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Original Article

Fungal decomposition of chicken-feather waste in submerged and solid-state fermentation

Decomposição fúngica de resíduos de pena de frango em fermentação submersa e de estado sólido

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Abstract

Poultry industry is expanding rapidly and producing million tons of feather waste annually. Massive production of keratinaceous byproducts in the form of industrial wastes throughout the world necessitates its justified utilization. Chemical treatment of keratin waste is proclaimed as an eco-destructive approach by various researchers since it generates secondary pollutants. Keratinase released by a variety of microbes (bacteria and fungi) can be used for the effective treatment of keratin waste. Microbial degradation of keratin waste is an emerging and eco-friendly approach and offers dual benefits, i.e., treatment of recalcitrant pollutant (keratin) and procurement of a commercially important enzyme (keratinase). This study involves the isolation, characterization, and potential utility of fungal species for the degradation of chicken-feather waste through submerged and solid-state fermentation. The isolated fungus was identified and characterized as *Aspergillus* (*A.*) *flavus*. In a trial of 30 days, it was appeared that 74 and 8% feather weight was reduced through sub-merged and solid-state fermentation, respectively by *A. flavus*. The pH of the growth media in submerged fermentation was changed from 4.8 to 8.35. The exploited application of keratinolytic microbes is, therefore, recommended for the treatment of keratinaceous wastes to achieve dual benefits of remediation.

Keywords: *Aspergillus flavus*, biodegradation, bioremediation, economical bioremediation, keratinase, poultry industry.

Resumo

A indústria avícola está se expandindo rapidamente e produzindo milhões de toneladas de resíduos de penas anualmente. A produção massiva de subprodutos queratinosos na forma de resíduos agrícolas e industriais em todo o mundo exige sua utilização justificada. O tratamento químico de resíduos de queratina é proclamado como uma abordagem ecodestrutiva por vários pesquisadores, uma vez que gera poluentes secundários. A queratinase liberada por uma variedade de micróbios (bactérias e fungos) pode ser usada para o tratamento eficaz de resíduos de queratina. A degradação microbiana de resíduos de queratina é uma abordagem emergente e ecológica e oferece benefícios duplos, ou seja, tratamento de poluente recalcitrante (queratina) e obtenção de uma enzima comercialmente importante (queratinase). Este estudo envolve o isolamento, caracterização e utilidade potencial de espécies de fungos para a degradação de resíduos de penas de frango por meio da fermentação submersa e em estado sólido. O fungo isolado foi identificado e caracterizado como *Aspergillus (A.) flavus*. Em um ensaio de 30 dias, constatou-se que 74% e 8% do peso das penas foram reduzidos por *A. flavus*, respectivamente, por meio da fermentação submersa e em estado sólido. O pH do meio de crescimento em fermentação submersa foi alterado de 4,8 para 8,35. A aplicação explorada de micróbios queratinolíticos é, portanto, recomendada para o tratamento de resíduos ceratinosos para obter benefícios duplos de remediação.

Palavras-chave: *Aspergillus flavus*, biodegradação, biorremediação, biorremediação econômica, queratinase, indústria avícola.

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1. Introduction

Keratin is an insoluble, fibrous, and structural protein which is recalcitrant in nature due to abundance of hydrogen and disulfide bonds. A variety of vertebrates like fishes, reptiles, birds, and mammals have compact keratin in their integuments and due to abundance in nature it occupies third number after chitin and cellulose (Kreplak et al., 2004; Bragulla and Homberger, 2009; Mckittrick et al., 2012). Keratins are divided into two types, i.e., α-keratin and β-keratin (Meyers et al., 2008; Lange et al., 2014; Huang et al., 2015). Keratin proteins are resistant to chemical and/or mechanical breakdown due to presence of several disulfide (S-S) cross-linkages (Korniłowicz-Kowalska, 1997b). Only keratinolytic microbes and some insects like moths can efficiently degrade keratin by secreting keratinolytic enzymes (keratinases) that can degrade complex cross-linked bonds of keratin in coordination with other enzymes (Lange et al., 2016; Jin et al., 2017).

More than 25 thousand chicken farms are efficiently working to meet protein needs of people in Pakistan. A large amount of poultry waste is produced as byproduct of poultry processing plants (Abedullah and Bukhsh, 2007; Hussain et al., 2015; Khan et al., 2015). Four million tons of feathers are collected as poultry slaughtering waste annually (Onifade et al., 1998; Gousterova et al., 2005). However, it must be treated because a variety of pathogens are associated with this waste. The poultry waste is generally heated or dumped in soil (Suzuki et al., 2006; Ghaffar et al., 2018). Production of keratinase by bacteria is focused mainly, however, only few studies presented fungal keratin degradation (Gupta and Ramnani, 2006; Brandelli et al., 2010; Korniłowicz-Kowalska and Bohacz, 2011; Gupta et al., 2013; Sahni et al., 2015). Fungal keratinases may play a crucial role in the economical and environment-friendly treatment of keratin waste (Gradisar et al., 2005; Huang et al., 2015).

Filamentous fungi produce keratinases by using two ways of fermentation. These fermentation processes include solid state (SSF) and submerged fermentation (SmF) (Battaglino et al., 1991; Krishna, 2005). SmF plays a major role in production of enzymes at industrial scale and contributes more than 75% of the overall enzymatic production (Subramaniyam and Vimala, 2012). SmF process is more suitable for that microbes which need high moisture contents for their growth (Subramaniyam and Vimala, 2012). SSF is a process in which microbes are cultured on solid and moist medium (Pandey, 2003; Hölker and Lenz, 2005; Mitchell et al., 2006). Therefore, keeping in view the notable remedial properties of fungi, the present study was designed to isolate, characterize and employ keratinolytic fungi for the treatment of poultry (keratin) waste following SmF and SSF processes.

2. Materials and methods

2.1. Sample collection and isolation of pure culture of keratinolytic fungal strain

For the isolation of keratinolytic fungus, decaying feathers were collected under hygienic conditions from feather-dumping sites in district Kasur. The collected samples were then transported to Applied and Environmental Microbiology Laboratory, Department of Wildlife and Ecology, University of Veterinary and Animal Sciences, Lahore (Ravi campus, Pattoki), Pakistan for further processing. Chicken feathers were thoroughly washed with distilled water and soaked in sterile water for 2–3 h. Then 0.5 mL of the feather-suspended water was spread over skim milk agar (CM0681, Oxoid) and incubated for 3 days at 30 °C. The isolated fungal strain was then pure cultured by streak-plate method.

2.2. Phenotypic characterization

Fungal pure culture was morphologically identified based on macroscopic and microscopic features. Macroscopic features like shape, size, color, and texture of the fungal colony were examined. Microscopic features like septation of hyphae, metulae, phialides, vesicle and conidiophore were observed by staining the fungus with lactophenol-cotton blue.

2.3. Molecular identification of the fungal isolate

The pure cultured keratinolytic fungal isolate was then identified at the molecular level by 18S rRNA gene sequencing (Jing et al., 2015; Saini et al., 2015). Required sequence was created from forward and reverse sequences and Bio-Edit sequence alignment editor version 7.0.9.0 was used for sequence edition (Tom Hall, Ibis Biosciences, Carlsbad, California). Initial analysis of Pakistani sequences was carried out through BLAST (Basic Local Alignment Search Tool). The Pakistani sequences from GenBank were arrayed accompanied by most resembled retrieved sequence using Clustal W program of Molecular Evolutionary Genetics Analysis (MEGA 6) software (Tamura et al., 2013). Neighbor-Joining method was used for deducing the evolutionary history (Saitou and Nei, 1987). Next to the branches, replicate trees percentages were combined with closely related taxa in bootstrap test (100 replicates) (Felsenstein, 1985). Branch lengths of the tree drawn on scale were in the same units so as evolutionary distances used to deduce phylogenetic tree. The calculated evolutionary distances were in the units of the number of base substitutions per site as explained by Tamura and Nei (1993). The rate difference between sites was shown with gamma distribution (shape parameter = 1). In the final dataset, the total positions were 734 and gaps referred as data.

2.4. Optimization of growth conditions of the fungal isolate

After molecular analysis, the fungal isolate was inoculated on malt extract agar (MEA) with one loop full of fungal spores and incubated at 30 °C for 3 days. Different parameters like temperature, pH, inoculum size and incubation period were then optimized. For the optimization of temperature, fungal spores were incubated at 20, 30 and 40 °C on petri plates containing MEA for 3 days and different number of colonies was shown at different temperatures. For pH optimization, petri plates were incubated at 30 °C having pH 5, 7 and

9 for 3 days and number of colonies was noted on each plate. Inoculum size was optimized by inoculating petri plates with 0.1, 0.2, 0.3, 0.4 and 0.5 mL of fungal spores and incubated at 30 °C for 3 days and colonies were counted. Inoculated petri plates were incubated at 30 °C and number of colonies was counted on daily basis for consecutive 5 days.

2.5. Determination of feather-degrading potential of the fungal isolate in SmF and SSF

Feather-degrading potential of the fungal isolate in SmF and SSF was determined by using basal medium [composition (gL^{-1}): K_2HPO_4, 1.5; NaCl, 0.01; $MgCl_2.7H_2O$, 0.05; H_2O, 1000 mL] and dried feather meal (2% w/v) as growth substrate. Inoculum was prepared by using basal medium and feather meal as a source of carbon and energy. Medium was autoclaved and 100 mL of the medium was poured into sterile 250 mL Erlenmeyer flask; 1 g of feather meal was used. Fungal spores were transferred into medium through sterile inoculating loop. Flask was incubated at 29 °C and pH was maintained at 4.8. Maximum growth was observed after 15 days. This culture was used as fungal inoculum for all further experiments.

Feathers were washed thoroughly with distilled water and then dried at 170 °C for 4 h for complete removal of moisture. Experiment was conducted in sterile 250 mL Erlenmeyer flasks containing 1 g of sterile feathers and 100 mL of basal medium. Flasks containing medium and feathers were autoclaved and then inoculated with fungal spores upon cooling. Flasks containing medium were inoculated with 0.5 mL of the fungal spores and incubated at 29 °C and pH of 4.8 (six sets of flasks). All the experiments were conducted in triplicates. The control flasks were kept un-inoculated. After every 5 days, feathers were washed with distilled water, dried at 80 °C for 4 h to remove moisture contents and weighed to determine degree of degradation of feathers by the isolated fungal strain in SmF up to 30 days. It was observed that weight was varied in each set of flasks.

For checking degradation potential of the isolated fungal strain in SSF, feathers were washed thoroughly with distilled water and then dried at 170 °C for 4 h for complete removal of moisture. Experiment was conducted in 250 mL Erlenmeyer flasks containing 5 g of sterile feathers and the moisture contents of basal medium was adjusted up to 60% (w/v). Flasks containing feathers with specific moisture contents were autoclaved and then inoculated with fungal spores upon cooling. Flasks were inoculated with 0.5 mL of the fungal spores, incubated at 29 °C and pH was maintained at 4.8 (six sets of flasks). After every 5 days, feathers were washed with distilled water, dried at 170 °C for 4 h to remove moisture contents and weighed to determine the degree of degradation of feathers by the isolated fungal strain in SSF up to 30 days. It was observed that weight was varied in each set. All the experiments were conducted in triplicates. One flask was kept as control (uninoculated) under the same experimental conditions.

2.6. Statistical analysis

The data were analyzed according to Completely Randomized Design (CRD) under factorial arrangement using General Linear Model (GLM) procedures. Means were separated out using Duncan's Multiple Range (DMR) test with the help of SAS 9.1 for windows (SAS Institute Inc., 2002). Differences between means were considered significant at $P < 0.05$.

3. Results

3.1. Isolation of pure culture of keratinolytic fungal strain

The present study was conducted to isolate keratinolytic fungal strain from feather waste. The isolated strain was pure cultured and further identified phenotypically and genotypically.

3.2. Phenotypic characterization

The isolated fungal strain depicted maximum growth in 3 days on MEA medium. Colony color was olive green with white edges and shape of the colony was circular having powdery texture. By staining of fungal hyphae rough spiny conidiophore bearing vesicles were observed. Hyphae were non-septate, conidia were terminal and vesicles were globose and loosely radiated uniseriate phialides were present all over the vesicles. Metulae was absent and conidia were directly attached with vesicles.

3.3. Molecular identification of the fungal isolate

BLAST search of 18S rDNA nucleotide sequence of the fungal isolate revealed that the fungal isolate belonged to genus *Aspergillus* having 98% similarity with *Aspergillus flavus* (ATCC 16883).

3.4. Optimization of growth conditions of the fungal isolate

At pH 5, maximum number of colonies was shown (Figures 1 and 2). The fungal strain showed different growth on different degrees of temperature, pH, inoculum size and incubation period. The fungal strain showed maximum growth at 30 °C (Figure 3). Temperature was then optimized by incubating the fungal strain at 28, 29, 31 and 32 °C. The maximum number of colonies was

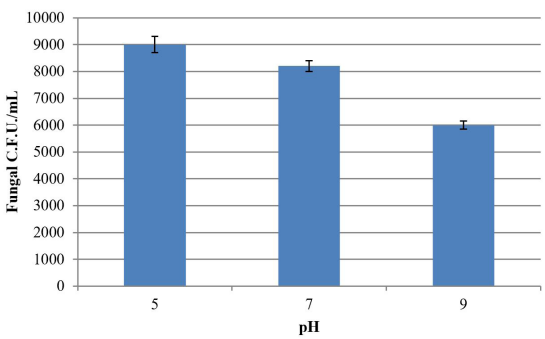

Figure 1. Optimization of pH for the fungal strain by using MEA.

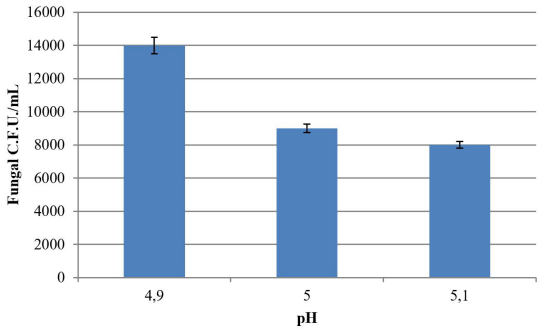

Figure 2. Optimization of pH for the fungal strain by using MEA.

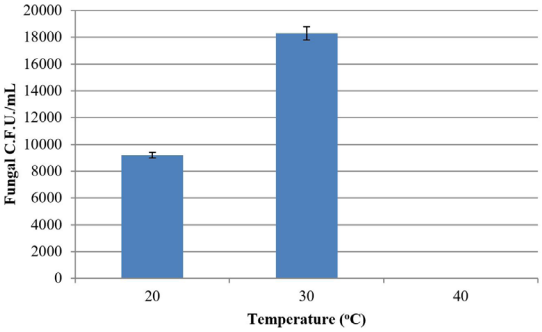

Figure 3. Optimization of temperature for the fungal strain by using MEA.

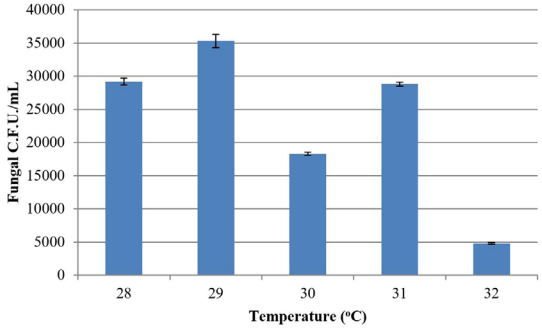

Figure 4. Optimization of temperature for the fungal strain by using MEA.

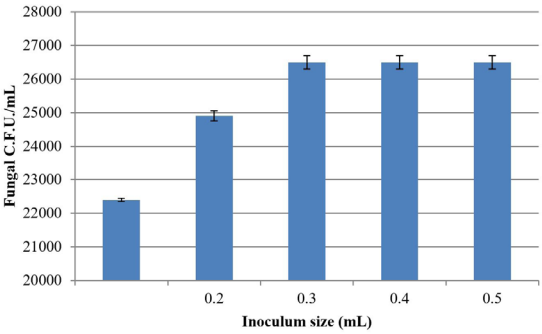

Figure 5. Optimization of inoculum size.

shown at 29 °C (Figure 4). Inoculum size was optimized by using previously optimized parameters and at maximum inoculum size maximum growth was observed (Figure 5). Fungal colonies showed maximum number of colonies in 3 days of incubation period at optimized temperature, pH and inoculum size.

3.5. Determination of feather-degrading potential of the fungal isolate in SmF and SSF

The utilization of keratin as substrate is an important factor for assessment of keratinolytic abilities of microorganisms. Ability of keratinolytic fungus to degrade chicken feathers was assessed in this study. With the passage of time, weight of feathers was reduced, and keratin degrading activity was increased. Different amounts of feathers were degraded among consecutive days in both fermentation processes. SmF and SSF showed drastic variation in feather loss. During SmF, initial amount of feathers was 1 g. Reduction of feathers was started after 5 days of incubation period. In the first 5 days no reduction was observed. The maximum amount of feather reduction was observed at 30th day of incubation period, i.e., 74%. Maximum reduction in weight of feathers was 0.74 g. Results showed keratinolytic activity was increased with the passage of time:

3.6. 30 days > 25 days > 20 days > 15 days > 10 days > 5 days

During the first 5 days of SSF, 0.2% reduction was observed. The maximum amount of feather reduction was shown at 20th day of incubation period, i.e., 8%. After 20 days, the feather reduction remained constant till 30th day of incubation period. Maximum reduction in weight of feathers was 8% on 20th day. Results showed that keratinolytic activity was increased with the passage of time:

3.7. 20 days > 15 days > 10 days > 5 days

During keratin degradation in SmF and SSF by *A. flavus*, pH changed from acidic to alkaline. As degradation of keratin releases S, N, O ions and several other compounds which cause pH to increase. In SmF, a remarkable increase in pH was observed on the day at which maximum feather reduction was shown (30th day), i.e., 8.35 (control pH = 4.8). In SSF, maximum pH was observed on the day at which maximum feather reduction was shown (20th day), i.e., 7.81 (control pH = 4.8). Results showed that pH increased with the increase of keratinolytic activity (Figures 6 to 9).

4. Discussion

The current study was conducted to evaluate the keratinolytic potential of *A. flavus* in SmF and SSF. A keratinolytic fungal species was isolated from chicken feather waste to check its potential for degrading chicken feathers (keratin) in both conditions (SmF and SSF). The isolated strain was morphologically identified as *A. flavus* and molecular characterization showed closer resemblance with *A. flavus*. The isolated fungal strain had following morphological features, i.e., green colored fungus with uniseriate phialides and conidia were globose. These features were almost same with the results of Hedayati et al. (2007) who reported that

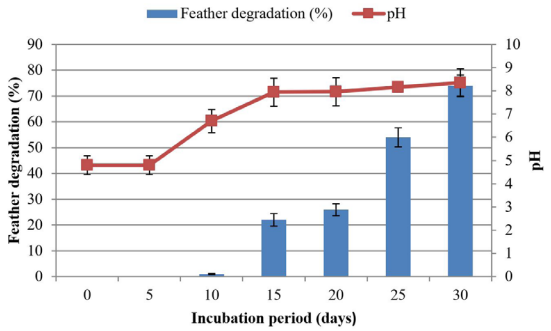

Figure 6. Feather degradation and pH changes in post-culture SmF medium.

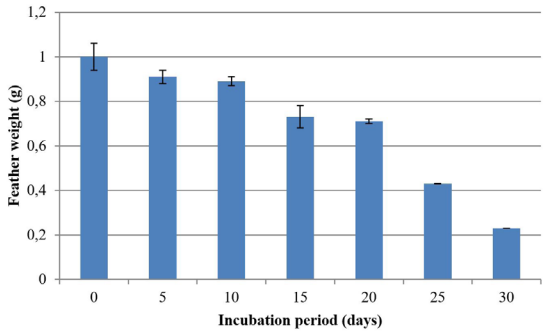

Figure 7. Feather weight after incubation during SmF.

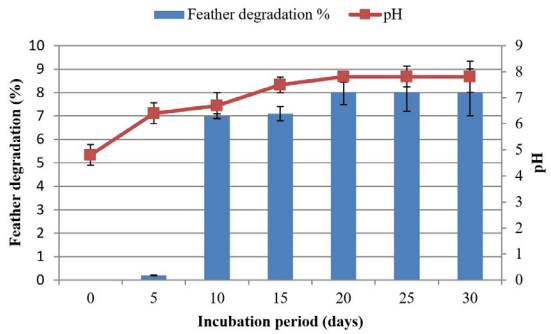

Figure 8. Feather reduction and pH change in post-culture medium during SSF.

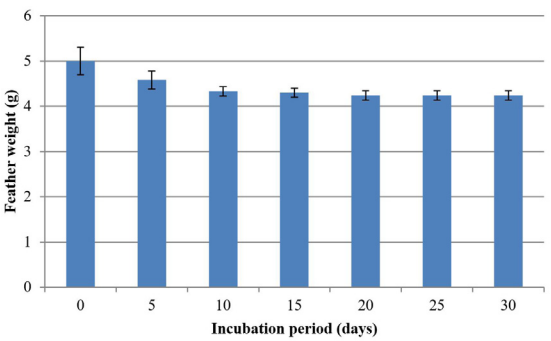

Figure 9. Feather weight after incubation in SSF.

A. flavus is either uniseriate or biseriate and yellow to green in color. Conidia are globose to sub-globose.

This isolated fungal strain showed maximum growth at 29 °C temperature and pH 4.8 and its activity was inhibited at 40 °C. The optimum temperature for the isolated fungus was in accordance with temperature range for *A. flavus* as reported by Samapundo et al. (2007). Cai and Zheng (2009) also reported that maximum keratinolytic activity was observed at 28 °C. Kote et al. (2009) reported that *A. flavus* showed maximum activity at pH 5. *A. flavus* is considered as an outstanding fungal producer of keratinase as compared to other fungal species (Friedrich et al., 1999). Mostly SmF is used for keratinase production (shaking conditions for bacteria and static conditions for fungi) (Riessen and Antranikian, 2001; Nam et al., 2002). Only a few reports are concerned with solid-state production of keratinase (De Azeredo et al., 2006; Esawy, 2007). The keratinolytic potential in sub-merged and solid-state fermentation was assessed in a trial of 30 days. There are a few reports on keratin degradation by *A. flavus* in SmF and fungal keratin degradation in SSF is also scarcely reported. Results showed that in SmF, *A. flavus* exhibited 9 times greater keratin degradation than that of SSF. In most of the studies, it has been reported that in SSF, microbes show more degradation than in SmF. Mazotto et al. (2013) reported 7 times greater keratinolytic activity of *A. niger* in SSF than in SmF. However, in the present study, results were completely opposite. In SmF, the keratinolytic activity started after 5 days of incubation period and increased with time up to 4 weeks of incubation period. The highest degradation (74%) of chicken feathers was shown at 30th day of incubation period. Korniłłowicz-Kowalska (1997a) reported that loss of substrate is a clear sign of keratinolytic activity. The keratinolytic activity of the *A. flavus* on chicken feathers in SmF in the current study was almost double than the activity of *A. flavus* in the study of Muhsin and Hadi (2002). Based on this percentage, the strain used in the present study can be defined as strongly keratinolytic. As reported by Kunert (2000), the microbes which can degrade keratin more than 40% within 60 days in submerged conditions are strongly keratinolytic. Bohacz (2017) reported that strain of *Chrysosporium articulatum* showed 63.7% of feather loss after 42 days in liquid culture conditions. This degradation is also less than the present results of *A. flavus*. A strain of *Chrysosporium keratinophilum* showed weaker keratinolytic activity, i.e., 35%,hile in SSF, *A. flavus* showed a weaker keratinolytic activity, i.e., 8%. The maximum degradation activity was exhibited on 20th day, afterwards it remained constant. This was the lowest keratin degradation in SSF reported till now.

Change in pH is an important characteristic of keratin degradation. Apparently, a remarkable increase in pH (4.8–8.3) was also observed in post-liquid culture medium. pH was also more inclined towards alkalinity in solid-state keratin fermentation. There was a direct relation between pH changes and keratinolytic activity. As keratinolytic activity increased, pH also became alkaline. In initial days of SmF, pH was low, i.e., 6.7 and a less keratinolytic activity was observed at this pH. Cai and Zheng (2009) also reported that at low pH less keratinolytic activity was

occurred. Maximum pH of 8.3 was recorded on the day of maximum keratinolytic activity in liquid culture. Cai et al. (2008) observed that pH level of the medium increased up to 8.5 during keratinase production. The change of pH towards alkalinity is due to degradation of keratin, release of keratinase and significant amount of ammonia, sulfur, and other compounds (Saparrat et al., 2007). It has been reported that fungi with more keratinolytic activity tend the post-culture medium more alkaline than the fungi with less keratinolytic activity (Kaul and Sumbali, 1999). Similar changes in pH have also been reported by Hasiia et al. (1990) and Elíades et al. (2010).

It is concluded that *A. flavus* can be efficiently employed for the degradation of feather waste and production of keratinase which is a valuable industrial enzyme. The present study was conducted at lab scale, however, future studies are required to explore remedial process kinetics at pilot and commercial scales for the viable implication of keratinolytic fungi.

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