Optimization of Cultural Conditions for Maximum Production of Fibrinolytic Enzymes from the Local Marine Bacterial Isolates and Evaluation of their Wound Healing and Clot Dissolving Properties

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Authors' contributions
This work was carried out in collaboration between both authors. Both authors are equally contributed of this study.

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ABSTRACT

Fibrinolytic enzymes find necessary applications to treat and prevent cardiovascular diseases. In this study, optimal conditions for enhancing the production of fibrinolytic enzyme from local marine bacterial strains were evaluated. The present study also focuses on screening of wound healing efficacy of the isolated fibrinolytic enzymes. Various physical parameters such as temperature, pH, incubation time and medium components viz. inoculum size, substrate (nitrogen and carbon) concentrations were optimized. A cultivation medium was designed using optimized conditions for mass production of fibrinolytic enzyme and specific activity of enzyme was analyzed. The maximum enzyme production was observed at 37 °C temperature, 8.0 pH, substrate concentration with 3 ml inoculum size and 32 h. of incubation time. Among the different carbon sources tested, Mannitol showed maximum enzyme activity i.e 538 U/ml. Yeast extract was found to be the best nitrogen source with an enzyme activity of 498 U/ml. The best substrate for the production fibrinolytic enzyme was found to be keratin with high
1. INTRODUCTION

Fibrinolytic enzymes are proteinases, which have an ability to degrade the fibrin clot (thrombus). Fibrin is the main component of blood clot, which is normally formed by fibrinogen with the involvement of thrombin. Thrombosis, the accumulation of blood clot in blood vessels in a heart chamber, causes myocardial infraction and other cardiovascular diseases. Cardiovascular diseases (CVDs) result in 17 million deaths every year as per the report of world health organization (WHO). Thrombosis also results in other serious medical problems leading to cerebral and myocardial infraction, arthritis, etc. In order to treat this problem, blood clot dissolving agents such as urokinase, streptokinase, and tissue plasminogen activator (t-PA) are administrated intravenously and orally. They activate the plasminogen into plasmin to dissolve the fibrin clot thereby dislodging the thrombin fastly and thoroughly. Currently, their high cost and undesirable side effects, prompt researchers to search for cheaper and safer resources [1]. The more researchers to search for the inexpensive and safer resources. Many researchers are searching for the inexpensive and safer resources which can interfere with blood clotting.

Fibrinolytic enzymes were found in a variety of foods, for example Nattokinase from Japanese natto, Tofu, Korean cungkook jang, soy sauce and mushroom [2]. Apart from these food sources, fibrinolytic enzymes were produced from non-food sources including earthworms, snakes, Microorganisms such as Bacteria, fungi, Actinomycetes. Bacteria such as Streptococcus pyogens, Staphylococcus, Bacillus, Bacillus amyloliquifacens produce fibrinolytic enzymes. Fungi such as Fusarium oxysporum, Mucor, Amillaria fumigatus were reported to produce fibrinolytic enzymes. In actinomycetes Streptomyces sp., Nocardia etc., Were reported to proce the fibrinolytic enzymes [3].

In the present study an attempt has been made to investigate the effect of physical factors, like temperature, pH, carbon sources, Nitrogen sources and different waste substrates for maximum production of enzymes from the potent isolate GPJ3 isolated from mangrove sediment of Pulicat lake.

2. MATERIALS AND METHODS

2.1 Potent Local Marine Bacterial Strain of GPJ3 and its Cultural Conditions

The bacterial strain GPJ3 isolated from mangrove sediments of Pulicat Lake was identified by standard methods as it was found to be the most potent producer of fibrinolytic enzyme among all the isolates. producing organisms. Stock culture were maintained in nutrient broth medium with 70% glycerol and cultures were preserved at -20°C. The inoculum was prepared by transferring a loopful of stock culture into 100ml of sterile nutrient broth. The medium was incubated for 24 hours at 37°C.

2.2 Production of Fibrinolytic Enzymes

Fibrinolytic enzyme production was carried out in a modified minimal medium composed of (gm/l): fibrin 2g, NH₄NO₃ 0.005; KH₂PO₄ 1.0and (NH₄)₂SO₄ at PH 7.0 and 1% of inoculum was added. The fermentation was carried out at 35°C for 48h. After the completion of fermentation, the whole fermentation broth was centrifuged at 10,000rpm at 4°C and the clear supernatant (crude enzyme) was subjected to recovery and purification process [4].

2.3 Optimization of Maximum Production of Fibrinolytic Enzyme by GPJ3 Strain

Fibrinolytic activity was carried out according to the method described by [5]. Fibrinogen solution 0.72 % (W/V) were mixed with 1.4 ml of 50Mm tris- Hcl and 20 units of thrombin and incubated and incubated at 37°C for 5mins. Then 0.1ml of sample was added and incubated for 60 mins. The reaction was stopped by addition of 0.2 trichloroacetic acid (TCA). After incubation the mixture was centrifuged at 8,000 rpm for 10 min. The absorbance of supernatant at 275nm was recorded. The values were calculated by considering one unit of fibrinolytic activity (FU)
as the amount of enzyme required to produce an increase in absorbance equal to 0.01 in 1 min at 275 nm [6].

Formula:

\[ FU = \frac{[(AR - AC) \times \text{Dilution ratio of sample}]}{[0.001 \times 60 \times 0.1]} \]

2.4 Effect of Incubation Period

Effect of incubation time were optimized by using different time intervals (24 to 72 hrs) using minimal media along with crude fibrin [7].

2.5 Effect of Temperature

Temperature optimization was done by incubating 20 ml of minimal media with 1.5% fibrin (crude fibrin) as a substrate at concentrations checked with different temperatures from 20°C to 80°C.

2.6 Effect of pH

The influence of pH on the enzyme production was determined by culturing the isolate in media with different pH values ranging from 4 to 12 separately. The pH was adjusted using 0.1 N NaOH. Fermentation carried out for 42 hrs incubation.

2.7 Effect of Incubation Period

The effect of incubation time on bacterial enzymatic activity was determined by incubating the culture broth for different time intervals (24 to 72 hrs) with an interval of 4 hrs and incubation period of 24 hrs culture was set as a control [8].

2.8 Effect of Carbon Sources

Various carbon sources such as Glucose, Fructose, Maltose, Mannitol and Sucrose were used to optimize and enhance the fibrinolytic enzyme activity using them as a sole source individually. The experiments were done in triplicate and mean values are represented.

2.9 Effect of Nitrogen Sources of Organic and Inorganic Substances

The fibrinolytic enzyme production by the isolated potent strain GPJ3 was also optimized by supplementing different organic and inorganic nitrogen sources individually. The organic nitrogen sources such as 1% yeast extract, soya meal, malt extract, Beef extract, peptone, Gelatin and inorganic Nitrogen sources such as 0.1% of MgSO₄, Na₂HPO₄, and ammonium sulphate, ammonium chloride, ferrous sulphate were added individually and incubated for 32 hrs [9].

2.10 Effect of Substrates on Fibrinolytic Enzyme Activity

Various substrates such as skimmed milk, wheat bran, soya bean meal, Rice bran, sesame oil cake, groundnut oil cake, Tamarind seed powder (kernel), chicken waste, shrimp waste, orange peel were used for submerged fermentation. Five grams of each substrate was added separately to 500 ml of main nutrient medium for the production of fibrinolytic enzyme. Fermentation was carried out after the addition of 2% inoculum. The media were incubated at 37°C for 42 hrs at 150 rpm. The pH and volume of the media were maintained at 8.0 and 100 ml respectively [10].

2.11 Characterization of Crude Fibrinolytic Enzyme

Clean grease free slides were taken and the clotted blood was placed on each slide to which the crude enzyme is added and observed for the lysis of blood clot.

2.12 Characterization of Wound Healing Properties of Fibrinolytic Enzyme of GPJ3 against H9C2 Cell Lines

The wound healing property of fibrinolytic enzyme of GPJ3 was evaluated by the scratch wound healing assay using H9C2 cell lines as in vitro models. In a typical scratch wound healing assay, a “wound gap” in a cell monolayer is created by scratching, and the “healing” of this gap by cell migration and growth towards the center of the gap is monitored and often quantitated. Factors that alter the motility and/or growth of the cells can lead to increased or decreased rate of “healing” of the gap.

3. RESULTS

3.1 Incubation Period

The enzyme production and growth were increased gradually with the increase in incubation period up to 24-32 hrs and then decreased with further incubation. Hence, the
incubation time selected for further experiments was 24-32hrs. The result was represented in Fig. 1 of both the enzymatic activity and growth of the GPJ3 strain. The fibrinolytic activity as shown in Fig. 3.

3.2 Effect of Temperature

The maximum production of the fibrinolytic enzyme by GPJ3 the was observed at 37°C as shown by the maximum activity (400 FU/ml of the enzyme represented in Fig. 2 using origin software 8.0 version.

3.3 Effect of pH

The classical approach for optimization of pH (4-12) demonstrated maximum activity at pH 8.0 and further increase in pH resulted in decrease

3.4 Effect of Carbon Source

Among the six carbon sources such as (Glucose, Sucrose, Fructose, Maltose, Lactose, and Mannitol) in that substrate sources. Mannitol was found to result in maximum production of fibrinolytic enzyme, whereas fructose and maltose resulted in the production of lower quantities of fibrinolytic enzyme. Therefore, further studies were conducted using the mannitol instead of glucose in the production media to enhance the production of enzyme. The activity was calculated and plotted in Fig. 4.

Fig. 1. Graphical representation showing enzymatic activity against incubation period

Fig. 2. Graphical representation showing enzymatic activity against temperature
3.5 Effect of Nitrogen Sources

In the present study, the organic and inorganic nitrogen sources were used to evaluate their ability to enhance enzymatic production. The results revealed that supplementation of soya meal in the basal medium as nitrogen source resulted in maximum (498.0FU/ml) enzyme production along with cell growth Fig. 5.

Inorganic Nitrogen source Ammonium sulphate (284.7FU/ ml) resulted in maximum production. Very poor production was observed in case of ferrous sulphate Fig. 6.

3.6 Effect of Substrates of Carbon and Nitrogen Sources

Among the 12 substrates used as Carbon and Nitrogen sources for the production of fibrinolytic enzyme, the maximum yield was observed in case of Tamarind seed powder (TSP) / kernel. The total activity of the enzyme was found to be 1054.8 FU/ml. Therefore, TSP was used for
production of fibrinolytic enzyme in Basal media for further work Fig. 7.

3.7 Partial Characterization of Crude Fibrinolytic Enzyme

In blood clot slide technique, the produced fibrinolytic enzyme dissolved the clot within 30 seconds. The results were indicated in Fig-8.

3.8 Wound Healing Properties of Partially Purified Fibrinolytic Enzyme

The partially purified enzyme exhibited remarkable wound healing property in in vitro models. It resulted in 24.03% growth of cells in the wound scratch after 24 hours of treatment and 96.57% of cell growth after 48 hours of treatment Table. 1 & Figs -9 and 10.

![Graphical representation showing enzymatic activity against mineral salts](image)

**Fig. 5. Graphical representation showing enzymatic activity against mineral salts**

![Graphical representation showing enzymatic activity against nitrogen sources](image)

**Fig. 6. Graphical representation showing enzymatic activity against nitrogen sources**
Fig. 7. Graphical representation showing enzymatic activity against substrates

Fig. 8. Blood clot & Dissolving of blood clot by crude enzyme

Fig. 9. Cell migration
A. 0 hrs                                                                                     B. 24 hrs

C. 48hrs

Fig. 10. Wound healing effect of partially purified enzyme on H9C2 cell lines

Table 1. H9C2 Overlay of % of Wound Closure Scored

| Incubation | Cell Control | Std         | Sample          |
|------------|--------------|-------------|-----------------|
| 0 hour     | 0            | 0           | 0               |
| 24 hour    | 13.87001263  | 62.31622941 | 24.03244352     |
| 48 hour    | 34.38710614  | 99.05062027 | 96.57327607     |

4. DISCUSSION

In past few years, many fibrinolytic enzymes have been reported from various microbes including Actinomycetes, Bacteria, Fungi, Animals, Marine algae and entophytic strain *Paenibacillus polymyxa* EJS-3 has also been reported to secrete two extra cellular fibrinolytic enzymes (118 and 49KDa) in culture broth [1].

In the present study, the optimization of various environmental and nutritional parameters for maximum production of fibrinolytic enzyme from the local marine isolate GPJ3 were reported. The isolate evidenced its own idiosyncratic physicochemical and nutritional requirements for growth and enzyme secretion and there is no general defined media for the purpose. With this perspective the present study was aimed at determination of ideal conditions for the growth of the isolate and maximum enzyme production.

In earlier studies optimization of Physical conditions such as Temperature, pH and incubation time for maximum production of fibrinolytic enzyme was reported in *B. altitudinis* S-CSR0020 and it showed high enzyme production at 40-60°C, [11]. In another study, *Bacillus sphaericus* MTCC3672 had shown higher enzyme activity at 30°C. *B. subtilis* and mutant *Bacillus cereus* GD55 were reported to produced fibrinolytic enzyme at 37°C [5] & [12].
Optimization of pH and nutrient content in any bio-process is primarily important to develop efficient process for enzyme production. Vijayaraghavan & Prakash 2016 reported maximum fibrinolytic enzyme production after 72 hr. of incubation, at pH 9 in Bacillus subtilis. Pathak et al. [13] reported that Bacillus subtilis utilizes crude substrates for fibrinolytic enzyme production. [14].

In the present study maximum production of enzyme by GPJ3 was observed at pH range 4-12 with an optimum of pH 8.0. Among the different carbon sources used for production of fibrinolytic enzyme from the isolated potent strain, Mannitol was found to be the best carbon source for the production of fibrinolytic enzymes. The temperature 37°C, the nitrogen sources yeast extract and the cheap agro waste tamarind seed powder (kernel) have shown high fibrinolytic enzymatic activity. The present study revealed the wound healing effect of partially purified fibrinolytic enzyme and also in vitro clot lysis .

5. CONCLUSION

In the present study the optimal conditions for the production of fibrinolytic enzyme by the bacterial strain GPJ3 were determined. The partially purified enzyme from the strain was found to exhibit wound healing property and also resulted in clot lysis.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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