Proceedings of the Pakistan Academy of Sciences: B Life and Environmental Sciences 60(1): 49-56 (2023)

ISSN (Print): 2518-4261; ISSN (Online): 2518-427X
http://doi.org/10.53560/PPASB(60-1)733

Pakistan Academy of Sciences

Research Article

Production of Fibrinolytic Enzyme by Soil *Actinobacteria*

Aminu Argungu Umar[1*], Aliyu Abdullahi Turaki[2], Ahmad Ibrahim Bagudo[3], and Rilwanu Yeldu Ibrahim[4]

[1]Department of Biochemistry, Kebbi State University of Science and Technology, Aliero, Nigeria
[2]Department of Biochemistry and Molecular Biology, Federal University, Birnin Kebbi, Nigeria
[3]Department of Microbiology, Kebbi State University of Science and Technology, Aliero, Nigeria
[4]Department of Biochemistry, Usmanu Danfodiyo University Sokoto, Nigeria

Abstract: Thrombotic diseases are increasingly becoming among the prominent causes of death across the globe. Researchers are now turning attention towards fibrinolytic enzymes as potential alternative treatment for thrombolytic diseases. This present study focused on the production of extracellular fibrinolytic enzyme from soil *Actinobacteria* and evaluation of its hydrolytic activity on blood clot. The Actinobacteria was isolated from compost soil of semi-arid climate in Nigeria. Bacterial isolation was achieved using starch casein agar. Potent fibrinolytic enzyme producing *Actinobacteria* were identified and subjected to enzyme production using submerged fermentation method. The interactive effects of incubation time, temperature, pH and media components on enzyme production were analysed. Extracellular fibrinolytic enzyme produced by the selected *Actinobacteria* was partially purified by ammonium sulphate precipitation and subsequently assayed for blood clot lysis activity. Results of these studies indicated that fibrinolytic enzyme was produced optimally at pH 8 and temperature of 40 °C after 72 hour of fermentation. Partially purified fibrinolytic enzyme was able to degrade blood clot comparable to the positive control. These results shows that soil Actinobacteria of unexplored semi-arid climate of Nigeria present a prospect in search of novel microorganisms with potentials in the production of fibrinolytic enzyme that can serve as an alternative blood clot buster in treating thrombolytic diseases.

Keywords: *Actinobacteria*, Semi-Arid, Fibrinolytic Enzyme, Yield optimization, Thrombolytic.

1. INTRODUCTION

Myocardial localized necrosis occurrence is on the increase globally, primarily due to Thrombosis and pulmonary embolism that develop inside the arteries [1, 2]. Thrombosis is a critical event and a common pathology underlying myocardial infarction [3]. Treatment of thrombosis is the usual way through which acute myocardial infarction (AMI) is combated. The use of synthetic therapeutics in treating thrombosis can result to negative effects such as allergic response, short half-life and they are expensive to acquire [4-6]. Therefore, it is critical to research and develop natural, safer, and cost effective thrombolytic agents. Nowadays, protein treatments are commonly used in combating AMI. In the last decade, microbial fibrinolytic proteases have shown great potential for therapeutic application [7]. It is envisaged that fibrinolytic enzymes of natural source are less likely to induce negative effects such as allergic response and they are inexpensive to produce. Therefore, therapeutic screening of fibrinolytic enzymes from microbial origin could be the most suitable method for fibrinolytic enzymes production [7].

Actinobacteria are widely distributed in nature inhabiting mainly soil and plants. They are filamentous Gram-positive microorganisms that are ubiquitously distributed in nature. They are significantly an important sources of bioactive metabolites of industrial and biotechnological importance [8]. There are literature reports on the production of fibrinolytic proteases by

Received: September 2022; Accepted: March 2023
*Corresponding Author: Aminu Argungu Umar <auargungu96@gmail.com>

the *Actinobacteria* from aquatic and terrestrial ecosystems [9-13].

The expanding applications of fibrinolytic proteases motivated this study to screen for novel fibrinolytic *Actinobacteria* from soil. One of the advantages of producing enzymes from microbial sources is that, it could be achieved using low cost substrates. From the industrial point of view, designing of a low cost production medium for bio products such as enzymes is one of the curial factors, because the market price of the particular bio products is directly influenced by the cost of production medium components. Therefore, utilizing a locally abundant cost effective inexpensive substrates could reduce the cost of production [14]. The present study aims to isolate soil *Actinobacteria* and induce extracellular fibrinolytic enzyme production by the isolated *Actinobacteria*. To the best of our knowledge, this is the first attempt to explore the potential of soil *Actinobacteria* of slaughter house for fibrinolytic enzyme production and characterization.

2. MATERIALS AND METHODS

2.1 Sample Collection

Soil sample was collected on 25th March 2021, from the abattoir compost soil in Yeldu town (12°47′06′′N 4°15′56′′E) at the depth of approximately 12 cm from the surface and brought to the laboratory in sterile polythene bag. Soil samples were incubated at 80 °C for 15 minutes to eliminate most of the unwanted gram-negative bacteria. Soils were allowed to cool and then 1 g of each soil sample was treated in a two-step process with 0.1 g $CaCO_3$ followed by 1.5 % phenol for 30 min.

2.2 Isolation and Identification of *Actinobacteria*

About 1g of the treated soil samples were suspended in 9 ml normal saline and vortexed. Serial dilution was carried out up to 10−5. Then 100µl of the serially diluted sample from 10−5 tube was inoculated on starch casein agar (SCA) supplemented with 50 µg/mL cycloheximide and 50 µg/mL nalidixic acid to restrict fungal and gram negative bacteria growth respectively. The resultant colonies were identified as *Actinobacteria* using conventional microbiology techniques and subsequently sub-cultured to obtain pure cultures. Screening for casein and fibrin hydrolysis was carried out based on the maximum zone of hydrolysis in both casein and fibrin, and the potent isolate was selected for further investigation [15-17].

2.3 Fibrinolytic Enzyme Production and Optimization of Production Media

Fibrinolytic enzyme production was initiated by transferring a single colony of *Actinobacteria* from the casein agar plate into a Erlenmeyer flask containing 50 mL of casein basal broth (Casein 10 gL^{-1}; K_2HPO_4 2 gL^{-1}; KNO_3 2 gL^{-1}; NaCl2 gL^{-1}; Casein 0.3 gL^{-1}, and 0.02 gL^{-1} of $MgSO_4$, $CaCO_3$, and $FeSO_4$ each, pH 7.5) [18], and incubated for 18 hrs at constant shaking of 200 rpm. The culture was then diluted at 1:100 (v/v) with the same but fresh Casein broth for three successive passages. Then 5 ml of the passaged culture of *Actinobacteria* was inoculated into another 250 ml of Casein basal broth, then incubated at 37 °C for 5 days on a shaker at 200 rpm.

To determine the required optimum physicochemical conditions in achieving maximum yield of keratinase through submerged fermentation (SF), a one-variable-at-a-time (OVAT) method was adopted and the effects of incubation period (24−120 h), incubation temperature (25−60 °C at 5 °C interval), pH (5.0−9.0), carbon and nitrogen sources were evaluated for optimal production of keratinase. Each experiment was carried out in triplicates. After 5 days of incubation, the crude extracellular fibrinolytic enzyme was obtained as a supernatant by centrifugation at 7,000 rpm for 10 min at 4 °C and then subjected to 85 % saturation of ammonium sulphate $(NH_4)2SO_4$. Using a dialysis tubing cellulose membrane obtained from Sigma-Aldrich, the ammonium sulphate precipitate was dialyzed against 10 mM Tris–HCl buffer (pH 7.5) [19].

2.4 Fibrinolytic Enzyme Assay

The reaction was setup with 3 mL of 0.1 M Tris- HCl containing 0.01 M $CaCl_2$ (pH 7.8), 1 mL (1 % w/v) of fibrin, and 1 mL of partially purified enzyme. The reaction was allowed to continue for 30 min at 37 °C after which 5 mL of trichloroacetic acid (TCA) containing 0.22 M sodium acetate and 0.33 M acetic acid was added to terminate the reaction. The

reaction mixture was centrifuged and absorbance of the supernatant was taken at 275 nm. One unit of fibrinolytic enzyme was defined as the amount of enzyme required to increase the absorbance at 275 nm equivalent to 1 µg of tyrosine per min [20]. The enzyme activity (EA) was calculated in U/mL according to the following Equation:

$$EA = (\Delta A \times V \times f)/(\varepsilon \times l \times v_o) \quad \text{(Equation 1)}$$

Where ΔA is the change in absorbance, V is the final volume of the reaction, *f* is the dilution factor, ε is the extinction coefficient, l is the path length of the cell and v_o volume of the enzyme used.

2.5 *In vitro* Assay of Blood Clot Lysis

Goat blood was obtained from a slaughter house in Aliero town. The blood was allowed to form a clot and then washed with phosphate-buffered saline to remove any impurities before adding the fibrinolytic enzyme. Positive and negative controls were prepared with clexane injection and a 0.9 % w/v saline solution respectively. A 5 mL of fresh goat blood was used for each test and all reaction mixtures were allowed to stand for 3 h at room temperature and checking after every 30 min [20].

2.6 Statistical analysis

Triplicates tests were performed in all the experiments and results were presented as Mean ± SD values. The significant differences of the data were evaluated via Analysis of Variance (ANOVA) and Duncan's Multiple Range Test (DMRT). Findings of all the experimental tests were significant at $p \leq 0.05$.

3. RESULTS AND DISCUSSION

3.1 Isolation and Identification of *Actinobacteria*

Actinobacteria of the slaughter house compost soil were isolated and identified based on the colonies morphological appearance on starch casein agar plate. The colonies were mainly white and some tiny, spherical and irregular ash-grey (Figure 1A). The isolated *Actinobacteria* was able to hydrolyse casein protein in the medium, indicating its proteolytic activity. Distinct colonies were selected and sub-cultured (Figure 1B) to have pure colonies which were subsequently subjected to production of fibrinolytic enzyme.

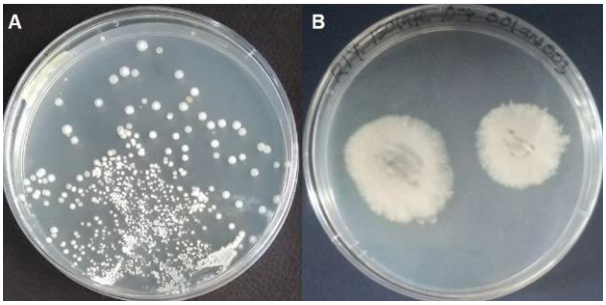

Fig. 1. (A) *Actinobacteria* isolates. (B) Sub-cultured *Actinobacteria* isolates.

3.2 Production of Fibrinolytic Enzyme and Optimization of Production Media

Various process parameters including incubation period, incubation temperature, pH, carbon and nitrogen sources influence fibrinolytic protease production. Depending on the microbial strains being studied, these production parameters varies for achieving maximum enzyme production. In this study, fibrinolytic enzyme production was initiated and allowed to continue for a period of 5 days under varying fermentation conditions including incubation period, temperature, pH, carbon and nitrogen sources. Experiments were carried out in triplicates under each fermentation condition. At 24 h intervals, an aliquot of the production media was collected and centrifuged. The supernatant was used as crude enzyme and the enzyme activity was assessed as described above.

Fibrinolytic enzyme production was determined by the activity of the crude enzyme, more activity indicate high concentration of the crude extracellular enzyme. Results of the OVAT experiments indicates that production of fibrinolytic enzyme started at 24 hour post inoculation and continued up to the fifth day, the enzyme production reached maximum (53.48 U/mL) at 72 h after which it begins to decline (Figure 2).

Previous study on Alcaligenes aquatilis PJS_1 reported 60 h incubation period as the optimum for maximum fibrinolytic enzyme production [14] whereas, *Bacillus* sp. IND7 was reported to achieve maximum production of fibrinolytic enzyme at 72 h [20].

Temperature is among the crucial factors that influences the ability of microorganisms to grow and synthesise metabolites. Each microorganism has its favourable temperature, therefore, metabolic reaction in microorganisms could be affected due to change in the fermentation temperature. This study assessed the temperature effects on fibrinolytic enzyme production by the isolated *Actinobacteria*. According to the results obtained, the optimum temperature for maximum enzyme production (68.28 U/mL) was 40 ^{o}C, and an increase in the incubation temperature to 45 ^{o}C causes a decline in the production of enzyme (Figure 3).

Previous studies on the production of fibrinolytic enzyme by marine *Actinobacteria* have reported the optimum temperatures for maximum enzyme production to be between 33 and 37 ^{o}C [12, 13]. Whereas, optimum fermentation temperature for fibrinolytic enzyme production by other bacterial strains was reported to be 35 ^{o}C [14, 21]. The variation in optimum fermentation temperature for fibrinolytic enzyme production between our study and the previous studies could be directly related to the fact that the sources of organisms and the bacterial strains are different from each study. In this study, the *Actinobacteria* was obtained from the compost soil of Yeldu town in Kebbi State, Nigeria which is a hot semi-arid climate where the daytime temperature can reach up to 45 ^{o}C during the warmest months of March to May. Hence, it is not surprising to have microorganisms from that habitat favouring high temperature for production of secondary metabolites.

Medium pH is another factor that affects the growth and synthesis of metabolites because metabolic reaction can be influenced as a result of change in the pH of the production media. The effect of pH on the production of fibrinolytic enzyme by the isolated *Actinobacteria* was evaluated and the results indicated that maximum enzyme production (57.69 U/mL) was achieved when the pH of the production media was 8 (Figure 4). Previous studies on the production of fibrinolytic enzyme by marine Streptomyces reported that maximum enzyme production was achieved when the pH of the production media was between 7 and 7.3 [12,13]. This shows that *Actinobacteria* fibrinolytic enzyme production is more favoured in a media of between neutral to near alkaline pH.

Carbon and nitrogen plays a role in the synthesis of microbial metabolites, hence the need to assess how sources of carbon and nitrogen can affect production of extracellular fibrinolytic enzyme by the *Actinobacteria* isolate. Results of these evaluations suggests that the *Actinobacteria* isolate was able to grow in all the media containing different sources of carbon and nitrogen, although the level of enzyme production varies with different source. For the carbon sources, the highest enzyme production of 71.13 U/mL was observed from the fermentation media containing glucose as the only source (Figure 5). Whereas, highest production of enzyme 65.11 U/mL was observed in the presence of tyrosine as nitrogen source (Figure 6).

Previous study reported a maximum fibrinolytic enzyme production by *Streptomyces rubiginosus* VITPSS1 in the presence of glycerol and soybeans as carbon and nitrogen sources respectively [12]. In another study, *S. radiopugnans* VITSD8 was reported to exhibit the highest yield of fibrinolytic enzyme when peptone and maltose were the sources of nitrogen and carbon respectively. Other strains of bacteria were also reported to having high yield of fibrinolytic enzyme in the presence of different

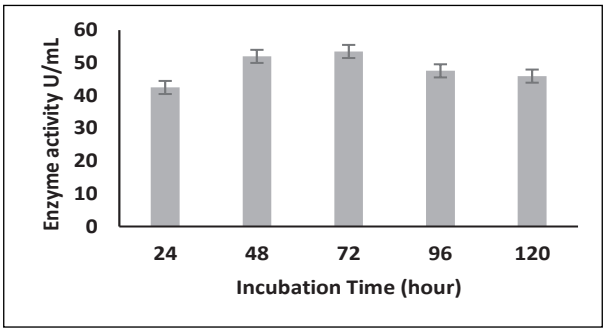

Fig. 2. Fibrinolytic enzyme activity of *Actinobacteria* isolate at different incubation period.

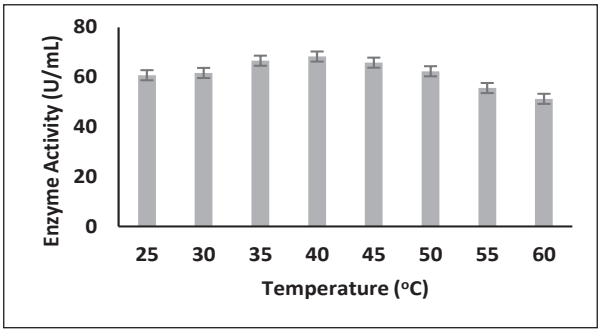

Fig. 3. Fibrinolytic enzyme activity of *Actinobacteria* isolate at different temperature.

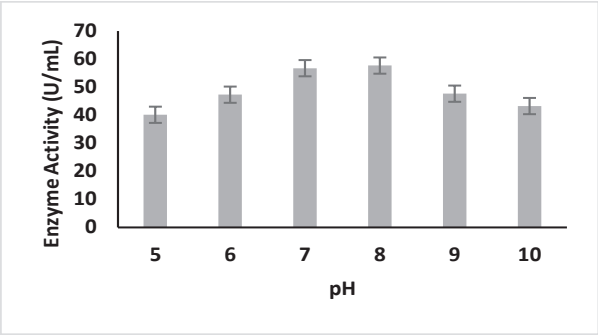

Fig. 4. Fibrinolytic enzyme activity of *Actinobacteria* isolate at different pH.

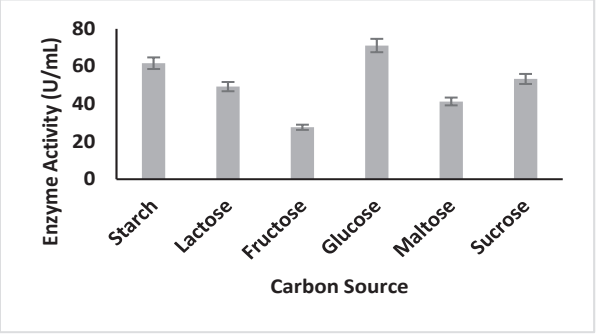

Fig. 5. Fibrinolytic enzyme production by soil *Actinobacteria* isolate under different carbon sources.

carbon and nitrogen sources [14, 21, 22]. This indicates that, the source of microorganisms and the strains can influence a variation in the synthesis of microbial metabolites as evidenced from these studies. This means that *Actinobacteria* of marine origin can be different from that of terrestrial origin in terms of factors that influence enzymes production, hence the need to explore more habitats in search of novel microbes for low cost production of fibrinolytic enzyme to convert thrombolysis disorder.

3.3 Partial Purification of Fibrinolytic Enzyme

The crude supernatant of the fermentation broth was partially purified with 85 % saturation of ammonium sulphate. From the purification results, it was observed that partially purified fibrinolytic enzyme exhibited a specific activity of 249.5 U/mg and 37 % yield after dialysis step (Table 1). The percentage yield was obtained using the formula

$$\% \text{ yield} = \frac{Total\ activity\ after\ dialysis}{Starting\ total\ activity} \text{ x } 100 \quad \text{(Equation 2)}$$

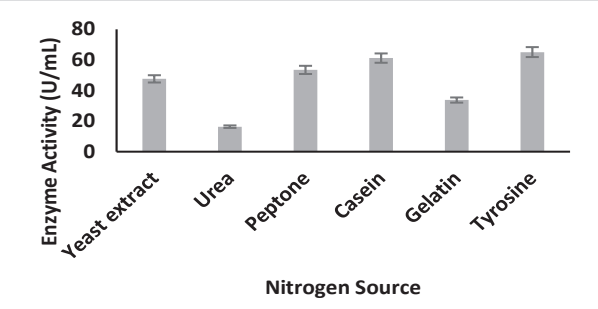

Fig. 6. Fibrinolytic enzyme production by soil Actinobacteria isolate under different carbon sources.

3.4 In vitro Assay of Blood Clot Lysis

Potential hydrolytic activity of the partially purified fibrinolytic enzyme (200 IU/mL) was evaluated on fresh goat blood in vitro. Clexane injection (200 IU/mL) and a 0.9 % w/v saline solution were considered as the positive and negative controls respectively. Results of these assays indicated that partially purified fibrinolytic enzyme achieved about 75 % clot lysis compared to 100 % achieved by the clexane injection (Figure 7).

Various studies have been reported on the blood clot lysis activity of fibrinolytic enzyme of microbial origins exhibiting different level of blood clot lysis. These include protease SFE1 [5], fibrinolytic protease of Streptomyces rubiginosus VITPSS1 [12], fibrinolytic protease of Streptomyces radiopugnans VITSD8 [13], the free protease BC1 and immobilized CLEA-Fib-mChi [23], fibrinolytic protease of Serratia marcescens subsp. sakuensis [24], and streptokinase [25]. Hence, our study further revealed the actinokinase-like activity of fibrinolytic enzyme from soil *Actinobacteria*. The fibrinolytic proteases are more

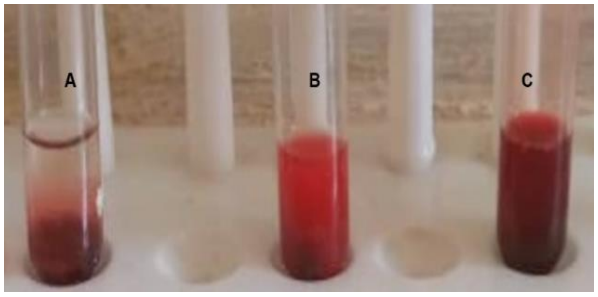

Fig. 7. Blood clot lysis activity. (A) Negative control (0.9 % saline solution) (B) Partially purified fibrinolytic enzyme (C) Positive control (Clexane injection)

Table 1. Purification table for fibrinolytic enzyme

Purification Step	Total Protein (mg)	Total Activity (U)	Specific Activity (U/mg)	% Yield	Purification Fold
Crude Supernatant	281	27,765.3	65.7	100	1
Ammonium Sulfate	85.6	19,429.2	151.2	46.6	2.3
Dialysis	41	10,229.5	249.5	37	3.8

of neutral enzymes that are produced mostly from *Actinobacteria*. Considering the high demand of fibrinolytic enzymes and the negative effects of the available drugs for the treatment of thrombosis, it is important to explore more fibrinolytic enzyme producing *Actinobacteria* especially from unexplored habitats. For the first time, the results of our study revealed that the soil compost of slaughter house from the semi-arid climate of Nigeria is a promising source of novel *Actinobacteria* with potentials in the production of fibrinolytic enzymes and possibly other industrially valuable secondary metabolites.

4. CONCLUSION

Actinobacteria was isolated from the compost soil of slaughter house obtained from Yeldu town and a proteolytic enzyme was produced by the isolated *Actinobacteria* strain. Due to its blood clot degradation activity in comparable with the standard clexane injection the protease was considered as fibrinolytic enzyme. It can be envisage that this fibrinolytic protease can be studied further towards developing it as a potential thrombolytic agent for treating heart diseases. Enzymes from unexplored natural habitats could be of immense benefits in various industrial applications. The findings of our study present more enlightenment that can lead to bioprospecting of fibrinolytic enzymes and even other enzymes of industrial importance from semi-arid habitats of Nigeria. We aim to further our study towards the molecular identification of the isolated *Actinobacteria* strain and carry out more purification steps to have enzyme of high purity in order to characterize the enzyme.

5. ACKNOWLEDGMENTS

We thank the Kebbi State University of Science and Technology, Aliero for providing us with conducive laboratories for the execution of this research work.

6. CONFLICT OF INTEREST

We declare no conflict of interest.

7. REFERENCES

1. E. Boersma, N. Mercado, D. Poldermans, M. Gardien, J. Vos, M.L. Simoons. Acute myocardial infarction. *Lancet* 361: 847–858 (2003).
2. S. Blix. The effectiveness of activators in clot lysis, with special reference to fibrinolytic therapy: A new method for determination of preformed clot lysis. *Acta Medica Scandinavica* 172: 1–24 (1962).
3. M.E.A. Alkhider, E.E. Elkhidir, and H.M. Ibrahim. Optimization of physical conditions of Actinokinase synthesis from local isolate using response surface methodology (RSM). *Journal of Applied Sciences* 20: 35-40 (2020).
4. J.R. Simkhada, P. Maner, S.S. Cho, and J.C. Ydoo. A novel fibrinolytic protease from Streptomyces sp. CS684. *Process Biochemistry* 45(1): 88–93 (2010).
5. X. Ju, X. Cao, Y. Sun, Z. Wang, C. Cao, J. Liu, and J. Jiang. Purification and characterization of a fibrinolytic enzyme from Streptomyces sp. XZNUM 00004. *World Journal of Microbiology and Biotechnology* 28(7): 2479–2486 (2012).
6. S. Pan, G. Chen, J. Zeng, X. Cao, X. Zheng, W. Zeng, and Z. Liang.. Fibrinolytic enzyme production from low-cost substrates by marine Bacillus subtilis: Process optimization and kinetic modelling. *Biochemical Engineering Journal* 141: 268–277 (2019).
7. V.G. Nielsen, J.K. Kirklin, W.L. Holman, and B.L. Steenwyk. Clot lifespan model analysis of the effects of warfarin on thrombus growth and fibrinolysis: Role of contact protein and tissue factor initiation. *ASAIO Journal* 55: 33–40 (2009).
8. S.N. Hazarika, and D. Thakur. Beneficial Microbes in Agro-Ecology. Bacteria and Fungi. *Elsevier*

chapter-21 *Actinobacteria* p. 443-476 (2020).

9. C.V. Dilip, S.S. Mulaje, and R.Y. Mohalkar. A review on actinomycetes and their biotechnological application. *International Journal of Biopharmaceuticals Science Research* 4: 1730–1742 (2013).
10. V. Mohanasrinivasan, S. Yogesh, A. Govindaraj, J.S. Naine, and S.C Devi. In vitro thrombolytic potential of actinoprotease from marine *Streptomyces violaceus* VITYGM. *Cardiovascular and Hematological Agents in Medicinal Chemistry* 14: 120–124 (2016).
11. B. Naveena, K.P. Gopinath, P. Sakthiselvan, and N. Partha. Enhanced production of thrombinase by Streptomyces venezuelae: Kinetic studies on growth and enzyme production of mutant strain. *Bioresource Technology.* 1: 417–424 (2012).
12. P. Verma, S. Chatterjee, M.S. Keziah, and S.C. Devi, S.C. Fibrinolytic Protease from Marine Streptomyces rubiginosus VITPSS1. *Cardiovascular & Hematological Agents in Medicinal Chemistry* 16: 44-55 (2018).
13. D. Dhamodharan, N.S. Jemimah, K.S. Merlyn, and D.C. Subathra. Novel fibrinolytic protease producing Streptomyces radiopugnans VITSD8 from marine sponges. *Marine Drugs* 17:164 (2019).
14. N. Prabhu, T. Gajendran, S. Karthikadevi, A. Archana, and R. Arthe. Utilization of sugarcane bagasse for enhancement production of fibrinolytic enzyme using statistical approach. *Cleaner Engineering and Technology* 5:100269 (2021).
15. S.A. Gopinath, and K. Lingappa. Isolation and screening of fibrinolytic enzymes producing bacterium strain from soil waste. *International Journal of Current Microbiology and Applied Sciences* 5(7): 969–976 (2016).
16. T. Astrup, and S. Müllertz. The fibrin plate method for estimating fibrinolytic activity. *Archive of Biochemistry and Biophysics* 40(2): 346–351 (1952).
17. S.B. Kim, D.W. Lee, C.I. Cheigh, E.A. Choe, S.J. Lee, Y.H. Hong, H.J. Choi, and Y.R. Pyun. Purification and characterization of a fibrinolytic subtilisin-like protease of Bacillus subtilis TP-6 from an Indonesian fermented soybean, Tempeh. *Journal of Industrial Microbiology and Biotechnology* 33(6): 436–444 (2006).
18. P.L. Bhargavi, and R.S. Prakasham, A fibrinolytic, alkaline and thermostable metalloprotease from the newly isolated Serratia sp RSPB11. *International Journal of Biological Macromolecules* 61: 479–486 (2013).
19. P. Hariharan, C.C. Naik, P. Vajpaye, and K. Srinivasa. Production, purification and characterization of thrombolytic enzyme from Cladosporium spp. through solid state fermentation. *International Journal of Engineering* 3: 585-589 (2014).
20. P. Vijayaraghavan, A. Arun, S.G.P. Vincent, M.V. Arasu, and N.A. Al-Dhabi. Cow dung is a novel feedstock for fibrinolytic enzyme production from newly isolated Bacillus sp. IND7 and its application in in vitro clot lysis. *Frontiers in Microbiology* 7:361 (2016).
21. K. Taneja, B. Kumar, S. Kumar, N. Dilbaghi. Process optimization for production and purification of novel fibrinolytic enzyme from Stenotrophomonas sp. KG-16-3. *Biocatalysis and Biotransformation* 37 (2): 124–138 (2019).
22. B.K. Bajaj, S. Singh, M. Khullar, K. Singh, and S. Bhardwaj. Optimization of fibrinolytic protease production from Bacillus subtilis I-2 using agro-residues. *Brazilian Archive of Biological Technology* 57 (5): 653–662 (2014).
23. S. Khankari, A. Badoei-dalfard, and Z. Karami. Cross-linked Enzyme Aggregates of Fibrinolytic Protease BC1 Immobilized on Magnetic Chitosan Nanoparticles (CLEAs-Fib-mChi): Synthesis, Purification, and Characterization. *Applied Biochemistry and Biotechnology* 193: 2004-2027 (2021).
24. A. Krishnamurth, and P.D. Belur. A novel fibrinolytic serine metalloprotease from the marine Serratia marcescens subsp. sakuensis: Purification and characterization. *International Journal of Biological Macromolecules* 112: 110–118 (2018).
25. S. Prasad, R.S. Kashyap, J.Y. Deopujari, H.J. Purohit, G.M. Taori, and H.F. Daginawal. Development of an in vitro model to study clot lysis activity of thrombolytic drugs. *Thrombosis Journal* 4: 1–4 (2006).