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Isolation, production and purification of new thrombolytic enzyme from C*ladosporium Spp.*

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Abstract
The organism isolated from an infected green leaf sample was selected based on the extracellular protease production using casein plate assay. The organism was identified as *Cladosporium species.* optimization of growth parameters using submerged fermentation, shake flask studies showed maximum enzyme activity at an inoculums size of 10^6 spores/ml, pH 9.0 (enzyme activity-228 U/ml/min), incubation temperature 28ºC, incubation time 72 hours (enzyme activity-420 U/ml/min). The thrombolytic enzyme was partially purified up to 1.8 fold by 60% $(NH4)_2SO_4$ fractionation then subjected to simple dialysis followed by ion exchange chromatography. Sodium dodecyl sulphate - polyacrylamide gel electrophoresis (SDS-PAGE) shows the partially purified thrombolytic enzyme at the molecular weight of ≈35kDa. The thrombolytic enzyme showed maximum activity at pH10 at 50ºC. This work reveals the potential of *Cladosporium species* as an unconventional and unexplored production alternative to already known thrombolytic agents.

Keywords: *Cladosporium spp*. fibrinolytic enzyme, submerged fermentation, dialysis, ion exchange chromatography

1. Introduction
Fibrin is the primary protein components of blood clots, which is formed from fibrinogen by thrombin (E.C.3.4.21.5) and are lysed by plasmin (EC3.4.21.7). The clot (thrombus) in blood vessels due to fibrin accumulation may lead to myocardial infarction or other cardiovascular diseases (Peng *et al*. 2005) [1]. Although, fibrin clot formation and lysis of clot is equilibrium in biological system, impairment of this phenomena leads to myocardial infarction, where in clots are not lysed properly (Sherry 1987) [2]. Fibrinolytic enzymes are the biological components which help to suppress the development of blood clots. These are extracted from microbial sources. 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) [3]. Hitherto, various types of therapeutically important fibrinolytic enzymes (clot-buster drugs) such as tissue pasminogen activators (tPA), Urokinase (UK) and Streptokinase (SK) and their derivatives have revolutionized the treatment of myocardial infarction in recent years (Nakajima et.al, 1993). Currently available thrombolytic agents are very expensive and possess significant shortcomings, including large therapeutic dose, short plasma half-life, limited, fibrin specificity and bleeding (Sherry 1987) [2]. Of late, researchers discovered several fibrinolytic enzymes from various sources such as bacteria, fungi, mushrooms, earthworms and snake venom etc, (Kim *et al*., 1996) [5]. Recently, Rovat *et al*. reported novel source of fibrinolytic enzyme from *Cladosporium cladosporioides* isolated from rain forest and the enzyme was able to degrade fibrin clots (Rovat *et al*. 2010) [10].
The present work aimed to produce and detect thrombolytic activity from *Cladosporium species* isolated from infected leaf sample. Fungal thrombolytic enzymes, mimicking the ability of enzymes from bacterial origin (streptokinase and staphylokinase) may have potential application in the pharmaceutical industry as thrombolytic agents.

2. Materials and Methods
Isolation of thrombolytic enzyme producing fungi
A fungal strain *Cladosporium species* used in the present study was isolated from Tectona grandis (Teak wood) infected leaf sample. It was grown on Potato Dextrose Agar (PDA) slants for at 28°**C** ± 2 for four days and maintained at 4^0C. Screening was performed by plate assay method by adding 0.5% casein to the Potato Dextrose Agar (PDA) media (Astrup& Mullertz, 1952) [7].

Preparation of pre – inoculums
Spore suspensions were prepared from 5 days old cultures grown on PDA slants by adding 10 ml of sterile distilled water containing 0.1% Tween-20 and suspending the spores with sterile loop. The spore suspension containing about 1 X 10^6 spores/ml was used to inoculate experimental substrates in the flasks.

Fermentation condition
100 ml of synthetic media containing soybean powder 5%, maltose 5%, polypeptone 0.5%, yeast extract 0.2%, KH_2PO_4 0.1%, $MgSo_4$ 0.1% was taken separately into 250 ml one Erlenmeyer flasks and adjusted to pH 7. The cotton plugged

flasks were then autoclaved at 121°Cfor 20 minutes and allowed to cool to about 30°C. The contents of the flasks were inoculated with 5 ml of spore suspension containing 10^6 spores /ml. Then the flasks were incubated in an orbital shaking incubator at 29°C for three days. Thrombolytic enzyme activities in the filtrate were determined by using Anson's method.

Extraction

After fermentation, the fermented media in the flask was extracted by the addition of around 50-60 ml of distilled water containing 0.1% Tween-20. The entire content was mixed thoroughly (110 rpm at 28°C for 1 hour) in orbital shaking incubator. The content was then filtered using Whatman Filter paper No. 1.Centrifugation at 8000rpm and 4ºC for 15 minutes was carried out. The enzyme from the supernatant was extracted by 60% $(NH_4)_2SO_4$ fractionation. The enzyme preparation was then subjected to dialysis using Dialysis membrane-70, LA393 by using method of G Ravikumar et.al.

Purification

The crude enzyme sample was then purified by ion exchange chromatography using DEAE Sephadex for the effective removal of impurities. The columns were packed with ion exchanger and these packed columns were then washed with distilled H_2O three times. Again they are washed with phosphate buffer of pH 7.Then enzyme sample was loaded and incubated for 30 minutes at room temperature for better binding. These loaded columns were subjected to centrifugation at 6000rpm and room temperature for 5 minutes. (C-4 Centrifuge REMI).Then supernatant was thrown and enzyme was eluted with 0.1M NaCl solution. The purity of the enzyme was checked by electrophoresis using 12% polyacrylamide gel. (Liu Xiao-lan, *et al*. 2005)

SDS Page

Using 12% separating gel (composed of stock acrylamide: 1.665ml, TrisHCl pH 8.8: 1ml, water: 2.26 ml, ammonium persulphate(5%): 50µl, TEMED: 50µl, SDS(10%): 100µl) was loaded to $3/4^{th}$ of the polyacrylamide gel electrophoresis setup and kept for 5min for gel formation. Then 6% stacking gel (composed of stock acrylamide: 0.83ml, TrisHCl pH 6.8: 0.5ml, water: 3.9ml, ammonium persulphate (5%): 50µl, TEMED: 50µl, SDS(10%): 100µl) was loaded to fill the setup. As soon as the stacking gel was loaded, the comb was applied for well formation. The comb was carefully removed after the gel formation and 10µl of the enzyme sample was loaded along with 10µl of loading buffer of 3X concentration (composed of TrisHCl pH 6.8: 5ml, SDS: 0.5g, sucrose: 5gmercaptoethanol: 0.25ml, bromophenol blue: 1ml, water: 10ml). Then electrode buffer (composed of 0.05M Tris: $6gl^{-1}$, 0.192 M glycine: $14.4gl^{-1}$, SDS: $1gl^{-1}$, water: 1L and pH: 8.2-8.4) was poured to the setup. Gel was run at 15mA current for 15 min and then at 20mA till the end. The samples were stained with Comassie Brilliant Blue R-250. The molecular weight was calculated according to the molecular weight standard (Sigma).

Fibrin Plate Assay

Quantitative analysis of fibrinolytic activity was determined by both the plasminogen-free fibrin plate method and the plasminogen-rich fibrin plate method (Astrup& Mullertz, 1952) [7]. Plasminogen –free fibrin plate was made up of the fibrinogen solution [2.5ml of 1.2% human fibrinogen (Sigma, USA) in 0.1M sodium phosphate buffer, pH 7.4], 10U of thrombin solution (Sigma, USA) and 1% agarose. Plasminogen-rich fibrin plate was made up 2ml of 1.5% fibrinogen and 5U plasminogen. The sterilized paper disc (5mm in diameter) was overlayed on the fibrin plate. To observe the fibrinolytic activity of the enzyme, enzymes taken in the range of 5-25µl was carefully dropped on the disc and incubated at 37ºC for 18hr. the activity of the fibrinolytic enzyme was determined by measuring the dimensions of the clear zone on the fibrin plate and plotting a standard curve made by varying the quantity of plasmin.

3. Results and Discussion

Initially 25 colonies were isolated on Potato Dextrose Agar. Two colonies were selected based on the screening and from the two colonies the one with maximum diameter of the zone of hydrolysis was selected for further studies. The source of the fungi was an infected green leaf sample from the green house unit in the campus. The fungal strain was identified as *Cladosporium species* by lactophenol cotton blue staining technique.

Fig 1: Zone of hydrolysis

Effect of enzyme activity on different substrates

Different substrates were used for the production of fibrinolytic enzyme such as coconut cake, peanut cake and soyabeen. The best substrate for obtaining maximum enzyme activity was found to be soyabean.

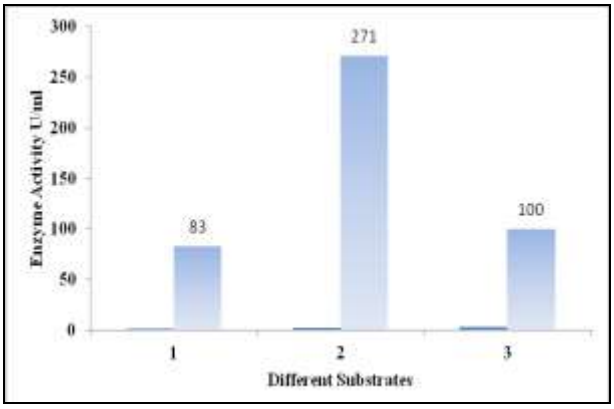


Fig 2: Effect of enzyme activity on different substrates

Effect of enzyme activity in different Substrate concentration

After soyabean was chosen as the desired substrate different concentration of the substrates were checked in order to get the maximum activity and it was found that 5% of soyabean gave the maximum enzyme activity as shown in the graph. Soyabean has a high protein content and is also easily available hence it also makes up a very cost effective substrate.

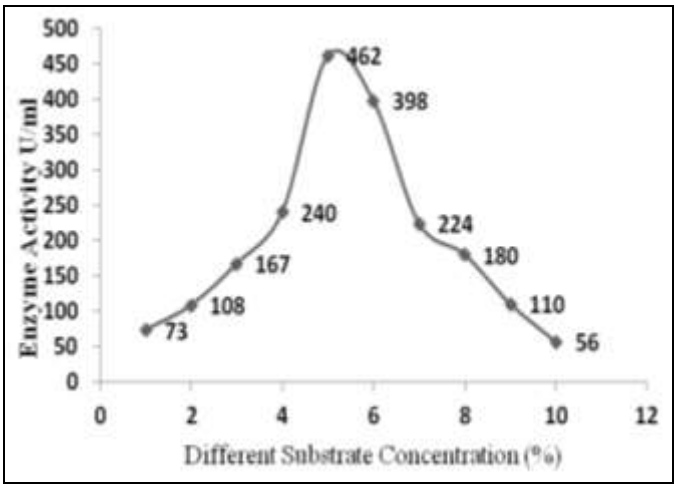


Fig 3: Effect of enzyme activity in different Substrate concentration

Effect of enzyme activity in different pH

pH is an important factor which effects the growth and enzyme production during submerged fermentation. The obtained results demonstrated that thrombolytic enzyme production was good at a wide range of pH 5-11, except for pH 9 which showed enzyme activity (228 mg/ml/min).

The pH of the fermentation media may change since organic acids are produced during fermentation. However, only 0.1 decrease or increase in the pH medium may be observed.

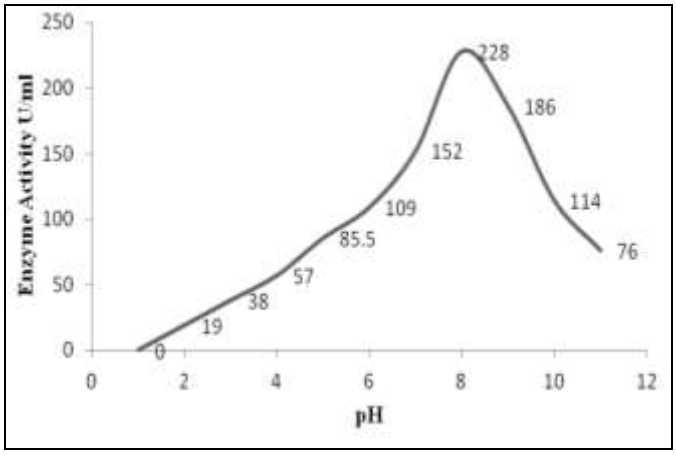


Fig 4: Effect of enzyme activity in different pH

Effect of enzyme activity in different Temperature

The optimum temperature for maximum thrombolytic enzyme production (1520 mg/ml/min) was found to be at 29ºC and a subsequent drastic decrease in growth and enzyme production was observed.

Table 1: Effect of enzyme activity in different Temperature

Temperature	Growth	Enzyme Activity (Mg/ml/min)
15 ºC	No growth	0
29 ºC	Growth	1520
35 ºC	Partial growth	760
45 ºC	No growth	0

Effect of enzyme activity in different Carbon Sources

Carbon sources are very important for microbial growth as they provide carbon skeletons needed for synthesis of new organic molecules (anabolism). Of the carbon sources tested maltose showed the maximum enzyme activity.

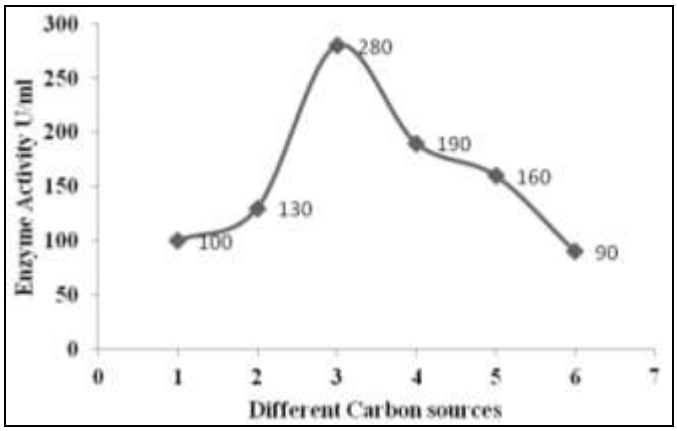


Fig 5: Effect of enzyme activity in different Carbon Sources

Effect of enzyme activity in different concentrations of maltose

Maltose that was chosen to give the highest enzyme activity was further studied as to which concentration of it would increase the enzyme activity. From the results obtained it was seen that 5% of maltose gave the maximum activity.

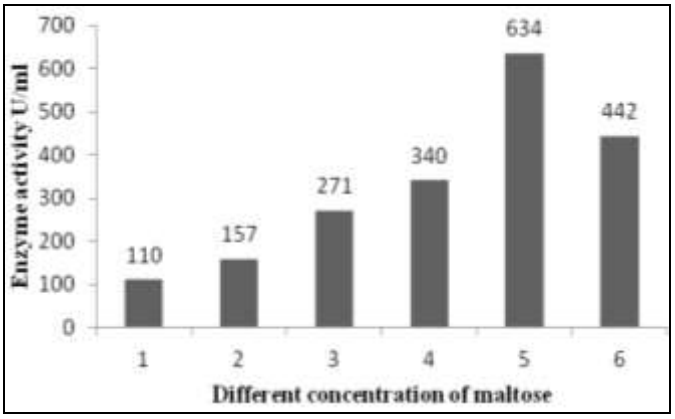


Fig 6: Effect of enzyme activity in different concentrations of maltose

Purification of thrombolytic enzyme

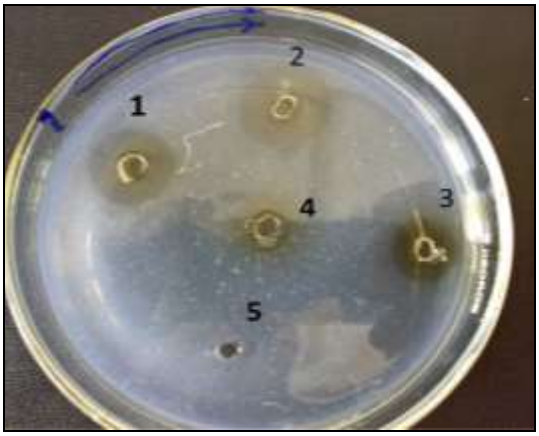
The extraction and purification of the thrombolytic enzyme from the optimized fermentation conditions resulted in gradual increase in specific activity at each stage of purification. The thrombolytic enzyme was purified upto 5 fold as compared to crude enzyme. In the final purification step, ion exchange chromatography gave a specific activity as shown in the table.

Table 2: Purification and recovery of fibrinolytic enzyme from *Cladosporium Species*

Steps	Protein (mg)	Activity (U)	Sp. Activity (U/mg)	Yield (%)	fold Purification
Centrifugation	0.195	1210	6205	100	1
$(NH_4)_2SO_4$	0.104	785	7548	64.87	1.21
Dialysis	0.055	510	9273	42.14	1.46
DEAE-cellulose	0.0135	448	33185	37.02	5.35

Fibrin plate assay

It was performed for the confirmation of fibrinolytic activity and the results are as shown in the diagram. This confirmed that the *Cladosporium* spp. is a source of fibrinolytic enzyme.



1.Fibrinolytic enzyme from *Bacillus Subtillis*
2.Fibrinolytic enzyme from *Cladosporium*
3.Plasmin
4.Fibrinolytic enzyme from fermented food
5.Control

Fig 7: Fibrin Plate Assay of purified enzyme

Molecular weight Determination

The molecular weight of the purified thrombolytic enzyme was found out by SDS-PAGE. The purified fraction of protein showed 3 distinguished bands in SDS-PAGE. Therefore further investigations were done by carrying out Native PAGE, to infer whether the protein is multimeric or monomeric. The partially purified thrombolytic enzyme molecular weight was found to be ≈35kDa.

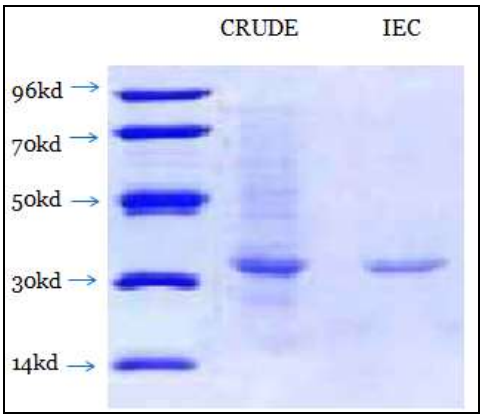


Fig 8: SDS – PAGE analysis of fibrinolytic enzyme from Cladosporium Species

4. Conclusion

Fibrin is a protein which forms clots in the body that can be broken down by fibrinolytic enzymes. The fungal isolate identified as a *Cladosporium* species was found to be capable of producing a potential thrombolytic enzyme which has a number of advantages over the currently used drugs. A suitable protein rich substrate was explored and the media standardization was done by submerged fermentation using shake flask studies to optimize the culture conditions. As seen from the tabulated results, the optimization of nutritional and environmental conditions significantly improved thrombolytic enzyme production. Recovery and purification of the protein was carried out by various downstream processing techniques. Characterization of the enzyme was done to determine the effect of various process parameters on the activity of the enzyme. Therefore, by carrying forward this work, a potential drug molecule for the treatment of cardiovascular diseases can be achieved.

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