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ISOLATION, PURIFICATION AND CHARACTERIZATION OF FIBRINOLYTIC ENZYME FROM BACTERIAL SOURCE

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ABSTRACT

Accumulation of fibrin in the blood vessels usually results in thrombosis, leading to myocardial infarction and other cardiovascular disease. For thrombolytic therapy, microbial fibrinolytic enzymes have now attracted. The culture PSPS02 selected on the basis of zone of hydrolysis during casienolytic and haemolytic activity. The fibrinolytic enzyme production and purification was carryout by salt precipitation and dialysis after submerged fermentation.

Keywords: Fibrinolytic, Casienolytic, Fermentation, Plasminogen activator, Haemolytic.

INTRODUCTION

Fibrinolytic enzyme is an extracellular protein, extracted from certain strains of beta hemolytic streptococcus**[1].** It is non-proteases plasminogen activator that activates plasminogen of plasmin, that enzyme that degrades fibrin clot through its specific lysine binding site; it is used therefore as a drug in thrombolytic therapy **[2].** Fibrinolytic enzyme activates human plasminogen by promoting its conversion to plasmin, is normally obtained from beta hemolytic streptococci **[3].** Streptokinase is currently used in clinical medicine as a therapeutic agent in the treatment of thromboembolic blockages, including coronary thrombosis **[4].** The clinical importance of streptokinase was first noted by Tiller and Garner in 1933, which 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 **[5].**

Plasmin is the active fibrinolytic component of the circulatory system, solubilizing the fibrin network in blood clots through limited proteolysis. Streptokinase is currently used in clinical medicine coronary thrombosis **[5].**Streptokinase is naturally produced and secreted by various strains of hemolytic streptococci **[6].**Haemostasis is a complex process obtained through an optimal balance between bleeding and clot formation. Blood clot is composed of fibrin **[7].** Fibrin is the main protein component of blood clot and is normally formed from fibrinogen by the action of thrombin **[7].** These fibrin clot are dissolved by the hydrolysis of fibrin is also known as fibrinolysis **[8].**

Fibrinolytic enzymes are one of the largest group of proteolytic enzyme involved in numerous regulatory process related to fibrinolytic action **[9].** These enzymes 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 **[1].** However these enzymes are often expensive thermo labile and can produce unwanted and undesirable side effects.Streptokinase has been used as an effective drug in case of thrombosis, arterial thromboembolism and critical stenosis **[10].** Mainly, this kind of treatment Handle in various animals like rat, cat, rabbit and dogs **[11].**

In human, streptokinase used as thrombolytic agent especially for the treatment of acute myocardial infarction as it is a cost effective, for a both animal and human disorder. These agents are used to treat heart attack, stroke, deep vein thrombosis, pulmonary embolism and occlusion of a peripheral artery **[12].**

These activators are of human origins 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 (haemostasis) **[13].**

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 types plasminogen activator. Urokinase and t-PA are widely used in thrombolytic therapy, but these agents have some undesirable side effect **[14].**

Thus the present study deals the production, purification and characterization of streptokinase enzyme.

METHODOLOGY:

Isolation of streptokinase producing bacteria:

The soil sample was collected from different slaughtering house situated in Vibhutikhand, Lucknow and serially diluted in 0.85% normal saline. After dilution the samples were spread on nutrient agar media to obtain mixed culture colony. Colony morphology was determined and mixedcultures were converted in to pure culture by streak plate methods. All isolates were streaked on nutrient agar media supplemented with 1% casein powder and incubated for 48 hours at 37°C. The positive cultures were shorted on the basis of measured zone of hydrolysis.Strains screened out from primary screening were restreaked in blood agar media supplemented with sterile clotted blood and incubated at 37°C for 48 hours and positive cultures were screened out on the basis of zone of hydrolysis **[1,15,16].**

Biochemical tests for bacteria:

Gram's staining, endospore staining, mannitol test etc. biochemical tests were performed for the bacterial culture by following the Bergey's manual.

3Media selection and its optimization:

One Factor at a time method was used to scrutinize the effects of different components on fibrinolytic enzyme production. Using this method the parameters were optimized for their different substitutes **[17].**

Table 1: Components of production media

Factors	**Media**	**Components**	**Quantity**
Media selection	PM 1	Nutrient broth	1.3 %
		Casein	1 %
	PM 2	Peptone	0.5 %
		NaCl	0.5 %
		Dextrose	0.8 %
		$MgSO_4.7H_2O$	0.02%
		Casein	1%
	PM 3	Na_2HPO_4	0.2 %
		K_2HPO_4	0.6 %
		NaCl	0.5 %
		$MgSO_4.7H_2O$	0.02 %
		NH_4Cl	0.5 %
		Dextrose	0.8 %
		Casein	1 %
Carbon sources	MM1	Dextrose	0.8 %
	MM2	Sucrose	0.8 %
	MM3	**Maltose**	0.8%
Nitrogen sources	**MM4**	**Peptone**	0.5 %
	MM5	NH_4Cl	0.5 %
	MM6	Yeast	0.5 %
Effect of pH	MM7	pH	4
	MM8	pH	7
	MM9	pH	9
	MM10	pH	11

Growth study of bacteria:

The culture was inoculated in sterilized optimized media and the growth study was done by taking the absorbance at 620 nm in colorimeter after respective time interval **[18].**

Fermentation and Downstream Processing:

The fermentative media was prepared and sterilized. The culture was inoculated and incubated at 37°C for 48-72 hours in shaker incubator. Further purification of enzymes was carried out by salt precipitation and dialysis **[20].**

Estimation of fibrinolytic enzyme by casienolytic assay:

The samples were added to sodium phosphate buffer (100mM) and casein (1%). After the incubation for 15 min at 37°C,trichloroaceteicaicd (TCA) was added to stop the reaction, again incubated at 4°C for 30 minutes.

5 ml reagent C (2% Naco3 + 0.1N NaOH + 0.5% CuSO4 + 1% sodium potassium tartrate) incubated at room temperature for 10 minutes. Further the samples were incubated in dark for 30 min after adding folins reagent to it. Optical density was taken at 680 nm **[20].**

Effect of temperature and P^H:

The best pH and temperature for the activity of fibrinolytic enzyme was resolute by measuring the optimum activity after incubating 100µl enzyme with 900µl, 20 mM Tris–HCl (pH 7.5) at different temperatures such as 4°C, 37°C, 20°C, 50°C for 1 hour. Similarly the optimum pH was measured by maintaining the pH of enzyme at 4,7, 9, 11. Further the caseinolytic assay was performed **[21].**

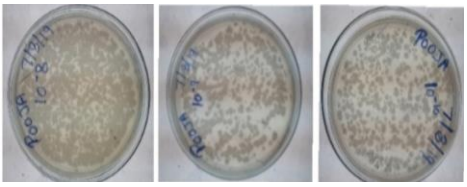
Enzyme activity test:

The enzyme was added to the clotted blood and incubated for observation of activity of enzyme.

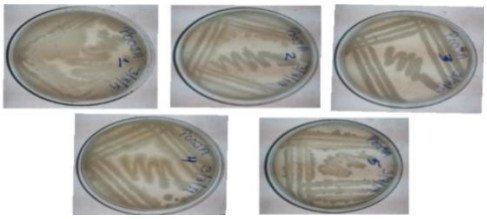
RESULTS:

Isolation and fibrinolytic enzyme producing bacteria:

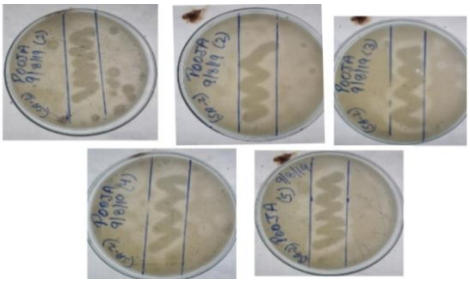
Firstly the sample was spread and then selected cultures were streaked on nutrient agar plates. Further the pure cultures were screened for the streptokinase production.



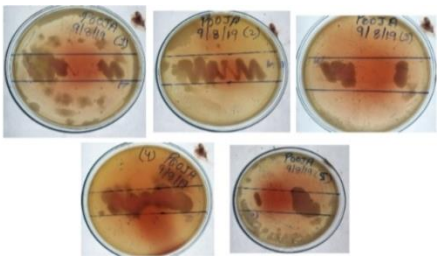
a. white colonies after spreading



b. pure cultures by streaking method



c. Caseinolytic activity test



c. Caseinolytic activity test d. haemolytic activity test

Figure 1: isolation, purification and screening of fibrinolytic enzyme producing bacteria.

Colony morphology:

Table 2: Morphological classification of selected colonies

S no.	Shape	Surface	Elevation
C1	Circular	Rough	Flat
C2	Circular	Rough	Convex
C3	Irregular	Smooth	Umbonate
C4	Punctiform	Smooth	Flat
C5	Circular	Smooth	Convex

S no.	Margin	Texture	Opacity	Pigmentation
C1	Entire	Hard	Opaque	Off white
C2	Entire	Hard	Translucent	Off white
C3	Discrete	Soft	Opaque	Cream
C4	Entire	Gummy	Opaque	Cream
C5	Discrete	Gummy	Opaque	Off white

Biochemical tests for bacteria:

For the identification of the bacteria the biochemical tests were carry out which is based on Bergy'sMannual.

Table 2: Biochemical tests for identification of culture

S no.	Biochemical Tests	Results
1.	**Endospore test:**	Positive
2.	**Gram staining:**	Gram's positive, *Bacillus*
3.	**Catalase test:**	Negative
4.	**Mannitol test:**	Positive
5.	**VP test:**	Negative
6.	**MR test:**	Positive
7.	**Urease test:**	Negative

Selection and optimizationof media.

For the growth of culture, the production media was selected and then optimization of the components was done on the basis of one factor at a time.

Table 3: Media Selection and its optimization for the growth of culture PSPS02

S no.	Production media	OD 620 nm
1.	**Selection of production media**	
	PM1	0.33
	PM2	0.66
	PM3	0.06

2.	**Effects of Carbon Sources**	
	Dextrose	0.57
	Maltose	0.66
	Sucrose	0.64
3.	**Effects of Nitrogen Sources**	
	Peptone	0.58
	Yeast	0.19
	NH_4Cl	0.03
4.	**Effects of pH**	
	pH 4	0.04
	pH 7	0.11
	pH 9	0.57
	pH 11	0.21

Effects of temperature:

Table 4: Effect of temperature on the growth of culture.

S. no.	Temperature (°C)	Bacterial Growth
1.	37	+++
2.	50	-
3.	4	-
4.	20	+

Growth study of culture PSPS02:

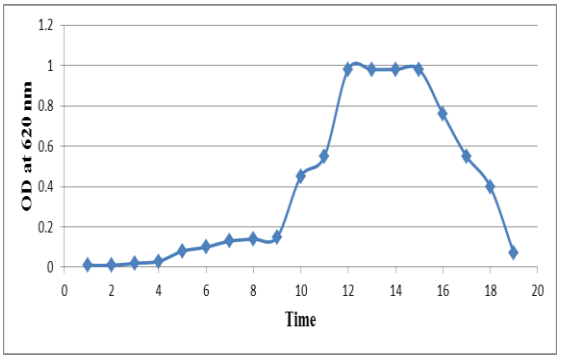


Figure 2: Graphical representation of growth study of bacterial culture PSPS02**.**

Fermentation and down stream processing:

The fermentation for the production of fibrinolytic enzyme was done at small scale, by using batch culture and submerged fermentation. Further down stream processing was carryout for the purification of enzyme by salt precipitation (40% ammonium sulphate) and dialysis.

3.8. Enzyme Assay:

Table 5: estimation of enzyme by casienolytic assay.

Sample	OD 680 nm	Concentration (µg/ml)	Enzyme activity (U/ml/min)
Crude enzyme	0.09	0.5	0.03
Pure Enzyme	0.15	0.9	0.054

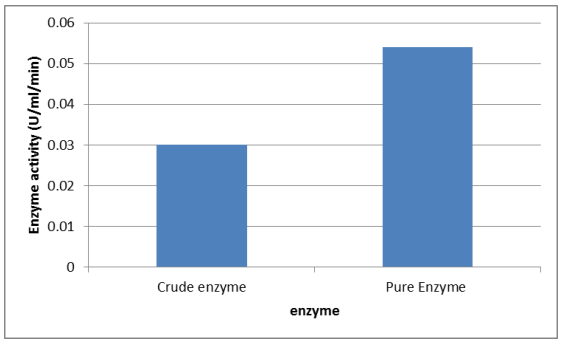


Figure 3: graphical respresentation of enzyme activity of pure and crude enzyme.

3.9 Effects of physiochemical factors on enzyme activity:

Table 6: Effect of pH and temperature on enzyme activity

Parameters	OD 680 nm	Concentration (µg/ml)	Enzyme activity (U/ml/min)
Effects of temperature (**°C**):			
RT	0.04	0.2	0.013
4	0.1	0.8	0.053
37	0.02	0.12	0.008
50	0.08	0.4	0.02
Effects of pH:			
4	0.01	0.05	0.003
7	0.03	0.19	0.012
9	0.04	0.2	0.013
11	0.02	0.12	0.008

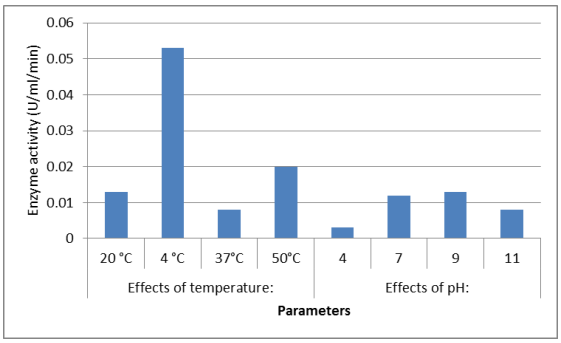


Figure 4: graphical respresentation of effect of pH and temperature on enzyme activity

3.10. Application of enzyme: for the lysis of the fibrin present in blood.

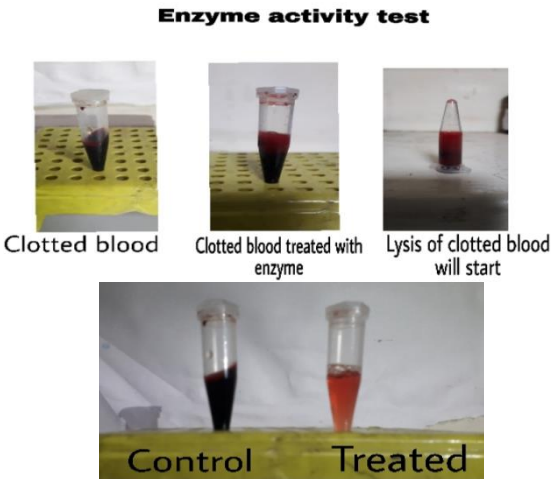


Figure 5:- Enzyme activity test

(positive result)

- Use of streptokinase is indicated in the treatment of pulmonary embolism, deep vein thrombosis, arterial thrombosis and embolism, arteriovenous cannula occlusion, and coronary artery thrombosis.
- It is recommended for the management and control of myocardial infarction (AMI) in adults to bring about various therapeutic benefits, such as
    - (i) Specific lysis of intracoronary thrombin.
    - (ii) Reduction of infect size and congestive heart failure associated with AMI .
    - (iii) Important in ventricular function.
    - (iv) Remarkable reduction of mortality associated with AMI.
- It is also indicated for the adequate lysis of objectively diagnosed pulmonary emboli , involving obstruction of blood flow to a lobe or multiple segments, with or without unstable thermodynamics.

Discussion and Conclusion:

This study is based on isolation of streptokinase from the bacteria. Streptokinase producing bacteria were isolated from slaughter house soil sample (only Slaughter house soil sample is choose because in Slaughter house many animals get died which blood is mixed in soil by with we can easily find out our specific bacteria which has fibrinolytic activity) , which was used to produce streptokinase. Microorganism was is isolated from soil sample using serial dilution and purification is done by streaking. The best growth of bacteria was obtained in production media 2 (PM2), in optimization of different components dextrose as carbon source and peptone as nitrogen source was selected and pH 9 and 37 °C temperature was selected for the fermentation.

The positive culture PSPS02 was Gram's positive and bacillus, the mannitol, endospore test were also positive but the catalase, MRVP and urease test were negative result shows. So according to BERGEYS MANUAL the isolated strain *is B.megaterium*.

By applying the purified enzyme on clotted blood to check the application it was found that clot lysis was completed within 30 minutes.

The streptokinase is an extracellular protein, currently used in clinical medicine as a treatment of thromboembolic blockages, including coronary thrombosis.

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