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Valorization of Brazilian Pequi Peel by Fungal Solid-state Fermentation for Protease Production, Aqueous Two-phase Recovery and Biochemical Characterization

Vinicius de Paula Taffarel
Universidade Federal do Piauí: Universidade Federal do Piaui https://orcid.org/0009-0002-8228-976X
Antônio Luís Sousa Junior
Universidade Federal do Piauí: Universidade Federal do Piaui https://orcid.org/0009-0008-8359-3521
Thalesram Izidoro Pinotti
Universidade Federal do Piauí: Universidade Federal do Piaui https://orcid.org/0009-0004-5063-0606
Edson da Silva Rodrigues
Universidade Federal do Piauí: Universidade Federal do Piaui https://orcid.org/0009-0005-8777-5179
Glayce Kelly da Costa
Universidade Federal do Piauí: Universidade Federal do Piaui https://orcid.org/0009-0001-3624-6729
José Pedro Martins Barbosa Filho
Universidade Federal do Piauí: Universidade Federal do Piaui https://orcid.org/0000-0003-3365-2569
Márcia Nieves Carneiro da Cunha
Universidade Federal do Piauí: Universidade Federal do Piaui https://orcid.org/0000-0003-2744-6216
Romero Marcos Pedrosa Brandão Costa
Universidade de Pernambuco https://orcid.org/0000-0001-7045-2975
Ana Lúcia Figueiredo Porto
Universidade Federal Rural de Pernambuco https://orcid.org/0000-0001-5561-5158
Alice Maria Gonçalves Santos
Universidade Federal do Piauí: Universidade Federal do Piaui https://orcid.org/0000-0003-0672-1709
Thiago Pajeú Nascimento
thiagopajeu@ufpi.edu.br
Universidade Federal do Piaui https://orcid.org/0000-0003-3480-6734

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Abstract

This study investigated the valorization of pequi (*Caryocar brasiliense*) peel as a substrate for protease production by *Penicillium* sp. 2A3, isolated from the Caatinga–Cerrado ecotone, and the subsequent recovery of the enzyme using a polyethylene glycol (PEG)/sodium sulfate aqueous two-phase system (ATPS). A (2^3) factorial design was used to evaluate the effects of PEG molar mass (1,500–20,000 g mol^{-1}), PEG concentration (18–30%, w/w), and sodium sulfate concentration (10–13%, w/w) on the partition coefficient (*K*), recovery yield (*Y*), and purification factor (*PF*). The selected conditions, consisting of PEG with a molar mass of 20,000 g mol^{-1}, 18% PEG, and 10% sodium sulfate, resulted in a *K* of 2.23, a Y_{PEG} of 164.1% in the PEG-rich phase, and a *PF* of 1.75. Biochemical characterization of the recovered fraction revealed optimal proteolytic activity at 40°C and pH 5.0. The enzyme exhibited differential responses to metal ions, with Mg^{2+}, Na^{+}, and K^{+} showing no significant effect or only slight inhibition, whereas Fe^{2+}, Zn^{2+}, Cu^{2+}, Mn^{2+}, and Ba^{2+} reduced residual activity. Inhibitor assays revealed sensitivity to EDTA, β-mercaptoethanol, and PMSF, although the results did not allow the catalytic class of the protease to be conclusively established. Overall, this study highlights the potential of pequi peel as a renewable feedstock for fungal enzyme production and demonstrates the applicability of ATPS as a recovery strategy, contributing to the valorization of agro-industrial biomass and the development of more sustainable bioprocesses.

Highlights

- Pequi peel was valorized as a substrate for fungal protease production.
- *Penicillium* sp. 2A3 reached 70.20 U/mL protease activity after 48 h.
- PEG/sodium sulfate ATPS favored protease partitioning into the PEG-rich phase.
- Selected ATPS conditions yielded 164.1% recovery and a 1.75 purification factor.
- The recovered protease showed optimum activity at 40°C and pH 5.0.

Statement of Novelty

This study presents an integrated approach to converting pequi (*Caryocar brasiliense*) peel, an underutilized agro-industrial residue from the Brazilian Cerrado, into a source of fungal proteases. Unlike studies focusing solely on enzyme production or recovery, this work combines residue valorization, fungal fermentation, aqueous two-phase system-based enzyme recovery, and biochemical characterization. The selected polyethylene glycol/sodium sulfate system achieved a protease recovery yield of 164.1% and a purification factor of 1.75, demonstrating its potential for recovering enzymatic activity. These findings contribute to the development of more sustainable bioprocesses, support the circular bioeconomy, and highlight the biotechnological value of regional biomass that would otherwise be discarded.

1. Introduction

The generation of agro-industrial residues represents a major environmental and economic challenge, particularly in regions where agricultural activities and fruit processing have significant socioeconomic importance. Although often regarded as low-value materials, these residues may have a composition favorable for microbial growth and the production of compounds of biotechnological interest. In this context, their use as substrates for fermentation processes represents an alternative to reducing dependence on synthetic media, decreasing production costs, and

transform underutilized biomass into higher-value products, thereby contributing to the principles of the circular bioeconomy [17, 24].

Among agro-industrial resources with potential for valorization, pequi (*Caryocar brasiliense* Camb.) stands out as a fruit characteristic of the Brazilian Cerrado and of considerable cultural, economic, and nutritional importance. In addition to its widespread use in regional cuisine, pequi contains bioactive compounds associated with antioxidants, anti-inflammatory, and functional properties, attracting interest for medicinal, cosmetic, and pharmaceutical applications [9, 23, 35]. However, its processing generates significant amounts of residues, particularly peel, whose improper disposal may contribute to environmental impacts. Conversely, recent studies have indicated the potential of pequi peel and other pequi-derived residues as renewable feedstocks for energy, chemical, and biotechnological applications [2]. Therefore, the use of pequi peel as a fermentation substrate may represent a strategy for integrating waste management with the production of bioproducts of industrial interest.

The use of agro-industrial residues as substrates for microbial fermentation has gained increasing attention as a strategy for producing value-added bioproducts while reducing the environmental impact associated with their disposal. In submerged fermentation (SmF), microorganisms grow in liquid media containing soluble nutrients and complex substrates, allowing greater control of physicochemical conditions and facilitating the recovery of extracellular metabolites and enzymes. Filamentous fungi are particularly relevant for this process due to their metabolic versatility, ability to utilize complex carbon sources, and capacity to secrete a wide range of extracellular enzymes. During the bioconversion of plant-derived substrates, these microorganisms can produce hydrolytic enzymes, including pectinases, cellulases, hemicellulases, and proteases. Among these enzymes, proteases are of particular biotechnological interest due to their broad functional diversity and applications in the detergent, food, leather, paper, waste treatment, biomedical, agricultural, and bioremediation sectors [4, 21, 31, 32].

Despite the potential of fungal fermentation for protease production using agro-industrial residues, the feasibility of the process also depends on the efficiency of enzyme recovery and purification. Conventional methods, such as precipitation, chromatography, and ultrafiltration, may present limitations related to cost, the number of processing steps, activity losses, and scalability. In this context, aqueous two-phase systems (ATPS) have been proposed as attractive alternatives for the recovery and partial purification of biomolecules due to their high-water content, mild separation conditions, operational simplicity, and compatibility with proteins [7, 12, 13, 27, 28]. Systems composed of polyethylene glycol (PEG) and salts may promote the selective partitioning of proteins between the phases, enabling enzyme recovery while preserving catalytic activity.

The recovery step should, however, be accompanied by biochemical characterization of the enzyme, since properties such as optimum pH and temperature, stability, and responses to different chemical agents are essential for assessing its application potential. Thus, integrating agro-industrial residue valorization, fungal fermentation, ATPS-based recovery, and enzyme characterization may contribute to the development of sustainable and cost-effective biotechnological processes.

Although studies have investigated fungal enzyme production from agro-industrial residues and the application of ATPS for biomolecule recovery, few studies have integrated these approaches for the valorization of regional Cerrado residues, particularly pequi peel, in association with protease production and recovery. Therefore, this study aimed to valorize pequi peel (*Caryocar brasiliense* Camb.) as a substrate for fungal solid-state fermentation and protease production, followed by enzyme recovery using an aqueous two-phase system composed of PEG and salt and biochemical characterization of the recovered protease.

2. Materials and Methods

2.1 Obtaining and origin of fungal isolates

The *Penicillium* spp. isolates (A7, 2A3, and 3A3) and *Trichoderma* sp. (UFPIT13) were obtained from the soil fungal collection of the Laboratory of Phytopathology at the Federal University of Piauí (UFPI), Professora Cinobelina Elvas Campus (CPCE), Brazil. These isolates were originally recovered from an ecotonal area between the Cerrado and Caatinga biomes, located in the municipality of Bom Jesus, Piauí, Brazil [25]. The isolates were cultured on 80-mm Petri dishes containing potato dextrose agar (PDA) and incubated at 28°C for seven days.

2.2 Production of the Pequi Peel-Based Culture Medium

Pequi peels (*Caryocar brasiliense* Cambess.) were obtained from private rural properties, including farms and smallholdings, located in the municipality of Bom Jesus, Piauí, Brazil. The collected material was confirmed by the donors as belonging to the species *Caryocar brasiliense* Cambess. Initially, the peels were washed under running water and dried in a forced-air circulation oven at 40°C for 48 h. Subsequently, the dried material was ground using a Wiley-type knife mill (SL-31) to obtain a fine powder.

The resulting material was used to formulate the MS-2 culture medium proposed by Porto et al [19], in which the soybean flour (2%, w/v) originally present in the medium was replaced with dried and ground pequi peel powder. The medium consisted of glucose (1%, w/v), $MgSO_4 \cdot 7H_2O$ (0.1%, w/v), NH_4Cl (0.435%, w/v), K_2HPO_4 (0.435%, w/v), and 0.1 mL of a mineral solution containing $FeSO_4$, $MnCl_2 \cdot 4H_2O$, $ZnSO_4$, and $CaCl_2 \cdot H_2O$ (100 mg of each compound in 100 mL of distilled water). The initial pH was adjusted to 7.2, and the medium was sterilized by autoclaving at 121°C and 1 atm for 20 min.

2.3 Protease Production by Submerged Fermentation

Suspensions of each of the fungal isolates described above, adjusted to a concentration of 10^7 spores mL^{-1}, were inoculated into Erlenmeyer flasks containing 125 mL of the modified MS-2 medium previously prepared with pequi peel powder as the substrate. The cultures were incubated in an orbital shaker (Tecnal TE-424) at 30°C and 120 rpm. Enzyme production was monitored over a 96-h cultivation period, with evaluations performed at 24, 48, 72, and 96 h. At each cultivation time, one independent Erlenmeyer flask for each fungal isolate was removed from incubation and centrifuged at 4,000 rpm for 10 min. The resulting supernatant was collected as the crude enzymatic extract and stored at − 18°C until enzymatic analyses were performed. The different cultivation times were evaluated to assess the proteolytic potential of the isolates and determine the most suitable cultivation condition for obtaining the enzymatic extract for subsequent experiments.

2.4 Determination of Protease Activity

Total protease activity was determined according to Ginther [11], with modifications. The reaction was performed in microcentrifuge tubes containing 250 µL of 1% (w/v) azocasein previously prepared in 0.2 M Tris-HCl buffer (pH 7.2), followed by the addition of 150 µL of the crude enzymatic extract obtained after fungal fermentation. The mixture was gently homogenized and incubated in the dark at 28°C for 1 h. After incubation, the reaction was stopped by adding 1 mL of 10% (w/v) trichloroacetic acid (TCA), followed by centrifugation at 8,000 rpm for 10 min.

Subsequently, 800 µL of the supernatant were transferred to a new microcentrifuge tube and mixed with 200 µL of 1.8 M NaOH. Absorbance was measured at 420 nm using a spectrophotometer. The reaction blank, used to adjust

the spectrophotometer, was prepared under the same conditions as the samples, replacing the enzymatic extract with Tris-HCl buffer. The absorbance values of the samples were corrected against the reaction blank before protease activity was calculated.

One unit of protease activity (U) was defined as a change of 0.1 absorbance unit at 420 nm per hour under the specified assay conditions. Protease activity was calculated using the blank-corrected absorbance value according to the following equation: protease activity (U/mL) = (Abs_{420}/0.1)/0.15, where 0.15 corresponds to the volume, in mL, of enzymatic extract used in the reaction. The results were expressed as U mL^{-1}.

2.5 Protein Quantification

Protein concentration was determined using the bicinchoninic acid (BCA) method, according to Smith et al [30], with bovine serum albumin (BSA) used as the protein standard. A calibration curve was prepared using BSA solutions at concentrations ranging from 0 to 1000 µg mL^{-1}. The samples and standards were subjected to the BCA reaction according to the conditions established for the method. After the incubation period, absorbance was measured at 562 nm using a spectrophotometer. Protein concentration in the samples was calculated from the calibration curve obtained using BSA as the standard and expressed as µg mL^{-1}.

2.6 Protease Recovery and Partial Purification Using an Aqueous Two-Phase System (ATPS)

The crude enzymatic extract obtained from the fungal isolate that exhibited the highest protease activity under the evaluated cultivation conditions was selected for the subsequent steps of enzyme recovery and partial purification using an aqueous two-phase system (ATPS).

2.6.1 Experimental Design

The experimental design was based on a 2^3 factorial design (Table 1), with the aim of evaluating the effects of three independent variables on the partitioning and recovery of protease present in the crude enzymatic extract: polyethylene glycol molecular mass (MM_{PEG}), polyethylene glycol concentration (C_{PEG}), and sodium sulfate concentration (C_{SALT}). Each factor was evaluated at two levels, defined according to the experimental conditions presented in Table 1. The factorial design allowed the evaluation of the individual effects of the independent variables, as well as their possible interactions, on the responses obtained in the ATPS.

Table 1
Levels of the variables evaluated in the complete 2^3 factorial design for protease recovery and partial purification using an aqueous two-phase system.

Variáveis	**Níveis**		
	Baixo (-1)	**Central (0)**	**Alto (+ 1)**
MM_{PEG} (g/mol)	1,500	10,000	20,000
C_{PEG} (%)	18.0	24.0	30.0
C_{SS} (%)	10.0	11.5	13.0
MM_{PEG} = Polietilenoglicol molecular mass;			
C_{PEG} = PEG concentration;			

C_{SS} = Sodium sulfate concentration.

2.6.2 Preparation of the Aqueous Two-Phase System

The aqueous two-phase systems (ATPS) were prepared in 15-mL graduated conical tubes using different concentrations of sodium sulfate solutions and 50% (w/w) polyethylene glycol (PEG) solutions, according to the conditions established in the factorial design. The components were maintained at 28 ± 2°C throughout the procedure. Initially, the PEG and sodium sulfate solutions were added to the tubes at the concentrations defined by the experimental design and homogenized using a vortex mixer for 1 min. Subsequently, 2 g of crude enzymatic extract, corresponding to 20% (w/w) of the total system mass, was added, followed by distilled water in an amount sufficient to adjust the total system mass to 10 g. The crude enzymatic extract was obtained from the fungal isolate selected as the best protease producer, using the cultivation time that resulted in the highest protease activity in the enzyme production curve.

After the addition of all components, the systems were again homogenized using a vortex mixer for 1 min and left undisturbed for 60 min at 28 ± 2°C to allow phase separation by settling. Subsequently, the volumes of the phases were measured, including the upper PEG-rich phase and the lower sodium sulfate-rich phase. Aliquots of each phase were carefully collected, avoiding mixing between the phases, and stored for subsequent analyses.

2.6.3 Calculation of the response variables

Aliquots from the phases obtained in the ATPS were analyzed for protein concentration and protease activity to determine the response variables: partition coefficient (*K*), purification factor (*PF*), and enzyme activity recovery (*Y*). After the experiments were performed, the results were subjected to statistical analysis using Statistica 8.0 software.

The protease partition coefficient (*K*) was determined as the ratio between protease activity in the upper polyethylene glycol-rich phase (PA_{PEG}) and protease activity in the lower salt-rich phase (PA_{SALT}), according to Eq. 1:

$$K = \frac{PA\mathrm{PEG}}{PASALT}$$

Enzyme recovery (Y) was determined for each of the two phases as the ratio between the total protease activity present in the upper phase (PAPEG), multiplied by the PEG volume (VPEG), or the total protease activity present in the lower salt-rich phase (PASALT), multiplied by the salt volume (VSALT), and the total protease activity present in the crude extract (PAcrude), multiplied by the initial volume (Vinitial) added to the system (2 mL). The result was subsequently multiplied by 100 to express enzyme recovery as a percentage, according to Eq. 2:

$$Y = \frac{PAPEG\ x\ VPEG\ or\ PASALT\ x\ VSALT}{PAcrude\ x\ Vcrude}\ x\ 100$$

The purification factor (PF) was defined as the ratio between the specific protease activity in the PEG-rich phase (SPA_{PEG}) or salt-rich phase (SPA_{SALT}) and the specific activity of the crude extract (*SPAcrude*) according to Eq. 2. Specific activity was calculated as the ratio between protease activity (U/mL) and protein concentration (mg/mL) in each phase and in the crude extract.

$$PF = \frac{SPAPEG\ or\ SPASALT}{SPAcrude}$$

2.7 Evaluation of other enzymatic activities

2.7.1 Amylolytic Activity

Amylolytic activity was determined using the 3,5-dinitrosalicylic acid (DNS) method, as described by Sun and Henson [34], with modifications according to the procedure proposed by Padilha et al [18]. For the assay, 500 µL of the enzyme extract, 500 µL of sodium acetate buffer (100 mmol L^{-1}) containing 5 mmol L^{-1} calcium chloride (pH 5.0), and 500 µL of a 2.5% (w/v) starch solution were used. The reaction mixture was incubated in a water bath at 40°C for 15 min. Subsequently, the reaction was stopped by adding 1000 µL of DNS solution, and the samples were incubated in a water bath at 95°C for 6 min. The samples were then immediately cooled in an ice bath, followed by the addition of 7.5 mL of distilled water. Absorbance was measured at 540 nm using a spectrophotometer, and amylolytic activity was expressed as units per milliliter (U mL^{-1}).

2.8 Enzyme characterization

2.8.1 Effect of temperature on protease activity

The effect of temperature on protease activity was evaluated in the crude extract and purified enzyme. Enzymatic reactions were conducted at different temperatures (10, 20, 30, 40, 50, 60, 70, 80, and 90°C) for 60 min. The assays were performed in triplicate, and the results were expressed as relative activity, with the highest activity considered as 100%, to determine the optimum temperature of the enzyme. For the evaluation of thermal stability, the crude extract and purified enzyme were incubated at 50°C, the optimum temperature previously determined for both enzymatic preparations, for up to 180 min. Aliquots were collected at regular intervals and subjected to residual activity determination, as described in Section 2.5.1. The results were expressed as residual activity (%) relative to the initial activity, determined before incubation.

2.8.2 Effect of pH on protease activity

The effect of pH on protease activity was evaluated in the crude extract and purified enzyme using different buffer systems, all at a concentration of 0.1 M: sodium acetate–acetic acid (pH 3.0–5.0), citrate–phosphate (pH 5.0–

7.0), and Tris-HCl (pH 7.0–9.0). For this purpose, 500 µL of the enzyme solution were mixed with 500 µL of the respective buffer and incubated for 1 h at 25°C. Subsequently, enzymatic activity was determined. As a control, the enzyme solution was incubated under the same conditions using 500 µL of 0.15 M NaCl instead of buffer. The assays were performed in triplicate, and the results were expressed as relative activity (%) as a function of pH.

For the evaluation of pH stability, the crude extract and purified enzyme were incubated at their respective optimum pH values (pH 9.0 for the crude extract and pH 5.0 for the purified enzyme) for up to 12 h. Aliquots were collected at regular intervals to determine residual activity, as described in Section 2.5.1. The results were expressed as residual activity (%) relative to the initial activity.

2.8.3 Effect of different metal ions on protease activity

The effect of different metal ions on protease activity was evaluated in the crude extract and purified enzyme. For this purpose, solutions of the following salts were prepared at a concentration of 10 mM: ferrous sulfate ($FeSO_4$), zinc sulfate ($ZnSO_4$), sodium sulfate (Na_2SO_4), magnesium sulfate ($MgSO_4$), copper sulfate ($CuSO_4$), manganese sulfate ($MnSO_4$), aluminum chloride ($AlCl_3$), potassium chloride (KCl), cobalt chloride ($CoCl_2$), and barium chloride ($BaCl_2$). The reaction mixtures were prepared at a 1:1 (v/v) ratio, using the enzyme extract and the respective 10 mM ionic solution, resulting in a final concentration of 5 mM of each metal ion in the reaction mixture. The samples were incubated for 60 min at room temperature (approximately 25°C). After incubation, aliquots were collected for determination of protease activity, as described in Section 2.5.1.

The experimental control was prepared by replacing the ionic solution with distilled water while maintaining the same incubation conditions. All assays were performed in triplicate, and the results were expressed as relative activity (%) compared with the control.

2.8.4 Effect of inhibitors on protease activity

The effect of different inhibitors on protease activity was evaluated using EDTA, β-mercaptoethanol, and PMSF, all at a concentration of 5 mM. The enzyme samples, corresponding to the crude extract and purified enzyme, were individually pre-incubated with each inhibitor for 60 min at room temperature (approximately 25°C). After pre-incubation, residual enzymatic activity was determined. The control was prepared under the same experimental conditions, but without the addition of inhibitors. All assays were performed in triplicate, and the results were expressed as residual activity (%) relative to control.

2.9 Statistical analysis

The data obtained from the factorial design were subjected to analysis of variance (ANOVA) to evaluate the effects of the independent variables—PEG molecular weight, PEG concentration, and salt concentration—on the response variables (*K*, *Y*, and *PF*), as well as the interaction effects among the factors. A significance level of 5% ($p < 0.05$) was adopted to determine the statistical significance of the evaluated effects. Statistical analyses were performed using Statistica 8.0 software (StatSoft Inc., Tulsa, OK, USA). All other analyses were performed in triplicate, and the results were expressed as mean ± standard deviation.

3. Results and Discussion

3.1 Valorization of pequi (*Caryocar brasiliense*) peel as a substrate

The valorization of agro-industrial residues through biotechnological processes represents a promising strategy for obtaining higher-value-added products while reducing the environmental impacts associated with biomass disposal. In this context, pequi (*Caryocar brasiliense*), a fruit native to the Brazilian Cerrado and of significant socioeconomic and cultural importance, presents considerable potential for the utilization of its different fractions. Its occurrence extends across several Brazilian regions and states, including the North (Pará and Tocantins), the Central-West (Goiás and Mato Grosso), and the Southeast (Minas Gerais and São Paulo) [16, 20]. The pequi fruit consists of external layers surrounding the pyrene, which is internally covered by an aromatic, oily, and edible pulp considered the main fraction of interest and consumption [16]. The pulp ranges in color from light yellow to deep orange, a characteristic associated with the presence of carotenoids, which contribute to its nutritional and functional value. The peel, consisting of the exocarp and outer mesocarp, accounts for approximately 75–84% of the fruit mass and is frequently discarded during processing, constituting an important source of agro-industrial residue.

Despite its residual nature, pequi peel exhibits a chemical composition that highlights its potential for biotechnological applications [14, 29]. Bemfeito et al [8] demonstrated that *C. brasiliense* peel flour contains a high dietary fiber content (42.09 g/100 g), as well as phenolic compounds and high antioxidant capacity, indicating the presence of an organic matrix with potential for various applications. Characterization studies of pequi residues have also highlighted the presence of lignocellulosic components, further supporting the potential of this biomass as a raw material for valorization and conversion processes.

In this context, the use of *C. brasiliense* peel as a substrate for the cultivation of *Penicillium* sp. 2A3 represents a strategy for utilizing low-value biomass to obtain a product of biotechnological interest. The composition of the peel, particularly its carbohydrate and fiber fractions, may provide structural components that can potentially be utilized by the fungus during growth and enzyme production. In addition, the use of pequi residues in technological applications has already been investigated, demonstrating that this biomass can be converted into higher-value-added products, contributing to waste reduction and the development of strategies associated with the bioeconomy and circular economy. Thus, the use of pequi peel for protease production by *Penicillium* sp. 2A3 represents an integrated approach to agro-industrial residue valorization and bioproduct production, adding value to a fruit fraction that would otherwise be destined for disposal.

3.2 Selection of the fungal isolate

Protease activity varied among the fungal strains evaluated throughout the fermentation period, revealing distinct patterns of enzyme production and maintenance of enzymatic activity (Fig. 1). During the first 24 h, the values were relatively similar among the strains, ranging from 56.83 U/mL for *Penicillium* sp. 2A3 to 62.33 U/mL for *Trichoderma* sp. T13. However, from 48 h onward, more pronounced differences were observed in the progression of protease activity among the isolates. The *Trichoderma* sp. UFPIT13 strain exhibited a protease activity of 62.33 U/mL after 24 h, followed by an increase to 66.06 U/mL at 48 h. Subsequently, activity decreased to 61.46 U/mL at 72 h, followed by a recovery at 96 h, when it reached 70.80 U/mL. This behavior indicates temporal variation in enzyme production, possibly associated with changes in the physiological and metabolic conditions of the fungus throughout the fermentation process. In contrast, strain A7 showed a moderate increase in activity up to 48 h (66.00 U/mL), followed by a progressive decrease at 96 h, reaching 42.00 U/mL at the end of the experimental period.

Strains 3A3 and 2A3 exhibited the highest protease activity values throughout the evaluated period. Strain 3A3 increased its activity from 57.30 U/mL at 24 h to 70.20 U/mL at 48 h, maintaining high activity at 72 h (66.00

U/mL) and showing a decrease at 96 h (61.60 U/mL). In turn, strain 2A3 showed a marked increase in activity during the first 48 h, reaching 70.20 U/mL, followed by maintenance of high activity at 72 h (66.93 U/mL). At 96 h, the activity was 63.73 U/mL.

These results demonstrate that the response of the strains to fermentation was not uniform, with protease activity being dependent on both the fungal strain and the cultivation time. The maintenance of high activity levels up to 72 h, particularly for strains 2A3 and 3A3, suggests a greater capacity of these strains to sustain protease production during the evaluated period. The decrease observed in some strains during the later stages of fermentation may be associated with changes in cultivation conditions, nutrient availability, or the dynamics of enzyme synthesis and stability. However, these hypotheses should be interpreted with caution, since such factors were not directly evaluated in the present study.

The ability of species of the genus *Penicillium* to produce proteases using alternative substrates and agro-industrial residues as nutrient sources has been previously described in the literature [1, 22]. In this context, the results obtained in the present study indicate that pequi (*Caryocar brasiliense*) peel has potential as a substrate for protease production by filamentous fungi, possibly by providing nutrients and compounds capable of supporting fungal metabolism and enzyme synthesis. The high activity observed for strain 2A3, particularly between 48 and 72 h, further highlights its potential for subsequent studies aimed at optimizing the fermentation process. Therefore, this strain was selected for the subsequent stages of the study based on its performance in protease production throughout fermentation, together with its high sporulation capacity observed experimentally. The combination of these characteristics is relevant to the continued development of bioprocess, as a strain exhibiting both high enzyme production and good propagation capacity may offer advantages for its use in subsequent fermentation processes.

3.3 Protease recovery using an aqueous two-phase system (PEG/sodium sulfate)

Following the selection of *Penicillium* sp. 2A3 based on its performance throughout the fermentation process, the enzymatic extract obtained after 48 h of cultivation was subjected to pre-purification using an aqueous two-phase system (ATPS). Table 2 presents the results of the 2^3 factorial design, in which different combinations of polyethylene glycol molar mass (MM_{PEG}), PEG concentration (C_{PEG}), and sodium sulfate concentration (C_{SS}) were evaluated. The response variables included the partition coefficient (*K*), enzyme recovery (*Y*), and purification factor (*PF*).

Table 2
Experimental conditions and results of protease recovery from *Penicillium* sp. 2A3 using an aqueous two-phase system (PEG/sodium sulfate), obtained according to a full factorial design 2^3

Assay	MM_{PEG} (g/mol)	C_{PEG} (%)	C_{SS} (%)	PA_{PEG} (U/mL)	PA_{SS} (U/mL)	K	Y_{PEG} (%)	Y_{SS} (%)	PF_{PEG}	PF_{SS}	MB (%)
1	1500	18.0	10.0	33.10	22.56	1.46	173.09	67.43	1.08	0.005	137.94
2	20000	18.0	10.0	52.30	23.40	2.23	164.09	97.89	1.75	0.004	112.46
3	1500	30.0	10.0	33.20	17.60	1.88	148.81	39.44	0.94	0.002	123.65
4	20000	30.0	10.0	28.60	16.83	1.69	149.55	50.30	0.77	0.003	148.75
5	1500	18.0	13.0	47.16	16.66	2.83	147.99	62.25	1.59	0.003	108.49
6	20000	18.0	13.0	40.50	25.40	1.59	114.97	102.46	0.95	0.004	123.47
7	1500	30.0	13.0	29.53	17.26	1.71	112.52	43.85	0.70	0.005	119.38
8	20000	30.0	13.0	24.60	16.10	1.52	73.51	48.11	1.13	0.002	87.78
9	10000	24.0	11.5	30.16	12.36	2.43	99.15	40.64	1.14	0.001	109.23
10	10000	24.0	11.5	24.46	13.33	1.83	73.11	35.85	0.80	0.005	86.79
11	10000	24.0	11.5	23.43	14.70	1.59	66.52	43.92	0.54	0.002	107.74
12	10000	24.0	11.5	24.73	16.53	1.49	70.21	54.34	0.60	0.002	109.67
MM_{PEG} - Molecular mass of polyethylene glycol;											
C_{PEG} - Polyethylene glycol concentration;											
CSS - Sodium sulfate concentration;											

PA_{PEG} - Protease activity in the PEG-rich phase;

PA_{SS} - Protease activity in the sodium sulfate-rich phase;

K - Partition coefficient;

Y_{PEG} - Protease recovery in the PEG-rich phase;

Y_{SS} - Protease recovery in the sodium sulfate-rich phase;

PF_{PEG} - Purification factor in the PEG-rich phase;

PF_{SS} - Purification factor in the sodium sulfate-rich phase;

MB - Mass balance

3.3.1 Partitioning behavior of protease

The partition coefficient (*K*) values ranged from 1.46 to 2.83 across all assays, with $K > 1$, indicating preferential partitioning of the protease into the upper PEG-rich phase. This behavior may be associated with the greater affinity of the enzyme for the PEG-rich phase, resulting from the characteristics of its surface and the physicochemical properties of the phases formed. According to Asenjo and Andrews [3], protein partitioning in aqueous two-phase systems is primarily determined by the surface properties of the protein, with hydrophobicity playing an important role, although the mechanism is complex and results from interactions between the protein and the components of both phases. In addition, enzyme distribution may be influenced by electrostatic interactions, hydrogen bonding, differences in chemical potential, solvation, and molecular size and conformation [3, 7]. In the PEG/sodium sulfate system, increasing the concentration of the components modifies the properties of the phases and, consequently, the relative solubility of the protease in each phase. Thus, the observed partitioning toward the PEG-rich phase is likely the result of a balance among these different interactions, favoring the retention of the protease in this phase over the sodium sulfate-rich phase.

This preferential partitioning of the protease into the PEG-rich phase is consistent with recent findings reported for fungal proteases in PEG-based aqueous two-phase systems. Silva et al. [28] investigated an extracellular protease produced by *Aspergillus* sp. UCP1287 using a PEG/phosphate aqueous two-phase system and observed preferential migration of the enzyme to the PEG-rich phase under the evaluated conditions. The authors also demonstrated that PEG molecular mass and phase composition influenced enzyme partitioning, highlighting the importance of the physicochemical characteristics of the ATPS in determining protease distribution between the phases. Similarly, in the present study, the protease consistently showed preferential partitioning into the PEG-rich phase ($K > 1$) across all experimental conditions, suggesting that the affinity of the enzyme for this phase is a reproducible behavior in PEG-based ATPS. The Pareto chart analysis (Fig. 2) showed that none of the individual factors or their interactions had a statistically significant effect on the partition coefficient (*K*), as all effects remained below the significance threshold.

Although not statistically significant, the effects with the greatest magnitudes were the three-way interaction between PEG molecular mass, PEG concentration, and sodium sulfate concentration (4.07), followed by the interaction between PEG molecular weight and sodium sulfate concentration (-4.05). The individual effects of PEG concentration, PEG molecular weight, and sodium sulfate concentration were − 2.64, -1.70, and 0.76, respectively, indicating that their effects on *K* were lower in magnitude than those observed for the interactions.

3.3.2 Protease recovery and purification

Since the proteases preferentially partitioned into the PEG-rich phase, with no detectable enzyme activity in the salt-rich phase, protease recovery and purification were evaluated only in the PEG-rich phase. The experimental protease recovery in the PEG-rich phase (YPEG) ranged from 66.52 to 173.09% across the experimental conditions. The highest recovery values were observed in Runs 1 and 2, both performed with a sodium sulfate concentration of 10%, indicating that lower salt concentrations favored protease recovery in the upper PEG-rich phase. In contrast to the partition coefficient (*K*), for which none of the investigated factors showed a statistically significant effect, the Pareto analysis of Y_{PEG} (Fig. 3A) revealed statistically significant effects for sodium sulfate concentration (C_{SS}), PEG concentration (C_{PEG}), PEG molecular weight (MM_{PEG}), and the $MM_{PEG} \times C_{SS}$ interaction. All these effects were negative, with standardized effects of − 19.97, − 12.39, − 8.59, and − 6.83, respectively. Thus, increasing the concentration of sodium sulfate, PEG, or PEG molecular mass tended to decrease protease recovery in the PEG-rich phase.

Among the individual factors, C_{SS} showed the greatest effect on Y_{PEG}, highlighting the importance of salt concentration in determining enzyme distribution between the two phases. Changes in sodium sulfate concentration modify the physicochemical properties of the system, including ionic strength and phase composition, which can consequently affect the relative solubility and partitioning of proteins between the PEG-rich and sodium sulfate-rich phases [12, 13]. The higher Y_{PEG} values observed at 10% C_{SS} therefore suggest that this condition provided a more favorable environment for protease recovery in the PEG-rich phase.

The interaction between MM_{PEG} and C_{SS} was also statistically significant (Fig. 3A), indicating that the effect of PEG molecular weight on protease recovery depended on the sodium sulfate concentration. At 13% C_{SS}, increasing MM_{PEG} from 1500 to 20,000 g/mol resulted in a marked decrease in Y_{PEG}, whereas this effect was less pronounced at 10% C_{SS}. This behavior indicates that the combined effects of polymer molecular mass and salt concentration can substantially modify the physicochemical properties of the phases and, consequently, enzyme distribution. PEG molecular mass can influence the composition and physicochemical characteristics of the polymer-rich phase, thereby affecting protein solubility and partitioning [3, 7].

The geometric interpretation of the interaction between MM_{PEG} and C_{SS} on Y_{PEG} is presented in Fig. 3B. The predicted Y_{PEG} values ranged from 76.34 to 143.05%, with the highest predicted protease recovery obtained at a PEG molecular mass of 1500 g/mol and a sodium sulfate concentration of 10.0%, whereas the lowest predicted value was observed at 20,000 g/mol and 13.0% sodium sulfate. The response surface illustrates the negative effects of both factors on protease recovery, with C_{SS} exerting the stronger effect. Overall, the results indicate that lower sodium sulfate concentrations and lower PEG molecular mass favor protease recovery in the PEG-rich phase.

The FP_{PEG} values ranged from 0.54 to 1.75 (Table 2). According to the Pareto chart of standardized effects (Fig. 4A), C_{PEG} concentration and the three-way interaction between MM_{PEG}, CSS, and C_{PEG} were the only statistically significant effects ($p < 0.05$), with absolute effects of 4.69 and 4.88, respectively. The effect of C_{PEG} was negative, indicating that increasing its concentration, when considered individually, tends to reduce the purification factor in the PEG-rich phase (FP_{PEG}). In contrast, the three-way interaction $MM_{PEG} \times C_{SS} \times C_{PEG}$ showed a positive effect, indicating that the simultaneous combination of these factors favored an increase in FP_{PEG}. The other effects had values below the established significance threshold.

Analysis of the cubic plot (Fig. 4B) revealed that the highest purification factor in the PEG-rich phase (FP_{PEG} = 1.752) was obtained in Run 2, under conditions of lower salt concentration (C_{SS} = 10%), lower PEG concentration (C_{PEG} = 18%), and higher PEG molar mass (MM_{PEG} = 20,000 g mol^{-1}). This result suggests that the combination of system composition and polymer molecular characteristics favored protease partitioning into the upper phase, resulting in greater relative purification compared with the total protein content of the extract. These effects may be associated with changes in phase composition, hydration, and the availability of free volume, which influence interactions between proteins and the components of the aqueous two-phase system. Longer PEG chains may intensify volume-exclusion effects, thereby modifying protein partitioning according to their physicochemical properties and interactions with the polymer-rich phase [7, 12]. Therefore, the performance of Run 2 highlights the importance of jointly adjusting PEG molar mass and polymer and salt concentrations to improve enzyme purification.

3.3 Characterization of the recovery enzyme

3.3.1 Effect of temperature on protease activity and stability

Figure 5A illustrates the effect of temperature on the activity of the protease produced by *Penicillium* 2A3, comparing the crude extract with the enzyme recovery by aqueous two-phase system (ATPS) under the conditions of Run 2 (PEG 20,000, 18% PEG, and 10% salt). The purified protease exhibited high relative activity over the temperature range of 10–40°C, reaching its optimum at 40°C, followed by a progressive decline at higher temperatures. In contrast, the crude extract exhibited a slightly higher optimum temperature of 50°C. This difference may reflect changes in the enzyme's microenvironment following purification, as components present in the crude extract may influence protein hydration and conformational stability, potentially contributing to the maintenance of catalytic activity at elevated temperatures. Nevertheless, the purified enzyme exhibited higher relative activity across most of the temperature range evaluated, indicating that the purification process did not compromise its catalytic performance under the tested conditions.

Regarding thermal stability (Fig. 5B), the crude extract retained substantially higher residual activity than the purified enzyme throughout the 180-min incubation period. This behavior suggests that components of the crude extract may contribute to maintaining protease stability under thermal stress. Non-target proteins, carbohydrates, and other compounds present in the fermentation medium may influence the enzyme's hydration environment and reduce conformational changes associated with thermal inactivation. Conversely, the removal of these components during purification may increase the protease's susceptibility to thermal denaturation and potentially to autolysis, particularly if the enzyme is not sufficiently stabilized in the purified preparation. Such effects are relevant when evaluating the thermal behavior of isolated proteases, whose stability depends on their structural characteristics and the physicochemical properties of the surrounding medium [4, 33]. However, further experiments would be necessary to establish the specific contribution of these mechanisms to the differences observed between the crude and purified preparations.

The optimum temperature of 40°C observed for the purified protease is consistent with the catalytic profiles commonly reported for proteases produced by mesophilic filamentous fungi, which frequently exhibit maximum activity between 30 and 50°C [21]. Similar temperature optima have been reported for proteases produced by *Penicillium* and *Trichoderma* species by Barros et al[6] and Ercilia et al[5], respectively. These findings support the suitability of the 2A3 protease for applications requiring catalytic activity at moderate temperatures. However, its potential for specific industrial applications should also be assessed considering its thermal stability, pH dependence, and performance under the intended process conditions.

3.3.2 Effect of pH on protease activity and stability

Figure 6A illustrates the effect of pH on the activity of the protease present in the crude extract and in the fraction recovered using the aqueous two-phase system (ATPS). The recovered fraction exhibited higher relative activity under acidic conditions, particularly at pH 3.0, 5.0, and 6.0, with pH 5.0 established as the optimum. In contrast, the crude extract exhibited higher activity under alkaline conditions, with an optimum at pH 9.0. This difference in activity profiles suggests that ATPS recovery may have altered the relative contribution of enzymatic components in each preparation, favoring the recovery of components with higher activity under acidic conditions. However, this hypothesis requires further investigation, as potential effects of the composition of the polyethylene glycol (PEG)-rich phase on catalytic activity should also be considered.

The predominance of activity under alkaline conditions observed in the crude extract is consistent with studies investigating fungal proteases from *Aspergillus tamarii* URM4634 and *Aspergillus heteromorphus* URM0269.

Proteases from these fungi exhibited alkaline pH optima, including values close to 8.0 following recovery using PEG/citrate aqueous two-phase systems [10, 26]. These findings demonstrate that fungal proteases can retain optimal activity under alkaline conditions even after recovery processes. In contrast, the optimum activity at pH 5.0 observed in the PEG-rich fraction in the present study indicates a distinct biochemical profile, which may be related to the characteristics of the enzyme preparation and the partitioning conditions employed.

Regarding pH stability (Fig. 6B), the recovered fraction, incubated at its optimum pH of 5.0, exhibited a gradual decrease in residual activity, with complete loss of activity after approximately 12 h. In contrast, the crude extract, incubated at pH 9.0, retained residual activity for a longer period, indicating greater stability under the respective conditions evaluated. This difference may be associated with the composition of the preparations, including potential protective effects of components present in the fermentation matrix. However, since the samples were incubated at their respective optimum pH values, the difference in stability cannot be attributed exclusively to the recovery process, as the incubation pH also differed between treatments.

The importance of jointly evaluating pH-dependent activity and stability is also highlighted by studies characterizing proteases from *Aspergillus tamarii* URM4634, which exhibited optimal activity under alkaline conditions and stability over a pH range compatible with these conditions [26]. Additionally, studies on proteases from *Aspergillus heteromorphus* URM0269 investigated stability following prolonged incubation at different pH values, demonstrating the importance of considering exposure time when characterizing the biochemical properties of these enzymes. These comparisons reinforce that the optimum pH for activity should not be interpreted in isolation as an indicator of prolonged stability. Although the optimum pH of 5.0 observed in the recovered fraction suggests potential applications in processes conducted under acidic conditions, the decrease in activity during incubation indicates a limitation for applications requiring prolonged exposure to this pH. Therefore, further studies on operational stability, the influence of the PEG-rich phase composition, and enzyme performance with specific substrates may help establish its biotechnological applicability.

3.3.3 Effect of different metal ions on protease activity

The effects of different metal ions (5 mM) on the activity of the protease produced by *Penicillium* sp. 2A3 revealed distinct response profiles between the crude extract and the fraction recovered using the aqueous two-phase system (ATPS) (Fig. 7). In the crude extract, all metal ions evaluated significantly inhibited protease activity compared with the control. This response may be related to the complex composition of the crude preparation, in which interactions between metal ions and different matrix components may influence the measured enzymatic activity. In contrast, the recovered fraction exhibited a more heterogeneous response, suggesting that the effects of metal ions differed between the two enzymatic preparations.

Among the ions evaluated, Mg^{2+}, Al^{3+}, Na^{+}, Co^{2+}, and K^{+} did not significantly affect or caused only slight inhibition of the residual activity of the recovered protease, indicating relative tolerance under the experimental conditions. Conversely, Fe^{2+} and Zn^{2+} significantly reduced residual activity, while Cu^{2+}, Mn^{2+}, and Ba^{2+} also caused moderate to pronounced inhibition. These findings indicate that the response of the recovered protease depends on the nature of the metal ion.

A relevant comparison can be made with the extracellular serine protease from *Aspergillus tamarii* URM4634 characterized by Silva et al [26], which exhibited increased activity in the presence of Mg^{2+} and inhibition by Zn^{2+}. Although the response to Zn^{2+} was consistent with that observed for the *Penicillium* sp. 2A3 protease, the effect of Mg^{2+} differed between the enzymes, as no significant change or only slight inhibition was observed in the

recovered fraction in the present study. These differences demonstrate that the effects of metal ions are enzyme-dependent and may vary according to the fungal species, the biochemical properties of the protease, and the experimental conditions. Therefore, the effects reported for one fungal protease cannot be directly extrapolated to another.

The contrasting responses observed between the crude extract and the recovered fraction also highlight the importance of considering the composition of the enzymatic preparation when interpreting metal-ion effects. Nascimento et al [15] demonstrated that ATPS based on PEG and sodium sulfate can promote the recovery and partial purification of a fibrinolytic protease from *Mucor subtilissimus* UCP 1262, illustrating the potential of this approach to modify the composition of enzyme preparations. In the present study, however, the differences in metal-ion responses cannot be attributed exclusively to ATPS recovery, as the contribution of matrix components and possible interactions with the recovered fraction were not directly investigated.

Overall, the results indicate that the protease from Penicillium sp. 2A3 exhibits differential sensitivity to metal ions, particularly in the recovered fraction. The relative tolerance to Mg^{2+}, Na^{+}, and K^{+} and the inhibitory effects of Fe^{2+}, Zn^{2+}, Cu^{2+}, Mn^{2+}, and Ba^{2+} provide preliminary information for evaluating its compatibility with different process formulations. Further studies involving metal-ion concentration gradients and enzyme kinetic analyses are required to determine whether these ions directly affect catalytic activity and to clarify their potential implications for biotechnological applications.

3.3.4 Effect of protease inhibitors on enzymatic activity

Table 3 presents the effects of different inhibitors on the residual activity of the protease produced by *Penicillium* sp. 2A3 in the crude extract and in the fraction recovered using the aqueous two-phase system (ATPS). The use of protease inhibitors provides preliminary information about the catalytic mechanism and the functional groups potentially involved in enzymatic activity.

Table 3
Effect of different inhibitors (5 mM) on the residual activity (%) of the protease in the crude extract and in the ATPS-recovered fraction.

Inibidors	**Residual activity in the ATPS-recovered (%)**	**Residual activity in the crude extract (%)**
Control	100 ± 0,56	100 ± 0,56
EDTA	62,91 ± 1,69	83,6 ± 0,37
B-Mercaptoetanol	66,83 ± 0,09	93,77 ± 0,70
PMSF	71,21 ± 0,04	96,23 ± 1,8

The ATPS-recovered fraction exhibited residual activities of 62.91%, 66.83%, and 71.21% in the presence of EDTA, β-mercaptoethanol, and PMSF, respectively, corresponding to activity reductions of 37.09%, 33.17%, and 28.79%. In comparison, the crude extract remained 83.60%, 93.77%, and 96.23% of its initial activity, respectively. Thus, the recovered fraction was more sensitive to all three inhibitors than the crude extract. EDTA caused the greatest reduction in activity in the recovered fraction. As a chelating agent, EDTA can bind metal ions that may contribute to catalytic activity or structural stability. The observed inhibition may therefore indicate that metal ions influence the activity of the recovered protease. However, EDTA sensitivity alone is insufficient to classify the enzyme as a metalloprotease, particularly because the residual activity remained above 60% and the effects of metal supplementation or other class-specific inhibitors have not been established.

β-Mercaptoethanol reduced the residual activity of the recovered fraction by 33.17%. Because this compound can alter disulfide bonds and the redox state of susceptible residues, the result suggests that reducing conditions may affect enzyme activity or conformation. Nevertheless, β-mercaptoethanol is not sufficiently specific to establish or exclude a cysteine-dependent catalytic mechanism. PMSF caused a 28.79% reduction in activity in the recovered fraction, indicating a measurable but incomplete inhibitory effect. PMSF is commonly used to investigate serine proteases; however, the magnitude of inhibition observed here does not, by itself, establish that the enzyme belongs to this class. This response differs from that reported for the fibrinolytic protease from *Mucor subtilissimus* UCP 1262 and the extracellular serine protease from *Aspergillus tamarii* URM4634, both of which exhibited strong inhibition by PMSF [15, 26]. These differences may reflect distinct catalytic properties and experimental conditions.

The lower sensitivity of the crude extract to the three inhibitors should also be interpreted cautiously. Because the crude extract contains a mixture of extracellular proteins and other compounds, its measured activity may reflect the combined contributions of multiple components. The higher sensitivity observed after ATPS recovery may be associated with changes in the composition of the enzymatic preparation. However, these results do not demonstrate that the recovered fraction contains a single protease or that ATPS alone caused the differences in inhibitor sensitivity. Overall, the inhibition profile provides preliminary evidence that the activity of the *Penicillium* sp. 2A3 protease is affected by EDTA, β-mercaptoethanol, and PMSF, particularly in the ATPS-recovered fraction. Nevertheless, its catalytic class cannot be conclusively assigned from these results alone. Additional assays using complementary inhibitors, such as pepstatin A for aspartic proteases and 1,10-phenanthroline for metalloproteases, together with appropriate concentration and incubation controls, would help clarify the enzyme's catalytic mechanism.

4. Conclusions

This study highlights the potential of pequi (*Caryocar brasiliense*) peel as a substrate for fungal protease production and the use of aqueous two-phase systems as a strategy for enzyme recovery. The approach integrates agro-industrial waste valorization with the production of biotechnologically relevant products, contributing to the development of more sustainable processes. The characterization of the protease from *Penicillium* sp. 2A3 provides a basis for further investigations into its properties and potential applications, although additional studies are needed to elucidate its catalytic mechanism and validate its applicability under process conditions.

Declarations

Competing Interests

The authors declare no conflicts of interest related to the publication of this article.

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Author Contributions

Vinicius de Paula Taffarel: Conceptualization, Methodology, Writing – original draft.; Antonio Luis de Sousa Junior: Formal analysis; Thalesram Izidoro Pinotti: Supervision, Writing – review & editing; Edson da Silva Rodrigues: Methodology, Visualization; Glayce Kelly da Costa: Visualization; José Pedro Martins Barbosa Filho: Methodology; Márcia Nieves Carneiro da Cunha: Formal analysis; Romero Marcos Pedrosa Brandão Costa: Methodology, Visualization; Ana Lúcia Figueiredo Porto: Resources; Alice Maria Gonçalves Santos: Resources; Thiago Pajeú Nascimento: Supervision, Funding acquisition, Resources.

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Data Availability

The data supporting the findings of this study are available from the corresponding author upon reasonable request.

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Figures

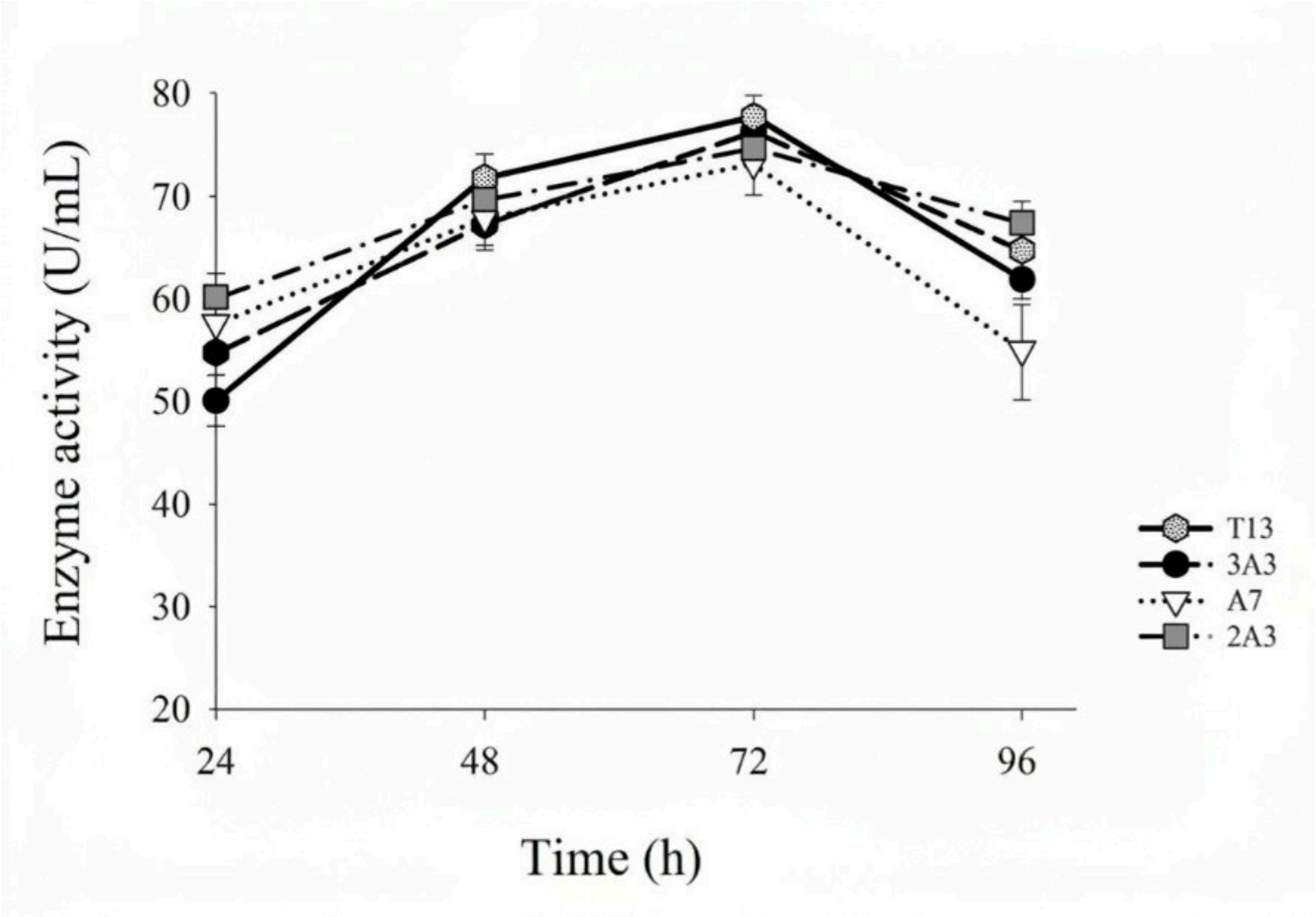

Figure 1

Protease activity (U/mL) of *Penicillium*spp. (A7, 2A3, and 3A3) and *Trichoderma* sp. UFPIT13 throughout the fermentation period

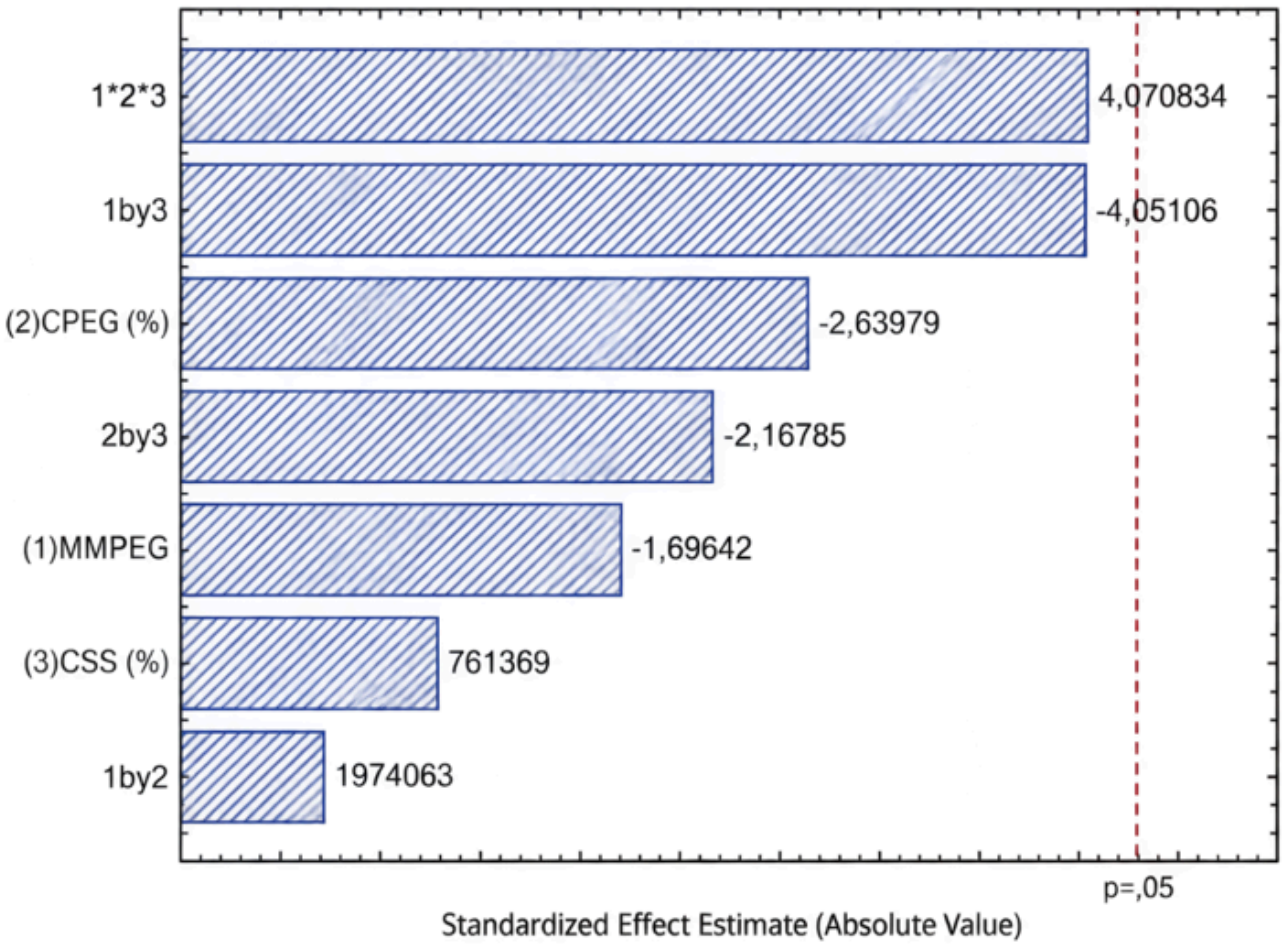

Figure 2

Pareto chart of the standardized effects of the variables: PEG molecular mass (MMPEG), PEG concentration (CPEG), and sodium sulfate concentration (CSS) on the protease partition coefficient (*K*)

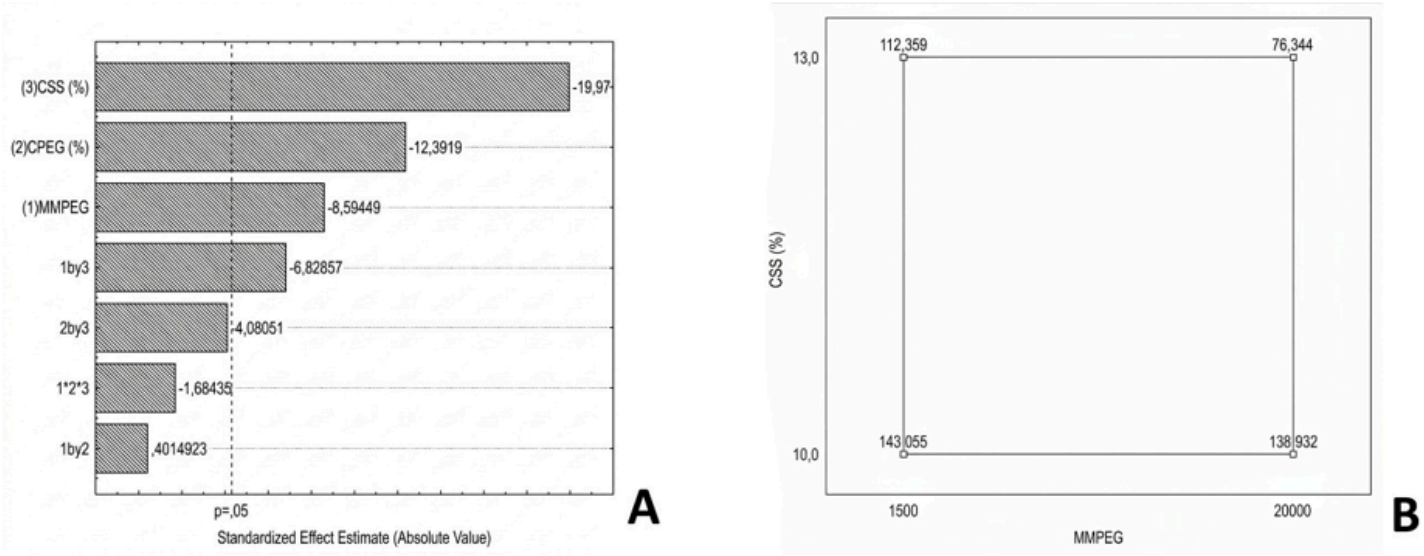

Figure 3

(A) Pareto chart of the standardized effects of the variables: PEG molecular mass (MM_{PEG}), PEG concentration (C_{PEG}), and sodium sulfate concentration (C_{SS}) on the recovery in the PEG-rich phase (Y_{PEG}). **(B)** Geometric interpretation plot of the significant interaction between the C_{SS} and MM_{PEG} variables on Y_{PEG}

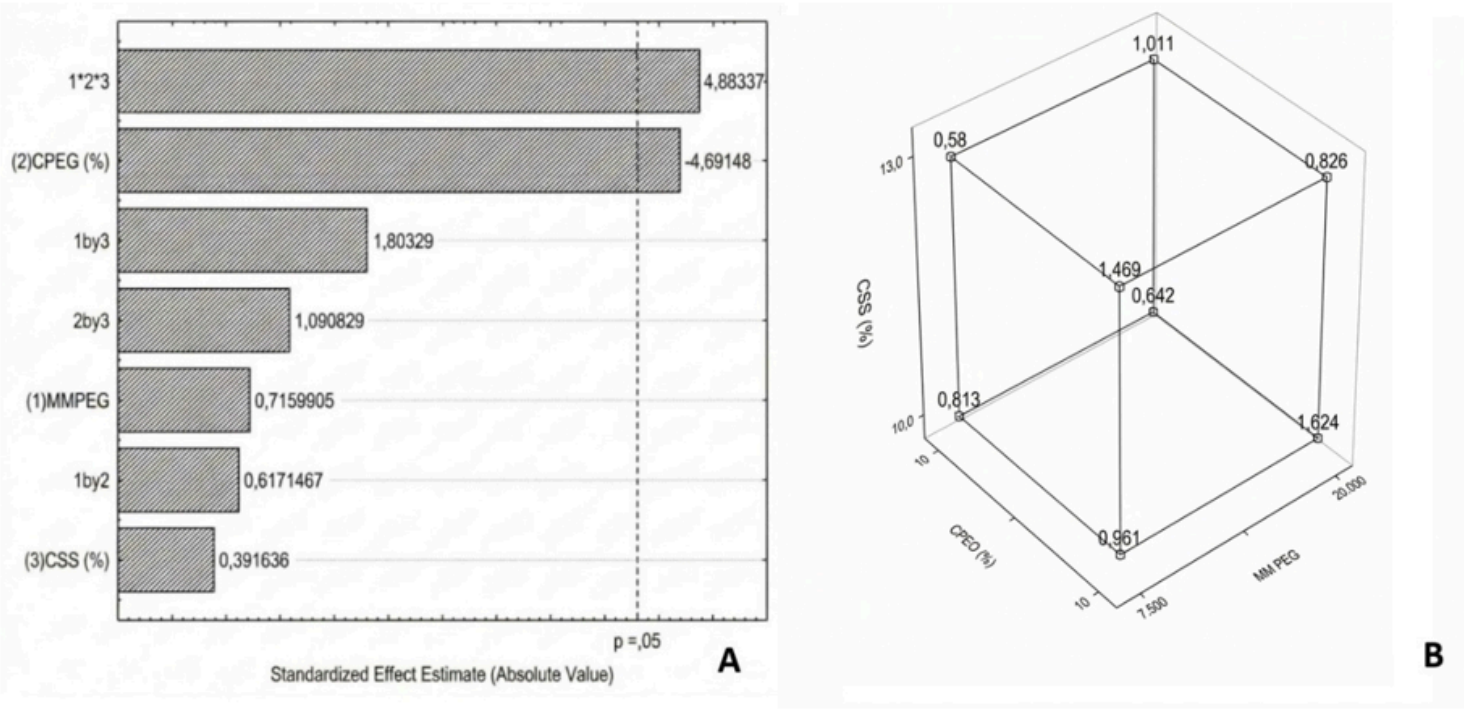

Figure 4

(A) Pareto chart of the standardized effects of the variables: PEG molecular mass (MM_{PEG}), PEG concentration (C_{PEG}), and sodium sulfate concentration (C_{SS}) on purification factor in the PEG-rich phase (FP_{PEG}). **(B)** Cubic plot of the variables MM_{PEG}, C_{PEG}, and C_{SS}, with recovery in the PEG-rich phase as the response variable

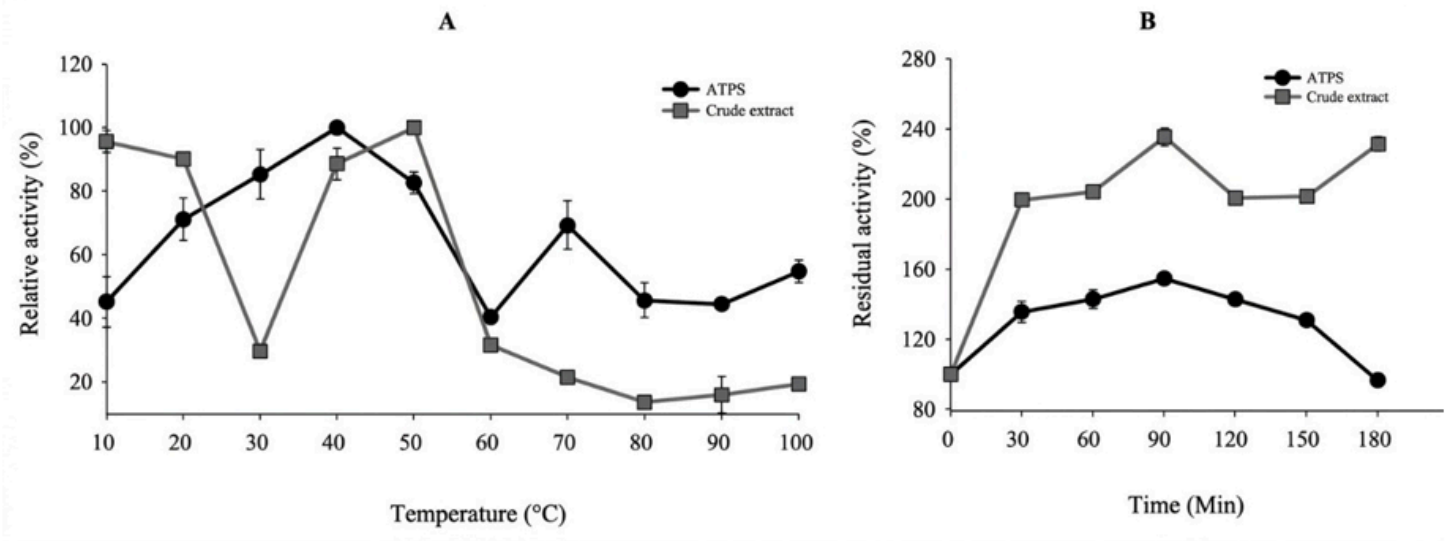

Figure 5

Effect of temperature and thermal stability of the enzyme in the crude extract and in the PEG-rich phase after recovery by aqueous two-phase system (ATPS). **(A)** Relative activity at different temperatures. **(B)** Residual activity over incubation time

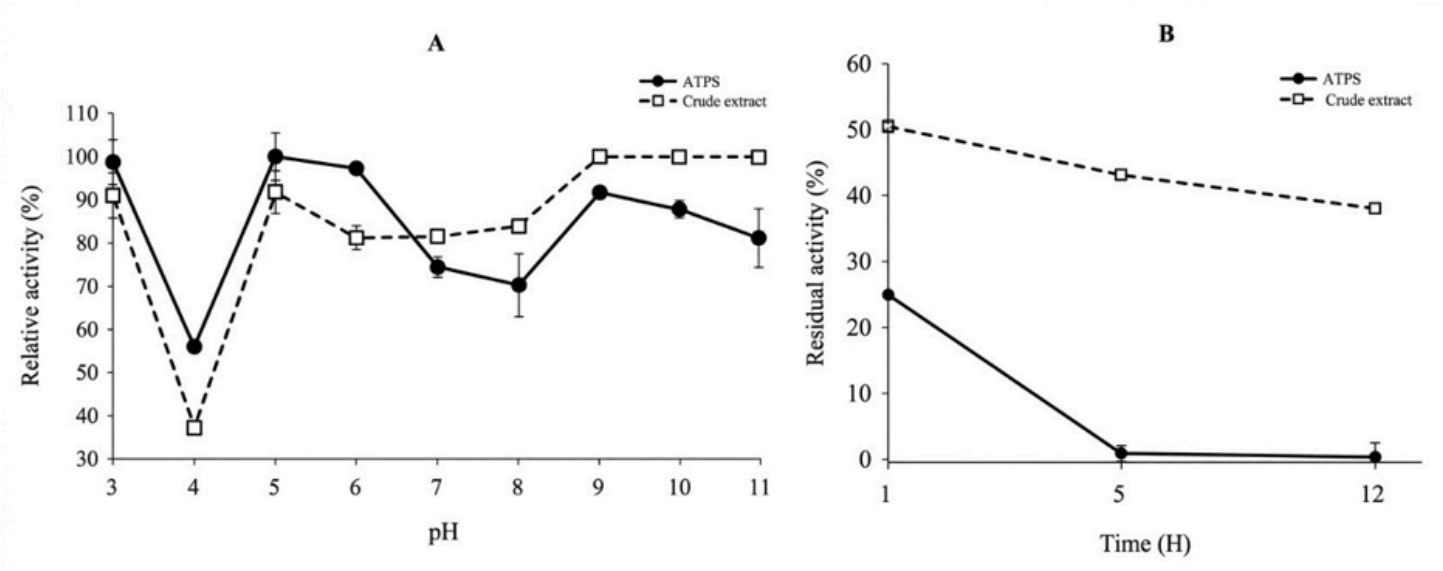

Figure 6

Effect of pH (**A**) and pH stability over time (**B**) on the activity of the protease recovered using an aqueous two-phase system (ATPS) and in the crude extract

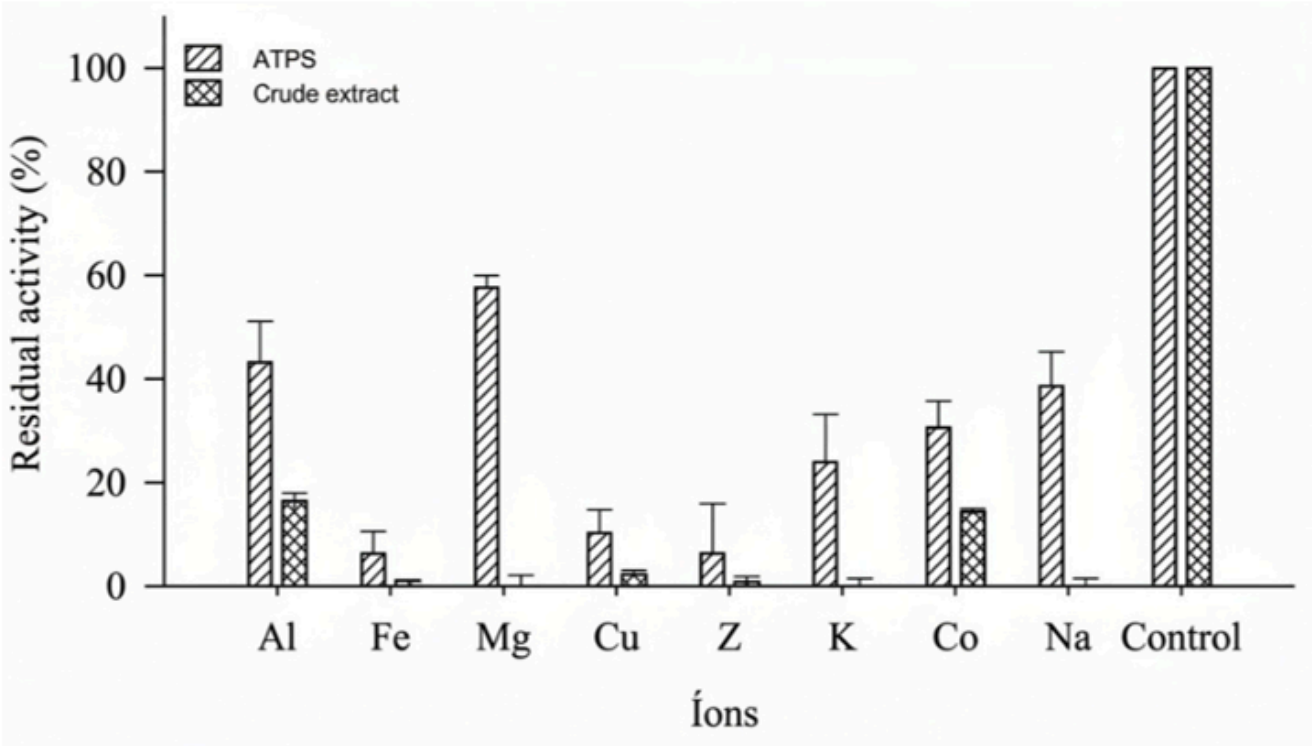

Figure 7

Effect of different metal ions (5 mM) on the relative activity (%) of the protease in the crude extract and in the ATPS-recovered fraction (PEG-rich phase)