Microbial bioremediation of feather wastes and eco-friendly production of keratinase and collagenase enzymes by Didymella keratinophila, the first-recorded fungus from Assiut, Egypt

Osama Abdel-Hafeez Mohamed Al-Bedak (osamaalbedak@gmail.com)
Assiut University Faculty of Science https://orcid.org/0000-0003-0465-619X
Ahmed Mohamed Moharram
Assiut University Faculty of Science
Nemmat Abdel-Gawad Hussein
Assiut University Faculty of Science https://orcid.org/0000-0002-8572-2437
Doaa Mohamed Taha
Assiut University Faculty of Science

Research Article

Keywords: Bioconversion, Collagenase, Didymella, Eco-friendly, Feather, Keratinase, Fermentation

Posted Date: July 12th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1645003/v1

Abstract

During a routine examination of Coelomycetes isolated from various fruit juices in Assiut Governorate, Egypt, a unique isolate was discovered and visually identified as a *Didymella* member. The fungus was confirmed as *Didymella keratinophila* based on sequencing of the internal transcribed spacer (ITS), since it had 100% identity to the type strain. This is the first time the fungus has been recorded in Egypt. The strain was able to flourish and hydrolyze native chicken feather in SmF to produce keratinase and collagenase enzymes. The keratinase enzyme performed best at pH 7.0 and 45 ^{o}C, yielding 44903 ± 1555 IU/mg keratinase activity, while collagenase enzyme be really at pH 6.0 and 8.0 at 35 ^{o}C and 60 ^{o}C, yielding 15753 ± 110 IU/mg and 16312 ± 21 IU/mg, respectively indicating two collagenases. The keratinases and collagenases produced were significantly inhibited by EDTA and the ions. Mn^{+2} had the most effective inhibitory effect on keratinases while K^{+} had the most potent inhibitory influence on collagenase. The current study identifies a feasible producer of keratinase and collagenase enzymes sourced from chicken feathers, providing a safe method for using these wastes to produce high-value commodities.

Introduction

Wool, hooves, horns, hair, nails, and feathers all include keratins as structural constituents. Each year, 40 million tonnes of keratinous wastes are produced in the United States, Brazil, and China (Sharma and Gupta 2016; Alwakeel et al. 2021). The meat industry produces millions of tonnes of keratinous wastes at slaughterhouses across the world per year (Verma et al. 2017). Up to two million tonnes of chicken feathers are produced worldwide (Reddy et al. 2021). Keratins are insoluble fibrillar proteins found on vertebrates' outer protective surfaces. Because of the solid stabilization of their polypeptide chains and the many disulfide connections that bridge these chains, keratinous materials are noted for their exceptional stability (Hassan et al. 2020a). Current keratin waste treatment methods include landfilling, chemical treatment, and incineration (Khumalo et al. 2020). These treatments are inadequate but since they 1) reduce soil space; 2) increase the amount of toxic chemicals such as halogens and salts in water bodies, endangering aquatic life; 3) increase greenhouse gas emissions, contributing to global warming; and 4) increase eutrophication due to the high amount of nitrogen and alkalinity in aquatic physiology (Vidmar and Vodovnik 2018).

Common protein-degrading enzymes, such as pepsin and papain, do not digest keratins. Keratinous wastes management requires a low-cost solution, particularly in underdeveloped nations. Fungi and bacteria are known to hydrolyze keratinous materials by producing extracellular keratinolytic enzymes (Gafar et al. 2020; Bohacz et al. 2020; Nnolim et al. 2020). Different fungi, such as *Aspergillus*, *Chrysosporium*, *Trichophyton*, and *Microsporum*, release keratinases that are generally extracellular inducible enzymes (Fang et al. 2013; Akhter et al. 2020; Akram et al. 2020). Keratinase powder, for example, is a commercial product made from the bacteria *Bacillus licheniformis* and the fungus *Parengyodontium album*. In the pharmaceutical industry, keratinases are utilized in the manufacturing of

vaccines and the creation of bioactive peptides and serums, as well as treatment of calluses, keratinized skin, psoriasis, and acne (Avdiyuk and Varbanets 2019). Keratinases are used in the production of cosmetics such as anti-dandruff shampoos, nutritious lotions, and creams, as well as in feed formulae, nitrogen fertilizers, and leather industry (Gafar et al. 2020; Abdel-Fattah et al. 2018; Moridshahi et al. 2020). Another unique application is the utilization of keratinases to remediate wastewater including keratin wastes (Vidmar and Vodovnik 2018).

Collagenases enzymes have the ability to hydrolyze peptide bonds in both native and denatured collagen. They are widely employed not only in the chemical and medical sectors, but also in food and fundamental biological science (Hisano et al. 1989). Collagen may be created in excess of the desired quantity, in inappropriate locations, or may not disintegrate after a given period of time. In such circumstances, injectable collagenase or collagenase ointment can aid in collagen decomposition. Collagenase-producing microorganisms and their collagenases play critical roles in collagen degradation and organic nitrogen recycling in the ocean. However, only a few collagenase-producing bacteria have been so far discovered over the last 40 years, including *Pseudomonas marinoglutinosa* (Hanada et al. 1973), *Clostridium histolyticum* (Van Wart and Steinbrink 1981), *Pseudomonas* sp. (Hisano et al. 1989), *Bacillus licheniformis* N22 (Asdornnithee et al. 1994), *Bacillus subtilis* FS-2 and *Bacillus subtilis* M2-4 (Tran and Nagano 2002), *Flocculibacter collagenilyticus* (Li et al. 2021), as well as from *Candida albicans* URM3622 (Lima et al. 2009).

Several fungal genera such as *Acremonium*, *Aphanoascus*, *Aspergillus*, *Chrysosporium*, *Cladosporium*, *Doratomyces*, *Fusarium*, *Lichtheimia*, *Microsporum*, *Paecilomyces*, *Scopulariopsis*, *Trichoderma*, and *Trichophyton* have been documented to have the keratinolytic ability. Many of which are human pathogens that have the capacity to destroy both hard and soft keratins (Köhler et al. 2015). On the other hand, the non-pathogenic fungi, on the other hand, have industrial use since they do not cause disease. As a result, more research into suitable microbes is needed to develop safe keratinase producers. However, because the majority of these microorganisms lacked keratinase and collagenase activity, their applications were limited. Thus, the purpose of this work was to find suitable and efficient fungal species capable of decomposing native chicken feather to create keratinase and, for the first time, collagenase for use in diverse biotechnological applications.

Materials And Methods

Strain isolation

The pour plate technique was used to isolate the strain from a mango juice sample taken from a local market in Assiut Governorate, Egypt. In Petri plates containing 20 mL Oat agar medium (OA, (Smith and Onions 1994), 1.0 mL of the sample was placed. The plates were then incubated for 15 days at 25°C. The developed fungi were then isolated and maintained as pure cultures at -86 C in 20% glycerol/water, as well as on cotton balls, as stated by Al-Bedak et al. (2019).

Morphological identification of the strain

Using inoculum size of 1 µl/spot, plates were inoculated in a three-point pattern on malt extract agar (MEA), potato dextrose agar (PDA) and oat agar (OA) (Smith and Onions 1994), using spore suspension (prepared in a 30% glycerol, 0.2% agar and 0.05% Tween 80 solution). Microscopic features on PDA were examined in lacto-phenol cotton blue after 7 days of incubation at 25°C using Zeiss microscope (Axio Star, Germany).

Molecular confirmation of the strain

DNA extraction, PCR and sequencing

For DNA isolation, a small piece of 7-day-old culture of *Didymella* sp. AUMC 15399 grown on MEA at 25 ºC was collected and transferred to 2 ml-Eppendorf tubes. DNA extraction was conducting according to Moubasher et al. (2019). The PCR reaction was performed using SolGent EF-Taq (Al-Bedak and Moubasher 2020). The universal primers ITS1 and ITS4 were used for ITS region amplification (White et al. 1990).

Phylogenetic analysis

The DNASTAR computer program (version 5.05) was used to produce a contiguous sequence of *Didymella* sp. AUMC 15399. The whole ITS dataset was utilized for phylogenetic analysis, which included one sequence for *Didymella* sp. AUMC 15399 in this work, 34 sequences from the genus *Didymella* downloaded from GenBank, and a sequence for *Boeremia lycopersici* CBS 161.47 which used as an outgroup. All sequences were aligned using MUSCLE (Edgar 2004), and optimized manually. Maximum-likelihood (ML) analysis was carried out under the following settings: heuristic searches with random stepwise addition of 1000 replicates (Felsenstein 1985), and Tree-Bisection-Regrafting (TBR) rearrangements, using MEGA X (version 10.2.6) (Kumar et al. 2018). The best optimal model of nucleotide substitution for the ML analyses was determined using akaike information criterion (AIC) as implemented in Modeltest 3.7 (Posada and Crandall 1998). Maximum-likelihood (ML) and Maximum parsimony (MP) phylogenetic analyses were performed using MEGA X (Kumar et al. 2018), and the resulting tree was edited using Microsoft Power Point (2016) and saved as TIF file (Al-Bedak 2020).

Keratin powder extraction

Native chicken feathers (100 g) were obtained from chicken farms in Assiut Governorate, Egypt. The obtained feather sample was defatted for 24 h using continuous agitation with chloroform–methanol (1:1), then washed three times with distilled water and oven-dried at 50 ºC. The procedure outlined in Alwakeel et al. (2021) was employed for keratin extraction. The keratin powder produced was employed in the keratinase assay procedures.

Production and partial purification of keratinase and collagenase in submerged fermentation (SmF)

Submerged fermentation was carried out in Erlenmeyer flasks (500 mL) containing 100 mL of 0.1% glucose- Czapek's supplemented with 1% native chicken feather as a fermentation substrate. Centrifugation (10,000 rpm at 4 ºC for 10 min) was used to produce the cell-free supernatant, which was then precipitated with 70% ammonium sulphate following the fermentation period (at 30 ºC and 150 rpm for 15 days). A freeze dryer (VirTis, model #6KBTES-55, NY, USA) was used to separate and lyophilize the precipitated total protein. Lyophilized protein was dissolved in citrate buffer (pH 5.0) and dialyzed twice for 2 hours at room temperature against the same buffer, eliminating the buffer each time, before being refrigerated overnight at 4 ºC to remove small molecules. The dialyzed protein was then lyophilized, weighed, and used in enzyme characterization experiments as a partly pure fungal keratinase and collagenase enzyme.

Keratinase assay

After 60 min, the reaction was terminated by introducing 2.0 mL of 10% trichloroacetic acid (TCA) and the precipitate was decanted after centrifugation at 10,000 rpm for 10 min. A 0.2 mL of the supernatant was diluted to 1.0 mL, and 5.0 mL alkaline cupper reagent (sodium carbonate, 40 g; tartaric acid, 7.5 g; copper sulfate, 4.5 g and distilled water, 1000 mL; final pH 9.9 ± 0.5) was added. Afterwards, 0.5 mL of the Folin–Ciocalteau reagent was applied and the tubes were kept in the dark for 30 min to allow the blue color formation at 660 nm (UV-visible spectrophotometer; T80+; UK). Using tyrosine as standard, the keratinase activity was calculated. One unit of keratinase is defined as the enzyme amount that releases 1 µmol tyrosine per mL per minute under the standard assay conditions (Alwakeel et al. 2021).

Collagenase assay

After 120 minutes, 0.2 mL of the reaction mixture was combined with 0.5 mL of 2% ninhydrin and boiled for 10 min (Bălan et al. 2013). After that, 5 mL of 50% isopropanol was added, and the degree of proteolysis was evaluated by colour development at 570 nm (UV-visible spectrophotometer; T80+; UK). Using leucine as standard, the collagenase activity was estimated. One unit of collagenase is defined as the enzyme amount that releases 1 µmol leucine per mL per minute under the standard assay conditions.

Impact of pH, temperature and some metal ions and inhibitors on keratinase and collagenase activity

A 0.01 g enzyme powder and 0.01 g chicken keratin powder or 0.01 g collagen powder (each dissolved in 1.0 ml of 50 mmol buffer solution), were included in the reaction mixture in a water bath. The impact of pH (3.0–10.0) and temperature (30–60 ºC) on keratinase and collagenase activities were estimated. The buffers used were citrate buffer (pH 3.0–6.0), phosphate buffer (pH 7.0–8.0), and borate buffer (pH 9.0–10.0). Monovalent and divalent metal ions (Na^+, K^+, Ca^{+2}, Co^{+2}, Ni^{+2}, Cu^{+2}, Fe^{+2}, Mn^{+2}, Mg^{+2}, and Zn^{+2}) were evaluated by introducing them at 5 mmol/ml concentrations as NaCl, KCl, $CaCl_2$, $CoCl_2$, $NiCl_2$, $CuSO_4$, $FeSO_4$, $MnSO_4$, $MgSO_4$, and $ZnSO_4$. A 5 mmol/ml ethylenediaminetetraacetic acid (EDTA) and sodium dodecyl sulfate (SDS) were used to evaluate an enzyme inhibitor. Under standard conditions, the activity of the keratinase and collagenase enzymes in the absence of ions or EDTA was evaluated to

define 100% activity. The various experiments were set up in three repetitions. Total protein was determined following Lowry et al. (1951).

Results

Molecular studies

Using blast search in GenBank, the ITS sequence of this strain was found to be 100% (525/525) identical to *Didymella keratinophila* UTHSC DI16-200 (type strain). The total number of sequences in the ITS dataset was 36. The maximum parsimony dataset had 493 characters, 448 of which could be aligned clearly, 35 variable characters that were parsimony-uninformative (7.8% of constant characters), and 14 characters that were parsimony informative (3.1% of constant). The Kimura 2-parameter (K2 + G + I) model was ideal for nucleotide substitution. Maximum Parsimony assessments yielded two trees with 74 steps, the most parsimonious of which is depicted in Fig. 1 was with the greatest log likelihood of -1138.60, consistency index of 0.380000, retention index of 0.630952, and composite index of 0.239762. The strain in this investigation was located at the same clade as *Didymella keratinophila* UTHSC DI16-200 (type strain) and *D. keratinophila* CBS 140826, although with a poor support value of < 50% ML/<50% MP (Fig. 1).

Brief description of Didymella keratinophila **AUMC 15399**

Hyphae pale brown, smooth- and thin-walled, septate, 3–8 µm wide. Conidiomata pycnidial, brown, solitary, superficial on OA, glabrous, broadly ellipsoidal, (50–) 150–170 (–250) µm, with a single papillate ostiolar neck. Conidiogenous cells phialidic, hyaline, smooth-walled, ampulliform or globose, 4–6 × 3–4 µm. Conidia aseptate, hyaline, smooth, thin-walled, ovoid to cylindrical, 3–6 × 2–3 µm. Chlamydospores present (Fig. 2).

3.3. Production of keratinase and collagenase from native chicken feather in SmF

After 15 days of incubation, the fungus was able to flourish in the fermentation media and totally dissolve the chicken feathers. After 60 percent ammonium sulphate precipitation, it was possible to get 7.13 g crude protein powder from *Didymella keratinophila* AUMC 15399 per liter of fermentation medium after the incubation time (Fig. 3).

3.4. Effect of pH and temperature on keratinase activity

The impact of pH (3–10) and temperature (30–60 ºC) on keratinase activity was investigated. The best pH for maximum keratinase activity was pH 7.0, which resulted in keratinase specific activity of 5835 ± 133 IU/mg protein (Fig. 4). At pH 7.0, the keratinase activity increased with temperature, reaching 44903 ± 1555 IU/mg protein at 45 ºC (Fig. 5).

3.4. Effect of pH and temperature on collagenase activity

The effect of various pH values (3–10) and temperatures (30–70 ºC) on collagenase activity was studied. Two peaks were seen at pH 6.0 and 8.0, indicating the synthesis of two collagenases. At pH 6.0 and 8.0, the specific activity was 13912 ± 30 IU/mg and 15550 ± 49 IU/mg, respectively (Fig. 6). Temperature had a substantial influence on the activity of the collagenase enzyme at pH 8.0, producing two peaks at 35 ºC and 60 ºC, when the activity increased to 15753 ± 110 IU/mg and 16312 ± 21 IU/mg, respectively (Fig. 7).

3.5. Effect of some ions and inhibitors on keratinase and collagenase activity

When examined under the optimal circumstances, EDTA and the ions had a substantial inhibitory impact on the activity of the keratinases and collagenases developed. The most potent inhibitory impact was shown with Mn^{+2} in the case of keratinases and K^{+} in the case of collagenase (Table 1).

Table 1

Effect of some ions and inhibitors (5 mmol mL^{-1}) on keratinase (at pH 7.0 and 45 ºC) and collagenase (at pH 8.0 and 35 ºC) activity produced by *Didymella keratinophila* AUMC 15399 (mean ± SD, n = 3). The results are expressed as the activity in the tested inhibitory conditions compared to the pectinase activity in the control without inhibitors (in bold).

Ions and inhibitors	Keratinase	Collagenase
	Specific activity	**Specific activity**
	U mg^{-1}	**U mg^{-1}**
Control	**44903 ± 1555**	**15753 ± 110**
Na^{+}	16728 ± 12	3532 ± 64
K^{+}	7619 ± 14	1439 ± 37
Fe^{+2}	16878 ± 64	14644 ± 37
Cu^{+2}	12430 ± 98	13358 ± 121
Ca^{+2}	14366 ± 128	13972 ± 18
Mg^{+2}	16093 ± 320	14600 ± 66
Zn^{+2}	19039 ± 149	13967 ± 21
Ni^{+2}	14680 ± 192	15426 ± 37
Co^{+2}	19495 ± 133	15550 ± 84
Mn^{+2}	7089 ± 18	6699 ± 36
EDTA	14209 ± 111	14644 ± 314
SDS	12720 ± 12	12744 ± 36

Discussion

In this study, which is the first of its type, natural chicken feather was demonstrated to be a viable substrate for collagenase enzyme production alongside keratinase. *Didymella keratinophila* AUMC 15399 was introduced as a new high-yielding keratinase and collagenase producer in this study. According to the current findings, keratinolysis can be boosted by combining keratinase with additional enzymes such as collagenase. Microbial keratinases are thought to be especially useful in a variety of biotechnological applications, such as plant growth promoters (Paul et al. 2013; Bhange et al. 2016), the treatment of keratinous waste generated in agriculture and the leather industry (de Menezes et al. 2021), and the

treatment of wastes from the cattle, poultry, and leather industries (Kang et al. 2018; Kalaikumari et al. 2019). Byproducts include amino acids, polypeptides, vitamins, and detergent additives are potential innovative uses that promote agricultural sustainability (Reddy et al. 2017; Peng et al. 2020).

More research is needed to understand the processes underlying the breakdown of keratinous materials. In this regard, keratinases generated by *Bacillus thuringiensis* isolated from donkey hairs were used to investigate the process of keratin breakdown using scanning electron microscopy (SEM) and Fourier transform infrared (FT-IR) spectrophotometry which revealed disintegration and breakage of the keratin structure's disulphide linkages (Hassan et al. 2020b). The utilization of natural microorganisms lowers the cost of enzyme synthesis while also providing cost-effective waste treatment techniques (Verma et al. 2017). The *Didymella keratinophila* AUMC 15399 investigated proved to be viable candidate for the production of active keratinase and collagenase. We report, for the first time, the potential of the strain to produce keratinase and collagenase from chicken feather, and its potential to be used in applications to degrade keratinous material because there is no proof of bioactivity or discussion of its potential application in biotechnology.

Keratinases are active throughout a wide temperature range (40–70°C) and pH range (6–11) (Tharwat et al. 2019; Sharma and Gupta 2016; Bohacz et al. 2020; Avdiyuk and Varbanets 2019; Abirami et al. 2020; Sousa et al. 2015; Paul et al. 2014; Bagewadi et al. 2018; Gradišar et al. 2005), as a result, optimal conditions for biotechnological solutions must be investigated. Keratinase from this study had an ideal pH of 7.0 and temperature of 45 ºC, whereas collagenases had optimal pH of 6.0 and 8.0 and temperatures of 35 ºC and 60 ºC, respectively. There are no reports of *Didymella* species producing neither keratinase nor collagenase, according to the published data. Various fungal species have extremely varying optimal conditions. For example, *Aspergillus terreus* grew best in a feather meal basal medium at pH 8 and 40 ºC (Koutb et al. 2012). *A. niger* strains produced varying amounts of keratinases with the highest activity at pH 5 (Mazotto et al. 2013). *A. flavus* grew best in a feather meal basal media at pH 8 and 28 ºC (Kim 2007). This implies that the ideal cultivation conditions for each species must be researched separately.

In this study, *Didymella keratinophila* hydrolyzed the chicken feather completely after 15 days at 30 ºC and initial pH 7.0 and produced the highest keratinase and collagenase enzymes. In this regard, *Cochliobolus hawaiiensis* produced the highest alkaline keratinase after 15 days incubation at 30 ºC and pH 9.5 (Isaac and Abu-Tahon 2016). *Chrysosporium tropicum* generated the maximum keratinase in a medium containing chicken feathers after 21 days at 25 ºC (Menon et al. 2020). *Trichophyton ajelloi* exhibited the highest enzyme activity (6.3 KU/mL) at 30 ºC (Kačinová et al. 2014). *Chrysosporium tropicum* keratinolytic activity peaked (8.6 KU/mL) on the 40th day of incubation Reddy (Reddy et al. 2017). The maximum activity for *Microsporum gypseum* (78 KU/mL) and *M. canis* (76 KU/mL) was seen on the 20th day of incubation (Peng et al. 2020). Because of minor changes in technique, it is difficult to compare enzyme activity results among research. As a result, comparisons should be interpreted cautiously.

Many investigations have demonstrated the metal requirement of microbial keratinase activity (Alwakeel et al. 2021; Tapia and Simões 2008; Riffel et al. 2007). This was also stated for our strain, *Didymella keratinophila* AUMC 15399. No previous information regarding our species has been found, however an *Aspergillus oryzae* feather-degrading culture was stimulated by Ca^{2+} and Ba^{2+} ions while inhibited by EDTA and Pb^{2+} ions (Farag and Hassan 2004). EDTA, Hg^{2+}, and Fe^{3+} significantly decreased *A. flavipes* keratinase activity during solid-state fermentation with chicken feathers, while the presence of Zn^{2+}, Mg^{2+}, and Cu^{2} + had no significant influence on *A. flavipes* keratinase.(El-Ayouty et al. 2012). The keratinases generated by *Aspergillus stelliformis* and *A. sydowii* had the greatest inhibitory impact with EDTA, while Ca^{2+}, Cu^{2+}, and Zn^{2+} were the inhibitory metal ions for *F. brachygibbosum* keratinase (Alwakeel et al. 2021).

Conclusion

Microbial keratinase-mediated degradation of keratin-rich substrates has accelerated the cost-effective and environmentally friendly valorization and bio-cycling of agro-industrial wastes to create high-value chemicals such as amino acids and bioactive peptides. The discovery of keratinases has drastically altered the industrial procedures for processing complex materials, moving away from traditional ways and toward more sustainable biobased solutions. In this work, *Didymella keratinophila* AUMC 15399 was presented as a producer of active microbial keratinase and collagenase for use in digesting troublesome and resistant keratinous wastes and producing sustainable agriculture.

Declarations

Ethical Approval: Not applicable

Consent to Participate: All the participants in this study consented to participate in the cohort and signed an informed consent approved by the review board.

Consent to Publish: Not applicable

Authors Contributions: Osama A.M. Al-Bedak: Conceptualization, investigation, molecular analysis, writing original draft, data curation. Ahmed M. Moharram: Supervision, reviewing and editing. Nemmat A. Hussein: Reviewing and editing, data curation. Doaa M. Taha: Investigation, methodology.

Funding: Not applicable

Competing Interests: The authors declare that there are no potential conflict of interest regarding the publication of this manuscript.

Availability of data and materials: The authors confirm that the data supporting the findings of this study are available within the article only.

References

1. Abdel-Fattah AM, El-Gamal MS, Ismail SA, Emran MA, Hashem AM (2018) Biodegradation of feather waste by keratinase produced from newly isolated *Bacillus licheniformis* ALW1. Journal of Genetic Engineering and Biotechnology 16 (2):311-318
2. Abirami S, Ragavi R, Antony VS (2020) Utilization of keratinolytic *Lichtheimia corymbifera* AS1 for degradation of cattle hoove–a slaughter house waste to use in plant growth. Biointerface Res Appl Chem 10:6417-6426
3. Akhter M, Wal Marzan L, Akter Y, Shimizu K (2020) Microbial Bioremediation of Feather Waste for Keratinase Production: An Outstanding Solution for Leather Dehairing in Tanneries. Microbiology Insights 13:1178636120913280
4. Akram F, Ul Haq I, Jabbar Z (2020) Production and characterization of a novel thermo-and detergent stable keratinase from *Bacillus* sp. NKSP-7 with perceptible applications in leather processing and laundry industries. International Journal of Biological Macromolecules 164:371-383
5. Al-Bedak OA, Moubasher AH (2020) *Aspergillus gaarensis*, a new addition to section Circumdati from soil of Lake El-Gaar in Wadi-El-Natron, Egypt. Studies in Fungi 5 (1):59-65
6. Al-Bedak OA, Sayed RM, Hassan SH (2019) A new low-cost method for long-term preservation of filamentous fungi. Biocatalysis and Agricultural Biotechnology 22:101417
7. Alwakeel SS, Ameen F, Al Gwaiz H, Sonbol H, Alghamdi S, Moharram AM, Al-Bedak OA (2021) Keratinases Produced by *Aspergillus stelliformis*, *Aspergillus sydowii*, and Isolated from Human Hair: Yield and Activity. Journal of Fungi 7 (6):471 *Fusarium brachygibbosum*
8. Asdornnithee S, Akiyama K, Sasaki T, Takata R (1994) Isolation and characterization of a collagenolytic enzyme from *Bacillus licheniformis* N22. Journal of fermentation and bioengineering 78 (4):283-287
9. Avdiyuk K, Varbanets L (2019) Keratinolytic enzymes: producers, physical and chemical properties. Application for biotechnology. Biotechnologia Acta 12 (2)
10. Bagewadi ZK, Mulla SI, Ninnekar HZ (2018) Response surface methodology based optimization of keratinase production from *Trichoderma harzianum* isolate HZN12 using chicken feather waste and its application in dehairing of hide. Journal of Environmental Chemical Engineering 6 (4):4828-4839
11. Bălan D, Israel-Roming F, Cornea P, Gherghina E, Luţă G, Matei F, Curta□u M (2013) Novel fungal collagenase from *Aspergillus oryzae*. Sci Bull Ser F Biotechnol 17:160-163
12. Bhange K, Chaturvedi V, Bhatt R (2016) Ameliorating effects of chicken feathers in plant growth promotion activity by a keratinolytic strain of *Bacillus subtilis* PF1. Bioresources and Bioprocessing 3 (1):1-10
13. Bohacz J, Korniłłowicz-Kowalska T, Kitowski I, Ciesielska A (2020) Degradation of chicken feathers by *Aphanoascus keratinophilus* and *Chrysosporium tropicum* strains from pellets of predatory birds and its practical aspect. International Biodeterioration & Biodegradation 151:104968

14. de Menezes CLA, Santos RdC, Santos MV, Boscolo M, da Silva R, Gomes E, da Silva RR (2021) Industrial sustainability of microbial keratinases: production and potential applications. World Journal of Microbiology and Biotechnology 37 (5):1-17
15. Edgar RC (2004) MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic acids research 32 (5):1792-1797
16. El-Ayouty YM, El-Said A, Salama A (2012) Purification and characterization of a keratinase from the feather-degrading cultures of *Aspergillus flavipes*. African Journal of Biotechnology 11 (9):2313-2319
17. Fang Z, Zhang J, Liu B, Du G, Chen J (2013) Biodegradation of wool waste and keratinase production in scale-up fermenter with different strategies by *Stenotrophomonas maltophilia* BBE11-1. Bioresource technology 140:286-291
18. Farag AM, Hassan MA (2004) Purification, characterization and immobilization of a keratinase from *Aspergillus oryzae*. Enzyme and Microbial Technology 34 (2):85-93
19. Felsenstein J (1985) Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39 (4):783-791
20. Gafar A, Khayat ME, Ahmad SA, Yasid NA, Shukor MY (2020) Response Surface Methodology for the Optimization of Keratinase Production in Culture Medium Containing Feathers by *Bacillus* sp. UPM-AAG1. Catalysts 10 (8):848
21. Gradišar H, Friedrich J, Križaj I, Jerala R (2005) Similarities and specificities of fungal keratinolytic proteases: comparison of keratinases of *Paecilomyces marquandii* and *Doratomyces microsporus* to some known proteases. Applied and Environmental Microbiology 71 (7):3420-3426
22. Hanada K, Mizutani T, Yamagishi M, Tsuji H, Misaki T, Sawada J (1973) The isolation of collagenase and its enzymological and physico-chemical properties. Agricultural and Biological Chemistry 37 (8):1771-1781
23. Hassan MA, Abol-Fotouh D, Omer AM, Tamer TM, Abbas E (2020a) Comprehensive insights into microbial keratinases and their implication in various biotechnological and industrial sectors: a review. International journal of biological macromolecules 154:567-583
24. Hassan MA, Taha TH, Hamad GM, Hashem M, Alamri S, Mostafa YS (2020b) Biochemical characterisation and application of keratinase from *Bacillus thuringiensis* MT1 to enable valorisation of hair wastes through biosynthesis of vitamin B-complex. International Journal of Biological Macromolecules 153:561-572
25. Hisano T, Abe S, Wakashiro M, Kimura A, Murata K (1989) Isolation and properties of a collagenase with caseinolytic activity from a *Pseudomonas* sp. Journal of fermentation and bioengineering 68 (6):399-403
26. Isaac GS, Abu-Tahon MA (2016) Dehairing capability of alkaline keratinase produced by new isolated *Cochliobolus hawaiiensis* AUMC 8606 grown on chicken feather. Romanian Biotechnological Letters 22 (6):12147-12154

27. Kačinová V, Kolčáková V, Petranová D (2014) Axiocentric media education as a strategy for the cultivation of media recipients. European Journal of Science and Theology 10 (1):103-116
28. Kalaikumari SS, Vennila T, Monika V, Chandraraj K, Gunasekaran P, Rajendhran J (2019) Bioutilization of poultry feather for keratinase production and its application in leather industry. Journal of cleaner production 208:44-53
29. Kang D, Herschend J, Al-Soud WA, Mortensen MS, Gonzalo M, Jacquiod S, Sørensen SJ (2018) Enrichment and characterization of an environmental microbial consortium displaying efficient keratinolytic activity. Bioresource technology 270:303-310
30. Khumalo M, Sithole B, Tesfaye T (2020) Valorisation of waste chicken feathers: Optimisation of keratin extraction from waste chicken feathers by sodium bisulphite, sodium dodecyl sulphate and urea. Journal of Environmental Management 262:110329
31. Kim J-D (2007) Purification and characterization of a keratinase from a feather-degrading fungus, *Aspergillus flavus* Strain K-03. Mycobiology 35 (4):219-225
32. Köhler JR, Casadevall A, Perfect J (2015) The spectrum of fungi that infects humans. Cold Spring Harbor perspectives in medicine 5 (1):a019273
33. Koutb M, Morsy FM, Bagy MMK, Hassan EA (2012) Optimization of extracellular keratinase production by *Aspergillus terreus* isolated from chicken's litter. Journal of Advanced Laboratory Research in Biology 3 (3):210-216
34. Kumar S, Stecher G, Li M, Knyaz C, Tamura K (2018) MEGA X: molecular evolutionary genetics analysis across computing platforms. Molecular biology and evolution 35 (6):1547-1549
35. Li J, Cheng J-H, Teng Z-J, Sun Z-Z, He X-Y, Wang P, Shi M, Song X-Y, Chen X-L, Zhang Y-Z (2021) Taxonomic and Enzymatic Characterization of *Flocculibacter collagenilyticus* gen. nov., sp. nov., a Novel Gammaproteobacterium With High Collagenase Production. Frontiers in microbiology 12:465
36. Lima CA, Rodrigues PM, Porto TS, Viana DA, Lima Filho JL, Porto AL, da Cunha MGC (2009) Production of a collagenase from *Candida albicans* URM3622. Biochemical Engineering Journal 43 (3):315-320
37. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. Journal of biological chemistry 193:265-275
38. Mazotto AM, Couri S, Damaso MC, Vermelho AB (2013) Degradation of feather waste by *Aspergillus niger* keratinases: comparison of submerged and solid-state fermentation. International Biodeterioration & Biodegradation 85:189-195
39. Menon S, Savur B, Kasat D, Mavani U, Singh S, Rane M (2020) Management and utilization of Keratin Waste–A review. Int J Adv Res Ideas Innov Technol 6:511-515
40. Moridshahi R, Bahreini M, Sharifmoghaddam M, Asoodeh A (2020) Biochemical characterization of an alkaline surfactant-stable keratinase from a new keratinase producer, *Bacillus zhangzhouensis*. Extremophiles 24 (5):693-704
41. Moubasher AH, Ismail MA, Al-Bedak OA, Mohamed RA (2019) *Ramophialophora chlamydospora*, a new species from an alkaline lake of Wadi-El-Natron, Egypt. Asian Journal of Mycology 2 (1):110-117

42. Nnolim NE, Udenigwe CC, Okoh AI, Nwodo UU (2020) Microbial Keratinase: Next Generation Green Catalyst and Prospective Applications. Frontiers in Microbiology 11:3280
43. Paul T, Das A, Mandal A, Halder SK, DasMohapatra PK, Pati BR, Mondal KC (2014) Production and purification of keratinase using chicken feather bioconversion by a newly isolated *Aspergillus fumigatus* TKF1: detection of valuable metabolites. Biomass conversion and Biorefinery 4 (2):137-148
44. Paul T, Halder SK, Das A, Bera S, Maity C, Mandal A, Das PS, Mohapatra PKD, Pati BR, Mondal KC (2013) Exploitation of chicken feather waste as a plant growth promoting agent using keratinase producing novel isolate *Paenibacillus woosongensis* TKB2. Biocatalysis and Agricultural Biotechnology 2 (1):50-57
45. Peng Z, Mao X, Zhang J, Du G, Chen J (2020) Biotransformation of keratin waste to amino acids and active peptides based on cell-free catalysis. Biotechnology for biofuels 13 (1):1-12
46. Posada D, Crandall KA (1998) Modeltest: testing the model of DNA substitution. Bioinformatics (Oxford, England) 14 (9):817-818
47. Reddy CC, Khilji IA, Gupta A, Bhuyar P, Mahmood S, AL-Japairai KAS, Chua GK (2021) Valorization of keratin waste biomass and its potential applications. Journal of Water Process Engineering 40:101707
48. Reddy MR, Reddy KS, Chouhan YR, Bee H, Reddy G (2017) Effective feather degradation and keratinase production by *Bacillus pumilus* GRK for its application as bio-detergent additive. Bioresource technology 243:254-263
49. Riffel A, Brandelli A, Bellato CdM, Souza GH, Eberlin MN, Tavares FC (2007) Purification and characterization of a keratinolytic metalloprotease from *Chryseobacterium* sp. kr6. Journal of biotechnology 128 (3):693-703
50. Sharma S, Gupta A (2016) Sustainable management of keratin waste biomass: applications and future perspectives. Brazilian Archives of Biology and Technology 59
51. Smith D, Onions AH (1994) The preservation and maintenance of living fungi. vol Ed. 2. CAB international,
52. Sousa M, Souza O, Maciel M, Cruz R, Rêgo M, Magalhães O, Pessoa-Júnior A, Porto A, Souza-Motta C (2015) Keratinolytic potential of fungi isolated from soil preserved at the Micoteca URM. Eur J Biotechnol Biosci 3:10-15
53. Tapia DM, Simões MLG (2008) Production and partial characterization of keratinase produced by a microorganism isolated from poultry processing plant wastewater. African Journal of Biotechnology 7 (3)
54. Tharwat N, Al-Bedak O, Hamouda R, El Shreif R, Mounir R, Sami A (2019) Antifungal effect of gold nanoparticles on fungi isolated from onychomycosis patients. Al-Azhar Journal of Pharmaceutical Sciences 60 (2):26-42
55. Tran L, Nagano H (2002) Isolation and characteristics of Bacillus subtilis CN2 and its collagenase production. Journal of food science 67 (3):1184-1187

56. Van Wart HE, Steinbrink DR (1981) A continuous spectrophotometric assay for *Clostridium histolyticum* collagenase. Analytical biochemistry 113 (2):356-365
57. Verma A, Singh H, Anwar S, Chattopadhyay A, Tiwari KK, Kaur S, Dhilon GS (2017) Microbial keratinases: industrial enzymes with waste management potential. Critical reviews in biotechnology 37 (4):476-491
58. Vidmar B, Vodovnik M (2018) Microbial keratinases: enzymes with promising biotechnological applications. Food technology and biotechnology 56 (3):312-328
59. White TJ, Bruns T, Lee S, Taylor J (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. PCR protocols: a guide to methods and applications 18 (1):315-322

Figures

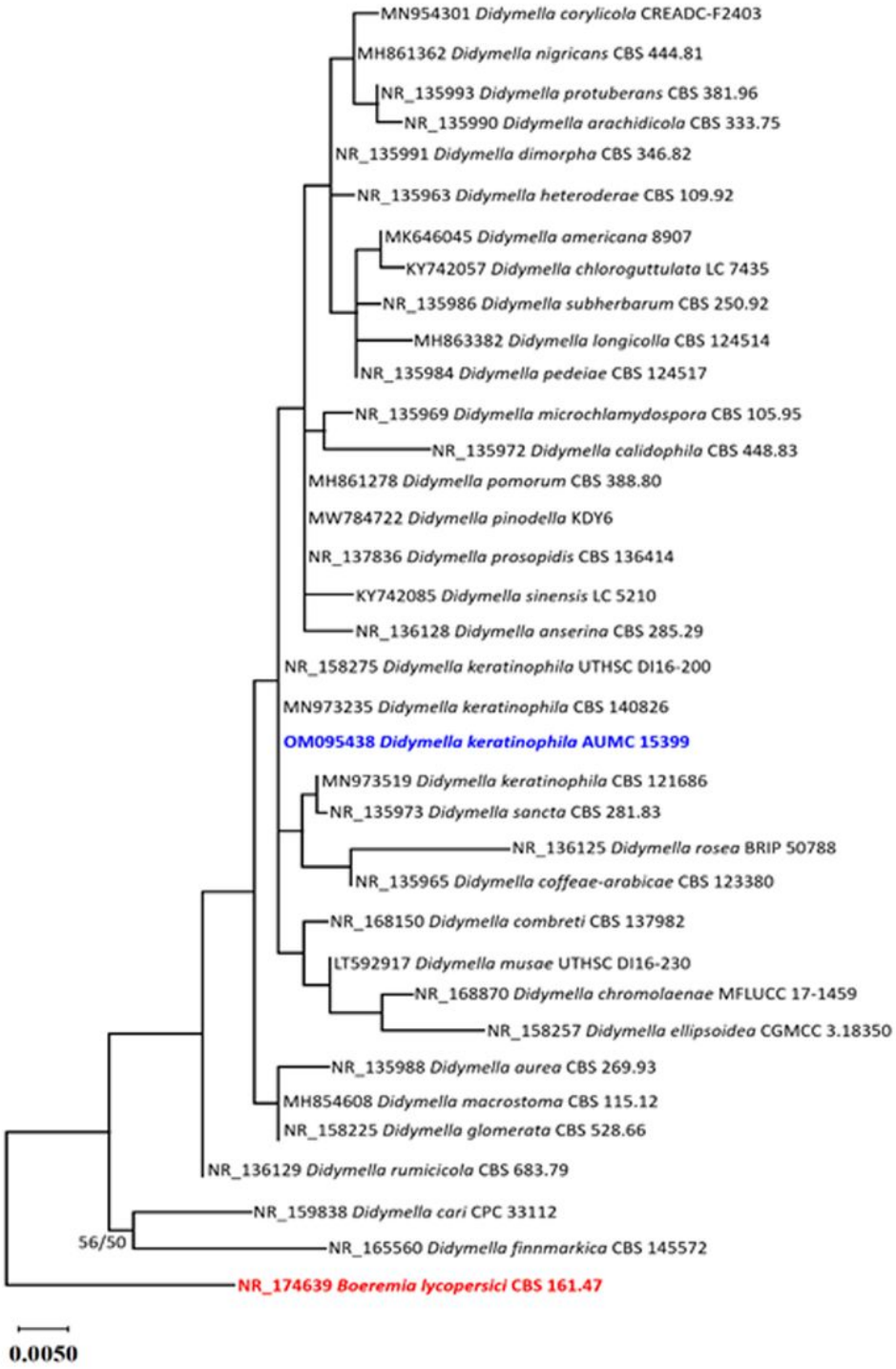

Figure 1

Maximum likelihood phylogenetic tree based on sequencing of the ITS region of *Didymella keratinophila* AUMC 15399 (in blue color) compared to the members of *Didymella* in GenBank. Bootstrap support values (1000 replications) for ML/MP ≥50% are indicated above/below the respective nodes. The tree is rooted to *Boeremia lycopersici* CBS 161.47 (in red color).

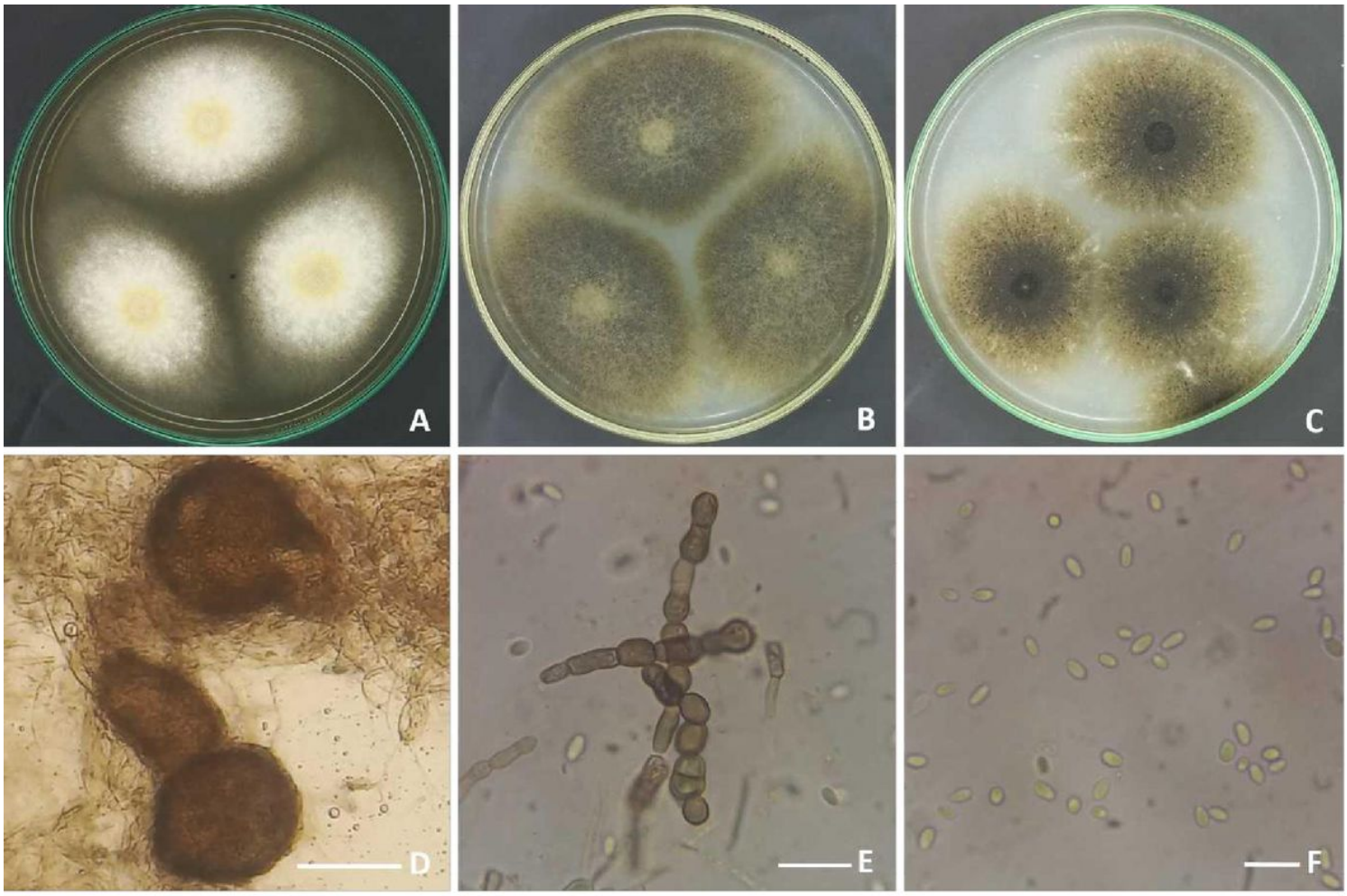

Figure 2

Didymella keratinophila AUMC 15399. A–C 7–day–old colonies on MEA, PDA and OA at 25 ºC. D Pycnidia. E, Chlamydospores. F, Conidia. Scale bar: D = 100 µm, E = 20 µm, F = 10 µm.

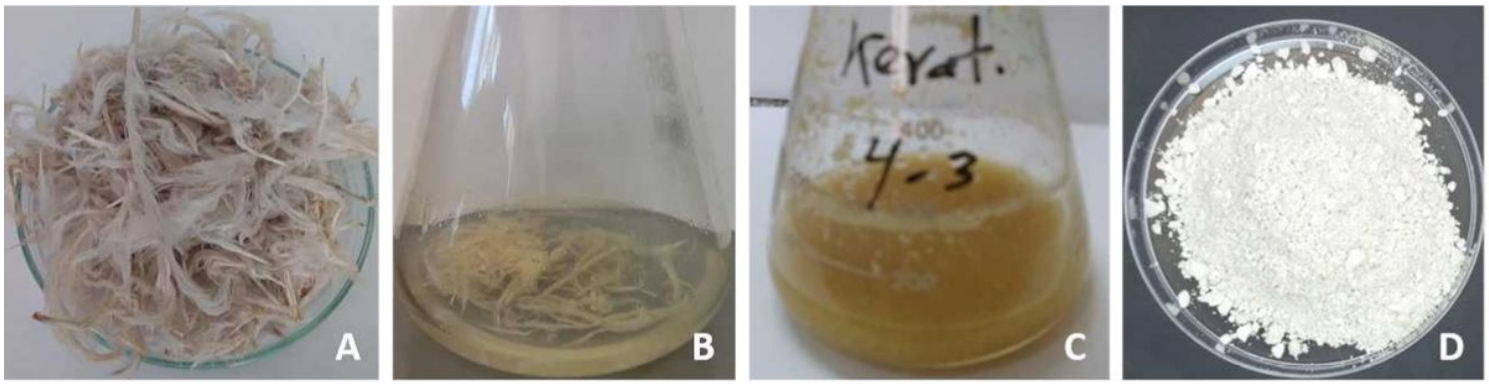

Figure 3

Stages of Keratinase and collagenase production by *Didymella keratinophila* AUMC 15399 in SmF. A Defatted chicken feather. B Fermentation medium containing native feather. C Fully hydrolyzed feather after 15 days of incubation. D Crude keratinase and collagenase enzymes lyophilized powder.

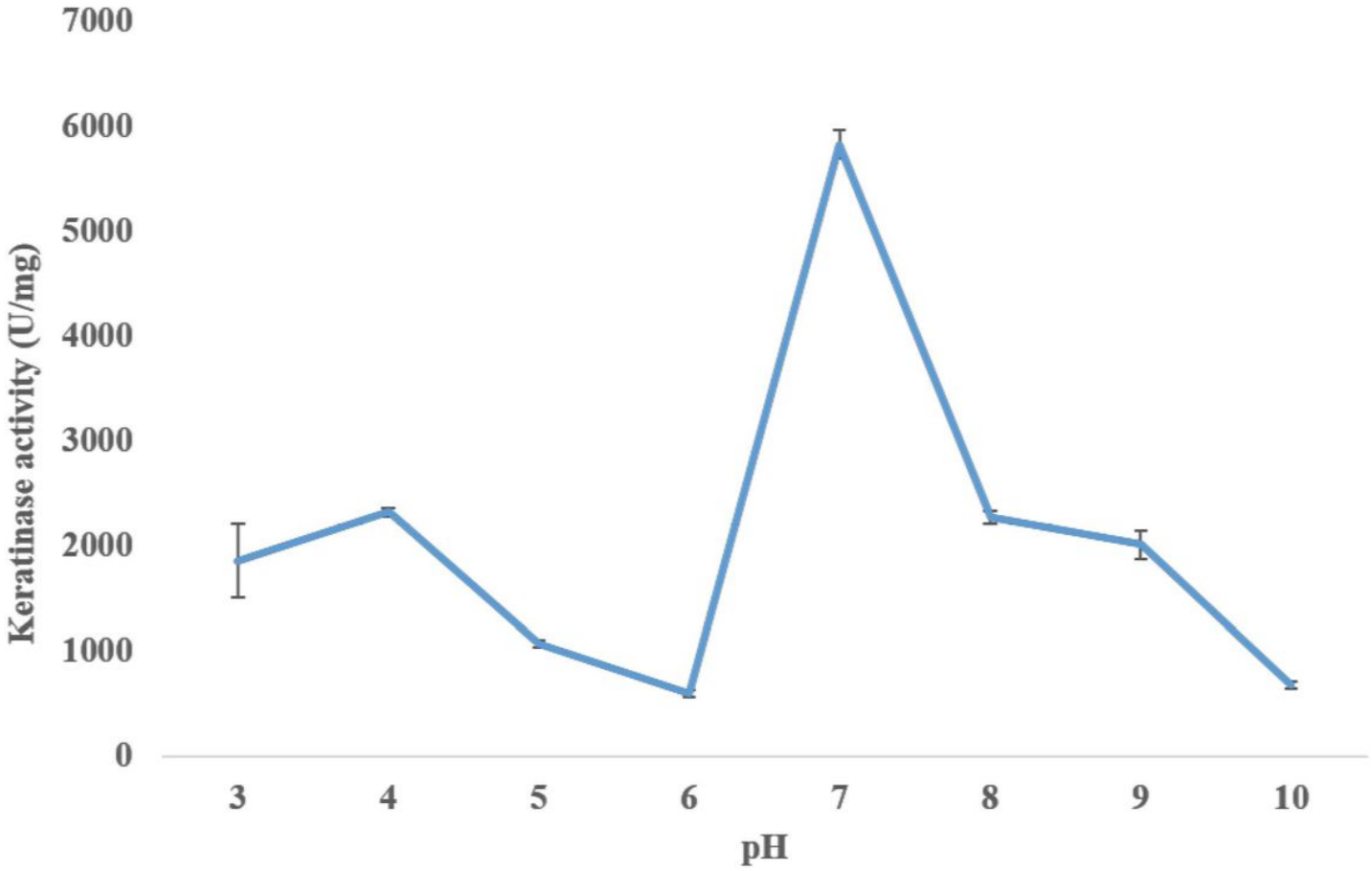

Figure 4

Effect of pH on the activity of keratinase produced by *Didymella keratinophila* AUMC 15399

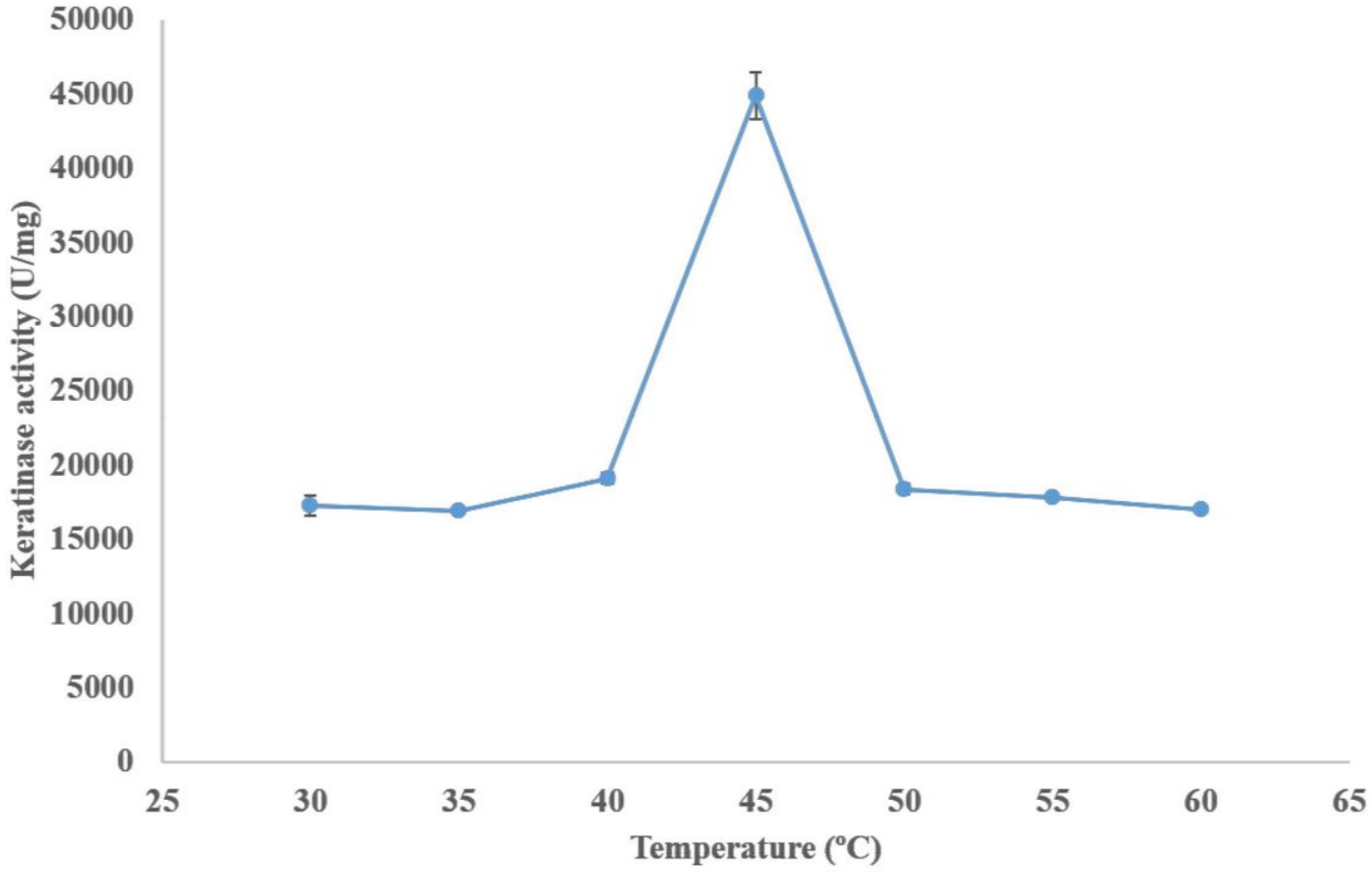

Figure 5

Effect of temperature on the activity of keratinase produced by *Didymella keratinophila* AUMC 15399 at pH 7.0

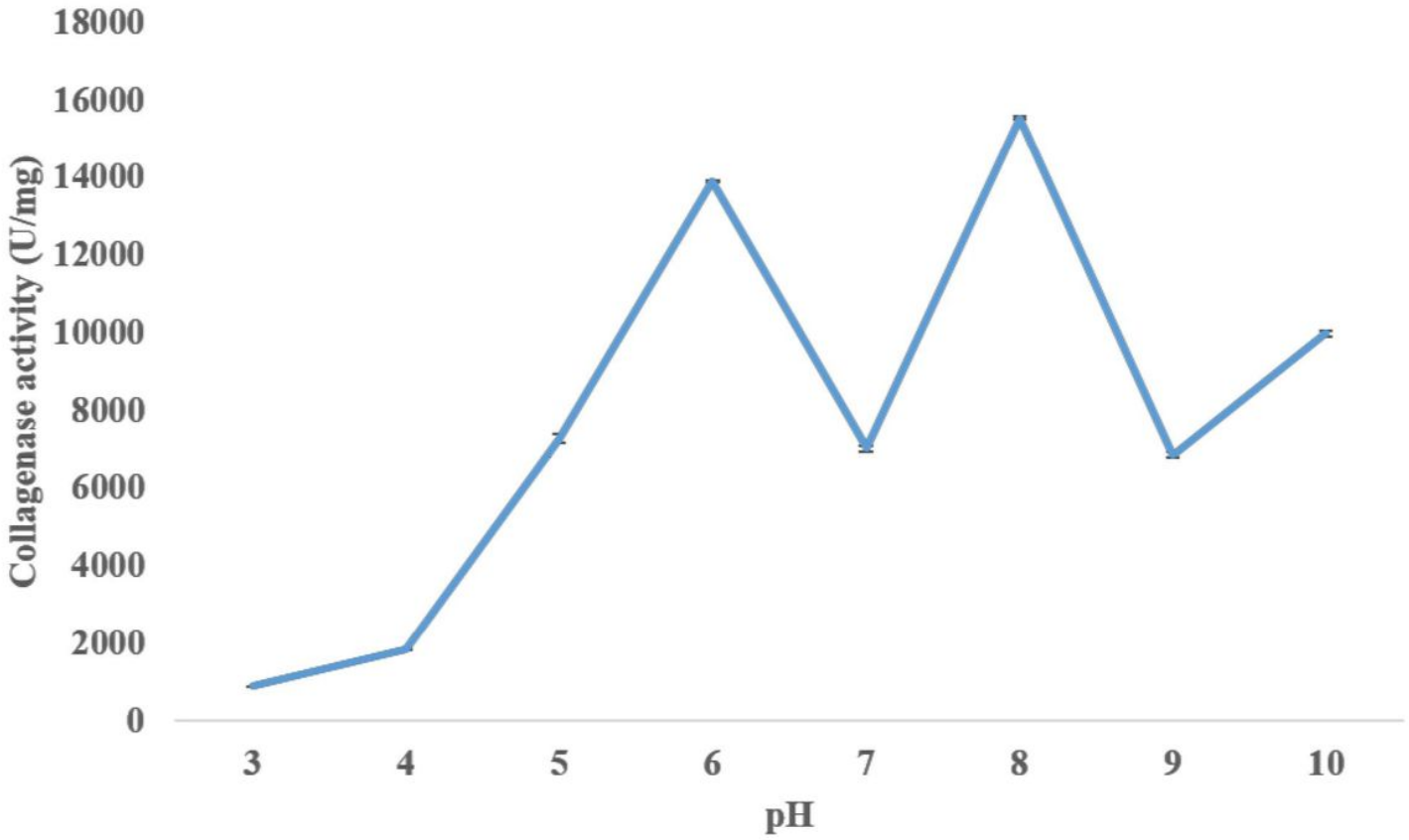

Figure 6

Effect of pH on the activity of collagenase produced by *Didymella keratinophila* AUMC 15399

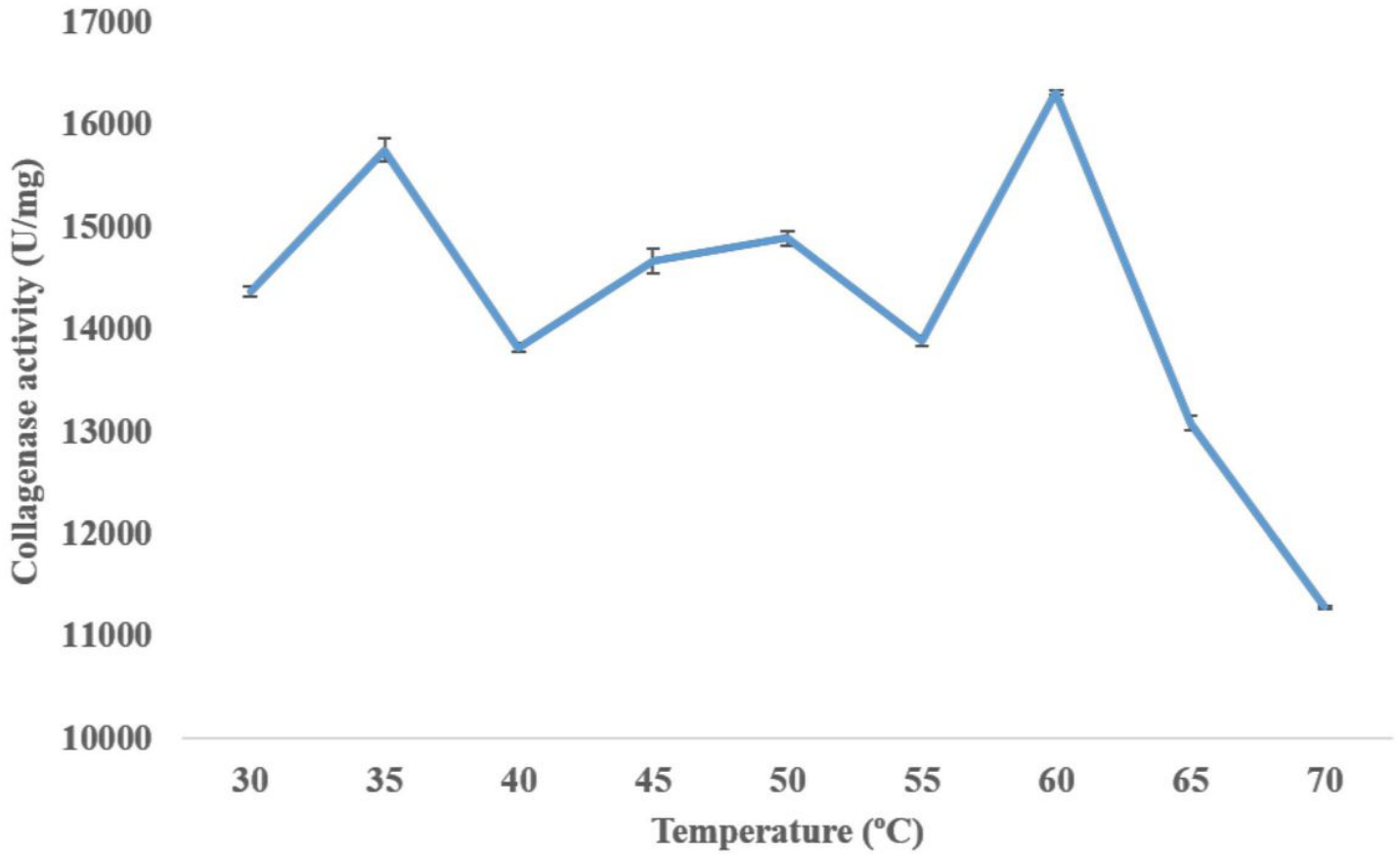

Figure 7

Effect of temperature on the activity of collagenase produced by *Didymella keratinophila* AUMC 15399 at pH 8.0.