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Exploration of Newer Bacterial Strain for Fibrinolytic Enzyme Production from Soil Waste

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A B S T R A C T

Fibrinolytic enzymes help in dissolving the fibrin clots and they have the potential use in the treatment of harmful cardiovascular diseases. High efficient fibrinolytic enzymes are produced from the fibrinolytic microbes under extreme environmental conditions. In the present work, microorganism strain that helps in the production of fibrinolytic enzymes were isolated and screened from the soil waste. Five isolates i.e., SWN-15, SWN -25, SWN -35, SWN -45 and SWN -55 were subjected to macroscopic characteristics The isolated organism was cultured in the sterile media at 37ºC for 24 hrs. The selected isolates were subjected to caseinolytic, gelatinolytic and fibrinolytic activities was done. The fibrinolytic activity of isolates SWN 25 and SWN 55 was higher than that of caseinolytic and gelatinolytic activities. The selected isolates were tested with modified media composition and at different pH values. Isolate SWN-55 showed good enzyme productivity (13.1 $\pm$ 2.6) U/ml at pH 8.0. Also the biochemical study indicated the isolate SWN 55 is similar to that of *Streptomyces sp. with* fibrinolytic function.

Introduction

Fibrinolytic protease is well known as a sub class of protease, it has an ability to degrade fibrin (Fujita *et al.*, 1993, 1995; Jeong *et al.*, 2001, 2004; Leonardi *et al.*, 2002; Sumi *et al.*, 1995; Wong *et al.*, 2004). Fibrin is normally formed from Fibrinogen by the action of Thrombin (EC 3.4.21.5). It is sliced by plasmin (EC 3.4.21.7) which is activated from plasminogen by tissue plasminogen activator (Voet *et al.*, 1990). In balanced condition, fibrin clots are hydrolysed by plasmin to avoid thrombosis in blood vessels. However, in unbalanced condition, as a result of pathophysiological disorders, the clots are not hydrolysed. Deposition of fibrin in blood vessels normally increases thrombosis and other cardiovascular diseases such as high blood pressure, acute myocardial infarction, ischemic heart disease, valvular heart disease, peripheral vascular disease, arrhythmias, stroke etc. (Bode *et al.*, 1996; Yoshinori *et al.*, 2005).

Fibrinolytic agents are used to treat a wide variety of venous and arterial thromboembolic disorders; especially against acute myocardial infarction (Torrens *et al.*, 1999). Based on working mechanism, thrombolytic agents are of two types, one is plasminogen activator which activates plasminogen into active plasmin to degrade fibrin and the other is plasmin like proteins which directly degrade fibrin (Koide *et al.*, 1982). Despite widespread uses, thrombolytic agents are expensive. They exhibit low fibrin specificity and have undesired side effects such as gastrointestinal bleeding, resistance to repercussion and allergic reactions (Pautov *et al.*, 1990). Therefore, continuous efforts have been focused in the search of safer and less expensive thrombolytic agents from diverse sources but one must respect biodiversity. Investigation of extracellular fibrinolytic enzymes is a essential due to their wide applications in clinical, pharmaceutical, food, and bioremediation process.

Materials and Methods

Chemicals

All chemicals and reagents used were of analytical grade obtained from Sigma USA.

Determination of fibrinolytic protease activity

The fibrinolytic protease activity was based on the hydrolytic power of the enzymes was determined by using the fibrin as substrate. Fibrinolytic protease activity was evaluated by the modified procedure of Greenberg (1957) using 2% bovine fibrin in 0.05 mM phosphate buffer (pH 8). 0.5ml fibrin solution with an equal volume of diluted enzyme solution was incubated for 10min at 37^0C. The reaction was stopped by the addition of 10% cold Tri Chloroacetic Acid (TCA). The mixture was centrifuged at 3,000rpm for l0 min and to the supernatant 5ml of 0.44M Na_2CO_3 was added, followed by the addition of 1ml of diluted Folin - ciocalteau reagent. The mixture was incubated for 30min at 37^0C, the sample develops color change, and the intensity of colour change will be measured at 660nm using Shimadzu UV-1700 (Japan) spectrophotometer against a blank reagent prepared without adding enzyme. Tyrosine is used as the reference standard.

Estimation of extracellular protein

Extracellular protein was estimated by Lowry method (Lowry *et al.*, 1951). To the 1ml of dilute protein sample (10-60 μg/ml), 5ml alkaline working solution was added and mixed well which was incubated at 37°C for 10min. To the above mixture, 0.5ml Folin Ciocalteau reagent was added, mixed well and incubated at 37°C for 30min. The absorbence was measured at 680nm in a spectrophotometer. The amount of protein present in the sample was calculated from the standard curve.

Screening and isolation of fibrinolytic protease producing bacteria

To isolate the potent fibrinolytic protease producing bactreria, soil samples were collected from various places in and around Bangalore, Karnataka, India. The soil samples were selected based on the varying characterisitics such as organic matter, particle size, colour of soil and geographical distribution. The samples were stored in sterile condition.

1g of the above samples was separately taken in a conical flask containing 100ml sterile water and the suspension was subjected to rotary shaker for 30 minutes. 1ml of the supernatant was serially diluted with sterile water and one ml of each of these dilutions

was added to 20ml of sterile molten starch casein-agar medium maintained at 40°C. It was mixed thoroughly and plated in 10cm diameter sterile petridishes and incubated at 37°C. 75µg/ml of Flucanazole was incorporated to control the fungal contamination. After 24h of incubation, the bacteria colonies with clear hydrolyzed zones were transferred onto starch casein agar slants.

Screening of isolates for proteolytic activity

Primary screening

The selected isolates were initially screened for their proteolytic activities i.e. caseinolytic, gelatinolytic and fibrinolytic activities.

Caseinolytic activity

Caseinolytic activity of the isolates was evaluated using casein-agar plate technique (Zerdani *et al.,* 2004; Li *et al.,* 2009; Gholamhossein *et al.,* 2013). To the sterilized agar, 10% of pasteurized skimmed milk was added aseptically and the media was transferred into sterile petridish and kept aside for solidification. Then a loopful of each culture was streaked onto the medium, incubated at 37°C for 24h. The diameters of hydrolyzed zones around the colonies and the growth zones were measured. The ratio of hydrolysis zone/growth zone was calculated which gives a measure of the caseinolytic activities of the isolates.

Gelatinolytic activity

20ml of sterile nutrient gelatin agar medium (McLaughlin *et al.,* 1996; Deshmukh *et al.,* 1997; Tran *et al.,* 2002; Shanmugasundaram *et al.,* 2012) was poured in sterile petridishes and inoculated with a loop full of spores from 24h old cultures and incubated at 37°C for 24h. The plates were flooded with mercuric chloride reagent (Williams *et al.,* 1971). After treating with mercuric chloride-HCl solution, the hydrolysis zone and growth zones were noted.

Fibrinolytic protease activity

To check the fibrinolytic protease activity, modified fibrin plate agar (Astrup *et al.,* 1952; Kumaran *et al.,* 2011) was poured in sterile petridishes and inoculated with a loop full of spores from 24h old cultures and incubated at 37°C for 24h. The diameters of hydrolyzed zones around the colonies and the growth zones were measured. The ratio of hydrolysis zone/growth zone was calculated which gives a measure of the fibrinolytic protease activities of the isolates.

Design of suitable basal medium

Fibrinolytic protease production in shake flasks was checked using different media composition (table.2). 1ml of the cell suspension from the isolates was aseptically transferred into 250ml Erlenmeyer flasks containing 50ml each of sterile medium. The contents were incubated at 37°C for 24h using rotary shaker at 70rpm. It is followed by centrifugation at 3000rpm for 10 min and the supernatant solution was tested for fibrinolytic protease activity by modified method of Greenberg 1957.

Determination of type of fibrinolytic protease produced by the isolates

To determine the whether the enzyme secreted by the isolate is of alkaline or acidic or neutral protease, fibrinolytic protease activity in the harvested broth was assayed by adding 0.5ml culture broth to 0.5ml of 2% fibrin solution. Further the activity was checked at different pH conditions using Citrate buffer (pH 4.0), Phosphate buffer (pH 7.0) and Carbonate buffer (pH 8.0, 10.0) by dissolving the fibrin in respective buffers. (Greenberg 1957)

Results and Discussion

From the ten soil samples collected 116 isolates were formed. Of the colonies formed, five isolates (Nos. 15, 25, 35, 45 and 55) were selected based on their macroscopic characteristics. They were sub cultured on starch casein agar media and subjected for secondary screening. They were designated as SWN-15, SWN -25, SWN -35, SWN -45 and SWN -55. Extent of growth and the proteolytic activities of the selected isolates were checked and the results were presented in Table.1. Fibrinolytic protease production was checked for the five isolates in shake flasks using different media composition (table.2). The results are presented in Table.3.

Study clearly indicated that isolates SWN 25 and SWN 55 are high fibrinolytic protease producers and they were further tested with medium IV and V for better fibrinolytic protease production.

Table.1

Sample	Source	Phsyical Characteristics
TMK -I	Chicken centre, Shivaji Nagar, Bangalore.	brown colour sticky semi solid soil sample
TMK -II	chicken centre, Tannery Road, Bangalore	black colour sticky semi solid soil sample
TMK -III	slaughterhouse, nearby Tannery Road, Bangalore	brown colour sandy semi solid soil sample
TMK -IV	dumping yard of chicken centre near Tannery Road, Bangalore	brown colour hard soil sample containing the mixture of sand and clay
TMK -V	slaughterhouse, nearby Soldevanahalli, Bangalore	brown colour semi solid sample
TMK -VI	chicken centre, Chikkabanavara, Bangalore	brick red sticky soil sample
TMK -VII	dumping yard of chicken centre near Devasandra lake, Bangalore	red colour semi solid consisting of sand and clay sample
TMK -VIII	slaughterhouse, nearby Tin Factory, Bangalore	red colour semi solid clay sample
TMK -IX	dumping yard of chicken centre near Tin Factory, Bangalore	black colour solid consisting of sand and clay sample
TMK -X	dumping yard of fish market near Yashwantpura Railway Station, Bangalore	black colour sticky sample consisting of sand and clay

Table.1 Growth pattern and proteolytic activities of selected isolates *Secondary Screening*

Sample No.	Isolate No.	Extent of Growth (24h)			Proteolytic activity		
		CA	GA	FA	Caseinolytic activity	Gelatinolytic activity	Fibrinolytic protease activity
TMK –II	SWN 15	++	+	++	8.4	2.6	**3.9**
TMK –IV	SWN 25	++	++	++	7.8	5.6	**6.9**
TMK –V	SWN 35	++	+	++	5.3	1.9	**3.1**
	SWN 45	++	+	+	5.8	2.6	**2.3**
TMK –VI	SWN 55	++	-	++	6.8	0	**8.4**
CA = Casein Agar Medium GA = Gelatine Agar Medium FA = Fibrin Agar Medium Hydrolysed zone (mm) / growth zone (mm) = Ratio					++ = Good growth ++ = Moderate Growth - = No Growth		

Table.2 Composition of different media for fibrinolytic protease production

Medium No.	Composition (g/100ml)
I	Glucose, 3.0; soyabean meal, 2.0; $CaCl_2$, 0.04; $MgCl_2$, 0.2.
II	Glucose 1.5; yeast extract, 0.5; $CaCl_2$, 0.2.
III	Glucose, 0.1; yeast extract, 0.5; tryptone, 0.5.
IV	Fructose, 1.0; fibrin; 0.2; peptone 1.0; salt solution 5ml*.
V	Fructose, 0.5; fibrin; 0.2; peptone 1.0; salt solution 5ml*.
VI	Fructose, 1.0; fibrin, 0.2; salt solution, 5ml*.
VII	Soluble starch, 1; Casein, 0.3; KNO_3 0.2; K_2HPO_4, 0.2; $MgSO_4$, $7H_2O$; $CaCO_3$, 0.002; $FeSO_4$, $7H_2O2$, 0.001.

* Salt Solution Composition (%): $KN0_3$, 0.2; K_2HPO_4, 0.2; $MgSO_4$, $7H_2O$, 0.005; $CaCO_3$, 0.002; $FeSO_4$, $7H_2O$, 0.001.

Table.3 Production of fibrinolytic protease (U/ml) by selected isolates in shake flask*

Medium No.	Isolate SWN15	Isolate SWN 25	Isolate SWN 35	Isolate SWN 45	Isolate SWN 55
I	3.7	2.5	4.2	2.6	**4.0**
II	4.2	4.6	2.0	4.1	**2.6**
III	5.1	4.8	2.8	1.2	**4.2**
IV	1.2	7.6	1.8	1.3	**8.6**
V	3.8	5.7	5.0	3.5	**7.0**
VI	4.7	3.6	3.2	2.0	**3.5**
vII	**1.7**	**2.0**	**2.4**	**1.9**	**2.0**

* Fibrinolytic Protease activity expressed in U/ml.

Table.4 Production of fibrinolytic protease in modified media

Medium No.	Composition of media	Enzyme yield (U/ml)	
		SWN-25	SWN -55
I	Fructose, 1%, fibrin, 0.2%; peptone 1%; $MgSO_4$,0.02%, KH_2PO_4, 0.2%; NH_4NO_3, 0.5% and $CaCl_2$ 0.04%	9.4 ± 2.5	**13.1 ± 3.6**
II	Fructose, 0.5%; fibrin, 0.2%, peptone 1%; $MgSO_4$,0.02%; KH_2PO_4; 0.2%, NH_4NO_3; 0.5% and $CaCl_2$ 0.04%	8.4 ± 2.8	**9.8 ± 3.2**
III	Fructose 0.1%, Yeast extract 0.5%, Tryptone 0.5%	7.9 ± 2.5	**8.9 ± 3.2**
IV	**Fructose 1%, Yeast extract 0.5%, Tryptone 0.5%**	**8.0 ± 2.0**	**6.0 ± 2.0**

Table.5 Fibrinolytic protease activity at different pH values

Isolate	Fibrinolytic protease activity (U/ml)			
	pH 4.0	pH 7.0	pH 8.0	pH 10
SWN - 25	1.2 ± 2.2	8.7 ± 1.4	9.2 ± 2.2	**1.3 ± 2.4**
SWN -55	**4.8 ± 2.5**	**10.6 ± 1.8**	**13.1 ± 2.6**	**3.5 ± 2.0**

Design of suitable basal medium

The composition of the medium IV and V were slightly changed in their fructose concentration and used for the production of fibrinolytic protease by isolates SWN 25 and SWN 55 to compare and design a suitable basal medium for efficient production. The results are presented in Table.4.

Maximum yield was obtained in medium IV. The composition of medium IV is Fructose, 1.0; fibrin, 0.2; peptone 1; $MgSO_4$, 0.02%; KH_2PO_4, 0.2%; NH_4N0_3, 0.5% and $CaCl_2$ 0.04%. This was designated as the basal medium and used for further studies.

Determination of type of fibrinolytic protease produced by the isolates

Isolates SWN -25 and SWN -55 indicated high enzyme activity at pH 8, indicating the enzyme is an alkaline protease. Further the isolate SWN-55 showed comparatively good enzyme productivity (13.1 ± 2.6) U/ml.

Also, results of classification and identification clearly showed that the isolate SWN -55 is having similar characteristics as of *Streptomyces aureus*.

Several effective thrombolytic agents have been identified and characterized from microorganisms, earthworms, plants, snake venoms, insects and leeches. Fibrinolytic proteases have wide applications in clinical, pharmaceutical, food, and bioremediation process. Among the various fibrinolytic proteases, bacterial extracellular fibrinolytic proteases are more attractive than those isolated from earthworms, plants, snake venoms, insects and leeches extracellular fibrinolytic proteases because of their high stability and unusual substrate specificity.

Different natural substrates collected from various places of Bangalore were screened for the isolation of potent fibrinolytic protease producing *Streptomyces sp.* A total of 116 cultures were isolated from 10 samples. All the isolates were tested for their proteolytic activity on milk casein agar medium. After preliminary studies, isolates were further screened in two different media for their caseinolytic, gelatinolytic and fibrinolytic activities. Five promising isolates (SWN 15, SWN 25, SWN 35, SWN 45 and SWN 55), which showed good fibrinolytic activity were further screened for their enzymatic activities by shake flask method. Among these 5 isolates, isolate SWN 55 exhibited good fibrinolytic activity, while other showed poor to moderate activity. This was subjected to biochemical studies. A close study indicated that the isolate (SWN 55) was related to *Streptomyces sp.* but different in some biochemical characteristics. In view of some significant differences from the reference culture, the isolate was considered to be the novel strains of *Streptomyces aureus.*

Strain development for enzyme production has been an essential prerequisite for efficient production process. The main aim of strain improvement programme is to increase the product litres. The potential productivity of the organism is controlled by its genome and therefore the genome must be modified to increase the potential yield. The process of strain improvement involves genetic modification of the culture, followed by process optimization. Therefore, the strain of *Streptomyces aureus* was subjected to strain improvement program with a view to obtain increased fibrinolytic protease production.

In conclusion, the isolate SWN 55 from the soil waste indicated the effective fibrinolytic activity at pH 8, indicating the enzyme is an alkaline protease with comparatively good enzyme productivity (13.1 $\pm$ 2.6) U/ml. Biochemcial studies clearly showed that the isolate SWN -55 is having similar characteristics as of *Streptomyces aureus*. In view of some significant differences from the reference, the isolate was considered to be the novel strains of *Streptomyces aureus*.

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