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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.

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 evaluated 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-satte 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× 2000*g*/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 $NaNO_3$, 1.0 KH_2PO4, 0.5 $MgSO_4 \cdot 7H_2O$, 0.5 KCl, 0.01 $FeSO_4 \cdot 7H_2O$, 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

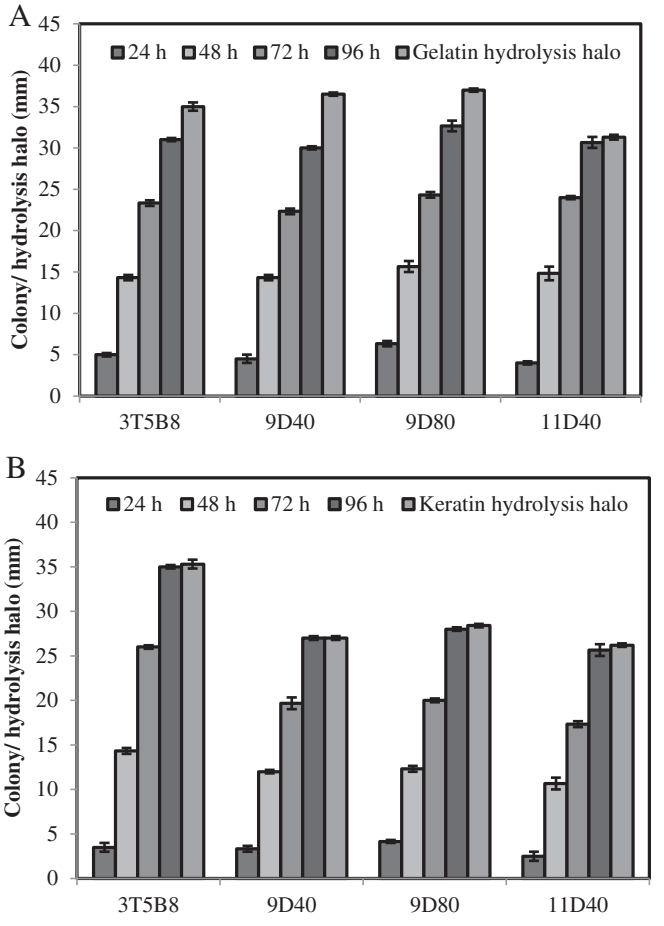

Fig. 1. Colony and hydrolysis zone diameters of different *Aspergillus niger* mutant strains on (a) gelatin agar plate or (b) keratin agar plate. Colony diameter was measured every 24 h for four days. Plates were then stained with Coomassie Brilliant Blue and hydrolysis zone was measured using a ruler.