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Research Paper

PRODUCTION AND PURIFICATION AND CHARACTERIZATION OF STREPTOKINASE USING *Bacillus licheniformis* UNDER SOLID STATE FERMENTATION

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Abstract

Streptokinase is a novel fibrionotic protein produced by several bacterial species. As a therapeutic, streptokinase can be used as thromboembolic disorders where it dissolves a blood clot by the activation of plasminogen to plasmin. In the present study, *Bacillus licheniformis* used for the production of streptokinase under soya bean as carbon source. The optimum day, pH and temperature were also optimized and were found to be 48hours, pH of 7 and temperature at 40^0C respectively. The enzyme was partially purified using ammonium sulphate followed by dialysis membrane and sephadex- G-100 column chromatography. The molecular weight of the streptokinase was determined using SDS-PAGE and was found to be 63 kDa. The enzyme was further confirmed its Proteolytic activity and thrombolytic activity.

Key words: Fibrinolytic enzyme, streptokinase, Proteolytic activity, thrombolytic activity.

INTRODUCTION

Streptokinase is an extra cellular protein, extractedfrom certain strains of beta hemolytic streptococcus. It is a non-protease plasminogen activator that activates plasminogen to plasmin, the enzyme that degrades fibrincloth 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 clinical importance of streptokinase was first noted by Tillet and Garner (Tillet and Garner, 1933), who discovered that this bacterial protein caused the lysis of human blood clots. It was later found that streptokinase is not an enzyme but rather a potent activator of plasminogen, the inactive precursor of plasmin. Plasmin is the active fibrinolytic component of the circulatory system, solubilizing the fibrinnetwork in blood clots through limited proteolysis. Streptokinase is currently used in clinical medicine as a therapeutic agent in the treatment of thromboembolic blockages, including coronary thrombosis. Streptokinase is naturally produced and secreted by various strains of hemolytic streptococci (Babashamsi, 2009).

Hemostasis is a complex process obtained through an optimal balance between bleeding and clot formation. Blood clot is composed of fibrin. Fibrin is the main protein component of blood clot and is normally formed from fibrinogen by the action of thrombin. These fibrin clots are dissolved by the hydrolysis of plasmin, which is activated from plasminogen by tissue plasminogen activator. The hydrolysis of fibrin is also known as fibrinolysis (Rashmi and Lini, 2013).

Fibrinolytic enzymes are one of the largest group of proteolytic enzyme involved in numerous regulatory process related to fibrinolytic action. (Jayalakshmi,*et.al,* 2012). These enzyme are currently used in managing in heart disease, effectively prevent in cardiovascular disease because it is a blood clot-dissolving agent and also used for the treatment of thrombosis. However this enzyme are often expensive thermo labile and can produce unwanted and undesirable side effects (ADR). (Rashad*et.al, 2012)*

Streptokinase has been used as an effective drug in case of thrombosis, arterial thromboembolism and critical stenosis. Mainly, this kinds of treatment handle in various animals like rat, cat, rabbit and dogs. In human, streptokinase used as a thrombolytic agent especially for the treatment of acute myocardial infarction as it is a cost effective, for a both animal and human disorder.This agents are used to treat heart attack, stroke, deep vein thrombosis, pulmonary embolism and occlusion of a peripheral artery (Zia *et.al,*2012)

These activator are of human origin which are generally safe, but very expensive and same thing as a microbial origin, have a great important application in pharmaceutical industry, health care and medicine. Recent years, streptokinase is one of the most powerful new dietary supplements in the market, it enhance the body ability to dissolve blood clots (Hemostasis). (RajaniGopal & Gad*et.al*,2014)

Bacillus genus is a well-known producer of a potent fibrinolytic enzyme like streptokinase, urokinase, nattokinase etc. Oral administration of the fibrinolytic enzyme like nattokinase and urokinase, can enhance fibrinolytic activity in plasma and the production of tissue type plasminogen activator. Urokinase and t-PA are widely used in thrombolytic therapy, but this agent have some undesirable side effect (Bin Chen *et.al*, 2013).

Thus the present study deals the production, purification and characterization of streptokinase enzyme using *Bacillus licheniformis* CBNR isolate.

MATERIAL AND METHODS

Source of organism

The organism *Bacillus licheniformis* was obtained from CBNR and it was sub-cultured in nutrient broth and incubated for 24 hours at 37^0C in orbital shaker. The grown culture was used for further studies. Similarly the culture was streaked in nutrient agar plate and incubated at 37^0C for 24 hours. The organism was stored and maintained at 4^0C.

Production and recovery of streptokinase

Streptokinase was produced using medium containing following in g/l, potassium di hydrogen phosphate 0.165, Di potassium hydrogen phosphate 0.06, Glucose 0.5, magnesium sulphate 0.05, thymine 0.01. To this add 0.5g of finely smashed soybean was added and autoclaved at 121^0C for 15 minutes. After sterilization *Bacillus licheneformis* was inoculated ($10^{-}6$) , incubated in orbital shaker (150rpm) for 96 hrs. After 96 h of fermentation, cell were removed by centrifugation.

Estimation of biomass

Biomass was estimated on dry weight basis , by centrifuging the culture filtrate on pre weighed centrifuge tube , dried at a constant weight and reweighed. The difference in weight denoted the growth of the bacteria.

Extraction and assay for streptokinase

The culture was centrifuged at 8000 rpm for 10 minutes (each day) and the enzyme assay was carried out by using the supernatant. To the supernatant 2.5ml streptavidinsolution and 0.1 M tris Hcl buffer was added and incubated at 37^0C for 20 minutes. After incubation period reaction was stopped by adding 2 ml of 1% TCA and incubated at 50^0C for 30 minutes. The solution was centrifuged and the supernatant (1ml) was taken. To that 1 ml of 2% sodium carbonate and 0.5% copper sulphate was added and incubated at room temperature for 10 minutes. To the mixture 1 ml folins phenol reagent was added, mixed well and incubated at room temperature for 30 minutes in dark condition. The colored solution was read at 660 nm in spectrophotometer. The enzyme

Effect of Incubation time on enzyme activity

The effect of incubation day was measured using production at various time interval (24 hrs to 96hrs). Each day enzyme activity and biomass were estimated.

Effect of pH on enzyme activity:

The effect of pH on the streptokinase production was carried out using the following pH values of 6,7,8 and 9 and the enzyme activity was measured.

Effect of temperature on enzyme activity:

The effect of temperature on streptokinase production was carried out using the following temperature values; 20°C 30°C, 40°C, and. 50°C, and the enzyme activity was measured.

Estimation of total protein (Lowry *method*)

The protein was estimated as prescribed by Lowry *et al* using BSA as standard.

Ammonium sulfate precipitation:

The protein part of the production medium was separated by ammonium sulphate precipitation. The production medium was centrifuged in 8000 rpm for 15 min and to the supernatant equal amount of ammonium sulphate (80%) was added and the mixture was kept overnight at 4°C for precipitation to occur. After complete precipitation, the clear solution in the upper part was discarded and the lower part with protein precipitate was centrifuged. The supernatant was discarded and the pellets were suspended in 0.1 M tris Hcl buffer (pH 7).

Dialysis:

The precipitate was desalted by dialysis following the standard protocol; 10 cm pre-treated dialysis bag was taken and activated by rinsing in double distilled water. One end of the dialysis bag was tightly tied and the precipitate recovered was taken inside the bag. The other end of the dialysis bag was tightly tied to prevent the leakage. After that, dialysis bag was suspended in a beaker containing 0.1 M Tris Hcl buffer.

Column chromatography:

1.0 gm of DMEM Sephadex G-100 was added to 50 ml of 0.1 M tris HCl buffer and kept for overnight soaking. To this 2 ml of dialyzed enzyme was added carefully to the top of the column and allowed to pass into the gel by running the column. Buffer was added without disturbing the gel surface and to the reservoir. After 30 minutes the samples were eluted and the fractions were collected in 6 eppendorf tubes and stored at -20º C.

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

In order to ascertain the presence of the enzyme, SDS PAGE was carried out using the sample. Resolving gel and Stacking were prepared and poured into the gel apparatus and allowed to polymerize. Sample buffer (β-merceptoethenol and bromophenol blue) was added into sample with volume 1:5 and the mixture was heated at 100 °C for 2 min and then spun down for a few seconds and then loaded in the wells of acrylamide polymerized gel in gel apparatus. The marker was also loaded in separate well to determine the molecular weight. The electrodes were attached to the gel apparatus and power supply was turned on at 50 V. Electrophoresis was carried out at a constant voltage until the dye reaches about 1 cm from the end of the gel. Power was turned off and the gel was taken off with the help of spacer. The gel was submerged in staining solution and allowed the gel to stain overnight. Gel was then placed in destaining solution to remove background stain. Protein bands were clearly visible after destaining.

Streptokinase has proteolytic activity:

To confirm the streptokinase has a proteolytic activity by prepared the nutrient agar plate with 0.25g of casein for 15ml. It was autoclaved at 121^0C for 15 minutes, then this allowed to cool in normal room temperature. The sterilized plate kept in laminar air flow chamber and 3 wells were made using cork borer and added 20 µl of crude, dialyzed and fractionated enzyme. Then the plate subjected to incubation for 24 hours in incubator.

Thrombolytic activity:

Streptokinase involved in cell aggregation process, it was done by using Widal test tubes as follows, 3 test tubes were marked as T1, T2 and T3 (represented as test and blank) and added 1ml, 2ml of enzyme and 3ml of distilled water in separate tubes, to this added 250 µl, 500 µl and 750 µl of serum sample. Same volume of NaClwas added in each tubes and kept for incubation in shaker at 100 rpm for half an hour and the tubes were allowed to incubate in room temperature for 24 hours.

After incubation period few drops of sample from each tubes was placed in separate and clean glass slides. The blood was smeared in those slides and allowed to dry. Then to the slides few drops of giemsa stain and allowed to incubate it for few minutes. The slides were then washed with tap water and it was visualized in light microscope at 10 X objective lens.

RESULTS

Subculture of *Bacillus species:*

The organism *Bacillus licheniformis* was obtained from CBNR and it was streaked on nutrient plate and incubated for 24 hours at 37^0 C. The grown organism was maintained at 4^0 C for further studies.

Figure 1: *Bacillus licheniformis*

Production of Streptokinase enzyme:

The subculture *Bacillus licheniformis* a loopful of culture was transfer to modified production medium. The organism can utilize the glucose and produced streptokinase enzyme successfully after 24 hours incubation at 37°C. The streptokinase enzyme production confirms that streptokinaseenzyme assay method.

Effect of time period:

Effect of incubation time on enzyme production was carried out and it was found to be 48hrs.

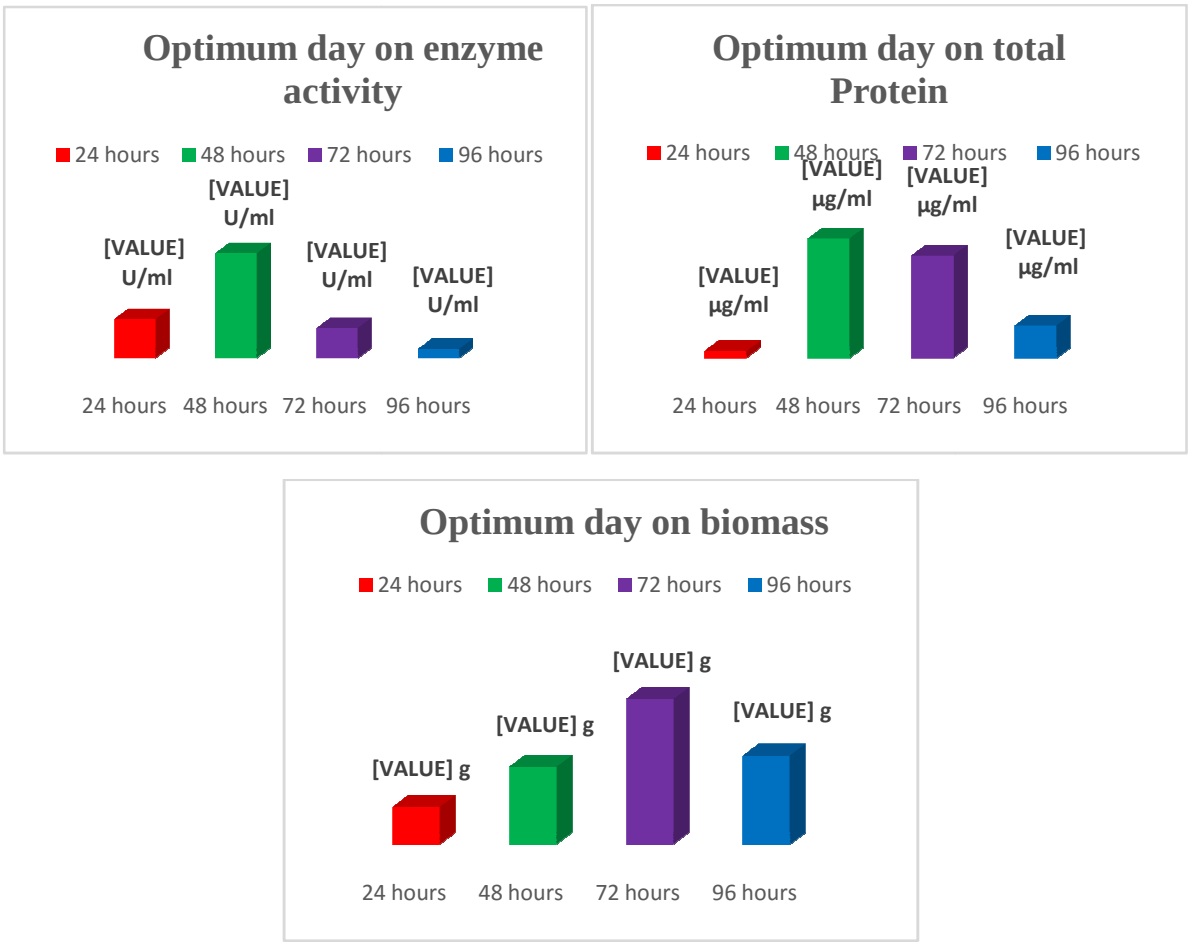


Chart 1. Effect of time period in enzyme activity and total protein.

Effect of pH

The enzyme activity was measured in Enzyme Assay methodwith various pH (pH 6.0, pH 7.0, and pH 8.0), spectrometric readings were taken and calculated enzyme units are given below. The optimum pH of the enzyme activity was obtained at pH 7 is 28.64 U/ml.

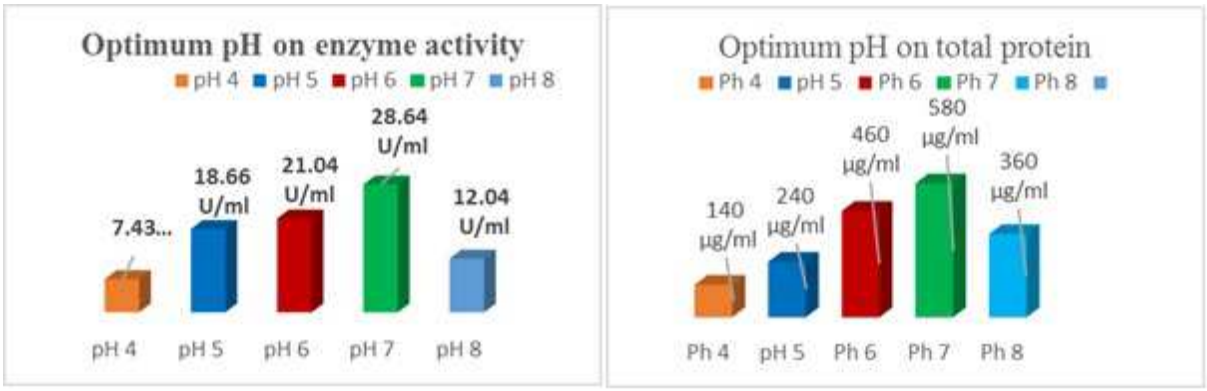
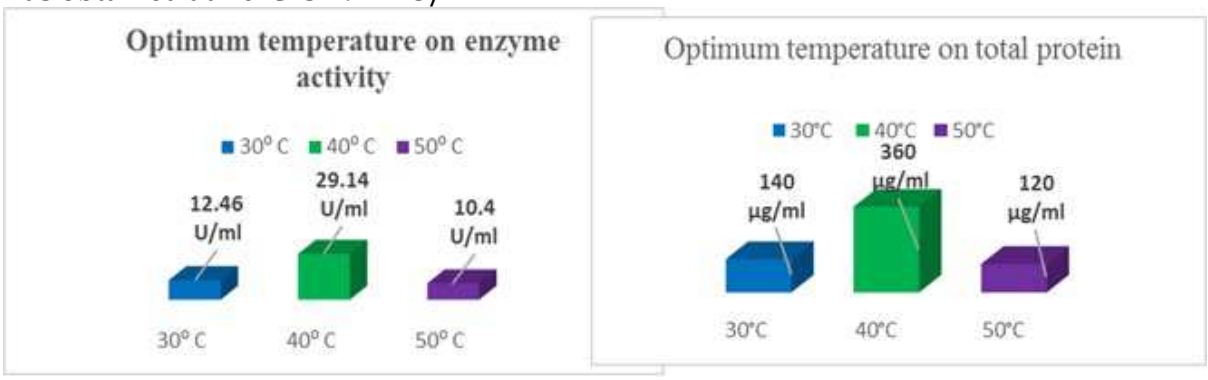


Chart 2. Effect of pH in enzyme activity and total protein.

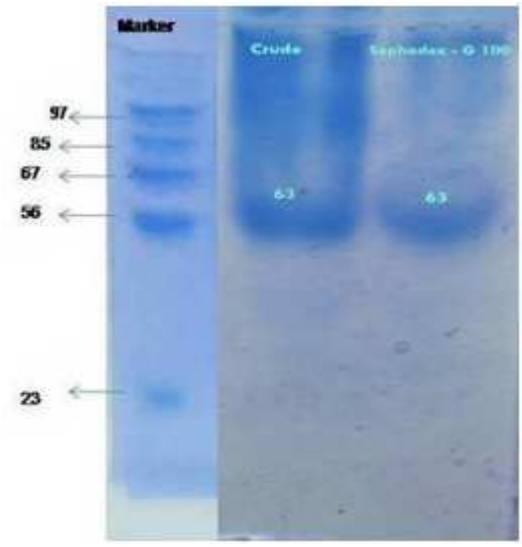
Effect of temperature

The enzyme activity was measured in Enzyme Assay methodwith various temperatures as 30° C, 40° C and 50° C, spectrometric readings were taken and calculated readings are given below. The optimum temperature of the enzyme activity was obtained at 40°C is 29.14 U/ml.



Determination of molecular weight

The streptokinase obtained after column chromatography was run on SDS-PAGE gel for *in situ* activity detection. Purified enzyme was run through 12% separating gel and 5% stacking gel for an hour at 50 mV and it was stained with Coomassie Brilliant Blue R-250 overnight. It was then destained using destainer solution for about 4 hours and bands were observed in the gel. Molecular weight of the enzyme was found to be 63kDa.



SDS-PAGE

Conformation of proteolytic activity:

Streptokinase enzymes such as crude, dialysed and column purified samples was added to the casein containing nutrient agar plate. After incubation period all crude, dialysed and column purified samples were lysed to the certain level confirming Proteolysis in streptokinase.

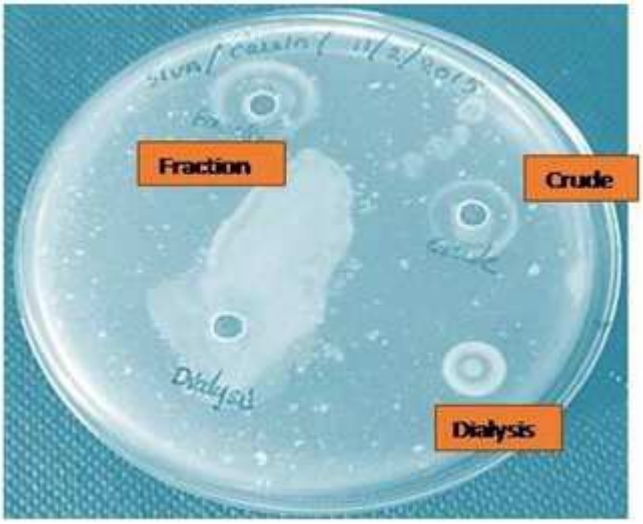


Figure 4: Proteolytic activity.

Thrombolytic activity:

In the present study the extracted streptokinase enzyme was used to remove the blood clot in the laboratory condition. The results showed that the produced streptokinase was successfully removed the blood clot. After 24 hours incubation it was seen the tubes were without clot and it was further confirmed under research compound microscope, the blood cells werenot aggregated to each other in test tubes (T1 and T2) and the blank tube cells are aggregated (T3).

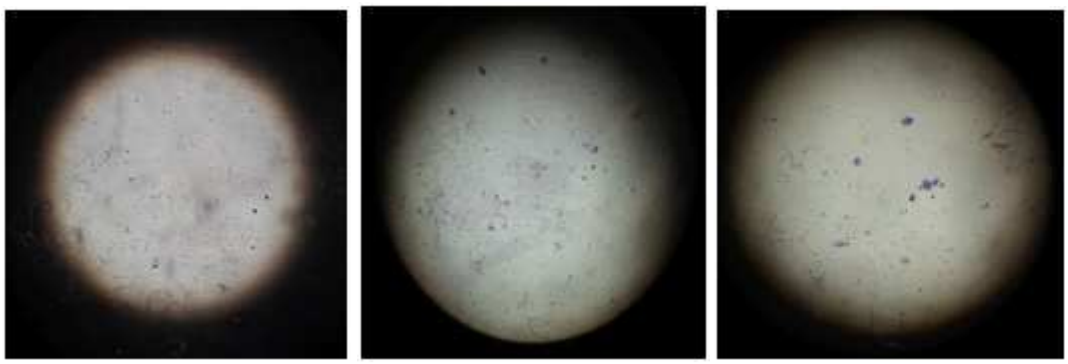


Figure 5 (a): Test 1**Figure 5 (b):** Test 2**Figure 5(c):** Blank

DISCUSSION:

Native streptokinase is useful for cost-effective thrombolytic therapy in clinical practice, but its use is not risk free. Large quantities of streptokinase can be produced inexpensively via bacterial fermentation. Cloning of the streptokinase gene in non-pathogenic microorganisms has enabled production of recombinant streptokinase that eliminates any risk of inadvertent inoculation of patients and production personnel with potentially pathogenic streptococci. Various chemical modifications of streptokinase have been used for extending its half-life in circulation, improving plasminogen activation, and reducing or eliminating immunogenicity. These objectives have been attained to various degrees also by producing mutated and engineered streptokinase. The streptokinase domains responsible for antigenicity, stability and plasminogen activation appear to overlap to some degree. Consequently, it may be impossible to produce a modified streptokinase that combines the every desired functional trait at the desired level (Anirban Banerjee*, et.al 2003).*

Effect of incubation period on streptokinase production

The incubation time for achieving the maximum enzyme level is governed by the characteristics of the culture and is based on growth rate and enzyme production. The enzyme production varies with incubation time (Kunamneni*et.al*, 2005). It is very essential to detect the optimum incubation time at which an organism exhibits highest enzyme production, since organisms show considerable variation at different incubation periods (Kumar *et.al*, 2012). Production of streptokinase from S. *dysgalactiae* subsp. *equisimilis* SK-6 was dependent on the incubation time. Streptokinase yield increased from 24 to 48 h of incubation, with the highest level of streptokinase production (0.238 U/ml) reported at 48 h. When the fermentation proceeded beyond 48 h there was a decrease in the streptokinase yield.

The probable reason for decrease in the streptokinase production beyond 48 h may be due to rapid depletion of nutrients in the medium, accumulation of excess acid in the media as a result of sugar utilization and developed oxygen tension. The reduction in the streptokinase production upon prolonged incubation may be attributed to the induction of Crabtree effect. Literatures suggest that Crabtree effect leads to accumulation of acetate which is inhibitory to cell growth and generation of toxic byproducts that often limit protein yields (Ferreira *et.al*, 2003). Lactic acid inhibition has been included in streptokinase production (Patnaik 1995).

Baewald*et.al*, (1975) used a simple and inexpensive medium to obtain high yields of streptokinase from S*. equisimilis*. The medium contained yeast autolyzate or corn steep liquor as nitrogen source, glucose and various salts. High titres of streptokinase were attained at 28 ^{0}C, pH 7.2–7.4, within 24 h in agitated cultures.

Production of streptokinase from S. *pyogenes* decreased while increasing the incubation time from 2 to 7 days (Patel *et.al*, 2011).

Chemically defined media for growing group A streptococci have been developed to require only small inocula and without the need for a prior adaptation regimen. The doubling times of the streptococci in such media can be comparable to those in complex media (McCoy *et.al,* 1991). Similar, Karimi*et.al,* (2011) found that the bacterial strain *S. equisimilis* H46A (ATCC 12449) produced relatively high yields of streptokinase by the use of 1 % inoculation, pH adjustment and glucose feeding as compared to 10 % inoculation and pH adjustment. While optimizing the batch fermentation parameters involved in streptokinase production from S. pyogenes, it was concluded that concentration of corn steep liquor and ageing period of the producing strain were the parameters which had positive significant effect on enzyme yield while inoculum volume had negative significant effect on its yield (Patel *et.al*, 2011).

CONCLUSIONS

Fibrinolytic enzymes such as Streptokinase, used as thrombolytic agent but too costly and also used through intravenous instillation, needs large scale production of streptokinase.In the present study streptokinase, a fibrinolytic enzyme was produced in a chemically defined medium using *Bacillus licheniformis CBNR* isolate. The streptokinase production was optimized. The optimum day, pH and temperature were found to be 48 hours, 7.2 and 40° C respectively.The enzyme was further partially purified using ammonium sulphate followed by dialysis (membrane and sephadex- G-100 column chromatography). The molecular weight of the streptokinase was calculated by using SDS-PAGE technique. It was found to be 63 kDa. The streptokinase was further confirmed its Proteolytic activity and thrombolytic activity.

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