

Screening, production and biochemical characterization of a new fibrinolytic enzyme produced by *Streptomyces* sp. (Streptomycetaceae) isolated from Amazonian lichens

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ABSTRACT

Thrombosis is a pathophysiological disorder caused by accumulation of fibrin in the blood. Fibrinolytic proteases with potent thrombolytic activity have been produced by diverse microbial sources. Considering the microbial biodiversity of the Amazon region, this study aimed at the screening, production and biochemical characterization of a fibrinolytic enzyme produced by *Streptomyces* sp. isolated from Amazonian lichens. The strain *Streptomyces* DPUA1576 showed the highest fibrinolytic activity, which was 283 mm². Three variables at two levels were used to assess their effects on the fibrinolytic production. The parameters studied were agitation (0.28 – 1.12 g), temperature (28 – 36 °C) and pH (6.0 – 8.0); all of them had significant effects on the fibrinolytic production. The maximum fibrinolytic activity (304 mm²) was observed at 1.12 g, 28 °C, and pH of 8.0. The crude extract of the fermentation broth was used to assess the biochemical properties of the enzyme. Protease and fibrinolytic activities were stable during 6 h, at a pH ranging from 6.8 to 8.4 and 5.8 to 9.2, respectively. Optimum temperature for protease activity ranged between 35 and 55 °C, while the highest fibrinolytic activity was observed at 45 °C. Proteolytic activity was inhibited by Cu²⁺ and Co²⁺ ions, phenylmethylsulfonyl fluoride (PMSF) and pepstatin A, which suggests that the enzyme is a serine protease. Enzymatic extract cleaved fibrinogen at the subunits A α -chain, A β -chain, and γ -chain. The results indicated that *Streptomyces* sp. DPUA 1576 produces enzymes with fibrinolytic and fibrinogenolytic activity, enzymes with an important application in the pharmaceutical industry.

KEYWORDS: Actinomycetes, fibrinolytic protease, inhibitor protease, fibrinogenolytic activity.

Seleção, produção e caracterização bioquímica de uma nova enzima fibrinolítica produzida por *Streptomyces* sp. (Streptomycetaceae) isolada de líquens da Amazônia

RESUMO

A trombose é uma doença patofisiológica causada pelo acúmulo de fibrina no sangue. Proteases fibrinolíticas com potente atividade trombolítica são produzidas por diversas fontes microbianas. Considerando a biodiversidade microbiana da região amazônica, o presente estudo teve como objetivo a seleção, produção e caracterização bioquímica da enzima fibrinolítica de *Streptomyces* sp. isolado de líquens da Amazônia. *Streptomyces* DPUA1576 foi a melhor produtora com atividade fibrinolítica de 283 mm². Três variáveis em dois níveis foram utilizadas para determinar as variáveis mais relevantes na produção da enzima fibrinolítica (FA). Os parâmetros estudados foram agitação (0.28 – 1.12 g), temperatura (28 – 36 °C) e pH (6.0 – 8.0) e todos obtiveram efeitos significativos na produção fibrinolítica. A maior atividade fibrinolítica (304 mm²) foi obtida a 1.12 g, 28 °C e pH 8.0. O extrato bruto da fermentação foi usado para determinar as propriedades bioquímicas da enzima. Atividades proteásica e fibrinolítica foram estáveis durante 6 horas no intervalo de pH entre 6.8 – 8.4 e 5.8 – 9.2, respectivamente. Temperatura ótima para a atividade proteásica foi entre 35 – 55 °C, enquanto que para a atividade fibrinolítica foi de 45 °C. Atividade proteásica foi inibida por íons Cu²⁺ e Co²⁺, fluoreto de fenilmetilsulfonil e pepstatina A, na qual sugere que a enzima é uma serino-protease. O extrato enzimático degradou o fibrinogênio nas subunidades A α , A β e γ . Os resultados apresentados indicam que *Streptomyces* sp. DPUA 1576 produz enzimas com atividade fibrinolítica e fibrinogenolítica, enzimas com aplicações importantes na indústria farmacêutica.

PALAVRAS-CHAVE: Actinomycetos, protease fibrinolítica, inibidor de protease, atividade fibrinogenolítica

INTRODUCTION

Thrombosis is one of the most widely occurring diseases in modern life. It occurs by the accumulation of fibrin when injury on tissue occurs. Normally, the fibrin formed in the blood is dissolved by the action of plasmin (E.C. 3.4.21.7). The unsolved clot by plasmin leads to the formation of thrombus, a serious problem because it induces cerebral and cardiovascular disease (Deepak *et al.* 2010). There are many drugs used on fibrinolytic therapy such as streptokinase (EC 3.2.1.35), staphylokinase (EC 3.4.21.35), urokinase (EC 3.4.21.31) but all of them have side effects. Thrombolytic therapy today is expensive and has undesirable side effects such as the risk for internal hemorrhage within the intestinal tract when orally administrated (Zhang *et al.* 2007).

Worldwide, over 29% of the total mortalities are due to thrombosis. By the year 2020, cardiovascular diseases (CVDs) may cause an estimated 25 million deaths per year, thus antithrombotic therapy is of great interest (Kotb 2014). Over the years, thrombolytic therapies via injecting or orally administrating thrombolytic agents to lyses thrombi in blood vessels have been extensively investigated (Peng *et al.* 2005). Therefore, continuous efforts have been focused in the search of safer and less expensive thrombolytic agents from diverse sources. Until recently, fibrinolytic enzyme with potential thrombolytic application has been purified from various sources such as fermented food, earthworms, mushrooms, snake venom and microbial sources (Kotb 2014). Microbial sources, such as bacteria, actinomycetes, fungi and algae are reported to produce fibrinolytic enzyme with few reports on the use of *Streptomyces* sp. (Chitte *et al.* 2011). *Streptomyces* sp. produces several extracellular enzymes of commercial interest, such as protease, pectinase, xylanase and cellulase. Proteases constitute two thirds of the total number of enzymes used in industry and this is expected to increase. Some of these proteases are fibrinolytic enzymes capable of digesting fibrin (Silva *et al.* 2015).

Considering the microbiological potential of the Amazon Region and the growing applicability of fibrinolytic proteases, the screening of *Streptomyces* sp. appears to be an interesting approach to finding novel fibrinolytic producers to reduce the drawbacks of marketed thrombolytic drugs. Therefore, this study aimed at the screening, production and biochemical characterization of a fibrinolytic enzyme produced by *Streptomyces* sp. isolated from Amazonian lichens.

MATERIALS AND METHODS

Screening of isolates with fibrinolytic activity

Fifty *Streptomyces* spp. isolates obtained from Amazonian lichens were used for fibrinolytic enzyme screening (Table 1). These *Streptomyces* isolates form part of the Culture Collection

of the Parasitology Department of the Federal University of Amazonas (DPUA/UFAM-Brazil).

The inoculum was produced following the International *Streptomyces* Project medium 2 (ISP-2) broth in orbital shaker (BR-300LF, TAITEC Co, Tokyo, Japan), agitation of 1.12 g for 48 h at 28°C. Enzyme production was carried out with initial cell concentration of 1×10^6 cells mL⁻¹ ($A_{600} = 0.1$) in 250-mL-Erlenmeyer flasks containing 50 mL of soybean flour medium (Porto *et al.* 1996), and incubated for 72 h at different conditions of agitation, temperature and pH as described in the Table 2. The samples were clarified by centrifugation (KR-20000T, Kubota, Osaka, Japan) at 8000 g and 4 °C for 10 min, and submitted to fibrinolytic activity determination.

Experimental design and statistical analysis

To assess the protease and fibrinolytic activity, a factorial arrangement with two levels and three variables (agitation, temperature and pH) was used to determine the most relevant combination. There were assessed agitation at 100, 150 and 200 rpm; these values corresponded to g forces of 0.28, 0.63 and 1.12, respectively. There were also evaluated temperature (ranged between 28 - 36°C) and pH, between 6 and 8 (Table 2). All statistical and graphical analyses were carried out with the *Statistica 8.0* software (StatSoft Inc., Tulsa, OK, USA).

Culture condition

The best fibrinolytic enzyme-producing *Streptomyces* sp. was cultivated in ISP-2 under the production conditions described in the experimental design (Table 2) during 144 h, in order to study cell growth, protease and fibrinolytic enzyme production. Cell concentration, pH and enzyme activity were analyzed every 12 h.

Effects of temperature and pH on protease and fibrinolytic activity

The optimal temperature was determined by relative activity after incubating 60 µL of crude extract and 100 µL of azocasein 1% (w/v) at different temperatures (25 – 75 °C) for 60 minutes. Thermal stability of crude extract was determined by measuring residual activity after incubating the enzyme for 120 min at the same range of temperatures, at pH 7.5. The effects of pH on protease activity were assayed with azocasein 1% (w/v) as substrate at various pH values (4.0 – 9.3) using standard buffers at 100 mM: citric acid/sodium citrate (4.0 – 5.2); citric acid/sodium phosphate (5.6 – 6.4), sodium phosphate (6.8 – 7.2) Tris-HCl (7.6 – 9.4). To determine the pH stability of the enzyme, aliquots of enzyme samples were incubated with their respective buffers (mentioned above) for 120 min at 25 °C. The percentage of residual activity was calculated by considering the initial enzyme activity as 100%. Each assay was carried out in triplicate. The effects of pH and temperature on fibrinolytic activity also were determined under the standard assay conditions described earlier.

Table 1. Screening of fibrinolytic activity obtained from different *Streptomyces* sp.

Sample	Halo (mm ²)	Sample	Halo (mm ²)
<i>Streptomyces</i> sp. DPUA 1541	113	<i>Streptomyces</i> sp. DPUA 1578	132.67
<i>Streptomyces</i> sp. DPUA 1543	50.24	<i>Streptomyces</i> sp. DPUA 1579	143.07
<i>Streptomyces</i> sp. DPUA 1545	201	<i>Streptomyces</i> sp. DPUA 1580	191.04
<i>Streptomyces</i> sp. DPUA1547	254	<i>Streptomyces</i> sp. DPUA 1581	153.86
<i>Streptomyces</i> sp. DPUA 1549	78.50	<i>Streptomyces</i> sp. DPUA 1582	132.67
<i>Streptomyces</i> sp. DPUA1550	254	<i>Streptomyces</i> sp. DPUA 1583	7.07
<i>Streptomyces</i> sp. DPUA 1551	28.26	<i>Streptomyces</i> sp. DPUA 1584	50.24
<i>Streptomyces</i> sp. DPUA 1553	78.50	<i>Streptomyces</i> sp. DPUA 1586	200.96
<i>Streptomyces</i> sp. DPUA 1554	201	<i>Streptomyces</i> sp. DPUA 1587	63.59
<i>Streptomyces</i> sp. DPUA 1557	0.79	<i>Streptomyces</i> sp. DPUA 1589	153.86
<i>Streptomyces</i> sp. DPUA 1559	113	<i>Streptomyces</i> sp. DPUA 1591	176.63
<i>Streptomyces</i> sp. DPUA 1560	113	<i>Streptomyces</i> sp. DPUA 1595	19.63
<i>Streptomyces</i> sp. DPUA 1561	50.24	<i>Streptomyces</i> sp. DPUA 1597	200.96
<i>Streptomyces</i> sp. DPUA 1563	133	<i>Streptomyces</i> sp. DPUA 1598	132.67
<i>Streptomyces</i> sp. DPUA 1565	113	<i>Streptomyces</i> sp. DPUA 1599	153.86
<i>Streptomyces</i> sp. DPUA 1566	154	<i>Streptomyces</i> sp. DPUA 1600	113.04
<i>Streptomyces</i> sp. DPUA 1567	30.18	<i>Streptomyces</i> sp. DPUA 1602	153.86
<i>Streptomyces</i> sp. DPUA 1568	154	<i>Streptomyces</i> sp. DPUA 1603	200.96
<i>Streptomyces</i> sp. DPUA 1570	133	<i>Streptomyces</i> sp. DPUA 1605	153.86
<i>Streptomyces</i> sp. DPUA 1571	0.00	<i>Streptomyces</i> sp. DPUA 1606	254.34
<i>Streptomyces</i> sp. DPUA 1572	30.18	<i>Streptomyces</i> sp. DPUA 1608	113.04
<i>Streptomyces</i> sp. DPUA 1573	189	<i>Streptomyces</i> sp. DPUA 1609	113.04
<i>Streptomyces</i> sp. DPUA 1575	18.09	<i>Streptomyces</i> sp. DPUA 1610	176.63
<i>Streptomyces</i> sp. DPUA 1576	283	<i>Streptomyces</i> sp. DPUA 1611	113.04
<i>Streptomyces</i> sp. DPUA 1577	177	<i>Streptomyces</i> sp. DPUA 1612	176.63

Effects of metal ions and inhibitors

The enzyme extract can be classified by their sensitivity to various inhibitors for 30 min at room temperature and the residual activity was measured. Effect of metal ion Zn²⁺, Na⁺, Mg²⁺, Fe²⁺, Cu²⁺, Ca²⁺, Mn²⁺, Co²⁺ and inhibitors phenylmethylsulfonyl fluoride (PMSF), ethylenediaminetetraacetic (EDTA), Pepstatin A and iodoacetic acid were determined. The activity of the enzyme assayed in the absence of inhibitors was taken as 100%. Each assay was carried out in triplicate.

Protease activity determination

Protease assay was conducted according to Alencar (2003). That is, a micro centrifuge tube was added of 100 µL of 1% azocasein (w/v, in 0.2 M Tris-HCl, pH 7.2), and 60 µL of crude extract. The reaction runs for 60 min at 25 °C. Then, 480 µL of 10% (w/v) trichloroacetic acid (TCA) were added to stop the reaction. After 15 min, centrifugation was carried out for 5 min at 8000g. The supernatant (320 µL) was added to 1M NaOH (560 µL) and the absorbance of this mixture was measured at 440 nm (B582 spectrofotometer, Micronal,

São Paulo, Brazil) against a blank similarly prepared except that 0.15M NaCl replaced the crude extract sample. One unit (U) of enzymatic activity was defined as the amount of enzyme capable to produce a 0.001 change in absorbance per minute. The analysis was performed in triplicate. Protein content was determined by after Bradford (1976) using bovine serum albumin as standard.

Fibrinolytic activity determination

Fibrinolytic activity was determined using the fibrin plate method of Astrup and Mullertz (1952), with minor modifications, as follows. The fibrin agarose plate was made by 1% agarose, 0.1% human fibrinogen, and 8 U mL⁻¹ of Human thrombin. The clot was allowed to stand for 1 h at room temperature. Then, 20 µL of sample solution were placed onto the plate. The plate was incubated at 37°C for 18 h and the diameter of the lytic circle measured. In the fibrin plate method, a clear transparent region is observed in which fibrin is hydrolyzed, and its diameter is directly proportional to the intensity of the fibrinolytic activity. The lysed area was evaluated as the circle area (in square millimeter). Plasmin from human plasma was used as positive control.

Table 2. Protease and fibrinolytic protease production by *Streptomyces* sp DPUA 1576 under the effect of agitation, temperature and pH treatments.

Runs	Agitation (g)	Temperature (°C)	pH	PA (U mL ⁻¹)	FA (mm ²)
1	0.28	28	6	1.76	0
2	1.12	28	6	1.94	0
3	0.28	36	6	1.94	0
4	1.12	36	6	21.7	123
5	0.28	28	8	20.9	13.6
6	1.12	28	8	105	304
7	0.28	36	8	7.41	0.35
8	1.12	36	8	96.6	268
9	0.63	32	7	3.43	0
10	0.63	32	7	3.61	0
11	0.63	32	7	2.13	0

PA- Proteolytic activity, FA- Fibrinolytic activity.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) of the crude extract and zymography

The molecular weights of the proteins of crude extract were determined using SDS-PAGE (12%) according to the method of Laemmli (1970). Electrophoresis was carried out at 20 mA/gel in Tris-glycine buffer, pH 8.3, containing 0.01% SDS. Zymography were performed with gelatin. Gelatin at 0.2% was copolymerized with 12% resolving gel. After electrophoresis, the gel was washed successively with Tris-HCL buffer (pH 7.5) containing 2.5% triton X-100 and glycine-NaOH buffer (pH 9.0). The gel was then incubated in glycine-NaOH buffer overnight at 37°C and stained with coomassie brilliant blue R-250 for visualization of the clear zones. The molecular-weight standard proteins used were phosphorylase b (97.4 kDa), albumin (66.2 kDa), ovalbumin (45.0 kDa), carbonic anhydrase (30.1 kDa), trypsin inhibitor (20.5 kDa), and α -lactalbumin (14.4 kDa). The slab gels were stained with Coomassie Blue R-250, 0.25% (w/v), in acetic acid : methanol : water (1 : 4.5 : 4.5, v/v). The molecular weight was estimated by LabImage 1D L340 program.

Fibrinogenolytic activity determination

Fibrinogenolytic activity was determined by modified fibrinogenolytic assay (Gao *et al.* 1998). The fibrinogen solution (0.05 ml of 2% human fibrinogen in 50mM Tris-HCl buffer, pH 7.6, 0.15 M NaCl) was mixed with 300 μ g of crude extract and it was incubated at 37 °C for 0, 5, 15, 30, 60 min and 5, 12, 24 h. After the indicated time intervals, an aliquot of 0.05 ml reaction mixture was transferred to 0.05 ml denaturing reagent (10 M urea, 4% SDS and 4% 2-mercaptoethanol) and incubated at 37 °C for 24h. The sample was then analyzed by 12.5% (w/v) SDS-PAGE.

RESULTS

Screening of fibrinolytic activity, cell growth and protease production

Screening on fibrin agar plates showed that forty-nine *Streptomyces* sp. isolates from Amazonian lichens were able to display extracellular fibrinolytic activity. *Streptomyces* DPUA1576 obtained the highest fibrinolytic activity of 283 mm² (Table 1).

The relationship between protease activity and *Streptomyces* sp. DPUA 1576 growth is shown in Figure 1. Protease production started at the stationary growth phase at 72 h and remained constant up to 132 h; then a linear decrease was observed. The highest extracellular protease activity of 11.5 U mL⁻¹ and fibrinolytic activity of 300 mm² obtained after 72 h of fermentation. Maximum cell concentration was 4.5 g L⁻¹ at 36 h of cultivation. The pH value decreased during cell growth until reaching stationary growth phase.

Effects of agitation, pH and temperature on protease and fibrinolytic production by multi-factorial experiments

The obtained values for protease and fibrinolytic activity for the different trials are shown in Table 2. Protease activity varied from 1.76 to 105 U mL⁻¹ and fibrinolytic activity from 0 to 304 mm² (Table 2). A first order (main effects) model was used to analyze the data from the factorial design. The main effects of the examined factors on the protease and fibrinolytic activities were calculated and are presented graphically in Figure 2A and 2B, respectively. Agitation, pH, interaction between agitation and pH, agitation and temperature had a positive effect ($p < 0.01$) on protease production while the interaction between temperature and pH has a negative effect ($p < 0.05$; Figure 2A). The main effect of temperature on protease production was not significant ($p > 0.05$) as shown on the Pareto chart (Figure 2A).

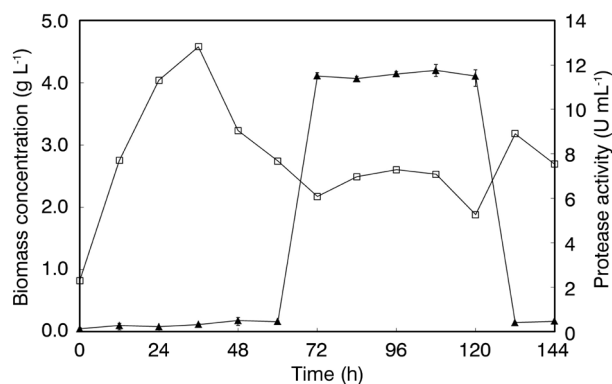


Figure 1. The time profile of cell growth of *Streptomyces* sp. DPUA 1576 and fibrinolytic activity during fermentation: (open square) Biomass (g L⁻¹), (closed triangles) Protease activity (U mL⁻¹);

Regarding the fibrinolytic enzyme production, only the main effect of agitation was positive and significant ($p < 0.05$, Figure 2B). As shown in Table 2, the increase in agitation of the flask shaker led to a remarkable enhancement of enzyme production and the highest enzyme activity was 304 mm² at 1.12 g (200 rpm). The highest fibrinolytic activity (304 mm²) was obtained under initial culture pH 8.0, agitation of 1.12 g and temperature of 28 °C (run 6; Table 2).

Effects of temperature and pH on protease and fibrinolytic activity

The protease was active at temperatures ranging from 25 to 55 °C and had an optimum temperature between 35 to 55 °C, while the activity decreased at a temperature higher than 55 °C (Figure 3). The relative activities at 25 and 75 °C were 59 % and 49 %, respectively. Protease activity was stable during 120 min at 25 and 35 °C and showed 98, 100, and 59% residual activity at 25, 35 and 45 °C, respectively. The enzyme in the crude extract lost its activity after 60 min between 55 to 75 °C. The effect of temperature on the

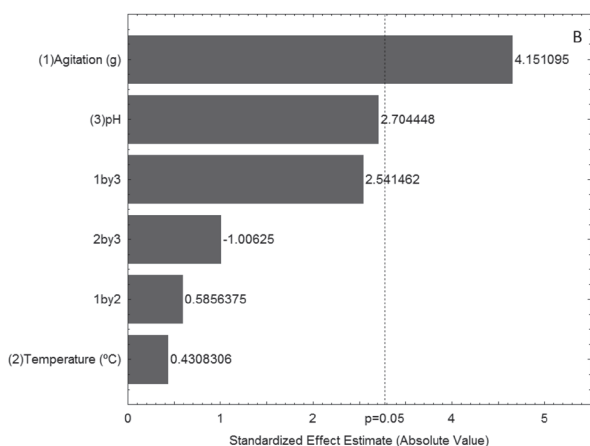
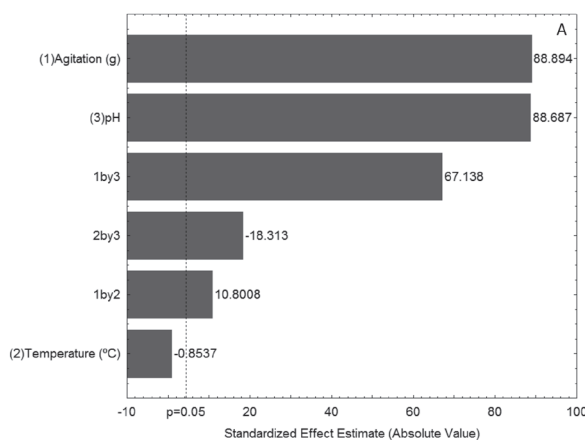


Figure 2. Pareto Chart showing variables and interactions on (A) protease and (B) fibrinolytic production by *Streptomyces* sp DPUA 1576.

fibrinolytic activity was examined. The temperature showing the maximal enzyme activity was 45 °C (Figure 3).

The pH between 4.0 to 9.2 has effect on the proteolytic activity. The highest proteolytic activities were obtained in the alkaline range and the protease was stable in the pH range from 6.5 to 9.2 during 120 min (Figure 4). The optimum pH for the fibrinolytic activity was 7.5, and the enzyme activity decreased rapidly at levels below pH 7.0 and above pH 8.0. The enzyme was stable between pH 6.0 and 9.5 for 360 min and the stability enzymatic decreased below pH 5.5 (Figure 4).

Effects of metal ions and inhibitors

The effects of metal ions and inhibitors on protease activity were investigated after incubation of the protease with different metal ions and inhibitors for 1 h at 25 °C. The activity of protease was slightly enhanced by the presence of Na⁺ ions.

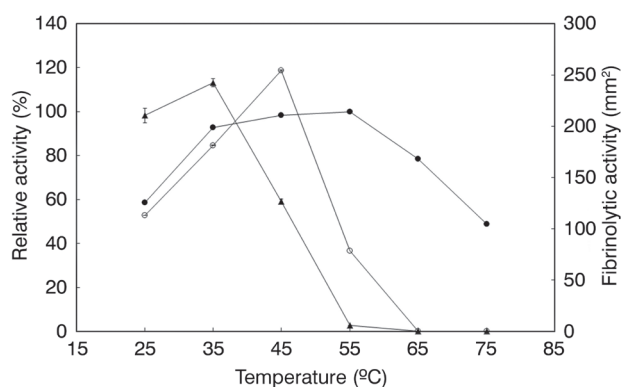


Figure 3. Effect of temperature on the proteolytic activity (closed circles), fibrinolytic activity (open circle) and proteolytic stability (closed triangles) from *Streptomyces* sp. DPUA1576. Optimum temperature of the enzyme was determined by assaying the activity at various temperatures. Thermal stability of the enzyme was determined by measuring the residual activity after the enzyme was incubated at selected temperatures for 6 h.

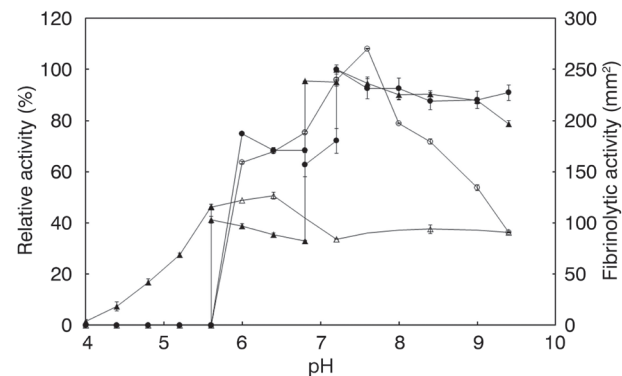


Figure 4. Effect of pH on protease activity (closed circles), Protease stability (closed triangles), fibrinolytic activity (open circles) and fibrinolytic stability (open triangles) from *Streptomyces* sp. DPUA1576. The determination of pH stability of the enzyme was measured by residual activity after the enzyme was incubated at the same pH ranges described above for 6 h.

On the other hand, the activity was significantly suppressed by Cu^{+2} and slightly inhibited by Co^{+2} . No significant effects were observed by the presence of other metal ions on the protease activity (Table 3). Proteolytic activity was slightly inhibited by 1mM of EDTA and phenylmethylsulfonyl fluoride (PMSF), but significantly inhibited by 50mM of PMSF and 10mM of EDTA showing a residual activity of $25.4 \pm 0.30\%$ and $39.9 \pm 0.65\%$, respectively. Pepstatin A at 1mM also inhibited the protease activity, resulting in a residual activity of $14.6 \pm 0.26\%$ (Table 2). Inhibition profile showed in the Table 2 indicates that protease from *Streptomyces* sp. L5A35 is a serine protease.

SDS-PAGE and fibrinogenolytic activity of crude fermentation extract

SDS-PAGE analysis of the crude fermentation extract indicates the existence of several proteins in the range of 31 to 45 kDa (line 2, Figure 5). A higher intensity band is observed at 39 kDa suggesting that it corresponds to the fibrinolytic enzyme that correlated with the clear hydrolytic bands observed in the gelatin zymogram (line 3, Figure 5).

The fibrinogenolytic reaction of the extracellular protease produced from *Streptomyces* sp. DPUA1576 was analyzed with SDS-PAGE (Figure 6). In this case, the subunit chains of fibrinogen were completely hydrolyzed after 0.5 h of incubation.

Table 3. Effect of metal ions and protease inhibitors on proteolytic activity of *Streptomyces* sp. DPUA1576.

Metal ion or inhibitor	Concentration (mM)	Residual activity %
None	0	100 ± 1.14
ZnSO ₄	1	81.4 ± 1.09
NaCl	1	106 ± 0.26
MgSO ₄	1	94.4 ± 0.60
FeSO ₄	1	95.3 ± 2.14
CuSO ₄	1	34.2 ± 0.43
CaCl ₂	1	88.8 ± 1.67
MnCl ₂	1	99.8 ± 2.28
CoCl ₂	1	60.8 ± 2.68
FeCl ₃	1	95.4 ± 0.81
Pepstatin A	1	14.6 ± 0.26
Phenylmethylsulfonyl fluoride (PMSF)	1	94.0 ± 0.26
	10	91.4 ± 0.31
	50	25.4 ± 0.30
EDTA	1	71.8 ± 0.52
	10	39.9 ± 0.65
IODO	1	105.7 ± 0.88
	10	100.1 ± 0.50

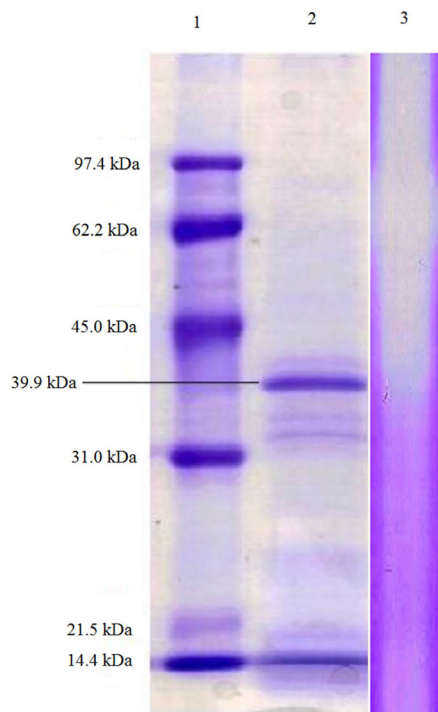


Figure 5. Electrophoresis SDS-PAGE of crude extract with fibrinolytic activity by *Streptomyces* sp. DPUA1576. 225 µg of protein was loaded on a 12.5% acrylamide gel. Lane 1 shows the standard molecular weight; lane 2 shows the protein content of crude extract, lane 3 shows zymography.

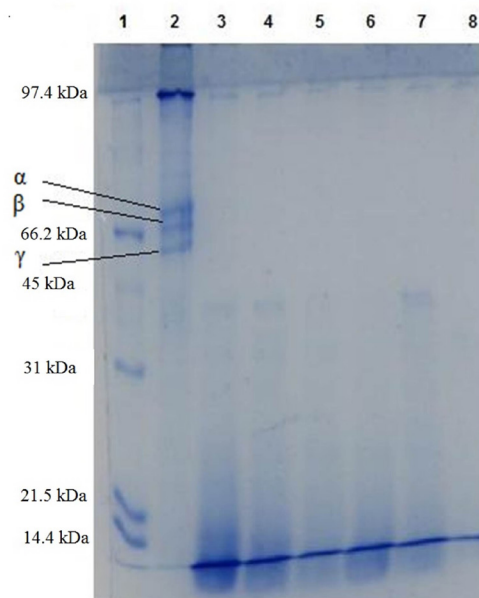


Figure 6. Hydrolysis of fibrinogen by *Streptomyces* sp. DPUA1576. (1) Fibrinogen (40µg) was incubated with fibrinolytic enzyme at 37 °C for the various time intervals and analyzed by 12 (%) SDS-PAGE. (2) Standard low molecular weight; (3) 0 (4) 0.5h (5) 1h (6) 2h (7) 3h (8) 24h.

DISCUSSION

Extracellular fibrinolytic activity showed that *Streptomyces* sp. isolated from Amazonian lichens presents clear haloes on fibrin plates indicating the ability of the produced enzymes to degrade fibrin in a direct way. *Streptomyces* DPUA1576 was selected as the best fibrinolytic producer (283 mm²) with enzyme activity higher than that obtained by Rovati *et al.* (2010) in fungal specimens isolated from the sub-tropical Las Yungas Pedemontana forest (Tucuman, Argentina). It reveals the potential of *Streptomyces* sp. strains from Amazonian lichens as an unconventional and unexplored production alternative to already known thrombolytic agents as well as a reservoir of microorganism species with promising biotechnological value could be also highlighted.

Streptomyces sp. DPUA 1576 showed maximum protease production after 72 h of incubation (stationary phase of cell growth) and the enzyme level declined with further incubation. Protease production during the stationary growth phase is in accordance with the studies of other authors (Miyaji *et al.* 2006; Gouda 2006). The protease production is directly related to biomass production (Gupta *et al.* 2002; Puri *et al.* 2002) and is associated with the stationary phase of cell growth (Puri *et al.* 2002; Rao 1998). The decline in protease production upon prolonged incubation may be due to autolysis or proteolytic activity of other proteases under starvation conditions (Puri *et al.* 2002).

At the growth stationary phase (Figure 1), the maximum proteolytic activity was observed after 72 h, and its production was not growth-associated.

The pH value decreased during cell growth until reaching stationary growth phase, after remaining constant between 72 to 132 h. The drop in pH may be attributed to the production of organic acids, which were consumed during later stages of growth resulting in a slight pH increase to 4.0 (Futamura *et al.* 2001). These results show that alkalization of the medium is closely related with the degradation of extracellular proteins by proteases produced from *Streptomyces* sp DPUA1576, suggesting that alkalization is adaptive. Our observations with *Streptomyces* sp. DPUA1576 suggest that this phenomenon may be widespread.

Temperature, agitation and pH are factors that influenced on protease and fibrinolytic productions. The decrease in the temperature caused an increase in protease production by *Streptomyces* sp DPUA1576. Other studies also showed that temperature is relevant factor for microbial protease production (Thys *et al.* 2006; Ellaiah *et al.* 2002) and an enhancement in protease production occurred when the temperature decreased from 50 to 25 °C (Thys *et al.* 2006).

An increase in agitation of the flask shaker to 1.12 g (200 rpm) led to a remarkable enhancement of enzyme

production. *Streptomyces* sp. is an aerophilic bacteria and increase the agitation providing a greater amount of oxygen for microorganism multiplication and metabolism. Al-Juamily and Al-Zaidy (2012) obtained a maximum fibrinolytic activity of 290 U, at 250 rpm.

The best pH for fibrinolytic enzyme production by *Streptomyces* sp DPUA 1576 was 8.0, similar to that reported by Bhavani *et al.* (2012) using *Streptomyces venezuelae*. The effect of pH is in agreement with previous reports on its effect on extracellular enzymatic activity (Caracuel *et al.* 2003).

In the initial culture, the highest fibrinolytic activity of 304 mm² was obtained at pH 8.0, agitation of 1.12 g (200 rpm) and temperature of 28 °C. This result corresponds to a 41% increase in the enzyme activity, when compared to fibrinolytic activity of 215 mm² using *Bacillus licheniformis* KJ-31 (Hwang *et al.* 2007). Silva *et al.* (2015) showed that *Streptomyces* sp. DPUA 1576 produced the highest fibrinolytic activity of 706.5 mm² at 1.7% soybean flour and 1.0% glucose concentration, which was 33% higher than plasmin.

Optimum temperature for protease was between 35 to 55 °C and to fibrinolytic was at 45 °C. Simkhada *et al.* (2010) reported that the optimum temperature of fibrinolytic protease from *Streptomyces* sp CS684 was 45 °C, but it was not stable at this temperature, losing more than 50% of its activity after 90 min. This enzyme was stable below 40 °C.

The highest proteolytic activities were obtained in the alkaline range. Several other researchers have also described alkaline proteases with broad pH activities and stabilities. A purified alkaline protease from *B. subtilis* was observed to be stable with activity $\geq 78\%$ in the pH range of 7.0–11.0, with the highest activity at pH 9.0 (Pandee *et al.* 2008)

The fibrinolytic enzyme was activated at neutral and alkaline pH values, and optimal reaction for fibrinolytic enzyme was obtained at pH 7.5, which is similar to the optimum observed for *Streptomyces* sp. CS684 (Simkhada *et al.* 2010), but higher than *Streptomyces* sp. P3 (Cheng *et al.* 2015). The fibrinolytic enzyme from *Streptomyces* DPUA1576 was stable at pH 6.0–9.5, and the activity was greatly decreased below pH 6.0, exhibiting a pH stability comparably wider than that of *Streptomyces* sp. P3 (Cheng *et al.* 2015) and similar to *Streptomyces* sp. CS684 (Simkhada *et al.* 2010). These results suggest that the enzyme is active over a very narrow range of pH values, which indicates that FSP3 may be suitable for use in the human in vivo environment

The optimal temperature and pH value of fibrinolytic enzyme from *Streptomyces* sp. DPUA1576 were found to be 45 °C and 7.5, respectively. This values was similar to optimal temperature of 40 °C and pH 8.0 obtained by *Streptomyces venezuelae* (Bhavani *et al.* 2012) and 50 °C and pH 8.0 obtained by *Shewanella* sp. IND20 (Vjayaraghavan and Vincent 2015).

Proteolytic activity significantly was inhibited by PMSF, an inhibitor of serine proteases, which indicates that the enzyme's catalytic site includes a serine residue. These results are in agreement with (Afifah *et al.* 2014; Mukherjee and Rai 2011).

SDS-PAGE of crude fermentation extract indicates the highest intensity band at 39 kDa suggesting it corresponds to the fibrinolytic enzyme, which is similar to that of fibrinolytic enzyme from *Streptomyces* megasporos SD5 (Chitte and Dey 2000) and *Streptomyces* sp. CS684 (Simkhada *et al.* 2010).

Another important activity of the fibrinolytic enzyme is its fibrinogenolytic reaction, by which fibrinogen is degraded. Extracellular protease produced from *Streptomyces* sp. DPUA1576 hydrolyzed completely fibrinogen after 0.5 h of incubation. This fibrinogenolytic pattern is completely different from the one obtained with the enzyme produced by *Streptomyces* sp. CS684, where degraded B β -chains and slowly released γ -chains and the A α -subunit of fibrinogen remained resistant to degradation (Simkhada *et al.* 2010) and by *Streptomyces omiyaensis* which degraded the A α and B β chains and γ chains after 3h of incubation (Uesugi *et al.* 2011).

CONCLUSIONS

Streptomyces sp. DPUA 1576 is a highly promising strain to be used in the production of fibrinolytic enzyme with a maximal enzyme activity of about 304 mm² and may be a potential source of new and unexploited fibrinolytic enzymes for different therapeutic purposes. It was also demonstrated that the protease present in the medium broth could act both on fibrinogen and fibrin blocking the activation process of fibrinogen to fibrin.

The obtained results demonstrate that microbial enzymes have the potential to be developed as drugs to prevent or cure cardiovascular diseases, as fibrinolytic enzymes in the treatment of thrombosis.

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