar^{1, 2#}, Baseer Ahmad^{3, 4*}, Maryam Akhtar⁵, Iqra Hussain¹, Quratulain Hanif⁶, Mirwaise Khan³, Rahat Ullah Khan⁷

¹Department of Biochemistry and Biotechnology, The Islamia University, Bahawalpur 63100, Pakistan

²College of Life Sciences, China Agriculture University, Beijing, China

³Faculty of Veterinary and Animal Sciences, Gomal University, D. I. Khan, Pakistan

⁴College of Animal Science and Technology, China Agriculture University, Beijing, China

⁵College of Life Sciences, Lanzhou University, China

⁶Computational Biology Laboratory, Agricultural Biotechnology Division, National Institute for Biotechnology and Genetic Engineering, Faisalabad, Pakistan

⁷Institute of Microbiology, Gomal University, Dera Ismail Khan, Khyber Pakhtunkhwa, Pakistan

Abstract

Streptokinase is a fibrinolytic enzyme and a product of β -hemolytic *Streptococci* strains. This enzyme is used as a medication to break down clots in some cases of heart disease. *Streptococcus equisimilis*, a species of group C *Streptococci*, is widely used for the production of streptokinase by fermentation technology. In this study, the sugarcane bagasse fermentation medium was optimized for metal ions (KH_2PO_4 , $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, CaCO_3 and NaHCO_3) at various levels to attain the maximal production of streptokinase. Sugarcane bagasse was used due to its profuse availability and as an ideal substrate for microbial processes for the manufacturing of value-added products. The results showed that maximal streptokinase production was found at 0.04% KH_2PO_4 , 0.04% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.15% NaHCO_3 and 0.04% CaCO_3 . Finally, the optimized medium resulted in 84.75 U/mg specific activity and 74.5% recovery. The purification process was carried out simultaneously using ammonium sulfate precipitation, ion-exchange chromatography, and gel filtration. Finally, a purified sample of streptokinase was run on SDS-PAGE and resolute 47 kDa molecular weight. The use of β -hemolytic *Streptococci* to obtain streptokinase is not free from health risks and is related to anaphylaxis. This study provides a way forward for the cost-effective ways to obtain streptokinase for the treatment of thrombosis.



SCAN ME



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Introduction

Cardiovascular diseases have become a leading cause of death worldwide. An acute coronary syndrome is a term, accepted for the reason that it was thought to be more noticeably revealing the disease development which is related to myocardial ischemia [1]. A vast variety of blood dissolving agents, including streptokinase, have been used for the cure of cardiac diseases. Due to pathogenesis such as unsuccessful obstruction of bleeding and the formation of thick blood needed an application of the thrombolytic agent to that particular site in veins, medical involvement is indispensable for directing thrombolytic mediator into veins [2]. Streptokinase is also among those mediators comprising 414 amino acid residues and 47 kDa of molecular weight [3]. In contrast with the supplementary plasminogen activators of other varieties, streptokinase holds a great affinity for circulatory plasminogen and has an extended period of half-life [4]. Hence streptokinase is principally and commonly utilized plasminogen activator in the deprived health care organization of the world due to its cheap price [5]. Streptokinase, a fibrinolytic enzyme, is obtained from many foods for achieving physiochemical properties. Other than these, there is a very famous Asian seasoning known as fermented shrimp paste that exhibited strong fibrinolytic activity [6]. These traditional Asian foods bestow these enzymes with a particular activity which is very valuable in thrombolytic therapy. For curing cardiac diseases, these gave an alternative to the costly fibrinolytic enzymes, which are presently used [6]. The *Streptococci* strains can produce streptokinase but good handling is needed due to the pathogenic activity of streptokinase [7].

Bagasse, the fibrous residues left behind when sugarcane is compressed in the refining factory of sugar to collect juice which in other countries is also recognized by the name bagasse. The word bagasse is originated from the Spanish and French word known as “*bagazo*” specified for the outer dry layer of olives or grapes taken as waste materials remained after extracting their fillings through compressing. This bagasse contained almost 1-3% dissolved solids, 47-50% moisture, and 48-50% fiber, including sugar, and is mainly utilized for the production of paper [8]. Furthermore, bagasse is composed of about 25% lignin, 25% hemicelluloses, and 50% cellulose. Hence, bagasse presents as an ideal substrate for microbial

processes for the manufacturing of value-added products because of its copious availability. From bagasse, efforts have been made to manufacture substrate protein-enriched organic acids, animal feed, amino acids, enzymes and compounds of pharmaceutical importance, etc. [9]. Improved substrate consumption is often attained through a pre-treatment process in the presence of microbes. Similarly, metal ions play an important role in microbial growth and metabolism. Therefore, the use of metal ions is recommended in culture medium for the optimum yields of bacterial products like antibiotics, enzymes, etc. [10, 11]. In this study, we optimized the production of the streptokinase enzyme by a β -hemolytic *Streptococcus equisimilis* in a liquid culture medium. The medium contained 4% bagasse and four metal ions were used at different levels to optimized the production of the streptokinase enzyme. Streptokinase extracted from culture media of *streptococci* strain was later purified.

Material and Methods

Microbial strain

The elected microorganism *Streptococcus equisimilis* (ATCC 12394, Gene Bank accession number CP002215) for the production of streptokinase in submerged fermentation was obtained from the Enzyme Biotechnology Laboratory (EBL), Department of Biochemistry, University of Agriculture, Faisalabad, Pakistan. Using the blood agar medium, *S. equisimilis* growth was recognized and used as a confirmation for streptokinase enzyme [10].

Enzyme production

The liquid medium used to obtain the highest production of streptokinase was composed of sugarcane bagasse 4% as a substrate along with glucose 2%, KH_2PO_4 0.04% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.04%, NaHCO_3 0.15% and CaCO_3 0.004% [11]. The pH was adjusted to 7 with 1M HCl or 1M NaOH. Optimization of various metal ions for the optimal production of streptokinase was done separately as described below to acquire the highest production of enzyme. For culturing, one ml of overnight inoculum of *S. equisimilis* was used and the incubation was sustained at 120 rpm in an orbital shaker having 37°C for 24 hours. Streptokinase enzyme was harvested from biomass by filtration and then centrifugation at 10,000 rpm for 20

minutes at 0°C. Enzyme was stored at -20°C and supernatant was assayed for enzyme activity [12].

Optimization of metal ions

We selected four salts as metal ions sources to optimize the production of streptokinase. The relative activity and quantitative estimation of the streptokinase enzyme were approximated as described by other researchers [13,14]. Biuret method was used for the protein estimation and spectrophotometric observation using Bovine serum albumin as standard [15].

Optimization of KH_2PO_4

Effects of five different concentrations of KH_2PO_4 (0.01, 0.02, 0.03, 0.04, 0.05, 0.06 and 0.07%) were evaluated for attaining maximal production of streptokinase enzyme.

Optimization of $MgSO_4.7H_2O$

To determine the maximal streptokinase production, five concentration levels of $MgSO_4.7H_2O$ (0.02, 0.04, 0.06, 0.08 and 0.10%) [16].

Optimization of $NaHCO_3$

For optimal production of streptokinase enzyme, the effect of five different concentrations of $NaHCO_3$ (0.10, 0.15, 0.20, 0.25, 0.30, 0.35, 0.40 and 0.45%) was deliberated in conjunction with optimal KH_2PO_4 and $MgSO_4.7H_2O$ concentrations in the fermentation medium of *S. equisimilis*.

Optimization of $CaCO_3$

Effect of seven different concentrations of $CaCO_3$ (0.01, 0.02, 0.03, 0.04, 0.05, 0.06 and 0.07%) for the maximum production of streptokinase were used in the liquid state fermentation medium along with the optimum levels of $NaHCO_3$, KH_2PO_4 and $MgSO_4.7H_2O$.

Enzyme assay

Different pre-weighed germ-free microcentrifuge tubes of 500 μ l were used for pouring venous blood taken from healthy humans after incubation of 60 minutes at 37°C. Entire removal of serum was done after clot formation and every single tube containing clot was weighed again to find out the weight of clot (weight of clot = weight of clot containing tube – weight of tube alone). Appropriate labeling of tubes was done and different dilutions were administered combined with 200 μ l streptokinase liquid culture or purified

enzyme in each tube. Water along with clot formation in one tube was set as a control group. Each of the tubes was then incubated for 90 minutes at 37°C to observe clot lysis. After incubating them all, the obtained liquid was removed to examine the difference in weight as the tubes were weighed again after disruption in the clot. Variation in weight after and before lysis of blood clot was articulated as a percentage of clot breakage. According to Madhuri et al. [16], streptokinase enzyme activity was considered I unit (U) if 0.12 ml enzyme solution completely lysed 1 ml of human clotted blood. Each assay was replicated three times [17].

Enzyme purification

Fibrinolytic enzyme was partially separated from liquid culture using the ammonium sulfate precipitation method. For purification, ion-exchange chromatography was carried out as DEAE-cellulose column chromatography. The crude enzyme was obtained from *streptococci* strain *S. equisimilis* showed a specific activity of 19.86 Umg^{-1} . After dialysis, the desalted sample showed increased activity of 387 $U ml^{-1}$. The substrate used gave the streptokinase-specific activity of 68.17 Umg^{-1} after the chromatography steps. The fibrinolytic enzyme was further purified by gel permeation chromatography using a Sephadex G-150 column based on the molecular difference of size, which has to eliminate. Later, the SDS-PAGE was carried out using 12% resolving gel, 4% stacking gel and 25mM Tris buffer (pH 8.3) having 0.1% SDS. Later for staining, Coomassie brilliant blue (R-250) was used to determine the molecular weight of the purified enzyme as described previously [18].

Results

Effect of KH_2PO_4

The effect of different concentrations of KH_2PO_4 was observed on the production of streptokinase. The results manifested that the maximum streptokinase production (335.2 Uml^{-1}) was obtained from *S. equisimilis* by using 0.04% concentration of KH_2PO_4 along with sugarcane bagasse (4%) as substrate in liquid culture after 24 hours at 37°C.

Effect of $MgSO_4.7H_2O$

The effect of different concentrations of $MgSO_4.7H_2O$ was observed on the production of

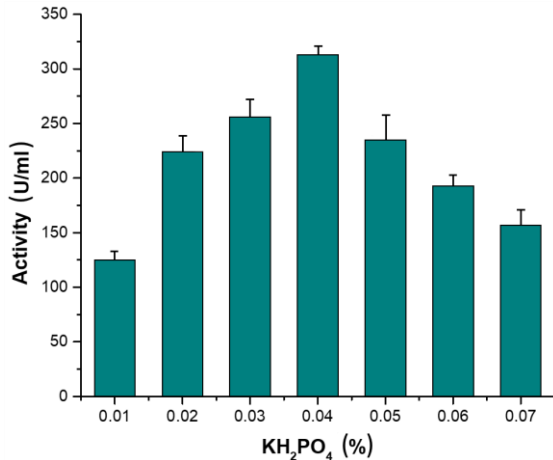


Fig. 1 Streptokinase production from *Streptococcus equisimilis* at different KH_2PO_4 concentrations by using sugarcane bagasse as a substrate.

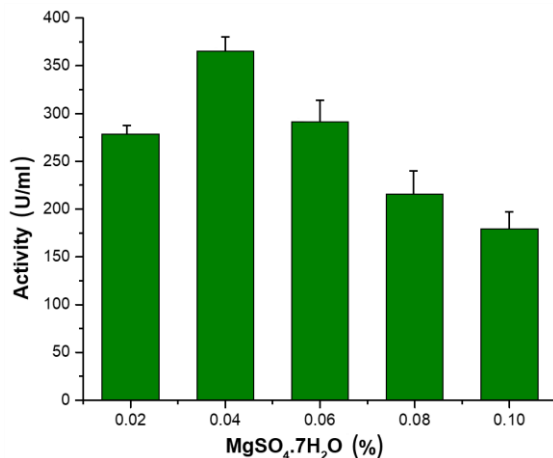


Fig. 2 Streptokinase production from *Streptococcus equisimilis* at different $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ concentrations by using sugarcane bagasse as a substrate.

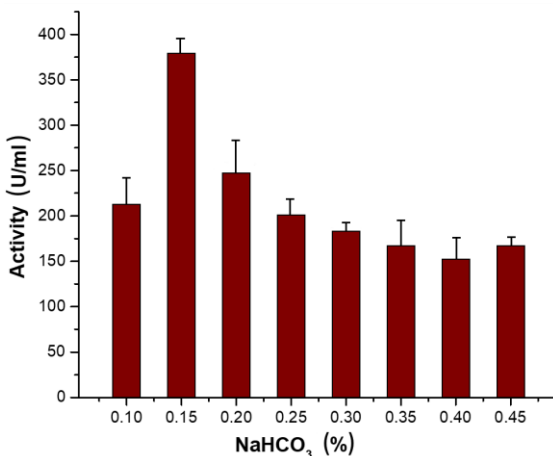


Fig. 3 Streptokinase production from *Streptococcus equisimilis* at different NaHCO_3 concentrations by using sugarcane bagasse as a substrate.

streptokinase. In the liquid culture medium containing optimum concentrations of sugarcane bagasse substrate (4%) and KH_2PO_4 (0.04%) for 24 hours at 37°C , 0.04% concentration of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ was selected for maximal streptokinase production (342.4 Uml^{-1}) from *S. equisimilis*.

Effect of NaHCO_3

The effect of different concentrations of NaHCO_3 was determined on the production of streptokinase. In the liquid culture medium containing optimum concentrations of sugarcane bagasse substrate (4%), KH_2PO_4 (0.04%) and $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.04%) for 24 hours at 37°C , 0.15% concentration of NaHCO_3 was selected for maximal streptokinase production (361.4 Uml^{-1}) from *S. equisimilis*.

Effect of CaCO_3

The effect of different concentrations of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ was observed on the production of streptokinase. In the liquid culture medium containing optimum concentrations of sugarcane bagasse substrate (4%), KH_2PO_4 (0.04%), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.04%) and NaHCO_3 (0.15%) for 24 hours at 37°C , 0.04% concentration of CaCO_3 was selected for maximal streptokinase production (342.4 Uml^{-1}) from *S. equisimilis*.

SDS-PAGE analysis

SDS PAGE was done to detect the molecular weight of enzyme streptokinase from *S. equisimilis* by employing 12% resolving gel and 4% stacking gel. It was accomplished that the molecular weight of obtained purified protein band of 47 kDa was in approximation to streptokinase enzyme.

Discussion

The *Streptococci* species are among the principal life forms reported for utilization of industrial production of streptokinase. The streptokinase enzyme has not only clinical importance but is also a very cost-effective plasminogen activator [2]. The enzyme streptokinase is a proteolytic enzyme and it is capable to dissolve fibrin production and constancy [1]. Most of the diseases related to heart and myocardial infarction are caused due to thrombosis which is a consequence of the amassing of fibrin in the blood vessels [8]. Microbial fibrinolytic enzymes used in the thrombolytic activity are currently attaining greater interest than classic thrombolytic agents having high cost

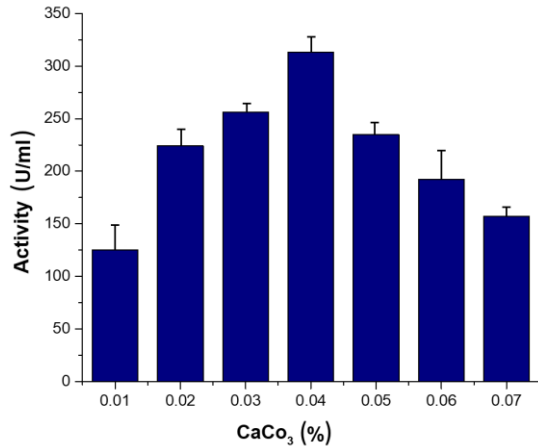


Fig. 4 Streptokinase production from *Streptococcus equisimilis* at different CaCO₃ concentrations by using sugarcane bagasse as a substrate.

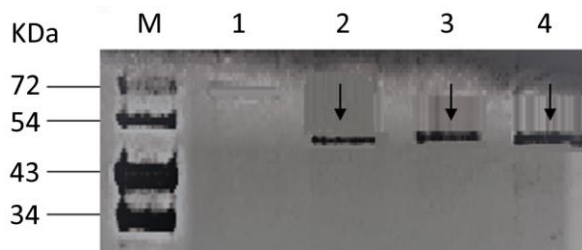


Fig. 5 SDS-PAGE analysis. Lane M, molecular weight markers (kDa); Lane 1, control and Lane 2-4 exhibit three replicates of 47 kDa streptokinase enzyme.

together with later on objectionable side effects [2]. Thrombolytic agents for therapy are used to activate the fibrinolytic system by converting the inactive plasminogen, proenzyme into the active enzyme plasmin, which degrades the fibrin target. There is a vast variety of microorganisms (*Staphylococcus*, *Bacillus*, *Escherichia coli*, and *Pseudomonas*) used for the production of fibrinolytic enzyme streptokinase [19]. However, bacterial species of *Streptococci*, especially *S. equisimilis* is considered an excellent source of streptokinase enzyme [20, 21].

Enzyme assay of streptokinase relies on its ability to activate plasminogen to plasmin, which in turn, hydrolyzes an indicator substrate, and the extent of hydrolysis over a given period is related to the concentration of streptokinase. According to Devi et al. [22] and Pratap et al. [23], substrates for plasmin may include the casein, fibrin clot, other proteins, and various synthetic esters, which have been utilized for the sensitive detection of the enzyme activity. Maximum clot lysis of about 67% was observed, which is very promising for

streptokinase production. The sugarcane bagasse showed the highest enzyme production as substrate in a previous study so it was used for this study [11]. The optimum pH of streptokinase was 7 and 47 kDa molecular weight was attained. These findings showed us that waste agro-industrial wastes can be considered as one of the abundant sources of fibrinolytic enzymes. Work on microorganisms producing fibrinolytic enzymes from a variety of traditional Asian fermented foods have also been reported and these organisms were mostly of the genus *Bacillus* [24]. *S. equisimilis* showed maximum production of thrombolytic (fibrinolytic) enzyme streptokinase by the use of agro-waste sugarcane bagasse. Therefore, fibrinolytic enzyme obtained from sugarcane bagasse exhibited 84.75 U mg⁻¹ specific activity along with 4.26 times fold purification and 74.5% recovery. Later, four metal ions were chosen to optimize their concentration and to further improve streptokinase enzyme production in the liquid culture medium. Our results showed that maximal streptokinase production was found at 0.04% KH₂PO₄, 0.04% MgSO₄·7H₂O, 0.15% NaHCO₃ and 0.04% CaCO₃. Stephani et al. [13] also reported optimal yield of streptokinase enzyme by using an increased amount of metal ions from bacterial strains. Similarly, Prasad et al. [14] used 0.01% MgSO₄·7H₂O for optimal production of streptokinase. We found an improved yield of streptokinase in this study with 0.15% level of NaHCO₃ which is in strong association with the work done by other researchers [25-28]. Madhuri et al. [16] reported that when the growth medium contains 0.004% CaCO₃, it gave hyper-production of streptokinase using bacterial strain. The CaCO₃ concentration level of 0.005% was also employed under similar conditions to enhance the yield of streptokinase by other researchers [11]. These findings correlate with the present research work for the maximal production of streptokinase. The SDS-PAGE was used to determine the molecular weight of purified streptokinase, which was found to be 47 kDa. This finding is in accordance with the findings of Felsia et al. [8] who also isolated 47 kDa streptokinase enzyme from the liquid culture of another bacterial strain, *S. pyogenes*.

Conclusions

The thrombolytic agents have numeral benefits in the world of presently used drugs. The *Streptococci* species have shown great potential for the production of thrombolytic enzymes in culture

medium. In this study, we optimized the liquid culture medium for the production of the thrombolytic enzyme “streptokinase” by a bacterium *S. equisimilis*. Our results showed that sugarcane bagasse appeared to be a good substrate and the production of enzyme was further improved by step-wise optimization of four metal ions (KH_2PO_4 , $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, CaCO_3 and NaHCO_3). The above-given results suggest that optimization of environmental and nutritional conditions significantly improved thrombolytic enzyme production in liquid culture. Later, the enzyme was purified using more than one purification step and its molecular weight was determined. So as per future aspects, in clinical practice, these findings can be applied due to their cost-effectiveness and cheap rates for the treatment of thrombosis.

Conflict of interest

The authors declare no conflict of interest.

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