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Screening of Fungi for Efficient Feather Degradation and Keratinase Production

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Abstract: ***Chicken feather waste generated by poultry industries represents a significant environmental challenge because of its high keratin content and resistance to natural degradation. This study investigated keratinophilic fungi isolated from poultry waste soils for their feather degradation and keratinase-producing abilities. Fungal isolates were obtained using hair-baiting and serial dilution techniques and identified by morphological characteristics. Keratinase production was screened on casein agar, after which selected isolates were evaluated under different incubation periods, temperatures, and pH conditions. Aspergillus niger and Aspergillus flavus demonstrated the highest keratinase activities and effectively degraded chicken feathers under optimized conditions. The findings indicate that these fungi possess considerable potential for environmentally friendly poultry feather waste management and production of value-added bioproducts through keratin biodegradation.***

Keywords: Keratinase; Keratinophilic fungi; Feather biodegradation; Aspergillus niger; Aspergillus flavus; Poultry waste; Bioconversion; Environmental biotechnology

1. Introduction

Large volumes of chicken feathers are produced as by-products of commercial poultry processing and natural shedding, and their disposal has become a significant global environmental challenge. (29). Approximately 400 million chickens are processed globally each week, and with an average feather yield of about 125 g per bird, this results in nearly 300 tons of feather waste being generated weekly. The management of this large volume of waste poses a serious environmental challenge, contributing to the contamination of air, soil, and groundwater resources. (40).

Feathers consist of more than 90% protein, primarily in the form of α- and β-keratins, which are fibrous, insoluble structural proteins stabilized by extensive disulfide, hydrogen, and hydrophobic cross-linkages. Keratin is composed of various amino acids, with cysteine, lysine, proline, and serine being the predominant constituents (31 and 50). These amino acids readily form intermolecular disulfide and hydrogen bonds, producing fibers that are mechanically strong, lightweight, and possess effective thermal and acoustic insulating properties. (39,31).

Keratin-rich waste generated by various industries mainly feathers, hair, horns, hooves, and nails are increasingly accumulating in the environment. In addition, substantial amounts of keratinous residues originating from routine shaving activities are present in sewage systems and river or canal sediments. These materials now constitute a significant component of solid waste streams and are resistant to natural degradation. (24).

Various methods have been employed for the management of feather waste, including landfilling, incineration, conversion to biogas, and processing for use as animal feed. However, disposal through landfilling or burning is costly and contributes to the pollution of air, soil, and water resources (35). Keratinases are serine or metalloproteases that catalyze the degradation of keratin, the primary structural protein of keratinous materials. The hydrolysis of keratin is widely recognized to occur through the action of these extracellular keratinolytic enzymes. (38).

Keratinase production has been reported in a wide range of microorganisms, including fungi such as *Doratomyces microsporus*, *Alternaria radicina*, *Trichurus spiralis*, *Aspergillus* spp., *Rhizomucor* spp., and *Stachybotrys alba*; actinomycetes such as *Streptomyces pactum*, *S. albus*, *S. thermoviolaceus*, *S. fradiae*, and *Thermoactinomyces candidus*; as well as several bacterial species, including *Fervidobacterium landicum*, *Pseudomonas aeruginosa*, *Microbacterium* spp., and multiple *Bacillus* species, notably *B. licheniformis* and *B. pumilus*. (21, 41).

In recent years, keratinases isolated from non-pathogenic microorganisms have gained significant biotechnological attention because of their ability to transform keratin into value-added products such as nitrogen-rich fertilizers, biodegradable films, adhesives, and foils (35, 7, 49). In addition, keratinolysis facilitated by these enzymes represents a potentially valuable protein source for animal feed applications (35).

Keratinases are applied in a wide range of fields due to their ability to hydrolyze keratin, including the leather and detergent industries, textile processing, waste bioconversion, as well as medical and cosmetic applications such as transungual drug delivery and the degradation of keratinized skin. (32).

2. Materials and Methods

2.1 Sample Collection

Soil samples were collected at random, with two samples obtained from each of five poultry waste dump sites within Michael Okpara University of Agriculture, Umudike, Umuahia. Soil was sampled to a depth of 10 cm at each site using a sterile spoon and transported in sterile polyethylene bags to the Central Service Laboratory of the National Root Crops Research Institute, Umudike, for fungal isolation. Chicken feathers used as bait were obtained from the poultry processing section of Orie Ugba market, Umuahia. The feathers were washed thoroughly to remove blood stains and extraneous materials, air-dried, and subsequently conveyed to the laboratory for analysis (29).

2.2 Media Preparations

Potato dextrose agar (39 g/L) was prepared according to the manufacturer's instructions, sterilized at 121 °C for 15 min, cooled, and dispensed into sterile Petri dishes.

2.3 Feather Meal Broth Preparation

Feathers were defatted with diethyl ether for 24 h, rinsed repeatedly with distilled water, oven-dried at 60 °C for two weeks, and ground into powder. Ten grams of the feather meal was mixed with mineral salts (0.5 g NaCl, 0.3 g K_2HPO_4, 0.4 g KH_2PO_4, 0.1 g $MgCl_2 \cdot 6H_2O$, 0.1 g yeast extract) in 100 mL of distilled water to prepare the feather-meal broth (2).

2.4 Casein Agar Media Preparation

Casein agar was prepared by combining skimmed milk (50 g/L), agar (15 g/L), and yeast extract (2.5 g/L), followed by sterilization at 121 °C for 15 min. After cooling, the medium was dispensed into sterile Petri dishes.

2.5 Isolation of Keratinophilic Fungi

Fungal isolation was carried out using both the serial dilution method and Vanbreuseghem's hair-baiting technique (HBT) (48), with HBT favored for its greater reliability in selectively isolating keratinophilic fungi.

2.5.1 Isolation using the serial dilution method

Each soil sample (I g) was serially diluted in sterile peptone water from 10^{-1} to 10^{-9}. Aliquots (1 mL) from the 10^{-3} and 10^{-4} dilutions were plated onto potato dextrose agar (PDA) using the pour plate method and incubated at 25 °C for 3-5 days (37).

2.5.2 Isolation using hair-baiting technique

The hair-baiting method (25) was performed using sterilized chicken feathers as a keratin source. Soil samples were moistened with 5 mL of sterile water and incubated at 25 °C for seven days. Emerging mycelia were aseptically transferred onto PDA for purification.

Figure 1: Showing Hair Baiting Method of Isolation.

2.6 Identification of the Purified Cultures

Fungal isolates were identified based on macroscopic characteristics, including colony morphology and pigmentation, as well as microscopic features using lactophenol cotton blue staining. Structures such as septate hyphae, conidiophores, fruiting bodies, and rhizoids were examined and compared with standard taxonomic keys (3).

2.7 Screening for Keratinophilic Fungi on Casein Agar Plates

Keratinase activity was assessed using a modified method described by Kanchana (27). Fungal isolates were inoculated onto casein agar and incubated at 25 °C for 3–5 days, with the diameter of the resulting clear zone serving as an indicator of proteolytic activity.

2.8 Submerged Cultivation of Fungi

Submerged fermentation was conducted following Mini et al. (32). Each 100 mL broth was inoculated with a 6 mm mycelial disc and incubated at 27 ± 2 °C, 37 °C, or 45 °C for five days. Cultures were filtered, centrifuged (4000 rpm, 5 min), and the supernatant used as crude enzyme extract.

2.9 Determination of Keratinase Activity

Keratinase activity was quantified by incubating 200 µL of enzyme extract with feather-meal substrate (20 mg) in 100 mM Tris-HCl buffer (pH 7.8) at 37 °C for 1 h. (19). Enzyme activity was expressed as one unit (KU) corresponding to an absorbance increase of 0.1 at 280 nm.

2.10 Effect of Incubation, pH, and Temperature on Enzyme Activity

The influence of incubation time (10-60 min), pH (4-10), and temperature (27-45 °C) on keratinase activity was evaluated to determine the enzyme's optimal conditions (28).

2.11 Degradation of Chicken Feather

The ability of fungal isolates to degrade feathers was evaluated by incubating each isolate with 10 intact feathers in feather-meal broth at 25 °C for seven weeks. Keratin degradation was assessed both visually and by measuring absorbance at 400 nm (27).

3. Results and Discussion

3.1 Isolation and Identification of Keratinophilic Fungi

Fungal colonies (13 colonies) were obtained from soil samples and identified through their colony morphology, cultural characteristics, slide culture observations, pigmentation, and the structure of hyphae and spores. The isolates were identified as *Aspergillus niger*, *Mucor* spp., *Rhizopus* spp., *Aspergillus flavus*, *Trichoderma* spp., *Fusarium* spp., and *Curvularia* spp. Comparable findings were reported by Ritesh et al. (43), who isolated *Aspergillus niger*, *Aspergillus flavus*, *Mucor* sp., *Rhizopus* sp., *Fusarium* sp., *Curvularia* sp., *Trichoderma* sp., *Penicillium* sp., and *Chrysosporium* sp. from garbage-contaminated soils in the Jharkhand region of India. Similarly, Nwadiaro et al. (36) observed a high prevalence of various *Aspergillus* species in soils impacted by tannery wastes in Jos, Nigeria. The elevated fungal counts suggest that contaminated soils support dense fungal populations, likely due to the presence of growth-promoting factors in the effluents. Additionally, the contamination may disrupt the soil's ecological balance, favoring the proliferation of these fungi, which are naturally indigenous to soil and capable of persisting in such environments. Micrographs of selected fungal isolates are presented in Plates 3.1–3.5. Table 3.1 provides the macroscopic and microscopic characteristics of the isolated fungal strains, as described using the mycology atlas of Barnett and Hunter (9).

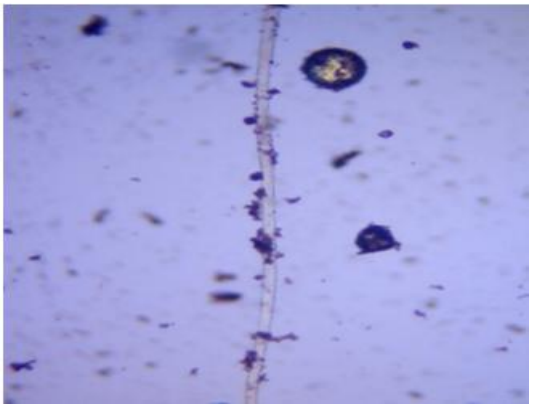

Plate 3.1: Micrograph of Aspergillus niger Isolated from Waste Dump Soil

Plate 3.2: Micrograph of Aspergillus flavus Isolated from Waste Dump Soils

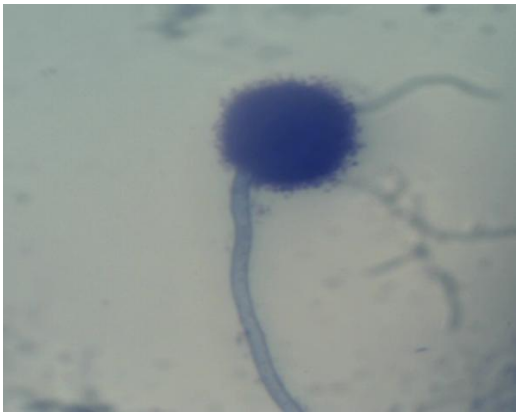

Plate 3.3: Micrograph of Mucor Isolated from Waste Dump Soils

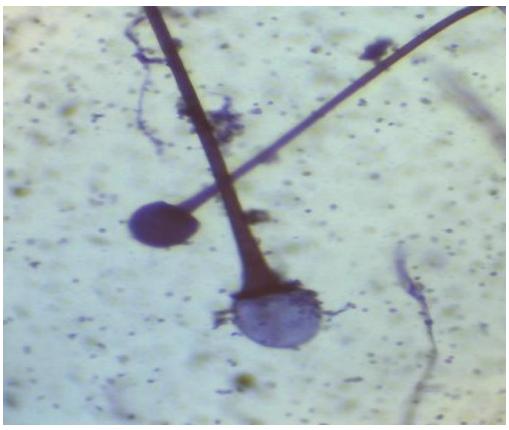

Plate 3.4: Micrograph of Rhizopus stolonifer Isolated from Waste Dump Soils

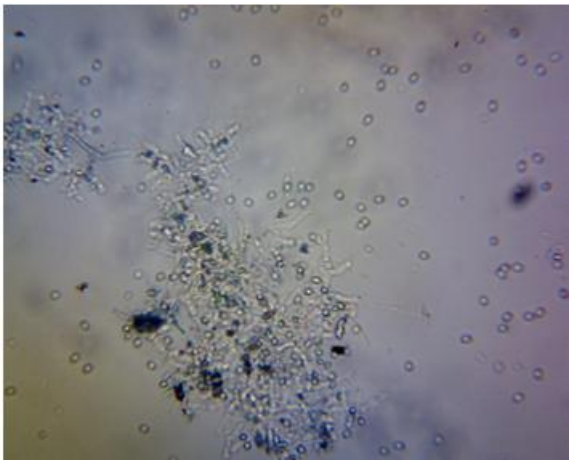

Plate 3.5: Micrograph of Trichoderma viridae Isolated from Waste Dump Soils

This method has been employed by Nwadiaro et al. (45), Kanchana and Divakar (28), Kanchana (27), Ritesh et al. (54), and Moallaei et al. (33) to isolate high counts of keratinophilic fungi. Similarly, Adesemoye et al. (1) reported elevated microbial counts in wastewater-contaminated soils at Agege and Odo abattoirs in Lagos, Nigeria. Additional studies from Egypt, Australia, Palestine, Spain, India, Kuwait, Ukraine, and Malaysia (34,7) confirm the widespread distribution of these fungi across diverse soil habitats.

Table 3.1: Macroscopic and Microscopic Description of Isolated Fungi

IsolateNo.	Morphology and Colour	Microscopic Description	Fungi
1.	Dark brown in colour, occurs in patches and gritty in appearance.	Conidiophores upright, simple, terminating in a globose or clarate swelling,bearing phialides at the apex or radiating from the apex or the entire surface; conidia 1-celled, and in dry basipetal chains.	*Aspergillus niger*
2.	Pinkish-White cotton-like in culture, fluffy in appearance.	Mycelium extensive and cotton-like in culture with some tinge of pink; conidiophores variable, slender and simple branched irregular bearing a whorl of phialides group into sporodochia, conidia hyaline, held in small moist heads; macroconidia 1-celled, ovoid, borne in chains, 2-3 celled, oblong or slightly curved.	*Fusarium Spp.*
3.	Brown in appearance, smooth dense appearance.	Conidiophores brown, simple, bearing conidia aptically or on new sympodial growing points; conidia dark end cells lighter, 3-5 celled, fusiform, typically bent with one of the central cells enlarged.	*Curvularia Spp.*
4.	Greenish, suspending scattered appearance, occurs in patches.	Conidiophores hyaline, much branched, not verticillate; phialides single; conidia hyaline, 1-celled, ovoid, borne in small terminal clusters, easily recognized by its rapid growth and green patches of conidia.	*Trichoderma spp.*
5.	Green in colour, occurs in patches, and gritty in appearance.	Conidiophores upright, simple, terminating in a globose or clavate swelling, bearing phialides at the apex or radiating from the apex or the entire surface; conidia hyaline, brightly coloured in mass, mostly globose in dry basipetal chains.	*Aspergillus Flavus*
6.	Grey in colour, fluffy appearance with numerous dark spores all over the culture.	Sporangiophores produced inside spherical sporangia, a large collumella that remains after the wall is broken; no rhizoids.	*Mucor spp.*
7.	Grey in colour, fluffy appearance with numerous dark spores round the colony and lesser at the center.	Rhizopus characterized by the presence of stolon and rhizoids, sporangiophores singly or in groups form nodes directly above the rhizoides and collumellate, multispored, generally globose sporangia. Colonies are fast growing and cover an agar surface with a dense cotton growth that is at first white becoming grey or yellowish brown.	*Rhizopus spp.*

3.2 Screening of Keratinophilic Fungi

All isolates were screened for enzyme activity using casein agar as the sole carbon and nitrogen source. Among the seven fungal strains, only two *Aspergillus niger* and *Aspergillus flavus* showed positive activity, as illustrated in Plates 3.6 and 3.7. *Aspergillus niger* produced the largest zone of inhibition (15 mm), while *Aspergillus flavus* showed a smaller zone (10 mm) (Table 3.3). The remaining isolates grew on the medium but did not produce inhibition zones. Fungi that formed clear zones on casein agar were classified as proteolytic.

The fungal isolates were screened for extracellular enzyme production on casein agar. *Aspergillus niger* showed the largest zone of casein hydrolysis on skim milk agar (Plates 3.7 and 3.8), while other isolates produced no hydrolytic zones, indicating limited protease activity. This aligns with findings by Thoomatti and Peramachi (47) and Kanchana and Divakar (28), who reported high protease activity in *Aspergillus* species. Although previously considered non-keratinolytic, these non-dermatophytic fungi may be keratinophilic or keratinolytic, utilizing fats on keratinous tissues as a carbon source (45).

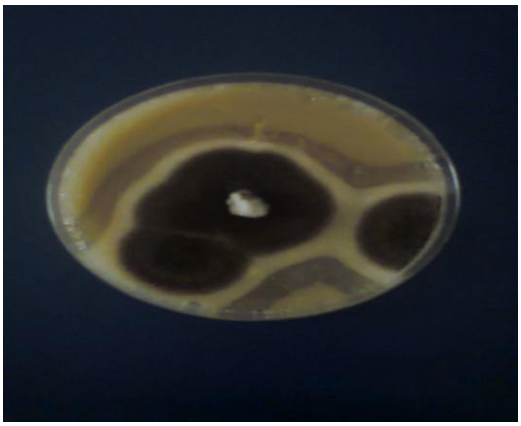

Plate 3.6: Aspergillus Niger with Zone of Inhibition on Casein Agar

Table 3.2: Clear Zone of Inhibition on Casein Agar by the Fungal Strains

Organisms	Clearance(mm)
Aspergillus niger	15
Aspergillus flavus	10

3.3 Enzyme Production

The effect of incubation time on enzyme production by *Aspergillus flavus* and *Aspergillus niger* is shown in Figure 3.1. Maximum enzyme yields were observed at 96 hours (4 days), with activities of 16.80 U/mL for *A. flavus* and 11.97 U/mL for *A. niger*, followed by a decline in production. Therefore, 96 hours represents the optimal harvesting time for maximum enzyme production in both species.

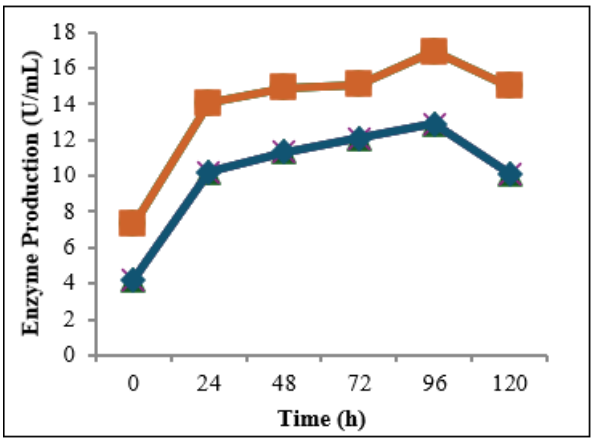

Figure 3.1: Effect of Incubation Period on Production of Keratinase by the Fungal Isolates

The reduction in enzyme production beyond this point was likely associated with nutrient depletion, accumulation of metabolic by-products, and the onset of the decline phase of fungal growth (42). Incubation time is a critical factor influencing enzyme production and varies among microorganisms because of differences in their growth characteristics, particularly the duration of the lag and exponential phases (10).

The optimum temperature for maximum enzyme production differed between the species: *Aspergillus flavus* produced 19.68 U/mL at 27 ± 2 °C, whereas *Aspergillus niger* reached 18.94 U/mL at 45 °C (Figure 3.2).

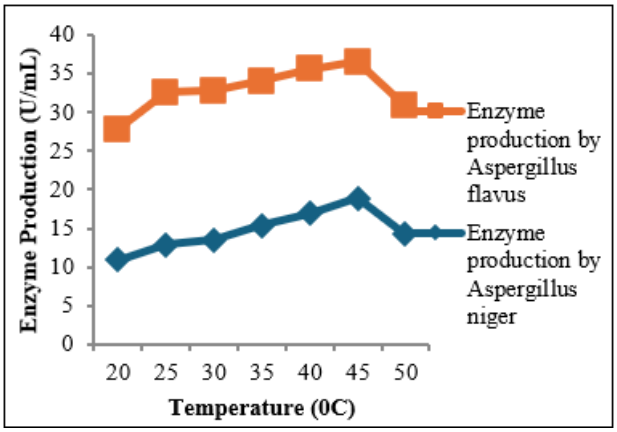

Figure 3.2: Effect of Temperature on Production of Keratinase by the Fungal Isolates

Temperature is a critical ecological factor influencing fungal growth, sporulation, and enzyme production, with each species exhibiting a distinct optimum range determined by its physiological and genetic characteristics. Although most fungi grow optimally between 15 °C and 35 °C (30,55), variations in enzyme production among fungal isolates are likely to reflect differences in their genetic makeup. Consistent with this, Daniel et al. (15) reported that enzyme activity increases with temperature up to an optimum level, after which it declines rapidly because of protein denaturation.

The present findings are consistent with Ali (4), who reported an optimum temperature of 30 °C for protease production by A. fumigatus, but differ from Hossain et al. (22) and Devi et al. (18), who observed optimum temperatures of 45 °C for Aspergillus spp. and A. flavus, respectively. Such variations may result from differences in fungal strains, culture conditions, or experimental methodologies. Temperatures below the optimum reduce fungal metabolic activity, whereas temperatures above the optimum can denature enzymes, impair respiration, and ultimately inhibit fungal growth or lead to cell death (52).

The effect of pH on enzyme production by the fungal isolates is shown in Figure 3.3. Enzyme production increased with pH until reaching the optimum, after which it declined. Aspergillus niger produced maximum enzyme activity (13.01 U/mL) at pH 9, whereas *Aspergillus flavus* reached peak production (17.01 U/mL) at pH 6. The control exhibited an enzyme activity of 11.17 U/mL.

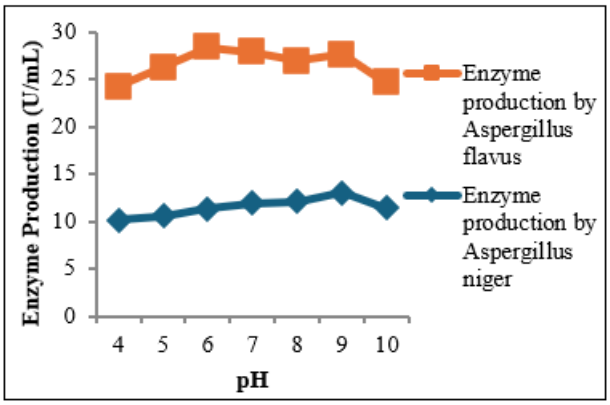

Figure 3.3: Effect of pH on Production of Keratinase by the Isolated Fungal Isolates

These findings indicate that the enzyme produced by *A. niger* is alkaline in nature, consistent with the alkalinophilic characteristics of the fungus, whereas *A. flavus* produces an enzyme with an acidic optimum. Similar optimum pH values of 9 for enzyme production by *A. niger* have been reported by Coral et al. (14) and Nwadiaro et al. (47).

Medium pH is a critical factor influencing fungal growth, metabolism, and enzyme production. Although fungi can tolerate a relatively broad pH range and may alter the pH of their environment during growth, keratinase production and feather degradation are generally enhanced under neutral to alkaline conditions (pH 7-9) (11). Da Silva and Oliveira (17) also reported that alkaline conditions favor the growth of keratinophilic fungi by increasing the accessibility of keratin through alterations in cystine residues. While most fungi grow within a pH range of 4.2-9.3, extreme acidic or alkaline conditions can disrupt protein stability and cytoplasmic organization, thereby impair cellular function and reduce fungal growth and enzyme production (44).

3.4 Enzyme Activity

3.4.1 Effect of pH on keratinase degradation of feathers

The effect of pH on keratinase activity within the range of pH 4-10 showed a gradual increase in activity, reaching a maximum at pH 9 for *Aspergillus niger* and pH 6 for *Aspergillus flavus*, followed by a decline at higher pH values (Figure 3.4).

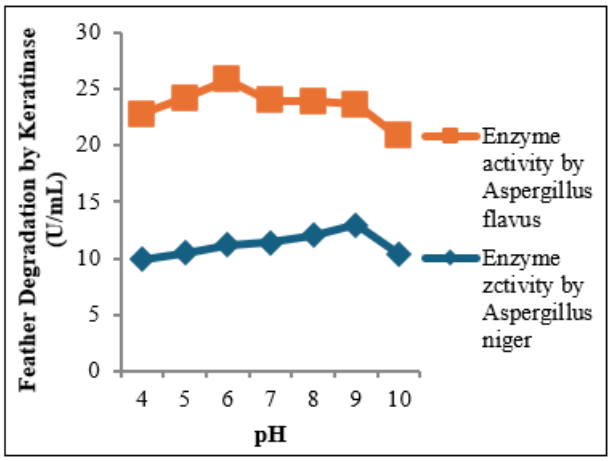

Figure 3.4: Effect of pH on Keratinase Degradation of Feather

These results indicate that the keratinases produced by the two species differ in their pH optima, suggesting potential applications in industrial processes operating under either alkaline or slightly acidic conditions.

Previous studies have shown that keratinases are generally active and stable over a broad pH range (5-13), with most bacterial, actinomycete, and fungal keratinases exhibiting optimum activity under neutral to alkaline conditions, although some remain active at pH values above 12 (27).

The alkaline optimum observed for *A. niger* is consistent with these reports and supports its potential use in industries such as feather degradation and leather processing, where alkaline conditions are common. In contrast, the lower pH optimum of *A. flavus* suggests suitability for applications requiring mildly acidic conditions. The influence of pH on keratinase activity is largely attributed to its effect on the ionization of amino acid residues, which alters enzyme conformation and catalytic efficiency (25).

3.4.2 Effect of temperature on keratinase degradation of feathers

The enzyme exhibited maximum activity at 45 °C for *Aspergillus niger*, indicating its thermotolerant nature, whereas *Aspergillus flavus* showed optimal activity at 27 °C. Enzyme activity declined at temperatures above these optima (Figure 4.5), likely because elevated temperatures disrupt enzyme structure through thermal denaturation, thereby reducing catalytic activity and impairing microbial metabolism and growth (5,16).

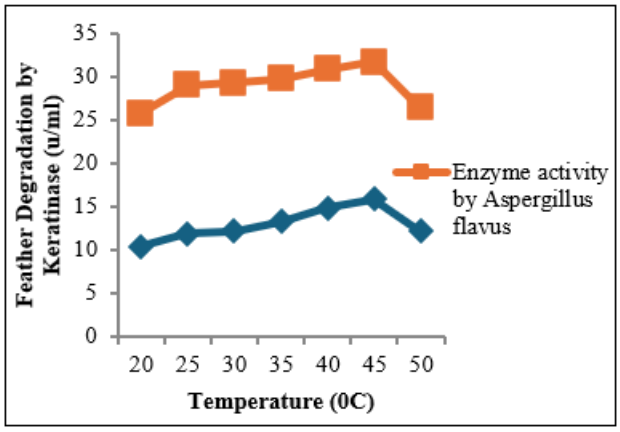

Figure 3.5: Effect of Temperature on Keratinase Degradation of Feather

These findings are consistent with reports that keratinases from most mesophilic microorganisms exhibit optimum activity between 28 and 45 °C (6), a temperature range that is advantageous for keratin hydrolysis because it reduces energy requirements during industrial processing (45). However, the thermal properties of keratinases vary considerably depending on the producing organism. For example, keratinase from the thermophilic *F. pennavorans* exhibits optimum activity at 80 °C, whereas that of the mesophilic *Stenotrophomonas maltophila* DHHJ is most active at 40 °C (11). Jeong-Dong Kim (2007) (26, 23) similarly reported reduced keratinase activity in *Aspergillus* species with increasing temperature. In comparison, other bacterial and fungal keratinases, although often active under alkaline conditions, generally exhibit lower temperature optima than that observed for *A. niger* (13).

3.4.3 Effect of incubation period on keratinase degradation of feathers

Following enzyme production, the crude keratinase was incubated for 1 hour at varying time intervals. A progressive decline in enzyme activity was observed for both fungal isolates, as illustrated in Figure 3.6.

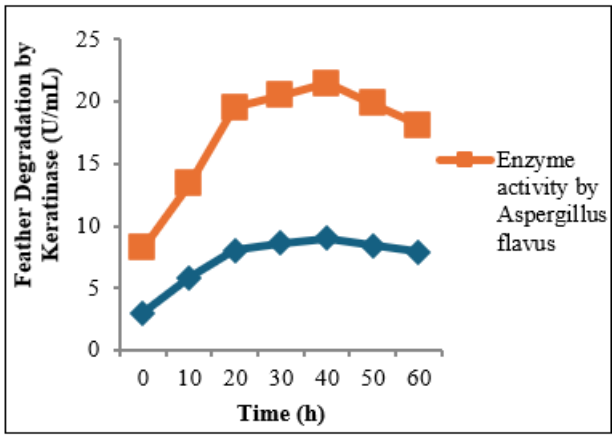

Figure 3.6: Effect of Incubation Period on Keratinase Degradation of Feather

3.5 Degradation of Chicken Feather by Keratinase

Plate 3.8b illustrates the degradation of chicken feathers by *Aspergillus flavus* and *Aspergillus niger*. Figure 3.8a shows the control flask containing sterilized chicken feathers and

feather meal broth without fungal inoculation, while Figure 3.8b presents the experimental setup after seven weeks of incubation. Complete degradation of the chicken feathers was observed. Feather degradation in this study was assessed visually, with increased turbidity of the feather broth indicating fungal growth and effective feather degradation.

Plate 3.8 (a) and 3.8 (b)

Plate 3.8: Degradation of Chicken Feathers by Keratinase produced from *Aspergillus flavus and Aspergillus niger***.**
(a): Control: (FMB + 10 intact raw feather), at 25°C, pH 7.0 for 7 weeks
(b): Test: (FMB + Inoculum + 10 intact raw feather), at 25°C, pH 7.0 for 7 weeks

Plate 3.8b illustrates the degradation of chicken feathers by *Aspergillus flavus* and *Aspergillus niger*. Plate 3.8a shows the control flask containing sterilized chicken feathers in feather meal broth without fungal inoculation, whereas Plate 3.8b presents the inoculated cultures after seven weeks of incubation.

Complete feather degradation was observed, accompanied by increased turbidity and a change in the fermentation broth from nearly colorless to yellow, indicating active fungal growth and keratin degradation. Although microbial keratin degradation may occur within 24 hours to several days depending on the organism and its keratinolytic mechanisms (55,58), the progressive macroscopic digestion observed throughout the incubation period demonstrates the effectiveness of keratinase produced by both fungal isolates. Similar findings have been reported for feather degradation by *Aspergillus* spp., *Chrysosporium* spp., and *Microsporum* spp. (27, 28).

Microbial keratin degradation is fundamentally a proteolytic process because keratin is a highly cross-linked structural protein whose resistance to degradation is largely due to extensive cystine disulfide bonds (34). In filamentous fungi, keratinolysis is achieved through the coordinated action of extracellular keratinases, mechanical penetration of the substrate by growing hyphae, sulfitolysis involving the reduction of disulfide bonds by fungal sulfite, and subsequent proteolysis (20, 58, 61).

Additional reducing agents, including disulfide reductases, sulfite, thiosulfate, and cellular membrane potential, further destabilize the keratin matrix, allowing keratinases and other proteases to hydrolyze the protein efficiently (61). These processes collectively involve deamination, sulfitolysis, and proteolysis, with keratinases serving as the principal inducible extracellular enzymes responsible for keratin breakdown (57,46).

The efficient degradation of chicken feathers observed in this study suggests that these keratinolytic mechanisms operated effectively in both fungal isolates. However, keratinase activity declined after prolonged incubation, possibly because of end-product inhibition by sulfur-containing degradation products or depletion of accessory factors required for sustained enzyme activity.

4. Conclusion and Recommendations

4.1 Conclusion

This study demonstrated that keratinophilic fungi isolated from poultry waste soils possess significant potential for the biodegradation of chicken feathers through keratinase production. Among the isolates, Aspergillus flavus and Aspergillus niger exhibited the highest keratinase activities under different optimal environmental conditions and effectively degraded feather waste. These findings support the use of fungal keratinases as sustainable alternatives for poultry waste management and highlight their potential application in the production of environmentally valuable bioproducts. Further studies involving molecular identification, enzyme purification, and pilot-scale production are recommended to facilitate industrial application.

4.2 Recommendations

Based on the findings of this study, the following recommendations are made:

Poultry feather waste should be reduced through biotechnological applications, such as their utilization in the production of adhesives, slow-release fertilizers, and feather meals.

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