

Effects of Different Parameters on the Production of Streptokinase Enzyme

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Abstract

Streptokinase is a novel bacterial enzyme that binds with plasminogen and activates it. Streptokinase is produced by different species of *streptococcus*. Activity of streptokinase is determined by different methods such as fibrin clot method, casein hydrolysis and Chromozym test. High production of streptokinase was obtained in experiments with batch cultures. Production of streptokinase increases 2-3 times in continuous culture. Different species of *streptococcus* was selected for Optimum production of streptokinase by using different concentration of substrates such as CSL, molasses, sugarcane bagass, rice polishing. Maximum activity was overserved by 0.3% CSL, 0.5% molasses, 0.4% sugarcane bagass and 0.5% rice polishing. Physical parameters such temperature, pH at optimum conditions (37°C, 7.0) enhance the streptokinase activity by using RSM. Nitrogen sources, carbon sources, incubation time, Growth factors such as glycine, thiamine and traces elements such as FeSO₄, MgSO₄, when uses in limited amount enhance the enzyme production. 1% (v/v) inoculum increase the yield of streptokinase (0.360 U/ml).

Keywords: Parameters Streptokinase Enzyme.

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INTRODUCTION

Enzymes are proteins in nature, and they act as a catalyst. Enzymes lower the activation energy that is required for a reaction to occur, without being used up. Fermentation is a method in which enzymes are produced for industrial purposes. In fermentation, microorganisms such as yeast and bacteria are used for enzyme production. There are two methods of fermentation i.e. solid-state fermentation and submerged fermentation. In liquid nutrient media, submerged fermentation involves in enzyme production by using microorganisms [1].

Streptococcus bacteria are used to produce streptokinase enzyme which is used as thrombolytic agent to dissolve the blood clot of fibrin, especially those clots that formed in the arteries of lungs and heart. It is also used during dialysis for removing the clot that formed in shunts. Various strains of streptococci and bacteria which contain the genetic material resulting from streptococcus of Lancefield groups A, C, G produced streptokinase. Hemolytic activity of streptokinase was firstly discovered by Tillet and Garner in 1933. They discover the hemolytic activity of

streptokinase. [2]Molecular weight of streptokinase is 47 kD and consist of 415 amino acids residues. It composed of 3 domains which denoted as a (residues 1 to 150), b (residues 151 to 287) and c (residues 288 to 414) [2].

Fermentation process optimized by using certain statistical tools such as Plackett-Burman design, Fed batch culture, Continuous culture, CCD and RSM. These tools increase the production of streptokinase as well as decrease in the production cost of enzyme.

For the optimization of multiple parameter RSM is used to optimize the multiple parameters. It determines the optimum process conditions during combining of experimental designs with interpolation by first and second order polynomial equations in sequential testing procedures. RSM optimize the culture conditions and increase the production of enzyme. Continuous culture method is an analytical tool and used to determine the instability of kinetics and product formation. It is also used to determine the specific growth rate, effect of antibiotics and substrate concentration on the expression of streptokinase [3].The

main purpose of present study was the streptokinase production from different streptococcus strains by using different substrates such as molasses, sugarcane Bagass, CSL and rice polishing and other important elements for fermentation process. In this study, we report the effect of different parameters like temperature, pH, growth factors, incubation time, coenzymes, inhibitors, activators, inoculum size, effect of nitrogen and carbon by using RSM, CCD, Continuous culture and casein hydrolysis method [4].

REVIEW OF LITERATURE

Enzyme production

Enzymes are proteins which are produced as intra and extra cellular compounds. Enzymes catalyze biochemical reaction with high specificity and increase the rate of reaction. After amylases and proteases, lipases are one of the highly commercialized enzymes. Lipases are found in microorganisms, plants and animals. Lipases are used in pulp and paper industry, pharmaceutical industry and detergent industry. It is also used to produce dairy and bakery products, biodiesel, fats and oil [5].

Amylases are the enzymes that can break down glycogen and starch. The amylases can be obtained from different sources such as microbes, animals and plants. Microorganisms are mostly used for amylases production because it has maximum production capacity and easily manipulate to obtain the enzyme of desired characteristics.

SSF involves in the production of microorganisms on a solid substrate. Solid-state fermentation has many advantages. These include high concentration of product, high volumetric productivity, less effluent generated and simple fermentation equipment [6].

Substrates selection depends on various factors, which is related to cost and availability of substrates. Other factors include level of moisture and particle size [7]. Smaller substrate particles have large surface area for microorganism production. If the substrate particles are too small then, the efficiency of respiration is affected, poor growth and poor production of enzyme will result. water contents of substrate must be optimized because lower and higher presence of water affect the activity of microbes [44].

Streptokinase

Streptokinase is an extracellular protein that extracted from the strains of beta hemolytic streptococcus. Streptokinase was first discovered by Tang and Jackson in 1982. The molecular mass of streptokinase is 47kD and it is made up of 141 amino acid residues [8]. Streptokinase contains several structural domains e.g. alpha domain, beta domain and gamma domains with different functional properties.

DSC study suggests that protein consist of two different domains [9].

Streptokinase is a thrombolytic agent. It is used as a drug for the treatment of clots that formed in the tubes by administering drugs for long period in patients with treatment of hemodialysis. The research on streptokinase continues to make the drug better as a thrombolytic agent. Urokinase and tissue-type plasminogen perform direct proteolysis but streptokinase form high affinity equimolar complex with plasminogen. It is now the best fibrinolytic agent in the treatment of thromboembolic conditions [10].

Techniques for streptokinase production

Continuous Culture

Continuous culture method is an analytical tool and used to determine the instability of kinetics and product formation. It is also used to determine the specific growth rate, effect of antibiotics and substrate concentration on the expression of streptokinase [11]. Streptokinase production at different temperature, pH and dilution rates were studied in the medium which contained excess glucose studies, which are a valuable analytical tool in the determination of the kinetics of instability and product formation, were conducted to determine the effect of antibiotic concentration, specific growth rate, and substrate concentration on the plasmid stability and expression of streptokinase [41]. The production of streptokinase is constant at pH 7.0 with respect to glucose. Streptokinase production increases with increasing the dilution rates ranging from 0.1 to 0.5 hours. pH range of 6.1 to 7.0 used for continuous culture method and favorable for streptokinase production [12]. When pH values below 6.3, production of streptokinase decreases.

Response Surface Methodology (RSM)

RSM is used for the optimization of multiple parameters. It determines the optimum process conditions during combining of experimental designs with interpolation by first and second order polynomial equations in sequential testing procedures. RSM optimize the culture conditions and increase the production of enzyme [12]. It is reused for the optimization of biological processes and help evaluating the immediate effects of different factors at optimum conditions [13].

Central Composite Design (CCD)

The optimization of factors that affecting the production of enzyme and medium components is done by using CCD. Second polynomial model gets from central composite design which is useful in response prediction. Design expert software 9.0.3.1 (Stat-Ease Inc., Minneapolis, MN, USA) of central composite design is applied for the statistical design of experiments and results interpretations [14].

Based on one factor at a time approach, central composite design is used to understand the interaction among tryptone concentration (g/l), glucose concentration (g/l) and incubation time (h) and their effects on the production of streptokinase. To evaluate

pure error, $-\alpha$, -1 , 0 , $+1$, and $+\alpha$ these variables are measured at five different levels in an experimental design which consist of 20 runs (fourteen experiments and six replicates). The range of these variables in terms of implied and actual values is showed in Table 1.

Table-1: Independent variables identified by conventional/one at a time

Responses	Independent variables	Coded Factors				
		$-\alpha$	-1	0	1	$+\alpha$
A	Glucose (g/l)	2.5	5.0	10.0	15.0	17.5
B	Tryptone (g/l)	3.75	7.5	15.0	30.0	60.0
C	Time (h)	12	24	48	72	84

The assay for activity of streptokinase is performed intraplate and the response (Y) is determined by taking the average of the experimental values. The

calculation of predicted response is done with the help of the polynomial equation of the second order.

$$Y = \beta_0 + \beta_1A + \beta_2B + \beta_3C + \beta_{11}A^2 + \beta_{22}B^2 + \beta_{33}C^2 + \beta_{12}AB + \beta_{13}AC + \beta_{23}BC$$

Where Y is the predicted response. β_0 is the intercept. β_1 , β_2 and β_3 are the linear coefficients. β_{11} , β_{22} and β_{33} are the squared coefficients. β_{12} , β_{13} and β_{23} are the interaction coefficients and A, B, C, A^2 , B^2 , C^2 , AB, AC and BC is the independent variables.

Production of Streptokinase

Producing Micro-organisms

Streptococcus was isolated first time from the blood in the scarlet fever. Classification of streptococcus species based on capacities how to hemolyze blood cells. In 1919, Brown represented the alpha, beta and gamma terms which describe the three types of hemolytic reactions that observed on blood agar plates [15]. Main source of streptokinase are the beta-hemolytic streptococci of groups A, G and C. To produce streptokinase, group C mostly preferred because they do not produce erythrogenic toxins.

Streptococcus mutans grown on blood agar media. Clear zones are formed around Streptococcus mutans colonies. These clear zones are confirmed the growth of streptococcus mutants. Then maintained this micro-organism on nutrient agar media. Yellowish growth is appeared on nutrient agar media [42].

Urokinase is produced from pseudomonas species are isolating from human urine. Bacteria is identified by biochemical tests and blood agar media. These tests show SK activity and can be stored at -20°C .

Streptococcus equisimilis H46A has been expressed in different gram negative and gram-positive bacteria including E. coli [16] and B. subtilis WB600 [17]. Plasmids that contained the streptokinase gene and erythromycin resistance genes have been introduced into Streptococcus equisimilis H46A for the selection of overproducer clones [18]. Plasmids designed for high-level secretory expression of streptokinase have been successfully evaluated to produce it [19]. Some

previous studies reveal that microorganisms are capable to be used as targeted delivery of different drugs [40].

Inoculum Preparation

For the streptococcus mutans, inoculum medium is prepared through different chemicals. Maintained the pH at 7 and autoclaved the medium at 121°C for 15 minutes. Culture of S. mutans is transferred into the flask of 250 mL and incubate it on shaker 120 rpm at 35°C for 24 hours.

Inoculum media of S. dysgalactiae contain glucose, KH_2PO_4 , $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, yeast extract, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ and pH 7.4. S. dysgalactiae was inoculated in 10 ml of MSM (mineral salt medium) and incubated at 37°C for 24 hours [20].

Fermentation media

To produce streptokinase, Streptococcus mutans was grown on liquid state fermentation culture media using CSL as a substrate at 37°C 120 rpm at pH 7 for 24 hours.

Table-2: Composition of fermentation media

Ingredients	Quantity (500ml)
Yeast extract	10 g
CaCO_3	2.5 g
KH_2PO_4	0.25 g
Corn steep liquor	0.1, 0.3, 0.5, 0.7, 0.8%
Glucose	1g
Distilled H ₂ O	500ml

Fermentation

Streptococci of group A Hemolytic require complex and nutritional factors with rich supplemented media for their growth [21]. Fermentation media containing yeast extract, CaCO_3 , glucose, KH_2PO_4 was used. CSL used as substrate to fulfill the nutritional requirements, in their optimized concentrations for 24 hours at 120rpm and 37°C at pH 7 in fermentation

media for streptokinase production from *S. mutans*. Streptokinase was isolated by centrifugation at 10,000 rpm at 0°C for 20 mins. From *Streptococcus mutans* culture. Enzyme is stored at 20°C because it is extracellular supernatant [21].

Rosenberger and Elsdén examined the limitation effect of both tryptophan and glucose on the growth of *S. faecalis* in continuous culture. They concluded that, energy sources should be limiting factor for obtaining the maximum yield of cell per unit energy source. Inexpensive and simple medium used to obtain high production of streptokinase from *Streptococcus equisimilis*. The medium contained corn steep liquor or yeast autolyzed as the nitrogen source, salts and glucose [22].

Enzyme Assay

Streptokinase assays depend on its ability to activate plasminogen to plasmin. Plasmin hydrolyzes the indicator substrate and increased the hydrolysis at given period which is related back to streptokinase concentration.

Fibrin Clot Method

In fibrin-clot method, lysis produced on the fibrin film in a petri dish is measured and related to the conc. of the fibrinolytic protein. It is a simple and easy method for the determination of enzyme activity. In fibrin-clot method, blood is taken from the human and allowed it to form a clot. Then applied enzyme suspension on the clot and noted the %lysis within 10 minutes. This percentage lysis shows the enzyme activity [23].

Casein digestion method

Casein digestion method is used to determine the activity of streptokinase. This method depends on the determination of released tyrosine from the digested casein. Casein digestion method is based on determination of the liberated tyrosine from digested casein.

One unit (U/ml) of SK activity is defined as the amount of enzyme releasing 1 mole of tyrosine eq/min. Bovine serum albumin is used for the preparation of standard curve Blank solution is used in spectrophotometer to standardize it [24].

Chromozym activity test

Activity of streptokinase is determined by colorimetric method by using N-p-tosyl-glycyl-lysine-p-nitroanilide acetate as a substrate for plasmin enzyme. Samples are mixed with plasminogen and incubated for 5 minutes at 37°C. Mixture of substrates containing the c that dissolved in 50mM tris-HCl pH 8 added in enzyme-substrate mixture. Reaction is incubated for 20 minutes at 37°C and change in absorbance was monitored [25].

Substrate for Streptokinase

Molasses

Molasses is the cheapest source of carbohydrates and protein production. Cane molasses is commercially available and byproduct of sugar manufacturing. It is used as a substrate for biomass production. Highest fibrinolytic activity is observed at the different concentrations of molasses. 0.5% concentration used for streptokinase production and showed 35.3% lysis. (Fig.1) Therefore, it is the optimum concentration for streptokinase production [27].

Rice Polishing

Rice polishing is the product of rice milling. It is used as a cheap source of vitamins, proteins and minerals. It is very suitable for microbial growth [28]. At different concentrations of rice polishing, maximum fibrinolytic activity is observed, when *Streptococcus* species used 0.5% concentration of rice polishing as substrate for streptokinase enzyme production and showed 36% lysis (Fig. 1). So, the optimum concentration of substrate for the streptokinase production is 0.5% [29].

Sugarcane bagasse

Sugarcane bagasse consists of 50% cellulose and 25% lignin and hemicellulose. It contains 30% pentosans and 2.4% ash. In liquid state fermentation, species of *S. mutans* grown by using 0.4% concentration of sugarcane bagasse as a substrate for Streptokinase enzyme production. Figure showed that 32.6% is the highest enzymatic activity at 0.4% concentration. So, the optimum concentration of sugarcane bagasse for streptokinase production is 0.4% [30].

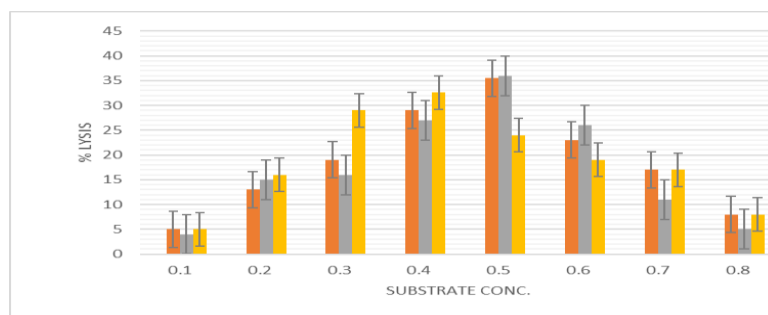


Fig-1: Activity of Streptokinase by Using Different Substrate Concentrations

- Orange color represents the concentration of Molasses.
- Gray color represents the concentration of Rice Polishing.
- Yellow color represents the concentration of Sugarcane Bagass [26].

Effects of parameters on Streptokinase production

Effect of temperature

Effect of temperature on streptokinase activity is observed at pH 7. Temperature that show maximum enzymatic activity is 27 to 37°C. Residual activity is showed at temperature 8, 20, 55 and 100°C. the optimal range of temperature is 27 to 45°C because in this range the enzyme remains active. Readings are taken from the spectrophotometer [31].

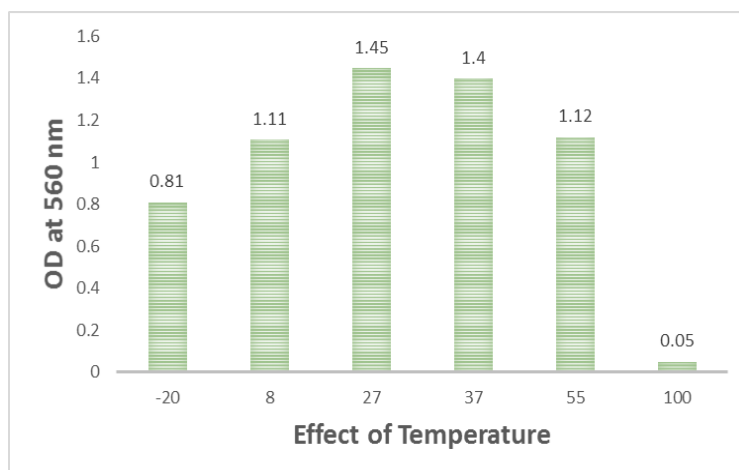


Fig-2: Activity of Enzyme in Different Temperatures.

Effect of pH

Through enzyme assay method, activity of the enzyme can be measured at different values of pH like pH 6, pH 7, pH 8. With the help of spectrophotometer, readings are taken and calculate the unit of enzymes.

28.64 U/ml activity of enzyme is observed at optimum pH 7. When pH below 3, the fibrinolytic activity of streptokinase decreases. The activity of enzyme abruptly decreases at pH 11[32].

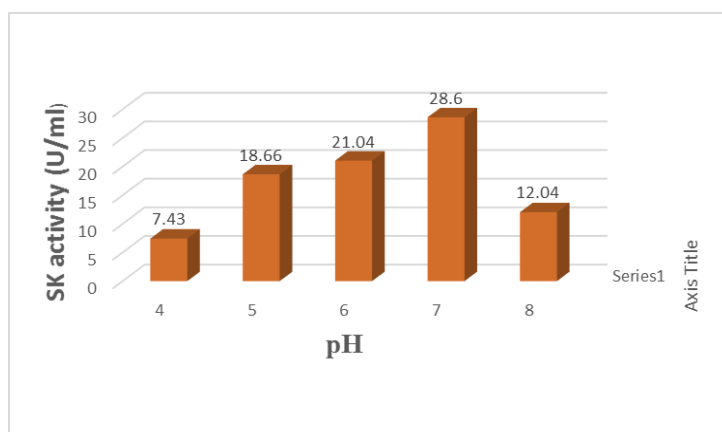


Fig-3: Effect of Different pH on Streptokinase Activity

Effect of MgSO₄

Magnesium ions play a role in cellular metabolism. These ions stabilize the structure of nucleic acids, cell membrane and proteins by binding to the surface of macromolecules. Addition of MgSO₄ in the production medium showed positive effects on the production of streptokinase [33].

Different concentrations of MgSO₄ affect the production of streptokinase. Maximum activity of streptokinase is 0.165 U/ml was noted for 0.02% MgSO₄. When concentration increases from 0.02% activity of streptokinase decreases. At highest concentration of MgSO₄ 0.1 w/v, production of streptokinase is severely affected [34].

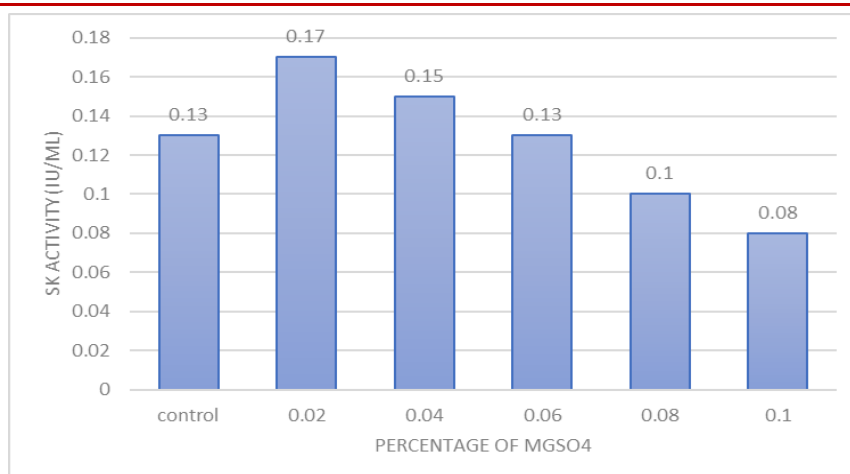


Fig-4: Effect of MgSO₄ on Streptokinase Activity

Effect of inoculum size

Bacterial inoculum size should be controlled to produce enzymes from the limited amount of nutrients. Inoculation of *Streptococcus dysgalactiae* with different inoculum sizes ranging from 0.05 to 3 % (v/v) influenced the streptokinase production. The maximum activity of streptokinase is 0.360U/ml that was achieved

with 1% inoculum size. The production of streptokinase reduced when the inoculum size is 3% (v/v) [35].

Due to high surface area to volume ratio, the production of enzyme increases by using small inoculum size. Thus, high inoculum sizes do not give high production of streptokinase [36].

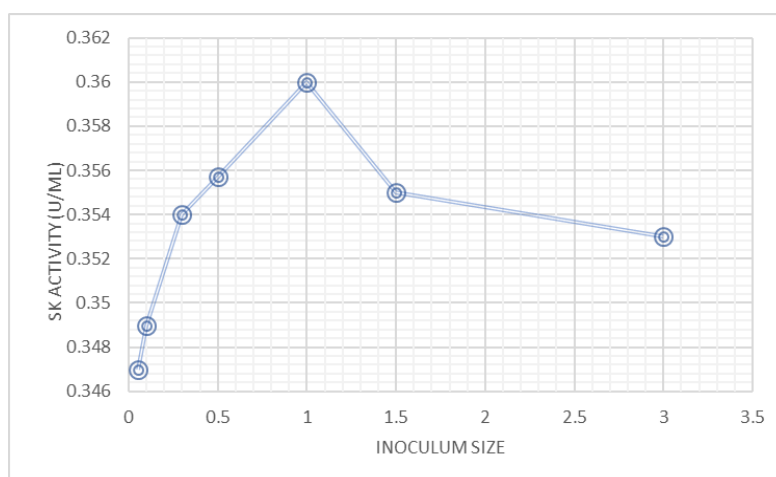


Fig-5: Effect of Inoculum Size on Streptokinase Activity

Effect of incubation period

Production of enzyme differs with incubation time [37]. It is very important to detect the optimum incubation time at which organisms give maximum production of enzyme. Organisms show significant variation at different incubation periods [38]. Streptokinase production increased from 24 to 48 h. the maximum level of production of streptokinase (0.238

U/ml) reported at 48 h. This medium contained glucose, yeast autolyzed, various salts and CSL as a nitrogen source. High yield of streptokinase was obtained at 28°C, pH 7.4, with in 24 h in cultures. When the incubation time increased from 2 to 7 days, production of streptokinase from *Streptococcus pyogenes* decreased [39].

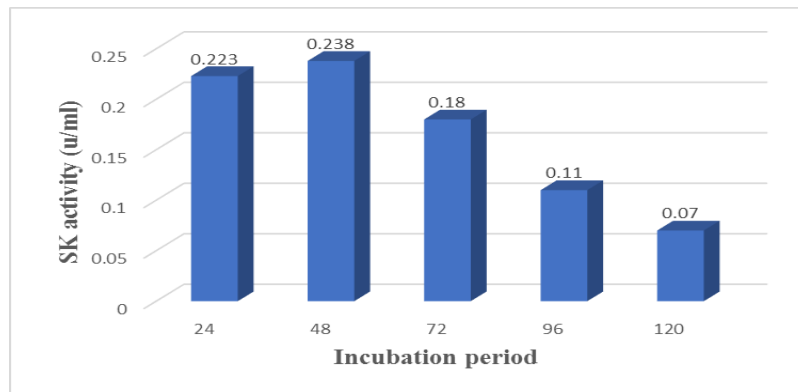


Fig-6: Effect of Incubation Period on Streptokinase Activity.

Effect of carbon and nitrogen sources

Placket-Birman design is used for testing the effect of nitrogen and carbon sources to produce streptokinase. This design is used for screening

different carbon sources such as dextran, glycine fructose etc. The difference between different sources of carbon for streptokinase production can be summarized and display graphically [14].

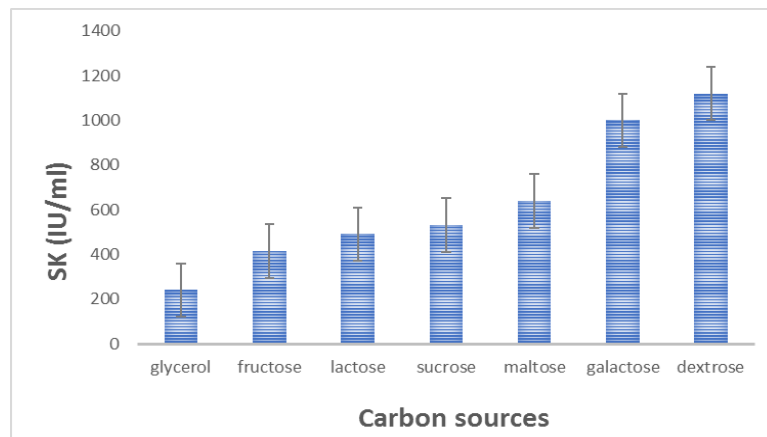


Fig-7: Effect of Carbon Sources on Streptokinase activity

Graph showed that dextrose is the important carbon source for streptokinase production of 1,120 IU/ml and glycine has less significant. The level of dextrose is further optimized by RSM in production media.

Similarly, graph below represented the effect of different organic sources of nitrogen such as

polypeptone, peptone, beef extract and yeast extract on streptokinase production. Peptone has significant effect on streptokinase production that resulted in 1080 IU/ml. yeast extract and polypeptide resulted in 1,025 and 1,015 of streptokinase production [12]. Different biological apps of smartphones may be used to obtain the result graph of streptokinase activity against nitrogen sources [43].

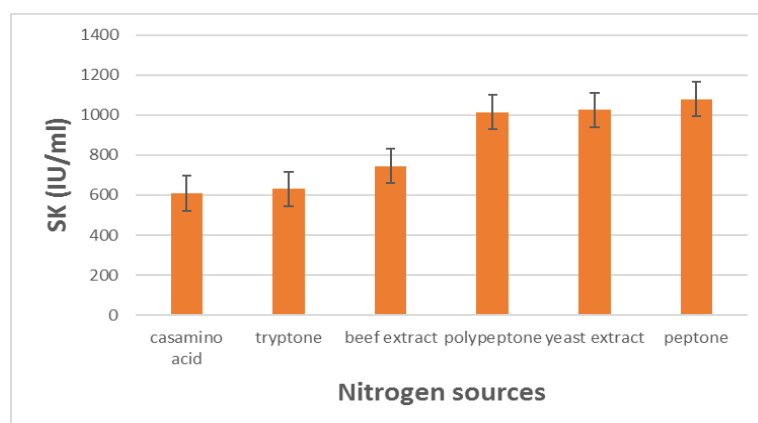


Fig-8: Effect of Nitrogen Sources on Streptokinase Activity

CONCLUSION

Streptokinase is used as a thrombolytic agent for human health care. Due to high cost and intravenous installation, different sources and methods are required for large scale production. So, the production of streptokinase by using bacterial sources i.e. streptococcus strains and RSM method is very useful and effective. We reported that streptokinase having better fibrinolytic activity can be produced by using traces elements, growth factors, co-factors, co-enzymes, inhibitors, activators, nitrogen and carbon sources, inoculum size and optimal conditions of physical parameters such as temperature, pH, incubation time and optimum concentration of substrates CSL, molasses, rice polishing, sugarcane bagass to fulfill the needs of this enzyme.

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