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Minelli Sousa
Odacy Souza
Marília Maciel
Roberta Cruz
Maria Gorete Rêgo
Oliane Magalhães
Cristina Souza-Motta
Department of Mycology, Federal University of Pernambuco, Cidade Universitária, Recife, Pernambuco, Brazil

Adalberto Pessoa-Júnior
Department of Biochemical and Pharmaceutical Technology, University of São Paulo, São Paulo, São Paulo, Brazil

Ana Porto
Animal Morphology and Physiology Department, Federal Rural University of Pernambuco, Dois Irmãos, Recife, Pernambuco, Brazil

Correspondence:
Cristina Souza-Motta
Department of Mycology, Federal University of Pernambuco, Cidade Universitária, Recife, Pernambuco, Brazil

Keratinolytic potential of fungi isolated from soil preserved at the Micoteca URM

Minelli Sousa, Odacy Souza, Marília Maciel, Roberta Cruz, Maria Gorete Rêgo, Oliane Magalhães, Adalberto Pessoa-Júnior, Ana Porto, Cristina Souza-Motta

Abstract
Keratinous materials are insoluble and resistant to degradation by common proteolytic enzymes; it is therefore important to study the microorganism producers of such enzymes for use in the biotechnology industry. Fifty isolates of fungi were checked to determine if they had the ability to use keratin as a substrate. They were grown in a submerged culture in a medium containing poultry feathers and salts. *Aspergillus sulphureus* URM 5029, *Trichoderma aureoviride* URM 5574, *A. avenaceus* URM 5051 and *A. sclerotiorum* URM 5586 gave the best results with 7.35 U/ml, 7.2 U/ml, 6.7 U/ml and 6.05 U/ml, respectively, of keratinolytic activity. The keratinase from *A. sulphureus* URM 5029 showed better activity at pH 10.0 and 35 ºC. The stability of keratinase from *A. sulphureus* URM 5029 over wide ranges of pH (6.5–9.0) and temperature (25–60 °C) suggests the feasibility of using this strain in commercial biotechnological processes

Keywords: *Aspergillus sulphureus*; enzyme; feather; filamentous fungi; keratin

Introduction
Degradation of keratinous material has mostly been associated with the activity of dermatophytic fungi, yeast and bacteria. A lot of information is available on the ability of these organisms to produce keratinases and on the catalytic properties of these enzymes [1, 2, 3]. However, there are relatively few reports on the production and characterisation of the keratinases produced by non-dermatophytic fungi [3, 4]. Some non-dermatophytic fungi have already been described [3, 5] for biotechnological purposes, mainly for the management of keratinous wastes [6, 7, 8]. However, bacterial keratinases, particularly those produced by *Bacillus*, have received more attention [9].
Keratinases are typical industrial enzymes. Keratinous waste, like chicken feather and horn, accumulates in the environment, raising environmental concerns that drive the development of new biotechnological recycling approaches [10, 11]. Keratinases have potential in keratinous waste processing and in the food and feed supplement industry, among other applications such as detergent additive and in the formulation of chemicals used in the leather industry [11, 12].
The preservation of fungal strains, as type material, as reference stocks for diagnostic or teaching purposes, and as samples in national culture collections, is an important aspect of mycology. Ideally, stored strains should remain viable over long periods but be dormant to prevent the accumulation of mutations resulting in morphological and biochemical alterations. Numerous methods for the storage of fungal cultures have been evaluated, and many have been found to be suitable for high quality storage of at least certain fungal strains [13].
This study aimed to evaluate keratinolytic potential of fungi isolated from soil preserved in mineral oil at the Micoteca URM in submerged cultivation.

2. Materials and methods
2.1 Microorganisms
Fifty isolates of fungi filamentous obtained from the Micoteca URM culture collection of the Federal University of Pernambuco, Recife, Brazil, were isolated from soil and stored under mineral oil for a period ranging from 3 to 14 years (Table 1). These isolates were analysed regarding the viability, taxonomic authentication and keratinolytic production.

2.2 Reactivation of cultures
The isolates were transferred for broth glucose [14] and maintained at 28 ºC ± 2 ºC. After growth, all isolates were transferred to specific media in test tubes and maintained at

the same temperature. For the taxonomic authentication, all isolates were submitted to the observation of macro- and micro-morphological traits. The taxonomic authentication followed the taxonomic keys and guides available for the genera [15,16,17,18, 19, 20, 21, 22].

2.3 Production of keratinase by submerged cultivation

Poultry feathers for use in the culture were cut into 1–2 mm pieces and defatted for 30 min at 37 °C with a mixture of chloroform and methanol (1:1 v/v). Then, the feathers were stored with detergent for 16 h at 42 °C, washed five times with distilled water and dried at 60 °C (48 h) [23]. The cultivation medium was prepared according to the method of Anbu et al. [24] with modifications and contained poultry feathers (15 g/l), K_2HPO_4 (1.5 g/l), $MgSO_4{\cdot}7H_2O$ (0.05 g/l), $CaCl_2$ (0.025 g/l), $FeSO_4{\cdot}7H_2O$ (0.015 g/l) and $ZnSO_4{\cdot}7H_2O$ (0.005 g/l) in phosphate buffer (0.02 mol/l, pH 7.8). Fractions of 50 ml of this medium were added in Erlenmeyer flasks of 250 ml capacity and autoclaved at 121 °C for 15 min. The inoculum was prepared by suspending spores in 10 ml of Tween 80-water (0.1%) (v/v). Then, 1 ml of this suspension, containing 10^6–10^7 spores, was used to inoculate each flask. The flasks were shaken at 120 rpm, 30 °C for 10 days. After this period of incubation, the broth culture was filtered with CELAB filter paper (9 cm in diameter). The pH value of the filtrate was measured using a digital pH meter. The culture filtrate was used as a crude enzymatic extract.

2.4 keratinase activity assay

Keratinolytic activity was determined by the method used by Anbu et al. [24]. Keratin powder (20 mg) was incubated for 1 h at 37 °C with Tris-HCl buffer (100 mM, pH 7.8; 3.8 ml) and crude enzymatic extract (0.2 ml). The samples were put in a 4 °C refrigerator for 10 min and then centrifuged for 10 min at 10,000 g at 4 °C. The absorbance of the supernatant fluid at 280 nm was measured by spectrophotometry against a blank. An increase of 0.100 in absorbance was taken to indicate one unit of enzyme activity. Keratinolytic activity of all the isolates was expressed in units of activity per milliliter (U/ml).

2.5 Enzyme characterization

The keratinase activity was measured at different pH and temperature values. The effect of pH on keratinase activity was measured using the following buffers at 100 mM concentration: acetate buffer (pH 4.0–6.0), sodium phosphate buffer (pH 6.5–7.5), Tris-HCl buffer (pH 8.0–9.0) and sodium carbonate-bicarbonate buffer (pH 9.0–11.0). Poultry feathers served as the substrate. To determine the pH stability, the crude enzymatic extract was pre-incubated in the buffers of different pH values (4.0–11.0) at 37 °C for 2 h. The optimum temperature for enzyme activity was also studied. The crude enzymatic extract was incubated at different temperatures ranging from 25–80 °C. For determination of thermostability, the crude enzymatic extract was incubated at different temperatures (25–80 °C) for 2 h. Then the enzyme assay was performed. The residual activity was estimated after incubation under the standard assay conditions and expressed as the percentage of the initial activity [25].

3. Results & Discussion

3.1 Viability of the isolates

All isolates of fungi filamentous evaluated were viable and confirmed taxonomically (Table 1). The results demonstrate that the preservation under mineral oil is appropriated for the maintenance these isolates. The isolates showed stability of morphological and physiological characteristics after storage for up to 13 years.

These results agree with those of Braz et al. [26], who assessed the viability of 31 isolates of *Acremonium*, where 26 remained viable when preserved under mineral oil at the Micoteca URM and 24 isolates were taxonomically authenticated after 31 years of preservation. Species of *Cladosporium* and *Trichosporon*, preserved by this method and maintained in the Micoteca URM, remain viable for up to 40 years [27]. According to Figueiredo [28] and Nakasone et al. [29], fungi cultures can be preserved under mineral oil for 20 to 32 years.

Deshmukh [30] obtained 239 keratinophilic fungi viables when stored in agar slants in tubes in refrigerator for 12 years (subcultured once every 4 months), in sterile distilled water for 7 and 10 years, under mineral oil for 7 and 10 years, in silica gel for 7 and 10 years, in soil for 7 and 10 years and for 12 years in freeze-dried. Thus, the methods of preservation were adequate for these fungi.

These studies showed the advantages of the preservation in mineral oil in the maintenance of the viability of some species of fungi over a prolonged period of storage. However, Bezerra et al. [31] evaluated 20 cultures of *Coccidioides immitis* preserved under mineral oil in the Culture Collection of Instituto Oswaldo Cruz (Rio de Janeiro, Brazil) and only five were viable, showing that this method of preservation was not adequate for these species.

The preservation under mineral oil has some advantages. It is considered a simple procedure and low-cost and low-maintenance method. However, some disadvantages also are observed, as growth rate slow down and requires enough storage space [32].

3.2 Production of keratinase

Of the species studied, all showed keratinolytic capacity. The strains *Aspergillus sulphureus* URM 5029, *Trichoderma aureoviride* URM 5574, *A. avenaceus* URM 5051 and *A. sclerotiorum* URM 5586 gave the best results with 7.35 U/ml, 7.20 U/ml, 6.70 U/ml and 6.05 U/ml, respectively, of enzyme activity after 10 days of fermentation at 120 rpm and 30 °C (Table 1).

Because of the high keratinolytic activity of the enzyme extracts, the pH increased (from 8.0 to 10.0) during cultivation and alkalinised the medium. This alkalinisation is a result of peptide deamination reactions and ammonium production [33]. In the work by Riffel et al. [33], an increase in pH (7.8 to 8.5) during cultivation and alkalinisation of the medium were observed.

Using poultry feathers as a substrate, Friedrich et al. [1] screened fungi for the synthesis of keratinolytic enzymes and obtained as the best producer *A. flavus*, which produced 0.781 U/ml of enzyme activity after seven days of submerged cultivation. The culture medium contained a solution of soluble minerals and had poultry feathers as the sole source of carbon and nitrogen. Anbu et al. [24] sought to improve the production of keratinase using a strain of *Scopulariopsis brevicaulis* and obtained their highest level of enzyme activity, 6.2 U/ml, with 35 days of cultivation. These values

are lower than those obtained in this study for overall keratinolytic activity. However, other authors have attained higher values of production. Sales et al. [34] studied the keratinase production by *A. carbonarius* URM 1546. They used poultry feathers as the substrate and obtained a maximum keratinolytic activity of 48.9 U/ml with seven days of cultivation at 120 rpm and with 0.5% (w/v) poultry feathers. Gioppo et al. [5] studied the filamentous fungus *Myrothecium verrucaria*, a non-dermatophyte able to grow and produce keratinase while submerged in liquid medium (93.0 ± 19 U/ml) or in semi-solid medium (98.8 ± 7.9 U/ml); in these cultures, poultry feathers were the sole substrate. Mazotto et al. [35] working with *A. niger* mutants obtained higher keratinase production when the strains were cultivated in a solid-state condition rather than a submerged condition. The keratinolytic activity of *A. niger* 3T5B8 was had the highest (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.

Using other substrates, Elíades et al. [3] studied 69 fungal strains for their ability to produce alkaline keratinolytic activity in submerged cultures supplemented with soybean meal and tryptone (FS), or supplemented with cowhide (PV), under conditions of solid-state fermentation. Among these strains, *A. niger*, *Cladosporium cladosporioides*, *Metarhizium anisopliae*, *Neurospora tetrasperma* and *Westerdikella dispersed* were the best producers, with levels of enzyme activity above 1.2 U/ml. Comparing the production of keratinase in these studies using other sources of keratin with the results obtained for overall keratinolytic activity in this study, we note that there is better production of the enzyme using poultry feathers, rich in keratin, as the sole source of carbon and nitrogen. However, Cavello and Cavallito [36] obtained the maximum keratinase yield (26.7 U/ml) studied the keratinolytic properties of *Purpureocillium lilacinum* LPS # 876, using only 7.10 g/l of glucose; 0.0065 mg/l of $CaCl_2$ and initial pH of 5.6 by submerged fermentation.

Table: 1 Preservation time, viability, taxonomic authentication and keratinolytic activity of fungi filamentous preserved under mineral oil in the Micoteca URM culture collection.

Species and access number at the Micoteca URM	Preservation time (years)	Viability	Taxonomic Authentication	Keratinolytic activity U/ml
Acremonium curvullum URM 4695	8	+	Confirmed	2.05
A. strictum URM 5031	5	+	Confirmed	3.7
Aspergillus acuelatus URM 4953	6	+	Confirmed	1.5
A. acuelatus URM 5240	5	+	Confirmed	1.55
A. avenaceus URM 5051	6	+	Confirmed	6.7
A. carbonarius URM 5012	6	+	Confirmed	1.4
A. carneus URM 5262	5	+	Confirmed	5.6
A. carneus URM 5577	4	+	Confirmed	4.55
A. clavatus URM 5841	3	+	Confirmed	5.2
A. clavatus URM 5076	6	+	Confirmed	2.2
A. granulosus URM 4641	8	+	Confirmed	5.45
A. janus URM 4456	9	+	Confirmed	1.15
A. japonicus URM 3840	14	+	Confirmed	3.45
A. japonicus URM 4599	8	+	Confirmed	2.7
A. japonicus URM 5242	5	+	Confirmed	2.6
A. japonicus URM 4533	9	+	Confirmed	2.25
A. japonicus URM 5751	3	+	Confirmed	2.65
A. nutans URM 4309	10	+	Confirmed	1.25

Table: 1 (continued)

Species and access number at the Micoteca URM	Preservation time (years)	Viability	Taxonomic Authentication	Keratinolytic activity U/ml
Paecilomyces farinosus URM 3896	14	+	Confirmed	1.95
P. variable URM 3835	14	+	Confirmed	1.85
P. variotii URM 5385	4	+	Confirmed	2.15
P. variotii URM 4657	8	+	Confirmed	2.0
Scolecobasidium humicola URM 4335	10	+	Confirmed	2.45
Scopulariopsis brevicaulis URM 5795	3	+	Confirmed	1.45
S. sphaerospermum URM 4603	8	+	Confirmed	3.05
Trichoderma aureoviride URM 5574	4	+	Confirmed	7.2
T. harzianum URM 4950	6	+	Confirmed	2.45
T. koningii URM 4649	8	+	Confirmed	1.95
T. virens URM 5007	6	+	Confirmed	2.2
T. viride URM 4996	6	+	Confirmed	5.1

+= viabal.

3.3 Effects of pH and temperature on keratinase activity and stability

For characterisation of the keratinase enzyme, crude enzymatic extract obtained from *A. sulphureus* URM 5029 that showed the highest keratinolytic activity was used.

The highest keratinolytic activities were obtained at pH 10.0 (10.95 U/ml) and pH 10.7 (10.55 U/ml). Stability of the keratinase from *A. sulphureus* URM 5029 was observed over

a pH range of 6.5–9.0, at which over 50% activity was maintained, and maximum stability was observed at pH 8.0, at which 94% activity was maintained. These results show that the keratinase is stable over a wide pH range (Figure 1).

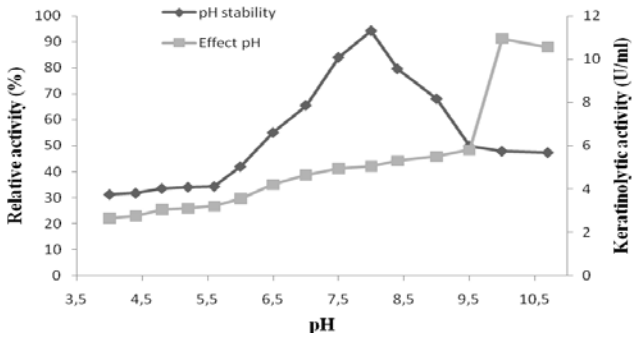

Fig: 1 Effect of pH (2 hours of incubation) on the stability and activity of the keratinase from *Aspergillus sulphureus* URM 5029.

Experiments to test the effect of temperature on keratinase activity showed that maximum activity was obtained at 35 °C (12.3 U/ml). The enzyme was found to be stable over a wide range of temperatures (25–60 °C), maintaining over 50% activity; maximum stability was observed at 35 ºC, at which 68% activity was maintained. These results show that keratinase is stable over a wide temperature range (Figure 2).

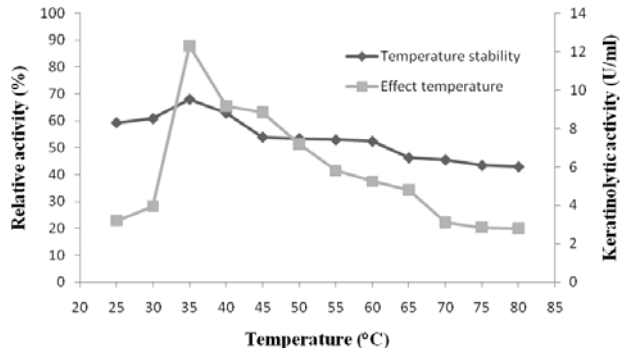

Fig: 2 Effect of temperature (2 hours of incubation) on the stability and activity of the keratinase from *Aspergillus sulphureus* URM 5029.

Keratinases from most bacteria, actinomycetes and fungi have an optimum pH within a neutral to alkaline range (pH 7.5-11.0) [33, 37, 38, 39]. Other keratinolytic enzymes have also been proven to be active at an alkaline pH; the enzymes secreted by *Streptomyces* sp. [40] and *Strepmomyces albidoflavus* [41] are examples. However, few keratinases show extreme alkalophilic optima at a pH>12 [7]. Although the optimum temperature for keratinases ranges from 30 to 80 ºC [7] the enzymes from *Chrysosporium keratinophilum* [42] and *Fervidobacterium islandicum* [43] have exceptionally high optimum temperatures of 90 and 100 °C, respectively.
Cao et al. [44] studied the keratinase produced by *Trichoderma atroviride*, which had an optimum pH of 8.0-9.0 and optimum temperature of 50 °C. Saber et al. [45] characterised the keratinolytic activities of cultures of *Alternaria tenuissima* K2 and *Aspergillus nidulans* K7. Keratinase activity was found to be greater in alkaline conditions ranging from pH 8.0 to 9.0 for *Alternaria tenuissima* K2 and from pH 7.5 to 9.0 for *Aspergillus nidulans* K7, with an optimum pH of 8.5 for both enzymes. The keratinase from *A. nidulans* K7 was active over a wide range of temperatures (from 25 °C to 65 °C) and showed maximum activity at 40 °C. It was found to be more active at higher temperatures than the keratinase from *Alternaria tenuissima* K2. Similary, Cavello et al. [46] observed that the extracellular keratinase of *P. lilacinum* LPS # 876 was stable over a broad pH range (from 4.0 to 9.0), and up to 65 °C.
Anitha and Palanivelu [47] characterised the purified keratinase of *A. parasiticus* isolated from poultry soil and obtained pH optimum of 7.0 and temperature optimum of 50 °C. Similary, Sankar et al. [48] working with keratinase produced by *S. brevicaulis* obtained optimum pH in the neutral region 7 to 7.5 and optimum temperature of 50 °C. In the present study, the results cited are in agreement with literature.

4. Conclusions

Soil is rich in keratinolytic fungi capable of degrading waste poultry feathers; this capability makes the fungi interesting in terms of their potential for bioconverting residues in food with high protein for animal feed and also for bioremediating environments contaminated with keratin residues. Preservation in mineral oil is sufficient to maintain the viability and taxonomic characteristics of cultures for long periods of time, as shown in our study; in addition, it could maintain the metabolic characteristics of these fungi, all of which retained keratinase activity. *Aspergillus sulphureus* URM 5029, *Trichoderma aureoviride* URM 5574, *A. avenaceus* URM 5051 and *A. sclerotiorum* URM5586 showed greater keratinolytic activity, indicating their suitability for keratinase production. The keratinase from *Aspergillus sulphureus* URM 5029 showed greater activity at pH 10.0 and 35 ºC. The ability of keratinase from *Aspergillus sulphureus* URM 5029 to be stable over wide pH and temperature ranges suggests the feasibility of using this strain in commercial biotechnological processes.

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