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AN APPROACH ON BIOSYNTHESIS OF FIBRINOLYTIC ENZYME BY SUPPLEMENTATION OF TRACE METAL IONS FROM ASPERGILLUS JAPONICUM

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ABSTRACT

Fibrinolytic enzymes are those enzymes that are able to digest fibrin, the major protein in blood clot formation. For therapeutic applications, three common fibrinolytic enzymes *viz.*, streptokinase, urokinase and tissue plasminogen activators have been used with some degree of success. But they elicit undesired effects like internal haemorrhage, short half-life and unintended immune responses. The exploration of fibrinolytic enzyme is utmost important. Fibrinolytic enzyme production was investigated in the filamentous fungi *Aspergillus japonicum* KGSY 05 a soil isolate of different regions from Bangalore. The process economization were employed to achieve higher yield of Fibrinolytic enzyme through submerged fermentation (SmF) and here as have made an attempt to incorporate metal ions as a trace elements sources such as copper sulphate ($CuSO_4$), iron sulphate ($FeSO_4$), Zinc sulphate ($ZnSO_4$) and magnesium sulphate ($MgSO_4$) as a source to the production medium in the range of 0.01%, 0.02% and 0.03%. Iron sulphate and Zinc sulphate acted as best source of metal ions for the production of fibrinolytic enzyme at 0.02% at 72 hrs of fermentation period, the enzyme production observed was 112 IU and 105 IU respectively. Magnesium sulphate and Copper sulphate were less inducers of Fibrinolytic enzyme production and the enzyme production observed was 95 & 85 IU respectively at 0.01% for 72 hrs of fermentation period.

KEYWORDS: Fibrinolytic Enzyme, Fibrin Plate, Submerged Fermentation and Metal Ion.

INTRODUCTION

Cardiovascular disease (CVDs) is caused by disorders of the heart and blood vessels, and includes coronary heart disease (heart attacks), cerebrovascular disease (stroke), raised blood pressure (hypertension), peripheral artery disease, rheumatic heart disease, congenital heart disease and heart failure (http://www.who.int/topics/cardiovascular_diseases/en/).

Cardiovascular diseases (CVDs) have now become the leading cause of mortality in India. A quarter of all mortality is attributable to CVD. Ischemic heart disease and stroke are the predominant causes and are responsible for >80% of CVD deaths. The Global Burden of Disease study estimate of age-standardized CVD death rate of 272 per 100,000 population in India is higher than the global average of 235 per 100,000 population (Prabhakaran, 2016).

Drugs using fibrinolytic enzymes are the most effective methods in the treatment of thrombosis. A variety of fibrinolytic enzymes such as tissue plasminogen activator (t-PA), urokinase (u-PA, EC 3.4.21.31), and bacterial plasminogen activator streptokinase (EC 3.2.1.35) have been extensively studied and used as thrombolytic agents (Moukhametova *et al*., 2002).

The microorganisms producing fibrinolytic enzymes include bacteria, actinomyces, fungi and algae. Microorganisms are important resources for thrombolytic agents. Some kinds of fungi have also been found to produce the protease with high fibrinolytic activity for example *Asperigillus ochraceus* 513, *Fusarium oxysporum*, *Penicillum chrysogenum*, *Rhizopus chinesis* 12**.** In addition, Matsubara et al, found the fibrinolytic enzymes from marine algae *Codiumlatum*, *Codiumdivaricatum*, and *Codiumintricatum* (Shilpa et al., 2007).

Present study highlights on supplementation of metal ions in fermentation medium for the biosynthesis of fibrinolytic enzymes from *Aspergillus japonicum.*

MATERIALS AND METHODS

Fungal strain

Aspergillus japonicum were isolated from soils collected from different regions in and around Bangalore. The *Aspergillus japonicum* were isolated by using Czapek Dox's media and tentatively identified in the laboratory and confirmed at Agharakar Research Institute, Pune.

Screening of Fibrinolytic Eenzyme producers by Plate Assay: Fibrinolytic activity was determined using the method described by Astrup and Mullertz. The fibrin agarose plate was made to a 1 mm thickness, and contained agarose (1.2% w/v), bovine fibrinogen (0.4% w/v), and bovine thrombin (20 U/mL) in a petridisc, and the clot was allowed to stand for 1 h at room temperature. Then, 10 µL of sample enzyme solution was carefully placed onto the plate. The plate was incubated for 5 h at 37ºC and the diameter of the lytic zone was measured and the clear transparent region was observed in which fibrin is hydrolyzed.

Effect of Metal ions as source for the biosynthesis of fibrinolytic enzyme

A set of conical flasks with 100 ml of production medium supplemented with a particular metal ions as a source such as copper sulphate ($CuSO_4$), iron sulphate ($FeSO_4$), Zinc sulphate ($ZnSO_4$) and magnesium sulphate ($MgSO_4$) were used for screening their effect on production of enzyme at 0.001 and 0.003% in production media devoid of $MgCl_2$. The culture was grown for 96 h with enzyme assay at every 24 h. The production medium consists (mg/100 ml) of Sucrose 3, di potassium hydrogen phosphate 0.1, KCl 0.05g, NaCl, and MgSO4 and FeSO4 are in the range of 0.001 and 0.003%. The condition of the fermentation medium is as follows .pH,6 temperature 40^0C and inoculums size is of 1.25ml.

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.

RESULTS AND DISCUSSION

The *Aspergillus japonicum* KGSY 05 were isolated from soil sample and screened for the fibrinolytic enzyme production and the fungal isolate were identified were screened for biosynthesis of fibrinolytic enzyme by plate assay.

The influence of metal ions for fibrinolytic enzyme production were carried out with concentration of 0.001 and 0.003%. The results revealed that all the metal ion sources employed under the present study have enhanced the production of fibrinolytic enzyme up to 0.02% of metal ions at 72 hrs of fermentation represented in Fig1-4, thereafter no significant production of fibrinolytic enzyme was observed on all the days of fermentation period. The Iron sulphate and Zinc sulphate acted as best source of metal ions for the production of fibrinolytic enzyme at 0.02% at 72 hrs of fermentation period The enzyme production observed was 112 IU and 105 IU respectively. Magnesium sulphate and Copper sulphate were less inducers of Fibrinolytic enzyme production and the enzyme production observed was 95 & 85 IU respectively at 0.01% for 72 hrs of fermentation period.

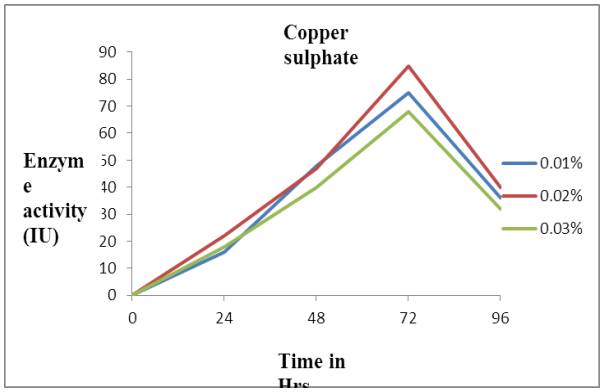

Fig. 1: Effect of CuSO4 on Fibrinolytic enzyme production.

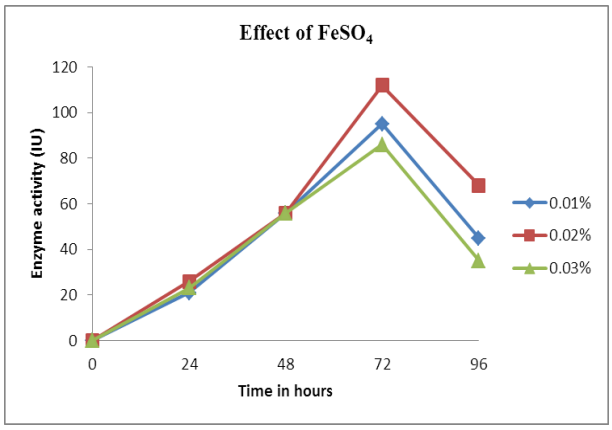

Fig. 2: Effect of FeSO4 on Fibrinolytic enzyme production.

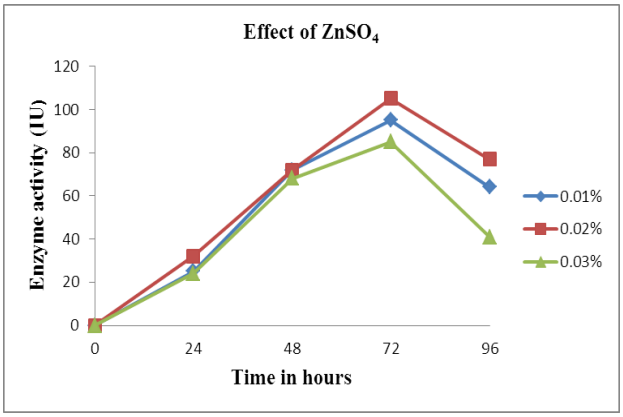

Fig. 3: Effect of ZnSO4 on Fibrinolytic enzyme production.

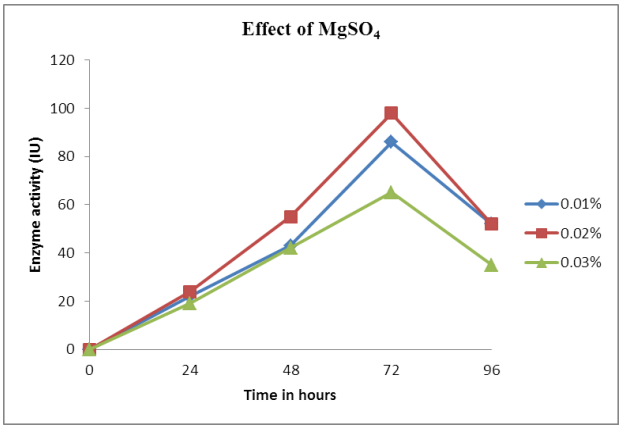

Fig. 4: Effect of MgSO4 on Fibrinolytic enzyme production.

Trace elements have profound effect on the growth and physiological activities of the organisms. In general trace elements play a key role in the metabolism of organisms. Few metal ions need to be supplemented to a fermenting medium, as they are essential for cell mass formation and also acts as a co factor for several biosynthetic enzymes.

Abdel-Naby et al., (1992), the effect of adding trace metals ($Zn^{2+,}$ Fe^{+2}, Mn^{+2}), separately or combined, to the medium had no significant effect on the production of fibrinolytic enzyme.

Venkata Naga Raju and Divakar (2013) divalent metal ions such as calcium, cobalt, copper, boron, iron, magnesium, manganese and molybdenum are required in the fermentation medium for optimum production of fibrinolytic proteases. However, the requirement for specific metal ions depends on the source of enzyme. The use of $MgSO_4$, $AgNO_3$, $CaCl_2$, $MnCl_2$ at a concentration of 0.1-0.5 mM or NaN_3 at a concentration of 0.1-0.5 mM resulted in an increase in fibrinolytic protease activity in *Bacillus subtilis*, β-hemolytic, *Oidiodendron flavum*, *Schizophyllum commune*, *Pseudomonas aeruginosa*, *Bacillus lichniformis* B4, *Rhizomucor miehei*, *Ganoderma lucidum* VK 12, *Escherichia coli*, *Candida guilliermondii*, *Bacillus cereus GD55*.

Chitte and Dey (2000) worked on the addition of trace metal solution had a negligible effect on the production of fibrinolytic enzyme. Calcium did not increase in enzyme production but played a significant role in maintaining thermostability. Cu^{2+}, Fe^{2+}, Zn^{2+} and Mn^{2+} did not affect the activity but Al^{2+} inhibited the enzyme activity.

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