



Degradation of feather waste by *Aspergillus niger* keratinases: Comparison of submerged and solid-state fermentation



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ABSTRACT

The isolation of native and/or the production of genetically modified enzyme-producing microorganisms may have substantial impacts on industrial processes. In this work twenty-eight *Aspergillus niger* mutants were screened for peptidases and keratinases production on a basal medium containing chicken feathers (1%). Four strains were selected after preliminary assays: 3T5B8, 9D40, 9D80, and 11D40. The keratinase production was higher when the *A. niger* strains were cultivated in a solid-state condition rather than a submerged condition: the keratinolytic activity of 3T5B8 strain was 7 times greater when cultivated by solid-state fermentation (SSF). *A. niger* 3T5B8 had the highest keratinase activity (172.7 U/ml) after seven days at pH 5.0 from solid-state fermentation, whereas the lowest activity was given by *A. niger* 9D40 after four days (21.3 U/ml) from submerged fermentation. Zymography of culture supernatant showed multiple bands migrating at 40–130, 14–130, displaying activity towards keratin and gelatin substrates, respectively. This is the first study to report production of high molecular mass peptidases using a feather-degrading *Aspergillus*. Peptidases from strains 3T5B8, 9D40, 9D80, and 11D40 were inhibited by PMSF, except the approximately 40-kDa peptidase, which was inhibited by phenanthroline, indicating the presence of serine and metallopeptidases. The results therefore suggest that the isolates are promising keratinase producers for biotechnological purposes.

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1. Introduction

Microorganisms harbor many different enzymes that are used in various industrial applications (Mitidieri et al., 2006). For instance, peptidases have been used in a wide range of applications in the fuel, pharmaceutical, brewing, food, animal feed, bioremediation, detergent, leather, paper and textile industries. In fact, the global enzyme market is expected to reach US\$ 7 billion by 2015. Thus, the isolation of native and/or the production of genetically modified enzyme-producing microorganisms may have substantial impacts

on present and future industrial processes (Yue et al., 2011; Vermelho et al., 2013).

Nowadays the most commonly used industrial enzymes belong to the hydrolase group, which exploits several natural substrates (Mitidieri et al., 2006). Keratin is an insoluble structural protein resistant to hydrolysis by common proteolytic enzymes such as trypsin, pepsin, and papain (Gupta and Ramnani, 2006). The mechanical stability of keratin and its resistance to microbial degradation are due to the tight packing of the protein chain either in α -helix (hair α -keratin) or β -sheet (feather β -keratin) structures, and their linkage by cystine bridges that have a high degree of cross-linkages by disulfide bonds, hydrogen bonding, and hydrophobic interactions (Gupta and Ramnani, 2006; Mazotto et al., 2011). However, keratin can be degraded by keratinases produced by some species of saprophytic and parasitic fungi (Gradišar et al., 2005), actinomyces (Jaouadi et al., 2010), and bacteria, especially of the genus *Bacillus* (Cedrola et al., 2012).

Because they degrade keratin, keratinolytic peptidases have a potential role in biotechnological applications such as enzymatic

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improvement of feather meal and feed additives (for instance, Versazyme; Odetallah et al., 2005), leather and detergents, and the production of amino acids or peptides from high molecular weight substrates for cosmetics (Gupta and Ramnani, 2006; Mazotto et al., 2011; Cedrola et al., 2012). Keratinases have been linked to prion degradation and investigated as active pesticide components against root-knot nematodes (Mitsuiki et al., 2006; Yue et al., 2011). In the poultry industry, feather can be converted to feedstuffs, fertilizers, and polymers after enzymatic hydrolysis or used in the production of rare amino acids such as serine, cysteine, and proline (Riffel et al., 2007).

The filamentous fungus *Aspergillus niger* is one of the most important industrial microorganisms and produces a variety of enzymes such as cellulase and xylanase (Couri et al., 2000; Farinas et al., 2010), phytases (Bhavsar et al., 2011), amylases (Mitidieri et al., 2006), and peptidases (Morya et al., 2012). Enzymes from *A. niger* have been used in food production for several decades, and even though their peptidases have been studied (Basten et al., 2003), there are few reports on keratinase production by *A. niger* strains (Lopes et al., 2011).

This study aimed to detect and evaluate the keratinase and peptidase production by mutants of *A. niger* in submerged and solid-state fermentation and also investigated their potential use to degrade feather keratin, an agro-industrial residue. Mutant strains of *A. niger* belonging to Embrapa Food Technology were selected for their ability of enzyme production and the enzymatic production was characterized. The genus *Aspergillus* has rarely been described for the production of keratinase, thus making this study important in the search for new functions of a major industrial microorganism.

2. Materials and methods

2.1. Chemicals

Gelatin was obtained from Merck (Darmstadt, Germany). Reagents used in electrophoresis and molecular mass standards were acquired from Amersham Life Science (Little Chalfont, England). Polyethyleneglycol 4000 (PEG 4000) was purchased from Vetec (Rio de Janeiro, Brazil). Peptidase inhibitors trans-epoxysuccinyl-L-leucylamido-(4-guanidino) butane [E-64], phenylmethylsulfonyl fluoride [PMSF], 1,10-phenanthroline, pepstatin A, and ethylenediaminetetraacetic acid (EDTA) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). All other reagents were of analytical grade.

2.2. Keratin substrate

Keratin derived from ground chicken feathers was used in the culture medium. Chicken feathers obtained from poultry waste were washed extensively with water and detergent, dried at 60 °C overnight, delipidated with chloroform:methanol (1:1, v/v) for 1 h and dried again at 60 °C. After drying, feathers were ball milled for use in the feather agar medium. Whole feather medium was used for submerged and solid-state cultivation.

For the enzymatic assays keratin was extracted from chicken feather based on the methodology described by Mazotto et al. (2011) for keratin powder production. Briefly, 10 g of feathers were heated with a reflux condenser at 100 °C for 80–120 min with 500 ml of DMSO. Keratin was then precipitated by the addition of two volumes of acetone and maintained at 4 °C for 24–48 h. The keratin precipitates were collected by centrifugation (2 × 2000g/15 min), washed twice with distilled water and dried at 4 °C. The white powder obtained was ground to homogeneity in a mortar. This keratin was used for keratinolytic activity assays and keratin zymography.

2.3. Selection of keratinolytic *A. niger* lineages

A. niger mutants were selected from the Embrapa Food Technology collection. These mutants were obtained by conventionally induced mutation techniques using chemical and physical agents as described by Couri and Farias (1995). The culture was kept on dry sand at –20 °C and activated by transferring spores twice to basic salt medium agar slants (g l⁻¹: 3.0 NaNO₃, 1.0 KH₂PO₄, 0.5 MgSO₄·7H₂O, 0.5 KCl, 0.01 FeSO₄·7H₂O, 20.0 agar at pH 5.0) containing either 10g l⁻¹ ball milled chicken feather or 10g l⁻¹ gelatin and incubation was performed at 32 °C for eight and six days, respectively. Then the lineages were spot plated on the media described above and incubated at 32 °C for four days. Colony diameter, sporulation, homogeneity, and the absence of sectors were determined at periodic intervals (24 h). After 96 h, Coomassie Blue solution (Coomassie Brilliant Blue R-250 2.5 g l⁻¹ in methanol – acetic acid – water 50:10:40) was added to the plates. The keratin/gelatin agar medium was destained with methanol – acetic acid – water (50:10:40) and the hydrolysis zone diameter was measured. The experiment was conducted in triplicate.

2.4. Inoculum preparation

The strains were cultivated on basic salt medium agar slants containing 10g l⁻¹ milled chicken feather or 10g l⁻¹ gelatin and incubated at 32 °C for five days. The conidia were suspended with

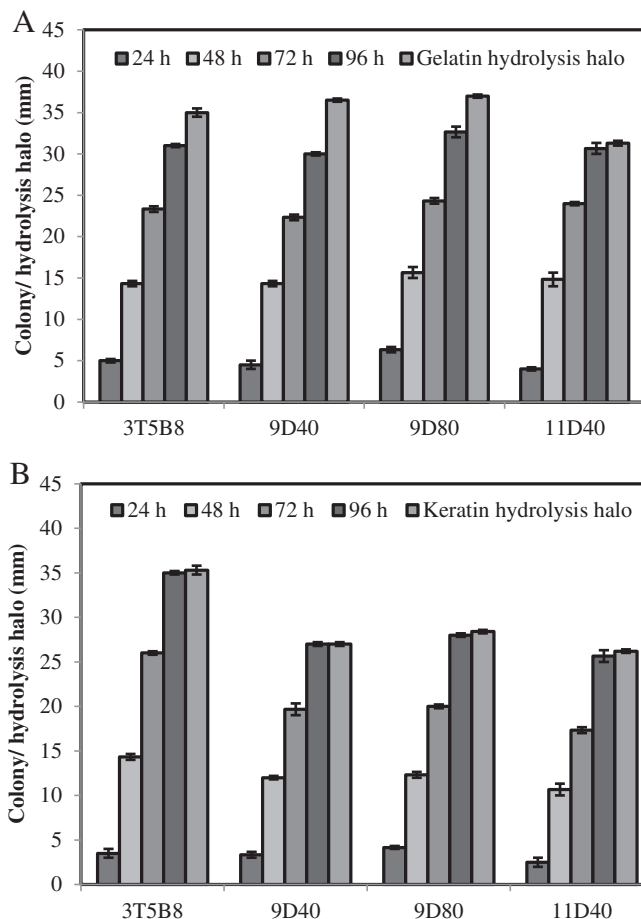


Fig. 1. Colony and hydrolysis zone diameters of different *Aspergillus niger* mutant strains on (a) gelatin agar plate or (b) keratin agar plate. Colony diameter was measured every 24 h for four days. Plates were then stained with Coomassie Brilliant Blue and hydrolysis zone was measured using a ruler.

3 ml 0.3% sterile Tween 80, and approximately 1 ml of suspension was dripped on ground corncob medium. Inoculation was carried out in 125 ml shake flasks containing 4.6 g ground corncob and 6 ml solution containing 56 g l^{-1} peptone, $0.76 \text{ g l}^{-1} \text{ K}_2\text{HPO}_4$, $36 \text{ g l}^{-1} \text{ ZnSO}_4$, $46 \text{ g l}^{-1} \text{ Na}_2\text{SO}_4$, $0.01 \text{ g l}^{-1} \text{ MnSO}_4$, and 0.5% H_2SO_4 . After incubation at 32°C for four days, 20 ml of 0.3% Tween 80 were added to the medium and the flask was stirred vigorously. Conidia were separated by filtration through gauze and spore concentration was determined using a Neubauer chamber. The concentration used for submerged and solid-state fermentation was 10^6 spores/ml.

2.5. Submerged fermentation (SmF)

The medium used for keratinase production by submerged fermentation contained the following composition (g l^{-1}): $3.5 (\text{NH}_4)_2\text{SO}_4$, $1.0 \text{ KH}_2\text{PO}_4$, $0.5 \text{ MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1 KCl , 5.10^{-3} ZnSO_4 , and 10 feathers at pH 5.0. Cultivation was performed using 125 ml Erlenmeyer flasks containing 50 ml medium for four and seven days at 32°C to check for production of enzymes at different growth phases. Subsequently, the cultures were filtered (Whatman 40 and Millipore $0.2 \mu\text{m}$) and used for enzymatic assays. The filtered culture supernatant was used in this study as a crude enzyme extract.

2.6. Solid-state fermentation (SSF)

For solid-state fermentation 0.4 g of whole chicken feather was mixed with 40 g of a wheat bran mixture in a 125 ml Erlenmeyer flask. The wheat bran contained 50 g powdered wheat bran in 30 ml of a solution of 0.9% $(\text{NH}_4)_2\text{SO}_4$ in $0.1 \text{ mol l}^{-1} \text{ HCl}$. After sterilization, the medium was inoculated with 10^6 conidia per ml of medium and incubated at 32°C for 4 and 7 days. After the fermentation periods, 100 ml of acetate buffer 200 mM (pH 4.2) was

added to each batch. The mixture was stirred for 1 h at 180 rpm and 32°C . The solid residue was separated from the crude enzymatic solution by filtration through a Whatman 40 filter paper and a Millipore $0.2 \mu\text{m}$ filter (Couri et al., 2000).

2.7. Keratinolytic assay

Keratinase activity was measured as described by Mazotto et al. (2011). The reaction mixture contained 1.0 ml of the culture supernatant diluted five times and mixed with 1.5 ml 0.67% (w/v) keratin suspension in 0.1 M phosphate buffer at pH 7.4. After 1 h incubation at 37°C the reaction was interrupted by adding 1 ml 10% trichloroacetic acid and placed in a refrigerator at 4°C for 30 min. An enzyme control was prepared by adding 1 ml trichloroacetic acid before incubation. The reaction mixture was centrifuged ($2000 \text{g}/10 \text{ min}$) and read at 280 nm in a spectrophotometer. One unit of keratinase activity was defined as the amount of enzyme required to produce an absorbance increase of 0.01 under the described assay conditions.

2.8. Protein assay

Protein content of crude enzymatic solution obtained was described in the Section 2.5 and 2.6 and was determined by the Lowry method (1951) with bovine serum albumin as a standard.

2.9. Feather keratin and gelatin zymography

For the zymography evaluation, the crude enzymatic extract were concentrated 25-fold by dialysis (cut-off 9 kDa) against PEG 4000 overnight at 4°C . Keratinases were assayed and characterized by electrophoresis on 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with

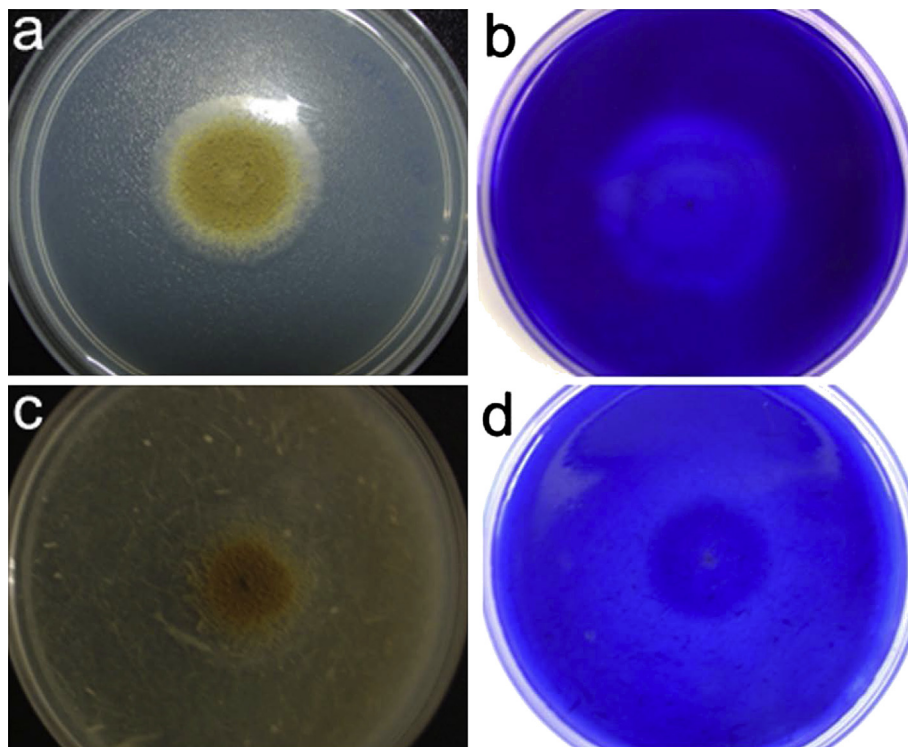


Fig. 2. Colony of *Aspergillus niger* strain 9D40 on agar plate with (a) 1% gelatin and (c) 1% ball milled feather after four days at 32°C , and respective clear zone indicating hydrolysis by *A. niger* strain 9D80 on (b) gelatin and (d) keratin substrates.

co-polymerized keratin feather powder as described by Cedrola et al. (2012). Proteolytic activity was evaluated in zymograms under the same conditions using 0.1% gelatin as substrate. Gels were loaded with 30 μl of enzyme extract in sample buffer per slot (125 mM Tris, pH 6.8, 4% SDS, 20% glycerol, 0.002% bromophenol blue) in a proportion of 9:1 (v/v). After electrophoresis at 170 V for 2 h at 4 °C gels were soaked for 1 h at 26 °C in 2.5% Triton X-100. The gels were then incubated for 48 h at 37 °C in citric acid proteolysis buffer pH 5.0 (0.05 M citric acid and 0.1 M Na_2HPO_4). Then, the gels were stained for 2 h with 0.2% Coomassie Brilliant Blue R-250 in methanol – acetic acid – water (50:10:40) and destained in the same solvent.

2.10. Determination of peptidase classes

Enzymes were classified by zymography by adding 3 mM phenylmethylsulfonyl fluoride (PMSF), 0.26 mM ethylenediaminetetraacetic acid (EDTA), 30 mM 1,10-phenanthroline (Phenan), 10 μM pepstatin A (Peps), and 5 μM trans-epoxysuccinyl-L-leucylamido-(4-guanidino) butane (E-64) to the proteolysis buffer. The absence of a hydrolysis band after staining, as described above, indicates that enzyme activity was inhibited (Mazotto et al., 2011).

2.11. Statistical analysis

The experiments were conducted in triplicate. The statistical significance of the terms in the regression equation was examined by analysis of variance (ANOVA). Significance was accepted at $p < 0.05$.

3. Results

3.1. Selection of keratinolytic and proteolytic *A. niger* mutants

All 28 mutants grew on both media (gelatin and keratin agar), but only strains 3T5B8, 9D40, 9D80, and 11D40 had larger hydrolysis zone diameters than colony diameter (Fig. 1). The largest hydrolysis zones were produced by *A. niger* 9D80 with 37 mm on gelatin agar plate and *A. niger* 3T5B8 (35 mm) on keratin agar plate. Fig. 2 shows an *A. niger* colony after incubation for four days in solid media with gelatin (Fig. 2a) and keratin (Fig. 2c) as the only carbon source. After growth, plates were stained with Coomassie Brilliant Blue for halo visualization, as shown in Fig. 2(b and d).

3.2. Enzyme production

A. niger strains 3T5B8, 9D40, 9D80, and 11D40 promoted partial feather degradation during submerged and solid-state fermentation using feathers as the only carbon source. The feathers were not completely degraded: barbules were degraded after fermentation for seven days, but rachides remained intact (data not shown). Keratinase production was higher in solid-state than in submerged condition. The keratinolytic activity of 3T5B8 strain was 7 times greater in crude enzymatic extract from solid-state fermentation than submerged fermentation in both four and seven days (Fig. 3). Strains 3T5D8 and 9D40 showed higher keratinolytic activity for the seven-day batch cultures than the four-day ones (Fig. 3), however, these differences were not significant. The keratinolytic activity of 9D80 was less in the seven-day cultures when compared with four-day culture batches. The keratinolytic activity of 11D40 showed no significant difference in the periods of fermentation evaluated. *A. niger* 3T5B8 had the highest keratinase activity (172.7 U/ml) after seven days at pH 5.0 in solid-state fermentation, whereas the lowest activity was observed for *A. niger* 9D40 after four days (21.3 U/ml) in submerged fermentation (Fig. 3a).

The concentration of soluble protein in the culture supernatant of *A. niger* strains was measured. As the substrate was feathers, which are a complex protein, a measure of the protein concentration in the supernatant can be an indirect indicator of the degradation of the substrate. The protein concentration in culture supernatant of *A. niger* 3T5B8 was 9.4 mg ml^{-1} after four days in solid-state fermentation, approximately 1.9, 3.0, and 1.6 times higher than the protein concentration in the supernatants of *A. niger* 9D40, 9D80, 11D40 under the same conditions (Fig. 3b). Corroborating with the result of keratinolytic activity, the concentrations of the proteins in extracts from solid-state fermentation were substantially higher than those from submerged fermentation.

3.3. Proteolytic activity on different substrates

Strains 3T5B8, 9D40, 9D80, and 11D40 showed enzymatic activity on gelatin and keratin substrates (Fig. 4a and b). Incubation of selected *A. niger* strains in feather medium resulted in the production of numerous peptidases. The proteolytic profiles of the strains were similar after fermentation for four and seven days. Additionally the proteolytic profiles showed few differences between submerged and solid-state fermentation. A band migrating at approximately 60 kDa was produced by all strains under all conditions. This band presented keratinolytic and gelatinolytic activity. The 60 kDa keratinase showed high activity and wide specificity, which are suitable characteristics for use as detergents. In the submerged fermentation strain 9D80 produced a band

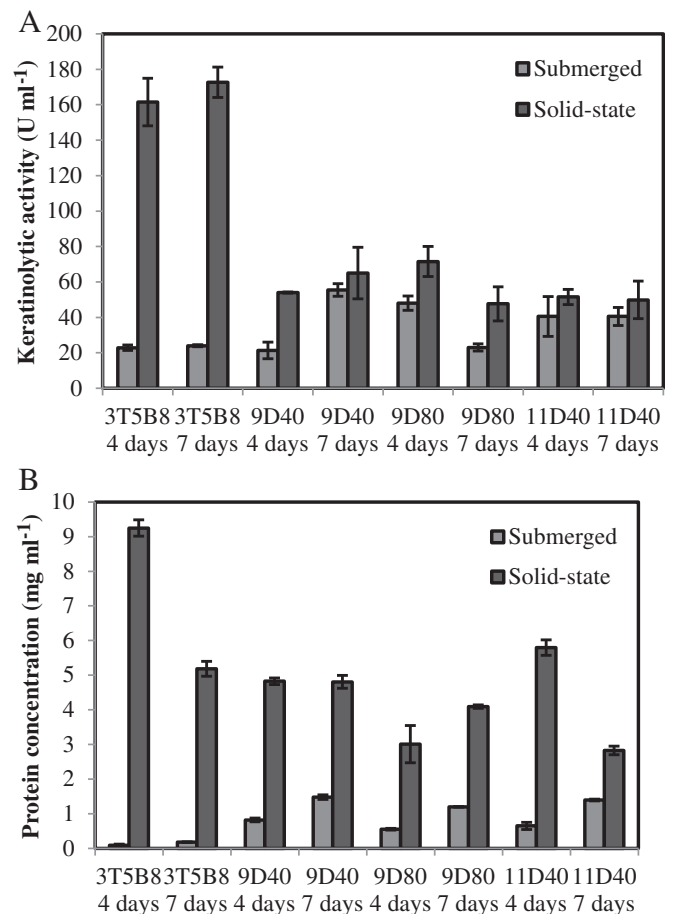


Fig. 3. Keratinolytic activity (a) and protein concentration (b) of culture supernatant of *Aspergillus niger* strains after cultivation for four and seven days under submerged and solid-state fermentation.

migrating at 14 kDa observed on the gelatin substrate (Fig. 4a) and a 130 kDa band on the keratin substrate. These bands were not observed in the other samples.

3.4. Effect of inhibitors

PMSF inhibited peptidases from strains 3T5B8 and 9D80 (Fig. 5). The peptidase migrating at approximately 40–45 kDa was inhibited by phenanthroline, whereas the peptidase migrating at approximately 60 kDa was inhibited by PMSF, indicating the presence of serine and metallopeptidase in the extracellular medium of *A. niger* 9D40 and 11D40 (Fig. 5). Conversely, peptidases from strain 11D40 were partially inhibited by E-64 and PMSF, except the one migrating at ~40 kDa (Fig. 5).

4. Discussion

In this study, we aimed to detect and investigated the production of keratinases with *A. niger* mutants and evaluated their potential use in degradation of feather keratin, an agro-industrial residue. We found that strains 3T5B8, 9D40, 9D80, and 11D40 partially degraded the substrate during growth on fermentation medium with feather as the only carbon source. Similar results were observed for keratinolytic *Bacillus* species isolated from the Amazon Basin (Giongo et al., 2007). Nevertheless, comparisons of keratinolytic activity are difficult due to the variety of keratin substrates used by investigators, which include azokeratin and azoproteins (Thys and Brandelli, 2006), keratin azure (Scott and Untereiner, 2004), guinea pig hair (Wawrzkiwicz et al., 1991), feathers, and other keratin structures found in nature (Dozie et al., 1994).

Zymograms indicated the occurrence of several peptidases and keratinases, suggesting that keratin degradation was not the

product of a single enzyme (Fig. 4). In fact, multiple bands were observed through zymography in culture supernatants of *Bacillus subtilis* AMR (Mazotto et al., 2011), *B. subtilis* SLC (Cedrola et al., 2012), *A. niger* (Lopes et al., 2011), and *Chryseobacterium* sp. kr6 (Riffel et al., 2007) when cultured on media with feathers or feather meal. Lopes et al. (2011) suggested that the synergistic activity of keratinase and peptidase may be responsible for keratin degradation.

This study is the first report of keratinase production by *A. niger* in solid-state fermentation (SSF). There are few reports on keratinase production under solid-state fermentation (De Azeredo et al., 2006; Rai et al., 2009). The production of enzymes by SSF is a field to be explored since it presents advantages such as lower production costs (Belmessikh et al., 2013). Solid-state fermentation could be explored for the production of keratinases by *A. niger*, specially with the 3T5B8 strain, that showed a keratinolytic activity 7 times higher in SSF than in submerged fermentation (SmF). *Aspergillus oryzae* NRRL 2220 showed a neutral peptidase production nine times higher in SSF compared to liquid culture (Belmessikh et al., 2013). In agreement with these results, De Azeredo et al. (2006) reported that *Streptomyces* sp. 594 had a keratinase production 2 times higher in SSF than SmF. Therefore, the fermentation condition can greatly affect the production of extracellular enzymes. However the advantages of SSF over SmF are not yet fully understood and hence more attention should be given to this process (Belmessikh et al., 2013).

All *A. niger* strains produced a peptidase with keratinolytic activity migrating at 60 kDa and with the ability to digest all substrates tested (Fig. 4b). Farag and Hassan (2004) isolated a 60 kDa keratinase from the feather-degrading *A. oryzae*. This enzyme hydrolyzed different substrates, showing the highest proteolytic activity on bovine serum albumin and casein, followed by keratin, chicken feathers, collagen, duck feathers, and sheep's wool. In our

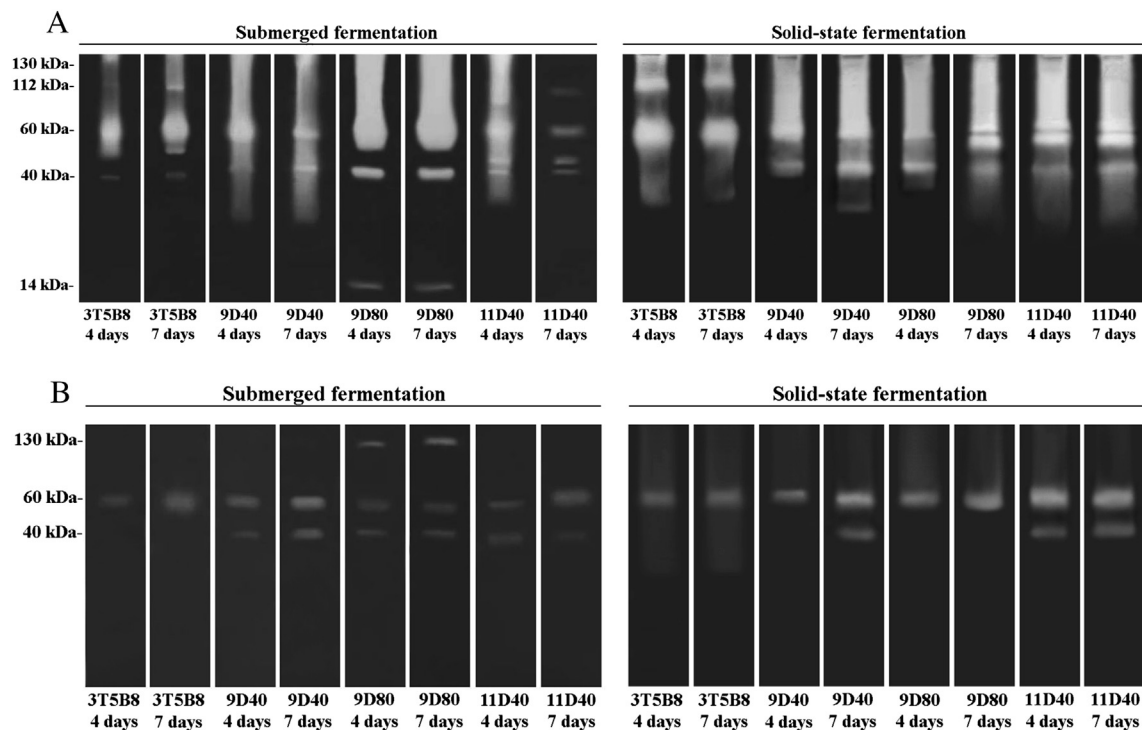


Fig. 4. Zymograms with co-polymerized (a) gelatin and (b) keratin of extracellular peptidases and keratinases from *Aspergillus niger* strains 3T5B8, 9D40, 9D80, and 11D40 produced under submerged and solid-state fermentation. Gel strips containing 30 μ l of concentrated culture supernatant were incubated for 48 h at 37 $^{\circ}$ C in 0.5 M citric acid buffer at pH 5.0. Molecular mass of peptidases, expressed in kDa, shown on the left.

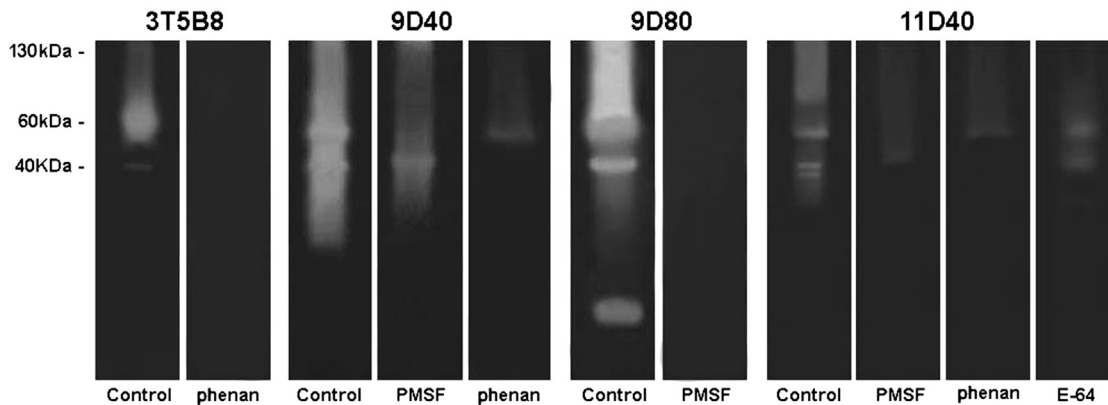


Fig. 5. Effect of proteolytic inhibitors on extracellular peptidases from *Aspergillus niger* strains 3T5B8, 9D40, 9D80, and 11D40. Gel strips containing concentrated culture supernatant after incubation for 48 h at 37 °C and pH 5.0 in the absence (control) or presence of different proteolytic inhibitors: 1,10-phenanthroline (phenan), phenylmethylsulfonyl fluoride (PMSF), and trans-epoxysuccinyl-L-leucylamido-(4-guanidino) butane (E-64). Approximate molecular mass of peptidases, expressed in kDa, is shown on the left.

study, *A. niger* strain 9D80 produced a ~130 kDa keratinase not previously reported in the keratinolytic genus *Aspergillus*. Similarly, a keratinase with molecular mass estimated at 130–150 kDa was purified from cultures of *Lysobacter* NCIMB 9497 (Allpress et al., 2002). However, few studies have detected the presence of high molecular weight keratinases in culture supernatants of keratinolytic microorganisms. Keratinases of high molecular mass have been identified in *Kocuria rosea*, *Fervidobacterium islandicum*, and *Bacillus cereus* 1268, with 240, 200, and ~200 kDa, respectively (Nam et al., 2002; Bernal et al., 2006; Mazotto et al., 2011).

The main proteolytic activity of keratinases is normally associated with serine peptidases (Cedrola et al., 2012). The inhibition of enzymatic activity by PMSF indicates that keratinases produced by *A. niger* strains 3T5B8 and 9D80 are serine peptidases (Fig. 5). These results are in agreement with those found for keratinases produced by *Aspergillus fumigatus* (Santos et al., 1996) and *Aspergillus flavus* K-03 strains (Kim, 2003). Different *Aspergillus* species have been described as serine peptidase producers (Morya et al., 2012) including *A. niger* (Kubota et al., 2005), which produced an extracellular serine peptidase with 54.5 kDa. Additionally, aspartic peptidase production has been identified in the crude enzyme extract of *A. niger* (Lopes et al., 2011) and as a serine peptidase. However, in our study, aspartic peptidase was not detected by the methodology employed. The peptidases migrating at approximately 40 kDa in crude enzyme extract of strains 9D40 and 11D40 was inhibited by phenanthroline, suggesting it is a metallopeptidase. A 95 kDa metallo-peptidase inhibited by EGTA, EDTA, and 1,10-phenanthroline was reported for a cellular extract of *A. niger* (Basten et al., 2001). Other keratinases such as 148 kDa keratinase produced by *Lysobacter* NCIMB 9497 (Allpress et al., 2002), 60 kDa keratinase from *A. oryzae* (Farag and Hassan, 2004), 45 kDa keratinase from *B. cereus* (Sousa et al., 2007), and 20 kDa keratinase from *Chryseobacterium* sp. kr6 (Riffel et al., 2007) have also been identified as metallopeptidases.

In this study, gelatin and milled feather agar plates were used for screening of keratinolytic fungi lineages (Fig. 3). Agar plates are a rapid, reproducible, and easy method to identify hydrolytic microorganisms. Due to the difficulty in incorporating insoluble substrates such as keratin in the medium, most studies use soluble substrates such as gelatin or casein to select keratinolytic microorganisms (Allpress et al., 2002). However, keratin-based solid media have been successfully used for screening of keratinolytic *Candida parapsilosis* mutants (Duarte et al., 2011). In addition, keratin-containing media have been used to induce keratinase production by keratinolytic microorganisms (Gupta and Ramnani, 2006).

Many keratinases hydrolyze a broad range of soluble and insoluble proteins, such as the enzymes produced by *B. subtilis* KD-N2 (Cai et al., 2008). This keratinase showed activity on casein, BSA, ovalbumin, feather meal, feather keratin, human hair, and sheep's wool. Finally, keratinases from *Paecilomyces marquandii* and *Doratomyces* microspores have also been shown to hydrolyze different substrates (Gradišar et al., 2005).

Keratinase production has rarely been described for the genus *Aspergillus* and the production of keratinase by *A. niger* strains under solid-state production has not been described until now. Our results here with *A. niger* could contribute to finding a biotechnological route for feather waste degradation.

5. Conclusions

A. niger mutants, especially strain 3T5B8 and 9D80, showed promising proteolytic activity for hydrolyzing feather waste. *A. niger* mutants cultured by submerged and solid-state fermentation on basal medium supplemented with chicken feather produced a peptidase mixture with high keratinolytic activity. SSF was the best fermentation condition for keratinase production. The keratinases and peptidases migrating at approximately 14–130 kDa are mostly serine peptidases. The keratinase produced by strain 9D80 migrating at 130 kDa seems to be a new keratinase. *A. niger* may have a potential to be used in biotechnological processes involving keratin hydrolysis, mainly because of its “generally recognized as safe” (GRAS) status and the vast knowledge already accumulated concerning its industrial use.

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