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Research Article

SOIL BACTERIAL ISOLATES SHOWING PROMISING FIBRINOLYTIC ACTIVITY

Suneeta Gireesh Panicker*[1], Avinash Lendave[2], Akshay Sabale[2]
[1]Faculty of Microbiology, Dr. D. Y. Patil Arts, Commerce and Science College, Pimpri, Pune, India
[2]Dr. D. Y. Patil Arts, Commerce and Science College, Pimpri, Pune, India
*Corresponding author: suneeta.panicker@dypvp.edu.in
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ABSTRACT
Directly acting fibrinolytic enzyme is found to be superior therapy for treatment of cardiovascular diseases. Hence, there is a need of a novel and highly potent fibrinolytic agent which can directly degrade fibrin clot in lesser time with improved specificity and overcome shortcomings of haemorrhage. Amongst various sources, microbial fibrinolytic enzymes have attracted attention of research community so the present study aimed to isolate fibrinolytic enzyme-producing bacteria from different soil samples. Caseinolytic activity and clot lyses activity of the crude enzyme produced by these isolates were determined. All together six caseinolytic organisms were isolated, 3 of them produced fibrinolytic protease which would lyse fibrin and whole blood clot. Caseinolytic activity in the crude enzyme preparation from Colony C_4 was detected to be 78.2 U/ml and was able to lyse 85.54% of whole blood clot in 90 mins. Colony 4 may be of potential interest for biotechnological use as it produces fibrinolytic proteases that degraded fibrin clot and the whole blood clot, to the maximum.

Keywords: Cardiovascular Diseases, Fibrinolytic protease, Caseinolytic activity, Fibrin clot, Whole blood clot.

1. INTRODUCTION
According to a report published by the World Health Organization (WHO) in 2016, 18 million people die every year of cardiovascular diseases (CVDs) and representing the 31% of all global death. The formation of a blood clot in a blood vessel (intravascular thrombosis) is one of the main causes of CVDs and thrombolytic therapy has become a conventional treatment for this. Clinically prescribed thrombolytic drugs demonstrate issues such as delayed action and other side effects [1] like bleeding, re occlusion etc. Various fibrinolytic enzymes, such as tissue plasminogen activator (t-PA), urokinase and streptokinase have been extensively used as thrombolytic agents [2]. Fibrinolytic enzymes from the microorganisms are known to be used in the treatment and prevention of CVDs. They are involved in different medicinal applications like anticoagulants, thrombolytics, anti-inflammatory, oncolyticus, etc. Fibrinolyticprotease can be used to dissolve the blood clot directly.

Bacteria grow faster and can utilize crude raw material as nutrients. They are also easily amenable to genetic manipulations and hence microbial fibrinolytic enzymes have attracted much more medical interest in recent decades. Most of the studies on fibrinolytic proteases involved purification and characterization of enzymes. Potential fibrinolytic enzymes with significant thrombolytic applications have been isolated and purified from different sources like snake venom, earthworms and fermented foods [3]. With a very well-known fact that bacteria are physiologically and biochemically diverse, the scope of finding better fibrinolytic proteases for potential therapeutic applications gets enhanced. Moreover, it is economically favourable to use microbes for bulk enzyme production.

In this study, naturally occurring microflora showing fibrinolytic activity, were screened from soil of blood outlet of various slaughter house (3 samples) and food garbage dumping area (1sample). Based on the extent of fibrinolytic activity in the fibrin clot and whole blood clot, 3 colonies were selected. Results of this study demonstrated a high possibility of developing an alternative thrombolytic agent.

2. MATERIAL AND METHODS

2.1. Isolation and Screening of Fibrinolytic Bacteria [4]

Soil samples from area surrounding the blood outlet of slaughter house in Chikhali, Kondhwa and Nigdi was collected. Soil sample from food garbage dumping area, Moshi was also collected and used for the isolation of bacteria producing fibrinolytic protease. Serial dilutions of the collected soil sample were carried out up to 10^{-7} and last three dilutions were spread on casein agar plate. The plates were incubated at 37°C for 18-20 hours. After incubation, the plates were observed for the zone of hydrolysis. Bacterial isolates, which exhibited caseinolytic activity, were then spot inoculated on casein agar plate for further confirmation and the isolates showing maximum zone of hydrolysis were subjected to enzymatic assays.

2.2. Production of the fibrinolytic protease enzyme

Fibrinolytic enzyme producing organisms were inoculated in to 100 ml sterile casein broth. The inoculated flasks were incubated at 37°C on a rotary shaker with 150 rpm for 48 hours. The broth containing enzyme was centrifuged at 12000 rpm for 10 minutes. The clear supernatant was treated as crude enzyme and used for the caseinolytic and fibrinolytic assay.

2.3. Caseinolytic assay

Caseinolytic assay was performed using casein as the substrate as described earlier [5] with slight modifications. 0.5ml of crude enzyme solution was mixed with 0.5ml of 0.5% casein dissolved in 0.1M sodium phosphate buffer and incubated for 20mins at37°C. After incubation, 3ml of Trichloroacetic acid (5%, w/v) was added to stop the reaction and allowed to stand at 37°C for 10 mins. This was then centrifuged at 4°C at 10,000 rpm for 15 mins. To the supernatant 5 ml of 0.44 M Na_2CO_3 and 1ml of two-fold diluted Folin Ciocalteau reagent was added. When the colour was developed after 30 min, it was read at 660 nm against a reagent blank prepared in the same manner. Tyrosine was used as the reference standard. The optical density of these solutions was measured in a Shimadzu (Japan) spectrophotometer. One unit of caseinolytic activity (CU) was defined as the amount of enzyme that released one µg of tyrosine per ml per min.

2.4. *In-vitro* whole blood clot lysis assay [6]

Empty sterile Eppendorf tubes were weighed and noted as their Initial weight. 500 µl of venous blood drawn from healthy volunteers was added to each of these pre-weighed sterile Eppendorf tubes and allowed them to stand by 37°C for 45 minutesto form the blood clots. After clot formation, serum was completely removed using a micropipette without disturbing the formed clot and then again each tube with the blood clot was weighed to determine the clot weight. 500 µl of crude enzyme was then added to each tube. The tubes were incubated 37°C for 90 minutes and clot lysis was observed. After incubation, lysis of clot resulted in fluid which was then removed and the tubes with the remaining blood clot were weighed again to determine the difference in the weight of the clot, before and after lysis. The obtained difference was expressed as percentage of clot lysis.

2.5. Fibrin clot lysis assay [7]

Fibrin clot was formed in the laboratory by using path lab reagent, uniplastin and sodium citrated plasma. Uniplastin is a ready to use highly sensitive, liquid Calcified Thromboplastin reagent, derived from rabbit brain with low opacity, procured from Tulip Diagnostics (P) LTD. 500µl of Uniplastin was taken in sterile test tube and 250µl of pre citrated plasma was added to it. This was incubated for 10 mins at 37°C. The clot was left undisturbed for 30 min at room temperature. 250µl of crude enzyme was added to the fibrin clot and incubated it at 37°C. To check the fibrinolytic activity of the enzymes, the clot size was examined after every three hours.

3. RESULTS AND DISCUSSION

3.1. Screening and Isolation of Fibrinolytic Bacteria

The soil samples spread on casein agar plates showed presence of bacterial isolates possessing proteolytic activity which was indicated by the zone of hydrolysis around the colonies (Fig. 1A). The caseinolytic activity of these bacterial isolates was confirmed by spot inoculation on casein agar. Different diameters of zone of casein hydrolysis were observed for different isolates as seen in Fig. 1B. Six isolates which were further inoculated in the casein broth showed confirmation of their extra cellular caseinolytic activity by well method and is shown in Fig.2. Zone of hydrolysis was observed for all the six isolates but colony 3 and 4 showed greater capability of casein hydrolysis.

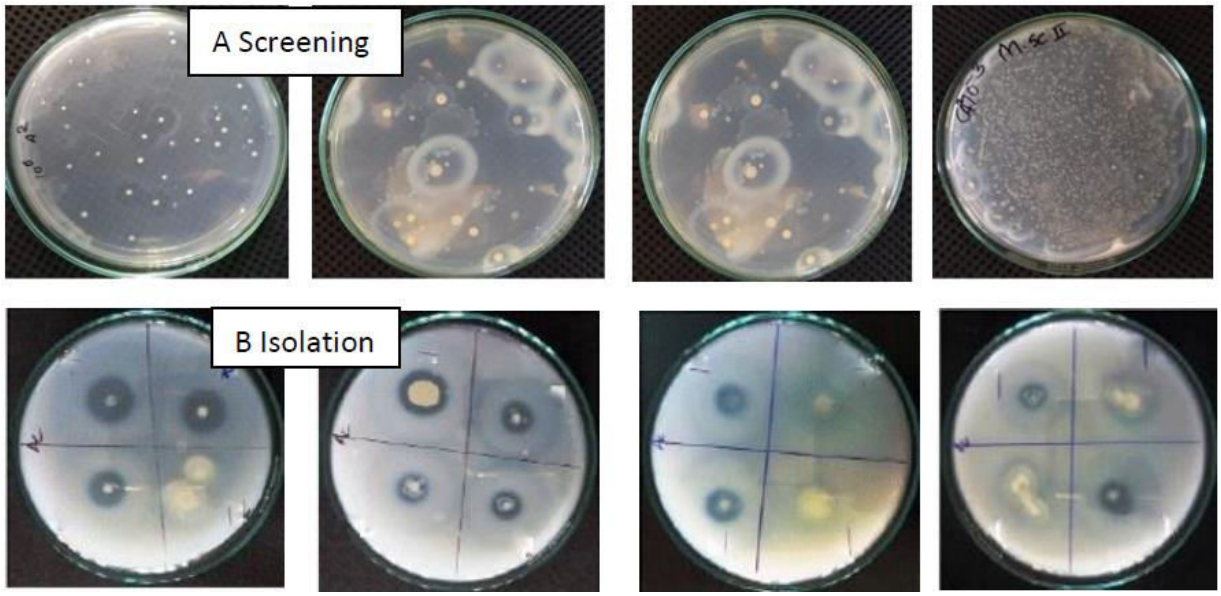


Fig. 1: Primary screening and isolation on casein agar plates: spot inoculated colonies showing zone of casein hydrolysis

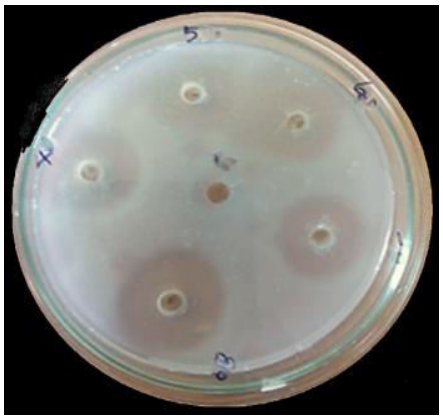


Fig. 2: Confirmation of caseinolytic activity by well diffusion method

3.2. Caseinolytic activity

Three selected colonies were tested for proteolytic activity. It was clear from the results seen in table 1, that isolate Colony No. 4 was the best protease producer among the three isolates.

Table 1: Caseinolytic activity [U/ml] of Colony No. 1, 3, 4 in casein broth

	Colony No.1	Colony No.3	Colony No.4
Protease activity expressed in U/ml	52.3	64.7	78.2

3.3. *In-vitro* whole blood clot lysis assay

Blood clot lysis activity was observed for the crude enzyme. 85.54% clot lysis was visually observed after 90 minutes in the Colony No. 4 tube. Similarly, colony 1 and 3 showed 84.49 and 82.58 percent clot lysis as seen in Fig. 3 and the observations are noted in the table 2.

3.4. Fibrinolytic activity

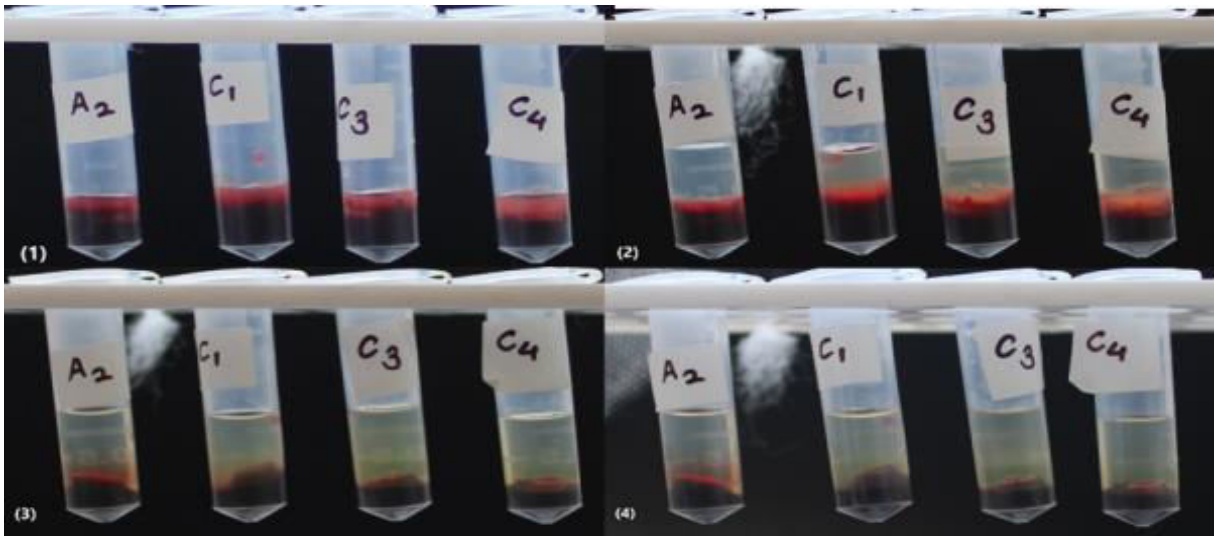
Fibrinolytic activity was determined by the in vitro fibrin lysis method. The crude enzyme preparation from colony no. 4 showed maximum hydrolysis of the fibrin clot, which was visually observed after three hours of incubation at 37°C (Fig. 4). Maximum clot was liquefied after 9 hours of incubation.

The results of the present study are supported by existing reports on enhanced production of various microbial fibrinolytic enzymes which significantly demonstrated better activity compared to existing thrombolytic agents. The enzyme activity was determined as the extent of fibrin clot lyses and one unit of enzyme activity was defined as the minimum amount of enzyme at which blood clot lyse occurred completely [8, 9]. Thereby, it was concluded from the results that after 90 min the enzymes were most effective to lyse the clot. Many reports presented microbial enzymes including the fibrinolytic alkaline protease of *Fusarium* sp. BLB (27 kDa), the metalloprotease of *C. militaris*, the serine protease of *Bacillus amyloliquefaciens* DC-4 (28

kDa) and the metalloprotease of *Armillariamellea* (21 kDa) [3,10,11]. A highly potent fibrinolytic serine protease from *Streptomyces omiyaensis* isolate, which had about 18-fold higher activity than that of plasmin was reported in a study [12]. The purification and characterization of these enzymes would open the possibility of production of a novel therapeutic agent.

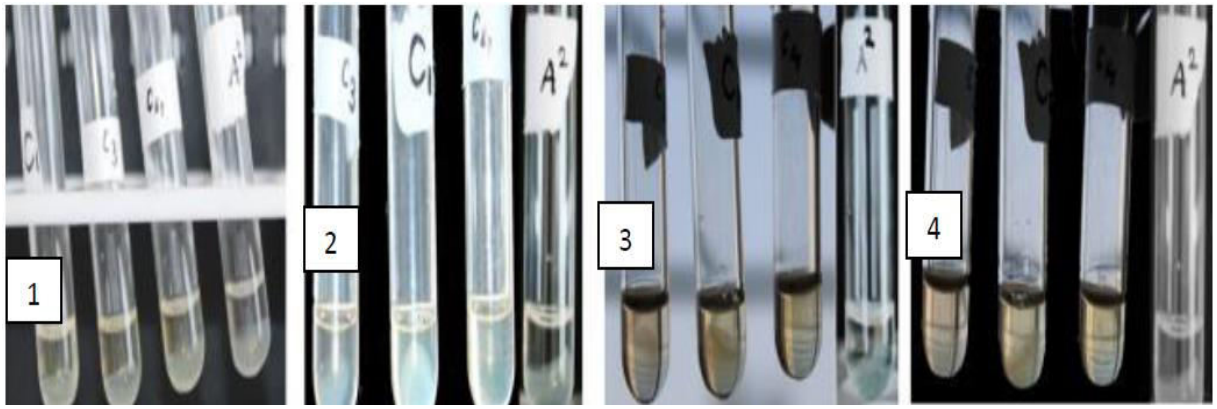
Table 2: Observations of whole blood clot assay

	CONTROL	Colony No.1	Colony No.3	Colony No.4
Initial weight of Eppendorf tubes [I_1]	1.176	1.147	1.195	1.16
Weight after addition of blood clots 500µl [I_o]	1.773	1.734	1.878	1.784
Weight after addition of 500µl enzyme and D/W in control [F_o]	2.284	2.212	2.293	2.303
Incubation for 30 mins at 37°C				
Weight after removal of enzyme [F_1]	1.581	1.48	1.588	1.47
Difference = [I_o- F_1]	0.192	0.254	0.29	0.314



A_2 is control with no enzyme. (1)whole blood clot, (2) whole blood clot + crude enzyme in C_1, C_3, $C_4$and D/w in A_2(3) Clot lysis after 30 minutes and (4) Clot lysis after 60 minutes.

Fig. 3: Whole Blood Clot Liquefaction by crude Enzyme from Colony No. 1, 3 and 4



(1) initial reading just after adding of partially filtered supernatant containing enzyme (2) observation after 3hrs, (3) observation after 6hrs and (4) observation after 9hrs

Fig. 4: Fibrin Clot Liquefaction by crude Enzyme from Colony No. 1, 3 and 4

4. CONCLUSION

The present work describes the production of fibrinolytic proteases from soil isolates. In conclusion, the colony 4 may be of potential interest for biotechnological use as it produces proteases that degraded fibrin clot and the whole blood clot, to the maximum. Further works should be done to purify and characterize the proteases.

5. ACKNOWLEDGEMENT

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Conflict of Interest

The authors declare that they have no conflict of interest.

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