# ISOLATION, PRODUCTION, PURIFICATION, CHARACTERIZATION OF FIBRINOLYTIC ENZYME FROM FUNGAL SOURCE

Thesis submitted to the Faculty of Medicine
BLDE (Deemed To Be University), Vijayapura, Karnataka, (India)
For the Award of

**DOCTOR OF PHILOSOPHY (Medical)**

Subject: Medical Biochemistry

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**2019**

# BLDE (DEEMED TO BE UNIVERSITY)

**Vijayapura, Karnataka, (India)**

## Certificate

This is to certify that the thesis entitled **"Isolation, production, purification, characterization of fibrinolytic enzyme from fungal source''** is a bonafide work of Mrs. Shilpa H K and has been carried out under our guidance and supervision.


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## DECLARATION

I declare that the thesis entitled **"Isolation, production, purification, characterization of fibrinolytic enzyme from fungal source"** has been prepared by me under the guidance of Professor Jeevan G. Ambekar, Department of Biochemistry, BLDE (Deemed To Be University), Shri B.M.Patil Medical College, Hospital & Research Centre Vijayapura, Karnataka, (India). No part of this thesis has formed the basis for the award of any degree or fellowship previously by me.

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# ACKNOWLEDGEMENT

I sincerely take an opportunity to acknowledge my gratitude to all the people that are mentioned here under without whom this thesis would not have been possible.

I am thankful to Dr. M.S.Biradar sir, Vice Chancellor, BLDE (Deemed to be University), Shri B.M.Patil Medical College, Hospital & Research Centre, Vijayapura.

Firstly, I express my sincere gratitude to my guide, Dr J.G Ambekar sir, Professor of Biochemistry and Registrar, BLDE (Deemed to be University), Shri B.M.Patil Medical College, Hospital & Research Centre, Vijayapura, for his masterly directions, constant support and eminent guidance, at every stage of this study.

I am sincerely indebted to my co-guide Dr K.G.Siddhalingeshwara sir, Research and Development head in Scientific Industrial research centre, Bangalore, Karnataka for his support and invaluable suggestions during the planning and development of this research work.

My Sincere thanks to Professor Kusal K. Das sir, Laboratory of Vascular Physiology & Medicine, Dept of Physiology and Dr. Nilima N. Dongre, Associate Professor, Dept. Of Biochemistry, BLDE (Deemed to be University), Shri B.M.Patil Medical College, Hospital & Research Centre, Vijayapura for their great ideas, untiring guidance, affectionate & constant encouragement to complete this research work.

I owe my most sincere gratitude to Dr. S.P. Guggarigoudar sir, Principal, BLDE (Deemed to be University), Shri B.M.Patil Medical College, Hospital & Research Centre, Vijayapura for his help and support.

I would like to express my very great appreciation to the Vice-principal Dr. Tejaswini Vallabha, Shri B.M.Patil Medical College, Hospital & Research Centre and Chairman of Doctoral committee, BLDE (Deemed to be University), for her support.

I would like to express my sincere gratitude to Prof Dr. Basavaraj Devaranavadgi sir, Head of the Department of Biochemistry, Dr. Basavaraj Aski sir, Associate professor and other supporting staffs in Department of Biochemistry, BLDE (Deemed to be University), Shri B.M.Patil Medical College, Hospital & Research Centre, Vijayapura for their help, support and encouragement during this study.

I would like to express my sincere gratitude to Mr. Satish B. Patil sir, Deputy Registrar, BLDE (Deemed to be University), Vijayapura, for their advice and instructions for all academic proceedings, during entire Ph.D course.

I am thankful to Dr. Manjula. M, Chief Librarian, Mr. Shivakumar Acharya, Asst. Librarian, for their help, support and encouragement during this study.

My heart full thanks to my parents for their great support. **MY MOTHER'S** inspiration and **MY FATHER'S** versatile opinion helped me to look forward during my study. I am indebted to my husband **Mr. Vinod kumar R** for his constant cooperation and support during my study. I am grateful to my sister **Ms. Megha Shree H K** and all my family members for their encouragement for my study. I am also thankful to my cute daughter **Pavika Vinod** for her valuable support.

My sincere gratitude to Dr.Renji.K, Group Director, U R Rao satellite centre, ISRO, Bangalore, for his financial support during my studies.

# ABSTRACT

**Background:**

Thrombus formation in the blood vessels is one of the major problems in today's modern life. Disturbance in haemostasis leads to cardiovascular diseases and causes disabilities and death. World Health Organization (WHO) says that every year about 17 million people die because of cardiovascular diseases. The tissue that is supplied with oxygen will die or severely gets damaged until the blockage is removed promptly.

Acute myocardial infarction, ischaemic heart diseases are the leading causes of death worldwide. According to WHO report by 2030 the death rate due to cardiovascular diseases will be increased to 23.3 million. To overcome these problems fibrinolytic enzymes are used. The fibrinolytic enzymes dissolve fibrin clots.

**Objective:**

The main objective of the study is to isolate, screen, produce (high yield) and extract the fibrinolytic enzyme from the fungal source and to evaluate its action on clot degradation/ hydrolysis.

**Methods:**

From the different regions of Karnataka, the fertile soil samples are collected. These soil samples were used for the isolation of fungi by serial dilution method. Thirty fungal strains were isolated on sabouraud dextrose agar and the cultures were maintained on Czapek Dox's agar slants. These fungal species were screened using fibrin plate assay.

Production media was prepared to carry out submerged fermentation method. Optimization of pH, temperature and inoculum size were done for the fermentation media. Carbon, nitrogen sources and metal ions evaluation was done to check the enzyme production during the fermentation process. The Purification of the crude extract was done by adopting filtration, centrifugation, Ammonium sulphate fractionation, Dialysis, Gel-filtration chromatography and Ion-exchange

chromatography. Molecular weight determination was done using SDS-PAGE with known standards.

The purified enzyme is characterized by determining the optimum pH, temperature, stability of temperature. Inhibitors and metal ions effects were determined Sequencing and analysis of PCR amplification was done with the suitable primer and its comparison was done with the existing sequences in NCBI database using the Blast N program.

Enzyme activity is compared between Aspergillus tamarii SAS02 and bacterial sp *(Bacillus, E.coli, and Pseudomonas)*. Enzyme obtained from Aspergillus tamarii SAS02 shows the highest activity than the bacterial sp *(Bacillus, E.coli, and Pseudomonas).*

Potentiality of the fibrinolytic enzyme (*Aspergillus tamarii* SAS02) is proved by comparing it with the commercially available enzyme. Mutational studies are done out by subjecting it to UV irradiation.

**Results:**

The fungal strains obtained from the soil sample were labelled as S1 to S20. All the 20 strains were subjected to screening using fibrin plate method. All the 20 isolates did not showed the clear zone around the colony. Only one strain showed the highest clear zone around the colony was chosen for further studies and labelled it as SAS02 and confirmed it as *Aspergillus* sp, identified at Agarkar Research Institute, Pune, India. By comparing it with the NCBI database ( in Bhat Bio-tech, Bangalore) the strain was named as *Aspergillus tamarii* SAS02.

Production media was designed and optimized for the pH-6, incubation temperature of about 40ºC and inoculum size of about 1.25ml.

The process economizations were employed to check the evaluation of the enzyme production by using carbon sources like glucose, sucrose and maltose. Among all, the glucose showed the highest fibrinolytic enzyme activity of 186 IU when compared to sucrose and maltose. Organic nitrogen sources like yeast extract, beef extract and peptone were used. Among all, peptone showed the highest activity of 193 IU. Ammonium sulphate, Ammonium chloride and Ammonium nitrate used as

a inorganic nitrogen sources. Ammonium chloride showed the maximum fibrinolytic enzyme production of 198 IU when compared to Ammonium sulphate and Ammonium nitrate. Traces of few metal ions like Zinc sulphate, Magnesium sulphate, Copper sulphate and Iron sulphate used to check the enzyme production of fibrinolytic enzyme. Organism showed the highest enzyme production of 152 IU with the presence of Zinc sulphate when compared to other metal ions.

Downstream process is carried out for the purification of the enzyme by Ammonium sulphate salt precipitation which showed the 185 IU/ml with 1% purity, followed with Dialysis obtained the activity of 180 IU/ml with 115.13% purity. The activity of 126 IU/ml was obtained by Gel-filtration with 132.48% purity. By adopting Ion-exchange chromatography 320.07% purity was obtained with the enzyme activity of 96 IU/ml and the Molecular weight of the obtained protein is 29kDa.

Characterization of purified fibrinolytic enzyme was done which showed the 100% activity at pH-8, temperature at 45ºC and showed the better stability at 55ºC within room temperature for 30min. Enzyme activity was assayed with the different concentrations of substrate (fibrin), the *Km* was 1.25 and *Vmax* was 2.1. In the inhibition studies purified enzyme displayed complete inhibition in the presence of Hg, PMSF, EDTA and in the presence of NaCl and $H_2O_2$ enzyme lost its activity but the highest activity was induced by Zn (114%), Mg (126%), SDS (38.2%).

The activity of the enzyme is compared with few bacterial strains upon all these, the fibrinolytic enzyme obtained from the *Aspergillus tamarii* SAS02 showed the highest activity of 180 IU. Blood clot hydrolysis was observed in the plates one which contains the fibrinolytic enzyme obtained from Aspergillus tamarii SAS02 and the other which contains the commercial enzyme. After the incubation period of 15-18hrs clot degradation was observed in the capillary which is filled with blood clots. Hence proved that our enzyme is a tool for obliteration of blood clots.

To improve the enzyme production the conventional mutagenesis was adopted by subjecting the strain under UV irradiation and observed for the enzyme activity. The mutated *Aspergillus tamarii* SAS02mu showed the highest activity of 213IU than that of the parental strain which showed 180IU.

**Conclusion:**

Isolation, Screening and Molecular characterization of fungal species from soil samples showed that *Aspergillus tamarii* SAS02 was the most suitable fungal source of fibrinolytic enzyme. This can be further utilized for the large scale production of the fibrinolytic enzyme with the optimization of various factors like pH, temperature, inoculum size, carbon source, nitrogen source, metal ions and controlling the effect of inhibitors. The enzyme is also found to be effective in obliteration of the blood clot *in vitro*, when compared with the commercially available enzyme (Myokinase). But this needs to be proved by clinical trials with this enzyme on human beings. This will help in developing an economically cheaper source of fibrinolytic enzyme for the treatment of blood clot obliteration.

**Key words:** Fibrinolytic enzymes, cardiovascular diseases, *Aspergillus sp*., invitro.

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## 1.1 Purpose of the study

In modern life, the most widely occurring disease is the formation of clot in the blood vessel which leads to cardiovascular diseases. World Health Organisation reported that every year there is death of 17 million people due to cardiovascular diseases[1].

A clot developed in the circulatory system causes vascular blockage and sometime even death. If the blockage is not removed, the tissue that is normally supplied with oxygen will die. If the damaged tissue region is large the normal electrical signal conduction is disturbed that leads to irregular heart beat. For treating these cardiovascular diseases enzyme therapy is most commonly used. Some bacterial species like *"Bacillus subtilis, Streptomyces megaspores, Bacillus pyocyaneus, Staphylococcus aureus"* are the few fibrinolytic enzyme producers[1-7].

These sources are widely used in thrombolytic therapy. But they are expensive and show undesirable side effect like internal haemorrhage. To minimize the side effect the research is going on to find other easily available and cheaper sources[4].

Hence we have made an attempt to use different types of soil samples and find a suitable fungal source of fibrinolytic enzyme. The soil is rich in different kinds of microorganisms and it is easily available everywhere. Isolation of microorganism from the soil as a source is cost effective due to this our ecology also will not be disturbed.

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## 2.1. Aims and Objectives of the study:

**Aim:**

As there are very scanty reports available on fibrinolytic enzymes from fungal source the present study is an attempt to isolate, produce, purify and characterize fibrinolytic enzyme from the soil sample.

**Objectives:**

1. Isolation of fibrinolytic enzyme producing organisms from the soil samples.
2. Screening of fibrinolytic enzyme producing organisms.
3. Production of fibrinolytic enzyme by suitable methods.
4. Purification and characterization of fibrinolytic enzyme.
5. Process economization for the biosynthesis of fibrinolytic enzyme.

### 3.1. Historical aspects of Fibrinolytic Enzymes

**Thrombosis-** "It is the formation of a blood clot inside a blood vessel, obstructing the flow of blood through the circulatory system".

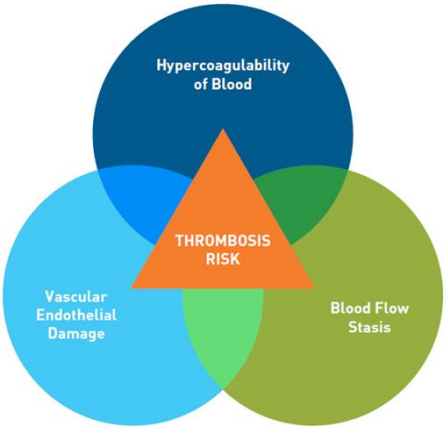


**Fig.3.1- Factors that are responsible for Thrombosis**

Endothelial injury, abnormal blood flow and Hypercoagulability are the three factors that lead to thrombus formation. These three factors are interrelated; endothelial injury predisposes to abnormal blood flow and Hypercoagulability, while abnormal blood flow can cause both endothelial injury and Hypercoagulability.

"Hypercoagulability also known as thrombophilia, refers to higher levels of coagulation factors in the blood that increase susceptibility to thrombosis"[49].

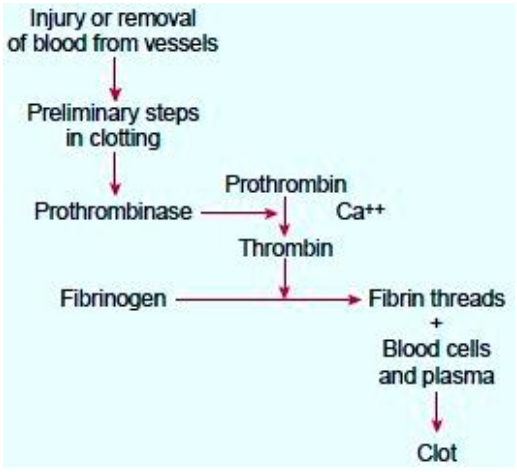


**Fig.3.2-Process of blood clot formation**

Risk factors for thrombosis-

a. Inheriting a blood-clotting disorder

b. Prolonged bed rest

c. Injury

d. Use of birth control pills

e. Being overweight

f. Smoking

g. Cancer

Blood clot formed in the circulatory system causes blockage in the vascular system which sometimes leads to end of the life. A healthy hemostatic system suppresses the blood clot formation. Improper blood clotting becomes the major risk factor for myocardial infarction and stroke. If the hemostatic system is failed it leads to cardiovascular diseases. To overcome these problem pathologists made a clinical intervention of administrating thrombolytic agents intravenously.

Enzymes are the biocatalysts which play the central role in every biochemical process. They enhance the rate of reaction without themselves getting altered during the process. They have high degree of specificity for substrates.

Fibrinolytic enzymes dissolve fibrin clots and are used effectively in thrombolytic therapy. Fibrinolytic enzymes catalyse by the breakdown of proteins (Protein Z, Factor VIII, and Factor XIII) by acting on interior peptide bonds[8].

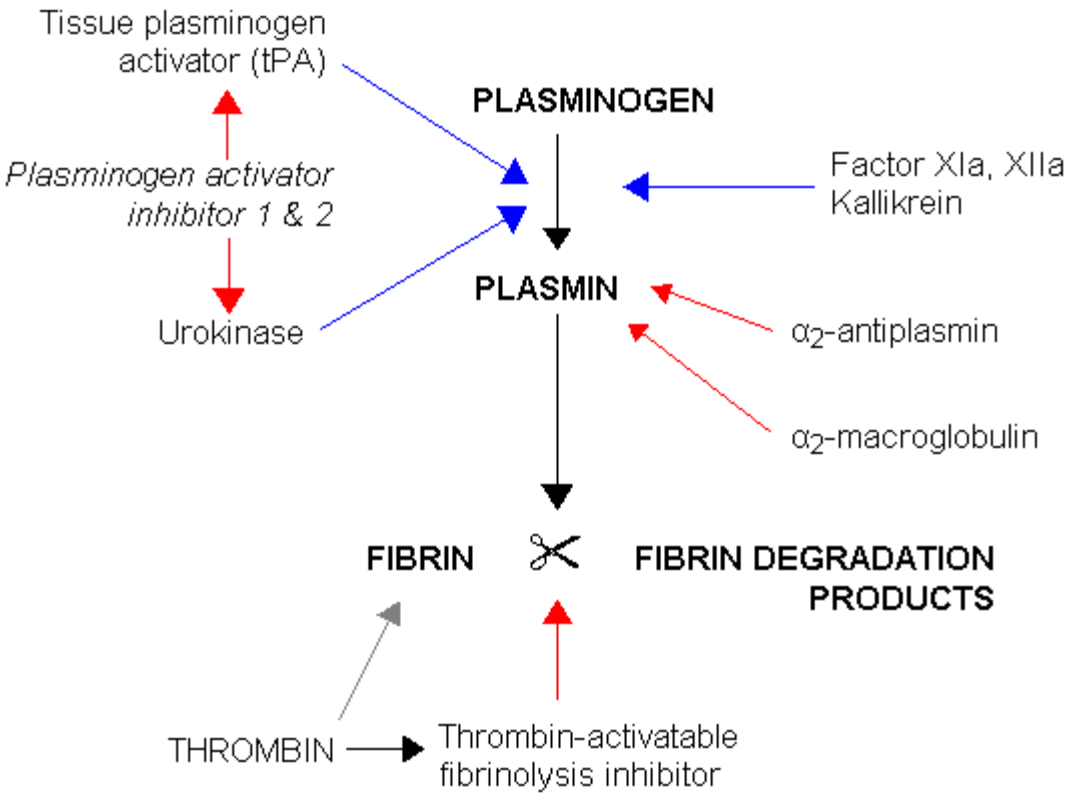


**Fig-3.3-Action of fibrinolytic enzyme on fibrin degradation**

Fibrinolytic enzyme when added to human plasma containing a fibrin clot poorly reacts with plasminogen but reacts with high affinity with plasmin. At the clot surface, the plasmin-fibrinolytic enzyme complex efficiently activates plasminogen to plasmin. The plasmin-fibrinolytic and uncomplexed plasmin bound to fibrin are protected from rapid inhibition by $\alpha_2$-antiplasmin, whereas their unbound counterparts, those are liberated from the clot or which is generated in plasma are inhibited rapidly by $\alpha_2$-antiplasmin. The process of plasminogen activation is confined to the thrombus, which prevents excessive plasmin generation, $\alpha_2$-antiplasmin depletion, fibrinogen degradation in plasma.

Fibrinolysis is a process that prevents blood clots from growing and becoming problematic. This can be achieved in vivo by two types: primary fibrinolysis and secondary fibrinolysis.

The primary fibrinolysis is a normal body process whereas secondary fibrinolysis is the breakdown of clots caused due to medical disorder by the use of therapeutic agents. This is how the blood clot is removed at various places[46].

Fibrinolytic enzymes are successfully discovered from different microorganisms like Bacteria, Fungi, Actinomycetes e.g. **Streptokinase, Urokinase, Nattokinase.**

R.Dubey et al. (2011) reported that Urokinase, streptokinase and other tissue plasminogen activators are extensively used to lyse the fibrin clots therapeutically[2]. Food and Microbes are the sources of thrombolytic agents.[3]

### 3.2. Fibrinolytic Enzyme sources

#### 3.2.1. From Bacteria

*Bacilluls subtilis* produces a fibrinolytic enzyme which has a high potentiality. The first fibrinolytic enzyme isolated from Japanese soyabean by Sumi et al. (1987) is nattokinase[4]. Collen and Lijnen (1994) proved that streptokinase and staphylokinase are the fibrinolytic enzymes used for thrombolytic therapy[9]. Kim et al (1996) isolated fibrinolytic enzyme producing organism *Bacillus sp*. CK from soybean sauce[9]. Kim et al. (1997) isolated *Bacillus sp*. also produces fibrinolytic enzyme from jeot-gal. Yoon et al. (2002) isolated fibrinolytic enzyme producing organisms from commercially fermented food[32]. Peng and Zhang (2002) isolated the organism *B.amyloliquefaciens* produces fibrinolytic enzyme from Chinese soybean[26]. Choi et al. (2005) isolated *Bacillus* strains DJ-4 which produces fibrinolytic enzymes from the Korean doenjang, this finding imply the possibility of consuming the fermented food to prevent cardiovascular diseases[36]. *Bacillus sp*. produces varieties of fibrinolytic enzymes proved by Wang et al. in the year 2009. *Pseudomonas sp*. TKU015 exhibited highest activity towards fibrin which is isolated from the soil. Sekar kumaran (2011) isolated (VK12) of *Ganoderma Lucidum* showed pulmonary thrombosis *invivo*[18]. Essam F.Al-Juamily isolated bacteria *Bacillus lichniformis*, (2012) showed highest activity under optimal conditions obtained from different parts of the soil from Baghdad university[38]. *Bacillus ceresus* SRM-001 by Manoj kumar Narasimhan (2015)[46], isolated from chicken dump yard proved it as a potential therapeutic thrombolytic agent in-vitro. The fibrinolytic enzyme isolated from *Bacillus sp.* IND18 by Ponnuswamy vijayaraghavan, (2016) activated plasminogen and degraded the fibrin mesh of blood clot and proved it as an effective fibrinolytic agent[42.] Bacillus sp. IND by Mohammed.A.Almalki (2017), isolated from fermented rice for the production of fibrinolytic enzyme, as good source for various biotechnological applications[10].

**Table-3.1- Bacterial Fibrinolytic Enzyme Producers**

| SL.No. | Microorganism | References |
|---|---|---|
| *1* | *B. natto, NK* | Fujita et al. (1993) |
| *2* | *Bacillus sp.* | Fujita et al. (1993) |
| *3* | *Katsuwonus pelamis* | Sumi et al. (1995) |
| *4* | *Bacillus sp. CK* | Kim et al. (1996a,b) |
| *5* | *Bacillus sp. KA38* | Kim et al. (1997) |
| *6* | *Bacillus sp.* | Noh et al. (1999) |
| *7* | *Armillaria mella* | Kim and Kim (1999) |
| *8* | *Bacillus sp. DJ-4* | Kim and Choi (2000) |
| *9* | *B. subtilis IMR-NK1* | Chang et al. (2000) |
| *10* | *Bacillus sp. KDO-13* | Lee et al. (2001) |
| *11* | *B. amyloliquefaciens DC-4* | Peng et al. (2003) |
| *12* | *B. subtilis QK02* | Ko et al. (2004) |
| *13* | *Bacillus firmus NA-1* | Seo and Lee (2004) |
| *14* | *Bacillus sp. DJ-2* | Choi et al. (2005) |
| *15* | *Bacillus sphaericus* | K.Balaraman (2006) |
| *16* | *Streptomyces omiyaensis* | Uesugi et al (2011) |
| *17* | *Bacillus lichniformis* | Essam F.Al-Juamily (2012) |
| *18* | *Bacillus Cereus SRM-001* | Manoj kumar Narasimhan (2015) |
| *19* | *Bacillus halodurans IND18* | Ponnuswamy vijayaraghavan (2016) |
| *20* | *Bacillus sp.IND6* | Mohammed.A.Almalki (2017) |

These sources are widely used in thrombolytic therapy. But they are expensive and show undesirable side effect like internal haemorrhage, other than this the possible risk factors are-

- Bleeding at the access site.
- Damaged to the blood vessel.
- Blood clot migration to another part of the vascular system.
- Kidney damage in the diabetes patients.

To minimize these side effects the research is going on to find other easily available, safe and cheaper sources.

### 3.2.2. From Fungi

*Streptomyces sp* SD5, isolated from the hot springs produced a strong thermostable enzyme proved by Chitte and Dey (2000). *Penicillium chrysogenum* by El-Aassar et al. (1990)[51] , *Fusarium oxysporum* by Sun et al. (1998) , *Aspergillus ochraceus* 513 by Batomunkueva and Egorov (2000)[8] and *Rhizopus chinensis* 12 by Xiao-Lan et al. (2005)[38] are the different fungi which produces proteases with fibrinolytic activity[25]. Nonaka et al. (1997) isolated the fibrinolytic enzyme from fruiting bodies and mushrooms which are used for medicines like Grifora frondosa aminopeptidase, Pleurotus astreatus metalloprotease. Choi et al. (1999)[38] isolated Perenniporia fraxinea proteases 1 and 2 and Cordyceps militaris .[10]

**Table-3.2- Fungi Fibrinolytic Enzyme Producers**

| SL.No. | Microorganism | References |
|---|---|---|
| *1* | *Cochliobolus lunatus* | Abdel-Fattah and Ismail (1984) |
| *2* | *P.chrysogenum H9* | El-Aassar et al (1990) |
| *3* | *Fusarium pallidoroseum* | El-Aassar (1995) |
| *4* | *F.oxysporum* | Tao et al.( 1998) |
| *5* | *Pleurotus ostreatus* | Chol and Shin (1998) |

| | | |
|---|---|---|
| *6* | *Armillaria mella* | Kim and Kim (1999) |
| *7* | *A.ochraceus 513* | Batomunkuev and Egorov (2001) |
| *8* | *Rhizopus chinensis 12* | Xiao-Lan et al (2005) |
| *9* | *Verticillium sp. Tj33* | Li et al (2007) |
| *10* | *Pleurotus eryngii* | Cha et al (2010) |
| *11* | *Cordyceps militaris* | Choi et al (2011) |
| *12* | *Paecilomyces tenuipes* | Kim et al (2011) |
| *13* | *Rhizopus microsporus* | Shulizhang (2015) |

### 3.2.3. From Actinomycetes

Actinomycetes thermovulgaris produces a fibrinolytic enzyme reported by. Sasirekha C (2012) isolated the Actinomycetes from soil samples collected from the slaughter house in and around salem. Streptoverticillium mobaraense isolated from soil sample proved its fibrinolytic activity by showing a clear zone around[12-13]. Fibrinolytic enzyme from Actinomycetes from different biotopes in Manipur is isolated by Debananda (2016)[27-28].

**Table-3.3- Actinomycetes Fibrinolytic Enzyme Producers**

| SL.No. | Microorganism | References |
|---|---|---|
| *1* | *Streptoverticillium mobaraense* | Moataza MS(2015) |
| *2* | *Actinomycetes* | Debananda (2016) |

### 3.2.4. Some Other fibrinolytic enzyme producing organisms

Nikai et al. (1984)[10] showed that hemorrhagic toxin of snake venoms produces a fibrinolytic enzyme[14]. These snake venoms also contain many components which are biologically active and effects hemostasis reported by White (2011)[15]. Fibrinolytic enzymes are also isolated from viperidae, elapidae and crotalidae snakes reported by Markland (1998), Eisenia fetida is the snake showed strong fibrinolytic activity than the other proved by Roch (1979)[21]. Cartwright (1974) and Gardell et al. (1989) isolated the fibrinolytic enzymes from vampire bat which showed the fibrinolytic activity[16].

**Table-3.4- Algae Fibrinolytic Enzyme Producers**

| SL.No. | Microorganism | References |
|---|---|---|
| *1* | *Codium intricatum* | Matsubara et al (1998) |
| *2* | *Codium latum* | Matsubara et al (1999) |
| *3* | *Codium divaricatum* | Matsubara et al (2000) |

**Table-3.5- Other Fibrinolytic Enzyme Producers**

| SL.No. | Microorganism | References |
|---|---|---|
| *1* | *Lumbricus rubellus* | Mihara et al (1993) |
| *2* | *Katsuwonus pelamis* | Sumi et al (1995) |
| *3* | *Earthworm* | Wang et al (2004) |
| *4* | *Agkistrodon blomhoffii ussurensis* | Sun et al (2006) |
| *5* | *Neries* | Zhang et al (2007) |
| *6* | *Eisenia andrei, Eisenia fetida and Lumbricus terrestris* | Verma and Pulicherla (2011) |

### 3.3. Microbial production of Fibrinolytic enzyme

Fungi were isolated from soil of rich vegetation using enrichment techniques. Fibrinolytic activity was also determined by artificial fibrin plate assay which synthesized by the method described by Astrup and Mullertz (1952)[17].

Soil is been collected from 10 to 12 cm deep from the earth surface. The soil samples are diluted in the Master Test Tubes. Diluted soil samples are inoculated by using pore plate method or spread plate method. Plates are incubated for 48 to 72 hrs or growth for 7 days for fungus. The fungi cultures were grown on Czapek's Dox Agar (CDA) + 0.5 percent yeast extract agar, acidified with phosphoric acid to pH 4.0 has been found a satisfactory medium for the growth and sporulation of many soil fungi and has been extensively used as an isolation medium .All the isolates were screened for both caseinolytic and fibrinolytic activities by measuring the lytic zone in a medium which may also include skim milk powder as the substrate using the methods described by Rajamani, Hilda and , Sekar Kumaran, (2011)[18]. Screenings of fibrinolytic enzymes was done using nutrient agar medium containing 2% casein and 2ml human serum by Dubey et al. (2011)[19].

Fibrinolytic activity was determined by serum, casein and fibrin plate method reported by Dubey et al. (2011)[19]. Fibrin plate was made at room temperature in a petridish containing 1.5% agarose, 0.2% human fibrinogen and 10U thrombin. Fungus culture containing mycelia was placed at the centre of the gel matrix of the fibrin plate and incubated for 24 hrs at 30ºC to observe the clot degradation. The plate which shows the clear zone was selected for further process.

### 3.4. Production of Fibrinolytic Enzyme

**"**Fermentation is the technique of biological conversion of complex substrates into simple compounds by various microorganisms such as bacteria and fungi". The products of fermentation are several antibiotics, peptides, enzymes and growth factors (Fibroblast growth factor, Nerve growth factor, harmones). They are called as bioactive compounds. They are also called as secondary metabolites.

Recently researchers have demonstrated that several of these secondary metabolites are industrially and economically important. They have been used in a variety of industries such as pharmaceutical and food, especially in the field of probiotics and preprobiotics.

Fermentation technique is carried out in two different processes:

1. Submerged fermentation

"Submerged Fermentation (SmF)/ Liquid Fermentation (LF) utilizes liquid substrates like molasses and broths. The bioactive compounds are secreted into the fermentation broth. The substrates are utilized quite rapidly; hence need to be constantly replaced/supplemented with nutrients"[21].

2. Solid state fermentation

"Solid state fermentation (SSF) is generally defined as the growth of the microorganism on solid material (moist) in absence or near absence of free water". In recent years SSF has gained and revealed much promise in the development of several bioprocess and products. It seems that two terms, Solid state fermentation and solid substrate have often been ambiguiously used[29].

### 3.4.1. Production of Fibrinolytic Enzyme by Submerged Fermentation (SmF).

Submerged fermentation is a method of manufacturing bio molecules in which enzymes and other reactive compounds are submerged in a liquid such as alcohol, oil or a nutrient broth. The process is used for a variety of purposes in industrial manufacturing.

"SmF is primarily used in the extraction of secondary metabolites that need to be used in liquid form. In case of SmF, the accumulation of a variety of intermediate metabolites results in lowering the enzyme activity and production efficiency".

The submerged fermentation technique is dominated by industrial enzymologists because it is well adapted for fermentation of aerobic bacteria which

are used to produce different types of enzymes like amylase, protease and other bacterial enzymes. SmF utilizes liquid substrates like molasses and broths. Purification of products is easier in SmF. Different microorganisms possess diverse physiological characteristics, so the selection of medium components is usually critical for fermentative production of fibrinolytic enzymes, it is necessary to optimize nutrient components and environmental conditions for cell growth and fibrinolytic enzyme production. Submerged liquid fermentations are traditionally used for the production of microbially derived enzymes[20].

The macrofungal fibrinolytic enzymes produced by a submerged mycelia culture are used in many medicina applicationsl. The optimal temperature of *Streptomyces megaspores* SD5 for enzyme synthesis is 55ºC, as the strain is isolated from a hot spring[22]. In some cases, fibrin was found to enhance the enzyme production.

"Fibrin as a substrate of fibrinolytic enzyme could activate or induce enzyme production during cultivation employed the statistical methods of fractional factorial design (FFD) and central composite design (CCD) to optimize the fermentation media for the production of Nattokinase and finally increased the fibrinolytic activity to 1300U/ml, which is about 6.5 times higher than the original value"[22].

The optimum temperature varies according to the kind of bacterium, the substrate used, as well as other environmental conditions during fermentation. Active production of the enzyme by *B.subtilis* LD.8547 was between 35ºC and 65ºC, with optimum activity at 50ºC, while the enzyme production by the marine bacterium *B.subtilis* A26 was maximum at temperature from 50ºC to 70ºC, with optimum of 60ºC [23].

Several workers have studied about the effect of optimum pH during the fermentation. The maximum production of the enzyme by *B.subtilis* LD.8547 was at a pH range of 7.0-10.0, with pH 8.0 as the optimum pH, while Ashipala and He (2008) reported pH 6.52 as the best for fibrinolytic enzyme production by *Bacillus subtilis* DC-2, using statistical programs[24].

Starch as a substrate with the concentration of 0.2% (w/v) played a active role in biosynthesis of fibrinolytic enzyme by *B.macerans* 3185. Liu et al, 2005 reported that the optimal carbon source for nattokinase production by *B.natto* NLSSE was maltose[10]. Glucose and sucrose had similar positive effects, while xylose and glycerol performed poorly. Deepak et al. (2008) reported glucose as the best C-source for *Bacillus subtilis*[25].

Liu et al. (2005) reported that, when inorganic nitrogen sources were used, very poor enzyme activities were achieved, and much higher activities were obtained with organic nitrogen sources, with soya bean being the optimal nitrogen source[10]. They also added that organic nitrogen sources (casein, soypeptone and soya meal) could bring out glutamine and other amino acids by enzyme catalysis, all of which were preferred nitrogen sources of *B.subtilis*[25].

### 3.4.2. Production of Fibrinolytic Enzyme by Solid state fermentation (SSF).

Solid substrate fermentation can be used to define only for those processes in which the substrate acts as a carbon or energy source, occurring in absence of or near absence of free water. Solid state fermentation can also be employed where an inert substrate is used as a solid support.

Peng Y (2003), studied production of the fibrinolytic enzyme from *F.oxysporum* using different SSF methods. This could help in increasing production and reducing the cost[26].

For the production of extracellular alkaline protease by *Bacillus subtilis* ASKK96 under solid state fermentation (SSF), different agro residues as substrate were used. The highest enzyme production was expressed with lentil husk as units per assay of dry substrate (3937.0 U/mg). Production parameters were optimized as incubation time 120h, extraction medium Triton-X100 1% initial moisture content 30%, initial pH 9.0. The high level of alkaline protease obtained in the medium containing arabinose followed by lactose, galactose and fructose. Among various nitrogen sources, beef extract was found to be the best inducer of alkaline protease, while

other nitrogen sources repressed enzyme production. Among metal salts $FeSO_4.7H_2O$ and MgSO4.7H2O was found to increase protease production. The maximum enzyme production (5759.2 U/mg) was observed with lentil husk in 1000mL of fermentation medium volume[31].

Namrata Sharma (2012) reported *Aspergillus terreus* JBPSI mustard oil cake being rich in protein offer potential benefits when utilized as substrate for fermentative production of enzymes. In this study, impact of diluents on the production of protease by *A.terreus* grown on mustard oil cake was done. Supplementation of D3 diluent increased the enzyme production significantly (110 U/g of dry solids). Enzyme production was maximum when mustard oil cakes inoculated with *A.terreus* was incubated at 40ºC temperature at 9.5 pH. Maximum protease production was obtained after 96 h of incubation period[47].

Kranthi et al. (2013) evaluated the two strains of *Aspergillus* sp.; *Aspergillus flavus* and *Aspergillus oryzae*. Wheat bran, sesame oil cake, groundnut oil cake, cotton seed oil cake and mustard oil cake are differen substrates used for the fermentation and results showed that enzyme production was maximum when wheat bran substrate for both *A.flavus* and *A.oryzae*. With *A.oryzae*, groundnut oil cake was also found to be effective substrate. Various process parameters including temperature, pH and nutrient supplements were optimized under solid state fermentation conditions. After 72hrs, *A.flavus* also proved to be a good producer of protease at pH 7.5 and 40ºC temperature and where as *A.oryzae* showed the maximum activity at pH 7.0 and 35ºC temperature after which the production was declined in both the cases. Among the various activators used *A.flavus* protease activity was enhanced with $ZnSO_4$ but for four *A.oryzae* the activity was increased with $FeSO_4$ whereas EDTA inhibited the protease activity of four *A.flavus* and PMSF inhibited *A.oryzae* protease activity[10].

Kranthi et al. (2013) highlighted that *Apergillus* species are known to produce various types of proteases. The isolated *A.flavus* found to be better producer of protease at pH 7.5 and temperature 45ºC indicating slight thermo stability. Various metal ions like $Zn^{2+}$, $Mg^{2+}$ , $Fe^{2+}$ , $Cu^{2+}$ , $Mn^{2+}$ shown to be effective activators of

protease activity and PMSF had given approximately 50% inhibition. Among the selected oil seed cakes sesame oil was shown to be a suitable substrate after wheat bran for the production of protease by *A.flavus*[10].

Thus in the present study SmF methodology is used because this helps in increasing fibrinolytic enzyme production with reducing the cost.

### 3.5. Purification of Fibrinolytic Enzyme

Purification of fibrinolytic enzymes is started by salting out the enzyme from its aqueous solution by means of a suitable electrolyte such as ammonium sulfate or sodium chloride. Organic solvents that are miscible with water are used for the precipitation from the broth but solvents like alkanes or alkenes should not be used for the enzyme precipitation. The precipitates obtained usually are amorphous, gummy masses[31].

The filtrate containing the enzyme also contains hundreds of other proteins of different molecular weight, it must be purified to know the clear enzyme property, reported by Dixon and Webb (1964)[31].

Kim et al. (1997) purified the fibrinolytic enzyme extracted from *Bacillus* sp. KA38 using ammonium sulphate treatment, diethylaminoethyl cellulose (DEAE) column and Mono Q column chromatography. The purity of the enzyme solution was further improved by liquid column chromatography[31].

K.Balaraman and G.Prabakaran (2006) used ion-exchange chromatography using Q-sepharose followed by gel filteration chromatography using sephacryl-S-300 to recover the fibrinolytic enzyme from *Bacillus sphaericus*[33]. Douchi is the fibrinolytic enzyme from Bacillus subtilis LD-8547 is purified by salting out, dialysis and gel filteration chromatography with sephadex-G-100 by Jun Yuan (2012)[41].

To purify the fibrinolytic enzyme obtained from *Bacillus pumilus* 2.g Diana Nur Afifah (2014) used ammonium sulphate precipitation and chromatography with phenyl sepharose 6 fast flow resin. Ponnuswamy vijayaraghavan (2015) purified the

fibrinolytic enzyme from the marine isolate pseudoalteromonas sp using ammonium sulphate precipitation, DEAE-cellulose ion exchange and casein agarose affinity chromatography[34].

Shulizhang (2015) used ammonium sulphate precipitation, DEAE-Sepharosefast flow and sephadex G-75 column chromatography to purify the fibrinolytic enzyme from Rhizopus microspores var.tuberosus. To achieve the recovery of fibrinolytic enzyme from tempen bongkrek IRA Sasmita (2018) used ammonium sulphate, ion-exchange chromatography on a column of DEAE-Sepharose and gel filteration chromatography using a sephadex G-100 column[35].

Sephadox G-100 is used mainly in protein chromatography. It is faster and alternative to dialysis; it requires a low dilution factor with high activity recoveries. DEAE-Cellulose is used for the separation and purification of proteins and nucleic acids. They lock the negatively charged proteins. The proteins are released into the resin by changing the pH of the solution as to change the charge on the protein[53-54].

### 3.6. Biochemical Characteristics of Purified Fibrinolytic Enzyme:

The biochemical characteristics of the purified Fibrinolytic Enzymes are studied by several researchers, here is the list given below in the table-3.6, which explains the various biochemical properties of purified fibrinolytic enzymes isolated from different microorganisms.

**Table-3.6- Biochemical Properties of Fibrinolytic Enzymes isolated and purified from various sources**

| Microorganism, Enzyme | Temperature stability and pH | Mol.wt, | References |
|---|---|---|---|
| B.*natto*, Nattokinase | Stable at less than 50ºC and at pH 7-12 | 27.2 kDa | Fujita et al (1993), Sumi et al, (1987) |
| *Bacillus* sp.CK, CK | Stable at less than 50ºC and pH at 7-10.5 | 28.2kDa | Kim et al (1996a,b) |
| *Bacillus* sp. KA38, *Jeot-gal enzyme* | Stable upto 40ºC and at pH7-9 | 41kDa | Kim et al.(1997) |
| *Streptomyces* sp Y405,SW-1 | Stable at 4-37º and pH at 4-9C, | 30kDa | Wang et al. (1999a) |
| *Bacillus* sp DJ-4, Subtilism DJ-4 | Stable at room temperature and at pH 4-11 | 29 kDa | Kim and Choi (2000) |
| *Bacillus* sp. KDO-13 | Stable at 50ºC and at pH 7-9 | 45kDa | Lee et al. (2001) |
| B.*amyloliquefaciens* DC-4,Subtilism DFE | Stable at less than 50ºC and at pH 6-10 | 28kDa, | Peng et al.(2003) |
| *Bacillus* sp QK02, Subtilism QK-2 | Stable at 40ºC for and at pH 3-12 | 28kDa | Ko et al.(2004) |
| B.*subtilis* A1,Bacillokinase II (BKII) | Stable upto 50ºC and at pH 4.0 | 31.4kDa | Jeong et al.(2004) |
| R. *Chinensis* 12 | Stable at 37ºC for and at pH 6.8-8.8 | 18kDa | Xiao-Lan et al.(2005) |

### 3.7. Application of Fibrinolytic Enzyme

Fibrinolytic enzyme (ex.*natto*) was consumed only for cardiovascular support but also to lower the blood pressure, it was the traditional belief. But in recent years this belief has been confirmed by several clinical trials proved by Sumi et al. and his collegues (1987). Serratiopeptidase which is isolated form serrapeptase breaks down artherosclerotic plaques those are accumulated in arteries. It digests non-living tissue and leaves live tissue alone, it effectively removes the deposition of fatty substances, cholesterol, calcium and fibrin inside the arteries[52].

The use of fibrinolytic proteases in laundry detergents accounts for approximately 25% of the total worldwide sales of enzymes. In the year 1956 under the trade name BIO-40 is the first detergent containing bacterial enzyme was introduced. Currently in the market all detergent which are in use are Serine proteases produced by *Bacillus* strains.

Shoemaker S (1986) fibrinolytic proteases are used in food industry for cheese making, baking, preparation of soya, hydrolysates and meat tenderization. Alkaline proteases solubilized proteins in waste through a multistep process to recover Liquid concentrates or dry solid of nutritional value for fish or livestock[47].

Fungal alkaline proteases are advantageous due to the ease of downstream processing to prepare a microbe-free enzyme. An alkaline protease from *Conidiobolus coronatus* was found to be compatible with commercial detergents used in India and retained 43% of its activity at 50ºC for 50 min in the presence of $Ca^{2+}$ (25mM) and glycine (1M). The use of enzyme as a alternatives to chemicals successfully proved in improving the leather quality, also in reducing the environmental pollution and also effectively saving the energy. The three different proteases namely Aquaderm, NUE and Pyase manufactures by Novo Nordisk are used in soaking, dehairing and bating of leather reported by Phadatare S U et al. (1993)[48].

These enzymes are excellent in blood- thinning and clot-dissolving. It also acts upon inflammation by thinning the fluids in the body that collected around injured areas and increasing fluid drainage, this also enhances tissue repair and reduces pain. It also has the ability to dissolve the dead and damaged tissue without harming living tissue, it guides the inflammatory cells to their targets reported by Klein and Kullich (2000), fibrinolytic proteases are also of major importance in food, leather, and detergent, pharmaceutical and waste management industries[10].

Potent fibrinolytic enzyme from a mutant *Bacillus subtilis* IMR-NK1 by Chen-Tien chang (2000)[39], can be used as natural agent for oral fibrinolytic therapy or thrombosis prevention. The enzyme obtained from *Bacillus subtilis* BK-17 by Yong kee Jeong (2001)[32], degraded the blood clot and was proved in thrombolytic therapy related to cardiovascular disease. K.Balaraman (2006) isolated Thrombinase which is obtained from *Bacillus sphaericus* converts inactive plasminogen to active plasmin *invivo*[33].

Sukpark (2010) reported that the fibrinolytic enzyme with the molecular weight of 17kDa from *Schizophyllum commune* is unique because it exerted a dual function in fibrin degradation which acts as a plasminogen activator. VK12 of *Ganoderma Lucidum* by Sekar kumaran (2011) showed a significant antithrombotic activity which is due to antiplatelet activity[40].

Douchi fibrinolytic enzyme obtained from *Bacillus subtilis* LD-8547 showed thrombolytic effect on carotid thrombosis in rabbits, it also increases the clotting and bleeding time, proved it as a useful tool for preventing and treating clinical thrombus[41]. Marine isolate, *Pseudoalteromonas sp*., IND11 a fibrinolytic agent have a potentiality to treat cardiovascular diseases such as heart attack and stroke[42]. Fibrinolytic enzyme from *Rhizopus microspores* var. tuberosus degrades fibrin directly and also activates plasminogen into plasmin to degrade fibrin, possible to use in the therapy of thrombosis[42].

Bacillus halodurans IND18 isolate used agro-industrial residues for the production of fibrinolytic enzyme, which digest the fibrin net of blood clot rapidly by Ponnuswamy vijiayaraghavan (2016). Hajra Ashraf (2017) illustrated that fibrinolytic enzymes like Nattokinase, Urokinase, Staphylokinase and Streptokinase are the therapeutic agent to control myocardial infarction, venous stroke and pulmonary clotting[42].

Since, fibrinolytic enzymes have wide varieties of application in various fields, in the present study an attempt is made to prove the therapeutic use of fibrinolytic enzyme extracted and purified from *Aspergillus tamarii* SAS02 which is isolated from the soil sample.

### 3.8. Mutational studies for the improvement of fibrinolytic enzyme production.

Biological production of proteins starts with the identification of strain followed by optimization process. To obtain a desired product with greater impact on economics strain improvement can be done.

In the year 1970 Hopwood and Ferguson insisted auxotrophic mutants with resistance to analogues used for production of aminoacids commercially. For isolating auxotrophic mutants penicillium is used. By using a classic mutagenesis for the mutation of two protease producing strains by *Bacillus licheniformis*. One strain was made insensitive by exposing it to UV and by using excess ammonia. The other strain synthesised high protease/ml independently of ammonium concentration in the medium reported by Bierbaum et al. (1994). Fleming et al. (1995) in his study showed that in vivo recombination can be used to prepare defined deletions and this deletion of the SPOIIAC gene in *B.licheniformis* gives rise to a sporulation deficient strain exhibiting extracellular serine protease synthesis[37].

Gupta et al. (2002) reported that microbial strain can be improved either by conventional mutagenesis (UV or chemical exposure) or by recombinant DNA technology (rDNA) for the increase in the protease production[31]. Conventional mutagenesis and current recombinant DNA technology have also been adopted to

improve enzyme production. Recently, Lai et al. (2004) showed successful doubling of the specific activity of fibrinolytic enzyme through random mutagenesis in vitro using Ethyl Methyl Sulphonate (EMS) [10]. Introducing genetically modified strain of *B.Pumilis* by rDNA technology (pBX96 plasmid) changed the carbon source from glucose to starch and also got rid of the inhibitory levels of high glucose concentration on protease production. Bai et al. (2004) enhanced the production of lactic acid by mutating the strain of *Rhizopus oryzae* using UV, diethyl sulphate (DES) [38].

*Streptococcus equisimilis* isolated from throat infected patients subjected to mutation using UV and N-methyl-N-nitro-N-Nitroso Guanidine (NTG), the strain showed the 146% increase in yield than the wild strain. This result indicated that the combination of UV and NTG were effective mutagenic agents for strain improvement reported by Abdelghani et al. (2005)[1].

Purohit et. al. (2006) used UV, heat and NTG (3-nitro, 5-methylguanidine) for the mutation of co-culture of *Aspergillus foetidus* and *Rhizopus oryzae* for the improvement of tannase and gallic acid production. Wild strain showed 44.2U/ml, mutated strain showed 53.6U/ml[34].

The strain *Bacillus subtilis* LD-8547 obtained from douche improved its activity from 2760 U/mL to 3280 U/mL to 3980 U/mL by mutating it with NTG and gamma radiation and proved it is a effective method for strain screening for high production by Wang et al. (2008)[10]. Tajima et al. (2009) mutated *Ashbya gossypii* which produces riboflavin using N-methyl-N-nitro-N-nitrosoguanidine increased its production of riboflavin 10 fold higher compared to the wild type[38].

Madhuri Doss et al. (2011) mutated *β-haemolytic streptococci* using UV radiation and showed an increase in 67% yield of streptokinase compared to the wild strain. It shows that UV irradiation is a effective mutagen for strain improvement. Penicillium oxalicum produces chitin deacetylase ($108.26 \pm 1.98 UL^{-1}$), when it is mutated using ethidium bromide (EtBr) and microwave irradiation improved its level to ($210.71 \pm 1.65 UL^{-1}$) was studied and proved by Pareek et al. (2011). Trichoderma

viride mutant was developed by using EMS followed by UV irradiation for cellulolytic enzyme production[10].

In the present study we have used UV irradiation as a mutagenic source for the strain improvement.

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54. Wikipedia,https://en.m.wikipeida.org/wiki/sephadex.

## 4. Materials and Methods:

In this study seven fertile soil samples were collected from different regions of Karnataka. These samples were used to isolate fungi strains for the extraction of fibrinolytic enzyme. Organisms were screened and maximum enzyme producing organism was identified by its molecular characterization, it was found to be *Aspergillus sp*. Further to enhance the production of fibrinolytic enzyme fermentation medium was prepared for submerged fermentation method. Fermentation medium was optimized for pH, temperature and inoculum size based on the characteristics of the strain. To enhance the production the process economization was done using carbon, nitrogen sources and metal ions. Enzyme inhibitors were identified. Purification and biochemical characterization of fibrinolytic enzyme was done, it was found to be *Aspergillus tamarii* SAS02. Enzyme activity of the fungal strain is compared with few bacterial species. Application of fibrinolytic enzyme as a therapeutic use is proved ex vivo in comparison with the commercially available myokinase. Mutational studies are carried out to check the potentiality between the parental strain and mutated strain.

**Study protocol is broadly classified into following categories:**

4.1. Isolation and screening of fungi from soil samples
4.2. Production of fibrinolytic enzyme
4.3. Optimization of fermentation conditions for biosynthesis of fibrinolytic enzyme
4.4. Purification of fibrinolytic enzyme
4.5. Characterization of fibrinolytic enzyme
4.6. Molecular characterization of fibrinolytic enzyme producing organism
4.7. Process economization for fibrinolytic enzyme production
4.8. Comparative studies on fibrinolytic enzyme from bacterial and fungal strains (*Aspergillus tamarii* SAS02)
4.9. Application of fibrinolytic enzyme on blood clot hydrolysis
4.10. Mutational studies of *Aspergillus tamarii* SAS02

**4.1. a. Isolation of fungi from soil samples:**

The fertile soil samples were collected from different regions of Karnataka like Bangalore (Bangaluru), Bijapur (Vijayapur), Raichur, and Tumkur from the places near garden, agricultural field, compost soil etc. The collected soil samples were used for isolation of fungi by serial dilution method on saboured dextrose agar (SDA). Twenty Fungi strains were isolated. The isolated strains were maintained on Czapek Dox's Agar (CZA) slants[1].

Further, the strains were labelled serially as S1 to S20. They were screened for fibrinolytic enzyme activity.

**4.1. b. Screening of fungi strains for fibrinolytic enzyme through plate assay:**

Twenty strains of fungi were screened for their fibrinolytic enzyme production by plate assay. The screening medium is as follows, a mixture consisting of 2ml of fresh healthy human plasma and 3ml of 1.2% molten agarose ($45^0$C) in 10 mM Tris-HCl buffer containing 70 mM $(NH_4)2SO_4$, 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 ($25^0$C to $27^0$C). 10 ml of sabouraud dextrose broth was incubated with the fungal strain and incubated at $30^0$C for 2 days. This culture was used for fibrin clot assay. 20 ml of fungal culture containing mycelia was placed at the centre of the gel matrix of the fibrin plate and incubated for 24 h at $30^0$ C. The diameters of the clear zones (plaque) were noted[2]. Among the twenty isolated fungi S1 and S2 showed fibrinolytic activity by showing its clear zone around the colony, S5 did not show any zone clearance around it, it was kept as fungal control strain.

The plates S1 and S2 with maximum zone clearance were selected as the fungal strain of maximum fibrinolytic enzyme producing strain from the soil. The S1, S2 and control - S5 strains were further sent to Agharkar Research Institute, Pune for its confirmation. There the fungal strain from the three plates was confirmed as *Aspergillus sp.*

The isolated strains from each of these three plates were labelled as SAS01, SAS02 and SAS05.

**4.1. c. Selection of isolate:**

The *Aspergillus sp* strain SAS02 was evolved as potential strain as it showed the maximum zone of clearance of 19 mm. Further this strain is used for estimating fibrinolytic enzyme activity and for the preparation of inoculum as mentioned later in 4.2a[2].

**4.1. d. Assay of crude extract of fibrinolytic enzyme:**

This was measured by the method of Anson (1939). The reaction mixture contained 1ml of 1.2% of bovine fibrin solution in Tris-HCl buffer (pH 8.0) and 1ml of cell-free supernatant (CFS). The reaction mixture was incubated for 2 h at $37^0$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[3]. The enzyme activity is carried out by tyrosine calibration curve[3,4] .

**Tyrosine Calibration Curve:**

The standard tyrosine solution is taken in different concentrations in different test tubes and diluted to 1 ml with distilled water. After addition of 5 ml alkaline copper reagent and 0.5 ml of Folin-Ciocalteu reagent (FCR) the reaction mixture is allowed to incubate for 20 min and OD was read at 660 nm. The result was plotted onto a graph[9]. A blank was prepared by the same procedure, except that TCA was added at '0' time i.e., in the control tube, TCA was added before the addition of the enzyme solution[9,10].

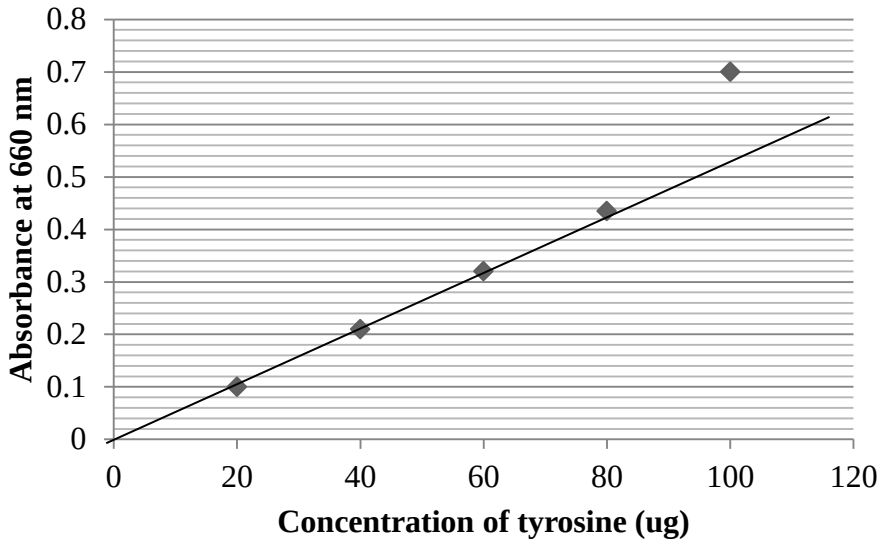


**Fig.4.1- Standard Tyrosine curve**

**Expression of the enzyme activity:**

One unit of fibrinolytic activity (IU) was defined as the amount of enzyme required to liberate 1µg of L-tyrosine/ml/min at $37^0$C.

**4.2. Production of fibrinolytic enzyme through submerged fermentation:**

Submerged fermentation was carried out for the production of fibrinolytic enzyme by employing *Aspergillus sp* SAS02 strain. Fermentation was carried out in 250 ml Erlenmeyer flask. The isolate was grown in Czapek-Dox media with the 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. The flasks containing 100ml of fermentation medium were autoclaved at $121^0$C for 20 minutes and cooled to room temperature. Then the flasks were inoculated with 1ml of spore suspension and the contents were thoroughly mixed and incubated for a period of 3-5 days at $30^0$ C[10].

**4.2. a. Preparation of inoculum:**

Spore suspensions were prepared on Czapek Dox's agar slants by adding 10ml of sterile distilled water containing 0.01% tween-80 and suspending the spores

with a sterile loop. One ml of the spore suspension containing about $1x10^7$ Spores/ml was used to inoculate experimental media in the flasks[10].

Fermentation media is optimized for its pH, temperature and inoculum size.

**4.3. Optimization of fermentation conditions for biosynthesis of fibrinolytic enzyme:**

The production of fibrinolytic enzyme under submerged fermentation mainly depends on various factors like initial pH, temperature, inoculum size etc. Hence, these parameters must be optimized in order to achieve higher yields of fibrinolytic enzyme. During this optimization process, once a particular parameter was optimized, the same optimum condition of that specific parameter was employed in the subsequent studies[5].

**4.3. a. Optimization of pH:**

Erlenmeyer flasks (250 ml) containing 100 ml of production medium was prepared by mixing with acid and alkali solution to obtain the required pH. The pH was adjusted in the range of 3-9 with increments of 1.0. Thus prepared flasks were cotton plugged and autoclaved at $121^0$C for 15 minutes. The flasks were inoculated and incubated with 72h 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 minutes and supernatant was assayed for the fibrinolytic enzyme activity as mentioned further in 4.1. d[6].

**4.3. b. Optimization of temperature:**

Production medium (100 ml) was separately taken in 250 ml Erlenmeyer flasks and prepared for submerged fermentation. The prepared flaks were inoculated with 72h old culture and incubated for 4-5 days at different temperature ranging from $30^0$C to $50^0$C. The cell free extract was assayed for the fibrinolytic enzyme activity as mentioned in 4.1.d[5].

**4.3. c. Optimization of inoculums size:**

The inoculums for fibrinolytic enzyme production were prepared using 72 h old culture of the test fungi at different levels ranging from 0.25 ml to 1.25 ml. The culture was grown at $40^0$C for 4-5 days and the cell free extract was assayed for the fibrinolytic enzyme activity as mentioned in earlier 4.1.d[6].

Fermentation media was optimized and the bulk production of the fibrinolytic enzyme was carried out using 500 ml fermentation medium with pH 6.0, incubating for 7days at $40^0$C. The crude enzyme extract was assayed for fibrinolytic enzyme activity as mentioned in 4.1.d and further used for the purification process[10].

**4.4. Purification of fibrinolytic enzyme:**

Protein purification is a process used to isolate proteins from cells, tissues or whole organisms. In the purification process protein and non-protein part will be separated to extract the protein of our interest[40].

The purification was carried out at $4^0$C on the crude extract. Purification measures adopted by us include:

- Ammonium sulphate fractionation.
- Dialysis
- Gel filtration chromatography
- Ion – exchange chromatography

After each step of enzyme purification fibrinolytic activity is calculated using the method explained in 4.1d and the protein content of samples is estimated at every stage of purification determined by the method of Bradford in 1976 to determine the specific activity of protein.

**BSA standard curve:**

The standard BSA solution is taken in different concentrations in different test tubes, it diluted to 5 ml with Bradford's reagent (10 mg Commassie Brilliant Blue G-250 dissolved in 0.5 ml of 95% ethanol and 10 ml of o-phosphoric acid was

added and the volume was made to 100 ml with distilled water) and OD was read at 595 nm. The standard BSA curve was plotted on OD against concentration of BSA[9].

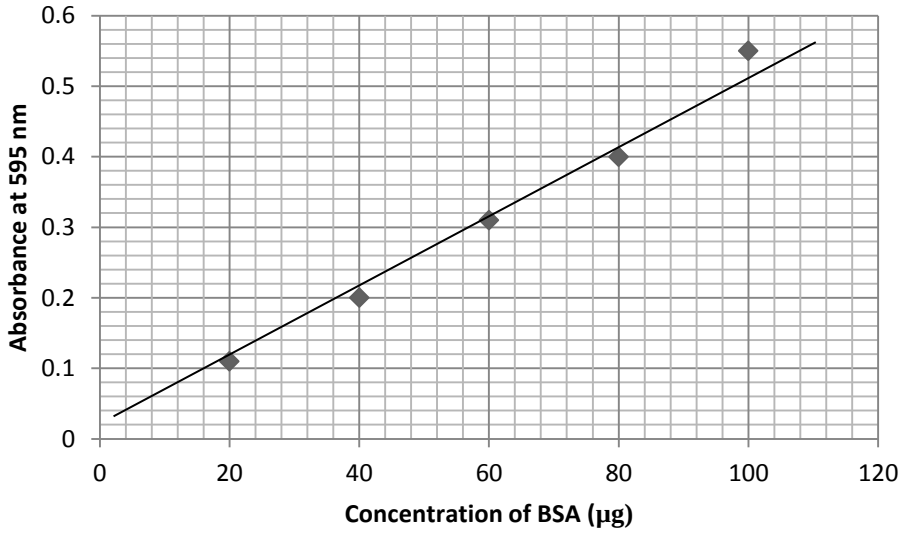


**Fig.4.2-Standard Bovine serum albumin curve**

### 4.4. a. Ammonium sulphate fractionation:

The first step in the protein purification process is ammonium sulphate precipitation; it is preferred for its high solubility and its dissolution in water. Standard Ammonium Sulphate Fractionation Table is referred is given below[49]. In this method the charges on a protein solution is neutralised by the addition of neutral salt like ammonium sulphate and protein is precipitated. This method is inexpensive and can be easily performed with the large volumes.

**Table: 4.1- Standard Ammonium Sulphate Fractionation**

| | Final percent saturation to be obtained | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 20 | 25 | 30 | 35 | 40 | 45 | 50 | 55 | 60 | 65 | 70 | 75 | 80 | 85 | 90 | 95 | 100 |
| Starting percent | Amount of ammonium sulfate to add (grams) per liter of solution at 20°C saturation | | | | | | | | | | | | | | | | |
| 0 | 113 | 144 | 176 | 208 | 242 | 277 | 314 | 351 | 390 | 430 | 472 | 516 | 561 | 608 | 657 | 708 | 761 |
| 5 | 85 | 115 | 146 | 179 | 212 | 246 | 282 | 319 | 358 | 397 | 439 | 481 | 526 | 572 | 621 | 671 | 723 |
| 10 | 57 | 86 | 117 | 149 | 182 | 216 | 251 | 287 | 325 | 364 | 405 | 447 | 491 | 537 | 584 | 634 | 685 |
| 15 | 28 | 58 | 88 | 119 | 151 | 185 | 219 | 255 | 293 | 331 | 371 | 413 | 456 | 501 | 548 | 596 | 647 |
| 20 | 0 | 29 | 59 | 89 | 121 | 154 | 188 | 223 | 260 | 298 | 337 | 378 | 421 | 465 | 511 | 559 | 609 |
| 25 | | 0 | 29 | 60 | 91 | 123 | 157 | 191 | 228 | 265 | 304 | 344 | 386 | 429 | 475 | 522 | 571 |
| 30 | | | 0 | 30 | 61 | 92 | 125 | 160 | 195 | 232 | 270 | 309 | 351 | 393 | 438 | 485 | 533 |
| 35 | | | | 0 | 30 | 62 | 94 | 128 | 163 | 199 | 236 | 275 | 316 | 358 | 402 | 447 | 495 |
| 40 | | | | | 0 | 31 | 63 | 96 | 130 | 166 | 202 | 241 | 281 | 322 | 365 | 410 | 457 |
| 45 | | | | | | 0 | 31 | 64 | 98 | 132 | 169 | 206 | 245 | 286 | 329 | 373 | 419 |
| 50 | | | | | | | 0 | 32 | 65 | 99 | 135 | 172 | 210 | 250 | 292 | 335 | 381 |
| 55 | | | | | | | | 0 | 33 | 66 | 101 | 138 | 175 | 215 | 256 | 298 | 343 |
| 60 | | | | | | | | | 0 | 33 | 67 | 103 | 140 | 179 | 219 | 261 | 305 |
| 65 | | | | | | | | | | 0 | 34 | 69 | 105 | 143 | 183 | 224 | 267 |
| 70 | | | | | | | | | | | 0 | 34 | 70 | 107 | 146 | 186 | 228 |
| 75 | | | | | | | | | | | | 0 | 35 | 72 | 110 | 149 | 190 |
| 80 | | | | | | | | | | | | | 0 | 36 | 73 | 112 | 152 |
| 85 | | | | | | | | | | | | | | 0 | 37 | 75 | 114 |
| 90 | | | | | | | | | | | | | | | 0 | 37 | 76 |
| 95 | | | | | | | | | | | | | | | | 0 | 38 |

The crude protein supernatant was precipitated with ammonium sulphate (till 75% saturation) at $4^0$C through constant stirring. The proteins were retrieved by centrifuging at 10,000 rpm for 10 min at $4^0$C. The pellets of proteins were dissolved with a minimum volume of 50 mM Tris-HCl buffer (pH 8.0)[38].

In this process the enzyme activity was 185 U/ml with 100% yield of the protein, 1% yield of fold purity; to increase the fold purity of the enzyme dialysis is carried out where the salts are removed.

**4.4. b. Dialysis:**

Small molecules like salts are removed by this process. The precipitates of proteins were dissolved with a minimum volume of 50 mM Tris-HCl buffer (pH 8.0)[38].

The protein sample was loaded onto the pre-activated dialysis membrane (Himedia, cutoff range 12 kD). The dialysis membrane was then hung into the 500 ml of buffer (Tris-HCl, pH 8.0) with 10 times lesser strength (i.e., 5 mM). The experiment was carried out for 8-12 h with 2 changes in buffer, at 4ºC[38]. The salts moves out from the semi permeable membrane and the proteins left behind in the dialysis membrane. The precipitates of proteins were dissolved with a minimum volume of 50 mM Tris-HCl buffer (pH 8.0)[38].

In this process the enzyme activity was 180 U/ml with 84.5% yield of the protein; 115.13% fold purity, to increase the fold purity of the enzyme gel filtration chromatography is carried out where the enzyme is purified based on its size.

**4.4. c. Gel filtration chromatography:**

In this method proteins are separated based on their size.

The Sephadex-G-100 (Sigma-Aldrich) column matrix was activated by heating at $50^0$C for 30 min with constant shaking in the presence of 50 mM Tris-HCl buffer (pH 8.0). The matrix was then loaded onto the column. After packing, the column was run with 10 times the volume of buffer as a washing step. The protein sample was loaded and column was connected to the buffer reservoir. 30 fractions of 2 ml were collected. The fractions were analyzed for protein and enzyme activity[38].

In this process the enzyme activity was 126 U/ml with 51.4% yield of the protein; 132.48% fold purity, to increase the fold purity of the enzyme ion exchange chromatography is carried out where the enzyme is purified based on its charges.

**4.4. d. Ion – exchange chromatography:**

This method is used particularly for the separation of proteins and enzymes on the basis of difference in their charges.

The matrix (DEAE-Cellulose) was then activated by mixing thoroughly with 50mM Tris-HCl buffer (pH 8.0) buffer as used and explained by Sekar et al., 2011. The pH was set to 8.0 by using the appropriate buffer component. The matrix was loaded onto a glass column fitted with glass wool. A volume of 500 ml of 50 mM Tris-HCl buffer (pH 8.0) was run through the column initially before loading the protein sample. A bridge tube was set between beakers containing 50 mM and 0.5 M Tris-HCl buffer (pH 8.0). This was set as a reservoir for the column and the column was run with the sample protein. Thirty fractions were collected of 2 ml and were analyzed for protein and enzyme activity[28].

Here we obtained 96 U/ml with the yield of 16.19%, fold purity is 320.07%.

The molecular weight of a protein (fibrinolytic enzyme) is determined by Polyacrylamide gel electrophoresis technique (PAGE).

### 4.4. e. Molecular weight determination by using PAGE:

**"**Polyacrylamide gel electrophoresis technique is widely used in biochemistry to separate biological macromolecules like proteins. PAGE uses a gel made by polymerizing acrylamide monomers with methylene bis acrylamide. These gels have a smaller pore size that enables the efficient separation of proteins. It separates protein based on their molecular weight"[24].

Clean and dry, grease free PAGE glass plates were taken and assembled using spacers and clips. The bottom of the assembly was sealed using agar. Stalking gel was cast on the separating gel with immediate insertion of comb. After the gel is polymerized, the comb was removed carefully. The gel was then assembled into PAGE unit and electrophoresis buffer (Tris- 3 g, Glycine- 14.3 g, SDS- 2.0 g for 1 litre) was added into upper and lower tank. The protein sample was mixed with sample buffer (1.5 M Tris-Cl (pH 8.0) 0.625 ml, 20% SDS 1.0 ml, Glycerol 1.0 ml, 2-mercaptoethanol 0.5 ml, 0.2% Bromophenol blue) in 1:1 ratio and boiled for 2-10 min. The sample was loaded into the gel and the gel was run at constant current of 50-150V for 3-4 hr[24].

**4.4. f. Silver Staining of Proteins:**

"Silver staining is used to detect proteins after electrophoretic separation on polyacrylamine gels".

The gel was removed carefully and stained using silver staining method[13].The proteins were visualized by silver staining according to Blum et al., (1987). The gel was incubated in pretreatment solution (0.08 g of sodium thiosulphate in 100 ml) for 5 min and washed for 5 times in distilled water with 5 min of interval. The gel was incubated with silver nitrate solution (0.2 g of $AgNO_3$ and 75 µl formaldehyde in 100 ml) for 20 min in dark followed by wash with distilled water for 2 min. The gel was then immersed in developer (2 g $Na_2CO_3$ and 75 µl of formaldehyde in 100 ml) till the development of brown bands. The reaction was stopped before the gel turns brown[33]. The gel was soaked in destaining solution (30 mM potassium ferricyanide, 100 mM sodium thiosulphate) for 10 min and replaced with distilled water and constantly washed for 5 min. This step was repeated for 5 times.

The purified enzyme is characterized for its optimum pH, optimum temperature and temperature stability study and enzyme kinetics.

**4.5. Characterization of fibrinolytic enzyme:**

**4.5. a. Determination of optimum pH:**

The optimum pH of Fibrinolytic enzyme was determined with 1% fibrin (w/v) as substrate dissolved in different buffers (acetate buffer pH 4.0, Citrate buffer, pH 5.0 and 6.0, phosphate buffer pH 7.0, Tris-HCl buffer, pH 8.0, glycine-NaOH buffer, pH 9.0, 10.0 and 11.0). The activity of the enzyme is assayed as mentioned in 4.1d[21].

**4.5. b. Determination of optimum temperature:**

After the optimum pH was known, the optimum temperature of fibrinolytic enzyme activity was determined by incubating the reaction mixture at different temperatures in a range from 37-65$^0$C in 50 mM Tris-HCl buffer (pH 8.0) for 10 min. The activity of the enzyme is assayed as mentioned in 4.1d[21].

**4.5. c. Temperature stability analysis:**

The stability of the enzyme was analyzed at 37, 40, 45, 50, 55, 60 and $65^0$C for 15, 30 min and 60 min. The enzyme was incubated in respective temperatures and assayed at respective time. The activity of the enzyme is assayed as mentioned in 4.1d[21].

**4.5. d. Kinetic studies:**

The study of enzyme kinetics is important to know about how enzymes work and how they behave in the living system.

The Km of fibrinolytic enzyme was determined against different concentrations of fibrinogen. The reciprocal velocity of the enzyme reaction (1/[V]) was plotted against reciprocal concentrations of fibrin (1/[S]) and Km was determined[15]. Km is the concentration of the substrate and Vmax is the substrate catalysed per second.

Few metal ions and inhibitors, inhibits the production of fibrinolytic enzyme, description is given below.

**4.5. e. Determination of metal ions and inhibitors for the inhibition of fibrinolytic enzyme production:**

The metal ions in the form of their salts like $HgCl_2$, $CaCl_2$, FeCl3, $AgNO_3$, $ZnSO_4$, $MgSO_4$, $MnSO_4$, and $CuSO_4$ were analysed. The effect of various enzyme inhibitors (at 2 mM) such as PMSF, 1-10, Phenanthroline, p-Chloromercuryl benzoate, EDTA, detergent like SDS (1%), bleaching agent like $H_2O_2$ (1%) and salt solution NaCl (1M)[21].

$HgCl_2$, PMSF, 1-10, Phenanthroline and EDTA are the inhibitors which showed complete inhibition of fibrinolytic enzyme production.

**4.6. Molecular characterization of fibrinolytic enzyme producing organisms:**

Molecular characterization is carried out to determine the strain of *Aspergillus sp.* SAS02 both by its phenotypic and genotypic identification.

**4.6. a. Phenotypic Identification:**

The fungal colonies grown on the plate were studied by colony characteristics and Lacto-Phenol cotton blue staining. Lacto-Phenol cotton blue is used because- Phenol-kills the live organisms; lactic acid -preserves structure, cotton blue –stains the chitin in the fungal cell wall[43].

**Staining procedure is as follows:**

1. A drop of seventy percent alcohol is placed on a clean microscopic slid
2. Filamentous fungi are removed from the culture plate using sterile loop and place it on the drop of the seventy percent alcohol
3. One or two drops of the lacto-phenol cotton blue stain is added before the alcohol dries out
4. Layer the cover slip gently onto the slide by avoiding the air bubbles
5. Slide is placed under the 10X lens in the microscope for the Initial examination
6. For the detailed examination of spores the lens power is switched to 40X lens[43].

The characteristics were compared with the known organisms from the Literature.

**4.6. b. Molecular (genotypic) Identification:**

**Colony PCR**

PCR was performed by picking a colony directly from the plate the care was take to avoid any contamination.

**PCR Amplification**

Polymerase chain reaction allows the amplification of DNA sequencing in an exponential way using repeated thermal cycling; it allows the generation of many millions of copies of DNA using heating and cooling cycles.

**PCR was performed using the standard protocols.**

PCR was performed as follows in a total volume of 50µl in a 0.2 ml thin walled PCR tube.

| Components | Volume |
|---|---|
| Nuclease free water | 32 µl |
| Colony from plate | 2.0 µl |
| Forward Primer (10µM) | 2.0 µl |
| Reverse Primer (10µM) | 2.0 µl |
| 5xReaction Buffer | 10 µl |
| dNTP Mix (10mM) | 1 µl |
| Taq DNA polymerase (2.5U/µl) | 1 µl |
| **Total volume** | **50 µl** |

Internal transcribed spacer (ITS) is the spacer present between the small subunit ribosomal RNA and large subunit ribosomal RNA. Amplification of the (ITS1, ITS4 and 5.8 S rRNA Gene) for fungus sample was performed using following primer. ITS1 is situated between 18S and 5.8S rRNA. These primers amplify a wide range of fungal strains and work to analyze DNA isolated from individual organism[44].

**The amplification was carried out in a Thermocycler (DNA-AMP Bhat Biotech) using the following program, in Bangalore.**

**ITS Primers (5´ to 3´ )**

ITS1 F TCCGTAGGTGAACCTGCGC

ITS4 R TCCTCCGCTTATTGATATGC

Initial denaturation was carried out at $94^0$C for 10 minutes followed by 35 cycles of denaturation at $94^0$C for 1 minute, annealing at $56^0$C for 1 minute and extension at $72^0$C for 1 minute. Final extension was carried out at $72^0$C for 10 minutes.

The PCR products were purified to remove unincorporated dNTPS and Primers before sequencing using PCR purification kit (GENEASY GEL ELUTION KIT, Bhat Biotech India Pvt Ltd).

**4.6. c. Sequencing:**

Both strands of the rDNA region amplified by PCR were sequenced by automated DNA sequence -3037*xl* DNA analyzer from Applied Bio systems using BigDye® Terminator v3.1 cycle sequencing Kit (Applied Bio systems). Sequence data were aligned and dendrograms were generated using Sequence Analysis Software version 5.2 from applied bio systems. The sequences obtained for plus and minus strands were aligned using appropriate software before performing bioinformatics.

**4.6. d. Bioinformatics analysis:**

**"**Bioinformatics is the science of collecting and analysing complex biological data. It develops the methods and software tools for understanding biological data".

Sequences were compared to the non-redundant NCBI database using BLASTN, with the default settings used to find sequences closest to each other. The Expected value and e values were noted for the most similar sequences. Ten similar neighboring sequences were aligned using programme CLUSTAL W2. The multiple-alignment file thus obtained was then used to create a Phylogram using the MEGA5 software.

E-value- "Is the probability due to chance, that there is another alignment with a similarity greater than the given S score"[45].

With this molecular identification the fungal stain is named as *Aspergillus tamarii* SAS02, which is further used for the process economization of the fibrinolytic enzyme production.

**4.7. Process economization for fibrinolytic enzyme biosynthesis**

The main objective of this study was to evaluate the effect of different carbon and nitrogen sources on the biosynthesis of fibrinolytic enzyme by *Aspergillus tamarii* SAS02 under submerged fermentation. The studies on nutrient requirements of *Aspergillus tamarii* SAS02 conducted and have shown that carbon and nitrogen were essential for cell growth as well as fibrinolytic enzyme biosynthesis, though the degree of production varied with different carbon and nitrogen sources present in the

fermentation medium. Under the present study nutrient sources like carbon, nitrogen (organic, inorganic) and metal ions were supplemented to the fermentation medium to evaluate the yield of fibrinolytic enzyme under submerged fermentation.

The proper supplementation of desired nutrients and favourable conditions are necessary for growth and physiological activities of an organism. The above said nutrients were provided for maximal enzyme production under submerged fermentation process[25].

**4.7. a. Enhancement for the production of fibrinolytic enzyme biosynthesis is carried out using carbon sources:**

A set of conical flasks with 100 ml of production medium supplemented with a particular carbon source with concentrations ranging from 0.25% to 1.25% with increments of 0.25%. The different carbon sources like monosaccharide's (glucose) and disaccharide's (sucrose and maltose) were used in the present study[25].

Glucose showed the better result than the sucrose and maltose.

**4.7. b. Enhancement for the production of fibrinolytic enzyme biosynthesis is carried out using nitrogen sources:**

Here both organic and inorganic nitrogen sources were supplemented for better yield.

A set of conical flasks with 100 ml of production medium supplemented with different organic nitrogen sources such as yeast extract, malt extract, and peptone extract were added to the Czapek's Dox media at concentration ranging from 0.25% to 1.25% with increments of 0.25%. The culture was grown for 96 h and production of enzyme was monitored for every 24 h[25].

Peptone showed the better result than the yeast extract and malt extract.

A set of conical flasks with 100 ml of production medium supplemented with different inorganic nitrogen sources like Ammonium sulphate, Ammonium chloride, Ammonium nitrate were screened for their effect on production of enzyme at 0.25% to 1.25% concentrations in Czapek Dox media with increments of 0.25%. The culture was grown for 96 h with a enzyme assay at every 24 h[25].

Ammonium chloride showed the better result than the Ammonium sulphate and Ammonium nitrate.

**4.7. c. Enhancement for the production of fibrinolytic enzyme biosynthesis is carried out using metal ions:**

The different trace metals in the form of their salts such as $CuSO_4$, $FeSO_4$, $ZnSO_4$ and $MgSO_4$ were supplemented with the concentration of 0.01% to 0.03% in Czapek Dox media. The salt solutions were prepared by using double distilled water, added separately in different conical flasks containing Czapek Dox media and kept for fermentation and assayed for fibrinplytic enzyme activity at every 24 hr. The effect of various metal ions on the fibrinolytic enzyme was assayed. The metal ions were tested at 20 mM concentration[25].

$ZnSO_4$ showed the better result than the $CuSO_4$, $FeSO_4$ and $MgSO_4$.

**4.8. Comparative studies on fibrinolytic enzyme from bacterial and fungal strains (*Aspergillus tamarii* SAS02):**

To prove that *Aspergillus tamarii* SAS02 is potential source of fibrinolytic enzyme than the bacterial strain. The fungal strain has been compared with bacterial strains such as *Bacillus sp*, *Pseudomonas sp* and *E.coli* for the biosynthesis of fibrinolytic enzyme. *Bacillus sp* showed 17.5 IU/ml; *Pseudomonas sp* showed 14.1 IU/ml; *E.coli sp* showed 12.5 IU/ml. *Aspergillus tamarii* SAS02 showed 180 IU/ml a high level of fibrinolytic enzyme production as compared to bacteria[22].

**4.9. Application of fibrinolytic enzyme on blood clot hydrolysis (ex-vivo):**

To check the efficacy of the isolated strain Aspergillus tamarii SAS02, the following procedure was carried out-

The blood clot was formed in capillary tube by spontaneous coagulation in glass capillary tube using human blood (fresh). The capillary tubes were kept in different petriplates containing saline, partially purified *Aspergillus tamarii* SAS02 fibrinolytic enzyme (200 IU/ml) and commercially available fibrinolytic enzyme (Brand name – Myokinase 200 IU/ml) were incubated for 15 – 18 hrs, during this

time petriplates were shaken 3-4 times and the saline were used as control. The results were observed that there is a clot hydrolysis by using partially purified enzyme and also in commercially used enzyme. Hence finally fibrinolytic enzyme showed clot hydrolysis[23].

### 4.10. Mutational studies of *Aspergillus tamarii* SAS02

The main aim of mutational studies is to check the enhancement or deterioration of the enzyme production in the mutated strain when compared to the parental strain. Mutation of Aspergillus tamarii SAS02 is carried out using few mutagens like UV radiation, Diethylstilbetrol(DES), N-methyl-N-nitro-N-nitrosoguanidine (NTG), Ethyl methanesulfonate (EMS).

In the present study strain *Aspergillus tamarii* SAS02 was subjected to UV irradiation to induce mutation.

Spore suspensions of the *Aspergillus tamarii* SAS02 were irradiated using a 15W Phillips UV lamp. Four plates of *Aspergillus tamarii* SAS02 were kept at varying distances of 5, 10, 15 and 20 cm one below the other for 15 min. The plate kept at 15cm distance is chosen for the determination of enzyme activity. The irradiation was performed in a dark room and the irradiated suspensions were protected from light until plating was done on Czapek Dox agar in order to minimize any photo-reactivation effects as described by Gardener et al. and Banik[43].

The mutated strain is labelled as *Aspergillus tamarii* SAS 02mu.

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## 5. Results and Discussion:

The result obtained after employing the methodology as mentioned in chapter four are presented with tables, figures and plates sequentially. This was explained and discussed under the suitable headings as below.

### 5.1. a. Isolation of *Aspergillus sp.* for fibrinolytic enzyme production:

Soil provides a heterogenic and complex environment for all soil inhabitants. In soil diverse group of different kinds of microorganisms are present. Soil is the rich sources for potential enzymes producing organisms especially for fungi. Therefore, in the present study soil has been chosen as a source. The soil samples were collected from various places of Karnataka, India for the isolation of fungi strains. Totally, 20 strains of fungi were isolated. After the isolation of fungi strains, they were subjected to rapid screening for the production of fibrinolytic enzyme by rapid plate assay method.

**Table -5.1- Isolates of fungi from different soil samples**

| SL.No | Soil samples collected place | Source | No. of isolates |
|---|---|---|---|
| 1. | Bangalore-IIHR | Field soil | 02 |
| 2. | Bangalore-KMF | Field soil | 02 |
| 3. | Bangalore-Rabbit farm | Compost soil | 01 |
| 4. | Bangalore university campus | Garden soil | 05 |
| 5. | Bijapur | Cultivated field soil | 03 |
| 6. | Raichur | Cultivated field soil | 03 |
| 7. | Tumkur | Cultivated field soil and coconut plantation soil | 04 |
| | **Total** | **_** | **20** |

The isolation pattern of fungi is presented in table – 5.1. In the present study, thirty strains of fungi were isolated from seven different fertile soil samples and the strains were named serially from SAS01 to SAS20. All the isolates were isolated from different regions of Karnataka such as Tumkur, Bangalore and Bijapur. The seven different kinds of soil samples were used for the isolation of fungi. Soil samples collected from different regions is stated in the table-5.1.

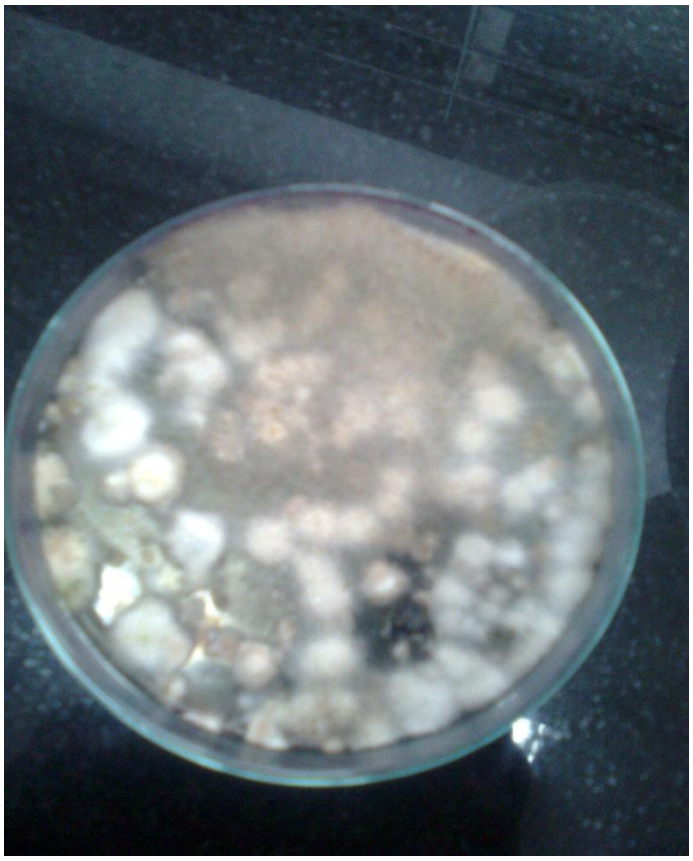


**Plate-5.1-Isolate of fungi**

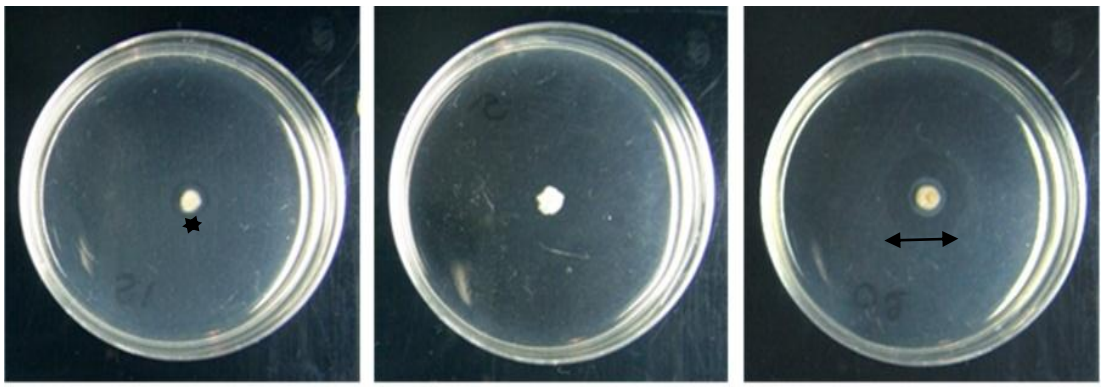
**5.1. b. Rapid screening of fibrinolytic enzyme producers by plate assay method:**

All the 20 isolates are isolated from 7 different soil samples, exhibited the zone of various diameters on rapid plate assay method. For the convenience the grouping of strains of fungi isolated from the soils has been done on the basis of zone diameter they exhibited. Therefore, it is proposed that the strains exhibiting zone diameter above 1 cm are referred as good fibrinolytic enzyme producer, those strains with zone diameter 0.5 to 1 cm and those having below 0.5 cm are referred to as

moderate or poor fibrinolytic enzyme producers. As per the grouping, the strain *Aspergillus sp.* SAS02 exhibited higher zone diameter of 19 mm (1.9cm) and it was considered as potential strain for fibrinolytic enzyme production. As such, strains SAS03, SAS06, SAS09, SAS16, and SAS20 are treated as moderate fibrinolytic enzyme producers and remaining isolates were treated as poor fibrinolytic enzyme producers. Therefore, on the basis of results observed on the rapid plate assay method, it was considered that the strain *Aspergillus sp.* SAS02 is a potential fibrinolytic enzyme producer.

**Table: 5.2 - Shows the three fungal samples which shows the zone of clearance**

| Sl.No | Sample Name | Diameters of the clear zone | Inference |
|---|---|---|---|
| 1 | S1 | 12mm | Positive |
| 2 | S5 | No clear zone | Negative |
| 3 | S2 | 19mm | Positive |



S1 S5 S2

***Aspergillus sp.*SAS02**

**Plate-5.2. of the 3 fungal samples, S1 and S2 are producing fibrinolytic enzymes and sample S5 (control) is not producing fibrinolytic enzymes.**

**5.1. c. Selection of isolate:** From the three fungal isolates S2 showed the higher zone clearance of about 19mm and it is chosen for our studies, the strain is named as *Aspergillus sp*.SAS02.

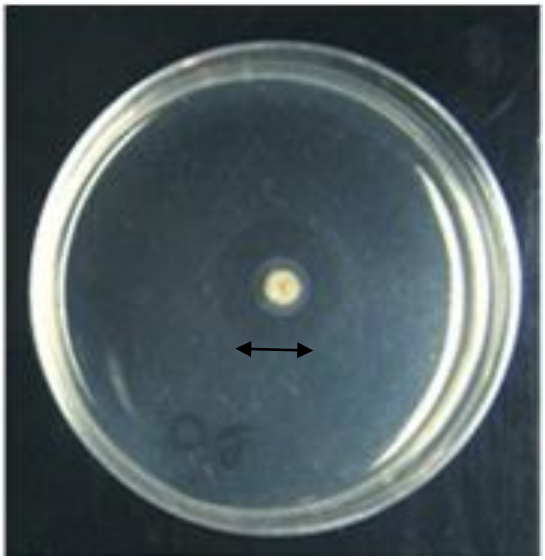


**Plate-5.3: Isolate of *Aspergillus sp*. SAS02**

**5.1. d.** Assay of crude extract of fibrinolytic enzyme is carried out using the method described in 4.1d. The amount of protein released during the assay is calculated using standard tyrosine calibration curve and the result is tabulated in the table-5.

**5.2. Production of fibrinolytic enzyme through submerged fermentation:**

Production media is prepared as described in 4.2. The flasks were inoculated with 1ml of spore suspension of *Aspergillus sp*.SAS02.

**5.3. Optimization of fermentation parameters:**

The appropriate selection of medium components based on both aspects of regulatory effects and economy is the goal in designing the chemical compositions of the fermentation media, where nutritional requirement for growth and production must be appropriate. Fast formation and high concentration of the desired product are the criteria for the qualitative and quantitative supplement of nutrients and other

ingredients. Bacteria, yeast, and fungi can grow well on submerged fermentation and found very good applications in submerged fermentation processes. Submerged fermentation (SmF) techniques have been successfully used for enzyme production by enzyme manufacturer's worldwide[18].

In general, production of fibrinolytic enzyme is either a constitutive or partially inducible property. The culture conditions that promote fibrinolytic enzyme production are found to be significantly different from that of promoting cell growth reported by Moon and Parulaker (1993). Usually, in the production of fibrinolytic enzyme at an industrial scale, technical media are used reported by Aunstrup (1980). There is no defined medium established for the production of alkaline fibrinolytic enzyme, since the constituents of a medium and there concentrations vary with organism and fermentation conditions[22].

Generally, the outcome of any fermentation process depends on the substrate, fermenting organism. A proper balance needs to be achieved among the three factors in order to achieve maximum product recovery. To maximize any product/enzyme production by isolated organisms, the basic need is to have preliminary information on growth conditions and its associated enzyme characteristics showed by Pandey *et al*. (2006), which helps in the designing of proper fermentation conditions for economic production. The important parameters that govern the solid state fermentation process and the product recovery are moisture, initial pH, and temperature and inoculum size of the fermenting organism. In order to achieve maximum fibrinolytic enzyme production these parameters need to be optimized[4].

**5.3.a. Effect of pH on fibrinolytic enzyme biosynthesis:**

An important factor that affects the performance of fermentation is the initial pH of the fermentation medium. Initial pH level of the medium is one of the crucial factor for the successful fibrinolytic enzyme production under submerge fermentation. It is noticed that for the industrial fermentation the control of pH of the medium at optimum level is essential for achieving maximum product formation[19].

The effect of different initial pH of synthetic medium on fibrinolytic enzyme production by *Aspergillus sp.* SAS02 is shown below. The effect of initial pH on fermentation medium revealed that the yield of fibrinolytic enzyme increased with the increase in the initial pH of the production medium up to pH 6. These increasing peaks were observed up to 72h of fermentation period and there after the yield decreased as pH levels and fermentation period increased. The maximum fibrinolytic enzyme activity 150 U/ml was obtained at pH 6 for 72h of fermentation period. The least fibrinolytic enzyme activity was observed at pH 3 with *Aspergillus sp.* SAS02 strain and it showed 10 U/ml at 72h of fermentation period.

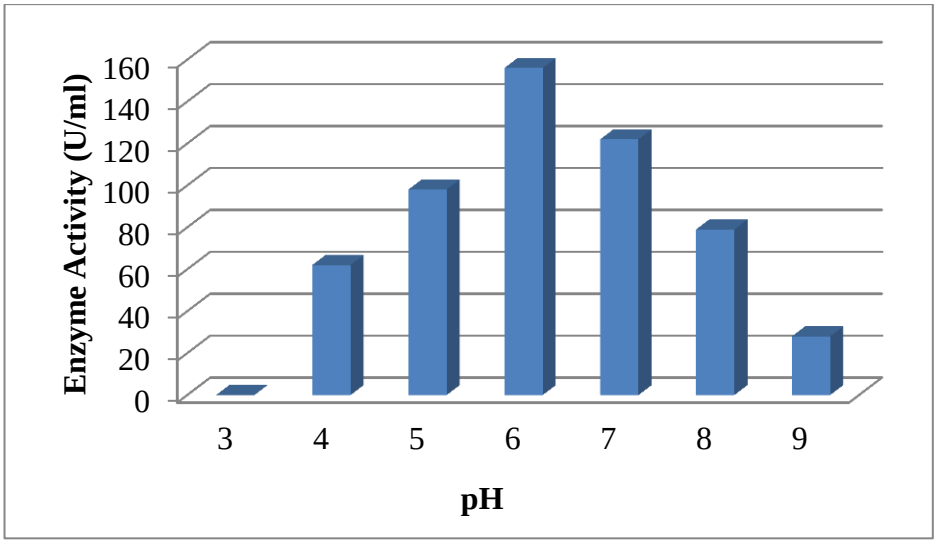



**Fig.5.1- Effect of pH on biosynthesis of fibrinolytic enzyme**

One organism may secrete variable amounts and types of enzymes depending upon the pH and composition of medium. Changes in pH may also cause denaturation of enzyme resulting in loss of catalytic activity. It may also cause changes in the ionic state of substrate which may result in the formation of charged particles which may not correspond with the ionic activity sites of the enzyme.

The kinetics of pH variations depends highly on the microorganisms with *Aspergillus sp*, *penicillin sp*, and *rhizopus sp*, the pH can drop very quickly below 3 for other types of fungi like *trichoderma*, *sporotrichum* and *pleurotu sp*, the pH is

more stable between 4 and 5. Most of the fungi are capable of growing in a wide range with pH 4-8. The response of the microorganism of same species differs by variation in pH[22].

The culture pH also strongly affects many enzymatic processes and transport of various components across the cell membrane. The decline in the pH may also be due to production of acidic products. In view of close relationship between fibrinolytic enzyme synthesis and utilization of nitrogenous compounds, pH variations during fermentation may indicate kinetic information about the fibrinolytic enzyme production, such as the start and end of the fibrinolytic enzyme production. When ammonium ions are used, the medium turned acidic, while it turned alkaline when organic nitrogen such as amino acids or peptides are metabolized[23].

The data obtained in the present study on the effect of initial pH in fermentation medium is shown in graph which reveals that the production of fibrinolytic enzyme increased with the increase in the initial pH up to pH 6 and thereafter the decrease in fibrinolytic enzyme activity occurred. The maximum production of fibrinolytic enzyme activity 150 U/ml was obtained at pH 6 and the minimum production of fibrinolytic enzyme activity 10 U/ml was observed at pH 3 with *Aspergillus sp.* SAS02 strain. Most microbial extracellular enzymes are produced at high levels when their growth pH is near to the optimal pH which is required for the maximal enzyme activity.

The initial pH of the medium mainly depends on the carbon sources used during the fermentation. However, the kinetics of pH variation depends highly on the microorganism. The fibrinolytic enzyme production increased as the initial pH of the medium increased and reached maximum at pH 6. This shows alkalophilic nature of the fungus. The present experimental results indicate that the isolate is sensitive to pH because the change in initial growth medium pH affects enzyme production. It has been noted that the most important characteristics of alkalophilic microorganisms is their strong dependence on extracellular pH for cell growth and enzyme production. Maximum alkaline fibrinolytic enzyme production by different *Aspergillus sp* has been reported at various optimum initial pH of the medium[5].

Most microbial extracellular enzymes are produced at high levels when the growth pH is near to the optimal pH which is required for the maximal activity. The response of the organisms to variations in pH defers in strains of the same species. Among the physical parameters studied, pH of the medium plays an important role by inducing morphological changes in microbes and in secretion of enzyme.

*Fusarium sp*.BLB is found to be stable at pH 9.5 in the production of fibrinolytic enzyme activity. It is well known that the initial pH of the medium is an important factor affecting the production of fibrinolytic enzyme. It can affect the growth of the microorganism either indirectly by affecting the availability of nutrients or directly by action on the cell surfaces[22].

The production medium is prepared of pH ranged from (6.2, 6.8, 7.2, 7.5 and 8) for estimating the enzyme activity of *Bacillus lichniformis B4*. Moataza MS (2015) noticed that fibrinolytic enzyme activity of S.Mobaraense GH4 was increased with increasing in pH value until it reaches the maximum level of pH 8[5].

The fibrinolytic enzyme obtained from Bacillus halodurans IND18 reported by Ponnuswamy vijayaraghavan (2016) maintained the optimum medium pH 8.32 which showed the enzyme activity of 6851 U/g. The production of fibrinolytic enzyme by Streptomyces lusitanus showed by S.Sudesh warma (2017) obtained by optimizing the media pH to 7[6].

**5.3. b. Effect of initial temperature on fibrinolytic enzyme production:**

Temperature is another critical parameter that has to be controlled and varied from organism to organism. The mechanism of temperature control of enzyme production is not well understood. However, studies by showed that a link existed between enzyme synthesis and energy metabolism in bacilli which was controlled by temperature and oxygen uptake[6].

The result on the effect of temperature on fibrinolytic enzyme production is shown below. The production of fibrinolytic enzyme increased significantly with the increase in fermentation temperature from $30^0$C-$50^0$C and decreased above $40^0$C.

The maximum fibrinolytic enzyme production obtained at $40^0$C was 170 IU/ml and the least production was observed at $30^0$C i.e (110 IU/ml) at 72 hours of fermentation period by using *Aspergillus sp.* SAS02. Any temperature behind the optimum range is found to have some adverse affect on the metabolic activities of the microorganisms and is also reported by various scientists that the metabolic activities of the microbes become slow at lower or higher temperature[4].

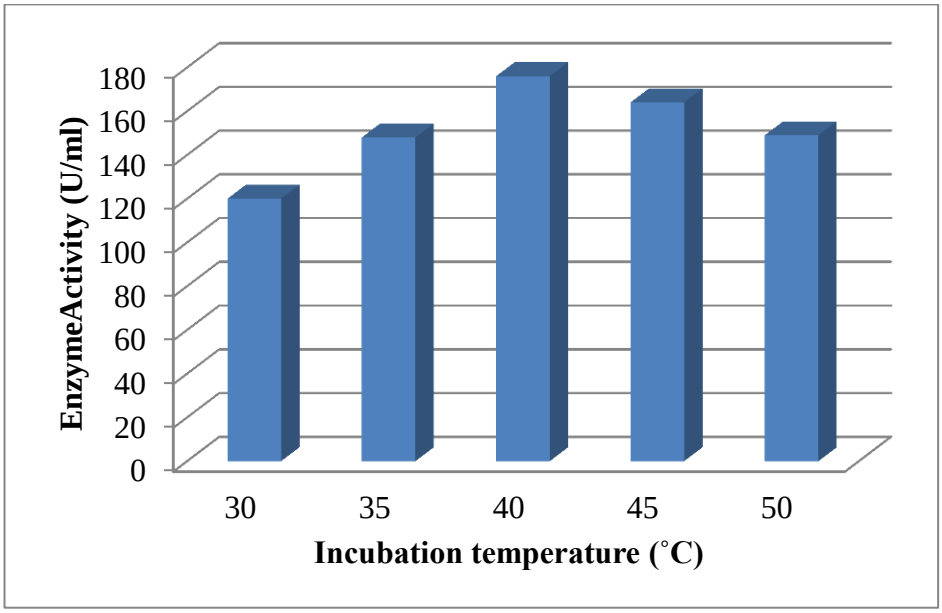



**Fig.5.2- Effect of incubation temperature on biosynthesis of fibrinolytic enzyme**

Incubation temperature is an important environmental factor for the production of fibrinolytic enzyme by microorganisms because it affects growth rate of microorganism, regulates the synthesis of the enzyme and also the enzyme production by changing the properties of the cell wall reported by Satyanarayana, (1994)[52].

The optimum temperature of the fibrinolytic enzyme is measured from $30^0$C to $70^0$C for 40min. Chen-Tien chang (2000), reported that the enzyme was relatively labile between $30^0$C and $40^0$C and became inactive above $40^0$C . However, the optimum pH and temperature of each culture has to be determined initially for the culture under study, since each culture has different pH and temperature

requirements. As such our findings are in close agreement with findings of Paik et al. (2004), Lee et al. (2001)[7].

Temperature is an important parameter that governs the process of fermentation as well as the recovery of desired product. It is widely accepted that the secondary metabolism of microorganisms represent an important pathway for survival and in turn depends on the incubation temperature by Lai et al. (2005). However, the influence of temperature-shift varied from strain to strain employed during fibrinolytic enzyme synthesis by the fungus. Since various temperature levels have been reported as optimum for the fermentation, it is desirable to check the influence of incubation temperature upon the fermentation[35].

Patcharaporn pandee (2008)[8] showed the influence of temperature on enzyme activity from $25^0$C to $60^0$C. The enzyme activity was increased with rise in the temperature up to $50^0$C and decreased at $60^0$C by *Schizophyllum commune* BL23. To determine the optimum temperature of the fibrinolytic enzyme the purified enzyme was kept in a 10mM phosphate buffer for 30min at various temperature ranging from $20^0$C to $60^0$C. The fibrinolytic enzyme showed the highest activity at 40ºC and decreased significantly at above $50^0$C Suk park (2010)[8].

The optimum temperature for fibrinolytic enzyme production varied from strain to strain. The potential strain of Actinomycetes showed its optimum temperature at $28^0$C for fibrinolytic enzyme production reported by Sasirekha (2012)[9]. The purified enzyme shows its stability at temperature up to $50^0$C, but the activity rapidly decreased at temperatures greater than or equal to $60^0$C. It shows that the partially purified enzyme is not thermotolerant, so the products containing these enzymes should not be subjected to heat treatment above $50^0$C by Diana Nur Afifa (2014). The fibrinolytic enzyme from *Bacillus halodurans* IND18 Ponnuswamy vijayaraghavan (2015) depicted an optimal temperature is $60^0$C and it was stable upto $50^0$C. This enzyme activates plasminogen which degrades the fibrin net of blood clot and proved its potential as a effective thrombolytic agent[9-10].

**5.3. c. Effect of inoculum size on fibrinolytic enzyme production:**

Optimization of inoculums size (spores/ml) is necessary in solid state fermentation because low density of spores leads to insufficient biomass and end product synthesis permit the growth of undesirable contaminants and too high densities of spores may cause a quick and too much biomass production, there by leading to fast nutrient depletion and ultimately reduction in the end product quality. An attempt has been made in the present study to optimize the inoculum size for maximum fibrinolytic enzyme production on synthetic medium.

The results of studies on the effect of inoculums size on the production of fibrinolytic enzyme by employing *Aspergillus sp.* SAS02 strain using production medium are presented below. The data revealed that the production of fibrinolytic enzyme by *Aspergillus sp.* SAS02 increased as the inoculum size increased up to 1.25 ml for 72 h fermentation period. Further increase in the inoculum size has not yielded significant increase in the production of fibrinolytic enzyme. The inoculum size of 1.25 ml showed maximum production of fibrinolytic enzyme 180 U/ml at 72h fermentation period. The lowest amount of fibrinolytic enzyme production of 105 U/ml was observed at inoculum size of 0.25 ml at 72 h of fermentation period.

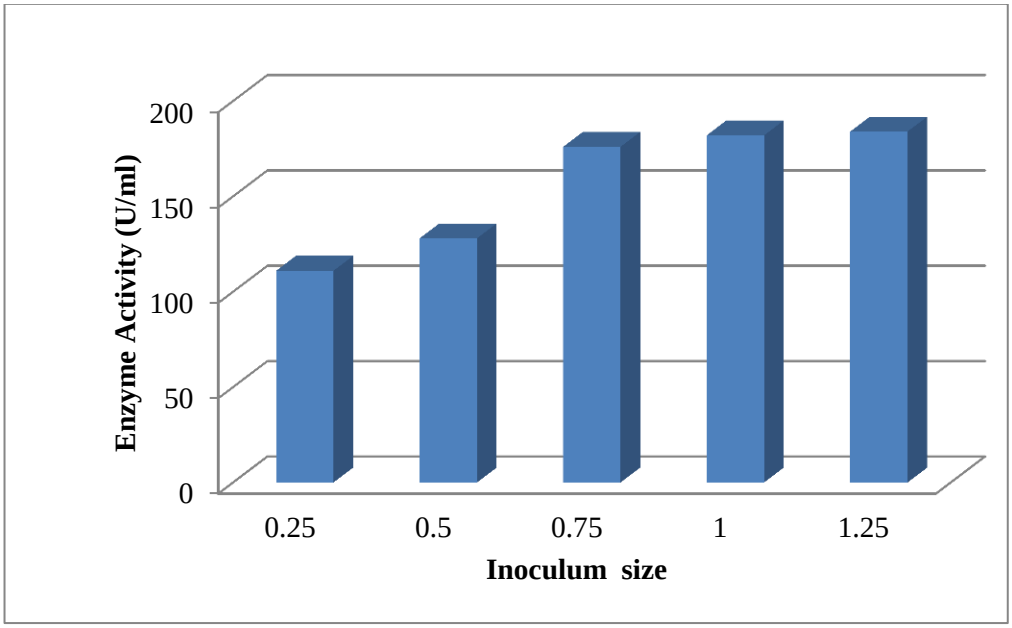



**Fig.5.3- Effect of inoculum size on biosynthesis of fibrinolytic enzyme**

Among all optimization factors like pH, temperature and inoculum size, type (spore or vegetative) and age of the inoculum are of prime importance. Attempts were made to standardize inoculum for citric acid production in submerged culture.

Biomass and enzyme production was also influenced by the initial inoculum concentration. Optimum inoculum size is required for fibrinolytic enzyme production. The increase in fibrinolytic enzyme production using small inoculum sizes was suggested due to the higher surface area of volume ratio resulting in increased fibrinolytic enzyme production. If the inoculum size is too small, insufficient number of bacteria would lead to reduced amount of secreted fibrinolytic enzyme. This may be due to the limitation in other fermentation medium components and reduced dissolved oxygen.

Inoculum density has some optimum value depending upon the microbial species and fermentation processes. The initial inoculum size controls the kinetics of growth and several metabolic functions leading to overall biomass and extracellular product formation. The age and size of inoculum have been reported which affect the yield of bacterial enzymes.

The highest inoculum level of 7% to 14% has been reported to be optimum for fibrinolytic enzyme production by *Penicillium chrysogenum* SGAD12 by Gopinath S M (2011), Essam.F.Al Juamily (2012) showed the production of the fibrinolytic enzyme from *Bacillus lichniformis* B4 which increases with increasing concentration of inoculums up to ($10^5$cell/g) as it was effective in showing 19.185 U/ml and the activity decreased with the increase in the inoculums[18].

Several authors showed that different inoculums size effects the production of enzymes by several microorganisms. *Bacillus cereus* GD55 showed the highest enzyme activity of 45 U/g with the inoculum size of 2.0 ml reported by Erumalla venkata Nagaraju (2013)[53].

Moataza M S (2015) reported that the inoculum age of *Streptoverticillium mobaraense* $GH_4$ was an important factor for enzyme production. 1ml of inoculum size shows the maximum activity of $908.02 \pm 0.037$ U/ml[5]. Manoj kumar in 2015 has

set the inoculum volumes by 1%, 2%,5% and 7% with respect to inoculum ages. The highest productivity of the fibrinolytic enzyme produced by *Bacillus cereus* SRM-001 showed the highest activity of 799 U/ml at 1% inoculum volume with 24hrs inoculum age[11].

**5.4. Purificational studies of fibrinolytic enzyme:**

Process optimization is often followed by downstream process for purification and characterization of the desired product. Various methods have been used for the recovery of extracellular fibrinolytic enzyme from a mixture of different proteins. To reach the required quality standards of the products an efficient and selective downstream processing is essential. The different stages of the purification process of extracellular bio-products include solid/liquid separation, concentration, purification and formulation. Each stage again consists of a number of well-defined process steps for recovery of extracellular product required purity and final form of the product[12].

Purification of proteins is vital in the study of function and expression. Separation involves removing any contaminants that are present in the mixture; these may be other proteins or completely different molecules altogether. For an enzyme to be employed as a reagent in any field, like clinical chemistry or organic synthesis, it must first be purified to a degree that removes any other enzyme capable of catalyzing undesirable side effects. Enzymes are unstable molecules with a definite physicochemical environment. Even a slight change in this environment reduces the activity of enzyme and sometimes the enzyme is totally inactivated. Therefore the enzymes have been isolated under controlled condition of pH, ionic strength and temperature since they are proteinaceous in nature[13].

Purification of enzymes are done by different methods, first by separation of the enzymes from its producer cells, secondly by the removal of excess of water and most of the non-protein materials has been removed. This may or may not mean purification to homogeneity. For the enzyme to be commercially viable, purification must yield tens or hundreds of grams of proteins. Many standard procedures for the purification of proteins in the laboratory do not readily lend themselves to scaling up,

whereas, on the other hand, some techniques are relatively satisfactory in the laboratory for the large scale production[14].

Ammonium sulphate precipitation method has been employed first for purification of enzymes followed by different chromatographic techniques like Gel-filteration chromatography, Ion-exchange chromatography and Polyacrylamide gel Electrophoresis under the present study. Jeong *et al*. (2001), purified the fibrinolytic enzyme by applying 75% ethanol followed by loading on to DEAE-Sephadex A-50 then subjecting the elutes to Sephadex G-100 column chromatography[15].

BSA curve is taken as a standard curve to measure the liberated protein (specific activity) during enzyme assay.

**5.4. a. Ammonium sulphate precipitation:**

The crude enzyme obtained from *Aspergillus tamarii* was precipitated with ammonium sulphate solution (till 75% saturation ) at $4^0$C with constant stirring. The centrifuged pellets of precipitated proteins were dissolved in 50 mM Tris-HCL buffer (pH 8.0).

**5.4. b. Dialysis:**

Thus the precipitate of proteins that is dissolved is loaded into the activated dialysis membrane dialysed for 8 h at $4^0$C against 5 mM Tis-HCl buffer (pH 8.0). The activity showed by dialysed enzyme sample and concentration of proteins are presented below.

**Table-5.3- Steps of purification of fibrinolytic enzyme**

| Steps | Activity (U/ml) | Protein Concentration (mg/ml) | Specific Activity (U/mg protein) | Yield (%) | Fold Purity (%) |
|---|---|---|---|---|---|
| **Crude** | **185** | **1.42** | **130.28** | **100** | **1** |
| **Dialysis** | **180** | **1.2** | **150** | **84.5** | **115.13** |
| **Gel filtration chromatography** | **126** | **0.73** | **172.6** | **51.4** | **132.48** |
| **Ion Exchange chromatography** | **96** | **0.23** | **417.39** | **16.19** | **320.07** |

**5.4. c. Gel-filtration Chromatography:**

The Sephadex G 100 column (1X50 cm) matrix was active by heating at $50^0$C for 30 min with constant shaking in presence of 50mM Tris HCL buffer (pH 8.0). Once the Sephadex G-100 column was ready, the dialysed enzyme fraction (3ml) was loaded into it. Through column filtration the protein eluted with 50 mM Tris HCL buffer (pH 8.0) at 2 ml per 3 mins. The different fractions (30) were collected at $4^0$C. The 5, 6 and $7^{th}$ fractions are rich in protein concentration, they were assayed for protein by Bradford method and the enzyme activity of each fraction was assayed. The 5,6 and $7^{th}$ fractions are called as active fractions, they were pooled, their yield was 51.4%, fibrinolytic enzyme activity was found to be 126 U/ml/min and further used for ion exchange column chromatography[17].

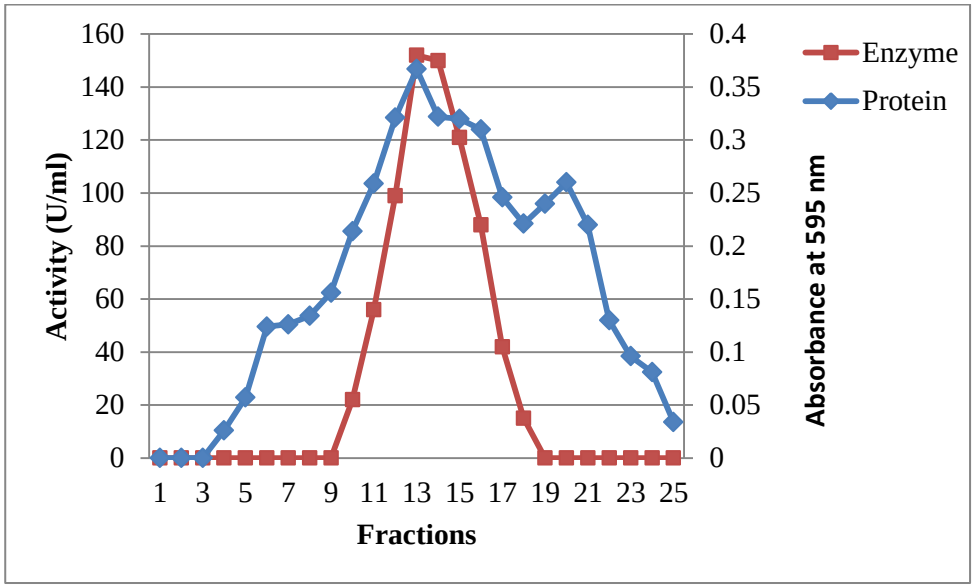



**Fig.5.4-The elution profile of fibrinolytic enzyme from Sephadex G-100 column**

### 5.4. d. Ion- exchange chromatography:

The column (1.5X30cm) was prepared by using DEAE-cellulose by dissolving in 50mM Tris-HCL buffer (pH-8.0). The column was allowed to set with elution of buffer. The protein elution was done with same buffer. The different protein fractions were collected at $4^0$C to check the quantity of protein using Bradford reagent and the enzyme activity was also estimated. The active fractions were pooled and used as partially purified enzyme solution for further characterization. Thus the activity of enzyme during Ion-exchange chromatography was very low with respect to the activity in previous purification step. The loss of activity could be due to the partial autolysis of the enzyme, which is seen in most of the fibrinolytic enzyme due to loss of cationic or anionic cofactors occurs during the ion exchange process[17]. In the present study, fibrinolytic enzyme was purified by 320.07 folds with a yield of 16.19%.

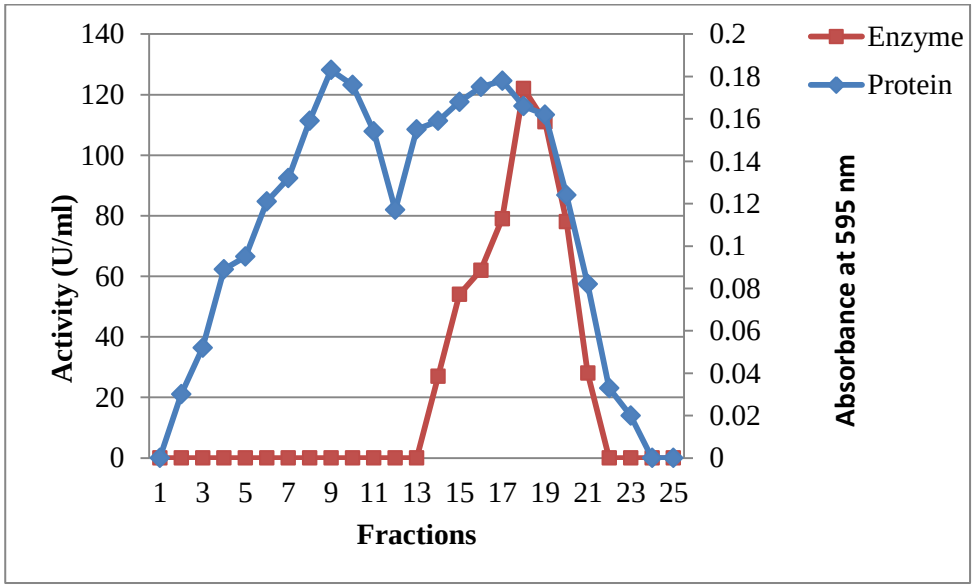



**Fig.5.5-The elution profile of fibrinolytic enzyme in Ion-exchange column**

### 5.4. e. Molecular weight determination of proteins by Polyacrylamide Gel Electrophoresis (PAGE):

In order to find out the molecular weight of the enzyme thus purified, it was electrophoresed with molecular marker. The gel displaced two bands with a prominent band corresponding to molecular weight of approximately 29kDa. It was inconclusive to find the fibrinolytic enzyme among those three proteins. The molecular weight of serine proteases ranges from 27.7kDa of Nattokinase from B. *natto*, 28kDa from B. *amyloliquefaciens*, 29kDa by Bacillus sp. Hence our studies are in close agreement with the molecular weight obtained from B.*natto* and B.*amyloliquefaciens*[16].

**5.4.f.** The brown colour bands are obtained on the gel due to silver staining of proteins.

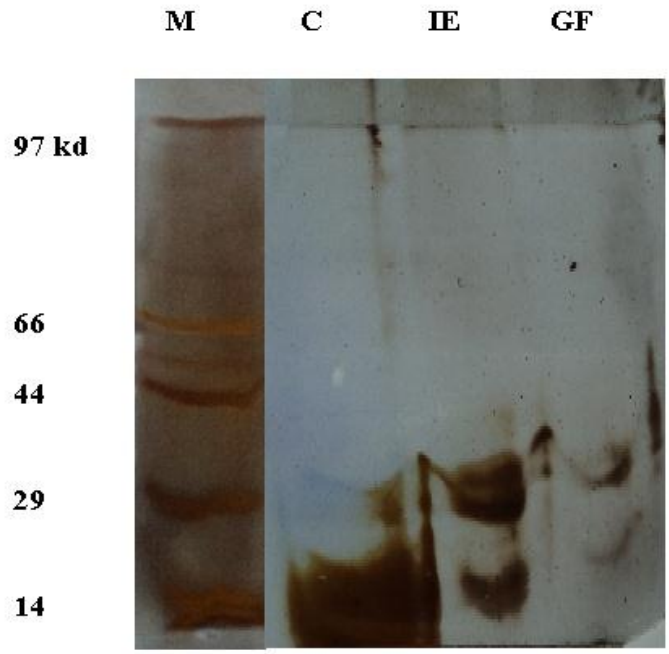



**Plate-5.4: SDS-PAGE for analysis of purified sample**

**(M-Molecular weight marker, medium range, IE-Ion exchange,**

**GF-Gel filteration)**

## 5.5. Characterization of fibrinolytic enzyme:

### 5.5. a. Determination of optimum pH of enzyme activity:

The below graph represents that the purified fibrinolytic enzyme for the determination of the optimum pH, Acetate (pH 4.0), Citrate (pH 5.0 and 6.0), Phosphate (pH 7.0), Tris-HCL (pH 8.0) and Glycine-NaOH (pH 9.0, 10.0 and 11.0) buffers were used. The highest fibrinolytic activity was found to be at pH 8.0 (100%) by using *Aspergillus sp.* SAS02[17].

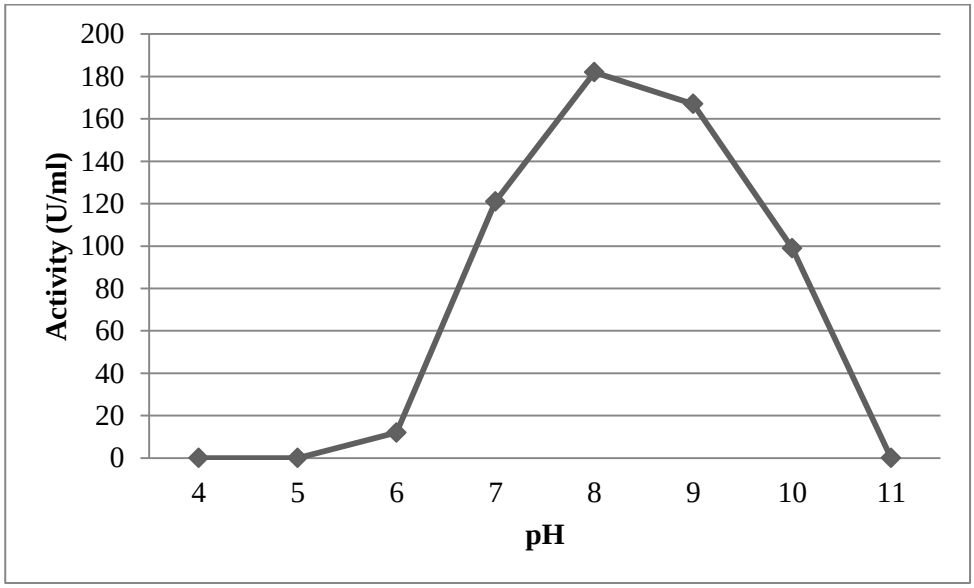



**Fig.5.6 –Determination of optimum pH of fibrinolytic enzyme**

Essam F. Al-Juamilly (2012) isolated *Bacillus lichniformis* from soil to produce fibrinolytic enzyme using the medium with different hydrogen numbers (6.2, 6.8, 7.2, 7.5, and 8)[18]. Maximum production was recorded at pH 7. Siraj (2011) reported the optimum pH of nattokinase is pH 7. The fibrinolytic enzyme isolated from oidiodendron flavum, the optimal pH of the fibrinolytic activity of the enzyme was determined within a pH range of 1.2-3.5 towards fermented medium incubated at $37^0$C. Over a wide range the enzyme showed its maximum activity at optimum pH 8 reported by Nagwa.a. tharwat (2006)[54]. We found the similar results with *Aspergillus sp.* SAS02.

Almalki et al. (2017) reported that the effect of pH on the fibrinolytic activity towards fermented medium was examined at various pH values (5-10). The enzyme isolated from *Bacillus sp*. IND6 showed maximum fibrinolytic activity at pH 9[19]. This optimum pH is similar to that of the fibrinolytic enzyme isolated from Bacillus halodurans IND18 in 2016 by Ponnuswamy. The same optimum condition was maintained for the production of fibrinolytic enzyme by *Bacillus subtilis* RJAS19 by D.J Mukesh kumar in 2013 which showed the maximum activity at pH 9[10].

**5.5. b. Determination of optimum temperature of enzyme activity:**

The optimum temperature of fibrinolytic enzyme from *Aspergillus sp.* SAS02 is shown blow. The activity of the fibrinolytic enzyme was determined at different temperatures ranging from $37^0$C to $55^0$C. The optimum temperature was recorded at $40^0$C for fibrinolytic activity. The enzyme activity gradually increased up to $45^0$C and thereafter decreases.

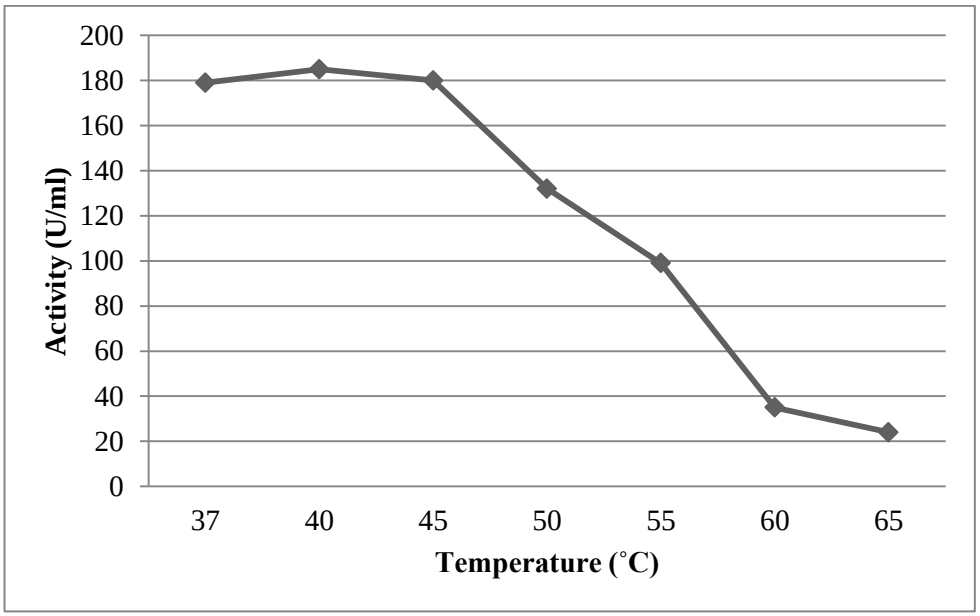



**Fig.5.7 –Determination of optimum temperature of fibrinolytic enzyme**

Fibrinolytic enzyme isolated from *Bacillus subtilis* BK-17 by Yong Kee Jeong in 2001 reported the optimum temperature at $50^0$C. And this was almost similar to the fibrinolytic enzyme isolated from *Bacillus subtilis* RJAS19 by D.J.Mukesh kumar (2013), which showed the optimum temperature at $45^0$C[20].

Nagwa.A.Tharwat reported that the fibrinolytic enzyme isolated from oidiodendron flavum (2006) was most active at $45^0$C and $55^0$C all over the incubation time which was similar to Yongkee Jeong (2001) and D.J.Mukesh kumar enzyme isolated from *Aspergilllus sp.* SAS02 in the present study[20-21].

S.Kumaran (2010), reported the effect of temperature on fibrinolytic enzyme activity isolated from *Ganoderma lucidum* at different temperature ranging from 20, 25,30,35,40 and $45^0$C. The mycelia grown at $30^0$C showed the highest enzyme

activity. Sasirekha.C.et al. showed the highest enzyme by isolating the fibrinolytic enzyme activity at $28^0$C from Actinomycetes (2012)[2].

**5.5. c. Analysis of temperature stability on enzyme activity:**

The purified fibrinolytic enzyme analyzed for its stability at room temperature by using *Aspergillus sp.* SAS02 is shown below. The results indicated that the enzyme shows its maximum activity at room temperature with in 30 min, at $55^0$C displayed better stability. But after 60 min enzyme loses its significant activity.

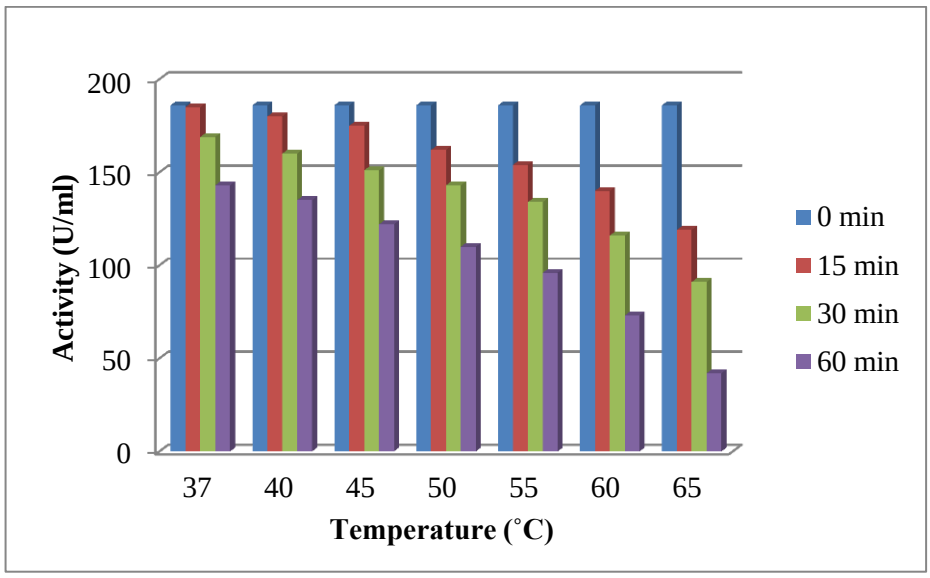



**Fig.5.8- Analysis of temperature stability study of fibrinolytic enzyme**

Yongkee Jeong (2001) showed that the fibrinolytic enzyme isolated from *Bacillus subtilis* BK-17 showed its pH stability between pH 4-7 with temperature stability from 30-$50^0$C[21]. Nagwa A Tharwat (2006) isolated fibrinolytic enzyme by Thermophilic fungus *Oidiodendron flavum* was most active at $45^0$C and $55^0$C over the incubation time, also lost its substantial activity upon incubation at more than $65^0$C for 30mins. D.J.Mukesh Kumar (2013)[20] isolated the enzyme by *Bacillus subtilis* RJAS19 showed the thermo stability high at $65^0$C to $85^0$C at pH of 9.5. The similar thermal stability was also observed in the strains *Bacillus subtilis* YJ1 (Yin et

al. (2010), *B.Amyloliquefaciens* DC-4 by Peng et al. (2003)[4] and *Bacillus sp*.KCK-7 by Kim et al. (1996). Fibrinolytic enzyme isolated from *Bacillus halodurans* IND18 showed its maximum activity at $60^0$C and decreased abruptly above $60^0$C and was stable upto $50^0$C for 1hr with the pH 9.0 reported by P.Vijayaraghavan et al. (2011)[19].

### 5.5. d. Determination of *Km* and *Vmax:*

The effect of different concentrations of substrate on enzyme activity was assayed and plotted according to Lineweaver and Burk. The *Km* was 1.25 and *Vmax* was found to be 2.1, as shown in Fig-5.11

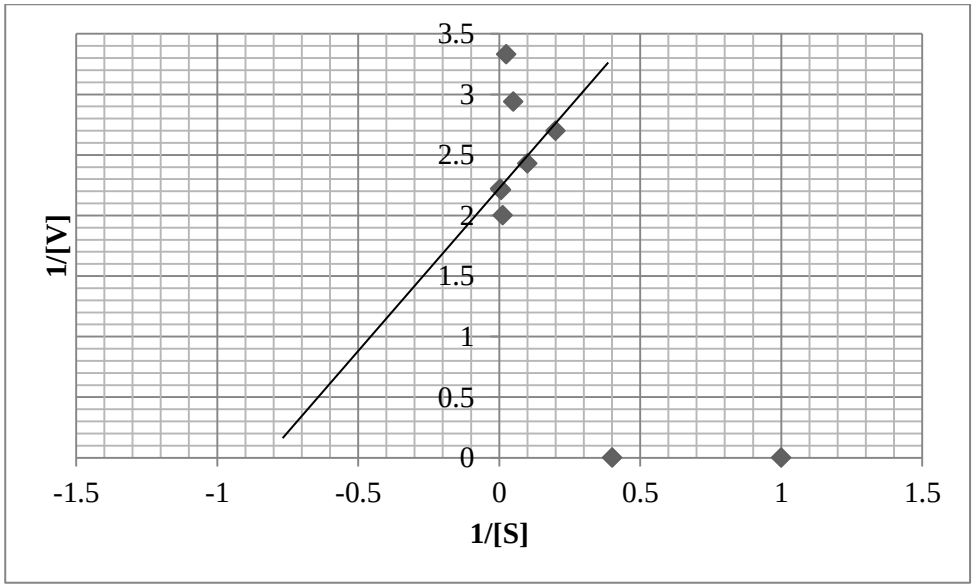



**Fig-5.9- Determination of *Km* and *Vmax* of fibrinolytic enzyme**

### 5.5 .e. Effect of Inhibitors and Metal Ions on Fibrinolytic enzyme:

The inhibition studies of the enzyme purified up to now gave a unique inhibitors and inducers list. The purified enzyme activity was induced up to 126% and 114% by the presence of Mg and Zn ions. But complete inhibition was displayed by Hg. Significant inhibition was also noted in presence of Ca, and Cu. Phenylmethylsulfonyl fluoride inhibited enzyme completely, indicating the serine

protease nature of the enzyme. EDTA and 1, 10- phenanthroline also inhibited the enzyme, so the enzyme is a metalloprotease. Enzyme did not display significant activity in presence of SDS, indicating the susceptibility of the enzyme for detergents. Enzyme obtained from *Aspergillus sp.* SAS02 was not saline stable and bleach resistant, as it loses the activity in presence of NaCl and $H_2O_2$[55].

**Table – 5.4- Effect of metal ions on enzyme activity**

| Metal ion | Residual activity (%) |
|---|---|
| Control | 100 |
| $HgCl_2$ | 0 |
| $CaCl_2$ | 22.54 |
| $FeCl_3$ | 89.20 |
| $AgNO_3$ | 78.61 |
| $ZnSO_4$ | 114.63 |
| $MgSO_4$ | 126.38 |
| $MnSO_4$ | 98.30 |
| $CuSO_4$ | 12.38 |

**Table – 5.5- Effect of inhibitors and other compounds on enzymes**

| Inhibitor | Residual activity (%) |
|---|---|
| Control | 100 |
| SDS | 38.2 |
| NaCl | 29.4 |

| | |
|---|---|
| $H_2O_2$ | 34.63 |
| PMSF | 0 |
| 1-10, Phenanthroline | 0 |
| p-Chloromercuryl benzoate | 1.37 |
| EDTA | 0 |

**5.6. Molecular Characterization of Fibrinolytic Enzyme:**

**5.6. a. Phenotypic Identification of *Aspergillus tamarii SAS02***

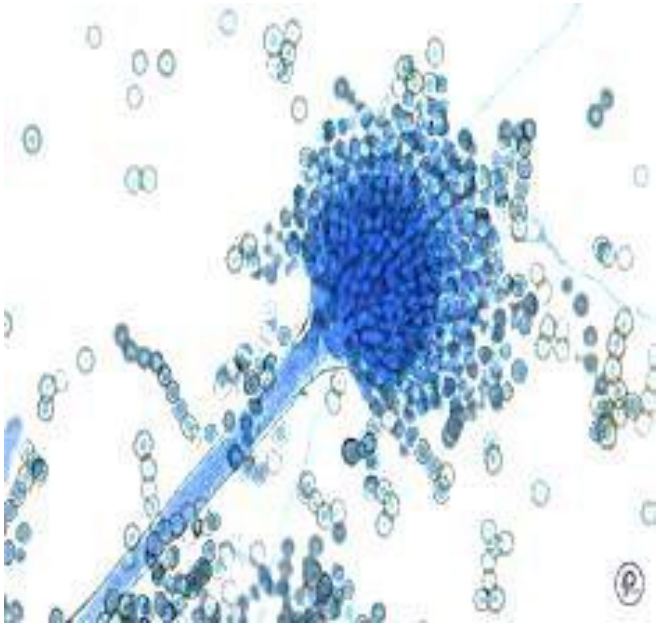
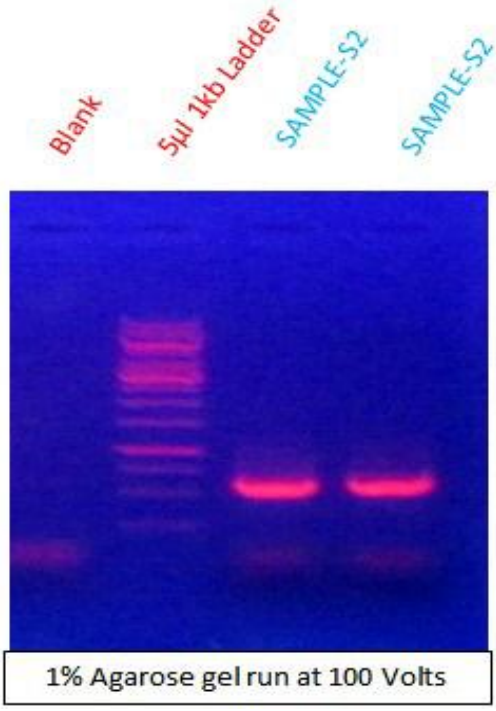


**Plate-5.5: Electron microscopic image of *Aspergillus tamarii* SAS02 at 100 µm**

**Lacto phenol cotton blue-stain micrograph of *Aspergillus tamarii* SAS02**

### 5.6. b. Genomic DNA isolation and PCR amplification of the ITS gene




### 5.6. c. Sequence analysis for sample1 (SAMPLE-S2):

**SAMPLE-S2 ITS1 F**

**TNGAGNGTAGGGTTCCTAGCGAGCCACCTCCCACCCGTGTTTACTGT**
**ACCTTTGTTGCTTCGGGGGGGCCGGCCTTTAAGGCCGCCGGGGGGG**
**TCCAATCGGGAAATAGTTCCCTCCGTGGTTGTTTTTTTGAGGCCGGC**
**GGCAACCCAGACCATCCTTTTATTAAAATTTTTAAACGGAAGCGATA**
**CAAATTCAAGTCGAATTCTANCAAATAAGATTTTGTTTTTTTTTCTGC**
**CGCGGGCCGGCCTGATTCACCCCCCCCCGGCGCCCCTGGAGGGGCC**
**GGCCCGCCGCAAGCAAATATACACAGACAACAGGGGGGGGGGGGGG**
**GGGCGGTAGACCCCACACTTAGTACTTCTTCCGTAGGACAACCTGAG**
**GAAGCATCATTACCGAGTGTGGGGTTCGTAGGGCCCCCAACCTCCCA**
**CCGGTTTTTATTACACTTTAGTTGTTTGGGGGGGCCCGCCTTTCATG**
**GCCGGGGGGGGTTATCACCCCGGGGCCCGCCCCCGCGGGAGACAAC**
**AAAAACTGTGTGATATATAGTGATGTGTGAGTTGATTG**

**SAMPLE-S2 ITS4R**

**TATCACAATCAGTTAAAATTTTAAAAAATGGATCTTTGGGTTCGGGC**
**ATCGGAAAAACGCAGAAAAATGAGAAAAATAGTGAAAATTGCAAAAT**
**TCCGAGAATCATAGAGTTTTTAAACACACATTGCGCCCCGGGGTATT**
**CGGGGGGACACGCGTGTCAGAGTCTCATTGGTGCCCAAAAAGCAGG**
**GTTTGTGTGTTGTGTTGTTGTCCCCTTTCGGGGGGGGAGGGGCCCAA**
**AAGGGAGGGGGGGCAGCGTGTCATCTCCTAGAGTATATGGGGTTTC**
**TCACCCTCTGTATAGGCCGGGCGGGCGCTTGGAAAACACAAAATTTT**
**TCCAGGTGACCTCGGATCAGTAGGANN**

**5.6. d. The Blast results and Phylogenetic Tree analysis these sequences are given below:**

**Table-5.6-Blast result**

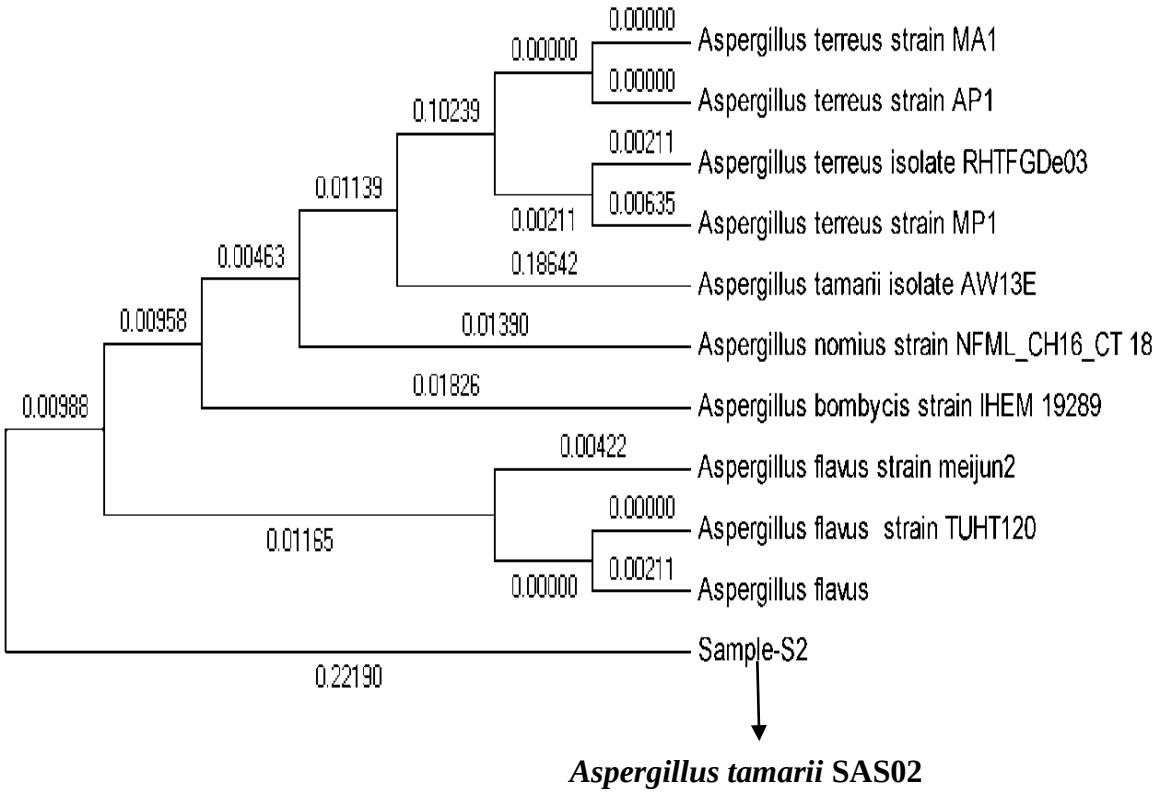
| Description | Max Score | Total Score | E Value | Score | Accession Number |
|---|---|---|---|---|---|
| *Aspergillus tamarii isolate AW13E* | 302 | 302 | 1.00E-77 | 90% | KP735243.1 |
| *Aspergillus terreus strain MA1* | 176 | 176 | 7.00E-40 | 90% | JQ387729.1 |
| *Aspergillus terreus strain AP1* | 169 | 169 | 1.00E-37 | 89% | KF860887.1 |
| *Aspergillus terreus isolate RHTFGDe03* | 158 | 158 | 2.00E-34 | 74% | GU564263.1 |
| *Aspergillus terreus strain MP1* | 150 | 150 | 4.00E-32 | 76% | HQ449678.1 |
| *Aspergillus bombycis strain IHEM 19289 isolate ISHAM-ITS_ID MITS112* | 117 | 117 | 4.00E-22 | 90% | KP131550.1 |
| *Aspergillus flavus* | 117 | 117 | 4.00E-22 | 90% | LN482517.1 |
| *Aspergillus flavus strain meijun2* | 111 | 111 | 2.00E-20 | 89% | KR076749.1 |
| *Aspergillus nomius strain NFML_CH16_CT 18* | 111 | 111 | 2.00E-20 | 89% | KM458794.1 |
| *Aspergillus flavus stra TUHT120 in* | 111 | 111 | 2.00E-20 | 89% | LN482516.1 |

BLAST – Basic Local Alignment Search Tool is a sequence comparison algorithm optimized to process or result of matching aminoacid residues of two or more biological sequences to achieve maximal levels of identity and in the case of aminoacid sequences, conservation for the purpose of assessing the degree of similarity and the possibility of homology[45].

**Phylogenetic tree for Sample- SAS02:**

**Table-5.7- Phylogenetic analysis**

| ID | Description | % Similarity |
|---|---|---|
| KP735243.1 | *Aspergillus tamarii isolate AW13E* | 90% |
| JQ387729.1 | *Aspergillus terreus strain MA1* | 90% |
| KF860887.1 | *Aspergillus terreus strain AP1* | 89% |




***Aspergillus tamarii* SAS02**

## 5.7. Process economization:

To check with the enhancement of fibrinolytic enzyme production the fermentation media is supplemented with carbon sources, nitrogen sources and metal ions.

### 5.7. a. Influence of carbon on fibrinolytic enzyme production:

The results of the studies pertaining to the production of fibrinolytic enzyme by *Aspergillus tamarii* SAS02 on synthetic medium supplemented with different concentrations of various carbon sources like glucose, sucrose and maltose are presented below.

**Table – 5.8- Effect of Glucose (Enzyme activity) - *Aspergillus tamarii* SAS02**

| SL.No | Concentration (%)/Time | 0.25 | 0.50 | 0.75 | 1.0 | 1.25 |
|---|---|---|---|---|---|---|
| 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| 2 | 24 | 15 | 18 | 25 | 39 | 23 |
| 3 | 48 | 36 | 37 | 46 | 98 | 68 |
| 4 | 72 | 74 | 91 | 152 | 186 | 105 |
| 5 | 96 | 56 | 65 | 112 | 115 | 86 |

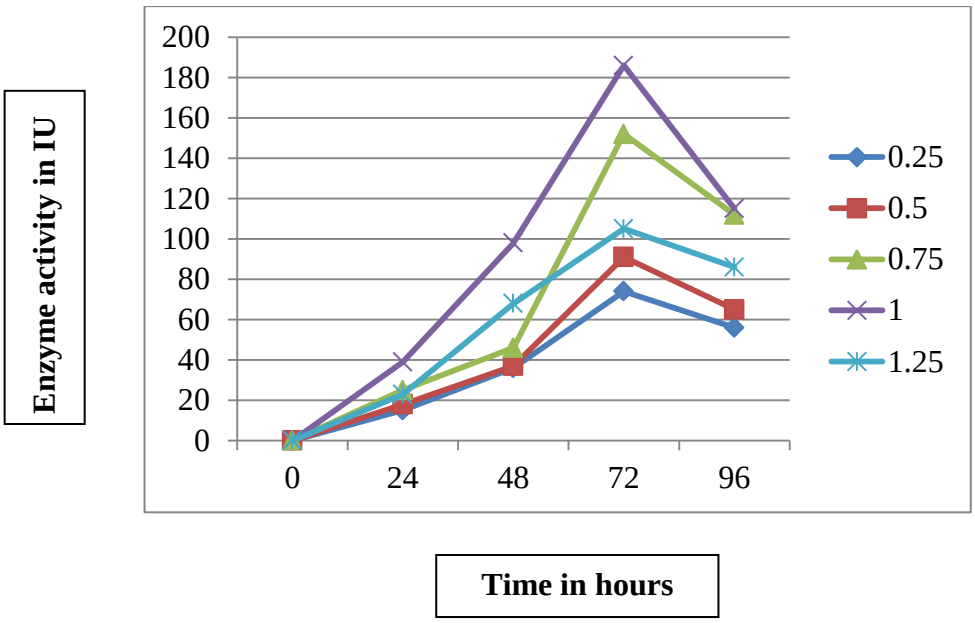



**Fig.5.10-Effect of Glucose on fibrinolytic enzyme production**

**Table – 5.9- Effect of Sucrose (Enzyme activity)- *Aspergillus tamarii* SAS02**

| SL.No | Concentration (%) /Time | 0.25 | 0.50 | 0.75 | 1.0 | 1.25 |
|---|---|---|---|---|---|---|
| 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| 2 | 24 | 13 | 19 | 25 | 28 | 23 |
| 3 | 48 | 26 | 38 | 40 | 47 | 35 |
| 4 | 72 | 67 | 43 | 65 | 86 | 68 |
| 5 | 96 | 33 | 37 | 54 | 56 | 42 |

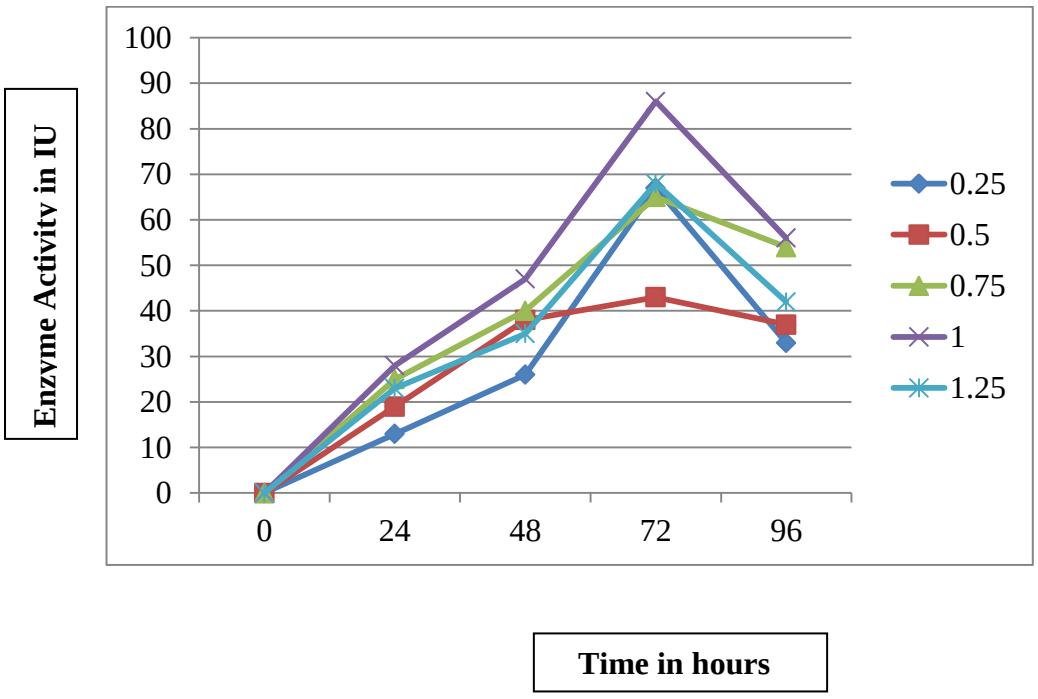



**Fig.5.11- Effect of Sucrose on fibrinolytic enzyme production**

**Table – 5.10- Effect of Maltose (Enzyme activity) - *Aspergillus tamarii* SAS02**

| SL.No | Concentration (0.5%) /Time | 0.25 | 0.50 | 0.75 | 1.0 | 1.25 |
|---|---|---|---|---|---|---|
| 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| 2 | 24 | 16 | 24 | 25 | 32 | 21 |
| 3 | 48 | 26 | 55 | 40 | 64 | 33 |
| 4 | 72 | 53 | 74 | 65 | 109 | 73 |
| 5 | 96 | 37 | 48 | 54 | 87 | 49 |

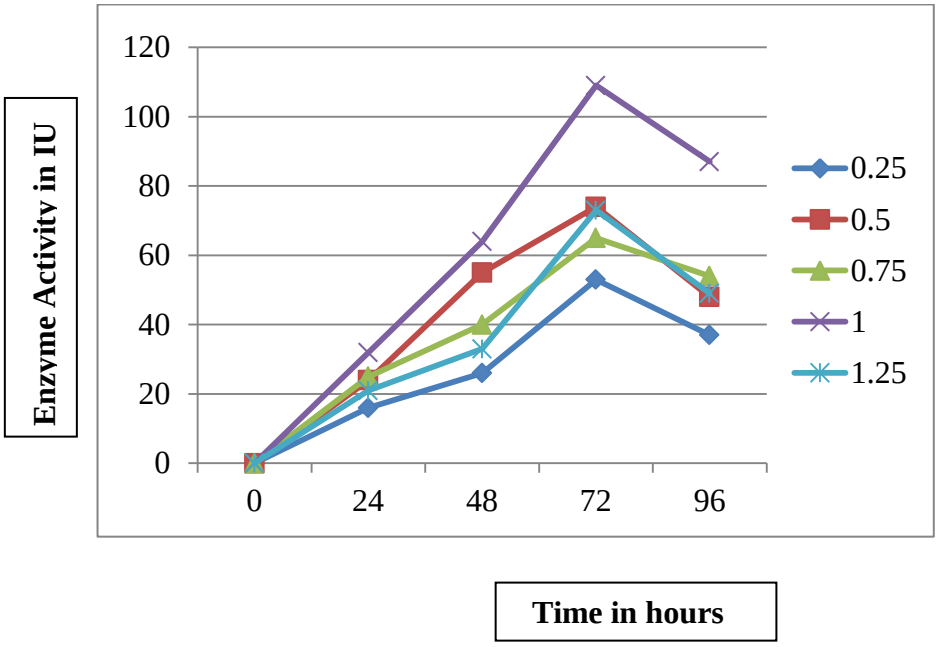



**Fig.5.12- Effect of Maltose on fibrinolytic enzyme production**

The process economization for fibrinolytic enzyme production with carbon sources supplemented to the production medium were carried out with concentration of 0.25%,0.5%, 0.75%,1.0% and 1.25%. The results revealed that all the carbon sources employed under the present study have enhanced the production of fibrinolytic enzyme from 0.25% to 1.0% for 96hrs of fermentation, thereafter no significant production of fibrinolytic enzyme was observed on all the days of fermentation period. Glucose (monosaccharide) yielded the maximum fibrinolytic enzyme production of 186 IU compared to sucrose and maltose (disaccharide) which yields less fibrinolytic enzyme production of 86 IU and 109 IU.

In general, carbon concentration has a positive effect on fibrinolytic enzyme production and high titers of fibrinolytic enzyme can be obtained in a medium rich in carbon source. Various researches have reported that the supplementation of different carbon sources to fermentation medium for fibrinolytic enzyme production, some of the carbon sources, either specifically or non-specifically are known to influence the activity of fibrinolytic enzyme in other microorganisms[28].

Starch stimulates the biosynthesis of proteolytic enzymes from several bacterial and actinomycetes species. In commercial practice high carbohydrate was added either continuously or in the small amounts throughout the fermentation to supplement the exhausted component and keep the volume minimum and thereby reduce the power requirements recorded the significant differences in enzyme production when the carbon sources are varied along with the nitrogen sources. Optimum glucose concentration plays a significant role in enhancing the production of alkaline fibrinolytic enzyme. Growth and enzyme production repression at higher concentration might be due to the catabolic repression or substrate inhibition.

M.A.Abdel-Naby (1991) showed that *Streptomyces sp*. NRC411 utilized starch at different levels for the production of fibrinolytic enzyme by increasing its concentration of about 5% to get the best yield. Dextrin, sucrose, lactose, glucose and ribose are used as a substitution of starch which showed the decrease in the synthesis of fibrinolytic enzyme[29].

The main product of the fermentation process will often determine the choice of carbon source, particularly if the product results from the direct dissimilation of it. Energy for growth comes from either the oxidation of the medium components or from light. Most industrial microbes utilize the commonest form of energy in the form of carbon sources such as carbohydrate, lipids and proteins. It is a common practice to use carbohydrates as the carbon source in microbial fermentation processes. The rate at which the carbon source is metabolized often influences the formation of biomass or production of primary or secondary metabolites by *Stanbury et al*. (1995)[22].

It is a common practice to use carbohydrates as the carbon sources in microbial fermentation processes. Further most of the industrial microorganisms are chemo organotrophs; therefore the commonest source of energy will be the carbon sources such as carbohydrates. However some microorganisms can also use hydrocarbons or methanol as carbon and energy sources. Hence, energy for the growth of desired microorganism during industrial fermentation derives either from the oxidation of medium components or from light by *Stanbury et al*. (1995). In general, the main product of fermentation process will often determine the choice of carbon source, particularly if the product results from the direct dissimilation of it. Hence, it is now well recognized that the rate at which the carbon source is metabolized can often influence the formation of biomass or production of primary or secondary metabolites. Addition of different carbon sources including glucose, sucrose and maltose inhibits the production of fibrinolytic enzyme in solid culture of *A.niger,* showed by Chakraborthy *et al*. (1995)[22].

Carbohydrates are the important source of energy for the fibrinolytic enzyme production by fungi. Different carbon sources are amended for 21 days to get the highest yield of fibrinolytic enzyme from *Ganoderma lucidum* by S.Kumaran in 2010. Glucose, starch, cellulose, lactose, sucrose, maltose and fructose are the carbon sources used. Among all these glucose showed the highest activity of 143.43 U/g.w.wt and maltose showed the least activity of 40.1 U/g.w.wt[2].

Various carbon sources like lactose, fructose, arabinose, sucrose, starch, maltose and glucose are used for the production of fibrinolytic enzyme from actinomycetes by Shasirekha (2012). Enzyme activity in the culture broth was measured after 5 days of cultivation. Sucrose showed the effective enzyme production. Fructose, arabinose and maltose showed the normal growth without inducing any enzyme production. Whereas starch, lactose and glucose exhibit moderate activity of the enzyme production[9].

Anjana Sharma reported (2015) used carbon sources like glucose, sucrose, xylose, lactose, melibiose, soluble starch and sodium citrate for the production of fibrinolytic protease from *Citobacter braakii*. Among all these carbon sources maximum production (79.3 FU/mL) with 1.09 fold increase was obtained when sucrose was used as sole carbon source[23].

Many carbon sources were used to determine the impacts on fibrinolytic activity of *Bacillus sp*.IND6. The carbon sources like trehalose, xylose, glucose and maltose enhanced the enzyme production but when it is compared with starch (1713.8±53.9 U/g) the activity was less reported by Mohammed.A.Almalki (2017)[19].

**5.7. b. Influence of nitrogen sources on fibrinolytic enzyme production:**

Most of the industrial microorganisms can utilize inorganic or organic nitrogen sources. The requirement of nitrogen source mainly depends on the strain and the substrate employed during fermentation showed by Pintado *et al*. (1993)[4]. Nitrogen can be a limiting factor in the microbial production of enzymes by Sabu *et al*. (2000). It is clear that the organism shows slight differences in the growth pattern in the presence of nitrogen sources than in the case of nitrogen-free medium[24].

Nitrogen is an important factor which plays a key role in fibrinolytic enzyme production. *Aspergillus tamarii* SAS02 can utilize nitrogen source either in organic form or inorganic form sometimes both. In many instances growth will be faster with supply of organic and inorganic nitrogen source. It is clear that the organism show slight differences in the growth pattern in the presence of nitrogen source than in the case of nitrogen free medium. Generally, for fibrinolytic enzyme production

inorganic nitrogen will be supplemented in the form of ammonium salts those were ammonium sulphate and ammonium chloride. In some instances, the organic nitrogen source in the form of proteinaceous nitrogen compounds such as yeast extract, malt extract, peptone and urea. Among these nitrogen sources, some were capable of influencing the growth of the organism and production of enzyme.

**Influence of organic nitrogen sources on fibrinolytic enzyme production:**

The organic nitrogen sources greatly influence the growth of an organism. In the present study various organic nitrogen sources like yeast extract, beef extract, peptone were supplemented at 0.25%, 0.50%, 0.75%, 1.0% and 1.25% levels for the production of *Aspergillus tamarii* SAS02.

**Table – 5.11- Effect of Yeast extract (Enzyme activity) - *Aspergillus tamarii* SAS02**

| SL.No | Concentration (%) /Time | 0.25 | 0.50 | 0.75 | 1.0 | 1.25 |
|---|---|---|---|---|---|---|
| 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| 2 | 24 | 12 | 13 | 22 | 27 | 20 |
| 3 | 48 | 27 | 45 | 40 | 68 | 49 |
| 4 | 72 | 53 | 82 | 65 | 96 | 71 |
| 5 | 96 | 37 | 34 | 54 | 72 | 53 |

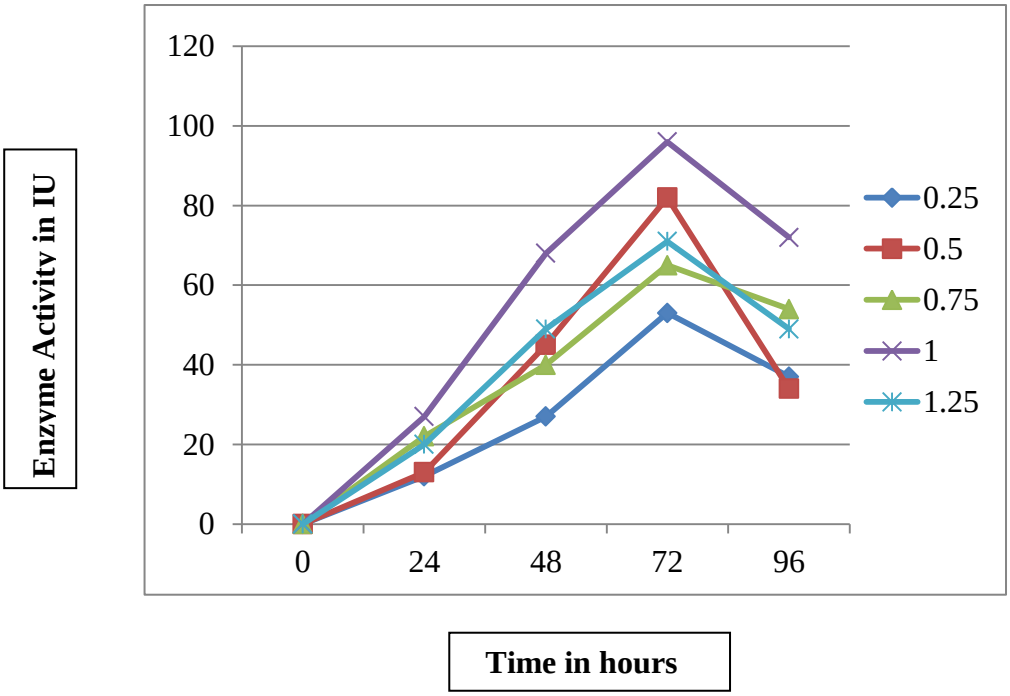



**Fig.5.13- Effect of Yeast extract on fibrinolytic enzyme production**

**Table – 5.12- Effect of Beef extract (Enzyme activity) - *Aspergillus tamarii* SAS02**

| SL.No | Concentration (%)/Time | 0.25 | 0.50 | 0.75 | 1.0 | 1.25 |
|---|---|---|---|---|---|---|
| 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| 2 | 24 | 12 | 14 | 22 | 24 | 18 |
| 3 | 48 | 17 | 38 | 40 | 64 | 39 |
| 4 | 72 | 53 | 49 | 75 | 103 | 78 |
| 5 | 96 | 37 | 40 | 54 | 72 | 57 |

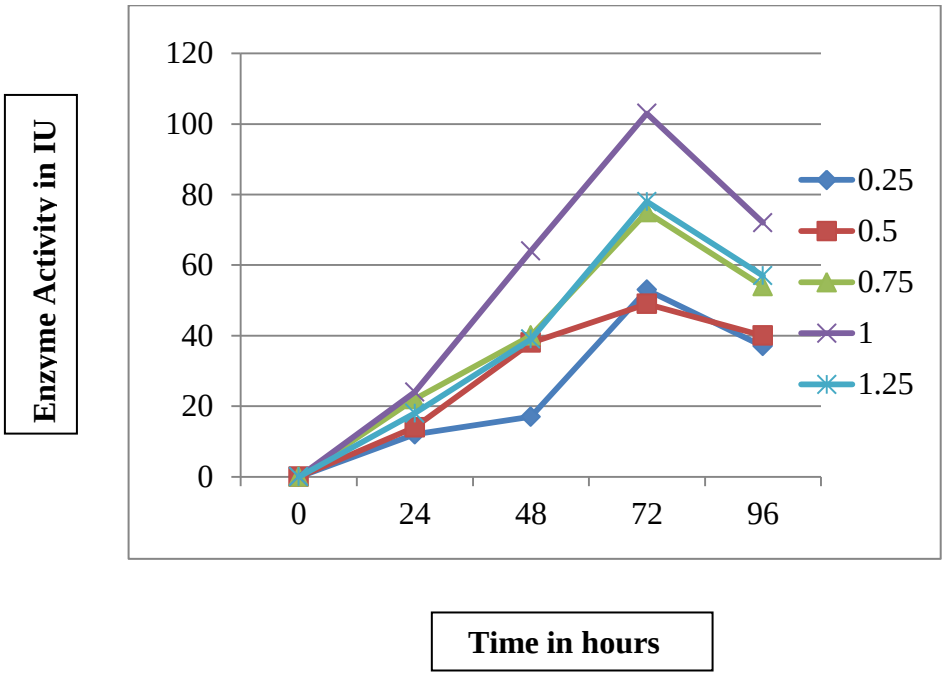



**Fig.5.14- Effect of Beef extract on fibrinolytic enzyme production**

**Table – 5.13 - Effect of Peptone (Enzyme activity) - *Aspergillus tamarii* SAS02**

| SL.No | Concentration (%) /Time | 0.25 | 0.50 | 0.75 | 1.0 | 1.25 |
|---|---|---|---|---|---|---|
| 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| 2 | 24 | 12 | 14 | 22 | 32 | 21 |
| 3 | 48 | 17 | 38 | 40 | 85 | 58 |
| 4 | 72 | 53 | 49 | 75 | 193 | 79 |
| 5 | 96 | 37 | 40 | 54 | 89 | 39 |

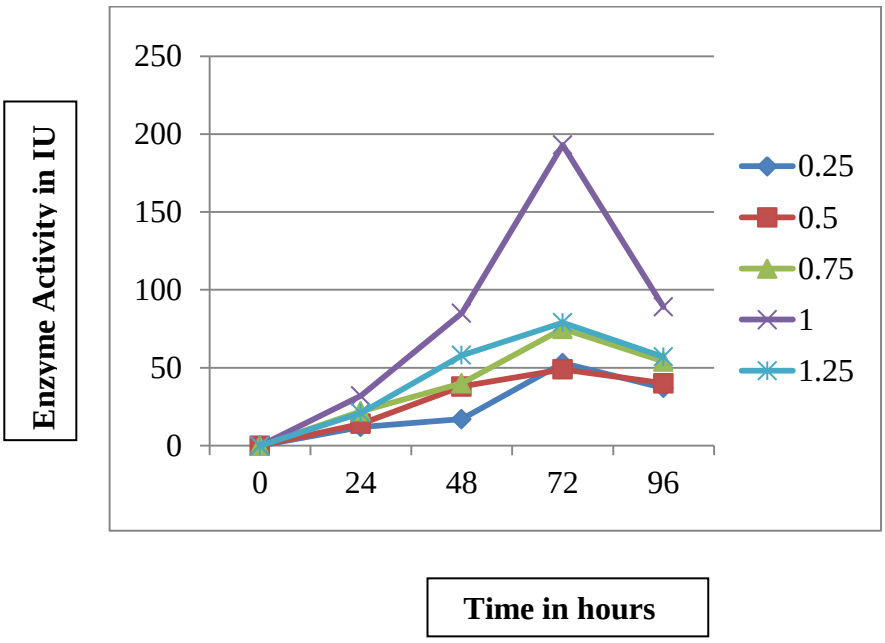



**Fig.5.15- Effect of Peptone on fibrinolytic enzyme production**

The results revealed that the production of fibrinolytic enzyme increased with the increase in the organic nitrogen concentrations up to 1.0%, there after no significant increase in fibrinolytic enzyme production was noticed during the fermentation period. The, nitrogen sources like yeast extract yielded 96 IU, beef extract 103 IU; peptone 193 IU influenced the fibrinolytic enzyme production respectively by *Aspergillus tamarii* SAS02 strain. Amongst various organic nitrogen sources tested, peptone produced maximum of 193 IU amount of fibrinolytic enzyme and emerged as a best organic nitrogen source.

Organic nitrogen sources have been found to be better source for the growth and fibrinolytic enzyme production in some organisms reported by Pthadatare *et al*. (1993), Aleksieva *et al*. (1981) where used as inorganic nitrogen sources gave better enzyme yields in other organisms by Sinha and Satyanarayn (1991). When compared to yeast extract, beef extract showed the rich fibrinolytic enzyme production, but in few cases yeast extract followed by casein showed the highest fibrinolytic enzyme production in termophilic *Bacillus sp*. JB-99[25].

Highest yield of fibrinolytic enzyme was recorded in medium supplemented with peptone followed by beef extract, casein, yeast extract, tryptone and $NaNo_3$. The organic nitrogen sources like peptone, gelatin, and malt extract at equivalent nitrogen concentration, among them peptone was found to be the best organic nitrogen source.

Most of the higher fungi prefer organic and ammonial nitrogen sources than inorganic nitrogen sources. Peptone showed the highest fibrinolytic enzyme activity (162.17 U/g) when compared to yeast extract, beef extract, malt extract, casein and gelatine at the incubation period of 21 days of Ganoderma lucidum by S.Kumaran et al. (2010)[2].

Essam.F.Al-Juamily (2012) used five kinds of organic nitrogen sources like casein, $NH_4Cl$, $KNO_3$, $NH_3PO_4$ and peptone. Among all the six soya peptone was found to the most promising one with fibrinolytic activity of 50 units/ml. This result was similar with the result shown by Liu *et al* who showed that soya peptone was found to be the best one for fibrinolytic activity. Actinomycetes showed the highest

zone inhibition using yeast extract when compared to other nitrogen sources like ammonium sulphate, beef extract, casein, soyabean flour, malt extract Sasirekha (2012)[26].

D.J.Mukesh kumar (2013) showed the highest fibrinolytic enzyme production by using peptone (3µ/ml) in *Bacillus subtilis* RJAS19[20]. Bacillus cereus SRM-001 showed the highest production of fibrinolytic enzyme by using soyabean powder (799 U/ml) when compared to peptone, tryptone and casein by Manoj kumar (2011)[11]. Anjana Sharma (2015) showed that soya flour were found to be the best nitrogen source for the production of fibrinolytic enzyme 140IU/ml by citrobacter braakii compared with other nitrogen sources like yeast extract, peptone, casein, urea and sodium nitrate[23].

The result obtained under the present study is in agreement the findings of Riyad Abdul Jabbar Abdul Sahib (2009) and Essam.F.Al-Juamily (2012)[26].

**Influence of inorganic nitrogen sources on fibrinolytic enzyme production:**

The inorganic nitrogen source does impart growth and physiological activities of micro organisms. In the present study various inorganic nitrogen sources such as Ammonium sulphate, Ammonium chloride, Ammonium nitrate were used at various concentration (0.25%, 0.50%, 0.75%, 1.0% and 1.25%) are supplemented to the synthetic medium for the production of fibrinolytic enzyme by *Aspergillus tamarii* SAS02.

**Table – 5.14 - Effect of Ammonium sulphate (Enzyme activity) - *Aspergillus tamarii* SAS02**

| SL.No | Concentration (%) /Time | 0.25 | 0.50 | 0.75 | 1.0 | 1.25 |
|---|---|---|---|---|---|---|
| 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| 2 | 24 | 19 | 23 | 22 | 28 | 25 |
| 3 | 48 | 39 | 43 | 56 | 74 | 65 |
| 4 | 72 | 55 | 74 | 87 | 106 | 86 |
| 5 | 96 | 42 | 38 | 43 | 77 | 46 |

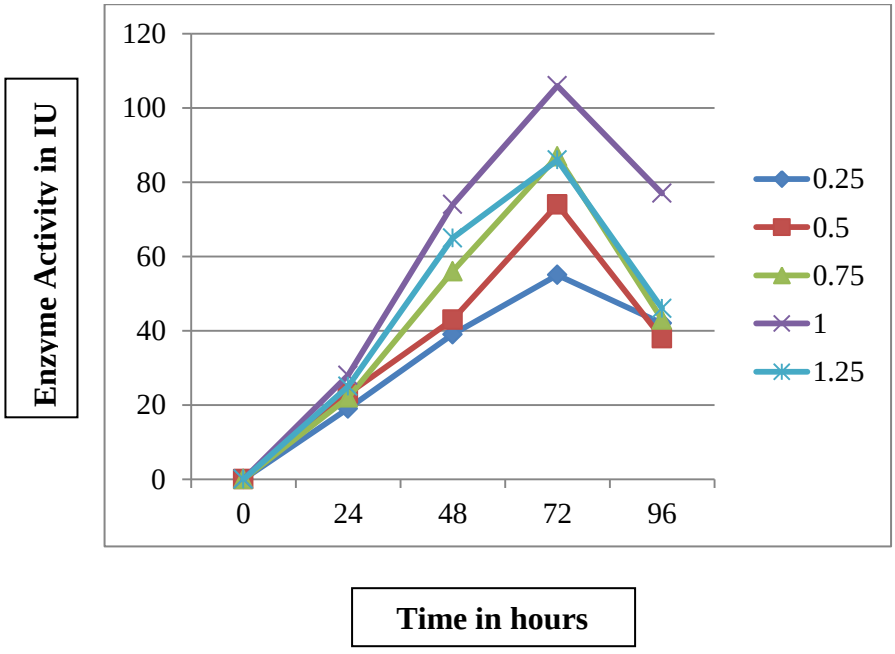



**Fig.5.16 - Effect of Ammonium sulphate on fibrinolytic enzyme production**

**Table – 5.15- Effect of Ammonium chloride (Enzyme activity) –**

***Aspergillus tamarii* SAS02**

| SL.No | Concentration (%) /Time | 0.25 | 0.50 | 0.75 | 1.0 | 1.25 |
|---|---|---|---|---|---|---|
| 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| 2 | 24 | 19 | 29 | 26 | 32 | 28 |
| 3 | 48 | 39 | 52 | 62 | 81 | 52 |
| 4 | 72 | 55 | 69 | 94 | 198 | 117 |
| 5 | 96 | 42 | 36 | 41 | 112 | 56 |

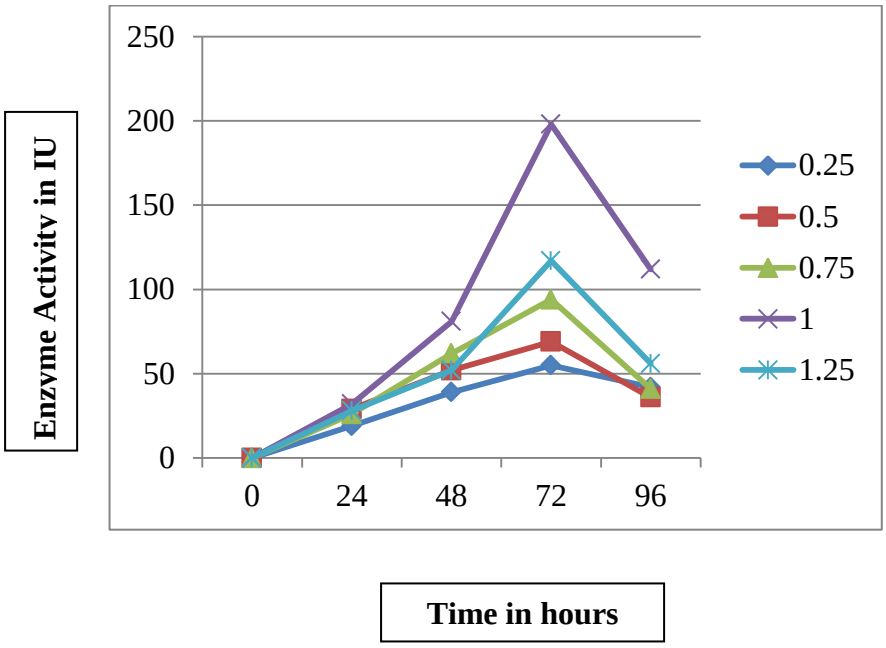



**Fig.5.17- Effect of Ammonium chloride on fibrinolytic enzyme production**

**Table – 5.16 - Effect of Ammonium nitrate (Enzyme activity) –**

***Aspergillus tamarii* SAS02**

| SL.No | Concentration (%) /Time | 0.25 | 0.50 | 0.75 | 1.0 | 1.25 |
|---|---|---|---|---|---|---|
| 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| 2 | 24 | 18 | 23 | 30 | 38 | 22 |
| 3 | 48 | 40 | 62 | 73 | 78 | 65 |
| 4 | 72 | 66 | 92 | 124 | 129 | 92 |
| 5 | 96 | 39 | 54 | 52 | 74 | 36 |

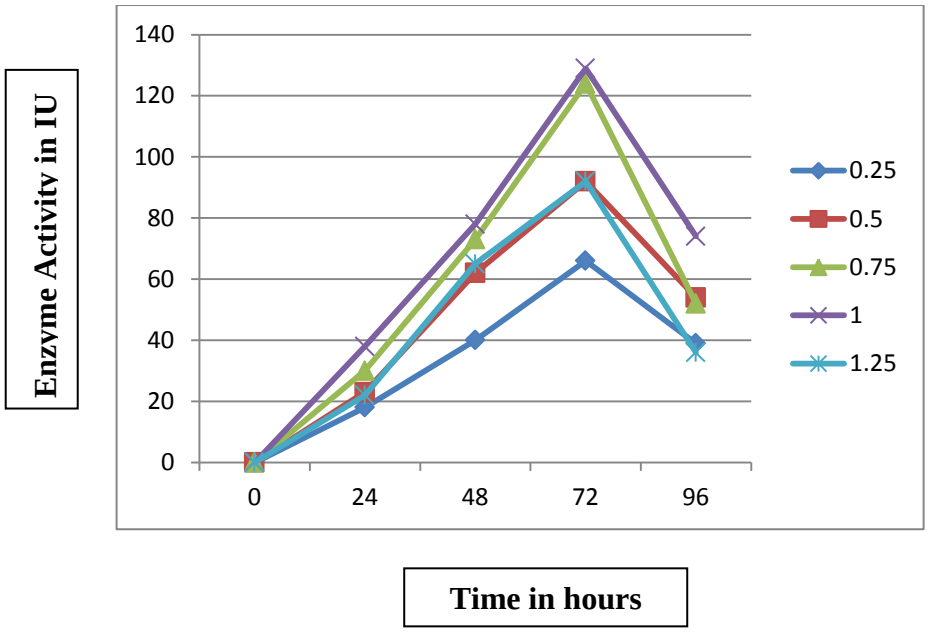



**Fig.5.18- Effect of Ammonium nitrate on fibrinolytic enzyme production**

The results pertaining to the above studies reveals that fibrinolytic enzyme production increased with the increase in the inorganic nitrogen concentration up to 1.0% and further increase in inorganic nitrogen source has revealed no significant change in fibrinolytic enzyme production. The production of fibrinolytic enzyme by Ammonium sulphate (106 IU), Ammonium chloride (198 IU), Ammonium nitrate (129 IU) was observed. The highest production of fibrinolytic enzyme 198 IU of Ammonium chloride at 72 h of fermentation period and this appear to be good inorganic nitrogen source under submerged fermentation process.

Increasing in the inorganic phosphate ($KH_2PO_4$) from 0.05 to 1.2 g/l stimulates the enzyme production from 14.3 U/ml to 19.4U/ml by M.A.Abdel-Naby (1991)[29]. Inorganic nitrogen sources showed the highest production of fibrinolytic enzyme than the organic nitrogen sources. Complex nitrogen sources are usually used for fibrinolytic enzyme production, the requirement for a specific nitrogen supplement differs from organism to organism. Sen and Satyanarayana, (1993) observed the low levels of enzyme production with the use of inorganic nitrogen sources, but Sinha and Satyanarayana (1991), observed the increased enzyme production with the use of inorganic nitrogen sources[22].

Essam.F.Al-Juamily (2012) used Ammonium chloride, Potassium nitrate and Ammonium phosphate as a inorganic nitrogen sources which showed very poor enzyme activity as compared to organic nitrogen sources. Ammonium chloride showed the highest activity of 19 U/ml by *Bacillus lichniformis* $B_4$[26].

Manojkumar, Narasimhan (2015) showed the effect of inorganic nitrogen sources of Ammonium sulphate, Ammonium-di-hydrogen phosphate and Sodium nitrite on the production of fibrinolytic enzyme by *Bacillus cereus* SRM-001. Among all the three different inorganic nitrogen sources Ammonium sulphate showed the activity of 462 U/ml, Ammonium- di- hydrogen phosphate (512 U/ml), Sodium nitrate showed the negligible activity. Moataza MS in 2015 showed that *Streptoverticillium mobaraense* showed the activity of 700 U/ml[28].

The results obtained under the present study are in accordance with the findings of Essam.F.Al-Juamily (2012)[26].

### 5.7.c. Influence of metal ions on fibrinolytic enzyme production:

Trace elements have profound effect on the growth and physiological activities of the organisms. Hence this experiment was conducted to evaluate the effect of different metal ions on fibrinolytic enzyme production. In general trace elements play a key role in the metabolism of organisms. The metal ions such as Zinc sulphate, Magnesium sulphate, Copper sulphate, Iron sulphate were employed for the production of fibrinolytic enzyme by *Aspergillus tamarii*.

**Table – 5.17 - Effect of Zinc sulphate (Enzyme activity) –**

***Aspergillus tamarii* SAS02**

| SL.No | Concentration (%)/Time | 0.01 | 0.02 | 0.03 |
|---|---|---|---|---|
| 1 | 0 | 0 | 0 | 0 |
| 2 | 24 | 29 | 35 | 22 |
| 3 | 48 | 76 | 84 | 68 |
| 4 | 72 | 115 | 152 | 76 |
| 5 | 96 | 72 | 98 | 32 |

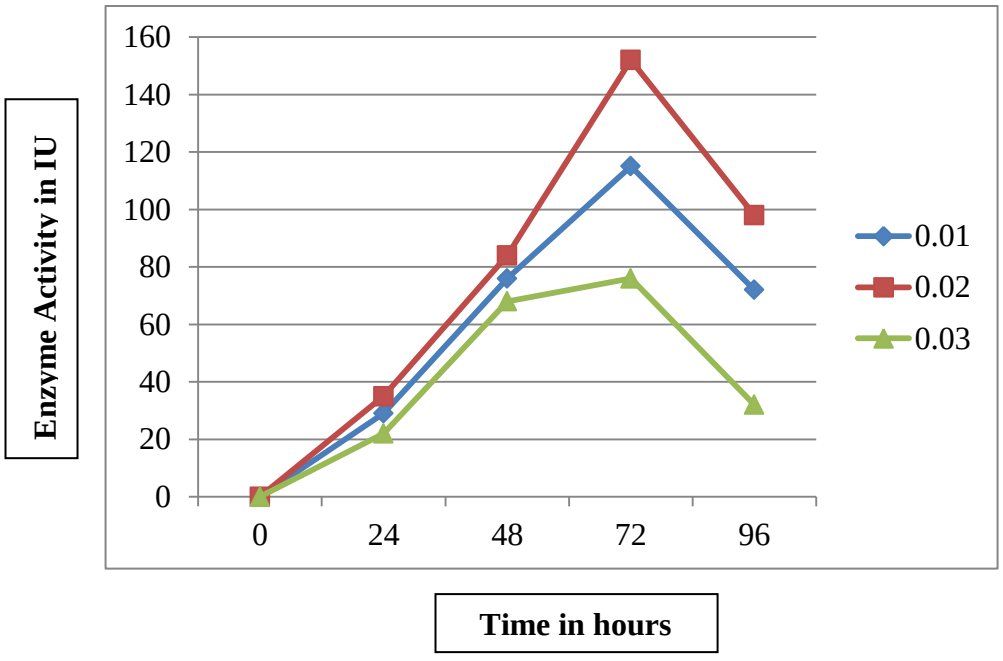



**Fig.5.19- Effect of Zinc sulphate on fibrinolytic enzyme production**

**Table – 5.18 - Effect of Magnesium sulphate (Enzyme activity) –**

***Aspergillus tamarii* SAS02**

| SL.No | Concentration (%) /Time | 0.01 | 0.02 | 0.03 |
|---|---|---|---|---|
| 1 | 0 | 0 | 0 | 0 |
| 2 | 24 | 21 | 25 | 18 |
| 3 | 48 | 46 | 54 | 45 |
| 4 | 72 | 82 | 95 | 68 |
| 5 | 96 | 41 | 48 | 32 |

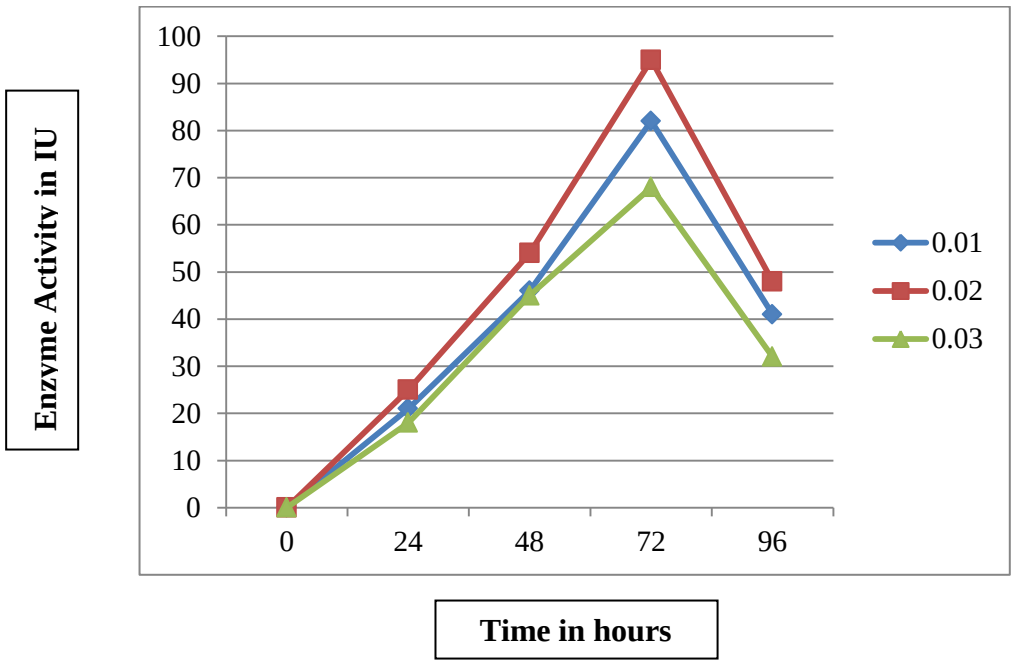



**Fig.5.20- Effect of Magnesium sulphate on fibrinolytic enzyme production**

**Table – 5.19- Effect of Copper sulphate (Enzyme activity) –**

***Aspergillus tamarii* SAS02**

| SL.No | Concentration (%) /Time | 0.01 | 0.02 | 0.03 |
|---|---|---|---|---|
| 1 | 0 | 0 | 0 | 0 |
| 2 | 24 | 16 | 15 | 18 |
| 3 | 48 | 48 | 41 | 40 |
| 4 | 72 | 66 | 71 | 68 |
| 5 | 96 | 39 | 40 | 32 |

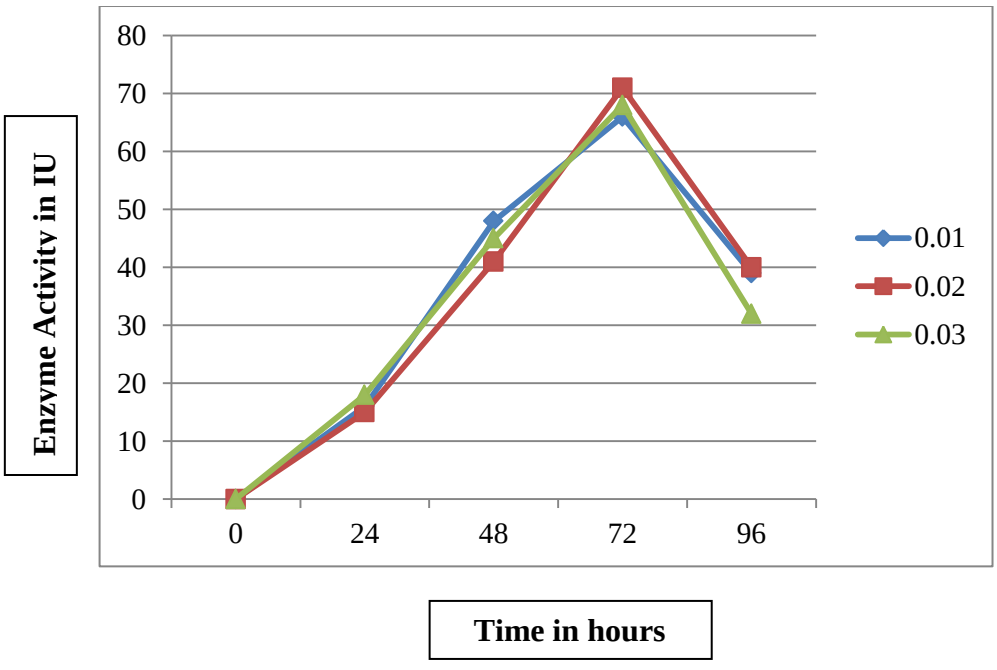



**Fig.5.21- Effect of Copper sulphate on fibrinolytic enzyme production**

**Table – 5.20 - Effect of Iron sulphate (Enzyme activity) –**

***Aspergillus tamarii* SAS02**

| SL.No | Concentration (%) /Time | 0.01 | 0.02 | 0.03 |
|---|---|---|---|---|
| 1 | 0 | 0 | 0 | 0 |
| 2 | 24 | 21 | 26 | 24 |
| 3 | 48 | 56 | 56 | 56 |
| 4 | 72 | 98 | 98 | 76 |
| 5 | 96 | 45 | 39 | 37 |

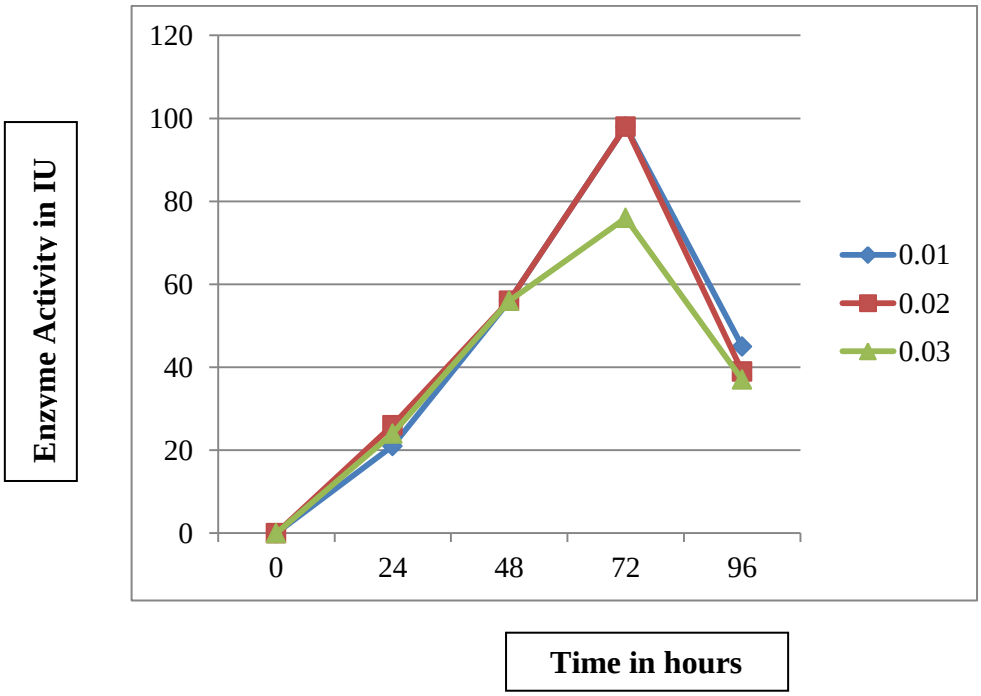



**Fig.5.22- Effect of Iron sulphate on fibrinolytic enzyme production**

The addition of the metal ions was done in the percentages of 0.01%, 0.02% and 0.03%. The production of fibrinolytic enzyme with Zinc sulphate (152 IU), Magnesium sulphate (95 IU), Copper sulphate (71 IU), Iron sulphate (98 IU). The highest production of fibrinolytic enzyme is with the presence of Zinc sulphate (152 IU) at 0.02% at 72h of fermentation period.

All microorganisms require certain mineral elements for growth and metabolism. Certain trace elements like magnesium, manganese, copper, zinc, iron, cobalt and calcium are needed at appropriate concentrations and must be added as distinct components to the fermentation medium. Further, 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.

A major determinant of their functional relevance in living systems is the requirement of metals for substantial fraction of enzymes for their catalytic activity. Trace elements have profound effect for the growth and physiological activities of the organisms; they are the important modulators of the biological response. The metals play their most important role as cofactors in enzyme production. A number of divalent metals like iron, copper, zinc, manganese and magnesium have been found to be essential in trace levels for a successful process reported by Kapoor *et al*., 1982[22].

M.A.Abdel-Naby (1991) showed that by adding trace metals $Zn^{2+}$, $Fe^{2+}$, $Mn^{2+}$) separately and also in the combined form does not shown any significant effect on the production of fibrinolytic enzyme by *Streptomyces sp*.NRC 411. Metal ions did not activate the enzyme produced by *B.subtilis* DC33 showed by Cheng Tao Wang in (2005)[29].

It is well established that a number of metal ions are essential to life Bertini (2006), Frausto da silva and Williams (2001). Metals are involved in redox (30%) and non-redox catalysis (70%). The most significant property is Lewis acidity, related to the power of a reaction centre to attract electrons. The enzyme produced by *S.commune* BL23 may be metalloprotease because it showed 30% inhibition in presence of $Cu^{2+}$ and 50% inhibition in presence of $Zn^{2+}$, the activity were

completely inhibited by $Hg^{2+}$ but the presence of $CO^{2+}$ stimulated the enzyme activity[56-57].

In fact, the metal removes electron density from the legend and causes polarization of the substrate/ cofactor reactive bonds, thereby increasing its electrophilic character reported by Sissi and palumbo (2009). In Suk Park (2010), reported that *Schiophyllum commune* showed the increase in enzyme activity by addition of $Zn^{2+}$ (63%), where as $Hg^{2+}$ decreased the activity by 63%. The other metals like $CO^{2+}$, $Cu^{2+}$, $Fe^{2+}$, $Mg^{2+}$ and $Mn^{2+}$ did not show any significant effect on the fibrinolytic activity[16].

Ravikumar *et al*. (2012) found that $Cu^{2+,}$ $Mg^{2+}$ and $Zn^{2+}$ are found to be the activator of fibrinolytic enzyme. Maximum enzyme production by *Citrobacter braakii* was observed along with cell growth in the presence of dipotassium hydrogen phosphate and also observed on incorporation of sodium chloride to soyaflour-based optimized medium by Anjana Sharma (2015)[23].

The fibrinolytic enzyme produced by *B.halodurans* showed the highest activity in presence of $Ca^{2+}$, $Mg^{2+}$,$CO^{2+}$ and $Na^{+}$ where as by the addition of $Cu^{2+}$, $Hg^{2+}$, $Fe^{2+}$and $Zn^{2+}$ enzyme activity was lowered reported by P.Vijayaraghavan et al. (2016)[10]. Mohammed A.Almalki (2017) reported that calcium chloride showed the highest production of fibrinolytic anzyme by *Bacillus sp*. IND6 whereas magnesium sulphate has been found to be the good inducer for fibrinolytic enzyme production[19].

Results obtained under the present study are similar with the findings of Ravikumar *et al*., in 2012.

### 5.8. Comparative studies of fibrinolytic enzyme *Aspergillus tamarii* SAS02 with few bacterial *sp*.

Fibrinolytic enzyme extracted from *Aspergillus tamarii* SAS02 showed the activity of 180 IU/ml. The activity of the enzyme is also compared with *Bacillus sp*, *Pseudomonas sp* and *E.coli*. As shown in the figures *Bacillus sp* showed the activity of 17.5 IU/ml, *Pseudomonas sp* showed the activity of 14.1 IU/ml, *E.coli* showed the

activity of 12.5 IU/ml. When the enzyme activity is compared between the *Aspergillus tamarii* SAS02 and with Bacterial sp, *Aspergillus tamarii* SAS02 showed the highest activity and thus we opted it for our research work.

**Comparative studies on fibrinolytic enzyme from bacterial and fungal strains (*Aspergillus tamarii* SAS02)**

**Table – 5.21- Fibrinolytic enzyme production by Bacillus sp:**

| SL.No | Inoculum Concentration(ml)/Time | 0.5 ml | 1.0 ml |
|---|---|---|---|
| 1 | 0 | 0 | 0 |
| 2 | 24 | 6.8 | 8.1 |
| 3 | 48 | 13.5 | 17.5 |
| 4 | 72 | 7.2 | 12.4 |
| 5 | 96 | 5.4 | 6.5 |

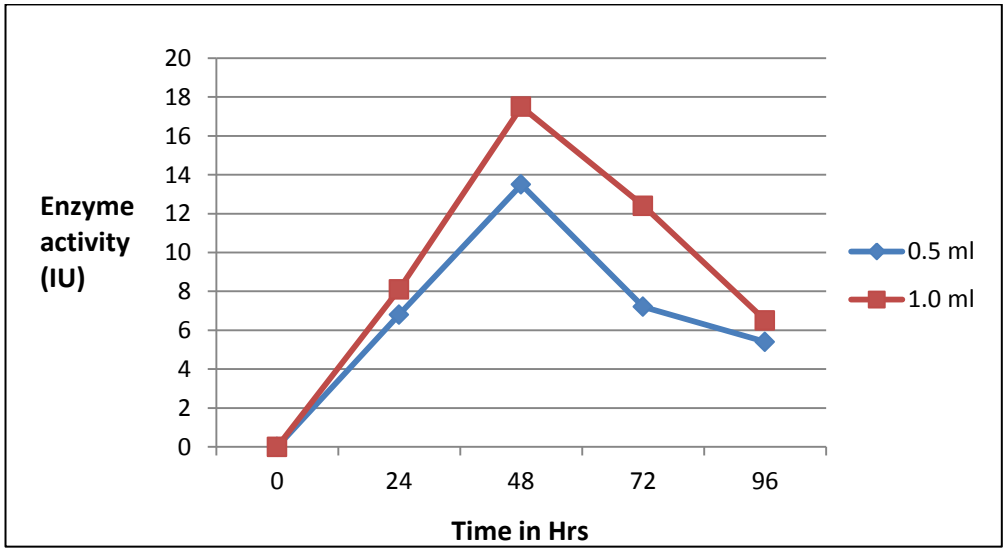



**Fig-5.23- Fibrinolytic enzyme production by *Bacillus sp.***

**Table – 5.22- Fibrinolytic enzyme production by *Pseudomonas sp*:**

| SL.No | Inoculum Concentration(ml)/Time | 0.5 ml | 1.0ml |
|---|---|---|---|
| 1 | 0 | 0 | 0 |
| 2 | 24 | 4.5 | 7.2 |
| 3 | 48 | 11.4 | 14.1 |
| 4 | 72 | 6.8 | 10.5 |
| 5 | 96 | 4.1 | 4.2 |

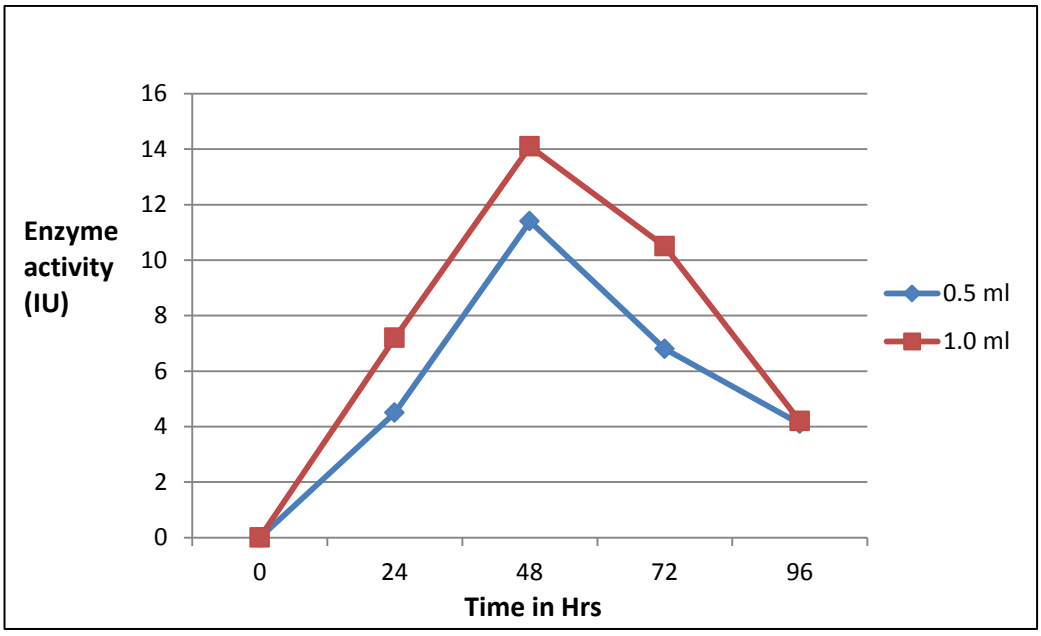



**Fig-5.24- Fibrinolytic enzyme production by *Pseudomonas sp*.**

**Table – 5.23- Fibrinolytic enzyme production by *E.Coli*:**

| SL.No | Inoculum Concentration(ml)/Time | 0.5 ml | 1.0ml |
|---|---|---|---|
| 1 | 0 | 0 | 0 |
| 2 | 24 | 3.5 | 4.2 |
| 3 | 48 | 8.4 | 12.5 |
| 4 | 72 | 6.2 | 7.5 |
| 5 | 96 | 2.4 | 5.5 |

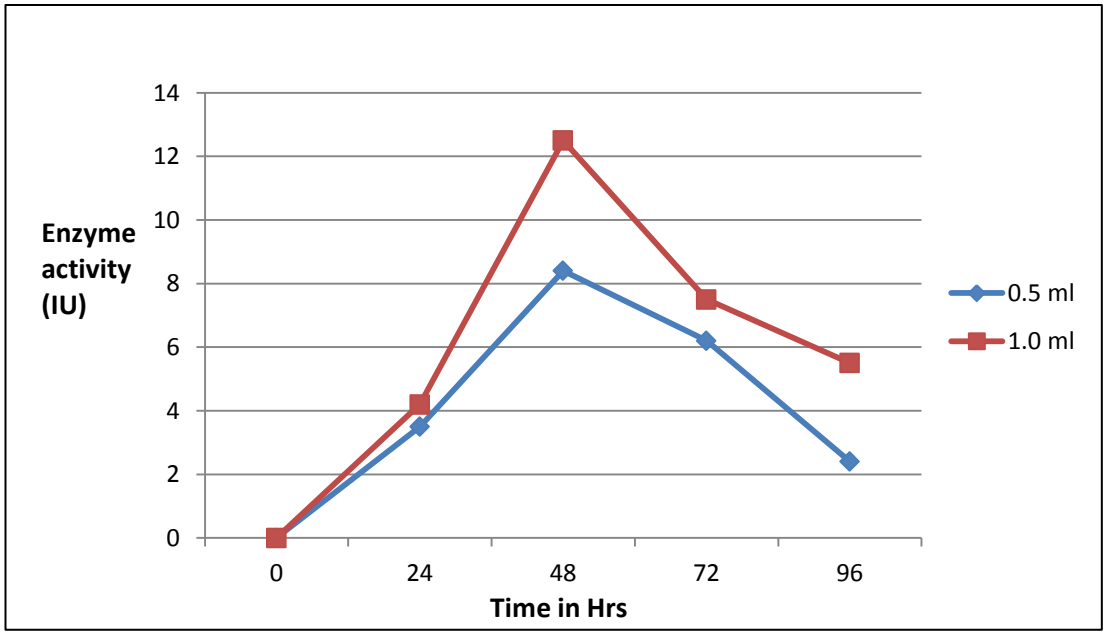



**Fig-5.25- Fibrinolytic enzyme production by *E.Coli*:**

### 5.9. Blood clot hydrolysis by fibrinolytic enzyme

In the present study blood clot hydrolysis was observed in both the plates, which contains capillary tubes filled with blood clots. One which contains purified enzyme extracted from *Aspergillus tamarii* SAS02 and in the other plate commercially available enzyme (Brand name – Myokinase) with the concentration of 200 IU/ml. Capillaries present in both the plates showed the action of blood clot degradation with the incubation period of 15-18 hrs, where the same quantity of degradation was observed in both the capillary tubes. Hence we say that our enzyme is a tool for obliteration of blood clots.

Serratopeptidase is an excellent blood thinning and clot dissolving agent. It acts upon inflammation by thinning the fluids in the body that collect around injured areas and increasing fluid drainage, this enhances tissue repair and reduces pain. Serratopeptidase breaks down atherosclerotic plaques accumulated in arteries. It is effective in removing deposition of fatty substances, cholesterol, cellular waste products, calcium and fibrin inside the arteries[22].

Jun Yang in 2012 showed the thrombolytic effect of dilated fundus Examination from Bacillus subtilis both invitro and invivo. Natural blood clots were done with the fresh animal blood samples, this clots was cut into same size and places on the petriplates , incubation was done at $37^0$C for 24hrs with the urokinase of 5000 U as a positive control followed with different concentrations of DFE on blood clots. Clot lysis was inspected on the petriplates for every 2.5hrs[32].

Sasirekha in 2012 found that fibrinolytic enzyme isolated from Actinomycetes showed the degradation of the blood clot within 2hrs. To determine the thrombolytic effect of the enzyme the control tube was kept only with the blood clot no enzyme in the other tube the blood clot was treated with the enzyme and inspected for every 15 minutes, the blood clot get decreased from initial size compared with the end of the experiment. This indicates the strong enzyme activity on fibrin[9].

Manoj kumar in 2015 showed that the fibrinolytic enzyme isolated from *B.cereus* at 180 minutes of incubation a residual 13% of the initial clot was obtained saying that 87% of the blood clot was removed due to lysis by the fibrinolytic enzyme. This proves that the enzyme secreted by *B.cereus* SRM-001 is a potent fibrinolytic enzyme[28].

Thrombosis which is formed due to the accumulation of fibrin in the blood vessels which leads to myocardial infarction and other CVD's, for these cases microbial fibrinolytic enzymes attracted for thrombolytic therapy than the typical thrombolytic agents because of the expensive prices and the undesirable side effects. Hence it can be used as drugs to prevent or cure thrombosis and other related diseases. Fibrinolytic enzyme plays key role in case of vascular injury and stops bleeding to overcome the threat to life. The enzymes show therapeutic potential in treating many diseases like myocardial infarction, venous stroke and pulmonary clotting reported by Hajra Ashraf, 2017[33].

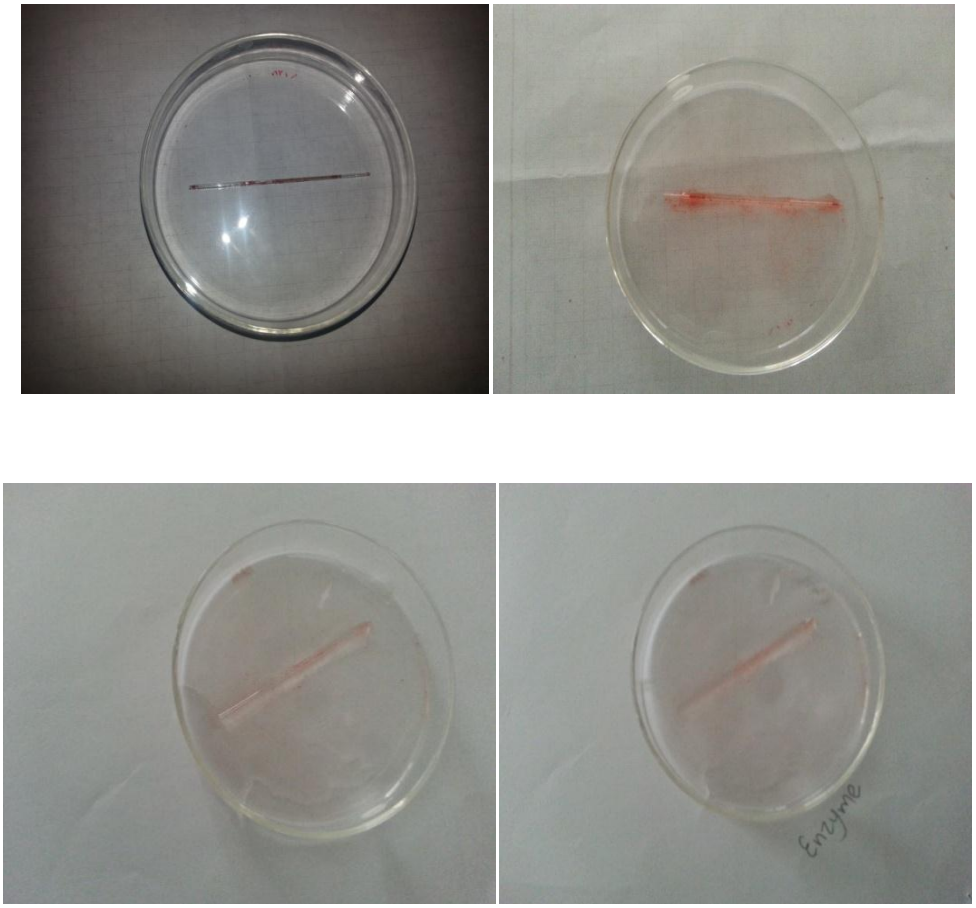



**Plate-5.6: Blood clot hydrolysis by fibrinolytic enzyme from**

***Aspergillus tamarii* SAS02**

**5.10. Mutational study of *Aspergillus tamarii* SAS02:**

The mutant strain obtained from *Aspergillus tamarii* SAS02, is *Aspergillus tamarii* SAS02 *mu* (obtained by irradiation at heights of 5cm, 10cm, 15cm and 20 cm respectively for 15 minutes). Results on the studies relating to the fibrinolytic enzyme production by the mutant strain of *Aspergillus tamarii* SAS02 mu on synthetic medium fermented for 72h are presented below. Results revealed that the mutant strain *Aspergillus tamarii* SAS02 mu produced better amount of fibrinolytic enzyme when compared to the parent strain *Aspergillus tamarii* SAS02. The mutant strain *Aspergillus tamarii* SAS02 mu produced 213 IU of fibrinolytic enzyme when compared to parent strain *Aspergillus tamarii* SAS02, which could yield only 180 IU of fibrinolytic enzyme on synthetic medium.

**Plate: 5.7-*Aspergillus tamarii* SAS02 mu**

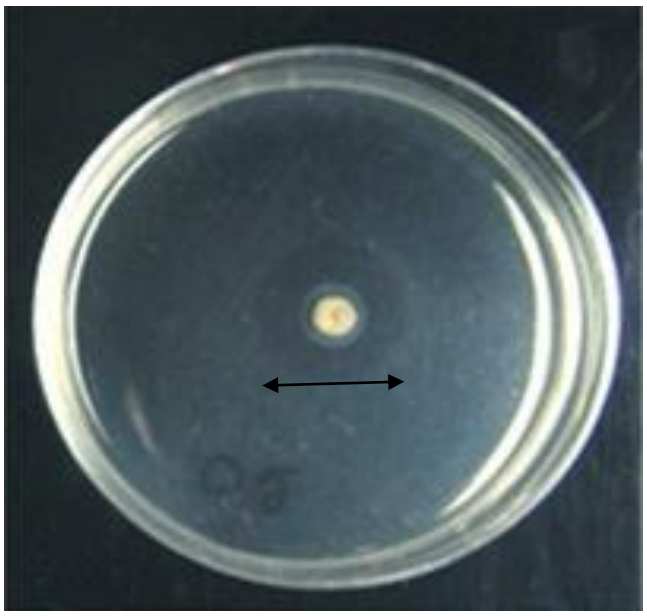


**Plate: 5.8-*Aspergillus tamarii* SAS02 mu**

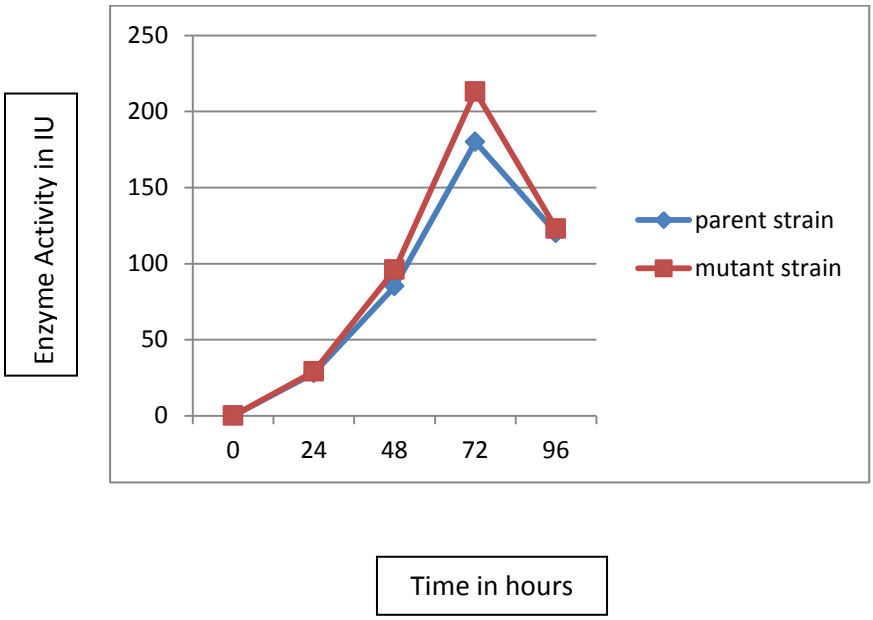



**Fig- 5.26- Comparative studies of Fibrinolytic enzyme production (*Aspergillus tamarii* SAS02 v/s *Aspergillus tamarii* SAS02mu)**

After the various mutagenic treatments by physical and chemical agents such as UV irradiation, Methyl methanesulfonate (MMS), EMS, Methylnitronitrosoguanidine (MNNG), Nitros acid ($HNO_2$) and Ethidium bromide (EtBr), the productivity of the enzyme was enhanced. Few reports were available on the development of mutant strains through conventional methods reported by Dutta and Banerjee (2006) Nadeem *et al*. (2010); Vasudea, 2010. Mutagenesis is also achieved by different methods like irradiation and using chemicals. The chemical mutagens are less potent, inefficient and also cause side effects to the researches. Therefore when mold are the target organisms irradiation mutagens were used for mutagenic study[31].

Generally X-rays, gamma rays and UV rays are frequently used as mutagens where as X-rays and gamma rays have high penetrating power and cause greater nuclear damages and also cause lethal effect in the organisms. So such a powerful mutagens which causes nuclear damage are avoided. But the UV rays with shorter wavelength than the visible light are mild mutagens, hence they are considered to be ideal for induction of mutations showed by Elander (1969); Hopwood (1970); Thoma (1971). Hopwood *et al*. in 1985 suggested that 99.9% kill is best suited for strain improvement as the fewer will still survive which require repeated mutations or multiple mutations which enhances the productivity of the culture[22].

Conventional mutagenesis and current recombinant DNA technology have been adopted to improve enzyme production and simplify downstream manipulation. Lai *et al* (2004) reported successful doubling of the specific activity of fibrinolytic enzyme through random mutagenesis invitro using the chemical ethyl methane sulfonate (EMS)[22].

In the present study the attempt was made to check the potentiality between parental strain *Aspergillus tamarii* SAS02 with the mutated strain *Aspergillus tamarii* SAS02mu. The mutated strain exhibits higher enzyme activity than the parental strain. This helps the researchers to carry out the production process using mutated stain in future.

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## 6.1. Summary:

Microorganisms are being used for thousands of years to supply fermented products. The importance of microbes was increased significantly during World War I during which development of bioconversion and fermentation of many useful products such as enzyme, amino acids, nucleotides, vitamins, organic acids, solvents, vaccines and polysaccharides are invented. A major segment of these is represented as enzyme. Enzyme is a major resource utilized by the food, chemical and allied industries to produce a wide range of biotechnology products and have already been recognized as valuable catalysts for various organic transformations and production of fine chemicals and pharmaceuticals.

Enzymes have attracted attention of researchers all over the world because of the wide range of physiological, analytical and industrial applications, especially form microorganisms, because of their broad biochemical diversity, feasibility of mass culture and ease of genetic manipulation. Despite the fact that more than 3000 different enzymes have been identified and many of them have found their way into biotechnological and industrial applications, the present enzyme toolbox is not sufficient to meet most of the industrial demands. In view of these limitations, researchers have diverted their attention for isolation and characterization of enzymes from different environments. Whenever required, due attention is also paid towards development of recombinant enzymes with desired characteristics and for specific applications.

Enzymes are the work and the life-force of the human body. Three thousand different enzymes have been identified in the human body that results in over 1000 enzymatic reactions. The main advantage of enzymes compared to most other catalysts is their stereo, region, chemo selectivity and specificity. Regular consumption of enzymes and enzyme-rich foods is a key to vibrant health, disease prevention and anti-aging process. Every cell in our body needs enzymes for its biochemical functions and a deficiency will accelerate the aging process. Some of the important functions of enzymes include: i) regulating the growth of the body from a single cell to a mature organism, ii) converting food to energy to satisfy the

body's needs and iii) breaking down or building up of certain substances within the cell.

Proteolytic enzymes are ubiquitous in occurrence, found in all living organisms, and are essential for cell growth and differentiation. There is renewed interest in the study of proteolytic enzymes, mainly due to the recognition that these enzymes not only play an important role in the cellular metabolic processes but have also gained considerable attention in the industrial community. Proteases execute a large variety of functions, extending from the cellular level to the organ and organism level, to produce cascade systems such as homeostasis and inflammation. They are responsible for the complex processes involved in the normal physiology of the cell as well as in abnormal patho physiological conditions.

Microbes are the goldmine of proteases and represent preferred sources of enzymes in view of their rapid growth, limited space required for cultivation and ready accessibility to the genetic manipulation. Initially all these microbes live in natural environment where most of the nutrients required for growth exist in the form of macromolecules. These nutrients cannot be utilized without degrading into smaller molecules. The degradation is carried by the secretion of extracellular enzymes.

The summary of the results obtained on the basis of the results presented in the present thesis are outlined briefly as below:

1. A brief introduction and purpose of the study is to find out the effective fibrinolytic enzyme from the fungal source which can be used to cure or minimize the development of blood clots in the normal blood circulation which is a main cause of CVD's.

2. All the objectives were evaluated with the following experimental procedures.

3. An exhaustive review of the relevant aspects of enzyme chemistry and historical aspects, the characteristics of the fibrinolytic enzyme and medium are presented and also an attempt has been made to evaluate the different medium were used in

fermentation for production of fibrinolytic enzyme and also application of the fibrinolytic enzyme.

4. The aspects covered are mainly collection of soil samples, isolation of fungi, screening using fibrin plate assay, selection based on the clear zone around the culture. Preparation of fermentation medium for the production of fibrinolytic enzyme using submerged fermentation method. Optimization of fermentation conditions for pH, temperature and inoculum size. Process economization through nutrient supplementation like carbon source, nitrogen sources and also few metal ions. Mutation of *Aspergillus tamarii* SAS02 for maximum production of fibrinolytic enzyme, purification and also with biochemical characterization of fibrinolytic enzyme, comparative studies with few bacterial strains and application of the enzyme on clot hydrolysis.

5. The results are indicated in brief as below:

a. The isolates of Aspergillus tamarii were initially subjected for fibrinolytic enzyme production through fibrin plate assay method by evaluating clear zone around the colony. Out of the 20 isolates SAS02 exhibited higher zone of diameter 19 mm and hence the same was considered as the promising strain for fibrinolytic enzyme production.

b. Optimization of submerged fermentation parameters like initial pH of the substrate the temperature and the inoculum size on fibrinolytic enzyme production was carried out, because the success and direction of fermentation depends on obtaining a proper balance between the components of the synthetic medium. Once the parameter was optimized, the optimum level of the parameter was continued in the next step of experiment.

i. The studies reveal that the optimum fermentation period required for the production of maximum fibrinolytic enzyme from synthetic medium was 72 hrs by employing *Aspergillus tamarii* SAS02.

ii. The maximum production of fibrinolytic enzyme by the organism was observed at pH 6.0 of 150 IU/ml, temperature of $40^0$C was 170 IU/ml and inoculum size of 11.25ml showed 180 IU/ml.

c. The studies on the purification of fibrinolytic enzymes were carried out and reveals that the crude enzyme extract showed lowest specific activity of 130.28 IU with 100% yield of protein and 1% purity, whereas in ammonium sulphate precipitation method there was about 150 IU with the 84.5% recovery of proteins and 115.13% fold purity. In gel filtration chromatography (Sephadex G-75 column) showed the specific activity of 172.6 IU with the recovery of 51.4% protein with 132.48 folds of purification. Among all these in Ion-exchange chromatography (DEAE-cellulose) there was a highest specific activity of 417.39 IU with the 16.19% protein recovery which showed 320.07% folds of purity. The molecular weight of fibrinolytic enzyme obtained from *Aspergillus tamarii* SAS02 was 29kDa.

d. The enzyme characteristics indicated that the enzyme activity is optimum at alkaline pH of 8.0 and the optimum temperature was found to be $40^0$C. The temperature stability showed by the fibrinolytic enzyme obtained from *Aspergillus tamarii* SAS02 at 30 and 60min is $55^0$C. At room temperature it showed maximum activity of $55^0$C for 30min and it displayed better stability. But at 60min at the same $55^0$C enzyme loses its activity significantly.

e. The LB-plot developed by plotting 1/[S] against 1/ [V] displayed a Vmax of 2.1 with a Km of 1.25.

f. The purified enzyme activity was induced up to 126.38% by $MgSO_4$ and all other metal ions were found to be inhibitors. The PMSF inhibited the enzyme, so the enzyme obtained from *Aspergillus tamarii* SAS02 is a serine protease. Enzyme displayed 38.2% activity in the presence of SDS indicating the susceptibility of the enzyme for detergents. The enzyme was not stable to saline and was not resistant to bleach so it lost activity in the presence of NaCl and $H_2O_2$.

g. Molecular characterization of fibrinolytic enzyme was done by taking SAS02 sample subjected it to PCR amplification from different primer and the sequencing result was observed. The sequence was compared with existing sequences in the NCBI database using the Blast N program it is best matched with that of *Aspergillus tamarii* isolate AW13E and named it as *Aspergillus tamarii* SAS02.

h. In the studies dealing with the process economization, attempts have been made to improve the production of fibrinolytic enzyme during submerged fermentation by using synthetic medium through supplementation of various nutrients and also by mutation of the fermenting organism.

In the studies involving nutrient supplementation, the synthetic medium was amended with varying concentration of different carbon sources, organic and inorganic nitrogen sources and metal ions individually as below:

i. Among all the three carbon sources tested for fibrinolytic enzyme production glucose proved to be beneficial. The optimum concentration of glucose needed to be supplemented to the synthetic medium to yield maximum fibrinolytic enzyme production by *Aspergillus tamarii* SAS02 was observed to be 1.0%, at this level of glucose supplementation *Aspergillus tamarii* SAS02 produced 186 IU of fibrinolytic enzyme at 72 hrs of fermentation.

ii. Peptone as an organic nitrogen source showed a maximum fibrinolytic enzyme production of 193 IU at 1.0% by *Aspergillus tamarii* SAS02at 72hrs of fermentation. And proved peptone is beneficial than yeast extract and beef extract.

iii. Among the inorganic nitrogen sources like Ammonium sulphate, Ammonium chloride and Ammonium nitrate tested for fibrinolytic enzyme production by *Aspergillus tamarii* SAS02, Ammonium chloride proved to be beneficial by producing 198 IU at 1.0% at 72hrs of fermentation.

iv. Zinc sulphate, magnesium sulphate, copper sulphate and iron sulphate are tested for the fibrinolytic enzyme production by *Aspergillus tamarii* SAS02. Among all the four metal ions Zinc sulphate proves to be beneficial by producing 152 IU at 1.0% at 72hrs of fermentation.

i. Fibrinolytic enzyme activity of *Aspergillus tamarii* SAS02 is been compared with few bacteria like *Bacillus sp, pseudomonas sp and E.Coli*. Enzyme obtained from *Aspergillus tamarii* SAS02 showed highest activity ranges from 150-200 IU in different stages.

j. Blood clot degradation was observed in the capillary tubes which contain blood clots placed in the petriplates one which contain the same concentration of commercially available enzyme and the other enzyme which is extracted from *Aspergillus tamarii* SAS02. In both the plated same amount of degradation was observed at the same time, proved it is a tool for obliteration of blood clots.

k. Induction of mutation was attempted by UV rays treatment at the distance of 5cm, 10cm, 15cm and 20cm for 15min. All the four plates showed maximum zone clearance around the colony when compared to the parent strain of *Aspergillus tamarii* SAS02 on synthetic medium. The strain which is at a distance of 15cm for 10min exhibited zone of clearance and exhibited 213 IU of enzyme activity for 72hrs of fermentation and thus proved *Aspergillus tamarii* SAS02mu strain showed maximum activity than *Aspergillus tamarii* SAS02 which exhibited for 72hrs of fermentation.

**Conclusion:**

- The Fibrinolytic enzyme isolated from *Aspergillus tamarii* SAS02 from the soil sample showed a clear zone appearance around it of about 19mm.
- For the production of this enzyme, the production media is standardized for the optimization of pH-6, temperature $40^0$C and the inoculum size of about 1.25ml.
- Tyrosine curve and BSA curve is plotted to standardize the liberation of tyrosine and protein during the enzyme assay.
- Purification of the enzyme is done using salt precipitation, gel filtration chromatography, ion-exchange chromatography and PAGE and obtained a yield of 320.07% and 16.19% folds of purification.
- Purified enzyme is characterized and showed its activity till pH-8, temperature stability of up to $55^0$C.
- Enzyme substrate concentration value is Km-1.25 and Vmax-2.1 is plotted against Lineweaver and Burk plot.
- Presence of EDTA and 1-10 Phenanthroline showed complete inhibition of action of enzyme. Characterization of the enzyme's ITS region is done using PCR amplification.
- Process economization is carried out using few carbon, nitrogen (organic and inorganic) source and metal ions. From the carbon source use of glucose showed the highest activity of 186 IU, Peptone as a organic nitrogen showed the highest activity of 193 IU, Ammonium chloride as a inorganic nitrogen source showed activity of 198 IU, Zinc sulphate as a trace element showed the activity of 152 IU.
- Blood clot degradation process was observed by comparing it with the commercially available enzyme, in both the plates same amount of degradation is observed at the same time and proved it is a tool for obliteration of blood clots.

- When the activity of the enzyme isolated from Aspergillus tamarii SAS02 is compared with few bacterial species like Bacillus sp, pseudomonas sp and E.Coli, Aspergillus tamarii SAS02 showed the activity of 180IU. Mutational studies is done using UV rays, the mutated strain showed the highest activity of 213 IU.

**PUBLICATIONS:**

1. Shilpa. H.K. An approach on biosynthesis of fibrinolytic enzyme from *Aspergillus sp*. JOCPR.2014, 6(12), 797-801.

2. Shilpa. H.K. Molecular confirmation and Role of carbon source for Biosynthesis of fibrinolytic enzyme by Aspergillus tamarii. Der Pharmacia Lettre, 2016, 8 (6):23-28.

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## 6.2. Limitations of the study:

Development of other microbial fibrinolytic enzymes is still ongoing and much work needs to be done intensively and extensively especially concerning thrombolytic effects in vivo.

### 6.3. Future directions:

The study finding reveals that enzymes can be used as natural medicines to treat cardiovascular disorders. Optimal modes, dosage of administration, combination of thrombolytic agents have to be explored further. And also improvement can be done by making use of these enzymes on patients with clotting disorders.