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Screening of Fibrinolytic Enzymes from Microorganisms Especially Actinomycetes from Different Biotopes in Manipur

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Abstract

Background: The present work was focused on screening fibrinolytic enzymes from actinomycete and bacterial strains obtained from different biotopes in Manipur.

Methods and Findings: The fibrinolytic enzyme activity of the isolates was screened on plasminogen-free fibrin plate. To determine the specific activity, the enzymes were partially purified through ammonium sulphate precipitation followed by dialysis. The stability of the enzymes at different pH and temperatures were also analyzed. A total of 33 isolates were screened for fibrinolytic activity, of which 12 showed positive results. Six strains (MBRL-575, NRB1-19, FS19, FS7, HA4 and SxL6), out of 12 fibrinolytic isolates, showed highly potent activity. MBRL 575 exhibited the highest enzyme activity of 33.66 U/ml, followed by NRB1-19, FS19, FS7, HA4 and SxL6 with specific activity of 33.48, 20.05, 19.38, 17.68 and 15.02 U/ml respectively. The enzymes were stable at pH ranging from 5-9 and temperatures from -20 to 55°C.

Conclusion: The fibrinolytic enzymes can withstand alkaline pH and cold temperature. They are stable at pH range of pH 5-9 and temperature ranging from -20 to 55°C.

Keywords: Actinomycetes; Fibrinolytic enzyme; pH stability; Temperature stability

Introduction

Intravascular thrombosis is a major precipitating factor for cardiovascular diseases, accounting for about 17.3 million deaths annually, representing 30% of total mortality rate globally [1-3]. Currently used fibrinolytic (thrombolytic) agents e.g. tissue-type plasminogen activator (t-PA), urokinase and streptokinase convert plasminogen to plasmin that degrades the fibrin clot but suffer from shortcomings such as low specificity and stability, allergic reactions, resistance to repercussion, haemorrhagic side effects, large therapeutic doses and high costs, though some fibrinolytic agents can be direct acting by mimicking plasmin [4]. This warrants the search for novel fibrinolytic enzymes from various sources with higher efficacy, safety, specificity and stability and preferably those direct acting activities. Though new fibrinolytic enzymes are being explored from microbes [5], animals [6], plants [7] and fermented foods [8], microorganisms remain the preferred source due to their biochemical versatility, feasibility of mass culture and ease of genetic manipulation.

Actinomycetes, in spite of being excellent producers of bioactive and structurally novel metabolites, only a few fibrinolytic *Streptomyces* species have been reported in the literature e.g. *Streptomyces* sp. NRC 411 [9], *Streptomyces megasporus* SD5 [10] and *Streptomyces* sp. CS684 [11]. Most studies so far have been focused on Bacillus species isolated from both food and non-food sources e.g. *Bacillus natto* from Japanese fermented soybean food, natto [12], *Bacillus* sp. KA38 from Korean fermented fish, jeot-gal [13] and *Bacillus amyloliquefaciens* DC-4 from Chinese's douchi [14]. Thus, the present work aims to screen and characterize fibrinolytic enzymes from microorganisms especially actinomycetes that are deposited in MBRL culture collection.

Materials and Methods

Strains and cultivation

The actinomycete strains for the present study were obtained from Microbial Biotechnology Research Laboratory (MBRL) culture collection, Manipur University, India and Lactic Acid Bacteria (LAB) strains from Microbial Biotechnology Laboratory, North Eastern Hill University, India. The cultures were revived on nutrient agar plate at 30°C for 7 days. A loopful of cultures were scraped from the plate and inoculated into seed medium [Glucose/Yeast extract/Peptone (GYP) broth] containing (% w/v): glucose, 1; yeast extract, 0.5; peptone, 0.5; NaCl, 0.5 and CaCl2, 0.02. They were grown for 5 days in shaker incubator (180 rpm) at 30°C. Cells were harvested by centrifugation at 10,000 rpm for 15 min at 4°C. The culture supernatants were filtered through sterile

Whatman filter paper no.1 and the cell free filtrates were assayed for enzyme activity.

Screening of fibrinolytic activity

Screening of the enzyme activity was done by plasminogen-free fibrin plate assay [15]. The plate was made up of fibrinogen solution (2.5 ml of 1.2% fibrinogen) in 0.1 M sodium phosphate buffer, pH 7.4), 10 U thrombin and 1% agarose. 100 µl of the cell free filtrates was carefully placed on the plates and the zones of clearance were checked after incubation at 37°C for 18 hrs.

Fibrinolytic enzyme extraction

The actinomycete and LAB isolates were grown on GYP broth for 5 day at 30°C on a shaker incubator. Cells were harvested by centrifugation at 10,000 rpm for 15 min at 4°C. The cell free supernatants were subjected to ammonium sulphate (20-80% w/v) precipitation with constant overnight stirring at 4°C. The precipitate formed was centrifuged at 10,000 rpm for 30 min and the precipitate obtained was dissolved in 50 mM Tris-HCl buffer (pH 7.2). The dark brown solution thus obtained was dialyzed at 4°C for 24-48 hr against 10 mM Tris-HCl buffer, pH 7.2 [16]. The dialyzed crude was stored at 4°C for further analysis of fibrinolytic enzyme activity.

Assay of fibrinolytic activity

Fibrinolytic activity was estimated according to protocols followed by Raju and Divakar [17]. Briefly, 1.4 ml of 50 mM Tris–HCl (pH 8.0) and 0.4 ml of 0.72% (w/v) fibrinogen solution were mixed and incubated at 37°C for 5 min. Then 0.1 ml (20 U/ ml) of thrombin was added and the mixture was kept at 37°C for 10 min. Enzyme sample (0.1 ml) was added and incubation was done for an additional 60 min. The reaction was stopped by addition of 0.2 M trichloroacetic acid. After centrifugation (10,000 rpm for 5 min), the absorbance of the supernatant (Ar) and blank (Ac) was measured at 275 nm and recorded. One unit of fibrinolytic activity (FU) is defined as the amount of enzyme required to produce an increase in absorbance equal to 0.01 in 60 min at 275 nm. Fibrinolytic unit (FU) = [(Ar-Ac) × dilution ratio of sample] / [0.001 × 60 × 0.1].

Effect of temperature on enzyme activity and stability

The effect of temperature on enzyme activity was determined by incubating the enzyme in 50 mM sodium phosphate buffer, pH 7.0 for 5 hrs at various temperatures (-20 to 100°C). The thermal stability of each enzyme solution was determined by measuring the residual activities after incubating the enzyme for 60 min at 37°C [18].

Effect of pH on enzyme stability

The effect of pH on enzyme activity was determined by incubating the enzyme (at ratio of 1:1) at different pH ranging from 3.0–11.0 at 37°C for 5 hrs [19]. The buffer systems used were 0.2 M citrate buffer (pH 3.0 and 5.0), 0.2 M Tris-HCl buffer (pH 7.0) and 0.2 M glycine NaOH buffer (pH 9.0 and 11.0). pH stability was determined by measuring the residual activities after incubating the enzyme for 60 min at 37°C.

Results

Screening of fibrinolytic enzyme

A total of 33 strains, obtained from different habitats of North-East India, were screened for fibrinolytic activity of which 12 isolates showed positive results (**Table 1** and **Figure 1**). Of the 12 fibrinolytic isolates, 5 strains (NRB1-19, MBRL-575 NRSI-19, SxF2, SxL6 and FS19) showed very potent activity with zone of clearance above 21 mm. SxF2 and SxL6 are endophytic actinomycetes, and FS19, NRBI-19 and MBRL-575 are LAB, actinomycete and Bacillus sp. respectively.

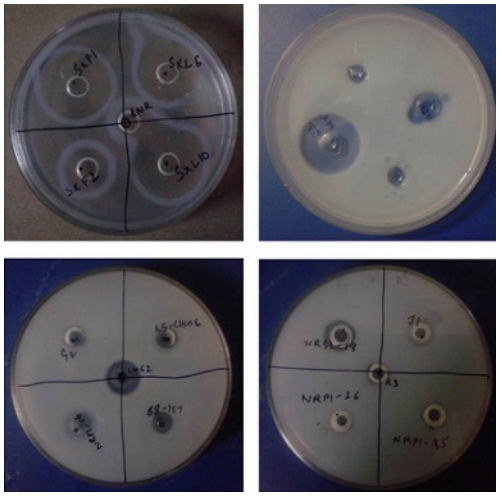

Figure 1: Plasminogen-free fibrin plate assay for screening fibrinolytic activity, Zone of clearance indicates hydrolysis of fibrin clots.

Extraction and assay of fibrinolytic activity

The fibrinolytic enzymes were extracted by ammonium sulphate precipitation followed by dialysis. It was found that, for most of the isolates, the precipitation occurred at 50% (w/v) saturation except for SxF1, SxF2, SxL6 and SxL10 (endophytic actinomycetes) for which precipitation was achieved at 80% (w/v) saturation. The obtained dialyzed crude enzymes were used for the assay of fibrinolytic activities. MBRL-575 and NRBI-19 strains showed the highest activity with 33.66 U/ml and 33.48 U/ml respectively (**Figure 2**). FS7 and FS19 also showed high activity with 19.38 U/ml and 20.05 U/ml respectively. SxF1 which had very high activity on plate showed less activity with 8.1 U/ml when assayed.

Effect of temperature

The activity and stability of the crude enzymes were determined at various temperatures between -20 and 100°C at pH 7 for 5 hrs. Most of the enzymes were active at

temperature range of -20 to 55°C (**Table 2** and **Figure 3**). The maximum activities were observed at 27 and 40°C, however as the temperature increases towards 55°C their activities gradually decreased and was completely lost at 100°C.

Table 1 Fibrinolytic activity profile.

Sl. No.	Isolates	Source	Fibrinolytic enzyme activity
1.	NRP1-14	Nambul river	-
2.	NRP1-26	Nambul river	-
3.	NRP1-35	Nambul river	-
4.	NRB1-19	Nambul river	+ ++
5.	NRB1-44	Nambul river	-
6.	NRS1-18	Nambul river	+
7.	NRS1-116	Nambul river	-
8.	LS1-81	Loktak lake	-
9.	LS1-88	Loktak lake	-
10.	LS1-128	Loktak lake	-
11.	LS1-145	Loktak lake	-
12.	LSCH-10C	Loktak lake	-
13.	LWG-2	Agricultural soil	+
14.	HA2	Hundung	-
15.	HA4	Hundung	+
16.	MBRL575	Hundung limestone deposits	+++
17.	SJ-1	Shirui jungle	-
18.	SJ-2	Shirui jungle	-
19.	SJ-3	Shirui jungle	-
20.	SL2	Shirui hills	-
21.	SL3	Shirui hills	-
22.	SK1-1	Salt springs	-
23.	SK1-3	Salt springs	-
24.	BLU-3	Bamboo leaves	-
25.	BLU-5	Bamboo leaves	-
26.	SxF1	*Solanum xanthocarpum*	+++
27.	SxF2	*Solanum xanthocarpum*	+
28.	SxL6	*Solanum xanthocarpum*	++
29.	SxL10	*Solanum xanthocarpum*	+++
30.	ANR	*Artemesia nilagarica*	-
31.	FS1	Fermented soybean food	++
32.	FS7	Fermented soybean food	++
33.	FS19	Fermented soybean food	+++

- : no activity; + : activity with zone size between 10-15 mm; ++ : activity with zone size between 16-20 mm; +++ : activity with zone size 21 mm and above

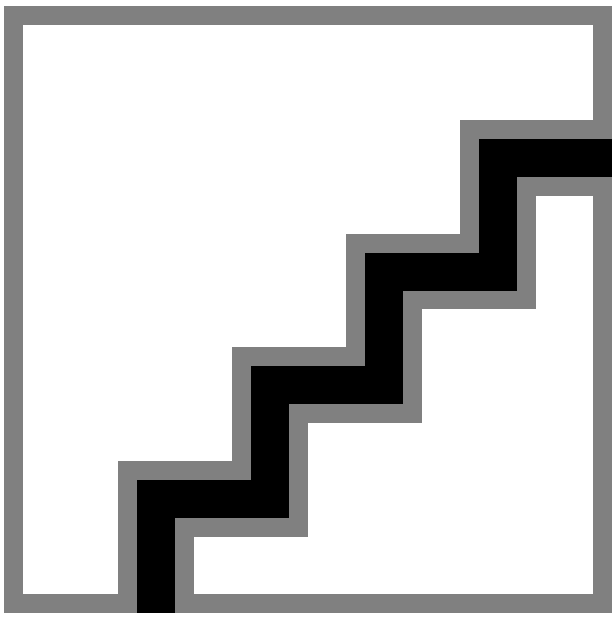

Figure 2 Fibrinolytic activities of different isolates at 37°C.

Figure 3 Effect of temperature on the fibrinolytic enzyme activity.

Effect of pH

To study the effect of pH on fibrinolytic activity and enzyme stability, assays were conducted at different pH ranging from 3.0-11.0 at 37°C for 5 hrs. The effects of pH on fibrinolytic activity and enzyme stability are shown in **Figure 4** and **Table 2**. The enzymes were highly stable at pH 7 to 9. At pH 3 and 5 the activities were very less. However, the activity increased as the pH rises from 7 to 9, and then decreased at pH 11. The optimum pH of the enzyme was 7.0 except for MBRL-575 which was 9.

Discussion

In the past few decades, many fibrinolytic enzymes have been reported from various microbes including actinomycetes eg. *Streptomyces* sp. NRC 411 [9], *Streptomyces* sp. [20], *Actinomycetes* [21,22], *Bacillus natto* [12], *Staphylococcus aureus* [23], *Bacillus* sp. CK [24], *Bacillus* sp. KA38 [13] and *Bacillus* sp. strain DJ-4 [1]. Fibrinolytic enzymes have also been reported from different, such as fermented foods [8,12], soils [22], animals [6] and marine algae [7]. Endophytic strain *Paenibacillus polymyxa* EJS-3 has also been reported to secrete

two extracellular fibrinolytic enzymes (118 and 49 kDa) in culture broth [25]. In the present study 1 LAB, 2 *Bacillus* sps. and 3 actinomycete strains were found to be potent producers of fibrinolytic enzymes. The specific activity of these isolates ranged from 15.02 to 33.66 U/ml. Simkhada et al. [11] reported a novel fibrinolytic protease from *Streptomyces* sp. CS684 that had a specific activity of 19 U/mg. Ju et al. [22] purified a fibrinolytic enzyme from *Streptomyces* sp. XZNUM 00004 that has a specific activity of 530.0 IU/mg. Hyeon-Deok et al. [26] and Chang et al. [27] reported fibrinolytic enzyme from *Bacillus subtilis* and *Bacillus amyloliquefaciens* MJ5-41 that had a specific activity of 21.6 U/mg and 3.44 U/mg respectively.

Table 2 Biochemical profile of fibrinolytic enzymes obtained from different sources.

Isolates	Organism	Source	Optimal		Stability		Specific activity (U/ml)
			Temperature (°C)	pH	Temperature (°C)	pH	
MBRL-575	Bacillus sp.	Hundung limestone deposits	40	9	-20 - 55	7 - 9	33.66
NRB1-19	Actinomycetes	Nambul river	40	7	-20 - 55	7 - 9	33.48
FS19	Bacillus sp.	Fermented soybean food	27	7	-20 - 40	7 - 9	20.05
FS7	Vagococcus sp.	Fermented soybean food	27	7	-20 - 40	7 - 9	19.38
HA4	Actinomycetes	Hundung limestone deposits	27	7	10 - 55	7 - 9	17.68
SxL6	Actinomycetes	*Solanum xanthocarpum*	27	7	-20 - 40	5 - 9	15.02
SxF2	Actinomycetes	*Solanum xanthocarpum*	27	7	-20 - 40	7 - 9	12.1
FS1	Lactococcus sp.	Fermented soybean food	27	7	-20 - 40	5 - 9	11.3
SxF1	Actinomycetes	*Solanum xanthocarpum*	27	7	10 - 40	5 - 9	8.1
NRSI-18	Actinomycetes	Nambul river	40	7	-20 - 40	5 - 9	4.93

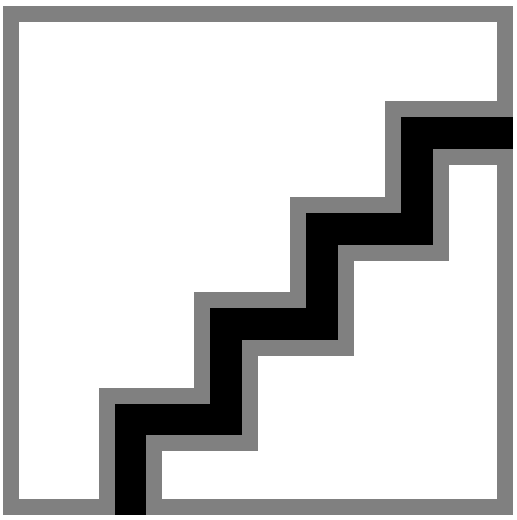

Figure 4 Effect of pH on the fibrinolytic enzyme activity.

The temperature stability profile of the enzymes ranged from -20 to 55°C. It was found that enzymes of MBRL-575, FS7, FS19 and SxL6 could retain more than 50% activity at 40°C even after 5 hrs of incubation. NRB1-19, however, could retain only 33% of its initial activity. At temperature 55°C the enzymes retained only about 23% activity. Mahajan et al. [5] reported that the purified enzyme of *Bacillus subtilis* ICTF-1, after 1 hr of incubation at 40°C and 50°C, retained 50% and 18% of its initial activity respectively. Agrebi et al. [28] reported a fibrinolytic enzyme from *Bacillus amyloliquefaciens* which had relatively higher thermal stability. This enzyme retained 62% of its initial activity at 50°C after 1 hr incubation but was completely inactivated after 20 min at 60°C and 2 min at 70°C. Chitte and Dey [10] extracted a fibrinolytic enzyme from a thermophilic Streptomyces megasporus strain SD5 that had more than 66% activity at 37°C.

The enzyme stability at different pH for the present study ranged from pH 5-9. The optimal pH was found to be 7.0 except for MBRL-575 which was 9. It was found that MBRL-575, which had the highest activity, could retain 50% of its initial activity at pH 9 after incubation for 5 hrs. NRB1-19, on the other hand, could retain only 21% of its initial activity. Many of the fibrinolytic enzymes reported are found to be stable at pH ranging from 4 -12, for instance Hassanein et al. [29] reported a fibrinolytic enzyme from *Bacillus subtilis* K42 that has maximum activity at pH 9.4 (stable at pH 6.5-10.5). Mander et al. [30] reported a fibrinolytic enzyme from *Streptomyces* sp. CS624 whose optimum pH was 7. This enzyme retained <90% activity at pH 6.5 and 7.5 after incubation at 4°C for 24 hrs. Mahajan et al. [5] reported an enzyme from *Bacillus subtilis* ICTF-1 that was stable at pH range of 7.0-11.0 but exhibited maximum activity at pH 9.0. The enzyme was also stable at pH range of 5.0-11.0 after incubation at 30°C for 60 min. Ko et al. [31] and Wang et al.

[32] also reported fibrinolytic enzyme from *Bacillus subtilis* that has optimal activity at pH 8.

From the present study, it can be concluded that the fibrinolytic enzymes, especially MBRL-575, can withstand alkaline pH and cold temperature, and can be preserved at -20°C. The enzymes are stable at pH range of pH 5-9 and temperature ranging from -20 to 55°C.

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Conflicting Interests

The authors declare that they have no conflicting interests.

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