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Full Length Research Paper

Optimization of metalloprotease production by *Eupenicillium javanicum* in both solid state and submerged bioprocesses

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Proteases are enzymes that have great importance in industries and can be produced through bioprocess by microorganisms. Changes in parameters can influence the production rate of these enzymes. The current study evaluated the influence of these parameters on the production of proteases by the fungus *Eupenicillium javanicum* under both solid state bioprocess (SSB) and submerged bioprocess (SmB), and determined the partial biochemical characterization of the crude enzymatic extract. Our studies have indicated that the best conditions for the production of proteases under SSB were a 10% albumin, 90% wheat bran incubated at 30°C. Under SmB the optimal conditions were medium supplemented with 0.25% casein, medium pH of 5.0 and incubation at 30°C. The biochemical characterization showed that the enzyme is a metalloprotease that presented collagenolytic activity, with optimal pH and temperature of 5.5 and 60°C, respectively.

Key words: Metalloprotease, *Eupenicillium javanicum*, bioprocesses, collagenolytic activity, biochemical characterization.

INTRODUCTION

Since the middle of the last century, the interest in fungi to produce commercially important products such as enzymes has increased (Papagianni, 2004). Proteases are proteolytic enzymes that catalyze the hydrolysis of protein peptide bonds and occupy an important position due to its use in various research fields and its commercial applicability in several areas (Rao et al., 1998).

One important kind of proteases is the collagena-ses that are used to remove stains and keloids, to treat burns and ulcers, and used in scientific studies (Sukhosyrova et al., 2003). Collagen has a rigid structure and only a few proteases can cleave (Watanabe, 2004).

The action of collagenases in the collagen fibers generates peptides that have diverse biological activities of interest to industry, leading to the establishment of a wide variety of applications such as for immunotherapeutic agents and in cosmetic development (Tsuruoka et al., 2003).

Mandl et al. (1953) identified collagenases produced by *Clostridium hystolyticum* and this microbial collagenase is the most known. Moreover, the production of microbial proteases with collagenolytic activities by *Streptomyces* sp. (Zhao et al., 2012), *Aspergillus fumigatus* (Monod et al., 1993) and *Aspergillus flavus* (Sukhosyrova et al., 2003) were described.

These studies show the importance of proteases produced by fungi with potential applicability in industry.

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Eupenicillium javanicum is a filamentous fungus which has shown potential for the production of amylases and proteases (Tanaka et al., 1986). However, there are no reports on the literature on the production of proteases with collagenolytic activity by the fungus *E. javanicum*. This can provide the production of enzymes with different characteristics.

The aim of this paper is to optimize the production of proteases produced by *E. javanicum* in both submerged and solid state bioprocesses that showed collagenolytic potential.

MATERIALS AND METHODS

Isolation, identification and maintenance of the organism

The fungus *E. javanicum* was isolated from silage and identified by research group of Dr. Cristina Maria de Souza Motta (Federal University of Pernambuco). This fungus belongs to a collection of microorganisms in the Enzyme Technology Laboratory under the responsibility of Dr. Hamilton Cabral (School of Pharmaceutical Sciences of Ribeirão Preto-University of São Paulo). The fungus was kept in Sabouraud medium at 4°C, up to one month.

E. javanicum inoculum preparation

The inoculum was obtained by peaking of the fungus in 250 mL Erlenmeyer flasks with Sabouraud medium and maintained for 7 days at 30°C, and the mycelium of the fungus was scraped from the surface of the culture medium in the presence of sterile distilled water for submerged bioprocess or in the presence of saline solution (0.1% (w/v) $(NH_4)_2SO_4$, 0.1% (w/v) NH_4NO_3 and 0.1% (w/v) $MgSO_4x7H_2O$) for solid bioprocess.

Submerged bioprocess (SmB)

In 250 mL Erlenmeyer flasks, 50 mL of liquid medium were added, its composition was 0.7% (w/v) KH_2PO_4, 0.2% (w/v) K_2HPO_4, 0.01% (w/v) $MgSO_4.7H_2O$, 0.01% (w/v) $CaCl_2.2H_2O$, 0.5% (w/v) NaCl, 0.1% (w/v) yeast extract, 0.5% (w/v) casein and 0.5% (w/v) peptone (Tran and Nagano, 2002), with modifications. After the preparation, the medium pH was adjusted to 6.0 and autoclaved at 121°C for 15 min. This medium was called standard liquid medium.

One milliliter of a mycelium suspension was inoculated in the Erlenmeyer flasks containing the standard liquid medium, and this solution was incubated for a period of 24 to 168 h at 30°C (except for the temperature variation study) in a shaker with a rotation of 120 rpm; at each 24 h time point, a flask of each experiment variable was removed. After each bioprocess, the material was filtered with Whatman No 1 filter paper. The filtrate was denominated as crude enzyme extract (CEE), and triplicate enzyme assays were performed for each bioprocess study.

Parameters variation in the SmB

Studies were performed to analyze the effects of bioprocess time, the organic nitrogen (casein) and carbon sources, source concentration, pH of culture medium and incubation temperature.

To evaluate of the effect of the nitrogen source on protease production, the standard liquid medium was supplemented with casein in the following concentrations: 0.25% (w/v), 0.5% (w/v) or 1.0% (w/v). To analyze the effect of the carbon source, the standard medium was supplemented with several carbon sources (glucose, fructose or sucrose) at several percentages (0.1% (w/v), 0.5% (w/v) or 1% (w/v)). To analyze the effect of pH, the pH of the standard liquid medium was adjusted to 5.0, 6.0, 7.0 or 8.0 with NaOH or HCl solutions.

To analyze the effect of temperature, the liquid medium was prepared with the best concentration of casein and pH medium and these culture media were incubated at temperatures of 30, 35, 40 and 45°C.

To determine the optimum time of bioprocess, the standard liquid medium was used with the best conditions of casein concentration, initial pH and temperature.

Solid state bioprocess (SSB)

Protease production by fungus *Eupenicillium javanicum* was eva-luated in 250 mL Erlenmeyer flasks containing 5 g of solid medium added with 9.0 mL of saline solution, and then the media were sterilized by autoclaving at 121°C for 40 min. To solid medium was added one milliliter of the inoculum in saline solution prepared with 0.1% (w/v) of $(NH_4)_2SO_4$, NH_4NO_3 and $MgSO_4x7H_2O$ (Merheb et al., 2007) and incubated in a chamber at 30°C. Every 24 h (over a total of 168 h), a flask of each experiment was removed, and 40 mL of distilled water at 4°C was added to each flask for enzymes solubi-lization. The latter process was aided by maceration with a plastic rod, and then the flasks were agitated in a shaker at 200 rpm for 30 min at 4°C. The material was filtered and centrifuged at 5000 x*g* for 20 min at 4°C. The supernatant thus obtained was called crude enzyme extract (CEE), and it was assessed for proteolytic activity. Initially, to determine the standard solid medium the following agro-industrial residues were evaluated: wheat bran (WB) and cotton-seed meal (CM) in the proportions WB 100%, CM 100% or WB 50% + CM 50%. The WB 100% was assumed as standard solid medium because it obtained the best protease production.

Parameters variation in the SSB

Using this standard medium, organic nitrogen source (casein and albumin), temperature and bioprocess time were evaluated for their influence on the production of proteases during solid state bioprocess. The effect of exogenous nitrogen sources was shown using casein and albumin, in concentrations of 5, 10 and 20% (w/w) in relation to WB and the final mass of the solids was equal to 5 g. The influence of temperature was evaluated using the medium that had the best enzymatic activity when incubated at 30, 35, 40 and 45°C.

The optimum time of bioprocess was determined with the solid medium (100%WB) under the best conditions of albumin as nitrogen source concentration and temperature.

Evaluation of proteolytic activity with a casein substrate

The protease production profile was evaluated in the SmB or SSB with casein substrate using the protocol described by others (Sarath et al., 1996), with some modifications. We used 1 mL of 1% (w/v) casein prepared in 50 mM monobasic sodium phosphate buffer pH 6.5, 100 µl of 50 mM monobasic sodium phosphate buffer pH 6.5 and 100 µl of the solution containing the enzyme (CEE). The reaction mixture was incubated for 60 min at 40°C. Upon completing the reaction time, 600 µl of 10% (w/v) trichloroacetic acid (TCA) was added to stop the reaction. The blank tube was prepared with denatured enzyme and in same conditions of reaction of test tube. After the enzymatic reaction, test tubes and blanks were centrifuged at 10000 x *g* at 30°C for 10 min. The supernatants of the test tubes were measured against their blanks in cuvettes at

280 nm in a Genesys 10S spectrophotometer (Thermo).

One unit of activity was defined as the amount of the enzyme required to cause an increase of 0.001 $A_{280\ nm}$ within the reaction conditions using casein as a substrate (Gupta et al., 2002).

Fractionation and concentration by ethanol

The partial characterization assays was performed with crude enzymatic extracts of the SmB and SSB concentrated and partially purified by precipitation with ethanol, in a ratio of 1 (enzymatic extract):2 (ethanol). The material was precipitated overnight at -20°C. The precipitate was centrifuged at 5000 x*g* for 20 min at 4°C for sedimentation and then the supernatant was discarded. The precipitate was stored at -20°C. The precipitate was dissolved in distilled water at 4°C for biochemical characterization and was called the partially pure extract (PPE).

Evaluation of proteolytic activity with the azocasein substrate

We added 100 µl of PPE, 100 µl of 50 mM adequate buffer and 200 µl of 1% (w/v) azocasein prepared in the same buffer. The reaction mixture was incubated for 60 min for SmB and 15 min for SSB at 40°C, and the reaction was stopped with 800 µl of 10% (w/v) TCA. The blank tube was prepared with denatured enzyme and in same conditions of reaction of test tube.

Test and blanks tubes were centrifuged at 10000 x*g* at 30°C for 15 min. A volume of 466.5 µl of the supernatant was separated and added 400 µl of 1 M NaOH, and this mixture was measured against the respective control using cuvettes at 440 nm in a Genesys 10S spectrophotometer (Thermo) (Ducros et al., 2009). All the enzymatic reaction were performed in triplicate.

One unit of activity was defined as the amount of the enzyme required to cause an increase of 0.001 $A_{440\ nm}$ within the reaction conditions using azocasein as a substrate (Morita et al., 1998).

Characterization of the partially pure extract: Effect of pH, temperature and stability

The optimum pH was determined by evaluating the activity of the PPE at various pH levels, from 4.5 to 10.5 with increments of 0.5 pH units. The buffers used were acetate (pH 4.5 - 5.0), MES (pH 5.5 - 6.5), HEPES (pH 7.0 - 8.0), BICINE (pH 8.5 - 9.0) and CAPS (pH 9.5 - 10.5), all at 50 mM. The enzymatic reaction was performed with different pH buffers at 40°C. The pH condition that proportioned the best enzymatic activity was called optimum pH.

The optimum temperature was determined by assessing various temperatures that ranged from 30 to 80°C, with 5°C increments. The enzymatic reaction was performed with optimum pH at different temperatures. The temperature condition that proportioned the best enzymatic activity was called optimum temperature.

The pH stability was assessed by incubating PPE for 60 min at 25°C at various pH levels over a range of values (4.0 to 10.5) with increments of 0.5 pH units. After the previous exposition to different pH the enzymatic reaction was performed with optimum pH buffer at 40°C.

The thermal stability was evaluated by incubating the PPE at temperatures of 30, 35, 40, 45, 50, 55 or 60°C for 5, 15, 30 or 60 min. The enzymatic reaction was performed with optimum pH and temperatures.

All the enzymatic reactions were performed in triplicate as described in evaluation of proteolytic activity with the azocasein substrate.

Characterization of the partially pure extract: Effect of ions and inhibitors on the enzymatic activity

The effect of ions and inhibitors on the enzymatic activity in each reaction was determined, with the addition of ion and inhibitor solutions in tubes containing the enzymatic extract (SmB or SSB) to obtain a final concentration of 10 mM. The ions tested were NaCl, $ZnSO_4$, $CoCl_2$, $CuCl_2$, $CaCl_2$, $MgCl_2$, $BaCl_2$ and $AlCl_3$ all at a concentration of 100 mM (stock). The inhibitors tested were phenylmethylsulfonyl fluoride (PMSF), ethylenediaminetetraacetic acid (EDTA) and iodoacetic acid (IAA), all at a concentration of 100 mM (stock), according to the protocol previously described (Dunn, 1989), with modifications. The tubes containing ions or inhibitors were previously incubated for 5 min at 4°C. The control tube was prepared without addition of ions or inhibitors and proteolytic relative activity was assuming as 100%. The enzymatic reactions were performed in triplicate as described in evaluation of proteolytic activity with the azocasein substrate.

Evaluation of collagenolytic activity with substrate azocoll

One milliliter of PPE was added to a suspension of 1.5 g/l of azocoll in 1 mL of MES buffer (50 mM, pH 5.5) and incubated in a shaking water bath at 40°C for 4.5 h. Samples from the reaction mixtures were transferred to tubes and centrifuged at 10000 x*g* for 10 min at 25°C. The supernatant was measured against its blank at 550 nm in a Genesys 10S spectrophotometer (Thermo). Blanks were prepared in a similar manner as the reaction; however, prior to mixing, the PPE was boiled for 10 min as previously described (Foroughi et al., 2006), with modifications.

RESULTS

Protease production profile by SmB using casein as nitrogen source

The analysis of nitrogen source effects in SmB was performed using casein. The fungus *E. javanicum* showed that the presence of 0.25% (w/v) of casein was adequate to promote protease synthesis and in this condition the peak production was 32.8 U/mL at 144 h. The increase in the casein concentration repressed protease secretion and detection (Figure 1a).

Protease production profile by SmB using different carbon sources

The carbon source effects in SmB were evaluated with glucose, fructose and sucrose. The results shown in Table 1 represent the peak production obtained with each carbon source and their respective concentrations. In the assessment of carbon source supplementation, we observed that the protease production by *E. javanicum* increased 37% only when the medium was supplemented with 0.5% (w/v) glucose at 144 h (Table 1). This higher level of protease production, as compared to medium without supplementation, suggests a dependence on the glucose for the production of proteases.

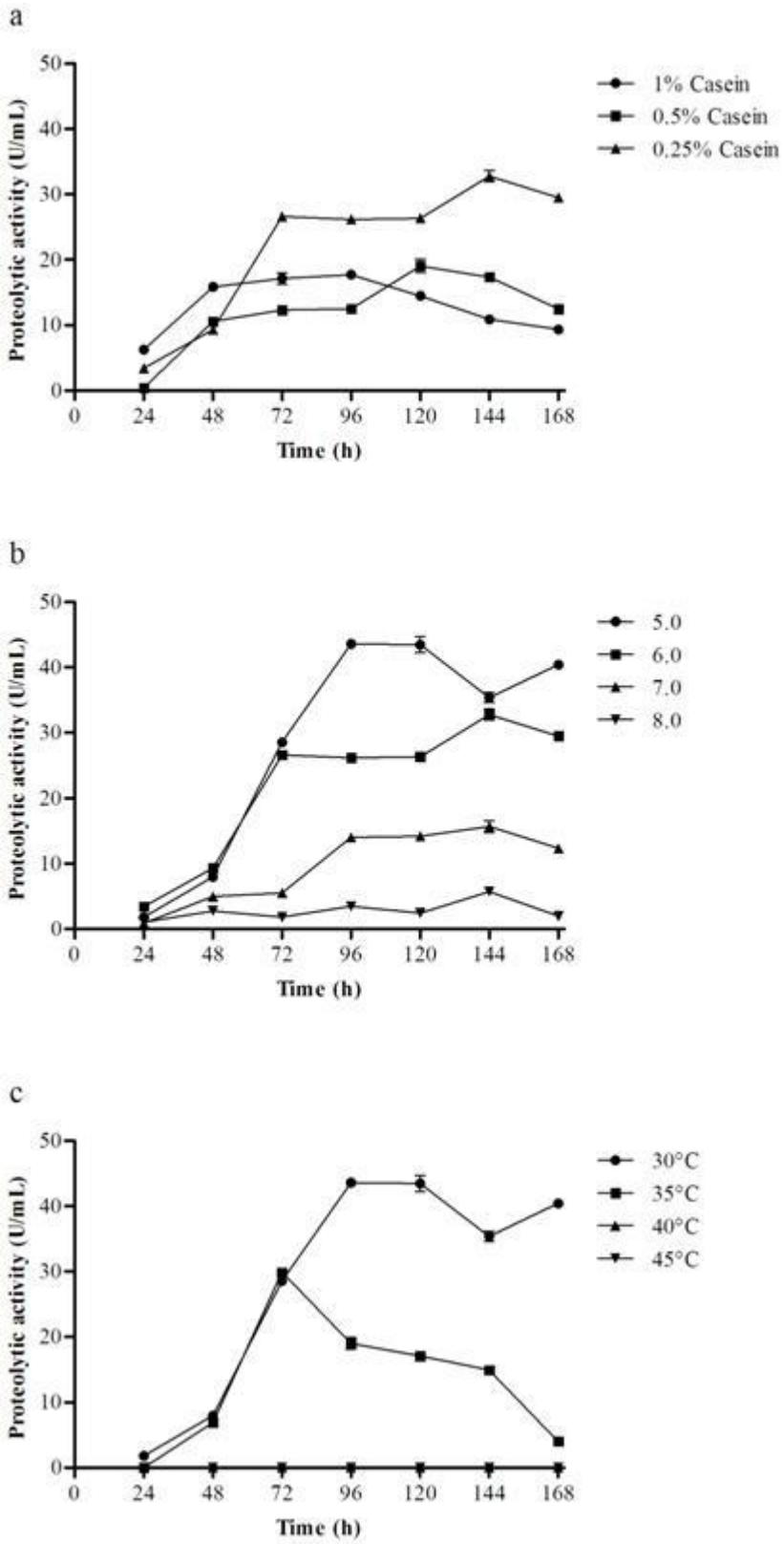

Figure 1. Effect of parameters variation on protease synthesis by the fungus *E. javanicum* under SmB. (a) Casein concentration at 30°C and 120 rpm. (b) Initial medium pH at 30°C and 120 rpm. (c) Temperature variations pH 5.0 and 120 rpm. Values shown are the averages (mean ± SD) of the triplicate samples from one representative experiment. SmB: submerged bioprocess.

Table 1. The effect of different concentrations of carbon source on protease synthesis by *E. javanicum* under submerged bioprocess at 30°C and 120 rpm.

Carbon source	Carbon source concentration (%)	Proteolytic activity (U/mL)
Control	-	19.05 ± 1.00
Fructose	0.1	15.86 ± 0.97
Fructose	0.5	18.35 ± 1.27
Fructose	1.0	18.35 ± 1.36
Glucose	0.1	16.39 ± 1.76
Glucose	0.5	26.28 ± 1.84
Glucose	1.0	16.18 ± 1.00
Sucrose	0.1	18.25 ± 0.88
Sucrose	0.5	10.32 ± 0.28
Sucrose	1.0	12.00 ± 0.18

The proteolytic activity was determined using casein 1% as substrate. The control sample represents medium without supplementation carbon sources. The values shown are the averages (mean ± SD) of the triplicate samples from peak production of each experiment.

Protease production profile by SmB in different initial medium pH

The effect of pH on protease production is related to the amount of available protons in the bioprocess medium that influence the transport of nutrients and enzyme reactions. In the assessment of the effect of pH on protease synthesis by the fungus *E. javanicum* in SmB, the highest production was observed at pH 5.0 (Figure 1b). The increasing pH levels proportioned the decrease in protease detection.

Protease production profile by SmB in different temperature of incubation

The temperature can influence thermal stability and fungal metabolism of the enzyme produced. In the analysis of the temperature effects, the best temperature for protease production was 30°C, since the fermentation process at 40 and 45°C was unsuccessful (Figure 1c). The temperature assessment revealed that an increase in temperature decreased protease secretion by *E. javanicum* in SmB.

The choice of wheat bran as agro-industrial residue to protease production by SSB

Agro-industrial residues are interesting resources to enzymes production in SSB once these substrates are inexpensive. Protease synthesis by the fungus *E. javanicum* in SSB was analyzed using the agro-industrial residues wheat bran (WB) and cottonseed meal (CM) and different proportions (100% WB, 100% CM and 50% WB + 50%CM). The results showed higher levels of pro-tease synthesis when 100%WB was used as the solid medium in SSB (Figure 2a). The condition 100% WB was assumed as standard solid medium.

Protease production profile by SSB using different nitrogen source

After determining the best agro-industrial residue, WB was supplemented with a nitrogen source trying to improve protease synthesis. The additional nitrogen sources were casein and albumin. The analysis of casein influence on protease synthesis in SSB by *E. javanicum* showed that 10% casein concentration proportioned similar profile production as those without a nitrogen source supplementation, 94 and 99 U/mL at 96 h, respectively (Figure 2b). The condition 80% WB+20% casein decreased protease synthesis. In the analysis of albumin influence on protease production in SSB by *E. javanicum* showed that high albumin concentrations improved production. In the conditions with 10 (w/w) and 20% (w/w) albumin increased protease secretion about 10% as compared to 100% WB, and the peak production was 110 U/mL at 72 h (Figure 3a).

The different nitrogen source (casein and albumin) proportioned different effects on proteases production. The casein supplementation had no effect, although 90% WB + 10% albumin had proportional increase in production and was selected further studies.

Protease production profile by SSB in different temperature of incubation

The temperature influences the fungal metabolism and other parameters of the culture. The optimal temperature for protease synthesis was 30°C, however the fermentation process at 40 and 45°C was unsuccessful different

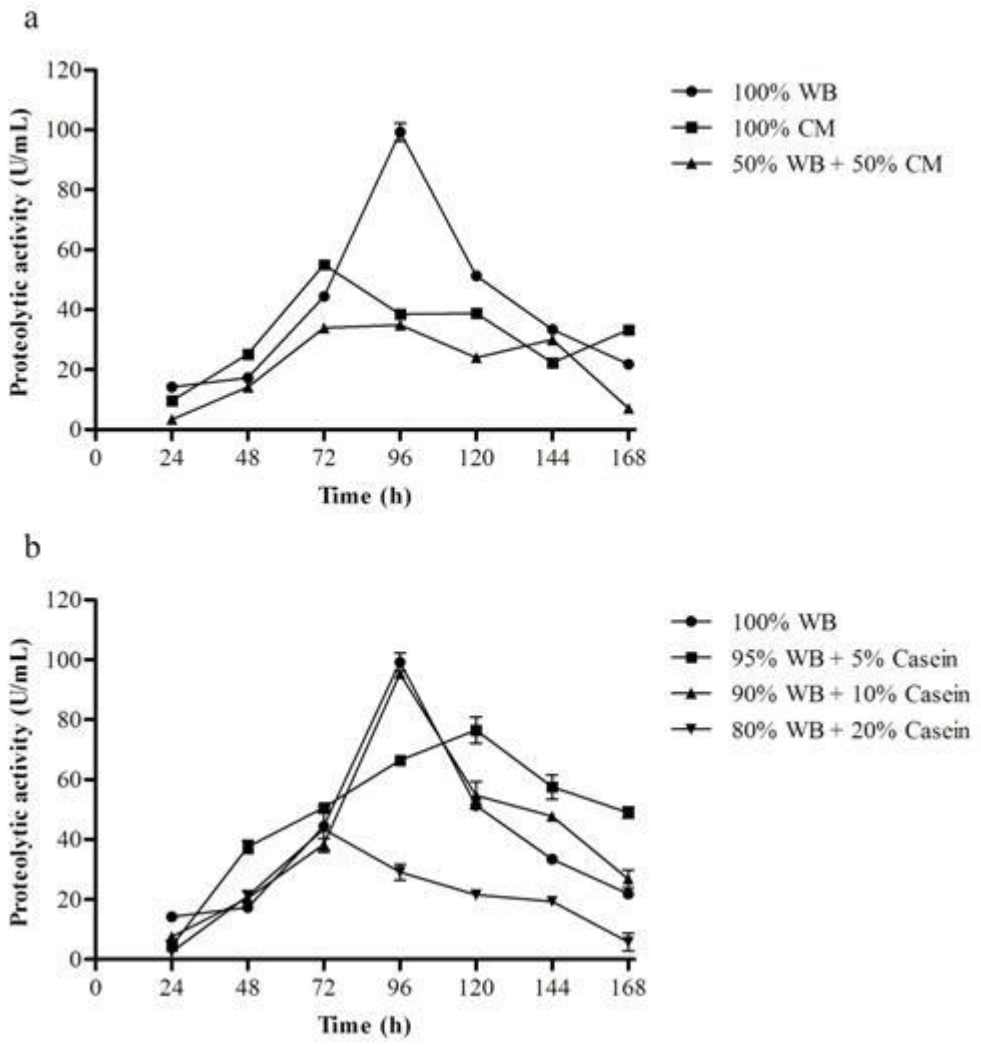

Figure 2. Effect of parameters variation on protease synthesis by the fungus *E. javanicum* under SSB. (a) Effect of agro-industrial residues at 30°C. (b) Nitrogen sources: casein concentration at 30°C. Values shown are the averages (mean ± SD) of the triplicate samples from one representative experiment. SSB: solid state bioprocess, WB: wheat bran, CM: cottonseed meal.

temperatures on production showed that increasing the bioprocess temperature decreased protease synthe-sis by the fungus *E. javanicum* in SSB.

Optimal conditions to activity of the protease produced by *E. javanicum* in SmB and SSB

Initially, we determined the pH in the one that obtained the best levels of protease activity. In both bioprocesses, the optimum pH was 5.5 (Figure 4a and b), however the protease produced in SmB showed other smaller peaks (at pH 8.0 and 10.0) (Figure 4a), indicating the possible presence of two other proteases produced by SmB. After determining the optimum pH, the enzymatic extract was submitted to various temperatures and the effect of this parameter was evaluated based on proteolytic activity. The results demonstrated that the optimal temperature was 60°C in both bioprocesses (Figure 5a and b).

Ions effects and subclass determination of proteases produced in SmB and SSB using inhibitors assay

The inhibitors assay is used to determine the subclass of proteases that were produced, according to the mechanism of catalytic action. This enzymatic test revealed that enzymes produced under both fermentative processes are inhibited by EDTA (Figure 6a), suggesting that the proteases of both bioprocesses belong to the metalloprotease subclass.

The ions can influence the proteolytic activity by interaction with amino acids residues in protease structure. In SmB the presence of $CoCl_2$ and $ZnSO_4$ increased 87 and

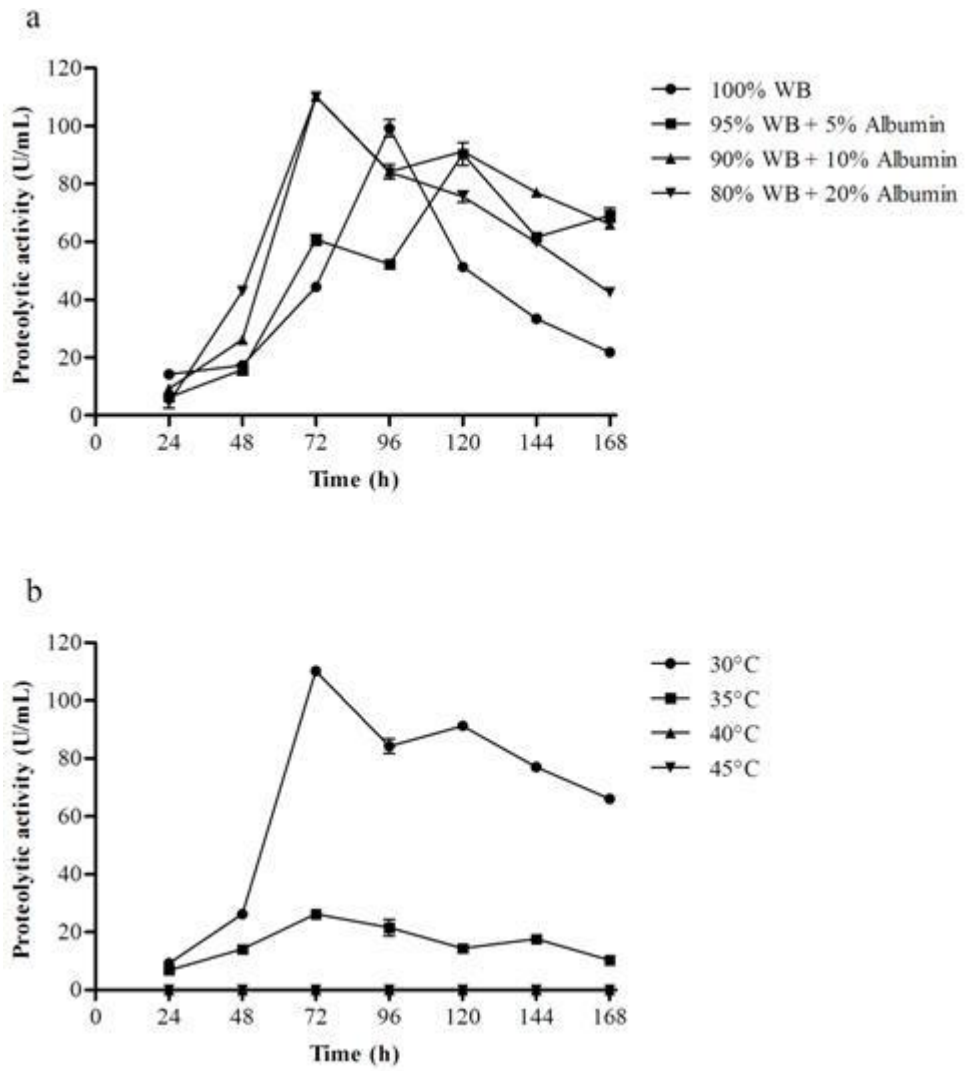

Figure 3. Effect of parameters variation on protease synthesis by the fungus *E. javanicum* under SSB. (a) Nitrogen sources: albumin concentration at 30°C. (b) Temperature variations (10% albumin+90%WB). Values shown are the averages (mean ± SD) of the triplicate samples from one representative experiment. SSB: solid state bioprocess, WB: wheat bran.

12% of the enzyme activity of proteases, respectively. The influence of ions on protease produced in SSB was more evident and different divalent metallic cations presented positive effects such as Co^{+2}, Mg^{+2}, Zn^{+2}, Ca^{+2} and Ba^{+2} or trivalent metallic ions Al^{3+}, however Cu^{+2} de-monstrated negative effect. The highest activities in SSB were obtained in the presence of $CoCl_2$ and $ZnSO_4$ that show an increase of 40 and 46% as compared to control sample (Figure 6b). The enzyme assay with the addition of various ions showed that ions can have positive or negative effects on proteolysis.

pH and temperature stability of the proteases produced by *E. javanicum* in SmB and SSB

The recognition of pH and thermal stabilities can guide the potential application of the enzymes. The exposition to various pH levels revealed that the proteases produced in SmB and SSB provides greater stability at acidic pH levels (Figure 7a and b). In the Figure 7a it is observed that the residual activities in all pH levels remained above 80%, although had shown a slight drop when the protease produced in SmB was exposed to a pH level above 4.5. Similarly, in the Figure 7b, the residual activities in all pH levels remained above 70% and the exposition to pH levels above 5.5 for protease produced under SSB also had showed a slight drop. The pH stability results indicate that proteases can be used or stored at several pH levels.

The exposition to different temperatures and times provides the recognition of temperature stability of the proteases produced in SmB and SSB. The proteases produced under SmB retained 40% of their activity when exposed at 55°C for 30 min and 35% when exposed to

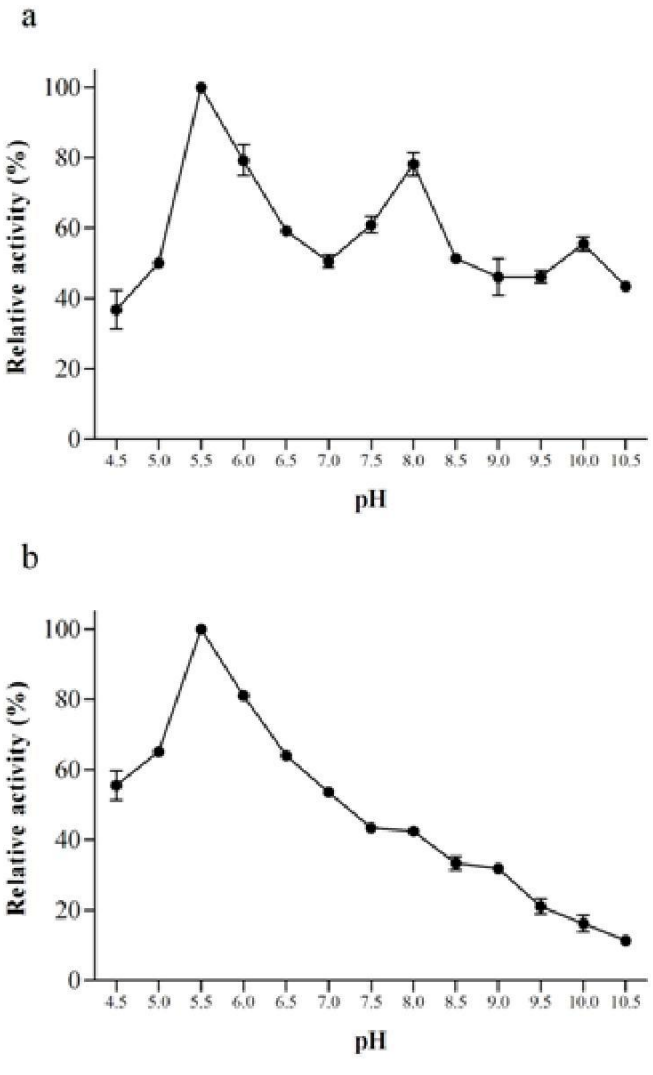

Figure 4. Biochemical characterization of enzyme extract precipitated. Effect of pH on the proteolytic activity of proteases at 40°C: (a) SmB and (b) SSB. Values shown are the averages (mean ± SD) of the triplicate samples from one representative experiment. SSB: solid state bioprocess, SmB: submerged bioprocess.

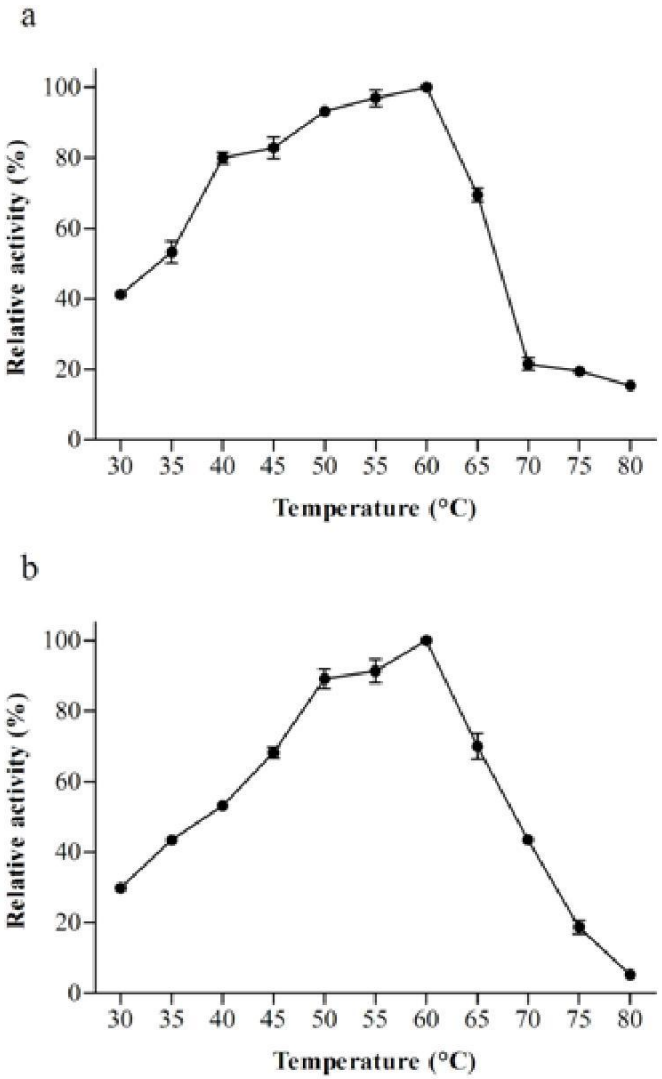

Figure 5. Biochemical characterization of enzyme extract pre-cipitated. Effect of temperature on proteolytic activity of proteases at pH 5.5: (a) SmB and (b) SSB. Values shown are the averages (mean ± SD) of the triplicate samples from one representative expe-riment. SSB: solid state bioprocess, SmB: submerged bioprocess.

60°C for 15 min (Figure 8a). The proteases produced under SSB maintained 40 and 35% of their activity when exposed at 55°C for 30 and 60 min, respectively (Figure 8b). The activity of proteases produced under both bio-processes maintained about 50% of initial activity for 60 min of exposition at 50°C.

Collagenolitic potential of proteases produced in SmB and SSB

The potential of proteases from both SmB and SSB to collagen degradation was shown using Azocoll substrate. The results were observed by hydrolysis of Azocoll, SSB and SmB obtained with A_{550} = 0.095 and A_{550} = 0.139 abs, respectively. The protease produced by SmB demonstrated more activity than SSB.

DISCUSSION

Here we found that *Eupenicillium javanicum* was able to produce proteases in both solid state and submerged bio-processes, although the SSB protease production was twice higher than SmB and this production can be opti-mized by parameters studies.

Furthermore, we showed that the proteases produced had collagenolytic potential. The optimal rate of meta-bolite production of a fungus is obtained through detailed knowledge of its growth and physiological conditions

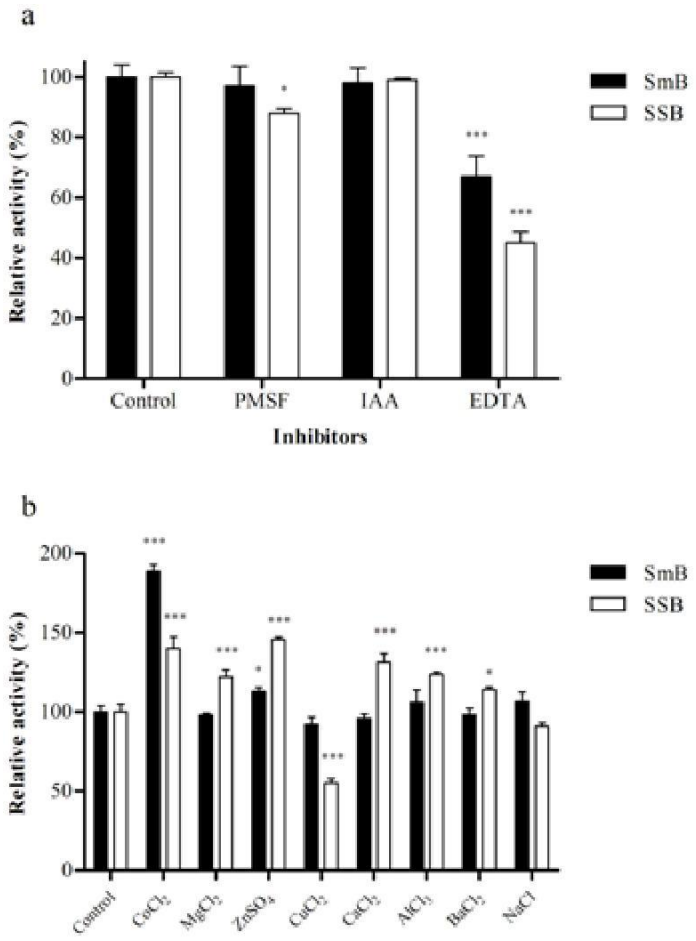

Figure 6. Effect of the addition of metallic ions and inhibitors on proteolytic activity of proteases produced by the fungus *E. javanicum* under SmB and SSB. (a) Metallic ions at 40°C and pH 5.5. (b) Inhibitors at 40°C and pH 5.5. Values shown are the averages (mean ± SD) of the triplicate samples from one representative experiment. Significance level: *p<0.05, ***p<0.001. SSB: solid state bioprocess, SmB: submerged bioprocess, PMSF: phenylmethylsulfonyl fluoride, IAA: iodoacetic acid, EDTA: ethylenediaminetetraacetic acid.

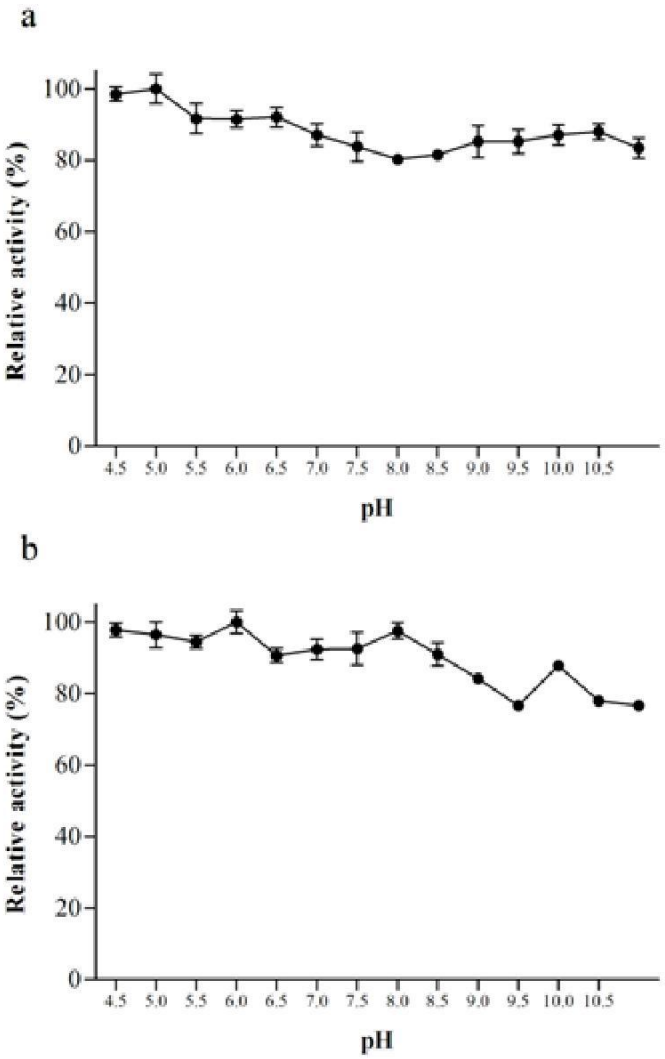

Figure 7. Stability of the proteases produced by the fungus *E. javanicum*. Exposition to various pH levels at 40°C: (a) SmB and (b) SSB. Values shown are the averages (mean ± SD) of the triplicate samples from one representative experiment. SSB: solid state bioprocess, SmB: submerged bioprocess.

(Papagianni, 2004).

The production of proteases can be induced with or without the addition of supplementary protein sources. In analysis of protease produced by SmB, the fungus *Eupenicillium javanicum* showed a repression of protease synthesis with casein concentration above 0.25%. However, other study demonstrated that the addition of 0.5% casein has been shown to increase protease production by the fungus *Mucor mucedo* DSM 809 (Yegin et al., 2010).

We showed that 0.5% glucose increased 37% of protease production by *E. javanicum* in SmB demonstrating the importance of this carbon source. However, the addition of a carbon source can cause catabolic repression, as was observed in the presence of sucrose, because increasing the percentage of sucrose decreased the production of proteases. Similarly, it has been shown that greater acid protease production by *Aspergillus awamori* MTCC 548 was observed when the medium was supplemented with glucose (Sinha and Sinha, 2009), but this situation was not observed in the protease production by the fungus *Botrytis cinerea*, once the addition of glucose to submerged media promotes a decrease in protease production (Abidi et al., 2008). Sinha and Sinha (2009) also showed that higher glucose concentrations decreased the protease production and other carbon sources did not increase production.

The study of effect of pH is important because the pH may influence any activities, yet this topic is rarely addressed (Papagianni, 2004). *E. javanicum* responded to an increase in pH levels with lower protease production, indicating that the absence of protons in the bioprocess

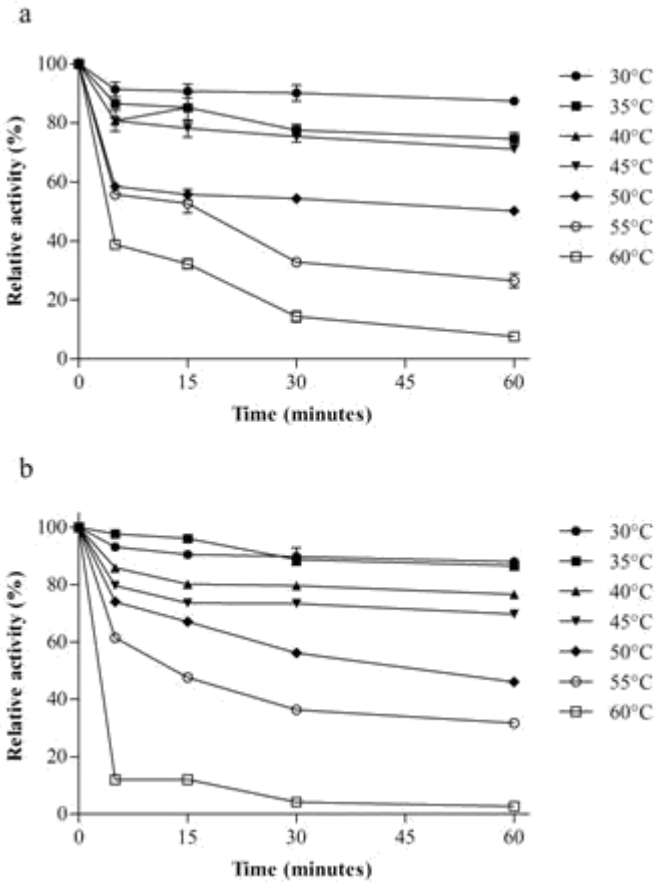

Figure 8. Stability of the proteases produced by the fungus *E. javanicum*. Exposition to various temperatures and times at pH 5.5: (a) SmB and (b) SSB. Values shown are the averages (mean ± SD) of the triplicate samples from one representative expe-riment. SSB: solid state bioprocess, SmB: submerged bioprocess.

media decreases the protease production. Likewise, the fungus *Aspergillus oryzea* has shown a lower produc-tion of proteases with increasing pH (Sandhya et al., 2005), confirming that pH has large effect on the pro-duction of proteases. The assay of the temperature effect on the protease production revealed that *E. javanicum* did not respond positively to increases in temperature. The results found during the optimization of protease production by *Bacillus* sp. I-312 also showed that increasing incubation temperature can cause a reduction in the protease production (Joo and Chang, 2005) and indicated the importance of temperature control in bio-processes.

The choice of agro-industrial residues in bioprocesses is very important. In SSB, the analysis of agro-industrial residues wheat bran (WB) and cottonseed meal (CM) showed better protease production when 100% of WB is used as a bioprocess medium. Sandhya et al. (2005) related that although many agro-industrial residues have been tested, the wheat bran showed the best results.

Other authors suggest that residues with higher amounts of protein in their composition (Madruga and Camara, 2000) as well as the porosity of the residue facilitate the mycelial dispersion under SSB (Sandhya et al., 2005; Pandey, 2004) and can improve the protease production.

Supplementation of the medium with a protein source can positively or negatively influence the production of proteases. In SSB, the addition of 10 and 20% of albumin promoted an improvement in the production of proteases, but the addition of 10% of casein showed no effect when compared with medium without additional nitrogen source. Casein concentrations above 10% demonstrated nega-tive effects on protease synthesis. These data show the importance of choosing the ideal protein source.

The effect of temperature in solid state bioprocess pro-cess was similar to submerged bioprocess process, the temperature increase decreased proteases production, and at temperatures above 35°C, there was no apparent fungus growth. According to other authors, high tempera-tures can negatively influence the metabolic activities of microorganisms and can inhibit the growth of fungus, in general, fungal proteases are thermolabile and show lower activities at higher temperatures (Haq et al., 2006).

The protease production under SSB (90% wheat bran + 10% albumin) at 30°C was twice higher than SmB (glu-cose) at 30°C; however this difference can be more ex-pressive. Zanphorlin et al. (2010) demonstrated that the pro-duction of proteases by thermophilic *Myceliophtora* sp. by solid state using wheat bran and casein was 4.5 times higher than submerged fermentation (Zanphorlin et al., 2010). Similar result was demonstrated by Silva et al. (2013) (accepted for publication), in this study the fungus *A. fumigatus* presented protease production 30 times higher under solid state fermentation than submerged fermentation.

In the current study, we determined the effect of pH and temperature on activity and stability, and the effect of metal ions and inhibitors. The reaction parameters are essential for the catalytic production of proteases from two bioprocesses. The effect of pH levels on activity show that pH 5.5 provide highest activities for proteases produced under both bioprocesses, suggesting that these has acidic characteristics. Additionally, in the SmB pro-cess, there are peaks at pH 8.0 and 10.0, which indicate the potential presence of another protease with alkaline characteristics. The effect of temperature was similar for proteases produced under both bioprocesses.

The results obtained in SSB and SmB using *E. javanicum* suggest that proteases produced have optimal temperature of 60°C and at temperatures above 60°C, the activity decreased. In solid state bioprocess, *Rhizopus oryzae* has been shown to produce acidic proteases with same optimal temperature and pH (Kumar et al., 2005), and similar results also have been seen with the fungus *Thermoascus aurantiancus* (Merheb et al., 2007). The optimal conditions of pH and temperature were similar to proteases produced in SmB and SSB.

The EDTA provides an inhibition of approximately 33

and 55% on proteolytic activity of proteases produced by *E. javanicum* in SmB and SSB, respectively. These results suggest metalloproteases. Despite this enzyme subclass shows optimal pH at near neutrality, some acidic metalloproteases have been described in other studies, including the fungi *T. aurantiancus* (Merheb-Dini et al., 2009) and *Streptomyces septatus* TH-2 (Hatanaka et al., 2005).

Metal ions can modulate the proteolytic activity positively or negatively. The addition of 10 mM cobalt chloride or zinc sulfate increased the enzyme activity of proteases produced under both bioprocesses. According to other authors, metalloproteases need divalent ions to improve their activity levels (Rao et al., 1998). However, other study related the opposite comportment, 1 mM of divalent metallic ions cobalt and zinc were responsible for 100% inhibition of a metalloprotease with collagenolytic activity secreted by *A. fumigatus*. Furthermore, similar to our results in SSB, the presence of copper chloride resulted in a loss of metalloprotease activity (Monod et al., 1993).

The protease produced under SmB and SSB presented greater stability at acidic pH levels which is optimal pH. A pH closer to the alkaline level tends to decrease the stability, and this trend was greater for proteases produced under SSB than under SmB. Similarly, the acidic proteases produced by *A. niger* I1 had greater stability near the optimal pH level, and stability decreased when the pH was shifted away from the optimal level (Siala et al., 2009); however, this drop was larger than that presented in this study.

The protease stability results in relation to temperature as a function of time revealed that the enzymes produced under SSB, when exposed to 55°C for 30 min, retained 40% of their activity. The enzymes produced under SmB were more stable with temperature changes, maintaining approximately 40% of their activity after 15 min of exposure to 60°C. Here, the proteases produced in SmB and SSB maintained about 60 and 50% activity after 30 and 60 min of exposition at 50°C, respectively. Shivakumar (2012) considered the thermostability at 50°C for 30 min of the protease produced by *Aspergillus* sp. with potential for food industries. In another study, the acidic proteases produced by *A. niger* I1 maintained approximately 30% of their activity following exposure to 50°C for 15 min (Siala et al., 2009).

In this study, the metalloproteases produced in SmB and SSB by *E. javanicum* were able to degrade Azocoll substrate. Others authors related collagenolytic metalloprotease with similar characteristics secreted by *A. fumigatus* (Monod et al., 1993). Another study measured the activity of several commercially available enzymes, after 24 h of reaction with an azocoll substrate finding values that ranged from 0.3 to 0.92 abs (Foroughi et al., 2006).

In the current study, after 4.5 h, we found value of 0.139 and 0.095 from metalloproteases produced by *E. javanicum* under SmB and SSB, respectively. The exposure time of the enzyme with a substrate was approximately 5.5 times lower than the previously mentioned study, showing the collagenolytic potential of these specific metalloprotease.

We found that the metalloprotease profile production can be explored to obtain proteases with different applications. Here, the metalloproteases produced by fungus *E. javanicum* showed potential to collagenolytic activity for proteases produced under both submerged and solid state bioprocess, and this type of enzyme can have potential uses in industry.

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