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MICROBIOLOGY

Purification and characterization of a protease from *Aspergillus sydowii* URM5774: Coffee ground residue for protease production by solid state fermentation

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Abstract: Solid state fermentation is a promising technology largely used in biotechnology process and is a suitable strategy for producing low-cost enzymatic products. At the present study, a novel enzyme obtained through solid state fermentation using *Aspergillus sydowii* was herein purified and characterized. The fermentations used coffee ground residue as substrate and the crude enzyme was submitted through further purification steps of: acetonic precipitation, DEAE-Sephadex and Superdex G-75 column. Both crude and purified enzymes were submitted to biochemical characterization of their thermostability, optimal temperature and pH, effects of inhibitors and metal ions. A purified protease was obtained with yield of 5.9-fold and 53% recovery, with maximal proteolytic activity of 352.0 U/mL. SDS-PAGE revealed a band of protein at 47.0 kDa. The enzyme activity was abolished in the presence of phenyl-methyl sulfonyl fluoride and partially inhibited against Triton X-100 (78.0%). The optimal activity was found in pH 8.0 at 45°C of temperature. Besides, the enzyme showed stability between 35°C and 50°C. It was possible to determine appropriate conditions to the obtainment of thermostable proteases with biotechnological interest associated with a method that concomitantly shows excellent production levels and recovery waste raw material in a very profitable process.

Key words: *Aspergillus sydowii*, biotechnology, proteases, solid state fermentation, waste coffee residue.

INTRODUCTION

Agricultural waste recovery associated with the development of techniques of conversion of raw material into economically useful products makes research of bioconversion by microorganisms increasingly relevant. Production of proteases using solid substrates through fermentative processes is pointed out in the present study as a low-cost alternative for waste recovery (Novelli et al. 2016, Albuquerque et al. 2020).

Proteases hydrolyze peptide bonds and provide essential modifications in proteins which are involved in the process of digestion, activation of enzymes, blood clotting and membrane transport. They have a variety of functions and represent approximately 60% of the enzyme world market, being often used in detergent, leather, pharmaceutical and food

industries (Castro et al. 2014, Albuquerque et al. 2020).

Processes for obtaining proteases derived from plants are slower than others, due to plant senescence. This period is a natural developmental process, but it is also closely linked to abiotic and biotic stresses. This physiological set of events can be modulated by endogenous and exogenous factors such as plant growth regulators, so the protease production aiming to industrial field should be difficult. At the same time, proteases from animal origin (for example pepsin, chymosin and trypsin) need to be prepared in larger scale and are dependent on the cattle for slaughter, what hinders its use. Microbial proteases, on the other hand, are considered commercial profitable enzymes, because their biochemical diversity, rapid growth, and fast and safe production (Mansor et al. 2019), presenting therefore higher economic potential.

Aspergillus is in the order Eurotiales also comprising the genera *Penicillium*, which are known to have a potential for producing various metabolites such as antibiotics, organic acids, medicines or enzymes, milk-clotting enzymes and extracellular proteases (Silva et al. 2018). Filamentous fungi are widely applied for production of enzymes as amylases, lipases, proteases and pectinases (Sethi et al. 2016), and the processes are advantageous for their low cost of materials, high production and recovery, since they are obtained from the extracellular medium (Vishwanatha et al. 2009, Souza et al. 2015). The genus *Aspergillus* is the most common filamentous fungi used in industry.

The solid-state fermentation (SSF) provides some advantages over the submerged (SMF) such as: the use of agricultural residues as substrates (wheat bran, soybean, rice, bagasse, and nuts); requires low amount of water; produces more concentrated metabolites; obtained by stationary process (causing no energy costs); and in most cases the enzyme yield is higher (Zenebon et al. 2008).

With high production of the world coffee industry, a lot of waste is generated, and it is estimated that 9.9 million solid waste are produced annually worldwide (Tang et al. 2020) which draws attention to a high waste of raw materials and environmental damage.

Disposal of residues in agro-industrial processes represents a large financial disadvantage that could be prevented from reuse of raw material for obtaining biotechnological enzymes (Albuquerque et al. 2020). The present work aims to follow the multistep process (production, purification, characterization, and application) for converting coffee ground residues into a purified protease through solid state fermentation by *Aspergillus sydowii*.

MATERIALS AND METHODS

Chemicals

Azocasein, DEAE-sephadex G50, trichloroacetic acid, ammonium sulfate, β-mercaptoethanol, Tris (hydroxymethyl) aminomethane, glycine, Phenylmethylsulphonyl fluoride (PMSF) and protein markers of molecular weights were purchased from Sigma Chemicals (St Louis, USA). Ammonium persulfate, N, N, N', N'-tetramethyl ethylene diamine (TEMED), acrylamide, sodium dodecyl sulfate (SDS) and Coomassie Brilliant Blue R-250 were obtained from Bio-Rad Laboratories (Hercules, CA, USA). All other reagents were of analytical grade.

Microorganism

The microorganism used was the *Aspergillus sydowii* URM5774, isolated from the coffee grounds and duly identified by the URM Culture Collection of the Federal University of Pernambuco. The cultures were stocked in the

medium Potato Dextrose Agar (PDA) at room temperature (±26°C).

Inoculum preparation

The culture was maintained in Potato Dextrose Agar (PDA) at 30°C for 7 days. Then, the spores were suspended in a nutrient solution previously sterilized that comprised the culture medium Potato Dextrose Agar (PDA) in 0.15 M NaCl solution (5 mL) according to Nascimento et al. (2017). The number of spores was standardized in a Neubauer chamber (Laboroptik, Lacing, United Kingdom) to obtain the final concentration of 10^7 spores/mL.

Solid state fermentation (SSF)

Solid state fermentation (SSF) was used as carbon and nitrogen source from coffee ground residues. The coffee residues were obtained from the university restaurant in the Universidade Federal Rural de Pernambuco (UFRPE), Recife, Brazil, and kept in oven for a period of 72h at 50°C, to ensure complete drying of the coffee to obtain a constant weight. The fermentations were carried out with 4 g of coffee residue, during 72h, at 30°C, according to Zenebon et al. (2008) and deposited in a germination chamber (BOD) until the end of the process. The compositions of the solid-state fermentation consisted of coffee residue (4.0 g) as a substrate (moisture content of 60%) which were sterilized by autoclaving at 121°C, for 20 min, inoculated with the suspension of *A. sydowii* spores (final concentration 10^7 spores/mL), and incubated at 30°C for 72 h. After incubation, 7.5 mL of 100 mM Tris-HCl buffer pH 8.0 were added per g of substrate and the flasks were placed in an orbital shaker at 150 rpm for 120 min at 25°C ± 2.

Enzyme extraction

For the protease extraction, 30.0 mL (0.1 M Tris-HCl buffer pH 8.0) were added to solid state fermentation (7.5mL/g of substrate), followed by orbital agitation (2h), and after to filtration with filter paper in using vacuum pump. The crude extract was centrifuged at 12,000 xg for 30min at 4°C, and the supernatant was used as crude enzyme to the subsequent purification steps (Nascimento et al. 2017).

Determination of protease activity

Protease activity was measured as described by Ginther (1979). Assay mixtures of 1.0 mL containing 0.2 M Tris-HCl, pH 7.2, 1 mM CaCl2, 1% Azocasein and 150 µL of enzymatic purified, were incubated at 28°C for 1 h. After stopping the reaction by adding 1.0 mL of 10% trichloroacetic acid, samples were centrifuged at 3,000 g for 15 min, and 0.8 mL of the supernatant was transferred into a second tube containing 0.2 mL of 1.8 M NaOH. Finally, samples were blended in a vortex mixer, and the absorbance was measured at λ420 nm. One unit of protease activity was defined as the amount of enzyme responsible for a 0.1 increase per hour in the absorbance.

Protein measurements

Protein concentration was determined according to the method of Smith et al. (1985). Bovine serum albumin was used as standard for protein determination and a standard curve was done using a range at 40 to 500 ug/mL.

Acetonic precipitation

The partial purification process was performed using the method of precipitation with acetone at 70% and centrifuged at 16.800 x *g* during 10 min (Nascimento et al. 2017). Then the precipitate was separated from the supernatant and resuspended in Tris-HCl buffer pH 8.0 to obtain the acetonic fraction.

DEAE-Sephadex Chromatography

The acetonic fraction was submitted through a DEAE-Sephadex G-50 column, equilibrated with 0.1 M Tris-HCl buffer, pH 8.0. In this case, the samples were eluted with different concentrations of NaCl solutions (0.3 M, 0.6 M, and 1 M), diluted in the same buffer. Fractions of 1.0 mL were collected at a flow of 1 mL/min after the addition of elution buffer, the process was monitored at 280 nm in spectrophotometer. The fractions with protease activity was pooled and the enzyme solution concentrated for further step in Gel Filtration Chromatography. All the steps were done according to Nascimento et al. (2017).

Gel Filtration Chromatography

An aliquot (1.0 mL) of the sample obtained from DEAE which presented activity was submitted to Gel filtration. The analysis was performed using Tris–HCl buffer 0.1 M (pH 8) added of 0.15 M NaCl in an ÄKTA Avant 25 System (GE Healthcare, Uppsala, Sweden) on Superdex G 75 (HR10/300GL), PC 3.2/30 column, as described in the manufacturer's instructions. The absorbance of the samples was evaluated at 215 nm and 280 nm. The column was calibrated using a mixture of molecular weight markers (1 mg/mL each): bovine serum albumin, carbonic anhydrase and albumin from chicken egg and a trypsin inhibitor. All the steps were done according to Nascimento et al. (2017).

Electrophoresis and protein staining

The SDS-PAGE method was performed according to Laemmli (1970) with a 10% resolving gel and a 5% stacking gel, under non-reducing conditions. The gels were stained with Coomassie Brilliant Blue and kept incubated for 15 h at room temperature under gentle shaking.

Biochemical characterization

Effect of inhibitors and metal ions on proteinase activity

To evaluate the effect of inhibitors and metal ions on the protease activity, the purified enzyme (150 µl) was lyophilized, then resuspended with different solutions (150 µL) containing: Phenylmethyl-sulfonyl-Fluor (PMSF); ethylenediamine tetra acetic acid (EDTA); β-mercaptoethanol and ions Fe^{+2}, Mg^{+2}, Ca^{+2}, Mn^{+2} and Co^{+2} at 0.2 M, during 30 min. All the steps were done according to Nascimento et al. (2017).

Effect of pH (optimum and stability) and temperature (optimum and stability) on purified protease activity.

The purified enzyme obtained from Gel filtration chromatography was subjected to different temperatures varying from 5°C, from 20°C to 100°C for 60 minutes to evaluate the thermostability and then performing the measurement of activities. All the steps were done according to Nascimento et al. (2017). The optimum temperature was assessment by incubation of enzyme and substrate in temperatures varying from 5°C, from 20°C to 100°C, where the reaction presenting and described in 2.6 topic was done.

The pH stability was determined by re-suspending the purified enzyme, previously lyophilized, in different buffers with different pH: acetate buffer 0.2M at pH 5.0 and 6.0 and 0.2 M Tris-HCl buffer at pH 7.0, 8.0 and 9.0. After, the samples were subjected to protease reaction described in topic 2.6. All the steps were done according to Nascimento et al. (2017). The optimum pH was achieved changing the pH buffer solutions described in topic 2.6. The concentration of enzyme and substrate was maintained. The optimum pH was determined

to be in the range of 4.0–12.0 using different buffers (Sodium citrate, NaOH and Tris-HCl) at 50 mM concentration. All the steps were done in triplicate according to Nascimento et al. (2017).

Potential hydrolysis of azocasein

To evaluate the potential hydrolysis of azocasein substrate, after FPLC, an aliquot of enzyme plus substrate solution was pooled at different temperature and lyophilized, and later resuspended with 1.0 mL of 0.1% trifluoracetic acid (TFA) solution, and an aliquot (50 µL) was submitted to high performance liquid chromatography (HPLC) and eluted using a 0 – 90% acetonitrile. The samples were applied sequentially to reverse - phase high performance liquid chromatography (RP - HPLC) coupled to an electro spray ionization mass spectrometer (ESI - MS) using an LC/ MS - 2020 EV (Prominence Shimadzu Corporation, Tokyo, Japan). The chromatography process was assessed on a C18 column (Ultra sphere, 5 µm, 4.6 × 250 mm) and monitored using a UV-detector (Shimadzu UV – vis SPD - 20AV) at 220 – 365 nm.

Effect of inhibitors on protease activity

To evaluate the effect of inhibitors on enzyme activity, the purified enzyme was exposed to the following protease inhibitors: PMSF (10 mM), β-mercaptoethanol (10 mM), ethylenediaminetetraacetic acid (EDTA) (10 mM), Pepstatin A (1mM) and Iodoacetic acid (10mM). Each inhibitor was dissolved according to the Sigma protocol and incubated for 30 min at 25°C with the enzyme. The enzyme activity without inhibitor was considered as control (100%).

Statistical analysis

All data were analysed statistically using the General Linear Models procedure of the Statistix 8.1 software package (Analytical Software, St Paul, MN, USA) and presented as the mean ± standard error (SE). All assays were done in triplicate.

RESULTS AND DISCUSSION

Enzyme production

Factors such as humidity, temperature, extraction type and time of incubation were studied aiming to optimize the production of protease. *A. sydowii* was able to produce a protease (crude extract) with high activity (specific activity of 8.59 U/mL and a content proteolytic 89.8 mg/mL). The humidity, temperature and the time of incubation showed the best production at 60%, 30°C and 5 days, respectively, in comparison with other tested conditions. The data also demonstrate that the coffee ground is a valuable substrate for protease production, since 4.0 g of coffee residue was enough to produce 1.16 mg/mL of protease with 256 U/mL of activity. Proteases are proteolytic enzymes that catalyze the cleavage of peptide bonds in proteins. These enzymes form an exceptionally large and diverse group of complex enzymes which have different properties such as a substrate, an active site and a catalytic mechanism, optimal pH, and a profile of stable temperatures (Silva et al. 2018).

Enzyme purification and electrophoresis (SDS-PAGE)

Partial purification by acetone precipitation concentrated and consequently increased the protease specific activity (see Table I). In this step, the enzyme was recovered by 93% with 2.11-fold purification. Novelli et al. (2016) using wheat bran and soybean meal as substrate for fermentations by *Aspergillus* did not exceed the value of 40 U/mL of protease activity with this pre-purification step.

The subsequent two chromatographic steps (anion-exchange and gel filtration chromatography) improved the enzyme purity,

Table I. Purification steps of protease from *Aspergillus sydowii.*

Purification step	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification (Fold)	Recovery (%)
Crude extract	500	89.8	59	1	100
Cetonic precipate	465	30	125	2.11	93
DEAE-Sephadex	136	0.95	242	4.09	27
Superdex 75	256	1.16	352	5.94	51

reflected in the enzyme recovery and yield and in the obtaining of a single active peak. For that, the partially purified enzyme was applied in a DEAE-Sephadex G-50 column, resulting of fractions eluted by NaCl (1 M). Figure 1 shows the arrows to comprise the pooled containing protein with enzymatic activity. The eluted protein fraction, correspondent to the peaks highlighted in the Figure 1, showed a 4.09-fold purification (specific activity of 242.0 U/mL). Further, the active pooled peaks were concentrated, lyophilized, resuspended, and then submitted through a Superdex G-75 column, what resulted in a varied pattern of protein, however with single protease fraction. That fraction was selected and re-submitted into the same Superdex G-75 column, obtaining a single active peak with 51% protease recovery and 5.94-fold purification (specific activity of 352 U/mL). The isolated peak obtained in the chromatogram 2 (Figure 2) was correlated to a band of ~47.0 kDa in the electrophoresis, showing a homogenous enzyme fraction under non-reducing conditions (Figure 3). The molecular mass of the enzyme purified from Gel filtration had the estimated mass compared with SDS-PAGE.

Effects of inhibitors and metals on the protease activity

Figure 4 shows the influence of metal ions on the purified protease activity. Among the metal ions, the presence of Cu^{+2} and Zn^{+2} provided the lowest residual protease activity, with 43.5% (53 U/mL) and 47.3% (58 U/mL), respectively. The ion Ca^{+2}, in contrast, increased by 30% the residual protease activity, acting as an inductor of activity. According to Purushothaman et al. (2019), the catalytic activity of many enzymes depends on smaller molecules called non-protein cofactors, which can be grouped into two classes: metal ions and the coenzymes. About the activity under presence of metal ions, Zn^{+2} promoted a slight increase in activity, different from the data presented in this paper.

Among the inhibitors, PMSF and EDTA presented the highest levels of inhibition, decreasing the residual activity to 30.4% and 45.7% of their initial value, respectively (Table II). An inhibition of 70% of the protease activity by PMSF indicates that the enzyme is a serine protease. Negi & Banerjee (2009) purified a protease from *Aspergillus awamori* and found no inhibition using PMSF and EDTA. Asker et al. (2013) have discussed the inhibition for both PMSF and EDTA, where the complete inhibition of protease by PMSF is highly indicative of the presence of serine residue in active site, although the inhibition by EDTA might lead a misinterpretation of the inhibition causes, since a large number of enzyme require calcium for their activity, and EDTA as chelator could hinder the calcium effect.

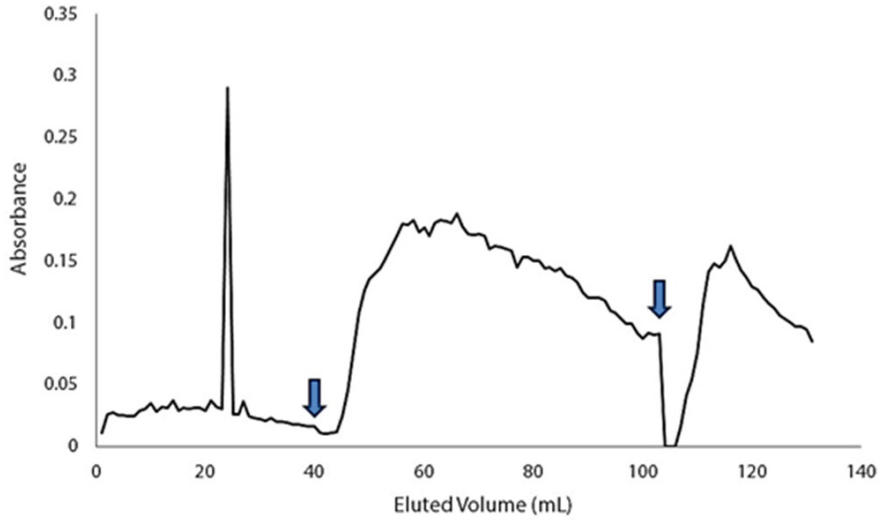

Figure 1. Elution profile from DEAE-Sephadex anion exchange chromatography of a sample obtained from acetonic precipitation of liquid metabolic from *Aspergillus sydowii* **URM5774** extract after solid state fermentation. The marks show the fractions containing the protease activity. The content of the protein was expressed as the absorbance at 280 nm.

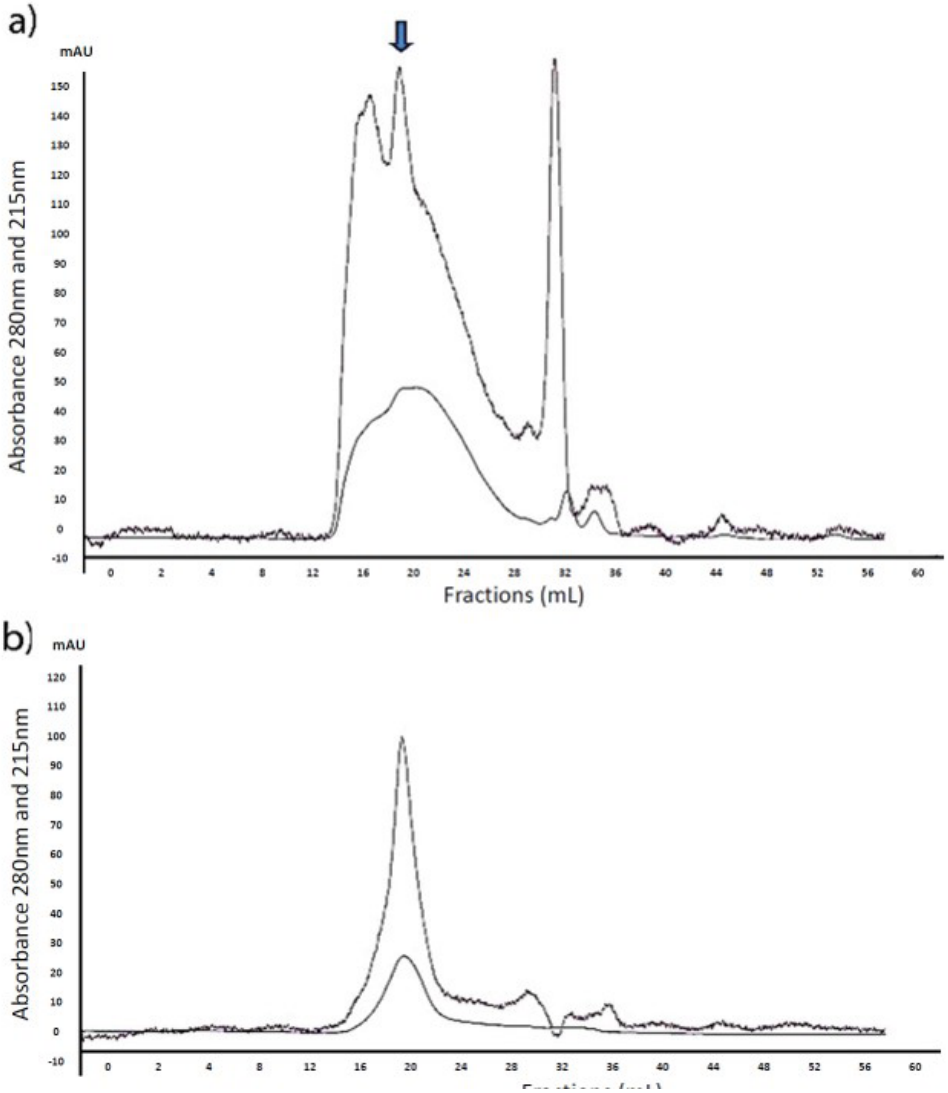

Figure 2. Elution profile from Superdex 75 FPLC system. a) First run: Chromatogram containing varied peaks of protein with the protease peak pointed out. b) Second run: Isolated fraction containing the peak of protease found in a). The content of the protein was expressed as the absorbance at 215 nm.

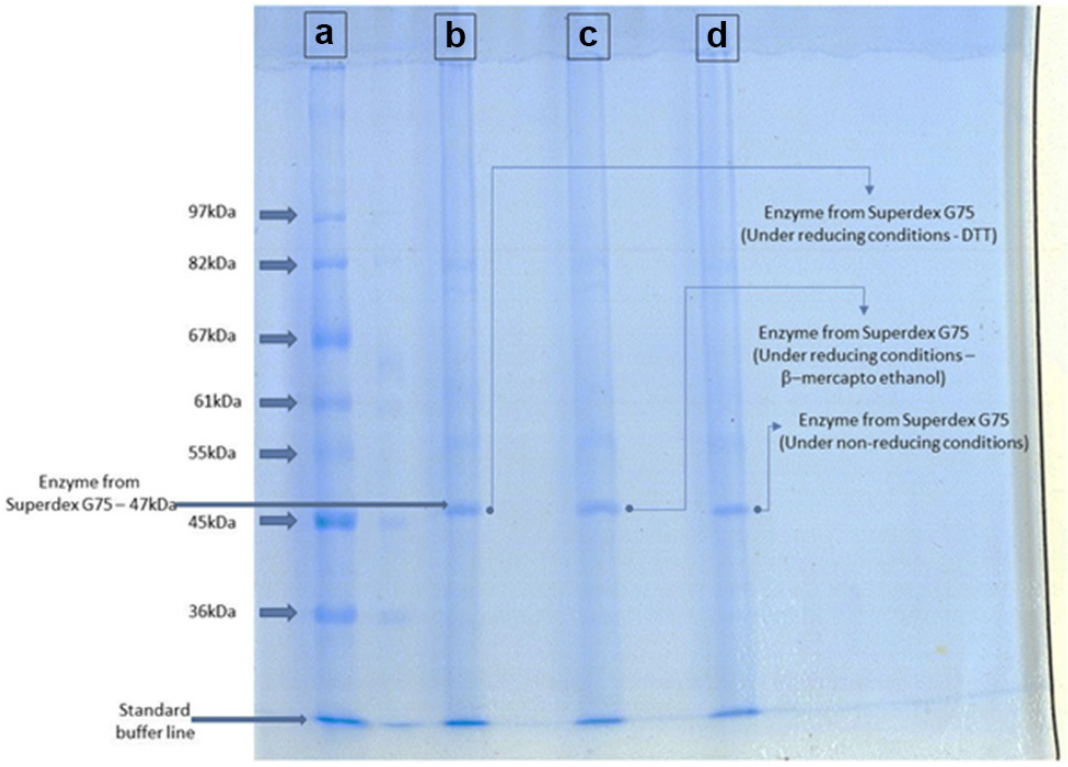

Figure 3. SDS-PAGE (10%) image of enzyme produced and purified from *Aspergillus sydowii* by FPLC system. (a) Molecular markers; (b) Enzyme purified under reducing conditions in presence of DTT; (c) Enzyme purified under reducing conditions in presence of b-mercapto ethanol; (d) Enzyme purified under non- reducing conditions. The standard proteins (×) used to calculate the linear regression: ribonuclease A (13.7kDa), carbonic anhydrase (29kDa), ovalbumin (43kDa), conalbumin (75kDa), aldolase (158kDa), ferritin (440kDa), and thyroglobulin (669kDa). The molecular mass of purified enzyme was calculated from the standard curve.

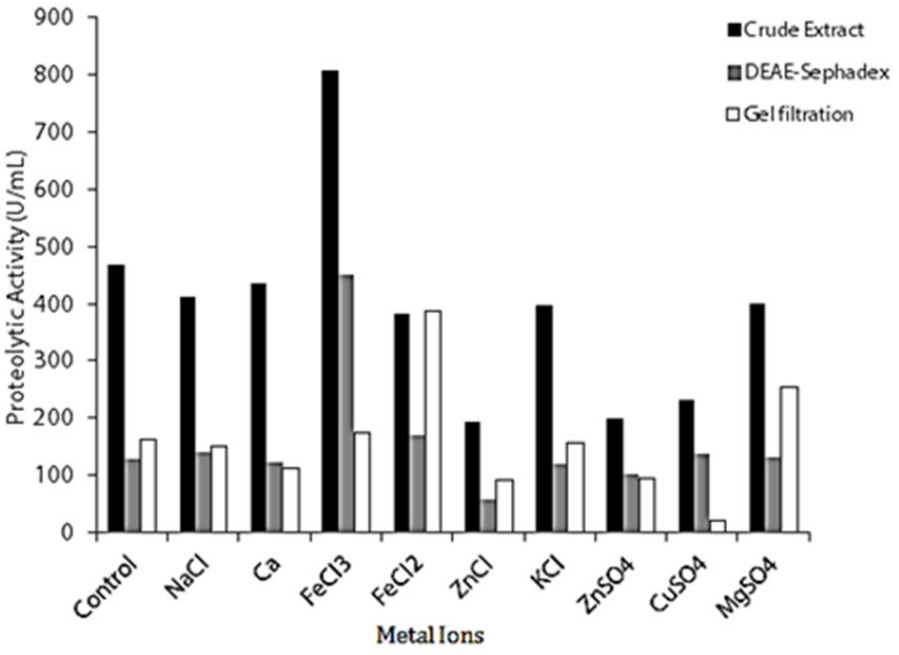

Figure 4. Effect of metal ions and inhibitors on the protease activity. Protease assays were carried out using purified protease (0.75µg).

Effects of temperature and pH, and inhibitors on the protease activity and stability

In Figure 5, we observed that the temperature for the maximum protease activity was 45°C, however the activity was abruptly decreased in the interval of 5°C, achieving a decrease of up 68%. The thermal stability is an essential factor for application of proteases. The protein herein studied was active and thermostable from 25°C to 55°C (low activity levels were kept until 60°C).

In work carried out by Singh (2017) with *Aspergillus oryzae*, the protease activity remained stable up to 50°C, when there was a sharp decline of the activity, possibly due to denaturation of the protease. These data corroborate the data presented in this paper.

Regarding the optimal temperature was observed to 40°C showed a proteolytic activity of 478 U/mL as shown in Figure 5. Similar results were found in fungal protease derived from fermentation of *Aspergillus flavipes* with the optimum temperature being 50°C Novelli et al. (2016), and *Myceliophthora* sp. with optimum temperature was between 40 to 45°C (Zanphorlin et al. 2011).

Previous studies indicated the improved thermostability of proteins with increased levels of some glycosylation (Lim et al. 2019). The protease purified in this work and confirmed by electrophoresis not shown glycosylation by Schiff technique (data not shown). Among the tested protease, the remaining activity of purified protease increased slightly at temperatures above 60°C but the activity declined to10% at 100°C.

Table II. Effect of specific protease inhibitors on catalytic activity of purified enzyme.

Type of inhibitor	Percentual of residual activity (%)
Enzyme control	100
Pepstatin A	100
Iodoacetic acid	44
EDTA	10
PMSF	0
Triton-X100	28

Figure 6 shows that the optimum pH for the purified enzyme. The enzyme maintained considerable activity during 1h of incubation in pH between 5.0 and 9.0, although under acidic conditions the activity was constantly

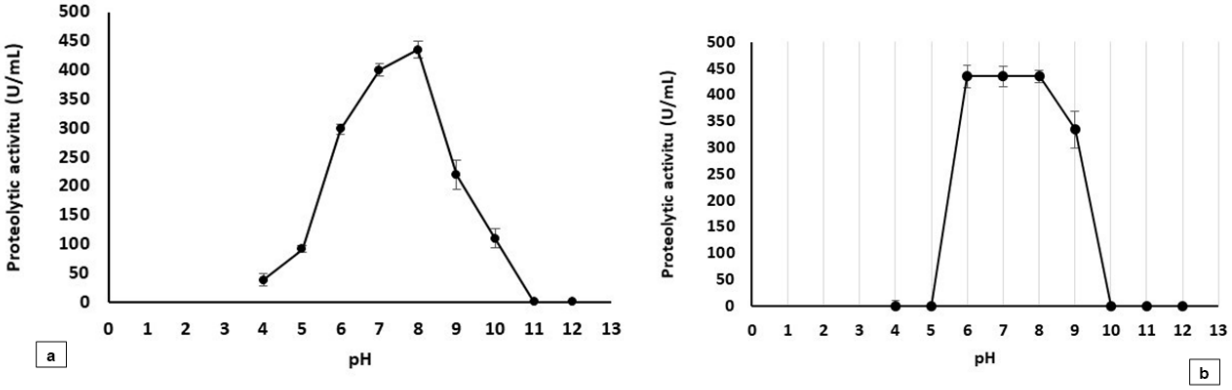

Figure 5. Effect of the pH on purified protease activity obtained from *Aspergillus sydowii* URM5774. Optimum pH (a) and pH stability (b) of the purified enzyme. Protease assays were carried out using purified protease (0.75µg). Error bars represent the standard deviation of triplicate measurements.

decreased. The pH 8 provided the best activity level for purified enzyme, what evidences the alkaline character of the enzyme, although next to neutral pH, since after pH 9 the activity of the enzyme decreased abruptly. This makes the protease suitable to applications in neutral-alkaline environments. Alkaliphiles have made a great impact in industrial applications. Biological detergents contain alkaline enzymes, such as alkaline cellulases and/or alkaline proteases, that have been produced from alkaliphiles (Derombise et al. 2009). The current proportion of total world enzyme production destined for the laundry detergent market exceeds 60%, for example. Besides, neutral-alkaline environments are important to polymer degradation and Alkaline environments benefit microbial K-strategists to efficiently utilize protein substrate and promote valorization of protein waste into short-chain fatty acids (Wang et al. 2020).

According to studies by Markaryan et al. (1994) using *Aspergillus fumigatus* was shown a pH optimum between 7.5 and 8 and Souza et al. (2017) *A. foetidus* with the optimum pH was between 8-9 but keeping a high rate of activity between pH 5-9 and *A. flavipes* the optimum pH was 8 having a good performance in the enzymatic activity in the pH range 7-9. Similar results to those found in this research. This broad pH range of proteases produced must be due to the genus *Aspergillus* ability to adapt to a wide pH range having as preferred pH acid being one acidophilus. This genus could modify the pH of the medium because of the ability to acidify the medium according to Straat et al. (2014).

In Table II, the purified protease was unaltered by all inhibitors, except Phenylmethyl sulfonyl fluoride (PMSF) is a well-known inhibitor of serine-protease (Table II). The purified protease from *Aspergillus sydowii* was strongly inhibited by PMSF, suggesting that the protease exhibits the serine amino acid near the active site, indicating to be a serine-collagenase. Effect of inhibition by PMSF has also been reported in serine-proteases. The moderate stability of protease in the presence of chelating agents like EDTA is a requirement for any detergent enzyme since EDTA is used in detergent formulation as a water softener.

The ability of the enzyme produced by *A. sydowii* to hydrolyze azocasein was accessed using the optimum parameters 45°C and pH 8.0 (Figure 7). Our findings agree with the report on fungi like *Aspergillus niger*, *Mucor disperses* NRRL 3103 and *A. elegans* NRRL 3104 which were studied for their substrate specificity. Hydrolysis of azocasein has been the source of the fermented compounds. Degradation of proteins like azocasein increases the free amino acid content during fermentation processing, particularly for glutamic acid and alanine, which are the most important substances for producing the characteristic taste and *flavour* for example in meats.

CONCLUSION

The enzyme from *Aspergillus sydowii* produced by solid state fermentation using coffee residues was herein purified and proved to have a biotechnological potential for use in the detergent industry and leather softening as well as a possible solution to the impact environment caused by the coffee grounds of disposal. The findings were presented can be useful for the enzyme industry since they represent a way to manage the waste of raw converting into a viable economic product.

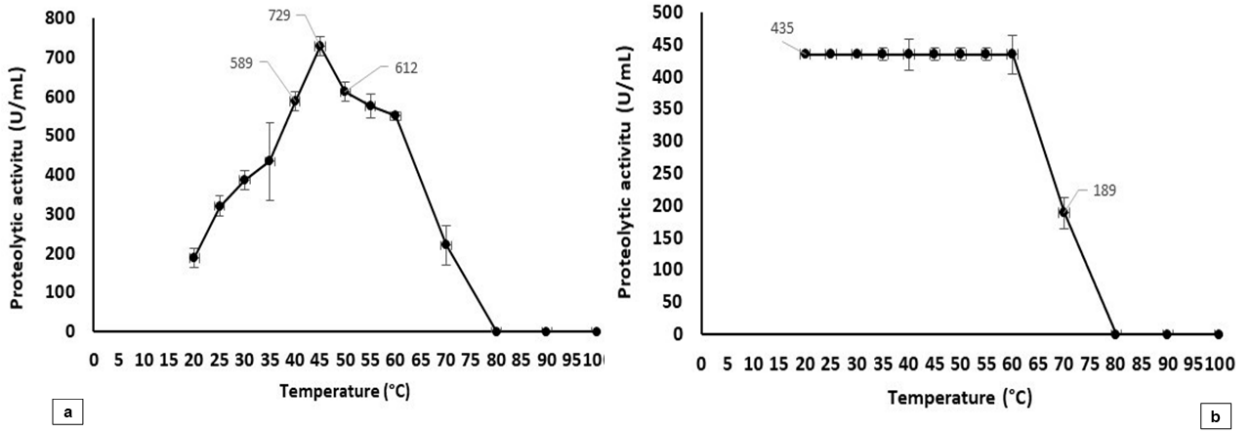

Figure 6. Optimum temperature (a) and temperature stability (b) assays to evaluate the activity of the purified enzyme obtained from *Aspergillus sydowii* URM5774. Protease assays were carried out using purified protease (0.75µg). Error bars represent the standard deviation of triplicate measurements.

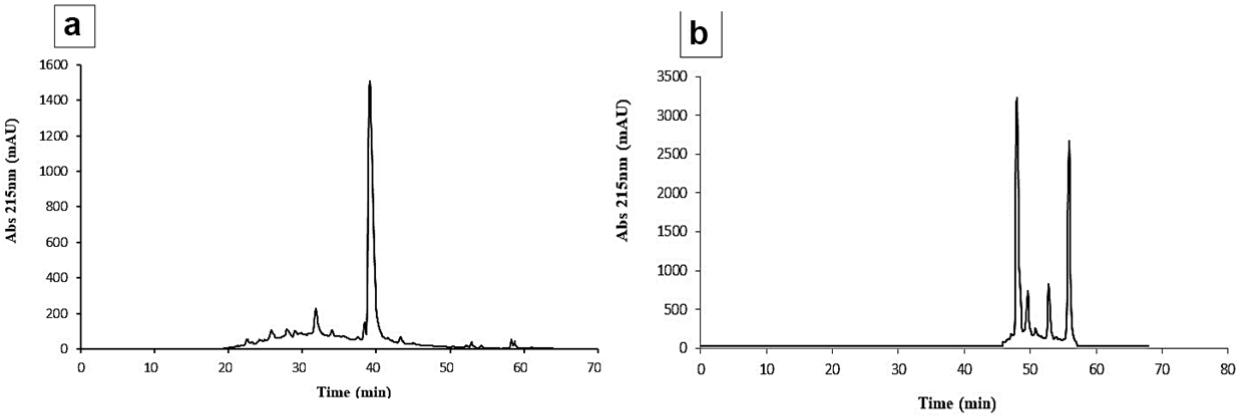

Figure 7. HPLC chromatogram profile of azocasein degradation process using a protease purified from *A. sydowii*. (a) HPLC profile of Azocasein purchased from Sigma; (b) HPLC profile of Azocasein hydrolysis using a protease purified through the optimum parameters.

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Author contributions

All authors contributed to the development of the manuscript: Felype T.B. Rocha and Romero M.P.B. Costa isolated the microorganism Aspergillus sydowii from the coffee grounds and identified by the URM Culture Collection of the Federal University of Pernambuco. Besides, they prepared the solid-state fermentation technique. Anna Gabrielly D. Neves did the enzyme extraction and determined the proteolytic activity. Kethylen B.B. Cardoso and Thiago P. Nascimento did partial purification process of the enzyme after extraction procedure. Follow, the sample was purified by DEAE-Sephadex Chromatography. Wendell W.C. Albuquerque used Gel Filtration Chromatography under AKTA AVANT equipment to purify the enzyme. Ana Lúcia F. Porto improved the research with statistical analyses. The authors declare that they have no conflict of interest.

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