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Integrated Process Production and Extraction of the Fibrinolytic Protease from *Bacillus* sp. UFPEDA 485

Amanda Emmanuelle Sales · Fabiana América Silva Dantas de Souza · José Antônio Teixeira · Tatiana Souza Porto · Ana Lúcia Figueiredo Porto

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Abstract Fibrinolytic proteases are enzymes that degrade fibrin; these enzymes are a promising alternative for thrombolytic therapy, and microorganisms produce them. The aim of this study was to evaluate the optimum conditions for the integrated production and purification of fibrinolytic protease from *Bacillus* sp. UFPEDA 485. Extractive fermentation was carried out in a culture medium containing soybean flour and by adding polyethylene glycol (PEG) and Na_2SO_4 according to a 2^3 experimental design. In all assays, the enzyme preferentially partitioned to the bottom phase ($K<1$), with an optimum activity of 835 U ml^{-1} in the bottom phase (salt-rich phase). The best conditions for extractive fermentation were obtained with 18 % PEG 8000 and 13 % Na_2SO_4. Characterization showed that it is a metalloprotease, as a strong inhibition—residual activity of 3.13 %—occurred in the presence of ethylenediaminetetraacetic acid. It was also observed that enzymatic activity was stimulated in the presence of ions: $CaCl_2$ (440 %), $MgCl_2$ (440 %), $FeSO_4$ (268 %), and KCl (268 %). The obtained results indicate that the use of a low-cost substrate and the integration of fermentation with an aqueous two-phase system extraction may be an interesting alternative for the production of fibrinolytic protease.

Keywords Fibrinolytic protease · *Bacillus* · PEG/sodium sulfate · ATPS · Extractive fermentation

A. E. Sales · F. A. S. D. de Souza · T. S. Porto · A. L. F. Porto (✉)
Department of Morphology and Animal Physiology, Federal Rural University of Pernambuco, 52171-900 Recife, PE, Brazil
e-mail: analuporto@yahoo.com.br

J. A. Teixeira
Institute for Biotechnology and Bioengineering, Centre of Biological Engineering, University of Minho, Campus of Gualtar, 4710-057 Braga, Portugal

T. S. Porto
Academic Unit of Garanhuns, Federal Rural University of Pernambuco, 55296-901 Garanhuns, PE, Brazil

Introduction

Proteases are hydrolytic enzymes which degrade proteins into amino acids and peptides. Such proteases are physiologically necessary for all living organisms and they are found in a wide variety of sources, including plants, animals, and microorganisms [1]. Fibrinolytic proteases are enzymes that have the potential to degrade fibrin, the major protein component of blood clots, which are formed from the activation of fibrinogen by thrombin [2]. Fibrinolytic proteases that are produced by bacteria of the *Bacillus* genus have attracted interest as thrombolytic agents due to their efficiency in the fibrinolytic process [3].

Cardiovascular diseases, such as acute myocardial infarction, ischemic heart disease, and high blood pressure, are the leading causes of death in the world. Among the different types of cardiovascular diseases, thrombosis is one of the most widely occurring diseases in modern life. Drugs using fibrinolytic enzymes are the most effective methods in the treatment of thrombosis [4].

Extractive fermentation and product recovery "in situ" have been suggested as a solution to overcome product inhibition and the low productivity of biotechnological processes [5, 6]. The concept of this "in situ" purification process involves the integration of an extractive step as the first stage of downstream processing to simultaneously synthesize and remove the product. This is not only to ensure primary recovery but also to increase the product formation rate by minimizing inhibition by the end product during fermentation [6].

A significant number of processes based on extractive fermentation using an aqueous two-phase system (ATPS) have been reported. Examples include the production of fibrinolytic protease [5], alkaline phosphatase [7], clavulanic acid [8], lipase [9], and products from cyanobacteria [10].

Aqueous two-phase systems are formed by adding two (or more) polymers or a water-soluble polymer and a specific salt in aqueous solution above certain critical concentrations and temperatures, resulting in two immiscible aqueous phases. Solutes of various sizes, such as inorganic ions, small organic molecules, biological macromolecules and inorganic colloidal particles, viruses, and cells, can be partitioned between the phases of ATPS [11].

There have been some prior attempts made by the industry to optimize procedures that ensure high yields with considerable purity [12]. In this regard, partitioning in ATPS provides a powerful method for separating and purifying biomolecules such as proteins. The ATPS also offers advantages such as short processing time, low power consumption, and a biocompatible environment for the biomolecule because of the large amount of water in the extraction systems. Thus, the ATPS has been recognized as an efficient and economical process for the recovery of biomolecules due to its relative low cost and ease of operation [12–14].

The objective of this study was to evaluate the optimum conditions for the integrated production and purification of fibrinolytic protease by *Bacillus* sp. UFPEDA 485 in extractive fermentation using a low-cost medium with polyethylene glycol (PEG)/Na_2SO_4, as phase-forming compounds, and the biochemical characterization of this enzyme.

Materials and Methods

Microorganism

The microorganism *Bacillus* sp. UFPEDA 485 was supplied by the Collection of Microorganisms, Department of Antibiotics at the Federal University of Pernambuco. This

microorganism was isolated from the fermentation of sugar cane mills native from the *Zona da Mata* of Pernambuco, Brazil. The microorganism was stored in nutrient broth (1 % peptone, 0.3 % meat extract, and 0.5 % NaCl) in cryotubes (10 % *v*/*v* glycerol) at −70 °C.

Culture Conditions

The microorganism was grown in nutrient broth, 1 % peptone, 0.3 % beef extract, and 0.5 % NaCl (pH 7.0). After 24 h growth in nutrient broth, 10-ml aliquots of this culture were transferred to conical flasks of 250 ml containing 90 g of extractive fermentation medium using ATPS.

For protease production (submerged fermentation and extractive fermentation), soybean medium (MS-2) described by Porto et al. [15] was used. The medium composition was soybean filtrate (2 % *w*/*v*), K_2HPO_4 (0.435 % *w*/*v*), and 1 ml of mineral solution containing $FeSO_4{\cdot}7H_2O$ (100 mg), $MnCl_2{\cdot}4H_2O$ (100 mg), and $ZnSO_4{\cdot}H_2O$ (100 mg) of distilled water q.s.p. 100 ml, NH_4Cl (0.1 % *w*/*v*), $MgSO_4{\cdot}7H_2O$ (0.06 % *w*/*v*), and glucose (1 % *w*/*v*).

Medium for Extractive Fermentation Using ATPS

The culture medium for extractive fermentation using ATPS was prepared by mixing PEG and sodium sulfate solutions according to the experimental design described in the succeeding section in this study and the culture medium MS-2. After adjusting the pH to 7.2, the system was autoclaved at 121 °C for 20 min.

Extractive fermentation lasted for 84 h and was performed at 150 rpm agitation and 37 °C under different conditions according to a factorial design. At the end of the process, the flasks were left to settle for 1 h and further centrifuged at 12,000×*g* for 15 min to separate both phases (PEG-rich or top and bottom or salt-rich). After that, the phases were subjected to the analytical determinations (protein content and fibrinolytic activity).

Factorial Design 2^3

The influence of phase-forming parameters in the fibrinolytic protease extractive fermentation process was performed according to a 2^3 factorial design shown in Table 1. The independent variables PEG molecular weight and PEG and salt (sodium sulfate) concentrations were selected according to Ashipala and He [5]. The response variables were the partition coefficient (*K*) and top (FA_t) and bottom (FA_b) phase activities. The experimental design consisted of 12 trials and 4 replicates in the central point, necessary to calculate the pure error.

Table 1 Levels of the independent variables of the full factorial design 2^3 for the extractive fermentation process of the fibrinolytic protease from *Bacillus* sp. UFPEDA 485

Variables	Levels		
	(−1)	(0)	(+1)
PEG molar mass (g mol^{-1})	4,000	6,000	8,000
PEG concentration (% *w*/*w*)	18	24	30
Sodium sulfate concentration (% *w*/*w*)	10.0	11.6	13.0

The effects were evaluated by an analysis of variance with a significance level of 95 % to make estimates of the main and second-order effects a linear, absolute value of the factors in relation to the response variables studied. Statistical analysis of the experimental design was performed using the software Statistica 8.0 (Statsoft Inc., Tulsa, OK, USA).

Determination of Total Protein

The protein content was determined by the Bradford method [16] using bovine serum albumin as a standard. Each experiment was performed in triplicate and the average value was then calculated after correction of the corresponding blank.

Determination of Fibrinolytic Activity on Fibrin Plates

Fibrinolytic activity was determined by the fibrin plate method [17]. The fibrin plate was composed of 2 mg ml^{-1} of fibrinogen solution (fibrinogen from bovine plasma in buffer 150 mM Tris–HCl and 150 mM NaCl, pH 7.75), and 200 μl of thrombin from bovine plasma solution (20 U ml^{-1} diluted in 150 mM sodium chloride solution) was added to 4 ml of fibrinogen solution, 4 ml of 2 % agarose, and 100 μl of 1 M $CaCl_2$ solution. The reaction mixture with the agarose was placed in plastic Petri dishes. After the polymerization of fibrin, 20 μl of enzymatic extract was added to 5-mm-diameter wells and then incubated at 37 ° C for 18 h. One unit of fibrinolytic activity corresponds to the correlation between the diameter of the degradation halos in the fibrin plate (in millimeters) and the standard curve made using plasmin from human plasma. The enzymatic activity was expressed as units per milliliter.

Calculating the pure error was difficult due differences in fibrinolytic activity values at the factorial design central points, so the determination of fibrinolytic activity was also carried out with the fibrin degradation assay. However, it is possible to establish an excellent correlation between the two methods.

Determination of Fibrinolytic Activity

Fibrinolytic activity was also measured using the fibrin degradation assay. First, 0.4 ml of 0.72 % fibrinogen was placed in a test tube with 0.1 ml of 245 mM phosphate buffer (pH 7.0) and incubated at 37 °C for 5 min. Then, 0.1 ml of a 20-U ml^{-1} thrombin solution was added. The solution was incubated at 37 °C for 10 min, 0.1 ml of diluted enzyme solution was added, and incubation continued at 37 °C. This solution was again mixed after 20 and 40 min. At 60 min, 0.7 ml of 0.2 M trichloroacetic acid was added and mixed. The reaction mixture was centrifuged at 15,000×*g* for 10 min. After that, 1 ml of the supernatant was collected and the absorbance at 275 nm was measured. Each experiment was performed in triplicate and the average value was then calculated after correction of the corresponding blank. In this assay, 1 U (fibrin degradation unit) of enzyme activity is defined as a 0.01/min increase in absorbance at 275 nm of the reaction solution [2].

Biochemical Characterization of Fibrinolytic Protease

After the integrated production and purification by the extractive fermentation process using ATPS, the sample that showed the best fibrinolytic activity was subjected to biochemical characterization to evaluate the optimum pH, optimum temperature, stability to pH and temperature, as well as the effect of enzyme inhibitors and metal ions.

Effect and Stability to pH and Temperature on Fibrinolytic Activity

To study the effect of pH and its stability, the fibrinolytic protease generated by extractive fermentation (salt ATPS phase) was mixed with different buffers: sodium acetate (pH 3.0 to 5.0), citrate phosphate (pH 5.0 to 7.0), Tris–HCl (pH 7.0 to 8.5), and glycine–NaOH (pH 8.5 to 11.0) and incubated at 37 °C for 60 min.

The temperature effect was determined by incubating the salt ATPS phase at temperatures ranging between 4 and 85 °C for 30 min. To determine the stability to temperature and pH, aliquots were withdrawn every 15 min for a period of 1 h. These aliquots were submitted to the determination of fibrinolytic activity by the fibrin plate method.

Effect of Metal Ions on Fibrinolytic Activity

Fibrinolytic activity samples from the bottom phase (extractive fermentation) were evaluated in the presence of ions. These ions are described as inhibitors or activators of fibrinolytic activity. The effect of ionic solutions was evaluated at a concentration of 5 mM. The bottom phase was exposed to the following ions: zinc sulfate [$(ZnSO_4)\cdot 7H_2O$], magnesium sulfate [$MgSO_4$], copper sulfate [$CuSO_4$], ferrous sulfate [$FeSO_4$], calcium chloride [$CaCl_2$], magnesium chloride [$(MgCl_2)\cdot 4H_2O$], potassium chloride [KCl], and cobalt chloride [$(CoCl_2)\cdot 2H_2O$] and incubated at 37 °C for 60 min. The ions were dissolved in Tris–HCl pH 7.75 with 0.15 M NaCl. Fibrinolytic activity was determined by the fibrin plate method.

Effect of Inhibitors in Fibrinolytic Activity

To evaluate the effect of inhibitors on enzyme activity, the bottom phase containing the fibrinolytic protease was exposed to the following inhibitors: PMSF (fluoride methylphenylsulfonyl—$C_7H_7FO_2S$), mercuric chloride ($HgCl_2$), 2-mercaptoethanol (2-hydroxy-1-ethanethiol—C_2H_6SO), and ethylenediaminetetraacetic acid (EDTA—acetic—$C_{10}H_{16}N_2O_8$) and incubated for 60 min at 37 °C. The inhibitors were dissolved in pH 7.75 Tris–HCl with 0.15 M NaCl, and the concentration of the solutions was standardized at 5 mM. The methodology for the determination of fibrinolytic activity was performed by the fibrin plate method.

Methodology for the Analysis of the Results

The distribution of the fibrinolytic protease between phases was expressed in terms of the partition coefficient (K), calculated as:

$$K = \frac{FA_t}{FA_b} \tag{1}$$

where FA_t refers to fibrinolytic activity in the top phase and FA_b refers to fibrinolytic activity in the bottom phase.

The specific fibrinolytic activity (SFA) was calculated by the ratio of fibrinolytic activity (FA) and the total protein (P) (in units per milligram):

$$SFA = \frac{FA_t}{P} \tag{2}$$

Results and Discussion

Extractive Fermentation of the Fibrinolytic Protease

Bacillus sp. UFPEDA 485 was the subject of this study on finding the best conditions for extractive fermentation using ATPS based on a complete experimental design. The matrix of the design variables and the results of the responses, the partition coefficient (K), and the activity on the top and bottom phases are shown in Table 2. In all the experiments, fibrinolytic activity ranged between 250 and 835 U ml^{-1}.

The fibrinolytic protease partitioned preferentially to the bottom phase in all experiments ($K<1$), while the cells were positioned at the interface. The system consisting of 18 % (*w*/*w*) PEG 8000 and 13.0 % (*w*/*w*) salt showed a partition coefficient of $K=0.3$.

The higher values in fibrinolytic activity (835 U ml^{-1}) were obtained in the bottom phase of the systems with the highest concentration of salt (13.0 % *w*/*w*). The highest activity values of fibrinolytic protease from *Bacillus* sp. UFPEDA 485 were observed at experimental runs 6 and 8, as shown in Fig. 2. High values of fibrinolytic activities were also obtained by Ashipala and He [5], but they found that, when using PEG 4000 (7 %) and sodium sulfate (4.8 %), the best obtained fibrinolytic activity was 1,224 U ml^{-1} with a partition coefficient of $K=0.44$. In the article of Ashipala and He [5], the authors only showed the fibrinolytic enzyme partition in ATPS, while in the current work, the simultaneous production and purification/partition in ATPS was described. In this paper, as the integrated production and purification of fibrinolytic protease was presented, the production medium, the cells, and ATPS together in one system may alter the cellular physiology, modify the viscosity of the medium due to the polymers inserted, and consequently, modify the production of the biomolecule and its partition. Moreover, each paper describes a different fibrinolytic enzyme, and the partition process is different, too.

Partition of penicillin acylase in ATPS composed of 10 % PEG 4000 and 7.5 % of sodium sulfate was evaluated by Gavasane and Gaikar [18], using similar conditions to this work,

Table 2 Matrix decoded and results of the full factorial design 2^3 for the responses: the partition coefficient (K) and activity in the ATPS top and bottom phases

Runs	MPEG (g mol^{-1})	CPEG (% *w*/*w*)	Csalt (% *w*/*w*)	FA_t (U ml^{-1})	FA_b (U ml^{-1})	K	V_r
1	4,000	18	10.0	250	605	0.4	1.0
2	8,000	18	10.0	410	685	0.5	1.0
3	4,000	30	10.0	360	675	0.5	1.8
4	8,000	30	10.0	280	485	0.5	1.0
5	4,000	18	13.0	350	740	0.4	1.0
6	8,000	18	13.0	300	835	0.3	0.8
7	4,000	30	13.0	345	430	0.8	1.2
8	8,000	30	13.0	415	770	0.5	1.0
9 (C)	6,000	24	11.6	390	495	0.7	1.0
10 (C)	6,000	24	11.6	360	455	0.7	1.0
11 (C)	6,000	24	11.6	375	380	0.9	1.0
12 (C)	6,000	24	11.6	295	415	0.7	1.0

FA_t fibrinolytic activity in the top phase, FA_b fibrinolytic activity in the bottom phase, K partition coefficient, V_r volume ratio of the phases, *(C)* central points

with the main difference that extraction was made separately from fermentation. The obtained partition coefficients of the enzyme and proteins were 0.01 and 0.07, respectively [19].

ATPS formed by PEG (2000, 4000, and 6000), polymers dextran T500 and dextran 40, magnesium sulfate salts, sodium sulfate, and trisodium citrate and were evaluated for the partition of alkaline phosphatase. The obtained partition coefficients (K=0.1 and K=0.06) for the PEG 6000/Na_2SO_4 and PEG 4000/Na_2SO_4 systems, respectively, indicated that the enzyme under study partitioned mostly to the bottom phase, as in this work [7].

The specific activity of the fibrinolytic protease obtained by conventional fermentation (data not shown) and extractive fermentation using ATPS was compared. The best result for enzyme-specific activity in conventional fermentation was of 1,054 U mg^{-1} of enzyme while using the same culture conditions, but in extractive fermentation (ATPS), the enzyme-specific activity was 9,240 U mg^{-1}. For this situation, the obtained enzyme recovery yield was 136 %. These results clearly demonstrate the advantages of using extractive fermentation as it was possible to obtain a ninefold concentration of the target enzyme as a result of process selectivity and reduced product inhibition.

Higher concentrations of enzyme were also obtained in a study with extractive fermentation using aqueous two-phase systems (PEG 4000, 6000, and 9000 with potassium phosphate) [19]. In the production of the alkaline protease of *Bacillus thuringiensis*, a 2.8 times larger activity was obtained with the 13.6 % (*w*/*w*) PEG 4000 and 13.7 % (*w*/*w*) potassium phosphate ATPS relative to conventional fermentation [19].

Statistical analysis showed that the variables concentration of PEG (CPEG), the interaction between PEG molar mass (MPEG) and concentration of sodium sulfate (Csalt), as well as the interaction between all variables were statistically significant for the fibrinolytic activity response in the bottom phase (FA_b) (Fig. 1), since the values were higher than the level of statistical significance, as shown in Table 3. However, the variable (CPEG) PEG concentration showed a negative effect on fibrinolytic activity in the bottom salt-rich phase. This indicates that the decrease in PEG concentration contributes to the increase in fibrinolytic activity in the bottom phase (Fig. 1).

The interaction between the variables MPEG and Csalt was statistically significant and revealed that an increase in their levels has improved the fibrinolytic activity values in the bottom phase (FA_b) (Fig. 2). So, the best condition for the response (FA_b) was obtained with the system MPEG 8000 18.0 % (*w*/*w*) and Csalt 13.0 % (*w*/*w*).

These results show that the enzyme can be entrapped by metal ions of the lower salt-rich phase due to ionic interaction between the metal ions and the anionic amino acids of the enzyme molecule [19]. Studies have been conducted to evaluate the interaction between the metal ions from the lower phase of the system and the biomolecule of interest aiming at partition of the biomolecule to the lower phase through the existing ionic interactions [20].

Similar results were obtained for purification in ATPS of a lipase produced by *Bacillus subtilis*, with the PEG molar mass being the most significant effect. The partition coefficient of the enzyme was reduced as a consequence of the increased hydrophobicity of the top phase due to the increase in chain length of PEG that had less hydroxyl groups for the same concentration of the polymer [21].

Partition Coefficient of the Fibrinolytic Protease

Table 3 demonstrates that no variable or interaction between them had a statistically significant effect for the response partition coefficient (K) because the values of the estimated effects are below the level of statistical significance (p=0.05). In all experiments,

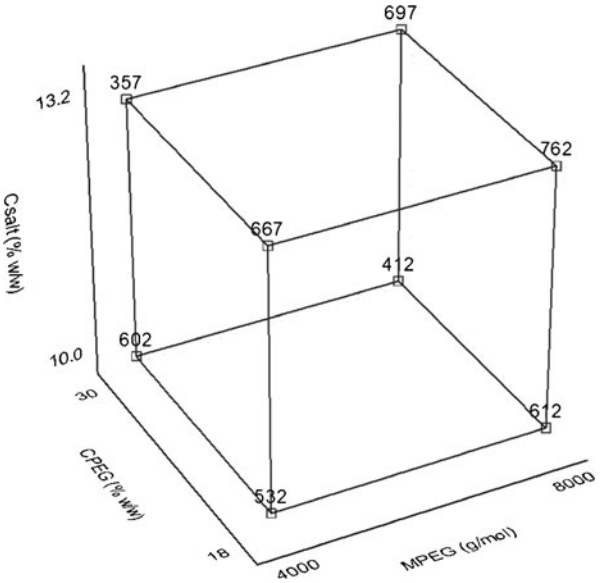


Fig. 1 Cubic plot of the interaction between the variables salt concentration (*Csalt*), PEG concentration (*CPEG*), and PEG molar mass (*MPEG*) for fibrinolytic activity response in the bottom phase of extractive fermentation

values of the partitioning coefficient (K) were <1 and ranged from 0.3 to 0.9, indicating that the fibrinolytic protease preferentially partitioned in the bottom phase (Table 3).

In contrast to these results, the PEG molar mass had no influence on the partition coefficient as the hydrophobic character of PEG increases with the chain length. In a study of phenylalanine ammonia–lyase purification, the system formed by PEG 1000 at a concentration of 13 % and sodium sulfate at 14 % was regarded as the best condition for partition. The partition of the enzyme decreased with increasing PEG molar mass (from 1,000 to 3,350) due to the excluded volume effect [22].

The presence of salts can affect partitioning by weakening or strengthening the interactions or the interaction between ionized groups with the opposite net charge of proteins. The effectiveness of the salt is mainly determined by the nature of the anion. Multicharged anions

Table 3 Calculated effect of the responses in the factorial design 2^3 for the integrated production and purification of the fibrinolytic protease by *Bacillus* sp. UFPEDA 485

Effects	Fibrinolytic activity		
	K	FA_t	FA_b
1. MPEG	−3.73	0.84	2.31
2. CPEG	1.74	0.76	−3.59*
3. Csalt	−2.72	0.93	2.31
1×2	−1.27	−1.01	−0.18
1×3	2.22	−0.51	3.87*
2×3	−0.38	1.09	−1.74
1×2×3	−0.23	3.04	3.66*

*$p<0.05$, data were statistically significant at the 95 % confidence level

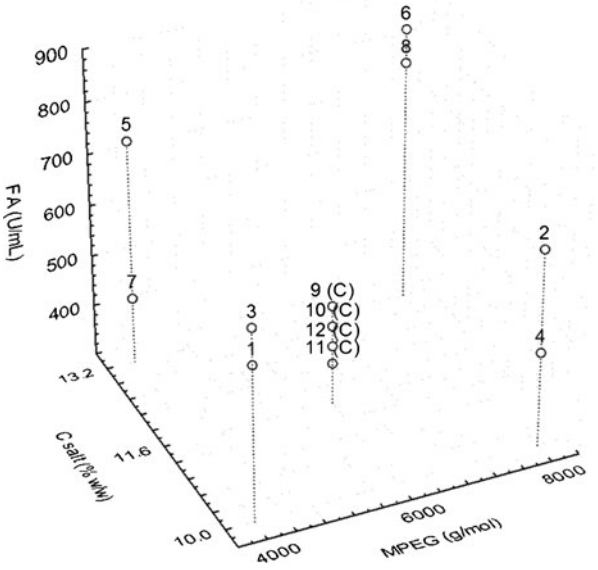


Fig. 2 Simultaneous effects of salt concentration (*Csalt*) and PEG molar mass (*MPEG*) for fibrinolytic activity in the bottom phase of extractive fermentation

are the most effective in the order: $SO_4^{2-}>HPO_4^{2-}>CH_3COO^->Cl^-$. The order of cations is usually given as $NH_4^+>K^+>Na^+>Li^+>Mg^{2+}>Ca^{2+}$ [23].

The potential difference between the phases will influence the partitioning of macromolecules and charged particles, especially those carrying a large number of electric charges on their surface. The electric potential created can be adjusted by changing the salt's composition and concentration; therefore, it can be used to control the partition coefficient [24].

Protease Inhibitors on Fibrinolytic Activity

The enzyme was subjected to the action of inhibitors and metal ions after pre-purification by extractive fermentation. Enzyme activity was significantly inhibited by the metalloprotease inhibitor, EDTA, showing a residual activity of 3.1 %. It has been also slightly inhibited by β-mercaptoethanol (98.9 %) but was not inhibited when subjected to PMSF, the inhibitor of serine proteases. These results allowed characterizing fibrinolytic protease as a metalloprotease. Similar results were obtained by Simkhada et al. [25] and Park et al. [26]. The effect of the different inhibitors can be seen in Table 4.

Table 4 Effect of inhibitors on the fibrinolytic activity

Inhibitors	Residual activity (%)
Control	100
EDTA	3.1
β-Mercaptoethanol	98.8
PMSF	100

Metal Ions on Fibrinolytic Activity

Enzyme activity was not affected by the ions $ZnSO_4$, $MgSO_4$, $CuSO_4$, and $CoCl_2$. On the other hand, enzymatic activity was stimulated in the presence of the following ions: $CaCl_2$ (440 %), $MgCl_2$ (440 %), $FeSO_4$ (268 %), and KCl (268 %) and inhibited only by the HgCl ion (94.20 %). The effect of metal ions can be seen in Table 5.

The $MgCl_2$, $FeSO_4$, and KCl ions also stimulated the activity of fibrinolytic protease produced by the *Paenibacillus polymyxa* EJS-3 bacterium whose residual activity was 111, 110, and 103 %, respectively [27]. The $CaCl_2$ ion also increased the fibrinolytic activity of the protease produced by *Streptomyces* sp. CS684; however, enzyme activity was inhibited by $ZnSO_4$ (35 %), $CuSO_4$ (1.6 %), and $CoCl_2$ (41.8 %) [25]. When incubated with the ions $CaCl_2$ and $MgCl_2$, the fibrinolytic protease activity produced by *Ganoderma lucidum* Vk12 [28] was also stimulated, as in this work.

Optimum Temperature and Stability of the Fibrinolytic Protease

The optimum temperature of the fibrinolytic protease was 37 °C, as can be seen in Fig. 3. At room temperature (25 °C), the enzyme showed 88 % activity; at 45 °C, the activity was 84 %, while it decreased to 76 % when subjected to 55 and 65 °C. These results show that the enzyme activity of the crude extract of conventional fermentation was reduced to 60 % at 55 °C and completely lost the activity at 65 °C (data not shown). These results show that, after extractive fermentation, the fibrinolytic protease became more stable at high temperatures.

Fibrinolytic activity was maintained at 80 % after 60 min of exposure to optimum temperature. These results differed from those obtained for the biochemical characterization of the fibrinolytic protease produced by *Bacillus* sp. nov. SK006, whose optimum temperature was 40 °C, had reduced activity at 50 °C, and was completely inactivated at 65 °C for 10 min of exposure [2]. Similarly, the optimum temperature of nattokinase produced by *B. subtilis* TKU007 was 40 °C. The enzyme retained 70 % activity at 60 °C and it was reduced to 52 % at 70 °C [2].

Optimum pH and Stability of the Fibrinolytic Protease

Results on the effect of pH on enzyme stability can be observed in Fig. 4. The optimum pH of the fibrinolytic protease obtained by extractive fermentation was detected between pH 7.0 and 8.5 in the presence of 0.05 M Tris–HCl and NaOH–glycine buffer, respectively. The

Table 5 Effect of the metal ions on the fibrinolytic activity

Metal ions	Residual activity (%)
Control	100.0
$MgSO_4$	100.0
$CuSO_4$	100.0
$CoCl_2$	100.0
$CaCl_2$	440.7
$MgCl_2$	440.7
$FeSO_4$	268.8
KCl	268.8
HgCl	94.2

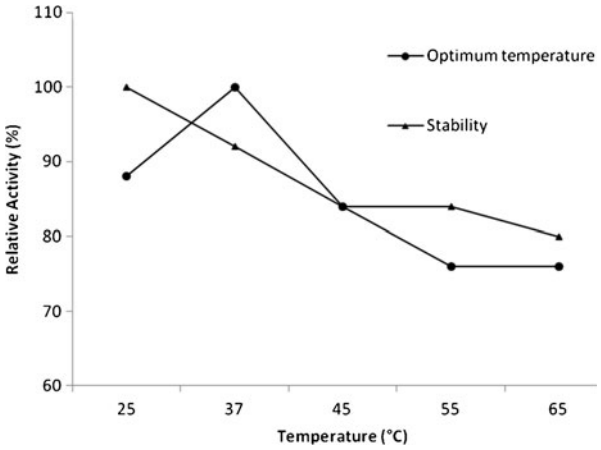


Fig. 3 Optimum temperature and stability of the fibrinolytic protease produced by *Bacillus* sp. UFPEDA 485 in the bottom phase of extractive fermentation

enzyme retained 95.2 % of its activity at pH 7.0 and 8.5. It has also maintained 92.8 % of its activity at pH 11.0, 85 % at pH 5.0, and 52 % at pH 3.0. The results show that, after pre-purification, the enzyme is stable over a broad range of pH values and a considerable lost in activity only occurs at acidic pH (3.0). The enzyme was characterized as a neutral to alkaline protease since it has maintained its activity when tested in the neutral–alkaline range (pH 7.0–8.5).

Similar results were obtained in the characterization of the fibrinolytic protease produced by *B. subtilis* ICTF-1 after purification, where the enzyme remained active in the pH range between 7.0 and 11.0, decreasing at pH 5.0 [4]. Fibrinolytic protease produced by *B. subtilis* also retained its activity between 5.0 and 11.0 [4]. In this work, the optimum pH of the enzyme was detected at pH 9.0, and at pH 7.0, the enzyme showed only 68 % activity [4].

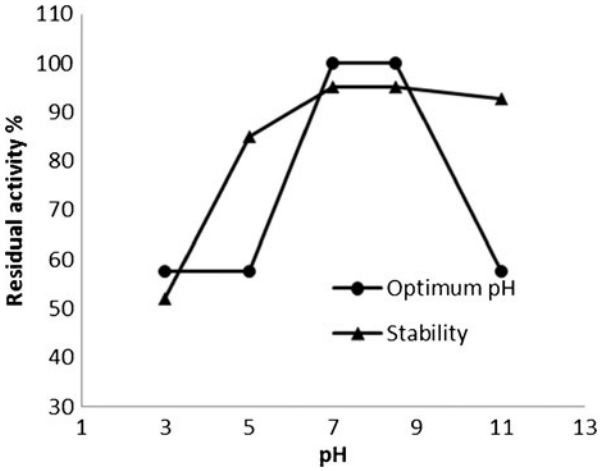


Fig. 4 Optimum pH and stability of the fibrinolytic protease produced by *Bacillus* sp. UFPEDA 485 in the bottom phase of extractive fermentation

Conclusions

It can be concluded that the integration of fermentation and purification made possible the purification of the fibrinolytic protease produced by *Bacillus* sp. UFPEDA 485. Furthermore, the use of ATPS in extractive fermentation can direct the development of a low-cost process by replacing the initial stages of conventional separation processes.

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