



Optimization of Extracellular Keratinase Production by *Aspergillus terreus* Isolated from Chicken's Litter

Mostafa Koutb^{1,2*}, Fatthy Mohamed Morsy¹, Magdy Mohamed Khalil Bagy¹ and Elhagag Ahmed Hassan¹

^{1*}Botany Department, Faculty of Science, Assiut University, Assiut-71516, Egypt.

²Umm Al-Qura University, Faculty of Applied Science, Biology Department, Mecca, Saudi Arabia.

Abstract: In this current study 45 fungal isolates were isolated from chicken's litter on Feather Agar Medium (FAM) were screened for determining the potent keratinase producing isolates. Out of these fungal isolates, twelve species and one species variety exhibited various degrees of keratinolytic activities from which *A. terreus* showed the highest keratinase production (12.6U/ml). The optimum temperature and initial pH for keratinase production by *A. terreus* were 40°C and 8, respectively. The highest keratinase production was observed for a period 25 days. The optimum ionic strength for the enzyme production was 80mM NaCl. Deprivation of K⁺, Fe²⁺, Mg²⁺, Ca²⁺ or Zn²⁺ from the culture medium drastically reduced the keratinase production by *A. terreus*. In contrast, sulfur deprivation did not significantly affect the keratinase production. The K_m and V_{max} values for *A. terreus* keratinase were 8.64mg keratin and 56.7U/mg proteins, respectively. The optimum temperature, pH and ionic strength for keratinase activity were 35°C, 7.8 and 80-100mM NaCl, respectively.

Keywords: *Aspergillus terreus*, Chicken's litter, Fungi, Keratinase production.

1. Introduction

Keratinases (E.C. No. 3.4.99.11), a group of proteinases, are important for hydrolyzing feather, hair, wool, collagen and casein to clean obstructions in the sewage system during wastewater treatment [1]. These enzymes are also used or could be applied in the food industry, textiles, medicine, cosmetics, leather and poultry processing industry [2, 3].

Keratinases are produced only in the presence of keratin-containing substrates. Microbial keratinases are predominantly extracellular; however, a few cells-bound and intracellular keratinases have also been reported [4-8]. Among non-dermatophytic fungi, keratinases showing attractive biochemical properties are *Aspergillus* [9, 10], *Trichoderma* [11], *Doratomyces* [12], *Myrothecium* [13], *Paecilomyces* [14], *Scopulariopsis* [15] and also *Acremonium*, *Alternaria*, *Beauveria*, *Curvularia*, and *Penicillium* [16]. Besides the biotechnological interest, these investigations may help in understanding the role of fungi in the degradation of complex keratinous substrates in nature

[16]. The objective of this study was to select highly keratinase producing non-dermatophytic fungi isolated from chicken's litter and further characterization of keratinase with optimizing its production process by the most potent keratinase producing fungal isolate.

2. Materials and Methods

2.1 Isolation of keratinolytic fungi from chicken's litter

The dilution-plate method (Johnson *et al.*, 1959) was used to isolate the keratinolytic fungi on Feather Agar Medium (FAM). The composition of (FAM) medium (g/l) is: Agar, 15.0; Feather powder (defatted with chloroform-methanol 1:1 (V/V) over 24 h, ground and sifted through a 0.2-mm screen.), 10.0; K₂HPO₄, 1.5; MgSO₄·7H₂O, 0.025; CaCl₂, 0.025; FeSO₄·7H₂O, 0.015; ZnSO₄·7H₂O, 0.005; Actidione, 0.5; Chloramphenicol, 0.25; Volume was adjusted to 1000ml with distilled water and pH was adjusted to 6.5. The growing colonies were isolated by aseptic transfer to Sabouraud dextrose agar medium (SDA) and incubated

*Corresponding author:

E-mail: moskoutb@yahoo.com; Phone: +9660597029270.

at 30°C. Fungal growth was identified based on the macroscopic aspect of colonies and micro-morphological examinations.

2.2 Screening for extracellular keratinase production

Extracellular keratinase production was tested in 45 fungal isolates on basal salt medium (BSM) containing (g/l): K_2HPO_4 , 1.5; $MgSO_4 \cdot 7H_2O$, 0.025; $CaCl_2$, 0.025; $FeSO_4 \cdot 7H_2O$, 0.015 and $ZnSO_4 \cdot 7H_2O$, 0.005. Defatted white chicken's feather powder (50mg) was added to each flask at initial pH 7.5. Media were inoculated with a spore suspension obtained from 10 days old cultures of each fungal isolate under investigation and incubated for 15 days at 30°C. The filtrate containing extracellular keratinase was centrifuged at 10000 x g for 15 min at 4°C. The supernatant containing keratinase was analyzed. The screened fungal isolates were ordered into 4 groups according to keratinase production. The fungal isolates producing (more than 10U/ml) designed as high (H) producers, (6-10U/ml) as moderate (M) producers, (1-6U/ml) as low (L) producers and (less than 1U/ml) as non producing). Extracellular keratinase production was expressed in a number of enzyme units per ml.

2.3 Assay for keratinase activity

Keratinase activity was measured as described by Yu *et al.*, [17] with some modifications. The reaction mixture contained 50mM Tris-HCl buffer (pH 7.8), 20mg keratin powder and 1ml of fungal filtrate containing crude extracellular keratinase in a total volume of 4ml. The reaction was left for 4 h at 37°C with shaking and subsequently, the released amino acids from keratin proteolysis were measured by the ninhydrin method as described by Muting and Kaiser [18]. One unit of activity was defined as the amount of enzyme that catalyzes the release of 1µg amino acids per hour. Keratinase specific activity was defined as the number of keratinase units per mg extracellular protein. The total extracellular protein produced by the keratinase producing fungi was measured in extracellular crude enzyme as described by Bradford [19].

2.4 Optimization of keratinase production

A. terreus was selected for further studies on optimization (incubation temperature, period, initial pH) of keratinase production and kinetics of keratinase activity.

2.5 Ionic strength and metal ions dependency

The Ionic strength dependency of the keratinase production was examined at different salt concentrations ranging from 0.0 to 2M NaCl in the BSM culture medium. The keratinase yield was

determined after two weeks of inoculation. *A. terreus* was grown in BSM medium as control and the same medium deprived of Mg^{2+} , K^+ , Ca^{2+} , Zn^{2+} or Fe^{2+} at both optimum temperature and initial pH. After two weeks the yield of keratinase produced was determined.

2.6 Kinetics of *A. terreus* keratinase

The K_m value of keratinase for keratin substrate was determined using Lineweaver-Burk plots of various keratin concentrations against keratinase activity.

2.7 The ionic strength, temperature and pH-dependency of the keratinase activity

The pH-dependency of the keratinase activity was examined in a pH range from 2.3 to 10. Different buffer systems were used in accordance with the respective pH ranges: 50mM Glycine-HCl buffer for the pH range from 2.3 to 5, 50mM citrate buffer for the pH range from 6 to 6.6, 50mM Tris-HCl buffer for the pH range from 7 to 9 and 50mM Glycine-NaOH buffer for the pH range from 9 to 10. The Ionic strength dependency of the keratinase activity was examined at different NaCl concentrations of 0 to 2M NaCl in the reaction mixture. The optimum temperature for the keratinase activity was determined in a temperature range of 20–50°C using the buffer system 50mM Tris-HCl (pH 7.8 at ambient temperature).

2.7 Scanning electron microscopy (SEM)

To determine the degree of degradation of feather, samples from 7 days culture were freeze-dried, incubated in 5% cold buffered glutaraldehyde for two days at room temperature. The samples were washed with sodium cacodylate buffer for three times (30 minutes each) and postfixed in 1% osmium tetroxide for two hours. Then, the samples were washed in the same buffer for three times (30 minutes each) and dehydration by using an ascending grade of ethanol 30, 50, 70, 90% for two hours and 100% for two days and in amyl acetate for two days. After that, the samples were dried in the critical point drainer using liquid carbon dioxide, and each sample stickled on a metallic block by using silver paint. In Gold Sputter Apparatus, the samples were evenly gold coated in thickness of 15nm. By JEOL JSM 5300 Lv scanning electron microscope, the samples then were examined at 15 kV and photographed.

3. Results and Discussion

3.1 Isolation and screening of keratinase producing fungi

Results in the keratinase production of fungi in submerged culture are shown in Table (1).

Table 1. Keratinase production and specific activity of fungal isolates from chicken litter.

Test Fungal species	Visual Growth	Extracellular keratinase production U/ml	Extracellular protein mg/ml	Specific activity U/mg protein
<i>Acremonium strictum</i>	+	5.38L	0.19	27.08
<i>Aspergillus flavus</i>	+	7.35M	0.18	41.13
<i>A. flavus</i> var. <i>columnaris</i>	+	6.48M	0.18	36.52
<i>A. fumigatus</i>	+	7.12M	0.26	27.05
<i>A. niger</i>	+	7.81M	0.19	41.21
<i>A. terreus</i>	+	12.60 H	0.30	42.27
<i>Chrysosporium keratinophilum</i>	+	7.88M	0.12	63.90
<i>C. tropicum</i>	+	11.63H	0.14	80.68
<i>P. funiculosum</i>	+	5.99L	0.23	26.37
<i>Scopulariopsis brevicaulis</i>	+	10.35H	0.21	47.82
<i>S. brumptii</i>	+	5.82L	0.18	31.78
<i>Trichoderma harzianum</i>	+	4.90L	0.17	29.22
<i>T. viridi</i>	+	5.91L	0.22	26.95

The keratinase production was measured after two weeks of inoculation of fungi; H= High production of keratinase (more than 10U/ml); M= Moderate production of keratinase (6-10U/ml); L= Low production of keratinase (less than 6U/ml).

Twelve species and one species variety out of 45 fungal isolates exhibited various degrees of keratinolytic activities when cultivated onto chicken feather liquid medium and 32 fungal isolates showed no keratinolytic activity (data not shown). Three out of the 13 keratinase producing fungal isolates were considered as highly keratinase productive. (First group) The highest keratinase activity was reached with *A. terreus* followed by *Chrysosporium tropicum* and *Scopulariopsis brevicaulis*. The second group contains moderate producers for keratinases such as *Aspergillus flavus*, *A. flavus* var. *columnaris*, *A. niger* and *Chrysosporium keratinophilum*. The remaining fungal isolates have low productivity of keratinase as shown in Table (1). *Aspergillus terreus* showed the highest keratinase productivity (12.6U/ml) with a specific activity (42.27U/mg proteins) and thus, it was selected for further optimization and kinetics experiments.

It can be concluded that keratinolytic activity is relatively widespread among common fungi. Many fungi excrete enzymes into the environment and can be used as producers of keratinolytic enzymes. *A. terreus* showed the highest keratinase productivity (12.6U/ml). Keratinases of non-dermatophytes are of particular interest because of safety in fungal handling and application. In this study, five potent keratinase producing non-dermatophytic fungi were isolated. In spite of its wide applications in the breakdown of keratinous wastes, keratinases of dermatophytes are dangerous to use in its crude form due to contamination of the crude enzyme with spores of such dangerous fungi. In contrast to dermatophytes, the crude extracts of keratinases isolated from non-dermatophytic fungi are completely safe to use without the expensive and laborious purification processes. It has been suggested that screening for non-pathogenic microorganisms with keratinolytic activity may prevent the need for isolation and purification of the enzymes [20]. It is worth to mention that all fungi isolated in this study from chicken litter are non-dermatophytes.

3.2 Optimization of keratinase production

Keratinase production by *A. terreus* was optimized. *Aspergillus terreus* showed an optimum pH range from 7-8 for keratinase production (Fig. 1A). The maximum enzyme production was recorded at 40°C, whereas below or above this temperature the keratinase production declined (Fig. 1B). The effect of different incubation periods of keratinase production was investigated (Fig. 2A).

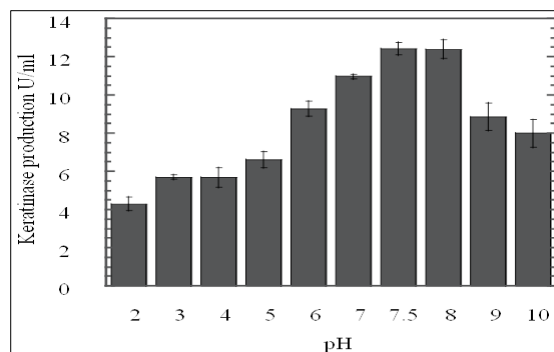


Fig. 1(a). Effect of pH on keratinase production by *Aspergillus terreus*. The experiments were repeated three times and mean values and standard errors are shown.

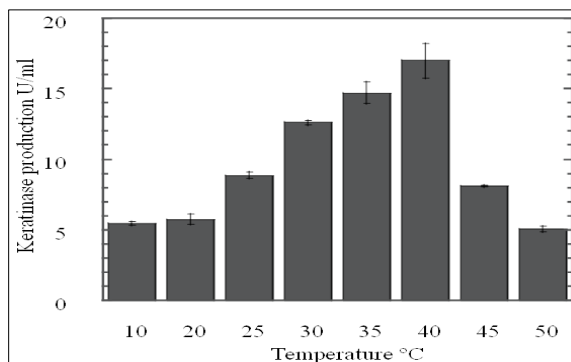


Fig. 1(b). Incubation temperature on keratinase production by *Aspergillus terreus*. The experiments were repeated three times and mean values and standard errors are shown.

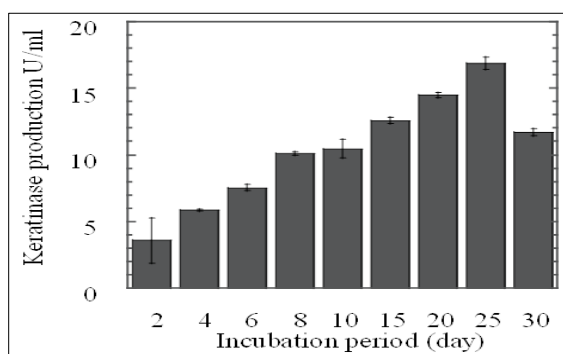


Fig. 2(a). Effect of incubation period on keratinase production by *Aspergillus terreus*. The experiments were repeated three times and mean values and standard errors are shown.

Keratinase production was detected after 2 days of incubation. The maximum productivity was detected after 25 days after which the production declined. The optimum Ionic strength for keratinase production was recorded at 80mM NaCl, and then sharply declined with increasing NaCl concentration in the culture medium (Fig. 2B). The effect of the basal salt medium constituents on keratinase production was studied by depriving the medium from each one of constituents separately. Deprivation of Ca, Mg, Fe, K⁺ or Zn²⁺ strongly affected the keratinase production, indicating that these elements are essential for the keratinase production. Sulfur deprivation did not significantly affect keratinase production (Fig. 2C). In the present study, the optimum production of keratinase enzyme(s) from *A. terreus* was recorded within incubation period 25 days. The production of keratinase was proportionally increased with the incubation time up to 25 days, after that the production of keratinase decreased. The optimum production of keratinase enzyme(s) was recorded within an incubation temperature of 35°C. The optimum pH ranges from 7-8 for keratinase production, indicating that the fungus productivity is not affected by the increase in pH value of the medium due to ammonium release as reported by Gupta & Ramnani [21]. The optimum concentration of NaCl for keratinase production by *A. terreus* is 80 to 100mM NaCl and then declined with increasing NaCl concentration in the culture medium. Keratin degradation processes a large amount of sulfur-containing amino acids [22]. Keratin proteolysis releases sulfur-containing amino acids and sulfhydryl groups [21]. Sulfur deprivation did not significantly affect the keratinase productivity by *A. terreus*, possibly because of the release of sulfur-containing amino acids and sulfhydryl groups from keratin degradation which might provide the fungus with its sulfur requirements for growth and keratinase production. Zn²⁺, k⁺ Ca²⁺, Mg²⁺ or Fe²⁺ deprivation strongly reduced keratinase productivity. These results indicate that these cations play an important role in the keratinase productivity and possible regulation of enzyme active conformation.

Ca²⁺ and Mg²⁺ have been reported to cause a 3-fold increase in the enzymatic activity [23]. These metal ions might be associated with the stabilization of the tertiary structure conformation of metalloproteases and protect these enzymes against autolysis [24].

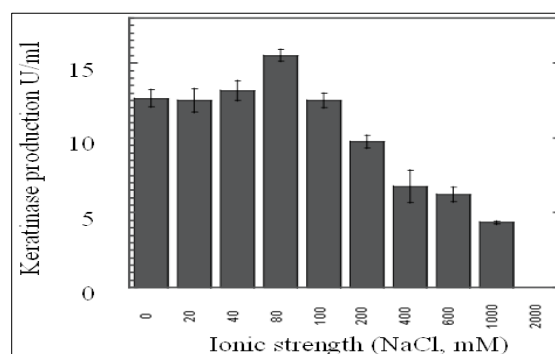


Fig. 2(b). Effect of Ionic strength on keratinase production by *Aspergillus terreus*. The experiments were repeated three times and mean values and standard errors are shown.

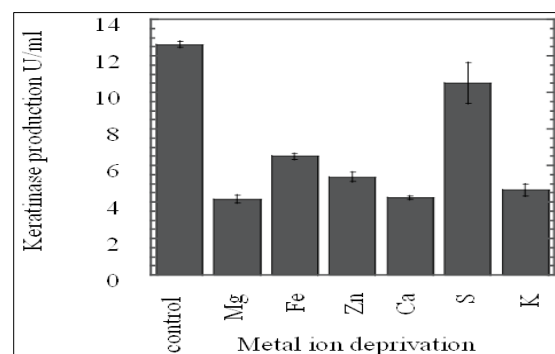


Fig. 2(c). Effect of metal ions deprivation on keratinase production by *Aspergillus terreus*. The experiments were repeated three times and mean values and standard errors are shown.

3.3 Kinetics of *A. terreus* extracellular keratinase

The K_m and V_{max} values for keratinase of *A. terreus* were determined using Lineweaver–Burk plots. The K_m and V_{max} values for *A. terreus* keratinase were 8.64mg keratin and 56.7U/mg proteins, respectively (Fig. 3). The optimum pH for keratinase activity was 7.8 (Fig. 4A). *A. terreus* keratinase showed an optimum temperature at 35-40°C (Fig. 4B). The optimum Ionic strength for *A. terreus* keratinase activity was 80-100mM NaCl. The keratinase activity sharply decreased in Ionic strength higher than 100Mm NaCl (Fig. 4C).

Although, increasing the enzyme specific activity by purification to homogeneity, is very useful for laboratory studies. There is no need for such purification when the target is to use the enzyme in commercial applications. That is because the high specific activity of the expensive purified enzyme will be lost again when mixing the enzyme with the crude keratin wastes in commercial applications. In addition, a large amount of the enzyme is always lost with

various degrees depending on the purification protocols.

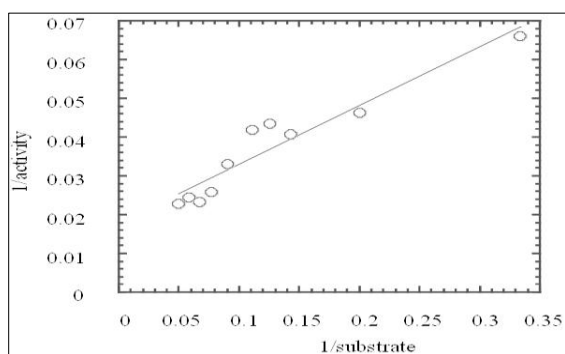


Fig. 3. Lineweaver-Burk plots of various keratin concentrations against *Aspergillus terreus* keratinase activity.

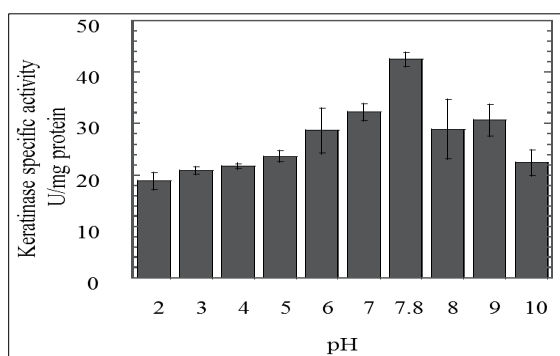


Fig. 4(a). Effect of pH on keratinase activity by *Aspergillus terreus*. The experiments were repeated three times and mean values and standard errors are shown.

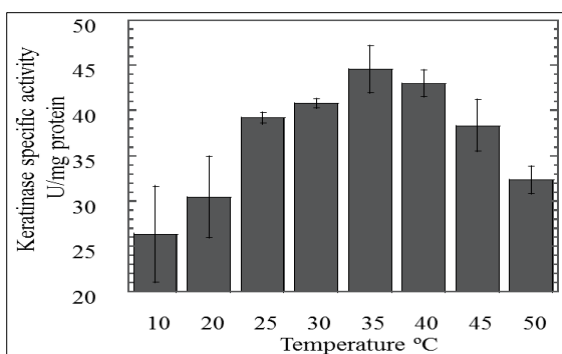


Fig. 4(b). Effect of temperature on keratinase activity by *Aspergillus terreus*. The experiments were repeated three times and mean values and standard errors are shown.

The K_m and V_{max} values for *A. terreus* crude extracellular keratinase were evaluated from a Lineweaver-Burk plot and found to be 8.64mg keratin and 56.7U/mg protein, respectively, indicating a high purity of the enzyme. The K_m and V_{max} values of the pure enzyme of *A. oryzae* were found to be 8.47mg/ml and 71.43U/ml, respectively [10]. The properties of microbial degrading enzymes appear to differ according to the producing species of microorganism. The

optimum pH for *A. terreus* keratinase activity was (7.8). In contrast keratinase of *Trichophyton schoenleinii* and *Trichophyton mentagrophytes* var. *erinacei* showed an optimum pH 5.5 [25].

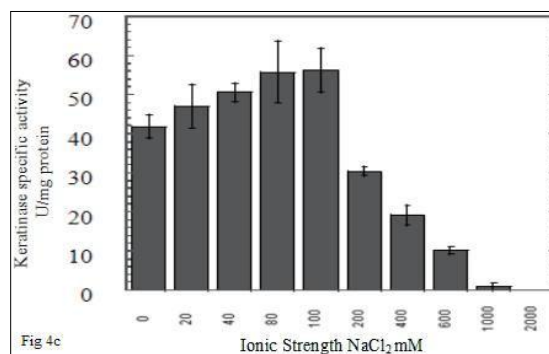


Fig. 4(c). Effect of Ionic strength on keratinase activity by *Aspergillus terreus*. The experiments were repeated three times and mean values and standard errors are shown.

In agreement with our results, Moallae *et al.*, [26] reported that keratinase activity from *Trichophyton vanbruseghemii* was optimum at pH 8. Similarly, the optimum pH for *A. oryzae* was 8 [10]. The optimum temperature for *A. terreus* keratinase were 35-40°C. Keratinases of *Scopulariopsis brevicaulis* and *Trichophyton* sp. HA-2 showed similar optimum temperature 35-40°C [27, 28]. In contrast, the optimum temperature for *A. oryzae* was 50°C [10]. The optimum Ionic strength for keratinase activity was 80-100mM NaCl. Higher NaCl concentrations sharply reduced the keratinase activity possibly by inhibiting its interaction with keratin substrate. Accordingly, the NaCl concentration higher than 100mM sharply reduced both growth and keratinase production by *A. terreus*.

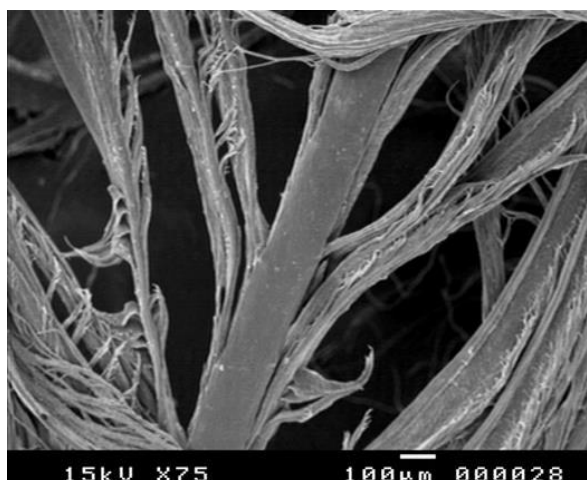


Fig. 5(a). Scanning electron micrograph of feather degradation by *A. terreus*. colonization of fungal mycelia on feather surface.

3.4 Scanning electron microscope

Scanning electron micrograph of feather degradation by *A. terreus* showed that most of the feathers were degraded after 7 days. The shaft of feathers with barbs (Fig. 5A) could be clearly observed in SEM micrographs of uninoculated feathers. A considerable degradation of feather shaft and barbs was observed and fungal mycelia aggregate with an extracellular matrix to degrade the surface (Fig. 5B, C).

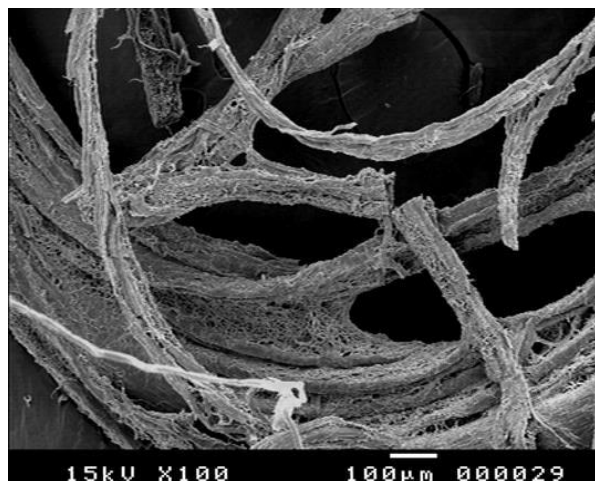


Fig. 5(b). Scanning electron micrograph of uninoculated chicken feather colonization of fungal mycelia on feather surface.

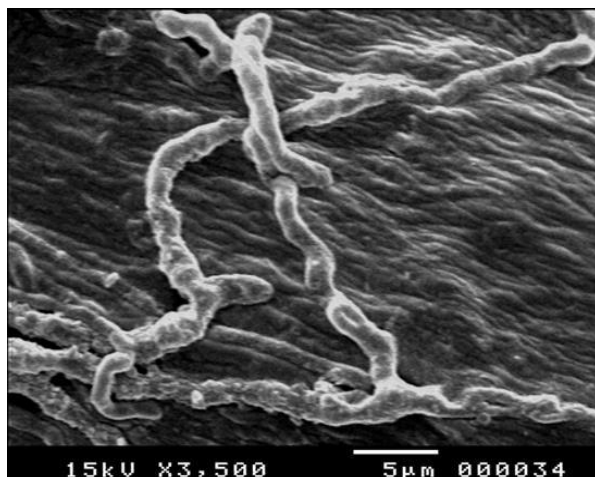


Fig. 5(c). Scanning electron micrograph of degradation of chicken feathers after 7 days and colonization of fungal mycelia on feather surface.

4. Conclusion

Aspergillus terreus was the best keratinase producing fungus in our screening. The optimization of keratinase production and its characterization showed potential characteristics for its possible application in commercial keratin degradation.

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