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Production of fibrinogenolytic and fibrinolytic enzymes by a strain of *Penicillium* sp. isolated from contaminated soil with industrial effluent

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ABSTRACT. Cardiovascular diseases associated with thrombosis are one of the main causes of death all around the world. Urokinase, streptokinase, and tissue plasminogen activator are the major thrombolytic agents used to treat thrombosis. However, the fact that these agents have several side effects and high prices has driven the search for safer and more economically viable compounds for the treatment of cardiovascular diseases. Thus, the aim of this study was to evaluate the potential of fungi isolated from industrial effluents to produce fibrino(geno)lytic enzymes. The selection of the protease-producing strains showed that only the BF20 strain was able to produce proteolytic halos in milk agar. This strain identified as belonging to the genus Penicillium was cultivated in submerged fermentation. Different media composition were tested to evaluate proteolytic activity, and the results showed that the medium containing 0.1% yeast extract and 1% skim milk, pH 5.0, present higher azocaseinolytic activity (0.24 U mL^{-1} $min.^{-1}$). This sample also showed the ability to degrade fibrinogen and fibrin after 15 and 120 min. of incubation, respectively. These results indicate that the BF20 strain has a thrombolytic potential, effectively degrading fibrinogen and fibrin, having great application in the health area.

Keywords: thrombosis; fungi; *Penicillium*, fibrin(geno)lytic enzymes.

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Introduction

Cardiovascular diseases are the main cause of death in the worldwide (Mathers & Loncar, 2006). The mortality rate caused by vascular disorders such as myocardial infarction, stroke, deep venous thrombosis, pulmonary embolism has significantly increased in recent years (Simkhada, Cho, Mander, Choi, & Yoo, 2012; Mahajan, Nayak, & Lele, 2012). Enzymatic therapies are becoming an alternative to surgeries in clinical practice (Sun et al., 2016), and thrombolytic agents are employed to dissolve non-hydrolyzed clots (Kunamneni, Abdelghani, & Ellaiah, 2007). Dissolution of thrombi is called fibrinolysis, which occurs by the action of an enzyme called plasmin. The plasminogen, the inactive form of plasmin, is activated by compounds called plasminogen activators. Thrombotic diseases can occur when fibrin is not hydrolyzed due to different hemostatic disorders (Holden, Lavigne, & Cameron, 1990).

There are three therapy options available for the treatment of thrombosis: anticoagulants, antiplatelet agents and fibrinolytic enzymes. However, the fact that anticoagulants and antiplatelet agents have a short half-life, undesirable side effects (Simkhada et al., 2012), low specificity and high production costs (Yong, Xiaojuan, & Yizheng, 2005; Flemmig & Melzig, 2012; Erdur et al., 2014), has motivated searching for safer and more economically viable compounds for the treatment of cardiovascular diseases.

Fibrinolytic enzymes, which directly degrade fibrin by dissolving the thrombi quickly and efficiently, is an alternative to the use of these thrombolytic agents. Fibrinolytic enzymes, unlike anticoagulants, promote the lysis of preexisting thrombi. These enzymes can be classified into two types: plasminogen activators and plasmin-type enzymes. Plasminogen activators are able to release plasmin, and the plasmin-like enzymes directly degraded the fibrin, leading to the rapid and complete destruction of the thrombi (Kotb, 2013). Urokinase and streptokinase are used in clinical practice and act indirectly in the degradation of fibrin converting the plasminogen to plasmin to degrade fibrin (Kunamneni et al., 2007). Fibrinolytic enzymes

belong to the class of proteases that constitute a large group of enzymes that catalyze the hydrolysis of peptide bonds of other proteins (Gupta, Beg, & Lorenz, 2002). Microbial proteases have also attracted great attention because of their great biotechnological potential and low production cost (Hernández-Martínez et al., 2011). They are widely used in pharmaceutical preparations (Souza et al., 2015a). These enzymes account for about 60% of the total sales of enzymes in the world (Zambare, Nilegaonkar, & Kanekar, 2011).

Research using direct thrombolytic agents has shown encouraging results in laboratory tests and in pre-clinical trials (Marder & Novokhatny, 2010). Nattokinase, one of the most studied microbial proteases, is a fibrinolytic enzyme produced by *Bacillus subtilis*. This enzyme was originally isolated from natto, a traditional Japanese fermented food and is commercially available for the treatment of thrombosis (Fujita et al., 1993). This enzyme is able to degrade thrombi and may also increase the activity of the tissue plasminogen activating factor, responsible for the activation of the fibrinolytic system (Sumi, Hamada, Nakanishi, & Hiratani, 1990; Fujita et al., 1995a; 1995b; Sumi, Yanagisawa, Yatagai, & Saito, 2004). Many nattokinase-like fibrinolytic enzymes were characterized from bacilli isolated from fermented soybean foods (Kim et al., 2009; Jo, Lee, Jeong, & Kim, 2011).

Penicillium genus also have great potential for the biotechnological production of proteases and other enzymes. The species include *Penicillium sp., P. camemberti, P. citrinum, P. griseoroseum, P. restrictum* and *P. roqueforti*. Most *Penicillium* fungi produce alkaline proteases under submerged fermentation conditions. However, *P. griseoroseum* and *P. camemberti* are known to produce acidic proteases in liquid medium. The *P. griseoroseum* IH-02 strain produced large amounts of an extracellular acid protease using medium containing wheat bran and soybean meal (Ul-Haq & Mukhtar, 2007). Fibrinolytic enzymes have also been isolated from *Aspergillus, Penicillium, Rhizopus, Mucor, Humicola, Thermoascus, Thermomyces*, and other genus (Souza et al., 2015b).

Since fibrinolytic enzymes are promising in the treatment of disorders associated with thrombosis, the objective of this work was to evaluate the ability of fungal strains isolated from soil contaminated with poultry slaughterhouse effluent to produce proteases with fibrinolytic activity and fibrinolytic activity.

Material and methods

Isolation of microorganisms

The fungi BF20, BF22, BF24 were isolated from contaminated soil of poultry slaughterhouse effluent located in the city of Rolândia, State Paraná. For the isolation was used Solid Minimum Medium ($NaNO_3$ 4.0; KH_2PO_4 1.5; $FeCl_3$ 0.05; MgSO4 0.2; CaCl2 0.01; Na_2HPO_4 0.5; yeast extract 0.05; and agar 15 g L^{-1}) and 0.001% of Tween 80 and 1% of olive oil. After isolation, the microorganisms were preserved in MEA (Malt Extract Agar) containing (g L^{-1}): malt extract (20), bacteriological peptone (1), glucose (20), agar (20) supplemented with 10 mg de $ZnSO_4.7H_2O$ and 5 mg de $CuSO_4.5H_2O$ in metal solution composed of 1 g $ZnSO_4.7H_2O$ and 0.5 g $CuSO_4.5H_2O$ in 100 mL distilled water. The strains were maintained at 4°C and replicated every 30 days.

Microorganism identification

Phenotypic identification of the fungal isolate was performed according to the identification key according to Pitt and Hocking (2009). The spreading was performed using CYA medium (Czapek yeast extract agar) containing (g L^{-1}): K2HPO4 (1), concentrated czapek (10), agar (15), yeast extract (5), sucrose (30), and in MEA containing (g L^{-1}): malt extract (20), bacteriological peptone (1), glucose (20), agar (20) supplemented with 10 mg of $ZnSO_4.7H_2O$ and 5 mg of $CuSO_4.5H_2O$.

Evaluation of protease production in solid medium

The proteolytic activity of strains was initially evaluated by the halo formation test in petri dishes containing 1% of skim milk, 18 of agar and 1 g L^{-1} of yeast extract in 0.05 M citrate buffer. The skim milk was sterilized separately from the rest of the medium in order to avoid coagulation of its components. Subsequently, the milk solution was added to the agar under aseptic conditions. BF20, BF22 and BF24 inoculum was added in the center of the plates, which were incubated for 7 days at 28°C. Enzyme production was observed by the formation of enzymatic halos, which were measured and

expressed in millimeters. The enzymatic activity was determined by the enzymatic index (EI) expressed as the ratio of the mean degradation halo diameter and the mean of the colony diameter. To verify the best conditions for protease production, an assay was performed by varying the incubation temperatures (24, 28, 32, 36 and 40°C) and different pH (5, 7 and 9), using 0.05 M of citrate buffer (pH 5.0), 0.05M phosphate buffer (pH 7.0) and 0.05M glycine buffer (pH 9.0). The experiments were performed in triplicate.

Production of proteases in submerged fermentation

For submerged fermentation, BF20 was initially cultivated in solid MEA medium at 28°C until the sporulation stage. Then, a spore suspension was prepared in 10 mL of 0.9% NaCl and Tween 80. Spore counting was performed in Neubauer's chamber and about 10^6 spores mL^{-1} were inoculated in Erlenmeyer flasks containing 25 mL of three different media: Medium 1:50 mM citrate buffer, pH 5.0, containing 0.1 of yeast extract and 1% casein; Medium 2: 50 mM citrate buffer, pH 5.0, containing 0.1 of yeast extract and 1% skim milk; Medium 3: Vogel medium containing (g L^{-1}): $Na_3C_6H_5O_7.2H_2O$ (3), KH_2PO_4 (5), NH_4NO_3 (2), $MgSO_4.7H_2O$ (0.2), $CaCl_2H_2O$ (0.1), biotin (5 µg) and trace element solution (100µL) + 1% casein. The flasks were incubated for 72, 96, 120 and 144 hours at 28°C, 180 rpm. Bottles inoculated with saline solution were used as negative controls. The fermentations were stopped by vacuum filtration and the filtrate was called the crude extract, which was used in protein quantification and fibrinolytic, and fibrinogenolytic activity.

Protein quantification

Protein concentration was determined by the method of Bradford (1976). In this method, one aliquot of each sample was dissolved in 100 µL with deionized water and 1 µl of Bradford's reagent (50 mg of Coomassie Brilliant Blue G-250 in 25 mL of 95% ethanol, 50 mL of concentrated phosphoric acid and distilled water). Quantification was performed in triplicates and the absorbance measured at 595 nm. The standard curve of bovine serum albumin (BSA) (1 mg mL^{-1}) was constructed, considering the molar extinction coefficient in 280 nm (0.665). The concentration of proteins was expressed in µg $µL^{-1}$ was determined from linear regression calculations based on the values obtained in the standard curve.

Proteolytic activity

The proteolytic activity was determined by the method described by Charney and Tomarelli (1947) with some modifications, using the azocasein as substrate. For this, 500 µL of the crude extract was incubated with 500 µL of azocasein solution (0.5%) (Sigma Aldrich) in 50 mM sodium acetate buffer, pH 5.0, for 40 min. at 37°C. The reaction was stopped by addition of 500 µL of trichloroacetic acid (10%). The samples were centrifuged for 10 min. at 3000 rpm. 1 mL of the supernatant was transferred to a test tube and 1 mL of 5.0M KOH was added. The absorbance of the samples was read at 430 nm.

Fibrinogenolytic activity

The fibrinogenolytic activity was evaluated by incubating 20 µg of human fibrinogen (Sigma Aldrich) with 5 µg of the crude extract for 15, 30, 45, 60 and 120 min. at 37°C. The reaction was then stopped by addition of Tris-HCl buffer (125 mM), pH 6.8 containing 10% of glycerol, 5 of β-mercaptoethanol, 2 of SDS and 0.001% of bromophenol blue. Samples were boiled for 5 min. and subjected to a polyacrylamide gel electrophoresis (SDS-PAGE) at the concentration of 12.5% on the separation gel, and 4% on the stacking gel (Laemmli, 1970). The run was performed at 25 mA at constant voltage. The run buffer containing (g L^{-1}): Tris (3), glycine (14.4), SDS (1), pH 8.3. The gels were stained with a solution containing 5% of acetic acid, 25% of methyl alcohol and 0.025% of Coomassie Brilliant Blue.

Fibrinolytic activity

The fibrinolytic activity was evaluated by incubating 20 µg of human fibrin (Sigma Aldrich) with 5 µg of crude extract for 15, 30 and 45 min., 1, 2, 4, 6 and 8 hours at 37°C. The reaction then stopped by the addition of Tris-HCl buffer (125 mM), pH 6.8 containing 10 of glycerol, 5 of β-mercaptoethanol, 2 of SDS and 0.001% of bromophenol blue. Samples were boiled for 5 min. The samples were added to a 12.5% polyacrylamide gel electrophoresis (SDS-PAGE), according to Laemmli (1970).

Results and discussion

The first step of this study was to evaluate the ability of strains isolated from soil contaminated with poultry effluent to produce proteolytic enzymes. These effluents have large amounts of residues such as blood and coagulated milk and have the potential to host microorganisms that express such enzymes. The results showed that the isolates BF22 and BF24 did not present any proteolytic activity, since no degradation halos were observed after 7 days of incubation. On the other hand, the isolate BF20 was able to produce enzymes that degraded the skim milk present in the culture medium. Enzymatic halos could be observed around the colony (Figure 1). The BF20 isolate presented the enzymatic index of 2.2 (Table 1). According to the literature, isolates exhibiting enzymatic indices greater than 2.0 are considered good protease producing strains.

BF20 was identified by morphological analysis as belonging to the genus *Penicillium*. After identification of the strain, our next step was to select the best conditions for the production of proteolytic enzymes in milk agar medium. The conditions of cultivation are essential for the success in the production of enzymes, and parameters such as pH, temperature and composition of the medium must be controlled (Abidi, Chobert, Haertlé, & Marzouki, 2011). The first parameter evaluated was the temperature, where we observed that the production of proteases by BF20 was higher at 28ºC with an enzymatic index of 2.2. In this assay the pH of the medium was set to pH 5.0. Next, we evaluated the effect of different pH values on the formation of the proteolytic halo by BF20. Using the incubation temperature of 28ºC, we observed a higher enzymatic index (2.2) at pH 9.0. However, expressive values of enzymatic activity were also observed at pH 5.0 with an enzymatic index of 1.4 (Figure 2).

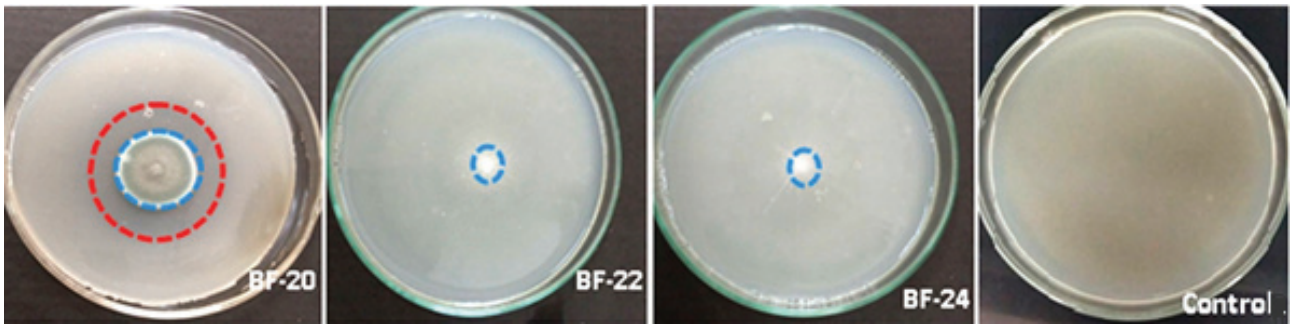

Figure 1. Formation of proteolytic halos by BF-20, BF-22 and BF-24. The red lines represent the diameter of the degradation halo and the blue lines the diameter of the colonies. The enzymatic activity was determined by the enzymatic index (EI) expressed as the ratio of the mean diameter of the degradation halo and the mean diameter of the colony.

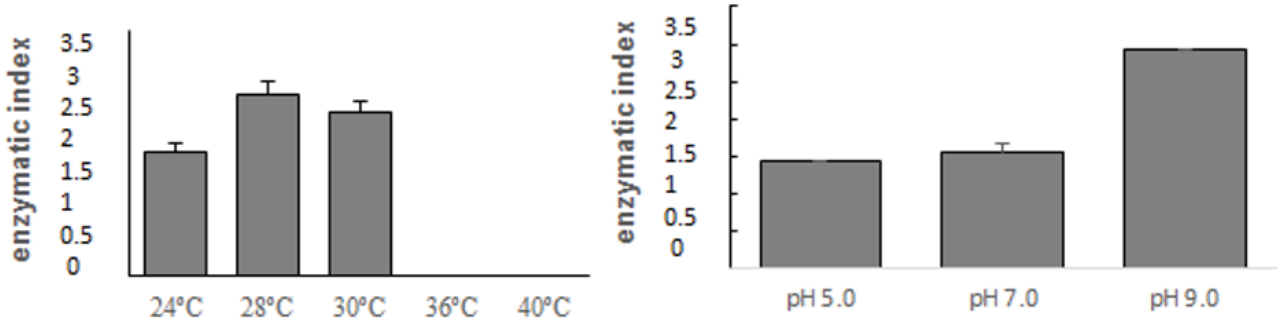

Figure 2. Evaluation of the proteolytic activity of the BF-20 strain in milk agar at different incubation temperatures and different pHs. The assay at different pH was performed at 28°C, and the assay varying the temperature was carried out at pH 5.0.

Table 1. Enzymatic index of fungal strains.

Strains	HD/CD (EI)
BF20	2,2 cm
BF22	0,0 cm
BF24	0,0 cm

HD: halo diameter, CD: colony diameter, EI: Enzymatic index.

In agreement with the literature fungi can produce acidic, neutral or alkaline proteases active in a wide range of pH. Acid proteases are of great commercial importance and are employed in the food and pharmaceutical industries (Aleksieva & Peeva, 2000). Thus, the acidic pH was selected for the production of proteases by submerged fermentation. Fungi of the genus Penicillium have great potential for the biotechnological production of proteases and other enzymes, and produce mostly alkaline proteases under submerged fermentation conditions. However, *P. griseoroseum* and *P. camemberti* are known to produce acidic proteases in liquid medium (Ul-Haq & Mukhtar 2007).

The production of proteolytic enzymes by BF-20 was then evaluated by submerged fermentation in different culture media using azocasein as substrate (Figure 3). Some authors consider azocasein as one of the best substrates for protease activity quantification, due to its higher solubility (Bendicho, Martí, Hernández, & Martín, 2002). Medium 2 containing skimmed milk and yeast extract diluted in citrate buffer pH 5.0, showed higher enzymatic activity reaching values of 0.24 U mL^{-1} $min.^{-1}$. The medium containing casein in Vogel (Medium 3) also resulted in similar enzyme activity values (0.2225 U mL^{-1} $min.^{-1}$). On the other hand, Medium 1 (50 mM citrate buffer, pH 5.0 containing 0.1 yeast extract and 1% casein) resulted in a lower azocaseinolytic activity (0.0394 U mL^{-1} $min.^{-1}$). Recently, Salihi, Asoodeh, and Aliabadian (2017) have described the ability of *Aspergillus oryzae* to produce proteolytic enzymes capable of degrading azocasein. *Aspergillus foetidus* proteases were also able to degrade azocasein (Souza et al., 2017). In addition, *Penicillium candidum* strain (PCA 1/TT031) was able to synthesize different types of extracellular proteases (Alhelli et al., 2016).

Then, the samples obtained in the fermentations were quantification by Bradford method and then tested for the ability to degrade fibrinogen. Fibrinogen is a plasma glycoprotein that has three peptide chains (α, β, γ), and its conversion to fibrin by thrombin is essential for thrombus formation (Hoffbrand & Moss 2012). After incubation of the fibrinogen with the crude extracts obtained in the three fermentations, it was observed a proteolytic activity against the human fibrinogen (Figure 4). After 60 minutes of incubation, it was possible to observe the degradation of the three chains of this molecule. On the other hand, the control incubated without extract (Figure 4, line 1) presented the three bands of fibrinogen intact. In addition, the controls obtained from fermentations where the inoculum was not added also did not have the capacity to degrade fibrinogen (Figure 4, lines 5, 6 and 7).

Based on these results, a new fermentation was carried out at different times, using the composition of Medium 2 which induced the highest enzymatic activity using azocasein (0.24 U mL^{-1} $min.^{-1}$). The highest enzyme activity achieved was 0.248 U mL^{-1} $min.^{-1}$ in 120 hours (Figure 5). The highest production of protease at this incubation time is in agreement with results in the literature (Souza et al., 2015b). The 72 and 144 hours incubation period resulted in a lower production of 0.212 and 0.218 U mL^{-1} $min.^{-1}$ respectively.

The samples resulting from 120 hours fermentation sample, which had the highest enzymatic production, was incubated with fibrinogen at different incubation times (Figure 6).

According to the results obtained, 5 µg of the enzyme extract were able to degrade the three bands of fibrinogen with only 15 min. of incubation, evidencing a great potential of the enzymes produced by BF20 to degrade this substrate. On the other hand, the controls did not present fibrinogenolytic activity. Enzymes with fibrinogenolytic activity were also isolated from *Bacillus cereus* (Majumdar, Dutta, Das, Chattopadhyay, & Mukherjee, 2015). Previous studies have also shown the presence of a fibrinogenolytic enzyme isolated from *Aspergillus fumigatus* (Larcher et al., 1992).

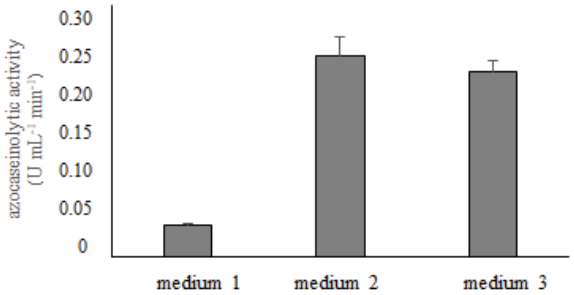

Figure 3. Proteolytic activity, using azocasein as substrate, in different culture media. 50 mM citrate buffer, pH 5.0 + 0.1% yeast extract + 1% casein (Medium 1); 50 mM citrate buffer, pH 5.0 + 0.1% yeast extract + 1% skim milk (Medium 2); Vogel medium + 1% casein (Medium 3). The fermentations were carried out in 96 hours, at 180 rpm, at 28ºC.

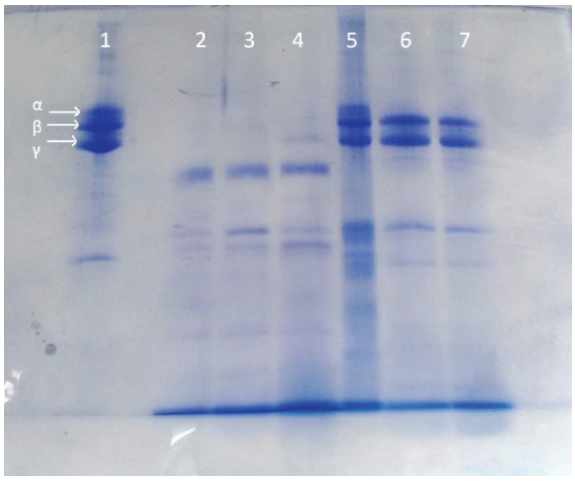

Figure 4. Fibrinogenolytic activity of the crude extracts produced by BF-20. Line 1 (fibrinogen control), 2 (Fibrinogen + enzyme extract medium3), 3 (fibrinogen + enzyme extract medium 2), 4 (fibrinogen + enzyme extract medium 1), 5 (fibrinogen + medium control 3), 6 (fibrinogen + medium control 2), 7 (fibrinogen + medium control 1).

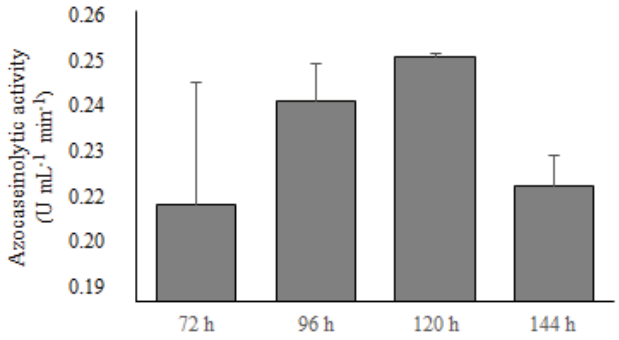

Figure 5. Proteolytic activity, using azocasein as substrate, at different incubation times, using 50 mM citrate Buffer, pH 5.0 + 0.1% yeast extract + 1% skim milk (Medium 2). The fermentations were carried out at different incubation times, at 180 rpm, at 28°C.

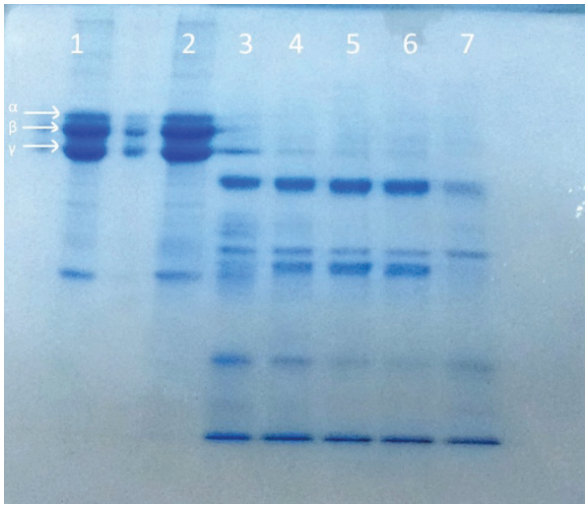

Figure 6. Fibrinogenolytic activity of the crude extract produced by BF-20 after 120 hours of fermentation. Line 1 (fibrinogen control), 2 (fibrinogen + medium control), Lines 3, 4, 5, 6 and 7 (fibrinogen + enzyme extract) incubated for 15, 30, 45, 60 and 120 min., respectively.

Interestingly, fungi are a major source for the production of fibrinolytic enzymes (Sandhya, Sumantha, Szakacs, & Pandey, 2005) and high reproducibility (Abo-Elmagd & Housseiny, 2012; Ravikumar, Gomathi, Kalaiselvi, & Uma, 2012). In this way, the ability to degrade fibrin was also evaluated for the enzymatic extract produced by BF20. According to Figure 7, about 5 µg of the enzyme extract was able to degrade 20 µg of fibrin, but not as fast as observed for fibrinogen. The proteolytic action started after 30 min. of incubation and the degradation of the three fibrin peptide chains was only completed after 8 hours of incubation (Figure 8).

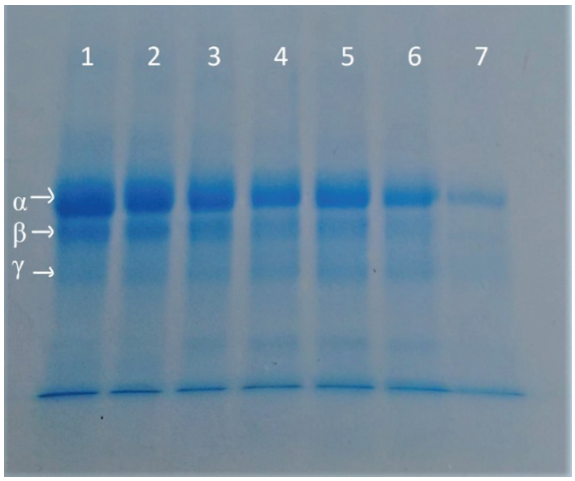

Figure 7. Fibrinolytic activity of the crude extracts produced by BF-20 in 120 hours. Line 1 (fibrin control), 2 (fibrin + medium control), Lines 3, 4, 5, 6 and 7 (fibrin + enzyme extract) incubated for 15, 30, 45, 60 and 120 min. respectively.

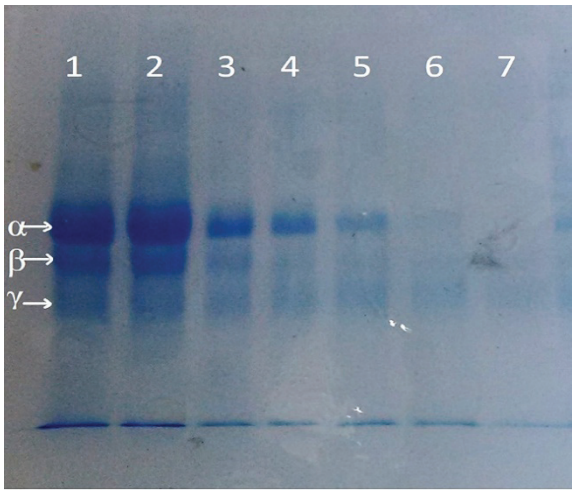

Figure 8. Fibrinolytic activity of the crude extracts produced by BF-20 in 120 hours. Line 1 (fibrin control), 2 (fibrin + medium control), Lines 3, 4, 5, 6 and 7 (fibrin + enzyme extract) incubated for 1, 2, 4, 6, 8 hours respectively.

The literature describes enzymes isolated from fungi that have fibrinolytic activity. Albuquerque, Nascimento, Brandão-Costa, Fernandes, and Porto (2017), reported that the crude extract of *Mucor subtilissimus* showed fibrinolytic activity presenting degradation halos in fibrin dishes. A new fibrinolytic protease, AfeE, with strong thrombolytic activity, was purified from *Streptomyces* sp. CC5 and presented higher substrate specificity for fibrin than fibrinogen, which has rarely been reported in fibrinolytic enzymes. AfeE also showed high thrombolytic activity in a rat tail thrombosis model induced by carrageenan (Sun et al., 2016). Studies evidencing the presence of fibrinolytic enzymes in fungi of the genus *Penicillium* are few described in the literature (El-Aassar, El-Badry, & Abdel-Fattah, 1990).

Conclusion

According to the results, the BF-20 belongs to *Penicillium genus* and has great capacity to produce proteolytic enzymes able to degrade fibrin and fibrinogen, presenting biotechnological potential for future applications in the health area. The data obtained are very promising for future studies of purification of these enzymes on a large scale, and *in vivo* tests for the evaluation of thrombolytic activity.

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