

INVITRO AND INSILICO ANALYSIS OF FIBRINOLYTIC ACTIVITY OF MICROBIAL ENZYMES

Submitted in partial fulfilment of the requirements for the award of

Bachelor of Technology degree in Biotechnology

by

SRINIVAS T (38230032)



**DEPARTMENT OF BIOTECHNOLOGY
SCHOOL OF BIO AND CHEMICAL ENGINEERING**

SATHYABAMA

**INSTITUTE OF SCIENCE AND TECHNOLOGY
(DEEMED TO BE UNIVERSITY)**

Accredited with Grade "A" by NAAC

JEPPIAAR NAGAR, RAJIV GANDHI SALAI, CHENNAI - 600119

MAY – 2022



AICTE

SATHYABAMA

INSTITUTE OF SCIENCE AND TECHNOLOGY
(DEEMED TO BE UNIVERSITY)

Accredited with “A” grade by NAAC | 12B Status by UGC | Approved by

Jeppiaar Nagar, Rajiv Gandhi Salai, Chennai – 600 119

www.sathyabama.ac.in

DEPARTMENT OF BIOTECHNOLOGY

BONAFIDE CERTIFICATE

This is to certify that this Project Report is the bonafide work of SRINIVAS T (38230032) who carried out the project entitled “**INVITRO AND INSILICO ANALYSIS OF FIBRINOLYTIC ACTIVITY OF MICROBIAL ENZYMES**” under our supervision from January 2022 to April 2022.

Dr . L. INBATHAMIZH

Internal Guide

Head of the Department

Dr. V Ramesh Kumar

Submitted for Viva voce Examination held on _____.

Internal Examiner

External Examiner

DECLARATION

I, **SRINIVAS T (38230032)**, hereby declare that the Project Report entitled “**INVITRO AND INSILICO ANALYSIS OF FIBRINOLYTIC ACTIVITY OF MICROBIAL ENZYMES**” done by me under the guidance of **Dr. Gracy Jenifer and Dr. Jerrine Joseph** at **Sathyabama Institute of Science and Technology, Chennai** is submitted in partial fulfilment of the requirements for the award of Bachelor of Technology degree in Biotechnology.

DATE:

A handwritten signature in black ink, appearing to read 'T. Srinivas', is written on a light blue rectangular background.

PLACE: CHENNAI

SIGNATURE OF THE CANDIDATE

ACKNOWLEDGEMENT

I am pleased to acknowledge my sincere thanks to the Board of Management of SATHYABAMA INSTITUTE OF SCIENCE AND TECHNOLOGY for their kind encouragement in doing this project and for completing it successfully. I am grateful to them.

I convey my thanks to **Dr. V Ramesh Kumar**, Head of the Department, Dept. of Biotechnology for providing me the necessary support and details at the right time during the progressive reviews.

I would like to express my sincere and deep sense of gratitude to my Project Guides **Dr. L. Inbathamizh** for her valuable guidance, suggestions and constant encouragement paved the way for the successful completion of my project work.

I wish to express my thanks to **Dr. P Prakash**, Year Coordinator, all Teaching and Non-teaching staff members of the Department of Biotechnology who were helpful in many ways for the completion of the project

LIST OF CONTENTS

CONTENTS	PAGE NO.
CHAPTER 1	
INTRODUCTION	
1.1 Fibrinolytic Enzymes	1
1.2 Thrombosis	1
1.3 Nattokinase	2
1.4 Streptokinase	2
1.5 Fibrinolytic enzyme from <i>Cordyceps militaris</i>	3
CHAPTER 2	
LITERATURE SURVEY	
2. 1 Nattokinase	4
2. 2 Streptokinase	5
2. 3 <i>Cordyceps militaris</i>	5
2. 4 Recovery of Streptokinase Dissolved Blood Clotting	5
2. 5 Recovery of Nattokinase Dissolved Blood Clotting	6
CHAPTER 3	
AIM AND OBJECTIVE	
3. 1 Aim	7
3. 2 Objective	7
3. 3 Scope	7
CHAPTER 4	
MATERIALS AND METHOD	
4. 1 Molecular Docking	8
4. 1. 1 Structures	8
4. 1. 2 Protein Data Bank	10
4. 1. 3 Biovia Discovery Studio	11
4. 1. 4 SWISS Model	11
4. 1. 5 Patchdock	11
4. 1. 6 Ligplot+	12

4. 2 Invitro Analysis	12
4. 2. 1 Sample collection	12
4. 2. 2 Partial Purification	15
4. 2. 3 Ammonium Sulphate Precipitaion	15
4. 2. 4 Dialysis	
15	
4. 2. 5 Clot Lysis Method	16
CHAPTER 5	
RESULT AND DISCUSSION	
5. 1 Docking Results	
17 5. 1. 1 Nattokinase – Fibrin	17
5. 1. 2 Nattokinase – Fibrinogen	19
5. 1. 3 Streptokinase – Fibrin	22
5. 1. 4 Streptokinase – Fibrinogen	
25	
5. 1. 5 Fibrinolytic Enzyme from Cordyceps militaris – Fibrin	30
5. 1. 6 Fibrinolytic Enzyme from Cordyceps militaris – Fibrinogen	32
5. 2 Invitro Results	36
5. 2. 1 Comparison between Human and Sheep Fibrinogen	36
5. 2. 2 Percentage of Clot Lysis	37
CHAPTER 6	
SUMMARY AND CONCLUSION	38

LIST OF FIGURES

Fig no.	Figure Name	Page
4. 1	Nattokinase	9
4. 2	Fibrinolytic enzyme from <i>Cordyceps militaris</i>	9
4. 3	Streptokinase	9
4. 4	Fibrin	10
4. 5	Fibrinogen	10
4. 6	Dried <i>Cordyceps militaris</i>	13
4. 7	Dried and powdered <i>Cordyceps militaris</i>	13
4. 8	Streptokinase	14
4. 9	Nattokinase	14
4. 10	Dialysis	16
4. 11	Blood clot before adding sample	16
5. 1	2D Visualisation of interaction between Fibrin Alpha(A, D) chain with Nattokinase	17
5. 2	2D Visualisation of interaction between Fibrin Beta(B, E) chain with Nattokinase	17
5. 3	2D Visualisation of interaction between Fibrin gamma(C, F) chain with Nattokinase	18
5. 4	2D Visualisation of interaction between fibrinogen alpha(A, D, G, J) chain with Nattokinase.	18
5. 5	2D Visualisation of interaction between fibrinogen Beta(B, E, H, K) chain with Nattokinase.	19
5. 6	2D Visualisation of interaction between fibrinogen gamma(C, F, I, L) chain with Nattokinase.	20
5. 7	2D Visualisation of interaction between fibrinogen gamma	20

	(C, F, I, L) chain with Nattokinase.	
5. 8	2D Visualisation of interaction between Fibrin alpha (A, D) chain with Streptokinase	21
5. 9	2D Visualisation of interaction between Fibrin beta (B, E) chain with Streptokinase	22
5. 10	2D Visualisation of interaction between fibrinogen gamma(C, F, I, L) chain with Nattokinase.	22
5. 11	2D Visualisation of interaction between fibrinogen gamma (C, F, I, L) chain with Nattokinase.	23
5. 12	2D Visualisation of interaction between Fibrin alpha (A, D) chain with Streptokinase	23
5. 13	2D Visualisation of interaction between Fibrin beta (B, E) chain with Streptokinase	25
5. 14	2D Visualisation of interaction between Fibrin Gamma(C, F) chain with Streptokinase.	25
5. 15	2D Visualisation of interaction between fibrinogen alpha(A, D, G, J) chain with Streptokinase	26
5. 16	2D Visualisation of interaction between fibrinogen Beta(B, E, H, K) chain with Streptokinase	26
5. 17	2D Visualisation of interaction between fibrinogen Beta(B, E, H, K) chain with Streptokinase	27
5. 18	2D Visualisation of interaction between fibrinogen gamma(C, F, I, L) chain with Streptokinase	27
5. 19	2D Visualisation of interaction between fibrinogen gamma(C, F, I, L) chain with Streptokinase	28
5. 20	2D Visualisation of interaction between fibrin alpha(A, D) chain with Fibrinolytic enzyme from Cordyceps militaris	30
5. 21	2D Visualisation of interaction between fibrin Beta(B, E) chain with Fibrinolytic enzyme from Cordyceps militaris	30
5. 22	2D Visualisation of interaction between fibrin gamma(C, F) chain with Fibrinolytic enzyme from Cordyceps militaris	31
5. 23	2D Visualisation of interaction between fibrin gamma(C, F)	31

	chain with Fibrinolytic enzyme from Cordyceps militaris	
5. 24	2D Visualisation of interaction between fibrinogen alpha (A, D, G, H) chain with Fibrinolytic enzyme from Cordyceps militaris.	32
5. 25	2D Visualisation of interaction between fibrinogen Beta(B, E, H, K) chain with Fibrinolytic enzyme from Cordyceps militaris.	33
5. 26	2D Visualisation of interaction between fibrinogen Beta(B, E, H, K) chain with Fibrinolytic enzyme from Cordyceps militaris.	33
5. 27	2D Visualisation of interaction between fibrinogen Gamma(C, F, I, L) chain with Fibrinolytic enzyme from Cordyceps militaris.	34
5. 28	2D Visualisation of interaction between fibrinogen Gamma(C, F, I, L) chain with Fibrinolytic enzyme from Cordyceps militaris.	34
5. 29	Similarity between Beta chain of human fibrinogen and sheep fibrinogen	36
5. 30	Similarity between Gamma chain of human fibrinogen and sheep fibrinogen	36
5. 31	Before adding sample	37
5. 32	After adding sample	37

LIST OF TABLES

Table no.	Table Name	Page
5. 1	Nattokinase - Fibrin Docking Results	19
5. 2	Nattokinase - Fibrinogen Docking Results	21
5. 3	Streptokinase - Fibrin Docking Results	24
5. 4	Streptokinase - Fibrinogen Docking Results	28
5. 5	Fibrinolytic enzyme from Cordyceps militaris - Fibrin Docking Results	31
5. 6	Fibrinolytic enzyme from Cordyceps militaris - Fibrinogen Docking Results	35
5. 7	Clot Lysis Percentage	38

ABSTRACT

Three Fibrinolytic enzymes were taken into study, Nattokinase from *Bacillus subtilis* Natto, Streptokinase from *Streptococcus* species and Fibrinolytic enzyme from medicinal mushroom *Cordyceps militaris*. The Fibrinolytic activity of the said enzymes were analysed and compared by Insilico and Invitro method. For Insilico analysis the interaction of enzymes with Human protein Fibrin and Fibrinogen were checked as they are responsible for Blood clotting. The Docking results were visualized and the Hydrogen interactions and the Docking score between them were noted. Streptokinase showed more interactions when compared with the other two enzymes. For Invitro the commercially available Streptokinase and Nattokinase and Fibrinolytic enzyme partially purified from Dried and powdered *Cordyceps militaris* were used. The Clot Lysis method is used to determine the Fibrinolytic activity of the mentioned enzymes. The Sheep's blood were allowed to clot and then the samples were added. Nattokinase obtained the highest clot lysis percentage whereas the fibrinolytic enzyme from *cordyceps militaris* obtained the second highest percentage. Though the Fibrinolytic enzyme from *Cordyceps militaris* showed average interactions in Insilico analysis, it was effective in Invitro analysis. Hence the Fibrinolytic enzyme from *Cordyceps militaris* can be used in place of any Fibrinolytic enzymes.

CHAPTER 1

INTRODUCTION

1.1 FIBRINOLYTIC ENZYMES:

Fibrinolytic compounds were distinguished and examined among numerous living beings including earthworms, and bacteria: *Streptococcus pyogenes*, *Aeromonas hydrophila*, *Serratia E15*, *B. natto*, *Actinomycetes* and fungi: *Fusarium oxysporum*; *Mucor sp*, *Armillaria mellea* (Jian Sha et al, 2003). Fibrinolytic compounds can be found in an assortment of food sources, for example, Japanese Natto, Tofuyo, Korean Chung kook Jang soy sauce and consumable honey mushroom.

Fibrinolytic compounds have been cleansed from these food sources and their physiochemical properties have been described. Matured shrimp glue, a well known Asian flavoring, was displayed to have solid fibrinolytic action. These fibrinolytic compounds from customary Asian food sources are valuable for thrombolytic treatment. They will give an assistance to the exorbitant fibrinolytic compounds that are right now utilized in overseeing coronary illness, since enormous amounts of chemical can be helpfully and effectively created.

Furthermore, these compounds have huge potential for food fortification and nutraceutical applications, with the end goal that their utilization could successfully forestall cardiovascular sicknesses (Yoshinori et al., 2005).

1.2 THROMBOSIS:

Records of cardiovascular sicknesses have turned into the main source of death in the Western world (Viles et al., 2004). Many blood clump dissolving agents, like urokinase, streptokinase, and tissue plasminogen activator (t-PA), have been used in clinical medicines for cardiovascular sicknesses. Hemostasis is a complicated cycle acquired through an ideal harmony among draining and blood clump development. In a lopsided state, fibrin clusters may not be lysed bringing about apoplexy. Thrombolytic specialists from different sources have been broadly examined. Proteins, for example, urokinase, streptokinase and tissues

plasminogen activators have been broadly utilized in the treating thrombosis. Nonetheless, these are frequently costly, thermolabile and can create unfortunate side effects (Chitte and Dey, 2000).

1. 3 NATTOKINASE(ORAL ADMINISTRATION):

Subtilisin Nattokinase (NAT) (previously assigned Subtilisin BSP), delivered by *Bacillus subtilis natto*, is a serine protease and is accounted for having powerful fibrinolytic action (Nakamura, et al., 1992). Other than in vitro trial of fibrinolytic activity, many in vivo examinations had been recorded (Sumi et al. 1990). Fujita et al. (1995) treated canines with Nattokinase by oral organization, and the fibrinolytic movement in plasma expanded and showed that subtilisin NAT could pass the rodent digestive system and break down the synthetically prompted thrombosis. Suzuki et al. (1990) tracked down that dietary supplementation of natto smothered intimal thickening and tweaked the lysis of mural thrombi after endothelial injury in rodent femoral artery. Sumi et al. (1990) additionally announced a comparative impact of dietary *Bacillus natto* productive protein on in vivo endogenous thrombolysis. Comparable fibrinolytic enzyme creating microbes have likewise been purified from Japanese shiokara, Korea chungkook-jang (Banerjee et al., 2004), and Chinese douchi. By the by, it is as yet the most steady and financial method for acquiring protein with fibrinolytic action by *B. subtilis natto*. Based on its food source, somewhat solid fibrinolytic movement, soundness in the gastrointestinal parcel, and helpful oral organization, subtilisin NAT enjoys benefits for industrially involved medication for protection and delayed effects (Uversky et al., 2004).

1. 4 STREPTOKINASE:

Streptokinase is an extra cellular protein, extracted from certain strains of beta haemolytic streptococcus. It is a non-protease plasminogen activator that activates plasminogen to plasmin, the enzyme that degrades fibrin cloth through its specific lysine binding site; it is used therefore as a drug in thrombolytic therapy (Mohammad et al., 2009). Streptokinase is currently used in clinical medicine as a therapeutic agent in the treatment of thromboembolic blockages, including

coronary thrombosis (Banerjee et al., 2004; Endrogan et al., 2006). Streptokinase (SK) a group of extracellular proteins produced by a variety of streptococci beta-hemolytic strains, and is a plasminogen activator composed of 414 amino acids with a molecular mass of 47 kDa. Unlike urokinase or tissue-type plasminogen activator that performs direct proteolysis, SK forms a high affinity equimolar complex with a plasminogen (Kim et al., 2000). Irrespective to other enzymes, streptokinase is still a significant agent in treatment of cardiovascular diseases. Streptokinase, being so economical, has an important value in treating cardiac diseases in developing countries.

1. 5 FIBRINOLYTIC ENZYME FROM MEDICINAL MUSHROOM:

A medicinal mushroom called *Cordyceps militaris* known for its anticancer property and various other properties may also contain Fibrinolytic activity. Though it was proved that it has fibrinolytic activity, it is not well known for it's fibrinolytic activity. So this study is just a try to check that fibrinolytic activity and compare it with some of the well known fibrinolytic enzymes such as Streptokinase, which is widely used in medicinal field for it's fibrinolytic activity, and Nattokinase, which is considered as oral administration, has proven effective when directly interacted with clotted blood. *Cordyceps militaris* is a possible harbor of bio-metabolites for natural medications and evidences are accessible about its applications for rejuvenation of different systems of the body from antiquated times. Among every one of the species, *C. militaris* is considered as the most established wellspring of a few helpful chemical constituents. Other than their well known applications for tonic medication by the all steps of the local area, the constituents of *C. militaris* are presently utilized broadly in current system of medication. Proof showed that the dynamic standards of *C. militaris* are valuable to go about as pro sexual, anti-inflammatory, anti-oxidant/anti-aging, anti-tumour/anti-cancer/anti-leukemic, anti-proliferative, anti-metastatic, immunomodulatory, anti-microbial, anti-bacterial, anti-viral, anti-fungal, anti-protozoal, insecticidal, larvicidal, anti-fibrotic, steroidogenic,, anti-fatigue, neuroprotective, liver-protective, reno-protective as well as pneumo-defensive, not to mention their other synergistic exercises, which let it be attractive in the western nations as over-the-counter medication.

CHAPTER 2

LITERATURE SURVEY

2. 1 NATTOKINASE:

The Protein arrangement of a fibrin disintegrating enzyme Subtilisin K2 like Nattokinase was docked with the Human fibrin and Fibrinogen utilizing HADDOCK. Several dynamic locales were found through this review and It created the impression that the Subtilisin K2 was a fibrin degrading enzyme than a fibrinogen corrupting enzyme.

Nattokinase has been concentrated widely all around the world for it's fibrinolytic action (hostile to coagulant action). Nattokinase (NK), a strong blood-clump dissolving protein utilized for the treatment of cardiovascular infections, is created by the bacterium *Bacillus subtilis* during the maturation of soybeans to deliver Natto.

Natto has been seen to gainfully affect CVDs(CardioVascular Disease) by bringing down BP. In an in vitro study, it was found that natto contained somewhat solid inhibitors that stifle angiotensin-changing over enzyme (ACE), a key enzyme responsible for the creation of a hypertensive peptide chemical, angiotensin II, in the renin-angiotensin system.

Low-dose aspirin (85-100mg everyday), as a powerful anticoagulant agent, is broadly utilized for the counteraction of respiratory failures, stroke, and therothrombotic illnesses. Nonetheless, the long term utilization of anti-inflammatory medicine accompanies genuine gastrointestinal (GI) secondary effects and dying. In a review looking at the antiplatelet impacts of NK and aspirin NK was displayed to show astounding antiplatelet caggregation and antithrombotic exercises in vitro and in vivo, restraining thromboxane B2 arrangement from collagen-enacted platelet. All the above information recommend that NK could be a decent applicant, with practically no undeniable unfavorable impacts, to improve blood stream and perhaps better than aspirin.

2. 2 STREPTOKINASE:

Streptokinase is a well known anticoagulant. It is used as a drug for cardiovascular diseases and any other thrombolytic diseases. Besides, being an excellent Fibrinolytic enzyme it also has some side effects and various restrictions due to its high fibrinolytic activity.

Based on a literature review, it was tracked down reports of 19 instances of low back torment during streptokinase infusion. Fortunately, No patient died because of this intricacy. The rate of this peculiarity is difficult to lay out in a dependable way because of the less number of cases announced.

2. 3 CORDYCEPS MILITARIS:

Cordyceps species are helpful natural fungi with an assortment of biological activities. *Cordyceps militaris*, which is a parasite on moth caterpillar hatchlings, is utilized extensively in East Asia as a customary medication. Notwithstanding, commercialization of this mushroom is troublesome in light of the fact that it is extremely rare in nature. As of late, a cultivation cycle for *C. militaris* was effectively settled. This mushroom has gotten consideration as a wellbeing supplement to further develop life span, perseverance, and human wellbeing and as an elective medication to improve sicknesses including stroke, sore throat, tuberculosis, epilepsy, and malignant growth. Numerous compounds like mannitol, ergosterol, polysaccharides, and cordycepin have been recognized from *C. militaris*, and studies showed variety of pharmacological Evidence-Based Complementary and Alternative Medicine exercises with antiviral, antioxidative, antidiabetic, mitigating, antiplatelet aggregation, and anticancer impacts.

2. 4 RECOVERY OF STREPTOKINASE DISSOLVED BLOOD CLOTTING:

According to a studies conducted on novel Fibrinolytic enzymes, The activity of enzyme was checked by clot lysis of human blood clotted. This experiment was done invitro in aseptic conditions; about 100 mg of coagulated blood was dissolved by 200 µl enzyme within 2 h at 37°C temperature.

2. 5 RECOVERY OF NATTOKINASE DISSOLVED BLOOD CLOTTING:

According to the above mentioned The activity of enzyme was checked by clot lysis of human blood clotted. This experiment was done invitro in aseptic conditons. About 100 mg of coagulated blood was dissolved by 200 µl enzyme within 2 h at 37°C temperature.

CHAPTER 3

AIM AND OBJECTIVE

3. 1 AIM:

To check and compare the Fibrinolytic activity of Cordyceps militaris with Streptokinase and Nattokinase using both Insilico and Invitro analysis.

3. 2 OBJECTIVES:

- To retrieve the 3D structures of the required proteins.
- To construct the 3D structure of Fibrinolytic enzyme from Cordyceps militaris.
- To Dock the enzymes with the substrates and Visualize the results.
- To purify the enzyme from Cordyceps militaris
- To perform the clot lysis test

3. 3 SCOPE:

The scope of this investigation is to check the Fibrinolytic activity of Cordyceps militaris and compare its fibrinolytic activity with that of Streptokinase and Nattokinase so that it can be used in place of the said enzymes.

CHAPTER 4

MATERIALS AND METHOD:

4. 1 MOLECULAR DOCKING:

Molecular docking is a critical instrument in structural molecular biology and PC assisted drug plan. The objective of ligand-protein docking is to anticipate the prevalent restricting mode(s) of a ligand with a protein of known three-layered structure.

4. 1. 1 STRUCTURES:

For Docking any molecules, their 3D structures were needed. So, the crystal structure of Human Fibrin and Fibrinogen were taken from the PDB(Protein Data Bank).

The PDB ID for the Human Fibrin is 2HLO

The PDB ID for the Human Fibrinogen is 3HGH

The crystal structures of the enzymes Nattokinase and Streptokinase were also taken from the PDB(Protein Data Bank)

The PDB ID for the Nattokinase is 4DWW

The PDB ID for the Strepokinase is 1BML

As the structure of enzyme Streptokinase was only available as Complex of catalytic domain of Human Plasmin and Streptokinase, the catalytic domain of human plasmin is removed from the complex using Biovia Discovery Studio. The structure of Fibrinolytic enzyme from *Cordyceps militaris* is not available on the PDB(Protein Data Bank), so its structure was made using homology modelling by SWISS MODEL. For SWISS MODEL, the FASTA sequence of the fibrinolytic enzyme from *Cordyceps militaris* was taken from NCBI database and that sequence is used for homology modelling. The enzyme structures were docked with the Human Fibrin and Fibrinogen using Patchdock.

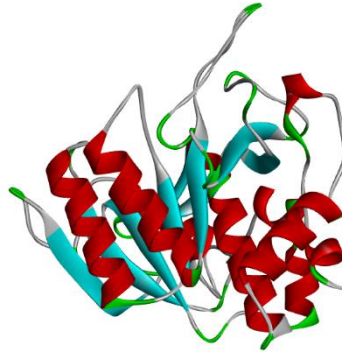


Fig 4. 1 :Nattokinase

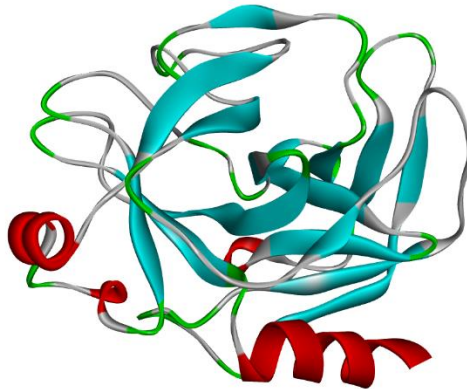


Fig 4. 2 :Fibrinolytic enzyme from Cordyceps militaris

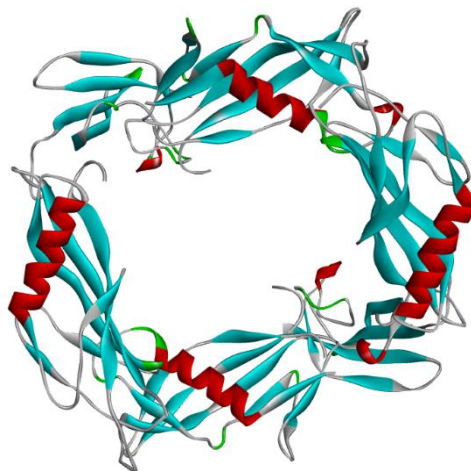


Fig 4. 3 :Streptokinase

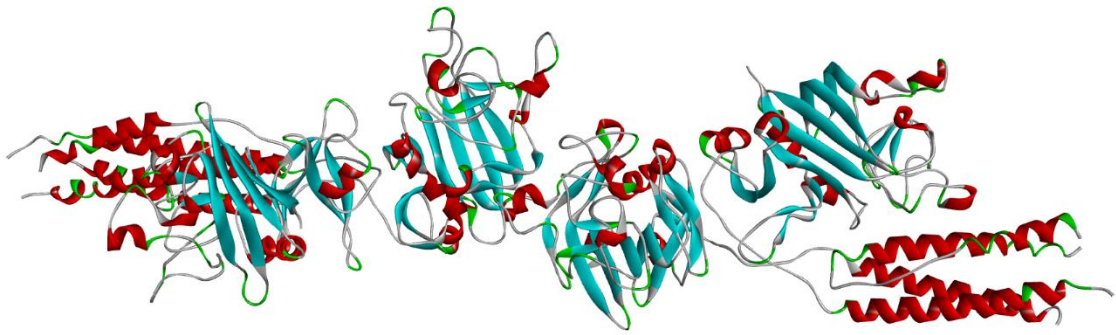


Fig 4. 4 :Fibrin

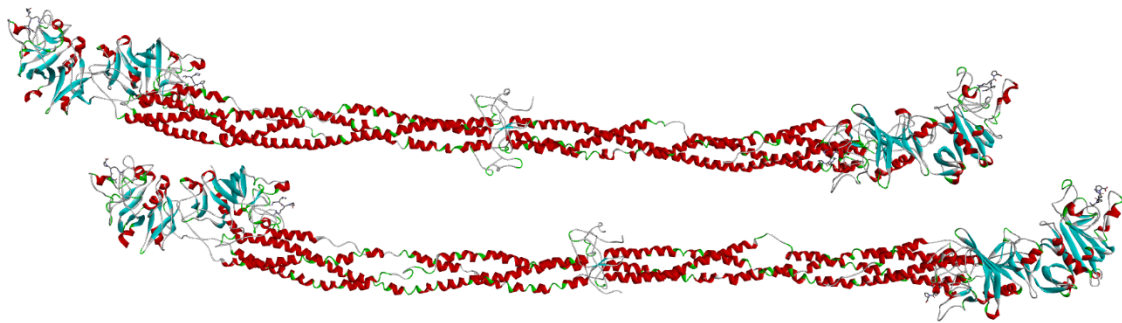


Fig 4. 5 :Fibrinogen

4. 1. 2 PROTEIN DATA BANK:

This resource is powered by the Protein Data Bank archive-information about the 3D shapes of proteins, nucleic acids, and complex assemblies that helps students and researchers understand all aspects of biomedicine and agriculture, from protein synthesis to health and disease. The RCSB PDB builds upon the data by creating tools and resources for research and education in molecular biology, structural biology, computational biology. (Berman, Westbrook et al., 2000).

4. 1. 3 BIOVIA DISCOVERY STUDIO:

The BIOVIA Discovery Studio Visualizer is a free, feature-rich molecular modeling application for viewing, sharing and analyzing protein and small molecule data. Experts and their colleagues can seamlessly and efficiently exchange results, without loss of either time or scientific information. BIOVIA modeling and simulation software allows scientists to perform computations of chemical, biological and materials properties; to simulate, visualize and analyze chemical and biological systems; and to communicate the results to other scientists.

4. 1. 4 SWISS MODEL:

SWISS-MODEL is a structural bioinformatics web-server dedicated to homology modeling of 3D protein structures. Homology modeling is currently the most accurate method to generate reliable three-dimensional protein structure models and is routinely used in many practical applications. It pioneered the field of automated modeling starting in 1993 and is the most widely-used free web-based automated modeling facility today.

4. 1. 5 PATCHDOCK:

PatchDock is an algorithm for molecular docking. The input is two molecules of any type: proteins, DNA, peptides, drugs. The output is a list of potential complexes sorted by shape complementarity criteria. PatchDock algorithm is inspired by object recognition and image segmentation techniques used in Computer Vision. Docking can be compared to assembling a jigsaw puzzle. When solving the puzzle we try to match two pieces by picking one piece and searching for the complementary one. We concentrate on the patterns that are unique for the puzzle element and look for the matching patterns in the rest of the pieces. PatchDock employs a similar technique. Given two molecules, their surfaces are divided into patches according to the surface shape. These patches correspond to patterns that visually distinguish between puzzle pieces. Once the patches are identified, they can be superimposed using shape matching algorithms.

4. 1. 6 LIGPLOT+:

LigPlot+ is a successor to our original LIGPLOT program for automatic generation of 2D ligand-protein interaction diagrams. It is run from an intuitive java interface which allows on-screen editing of the plots via mouse click-and-drag operations. LigPlot+ is a graphical front-end to the LIGPLOT and DIMPLOT programs. The plots generated by these programs can be interactively edited on screen and then written to a PostScript file for printing or conversion to various image formats.

The results were visualized using a software called Ligplot+. The interaction strength is evaluated by checking the number of Hydrogen bonds and Hydrophobic interactions. The amino acids that takes place in the interactions were noted. The Hydrogen Bonds below the distance of 3. 0A were only taken into account as they were considered to be strong interactions.

4. 2 INVITRO ANALYSIS:

4. 2. 1 Sample collection:

Commercially available streptokinase and Nattokinase were bought from well known manufacturers. As the fibrinolytic enzyme from *Cordyceps militaris* is not commercially available, the medicinal mushroom *Cordyceps militaris* is bought commercially. The mushroom was dried under sunlight for a few days, then they were powdered.



Fig 4. 6 :Dried Cordyceps militaris



Fig 4. 7 :Dried and powdered Cordyceps militaris

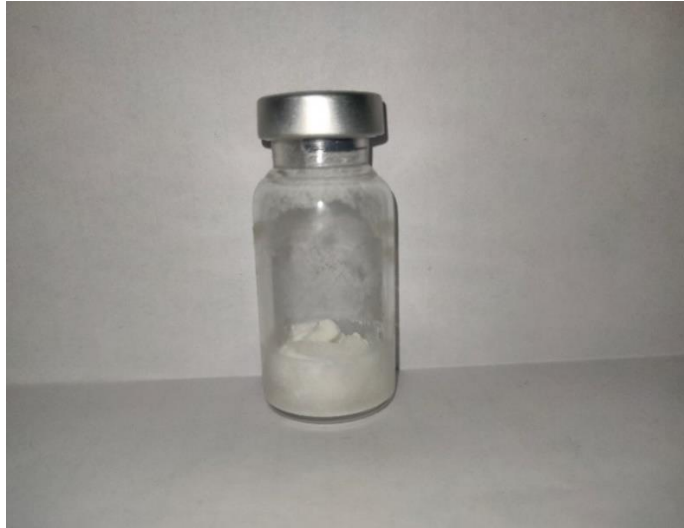


Fig 4. 8 :Streptokinase

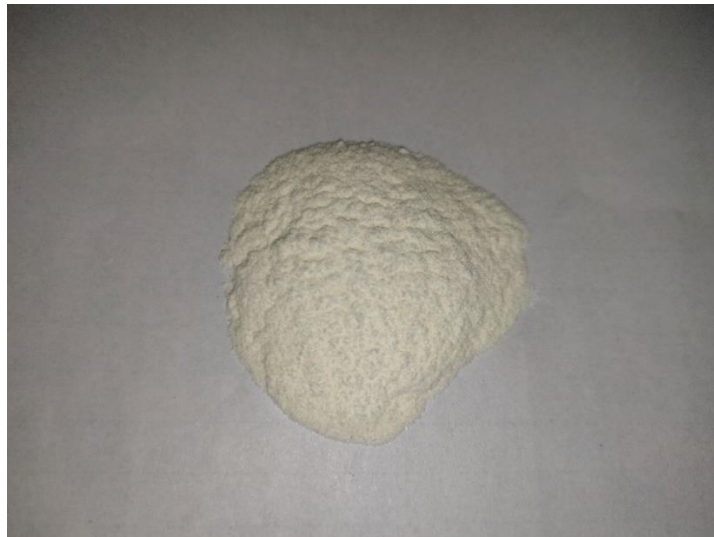


Fig 4. 9 :Nattokinase

4. 2. 2 Partial purification:

The partial purification of the fibrinolytic enzyme from *Cordyceps militaris* was done by using Ammonium sulphate precipitation method. The solubility of globular proteins increases upon the addition of salt, an effect termed salting-in. At higher salt concentrations, protein solubility usually decreases, leading to precipitation; this effect is termed salting-out ((Green and Hughes, 1955).

4. 2. 3 Ammonium sulphate precipitation:

1. Pour the sample into a beaker with a magnet bar and place it in an ice bath or at 4°C on a stirrer.
2. 60% Ammonium sulphate is added to sample.
3. Start stirring the solution and add salt to it in small portions, allow salt to dissolve before adding the next portion.
4. When all the salt has been added take beaker off stirrer and leave it at 4°C for some time, ideally overnight.

As a rule, you can store protein this way for a very long time. This is true for the majority of proteins, but not for all of them.

4. 2. 4 DIALYSIS:

Having precipitated a protein fraction that contains most of your enzyme, and redissolved it in buffer, it is necessary to remove the ammonium sulphate before you can proceed to subsequent steps in the purification process. The simplest way to achieve this is to dialyse the solution.

the enzyme solution is placed in a bag of selectively permeable membrane (e. g. cellophane), immersed in a large volume of buffer that is stirred and maintained at about 4°C. The membrane has pores that will permit small molecules such as ammonium and sulphate ions to cross, and hence equilibrate in the larger volume of buffer outside, while not permitting large protein molecules to cross. If the buffer is changed several times, allowing several hours each time for the ammonium sulphate to equilibrate, more or less all of it will be removed from the protein solution.

Dialysis will increase the volume of the enzyme solution, because of the initial osmotic effect of the ammonium sulphate; (this is why it is important to leave an air gap at the top of the membrane tube, to prevent it bursting)



Fig 4. 10 :Dialysis

4. 2. 5 CLOT LYSIS METHOD:

Blood drawn from healthy Sheep was transferred in different pre weighed sterile microcentrifuge tube (500 µl/tube) and incubated at 37°C for 45 minutes. After clot formation, serum was completely removed (aspirated out without disturbing the clot formed) and each tube having clot was again weighed to determine the clot weight (clot weight = weight of clot containing tube – weight of tube alone). Each microcentrifuge tube containing clot was properly labeled and 100 µl of sample were added to the tubes. Water was also added to one of the tubes containing clot and this serves as a negative thrombolytic control. All the tubes were then incubated at 37°C for 90 minutes and observed for clot lysis. After incubation, fluid obtained was removed and tubes were again weighed to observe the difference in weight after clot disruption. Difference obtained in weight taken before and after clot lysis was expressed as percentage of clot lysis. This process was done two times with different concentrations of the sample(1g/ml and 0.5g/ml)

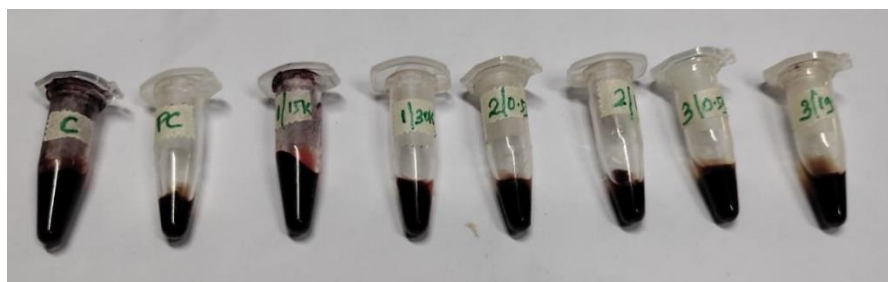


Fig 4. 11 :Blood clot before adding sample

CHAPTER 5

RESULTS AND DISCUSSIONS

5. 1 DOCKING RESULTS:

The docking results were visualized using a software called Ligplot+.

5. 1. 1 Nattokinase – Fibrin:

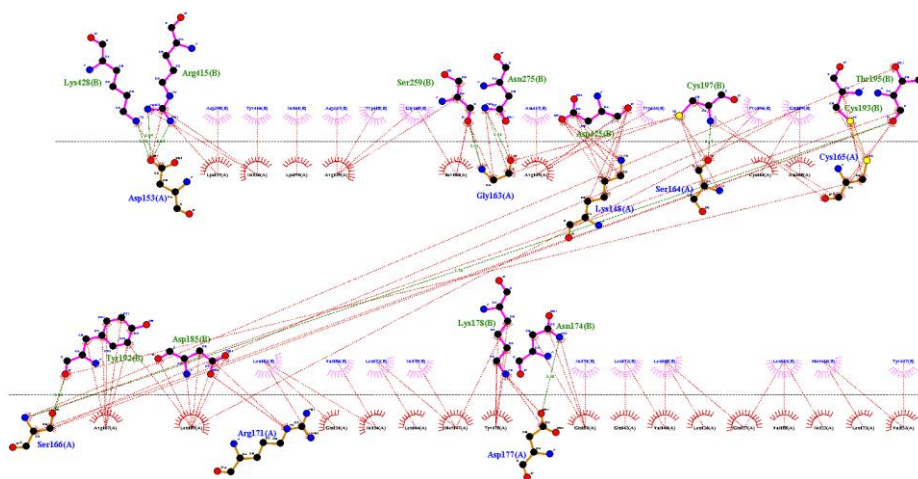


Fig 5. 1 :2D Visualisation of interaction between Fibrin Alpha(A, D) chain with Nattokinase
(D chain of substrate interacted with A chain of the enzyme)

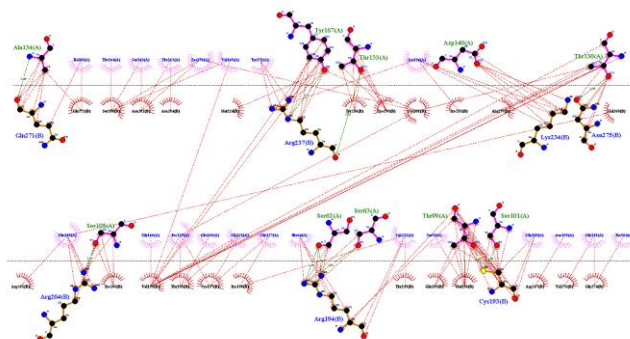
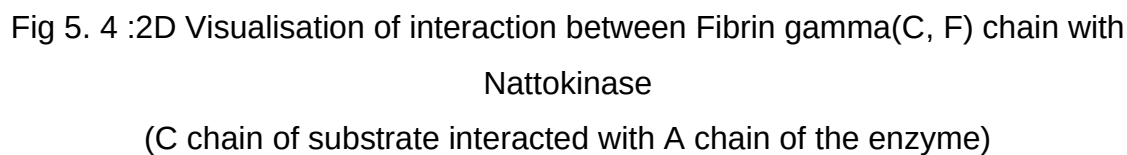
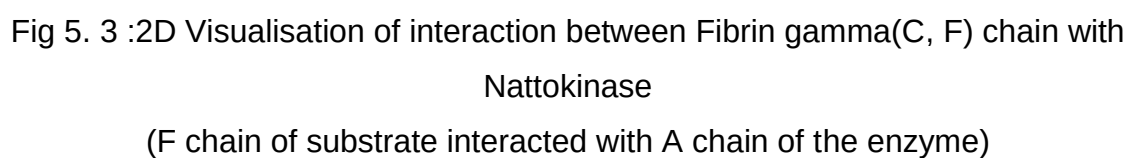


Fig 5. 2 :2D Visualisation of interaction between Fibrin Beta(B, E) chain with Nattokinase
(B chain of substrate interacted with A chain of the enzyme)



As the figures show the enzyme, Nattokinase show some favourable interactions with the Human fibrin molecule. Especially the both chains of Gamma chain in fibrin interact with our enzyme. More interactions was found with the Beta chain of fibrin when compared to Alpha and Gamma chains.

TABLE 5. 1

S. NO	COMPOUND	FIGURE	SCORE	AMMINO ACIDS INVOLVED IN HYDROGEN BONDING
1.	NATTOKINASE -FIBRIN ALPHA CHAIN		12738	A:TYR217 - A:GLU151 A:ASN218 - A:VAL140 A:GLN184 - A:GLU156
2.	NATTOKINASE -FIBRIN BETA CHAIN		13648	B:ARG194 - A:SER62 B:ARG264 - A:GLN103 B:ARG237 - A:TYR167 B:ARG264 - A:SER105 A:THR130 - B:ASN275
3.	NATTOKINASE -FIBRIN GAMMA CHAIN		15454	C:LYS151 - A:ASN118 C:ASN158 - A:SER116 F:LYS302 - A:GLN245

5. 1. 2 Nattokinase – Fibrinogen:

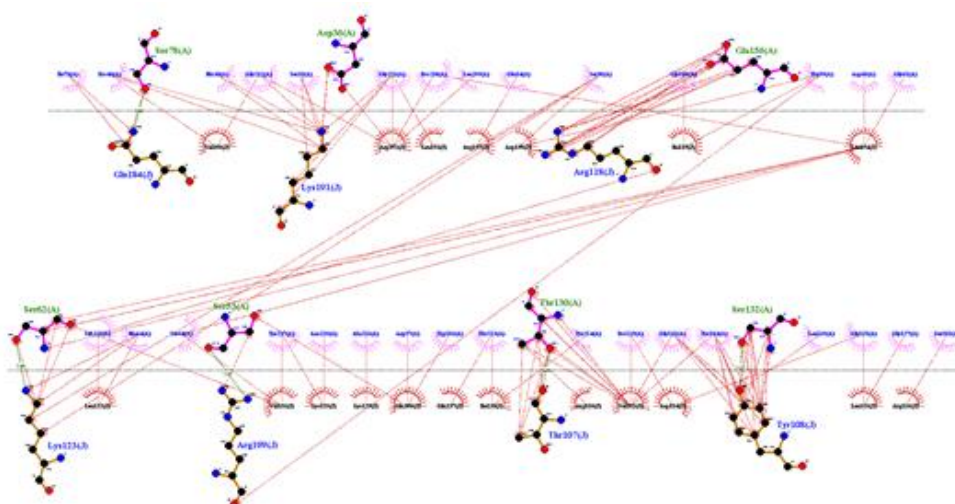


Fig 5. 5 :2D Visualisation of interaction between fibrinogen alpha(A, D, G, J) chain with Nattokinase.

(J chain of substrate interacted with A chain of the enzyme)

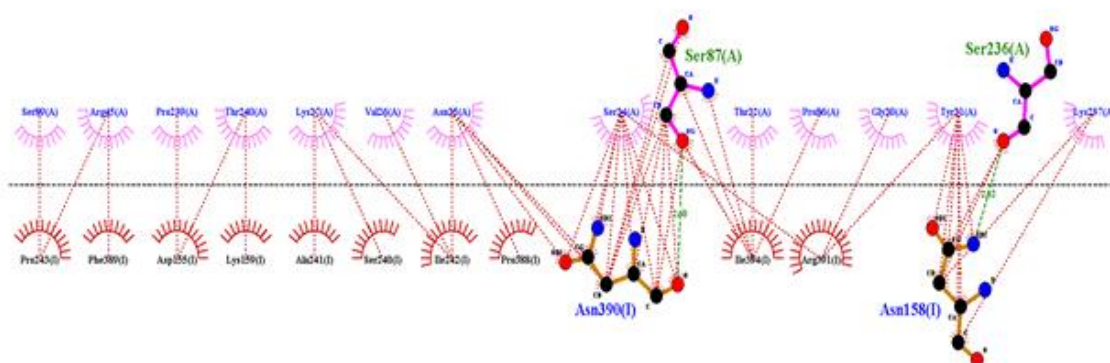


Fig 5. 8 :2D Visualisation of interaction between fibrinogen gamma (C, F, I, L) chain with Nattokinase.

(I chain of substrate interacted with A chain of the enzyme)

TABLE 5. 2

S. NO	COMPOUND	FIGURE	SCORE	AMMINO ACIDS INVOLVED IN HYDROGEN BONDING
1.	NATTOKINASE – FIBRINOGEN ALPHA CHAIN		15252	J:GLN184-A:SER78 J:LYS123 - A:SER62 J:ARG199 - A:SER53 A:THR130 - J:THR107
2.	NATTOKINASE – FIBRINOGEN BETA CHAIN		17850	K:ARG391 - A:ARG45 A:ASN56 - K:ALA427
3.	NATTOKINASE – FIBRINOGEN GAMMA CHAIN		17336	A:TYR262 - C:ASP141 I:ARG391 - A:GLY20 C:THR224 - A:GLU156 C:THR142 - A:TYR262

5. 1. 3 Streptokinase – Fibrin:

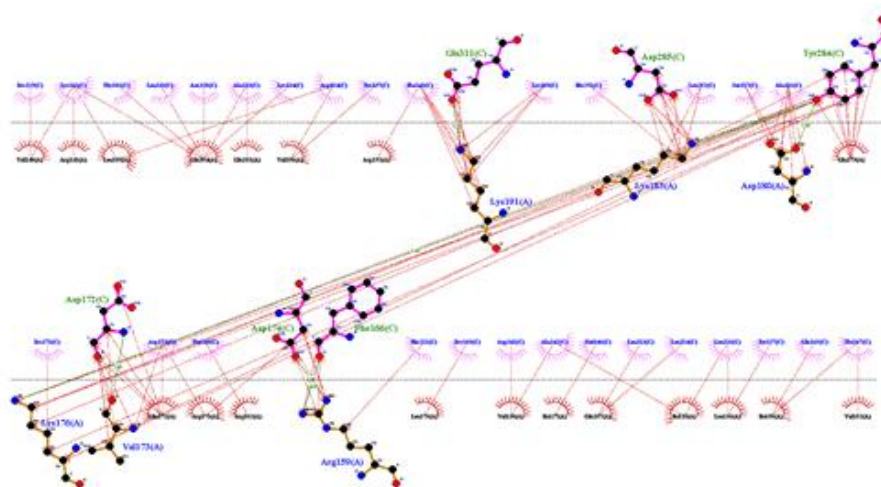
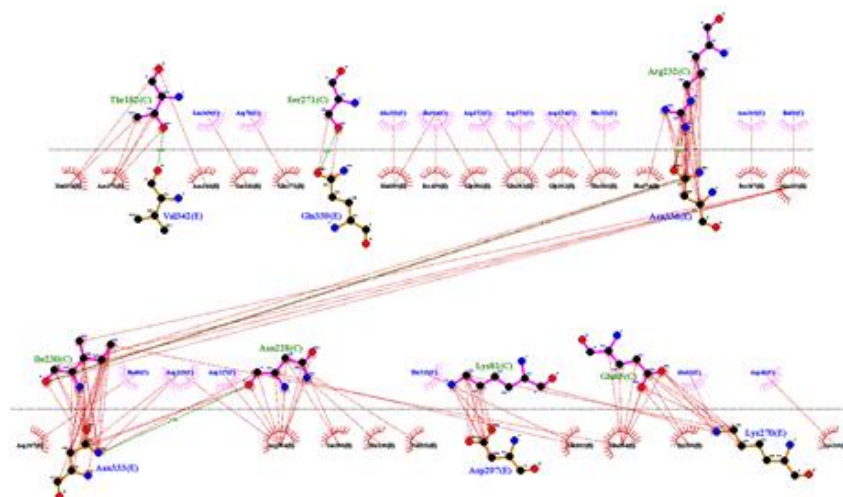


Fig 5. 9 :2D Visualisation of interaction between Fibrin alpha (A, D) chain with Streptokinase

(A chain of substrate interacted with C chain of the enzyme)



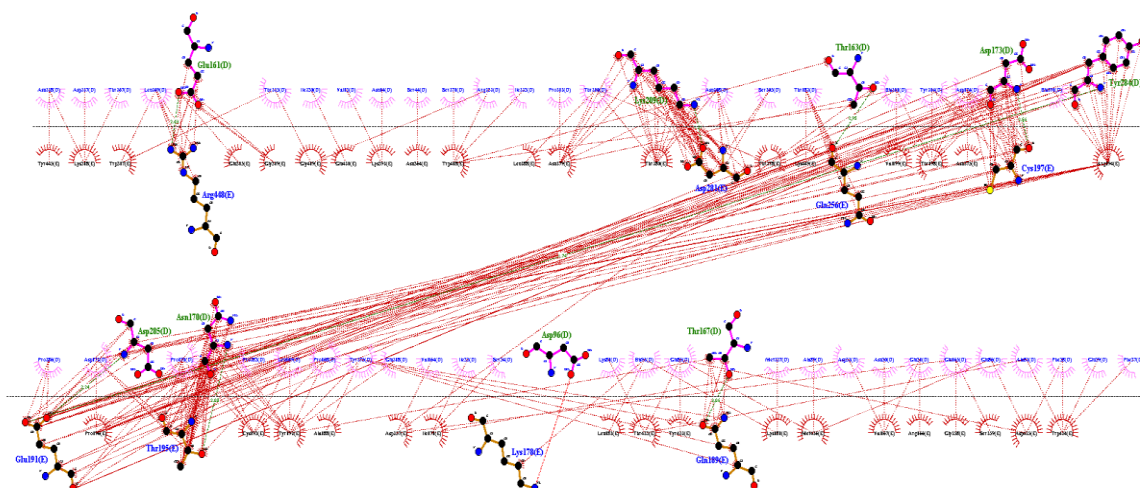


Fig 5. 11 :2D Visualisation of interaction between Fibrin beta (B, E) chain with Streptokinase
(E chain of substrate interacted with Dchain of the enzyme)

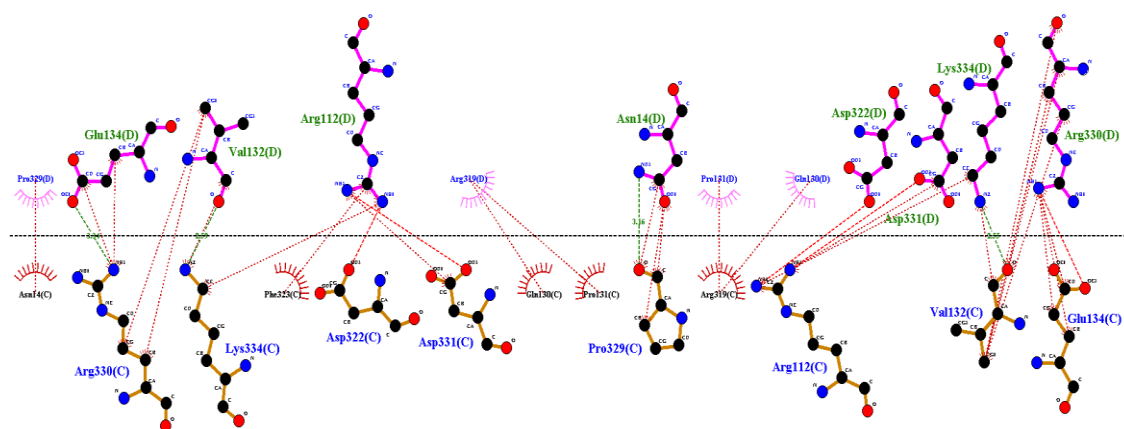


Fig 5. 12 :2D Visualisation of interaction between Fibrin Gamma(C, F) chain with Streptokinase.
(C chain of substrate interacted with D chain of the enzyme)

TABLE 5. 3

S. NO	COMPOUND	FIGURE	SCORE	AMMINO ACIDS INVOLVED IN HYDROGEN BONDING
1.	STREPTOKINASE - FIBRIN ALPHA CHAIN		17124	A:ARG159 - C:PHE166 C:LYS334 - D:VAL132 D:LYS334 - C:VAL132
2.	STREPTOKINASE FIBRIN BETA CHAIN		23094	E:ASN336 - C:ILE230 E:TYR338 - C:GLU85 E:LYS449 - D:THR182 C:LYS334 - D:VAL132 D:ASP285- E:GLU191 E:TYR192 - D:GLU218 E:THR198 - D:TYR214 D:ASN170 - E:TYR192 D:TYR284 - E:GLU191
3.	STREPTOKINASE - FIBRIN GAMMA CHAIN		24144	C:GLN134 - D:THR182 C:ARG391 - C:TYR284 C:LYS334 - D:VAL132

From the visualized docking results and the docking scores, it is shown that streptokinase also interact well with the both chains in beta chain when compared to the alpha and gamma chains, though the docking score is slightly higher with gamma chain when compared with the beta chain.

5. 1. 4 Streptokinase – Fibrinogen:

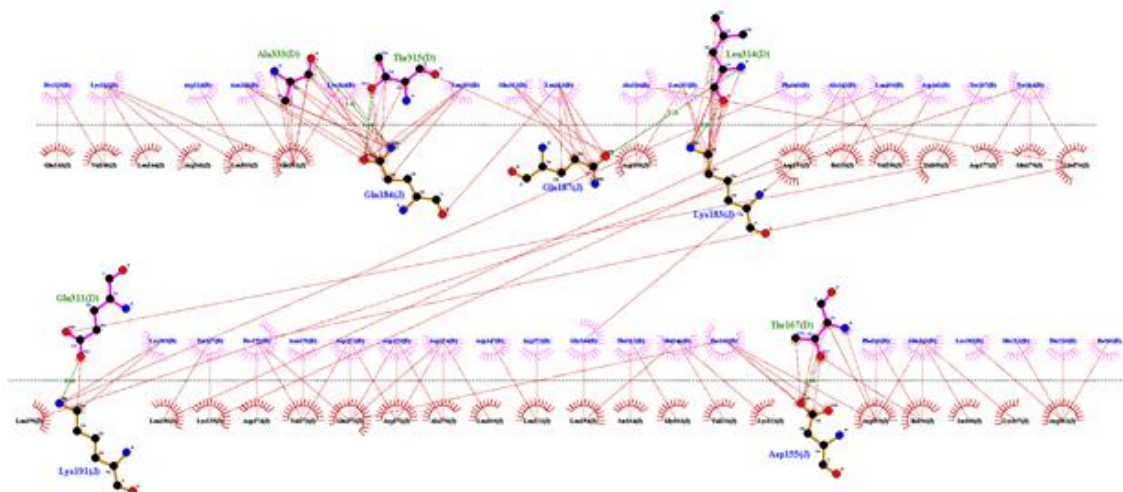


Fig 5. 13 :2D Visualisation of interaction between fibrinogen alpha(A, D, G, J) chain with Streptokinase
(J chain of substrate interacted with D chain of the enzyme)

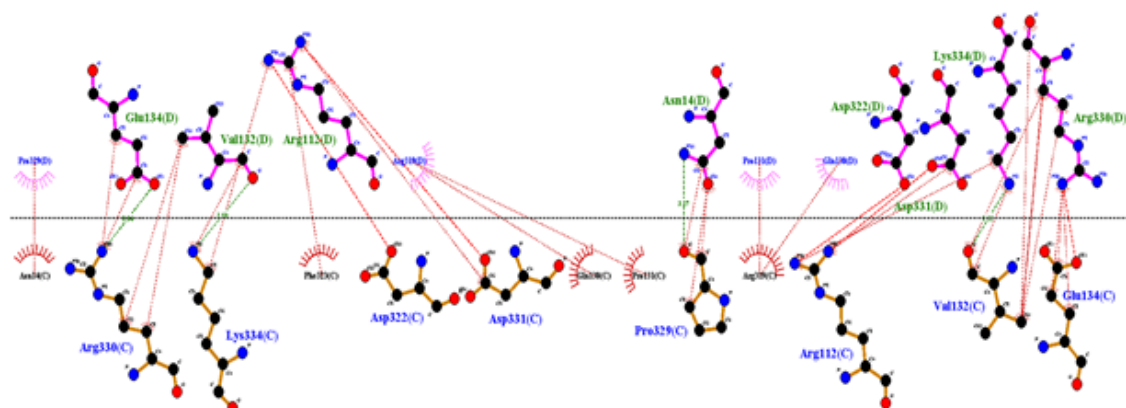


Fig 5. 14 :2D Visualisation of interaction between fibrinogen alpha(A, D, G, J) chain with Streptokinase
(D chain of substrate interacted with C chain of the enzyme)

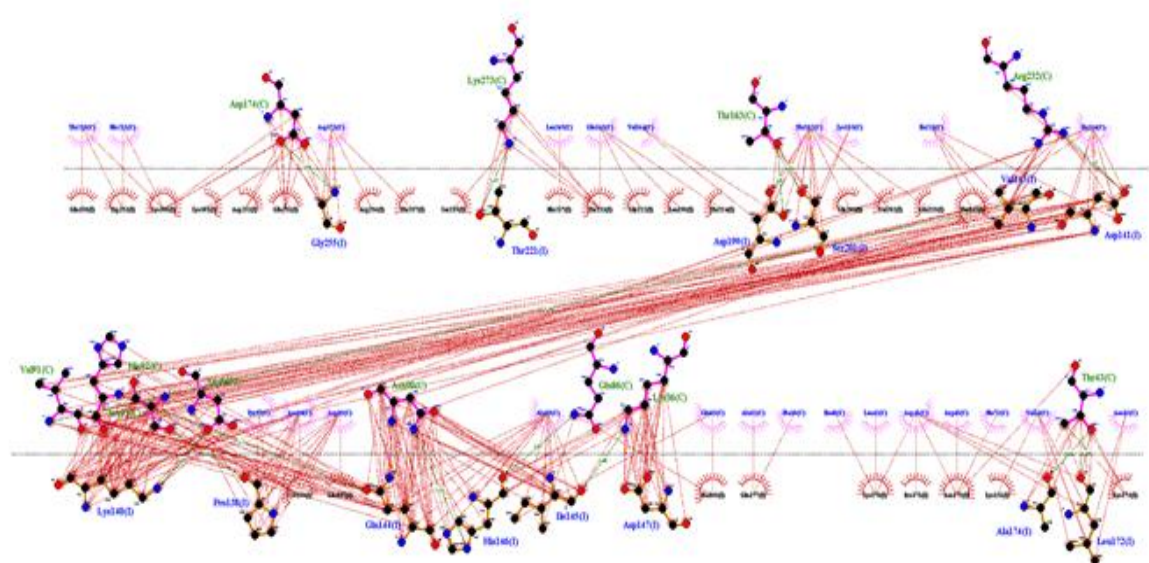


Fig 5. 17 :2D Visualisation of interaction between fibrinogen gamma(C, F, I, L) chain with Streptokinase
(I chain of substrate interacted with C chain of the enzyme)

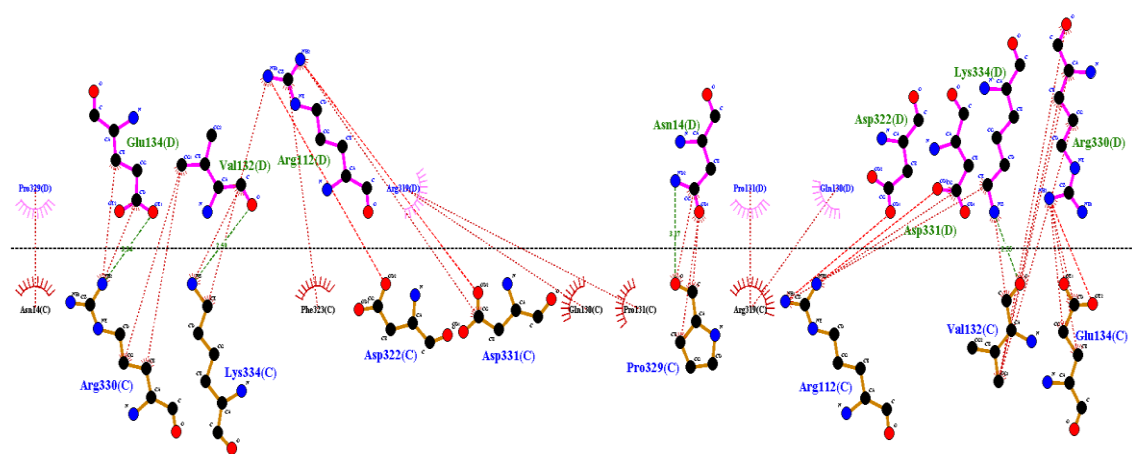


Fig 5. 18 :2D Visualisation of interaction between fibrinogen gamma(C, F, I, L) chain with Streptokinase
(C chain of substrate interacted with D chain of the enzyme)

				C:LYS334 - D:VAL132 D:LYS334 - C:VAL132 B:ALA335 - C:ASP174 B:TYR345 - D:ASN228 B:SER358 - D:THR225 D:ARG232 - B:SER358
3.	STREPTOKINASE – FIBRINOGEN GAMMA CHAIN		17706	I:SER201 - C:THR163 I:ARG247 - D:THR43 I:GLN311 - D:THR163 C:HIS92 - I:GLN144 C:THR182 - I:ASP199 C:LYS334 - D:VAL132 D:ARG45 - I:THR257 D:LYS81 - I:ASP288 D:THR182 - I:GLN311 D:LYS334 - C:VAL132 C:THR43 - I:LEU172 C:TYR97 - I:ASP141 I:SER300 - D:GLU249 I:THR259 - D:THR43

From the visualization results as well as the docking score, it is obvious that Streptokinase acts as a fibrinogen degrading enzyme than Fibrin degrading enzyme. It shows various interactions with the beta and gamma chains of the Fibrinogen. Even the Docking score is best with the Beta chain when compared with Nattokinase and the Fibrinolytic enzyme from *Cordyceps militaris*.

5. 1. 5 Fibrinolytic enzyme from *Cordyceps militaris* – Fibrin:

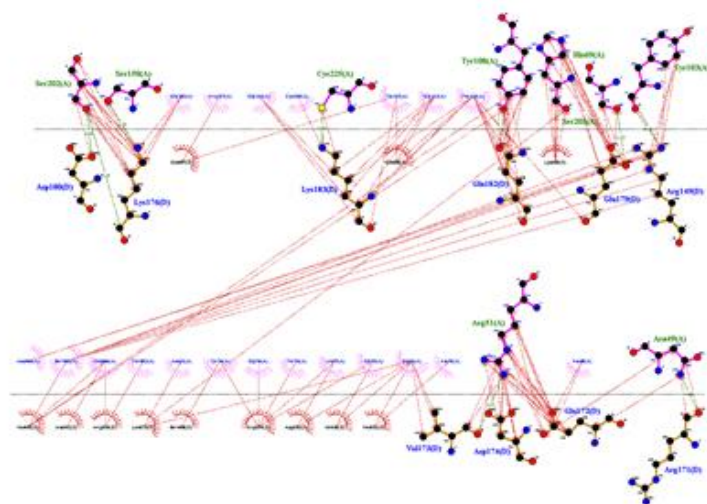


Fig 5. 20 :2D Visualisation of interaction between fibrin alpha(A, D) chain with Fibrinolytic enzyme from *Cordyceps militaris*
(D chain of substrate interacted with A chain of the enzyme)

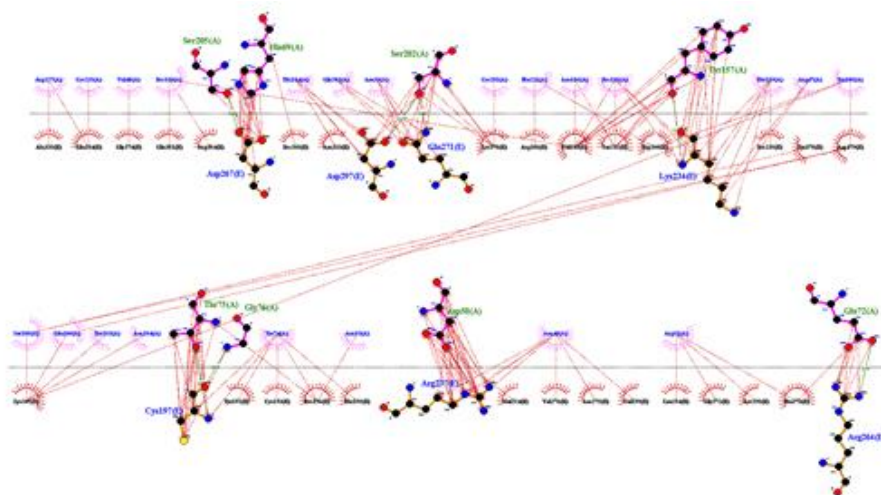


Fig 5. 21:2D Visualisation of interaction between fibrin Beta(B, E) chain with Fibrinolytic enzyme from *Cordyceps militaris*
(E chain of substrate interacted with A chain of the enzyme)

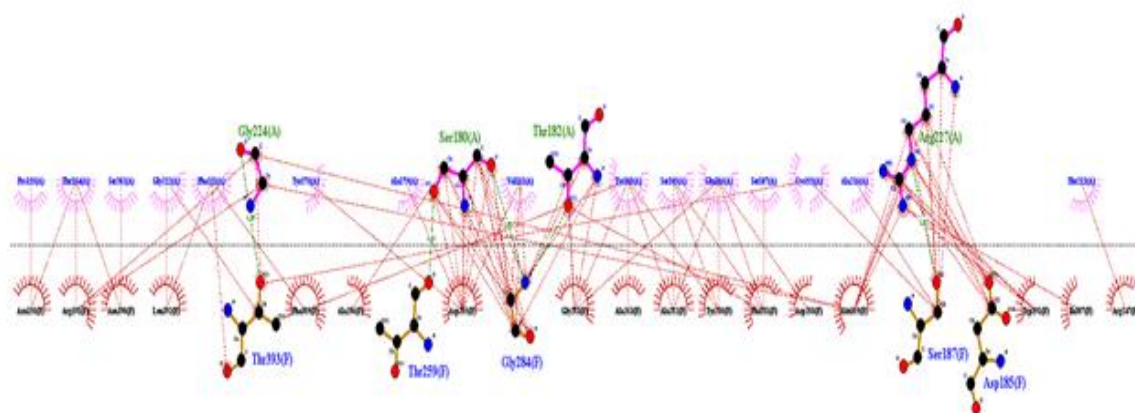


Fig 5. 22 :2D Visualisation of interaction between fibrin gamma(C, F) chain with
Fibrinolytic enzyme from Cordyceps militaris
(F chain of substrate interacted with A chain of the enzyme)

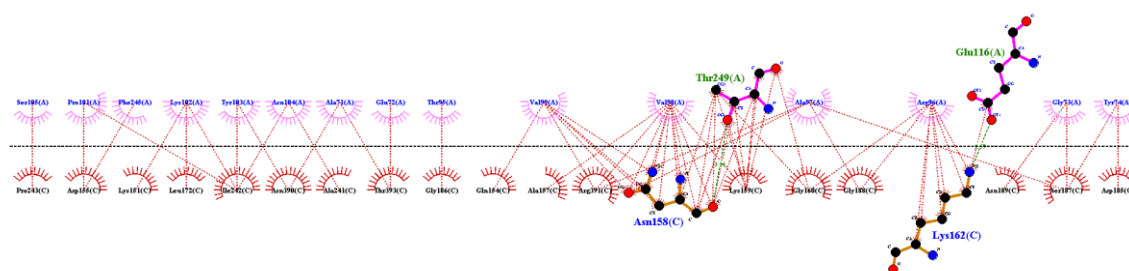


Fig 5. 23 :2D Visualisation of interaction between fibrin gamma(C, F) chain with
Fibrinolytic enzyme from Cordyceps militaris
(C chain of substrate interacted with A chain of the enzyme)

TABLE 5. 5

S. NO	COMPOUND	FIGURE	SCORE	AMMINO ACIDS INVOLVED IN HYDROGEN BONDING
1.	FIBRINOLYTIC ENZYME FROM CORDYCEPS MILITARIS – FIBRIN ALPHA		12844	A:ASN49 - D:ARG171 A:TYR108 - D:GLN182 D:LYS183 - A:CYS201 D:LYS183 - A:CYS225 A:ARG51 - D:VAL173

	CHAIN			A:SER205 - D:GLU179
2.	FIBRINOLYTIC ENZYME FROM CORDYCEPS MILITARIS – FIBRIN BETA CHAIN		15770	E:GLN271 - A:SER202 A:TYR108 - E:ASP379
3.	FIBRINOLYTIC ENZYME FROM CORDYCEPS MILITARIS – FIBRIN GAMMA CHAIN		15368	C:ASN158 - A:VAL99 C:ARG391 - A:ALA97 F:ARG247 - A:THR223 F:THR393 - A:GLY224 F:THR393 - A:CYS225

It is obvious that even though the Fibrinolytic enzyme from Cordyceps militaris does not show that much of interactions with beta chain it shows considerable amount of interactions with Alpha and gamma chains

5. 1. 6 Fibrinolytic enzyme from Cordyceps militaris – Fibrinogen:

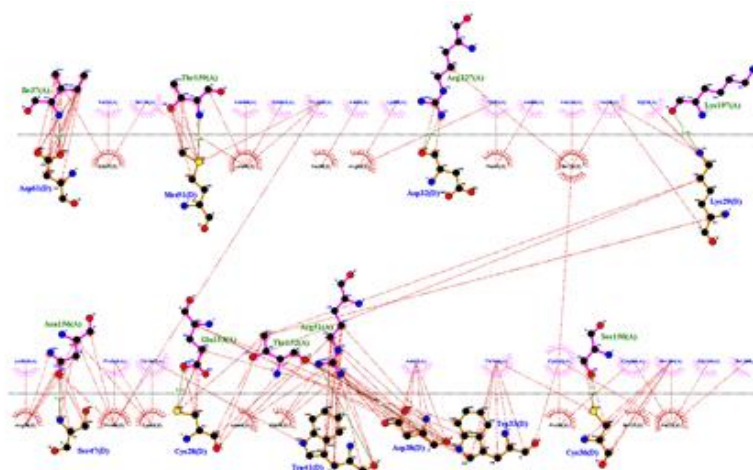


Fig 5. 24 :2D Visualisation of interaction between fibrinogen alpha (A, D, G, H) chain with Fibrinolytic enzyme from Cordyceps militaris.
(D chain of substrate interacted with A chain of the enzyme)

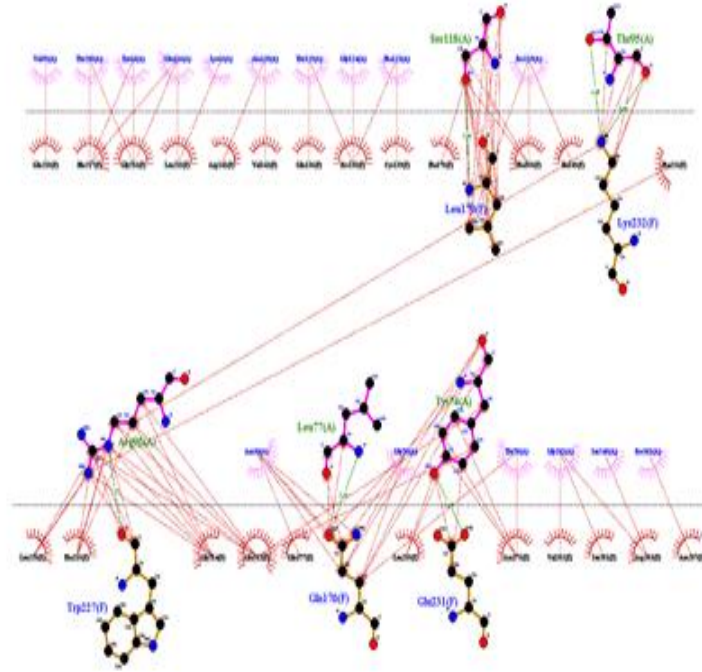


Fig 5. 27 :2D Visualisation of interaction between fibrinogen Gamma(C, F, I, L) chain with Fibrinolytic enzyme from Cordyceps militaris.
(F chain of substrate interacted with A chain of the enzyme)

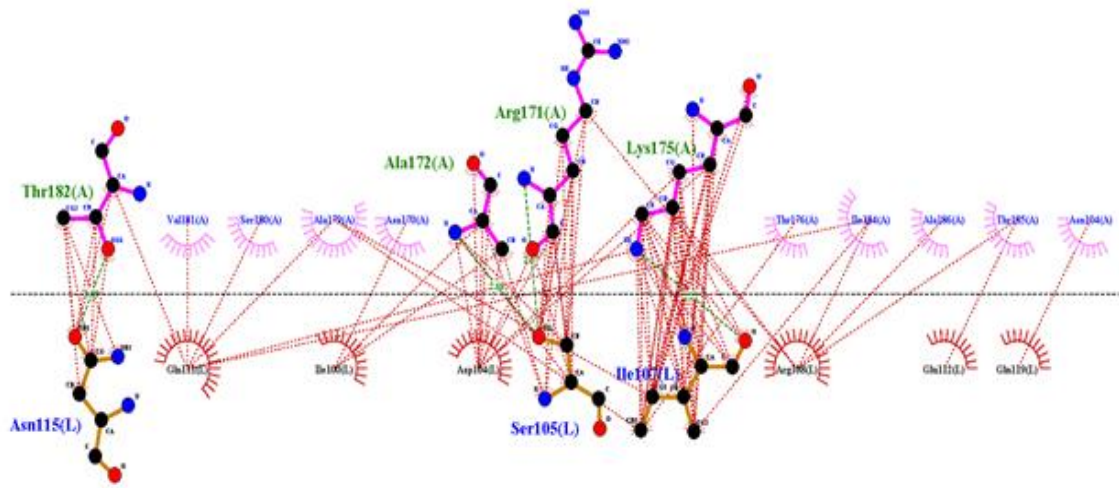


Fig 5. 28 :2D Visualisation of interaction between fibrinogen Gamma(C, F, I, L) chain with Fibrinolytic enzyme from Cordyceps militaris.
(L chain of substrate interacted with A chain of the enzyme)

TABLE 5. 6

S. NO	COMPOUND	FIGURE	SCORE	AMMINO ACIDS INVOLVED IN HYDROGEN BONDING
1.	FIBRINOLYTIC ENZYME FROM CORDYCEPS MILITARIS- FIBRINOGEN ALPHA CHAIN		15608	A:SER34 - D:GLU57 D:CYS36 - A:SER150 D:CYS36 - A:THR154 D:SER47 - A:ASN156 A:ARG227 - D:ASP32
2.	FIBRINOLYTIC ENZYME FROM CORDYCEPS MILITARIS- FIBRINOGEN BETA CHAIN		17806	K:ARG176 - A:CYS225 A:GLY222 - K:GLU183 A:ASP142 - E:ARG216 E:ARG346 - A:ILE37
3.	FIBRINOLYTIC ENZYME FROM CORDYCEPS MILITARIS- FIBRINOGEN GAMMA CHAIN		16472	F:LYS232 - A:THR95 A:LYS62 - F:LEU218 A:ASN104 - L:GLN119 A:LYS175 - L:ILE107 F:ASN175 - A:THR75 F:LYS232 - A:THR95 A:ASN93 - F:GLN177 A:ARG96 - F:TRP227 F:GLY24 - C:PHE15

Though Fibrinolytic enzyme from Cordyceps militaris has average docking score, it has formed many interactions with gamma chain and it is clear that Fibrinolytic enzyme from Cordyceps militaris acts as a fibrinogen degrading enzyme than being a fibrin degrading enzyme.

5. 2 INVITRO RESULTS:

Due to ethical reasons, the invitro analysis is done using sheep's blood in place of Human blood. Though it is done using Sheep's blood, the results will be helpful as the Human fibrinogen sequence and sheep's fibrinogen sequence were showing greater percentage of similarities.

5. 2. 1 Comparison between human and sheep fibrinogen:

```
# Length: 494
# Identity:   385/494 (77.9%)
# Similarity: 418/494 (84.6%)
# Gaps:      33/494 ( 6.7%)
```

Fig 5. 29 :Similarity between Beta chain of human fibrinogen and sheep fibrinnogen

```
# Length: 443
# Identity:   345/443 (77.9%)
# Similarity: 379/443 (85.6%)
# Gaps:      33/443 ( 7.4%)
```

Fig 5. 30 :Similarity between Gamma chain of human fibrinogen and sheep fibrinnogen

The protein sequence of both human fibrinogen and sheep fibrinogen were retrieved as FASTA sequence from NCBI database. The similarities between them were checked using EMBOSS Needle.

EMBOSS Needle reads two input sequences and writes their optimal global sequence alignment to file. It uses the Needleman-Wunsch alignment algorithm to find the optimum alignment (including gaps) of two sequences along their entire length.

Any percentage of similarity above 75% is considered to be a good similarity percentage. The human fibrinogen and sheep fibrinogen have a similarity percentage around 85% on maximum number of chains. Hence, the results obtained by using the sheep's blood should not differ that much from the results obtained by using human's blood.

5. 2. 2 Percentage of clot lysis :

The water was used as control. From the table, it is obvious that Nattokinase with high concentration has the highest percentage of clot lysis. Surprisingly, The fibrinolytic enzyme from *Cordyceps militaris* has the second highest percentage of 14. 29% of clot lysis which is higher than that of streptokinase. The fibrinolytic enzyme from *Cordyceps militaris* shows a good percentage of clot lysis even though it is only partially purified. If the enzyme is specifically purified then the fibrinolytic actiity may also increase with respect to the purity and the quantity of the enzyme.

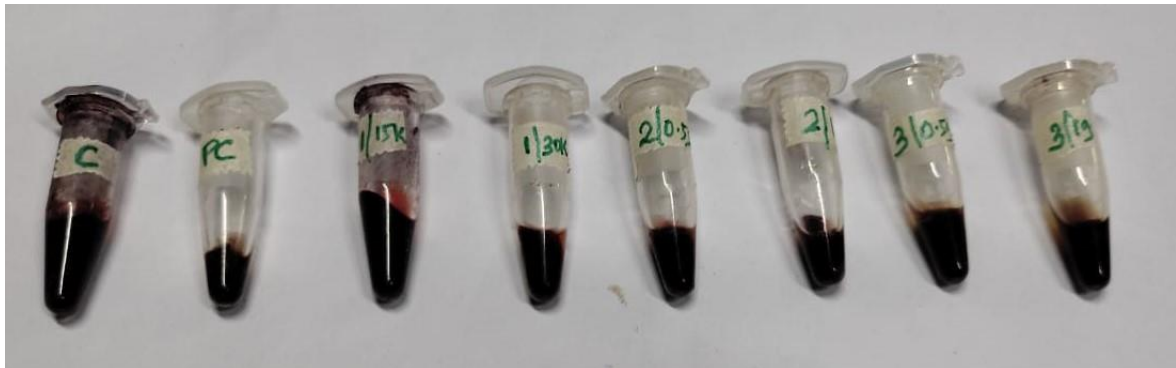


Fig 5. 31 :Before adding sample

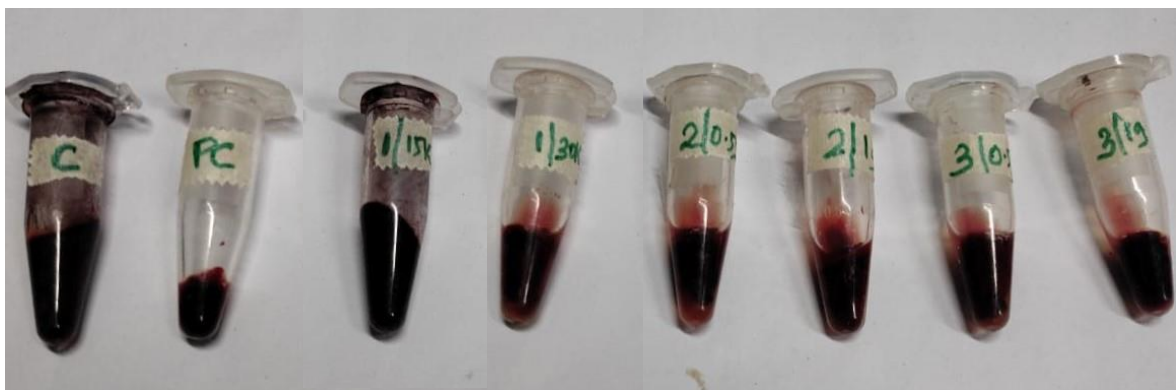


Fig 5. 32 :After adding sample

Table 5. 7:Clot lysis percentage

Sample	Initial Weight(g)	Final weight(g)	Weight difference(g)	Percentage of Clot lysis
Control	1. 53	1. 53	0	0%
Streptokinase 15K	1. 64	1. 52	0. 12	7. 32%
Streptokinase 30K	1. 62	1. 45	0. 17	10. 49%
Nattokinase 0. 5g/ml	1. 49	1. 3	0. 19	12. 75%
Nattokinase 1g/ml	1. 57	1. 31	0. 26	16. 56%
Fibrinolytic enzyme from Cordyceps militaris 0. 5g/ml	1. 43	1. 32	0. 11	7. 69%
Fibrinolytic enzyme from Cordyceps militaris 1g/ml	1. 33	1. 14	0. 19	14. 29%

CHAPTER 6

SUMMARY AND CONCLUSION

This study was solely focused on Fibrinolytic activity of a three selected enzymes and comparing them via both computational and invitro methods. The enzyme streptokinase is a well known fibrinolytic enzyme, the Nattokinase is a not so famous yet effective fibrinolytic enzyme whereas the *Cordyceps militaris*, well known for it's anticancer properties, was checked whether it has fibrinolytic activity. The molecular docking results suggest that the streptokinase performs very well when compared with other two enzymes. The fibrinolytic enzyme from *Cordyceps militaris* interacted comparable well with gamma chain of Fibrin. It also interacted well with alpha and gamma chain of Fibrinogen when compared with Nattokinase. Though the fibrinolytic enzyme from *Cordyceps militaris* performed average in molecular docking, it was very effective in invitro analysis. The lack of 3D structure of Fibrinolytic enzyme from *Cordyceps militaris* might be a reason for the average interactions with fibrin and fibrinogen. In laboratory test Fibrinolytic enzyme from *Cordyceps militaris* showed effective results eventhough it was partially purified. There are no records of any side effects due to the intake of *Cordyceps militaris*. Hence it might be able to supplant the place of many recombinant fibrinolytic enzymes that are used for thrombosis, cardio vascular diseases and any blood clot related issues. Through this study, It is confirmed that this medicinal mushroom *Cordyceps militaris* not only has anticancerous property but also fibrinolytic activity. It even performs equal to the well known fibrinolytic enzymes.

REFERENCES

- 1) Bienert, S., Waterhouse, A., de Beer, T.A.P., Tauriello, G., Studer, G., Bordoli, L., Schwede, T. The SWISS-MODEL Repository - new features and functionality. *Nucleic Acids Res.* 45, D313-D319 (2017)
- 2) Chitte RR, Dey S (2000). Potent fibrinolytic enzyme from a thermophilic *Streptomyces megasporus* strain SD 5. *Lett. Appl. Microbiol.* 31: 405-410.
- 3) Duhovny D, Nussinov R, Wolfson HJ. Efficient Unbound Docking of Rigid Molecules. In Gusfield et al., Ed. *Proceedings of the 2'nd Workshop on Algorithms in Bioinformatics(WABI)* Rome, Italy, *Lecture Notes in Computer Science* 2452, pp. 185-200, Springer Verlag, 2002 [PDF file]
- 4) E. Gidron, r. Margalit, and y. Shalitin A rapid screening test for reduced fibrinolytic activity of plasma: streptokinase activated lysis time .*Journal of Clinical Pathology*, 1978, 31, 54-57
- 5) Fujita M, Hong K, Ito Y, Fujii R, Kariya., K. Nishimuro S (1995). Thrombolytic effect of Nattokinase on a chemically induced thrombosis model in rat. *Biol. Pharm. Bull.* 18:1387-1391.
- 6) Girdhar M Taori¹ and Hatim F Dagainawala (2006), Development of an in vitro model to study clot lysis activity of thrombolytic drugs.
- 7) Guex, N., Peitsch, M.C., Schwede, T. Automated comparative protein structure modeling with SWISS-MODEL and Swiss-PdbViewer: A historical perspective. *Electrophoresis* 30, S162-S173 (2009)
- 8) Herrick, S., Blanc-Brude, O., Gray, A., & Laurent, G. (1999). Molecules in focus fibrinogen. *The International Journal of Biochemistry & Cell Biology*, 31(7), 741-746. [http://dx.doi.org/10.1016/S1357-2725\(99\)00032-1](http://dx.doi.org/10.1016/S1357-2725(99)00032-1). PMID:10467729.
- 9) Jian Sha CL, Galindo V, Pancholi VL, Popov Y, Zhao CWH, Chopra AK (2003). Differential expression of the enolase gene under in vivo versus in vitro growth conditions of *Aeromonas hydrophila*. *Microbial pathogenesis*, 34: 195-204.

- 10) Kim DM, Lee SJ, Kim IC, Kim ST, Byun SM (2000). Asp14-His48 region of streptokinase is important in binding to substrate plasminogen. *Thromb Res.* 99: 93-98
- 11) La Corte, A. L. C., Philippou, H., & Ariëns, R. A. S. (2011). Role of fibrin structure in thrombosis and vascular disease. *Advances in Protein Chemistry and Structural Biology*, 83, 75-127.
- 13) Laskowski R A, Swindells M B (2011). LigPlot+: multiple ligand-protein interaction diagrams for drug discovery. *J. Chem. Inf. Model.*, 51, 2778-2786
- 14) Li Cui, Ming Sheng Dong, Xiao Hong Chen, Mei Jiang, Xin Lv, Guijun Yan(2007), A novel fibrinolytic enzyme from *Cordyceps militaris*, a Chinese traditional medicinal mushroom.
- 15) Nakamura T, Yamagata Y, Ichishima E (1992). Nucleotide sequence of the subtilisin NAT gene, aprN, of *Bacillus subtilis* (natto). *Biosci. Biotechnol. Biochem.* 56: 1869-1871.
- 16) Needleman S.B. and Wunsch C.D. (1970) A general method applicable to the search for similarities in the amino acid sequence of two proteins *J. Mol. Biol.* 48(3):443-53
- 17) National Center for Biotechnology Information – NCBI. (2020). Retrieved from <https://www.ncbi.nlm.nih.gov/Structure/lexington/lexington.cgi>
- 18) R. Dubey, J. Kumar, D. Agrawala, T. Char and P. Pusp (2011), Isolation, production, purification, assay and characterization of fibrinolytic enzymes (Nattokinase, Streptokinase and Urokinase) from bacterial sources.
- 19) Research Collaboratory for Structural Bioinformatics – RCSB. (2020). Protein Data Bank (PDB). Retrieved from <https://www.rcsb.org/>
- 20) Ronald Feitosa Pinheiro, Adriana Sanches Pedrolo, Marcelo Nigri, Antonio Carlos de Camargo Carvalho(2002), Low Back Pain During Streptokinase Infusion.
- 21) Schneidman-Duhovny D, Inbar Y, Nussinov R, Wolfson HJ. PatchDock and SymmDock: servers for rigid and symmetric docking. *Nucl. Acids. Res.* 33: W363-367, 2005.
- 22) Shonkor Kumar Das, Mina Masuda, Akihiko Sakurai, Mikio Sakakibara(2010) Medicinal uses of the mushroom *Cordyceps militaris*: Current state and prospects.

- 23) Sweta Prasad, Rajpal S Kashyap, Jayant Y Deopujari, Hemant J Purohit, Wallace A C, Laskowski R A, Thornton J M (1996). LIGPLOT: a program to generate schematic diagrams of protein-ligand interactions. *Protein Eng.*, 8, 127-134.
- 24) Swiss Institute of Bioinformatics – SIB. (2020). Swiss-Model. Retrieved from <https://swissmodel.expasy.org/>
- 25) Syahbanu, F., Kezia, E., Puera, N., Giriwono, P. E., Tjandrawinata, R. R., & Suhartono, M. T. (2020a). Fibrinolytic bacteria of Indonesian fermented soybean: preliminary study on enzyme activity and protein profile. *Food Science and Technology*, 40(Suppl. 2), 458-465. <http://dx.doi.org/10.1590/fst.23919>.
- 26) Tsurupa, G., Hantgan, R. R., Burton, R. A., Pechik, I., Tjandra, N., & Medved, L. (2009). Structure, stability, and interaction of the fibrin(ogen) α C-domains. *Biochemistry*, 48(51), 12191-12201. <http://dx.doi.org/10.1021/bi901640e>.
- 27) Waterhouse, A., Bertoni, M., Bienert, S., Studer, G., Tauriello, G., Gumienny, R., Heer, F.T., de Beer, T.A.P., Rempfer, C., Bordoli, L., Lepore, R., Schwede, T. SWISS-MODEL: homology modelling of protein structures and complexes. *Nucleic Acids Res.* 46, W296-W303 (2018)
- 28) Viles-Gonzalez JF, Fuster V, Badimon JJ (2004). Atherothrombosis: A widespread disease with unpredictable and life-threatening consequences. *Eur. Heart J.* 25: 1197-1207.
- 29) Yang, Z., Mochalkin, I., & Doolittle, R. F. (2000). A model of fibrin formation based on crystal structures of fibrinogen and fibrin fragments complexed with synthetic peptides. *Proceedings of the National Academy of Sciences of the United States of America*, 97(26), 14156-14161. <http://dx.doi.org/10.1073/pnas.97.26.14156>. PMID:11121023.
- 30) Yunqi Weng, Jian Yao, Sawyer Sparks and Kevin Yueju Wang (2017) Nattokinase: An Oral Antithrombotic Agent for the Prevention of Cardiovascular Disease.
- 31) Yoshinori M, Ada HKW, Jiang B (2005). Fibrinolytic enzymes in traditional fermented food. *Food Res. In.* 38:243-250.