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Research Article

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# Production and Purification of Pharmaceutically Important Fibrinolytic Enzyme from Bacillus Species

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**ABSTRACT**

The medicinal and pharmaceutical importance of currently available thrombolytic agents like urokinase, t-PA, streptokinase, staphylokinase and others, demonstrated repeatedly since 1970s, however sometimes they cause undesirable side effects like bleeding and allergic responds. The present findings reports isolation, screening and identification of soil bacterium for production of fibrinolytic enzyme. Samples for the study were collected from different locations were first screened for proteolytic activity using skimmed milk agar plate and lastly fibrin plate method was used to evaluate fibrinolytic activity. The strain capable of producing fibrinolytic protein was identified as Bacillus Spp. Using both Bergery's manual of systemic bacteriology and biochemical characterization simultaneously. Selected strain was than subjected to the process of fermentation using basal media for 5 days, 37°C and at 180rpm. Protein content and fibrinolytic activity were measured by Biuret method using bovine serum albumin as standard and fibrinolytic assay respectively. Three stage purification was done, that includes salting out with ammonium sulphate, followed by gel filtration chromatography and finally separated by RP-HPLC, proteins were eluted in peaks with a retention time of 2.092, 3.188, 5.178, 7.295, and 11.32 minutes. The fraction with retention time 7.295 minutes shows a maximum activity. The enzyme found to be having an optimum pH between 7.0 and 7.5. Enzyme is also stable at the optimum pH and found to lose its activity on higher side of acidity or alkalinity. It is more active at 40°C and is stable at 37°C to 43°C with slight modification in activity.

**Keywords:** Bacillus Spp. Thrombus, clot, fibrin, fibrinolytic, fibrinogen.

## INTRODUCTION

Beating inside in every one of us the very kernel of our life force and our humanity is the heart. Power of the heart sends the blood in our body and brain, even stream of blood is vital, if a simple clot forms in the wrong place, in the vein or in the artery, it can quickly place the body in serious danger, avoiding the passage of blood, straining the heart, starving the brain of oxygen. [1] A blood clot is pathologically identified as thrombus and is major factor in 95% cased of the heart diseases and stroke. [2-3] The vascular system is massive network of vessels through which blood circulates in the body. Arteries, arterioles, veins, venules, capillaries and lymphatic elements contribute the structure of vascular system. A blood clot (thrombus) advanced in the circulatory system can cause vascular obstruction leading to serious significances including death. [4] A fit hemostatic system overwhelms the blood clot in the

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usual circulation and reacts extensively in the incident of vascular injury to avoid blood loss. [4-5] When this system get disturbed or failed, out comes contain stroke, pulmonary embolism, deep venous thrombosis, and circulatory disorders. Pathologies connecting a failure of homeostasis and creation of clot require a clinical involvement consisting of administration of thrombolytic agents. [6] A blood clot or thrombus as it is called consists of blood cells sealed in fibrin mesh. Dissolution of blood clot through mediated breakdown is called as thrombolysis or fibrinolysis. [7-9] Thrombolysis therapy is most common management accepted in the form of complaints caused by occlusion of blood vessels by thrombus.it is the therapy that uses a agents that breaks up or dissolves the clot and it includes alteplase (activase), streptokinase (streptase, kabikinase), urokinase (abbokinase), and tissue plasminogen activator (TPA). [10] So far several investigators had concentrated their hard work on the isolating and screening of microorganisms for enzyme production with high fibrinolytic activity and on purifying and characterizing afresh found enzyme. Though most of the work recommended that Bacillus Spp. also produce a variety of extracellular and intracellular fibrinolytic enzymes. [11-13]

## MATERIALS AND METHODS

### Isolation and screening of bacteria for fibrinolytic enzyme producing strain

About 40 diverse samples were selected from the different regions of Vadodara city, Gujarat, India. The area selected for screening were garden soil, industrial soil, mud water, body swabs and lake water. The samples were collected according to standard microbiological procedures and maintained in the freeze (4°C) until use. Samples were first diluted with the sterile water and screened for protease production using skimmed milk agar plate. Samples collected were first transferred on skimmed milk agar plate containing (g/l): peptone 5, yeast extract 3, bacteriological agar 12.5 and skimmed milk 250 ml. [14] Clear zones after incubation of 72 hours show the protease production. Colonies were isolated by repetitive streaking on fresh agar plates. [15-17] Isolated colonies were than subjected to fibrinolytic screening by fibrin plate method using fibrin as a substrate. The fibrin plate prepared was composed of 2.5 ml of 1.2% bovine fibrinogen in 0.1M phosphate buffer (pH 7.4), 7.6 ml 1% agarose solution, and 0.1 ml of bovine thrombin (10NIH unit/ml) in to petri dish. The solution in petri dish was set aside for 1 h [18] to form fibrin layer. One ml of sample was spread on the plate and was kept for incubation. Isolate producing clear zones in fibrin plate was selected and identified as Bacillus spp. by colony morphology, gram staining biochemical test and selective media. [19]

### Enzyme production

Bacillus Spp. was grown on the basal media containing (g/l): glucose 20, sucrose 30, yeast extract 5.0, beef extract 5.0, meat extract 5.0, peptone 5.0, $KH_2PO_4$ 0.5, $K_2HPO_4$ 0.5, slight traces of salts of Mg, Cu, and Fe, $CaCl_2$ 0.5, amphotericin B 2.5mg and 1000 ml distill water. Media autoclaved for 20 minutes at 121°C and 15Lb pressure. [20] Media cooled to room temperature and inoculated by two ml of uniformly prepared suspension of Bacillus Spp. Inoculated media kept on orbital shaker incubator at 37°C, 180 RPM for 5 days. [21]

### Protein analysis

At regular interval of 24 hours sample was withdrawn from the flask, centrifuged at 10,000 rpm for 10 minutes at 4°C, supernatant taken and protein content was determined by biuret method using bovine serum albumin as a standard.

### Enzyme purification

Every step was performed at low temperature except other vice stated. Centrifugation at a speed of 10,000rpm for 20 minutes at 4°C was used to separate out the cells from the broth. Supernatant was fractionated by slow addition of ammonium sulphate at 4°C with continuous stirring. The precipitates obtained in 0-70% saturation of ammonium sulphate were collected by centrifugation, dissolved in 20mM potassium phosphate buffer and checked for the activity. Fraction showing maximum activity was selected and subjected to dialysis for overnight at 4°C using the same buffer. [22] Sephacryl s-200 gel filtration column (1.0×64 cm) previously equilibrated with potassium phosphate buffer pH 7.4 was used for the further purification of enzyme dialysate. Fractions collected were subjected to determination of activity and fraction with maximum activity was selected. Selected fraction was further purified by reverse phase high performance chromatography (RP-HPLC) on a water reverse phase XR ODS column (3.9×300 mm). Proteins were eluted with linear gradient from 5% to 70% v/v acetonitrile containing 0.1% v/v trifluoro acetic acid. Protein elution monitored at 285nm and peaks were screened for the activity. [19]

### Assay of fibrinolytic activity

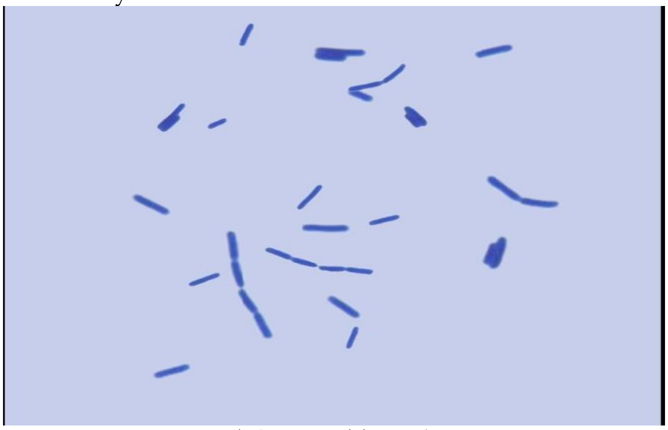
Fibrinolytic activity was performed according to the method described formerly. [23] To form a fibrin clot, briefly 3.0µl of thrombin (10NIH/ml) was mixed with the 40.0µl of 0.6% (W/V) solution of bovine fibrinogen prepared in 100mM potassium phosphate buffer pH 7.4. Mixture was kept to stand at room temperature and clot was formed. Enzyme sample added at a dose of 5.0µg/ml and reaction mixture incubated at 37°C for different time interval. 10.0µl of freeze cold trichloro acetic acid 10%v/v was added to terminate the reaction. Mixture obtained from above step was centrifuged, supernatant was used to determine the quantity of amino acid (tyrosine at 660 nm) released using folin-ciocalteu's reagent spectrophotometrically. Fibrinolytic activity was calculated from the standard curve of tyrosine. [24-25] One unit of fibrinolytic activity is defined as 1.0µg of tyrosine liberated per minute per ml of enzyme. [15, 26-29]

### *In vitro* thrombolytic assay

*In vitro* fibrinolytic activity was detected by artificial blood clot method given by omura. Synthetic blood clot was prepared using citrated goat blood (0.3 ml) and 10µl of thrombin (10NIH unit/ml). [30] The above mixture was kept at room temperature to form clot and incubated with definite amount of sample for altered time intervals at 37°C. After a required time period the remaining clot was weighed and the activity was stated as mg of clot lysed per µg of enzyme. [19, 21, 31]

**Biochemical characterization**

Activity of the enzyme was also checked for its optimal pH and temperature by inoculating enzyme at changed pH 5-12 and temperature ranges 30-55°C. [32] Specificity for substrate of enzyme was likewise checked by incubating enzyme with different substrate including casein, bovine serum albumin, globulin, fibrin, haemoglobin in buffer at 37°C for 30 minutes. 10µl ice cold trichloro acetic acid 10% v/v added to terminate the reaction and amount of free amino acid (tyrosine) released was determined at 660nm using folin-ciocalteu's reagent. One unit of fibrinolytic activity is defined as 1.0µg of tyrosine liberated per minute per ml of enzyme and which was obtained from the standard curve of tyrosine.



**a) Gram positive rods**

**b) Catalase test**

**Fig. 1: Biochemical characterization**

**RESULT**

**Isolation and screening of fibrinolytic enzyme producers**

In the current work samples were taken from garden and industrial soil, body swabs, and water from mud and lake. Samples first plated on skimmed milk agar for protease activity and around 10 microbial strains produced clear zones around their growth. Further isolation done to get pure colony by repeated streaking and finally all strains were subjected to screen for fibrinolytic activity. Out of 10 strains only one strain produces a clear zone of fibrin clearance. It was finally selected and further purified by repeated streaking on agar plate.

Identification of the isolated strain was done according to the Bergery's, manual of systemic bacteriology and prokaryotes. Culture gave creamy off white colonial morphology with slight promotion and uneven internal characteristic on the internal surface. Microscopically gram positive and rod shaped. Outcomes of biochemical test performed typically indicates characteristic of Bacillus Spp. [19]

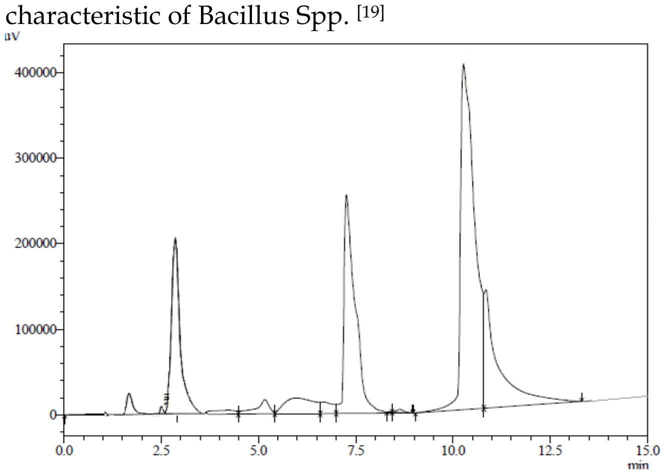



**Fig. 2: RP-HPLC profile**

**Table 1: Purification summary**

| Purification step | Total protein | Specific activity | Total activity | Yield | Purification yield |
|---|---|---|---|---|---|
| Crude extract | 510 | 867 | 1.7 | 100 | 1 |
| $NH_2SO_4$ | 310 | 775 | 2.5 | 89 | 1.47 |
| Sephacryl-200 | 144 | 662.4 | 4.6 | 76 | 2.70 |
| RP-HPLC | 4.3 | 78.26 | 18.2 | 9 | 10.70 |

**Enzyme Production and purification**

The fibrinolytic enzyme from Bacillus spp. was purified using a combination of salting out method by ammonium sulphate precipitation and chromatographic method. Protein concentration and activity assay were kept as indicator for the purification progress.

The 60% fraction showed a maximum activity, when this fraction was further purified by gel chromatography resulted in six fractions. All the fractions were checked for the activity and fraction four found to be more active. It was later separated by RP-HPLC, proteins were eluted in peaks with a retention time of 2.092, 3.188, 5.178, 7.295, and 11.32 minutes. The fraction with retention time 7.295 minutes shows a maximum activity. The fraction was found to homogenous, when it was re run on RP-HPLC using XR OSD column. A summary of purification scheme is given in the following Table 1.

**Fibrinolytic activity Assay**

The fibrinolytic action of the Bacillus Spp. was evaluated by incubating crude enzyme extract with artificial fibrin clot prepared by mixing thrombin and fibrinogen in test tube. Activity was calculated in terms of tyrosine amino acid released, that reacts with folin-ciocalteu's reagent and produce color. From the Table 1 listed above clearly indicates that each purification step the activity of enzyme increase and protein concentration decreases. It is also clear from the table that purification fold is much higher with less yield at final stage of purification.

**Evaluation of *in vitro* fibrinolytic assay**

Assessment of *in vitro* thrombolytic activity carried out using the fermented broth of Bacillus Spp. containing crude enzyme as a sample and saline solution and blood clot as a control activity evaluator respectively. Blood clot degradation observed in test tube containing sample. Clot was completely degraded after 1 hr. at 37°C and pH 7.0 in contrast; in control solution no clot degradation was observed.

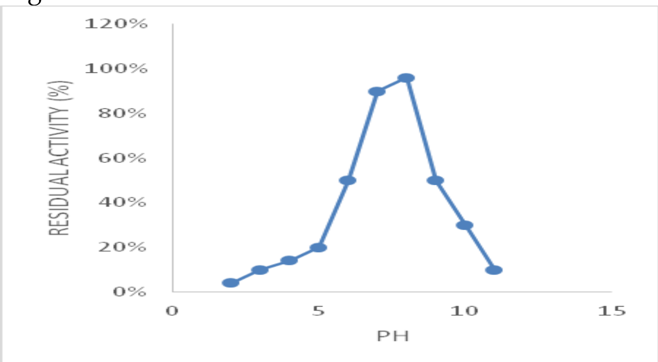



**Fig. 3: Optimum pH activity was determined by assessing the activity at different pH**

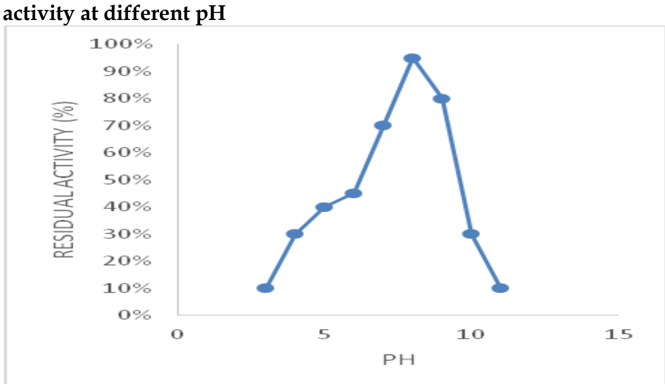



**Fig. 4: pH stability was determined by measuring the residual activity after the incubation with respective pH at 1°C for 3 h**

**Biochemical characterization**

pH and temperature greatly influenced the action of enzyme. Enzyme was found to be very active among pH 7.0 and 7.5. There is complete loss of action also experienced in very acidic and very alkaline situations. Activity was also found to be steady when incubated in optimum pH for 3 hour at 1°C. Similarly enzyme action was also analyzed for the effect of temperature and found that it was very active at 40°C and activity also persisted stable between 40°C and 45°C. Above and below the optimum temperature, lessening in the activity was observed. From the substrate study it also shows that the enzyme is more active against fibrin matched to all other.

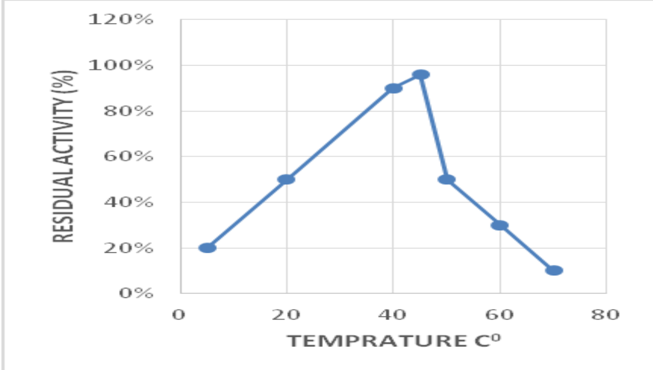



**Fig. 5: Optimum temperature was determined by assaying activity at different temperature for 20 minutes**

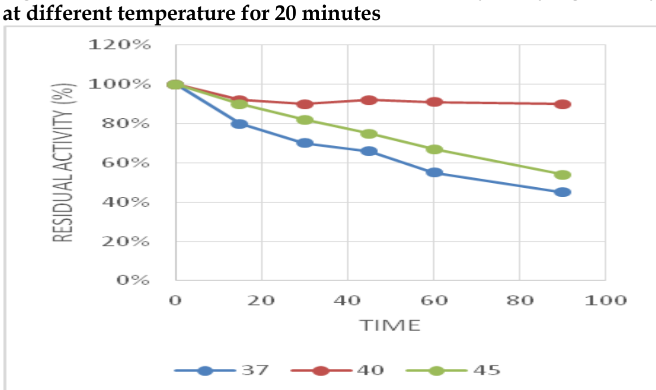



**Fig. 6: Thermal stability was determined by measuring the activity at 37°C, 40°C and 45°C for 15 to 90 minutes**

## DISCUSSION

Current study stated the screening and isolation of bacteria those can produce fibrinolytic enzymes. The work presented here reported the production and purification of fibrinolytic enzymes using multiple levels of purification methods. Further attempt was also made to find some biochemical properties of the purified enzymes. Form the study it indicates, Bacillus Spp. Are a potential candidate for the fibrinolytic enzymes and can be isolated and screened using a simple media. Moreover the enzyme produced was first checked for its proteolytic property and secondly fibrinolytic property, in both the cases the enzyme is powerful proteolytic and fibrinolytic agent. Biochemical characterization also suggests; it can be a novel agent for future thrombolytic therapy. Substrate specificity also indicates affinity of enzyme is more towards fibrin than other proteins, thus nature of enzyme if more fibrinolytic compared to proteolytic. *In vitro* anti-coagulant property of enzyme was also studied for the development of an anti-coagulant agent to prevent thrombosis and additional cardio vascular illnesses.

Literature review displays that several reports unfolds the purification and biochemical characterization of bacterial fibrinolytic enzymes available, very slight effort made to see the toxicity and pharmacological properties. [33-35] Work presented above powerfully recommend that enzyme produced from Bacillus Spp. can be strong applicant for the safer anti thrombotic drug and it should considered for preclinical studies by means of animal model to judge it's *in vivo* thrombolytic property. [36-40]

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