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CHICKEN FEATHER DEGRADATION BY KERATINOLYTIC *ASPERGILLUS* SPECIES ISOLATED FROM FEATHER WASTE DUMPING SOIL

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Abstract– Slaughterhouses and poultry processing units worldwide dispose of huge amount of chicken feathers that accumulate as solid waste in the environment and cause serious consequences. Keratinases are a group of enzymes, produced by microorganisms have the ability to degrade tough recalcitrant keratin that is abundantly present in chicken feathers. Soil sample was collected from a feather dumping site at Virudhunagar (9° 35' 13.9524'' N Latitude and 77° 57' 5.1516'' E Longitude), Tamil Nadu, India. Soil sample was serially diluted, inoculated on to feather meal agar plates and incubated at 37 °C for 7 days to isolate keratinolytic fungal strains. The isolated strains were screened for proteolytic activity on skim milk agar for 8 days and three better strains were identified based on morphology, mycelial growth and structure of hyphae using lactophenol cotton blue staining and confirmed by ITS primer sequencing method as *Aspergillus niger, Aspergillus terreus* and *Aspergillus flavus* respectively. Different range of pH (7 to 11), temperature (25 to 45 °C), substrate (bovine serum albumin, feather, peptone and gelatin) and feather concentration (0.5g, 1g, 1.5g and 2g) were tested to find out the optimum conditions for the growth and keratin degradation of the chosen fungal strains. The temperature and feather concentration for the optimum growth of *A. niger*, *A. terreus* and *A. flavus* were 25 °C and 1.5g, 45 °C and 1g and 30 °C and 1.5g respectively. All three *Aspergillus* species exerted better growth at pH 9 and with bovine serum albumin as the substrate.

INTRODUCTION

Poultry industries are rapidly expanding to meet out the demand of animal protein by the people. The increase in poultry industries generates huge amount of wastes such as feather, viscera, bones and dead animals (Reddy *et al*., 2021) that is difficult to dispose and results in protein wastage apart from causing environmental pollution (Shah and Vaidya, 2015) and in the outbreak of H5N1 viruses (Saber *et al*., 2010). Chicken feathers predominantly consist of a highly insoluble, mechanically stable fibrous protein called as keratin, hence further processing is needed to degrade keratin into simpler forms (Cheong *et al*., 2017; Fitriyanto *et al*., 2022). Keratin is rich in glycine, alanine, serine, cysteine, arginine and valine and small quantities of lysine, methionine and tryptophan (Elsayed *et al*., 2016). The structural organization of keratin, its bonds and hydrophobic interactions make it difficult to degrade by nature (Adelina *et al*., 2021).

Conventional feather disposal methods such as burning, land filling, alkali hydrolysis, and steam pressure cooking (Rao and Arthi, 2012) consume more energy, destroy the essential amino acids (Duffeck *et al*., 2020) and leads to severe air, land and water pollution. Keratinase is an extracellular enzyme produced by diverse group of microorganisms that can hydrolyse keratin and this enzyme finds applications in tannery, food processing and pharmaceutical industries (Kumar and Kushwaha, 2014; Banerjee, 2019). Biodegradation of chicken feather waste by keratinase is ecofriendly, labor and cost effective that improves the nutritional value and can be used for the production of fertilizers and animal feed (Arokiyaraj *et al*., 2019).

Microorganisms such as bacteria, fungi and

actinomycetes have been reported to utilize chicken feather keratin as source of food. Fungal keratinases receive attention as they can be grown on low cost substrates, secretion of large quantity of keratinase and can be extracted by downstream process (Alwakeel *et al*., 2021). Research has been going on regarding identification, characterization and production of keratinases from keratinophilic microorganisms. Keratinophilic fungi are commonly found in diverse habitats due to their potential to generate and secrete a large amount of extracellular enzymes (Sivakumar and Raveendran, 2015). Studies on microbial keratinase have been focused mainly on their production from fungi due to the ease of downstream processing and the ability of fungi to grow on low-cost substrates. Many fungi especially that belong to the genera: *Chrysosporium, Aspergillus, Alternaria, Trichurus, Curvularia, Cladosporium, Fusarium, Geomyces, Gleomastis, Monodictys, Microsporum, Myrothecium, Paecilomyces, Stachybotrys, Urocladium, Scopulariopsis, Sepedonium, Trichophyten, Penicillium and Doratomyces* have high keratinolytic activity (Issac and Tahon, 2016; Gunes *et al*., 2018). *Aspergillus* species are good producers of several enzymes including keratinase and can be explored further. Hence, the utilization of keratinous wastes as a fermentation substrate (carbon and nitrogen sources) by kerain degrading fungi offers a viable microbial technology for obtaining keratinolytic enzymes (Lima *et al*., 2020). Keratinases have suitable tendencies for green technology (Almahasheer *et al*., 2022). The keratinolytic microorganisms and technologies developed for feather degradation not only remove the waste feathers efficiently from the nature but also have important applications in food and leather industries, manufacturing of textiles and biodegrade able film and cosmetics and nitrogen fertilizer for plants (Nigam, 2013).

MATERIALS AND METHODS

Sample collection

Soil sample was collected from a feather dumping site at Virudhunagar district (9° 35' 13.9524'' N Latitude and 77° 57' 5.1516'' E Longitude), Tamil Nadu, India. Sample was collected from 3 to 4 cm depth, transferred to sterile zipper polythene bags and brought to the laboratory for further study.

Preparation of chicken feather powder

Chicken feather was collected from a slaughter house at Virudhunagar district (9° 35' 13.9524'' N Latitude and 77° 57' 5.1516'' E Longitude), India, washed with detergent well and then three to four times with water, dried and cut into small pieces. This feather was used for further studies.

Isolation and identification of keratinolytic fungi

One gram of the collected soil sample was mixed with 10 ml sterilized distilled water, serially diluted (10^{-2}, 10^{-4}, 10^{-6}, 10^{-8} and 10^{-10}) and inoculated (spread plate technique) on to feather meal agar plates individually with chicken feather. The fungal isolation medium contains (composition in g/L): $MgSO_4.H_2O$- 0.5; KH_2PO_4- 0.1; $FeSO_4.7H_2O$- 0.01; $ZnSO_4.7H_2O$- 0.005; agar- 15; and chicken feather powder- 10. The pH of the medium was adjusted to 7.5. Plates were incubated at 37 °C for 7 days. The medium was supplemented with 1% chicken feather as the sole source of carbon, sulfur and nitrogen. Proteolytic activity of the fungi was carried out by inoculating them in skim milk agar plate and incubated up to 8 days at room temperature and the detected colony were identified. The isolated fungi was stained with lacto phenol cotton blue, identified based on morphological features like presence of spores, septation of hyphae and conidiophores.

Molecular Identification of Fungi

DNA extraction of fungal strains

The selected fungus was grown in Potato Dextrose Agar medium for seven days at 28 °C and pure fungus has been used for DNA isolation by CTAB (Cetyl trimethylammonium bromide) solution method. 100 mG mycelium was approximately taken with 700 µl CTAB solution, sterile glass beads and well homogenized using pestle and then incubated at 60 °C for one hour in water bath (every 20 minutes sample were vortex).

18S rRNA gene for sequence, PCR amplification comparisons and Phylogenetic tree analyses of fungus

Fungal species were identified using ITS (internal transcribed spacer) region sequencing method. The 18S rRNA gene amplification was performed by polymerase chain reaction (PCR) technique using the universal primers such as ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (Ghonomy and Ali, 2017). PCR was performed in 25 µl as a final volume, which composed of 12.5 µl Taq PCR Master Mix containing 0.5 U μl^{-1} Taq DNA polymerase, 500

μM of each dNTP, 20 mM Tris-HCl (pH 8.3), 100 mM KCl, 3 mM of each $MgCl_2$ and Bromophenol Blue, 1 μl of fungal culture as a template DNA, 1 μl of each primer (10 μM) and 9.5 μl of double distilled water.

PCR reaction was carried out as follows: denaturation at 95 °C for 1 min followed by annealing at 55 °C for 30 S and extension for two minutes at 72 °C; and final extension for ten minutes at 72 °C. The products of PCR were detected in 1% agarose gel electrophoresis, stained with ethidium bromide (1 μG ml^{-1}) and visualized under UV light, to confirm its purity and size. The fungal gene sequence was compared with National Center for Biotechnology Information GenBank database nucleotide through Basic Local Alignment Search Tool (BLAST).

Influence of pH and temperature on fungal growth

The growth and culture conditions were optimized and the parameters such as pH, temperature, were incorporated in feather minimal medium containing feather powder (1%) for five days in shaking condition (150 rpm for 120 hours) to determine the efficacy of the selected and identified fungi. The fungal strains were inoculated separately into different pH such as 7, 7.5, 8, 8.5, 9, 9.5, 10, 10.5 and 11, and incubated in different temperatures (25 °C, 30 °C, 35 °C, 40 °C and 45 °C) respectively for five days in shaking condition for feather degradation reaction. The optimum pH and temperature influence on growth and enzyme production of the fungi was detected.

Influence of feather concentration and substrates on fungal growth

The effect of growth and feather degradation was investigated by chicken feather concentrations ranging from 0.5, 1.0, 1.5 and 2.0 g/100 ml in the minimal medium. The growth and enzyme production was investigated by various substrates like, bovine serum albumin grown, feather, peptone and gelatin (by adding 1% in the minimal medium) with different sole sources of carbon and nitrogen supplements and the cultivation was performed at 150 rpm and 37 °C for 120 hours. At every 24 hrs interval samples were collected aseptically by removing the cells through filter paper and the cell free filtrate was used for further study.

Protein and fungal growth determination

The feather degradation activity was identified by the soluble protein content of the cell free supernatant. The highest feather degradation was measured as soluble protein and the protein content present in the cell free culture medium was determined following Lowry *et al*. (1951) method with bovine serum albumin as standard protein incubated for five days. The fungal growth was determined following colony diameter method.

RESULTS

Isolation and identification of keratinolytic fungi

Five feather degrading fungi were isolated from chicken feather dumping site soil, on agar medium substituted with feather meal. The primary screening showed that most of the isolated fungi were able to grow and some of them showed weak growth but did not clarify the keratin agar, presumably due to the lack of extra cellular keratinase. Five morphologically distinct isolates exhibited growth on the feather meal agar plates and were identified as *Alternaria* sp., *Aspergillus* sp., *Aspergillus* sp., *Aspergillus* sp. and *Rhizopus* sp. respectively.

The fungal strains were active in feather meal agar by exerting maximum growth on feather meal agar plate and screened in skim milk agar plates have a clear zone around the colony could have potential keratinolytic activity. These isolates were also quantitatively tested and optimized in submerged culture condition by protein concentration using chicken feather powder as the only source of carbon and nitrogen.

Phylogenetic analysis

The three isolates were sequenced by using ITS universal primer and based on the sequences, they were identified as *Aspergillus niger*, *Aspergillus terreus* and *Aspergillus flavus* respectively. The sequence of fungus were also deposited in NCBI and obtained GENBANK accession number MH064151.1 (Figure 1), GENBANK accession number MH065614.1 (Figure 2), GENBANK accession number MH064167.1 (Figure 3).

Influence of pH and Temperature on Fungal Growth

In the present study, growth was found to be better in alkaline condition at pH 9 and over a wide range of temperature. Fungi grown at pH 9 produced maximum amount of protein in the culture filtrates

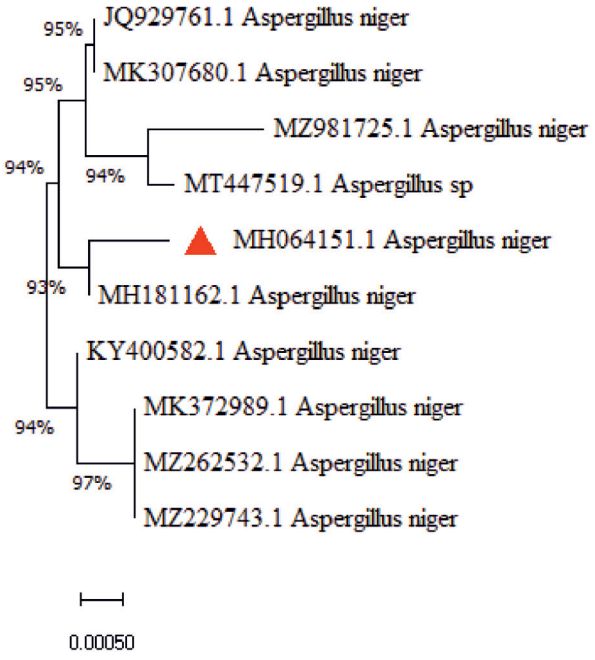

Fig. 1. Phylogenetic tree of the isolated fungus within the genus *Aspergillus*. The branching pattern was generated by neighbor-joining method

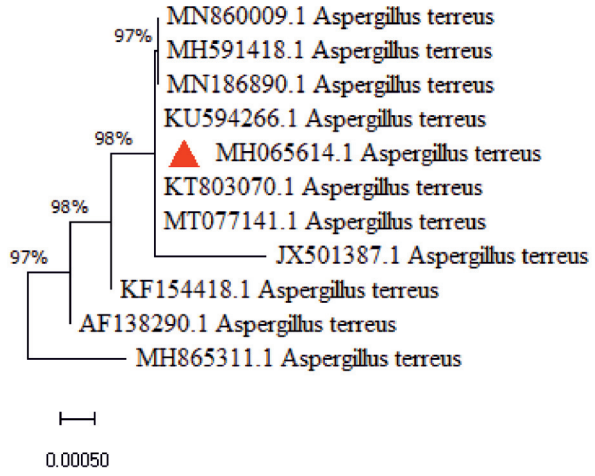

Fig. 2. Phylogenetic tree of the isolated fungus within the genus *Aspergillus*. The branching pattern was generated by neighbor-joining method

(Figure 4). The protein production of *A. niger* was maximum at 25 °C and pH 9.0 respectively. Hence remarkable amount of extracellular protein from fungi becomes interrelated with increased keratinolytic activity. The fungal growth efficiency and metabolism leads to changes in the hydrogen ion balance of the culture medium. The alkalinity of the medium increased the degradation of keratin substrate by the keratinolytic fungi. The optimum temperature for *A. terreus* has 45 °C whereas *A.*

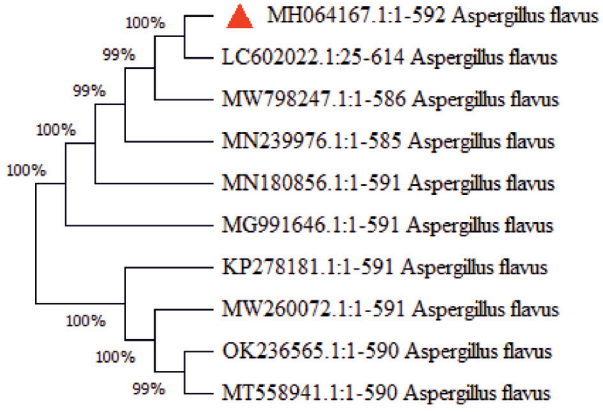

Fig. 3. Phylogenetic tree of the isolated fungus within the genus *Aspergillus*. The branching pattern was generated by neighbor-joining method

flavus showed 30 °C for higher degradation of chicken feather estimated with protein content (Figure 5).

Influence of feather concentration and substrates on fungal growth

Of the various feather concentrations, the optimum feather concentration for the growth of *Aspergillus niger* and *A. flavus* was 1.5g/100 ml, in which the production of protein was high and the feather

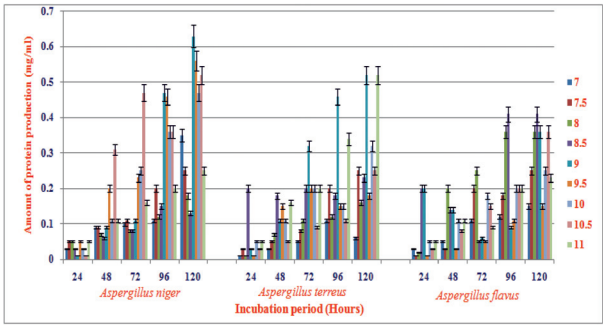

Fig. 4. Effect of pH on the production of protein by the isolated feather degrading fungi

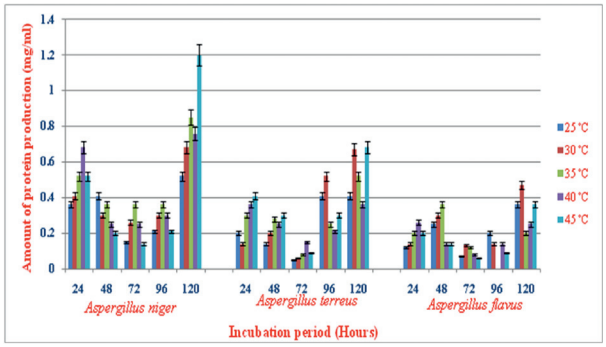

Fig. 5. Effect of temperature on the production of protein by the isolated feather degrading fungi

requirement for the optimum growth and degradation of *Aspergillus terreus* by protein production was 1g (Figure 6). The high concentration of feather might be cause substrate inhibition or repression of enzyme production. In various substrates, BSA showed maximum activity at 32 °C and pH 9 for 120 hrs (Figure 7). Among the different substrates tested, BSA was found to be suitable for the maximum growth and protein production in all *Aspergillus niger*, *A. terreus* and *A. flavus.* Next to BSA, chicken feather was recorded ability to degrade by fungi compared to others substrate sources.

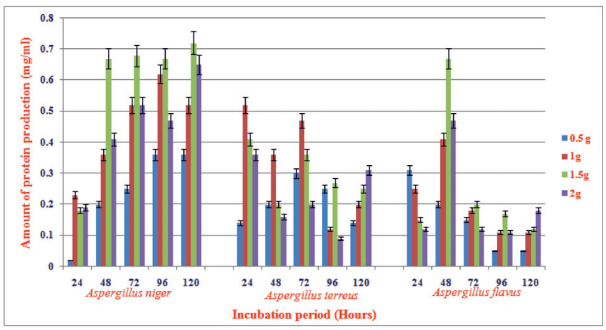

Fig. 6. Effect of feather concentration on the production of protein by the isolated feather degrading fungi

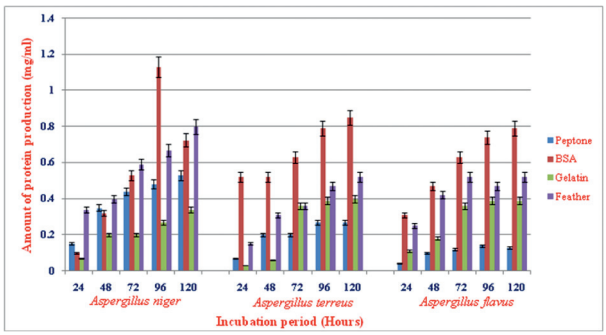

Fig. 7. Effect of various substrates on the production of protein by the isolated feather degrading fungi

DISCUSSION

Poultry industries and slaughter houses dispose feathers wastes into the environment and degradation of chicken feather is a complex phenomena that is associated with extracellular keratinase produced by keratinolytic microbes along with various abiotic factors (Tamilkani *et al*., 2017). Although keratinolytic fungi are relatively more interesting, studies are scarce when compared to bacteria. In the present study, five keratinolytic fungal strains were isolated from feather dumping site of Virudhunagar district (9° 35' 13.9524'' N Latitude and 77° 57' 5.1516'' E Longitude) and among them, three fungi were chosen, identified and taken for further studies. ITS primer sequencing confirmed them as *Aspergillus niger, Aspergillus flavus* and *Aspergillus terreus* respectively. The *Aspergillus* species are known human and plant pathogens and food spoilers by producing mycotoxin. Human pathogenic fungi are not good enough as producers of enzymes, however in producing antibiotics and organic acids, *Aspergillus* species plays a very significant role (Souza *et al*., 2015). The human pathogens are preferred an alternative sources of genes for recombinant expression of technologically significant enzymes due to the resulting enzymes may have strong inherent health risks (Huang *et al*., 2015).

Different species of *Aspergillus* have been reported as probable producers of keratinases but growth conditions have not been optimized for large scale production. In the present study, *Aspergillus* species showed notable keratinolytic activity by producing the enzymes and it could be exploited for keratin waste management (Anitha and Palanivelu, 2013). Generally the difference in growth and production of metabolites may be influenced by several factors such as biomass and viability. Fundamentally, microbial keratinolysis is a proteolytic process as keratin is a protein. The microorganisms produce keratinolytic protease through feather degradation and also release thiol group during microbial growth supports the essential role of disulfide bond reduction (Preczeski *et al*., 2020). The feather degradation appeared to be related to an increase of soluble protein which indicates that degradation of feather by depends on growth of fungi. The fungal strains showed different growth on different degrees of temperature, pH, various substrates and various substrate concentrations (Koutb, 2012). During the cultivation, an accumulation of soluble proteins may be caused by enzyme secretion during chicken feather degradation. Therefore the alkaline environment volunteer to some extent in keratinolysis but it also seems to be making the feathers more accessible for degradation by the fungal proteins. Besides, keratinases generally from most bacteria, actinomycetes and fungi have optimum pH ranging from neutral to alkaline (Gradrisar *et al*., 2005).

Saber *et al*. (2010) and More *et al*. (2013) elucidated the potential of filamentous fungi in

keratin degradation to be a combination of extracellular keratinase (release of thiol groups during microbial growth) such as mechanical keratinolysis (mycelia pressure and/or penetration of keratinous substrate), cell-bound redox system which leads to sulphitolysis (reduction of disulphide bonds with sulphite excreted by mycelia and release of thiosulfate) and proteolysis. The protein sulphitolysis is one of the fundamental characteristics of fungal dermatophyte degradation of keratin to furnish easy access for proteases/ keratinases to degrade the keratin protein still further (Lange *et al*., 2016). Similarly, Banerjee (2019) found three *Aspergillus* species namely *A. terreus, A. fumigatus* and *A. oryza* to be positive for protease hydrolysis by removing excess nitrogen by ammonia production and deamination process. The rise in pH of protein hydrolysates is one of the indicators of keratinolysis by deamination from ammonia of protein, amino acids and peptides through degradation process (Marcondes *et al*., 2008). Thus the results show the concentration of degradation products which reached maximum of micrograms per ml in the protein hydrolysis.

Alkalophilic microorganisms mostly depend on extracellular pH in a strong way and have a huge effect on the cell growth and enzyme production (Pandian *et al*., 2012). Result shows that poultry feathers are the main source of carbon under submerged fermentation. The optimum pH for isolated *Aspergillus* species during feather degradation in the present study was found to be 9 due to release of keratinase with significant amount of ammonia, sulfur and other compounds and the optimum temperature of 25 °C. In addition, the pH of the medium will become alkaline at the end of keratinase production due to the release of thiol groups in the form of cysteine and S-sulfocysteine (Gioppo *et al*., 2009). Bohacz (2017) also reported that during the biodegradation of feather keratin, the medium will be alkalinized because of release of proteins and peptides in tune with the keratinolytic activity.

Kumar and Kushwaha (2014) identified two hundred thirty four non dermatophytic strains from different habitats by hair baiting technique. Among them 52% of fungi only exhibited maximum growth and clear zone on skimmed milk agar. *Chrysoporium indicum* made maximum of clear zone (7 mM) and highest keratinase production (110.10 U ml^{-1}) in submerged conditions while other fungi were showed moderate enzyme production. Hence, the keratinase identified not only degrade keratin and also capable of degrading casein protein due to their wide range of substrate specificity. The non-pathogenic ascomycetes fungus *Onygena corvina*, secreted hightly active keratinolytic proteases under the optimal conditions for feather keratinolysis were found to be 25 °C, initial pH 8 and feather concentration of 15g L^{-1} (Issac and Tahon, 2016). Lima *et al*. (2020) also studied keratinolytic activity of *Aspergillus* sp. and *Penicillium* sp. in the medium containing different feather concentrations (0.5%, 1.0% and 1.5%) at 28 °C and 35 °C. *Penicillium* sp. showed better production of keratinase in culture medium containing chicken feather residue. Ai and Cic (2019) isolated and identified *A. niger* and *Penicillium purpurogenum* from Nigerian soil with keratin degrading potential by skim milk casein hydrolyzed by halo zones of hydrolysis (62 mM and 64 mM) and keratinase activity were recorded (13.5 and 12.0 U ml^{-1}) by hydrolyzed human hair and chicken feather into reducing sugar and proteins. Preczeski *et al.* (2020) reported that *Aspergillus* sp. showed higher enzymatic activity on 9th day of fermentation where degradation was maximum (113.50 U ml^{-1}) with feather as the substrate. Dalee *et al.* (2018) report that maximum activity of keratinase production of *Aspergillus* sp. attained on 4th day of incubation and established growth and keratinase production was parallel. Mini *et al*. (2015) reported that *Aspergillus flavus* S125 in solid state fermentation, maximum enzyme production was obtained at pH 9 and 55 °C on the 4th day of incubation. Kim (2007) also observed that the *Aspergillus flavus* K-03 was active in a broad range of pH and temperature with most favorable for keratinase activity at pH 8 and 45 °C. Each fungi have specific temperature requirement for optimal production of keratinases as observed in *Cochilobolus lunatus*, a non dermatophyte keratinolytic fungi had optimum activity at 30 °C with 9 days for keratinase production (Kazi *et al*., 2013). The results suggest that the wide range of pH and temperature might be useful for industrial applications.

The keratinolytic *Aspergillus flavipes* degraded chicken feather under solid state fermentation had wide proteolytic activity on the substrates of casein, albumin and gelatin (Ayouty *et al*., 2012). The results obtained in the present study shows that *Aspergillus* species has highest activity in BSA protein among various substrates provided as reported and Kumar and Mahal (2021) also recorded *Chrysosporium indicum* that exerted maximum activity on BSA

protein in diverse substrate degradation such as keratin powder, keratin azure and feathers and BSA protein. More *et al*. (2013) also reported that *Aspergillus flavus* and *Cunninghamella echinulata* produced high amount of keratinase after 16 and 14 days of incubation respectively. The biochemical features of α- keratinase such as therma-stability, high specificity activity, high temperature optima, stability againt surfactants and activity at high alkaline pH even in the presence of EDTA advocated the importance of protease in industrial purposes and other biotechnological processes (Brandelli, 2015). Mazotto *et al*. (2013) screened twenty eight *Aspergillus niger* mutants on the chicken feather as a basal medium for peptidase and keratinases production and the *A. niger* 3T5B8 keratinase activity was seven times (172.7 U ml^{-1}) higher than *A. niger* 9D40 (21.3 U ml^{-1}) on the solid-state condition. Hence *Aspergillus* species is "generally recognized as safe" and have very good potential to degrade keratin.

CONCLUSION

Five keratinolytic fungi have been isolated from soil collected from feather waste dumping site at Virudhunagar district, Tamil Nadu, India, from that three better strains were chosen for further studies. Based on morphology, mycelial growth and structure of hypae, they were identified as *Aspergillus niger*, *A. terreus* and *A. flavus* that were further confirmed by ITS primer sequencing method. Among the three, *Aspergillus niger* exerted better growth at 25 °C, pH 9 and 1.5 g of feather concentration and with bovine serum albumin as substrate. Further research is required to explore the possibility of utilizing *A. niger* for large scale degradation of feather waste.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

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