

Stability study of pre-purified protease obtained from *Aspergillus oryzae* NRRL 1911 by solid state fermentation with canola cake as substrate at different pH and temperature**Estudo de estabilidade de protease pré-purificada obtida de *Aspergillus oryzae* NRRL 1911 por fermentação em estado sólido com torta de canola como substrato em diferentes pH e temperatura**

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ABSTRACT

The use of agroindustrial waste to obtain products with higher added value has become an alternative to reduce the problems that can be generated to the environment as contamination. One of the high value products that can be obtained by solid state fermentation of this biomass are enzymes, among which proteases stand out for representing about 60% of the world enzyme market and due to the wide variety of applications and sources of enzymes it is necessary to characterize biochemically in order to predict in which processes they can be applied. Thus, the objective of this work was to

evaluate the stability of pre-purified protein extract in Ammonium Sulphate (80%) at different pH and temperatures. The pH range used ranged from pH 3.0 - pH 10.0 and the temperature range ranged from 30°C to 80°C. The pH stability range ranged from pH 4.5 - pH 7.5 with residual activity of 350.63 U/g to 309.38 U/g of canola cake, respectively. While the study of stability at different temperatures ranged from 30 °C to 45 °C with residual activity of 393.33 U/g - 311.67 U/g of canola cake, respectively. Thus, the pre-purified proteolytic extract can be used in industrial processes both at long-term mild temperatures and at shorter temperatures at higher temperatures. As in processes where the pH is close to neutral or slightly acidic pH.

Keywords: solid state fermentation; canola cake; stability

RESUMO

The use of agroindustrial waste to obtain products with higher added value has become an alternative to reduce the problems that can be generated to the environment as contamination. One of the high value products that can be obtained by solid state fermentation of this biomass are enzymes, among which proteases stand out for representing about 60% of the world enzyme market and due to the wide variety of applications and sources of enzymes it is necessary to characterize biochemically in order to predict which processes they can be applied to. Thus, the objective of this work was to evaluate the stability of pre-purified protein extract in Ammonium Sulphate (80%) at different pH and temperatures. The pH range used ranged from pH 3.0 - pH 10.0 and the temperature range ranged from 30 °C to 80 °C. The pH stability range ranged from pH 4.5 - pH 7.5 with residual activity of 350.63 U / g to 309.38 U / g of canola cake, respectively. While the study of stability at different temperatures ranged from 30 °C to 45 °C with residual activity of 393.33 U / g - 311.67 U / g of canola cake, respectively. Thus, the pre-purified proteolytic extract can be used in industrial processes both at long-term mild temperatures and at shorter temperatures at higher temperatures. In processes where the pH is close to neutral or slightly acidic pH.

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1 INTRODUCTION

Proteases are important biomolecules produced by biotechnology industries, currently accounting for 60% of the world enzyme market, due to their specificity of hydrolyzing peptide bonds contributing to a wide variety of applications such as the food, beverage, cosmetics, leather and other industries [1,2]. They can be classified depending on the type of amino acid linked to the active site being aspartic proteases, cysteine, glutamic, serine and threonine [3]. Proteases can be obtained from a variety of sources, whereas origins can be classified into plant, animal or microbial [4]. From these sources, microorganisms have potential for protease production because of their biochemical diversity and easy genetic manipulation [5].

Although proteases are commercially available, the use of these enzymes on an industrial scale has been limited due to their high production costs [4]. Although most enzymes are produced via submerged fermentation, which the enzyme recovery steps make the process costly, great emphasis has been placed on the production of enzymes by solid state fermentation. Where this type of fermentation uses agroindustrial waste producing these biomolecules at a lower cost, which will

reflect in the final product [6]. Chutmanop et al., (2008) [7] when comparing submerged fermentation versus solid state fermentation (SSF) suggest some characteristics that make SSF more attractive by listing in: simplicity, low cost, high yields, high enzyme concentrations and use agroindustrial waste.

With all these advantages one of the most important for SSF is the greater adaptability of the fungi in this type of substrate due to the filamentous fungi morphology that allows the colonization of the substrate surface and the matrix in search of nutrients and as a positive consequence the release of higher levels of metabolites and enzymes [8]. The SSF method resembles the natural habitat of filamentous fungi, assisting in the microbial decomposition of various substrates and requires minimal maintenance demand [9].

Filamentous fungi include several species of *Aspergillus* sp. They are able to adapt to various substrates producing metabolites of high biological interest and are recognized as FDA (Food and Drug Administration) safe microorganisms known as Generally Recognized as Safe (GRAS) [10]. Among the various species of *Aspergillus*, *Aspergillus oryzae* has been used in the FSS and is listed as GRAS. *Aspergillus oryzae* has been reported throughout history due to its use in the food industry for its high proteolytic and amylolytic activities and also for producing fermented foods [11].

Proteases can be obtained from various substrates such as wheat bran, soybean meal, cottonseed meal and orange peel [12] food residue [13] and canola meal [14]. It can also be obtained from various microorganisms such as *Bacillus* sp. [2], *Streptomyces* sp. [4], *Aspergillus oryzae* [5,7,9,10] among others. Due to the wide variety of enzymes, their biochemical characterization is necessary to evaluate their biotechnological potential. Within this characterization stands out the stability at different pHs and temperatures, which can be used to predict the successful application of the enzyme [5].

In this context the present work investigates the stability of *Aspergillus oryzae* proteolytic extract obtained from the semisolid fermentation of canola cake derived from oil extraction under different pH and temperature conditions.

2 MATERIAL AND METHODS

2.1 SAMPLE

The canola cake used was kindly provided by the company Celena Alimentos S/A, located in Eldorado do Sul, Rio Grande do Sul. Wheat bran used to obtain the inoculum was obtained in the commerce of the city of Imperatriz, Maranhão.

2.2 FERMENTATION MEDIUM

The canola cake was humidified using 100 g of bran to 40 ml of distilled water. For wheat bran a ratio of 100 g of wheat bran to 120 mL of water was used.

2.3 MICROORGANISM

The filamentous fungus strain used was *Aspergillus oryzae* NRRL 1911 donated by the US Department of Agriculture's Crop Collection and Agricultural Research Service.

2.4.1 Maintenance of culture

Spores of the *Aspergillus oryzae* NRRL 1911 strain were aseptically transferred to test tubes containing sterile soil and stored at -18°C. The spores were reactivated in three steps described below.

2.4.2 Culture activation

In the first stage, the fungus strains were transferred to slanted potato dextrose agar, previously sterilized at 121°C for 15 min, after inoculation they were incubated at 30°C for 7 days in an oven and then stored at 4 °C for 3 months.

The second stage consisted of transferring the spores from the first stage to a new slanted dextrose potato agar with the same composition. After inoculation they were incubated at 30 °C for 7 days in a incubator.

The last activation step aimed at the production of spores used in the inoculation of the fermentation medium. For this purpose, a medium consisting of 10.0 g of wheat bran and 4 ml of a 1.7% (w/v) NaPO₄ solution and 2.0% (w/v) of (NH₄)₂SO₄ was prepared. The medium was sterilized at 1atm at 121°C for 15 minutes.

The spores of the second activation step were then suspended by the addition of 5 mL of sterile water to the test tubes. The 1 mL volume of the spore suspension was used as inoculum in the wheat bran medium in 125 mL Erlenmeyer flasks, the inoculated medium was incubated at 30°C for 5 days in stove. After sporulation in wheat bran, the inoculum was stored at 4°C for use in fermentations.

2.4.3 Inoculum production

To produce the inoculum, 40 mL of sterile water was added to the wheat bran medium of the third step, followed by manual glass rod stirring for 5 minutes to transfer the spores into the water. Subsequently, sterile gauze filtration was performed and 1 mL of the spore suspension was added to the media to initiate the fermentation process.

2.5 SOLID STATE FERMENTATION

The substrate mass was placed in a polypropylene beaker, distilled water was slowly added to the substrates in the proportions mentioned in item 2.2. Subsequently, 40 g of the humidified medium was transferred to 500 ml Erlenmyer medium, 50 mm internal diameter of the mouth, and autoclaved at 121°C for 15 minutes. The media was inoculated with a 1 mL spore suspension and incubated at 30°C for 72 hours.

2.6 OBTAINING ENZYME EXTRACTS

The enzyme was extracted from the fermented medium by addition of 100 mL phosphate buffer (pH 7.0) and manual homogenization for 5 minutes. After homogenization the Erlenmeyers were incubated again at 30°C for 1 hour. Then vacuum filtration was performed using qualitative filter paper and the supernatant used in the determination of enzymatic activity.

2.7 PROTEASE PURIFICATION

The proteolytic extract (500 mL) was pre-purified with 80% ammonium sulfate in an ice bath. Then the saturated medium was centrifuged at 10000 x g for 20 min at 4°C, the precipitate was collected and dissolved in phosphate buffer pH 7.0. Then dialyzed on cellulose membrane for dialysis for 24 h in phosphate buffer. This dialyzed material was used for the analyzes.

2.8 PROTEASE ENZYME ACTIVITY ASSAY

Protease activity was performed according to the methodology described in the work colorimetric method for determination of proteolytic activity [15], with adaptation in the study of proteolytic enzyme production [14]. Analyzes were performed in triplicate. The reaction mixture consists of 1mL 0.5% (w/v) azocasein solution in 100 mM phosphate buffer pH 7.0 and 1mL enzyme extract, diluting 1 mL enzyme extract to 100 mL distilled water.

The reaction mixture was incubated at 40°C for 40 minutes. After this time, the reaction was stopped by adding 1mL of 10% (w / v) trichloroacetic acid (TCA) solution.

Then the samples were centrifuged at 3000 rpm for 15 minutes. Two mL of the supernatant containing low molecular weight amino acids and oligopeptides was transferred to a test tube and 2 mL of 5N KOH was added, forming a characteristic color compound that was quantified by a 428 nm wavelength spectrophotometer against a blank prepared under identical conditions with modification in the order of addition of TCA and only after the extract. One unit of protease activity (U) was defined as the amount of enzyme that produced a 0.01 absorbance difference between the reaction control and the sample per minute of reaction.

2.9 STABILITY OF THE ENZYME AT DIFFERENT TEMPERATURES

Initially 10 mL of extract pre-purified (2.7) was added into an Erlenmeyer (25 mL) with 3 repetitions and subjected to a temperature of 30°C to 80°C, at 5°C intervals, on an orbital shaker until 2.5 h was completed.

We collected 1 mL of extract from each Erlenmeyer at times 0, 30, 60, 90, 120, 150 min and transferred to previously identified Erlenmeyers with the corresponding repeat number and time described above, and kept refrigerated until complete. of analysis.

Then 99 mL of distilled water was added to the Erlenmeyer containing 1 mL of the pre-purified crude extract. The reaction mixing process and the determination of residual enzymatic activity was performed according to the description of item 2.8.

2.10 STABILITY OF THE ENZYME AT DIFFERENT PH

Initially buffer solutions of different pH were prepared. For pH 3-6 acetate buffer was used, pH 6.5 to 8.0 was used phosphate buffer and for pH 8.5 to 10 were prepared with borate buffer. The experiments were performed for pH ranging from 3 to 10, with a 0.5 unit interval, totaling fifteen experiments. Evaluation of the pH effect was performed by adding 1 mL of extract pre-purified in 99 mL of the pH buffer and shaken in orbital shaker for two hours at 30°C. Then 1 ml of the diluted Erlenmeyer extract was taken and transferred to centrifuge tubes containing 1 ml of azocasein previously set in a 40°C water bath in triplicate. To determine the enzymatic activity at different pH, following the same steps described in item 2.8 at the reaction temperature, which was 40°C for all samples [15].

3 RESULTS AND DISCUSSION

The research in this paper is based on previously tested studies to verify which microorganism available in the laboratory bank best produced protease and also based on the best time (72 h) and optimization temperature (30°C) conditions for protease production. As well as work on optimal pH (pH 7.0) and optimal temperature (40°C) conditions of proteolytic extract, unpublished results.

3.1 STABILITY IN PH DIFFERENT

The influence of pH on protease stability was examined at different pH ranges (3.0-10.0) at room temperature for 2h and the residual proteolytic activity evaluated. The results stability protease pre-purified in ammonium sulfate (80%) at different pHs are presented in Table 1, with their respective means \pm standard deviation obtained from 3 replicates of each experiment totaling 45 experiments. The protease pre-purified showed activity of 399.92 U/g of canola cake. The proteolytic

activity obtained by solid state fermentation of canola cake ranged from 0.0 U/g (pH 3) to 384.17 U/g of canola cake.

Table 1 – Mean \pm standard deviation of proteolytic activity obtained from three replicates of each treatment as a function of pH and temperature

pH	Mean \pm standard	Temperature (°C)	Mean \pm standard
7	384.17 \pm 9.97 ^a	30	393.33 ^a
6.5	360 \pm 0.29 ^{ab}	35	386.04 ^a
4.5	350.63 \pm 8.75 ^{abc}	40	358.13 ^a
6	332.92 \pm 8.04 ^{bcd}	45	311.67 ^b
5	330 \pm 4.37 ^{bcd}	50	77.92 ^c
5.5	318.13 \pm 4.15 ^{cd}	55	71.88 ^c
7.5	309.38 \pm 5.45 ^d	60	36.04 ^{cd}
8	267.09 \pm 0.76 ^e	65	3.54 ^d
8.5	255.21 \pm 6.49 ^e	70	2.92 ^d
4	250.63 \pm 8.49 ^e	75	2.71 ^d
9	236.21 \pm 1.25 ^e	80	0.0 ^d
9.5	142.29 \pm 0.27 ^f		
10	94.17 \pm 3.08 ^g		
3.5	2.29 \pm 0.95 ^h		
3	0 \pm 0 ^h		

Analysis of variance ANOVA and Tukey test (Table 1 and Table 2) show significant difference ($p < .0001$) between the proteolytic activities of each assay. This is because pH mainly influences the structure of the enzyme causing it to lose its conformation and thus its specific activity. With the average residual proteolytic activity pH 7 presented the highest average (384.17 \pm 9.97 U/g canola cake) among all experiments, but it was not significantly different from the residual proteolytic activity of pH 6.5 and pH 4.5 with activity 360 \pm 0.29 U/g and 350.63 \pm 8.75 U/g canola cake, respectively.

Table 2 - Analysis of variance (ANOVA) for proteolytic residual activity at different pH and temperature

	Source	DF	SS	MS	F value	pr>F
pH	Model	14	663037.65	47359.83	338.58	<.0001
	Error	30	4196.32	139.88		
	Corrected Total	44	667233.98			
	R²=0.9937					
Temperature	Model	10	887820.67	88782.07	370.73	<.0001
	Error	22	5268.49	239.48		
	Corrected Total	32	893089.16			
	R²=0.9948					

The stability range can be better visualized according to Fig 1, considering the residual activity above 70% it is possible to verify the proteolytic stability of the pre-purified extract in a wide pH range between 4.5 to 7.5 with 87.68% and 77.35% residual proteolytic activity, respectively.

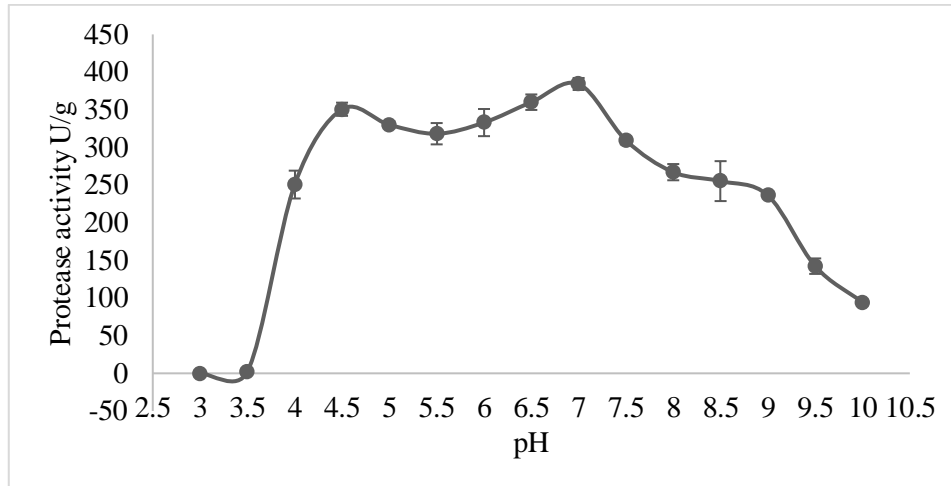


Fig 1. Proteolytic residual activity at different pH to verify stability after 2 h incubation at room temperature

Beltagy et al., (2018) [3] investigating the stability of a terohalophilic chitinase produced *Aspergillus flavus* previously purified exposed at a wide pH range (2.0-9.0) found that the enzyme was more stable at pH 7.5 with residual activity 490 U/L and activity began to decrease from pH 8 to pH 9. These results roughly coincide with the data obtained in the present research.

In the present work the proteolytic stability of protease pre-purified of *Aspergillus oryzae* NRRL 1911 ranged from pH 4.5 to 7.5 considering residual activity above 70%, results that are close to the results obtained by Farag et al., (2016) [4], by isolating a strain of *Aspergillus terreus* of marine origin and evaluating the stability of chitinase produced by the microorganism by subjecting it to different pHs (4-9) at 50°C/60 min, they observed that purified chitinase retained more than 80% of its residual activity at pH 5.0 to 8.0 after 60 min incubation. May be related to the conformation of the enzyme that depends mainly on the microorganism used [5].

Dias et al., (2016) [6] investigating the stability of *Aspergillus oryzae* CCT 3940 L-asparaginase using a central rotational composite design with pH ranging from 3.0 to 10.0 with optimum activity at pH 8.0, found that L-asparaginase purified was more active at pH 7.5 to 8.0 and the best temperature stability range was 45°C to 50°C. Observing the response surface and the contour plot concluded that the temperature had a positive effect, while the pH had a negative effect. These results corroborate the data obtained in the present study, which has stability close to that obtained by the referred authors.

By characterizing the stability of an enzyme of *Aspergillus niger* with pH between 3.0 and 7.0 at 20 °C for 1 h, Wang et al. (2018) [7] found with based on the results residual activity that the enzyme had stability pH 3 and 6. It is important to note that the enzymes obtained from *Aspergillus* fungi both in the present work and in the literature present stability at slightly acidic pH and near neutral pH.

3.2 STABILITY IN TEMPERATURE DIFFERENT

Stability has also been studied at different temperatures ranging from 30 °C to 80 °C at 5 °C intervals. The protease was kept at optimal pH (pH 7.0) and the temperature varied, with the experiment being kept for 2.5 h and the residual activity evaluated. The data residual activity of purified protease is presented in Table 1 with their means \pm standard deviation obtained from 3 replicates of each assay totaling 33 experiments. The initial activity of the proteolytic extract was evaluated at 399.92 U/g of canola cake. According to Table 1 the proteolytic residual activity ranged from 0.0 U/g to 393.33 U/g of canola cake.

From Table 1 and Table 2 showing the analysis of variance (ANOVA) and Tukey test it is possible to observe that besides the pH, the temperature also has an impact on the activity of the protease. In the present work, the temperature had a significant negative influence ($p < .0001$), showing that the higher the temperature, the lower the residual activity of the protein extract will be Fig 2. The average results suggest that the temperature of 30 °C had the highest proteolytic residual activity with 393.33 U/g of canola cake, however, no significant difference was observed between samples submitted to 35°C and 40°C with activities 386.04 U/g and 358.13 U/g canola cake, respectively.

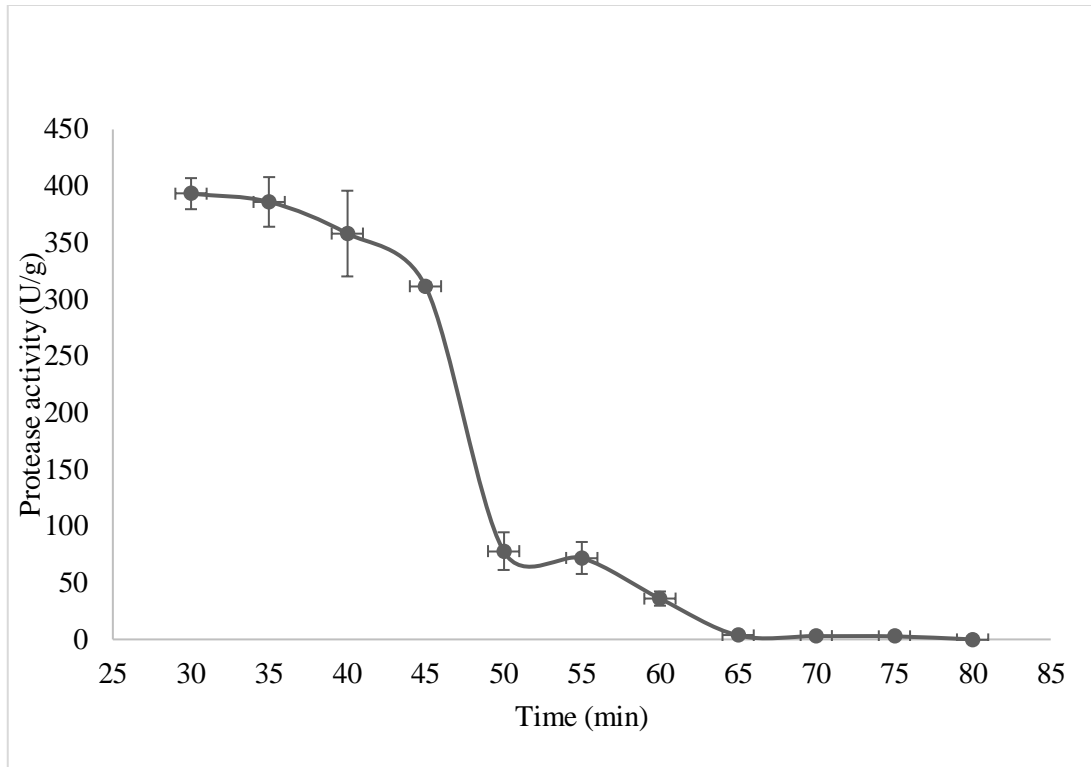


Fig 2. Residual protease activity at different incubation temperatures analyzed after 2.5 h incubation at pH 7.0

Considering as a good condition that the protein extract has stability when maintaining its activities above 70% it can be seen that after 2.5 h of incubation at different temperatures it was possible to verify that the proteolytic extract had a residual activity of 98.35%, 92.53%, 89.55% and 77.93% at temperatures of 30°C, 35°C, 40°C and 45°C, respectively.

Stability results as a function of different temperatures were also evaluated over 2.5h and protein activity evaluated every 30 min is shown in Fig 3. It can be observed that at temperatures of 30°C, 35°C and 40°C, the data obtained for protease activity over time are stable and do not change forming a constant line and quite similar. Activity at a temperature of 45°C is practically stable for the first 30 min and thereafter begins to have a steady drop in activity, yet maintaining activity above 70% after more than two hours of incubation.

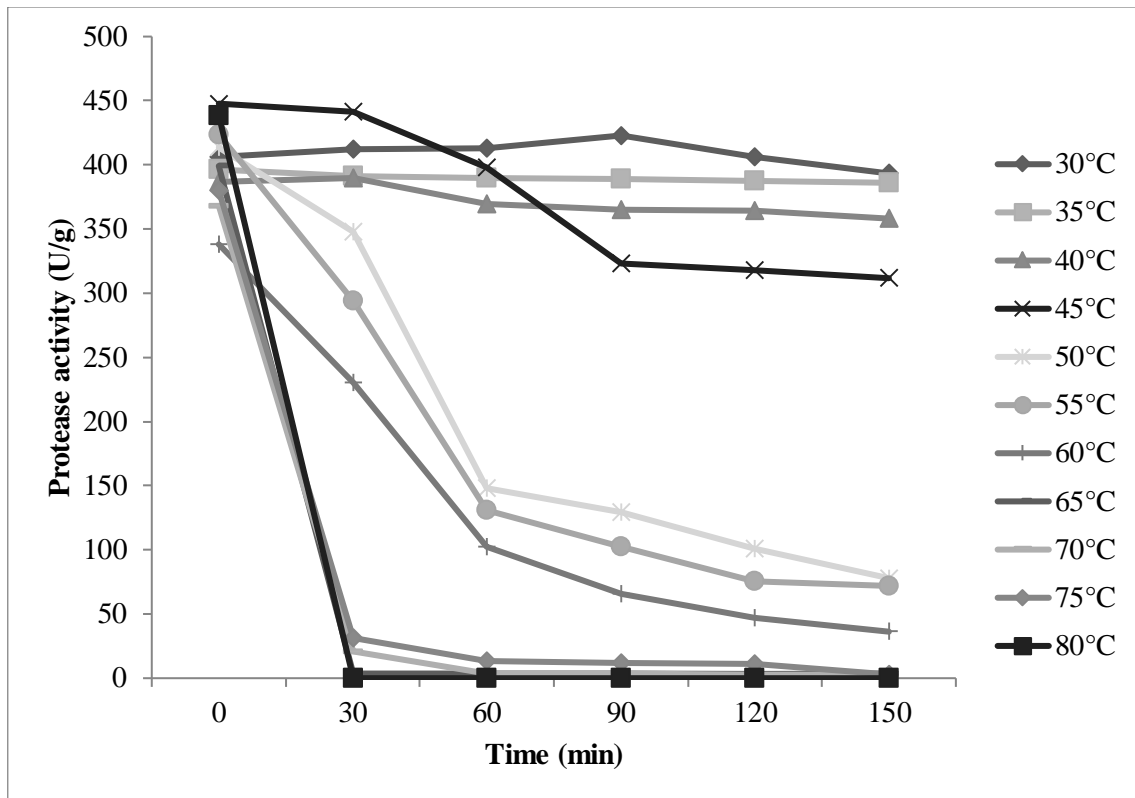


Fig 3. Monitoring of residual activity of different incubation temperatures at times 0, 30, 60, 90, 120 and 150 min.

At subsequent temperatures the reduction in proteolytic activity is more pronounced during the first 30 min, yet at 50 °C, 55°C and 60°C the protein activity is maintained above 50%, but from 30 min this activity falls to almost 25% both exhibiting the same behavior of decay. Temperatures of 65°C, 70°C, 75°C and 80°C are sufficient to lead to 90% more loss of activity in the first 30 minutes.

Wahab and Ahmed (2018) [8] using the response surface methodology tool to characterize alkaline protease obtained from *Aspergillus niger* WA 2017 assessing stability as a function of temperature (50-80°C) for 90 min, observed that the enzyme was slightly inhibited with residual activity of 76.2% when incubated at 60°C for 60 min, whereas under the same condition the result of residual activity was lost by 74.44%. The same authors found that at 50°C for 30 min residual activity was strongly inhibited and maintained only 40.3% of its activity, results which contrasted with the present investigation that at 50°C for 30 min it maintained residual activity at 86.99%.

Silva et al., (2018) [9] purifying and characterizing a serine protease obtained from *Aspergillus tamarii* URM4634 by assessing the thermostability of the enzyme ranging from 20°C to 80°C for up to 180 min, concluded that the enzyme had stability of 20-40°C maintaining residual activity at more than 60%. Similar result to that observed in the present work, although the stability of the protease purified obtained residual activity higher than 70%.

Bhardwaj et al. (2019) [10] evaluated the xylanase thermostability obtained from *Aspergillus oryzae* LC1 with temperature ranging from 30 to 70°C at pH 5.0 (optimal enzyme pH) and suggested

that the best temperature range was 30°C to 50°C with residual activity evaluated in 12 hours of experiments. In this condition the enzyme showed 50% residual activity. They concluded that at higher temperatures the enzyme loses its activity very quickly, because the temperature helps break the weak bonds of the enzyme structure and with that its activity is also lost, after finding that at 60 and 70°C the enzyme lost its activity by more than 50% after 4 h. This result is similar to that observed in the present study in which the protease showed stability at a temperature of 30°C to 45°C over a wide pH range and could also be considered an acidic enzyme.

Bazaraa et al., (2016) [11] purified and analyzed various properties of extracellular glutaminase of *Aspergillus oryzae* NRRL 32567 using L-glutamine as substrate and among the various properties analyzed verified the effect of different temperatures on protease stability with temperatures between 30 to 70°C in two hours of incubation and found optimal stability at 30 and 40°C. They concluded that at 30°C glutaminase was very stable, without losing its activity at 120 min, while at 40 ° C its residual activity was lost by 5%. They emphasized that the increase in temperature favors the loss of activity in a shorter time by modifying the enzyme structure. Information also observed in the present work because the higher the applied temperature, the smaller the residual activity in shorter time.

Watanabe et al., (2016) [12] observed that glucosidase thermostability maintained residual activity at almost 100% when incubated at 4 ° C and in the range 20-60°C, losing all of its residual activity between 70-80°C. These results were obtained when studying the purified glucosidase obtained from *Aspergillus oryzae* at temperatures of 4-80°C in phosphate buffer (pH 6.8).

The thermal stability results of enzymes obtained from *Aspergillus oryzae* show a wide range of residual activity at different temperatures because each enzyme has its specificity, its structural conformation characteristics that can quickly change due to the disruption of interactions caused by temperature and thus lose its conformation and thus its specificity to bind to the substrate to form new products.

4. CONCLUSION

The protease purified obtained from canola cake fermentation presented stability in a wide range of pH and temperature, and can be considered of good performance in industrial processes with mild temperatures (30-40°C) for long time. In addition, it can be applied in processes at higher temperatures (45-55°C) for short times. Regarding pH, the enzyme also showed stability in a wide pH range (4.5-7.5) and can be applied in processes close to neutrality, as well as in milk coagulation to obtain cheese.

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