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Peptidase with Keratinolytic Activity Secreted by *Aspergillus terreus* During Solid-State Fermentation

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ABSTRACT

The aim of this study was to evaluate peptidase production by Aspergillus terreus *in solid-state bioprocess and evaluate its parameters. The best conditions were 5.0 g of wheat bran as substrate, incubation temperature 30°C, inoculum 2.0x10[5] spores/g and 75% saline volume, with production reaching 677 U/mL (5400 U/g culture medium) after 72 h of fermentation. Biochemical characterization of the crude enzymatic extract showed the optimum pH and temperature of 6.5 and 55°C, respectively. The stability at different temperatures and pH values showed that the extract could endure different pH. The evaluation of the ions influence and inhibitors proved that the enzyme required an ion for better activity, which was corroborated with the inhibition of EDTA and PMSF, characterizing serine and/or metallo peptidase. The extract was also tested for specific activities and showed promising results for keratinolytic and collagenolytic activities (0.252 and 0.165 OD/mL, respectively).*

Key words: protease, solid-state fermentation, keratinase, wheat bran, *Aspergillus terreus*

INTRODUCTION

Fermentation uses a microorganism to obtain biotechnological products, such as enzymes (Pereira et al. 2008). In solid-state fermentation processes, there is lack of free water in the culture medium, with only enough amount to maintain the growth of the microorganism (Pandey et al. 2000). In these processes, the solid material most often used is agro-industrial waste, which serves both as physical support and as a nutrient source (Pandey 2003). This type of process has advantages, such as better biomass production in a short period of time and similarity to the natural habitat. The downstream processes are facilitated by the high concentration of the product, thereby potentially increasing the earned value to the final product. Hence, this process is a great technology for obtaining the products and potential use of solid residues (Soccol and Vandenbergh 2003; Graminha et al. 2008).

Enzyme production by solid-state fermentation has been studied extensively. Peptidases are enzymes that have been found in different types of culture medium using agro-industrial waste, such as wheat bran (WB) (Tunga et al. 1998; Uyar and Baysal 2004), wheat and soybean flours (Wang et al. 2005) and rice straw (Chapla et al. 2010). These enzymes have applications in different areas of industry, including pharmaceuticals and cosmetics, food, leather, detergents and bioremediation and waste treatment (Anwar and Saleemuddin 1997; Rao et al. 1998; Sharma et al. 2001; Gupta et al. 2002; Novozymes 2008).

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Microorganisms are interesting sources of peptidases because they have wide biochemical diversity, easy cultivation and maintenance, a reduced cost and the possibility of genetic manipulation for the improvement or modification of the final product. Therefore, microbial peptidases are among the most important hydrolytic enzymes currently used. There is, thus, a constant search for new peptidase producers, including filamentous fungi and new culture media and conditions for cultivation that can increase the yields and decrease the costs of production (Ladeira et al. 2010).

The filamentous fungus *Aspergillus terreus* is known for producing important biotechnological products, such as lovastatin and itaconic acid (Benett 2010). There is an interest in generating other products, such as enzymes, including peptidases. The goal of this study was to determine the best parameters for peptidase production by *A. terreus*, its characterization and potential applications.

MATERIALS AND METHODS

Microorganism and maintenance

A. terreus was obtained from the culture bank of the enzyme technology laboratory of the Faculty of Pharmaceutical Sciences of Ribeirão Preto. The fungus was isolated from a feather found in the soil in Miguelópolis, São Paulo state, Brazil. The strain was identified by Dr Cristina Maria S Motta at the Mycology Department of the Federal University of Pernambuco – UFPE and was grown on potato dextrose agar (PDA) in culture tubes by incubating at 30°C for 168 h.

Inoculum preparation

An inoculum was prepared by adding sterile saline solution (0.1% w/v $(NH_4)_2SO_4$, 0.1% w/v NH_4NO_3 and 0.1% w/v $MgSO_4.7H_2O$) and scraping the surface to form a spore solution. The spore concentration was determined by counting in a Neubauer chamber. The volume added was variable and the total volume of the solution was 10 mL (Merheb-Dini et al. 2007).

Solid-State Fermention (SSF)

Adapted from the techniques of Lotong and Suwanarit (1985) and Merheb-Dini et al. (2007), the method for peptidase production in SSF used autoclavable polypropylene bags of 12x20 cm to which 5.0 g of the substrate and 8.0 mL of saline solution as above was added and then autoclaved at 121°C for 40 min. The bags after inoculation were incubated at 30°C. Every 24 h, one bag was removed and 40 mL of distilled water at 4°C was added. The extraction of enzyme was performed by soaking and agitation. The material was filtrated and centrifuged at 5,000xg at 4°C for 20 min. The supernatant was considered as crude enzymatic extract and was used for proteolytic activity tests.

The composition of the culture medium included 5.0 g of WB only or supplemented with 5, 10 or 20% casein or albumin or 5.0 g of cottonseed (CS) meal only or supplemented with 5, 10 or 20% soy protein, gluten or WB. After determining the best agro-industrial waste for peptidase production, other parameters were varied to define the optimum conditions for the production of enzyme, including the spore concentration ($5.0x10^6$, $2.5x10^6$ or $1.0x10^6$ spores/mL), temperature (30, 35, 40 and 45°C) and saline volume (65, 75, 85 or 100%, w/v), which was calculated based on the grams of the WB and saline (15 g) as 100%.

Proteolytic activity using casein as substrate

Proteolytic activity was determined using the protocols described by Sarath et al. (2001), with modifications. The assay used was performed in triplicate using 1% casein dissolved in monobasic sodium phosphate buffer (50 Mmol/L, pH 6.5). The assay was carried out using 150 µL of crude enzymatic extract, 100 µL of monobasic sodium phosphate buffer (50 Mmol/L, pH 6.5) and 1000 µL of 1% casein, incubating the mixture at 40ºC for at least 60 min, after which the reaction was stopped using 600 µL of 10% trichloroacetic acid (TCA). TCA was added to blank tubes before casein to precipitate the enzyme. The tubes were centrifuged at 10,000xg at room temperature for 15 min. The supernatants were measured against blanks in quartz cuvettes at 280 nm. The amount of enzyme required to result an increase of $0.001A_{280nm}$ under assay conditions was defined as one unit of enzyme (Sarath et al. 2001).

Protein precipitation

The crude enzymatic extract was subjected to precipitation using fractionation at proportion of 1:3 of crude enzymatic extract and ethanol 92.6°GL. The material was kept at -20°C for 24 h and then centrifuged at 10,000xg at 4ºC for 30 min. The supernatant was discarded and the pellet was resuspended under mild agitation in

monobasic sodium phosphate buffer (50 Mmol/L, pH 6.5) at 4°C. This new extract was used for proteolytic activity assay using azo-casein as substrate.

Biochemical Enzyme Characterization

Proteolytic Activity using Azo-casein as Substrate

The protocol for assessing the proteolytic activity using azo-casein as a substrate was based on an assay reported by Sarath et al. (2001) with modifications. The reaction was performed using 100 µL of precipitate extract, 100 µL of indicate buffer and 200 µL of 1% azo-casein. This mixture was incubated for 10 min at indicated temperature. The reaction was stopped by adding 800 µL 10% TCA. TCA was added to blank tubes before azo-casein to precipitate the enzyme. The tubes were centrifuged at 10,000 xg at room temperature for 15 min. Then, 800 µL of supernatant was added to 933 µL of 1M sodium hydroxide. This mixture was measured against blank in plastic cuvettes at 440 nm. All the experiments were performed in triplicate. The amount of enzyme required to result an increase of $0.001A_{440nm}$ under the assay conditions was defined as one unit of enzyme (Sarath et al. 2001).

Optimum pH and temperature

To optimize the pH of the precipitated extract following buffers were used: acetate (pH 4.5 and 5.0), 2-(*N*-morpholino)ethanesulfonic acid (MES) (pH 5.5, 6.0 and 6.5), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (pH 7.0, 7.5 and 8.0), bicine (pH 8.5 and 9.0) and CAPS (pH 9.5, 10.0 and 10.5), all at 50 mM/L. After the determination of the optimum pH, the influence of temperature was examined for a range of 30 to 75ºC, with increments of 5ºC.

Enzymatic extract stability

Thermal stability was determined by incubating the enzyme at 30 to 65ºC for 5, 10, 15 and 60 minutes, after which proteolytic activity was assayed at 55ºC and pH 6.5. pH stability was assessed by pre-incubating the precipitate extract at 25°C for 1 h in different buffers ranging from pH 4.5 to 10.5, with increments of 0.5. Proteolytic activity was then assayed at 55°C and pH 6.5.

Effect of inhibitors and ions

To determine the mechanism of action of the enzyme, the effects of inhibitors were determined according to the protocol of Dunn (1989), with modification. The inhibitors used were iodoacetic acid (IAA), phenylmethylsulfonyl fluoride (PMSF) and ethylene-diaminetetraacetic acid (EDTA), each at a final concentration of 10 mM/L. The effect of ions was determined using NaCl, $MnCl_2$, KCl, LiCl, $ZnSO_4$, CoCl, CuCl, $CaCl_2$, $MgCl_2$, $BaCl_2$ and $AlCl_3$, each at a final concentration of 10 mM/L.

Evaluation of collagenase activity

Collagenolytic activity was based on a protocol described by Jiang et al. (2007), with modifications. In brief, 200 µL of crude enzymatic extract was added to a suspension of 1.5 g/L azocoll in 1.0 mL of HEPES buffer (100 mM/L, pH 7.0) and incubated at 45°C for 70 min, in triplicate. Samples of this mixture were transferred into tubes and centrifuged at 10,000 xg at room temperature for 10 min. The supernatants were measured against the blanks at 550 nm. The blanks were prepared similarly to the sample tubes, but the crude enzymatic extract was boiled for 10 min to inactivate the enzyme. The results were expressed as optical density (OD) per mL of crude enzymatic extract at 550 nm according to Jiang et al. (2007).

Evaluation of keratinase activity

The assay for keratinase activity was performed in triplicate using keratin-azure prepared in aqueous solution at 4 mg/mL according to Bressollier et al. (1999), with modification. The tests were performed using 200 µL of crude enzymatic extract, 250 µL of substrate suspension and 750 µL of HEPES buffer (100 mM/L, pH 7.0). This mixture was incubated at 45°C for 24 h. The tubes were centrifuged at 10,000 xg at room temperature for 10 min and measured against the blanks at 595 nm. The blanks were prepared similarly to the sample tubes, but the crude enzymatic extract was boiled for 10 min to inactivate the enzyme. The results were expressed as OD per mL of crude enzymatic extract at 595 nm (Foroughi et al. 2006).

RESULTS AND DISCUSSION

Figure 1 shows the production of peptidases by *A. terreus* using agro-industrial residues, WB and CS meal, combined with certain other protein sources

(casein, albumin, gluten and soy protein). Under the conditions tested, the best medium for peptidase production was WB without supplementation, resulting in the production of approximately 400 U/mL after 72 h.

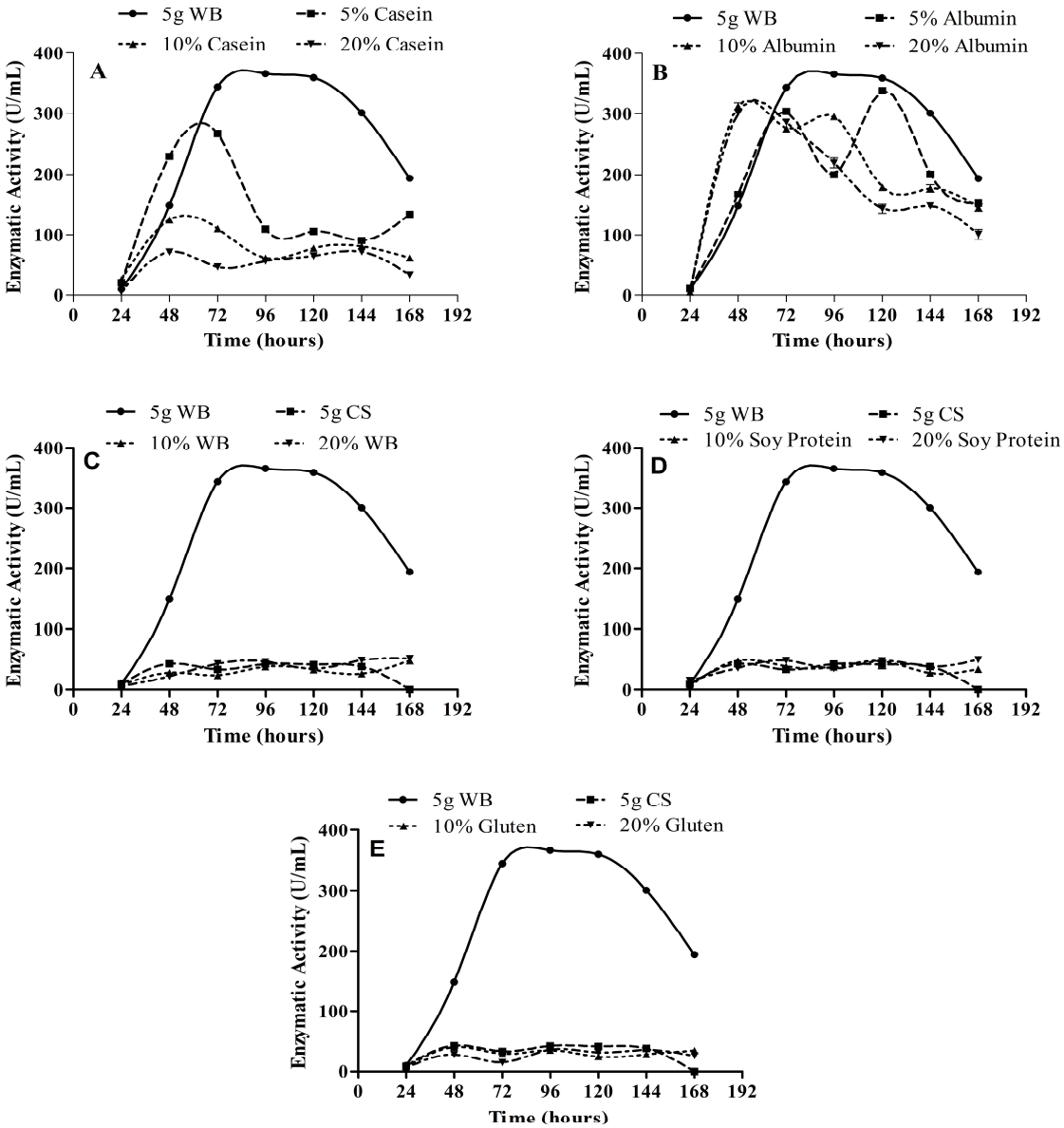

Figure 1 - Influence of different media on the production of peptidase by *Aspergillus terreus*. (A) WB and supplementation with casein; (B) WB and supplementation with albumin; (C) WB and CS and supplementation with WB; (D) WB and CS and supplementation with soy protein; (E) WB and CS and supplementation with gluten. These tests were performed at 30°C, 65% of saline volume and $5x10^5$ spores/g.

The results showed that the use of protein sources other than WB was not efficient for the production of peptidase. According to Abidi et al. (2008), the presence of protein sources as inducers in the culture medium affects the production and secretion of peptidases by *Botrytis cinerea*. In this study, the supplementation with casein (Fig. 1A) or albumin (Fig. 1B) decreased peptidase production when used along with WB, except in 48 h production where 5% of casein, 10 and 20% of albumin showed better results than the use of WB only. In comparison of supplemental albumin

or casein, albumin showed better result than casein. However, the production by WB alone was the highest.

In the bioprocess using CS meal, the production of peptidase was lower than with WB (10-times difference). The protein composition of the substrates was not the best explanation for these results because WB had approximately 16.58% protein and CS had 30.76% (Filho et al. 2000). This could be due to a difference between the particle sizes of the two residues, which might reduce the accessibility of the substrate or the voids occupied by oxygen (Krishna 2005).

Spore concentration is important in the fermentation process because it can modulate the production of enzyme in the culture medium due to the availability of material to be degraded. Based on production profiles, concentrations of $5.0x10^5$ and $2.0x10^5$ spores/g resulted significant increase in the production compared with $1.0x10^6$ spores/g (Fig. 2). Thus, the spore concentration selected for further experiments was $2.0x10^5$ spores/g because this condition combined the best peptidase production after 72 h.

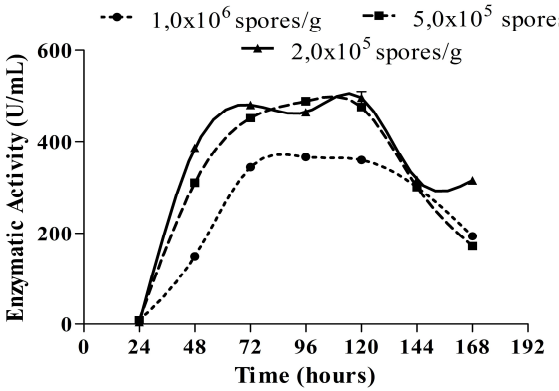

Figure 2 - Influence of spore concentration on peptidase production by the fungus *Aspergillus terreus*, in culture medium with 5 g of WB incubated at 30°C and 65% of saline volume.

An increase of spore concentration might have caused a faster depletion of essential nutrients so that there was no need for more peptidase production, whereas with a decrease in the spore concentration, enzyme production was enhanced.

The cultivation temperature interferes with the growth and metabolism of the fungus, and consequently, peptidase production may vary. The best temperature was 30°C (Fig. 3), at which best results were obtained at all the sampling periods with maximum being 500 U/mL. At 35°C, only one peak of approximately 420 U/mL and a decrease in activity beginning at 96 hs was observed. The results indicated that the fungus was sensitive to temperature. An increase in temperature promotes the higher evaporation of water from the fermentation, which causes cell death or an absence of spore germination. Thus, there was a decrease in peptidase production and secretion, with low levels at 40 and 45°C.

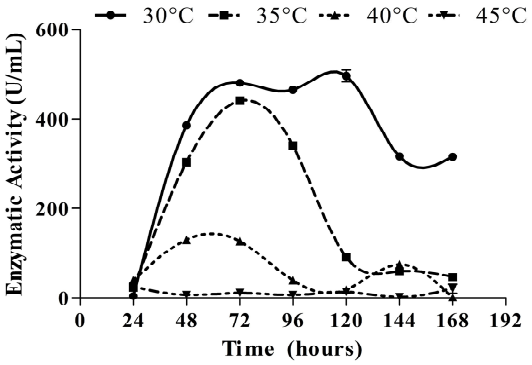

Figure 3 - Influence of the bioprocess temperature on peptidase production by the fungus *Aspergillus terreus* in culture medium containing 5 g of WB and $2.0x10^5$ spores/g and at 65% saline volume.

The variation of saline volume presented in solid medium is proportional to the content of free water and may also affect the levels of peptidase production by *A. terreus*. As shown in Figure 4, 75% of saline volume provided the best performance, but 65% showed similar levels in certain periods of the bioprocess. Increase in the saline volume to 85 or 100% affected peptidase production, which was decreased to nearly half of the production under the best condition (75%).

The above results could be related to the fact that humidity affected the porosity of the culture medium, and therefore, compromised oxygenation, fungal growth and consequently peptidase or other metabolite production (Pandey 1992). The peak of peptidase production was 677 U/mL (5400 U/g medium) after 72 h of fermentation, using 5 g of WB at 30°C, a spore concentration of $2.0x10^5$ spores/g and 75% of saline volume. The first production obtained a peak around 370 U/mL after 96 h of fermentation and the optimization led to an increase of 1.8 times of activity and shifted the peak for 72 h of the bioprocess. Different species

of *Aspergillus* sp in similar culture medium showed peptidase production similar to the production in this work, with peaks of 400 to 800 U/mL, as reported by Macchione et al. (2008).
In a study using the same culture medium (5 g of WB) with supplementation with egg albumin and casein, the production of peptidase by *Penicillium corylophilum* reached a peak of 520 U/mL after 96 h using only WB, and the production by *Penicillium waksmanii* was slightly increased using supplementation, with peak of 545 U/mL after 72 h. However, the supplementation could be removed to generate cheaper medium because the peak with 5 g of WB alone was approximately 500 U/mL (Silva et al. 2013a). Similar to this, the present work showed that *A. terreus* did not require an external source of nitrogen for the production of peptidase and possessed the potential as a peptidase producer.

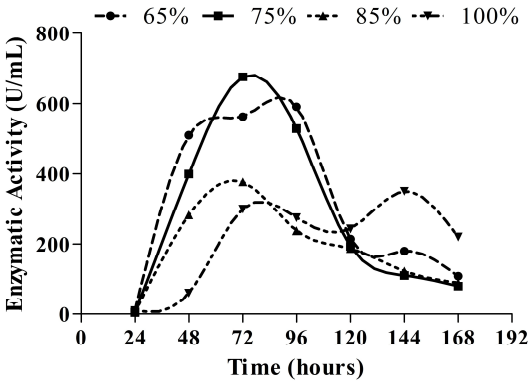

Figure 4 - Influence of the saline volume on peptidase production by the fungus *Aspergillus terreus* in culture medium containing 5 g of WB and $2.0x10^5$ spores/g and incubated at 30°C.

Biochemical characterization – optimum pH and temperature

Enzymes exhibit structural effects following pH variation. Changes in pH may modify the chemical structure of an enzyme due to protonation and deprotonation of the amino acid residues. The effects of the protonation and deprotonation of the catalytic site reflect the optimum pH of an enzyme (Tipton et al. 2009). In this study, azo-casein was used as a substrate in a pH range of 4.5 to 10.5. The higher activities were reached at pH 5.5 to 8.0, and optimal activity was at pH 6.5. The decrease in activity started at pH 8.5 indicating better tolerance to alkaline pH. This decrease might be due to the protonation of several amino acid residues, or a structural modification that promoted the inactivation, or denaturation of the peptidase (Fig. 5).

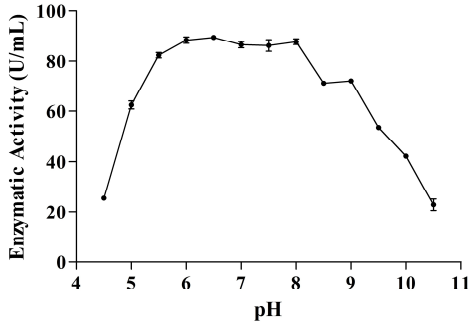

Figure 5 - Influence of pH (range of 4.5 to 10.5) on enzymatic activity. Assays with azo-casein at 45°C.

The effect of temperature on the proteolytic activity was determined based on the hydrolysis of azo-casein incubated at different temperatures and at an optimum pH. The optimum temperature for the reaction was determined at 55ºC (Fig. 6). Above 55°C, there was a decrease in proteolytic activity, possibly by denaturation of the enzyme by breaking the chemical bonds with thermal energy. Below 55°C, with increasing temperature, proteolytic activity also increased, which was related to the increased agitation of the molecules colliding with the substrate, promoting the better formation of products.

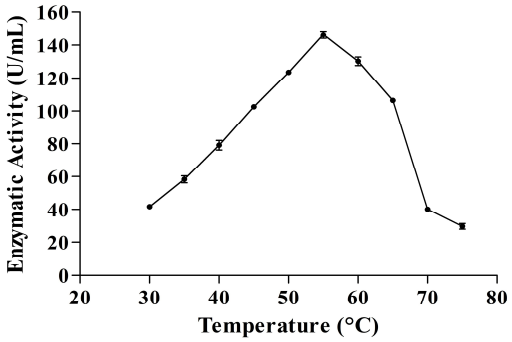

Figure 6 - Influence of temperature (range of 30°C to 75°C) on enzymatic activity. Assays with azo-casein on pH 6.5.

Enzymatic extract stability

The effects of temperature and pH on the stability of the enzymatic extract were determined based on the hydrolysis of azo-casein incubated at different temperatures and pH values. These assays are

important for the certification of enzyme stability for a subsequent application. The enzymatic extract was only partially purified, therefore, did not contain just the enzyme. The presence of different other proteins, carbohydrates, or other particles could affect the enzyme activity and stability, interfing positively, or negatively. The temperature stability of the enzymatic extract was 30°C, maintaining activity close to 50% for 1 h (Fig. 7A). Figure 7B shows the performance of the extract in a pH range of 4.5 to 10.5. This showed better stability at an acidic pH (4.5 to 6.0). Despite the decrease in the activity with an increase in pH, the decrease was not under 50%.

Stability is an important parameter given the different applications of enzymes and extracts in industrial processes. From these tests, it was obvious that an extract could be used in complex processes, or be preserved by stabilization techniques (Ameri and Maa 2006; Namaldi et al. 2006).

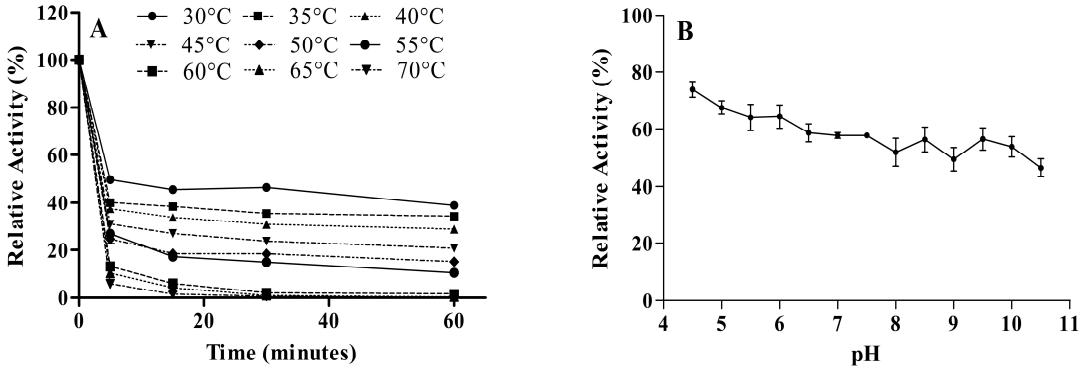

Figure 7 - (A) Temperature stability at exposure times of 5, 15 and 60 min; **(B)** pH stability (range of 4.5 to 10.5) and enzymatic activity over 1 h.

Effect of inhibitors and ions

The classification of the peptidase based on the nature of the catalytic site was determined by an inhibitor assay. This assay showed that there might be more than one peptidase in the extract or only one metal-dependent peptidase because EDTA and PMSF were inhibitory. These inhibitors indicated serine and metallo peptidases, respectively (Fig. 8).

The modulation of enzymatic activity in the presence of ions may be positive when there is an increase in proteolytic activity compared with a control, or negative when this activity decreases. Ions can interact with the enzyme through residues in the catalytic site, modifying the residues' disposal promotes the activation or inactivation of the enzyme. Another explanation for the modulation by ions is interaction with amino acids outside of the catalytic site, promoting the modification of tertiary structure and improving or not improving proteolytic activity. As noted, the peptidase in the precipitate extract presented positive modulation, with increases of 120 and 110% in the presence of Ca^{2+} and Al^{3+}, respectively. There was negative modulation when the enzyme was exposed to lithium and copper, which possibly bonded to the serine residue in the catalytic site of the peptidase, causing a loss of activity (Fig. 9). Hajji et al. (2008) observed positive modulation when a serine peptidase produced by *A. clavatus* was incubated with calcium (124%), with performance similar to the present study.

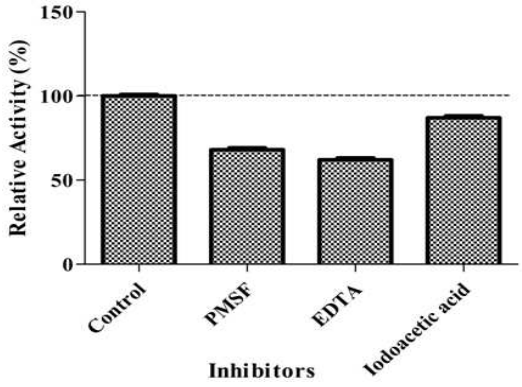

Figure 8 - Influence of the addition of inhibitors on the proteolytic activity of the peptidase produced by *A. terreus*. Proteolytic activity was determined using azo-casein at 55°C in MES buffer at pH 6.5 and a final concentration of 10 mM/L.

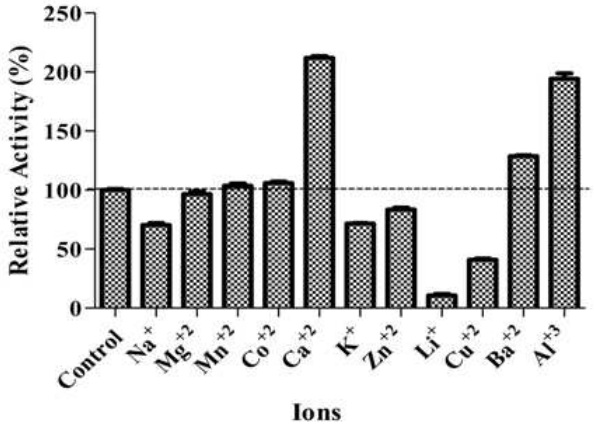

Figure 9 - Influence of the addition of ions on the proteolytic activity of the peptidase produced by *Aspergillus terreus*. Proteolytic activity was determined using azo-casein at 55°C in MES buffer at pH 6.5 and a final concentration of 10 mM/L.

A study by Silva et al. (2013b) characterized a serine peptidase produced by *A. fumigatus* in SSF using WB; the enzyme showed similar characteristics to the peptidase in the current study. In another study by Stefanova et al. (1997), *A. terreus* produced two classes of peptidases, serine and metallo, which were responsible for the cleavage of low-mass endoglucanases. These studies showed the possibilities presented in this study, the presence of one ion-dependent serine peptidase, or two classes of peptidase, metallo and serine.

Collagenolytic and keratinolytic potential

The keratinolytic activity obtained was 0.252 OD/mL in 24 hours, showing the potential of this extract in the degradation of keratin. Foroughi et al. (2006) reported that the extracts of different fermentations obtained results of 0.02 to 0.09 OD/mL over 24 hours, showing that under the conditions in the present study the result was better. The collagenolytic activity showed a result of 0.165 OD/mL in 70 min, proving the extract's potential.

CONCLUSION

The agro-industrial residues were proven to be efficient substrate for peptidase production by *A. terreus*. The level of the proteolytic activity presented might be viable for use in industrial processes because of the low cost of the culture media, adding value to the product. The biochemical characterization of the precipitate extract showed the presence of two different peptidases (serine and metallo peptidases), or the existence of only one metal-dependent serine peptidase and a favorable behavior in a wide pH range. Thus, it could be possible the use of this enzyme in detergent industry, as it tolerated different pH. It also could be used in residues treatment, as the ions did not have a negative influence in the enzymatic activity, except for lithium and cooper. The keratinolytic and collagenolytic potential proved to be important for the possible application of this extract, showing promise for others applications.

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