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Microbial Exploitation of Feather Wastes for Sustainable Production of Keratinase and Collagenase Enzymes by *Didymella keratinophila* AUMC 15399 in Submerged Fermentation

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Abstract: A distinctive isolate was discovered and visually recognized as a member of the genus *Didymella* during a routine examination of Coelomycetes isolated from diverse fruit juices. Based on sequencing of the internal transcribed spacer (ITS), the fungus was identified as *Didymella keratinophila* since it showed a 100% identity to the type strain. The strain thrived and produced keratinase and collagenase enzymes by hydrolyzing native chicken feathers in submerged fermentation (SmF). After 10 days of fermentation at 30 °C, pH 9 using sodium nitrate as a nitrogen supply produced the highest keratinase activity of 8780 ± 620 U/mL/min, while pH 6 and beef extract produced the maximum collagenase activity of 11,230 ± 1290 U/mL/min. The partially-purified keratinase enzyme worked best at pH 7.0 and 45 °C, exhibiting a specific activity of 44,903 ± 1555 U/mg protein. The activity of the partially-purified collagenase enzyme was excellent at pH 6.0 at 35 °C, generating 15,753 ± 110 U/mg enzyme-specific activity. Mn^{2+} and K^{+} were the most efficient inhibitors of keratinases and collagenase, respectively. Both EDTA and metal ions significantly decreased the activity of keratinase and collagenase. This report identified a workable supplier of collagenase and keratinase enzymes derived from chicken feathers, offering a reliable way to exploit and manage these wastes for obtaining high-value products.

Keywords: bioconversion; collagenase; *Didymella*; eco-friendly; feather; keratinase; fermentation

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1. Introduction

Wool, hooves, horns, hair, nails, and feathers all include keratins as structural constituents. Each year, 40 million tonnes of keratinous waste are produced in the United States, Brazil, and China [1,2]. The meat industry alone produces millions of tonnes of keratinous waste per year at slaughterhouses across the world [3]. Up to two million tonnes of chicken feathers are produced worldwide [4]. Keratins are insoluble fibrillar proteins found on the outer protective surfaces of vertebrates. 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 [5]. Current keratin waste treatment methods include landfilling, chemical treatment, and incineration [6]. These treatments are inadequate since they (1) reduce soil space; (2) increase the amount of toxic chemicals such as halogens and salts in bodies of water, thus endangering aquatic life; (3) increase greenhouse gas emissions, contributing to global warming; and (4) increase eutrophication in aquatic ecosystems due to the high amount of nitrogen and the alkalinity of the water [7].

Common protein-degrading enzymes, such as pepsin and papain, do not digest keratins. Keratinous waste management requires a low-cost solution, particularly in underdeveloped nations. Fungi and bacteria are known to hydrolyze keratinous materials by producing extracellular keratinolytic enzymes [8–10]. Different fungi, such as members of the genera *Aspergillus*, *Chrysosporium*, *Trichophyton*, and *Microsporum*, release keratinases that are generally extracellular inducible enzymes [11–13]. Keratinase powder, for example, is a commercial product made from the bacteria *Bacillus licheniformis* (K4519-500UN; Merck, Darmstadt, Germany) and the fungus *Parengyodontium album* (P6556; Merck, Darmstadt, Germany). In the pharmaceutical industry, keratinases are utilized in the manufacturing of vaccines and the creation of bioactive peptides and serums, as well as the treatment of calluses, keratinized skin, psoriasis, and acne [14]. Keratinases are also used in the production of cosmetics such as anti-dandruff shampoos, nutritious lotions, and creams, as well as in feed formulae, nitrogen fertilizers, and the leather industry [8,15,16]. Another unique application is the utilization of keratinases to remediate wastewater including keratin wastes [7].

Collagenase enzymes could 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 [17]. Collagen may be created over of the desired quantity, in inappropriate locations, or may not disintegrate after a given 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 discovered thus far over the last 40 years, including *Pseudomonas marinoglutinosa* [18], *Clostridium histolyticum* [19], *Pseudomonas* sp. [17], *Bacillus licheniformis* N22 [20], *Bacillus subtilis* FS-2 and *B. subtilis* M2-4 [21], *Flocculibacter collagenilyticus* [22], as well as *Candida albicans* URM3622 [23].

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 these are human dermatophytes that infect and destroy both hard and soft keratins [24]. On the other hand, non-pathogenic fungi 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 are limited. Thus, the purpose of the research described herein was to find suitable and efficient fungal species capable of decomposing native chicken feathers to create keratinase and, for the first time, collagenase for use in diverse biotechnological applications.

2. Materials and Methods

2.1. Strain Isolation and Preservation

The pour plate technique was used to isolate the strain of *Didymella* from a mango juice sample taken from a local market (55JM+GR4 2073028) in Assiut Governorate, Egypt. In Petri plates containing 20 mL Oat agar medium (OA, [25]), 1.0 mL of the sample was inoculated and 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 described by Al-Bedak et al. [26].

2.2. Morphological and Molecular Identification of the Strain of Didymella

Using an 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) [25], using spore suspension (prepared in a 30 % glycerol, 0.2 % agar and 0.05 % Tween 80 solution). Microscopic features on PDA were examined in lactophenol cotton blue after 7 days of incubation at 25 °C using Zeiss microscope (Model: Axio Star; Jena, Germany). DNA extraction was carried out following the method described by Moubasher et al. [27], and

the PCR reaction was performed using SolGent EF-Taq [28]. The universal primers ITS1 and ITS4 were used for the ITS region amplification [29]. DNASTAR (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 was used as an outgroup. All sequences were aligned using MUSCLE [30] and optimized manually. Maximum-likelihood (ML) analysis was carried out under the following settings: heuristic searches with random stepwise addition of 1000 replicates [31], and Tree-Bisection-Regrafting (TBR) rearrangements, using MEGA X (version 10.2.6) [32]. The best optimal model of nucleotide substitution for the ML analyses was determined using the Akaike information criterion (AIC) as implemented in Modeltest 3.7 [33]. Maximum-likelihood (ML) and Maximum parsimony (MP) phylogenetic analyses were performed using MEGA X [32], and the resulting tree was edited using Microsoft Power Point (2016) and saved as a TIF file [34].

2.3. Extraction of Keratin Powder

Native chicken feathers (100 g) were obtained from chicken farms (FM89+Q78 2140001) in Assiut Governorate, Egypt. The 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 °C. The procedure outlined by Alwakeel et al. [2] was employed for keratin extraction. The keratin powder produced was employed in the keratinase assay procedures.

2.4. Optimization of Fermentation Conditions

The respective pH, temperature, nitrogen supply, and fermentation duration were varied under one factor at a time (OFAT) conditions. The experiments were carried out in 250 mL Erlenmeyer flasks with 50 mL of sucrose-free Czapek's broth as the fermentation medium. The native chicken feathers were added as a sole carbon source at a 1 % concentration. *Didymella keratinophila* AUMC 15399 culture that was 7 days old was used to produce spore suspension (2.0 %; v/v) that was used to inoculate the flasks. The flasks were then incubated under various operating conditions, including pH (3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, and 10.0), nitrogen source (peptone, yeast extract, beef extract sodium nitrate, ammonium chloride, and ammonium sulfate; each at 0.2 %), temperature (25, 30, 35, 40, and 45 °C), and incubation duration (1–12) days. For pH adjustment, citrate buffer (pH 3.0–6.0), phosphate buffer (pH 7.0–8.0), and glycine/NaOH buffer (pH 9.0–10.0) were the buffers employed. Three different experiments were conducted.

2.5. 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 °C for 10 min; Hermle Labortechnik; Wehingen, Germany) was used to produce the cell-free supernatant, which was then precipitated with 70 % ammonium sulphate following the fermentation period (at 30 °C and 150 rpm for 15 days). A freeze dryer (VirTis, model #6KBTES-55, Albany, 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 h at room temperature against the same buffer, eliminating the buffer each time, before being refrigerated overnight at 4 °C 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.

2.6. Keratinase Assay

The reaction contained 0.01 g keratin and 0.01 g enzyme powder (each was dissolved in 1 mL of phosphate buffer solution, pH 8.0). After 60 min at 50 °C, the reaction was terminated by introducing 2.0 mL of 10 % trichloroacetic acid (TCA) and the precipitate was decanted after centrifugation (Hermle Labortechnik; Wehingen, Germany) 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 copper reagent (sodium carbonate, 40 g; tartaric acid, 7.5 g; copper sulfate, 4.5 g and distilled water, 1000 mL; pH 10) 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+; Leicestershire, UK). One unit of the enzyme (U) was defined as the amount needed to release 1 µmol tyrosine per min. Using tyrosine as the standard, the keratinase activity was calculated according to Equations (1) and (2).

$$\text{Concentration of L-tyrosine} = (\text{Absorbance}/0.0019) \times 1000 \text{ mg/mL} \quad (1)$$

$$\text{Keratinase activity} = (\text{Concentration of L-tyrosine})/0.0001812 \text{ IU/mL/min} \quad (2)$$

2.7. Collagenase Assay

A 0.01 g enzyme powder was mixed with 1 mL of 1 % collagen each was dissolved in 1 mL citrate buffer (pH 6.0). After 120 min at 35 °C, 0.2 mL of the reaction mixture was combined with 0.5 mL of 2 % ninhydrin and boiled for 10 min [35]. After that, 5 mL of 50% isopropanol was added, and the degree of proteolysis was evaluated by color development at 570 nm. One unit of collagenolytic activity corresponds to the enzyme amount that releases 1 µmol leucine per mL. Using leucine as the standard, collagenase activity was calculated according to Equations (3) and (4).

$$\text{Concentration of L-leucine} = (\text{Absorbance}/0.0139) \times 1000 \text{ mg/mL} \quad (3)$$

$$\text{Collagenase activity} = (\text{Concentration of L-leucine})/0.000132 \text{ IU/mL/min} \quad (4)$$

2.8. Impact of pH, Temperature and Some Ions and Inhibitors on Keratinase and Collagenase Activity

A 0.01 g amount of enzyme powder and 0.01 g of keratin powder or 0.01 g of 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 °C) 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) was 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 the method described by Lowry et al. [36].

3. Results

3.1. Morphological and Molecular Identification of the Strain of Didymella

The isolated fungal strain in this study was morphologically identical to published descriptions of *D. keratinophila*. Hyphae pale brown, smooth- and thin-walled, septate hyphae. Conidiomata pycnidial, brown in color, solitary, superficial, 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 (Figure 1A–C).

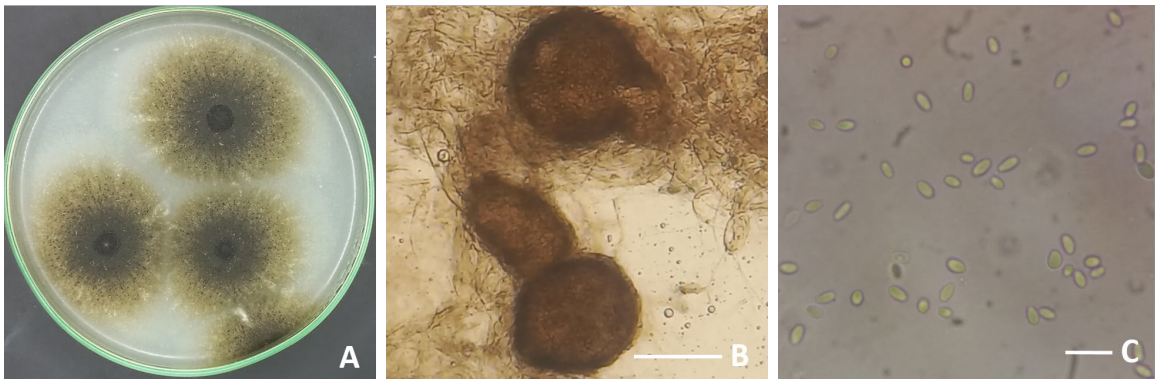

Figure 1. *Didymella keratinophila* AUMC 15399. (**A**), 7–day–old colonies on OA at 25 °C (**B**), Pycnidia with single papillate ostiolar neck (**C**), Ovoid to cylindrical conidia. Scale bar: B = 100 µm, C = 10 µm.

Using the blast search in GenBank, the ITS sequence of this strain was found to be 100 % (525/525) identical to *D. 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 Figure 2 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 in the same clade as *D. keratinophila* UTHSC DI16-200 (type strain) and *D. keratinophila* CBS 140826 (Figure 2).

3.2. Maximization of Keratinase and Collagenase Production

Didymella keratinophila AUMC 15399 produced keratinase and collagenase after 7 days of fermentation at 25 °C, with production producing 2440 ± 230 and 1548 ± 200 U/mL of each enzyme, respectively, at pH 9 and 6 (Figure 3A). The best nitrogen sources were sodium nitrate and beef extract, which exhibited keratinase and collagenase activity to 2440 ± 230 U/mL and 9230 ± 840 U/mL, respectively (Figure 3B). The optimal temperature was found to be 30 °C, and the peak values of keratinase and collagenase activity were 5380 ± 840 and 9342 ± 1950 U/mL, respectively (Figure 3C). After 10 days of fermentation, the chicken feathers were completely hydrolyzed and the maximum levels of keratinase and collagenase activity were attained reaching 8780 ± 620 and 11,230 ± 1290 U/mL, respectively (Figure 3D).

3.3. Production of Keratinase and Collagenase from Native Chicken Feathers in SmF

After 10 days of incubation, the fungus was able to flourish in the fermentation media and totally dissolve the chicken feathers. By employing 60 % ammonium sulphate precipitation, it was possible to get 7.13 g of crude protein powder from *D. keratinophila* AUMC 15399 per liter of fermentation medium after the incubation time (Figure 4A–D).

3.4. Effect of pH on Keratinase and Collagenase Activity

The best pH for maximum keratinase activity was pH 7.0 (Figure 5A), which resulted in keratinase specific activity of 5835 ± 383 U/mg protein at 50 °C. While, the specific activity of the collagenase enzyme peaked at pH 6.0 and 40 °C producing 13,912 ± 1130 U/mg (Figure 5B).

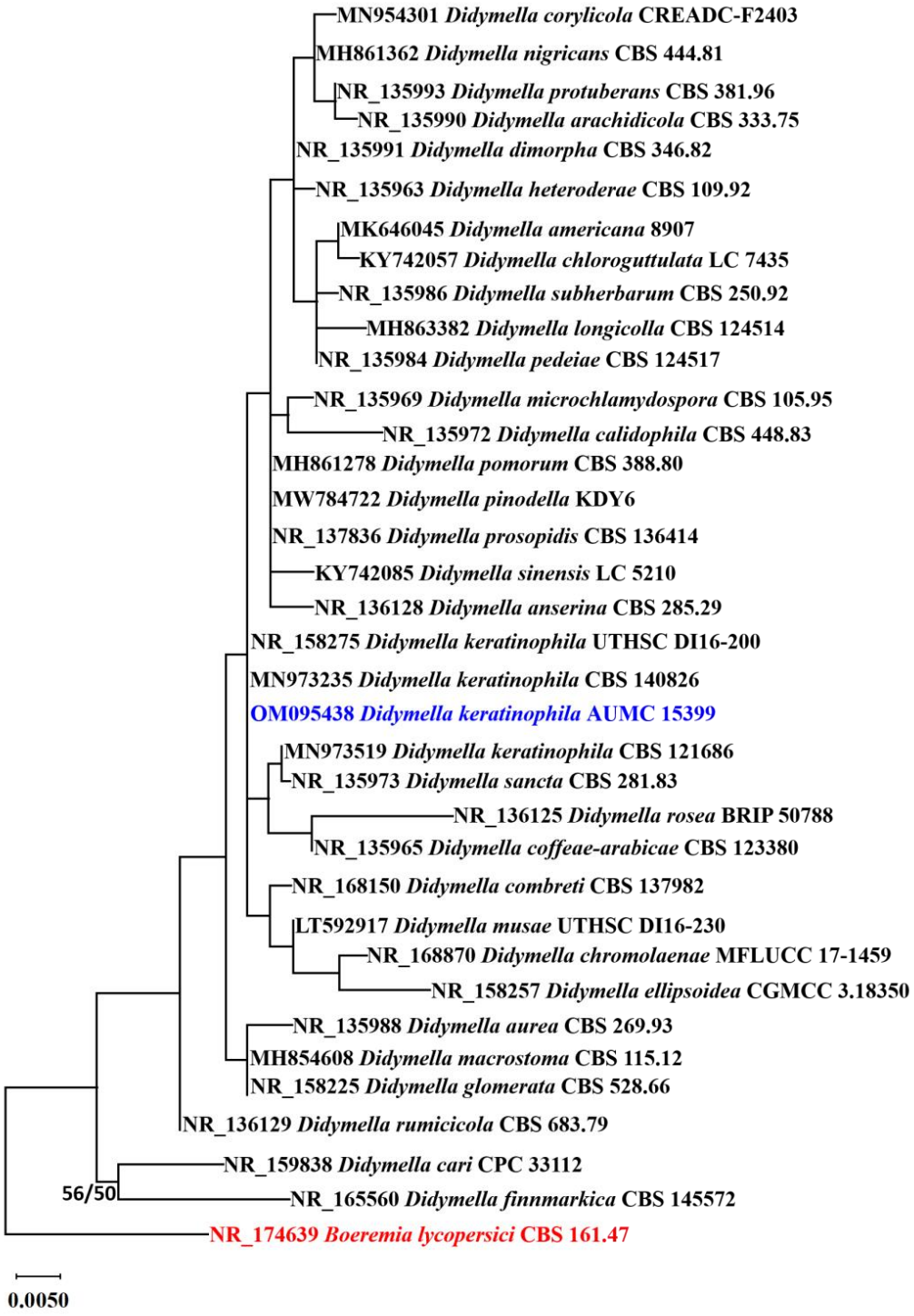

Figure 2. Maximum likelihood phylogenetic tree based on sequencing of the ITS region of *D. keratinophila* AUMC 15399 (in blue color) compared to the members of the genus *Didymella* in GenBank. Bootstrap support values (1000 replications) for ML/MP $\geq$ 50 % are indicated near the respective nodes. The tree is rooted to *Boeremia lycopersici* CBS 161.47 (in red color).

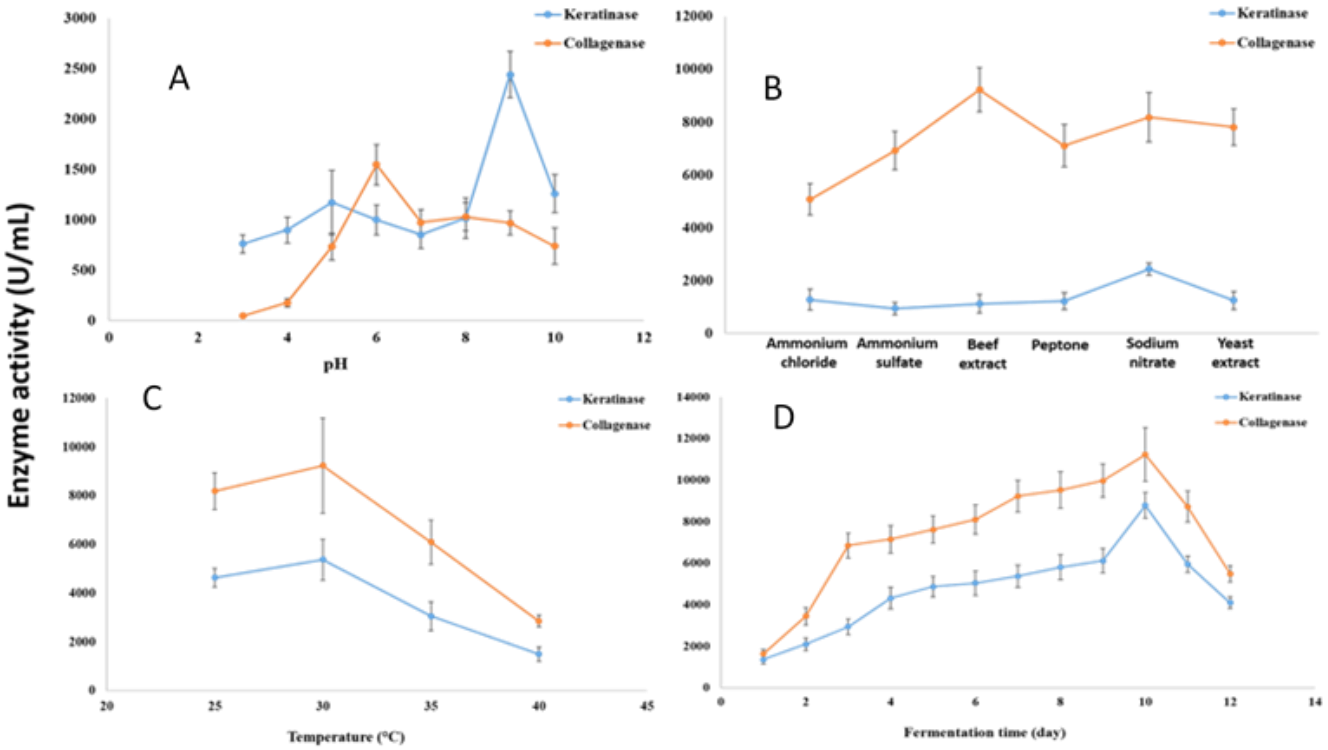

Figure 3. Fermentation conditions (**A**) pH, (**B**) Nitrogen source, (**C**) Temperature, (**D**) Fermentation time optimized for maximization of keratinase and collagenase production by *D. keratinophila* AUMC 15399 in SmF.

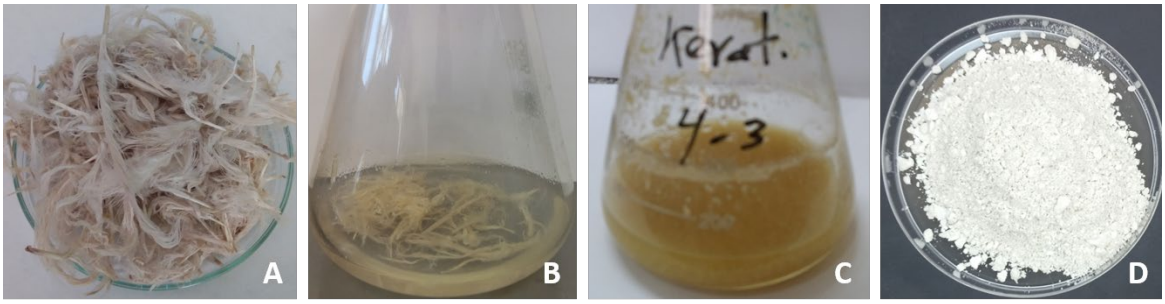

Figure 4. Stages of keratinase and collagenase production by *D. keratinophila* AUMC 15399 in SmF. (**A**), Defatted chicken feathers (**B**), Fermentation medium containing native feathers (**C**), Fully-hydrolyzed feathers after 10 days of incubation (**D**), Lyophilized powder of the crude keratinase and collagenase enzymes.

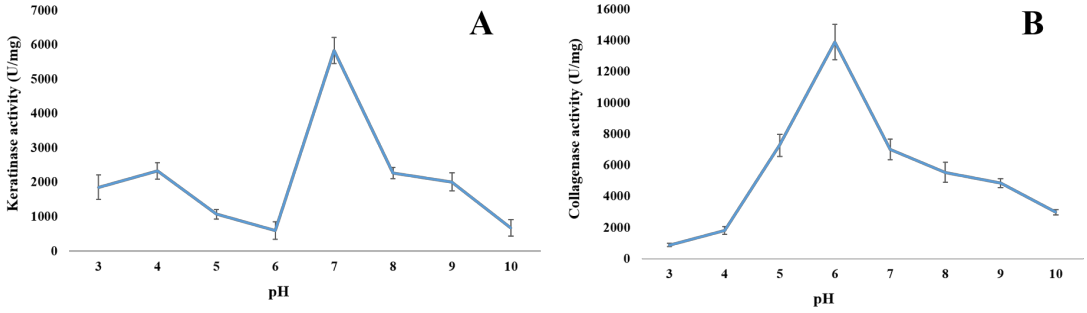

Figure 5. Effect of pH on the activity of (**A**) keratinase, and (**B**) collagenase produced by *D. keratinophila* AUMC 15399.

3.5. Effect of Temperature on Keratinase and Collagenase Activity

At pH 7.0, the keratinase activity increased to 44,903 ± 1555 U/mg protein at 45 °C (Figure 6A). The activity of the collagenase enzyme at pH 6.0 was significantly influenced by temperature, reaching a high at 35 °C and 15,753 ± 510 U/mg of specific activity (Figure 6B).

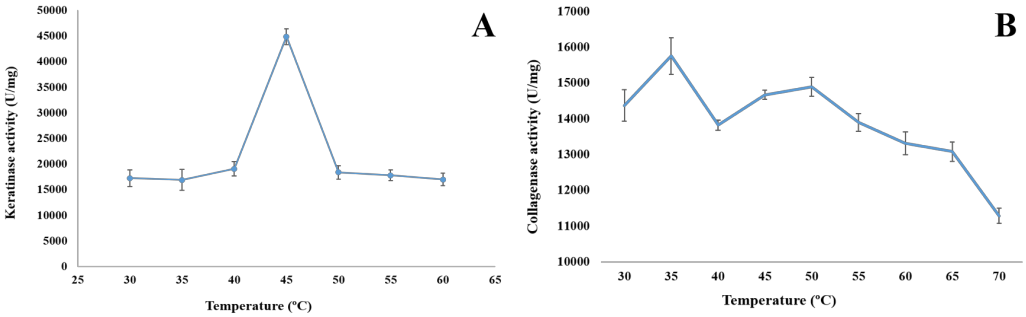

Figure 6. Effect of temperature on the activity of (**A**), keratinase and (**B**), collagenase produced by *D. keratinophila* AUMC 15399 at pH 7.0.

3.6. Effect of Some Ions and Inhibitors on Keratinase and Collagenase Activity

When examined under 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 the keratinases and K^{+} in the case of the collagenase (Table 1).

Table 1. Effect of some ions and inhibitors (5 mmol/mL) on keratinase (at pH 7.0 and 45 °C) and collagenase (at pH 6.0 and 35 °C) activity produced by *D. 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 Specific Activity (U/mg)	Collagenase Specific Activity (U/mg)
Control	**44,903 ± 1555**	**15,753 ± 110**
Na^{+}	16,728 ± 12	3532 ± 64
K^{+}	7619 ± 14	1439 ± 37
Fe^{2+}	16,878 ± 64	14,644 ± 37
Cu^{2+}	12,430 ± 98	13,358 ± 121
Ca^{2+}	14,366 ± 128	13,972 ± 18
Mg^{2+}	16,093 ± 320	14,600 ± 66
Zn^{2+}	19,039 ± 149	13,967 ± 21
Ni^{2+}	14,680 ± 192	15,426 ± 37
Co^{2+}	19,495 ± 133	15,550 ± 84
Mn^{2+}	7089 ± 18	6699 ± 36
EDTA	14,209 ± 111	14,644 ± 314
SDS	12,720 ± 12	12,744 ± 36

4. Discussion

In this first report, natural chicken feathers were 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 [37,38], the treatment of keratinous waste generated in agriculture and the leather industry [39], and the treatment of wastes from the cattle, poultry, and leather in-

dustries [40,41]. By-products including amino acids, polypeptides, vitamins, and detergent additives are potential innovative uses that promote agricultural sustainability [42,43].

More future work is 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 structure of the disulphide linkages of keratin [44]. The utilization of natural microorganisms lowers the cost of enzyme synthesis while also providing cost-effective waste treatment techniques [3]. *Didymella keratinophila* AUMC 15399 investigated proved to be a viable candidate for the production of active keratinase and collagenase. We report, for the first time, the potential of the strain *D. keratinophila* AUMC 15399 to produce keratinase and collagenase from chicken feathers, 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) [1,9,14,45–50]. As a result, optimal conditions for biotechnological solutions need to be investigated. Keratinase from this study had an ideal pH of 7.0 and temperature of 45 °C, whereas collagenases had optimal pH of 6.0 and temperatures of 35 °C, respectively. At these optimum parameters of keratinase and collagenase, the specific activity was improved 44,903 ± 1555 U/mg and 15,753 ± 110 U/mg for both enzymes respectively.

There are no reports of species of *Didymella* producing either keratinase or collagenase, according to the published data. Various species of fungi have extremely varying optimal conditions. For example, *Aspergillus terreus* grew best in a feather meal basal medium at pH 8 and 40 °C [51] while strains of *Aspergillus niger* produced varying amounts of keratinases with the highest activity at pH 5 [52]. *Aspergillus flavus* grew best in a feather meal basal media at pH 8 and 28 °C [53]. This implies that the ideal cultivation conditions for each species must be determined separately.

In this study, *D. keratinophila* completely hydrolyzed the chicken feathers after 10 days at 30 °C and produced the highest keratinase and collagenase enzymes at pH 9 and 6 using sodium nitrate and beef extract as nitrogen supply, respectively. In this regard, *Cochliobolus hawaiiensis* produced the highest alkaline keratinase after 15 days incubation at 30 °C and pH 9.5 [54]. *Chrysosporium tropicum* generated the maximum keratinase in a medium containing chicken feathers after 21 days at 25 °C [55]. *Trichophyton ajelloi* exhibited the highest enzyme activity (6.3 kU/mL) at 30 °C [56]. Keratinolytic activity for *Chrysosporium tropicum* peaked (8.6 kU/mL) on the 40th day of incubation [42]. The maximum activity for *Microsporum gypseum* (78 kU/mL) and *M. canis* (76 kU/mL) was observed on the 20th day of incubation [43]. Because of minor changes in technique, it is difficult to compare enzyme activity results among different research efforts. As a result, comparisons should be interpreted cautiously.

Many experiments have established the metal requirement of microbial keratinase activity [2,57,58]. This was also noticed for our strain, *D. keratinophila* AUMC 15399. There is no prior knowledge in the literature about our species, as EDTA and the ions had a substantial inhibitory influence on the activity of the keratinases and collagenases produced. With Mn^{2+} for the keratinases and K^{+} for the collagenase, the strongest inhibitory effects were seen. However, an *Aspergillus oryzae* feather-degrading culture was enhanced by Ca^{2+} and Ba^{2+} ions while inhibited by EDTA and Pb^{2+} ions [59]. During solid-state fermentation with chicken feathers, the addition of EDTA, Hg^{2+}, and Fe^{3+} greatly reduced the keratinase activity in *A. flavipes*, although Zn^{2+}, Mg^{2+}, and Cu^{2+} had no discernible effect [60]. The keratinases generated by *Aspergillus stelliformis* AUMC 10920 and *A. sydowii* AUMC 10935 had the greatest inhibitory impact with EDTA, while Ca^{2+}, Cu^{2+}, and Zn^{2+} were the inhibitory metal ions for *F. brachygibbosum* AUMC 10937 keratinase [2].

On the other hand, the development of novel microbial collagenases has grown throughout time, making it one of the largest enzyme industries today [61,62]. Since collagenase has a wide range of applications and high biotechnological potential, the devel-

opment of new production techniques, such as the hunt for producing microorganisms, alternative substrate sources, better extraction conditions, and purification of collagenase, has been of paramount importance. Aside from that, bacterial sources are the only ones included in the majority of published review publications on microbial collagenolytic enzymes. [63–65]. Consequently, it was necessary to have a better understanding of the state of the art in terms of fungi's synthesis, characterization, and purification of collagenolytic enzymes. *Didymella keratinophila* AUMC 15399 was used in this investigation to hydrolyze the native chicken feathers and produce collagenase enzyme with high specific activity.

Only a few studies have reported on enzymes that strongly prefer collagen to other protein substrates. *Arthrobotrys amerospora* [66], *Arthrobotrys tortor* [67], *Aspergillus oryzae* Mi 156 [68], *Candida albicans* URM3622 [23], *Entomophthora coronata* [69], *Penicillium aurantiogriseum* URM 4622 [70], *Penicillium* sp. [71], and *Rhizoctonia solani* [72], which are extracellular enzyme makers, stood out among the microorganisms investigated for their volumetric and specific collagenase activity. This work is a global first in employing *Didymella keratinophila* AUMC 15399 to hydrolyze native chicken feathers and create high levels of enzymes with high specific activity for keratinase and collagenase.

5. Conclusions

The primary source of several biotechnological processes and products is microbial biodiversity. In order to produce high-value compounds like amino acids and bioactive peptides, microbial keratinase-mediated degradation of keratin-rich substrates has expedited the cost-effective and environmentally friendly valorization and bio-cycling of keratinous wastes. The industrial processes for processing complex materials have undergone a significant change as a result of the discovery of keratinases, shifting away from conventional techniques and towards more environmentally friendly biobased alternatives. *Didymella keratinophila* AUMC 15399 shown effective cleavage of chicken feather residue in the current investigation. It can grow on simple medium using feathers as its only carbon and nitrogen sources and doesn't require a high substrate concentration. Results of *D. keratinophila* AUMC 15399's feather degradation point to low-cost future use in agro-industrial residues. It is described here as a prospective source of active keratinase and collagenase produced by microorganisms, which may be used to break down difficult-to-digest and resistant keratinous wastes necessary for sustainable development. A significant amount of collagenase was also produced, and this proved to be an excellent component for use in the formulation of nutrient-rich animal feed. Collagenases are being utilized more often in medical applications to create slow-releasing versions that may be used to burn therapy and digestive assistance.

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