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Agro Waste Mediated Production of Fibrinolytic Enzyme and Its Optimization and Application

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Abstract: ***Cardiovascular diseases (CVDs) are the leading cause of death globally. At least three-quarters of the world's deaths from CVDs occur in low-and middle-income countries. People living in low-and middle-income countries often do not have the benefit of primary health care programmes for early detection and treatment of people with risk factors for CVDs. Microbial fibrinolytic enzymes are very much considered as novel therapeutic candidate for the treatment of CVDs. The aims of this study were to screened and characterized Bacillus spp. isolate that could produce a natural Fibrinolytic enzyme with high activity. To develop safe and cheap thrombolytic agents, a fibrinolytic enzyme agro waste was used. BR1 and DGS1 was found to be potential strains for the production of enzyme production. After optimization potato peel showed better result by exhibiting maximum enzyme production i. e.2513 ± 49.2 U/g through SSF. The fibrinolytic enzyme were stable at pH 8 when incubated at 40°C. In vitro clot lysis showed digestion of on goat blood clot. The method has proven to be highly simple and can be implemented to other enzymes also.***

Keywords: Fibrinolytic enzyme, Cardiovascular disease, Bacillus sp., fibrin clot, casein

1. Introduction

Cardiovascular diseases (CVDs) are a rapidly emerging health concern in the modern world. It was estimated that more than 23 million deaths by 2030 would be due to CVDs alone and these deaths would be mostly from India, Southeast Asia, and the Mediterranean region (Singh T. A. et al., 2013). Accumulation of fibrin clots in the blood vessels often increases thrombosis, a lethal complication of medical diseases, resulting in myocardial infarction and other serious cardiovascular diseases (Wang S. H. et al., 2007). Among the different types of cardiovascular diseases, thrombosis is one of the most widely occurring diseases in our modern life (Kotb E., 2014). Thrombotic diseases result from the accumulation of fibrin inside blood vessels. The increase in the incidence of CVDs is often related to a change in food habits. In clinical practices, external fibrinolytic enzymes are administered to patients with CVDs (Singh T. A. et al., 2013).

Typical thrombolytic agents, including urokinase, streptokinase, and tissue plasminogen activator (t-PA), have been widely applied in clinical therapy. However, these drugs are expensive and have a short half-life after intravenous administration, and there is excessive risk of hemorrhagic complication (Wei X. et al., 2011). Currently used fibrinolytic agents e. g. tissue-type plasminogen activator (t-PA), urokinaseand streptokinase convert plasminogen to plasmin that degrades the fibrin clot but suffer from shortcomings such as low specificity and stability, allergic reactions, resistance tore percussion, hemorrhagic side effects, large therapeutic doses, and high costs, though some fibrinolytic agents can be direct acting by mimicking plasmin. This warrants the search for novel fibrinolytic enzymes from various sources with higher efficacy, safety, and stability and preferably those direct-acting activities. Though new fibrinolytic enzymes are being explored from microbes, animals, plants, and fermented foods, microorganisms remain the preferred source due to their biochemical versatility and feasibility of mass culture, and ease of genetic manipulation (Ningthoujam D. S. et al., 2016). Therefore, a safer and cheaper fibrinolytic enzyme has been attracting more interest during these past few decades (Wei X. et al., 2011).

Fermented rice (Vijayaraghavan P. et al., 2016), shrimps (Anh D. B. Q. et al., 2014), soybean, soil (Obeid A. E. F. E. et al., 2015) have been used for screening of fibrinolytic enzyme-producing organisms. The microorganism used for fibrinolytic enzyme production depends on the type of culture required for enzyme production, (SSF or SMF), pH, and thermal stability of the enzyme. Optimization of process conditions is one of the most critical stages in the development of an efficient and economic bioprocess (Bibi N. et al., 2015).

As cost is one of the important factors determining the use of enzymes in therapeutics, optimization of media for maximum production is considered important to reduce cost. Components of media such as carbon, nitrogen sources, and fermentation conditions such as temperature, pH, and incubation time significantly affect the physiology, production pattern, and yield of the metabolites and thus need to be optimized. Despite the availability of several media, the production cost is still a major hindrance for the wide range of applications of the enzyme at an industrial scale. Consequently, search for new optimized media to enhance yield which in turn may add economy to the process is an ongoing vogue. The use of statistical models helps in overcoming the limitations of the traditionally used method of one-factor-at-a-time being more economical, time-saving, less laborious, and most importantly more reliable as they provide a view of the interactive effect of different parameters (Taneja K. et al., 2017).

In recent years, microbial fibrinolytic enzymes have been reported to treat and prevent CVDs. These enzymes have various therapeutic applications, including, anticoagulants,

and thrombolytic. There are various reports on fibrinolytic enzymes with thrombolytic applications from many sources, such as fermented brown rice, fermented shrimps, and soil from dumping ground area. Thus, the proposed investigation aimed to isolate fibrinolytic enzyme-producing organisms from fermented brown rice, fermented shrimps, and soil from dumping ground and vegetable markets. Identification of Fibrinolytic enzyme-producing bacteria and suitability of the synthesized fibrinolytic enzymes for application in clot lysis activity were investigated.

2. Methods and Materials

1) Sample Collection and preparation:

a) **Collection of brown rice:** Brown rice was collected from local grocery stores in Thane district, Maharashtra, India.100gms of brown rice was cooked individually for 1hr with double the amount of drinking water and allowed for aerobic fermentation at room temperature for 48-72hr.

b) **Collection of Shrimps:** Shrimps were collected from a local fish market in Thane district, Maharashtra, India.50gms Shrimps paste was made and kept in a beaker with 48-72hr of incubation.

c) **Collection of Soil Sample:** Soil sample was collected in a sterile zip lock bag from the waste dumping area of the Vegetable market, and Slaughterhouse in Thane district, Maharashtra, India.

2) Isolation of microorganisms:

a) **Isolation of bacteria from brown rice sample**: Rice sample was boiled for 50mins, excess water was drained out and the rice was kept for aerobic fermentation for 72h. After incubation 1gm of cooked brown rice was suspended in 99ml double distilled water and was serially diluted. Dilutions10^{-4}, 10^{-5}, 10^{-6}were spread onto sterile Casein Agar plate (CSA) and Skim Milk Agar (SMA). Plates were then incubated at 37˚C for 24-72hrs. After incubation, the potent colonies showing halo zone around the colonies were selected and further screened on the Casein Agar plate and Skim Milk Agar to obtain a pure colony.

b) **Isolation of bacteria from Soil sample:** 1gm of different soil samples (vegetable market, slaughterhouse, and garden) was suspended in 99ml double distilled water, serially diluted. Selected dilutions10^{-4}, 10^{-5}, 10^{-6}were spread onto sterile Casein Agar Plate. Plates were then incubated at 37˚C for 24-72hrs. After incubation, the potent colonies showing halo zone around the colonies were selected and further streaked on the Casein Agar plate and Skim Milk Agar to obtain a purified colony.

c) **Isolation of bacteria from Shrimp paste sample:** 1gm of shrimp paste was centrifuged at 3000rpm for 10-15mins. The clear supernatant was mixed with nutrient broth and kept for incubation for 24hrs. After incubation, it was streaked on Nutrient Agar Plate and kept for incubation at 37˚C for 24 hrs. After incubation, the well-isolated colonies were selected and further screened by streaking on Casein Agar plate and Skim Milk Agar.

3) Quantitative analysis of Fibrinolytic activity by Enzyme Assay:

The bacterial isolates were cultured individually on skim milk agar and casein agar. The colony showing halo zones were selected and inoculated in a sterile liquid medium containing, casein 10g/L, yeast extract 5g/L, peptone 5g/L, and NaCl 1.5g/L. The liquid medium was kept in an orbital shaker at 175rpm for 48hr at 37oC. The culture medium was then centrifuged at 10, 000rpm for 10 minutes. The supernatant obtained was used as the Crude enzyme. The Fibrinolytic enzyme-producing capability of the isolate was assayed using fibrin as a substrate. A 0.1-ml aliquot of the enzyme was mixed with 2.5 ml of Tris-HCl buffer (0.1 M, pH 7.8) containing calcium chloride (0.01 M). Then, 2.5 ml fibrin (1.2%, w/v) was added and incubated for 30 min at 37◦C. The enzymatic reaction was then terminated by adding 5.0 ml trichloroacetic acid (0.11 M) containing sodium acetate (0.22 M) and acetic acid (0.33 M). This mixture was then allowed to stand for 30 min at room temperature and centrifuged at 10, 000 rpm for 10 min.

The absorbance was then measured with the clear supernatant at 275nm against individual sample blank. One unit of fibrinolytic activity is defined as the amount of enzyme that liberates 1 µg of L-tyrosine per minute under the standard assay conditions (Vijayaraghavan P. et al., 2016). Total protein concentration was measured by Lowry assay using bovine serum albumin as the standard (Lowry et al., 1951). The selected strain exhibiting the highest fibrinolytic activity was subjected to morphological and biochemical characteristics.

4) Identification of Fibrinolytic enzyme-producing bacteria:

Bacterial isolates were characterized based on morphological, cultural, and biochemical tests and were further identified using Bergey's Manual of Systematic Bacteriology.

5) Optimization of fibrinolytic enzyme production:

a) Optimization of substrate by solid state fermentation:

5g of substrate (Potato peel, Banana Peel, and Corn Husk) was added in 100ml Erlenmeyer flasks. It was moistened with 4ml 0.1M Tris-HCl buffer having pH 8.0. The mixture was then autoclaved at 121^{o}C for 20mins. It was then incubated after addition of 5% inoculums at 37^{o}C for 48-72h under static condition. After incubation the enzyme was extracted by 50ml double distilled water and placed in orbital shaker at 150rpm for 30mins at room temperature. Centrifugation was carried out at 10, 000 rpm for 10 minutes at 4^{o}C and the supernatant was collected as the Crude enzyme produced by the substrate. After that Total protein concentration of this crude enzyme was measured by Lowry assay using bovine serum albumin as the standard (Lowry et al., 1951)

b) Optimization of incubation period:

The selected isolates were grown on nutrient deficient broth containing best agro residues for different incubation period (20, 30, 37, 45, 50hr). After incubation, enzyme produced by substrate was quantified by method mentioned by **Vijayaraghavan P. et al., 2016**.

c) Optimization of Temperature:
The optimum temperature was determined by incubating the substrate and enzyme mixture at different temperatures (20, 30, 40, 50, 55℃). After incubation, enzyme produced by substrate was quantified by method mentioned by **Vijayaraghavan P. et al., 2016**. All experiments were carried out at least three times.

d) Optimization of pH:
The optimal pH for the production of the enzyme was determined within a pH range of 3.0–11.0, using the following buffer systems: 50 mM sodium acetate buffer (pH 3.0–5.0), sodium phosphate buffer (pH 6.0–8.0), carbonate buffer (pH 9.0–10.0), and glycine-NaOH buffer (pH 11.0), respectively. The pH of the substrate and enzyme was adjusted to the same value as that of the buffer range. After incubation, enzyme produced by substrate was quantified by method mentioned by **Vijayaraghavan P. et al., 2016**. All experiments were carried out at least three times.

6) In vitro application of Fibrinolytic enzyme for lysis of blood clot:
The Goat blood was collected from a slaughterhouse in Thane, India. Furthermore, the clot was washed several times with phosphate-buffered saline (PBS) (pH 7.2) and cut into small pieces aseptically. The enzyme was diluted appropriately (200 U/ml) and incubated with a blood clot (500 ± 50mg) at 30 ± 2℃. To the control vial, PBS was added instead of the enzyme. The tubes were then incubated for 4h and blood clot lysis was observed (Vijayaraghavan P. et al., 2016).

3. Results

a) Isolation of microorganisms:
Samples were enriched and total 7 morphologically different isolates i. e. BR1, BR2, SMP1, SMP2, VMS1, VMS2, & DGS1 were obtained exhibiting halo zone around the colonies on sterile Casein Agar Plate and Skim milk Agar (Figure 1.). Each of them was stored on Casein agar slants at 4°C till further use.

Figure 1: Isolates on Skim Milk Agar plate

b) Quantitative analysis of Fibrinolytic activity:
Fibrinolytic enzyme-producing capability of purified isolates were assayed and the absorbance of each isolate is mentioned in Table 1. Out of 7 isolates only two samples i. e. BR1 & DGS1 exhibited highest fibrinolytic activity of 3.58 U/ml and 3.05 U/ml respectively.

Table 1: Fibrinolytic activity of isolates

S. No.	Isolates	Fibrinolytic activity (U/ml)
1	BR1	3.58
2	BR2	0.95
3	SMP1	0.49
4	SMP2	1.2
5	VMS1	0.80
6	VMS2	1.40
7	DGS1	3.05

c) Identification of Fibrinolytic enzyme-producing bacteria:
The biochemical tests of two potential isolates i. e. BR1 and DGS1 were carried out. The results of test lead us to know the closest genus of our isolates according to Bergey's Manual of Systematic Bacteriology (Table 2).

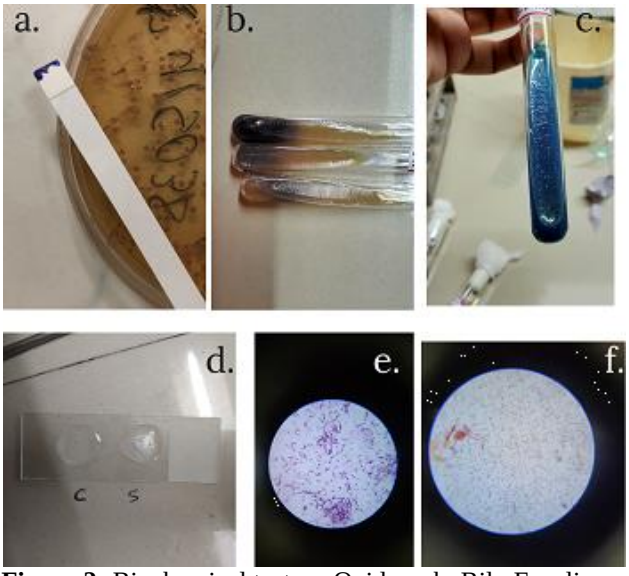

Figure 2: Biochemical tests a. Oxidase, b. Bile-Esculin, c. Citrate Utilization, d. Catalase, e. Gram stain of BR1, f. Gram stain of DGS1

The morphological characteristics and biochemical tests results of potent isolates exhibited similarity with standard biochemical of Bacillus sp. and Pseudomonas sp. (Figure 2).

Table 2: Biochemical tests of Potent Isolates

Sr No.	Biochemical tests	Samples	
		BR1	DGS1
1.	Gram Nature	+ve	-ve
2.	Indole	-ve	-ve
3.	Methyl Red	-ve	-ve
4.	Voges-Proskauer	+ve	-ve
5.	Catalase	+ve	+ve
6.	Citrate Utilization	+ve	+ve
7.	Bile-Esculin	+ve	-ve
8.	Sucrose Fermentation	+ve	-ve
9.	MannitolFermentation	+ve	+ve
10.	Maltose Fermentation	+ve	-ve
11.	Lactose Fermentation	+ve	-ve
12.	Glucose Fermentation	+ve	-ve
13.	Oxidase	+ve	+ve

d) Optimization of fibrinolytic enzyme production:
The appropriate selection of medium components based on both aspects of regulatory effects and economy is the goal in designing the chemical compositions of the fermentation

media, where nutritional requirement for growth and production must be appropriate. Fast formation and high concentration of the desired product are the criteria for the qualitative and quantitative supplement of nutrients and otheringredients. Bacteria, yeast, and fungi can grow well on submerged fermentation and found very good applications in submerged fermentation processes. Solid state fermentation (SSF) techniques have been successfully used for enzyme production by enzyme manufacturer‘s worldwide.

e) Optimization of substrate by solid state fermentation:

In the present study, various agricultural residues were screened for maximum production of fibrinolytic enzymes. The present results revealed that fibrinolytic enzyme production by depends on the substrate used in SSF. Maximum fibrinolytic enzyme production was observed with pota to peel substrate (2513±49.2 U/g). Enzymeproductionwas941±24.6 and1, 312±64.4 U/g for corn husk and banana peel respectively (Figure 3.).

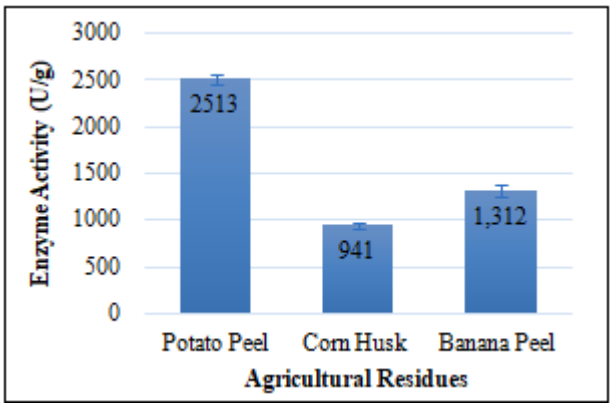

Figure 3: Optimization of substrate by SSF

f) Optimization of incubation period:

SSF was carried out in a 150ml Erlenmeyer flask containing 10gm potato peel moistened with 10ml 0.1M Tris-HCl buffer having pH 8.0 to measure the time when yield of enzyme is maximum. Thefibrinolyticactivity of the selected strain was subjected at various incubation periods (20, 30, 37, 45, 50hr); however, maximum fibrinolytic activity (3153 ± 34.3 U/g) was observed after 45hr incubation at 37℃ (Figure 4).

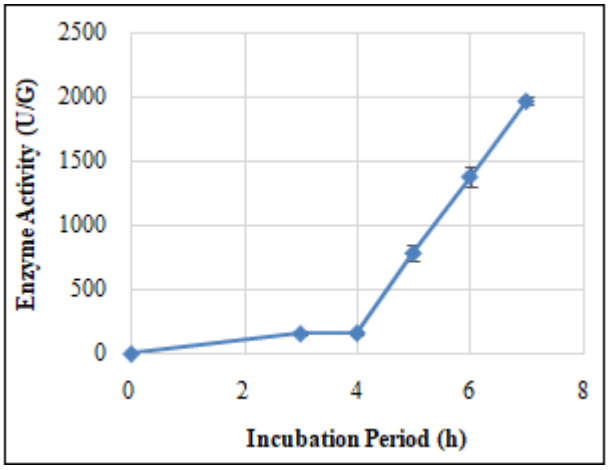

Figure 4: Optimization of Incubation Period

g) Optimization of Temperature:

Temperature is another critical parameter that has to be controlled and varied from organism to organism. The mechanism of temperature control of enzyme production is not well understood. However, studies by showed that a link existed between enzyme synthesis and energy metabolism in bacteria which was controlled by temperature and oxygen uptake. The result on the effect of temperature on fibrinolytic enzyme production is shown below in Figure 5. The production of fibrinolytic enzyme increased significantly with the increase in fermentation temperature from 20-50℃ and decreased above 40℃. The maximum fibrinolytic enzyme production obtained at 40℃ was 2708.6 ±79.4 U/g and the least production was observed at 20℃i. e (210 ± 19.6 U/g) at 45 hours.

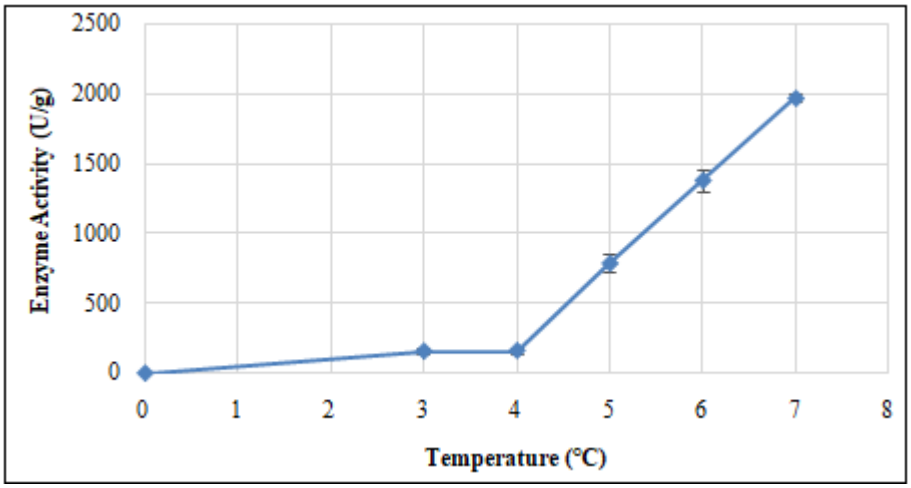

Figure 5: Optimization of temperature

h) Optimization of pH

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

The effect of different initial pH of synthetic medium on fibrinolytic enzyme production is shown below in Figure 6. The effect of initial pH on fermentation medium revealed that the yield of fibrinolytic enzyme increased with the increase in the initial pH of the production medium up to pH 8 and decreased furthers. The maximum fibrinolytic enzyme activity 2467 ± 36.1U/g was obtained at pH 8 for 45hr of fermentation period while the least fibrinolytic enzyme activity was observed at pH 3 (158.3 ± 16.8 U/g).

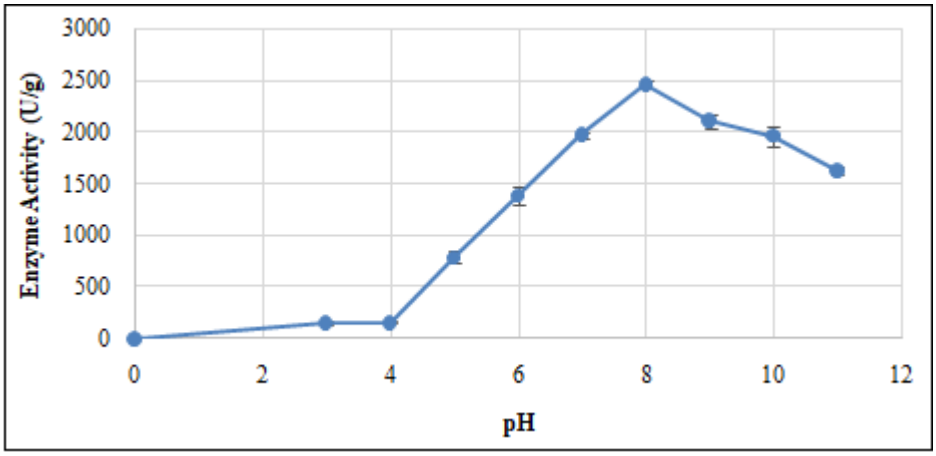

Figure 6: Optimization of pH

i) In vitro application of Fibrinolytic enzyme for lysis of blood clot:

The clot lysis activity of the fibrinolytic enzymes secreted by isolate BR1 was determined. The lysis activity increased with an increase in the incubation time exhibiting potent fibrinolytic activity.

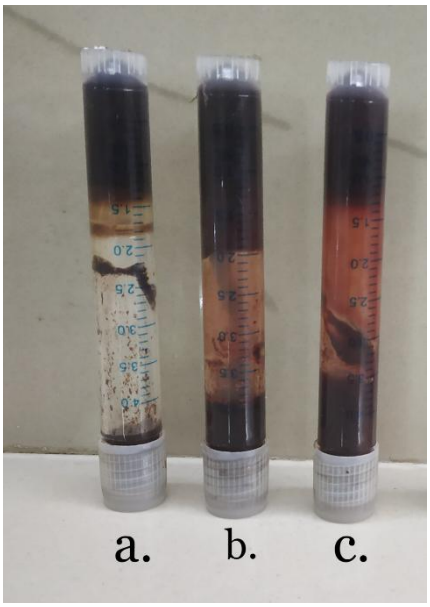

Figure 7: Blood Clot Lysis a. Control, b. with enzyme after 2hrs, c. with enzyme after 4hrs

4. Conclusion

Hence, in this study successful isolation of Fibrinolytic producing organism from different sources was done. Out of the isolated species BR1 and DGS1 had maximum fibrinolytic activity exhibiting immense potential to lyse blood clots. Different agro-waste were investigated as a cheap alternative for SSF of which Potato peel was found to be better than other agro-substrates by exhibiting maximum Enzyme production i. e.2513 ± 49.2 U/g. Optimization of parameters like Incubation period, Temperature, and pH which are critical in SSF were scrutinized and the best values for these parameters were determined. The maximum fibrinolytic enzyme production were obtained at pH 8 when incubated at 40°C for 45hrs. This clearly implies that enhanced production of fibrinolytic enzyme can be achieved by using cheap agro-waste instead of costly substrates. These kinds of studies help to utilize agro-waste substrates in enzyme bioprocess. In vitro clot lysis revealed its activity on blood clot. Hence, this study concludes that fibrinolytic enzymes can be obtained by using cheap agro-waste residues and thus can act as an alternative for the costly thrombolytic agents. Further studies needs to be done to check the efficacy of the extracted fibrinolytic enzyme for application in CVD's treatment and prevention.

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