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Biomedical analysis of fibrinolytic enzyme produced by microorganism from poultry farm; an in vitro study

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Abstract

Fibrinolytic enzymes have immense importance in the field of biomedicine due to their potential therapeutic applications. In recent years, microbial fibrinolytic enzymes have been reported to treat cardiovascular diseases. The aim of the present study is to analyze the biomedical application of fibrinolytic enzyme produced by microorganism isolated from poultry farm. The isolated bacterial strains from soil samples enriched with poultry waste were screened for their fibrinolytic activity. The fibrinolytic enzyme was produced from the screened isolate by enriching the medium with nutrient sources such as carbon sources, nitrogen sources, natural sources etc. Our finding clearly depicts the clot lytic activity and thrombolytic activity of the fibrinolytic enzyme in a dose dependent manner. The fibrinolytic enzyme exhibited cardioprotective and wound healing activities.

Key words: fibrinolytic enzyme, isolation, poultry farm, cardiovascular diseases

Introduction

Cardiovascular diseases such as myocardial infarction, arrhythmia and stroke are the leading cause of morbidity and mortality worldwide with 17 million deaths every year (Jemimah *et al*.,2016). Intravascular thrombosis is one of the major etiological conditions for such disorders. Traditionally, the treatment of thrombosis was relied, either on the use of antiplatelet and anticoagulating agents such as heparin and warfarin or on surgical treatments. The thrombus in blood vessels due to fibrin accumulation may lead to myocardial infarction or other cardiovascular diseases (Peng*et al.*, 2003).

Fibrinolytic enzymes have gained considerable attention in the field of biomedicine due to their ability to break down fibrin ,the principal component of blood clots formed from fibrinogen by thrombin (E.C.3.4.21.5) , lysed by plasmin (EC3.4.21.7). They help to dissolve clots and prevent any adverse effect due to clot formation, such as stroke,pulmonary embolism, deep vein thrombosis and acute myocardial infarction (Anirban Banerjee *et al.*, 2004).Currently available thrombolytic agents are very expensive and possess significant shortcomings, includinglarge therapeutic dose, short plasma half-life, limited fibrin specificity and bleeding. A variety of fibrinolytic enzymes are obtained from various sources viz. plants (Chung *et al.*, 2010); animals and microorganisms (Ju*et al*.,2012).

Microbes are capable of producing a variety of fibrinolytic enzymes. Due to high production capacity, there is unlimited supply of enzymes. Production capacity can be expanded by strain improvement. They can be manipulated easily in the laboratory.Large number of enzymes can be obtained economically from microorganism. Only well- designed and intensive search among microbial strains can usually find an appropriate organism to produce any enzyme. It is possible to introduce genetic changes in them rather easily, due to simplicity of their genome. Their growth requirements are simple and can be precisely defined which is of immense importance in industrial production to maintain consistency of product quality. In recent years, microbial fibrinolytic enzymes have been reported to treat and prevent CVDs. These enzymes have various therapeutic applications, including anticoagulants, thrombolytics and anti- inflammatories. There are various reports on fibrinolytic enzymes with thrombolytic applications from many sources, such as earth worm, snake venom, and fermented foods (Peng *et al.*, 2003).

Many studies were conducted on agro-residues, such as pigeon pea (Johnvesly *et al*., 2002), potato peel (Mukherjee *et al.*, 2008), *Jatropha curcas* seed cake (Mahanta*et al.*, 2008), rice chaff (Tao *et al*., 1997), Sesame oil cake (Rajendran and Thangavelu, 2013), Ground nut husk (Salihu*et al.*, 2014) and agro-industrial wastes for the production of enzymes. Selection of medium components is critical for the fermentative production of fibrinolytic enzymes. Enzyme therapies is becoming more prevalent in the medical world today and many manufacturers targeting their advantages in disease treatment. The present study focuses the cardiac protective and wound healing activity of the fibrinolytic enzyme isolated from poultry farm microorganism.

1. Materials and methods

1.1. Sample collection

Soil samples were collected from various locations of poultry farm at Villukuri Town Panchayat in Kanyakumari district. 10 gm of soil was obtained from a depth of 25 cm from eachsite.

1.2. Isolation of microorganisms

The soil was serially diluted and 10^{-8} dilution was used for plating by spread plate method on nutrient agar medium. The plates were incubated at 37˚C for 24 hours. Pure bacterial isolates were obtained by repeated sub culturing on nutrient agar plates, and were stored in agar slants with the help of 15% glycerol.

1.3. Screening of fibrinolytic bacteria

Fibrinolytic activity was investigated by fibrin plate assay (Astrup and Mullertz, 1952, Kumaran *et al*., 2011) with minor modifications. The fibrin agarose gel (5-mm thick) contained 2.0% agarose, 0.12%(w/v) fibrinogen, 0.5 U/ml thrombin and 0.1 M phosphate buffer (pH 7.4) containing 0.15 M NaCl. The clot was allowed to set for 30 min at room temperature, after whichcell-free bacterial culture supernatant of the selected proteolytic strain was carefully loaded onto the fibrin agar plate. Un-inoculated broth medium was used as the negative control. The loaded plates were incubated at 37°C for 24 h.

1.4. Biochemical characterization

The culture characterization was identified by performing the biochemical tests such as gelatinase production test, cellulase production test, catalase test, oxidase test, urease test, H_2S production test, gas production test, Indole production test, carbohydrate test, Nitrate reduction test, citrate utilization test, Gram staining and motility test.

1.5. Optimization of enzyme production

Production of fibrinolysis enzyme was optimized by supplementing the nutrient broth with various carbon sources such as glucose, fructose, maltose, lactose, sucrose as well as starch and nitrogen sources such as protein, methionine, alanine, glycine and asparagines etc. Media Optimization has been successfully and efficiently applied for the production of numerous compounds and fermentation process (Venkataraman *et al*., 2010).

1.6. Thrombolytic activity:

The clot lysis and thrombolytic activity was checked using modified Holmstrorm method(Swetha Prasad *et al.,* 2006). Clean grease free glass slides were taken and human clotted blood was placed on each slide to which fibrinolytic enzyme was added and observed for the lysis of blood clot (K.Gowthami and R. JayaMadhuri, 2021).

1.7. Invitro blood clot lytic activity of fibrinolytic enzyme:

About 50 ml of goat blood was collected from Slaughter house at Monday Market, in Kanya Kumari District and allowed to form clot. Different aliquots of blood clot were placed in separatetubes. The clots were washed twice with phosphate buffered saline and incubated with different concentrations of fibrinolytic enzyme at room temperature for 6 hours and the results were notedfor every 30 minutes.

1.8. Determination of invitro cardiac protective activity of fibrinolytic enzyme on cultured H9C2 cell lines

H9C2 cardiacmyoblast cell lines were purchased from NCCS Pune was maintained in Dulbecco's modified Eagles media and grown to confluency at 37°C and 5
% CO_2 in a humidified atmosphere in CO2 incubator. The cells were trypsinized (500µl of 0.025% Trypsin in PBS/ EDTA solution) for 2 minutes and passaged to T flasks in complete aseptic conditions. Doxorubicin was added at a final concentration of 0.1% to induce toxicity. Extracts were added to grown cells treated with Isopropanol at a final concentration of 1.5µl, 3.1µl, 6.2µl, 12.5µl and 25µl from a stock of 1mg/ml and incubated for 24 hours. The % difference in viability was determined by standard MTT assay after 24 hours of incubation. (Arung *et al*., 2000)

1.9. Scratch wound assay

RAW H9C2 cell line (monocyte /macrophage-like cells) was procured from the National Centre for Cell Sciences (NCCS), Pune, India.Wound healing property of the fibrinolytic enzyme produced by the poultry farm microorganism was evaluated by the scratch wound healing assay using raw cell lines (Gowthami and Jaya Madhuri ,2021). A wound gap is created by scratch wound assay and healing growth towards the center of the gap is monitored photographically..

2. Results and Discussion:

2.1. Isolation of microorganism

The soil samples collected were serially diluted and 10^{-8} dilution was used for plating by streak plate method. The inoculated plates were incubated at 37˚C in an incubator for 24 hours. Visible colonies were formed after incubation (fig 1).

Fig: 1: Isolated colonies on nutrient agar plate

2.2. Screening of fibrinolytic activity

Among the 5 isolates checked two isolates I1 and I2 showed mximum halozone in fibrin agar medium using the culture supernatant (Fig.2.A). To isolate a potent fibrinolytic enzyme producer stain the samples were screened on skim milk agar and fibrin plate (Kapila Taneja *et al*., 2017) .Among the two isolates , the IS1 exhibited the maximum clear zones around the paper discs was used for further study (Fig.2.B) . Our study colloborates with the results of Raju E V N and Divakar G ,(2013).

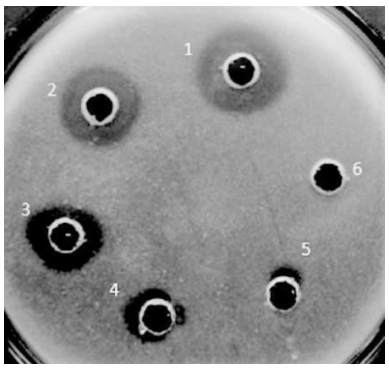

Fig.2.A. Growth on fibrin plate(1-I1, 2-I2, 3-I3, 4-I4, 5-I5 ,6-control)

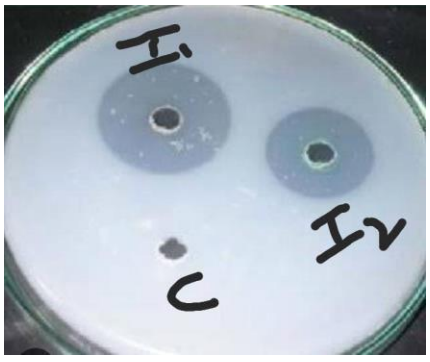

Fig .2.B.Growth of I1 and I2 onfibrin agar plate

2.3. Biochemical characterization of the isolate

The isolated pure culture was subjected to various biochemical characterizations (Kamekura *et al.*, 1995) and the results were shown in table 1. The results clearly indicated that the strain is colourless, Gram positive rod with evenly spread colonies. It shows motility and positive reaction in catalase, oxidase, gelatin liquefaction, starch hydrolysate, casein production,glucose, sucrose, dextrose, arabinose and mannitol tests.

Table 1: Biochemical Characterization of the isolated strain

Sl. No	Characteristics	Reaction
1	Source	soil
2	Colony Morphology	colourless
3	Gram reaction	+ive
4	Shape	rod
5	Arrangement.	evenly spread
6	Catalase	+
7	Oxidase	+
8	Motility	+
9	Indole production	-
10	Methyl red	-
11	Vogesproscauer test	-
12	Citrate utilization	-
13	TSI	-
14	Urease production	-
15	Gelatin liquefaction	+
16	Starch hydrolysate	+
17	Casein production	+
18	Glucose	+
19	Sucrose	+
20	Dextrose	+
21	Lactose	+
22	Arabinose	+
23	Mannitol	+

(+) – positive (-) - negative

2.4. Enzyme production in the optimized medium

Activity of fibrinolytic enzyme was determined from control and optimized medium. The results were recorded and tabulated in table (2). The results revealed that the activity of fibrinolytic enzyme was found to be more (516.98 U/ml) in optimized medium. The production of enzymes significantly depends upon the media components; central composite design was employed to

determine their optimum level aiming for maximum enzyme productionas reported by Kapila Taneja *et al*., (2017).

Table 2: Activity of fibrinolytic enzyme

PARTICULARS	ENZYME ACTIVITY (U/ml)
Control Medium	104.82
Optimized Medium	516.98

2.5. Thrombolytic activity

Thrombolytic activity of fibrinolytic enzyme was assessed and the results are given intable 3.In blood clot slide technique the fibrinolytic enzyme dissolved the clot in a dose dependent manner (Fig .4 and Table 3), Similar results were obtained by Gowthami and Jaya Madhuri (2021).

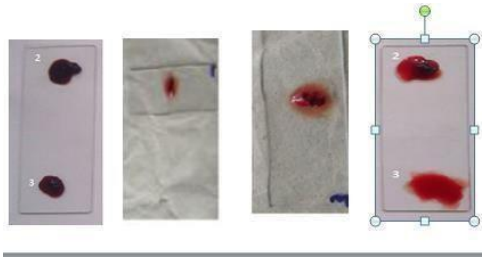

Fig: 4: Clot bursting activity in a dose dependent manner

Particulars	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	Blank
Blood clot	10µl	10µl	10µl	10µl	10µl	10µl	10µl	10µl	10µl	10µl	10µl
Purified Fibrinolytic enzyme	10µl	20µl	30µl	40µl	50µl	60µl	70µl	80µl	90µl	100µl	0
Lysis of clot	-	-	-	+	+	++	++	+++	++++	+++++	No Lysis

Table 3.Clot bursting ability of the fibrinolytic enzyme

2.6. Invitro blood clot lytic activity of fibrinolytic enzyme:

The results revealed that the clot lysis was increased significantly as the concentration of enzyme increased (Fig .5). In the present investigation the fibrinolytic enzyme digested the clot completely within 5 hours at room temperature. Similar results were also previously reported by Deepak *et al.*, (2008) , Mahajan *et al., (*2012) , Yuan *et al.*, (2012), Vijayaraghavan and Vincent (2014), their results revealed that the invitro clot lytic activity of the fibrinolytic enzyme increases with increased concentration of the enzyme.

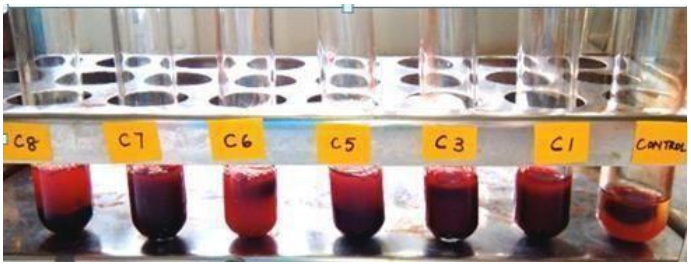

Fig.5.Clot lytic activity of the fibrinolytic enzyme

2.7. Determination of invitro cardiac protective effect of Fibrinolytic enzyme on cultured H9C2 cell lines

Cardiac protective activity of fibrinolytic enzymeon cultured H9C2 cells was examined and the results were tabulated from the table 4. The results revealed that viability of cardiac cells was significantly increased to 34.6% at 6.25µ L, 40.05% at 12.5µ L, 55.6% at 25µ L, 64.9% at 50µ L and 74.6% at 100µ L of fibrinolytic enzymerespectively, compared with percentage viability of cells treated with doxorubicin (Fig.6). Based on the observation it can be agreed that increased volume of the fibrinolytic enzyme increases the percentage viability of H9C2 cells (Fig.7).

Table: 4 Invitro cardiac protective effect of fibrinolytic enzyme on cultured H9C2 cell lines

Sample Volume (µl)	Average OD at 540nm	Percentage Viability
Control	**0.1626**	
Doxorubicin	0.0406	24.9
6.25	0.0563	34.6
12.5	0.0658	40.05
25	0.0904	55.6
50	0.1056	64.9
100	0.1213	74.6

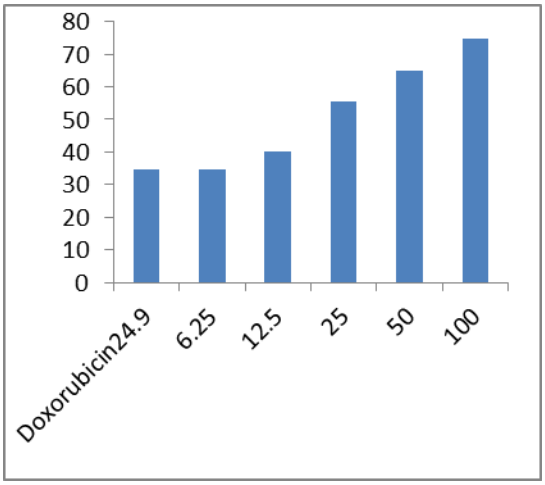

Fig.6: Graphical representation of percentage viability

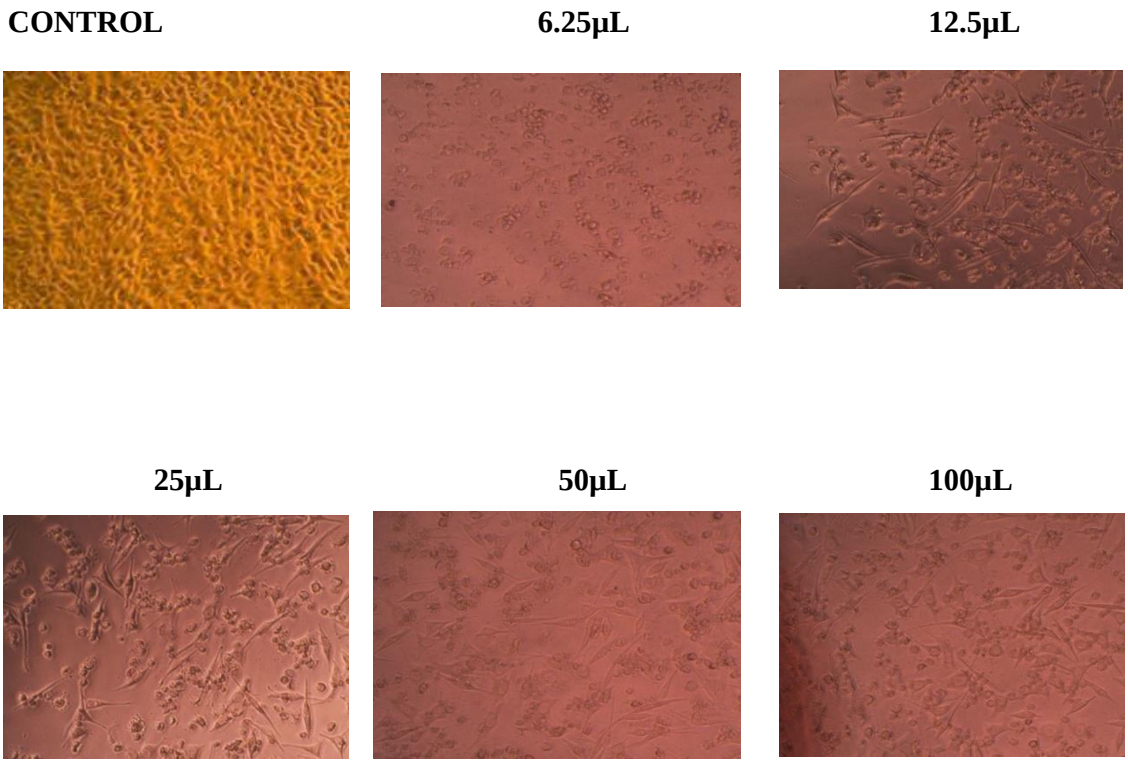

Fig .7.Invitrocardioprotective activity in a dose dependent manner

2.8. Scratch wound assay

The test compound synthesized fibrinolytic enzyme was found to elicit wound healing property at higher concentrations (50 µgand 100 µg) in a time dependent manner (Fig 8). The most effective concentration was found to be 100 µg.Similar results were also reported by Gowthami and Jaya Madhuri, 2021. Liang CC & Park AY (2007) reported that in vitro scratch assay is a convenient and inexpensive method for analysis of cell migration.

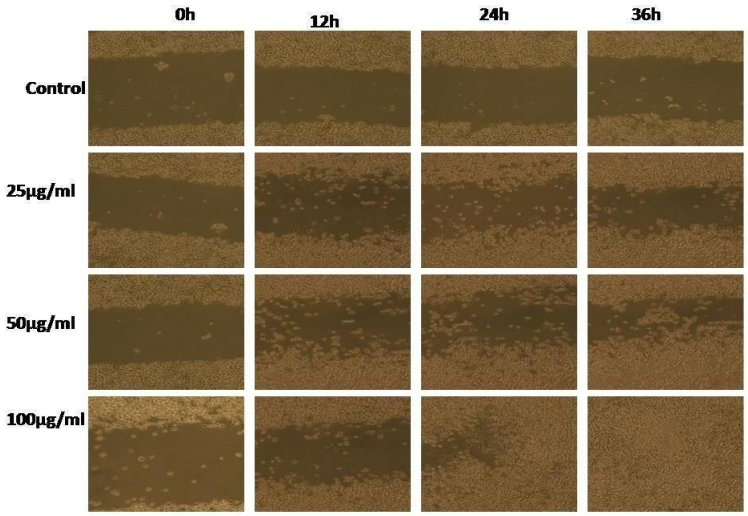

Fig.8. Scratch wound assay of synthesized fibrinolytic enzyme

Conclusion

Fibrinolytic enzymes are the biological component which helps to suppress the development of blood clots. Our findings demonstrate the potential of fibrinolytic enzymes derived from microorganisms in poultry farms and provide valuable insights for their potential applications in biomedical research. As currently available thrombolytic agents are very expensive, the present study reveals that the microbial fibrinolytic enzymes can be produced from cheaper sources. The Partially purified enzyme from the strain was found to exhibit clot lytic , Cardio protective and has wound healing activities.

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