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Optimization of Production, Biochemical Characterization and *In Vitro* Evaluation of the Therapeutic Potential of Fibrinolytic Enzymes from a New *Bacillus Amyloliquefaciens*

Fabiana América Silva Dantas de Souza*[,1,2], Amanda Emmanuelle Sales[1], Pablo Eugênio Costa e Silva[3], Raquel Pedrosa Bezerra[1], Germana Michelle de Medeiros e Silva[4], Janete Magali de Araújo[5], Galba Maria de Campos Takaki[6], Tatiana Souza Porto[7], José António Couto Teixeira[8], and Ana Lúcia Figueiredo Porto[1,3]

[1]*Department of Morphology and Animal Physiology, Federal Rural University of Pernambuco, 52171-900, Recife, PE, Brazil.*
[2]*Department of Natural Sciences, University of Pernambuco (Campus Mata Norte), 55.800-000, Nazaré da Mata, PE, Brazil*
[3]*Laboratory of Immunopathology Keizo Asami, Federal University of Pernambuco, 50670-901, Recife, PE, Brazil*
[4]*Department of Nanotechnology, Center of Strategic Technologies of the Northeast, 50740-540, Recife, PE, Brazil*
[5]*Department of Antibiotics, Federal University of Pernambuco, 50670-901, Recife, PE, Brazil*
[6]*Center for Research in Environmental Sciences, Catholic University of Pernambuco, 50050-900, Recife, PE, Brazil*
[7]*Academic Unit of Garanhuns, Federal Rural University of Pernambuco, 55296-901, Garanhuns, PE, Brazil.*
[8]*Centre of Biological Engineering, University of Minho, Campus Gualtar, 4710-057, Braga, Portugal.*

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Abstract: The capacity of fibrinolytic enzymes to degrade blood clots makes them of high relevance in medicine and in the pharmaceutical industry. In this work, forty-three microorganisms of the genus *Bacillus* were evaluated for their potential to produce fibrinolytic proteases. Thirty bacteria were confirmed as producers of fibrinolytic enzymes, the best results obtained for the strain *Bacillus amyloliquefaciens* UFPEDA 485. The optimization of the enzyme production conditions was done by a central composite design (CCD) star 2^3 that allowed to define the optimal conditions for soybean flour and glucose concentrations and agitation rate. The highest fibrinolytic activity (FA) of 813 U mL^{-1} and a degradation of blood clot *in vitro* of 62% were obtained in a medium with 2% (w/v) of soybean flour and 1% (w/v) glucose at 200 rpm after 48 h of cultivation, at pH 7.2 and 37 °C. The obtained fibrinolytic enzyme was characterized biochemically. Fibrinolytic activity was inhibited by PMSF (fluoride methylphenylsulfonyl - $C_7H_7FO_2S$) 91.52% and EDTA (ethylenediaminetetraacetic acid - $C_{10}H_{16}N_2O_8$) 89.4%, confirming to be a serine-metallo protease. The optimum pH and temperature were 7.0 and 37 ºC, respectively, and the enzyme was stable for 12 h. The fibrinolytic activity at physiological conditions of this enzyme produced by *Bacillus amyloliquefaciens* UFPEDA 485, as well as its long term stability, demonstrate that it has suitable characteristics for human and veterinary applications, and promises to be a powerful drug for the treatment of vascular diseases.

Keywords: screening, *Bacillus amyloliquefaciens*, fibrinolytic enzyme, optimization, characterization.

Introduction

The genus *Bacillus* is considered one of the largest producers of biotechnological molecules. The characterization of the *B. subtilis* 168 genome and other species of the genus facilitated their exploitation in industrial processes. Among numerous advantages, *Bacillus* present short fermentation cycles, and some species are considered safe in food and drug administration.[1,2] Research shows that the genus *Bacillus*, has contributed to the development of new therapeutic bioproducts: hydrogel,[3] vaccine,[4] antibiotic,[5] biosurfactant,[6] and fibrinolytic proteases.[7,8]

Proteases from microbial sources are hydrolytic enzymes that play an important role in cell metabolism and have attracted great interest from the pharmaceutical industry. Although several proteolytic enzymes are being produced, its production is not sufficient to meet the growing demand in the world market.[9] Among the several proteases, enzymes with fibrinolytic activity have gained importance in the medical and pharmaceutical industry due to their effectiveness to degrade blood clots *in vitro* and *in vivo*.[10,11]

Fibrin, a protein component of blood clots is responsible for the occurence of thrombolytic disorders. The human body produces various kinds of enzymes that assist the formation

*Corresponding Author. E-mail: fabiana.americasouza@yahoo.com.br

of thrombus, but only one enzyme, plasmin (EC 3.4.21.7), may break and remove the clot, because it acts directly by dissolving the blood clot and maintaining blood flow at sites of vascular injury.[12]

Fibrinolytic agents are commercially available for clinical use and are plasminogen activators, such as urokinase and streptokinase (bacterial origin). They present several disadvantages such as short half-life, high cost, need for high doses, collateral effects, including allergic reactions and hemorrhagic complications.[13] On the other hand, plasmin and enzymes such as nattokinase, lumbrokinase (EC 3.4.17.13) and fibrolase are fibrinolytic enzymes which directly degrade fibrin thrombus dissolving blood clots quickly and completely. Thus, enzymes with such properties are required as alternatives for a superior therapy in the treatment of cardiovascular diseases and a continued research for new fibrinolytic agents is needed.[14]

Fibrinolytic enzymes were discovered in insects,[15] snake venom,[16] fruits such as *Campomanesia xanthocarpa*, popularly known as "guavirova",[17] brown algae,[18] fermented foods *Bacillus subtilis* natto[19] and are produced by various microorganisms, mainly bacteria of the genus *Bacillus*, widely studied due to their potential to produce potent fibrinolytic proteases.[20-22]

The definition of the time required for the enzymes to be produced by microorganisms demands the use of methods that can accelerate the production process and the effect of the main process variables. Among others, central composite design (CCD) and response surface methodology (RSM) are statistical tools widely used in the optimization of fermentation processes involving enzyme production.[23]

Thus, considering the biotechnological potential of *Bacillus* strains for the production of bioactive compounds, this work presents results on the screening, production and biochemical characterization of a fibrinolytic protease produced by *Bacillus* spp. as well as the *in vitro* evaluation of its therapeutic potential.

Experimental

Screening and Culture Conditions. Forty-three *Bacillus* sp. were obtained from Cultures Collections of Department of Antibiotics (Federal University of Pernambuco) and Catholic University of Pernambuco, Brazil. The stock culture was stored in 10% v/v glycerol solution at -80 ºC. Stock culture was inoculated in nutrient broth at 37 ºC, 150 rpm. After 24 h of growth, the inoculum was standardized for an optical density of 0.1 at 600 nm using a spectrophotometer (Ultrospec 9000, GE Healthcare Life Sciences, Cambridge, UK) and the screening experiments done.

Soybean medium (MS-2)[24] was used for the production of fibrinolytic proteases. The medium is composed by: filtered soybean flour (2% w/v), K_2HPO_4 (0.435% w/v), NH_4Cl (0.1% w/v), $MgSO_4 \cdot 7H_2O$ (0.06% w/v), glucose (1% w/v) and 1% of mineral solution (v/v) containing: $FeSO_4 \cdot 7H_2O$ (100 mg); $MnCl_2 \cdot 4H_2O$ (100 mg), and $ZnSO_4 \cdot H_2O$ (100 mg) in 100 mL of distilled water. The production was done in shaken flasks of 250 mL with 100 mL of culture medium, pH 7.2, for 48 h, at 150 rpm and 37 ºC. At the end of the production process, the culture medium was centrifuged at 10,000 *g* for 10 min to obtain the enzyme extract.

Microbial Identification. Microbial identification was done using 16S and rpoB genes and the sequencing was performed by STAB VIDA in Lisbon, Portugal. A sequence similarity search was performed in the National Center for Biotechnology (NCBI) database using Basic Local Alignment Search Tool (BLAST) (http://www.ncbi.nih.gov/BLAST/). For 16S rRNA (ribosomal ribonucleic acid) sequencing, total genomic DNA (deoxyribonucleic acid) was extracted from the nutrient broth. The partial sequence of the 16S rRNA gene was amplified using a polymerase chain reaction (PCR) and bacterial universal primers specific to 16S rRNA gene. For further characterization of the microorganism the rpoB gene fragment which encodes the β subunit of RNA polymerase was investigated. The rpoB gene fragment was amplified from the genome DNA of *B. amyloliquefaciens* and *B. subtilis* by PCR using the following primers 5'-ATC GAA ACG CCT GAA GGT CCA AAC AT-3' and 5'-ACA CCC TTG TTA CCG TGA CGA CC-3'. The data base used was the Blast NCBI, and sequence comparison program used was Chromas Lite.

Central Composite Design (CCD) and Response Surface Methodology (RSM). The CCD using two levels and three factors was composed of 17 runs with 3 repetitions at the central point, needed to calculate the pure error. The goodness of fit was evaluated by the adjusted coefficient of determination (R2 adj) and the analysis of variance (ANOVA), as well as comparative analysis by the plot of the predicted values with the observed experimental values. The independent variables were soybean flour concentration (1.0, 2.0, and 3.0% w/v); glucose concentration (0.5, 1.0, and 1.5% w/v) and agitation rate (100, 150, and 200 rpm) and fibrinolytic activity (U mL^{-1}) was the response variable.

Statistical significance of the variables was determined at 5% probability level ($p<0.05$). The analysis of the results was carried with the program Statistic version 8.0. RSM using CCD was employed to optimize the selected three variables by the second order polynomial Eq. (1):

$$Y = b_0 + \sum b_i x_i + \sum b_{ii} x_{ii}^2 + \sum b_{ij} x_i x_j \quad (1)$$

where Y is the response (fibrinolytic activity); x_i and x_j represent the independent variables; b_0, b_i, b_{ii}, b_{ij}, were the coefficients of linear terms, square terms, and coefficients of interactive terms, respectively.

Determination of Total Protein. Total protein concentration was determined by the Bradford method using as standard bovine serum albumin (BSA).[25]

Degradation of Blood Clots. The used blood was extracted from the jugular of healthy young horses. The required volume of blood for clot formation was standardized and added

without anticoagulant in test tubes having its walls previously wetted with saline solution. After blood clotting, the tubes were placed in water bath at 37 °C for 1 h to separate the serum from the clot of fibrin. The retracted clots were washed with saline solution up to the obtention of a translucent solution. Then, the clots were transferred to test tubes with enzyme extract and kept at rest for 1 h at 37 °C. The sizes of the clots were standardized to 50% (w/v) in relation to the volume of enzyme extract. A saline solution was used as a negative control. The percentage of degradation of the clot (PDC) was calculated according to Eq. (2), where weight$_{t_0}$ (zero) is the weight of the clot formed spontaneously before the treatment with the enzyme extract. The weight$_{t_r}$ is the weight of the residue remaining after treatment with the enzyme extract.[26]

$$\mathrm{PDC} = \left(\frac{\mathrm{Weight}_{t_0} - \mathrm{Weight}_{t_r}}{\mathrm{Weight}_{t_0}}\right) \times 100 \quad (2)$$

Fibrinolytic Activity Determination by Fibrin Plate. The fibrin plate method[27] was used to evaluate the fibrinolytic activity in the screening process. The fibrin plate contained 4 mL of 2 mg mL^{-1} fibrinogen solution (Fibrinogen from bovine plasma in Tris-HCl 150 mM containing NaCl 150 mM pH 7.7), 200 μL solution of thrombin from bovine plasma (20 U mL^{-1} diluted in saline); 4 mL of agarose solution (2%) and 100 mL of $CaCl_2$ solution (1 M). The reaction mixture was placed in plastic Petri dishes. After fibrin polymerization, 5 mm diameter holes were made, where 20 μL of enzymatic extract were placed. Then the plates were incubated at 37 °C for 18 h and the diameter of the halos measured. Assays for the standard curve were performed in triplicate and the values of the diameters of the halos were obtained by average of the repetitions. One unit (U) of fibrinolytic activity corresponds to the correlation between the diameter of the degradation halos in fibrin plate (mm) and the standard curve made using plasmin from human plasma. The activity was expressed in U·mL^{-1}.

Fibrinolytic Activity (FA) Determination by Spectrometry. The fibrinolytic activity[28] of the enzyme extract obtained after the optimization experiments was measured as follows: a reaction mixture containing 0.1 mL of 245 mM phosphate buffer (pH 7.0) and 0.4 mL of 0.72% fibrinogen solution was incubated at 37 °C for 5 min; then, 0.1 mL of a 20 U·mL^{-1} thrombin solution was added and the reaction mixture was again incubated at 37 °C for 10 min. After clot formation, 0.1 mL of diluted enzyme solution was added, and incubation continued at 37 °C for 60 min, the reaction mixture being shaken every 20 min. The reaction was stopped by adding 0.7 mL of 0.2 M trichloroacetic acid (TCA). The reaction mixture was centrifuged at 15000 *g* for 10 min, and the absorbance of the supernatant was measured at 275 nm by spectrophotometry. In this assay, one unit of fibrinolytic protease activity was defined as the amount of enzyme required to produce an increase in absorbance equal to 0.01 per minute, equivalent to the release of tyrosine. All experiments were performed in triplicate.

Determination of Optimum pH and Temperature of the Enzyme. Optimum pH and temperature of the fibrinolytic enzyme activity was determinated. The optimum pH was determinated at 37 °C at pH values between 3.0 and 10.0. The buffers used were Glycine-HCl (pH 3.0), Sodium acetate (pH 4.0-5.0), Citrate phosphate (pH 6.0), Tris-HCl (pH 7.0-8.0), Glycine-NaOH (pH 9.0-10.0). The concentration of the buffers was standardized at 20 mM. The optimum temperature was determinate at pH 7.0 and the range of values was from 4 to 80 °C.

Effect of pH and Temperature on the Stability of the Enzyme. The effect of pH and temperature on enzyme stability was evaluated for 12 h; aliquots were removed at intervals of 2 h to determinate the residual enzyme activities.

Effect of Metal Ions and Protease Inhibitors on Fibrinolytic Activity. The inhibitors and metal ions were dissolved in 150 mM Tris-HCl buffer (NaCl 150 mM pH 7.75); the concentration of the solutions was standardized at 5 mM. The enzyme extract was incubated for 1 h at 37 °C. The inhibitors investigated were PMSF (fluoride methylphenylsulfonyl), EDTA (ethylenediaminetetraacetic acid), *β*-mercaptoethanol (2-hydroxy-1-ethanethiol - C_2H_6SO), Pepstatin A (4-amino-3-hidroxy-6-methyl-heptanoic - $C_{34}H_{68}N_5O_9$). The metal ions evaluated were $FeSO_4$, $CuSO_4$, $CaCl_2$, $MgSO_4$, $CoCl_2$, $ZnSO_4$, KCl, and $MgCl_2$.

Results and Discussion

Screening of Fibrinolytic Enzyme Producer. Forty-three strains of *Bacillus* were evaluated, among which thirty (70%) exhibited fibrinolytic activity (Table I). These results show the potential of the genus *Bacillus* as a producer of fibrinolytic enzymes. Other researchers[29] isolated microorganisms from the Chinese soybean cheese doufuru as well as Japanese natto and Chinese douche and, among the isolates obtained, sixteen microorganisms showed fibrinolytic activity, the highest value of 1833 U mL^{-1} obtained by *Bacillus subtilis*. In another work,[30] *Bacillus* sp. strain AS-S20-I was the best producer of fibrinolytic proteases with a maximum activity of 145.8 U mL^{-1}. This value was lower than the one obtained by *Bacillus* sp. UFPEDA 485, the highest producer in this work, that presented a fibrin degradation halo with 29 mm diameter (Figure 1), corresponding to a fibrinolytic activity of 720 U mL^{-1}.

Bacteria Identification Using 16S and rpoB Genes. The identification based on 16S rRNA gene sequence analysis showed 99% similarity homologies with the species *Bacillus amyloliquefaciens* and *Bacillus subtilis*. To differentiate between these two *Bacillus* genus, rpoB region analysis was carried out which showed higher identity to *Bacillus amyloliquefaciens* species. The Blast from the consensus sequence revealed about 98% homology with the region DNA-directed RNA polymerase *β*-subunit of the species *Bacillus amyloliquefaciens* and 89% homology to the same region of the *Bacillus subtilis* species. Identification by the rpoB region sequencing analysis offered

Table I. Microorganisms of the Genus *Bacillus* and Their Fibrinolytic Activity after 48 h of Cultivation in Soybean Medium (MS-2), pH 7.2, 150 rpm, and 37 ºC

Microorganisms	FA (U mL^{-1})[a]	Microorganisms	FA (U mL^{-1})[a]
B. subtilis UCP 999	-[b]	*B. circulans* UFPEDA 436	267 ± 0.83
B. licheniformis UCP 1008	342 ± 1.01	*Bacillus* sp. UFPEDA 437	22.5 ± 0.93
B. licheniformis UCP 1009	1.1 ± 0.02	*Bacillus* sp. UFPEDA 449	342 ± 1.61
B. licheniformis UCP 1010	-[b]	*Bacillus* sp. UFPEDA 450	99.3 ± 0.54
B. licheniformis UCP 1013	60.6 ± 1.07	*Bacillus* sp. UFPEDA 451	-[b]
B. licheniformis UCP 1016	163 ± 0.92	*B. cereus* UFPEDA 452	-[b]
B. licheniformis UCP 1033	163 ± 0.82	*B. alvei* UFPEDA 461	99.3 ± 1.33
B. licheniformis UCP 1477	163 ± 0.82	*Bacillus* sp. UFPEDA 464	163 ± 0.73
B. licheniformis UCP 1482	99.3 ± 0.89	*Bacillus* sp. UFPEDA 465	36.9 ± 0.10
B. cereus UFPEDA 11	-[b]	*Bacillus* sp. UFPEDA 466	-[b]
Bacillus. sp. UFPEDA 12	-[b]	*Bacillus* sp. UFPEDA 469	267 ± 0.00
Bacillus. sp. UFPEDA 13	13.7 ± 0.74	*B. licheniformis* UFPEDA 470	-[b]
B. mycoides UFPEDA 14	8.4 ± 0.08	*B. firmus* UFPEDA 471	-[b]
B. subtilis UFPEDA 15	-[b]	*B. pumilus* UFPEDA 472	209 ± 1.49
B. subtilis UFPEDA 16	-[b]	*B. pumilus* UFPEDA 474	60.6 ± 1.46
B. subtilis UFPEDA 86	8.4 ± 0.09	*Bacillus* sp. UFPEDA 483	267 ± 0.08
B. megaterium UFPEDA 108	3.1 ± 0.09	*Bacillus* sp. UFPEDA 484	-[b]
B. subtilis v. aterrimus UFPEDA 170	0.51 ± 0.08	*Bacillus* sp. UFPEDA 485	720 ± 0.08
Bacillus. sp. UFPEDA 189	267 ± 1.51	*Bacillus* sp. UFPEDA 486	4.0 ± 0.08
Bacillus. sp. UFPEDA 194	47.3 ± 0.74	*Bacillus* sp.UFPEDA 487	-[b]
B. subtilis UFPEDA 260	5.1 ± 0.10	*Bacillus* sp.UFPEDA 488	1.5 ± 0.03
B. subtilis UFPEDA 404	209 ± 1.03		

[a]FA - Fibrinolytic activity by fibrin plate method and correlated with a standard curve of plasmin. [b]Absence of FA (-).

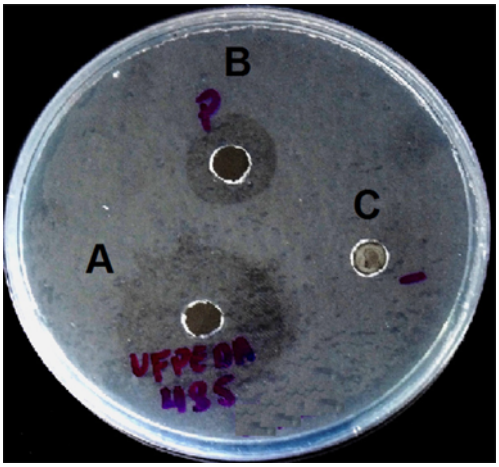

Figure 1. Halo fibrin degradation of the fibrinolytic enzyme of *Bacillus* sp. UFPEDA 485 after 48 h incubation (A), positive control with plasmin (B), and negative control with physiological solution (C).

advantages over the 16S because the rpoB gene has a higher degree of polymorphism compared to 16S, making it more accurate for species identification. The heterogeneity of the 16S rRNA gene hampers the quantification of bacterial species by PCR based assays. In contrast, the rpoB gene is common to all bacteria and occurs as a single copy in the genome. This also was observed by Ki[31] that identified thirteen different *Bacillus* species by 16S and rpoB genes and observed levels of similarity of 90.3% to *B. hwajinpoensis* and *B. sporothermodurans* and 99.8% to *B. anthracis* and *B. cereus*.

In this work, analysis of 16S and rpoB region confirmed that the sample tested has a higher homology to the *Bacillus amyloliquefaciens* and demonstrate that the polymorphism of the *Bacillus* rpoB gene can be used to identify *Bacillus* species, providing improvement over conventional methods in the identification of *Bacillus* species. The result was submitted to the GenBank database.

Optimization of the Conditions for Fibrinolytic Enzyme Production from *Bacillus Amyloliquefaciens* UFPEDA 485. The optimization of the conditions for the production of the

Table II. Matrix of the Central Composite Design (CCD) Star for the Optimization of the Conditions for Production of the Fibrinolytic Enzyme from *Bacillus Amyloliquefaciens* UFPEDA 485

Runs	Soybean Flour (%)	Glucose (%)	Agitation (rpm)	Fibrinolytic Activity (U mL^{-1})
1	1.0 (-)	0.5 (-)	100 (-)	462 ± 0.014
2	1.0 (-)	1.5 (+)	200 (+)	682 ± 0.003
3	3.0 (+)	0.5 (-)	200 (+)	666 ± 0.013
4	3.0 (+)	1.5 (+)	100 (-)	492 ± 0.004
5 (C)	2.0 (0)	1.0 (0)	150 (0)	702 ± 0.006
6	1.0 (-)	0.5 (-)	200 (+)	710 ± 0.008
7	1.0 (-)	1.5 (+)	100 (-)	419 ± 0.005
8	3.0 (+)	0.5 (-)	100 (-)	413 ± 0.009
9	3.0 (+)	1.5 (+)	200 (+)	696 ± 0.011
10 (C)	2.0 (0)	1.0 (0)	150 (0)	701 ± 0.003
11	1.0 (-)	1.0 (0)	150 (0)	636 ± 0.004
12	3.0 (+)	1.0 (0)	150 (0)	613 ± 0.004
13	2.0 (0)	0.5 (-)	150 (0)	607 ± 0.026
14	2.0 (0)	1.5 (+)	150 (0)	604 ± 0.007
15	2.0 (0)	1.0 (0)	100 (-)	538 ± 0.006
16	**2.0 (0)**	**1.0 (0)**	**200 (+)**	**813 ± 0.014**
17 (C)	2.0 (0)	1.0 (0)	150 (0)	701 ± 0.004

fibrinolytic enzyme from *Bacillus amyloliquefaciens* UFPEDA 485 was conducted according to a CCD star 2^3 with two levels and three factors and the response variable was fibrinolytic activity (FA) after 48 h of cultivation (Table II).

The increase of the concentration of the independent variables - soybean flour and glucose and its interaction had a linear effect positive and statistically significant on the increase of the fibrinolytic activity. For the quadratic effect, the increase of the concentration of the independent variables soybean flour, glucose and its interaction was statistically significant and had a negative effect on the increase of the fibrinolytic activity. The results from the analysis of variances and effect estimates for the main factors are shown in Table III. The variation around the mean of the residuals is fully explained by pure error, because there is no evidence of lack of fit, and a good model fit is obtained. The *p* value for lack of fit was < 0.00. The validity of the model is confirmed by the ANOVA analysis.

The quality of the model can be confirmed by the proximity of the data with the straight line shown in Figure 2. The distribution of the data points along the diagonal line indicates the good fit of the model.

Using the obtained values in presented the Table II, the corresponding multiple regression equation was calculated. The quadratic effect of the variable (X_3) was ignored in Eq. (3) as its effect was not statistically significant.

From the regression analysis, Eq. (3) was obtained:

Table III. Analysis of Variance (ANOVA) for the Response Fibrinolytic Activity, Over the Independent Variables Soybean Flour (%), Glucose (%) and Agitaton (rpm), According to CCD Star, with 95% Confidence Level

Factor	Fibrinolytic Activity	
	Estimates Effect	*p* Value
(1) Soybean flour (%) (L)	178	0.00
Soybean flour (%) (Q)	-51.3	0.00
(2) Glucose (%) (L)	506	0.00
Glucose (%) (Q)	-282	0.00
(3) Agitation (rpm) (L)	2.93	0.00
1L by 2L	45.0	0.00
1L by 3L	-0.14	0.00
2L by 3L	-0.17	0.00
Regression Coefficient ANOVA		
Pure Error = 0.18	$R^2 = 0.99$	$R^2_{adjusted} = 0.97$

$$Y = -151 + 178\,X_1 - 51.3\,X_1^2 + 506\,X_2 - 282\,X_2^2 + 2.93\,X_3 + 45\,X_1{\cdot}X_2 - 0.14\,X_1{\cdot}X_3 - 0.17\,X_2{\cdot}X_3 \quad (3)$$

The optimum conditions for the production of FA were 1.88% (w/v) of soybean flour and 0.988% (w/v) of glucose at 200 rpm. Under these conditions the calculated value was

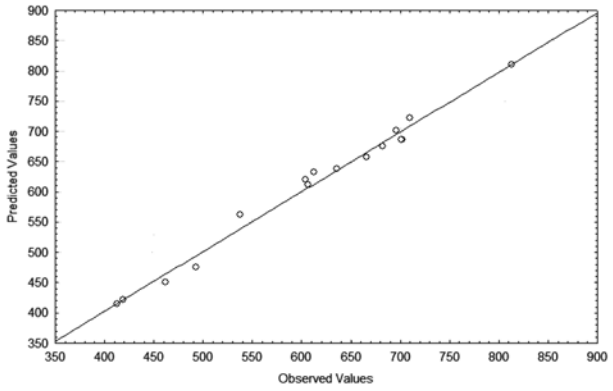

Figure 2. Relationship between the experimental values and the predicted values by the model for fibrinolytic activity (FA), according to central composite design (CCD) star.

808 U mL^{-1}, very close to the value of 813 U mL^{-1}, observed when the FA was calculated for the experimental conditions - soybean flour (2%), glucose (1%), and agitation (200 rpm). This makes irrelevant to adjust the optimum conditions from 1.88% to 2% in soybean flour concentration and of 0.99% to 1% in glucose concentration.

Response surface plot showing the effect of the independent variables soyben flour (% w/v) and glucose (% w/v) concentration with agitation at 200 rpm, after 48 h of cultivation on the response variable fibrinolytic activity of *Bacillus amyloliquefaciens* UFPEDA 485 is displayed in Figure 3.

Previous researches have proved that glucose is a preferred carbon source for the growth of *Bacillus* spp. Other carbon

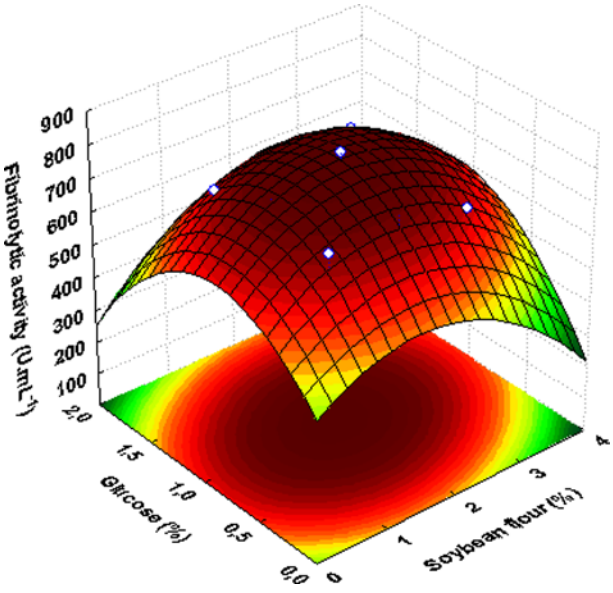

Figure 3. Response surface plot showing the effect of independent variables: soyben flour (%) and glucose (%) with agitation at 200 rpm, after 48 h of cultivation on the response variable fibrinolytic activity of *Bacillus amyloliquefaciens* UFPEDA 485.

sources such as fructose, maltose, lactose, sucrose and glycerol have shown minimum solubility, poor cellular uptake and unsuitable metabolic pathway reducing the production of fibrinolytic enzymes.[14] An optimum glucose concentration of 1.25% w/v for maximum fibrinolytic activity from *Bacillus sphaericus* MTCC 3672[14] was reported, a result similar to the value obtained in this work.

Other researchers optimized the production of fibrinolytic enzyme by Plackett-Burman Design, using soybean as a nitrogen source. In this case, maximum activity obtained in submerged fermentation was 797.28 U mL^{-1} at 72 hours, using soybean meal at a concentration of 1.5% (w/v).[32] In another study, the optimum conditions at laboratory scale for the maximal production of the fibrinolytic enzyme were obtained at pH 7.2, temperature 37 ºC and agitation 200 rpm, for a maximal activity of 28.98 U mL^{-1} at 72 h.[33] Unlike this study, using a central composite design (star) 2^3 in shake flasks that achieved the best result for fibrinolytic activity using soybean flour (2% w/v) and glucose (1% w/v) in 48 h and showed better performance in production process.

Evaluation of Fibrinolytic Activity by Degradation Of Blood Clots. The enzyme extract produced by *Bacillus amyloliquefaciens* UFPEDA 485 showed a high effectiveness in degrading blood clots *in vitro*. After 1 h, the results showed a PDC of 62% while the negative control containing saline solution kept the clot intact (Figure 4). The degradation of blood clots goat after 24 h using crude extract from the *Paenibacillus* sp. IND8[34] was also evaluated and a complete degradation of the human blood clot after 18 h using the enzyme from *Bacillus* sp. SFN[35] was observed. Fibrinolytic protease from *Fusarium* sp. was able to degrade 36.5% of mouse blood clots at room temperature for 1 h.[26] The potencial of the enzyme from *Fusarium* sp. to degrade blood clots is much lower when compared with the fibrinolytic enzyme from *Bacillus amyloliquefaciens* UFPEDA 485 produced in this work, that shows a two times higher capacity for the degradation of blood clots within 1 h.

Effects of pH and Temperature on the Activity and Stability of the Enzyme. The enzyme showed higher values of fibrinolytic activity at neutral pH, with an optimum at pH 7.0 (Figure 5(a)). After incubation for 12 h, the enzyme retained

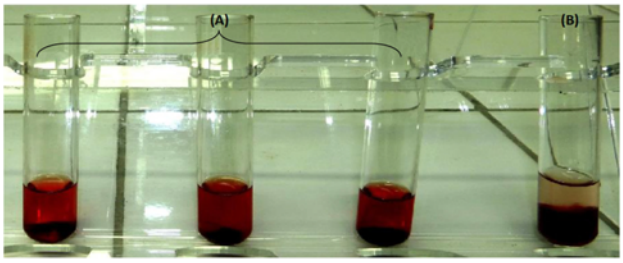

Figure 4. Degradation of blood clots by dispersion of red blood cells. (a) Blood clots after 1 h in the enzyme extract from *Bacillus amyloliquefaciens* UFPEDA 485 (in triplicate), (b) blood clot after 1 h in physiological solution.

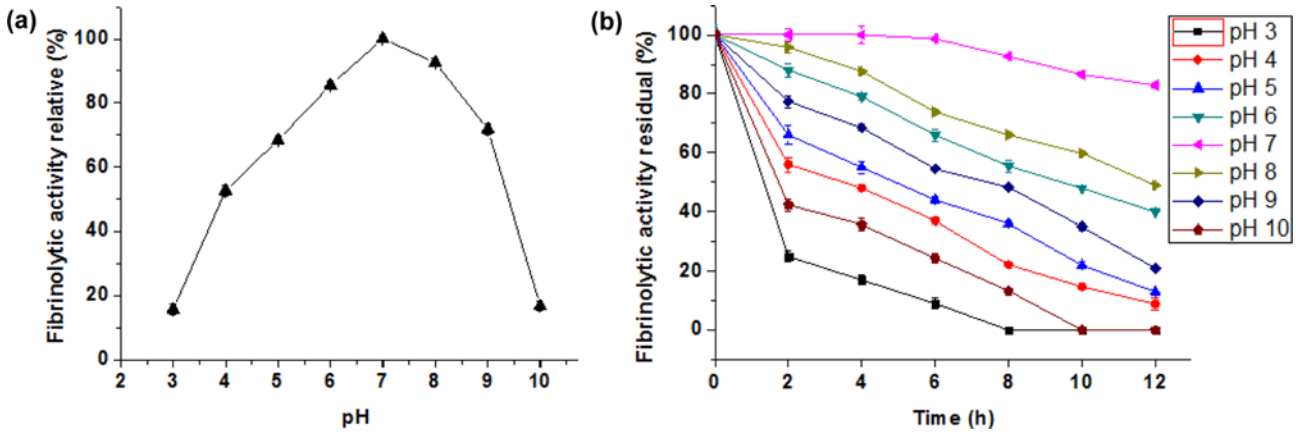

Figure 5. (a) Effect of pH on the fibrinolytic activity relative of the enzyme from *Bacillus amyloliquefaciens* UFPEDA 485 after 1 h of incubation. (b) Effect of pH on the stability of enzyme measured at intervals of 2 h per 12 h of incubation of the enzyme and expressed as percentage of residual activity. Buffers used: Glycine-HCl (pH 3.0), Sodium acetate (pH 4.0-5.0), Citrate phosphate (pH 6.0), Tris-HCl (pH 7.0-8.0), Glycine-NaOH (pH 9.0-10.0). All buffer concentrations were 20 mM. Each value is the average of the results of three experiments, and the error bars show the standard deviations.

82.89% of its activity at pH 7.0, 66.03% at pH 8.0, and 50% at pH 6.0 and 9.0 after 8 h of incubation (Figure 5(b)). Results corroborate with other researchers,[12] that studied a fibrinolytic enzyme produced by *Bacillus licheniformis* B4 - an optimum pH of 7.5 was observed with the enzyme retained 100% of its activity at pH 7.0 after 30 min of incubation. Fibrinolytic protease from *Bacillus cereus* NS-2 had an optimum pH of 9.0 and its activity was lost in just 30 min.[22] The fibrinolytic enzyme produced by *Bacillus amyloliquefaciens* UFPEDA 485 is very stable in physiological conditions, unlike other fibrinolytic enzymes produced by other species of the genus *Bacillus*.

The optimum temperature of the obtained fibrinolytic enzyme was 37 °C (Figure 6(a)). After 12 h of incubation, the enzyme remained stable at 4, 25 and 37 °C, maintaining activities of 96.04, 95.88, and 98.26%, respectively, and still maintained 58.84% of its activity at 40 °C (Figure 6(b)). Juamily and Al-Zaidy[12] related that fibrinolytic enzyme from *Bacillus lichniformis* B4 has an optimum temperature of 37 °C and retained its total activity after 1 h of incubation when maintained at 20-40 °C; then the activity decreased with the increase of temperature. in other study,[22] an optimum temperature of 40 °C and the enzyme lost 40-60% of its activity at each temperature of incubation. These results confirm that the fibrinolytic enzyme produced by *Bacillus amyloliquefaciens* UFPEDA 485 has an excellent stability at the physiological temperature of the blood, different from enzymes produced by others *Bacillus* strains cited in the literature.

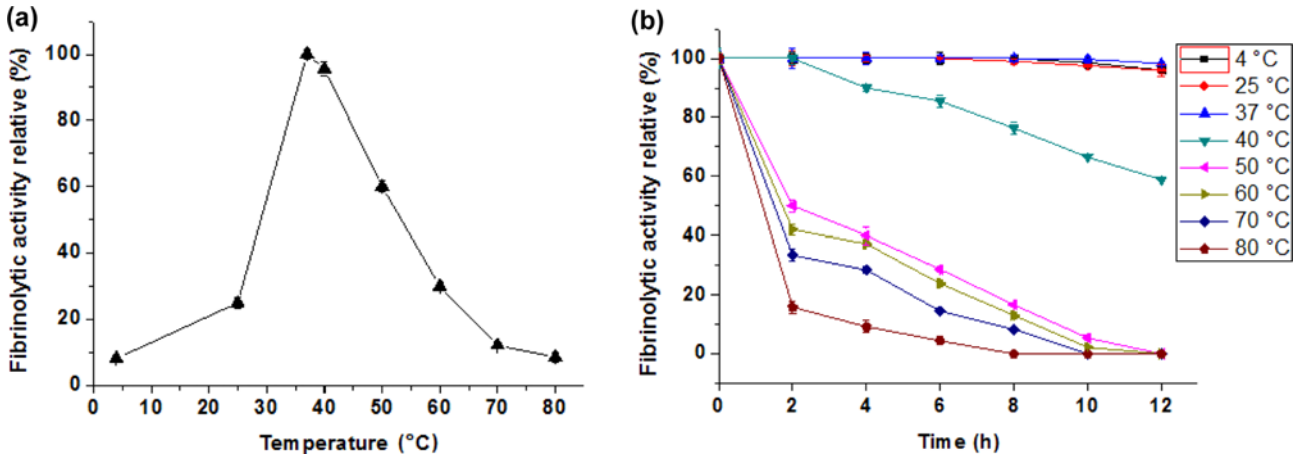

Figure 6. (a) Effect of temperature on the fibrinolytic activity relative of the enzyme from *Bacillus amyloliquefaciens* UFPEDA 485 after 1 h of incubation. (b) Effect of temperature on the stability of enzyme measured at intervals of 2 h per 12 h of incubation of the enzyme and expressed as percentage of residual activity. Each value is the average of the results of three experiments, and the error bars show the standard deviations.

Table IV. Effects of Inhibitors on the Fibrinolytic Activity of the Enzyme

Inhibitors and Metal Ions	Relative Activity[a] (%)
Control	100
PMSF	8.48 ± 0.01
EDTA	10.4 ± 0.02
Pepstatin A	48.2 ± 0.04
β-Mercaptoethanol	36.2 ± 0.02

[a]The values are presented as means ± SD (standard deviation).

Effects of Inhibitors and Metal Ions on the Fibrinolytic Activity. The fibrinolytic activity was almost completely inhibited after 60 min of incubation with PMSF (8.48%) and EDTA (10.4%) in Table IV. The results show that the enzymatic extract from *Bacillus amyloliquefaciens* UFPEDA 485 contains serine-metallo proteases, indicating that the hydroxyl (serine) group is located at or near the active site of one of the enzymes contained in the extract, hence being inhibited by PMSF (serine protease inhibitor); moreover, divalent metals seem to be required for the enzyme to maintain its activity, as an inhibition by chelating agents such as EDTA (metallo protease inhibitor) occurs. Enzyme activity was less inhibited by Pepstatin A (48.2%), an aspartic protease inhibitor and β-mercaptoethanol (36.2%), a cysteine protease inhibitor.

Enzyme activity was significantly inhibited by $FeSO_4$ (6.82%) and slightly increased by $CaCl_2$ (110%) (Table V). Both the activation as the inhibition of the enzyme activity occurs due to allosteric effects. The connection of a chemical substance with the allosteric site of an enzyme may induce conformational changes in the spatial structure of the enzyme, changing the affinity by its substrate.

The influence of protease inhibitors in fibrinolytic enzyme activity was also reported by other authors[36] that observed a significant inhibition in the presence of PMSF and EDTA; in these situations the enzyme activity was 0% and 3.35%, respectively. The enzyme was also classified as a serine-metallo protease and similar results to those reported in this work were obtained concerning the effect of metal ions as the enzyme when incubated with $CaCl_2$, increased slightly its fibrinolytic activity to 118.15%.

Table V. Effects of Metal Ions on the Fibrinolytic Activity of the Enzyme

Inhibitors and Metal Ions	Relative Activity[a] (%)
Control	100
$FeSO_4$	6.82 ± 0.04
$CuSO_4$	50.0 ± 0.05
$MgSO_4$	94.5 ± 0.03
$ZnSO_4$	52.6 ± 0.04
$CoCl_2$	48.3 ± 0.06
$MgCl_2$	96.8 ± 0.06
$CaCl_2$	110 ± 0.02
KCl	98.6 ± 0.01

[a]The values are presented as means ± SD (standard deviation).

The influence of protease inhibitors was also evaluated[37] and unlike this work, the enzyme when incubated with PMSF and EDTA, showed an activity of 2% and 92%, respectively, being considered a serine protease. For the effect of metal ions similar results to those reported in this work were obtained as the enzyme activity slightly increased (to 111%) in the presence of $CaCl_2$ and was reduced to 41% and 0%, respectively, when incubated with $CoCl_4$ and $FeSO_4$.

Conclusions

From the 43 strains evaluated, *Bacillus amyloliquefaciens* UFPEDA 485 was selected as the one producing the highest fibrinolytic activity. The optimized culture medium for the production of the fibrinolytic enzyme from *Bacillus amyloliquefaciens* UFPEDA 485 contained 1.88% (w/v) of soybean flour and 0.99% (w/v) of glucose. The fibrinolytic protease from *Bacillus amyloliquefaciens* UFPEDA 485 was shown to have biochemical characteristics suitable for human and veterinary applications, namely the best activity at physiological conditions as well as the high thermal stability. These characteristics differ from fibrinolytic enzymes produced by other microorganisms described in the literature up to date. These results demonstrate the biotechnological potential of *Bacillus amyloliquefaciens* UFPEDA 485 for the production of a protease suitable for the pharmaceutical industry and its possible application on the treatment of vascular disorders.

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