Production, Purification and Characterization of Thrombolytic Enzyme From Cladosporium Spp.Through Solid State Fermentation

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ABSTRACT-: **Solid state fermentation (SSF) isa process whereby an insoluble substrate is fermented with sufficient moisture but without free water. It has gained renewed interest owing to its importance in recent development in biomass conservation, in solid waste treatment, upgrading the usage of agro-based waste and its application to produce industrially important primary and secondary metabolites. Different types of fungi were isolated from local soil and leaf samples and were screened for thrombolytic enzyme production. *Cladosporium spp.* was selected for the production of enzyme on the agro-based waste, such as, silk worm pupa, egg shell and banana peel etc. The production of thrombolytic enzyme was carried by employing optimized conditions, supplement of mineral salt solution of pH 2.0, moisture content of 60%, temperature of 28±2^{o} C, with spore concentration of 1X10^{6}/ml and incubation time120 hr. The enzyme was purified and determined its molecular weight 35 kd. Enzyme was further purified by ion exchange chromatography to obtain specific activity of 15090U/mg of protein. Further enzyme is inhibited 100% by PMSF, a serine protease inhibitor. Hence enzyme is belongs to serine protease.**

Key words: Cladosporium Spp., thrombolytic enzyme, Silkworm pupa, egg shell, banana peel, SSF, etc.

1. INTRODUCTION

Proteases are class of enzymes which are very important as their application is found in physiological, commercial, pathophysiological and biological fields. About 60% of the proteolytic enzyme finds its application in industrial market world. There are number of biotechnological applications such as: pharmaceutical industry, food processing, leather industryetc [1]. These have been used since ages in different therapies. According to several chemical studies it has indicated benefits in inflammatory condition, oncology etc.[2]

The use of proteases plays a very important role in case of fibrinolytic enzymes which are used as "Life Savior enzymes" in thrombolytic therapy [3]. Fibrin is the primary component found in blood clot formed from fibrinogen by thrombin. In normal body conditions proper functioning of thrombin and plasmin helps in the proper degradation of fibrin. Once this mechanism fails then it results in accumulation of blood clots in blood vessels leading to several cardiovascular diseases such as: Myocardial infarction, Atrial fibrillation, Stroke etc [4]. The blood clot fibrin is lysed using fibrin using plasminogen which is activated using tissue plasminogen activator (tPA) [5].

The thrombolytic enzyme which are typically used for therapeutic purposes include: Urokinase, Streptokinase [6,7] and tPA(tissue plasminogen activator) [8]. These are plasmin which help in the conversion of plasminogen to plasmin and helps in the lysis of fibrin clots and these are of human origin and are safe for use, but they are very expensive.

Several scientists have done production of fibrinolytic enzymes using bacteria [9,10,11,12], fungi [13], earth worm [17,18,19] and fermented food products[14,15,16] for therapeutic purposes.

According to journal papers the authors have reported the production of proteolytic enzyme using Solid State Fermentation method(SSF) using several agro based industrial wastes such as : rice bran, wheat bran, sugarcane bagasse etc [20,21,22,23,24,25].

For the present research Solid State Fermentation(SSF) [26,27,21,22] have been used using fungal strains as it has resulted in increased production when compared to Submerged Fermentation. Economically this type of fermentation process has many advantages such as : Simpler downstream processing, low energy requirements, superior volume productivity etc [28].

The banana peel, egg shell and pupa these are generally considered as waste products which have been used as substrate in our research as it contains many nutritional values like : Protein, Carbohydrate, vitamin and several other mineral components which is valuable source of raw material in fermentation process. Presently these products are of no use and considered as waste which causes environmental pollution.

Hence several attempts were made to use this as enzyme for production of fibrinolytic enzyme using Solid State Fermentation (SSF) with the help of cladosporium sp isolated from soil.

2. MATERIALS AND METHOD :

2.1 *Microbial strains:*
The organism used in the present study *Cladosporium sp* was isolated from soil and grown on Potato Dextrose Agar(PDA) medium. The cultures were continuously maintained on PDA slants. The cultures were everytime cultured freshly on PDA slants and incubated at 28°C for 7 days before performing each experiment.

2.2 *Raw Materials:*
The banana peel and egg shell were collected from college canteen and were pre-treated and dried and powdered using warring blender, pupa waste were collected from silk factory and dried. The biochemical studies were studied by performing several chemical tests.

2.3 *Preparation Of Inoculum :*
Spore suspension cultures were prepared using 7 days old cultures which were grown on PDA slants by adding 90ml of sterile distilled water 0.1% Tween-20 and the spores were suspended using sterile loop. The spore suspension culture containing about $1X10^6$spores/ml was used for inoculating in the experimental media in flasks for all SSF studies.

2.4 *Fermentation Condition :*
5g of banana peel powder, egg shell powder and pupa waste powder was taken in 500ml Erlenmeyer flask and required amount of salt solution was added for adjusting the moisture. The media was sterilized at 15lb/$in^2$for 30 minutes. The sterilized substrates were cooled and 2ml of the suspension culture was inoculated and the contents were thoroughly mixed and kept in slant position in an incubator at 30°c with 65-70% humidity for 1 week and periodically assayed for enzyme activity.

2.5 *Effect Of Incubation Period On Enzyme:*
5g of substrates were impregnated into mineral salt solution in 500ml Erlenmeyer flask, which were autoclaved and suspension cultures were inoculated and same step as described above were followed and incubated. The contents of the flasks were harvested and assayed at every 24hr interval in triplicates.

2.6 *Effect Of Temperature:*
The influence of temperature on the production of enzyme using cladosporium species were studied by incubating at different temperatures ranging from 20°c-50°c.

2.7 *Effect Of Moisture Content:*
5g of ground substrate was taken and dried at 60°c for 6hrs. Cooled and weighed till a constant weight was obtained and the moisture level was adjusted to 40%-80% by the addition of required amount of salt solution and were sterilized at 15lb/in^2 for 15 min.

2.8 *Effect of pH:*
Substrate was mixed with mineral salt solution whose pH was adjusted, mineral salt solution was used as moistening agent which is used to adjust the pH. The pH was adjusted in the range of 2-7.5 with an increment of 0.5. The flasks which were prepared were sterilized, inoculated and incubated as per the protocol described before.

2.9 *Effect Of Particle Size:*
The ground substrate was sieved using different diameter of sieves ranging from 2, 4, 6, 8, 10, 12 and 14mm. The substrates of different sizes were used separately and were studied.

2.10 *Effect Of Inoculum Size:*
The inoculum was prepared as described in the earlier protocol and inoculated separately into the substrates at different levels i.e. from 1 X 10^5 to 1 X 10^{12} spores / ml. The flasks were incubated at 120h and the enzymes were extracted and assayed from each set.

2.11 *Extraction Of Enzyme:*
At the end of fermentation period, the entire quantity of substrates were homogenized with 5 volumes of 0.1%Nacl solution and mixed using orbital shaker at 200rpm for 1 hr at 25°c. Then the enzymes were filtered using double fold cheese cloth, the extraction was centrifuged at 15000rpm for 30min and the clear supernatant was collected and enzymes were assayed.

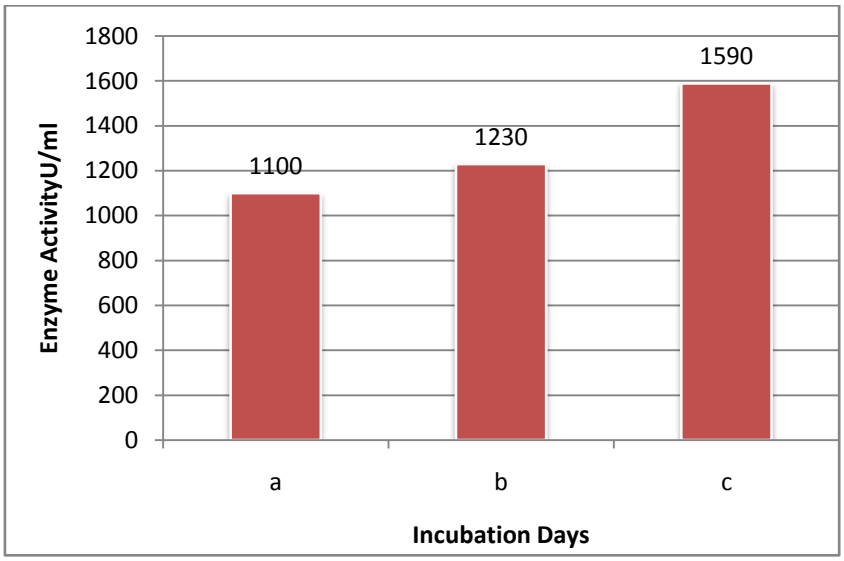
2.12 *Enzyme Assay:*
Quantitative analysis of fibrinolytic enzyme was assayed using both plasminogen rich and plasminogen free fibrin plate fibrin plate method. Plasmin (Sigma USA) 100U/ml was used as standard.

3 RESULTS AND DISCUSSION:

3.1 *Effect of Incubation Period on Enzyme Activity:*

The production of enzyme in SSF condition is very sensitive; and few selected parameters were optimized to achieve higher yield of enzyme. Once a fermentation parameter was optimized, the optimum level of that parameter is employed in the subsequent studies wherein another parameter is to be optimized. The production of fibrinolytic enzyme by *Cladosporium sp* was tested in solid state fermentation using egg shell, banana peel and pupa waste shows the fibrinolytic activity at different time of incubation. The highest activity 15090U/mg of proteinwas obtained at 120 h of incubation. The suitability of egg shell, banana peel and pupa waste may be due to insolubility in water, sufficient nutrients etc and able to loose even water is absorbed on to the substrate particle in moist conditions thereby providing good aeration and large surface area which can be utilized by microbes for their growth and metabolic activity. A low level of fibrinolytic enzyme activity was detected in the early stages of incubation; there was a steep increase in enzyme activity from 48 h on, with maximum being reached at 120 h of growth. Beyond 120 h a sharp

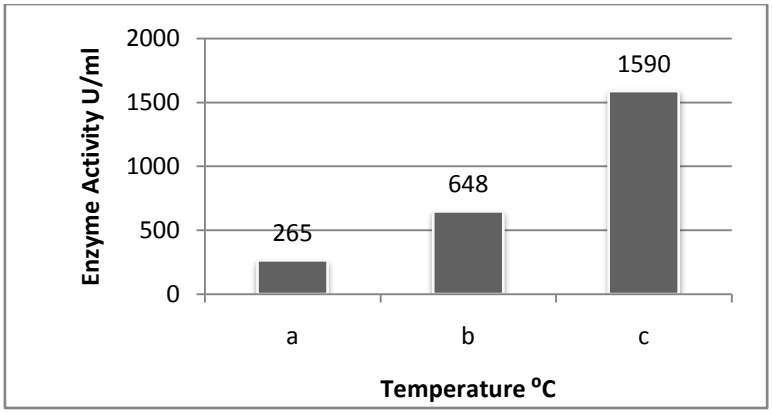
decrease in enzyme production was observed which could be due to loss of moisture after prolonged incubation upto 192 h.



Effect of incubation period on enzyme activity

3.2*Effect of Temperature:*

The effect of temperature on the production of enzyme was summarized.The maximum enzyme activity increased with increase in temp of fermentation up to 30^0C, thereafter gradual decrease. The maximum activity (1059U/ml) was obtained at 30^0C for 120 h of incubation. The lowest activity (265U/ml) was obtained at 50^0C for 120 h. An attempt to shorten the growth period was made at higher temperature fallowed by secondary period at 30^0C. The spore germination and formation of mycelium was accelerated by this method, no appreciable effect on enzyme secretion was apparent. The incubation temperature undoubtedly depends on mold strain and medium used. The lower yield of enzyme on egg shell, banana peel and pupa waste at lower and higher temperature than the optimal may be due to delayed microbial activity, dehydration of the medium and undesirable metabolic deviation.



Effect Of temperature on enzyme activity

3.3 *Effect of Moisture:*

The effect of different moisture levels on the yield of fibrinolytic enzyme under SSF is depicted in .The present study reveals that the *Cladosporium sp* shows maximum enzyme activity (1059U/ml) at 60% of moisture content of the substrate at 120 h of incubation. It is known that the water content of the medium has a profound influence on the growth and product formation in SSF .The moisture content beyond the optimum level inhibits the enzyme production because the higher moisture level of the substrate leads to decreased porosity , leads to lower oxygen transfer and decrease the diffusion. The lower moisture level than the optimum leads to the poor solubility of the nutrient of the solid substrate, improper swelling and higher water retention.

3.4 *Effect of pH:*

Initial pH level of the substrate is one of the crucial factor for successful of enzyme production in SSF. The effect of pH was studied in the range of 2.0 to 7.5. Maximum enzyme activity (1059U/ml) was obtained at pH 5.5. The initial pH of the substrate mainly depends on the carbon source used during the fermentation. When glucose and sucrose or other relatively pure materials are used for fermentation, then the substrate pH needs to be adjusted to 2.0 - 3.0. If crude substrates are used, a high initial pH is required because the presence of non-ion nutrient prevents germination. The lower yield of enzyme at lower and higher pH than optimum could be inhibition of condition. Fibrinolytic activity and condition could not be correlated although high enzyme activity always accompanied by condition.

3.5 *Effect of Particle size:*

The particle size of the substrate is greatly influences on enzyme production under SSF conditions. The maximum activity (1059U/ml) was obtained at 8 mm particle size. Several workers have been reported influence of particle size on production of proteases under solid state fermentation. Lower yield of enzyme in smaller particle size due to closer packing densities and reduction in the void space leading to reduced heat transfer. Where as in case of beyond the optimum size nutrient transfer is limited. Hence optimum particle size is needed to be study at which maximum productivity can be achieved.

3.6 *Effect of Inoculum:*

The fibrinolytic activity was maximum (1302) at $1X10^6$spores /ml. Further increase in the inoculum size did not elicit any significant increase in the yield of enzyme. The low density of spore leads to insufficient biomass and end products, where as too high densities of spores may cause a quick and much biomass production there by leading to fast nutrient depletion and ultimately reduction in the end product quantity.

CONCLUSION:

Although many reports on production of fibrinolytic enzyme have been published, very little information on solid state fermentation with agro based wastes. In this work it was demonstrated that pupa waste is a novel substrate because of cheap and easily available, on the other hand it does not require any external addition of nutrients like proteins, minerals etc, because it contain inherently sufficient to boost up enzyme production through solid state fermentation. Further, the novelty of fibrinolytic enzyme secreted by *Cladosporium* spit lyses the fibrin with and without plasminogen. This result reflected that the fibrinolytic enzyme was able to degrade fibrin clots in two ways: one forming plasmin from plasminogen (i.e. plasminogen activating type) and other one is without plasmin (direct fibrinolysis), for instance purification and characterization of enzyme currently under investigation.

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