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APPLICATION OF FIBRINOLYTIC ENZYME FROM *ASPERGILLUS TAMARII* – IN-VITRO STUDIES

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ABSTRACT
Fibrinolysis is the process in which fibrin clots are broken down to soluble products (Casarman-Maus and Hajjar, 2005). The main enzyme responsible for fibrinolysis is plasmin which cuts the fibrin mesh and converts it to soluble form which will be cleared from the body by kidney and liver. Fibrinolytic enzyme production were studied in the filamentous fungi *Aspergillus tamarii* SAS 02 a soil isolate from different regions of Karnataka. The enzyme tested for fibrinolytic activity for in-vitro studies by clot hydrolysis in capillary tube with standard enzyme which are available in market.

KEYWORDS: Fibrinolytic enzyme, Fibrin, blood clot, *in vitro study* and submerged fermentation.

INTRODUCTION
Blood clot if it gets developed in the circulatory system causes vascular blockage. Healthy hemostatic system minimizes the formation of blood clots in its normal blood circulation and also prevents blood loss by vascular injury. After the vascular damage the integrity of a closed, high pressure in the circulatory system can be maintained by the process called Hemostasis.

Blood clot formed in the circulatory system causes blockage in the vascular system which sometimes leads to the end of life. A healthful hemostatic system suppresses the blood clot formation. If the hemostatic system is failed it leads to a chain of CVD's, to overcome this problem pathologies made a clinical intervention of administrating a thrombolytic agents intravenously (Francis and Marder,1991).

Enzymes are biological molecules that perform a multitude of chemical reactions (Mabrouk et al., 1999). With an increasing industrial demand for enzymes produced by novel microbial strains, there is an increased demand for isolation of enzymes with novel properties that can be used as biocatalysts (Mehrotra et al., 1999). Microorganisms form an ideal source of fibrinolytic enzyme which are predominantly extracellular in nature.

Fibrinolysis process is carried by: primary fibrinolysis and secondary fibrinolysis. In primary fibrinolysis type plasmin is activated by plasminogen naturally without any medicine, whereas secondary fibrinolysis is by use of medicine. In fibrinolysis, the plasmin cuts the fibrin mesh. This is how the blood clot is removed at various places. Bacteria, earthworm and snake venom secretes the fibrinolytic enzymes (Sumi et al,1987) but till now only few evidences are there about the fungi which secretes the fibrinolytic enzyme (S.A.El-Aassar etal,1990; Sun Tao et al,1997). Present research work were covered majorly on application of fibrinolytic enzyme in vitro studies from *Aspergillus tamari*

MATERIALS AND METHODS
Fungal isolation and identification
The fungi *Aspergillus tamarii* SAS 02 (Plate-1) strain were isolated from different regions of soil samples. Were taken from different from regions from Karnataka, such as Tumkur, Bangalore and Bijapur and confirmed by molecular level identification

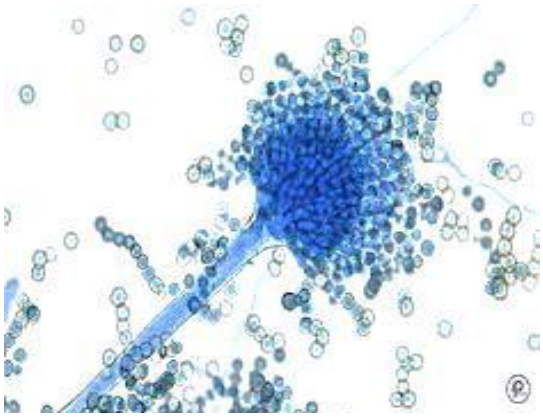


Plate 1: Electron microscopic image of *Aspergillus tamarii* SAS02 at 100 µm.

Screening of *Aspergillus tamarii* for fibrinolytic enzyme through plate assay

Thirty strains of *Aspergillus sp* were screened for their fibrinolytic enzyme production by plate assay. The screening medium is as follows, a mixture consisting of 2ml of fresh healthy human plasma and 3ml of 1.2% molten agarose (45^0C) in 10mMTris-Hcl buffer containing 70mM $(NH_4)2SO_4$, 90mM NaCl, 0.70mM $MgCl_2$ and 200µl of 0.2M $CaCl_2$ was poured into sterile 60mm petridish and allowed to stand for 2h at room temperature (25^0to27^0C). 10ml of sabouraud dextrose broth was incubated with the fungal strain and incubated at 30^0C for 2 days. This culture was used for fibrin clot assay. 20ml of fungal culture containing mycelia was placed at the centre of the gel matrix of the fibrin plate and incubated for 24h at 30^o C. The diameters of the clear zones (Plaque-Plate-1) were noted.

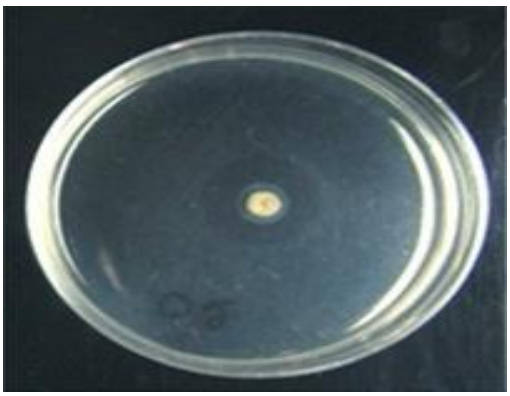


Plate 2: Rapid screening of fibrinolytic enzyme producers by plate assay.

Preparation of fermentation medium

The isolate was grown in Czapek-Dox media composition(g/l) sucrose-30.0, sodium nitrate-2.0, K_2HPO_4-1.0, $MgSO_4.7H_2O$-0.5, KCl-0.5, $FeSO_4$-0.01 for 96h on a shaker with constant 140rpm at room temperature.

Submerged fermentation methodology

Submerged fermentation was carried out in 250ml Erlenmeyer flasks. The flasks containing 100ml of the above said fermentation medium were autoclaved at 121^oC for 20 minutes and cooled to room temperature. Then the flasks were inoculated with 1ml of spore suspension and the contents were thoroughly mixed and incubated for a period of 3-5 days at 30^oC.

Extraction of fibrinolytic enzymes

The samples were withdrawn periodically at 24 hrs in aseptic condition. The extract was filtered through Whatman's filter No.1. The clear extract was centrifuged at 2000-3000 rpm for 15 min, supernatant were used as enzyme preparation. Thus prepared crude enzymes were used for assay of fibrinolytic enzyme.

Enzyme Assay

This was basically measured by the modified method of Anson (1939), but with a few modifications. The reaction mixture contained 1 ml of 1.2% of bovine fibrin solution in Tris-HCl buffer (pH 8.0) and 1 ml of cell-free supernatant (CFS). The reaction mixture was incubated for 2 h at 37°C. Then the reaction was stopped by addition of 2 ml of 10% (w/v) trichloroacetic acid. This was followed by centrifugation and assaying the solubilized proteins for tyrosine in the supernatant by measuring the absorbance at 750 nm (Mukesh Kumar, et al., 2013)**.**

Unit

One unit of fibrinolytic activity (U) was defined as the amount of enzyme required to liberate 1 µg of L-tyrosine/ ml/min at 37°C.

Application: Blood clot hydrolysis by fibrinolytic enzyme

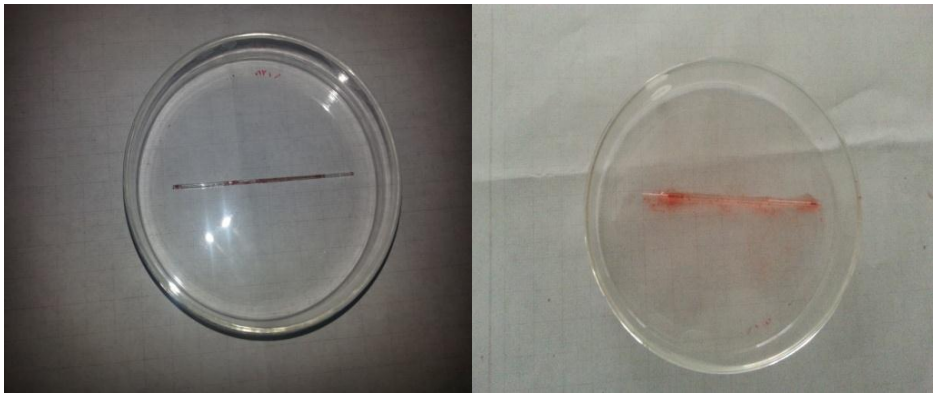
The blood clot was formed in capillary tube by spontaneous coagulation in glass capillary tube using human blood (fresh). The capillary tubes were kept in a different petridishes containing saline, partially purified *Aspergillus tamarii* SAS02 fibrinolytic enzyme (Approximately 200 IU/ml) and commercially available fibrinolytic enzyme (Brand name – Myokinase) incubated plated were incubated for 15 – 18 hrs of incubation during this time petriplates were shaken 3-4 times and the saline were used as control.

The results were observed that there is a clot hydrolysis by using partially purified enzyme and also in commercially used enzyme. Hence finally fibrinolytic enzyme showed clot hydrolysis.

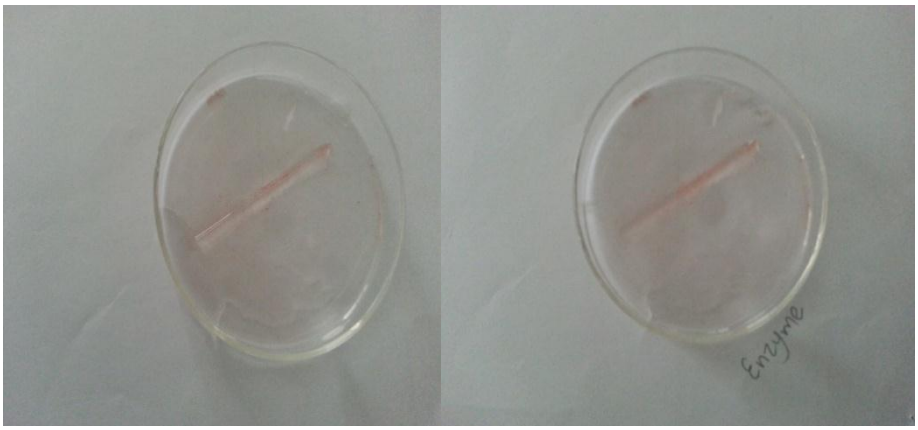
RESULTS AND DISCUSSION

Soil is the exceptionally rich sources for potential enzyme producing organisms especially for fungi. Therefore, in the present study soil has been chosen as a source and the soil samples were collected from various places of Karnataka. Totally, 30 strains of *Aspergillus sp* were isolated. After the isolation of *Aspergillus sp* strains, they were subjected to rapid screening for the production of fibrinolytic enzyme by rapid plate assay method. The *Aspergillus tamarii* SAS 02 were identified as potent strain for the fibrinolytic enzyme production. The produced enzymes were used for *in-vitro* studies of fibrinolytic activity on blood clot hydrolysis

In the present study blood clot hydrolysis was observed in both the plates, which contains capillary tubes filled with blood clots (Plate-3). One which contains purified enzyme extracted from *Aspergillus tamarii* SAS02 and in the other plate commercially available enzyme (Brand name – Myokinase) with the concentration of 200 IU/ml. Capillaries present in both the plates showed the action of blood clot degradation with the incubation period of 15-18 hrs, where the same quantity of degradation was observed in both the capillary tubes. Hence we say that our enzyme is a tool for obliteration of blood clots.



a. Capillary with Saline **b. Capillary with blood**



c **d**

Plate 3: Blood clot hydrolysis by fibrinolytic enzyme from c. *Aspergillus tamarii* SAS02 d. standard enzyme.

Jun Yang et al., (2012) showed the thrombolytic effect of DFE from *Bacillus subtilis* both in-vitro and in-vivo. Natural blood clots were done with the fresh animal blood samples, this clots was cut into same size and places on the petriplates, incubation was done at 37ºC for 24hrs with the urokinase of 5000U as a positive control followed with different concentrations of DFE on blood clots. Clot lysis was inspected on the petriplates for every 2.5hrs.

Manoj kumar (2015) showed that the fibrinolytic enzyme isolated from *B.cereus* at 180 minutes of incubation a residual 13% of the initial clot was obtained saying that 87% of the blood clot was removed due to lysis by the fibrinolytic enzyme. This proves that the enzyme secreted by *B.cereus* SRM-001 is a potent fibrinolytic enzyme. Our results were good agreement with Manoj kumar (2015).

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