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Research Article

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An approach on biosynthesis of fibrinolytic enzyme from *Aspergillus Sp.*

Shilpa H. K.[1], Jeevan G. Ambekar[1], Siddalingeshwara K. G[2] and Nilima N. Dongre[1]
[1]*Department of Biochemistry, BLDE University, Vijayapur*
[2]*Padmshree Institute of Information Sciences, Nagarabhavi Circle, Bengaluru*

ABSTRACT

Today, thrombosis is one of the most widely occurring diseases in modern life. Drugs with thrombolytic functions are the most effective methods in the treatment of thrombosis. This study investigates isolation of fibrinolytic enzyme producing Aspergillus sp and screening by fibrin plate. The production of extracellular fibrinolytic enzyme synthesis were carried out by using Aspergillus sp S2 were evaluated under different fermentation parameters by employing submerged fermentation method. The fibrinolytic enzyme producers were detected by the enzyme hydrolytic zone around the colony by fibrin plate assay method. Aspergillus sp S2 is the potential strain among the fungal isolates. The yield of fibrinolytic enzyme synthesis were increased after the optimization of fermentation parameters. The optimum pH 6.0, temperature 40^{0}C and inoculum size 1.25 ml and it showed 180 U/ml.

Key words: Fibrinolytic enzymes, fibrin, *Aspergillus* sp and Fibrin plate assay

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INTRODUCTION

Earlier days treatment of thromboembolic vascular disease was relied on the use of anticoagulants, such as warfarin (coumarin) and heparin to inhibit the formation of fibrin clots. However, recognition that lysis of preformed fibrin could be accomplished in vivo by a process involving the conversion of inactive plasminogen to active plasmin enzyme led to an alternative enzyme based approach.

Fibrin is the main protein component of the blood clot, and it is normally formed from fibrinogen by the action of thrombin (EC. 3. 4. 21. 5) after trauma or injury. Accumulation of fibrin in blood vessels usually increases thrombosis, leading to myocardial infarction and other cardiovascular diseases (CVDs). Variety of fibrinolytic enzymes such as tissue plasminogen activators (t-PA), urokinase (u-PA), and streptokinase were extensively studied and used as thrombolytic agents **[1].**

The microorganisms producing fibrinolytic enzymes include bacteria, actinomyces, fungi and algae. Microorganisms are important resources for thrombolytic agents. Streptokinase from *Streptococcus hemolyticus* and Staphylokinase from *Staphylococcus aureus* were earlier proved to be effective in thrombolytic therapy **[2].**

Some kinds of fungi have also been found to produce the protease with high fibrinolytic activity for example *Asperigillus ochraceus* 513 **[3]**, *Fusarium oxysporum***[4]**, *Penicillum chrysogenum***[5]**, *Rhizopus chinesis* 12 **[6].** In addition, Matsubara et al **[7],** found the fibrinolytic enzymes from marine algae *Codiumlatum*, *Codiumdivaricatum*, and *Codiumintricatum*. Lee et al **[8]** recently purified the fibrinolytic enzyme, designated as AMMP, from cultural mycelia of the mushroom *Armillariamella*.

In the present study, isolation of fungal strains was carried out from different soil samples collected from various places of Karnataka and also evaluated the fermentation kinetics for the production of fibrinolytic enzyme by *Aspergillus* sp.

EXPERIMENTAL SECTION

Collection and Isolation of Fungal strains

The *Aspergillus* strains were isolated from different soils samples. The soil samples were collected from different regions from Karnataka, such as Tumkur, Bangalore and Bijapur.Totally thirty strains of *Aspergillus* sp were isolated further confirmations were carried out at Agarkar research Institute (ARI), Pune.

Screening of fibrinolytic enzyme producing *Aspergillus* sp

A mixture consisting of 2 ml of fresh, healthy human plasma and 3 ml of 1.2% molten agarose (45^0C) in 10 mM Tris-HCl buffer containing 70 mM $(NH_4)_2 SO_4$, 90 mM NaCl, 0.70 mM $MgCl_2$ and 200 µl of 0.2 M $CaCl_2$ was poured into sterile 60 mm petridish and allowed to stand for 2 h at room temperature (25 to 27^0C). 10 ml of Sabouraud dextrose broth was inoculated with the given fungal strain and incubated at 30^0C for 2 days. This culture was used for fibrin clot assay. 20µl of fungal culture containing mycelia was placed at the center of the gel matrix of the fibrin plate and incubated for 24 h at 30^0C. The diameters of the clear zones (plaque) were noted.

Production medium for fibrinolytic enzyme

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 96 h on a shaker with constant 140 rpm, at room temperature.

Effect of pH

The above media was set at different pH like 3, 4, 5, 6, 7, 8 and 9 and inoculated with 72 h old culture and grown for 4-5 days at room temperature. The culture was filtered and filtrate was centrifuged at 10,000 rpm for 10 min and supernatant was assayed for the fibrinolytic enzyme activity.

Effect of temperature

The media (pH 6.0) in different flasks were inoculated with 72 h old culture and incubated for 4-5 days at different temperature like 30, 35, 40 and 50^0C. The cell free extract was assayed for the fibrinolytic enzyme activity.

Effect of inoculum volume

The 100 ml media with pH 6.0 in different flasks were inoculated with varying volume of 72 h old inoculum. The incolum volumes studied were 0.25, 0.5, 0.75, 1.0 and 1.25 ml. The culture was grown at 40^0C for 4-5 days and enzyme assay was done as mentioned before.

Enzyme assay

This was basically measured by the modified method of Anson **[9]**, 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 the 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 **[10].**

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.

RESULTS AND DISCUSSION

Fungal isolates were identified as *Aspergillus sp* in Agrakar Research Institute, Pune. All thirty strains of *Aspergillus sp* produced clear zone around colony in fibrin plate medium; those were selected from the soil sample. Of the thirty isolates *Aspergillus sp* S2 was considered to be the best and high fibrinolytic enzyme producing strain. It showed 1.9 cm of cleared zone around the colony (Plate-1). The data obtained in the present study on the effect of pH and temperature on submerged fermentation is shown in (Fig.1 and 2) which reveals that the production of fibrinolytic enzyme increased with the increase in the pH of the medium up to pH 6.0 temperatures 40^0C and thereafter the decrease of fibrinolytic activity was observed.

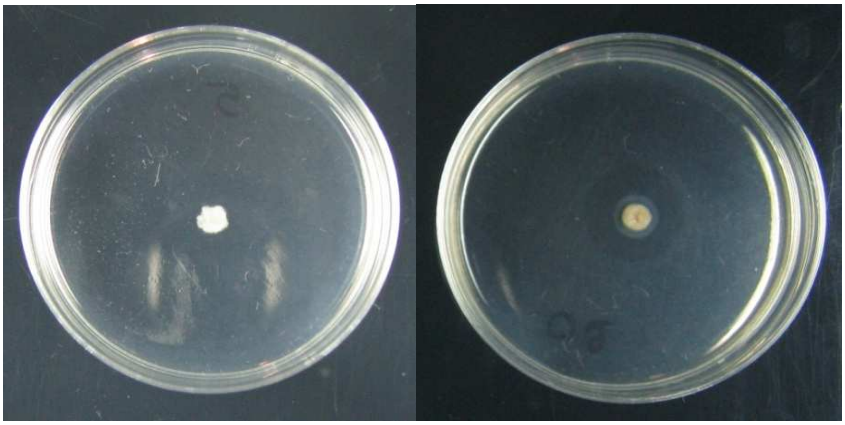

Plate-1: a. Control *Aspergillussp* **b. Fibrinolytic activity by *Aspergillussp***

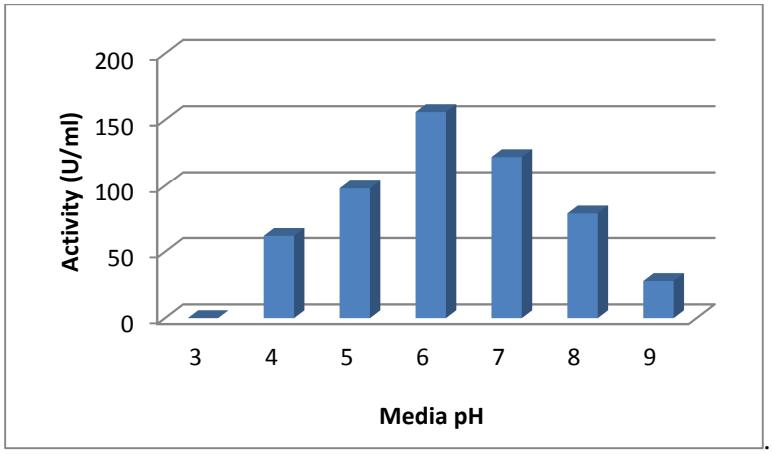

Fig-1. Effect of pH on fibrinolytic enzyme production

The maximum production of fibrinolytic enzyme is 160 U/ml was obtained at pH 6.0 (Figure 1) and the minimum production of fibrinolytic activity 20 U/ml was observed at pH 3.0. The production of fibrinolytic enzyme increased significantly with the increase in fermentation temperature from 25-40^{0}C and decreased above 40^{0}C. The maximum fibrinolytic enzyme production obtained at 40^{0}C was 180U/ml and the least production was observed at 30^{0}C resulted in only 120 U/ml of fibrinolytic enzyme at 72 hrs of fermentation period. Any temperature beyond the optimum range is found to have some adverse effect on the metabolic activities of the microorganisms and it is also reported by various scientists that the metabolic activities of the microbes become slow at lower or higher temperature **[11].**

The pH of the medium is one of the most critical environmental parameter affecting the mycelial growth, enzyme production and the transport of various components across the cell membrane **[12].**

In our study, the data revealed that the pH of 6.0 was found as suitable for maximum production of fibrinolytic enzyme with *Aspergillus* sp S2 strain under submerged fermentation. Fungal strains are noted for their best performance in the range of 3.5-7.0 and also low pH avoids the contamination by other microbes **[13].**Usama et al. **[14]** reported that pH 8 is the optimum for maximum fibrinolytic enzyme production and it showed 2.29 unit/mg protein. On the other hand Yong Peng *et al*. **[15]** found that the optimal pH 9.0 of the fibrinolytic enzyme of *Bacillus amyloliquefaciens*. Our findings are in close agreement with the earlier findings of Yong Peng *et al*., **[15]** showed that pH 9 was the suitable for maximum fibinolytic enzyme production.

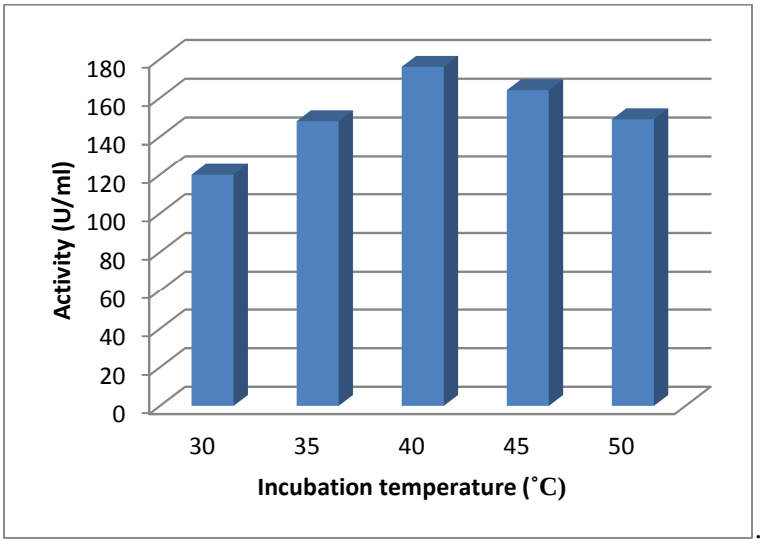

Fig-2. Effect of temperature on fibrinolytic enzyme production

Usama et al. **[14]** showed that incubation temperature 35^0C is optimum for maximum production of fibrinolytic enzyme and it showed 2.30 unit/mg protein. Keeping this in view, experiments were conducted to find out the effect of temperature on fibrinolytic enzyme production by *Aspergillus sp* S2. The present study revealed that the 40^0C (Figure 2) is suitable for maximum production of fibrinolytic enzyme with *Aspergillus sp* S2. Yong Peng *et al*., **[15]** reported that the maximum production of fibrinolytic enzyme was observed at temperature 48^0C by using *Bacillus amyloliquefaciens*. Similar observations were reported for fibrinolytic enzyme production by Usama et al. **[14]**.

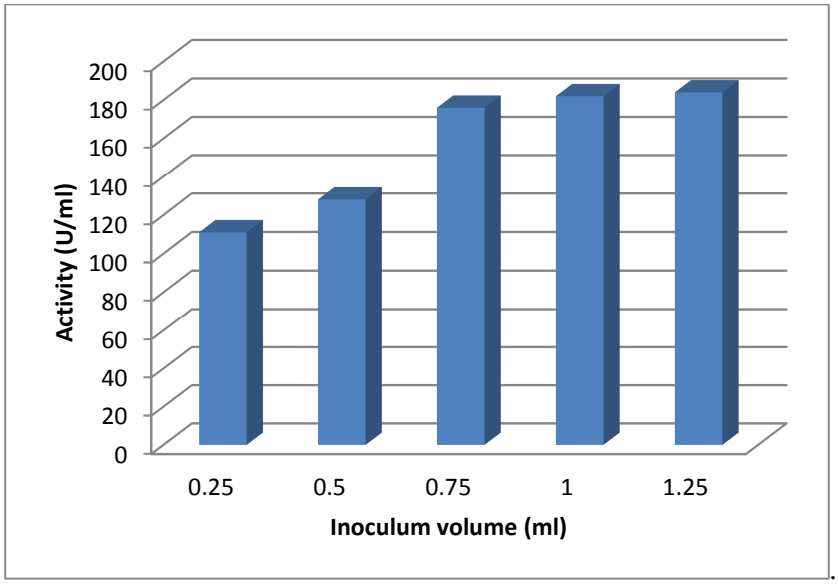

Fig-3. Effect of inoculums size on fibrinolytic enzyme production

It is generally necessary to optimize age and size of the inoculums, because low density gives insufficient biomass and high density produces too much biomass and resulting in depletion of nutrients necessary for fibrinolytic protease production. The earlier studies reported that inoculums size has crucial effect in fermentation process through microorganisms **[16]**. Importance of inoculum size on microbial fermentation process is widely accepted. Out of five inoculum size tested 0.25, 0.50, 0.75, 1.0 and 1.25 ml; the 1.25 ml inoculum size was found to be the most suitable for high production of fibrinolytic enzyme by *Aspergillus sp* S2 in submerged fermentation at 72 hrs of fermentation and it showed the yield of 180 U/ml. From Figure 3, it is clear that the fibrinolytic enzyme

production steadily increased with the increasing in the size of the inoculum until it reaches to the magnitude. When enzyme productivity became maximum, thereafter no appreciable change in production of fibrinolytic enzyme with high inoculum size could be observed. The maximum enzyme activity was showed at 180 U/ml at 1.25 ml inoculum size and least enzyme activity 100 U/ml was showed at 0.25 ml of inoculum size.

Venkatanagaraju and Divakar **[16]** reported that 2 % of mutant *Bacillus cereus* spores as an inoculums from one week old culture were inoculated for the maximum production of fibrinolytic enzyme and our results are consistant with Venkatanagaraju and Divakar**[16]**.

From this study we conclude that the isolated Fibrinolytic enzyme from the *Aspergillus* sp shows its maximum activity at optimum pH 6.0, temperature of 40^0C and inoculum size of 1.25ml gave the maximum yield.

CONCLUSION

Aspergillus Sp were isolated from different soil samples from different regions from Karnataka and screened for the production of fibrinolytic enzyme by plate assay. Optimization studies were also carried out for the maximum production of fibrinolytic enzyme.

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