Production, Purification and Characterization of Fibrinolytic Protease from *Fusarium spp.* CSN-6 Through Solid State Fermentation

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Abstract:- Solid state fermentation studies were conducted to find out the suitability of coconut cake for fibrinolytic enzyme production by a new *Fusarium spp.* CSN-6 isolated from soil. Further, the influences of various factors on maximum enzyme production are also reported. The study revealed that the incubation period of 120 h, growth temperature of 30°C, moisture of 60%, pH of 5.5, inoculum of 1×10^8 spores/ml and particle size of 8 mm were found as optimum conditions for maximum production (1302U/ml) of enzyme. The study also revealed that the coconut cake can serve as a good substrate for the production of fibrinolytic enzyme under SSF process by employing *Fusarium spp.*CSN-6.

Key words: Fibrinolytic enzyme; Fusarium spp.; Solid state fermentation; Coconut cake; Thrombolytic agent

1. INTRODUCTION

Proteases are the single class of enzymes, which occupy pivotal positions with respect to their applications in both physiological and commercial fields. Proteolytic enzymes account for nearly 60% of the industrial market in the world. They find applications in number of biotechnological processes Viz., food processing, pharmaceuticals, leather industry, detergent industry etc, [Godfrey and West 1996]. Additionally proteolytic enzymes have been used for a long time in various forms of therapy. Several clinical studies have indicated their benefits in oncology, inflammatory conditions, blood rheology control and immune regulations [Nicholas and Lewis Stevens 1992].

Fibrin is the primary protein component of blood clot, which is formed from fibrinogen by thrombin (E.C.3.4.21.5). Under normal body conditions, thrombin and plasmin controls the formation and degradation of fibrin. However once the balance fails, blood coagulates in the blood vessels, resulting in myocardial infarctions and other cardiovascular diseases [Lee, Jin wook et al 1999]. Cardiovascular disease is the world's leading killer, accounting for 16.7 million or 29.2 per cent of total global deaths *(Atlas of Heart Disease and Stroke, WHO, September 2004)*. Pharmacological dissolution of an established thrombus has become an accepted therapeutic approach for many patients who develop thrombotic occlusive disease (de Bono 1995).

Fibrinolytic enzyme or clot-busters are vital products that are used as "life saving enzymes" in thrombolytic therapy since 1980 (Collen and Lijnene 1991). These agents became part of the clinical care of patients with acute myocardial infarction, deep vein thrombosis and other thrombotic occlusive diseases (de Bono 1995). The clot-buster drugs such as tissue pasminogen activators (tPA), Urokinase (UK) and Streptokinase (SK) & derivative anisoylated plaminogen streptokinase-activator complex (APSAC) have revolutionized the treatment of myocardial infarction in recent years (Armstrong and Collen 2001). However, all these thrombolytic agents still possess significant shortcomings, including large therapeutic dose, short plasma half-life, limited fibrin specificity, reclusions and bleeding complications. The need for newer and better clot-busters with fewer side effects and higher target specificity remains a great challenge worldwide.

Urokinase [Wun and Voet 1982; Sasaki et al 1985] and a tissue type plasminogen activator (tPA) [Pennica et al 1983] are of human origin and generally safe, but are expensive. Therefore, the search for other fibrinolytic enzymes from various sources continues. Many researchers over the last decade, have discovered the fibrinolytic enzyme from variety of sources, such as earthworms (Mihara et al 1991; Wang et al 2005), snake venom (Jia et al 2001; De-Simone et al 2005), insects (Ahn et al 2003), food grade microorganisms (Jeong et al 2001), marine creatures (Sumi et al 1992), Mushrooms (Seu-eun Park et al 2007) and fermented food products such as Japanese natto (Sumi et al 1987), Korean chungkook-jang (Kim et al 1996) and Chinese douche (Wang et al 2006).

The production of fibrinolytic enzymes is usually carried out by submerged fermentation process. But of late many authors have reported production of proteolytic enzymes under SSF conditions by using different agro based solid wastes Viz., rice bran, wheat bran, wheat flour, sugarcane baggasse etc., (Malathi 1991; pandey 1999; Mulimani 1999; Rouhang wang 2003; Veerupakshi 2004). Recently Sun tao et al has reported that production of fibrinolytic enzyme from rice chaff under SSF. Economically SSF process many advantages, including superior volumetric productivity, use of simpler downstream processing and lower energy requirements (Lonsane et al 1985; Ashok pandey 1999).

The coconuts are mainly used for extraction of oil, the huge quantity of coconut cake (oil cake) generated still contains considerable amount of fermentable sugars, proteins fats and other nutrients that can serve as a valuable raw material for a number of fermentation process (Naik et al 2002). Presently this product is used as either cattle feed or disposed without treatment, which cause environmental pollution.

Hence attempts were made in this study to optimize the culture conditions for thermostable fibrinoytic metalloprotease production through solid state fermentation using new *Fusarium* spp.CSN-6 using coconut cake as a substrate

2. MATERIALS AND METHODS

2.1. *Microbial strain*

The organism used in the present study was isolated from the soil and designated as *Fusarium spp.* CSN-6. Thus isolated culture was routinely maintained on potato dextrose agar (PDA) slants. Before each experiment, the organism was transferred to fresh slants and incubated at 28ºC for 7 days.

2.2. *Collection of raw material*

The coconut cake collected from local oil mills were ground in a warring blender and passed through a sieve (4 to 16 mm) to remove dust and large aggregates.

2.3. *Preparation of pre- inoculums*

Spore suspensions were prepared from 5 d. old cultures grown on PDA slants by adding 10 ml of sterile distilled water containing 0.01% tween 80 and suspending the spores with sterile loop. The spore suspension containing about 1×10^7 spores/ml was used to inoculate experimental substrates in the flasks [Fernandez Vergano et al 1996].

2.4. *Fermentation condition*

40 g. of coconut cake was thoroughly mixed with 60 ml of salt solution [containing gl^{-1}, $NaNO_3$ (2g), KH_2PO_4 (1g), $MgSO_4 .7H_2O$ (0.5g), KCl (0.5g), $FeSO_4 .7H_2O$ (trace) and $ZNSO_4. 7H_2O$ (trace) adjust to pH 6.0] in 500 ml Erlenmeyer flask. The substrate was sterilized at 15 lb/in² for 30 min. The sterilized substrate was cooled and inoculated with 2 ml of spore suspension; the contents in flasks were mixed thoroughly and incubated in slanting position at 30ºC in an incubator with 65-70% relative humidity for 1 week and periodically assayed for enzyme activity.

2.5. *Effect of incubation period*

40 g of substrate was impregnated with mineral salt solution in 500 ml Erlenmeyer flask, autoclaved and inoculated with spore suspension of 5 d old culture as described earlier and incubated. The contents of the flasks were harvested and assayed at every 24 h interval in triplicates.

2.6. *Effect of temperature*

The influence of temperature on enzyme production by *Fusarium sp*. CSN-6 was studied by incubating at different temperature ranging from 20 to 50⁰C.

2.7. *Effect of moisture content*

40 g of ground substrate was taken separately and dried at 60⁰C for 6 h. Cooled and weighed until the constant weight was obtained. Then the different levels of moisture in the substrate were adjusted to 40 to 80% by addition of required amount of salt solution and sterilized at 121⁰C for 15 min.

2.8. *Effect of pH*

Substrate was mixed with pre-adjusted pH of mineral salt solution as a moistening agent to obtain required pH. The pH was adjusted in the range of 2 to 7.5 with an increment of 0.5. Thus prepared flasks were sterilized, inoculated and incubated as described earlier.

2.9. *Effect of particle size*

The ground substrate was sieved through sieves having different sizes like 2, 4, 6, 8, 10, 12, and 14 mm. And substrate media of each particle size was separately used in these studies.

2.10. *Effect of inoculum size*

The inoculum was prepared as described earlier and inoculated to the substrate separately at different levels i.e. 1×10^5 to 1×10^{12} spores / ml. The inoculated flasks were incubated at 120 h and enzyme was extracted and assayed from each set.

2.11. *Extraction of enzyme*

At the end of the fermentation period, the entire quantity of moldy cake was homogenized with five volumes of 0.1% NaCl solution, shaking on orbital shaker at 200 rpm for 1h at 25ºC**.** The enzyme was filtered from moldy substrate by using two-fold cheesecloth; the extraction was centrifuged at 15000 rpm for 30 min. and the clean supernatant was used for enzyme assay.

2.12. *Enzyme assay*

Quantitative analysis of fibrinolytic activity was conducted by both plaminogen free and plaminogen rich fibrin plate method [Astrup and Mullertz 1952]. Plasmin (Sigma USA) 100U/ml was used as a standard.

3. RESULTS AND DISCUSSIONS

The production of enzyme in SSF condition is very sensitive. Therefore only a few selected parameters were optimized to achieve higher yields of the enzyme. Once the fermentation parameter is optimized, the optimum level of that parameter is employed in the subsequent studies wherein another parameter is to be optimized.

3.1. *Effect of incubation period on enzyme yield*

The production of fibrinolytic enzyme by *Fusarium sp.* CSN-6 was tested under solid state fermentation using coconut cake as a substrate. The highest enzyme activity, 956 and 480 U/ml was observed with and

without plasminogen at 120 h of incubation respectively. Fig. 1 indicates that 120 h of incubation period is optimum for enzyme production. 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 onwards, with maximum being reached at 120 h of growth. Beyond 120 h a sharp decrease in enzyme production was observed in both the cases which could be due to loss of moisture and nutrients after prolonged incubation up to 192 h.

3.2. *Effect of Temperature on enzyme yield*

The highest enzyme activity with plasminogen 1024 and 510 U/ml without plasminogen was obtained at 30^0C for 120 h of incubation (Fig. 2). The lowest activity 265 and 79 U/ml, was obtained at 50^0C for 120 h with and without plasminogen respectively. The incubation temperature undoubtedly depends on mold strain and medium used [Fernandez Vergano et al 1996]. The lower yield of enzyme on coconut cake at lower and higher temperature than the optimal may be due to delayed microbial activity, dehydration of the medium and undesirable metabolic deviation (Raimbault and Alzard 1980; Rathbun and Shuler 1983; Saucedo Castaneda et al 1990)

3.3. *Effect of moisture on enzyme yield*

The present study revealed that the newly isolated *Fusarium sp*. CNS-6 showed maximum enzyme activity (1236U/ml) (Fig.3) at 60% of moisture content of the substrate at 120 h of incubation. The low enzymatic activity has been observed at early and later stages of fermentation. The moisture content beyond the optimum level inhibits the enzyme activity. The higher moisture level of the substrate leads to decreased porosity and alters the coconut particles due to its gummy texture, which leads to lower oxygen transfer and decreased diffusion. While the lower moisture level than the optimum leads to the poor solubility of the nutrients, improper swelling and higher water retention [Narahara et al 1982; Silman et al 1979)

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 maximum enzyme activity (1240U/ml) was obtained at pH 5.5 [Fig. 4]. The lower yield of enzyme at lower and higher pH than optimum is due to inhibition of conidiation. Fibrinolytic activity and conidiation could not be correlated although high enzyme activity was always accompanied by conidiation [Ruohang Wang et al 2003].

3.5. *Effect of particle size*

The particle size of the substrate greatly influences the enzyme production under SSF conditions [Lonsane and Ramesh 1990]. The maximum activity (1252U/ml) (Fig.5) was obtained at 8 mm particle size. Several workers have reported the influence of particle size on the production of proteases under solid state fermentation [Mlathi and Chakraborthy 1991; Ashok Pandey et al 1999;Mulimani and Patil 1999]. Lower yield of enzyme with smaller particle size of the substrate may be due to closer packing densities and reduction in the void space leading to reduced heat transfer.

Whereas, in the case of larger particle size, nutrient transfer is limited [Mudgett 1986] and hence decrease in enzyme activity is observed. Thus an optimum particle size is necessary to achieve maximum enzyme activity.

3.6. *Effect of inoculum*

The fibrinolytic activity was found to be maximum (1302) at 1×10^8 spores /ml (Table.1.). Increase in the inoculum size did not elicit any significant increase in the yield of enzyme. The low density of spores leads to insufficient biomass and end products; whereas high densities of spores may cause a quick biomass production there by leading to fast nutrient depletion and ultimately reduction in the end product quantity. This optimal density is in agreement with findings of Sun Tao et al. [Sun Tao et al 1997].

CONCLUSION

Although many reports on production of fibrinolytic enzyme have been published, very little information is available on production of fibrinolytic enzymes under solid state fermentation employing agro-based wastes. In this work, it has been demonstrated that coconut cake can be used as novel substrate for the production of fibrinolytic enzymes. Further, the novelty of fibrinolytic enzyme secreted by *Fusarium sp*. CSN-6 is that it lyses the fibrin with and without plasminogen. Currently purification and characterization of enzyme is under investigation

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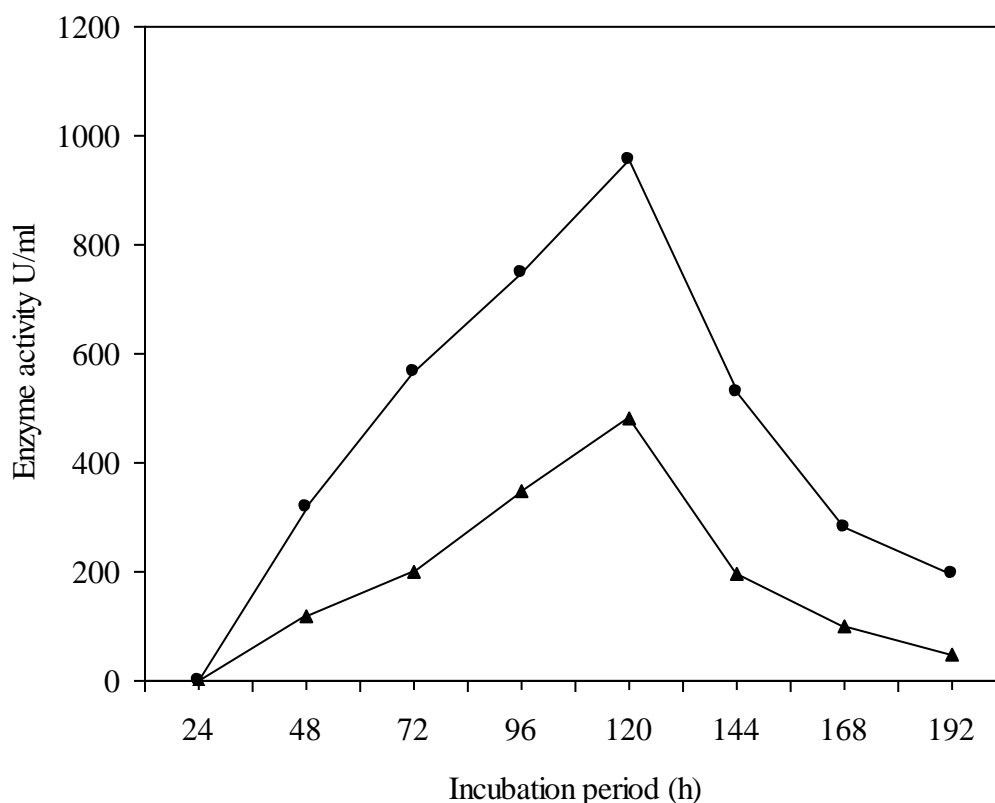
Fig.1. Effect of incubation period on the production of fibrinolytic enzyme by *Fusarium sp*. CSN-6 . (●) with plasminogen; (▲) without plasminogen. Results are mean of three experiments.

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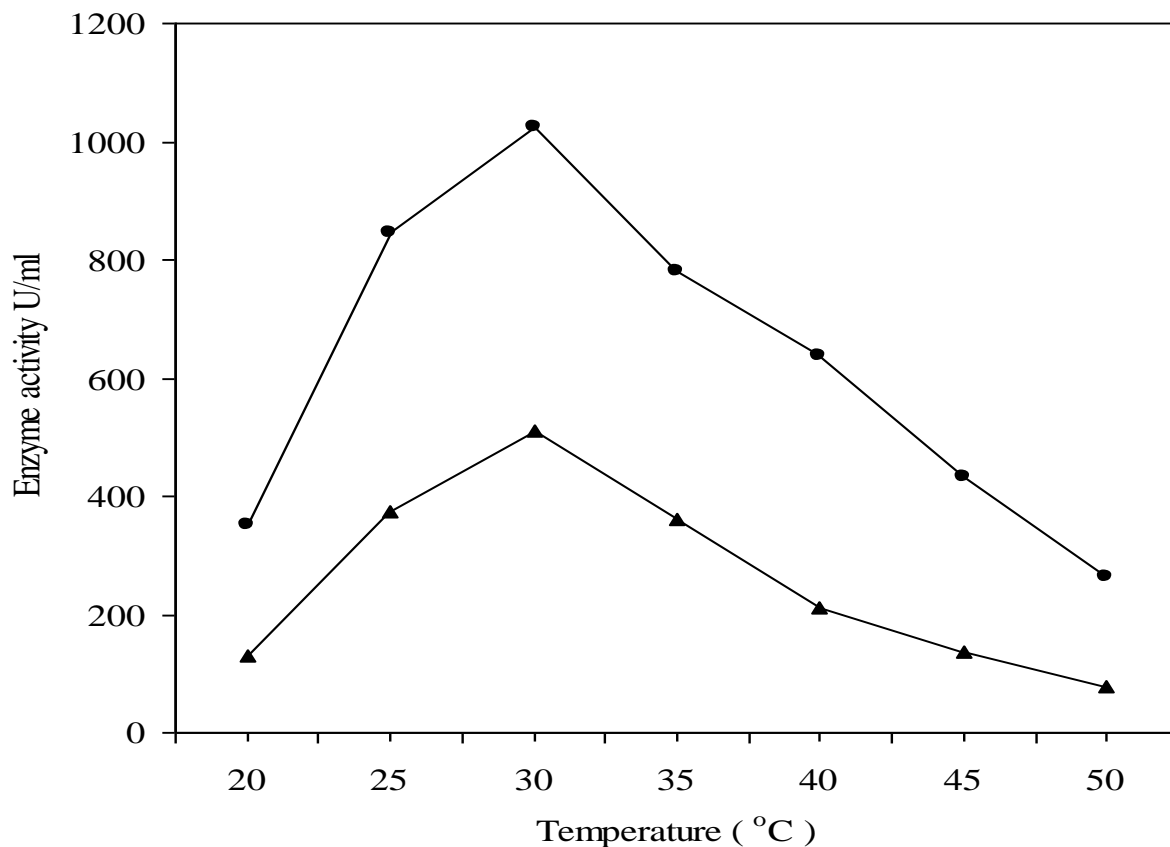(This work is licensed under a Creative Commons Attribution 4.0 International License.)

Fig.2. Effect of temperature on the production of fibrinolytic enzyme by *Fusarium sp*. CSN-6 . (●) with plasminogen; (▲) without plasminogen. Results are mean of three   experiments.
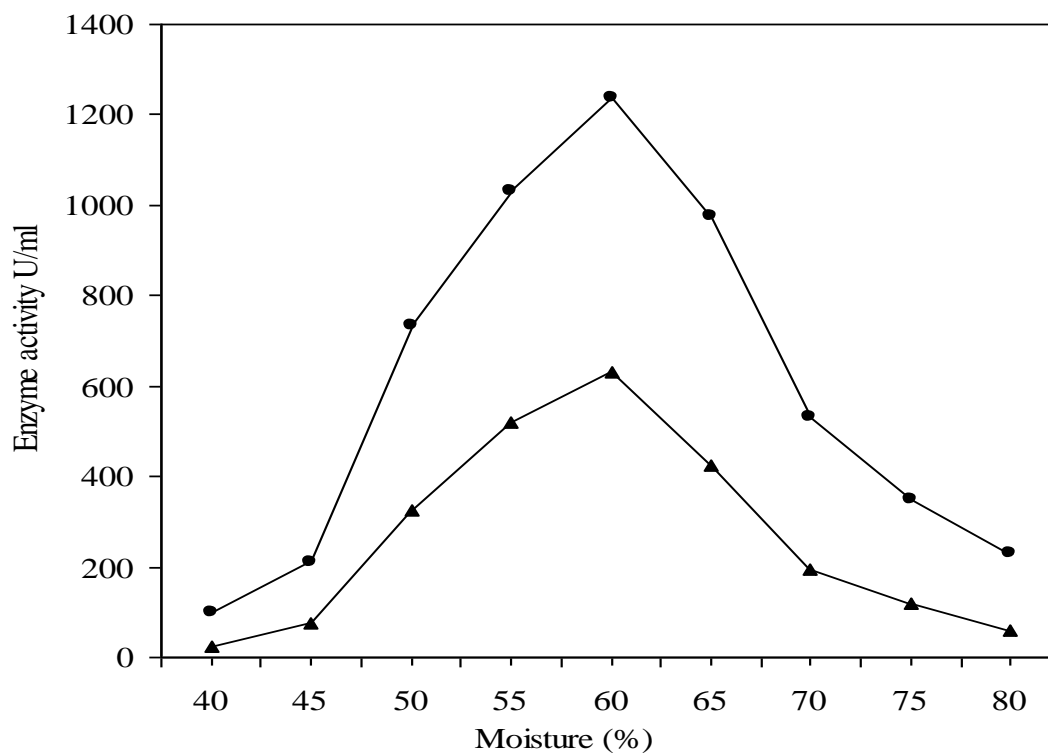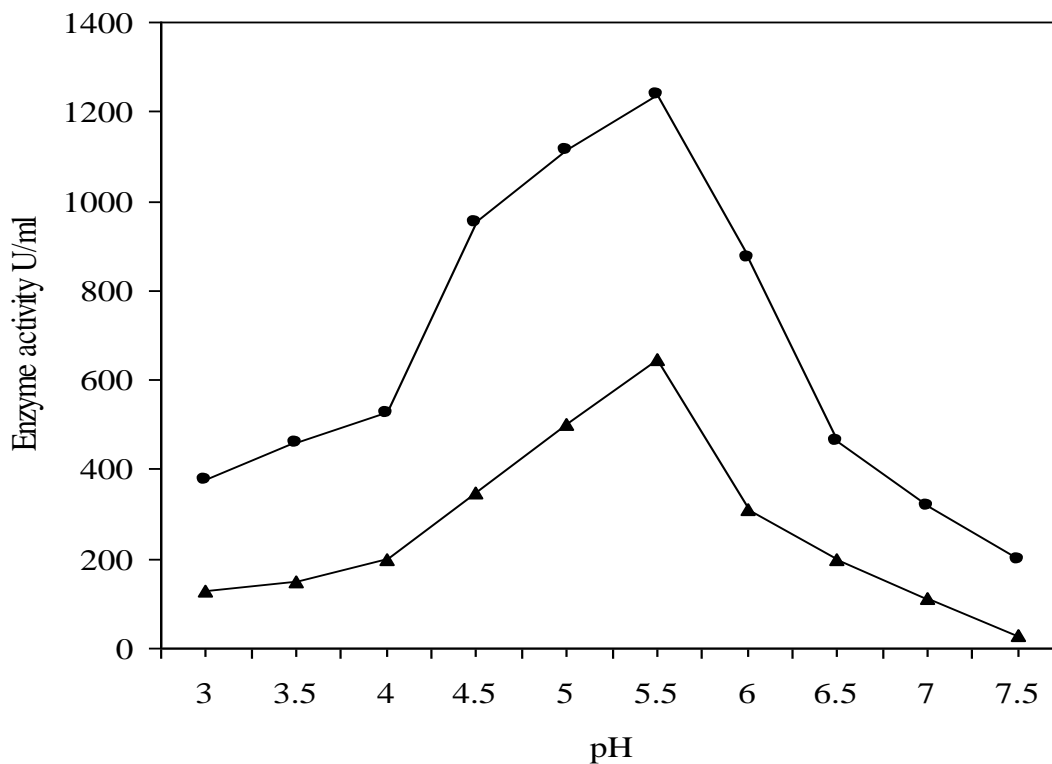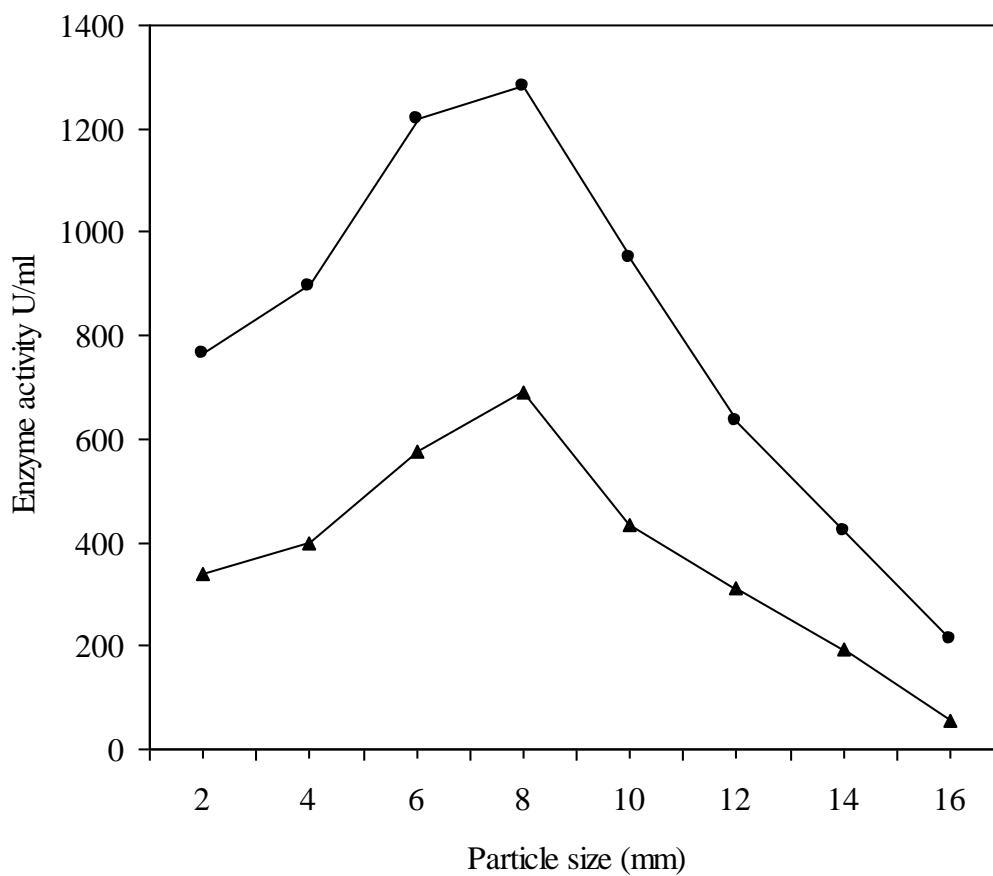

Fig.3. Influence of moisture on the production of fibrinolytic enzyme by   *Fusarium sp*. CSN-6 . (●) with plasminogen; (▲) without plasminogen. Results are mean of three experiments.

Fig.4. Effect of pH on the production of fibrinolytic enzyme by *Fusarium sp*. CSN-6 . (●) with plasminogen; (▲) without plasminogen. Results are mean of three experiments.

Fig.5. Effect of particle size on the production of fibrinolytic enzyme by *Fusarium sp*. CSN-6 . (●) with plasminogen; (▲) without plasminogen. Results are mean of three experiments.

Inoculum size (Spores/ml)	Enzyme activity (U/ml)	
	With plasminogen	Without plasminogen
1×10^5	958	450
1×10^6	1026	525
1×10^7	1285	650
1×10^8	1302	720
1×10^9	1030	495
1×10^{10}	940	300
1×10^{11}	731	210
1×10^{12}	532	125

Table.1. Effect of inoculum size on production of fibrinolytic enzyme by *Fusarium sp*. CSN-6 .Results is mean of three experiments.