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Research Paper

Production and partial characterization of serine and metallo peptidases secreted by *Aspergillus fumigatus* Fresenius in submerged and solid state fermentation

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Abstract

Enzyme production varies in different fermentation systems. Enzyme expression in different fermentation systems yields important information for improving our understanding of enzymatic production induction. Comparative studies between solid-state fermentation (SSF) using agro-industrial waste wheat bran and submerged fermentation (SmF) using synthetic media were carried out to determinate the best parameters for peptidase production by the fungus *Aspergillus fumigatus* Fresen. Variables tested include: the concentration of carbon and protein nitrogen sources, the size of the inoculum, the pH of the media, temperature, and the length of the fermentation process. The best peptidase production during SSF was obtained after 96 hours using wheat bran at 30 °C with an inoculum of 1 x 10^6 spores and yielded 1500 active units (U/mL). The best peptidase production using SmF was obtained after periods of 72 and 96 hours of fermentation in media containing 0.5% and 0.25% of casein, respectively, at a pH of 6.0 and at 30 °C and yielded 40 U/mL. We also found examples of catabolite repression of peptidase production under SmF conditions. Biochemical characterization of the peptidases produced by both fermentative processes showed optimum activity at pH 8.0 and 50 °C, and also showed that their proteolytic activity is modulated by surfactants. The enzymatic inhibition profile using phenylmethylsulfonyl fluoride (PMSF) in SmF and SSF indicated that both fermentative processes produced a serine peptidase. Additionally, the inhibitory effect of the ethylene-diaminetetraacetic acid (EDTA) chelating agent on the peptidase produced by SmF indicated that this fermentative process also produced a metallopeptidase.

Key words: metallopeptidase, serine peptidase, fermentative parameters, *Aspergillus fumigatus*.

Introduction

Aspergillus fumigatus Fresen. is a saprophytic organism that produces many peptidases (Bouchara *et al.*, 1993; Gifford *et al.*, 2002). Proteolytic enzymes are a major group of extracellular enzymes found in all forms of life, such as plants, animals and microorganisms (Haq *et al.*, 2006). Peptidases from bacteria and fungi have many uses: the production of pharmaceuticals, food production, cleaning agents and the removal of waste proteins such as fur and feathers (Rao *et al.*, 1998).

Microbial peptidase secretion varies between species. Each organism or strain has specific conditions for maximum enzyme production, resulting in large differences in peptidase production between submerged fermentation (SmF) and solid-state fermentation (SSF). In this context,

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filamentous fungi have been explored as a source of peptidases because they are able to grow under a range of growth conditions and they produce a variety of enzymes (Sandhya *et al.*, 2005).

Peptidase production by species of *Aspergillus*, in particular, has largely been studied because these species are known for their ability to secrete high levels of enzymes during growth in their natural environments (Sandhya *et al.*, 2005). *Aspergillus fumigatus* is a filamentous fungus commonly found in the soil and is an important agent in the decomposition organic matter. It is able to grow at high temperatures and is an opportunistic pathogen (Tekaia *et al.*, 2005).

Differences in fermentation methods (SSF and SmF) and in the compositions of culture media, especially the carbon and protein nitrogen source, greatly influence peptidase production. In addition, other factors such as temperature, pH and fermentation time can also affect this production. Thus, this study was conducted to investigate the most favorable parameters for *A. fumigatus* peptidase production in submerged and solid state fermentation and to biochemically characterize the peptidases obtained from both fermentative processes.

Materials and Methods

Microorganisms, strain identification and maintenance medium

The fungus *Aspergillus fumigatus* was isolated from decaying wood found in soil from São José do Rio Preto, SP-Brazil. It was maintained on potato-dextrose agar (PDA) slants at 30 °C for 168 hours to allow for complete growth. The strain was identified by a polyphasic approach using morphological and molecular markers. Preliminary microscopic examination indicated the strain belonged to the genus *Aspergillus*. To identify the species, we followed the standard plate regime established by Klich (2002). Briefly, the strain was cultivated for seven days in three culture media (CYA, MEA, CY20S) under different incubation temperatures (5, 25, 37 °C). Colony morphology as well as microscopic characteristics were observed and measured (n = 10 measurements in μm). All characteristics were compared to detailed descriptions of common *Aspergillus* species (Klich, 2002) and to descriptions of *A. fumigati* (Samson *et al.*, 2007).

DNA sequencing was used to assist strain identification. Prior to DNA extraction, the strain was cultured in 2% malt extract broth for five days at 25 °C. Then, the mycelium was recovered on a paper filter and freeze-dried overnight. DNA extraction followed the protocol of Sampaio *et al.* (2001) and the internal transcribed spacer region (ITS) was amplified and sequenced using the universal primer pair ITS4 and ITS5 (White *et al.*, 1990). Both forward and reverse sequences were generated using an ABI 3500 (Applied Biosystems) and assembled into a contig using Bioedit v.7.0.5.3 (Hall, 1999). The contig sequence was compared with homologous sequences available at NCBI-GenBank by BLASTN (Altschul *et al.*, 1997).

Sequences of close relatives were retrieved from GenBank and aligned in Muscle v.3.6 (Edgar, 2004). Phylogenetic analyses were carried out under the neighbor-joining algorithm using the Kimura 2-parameters as the substitution model and sites with gaps/missing data were pairwise excluded from the analyses. A neighbor-joining tree was generated using MEGA5 (Tamura *et al.*, 2011).

Solid-state fermentation process

Peptidase production by *A. fumigatus* in SSF was evaluated using agro-industrial waste wheat bran (W.B.). Five grams of W.B. was placed in a 250 mL Erlenmeyer flask and moistened with 10 mL of saline solution containing 0.1% (w/v) of the following salts: $(NH_4)_2SO_4$, $MgSO_4x7H_2O$ and NH_4NO_3. The flasks were autoclaved for 40 min at 121 °C and the media was then inoculated with 5×10^6 spores for each 5 g of W.B. The fermentation was carried out for 168 hours, and every 24 hours one material (flasks) was removed and then was held to solubilize the enzyme in 40 mL of distilled water at 4 °C. The solubilization was performed in a shaker for 30 min at 200 rpm at 4 °C. The solubilized material was then centrifuged at 8,000x*g* for 20 min at 4 °C. The supernatant was used to quantify proteolytic activity.

The following variables were tested in SSF: the addition of casein and egg albumin inductors (5, 10 and 20%), the concentration of spores (0.1×10^6, 1×10^6, 2.5×10^6 and 5×10^6) and the fermentation temperature (30, 35, 40 and 45 °C).

Submerged fermentation process

The media for SmF was prepared in 250 mL Erlenmeyer flasks and consisted of 50 mL of liquid media (standard) containing 0.7% (w/v) KH_2PO_4, 0.2% (w/v) K_2HPO_4, 0.01% (w/v) $MgSO_4.7H_2O$, 0.05% (w/v) citrate $2H_2O$, 0.1% (w/v) yeast extract, 0.01% (w/v) $CaCl_2.2H_2O$ and 1% (w/v) peptone (Tran and Nagano, 2002). The pH of the media was adjusted to 6.0, and the flasks were autoclaved for 15 min at 121 °C.

The following variables were tested in SmF: the addition of sucrose, glucose and fructose (0.1, 0.5, and 1%) as carbon sources, the use of casein inductor (0.25, 0.5 and 1%), the concentration of spores/mL of medium (1×10^5, 2.5×10^5, 5×10^5 and 10×10^5), the fermentation temperature (30, 35, 40 and 45 °C) and the initial pH of the media (5.5, 6.0, 6.5, 7.0, 7.5, 8.0 and 8.5).

The medium was inoculated with the spore suspension and incubated in a shaker at 30 °C and 120 rpm (orbital rotation) for 168 hours; every 24 hours one material (in flasks) was removed. The material was filtered with Whatman paper nº 1, and centrifuged at 8,000x*g* for 20 min at 4 °C. The supernatant was used to quantify proteolytic

activity and the fungal mycelium was dried at 45 °C in hot air oven (to a constant weight) to evaluate the growth of the culture.

Determination of proteolytic activity

Proteolytic activity was determined according to the protocol described by Sarath *et al.* (1996), with modification. The reaction mixture was composed of 1 mL of 1% (w/v) casein (Sigma) in a 0.05 M monobasic sodium phosphate buffer, pH 6.5, to which 0.2 mL of the enzyme solution was added. The reaction was carried out at 37 °C and stopped with the addition of 0.6 mL of 10% TCA (trichloroacetic acid) after 20 min for SSF samples or after 60 min for SmF samples. Reactions were then centrifuged at 10,000x*g* for 15 min at 25 °C, and the absorbance of the supernatant was measured at 280 nm. An appropriate control was prepared in which TCA was added before the enzymatic solution. One activity unit (U) was defined as the amount of enzyme required to liberate 1 μmol tyrosine/min under the assay conditions (Meyers and Ahearn, 1977).

According to peptidase production the crude enzyme extracts that presented the best proteolytic activities in each fermentation process were concentrated by precipitation with ethanol (92.8º GL) using a ratio of 1 volume of extract to 2 volumes of ethanol. Biochemical studies were performed using 1% azocasein as a substrate, according to the protocol described by Ducros *et al.* (2009). The reaction mixture consisted of 0.1 mL of partially purified enzyme extract, 0.1 mL of 0.05 M HEPES buffer, pH 8.0, and 0.2 mL of 1% azocasein. The mixture was incubated at 40 °C for 10 min and the reaction was stopped with the addition of 0.8 mL 20% TCA. Reaction and control tubes were centrifuged at 10.000x*g* for 15 min at 25 °C, and 0.8 mL of supernatant was removed and added to 0.9 mL of 1 M sodium hydroxide. The tubes were shaken, and the absorbance was measured with a spectrophotometer at 440 nm and compared to their respective controls. One activity unit (U) was defined as the amount of enzyme required to yield an increase of $0.001A_{440nm}$ under the conditions of the assay, as described by Morita *et al.* (1998), with modifications.

The effects of pH on the proteolytic activity was studied using the following buffers: acetate (pH 4.5 and 5.0), MES (pH 5.5, 6.0 and 6.5), 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 0.05 M and using azocasein as substrate. The influence of temperature on the activity of the peptidase was examined between a range of 25 °C to 70 °C, with increments of 5 °C. Thermal stability was determined by incubating the enzyme at 35 °C to 60 °C for periods of 5, 10, 15, 30, 45 and 60 min, after which proteolytic activity was assayed at 40 °C and pH 8.0. The study about effect of surfactants on proteolytic activity was carried out in different concentrations, using the following surfactants, Tween 20, Triton X-100, cetyltrimethylammonium bromide (CTAB) and sodium dodecil sulfate (SDS).

The mechanism of action was determined according to the protocol described by Dunn (1989), with modification. The following inhibitors were used: iodoacetic acid (IAA), phenylmethylsulfonyl fluoride (PMSF) and ethylene-diaminetetraacetic acid (EDTA), each at a final concentration of 10 mM. The reaction contained 1% (w/v) casein (Sigma) as a substrate and was carried out at 37 °C, pH 6.5.

Results and Discussion

Strain identification

Fungal colonies grown at 25 °C on different types of media reached a diameter of 47-48, 53-54 and 30-32 mm after growth on CYA, MEA and CY20S media, respectively. At 37 °C, colony growth reached 48-49 mm in diameter on CYA media; no growth was observed at 5 °C. Colonies grown on CYA were dark turquoise in color, with no visible exudate or soluble pigment. Stipes were light green in color, smooth, and 5.65-9.04 μm in diameter. Pyriform vesicles grew to 16.8-36.4 μm in diameter. Phialides reached 6.78-12.43 x 2.26-3.39 μm, and gave rise to conidia ranging in shape from globose to ellipsoidal with smooth to finely rough walls (2.8-3.39 μm in diameter). Overall, colony morphology and microscopic characteristics matched the descriptions of *Aspergillus* section *fumigati* (Klich, 2002; Samson *et al.*, 2007).

The complete ITS region (560 base pairs) was sequenced for the strain. BLASTN results indicated that available sequences of *A. fumigatus* in the public database were 99% identical to our sequence. Furthermore, phylogenetic analysis classified our strain as co-specific to other *A. fumigatus* strains (Figure 1).

Peptidase production profile by submerged and solid-state fermentation

The fermentative process is greatly influenced by the nature of the substrate. Several additional parameters affect the production of enzymes in the fermentation process, including time, moisture content, temperature and nutrition supplements such as carbon and nitrogen (Sumantha *et al.*, 2005).

Effect of inductor in the peptidase production

We investigated the influence of an inductor, casein, on peptidase production in submerged fermentation. The use of 0.5% casein induced maximum peptidase production in 72 hours, and the addition of 0.25% casein induces maximum peptidase production after 96 hours. The amount of peptidase produced under both conditions was 40 U/mL (Figure 2a).

In SSF, the greatest peptidase yield was achieved using W.B. (100%) as substrate for a fermentation period of

96 hours, with 1300 U/mL (Figure 2b). Notably, the peak peptidase production in SSF was obtained using W.B. and was approximately two-fold higher than the peak production reached with 5% egg albumin or casein protein supplements. Other recent studies also found the highest production of peptidases using W.B. as substrate for SSF (Macchione *et al.*, 2008, Sandhya *et al.*, 2005). Wheat bran is an excellent substrate for SSF because of its composition,

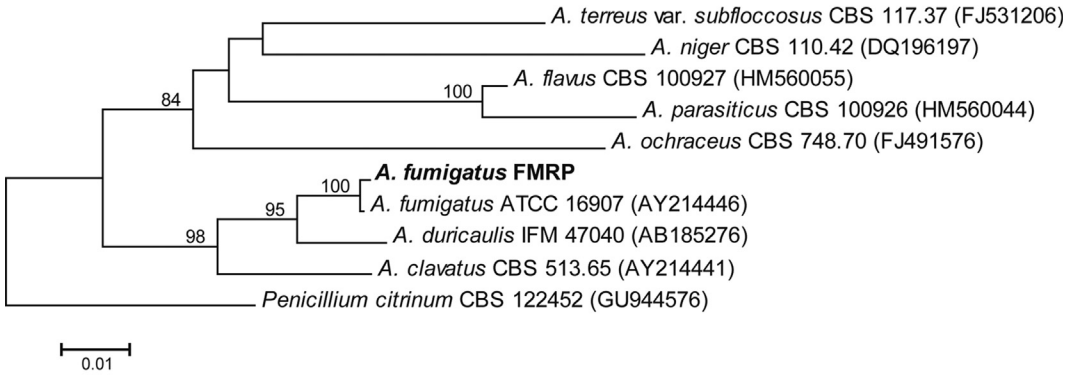

Figure 1 - Neighbor-joining tree inferred from a 531-base pair fragment of the internal transcribed spacer region (ITS) of selected *Aspergillus* spp. The tree was inferred under the Kimura 2-parameters substitution model. Numbers on branches are bootstrap support values obtained from 1,000 pseudoreplicates. The strain FMRP evaluated in this study is denoted in bold. Sequences are followed by the culture collection voucher number and the GenBank accession numbers in parentheses. An ITS sequence of *Penicillium citrinum* was used as an outgroup.

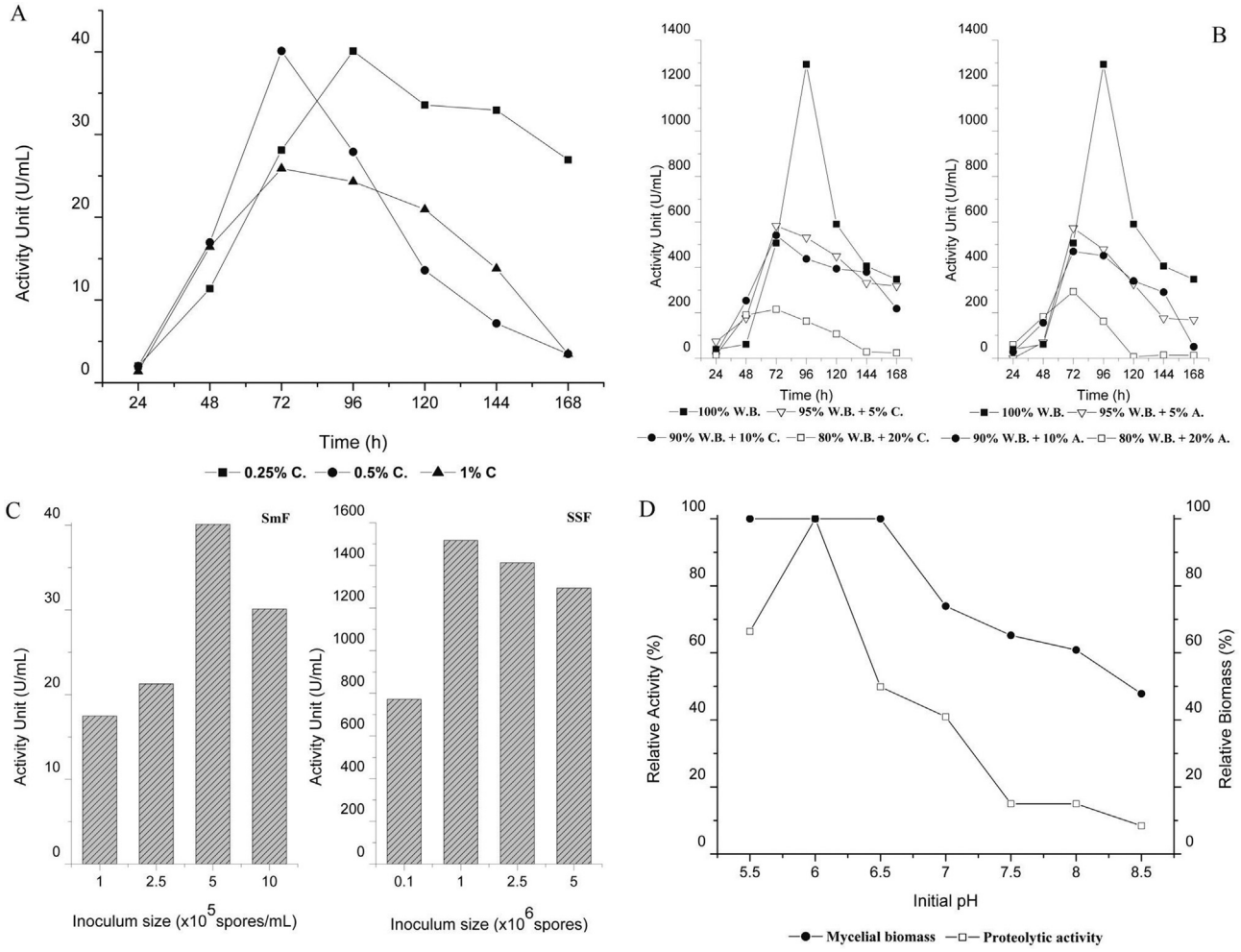

Figure 2 - Peptidase production by *A. fumigatus* in SmF and SSF. Effect of inductors (Casein (C) and egg albumin (A)) in SmF (a) and SSF (b). Effect of the inoculum size (c) and effect of the initial pH of the media in SmF (d).

protein content, texture and porosity facilitate the access and the dispersion of fungi (Pandey, 2003). The high protein content of W.B. (18% protein) may have contributed strongly to the amount of peptidase produced (Madruga and Camara, 2000).

However, in SSF media with 20% protein supplementation, the maximum peptidase production was approximately 200 U/mL for casein and 300 U/mL for egg albumin. Interestingly, this trend was observed in both fermentation methods, indicating that the increased concentration of inductor promoted a reduction in proteolytic activity. In comparison to the best peptidase production conditions, in SmF in the presence of 1% casein, the decline of 1.5 times in the proteolytic activity proves that the rise in the concentration of protein inductor also acts as a repressor in the production of peptidases. A similar result has also been shown with *Actinomycete* in SSF (Mehta *et al.*, 2006).

The composition of the growth media is a factor in determining the amount and the type of peptidase produced. In this work there was a highest peptidase production in SSF, we found that SSF yielded approximately 30 times more peptidase than SmF, probably because the high protein content of W.B and due to the media is closer of the natural microbial growth conditions (Pandey, 2003). Gifford *et al.* (2002) observed that proportion of serine peptidases and metallopeptidases produced depends on the proteic substrate utilized by the fungus *A. fumigatus*.

Effect of the inoculum size and temperature in the peptidase production

The inoculum size and growth temperature are important biological factors that determine the metabolic production and biomass of the fermentation process. Thus, the density of spores used to inoculate fermentation cultures is a fundamental parameter in fungal growth (Sandhya *et al.*, 2005). Among the studied concentrations of spores, we found that the maximum peptidase production in SmF and SSF was with $5x10^5$ spores/mL and $1x10^6$ spores, respectively (Figure 2c).

These results indicate that the concentration of nutrients was sufficient for fungal growth and peptidase production, allowing a better hydrolysis of proteins and an increase in amino acids available as nitrogen sources. However, it was also observed that concentrations greater than and less than the ideal spore concentration resulted in decreased peptidase production. A similar result was shown with *Aspergillus oryzae* in SSF (Sandhya *et al.*, 2005).

The effect of the temperature in SmF and SSF revealed that the best peptidase production occurred at 30 °C and that increasing the temperature resulted in a decrease in peptidase production in both fermentative processes (Table 1). The correlation between increased temperature and decreased proteolytic activity has also been shown in *Penicillium chrysogenum* (Haq *et al.*, 2006).

Table 1 - Effects of different temperatures in SmF and SSF on peptidase production.

Temperature (°C)	Peak production SmF	Peak production
SSF		
30	40	1300
35	39	477
40	16.3	425
45	6.47	84

Peak peptidase production (U/mL) at different temperatures (30, 35, 40 and 45 °C) in SmF and SSF. SmF process were performed in medium with 0.5% casein, 5 x 10^5 spores/mL, and pH 6.0. SSF process used wheat bran as substrate and an inoculum of 5 x 10^6 spores.

Effect of carbon sources on peptidase production in submerged fermentation

The effect of the addition of carbon sources (glucose, fructose and sucrose) at different concentrations (0.1, 0.5 and 1%) to SmF process was also studied. The results showed that peptidase production was repressed in all samples that contained glucose, fructose or sucrose. The highest level of peptidase production (28 U/mL) was obtained in the presence of 0.5% glucose after 168 hours of fermentation, and the lowest level of production (4.5 U/mL) occurred after 168 hours of fermentation in the presence of 1% sucrose. A comparison between these results and the results of fermentation using standard medium (0.5% casein without carbon source), which yielded an enzymatic activity of 40 U/mL, shows that the addition of these sugars as a carbon source resulted in the repression of peptidase production (Table 2). The repressive effect of the sugars is due to the presence of a carbon source that is easy to absorb; the fungus *A. fumigatus* produces peptidases slowly, after 168 hours of fermentation, and when the carbon sources in the media have been depleted. Catabolite repression by the addition of carbon sources has also been reported by others researchers (Abidi *et al.*, 2008; Haddar *et al.*, 2011).

Effect of the initial pH of the media on peptidase production in submerged fermentation

The pH of the media greatly influences fungal growth, affecting mechanisms such as enzyme secretion and cellular

Table 2 - Effect of different concentrations of carbon sources on peptidases production.

Carbon sources	0%	0.1%	0.5%	1%
Standard medium	40	-	-	-
Glucose	-	18	28.1	13.6
Fructose	-	26.5	24.8	6.7
Sucrose	-	19.7	15.7	4.5

Peak peptidase production (U/mL) after 168 hours of fermentation with different percentages (0.1%; 0.5% and 1%) of glucose, fructose and sucrose. The fermentations were conducted in medium with 0.5% casein, 5 x 10^5 spores/mL and carried out at 30 °C.

transport (Moon and Parulekar, 1991; Sandhya *et al.*, 2005). In this study, peak peptidase production was obtained with growth in media at a pH of 6.0 (Figure 2d). At this pH, the fungus reached the highest levels growth and peptidase production, showing an association between fungal growth and production of proteolytic enzymes. Increasing the initial pH of the medium resulted in a significant decrease in enzymatic production. Similar results have been demonstrated for the fungi *Penicillium chrysogenum* (Haq *et al.*, 2006) and *Aspergillus oryzae* (Sandhya *et al.*, 2005).

Partial characterization of the produced peptidases

Effect of the pH in the activity and stability on the peptidase

The effect of pH on the activity and stability of the peptidases obtained from SmF and SSF was determined using 1% azocasein as a substrate. Peptidases obtained from both fermentation processes demonstrated a wide range of activity with a strong alkaline tendency. They showed maximum activity at a pH of 8.0 and were stable at all pH levels tested (Figure 3a). The ability of others species of *Aspergillus* to secrete alkaline peptidases was described by Tunga *et al.* (2003) and Hajji *et al.* (2008); optimum activity was obtained at pH 8.0 and 8.5, respectively. The production of alkaline peptidases by *A. fumigatus* has also been noted in other studies (Larcher *et al.*, 1992; Wang *et al.*, 2005).

Effect of the temperature in the activity and stability of the peptidase

The effect of temperature on the activity of the peptidases was studied at pH 8.0 using 1% azocasein as a sub-

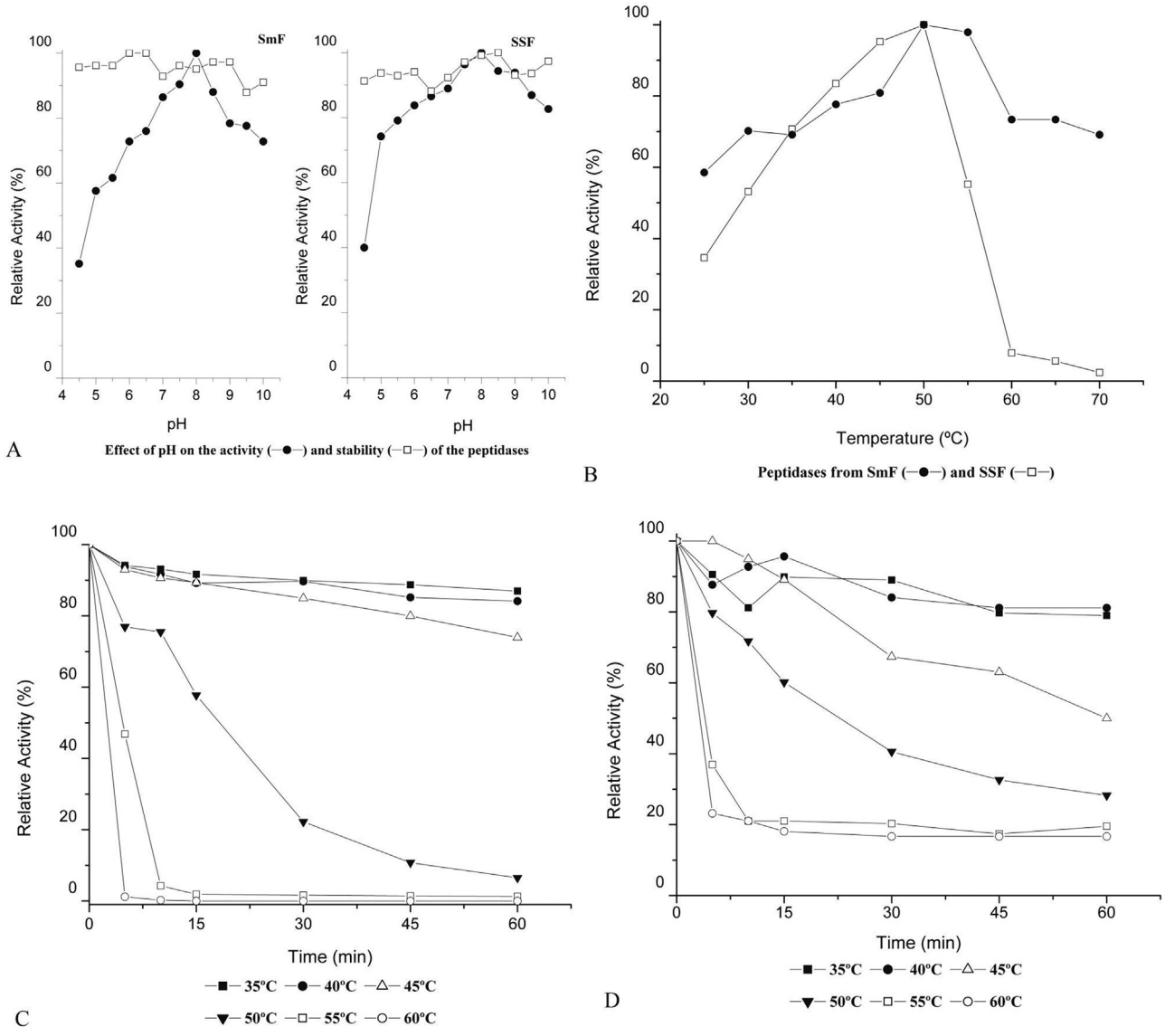

Figure 3 - Partial characterization of the peptidases produced in SmF and SSF. Effect of the pH in the activity and stability (a). Effect of the temperature in the activity (b) and stability of the peptidase produced in SSF (c) and SmF (d).

strate. The activity was examined for temperatures between 25 to 70 °C, with the optimum activity occurring at 50 °C for both peptidases. The peptidase produced by SmF maintained 70% of relative activity at 70 °C, in contrast to the peptidase obtained in SSF, which showed an abrupt decrease in activity at temperatures above 50 °C (Figure 3b). The results of the thermal stability assay showed that the peptidase obtained by SSF had a residual activity of 80% after 60 min of exposure to a temperature of 45 °C, but it maintained only 20% of the initial proteolytic activity after 30 min of exposure to a temperature of 50 °C (Figure 3c). The peptidase obtained by SmF showed a higher stability than the peptidase from SSF; after 30 min of exposure to a temperature of 50 °C, the peptidase maintained 40% of its initial activity (Figure 3d).

Point out that each method used for enzyme production presents self characteristics, leading to a different production between the systems of fermentation, justifying the induction of peptidases with unique biochemical properties. In addition, when referring it to thermal stability of enzyme extracts; the enzyme relation with other compounds (proteins and carbohydrates) is a factor of great influence on peptidase stability.

Effect of surfactants in the activity of the peptidase

The influence of different concentrations of several surfactants on enzymatic activity was assessed. The peptidases were highly stable in the presence of non-ionic surfactants (Tween 20 and Triton X-100). However, the proteolytic activity of both fermentative extracts (SSF and SmF) was reduced in the presence of cationic (CTAB) and anionic (SDS) surfactants. In comparison with the control reaction, the addition of 0.8% CTAB reduced proteolytic activity by approximately 60% for SSF and 25% for SmF, and the addition of 0.8% SDS reduced proteolytic activity by approximately 70% for SSF and 65% for SmF (Figure 4a and b). These results are likely due to the denaturing activity of surfactants, which releases non-covalent bonds, thus decreasing proteolytic activity (Berg *et al.*, 2002). Decreased proteolytic activity in the presence of SDS has also been shown in peptidases from *Aspergillus clavatus* ES1 (Hajji *et al.*, 2008) and *Penicillium* sp. (Agrawal *et al.*, 2004).

Effect of inhibitors on the activity of the peptidase

The catalytic mechanism of action of peptidases can be identified by their responses to different enzyme inhibitors. In the study about effect of inhibitors, in presence of IAA (10 mM) and EDTA (10 mM), the peptidase obtained from *A. fumigatus* SSF did not present inhibition of the proteolytic activity, whose residual activities were 98% and 93%, respectively. However, in presence of PMSF (10 mM), the peptidase was almost completely inhibited (residual activity of 5%) indicating that it is a serine peptidase. Differently, the proteolytic activity of the peptidase

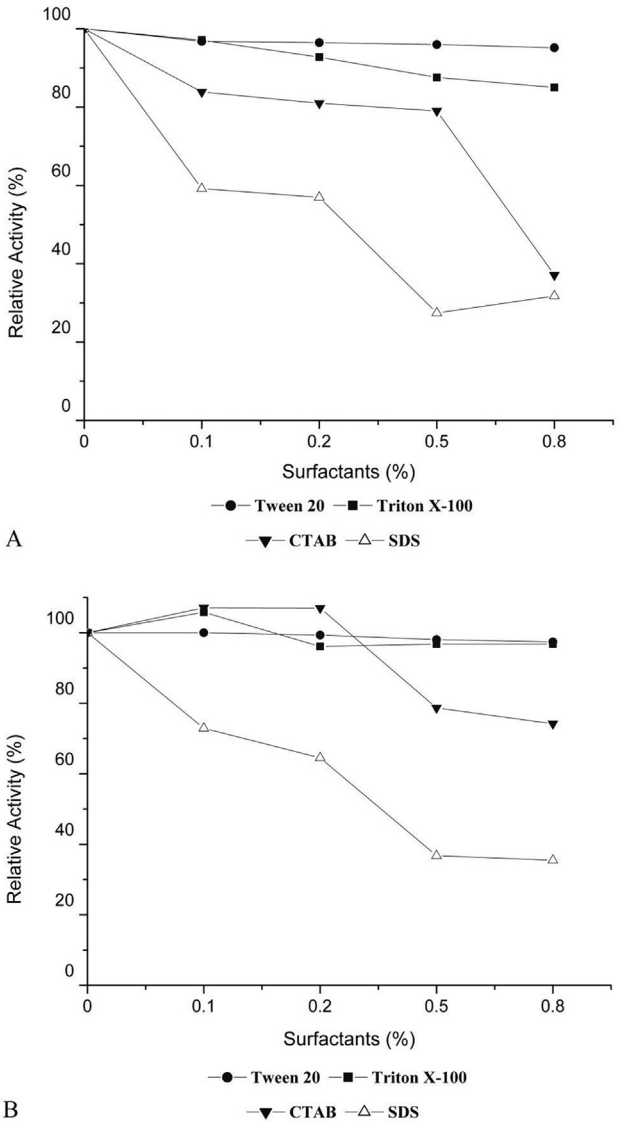

Figure 4 - Effect of surfactants on SSF peptidases (a) and SmF peptidases (b).

produced by SmF was approximately 50% and 45% of the original activity after incubation with PMSF (10 mM) and EDTA (10 mM), respectively. In addition, studies shown that increasing the concentration of PMSF (5, 10, 15 and 20 mM) did not further inhibit the proteolytic activity of the SmF peptidase, and the mixture between PMSF (10 mM) and EDTA (10 mM) promoted highest reduction of the proteolytic activity (residual activity of 5%) indicating that more than one peptidase class was present. This inhibition profile indicates that SmF produces both serine peptidases and metallopeptidases. The production of serine peptidases in SSF by the fungus *A. fumigatus* have also described by Wang *et al.* (2005) and Larcher *et al.* (1992), and Markaryan *et al.* (1994) has described metallopeptidase production by *A. fumigatus*.

In conclusion, fungal growth parameters influence peptidase production by *A. fumigatus.* Interestingly, we

found that SSF yielded approximately 30 times more peptidase than SmF, being the wheat bran an excellent substrate for peptidase production. In this work there was production of alkaline peptidases with optimum activities at 50 °C, wide range pH stability and more stable in presence of non-ionic surfactants. We also found differences in the types of peptidases produced by the different fermentative processes; serine peptidase and metallopeptidase were produced by SmF but SSF extract contained only serine peptidases. These results are important for future studies concerning the induction of peptidase production by the type of fermentative medium and indicate that enzyme production not only varies between fungal species but also can be influenced by the use of different fermentative processes.

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