Fibrinolytic Enzyme from *Bacillus Pumilus* Strain (MPMBJ1) through Solid-State Fermentation

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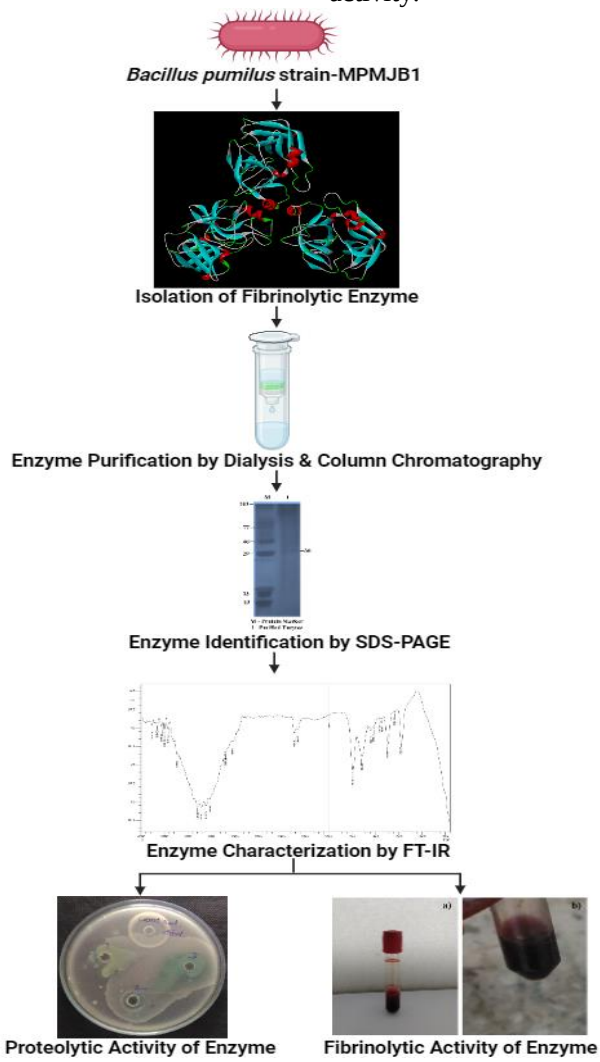
Abstract
Fibrinolytic enzymes are produced by several Bacillus species. For the therapeutic purpose, the fibrinolytic enzyme can be used as thrombolytic agent for the clot resolution. In the study, fibrinolytic enzymes are produced in the modified nutrient medium under solid-state fermentation using Bacillus pumilus strain-MPMBJ1 from CBNR isolate. The presence of fibrinolytic enzyme in the fermentation media was confirmed by salting-out methods in conjunction with SDS-PAGE. The enzyme was purified by using dialysis method followed by the chromatographic technique. Biological activities of the enzyme were assessed through proteolytic and fibrinolytic functional assays. The results showed that the optimum incubation period for fibrinolytic enzyme production is 24 hrs. We have obtained the yeilds of fibrinolytic enzyme as 64.55 ± 1.25 and 22.75 ± 0.75 mg/ml by using acetone and ammonium sulfate precipitation methods respectively. The molecular weight of the fibrinolytic enzyme was found to be 30kDa from SDS-PAGE analysis. The enzyme was found to exhibit potential proteolytic and fibrinolytic properties. Hence, further in vivo and clinical analysis on this enzyme may lead to the production of an effective drug for heart attack patients.

Keywords: *Bacillus pumilus* strain-MPMBJ1, Fibrinolytic enzyme, Column chromatography, Dialysis, Proteolytic activity.



Graphical Abstract

Introduction

Enzymes are biological molecule of biological origin. They are protein molecules that react with substrates to form products. Enzymes are very much required to speed up or inhibit the reactions in the biological systems. The enzymes exhibit specificity to their substrate. Enzyme-substrate complex is formed when the enzyme reacts with the substrate and it is a reversible reaction. The enzyme-substrate complex leads to the formation of a product which is an irreversible reaction. When the product is formed, the reacted enzyme is released which is ready for further reaction with other substrates. Several factors such as temperature, pH, pressure, substrate concentration and chemical environment can affect the enzyme activity[9,28]. The structural configuration of enzymes is much needed for their catalytic activity.

Enzymes are organic, soluble and colloidal substances used for various purposes such as food, paper, leather, textiles, agriculture and pharmaceutical fields. They remain active even *in vitro* conditions and because of their non-toxic and biodegradable nature, they can be produced in large amounts by microorganisms used for several industrial applications. The term holoenzyme is used to represent the total enzyme and contains apoenzyme which is a protein part and prosthetic group which has co-enzyme or metal ions. Prosthetic groups are classified into two groups: co-enzymes and co-factors. Nicotinamide adenine dinucleotide, flavin adenine dinucleotide, Co-enzyme A are co-enzymes, K^+ and Mg^+ are some examples for co-factors.

The divalent cations such as Mg^{2+}, Zn^{2+} and Mn^{2+} are generally called as enzymes activators. There are two different models for the mechanism of enzyme action: lock and key model which has a presumed active site so that substrate fits into the enzymes as keys fits into the lock and induced fit model where substrate changes the active site to its convenience so that it can fit into the enzyme to carry out the process. There are several types of enzymes based on their functions[5,24].

Blood clotting during inflammation is a defence mechanism of a body. Fibrinolysis is a process of resolving the blood clot. In the normal condition of the body, both blood clotting and fibrinolysis process were maintained in an equilibrium by enzymes, ribozymes, pro-enzymes, activators and pro-activators. When these factors are imbalanced, both processes will get disturbed. Clot formation occurs during fibrogenesis and clot formation is done by the plasma protein which is soluble fibrinogen and thrombin converts this protein into insoluble fibrin. Prothrombin is converted into thrombin to carry out the clot formation process. Vasoconstriction, formation of a fibrin and clot platelet aggregation are the biological processes which regulate blood clot dissolution by coverting the pro-enzyme plasminogen into plasmin which is a blood clot dissolving enzyme. Various thrombolytic agents are present in organisms. Different thrombolytic agents such as streptokinase from bacteria, arvin from the venom of the Malayan pit viper, brinase from a filamentous fungus Koji mold *Aspergillus oryzae*, reptilase from a South American snake and urokinase from human urine are used[5]. Fibrinolytic enzymes are produced from many microorganisms[2]. Fibrinolytic enzyme comes under a sub class of protease which is capable of degrading the fibrin (blood clots) whereas blood clots occur in the blood by thrombin[10,21,26]. Cardiovascular diseases affect many people leading to serious condition or death. Cardiovascular diseases are caused due to increased thrombosis.

Thrombosis is increased when the fibrin deposits in the blood vessel. There are three fibrinolytic enzymes, urokinase, streptokinase and tissue plasminogen activator (t-PA). The fibrinolytic enzyme which is extracted from bacteria is streptokinase and from kidney, it is urokinase. The t-PA is genetically engineered to undergo clot resolution. The patients who had pulmonary embolism subjected to urokinase and streptokinase showed three times better clot resolution than the patients who took only heparin[16,26]. Clot resolution can be done by fibrinolytic enzymes. Hence it has a main role in the treatment associated with blood clot. Some damages can be prevented by these enzymes by removal of blood clot immediately after their formation. Hence they have wide applications in the disease treatment[15,27].

Bacteria are prokaryotic microorganisms which are ubiquitous in nature. The products from the metabolic activities of bacteria are of much importance in industries. The product from the *Bacillus* organism has more industrial significance. Several important products of medical, pharmaceutical, agricultural and other industrial products are produced from different species of *Bacillus.* The products include sugars, enzymes, antibiotics and amino acids. These elements are very much important for agriculture, medicines and industries[11,14]. Fibrinolytic enzymes are broadly produced from various *Bacillus* species[13,29].

Bacillus pumilus is a type of bacteria found in soil. These species come under Gram-positive type of bacteria and are aerobic so they are grown only in the presence of oxygen. They are rod shaped bacteria. Due to the ability of forming spores inside the cell, these bacteria are called as endospore-forming bacteria. They are found to exhibit high lipase and protease activity. Hence fibrinolytic enzymes are more required in case of cardiac arrest due to myocardial infarction and massive pulmonary embolism as an immediate therapy. By injecting the enzyme into the patients intravenously, blood clot can be dissolved and patients life can be saved[18].

In our present study, isolation of fibrinolytic enzyme from the bacteria *Bacillus pumilus* strain-MPMBJ1 was carried out and was tested for its blood clot resolving effect to be used as a drug.

Material and Methods

Source of bacteria: The bacterial culture of *Bacillus pumilus* strain-MPMBJ1 was obtained from Centre for Bioscience and Nanoscience Research (CBNR), Coimbatore, Tamil Nadu. The obtained organism was sub-cultured in nutrient broth. It was incubated at 37˚C for 24 hours in orbital shaker and was used for further studies.

Production of fibrinolytic enzyme by *Bacillus pumilus* strain-MPMBJ1: Fibrinolytic enzyme produced from the *Bacillus pumilus* strain-MPMBJ1 was done using the procedure conducted by Dubey et al[8]. Fibrinolytic enzyme was obtained from the overnight grown culture. The medium used to grow the *Bacillus* and to isolate fibrinolytic enzyme contains the following in g/l: D-glucose 0.25, NaCl 0.125, beef extract 0.125 and finely smashed soybean 0.5. The medium should be checked for pH at 7.0 ± 0.2 autoclaved at 121˚C for 15 minutes. After sterilization, the medium was allowed to cool and then inoculated with 200 µl of *Bacillus pumilus* strain-MPMBJ1. The inoculated medium was incubated in orbital shaker for 48 hours. After the fermententation of 48 hrs, centrifugation was done to remove the cells[2,8].

Estimation of biomass: The estimation of biomass was done on dry weight basis (mg/g). The culture filtrate was centrifuged in a pre-weighed tube, then dried to a constant weight and re-weighed. The difference in weight indicates the bacterial growth[1,4].

Extraction of fibrinolytic enzyme from *Bacillus pumilus* strain-MPMBJ1 and enzyme assay: The produced fibrinolytic enzyme was extracted from the *Bacillus pumilus* strain-MPMBJ1 using the standard procedure given by Ajithkumar et al. The culture was centrifuged at 5000 rpm for 5 minutes and the supernatant was collected. The supernatant was used for the enzyme assay. To the 250µl supernatant, 1.5 ml of 0.6% fibrin and 0.1M phosphate buffer were added. The pH of the phosphate buffer should be maintained at 7.0. The solution was heated at 50˚C in a water bath for 10 minutes. After heating, 1.5ml of 0.4M TCA was added to stop the reaction and allowed to stand for 30 minutes.

Filter the solution through Whatmann no. 2 filter paper. About 2.5ml of 1M sodium carbonate and 0.5 ml of 1N folins phenol reagent were added to 1ml of filtrate. The solution was allowed to stand for 30 minutes in the dark condition and absorbance was measured for the coloured solution at 280 nm in double beam UV-VIS spectrophotometer[1, 19].

Effect of incubation period on enzyme activity: The maximum enzyme activity was found by the enzyme assay process. The extraction of fibrinolytic enzymes was done for the cultures incubated for 24 hrs to 48 hrs period in order to found the optimum period of enzyme activity[1].

Partial purification of isolated fibrinolytic enzyme: The purification of enzyme was followed as per the procedure performed using standard procedures[1,23].

Ammonium sulphate precipitation: The protein from the solution was separated by ammonium sulfate precipitation. To 10ml of crude enzyme, 5g of ammonium sulfate was added. The mixture was kept at 4˚C for 24 hours. After complete precipitation, all the tubes were centrifuged at 6000 rpm for 5 mins. After centrifugation, the supernatant was discarded. Pellets were dissolved in 1ml of phosphate buffer (pH 7.0) and absorbance was measured at 280nm in double beam UV-VIS spectrophotometer.

Acetone precipitation: The protein from the solution was separated by acetone precipitation. To another 10 ml of crude enzyme, 20ml of 95% pre-chilled acetone was added. The mixture was kept at 4˚C for 24 hours. After complete precipitation, excess of acetone was removed. All the mixtures were centrifuged at 6000 rpm for 5 mins. After centrifugation, the supernatant was discarded. In each tube, pellets were dissolved in 1ml of phosphate buffer (pH 7.0). Absorbance was measured for the sample at 280 nm in double beam UV-VIS spectrophotometer.

Dialysis: The solution was subjected to dialysis process in order to remove the impurities and salts. The pretreated dialysis bag (75kDa) was taken and rinsed in double distilled water for activation. The activated dialysis bag was tightly tied at one end and the solution which was treated with acetone, was taken inside the bag. After pouring the solution, the other end of the dialysis bag was also tied tightly and checked for leakage. The dialysis bag was kept inside 20ml of distilled water in a beaker[1].

Column Chromatography: About 1.0 g of Sephadex G-100 was added to 50 ml of distilled water and left overnight. The gel was packed into the column and distilled water was carefully added on top. After allowing the column to stand for 10 minutes, the water was drained dropwise and process was repeated twice. Further, 2 ml of dialyzed enzyme was added to the top of the gel. After waiting for 10 minutes, the enzyme was allowed to pass through the gel and then purified enzyme was collected. Phosphate buffer was added to the column during the procedure. The absorbance of all collected fractions was measured at 280 nm using a double beam UV-VIS spectrophotometer to detect the enzyme. The samples were stored at room temperature[1].

Sodium Dodecyl Sulphate - Polyacramide Gel Electrophoresis (SDS-PAGE): The sample had undergone SDS-PAGE to identify the molecular weight of the enzyme. SDS-PAGE apparatus setup was prepared using standard procedure. Resolving and stacking gels were prepared and loaded into the SDS-PAGE gel apparatus and left to polymerize. A sample buffer containing β-mercaptoethanol and bromophenol blue was added to the sample in a 1:5 ratio. The mixture was heated at 100°C for 2 minutes, briefly

rotated and then loaded into the wells of the polymerized acrylamide gel. Samples mixed with sample buffer were loaded in the wells. A molecular weight marker was also loaded into a separate well for reference. Electrodes were connected to the SDS-PAGE apparatus and 50V of power supply was given.

Electrophoresis was stopped when the dye reaches just above the end point of the gel. The gel which undergone electrophoresis, was taken out from the apparatus and kept in staining solution overnight. Gel was taken from the staining solution and kept in destaining solution. It removes the background stain. After destaining, protein bands were visualized under UV transilluminator[1].

Fourier-Transform Infrared Spectroscopy (FTIR) analysis of fibrinolytic enzyme: FTIR analysis of fibrinolytic enzymes was performed by following the procedure given by Sudesh et al[25]. By Fourier-transform infrared spectroscopy technique, functional groups present can be identified from the crude enzyme and purified enzyme of *Bacillus pumilus* strain-MPMJB1[25].

Proteolytic analysis of fibrinolytic enzyme: Proteolytic activity analysis was carried out for the purified enzyme. About 15 ml of nutrient agar was prepared with purified enzyme as samples and 2 gm of casein as a standard for the conformation of the proteolytic activity of fibrinolytic enzyme. The medium and Petri dish were autoclaved for 15 mins at 121˚C. After the cooling of the medium, it was poured into the plate in a laminar air flow chamber and was allowed to solidify. Using the cork borer, 4 wells were created and marked as standard, 1, 2 and 3. 20 µl of standard, crude (1) and purified (2 and 3) enzymes were added to the wells respectively. Then the plate was incubated at 37˚C for 24 hours[1].

Thrombolytic analysis of fibrinolytic enzyme: Fibrinolytic activity analysis was carried out for both crude enzyme and purified enzyme based on the procedure given by Ningthoujam et al[17] and de Souza et al[6]. Blood was obtained without adding EDTA from local clinical laboratory. It was separately added into 4 tubes and marked as control, crude, purified enzyme, protein standard respectively. 500µl of crude extract, purified enzyme and protein standard were added to their respective tubes. The tube which was marked as control, was kept without adding anything. The tubes were allowed to incubate for 24 hours[6,17].

Results

Subculture of *Bacillus pumilus* and fibrinolytic enzyme production: The microbe *Bacillus pumilus* strain-MPMBJ1 was obtained from CBNR. The bacteria were subcultured in the sterilized nutrient broth. In the modified nutrient medium, 200 µl of bacillus culture was inoculated. The bacteria successfully produced fibrinolytic enzyme by absorbing nutrients from soy waste and glucose after incubation of 24 hours at 37˚C.

Optimum period on enzyme activity: The optimum incubation period for the growth of *Bacillus pumilus* strain-MPMBJ1 was found to be 24 hrs by comparing the absorbance obtained for 24 and 48 hrs incubated bacteria (Figure 1).

Purification of enzyme: The mixture obtained from the enzyme assay underwent a precipitation process. The protein was separated by ammonium sulfate and acetone precipitation method. The white precipitate was obtained after refrigerating the samples at 4˚C for 24 hours. Hence the presence of protein was confirmed. The yeilds of fibrinolytic enzyme using acetone and ammonium sulfate precipitation method were found to be *64.55 ± 1.25* and *22.75 ± 0.75 mg/ml* respectively (Figure 2). Hence the acetone precipitated enzyme has more concentration than the ammonium sulfate precipitated enzyme. Therefore, acetone precipitated enzyme was taken for further analysis.

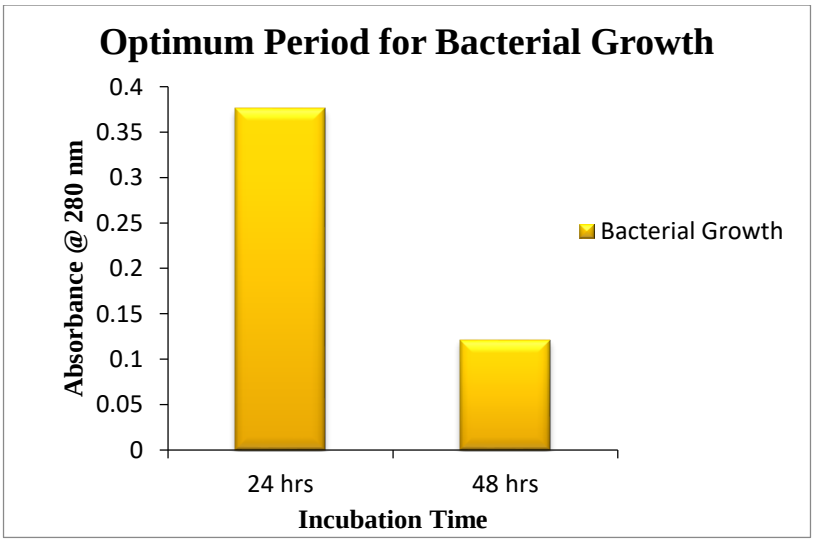


Figure 1: Optimum incubation period for *Bacillus pumilus* strain-MPMBJ1

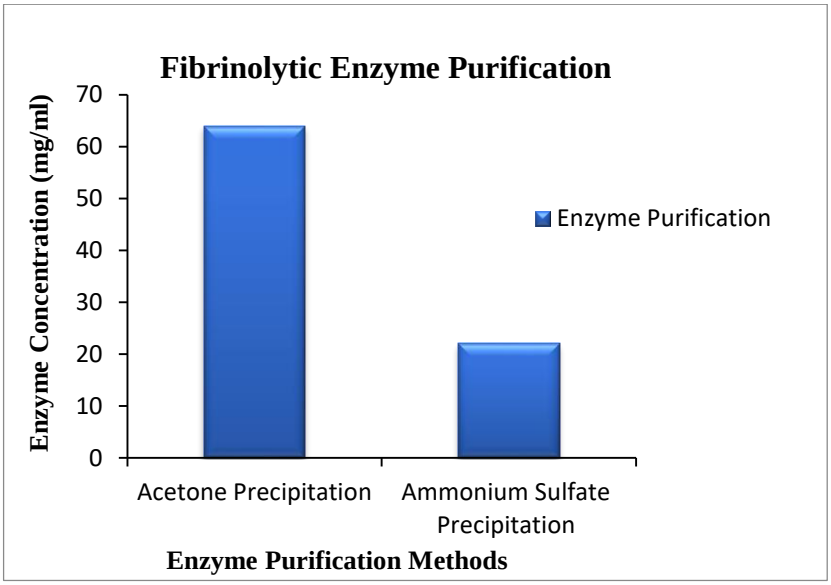


Figure 2: Enzyme purification from *Bacillus pumilus* strain-MPMBJ1 using precipitation methods

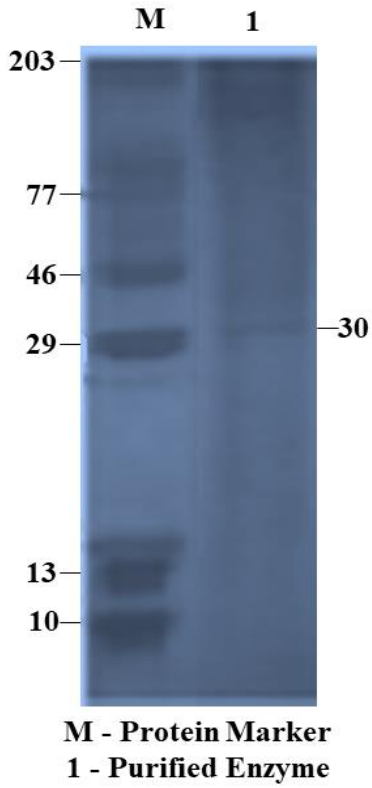


Figure 3: Identification of molecular weight of fibrinolytic enzyme using SDS-PAGE

Molecular weight detection: The acetone precipitated enzyme was dialysed to remove impurities. The dialysed sample underwent column chromatography to remove further impurities from the dialysed sample. The enzyme obtained from column chromatography was made to run in a SDS-PAGE gel and band was obtained. The molecular weight of the fibrinolytic enzyme was found to be 30kDa (Figure 3).

Fourier-Transform Infrared Spectroscopy analysis (FTIR): From the FT - IR analysis, several absorption peaks were obtained for the crude sample of fibrinolytic enzyme from *Bacillus pumilus* strain-MPMBJ1. The peak at 3362.28, 3309.85, 1739.79, 1643.35, 1365.60, 1219.01 Cm^{-1} with respect to the functional group of OH, N-H, C=O, C=C, C=O and C=C. The same kind of bandwidth was observed in purified enzymes also as 3360, 3309.85, 1739.79, 1643.35, 1369.46 and 1222.87 concerning the functional group of OH, N-H, C=O, C=C, C=O and C=C (Figures 4 and 5).

Proteolytic activity conformation of fibrinolytic enzyme: The crude and purified samples of fibrinolytic enzyme were added to the nutrient agar plate with casein as standard to

identify the proteolytic activity of the samples. Lysis was found in the crude and purified samples of fibrinolytic enzyme which conform its proteolytic activity (Figure 6).

Fibrinolytic activity: The crude, purified and protein standard were analyzed for its fibrinolytic property using blood clot lysis method. Incubation of blood with the samples showed the clot lysis after 24 hrs when the control is completely blood clotted. Hence, it was confirmed that the isolated enzyme has fibrinolytic property (Figure 7).

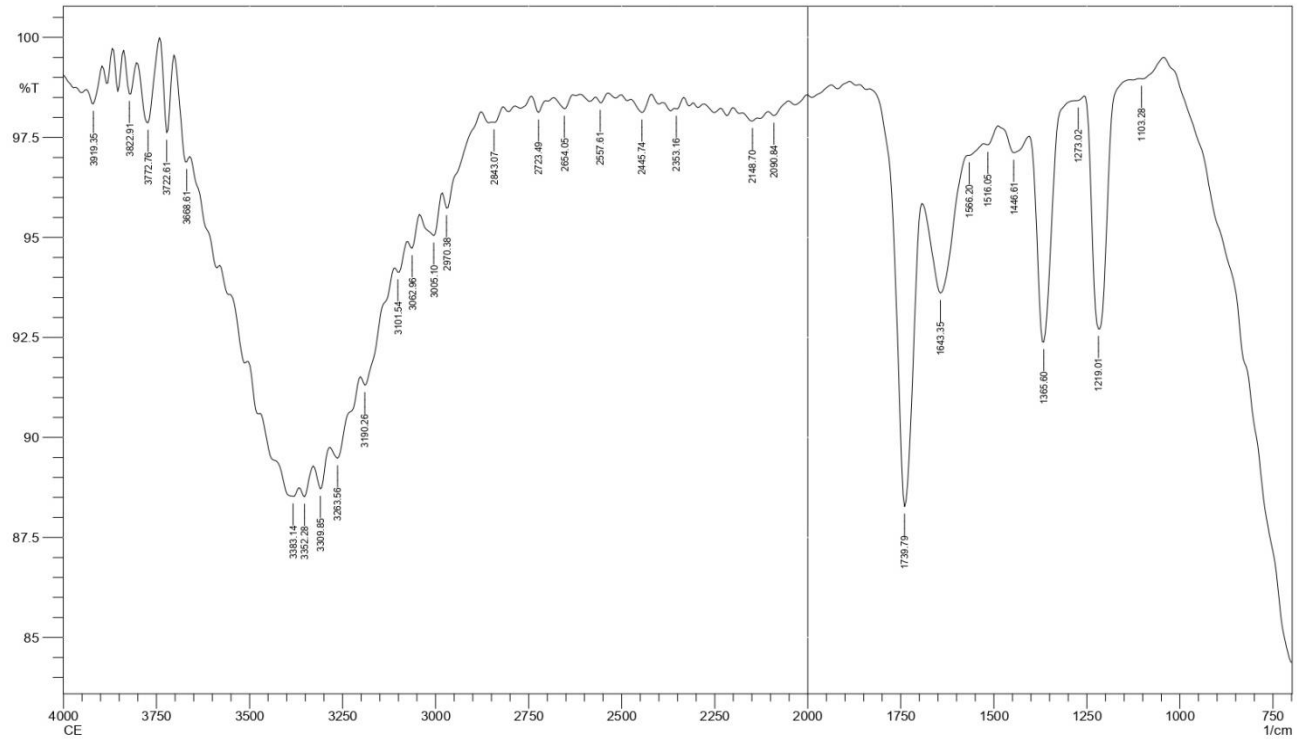


Figure 4: FT-IR Absorption peaks of crude enzyme

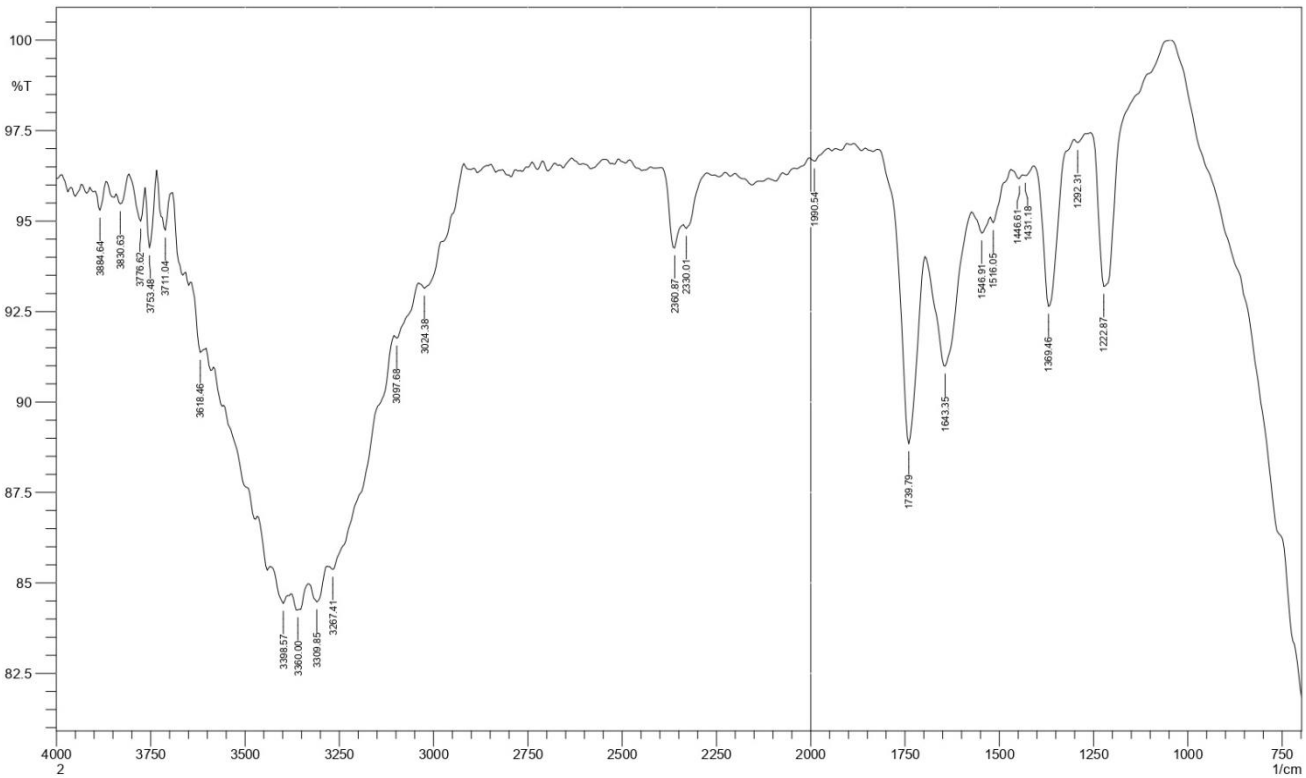


Figure 5: FT-IR Absorption peaks of purified enzyme

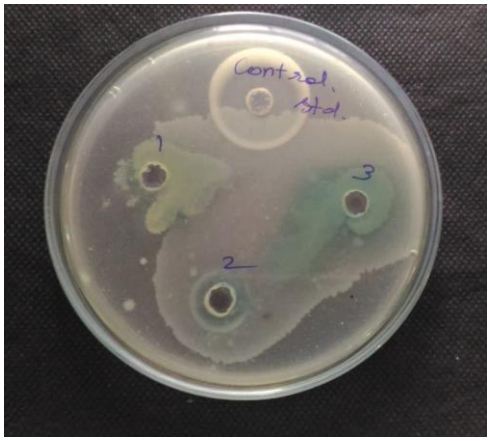


Figure 6: Proteolytic activity of fibrinolytic enzyme

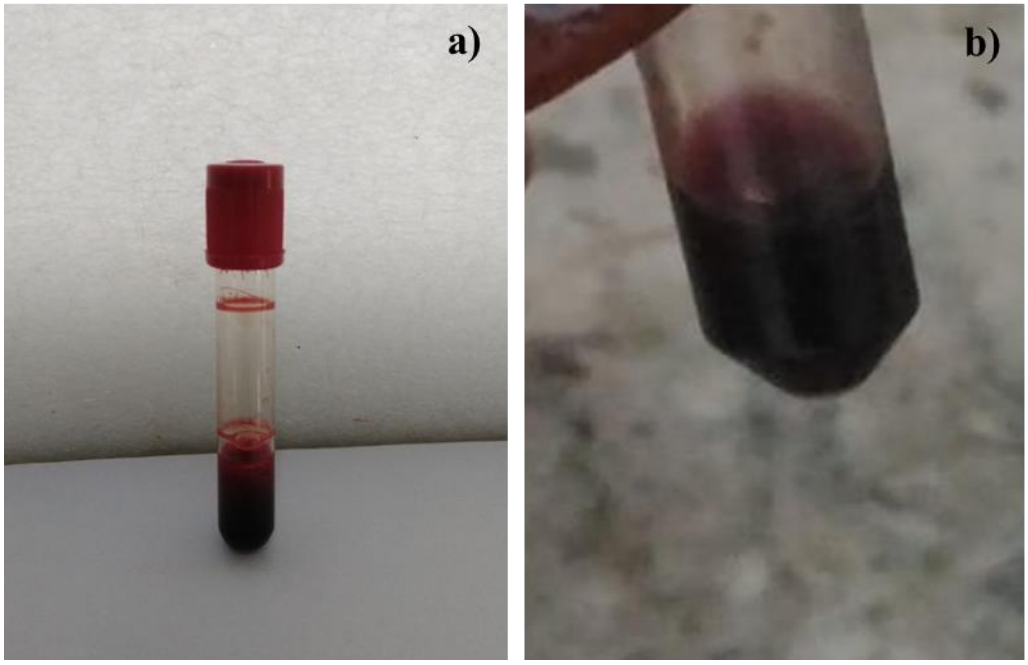


Figure 7: Fibrinolytic activity of a) control and b) fibrinolytic enzyme

Discussion

A fibrinolytic protease was isolated from *Bacillus amyloliquefaciens* strain KJ10 which was obtained from the fermented soy bean. The enzyme was found to have 29kDa molecular weight and exhibited fibrinolytic activity of 28 ± 1.8% within 60 mins of incubation[20]. Six Bacillus species isolated from fruit and vegetable waste such as *Bacillus altitudinus* HEM05, *Bacillus halotolerance* ARKH1, *Bacillus safensis* CO05, *Bacillus subtilis* subsp. *inaquorum* BE1, *Bacillus tequilensis* SAS23 and *Bacillus siamensis* BHO27 were tested for their thrombolytic activity and significant degradation in clot fibrin was found[22]. Similarly, in this study, fibrinolytic enzyme isolated and purified from *Bacillus pumilus* strain-MPMBJ1 also showed promising fibrinolytic activity.

The fibrinolytic enzymes play main role in the clot resolution. It is subjected to intravenous injection in the patients to lyse the blood clot. Hence it is required in large amount and it is too costly. Producing fibrinolytic enzymes from bacteria such as *Bacillus pumilus* is cost effective and can be produced in large amount by the fermentation process[3]. Fibrinolytic enzymes produced from microorganisms especially from bacteria are found to be cost effective with less time for production and produced in large amount. These enzymes have an important role in clinical practice involved in a thrombolytic therapy. It is risk free to be used as a drug for the clot resolution in the patients[7]. Hence fibrinolytic enzymes can be recommended to use in the clot resolution of brain and heart.

By recombinant DNA technology methods, fibrinolytic enzyme gene can be cloned in non- pathogenic bacteria to produce recombinant fibrinolytic enzyme to avoid any risk to the patients, to grow in normal conditions and to increase half life period. In addition to this technology, mutation, chemical modifications can lead to produce desirable character of the fibrinolytic enzyme. But proper analysis is a must to avoid unintentional risk to the patients. Further studies on fibrinolytic enzymes should concentrate on cost

effectiveness, easy production and availability of clot resolution drug for a serious brain, heart and other diseases regarding blood clot[12].

Conclusion

In the study, fibrinolytic enzymes are produced in the modified nutrient medium under solid-state fermentation using *Bacillus pumilus* strain-MPMBJ1. For the successful production of the organism, optimum temperature, period, pH and environmental factors are required. The optimum time for the enzyme activity was 24 hours. The presence of the enzyme was confirmed by ammonium sulfate precipitation and acetone precipitation methods and was purified by dialysis and column chromatography. The fibrinolytic enzymes were confirmed to have proteolytic activity and fibrinolytic activity.

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