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Keratinase Production and Biodegradation of Some Keratinous Wastes by *Alternaria tenuissima* and *Aspergillus nidulans*

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Abstract: The rapid growth of poultry industry has linked with increased output of keratin containing wastes. Keratinous wastes can be readily fermented to useful products and commodity chemicals by the appropriate microbes. The present research concerning biodegradation of keratinous wastes. From 82 fungal isolates, 27 isolates have keratinolytic activity. Identification tests indicated that the potent isolates were *Alternaria tenuissima* K2 and *Aspergillus nidulans* K7. Using chicken feather powder as a sole source of carbon and nitrogen, keratinase productivity were 53.4 and 55.8 U mL^{-1} by *Alt. tenuissima* K2 and *A. nidulans* K7 at the 6th and 5th day of incubation, respectively. Using additional carbon and nitrogen sources were not found to promote keratinase productivity, except when using starch and maltose. pH 7.5, 35°C and 7.5% inoculum ratio were the best for both keratinase production and feather solubilization by both fungi. Among different keratin containing wastes, chicken, duck and goose feathers were the most degradable keratinous wastes by *Alt. tenuissima* K2 and *A. nidulans* K7. During the course of investigation, keratinase production and degradation of keratinous wastes were positively and significantly correlated. Incubation of the produced keratinases at the optimum pH (8.5) and temperature (40°C) with different keratinous wastes led to about 70% hydrolysis of chicken, duck, goose and turkey feathers after 24 h of incubation. Goat hair, sheep wool and buffalo horn showed lower response towards keratinolytic hydrolysis. Therefore, keratinous wastes can be biologically degraded by either isolated fungi or their keratinases into useful products.

Key words: Keratinous wastes, keratinse, *Alternaria tenuissima*, *Aspergillus nidulans*, biodegradation

INTRODUCTION

Keratinous wastes constitute a troublesome environmental contaminant that is produced in large quantities in commercial poultry processing plants and their utilization is of economic value as well as ecological significance. Feather wastes make a serious problem as environmental pollutant and recently in outbreaks of H5N1 virus. Currently, feather waste is utilized on a limited basis as a source of nitrogen for plants or as a dietary protein supplement for animal feedstuffs, prior to its use for that purpose, feathers were chemically treated to increase the digestibility and reduce the rigidity; this procedure has a disadvantage, in that,

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certain heat-sensitive amino acids, such as methionine, lysine and tryptophane are degraded. The degradation products may generate non-nutritive amino acids, such as lysinoalanine and lanthionine. That is why the enzymatic biodegradation may be a better alternative to improve their nutritional value and offers cheap and mild reaction conditions for the production of valuable products (El-Naghy *et al*., 1998; Gushterova *et al*., 2005; Marcondes *et al*., 2008). The main content of feather is keratin, which are insoluble proteins resistant to degradation by common proteolytic enzymes such as trypsin, pepsin and papain because of a high degree of cross-linking by disulphide bonds, hydrogen bonding and hydrophobic interactions (Ignatova *et al*., 1999; Marcondes *et al*., 2008). Many fungi especially that belongs to fungi imperfecti have high keratinolytic activity including the following genera: *Chrysosporium*, *Aspergillus*, *Alternaria*, *Trichurus*, *Curvularia*, *Cladosporium*, *Fusarium*, *Geomyces*, *Gleomastis*, *Monodictys*, *Myrothecium Paecilomyces*, *Stachybotrys*, *Urocladium*, *Scopulariopsis*, *Sepedonium*, *Penicillium* and *Doratomyces* (Santos *et al*., 1996; Gradisar *et al*., 2000; Farag and Hassan, 2004; Marcondes *et al*., 2008). However, the isolation of filamentous fungi that efficiently degrade keratinous wastes is very interesting.

Therefore, the utilization of keratinous wastes as a fermentation substrate (carbon and nitrogen sources) by keratin-degrading fungi offers a feasible microbial technology for obtaining keratinolytic enzymes. These enzymes, besides waste feather elimination, could find their application in the food industry, manufacturing of textiles, biodegradable films, glues and foils, cosmetics, leather industry and nitrogenous fertilizer for plants (Onifade *et al*., 1998; Schrooyen *et al*., 2001; De Toni *et al*., 2002; Gushterova *et al*., 2005).

This study is a report on isolation, screening and optimizing conditions of keratinolytic fungi for biodegradation of keratinous wastes using native isolates and their keratinases.

MATERIALS AND METHODS

Isolation and Screening of Keratinolytic Fungi on Agar Plates

Samples (10 g each) collected from chicken farm wastes containing decayed feathers were serially diluted in 90 mL sterilized tab water, shaken at 100 rpm for 10 min the resulted suspension was used as a source for fungal isolation on agar plates medium, which contain (g L^{-1}); agar; 15, $MgSO_4.H_2O$; 0.5, KH_2PO_4; 0.1, $FeSO_4.7H_2O$; 0.01 and $ZnSO_4.7H_2O$; 0.005, the pH was adjusted to 7.5. The medium was supplemented with 1% chicken feather powder as a sole source of carbon and nitrogen (Wawrzkiewicz *et al*., 1991). Keratinolytic activity of the fungi was detected as a clear zone around the colony after incubation up to 5 days at room temperature. The diameter of the clear zone was measured to quantify activity. By this way, 82 fungi were isolated. The most active fungi were identification according to Domsch *et al*. (1980).

Preparation of Chicken Feather Powder

Poultry feather was cut into small fragments washed extensively with water and detergent and dried in a ventilated oven at 40°C for 72 h. To prepare feather powder, the feathers were milled in a ball mill and passed through a small mesh grid to remove coarse particles. This feather powder was used during optimization of keratinase production, unless otherwise stated.

Inoculum Preparation

Spore suspension of the fungal isolates was prepared by adding 10 mL of sterilized water to 7 days old fungal isolates growing on plates of potato dextrose agar. The final concentration of the spore suspension was adjusted to about 2×10^6 mL^{-1}.

Culture Technique and Tested Parameters

In 500 mL Erlenmeyer flasks, 100 mL of the previously mentioned broth basal medium of Wawrzkiewicz *et al.* (1991) was added separately to 1.5 g of poultry feather powder in each flask then autoclaved. After cooling, the flasks were inoculated by 5 mL of spore suspension and incubated under shaking (120 rpm) at 28°C. At periodic intervals, final culture pH was determined and the cultures containing the hydrolysates were centrifuged (2000 rpm) and filtered through filter paper. The filtrate was recovered to determine the keratinase activity. The residual which containing the cells and undigested feather was dried to a constant weight. The feather solubility was determined as: (The dry weight of residual feather after incubation/Initial dry weight of feather)×100. For optimization studies, the following parameters were investigated; (1) time course of keratinase production (1 to 10 days), (2) the supplementation of the production medium with additional different carbon sources at 1% (w:v) which were sterilized separately and added aseptically to the sterilized medium, (3) additional nitrogen sources at 0.5% (w:v), (4) different values of initial medium pH (5.0 to 9.0) which was adjusted before autoclaving (5) different incubation temperatures (20 to 50°C) and (6) inoculum ratio (2.5 to 20%, v/v).

Degradation of Different Keratinous Wastes by the Isolated Fungi

For studying the biodegradation of different keratinous materials, the keratinous wastes (chicken feather, duck feather, goose feather, turkey feather, sheep wool, goat hair and buffalo horn) were fragmented into pieces with about 1 cm long and added to the fermentation media as a sole source of carbon and nitrogen instead of poultry feather powder. These sources were added separately to the fermentation media at 0.5, 1.0, 1.5 and 2%, w/v. All other optimum conditions were used. Keratinase activity and the percent of keratinous waste solubilization were determined after incubation.

Determination of Keratinolytic Activity

Keratinase activity was assayed by the modified method of Yu *et al.* (1968). In brief, 20 mg of chicken feathers powder were suspended in 3.8 mL of 100 mM Tris-HCl buffer (pH 7.8) to which 300 μL of the culture filtrate (enzyme source) was added. The reaction mixture was incubated at 37°C for 1 h. After incubation, the assay mixture was dipped into ice-cold water for 10 min and the remaining feathers were filtered out. Then the absorbance of the clear mixture was measured at 280 nm using UV-spectrophotometer. The keratinase activity was expressed as one unit of the enzyme corresponding to an increase in the absorbance value 0.01 h^{-1}.

Keratinase Activity as Influenced by pH and Temperature

The effect of pH (5 to 10.5) on the keratinase activity was determined in 0.1 mol L^{-1} sodium acetate (pH 5.0 and 5.5), 0.1 mol L^{-1} phosphate (pH 6.0 to 7.5), 0.1 M Tris-HCl (pH 8.0 to 9.0) and 0.1 M borate (pH 9.5 to 10.5) buffers. The effect of temperature on the keratinase activity was measured at temperatures ranging from 25 to 65°C.

Hydrolysis of Keratin Containing Wastes by the Crude Enzyme

Keratinous wastes were cut into small fragments, washed extensively with water and detergent and dried in a ventilated oven at 40°C for 72 h, milled in a ball mill and sieved to remove coarse particles. 500 unites of enzyme was added to 0.5 g of each of the milled keratinous materials in 45 mL of 0.1 M Tris-HCL buffer, pH 8.5 and incubated at 40°C in a shaking water bath at 130 rpm for up to 24 h. Controls of keratinous materials were

done in the same conditions, using culture filtrate previously boiled for 5 min. The dry weight of the remaining keratinous materials was determined on membrane filters (pore size, 0.2 mL) after drying at 105°C for 12 h (Moreira *et al*., 2007).

RESULT AND DISCUSSION

Isolation and Identification of Keratinolytic Fungi

Concerning the isolation trials, 82 fungi belonging to more than six genera were isolated from chicken farm wastes containing decayed feathers on agar plates containing feather keratin (Table 1). The primary screening showed that most of the isolated fungi were able to grow in the given environmental conditions. Some of them showed weak growth but did not clarify the keratin agar, presumably due to the lack of extracellular keratinase. Meanwhile, the others (27 isolates) exhibited growth and clear zone on agar plates. It appeared that *Alternaria*, *Aspergillus*, *Fusarium, Penicillium* and *Trichoderma* were the most common active keratinolytic genera, representing more than 88% of the total positive keratinolytic fungi. The results are in accordance with those obtained by Friedrich *et al.* (1999).

As described by Onifade *et al.* (1998) and Farag and Hassan (2004), most of keratinolytic fungi are belonging to fungi imperfecti. The identification and characterization of new fungal species able to degrade keratinous wastes may help to understand the role of fungi in the degradation of complex keratinous substrates in nature, In addition, the ability of such fungi to grow and to produce appreciable levels of keratinase using keratinous wastes as substrates could be potentially useful for the development of biotechnological methods aiming to obtain useful hydrolysis products (Gioppo *et al*., 2009).

Table 1: The common fungal genera and percent of keratinolytic fungi isolated from decayed feathers on agar plates

Genus	No. of isolated species	Positive keratinolytic fungi	
		Number	%
Alternaria	3	2	7.4
Aspergillus	13	9	33.3
Fusarium	15	7	25.9
Penicillium	24	5	18.5
Trichoderma	17	1	3.7
Others	10	3	11.1
Total	82	27	100.0

Screening of the Most Active Keratinolytic Fungi

Based on the previous results, the most active isolates on keratin plates, were identified as *Aspergillus nidulans* K7, *Fusarium culmorum* K23, *Alternaria tenuissima* K2, *Aspergillus niveus* K11, *Aspergillus flavus* K6, *Trichoderma viride* K63, *Alternaria alternate* K1 and *Penicillium* sp. K40, the corresponding clear zone diameter on feather keratin agar plates were 43, 42, 41, 41, 35, 26, 16 and 14 mm, respectively. These isolates were also quantitatively tested in submerged culture for keratinolytic activity using poultry feather powder as the only source of carbon and nitrogen (Fig. 1). It was observed that the highest activity was reached with *A. nidulans* K7, *Alt. tenuissima* K2, *A. niveus* K11 and *F. culmorum* K23, which have the highest extracellular keratinase production as they recorded 45.2, 41.7, 39.8 and 38.3 U mL^{-1}, respectively.

In this respect, Marcondes *et al*. (2008) found that among 106 filamentous fungi isolated from poultry farm waste, 13 species belonging to seven genera (*Aspergillus*, *Acremonium*,

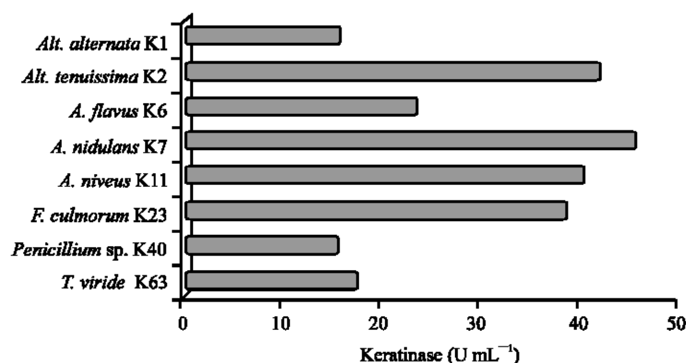

Fig. 1: Keratinolytic activity of the most active fungal isolates in submerged culture

Alternaria, *Beauvaria*, *Curvularia*, *Paecilomyces* and *Penicillium*) were able to grow and produce keratinase in stationary cultures using poultry feather powder as the only substrate. Friedrich *et al*. (1999) screened 300 common fungi for synthesis of extracellular keratinase, about 54% of the fungi grew on agar plates with soluble keratin and excreted the enzyme. Some representatives of *Fusarium*, *Acremonium* and *Geotrichum* were the most active and others (*A. flavus*, *Alt. radicina*, *Trichurus spiralis* and *Stachybotrys atra*) proved to be powerful producer of extracellular keratinases when cultivated in submerged conditions in a medium with porcine nail as the sole source of carbon and nitrogen. Some species of fungi are already known to colonize feathers or wool and some, such as *Alt. alternata* and *Alt. tenuissima, A. flavus, Stachybotrys chartarum* and *Trichurus spiralis*, are known as protease producers (Domsch *et al*., 1980). In the present research, quite important differences were detected among isolated fungi, hereby; *A. nidulans* K7, *Alt. tenuissima* K2, *A. niveus* K11 and *F. culmorum* K 23 were selected according to their keratinolysis.

Time Course of Keratinase Production by Selected Fungi

After the previous screening, the selected fungi were cultivated in submerged shaken cultures. As shown in Fig. 2, *A. nidulans* K7 and *Alt. tenuissima* K2 started with relatively high activity in first day (13.8 and 10.2 U mL^{-1}, respectively), with gradual increase to reach their maximum activities at the 5th day for *A. nidulans* K7 (55.8 U mL^{-1}) and 6th day for *Alt. tenuissima* K2 (53.4 U mL^{-1}), with slight decrease in activity to reach 45.2 and 39.8 U mL^{-1} at the 10th day for *A. nidulans* K7 and *Alt. tenuissima* K2, respectively. On the other hand, *A niveus* K11 and *F. culmorum* K23 started with low level of activity i.e., 3.3 and 6.6 U mL^{-1} and reached to the maximum activity after 6 and 7 days of incubation, recording 44.5 and 45.6 U mL^{-1}, respectively. Noronha *et al.* (2002) found the maximum yield of keratinase after 3 days by *A. fumigates*, meanwhile, Gradisar *et al.* (2000) and Gioppo *et al.* (2009) reported 4 days as the optimum incubation period and they considered this period as a very short time, whereas, 5 days was found to be the optimum for *Penicillium* sp. keratinase production (El-Gendy, 2009).

It is apparent (Fig. 2) that there was a change in the final culture pH of the medium towards alkalinity. The four fungal isolates showed significant positive correlation coefficient (r) between pH and production of keratinase, the values of r ranged from 0.88 to 0.98 at $p = 0.01$. Several studies (Malviya *et al*., 1992; Kaul and Sumbali, 2000; Muhsin and Hadi, 2001) documented that saprophytic fungi grown on keratinous substrates have the ability to extract nutrients from such substrates; apparently, there is a change in the pH of the media towards alkalinity after keratinase production and protein releasing by the tested fungi. The

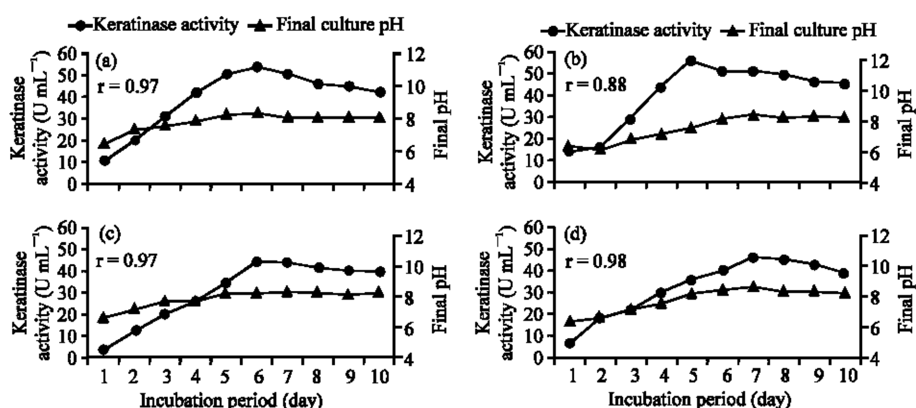

Fig. 2: Keratinase production by (a) *Alt. tenuissima* K2, (b) *A. nidulans* K7, (c) *A. niveus* K11 and (d) *F. culmorum* K23 as a function of cultivation time

ability of such fungi to alkalinize culture media, can be explained by accumulation of ammonium (the products of deamination) and other keratin degradation products (Marcondes *et al*., 2008; Gioppo *et al*., 2009) this variation in pH towards alkalinity also suggest keratinolytic activity. From the previous results and according to keratinase production, *A. niveus* K11 and *F. culmorum* K23 were excluded from this investigation.

Additional Carbon and Nitrogen Sources

Most of keratinolytic fungi can utilize feather as the sole source of carbon and nitrogen. To improve the enzyme production, many additional carbon sources were examined. In Table 2, the highest keratinolytic activity and feather solubilization were recorded with starch and maltose. It is worthy to note that, with the exception of starch and maltose, the extra addition of carbon sources to the fermentation medium led to remarkable reduction in feather solubilization (Fig. 3), in spite the obvious improvement in keratinase production. On the other hand, CMC and pectin negatively modulated keratinase production and feather solubilization in both fungal isolates. This means that this enzyme is inducible. In general, enzyme production is not totally repressed by the tested carbon sources and the major regulatory mechanism is induction of keratinase production by the substrate. These results are in accordance with the findings of Ignatova *et al*. (1999) and Ramnani and Gupta (2004). Simple sugars have been reported to suppress the synthesis of keratinase (Moreira *et al*.,

Table 2: Effect of additional carbon sources on keratinolytic activity of *Alt. tenuissima* K2 and *A. nidulans* K7

	Alt. tenuissima K2		*A. nidulans* K7	
Additional carbon source	Keratinase activity (U mL^{-1})	Feather solubilization (%)	Keratinase activity (U mL^{-1})	Feather solubilization (%)
Control	53.4	30.1	55.8	32.1
Glucose	69.9	20.1	68.8	24.3
Fructose	56.5	21.5	69.1	22.5
Mannose	60.1	23.4	50.9	24.0
Maltose	73.8	31.6	75.3	35.4
Lactose	71.6	29.8	73.0	22.6
Sucrose	55.1	19.5	67.9	23.5
Starch	75.4	31.5	77.4	38.6
CMC	50.6	22.3	50.1	26.5
Pectin	51.1	19.5	51.6	23.4

2007). Mabrouk (2008) reported that starch and glucose highly inhibited keratinase production by *Streptomyces* sp. MS-2. However, in some organisms the opposite was found, since increasing in keratinase production with the extra addition of carbon sources was previously reported (El-Naghy *et al*., 1998; Anbu *et al*., 2008; Son *et al*., 2008). The absence of carbon source drive the fungus to assimilate the keratin as carbon source which increase the percentage of solubilization as the keratinolytic organisms are capable of using keratin as the sole source of carbon and nitrogen (Szabo *et al*., 2000; Gousterova *et al*., 2005). Malviya *et al*. (1992) on *Chrysosporium queenslandicum* and El-Naghy *et al*. (1998) on *Chrysosporium georgiae* noted that the keratinase enzyme was inducible by keratin and its production was stimulated by glucose and inhibited by ammonia.

Concerning the use of nitrogen sources different from keratin, the extra addition of nitrogen sources have no or depressive effect on keratinase production as well as solubilization of faether keratin (Table 3 and Fig. 4). Among nine different nitrogen sources,

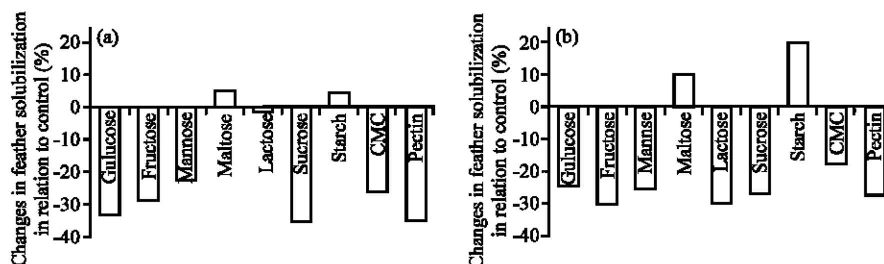

Fig. 3: Changes in feather solubilization (%) by (a) *Alt. tenuissima* K2 and (b) *A. nidulans* K7 in relation to control as a response of additional carbon source

Table 3: Effect of additional nitrogen source on keratinolytic activity of *Alt. tenuissima* K2 and *A. nidulans* K7

	Alt. tenuissima K2		*A. nidulans* K7	
Additional N source	Keratinase activity (U mL^{-1})	Feather solubilization (%)	Keratinase activity (U mL^{-1})	Feather solubilization (%)
Control	75.4	33.5	77.4	38.6
$(NH_4)_2SO_4$	63.6	24.3	61.6	26.6
$NH_4H_2PO_4$	47.4	15.8	44.1	13.4
NH_4Cl	52.1	17.1	53.7	20.6
$NaNO_3$	60.7	22.9	60.5	25.8
KNO_3	49.2	17.5	46.3	15.1
$(NH_4)_2C_6H_6O_7$	49.2	14.9	50.8	18.5
Gelatin	64.8	30.1	61.0	26.2
Peptone	63.0	28.8	59.3	24.9
Corn steep liquor	79.8	31.5	76.3	37.7

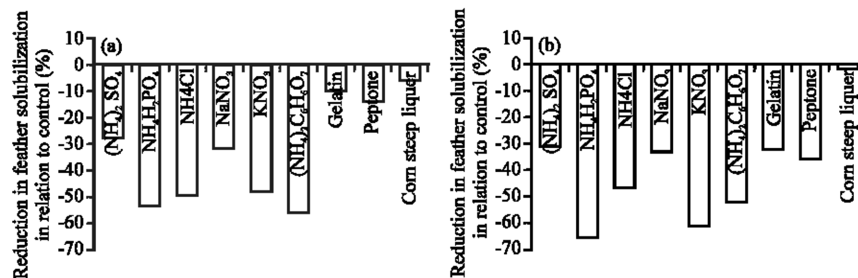

Fig. 4: Reduction in feather solubilization (%) by (a) *Alt. tenuissima* K2 and (b) *A. nidulans* K7 in relation to control as a response of additional nitrogen source

$NH_4H_2PO_4$, ammonium citrate ($(NH_4)_2C_6H_6O_7$), NH_4Cl and KNO_3 highly inhibited keratinase production and feather solubilization by *Alt. tenuissima* K2 and *A. nidulans* K7. These data confirm the inducible nature of keratinase. Malviya *et al.* (1992) and El-Naghy *et al.* (1998) noted that the keratinase enzyme was inhibited by ammonia. Santos *et al.* (1996) reported that additional nitrate supported the mycelial growth, but it repressed keratinase production in *A. fumigates*. However, most reports describe a partial or complete repressive effect of the supplementation of cultures with small nitrogen molecules (Malviya *et al.*, 1992; El-Naghy *et al.*, 1998; Son *et al.*, 2008). However, the drastic reduction in the keratinase production is due, or at least overwhelmingly due to catabolite repression by the nitrogen sources (Gioppo *et al.*, 2009). Contrary, when Veselal and Friedrich (2009) used microorganisms directly for the biodegradation of different keratin containing wastes, less solubilization of keratinous wastes could be observed than with the use of additional carbon and nitrogen sources since the microorganism itself consumes the released products. An inductive effect of soybean meal on keratinase production has been also reported to occur (Gradisar *et al.*, 2000).

Effect of pH on Keratinase Production

Regarding to the keratinase production vis pH, Fig. 5 shows that *Alt. tenuissima* K2 and *A. nidulans* K7 yielded the maximum amount of keratinase as well as feather solubilization at pH 7.5 and declined, thereafter, with the initial medium alkalinity. Keratinase production and feather solubilization was positively and significantly correlated ($r = 0.99$ and 0.98, at $p = 0.01$, respectively). Several reports are available on keratinolytic microorganisms and their biotechnological potential with respect to keratinases production at pH 6.0 to 9.0 (Gupta and Ramnani, 2006; El-Gendy, 2009) with high activity at 8.0 for *Trichophyton* sp. HA-2 (Anbu *et al.*, 2008). However, very few microorganisms were reported to be active above pH 11.0 (Gessesse *et al.*, 2003).

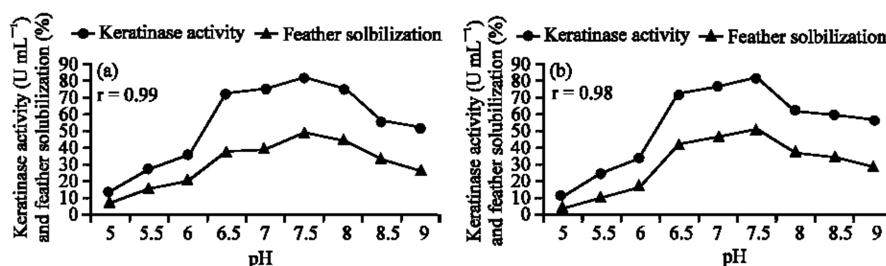

Fig. 5: Keratinase production by (a) *Alt. tenuissima* K2 and (b) *A. nidulans* K7 at different initial culture pHs

Keratinase Production in Relation to Incubation Temperature

As depicted in Fig. 6, the keratinolytic activity was detectable between 20 and 50°C, but showing a maximum activity at 35°C for both *Alt. tenuissima* K2 (80.8 U mL^{-1}) and *A. nidulans* K7 (79.7 U mL^{-1}). At the same manner, feather solubilization (%) increased with temperature and recorded 44.8 and 46.5%, respectively, at 35°C. Both isolates still show a positive significant correlation between keratinase production and feather solubilization. These results are similar to that reported for keratinases of other microorganisms, such as *Trichophyton* sp. (Anbu *et al.*, 2008), *Streptomyces pactum* (Bockle and Muller, 1997) and *Bacillus licheniformis* (Lin *et al.*, 1992), the above-mentioned studies reported optimum temperatures near 40°C. Whereas, El-Gendy (2009) reported that 26°C was the optimum for *Penicillium* sp.

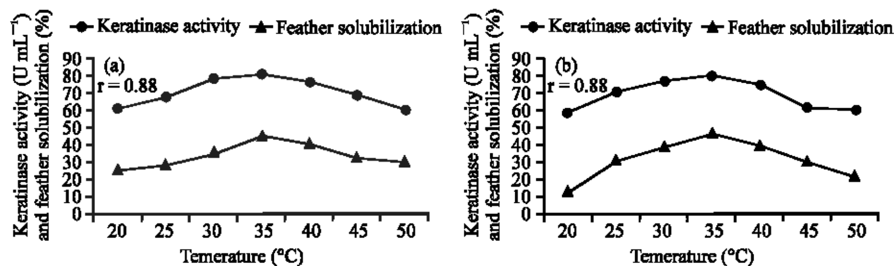

Fig. 6: Keratinase production by (a) *Alt. tenuissima* K2 and (b) *A. nidulans* K7 at different incubation temperatures

Keratinase Production at Different Inoculum Ratios

Inoculum ratio is one of important factor affecting keratinase production and feather solubilization. The pattern of keratinase production with respect to inoculum ratio indicated that, with the gradual increase in inoculum ratio, *Alt. tenuissima* K2 and *A. nidulans* K7 showed positive improvements in their activities and reached the maximum keratinase productivity (83.2 and 80.9 U mL^{-1}, respectively) and feather solubilization (56.3 and 53.4%, respectively) at 7.5% inoculum ratio (Fig. 7), both of the tested criteria, slightly, decreased when inoculum ratios were out of this point. Larger inoculum ratio has been shown to affect adversely the yield of keratinase by *Beauveria bassiana* (Suresh and Chandrasekaran, 1999) and *Penicillium* sp. (El-Gendy, 2009). Data (Fig. 7) of the analysis of correlation of coefficient continue providing evidence for the significant positive correlation between keratinase production and feather solubilization for both isolates. Conversely, Singh (1997) did not find any relation between the rate of enzyme production and keratin degradation.

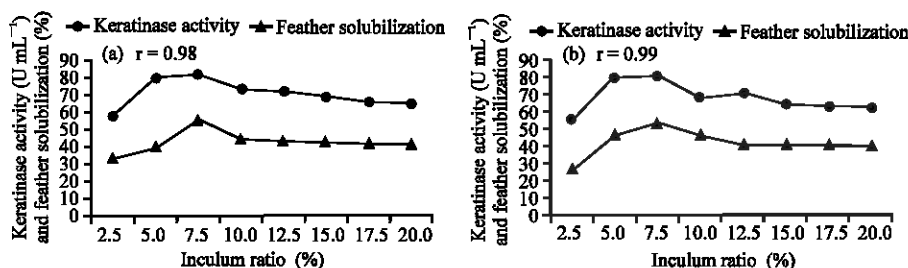

Fig. 7: Keratinase production by (a) *Alt. tenuissima* K2 and (b) *A. nidulans* K7 as affected by inoculum ratio

Biodegradation of Keratinous Wastes by *Alt. tenuissima* K2 and *A. nidulans* K7

Different keratinous wastes were used as a sole source of carbon and nitrogen in the growth medium of the tested fungi. The fungi under investigation were able to grow normally, using all keratin-containing wastes as their sole source of carbon and nitrogen (Tables 4 and 5). Generally, *A. nidulans* K7 possessed better keratinase production and solubilization percent than *Alt. tenuissima* K2 on different keratinous wastes. Chicken feather recorded high ability to be degraded by both fungi compared to the other keratin sources. Additionally 1.5% of different keratinous wastes was found to be optimum for the highest keratinase production and waste solubilization percent. Moreover, the mathematical relation between enzyme production and biodegradation of keratinous wastes at 1.5% waste concentration is depicted in Fig. 8. It is clear the positive correlation coefficient which shown

Table 4: Biodegradation of different keratinous wastes by *Alt. tenuissima* K2

	Keratinous waste concentration (%)							
	Keratinase activity (U mL^{-1})				Solubilization (%)			
Source	0.5	1.0	1.5	2.0	0.5	1.0	1.5	2.0
Chicken feather	33.0	48.6	68.6	61.9	22.2	43.1	50.3	47.2
Duck feather	34.5	50.4	66.3	63.1	25.3	40.3	49.3	43.2
Goose feather	29.5	48.6	65.4	64.4	21.4	35.1	46.3	40.2
Turkey feather	29.5	46.3	60.9	63.5	21.1	36.0	41.2	43.5
Goat hair	26.5	36.6	55.3	56.9	19.3	29.6	33.6	37.1
Sheep wool	35.6	40.5	58.9	62.2	21.6	33.2	41.0	43.4
Buffalo horn	44.3	51.3	41.6	40.0	30.2	33.8	30.5	30.1

Table 5: Biodegradation of different keratinous wastes by *A. nidulans* K7

	Keratinous waste concentration (%)							
	Keratinase activity (U mL^{-1})				Solubilization (%)			
Source	0.5	1.0	1.5	2.0	0.5	1.0	1.5	2.0
Chicken feather	43.3	51.9	73.6	66.2	33.5	40.5	53.4	44.5
Duck feather	40.5	51.2	66.8	55.5	33.0	40.5	50.2	41.2
Goose feather	31.2	50.3	67.6	60.3	26.3	37.8	40.6	40.0
Turkey feather	30.1	48.8	61.1	60.1	25.5	35.3	39.5	39.4
Goat hair	25.6	37.6	57.8	58.2	23.6	33.1	40.3	40.5
Sheep wool	40.2	50.2	59.3	64.6	30.3	40.2	43.5	46.6
Buffalo horn	46.6	56.4	50.6	50.1	36.3	43.6	40.0	40.2

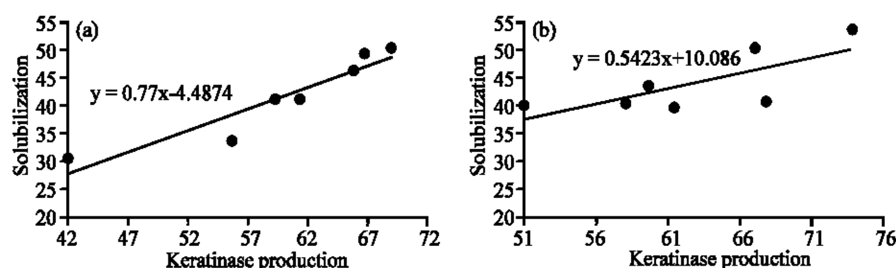

Fig. 8: Linear regression shows the relation between keratinase production and solubilization of keratinous wastes at 1.5% concentration by (a) *Alt. tenuissima* K2 and (b) *A. nidulans* K7

on the linear regression; as the enzyme production increased, remarkable degradation of keratinous wastes was induced by both fungi. *Alt. tenuissima* K2 recorded positive significant correlation while, *A. nidulans* K7 recorded positive non-significant correlation. Marcondes *et al.* (2008) found similar results.

The complete mechanism of keratin degradation is not fully understood. Basically, microbial keratinolysis is a proteolytic, protein-degrading process for the simple reason that keratin is a protein (Gupta and Ramnani, 2006). The high mechanical stability of keratin and its resistance to proteolytic degradation is due to the tight packing of the protein chains through intensive interlinkage by cystine bridges (Bockle and Muller, 1997). The capability of filamentous fungi to degrade keratin may be the result of a combination of extracellular keratinase, mechanical keratinolysis (mycelial pressure and/or penetration of the keratinous substrate), sulphitolysis (reduction of disulphide bonds by sulphite excreted by mycelia) and proteolysis (Bockle and Muller, 1997; Onifade *et al.*, 1998; Gupta and Ramnani, 2006).

Enzymatic or chemical reducing agents in form of disulfide reductases, sulfite, thiosulfate or cellular membrane potential may play a significant role in the degradation of this insoluble protein, additionally, the initial attack by keratinases and disulfide reductases may allow other less specific proteases to act, resulting in an extensive keratin hydrolysis (Gioppo *et al.*, 2009).

Effect of pH and Temperature on Enzymatic Reaction

The keratinolytic activities of the culture fltrates of both fungi were detectable over a wide range of pH and temperature. Keratinase activity was found to be better in the alkaline conditions ranging from pH 8.0 to 9.0 for *Alt. tenuissima* K2 and from pH 7.5 to 9.0 for *A. nidulans* K7, with an optimum at 8.5 for both enzymes (Fig. 9). Over a wide range of temperatures (from 25 to 65°C), keratinase showed maximum activity at 40°C (Fig. 10). Keratinase from *A. nidulans* K7 was found to be more active at the higher temperatures than keratinase of *Alt. tenuissima* K2.

Keratinases from most bacteria, actinomycetes and fungi have pH optima in neutral to alkaline range (Riffel *et al.*, 2003; Farag and Hassan, 2004; Thys *et al.*, 2004; Anbu *et al.*, 2005). Other keratinolytic enzymes have been also proved to be active at alkaline pH, as those secreted by *Streptomyces* sp. (Letourneau *et al.*, 1982) and *Streptomyces albidoflavus* (Bressolier *et al.*, 1999). However, few keratinases possess extreme alkalophilic optima of pH>12 (Gupta and Ramnani, 2006). Moreover, the optimum temperature of keratinases ranges from 30 to 80°C (Gupta and Ramnani, 2006) but, the enzyme from *Chrysosporium*

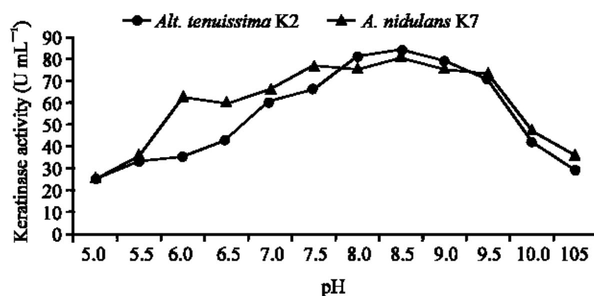

Fig. 9: Activity of keratinase of both *Alt. tenuissima* K2 and *A. nidulans* K7 at different pHs

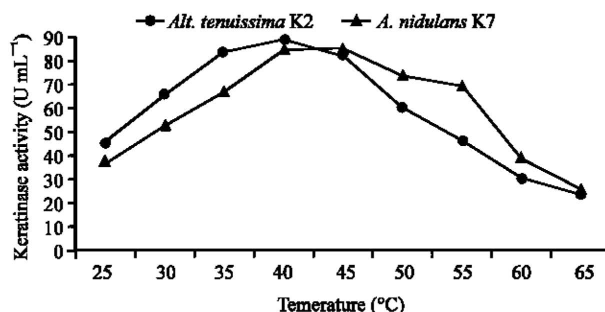

Fig. 10: Activity of keratinase of both *Alt. tenuissima* K2 and *A. nidulans* K7 at different temperatures

keratinophilum (Dozie *et al*., 1994) and thermophile *Fervidobacterium islandicum* (Nam *et al*., 2002) showed exceptionally high temperature optima of 90 and 100°C, respectively.

Degradation of Keratinous Wastes by the Crude Keratinase

The crude culture filtrates of both fungi were able to catalyze the biodegradation of native keratin of different keratinous wastes. At pH 8.5 and 40°C, both enzymes were able to hydrolyze efficiently keratin of all tested feather (Table 6). The degradation of different keratinous wastes by the two tested enzymes increased with the prolongation of time up to 24 h. More than 70% hydrolysis of chicken, duck, goose and turkey feathers was observed, after 24 h of enzyme-waste incubation. Goat hair, sheep wool and buffalo horn poorly hydrolyzed and showed lower response towards keratinolytic hydrolysis. The keratinolytic activity of crude enzyme was confirmed because the action of enzyme into poultry feather meal reduces its weight (Moreira *et al*., 2007). The keratinolytic activity of microorganisms is normally associated with the production of serine proteases or metalloproteases, irrespective of the microorganism (Gupta and Ramnani, 2006), with the exception of yeasts, which produce keratinases belonging to the aspartic proteases (Monod *et al*., 2002). The lower level of degradation of buffalo horns and goat hair at the expense of higher release of enzyme might be due to the restricted substrate specificity of the enzyme or removal of some accessory proteins capable of splitting the disulphide bonds present in the keratin molecules during the hydrolysis process (Singh, 1997).

Table 6: Hydrolysis of various keratinous wastes using the crude keratinase of *Alt. tenuissima* K2 and *A. nidulans* K7

	Residual keratinous wastes (%)			
	Alt. tenuissima K2		*A. nidulans* K7	
Source	12 h	24 h	12 h	24 h
Chicken feather	60.1	29.0	51.3	23.5
Duck feather	56.4	31.2	55.3	25.6
Goose feather	58.6	29.3	55.3	24.9
Turkey feather	63.1	33.7	60.1	25.3
Goat hair	70.4	64.9	71.5	67.9
Sheep wool	60.2	57.5	66.6	56.2
Buffalo horn	86.6	77.9	81.3	73.2

Finally, in Egypt like other many countries with high population and limited resources, recycling of wastes like keratin containing wastes is very important, in this study *A. nidulans* and *Alt. tenuissima* showed good result in this trend. The association of cheap and readily available keratinous wastes could result in a substantial reduction in the costs of enzyme production. Additionally, the hydrolysis of such wastes provide beneficial product that could find their application in several industries e.g., biodegradable films, glues and leather as well as in agriculture as nitrogenous fertilizer for plants.

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