JERE, *Volume 10, No.1 (85-92),* Special Issue on Bioprocess & Biosystem Engineering and their Downstream Processing

# Keratinolytic Potential of Fungal Strains Isolated from Soils of Public Places

Amira Nasyrah Husin[1], Subash C. B. Gopinath[1,2*] and M. N. A. Uda[1]

[1]*School of Bioprocess Engineering, Universiti Malaysia Perlis, 02600 Arau, Perlis, Malaysia.*
[2]*Institute of Nano Electronic Engineering, Universiti Malaysia Perlis, 01000 Kangar, Perlis, Malaysia.*

**ABSTRACT**

*Keratin is an insoluble macromolecule, comprised of long-polypeptide chains that are commonly found in hair, wool, feather, nail, horn and feather, and the stability of keratin depends on the adjacent chains linked by disulphide bonds. Several bacteria, fungi and actinomycetes are microorganisms, which are associated with secreting the keratinolytic enzymes called 'keratinases' play a significant role in the degradation of keratin. In this study soil samples were collected from different public places (poultry farm, school, mosque and park) and analysed the incidence of keratinophilic fungal strains. Specific fungal strains were isolated by keratin-baiting techniques and analysed for the production of keratinases. Poultry farm soil has the highest number of fungal species followed by the soil obtained from the school.*

**Keywords**: Keratinophilic Fungi; Feather; Keratinase; Baiting-Technique.

## 1. INTRODUCTION

Keratin is a biopolymer exists in large quantities and found widely in nature and represent as the major constituents of skin and its appendages, such as hair, nail, feather and other wastes (Teresa and Justyna, 2011; Godheja and Shekhar, 2014). Keratins are very specific sinewy proteins known as "Scleroproteins" (Teresa & Justyna, 2011). Keratin contributes to maintaining the stability of the cells as it functions as an outer layer and a protective barrier of human and animal organs. This characteristic is essential in preventing the loss of body fluids. Keratin is mostly found in tissues of reptiles, birds, amphibians and mammals. Very few organisms are able to utilize it by breaking down due to the strength and stability of keratin (Sharma & Rajak, 2003). It comprises two groups; they are alpha-keratins (alpha-helix) and beta-keratin (beta-sheets). Alpha-keratin can be found in the hair, wool, horn, nail, claw and hooves of mammals, whereas beta-keratin is found in bird feather, beak and claw (Gopinath *et al*., 2015).

Keratinophilic fungi or bacteria are parasitic, living on keratinous materials to obtain carbon and nitrogen sources (Gugnani *et al*., 2012; Kumar *et al*., 2013). They are considered to be the key players in the decomposition of keratinic waste materials using the secreted proteolytic (Keratinolytic) enzymes. Hyphomycetes include both dermatophyte and non-dermatophytic keratinophilic fungi and several other taxa belong to these are keratinophilic fungi. The keratinophilic species can be identified based on the morphological features (macroconidia and microconidia) besides using molecular methods and DNA sequence analysis. Similar to the isolates of fungi, bacterial strains that are capable of degrading keratins can also be isolated from the soil samples. Keratinolytic enzymes (proteases and keratinases) produced by fungi, actinomycetes and bacteria, are predominantly reside in the soil (Anbu *et al*.,2004; 2005; 2007; Gopinath *et al*., 2015).

*Corresponding Author: *subash@unimap.edu.my*

Microorganisms are ubiquitous in nature due to their successful survival rate in a wide range of environment, by the secretion of a wide range of enzymes depends on the available substrate(s) (Gopinath *et al*., 2000; 2002; 2003a; 2003b; Kumarevel *et al*., 2005). Because of the efficient enzyme secretion, microbes have adapted to inhospitable habitats. Similar way, keratinase producing microorganisms can be isolated from a wide range of habitats, where plenty of keratin substrates are available, such as slaughter-house, feather dumping areas, a place where higher numbers of animal bird and human are living.

**Figure 1.** Common sources of keratin. Feather and hair are shown.

## 2. MATERIALS AND METHODS

### 2.1 Material

To carry out the experiments, cycloheximide, chloramphenicol and streptopenicillin were used to prepare the antibiotic solution, which is needed to prevent the bacterial contamination. Besides, sterile deionized water was used to remoisten the soil in the Petri dish. In addition, Sabouraud dextrose agar (SDA) medium and LB media were used to cultivate the fungi. Ammonium sulfate precipitation was also used to purify the enzyme. Bovine serum albumin (BSA) was used as the standard to determine the content of the crude enzyme. Feather and hair were collected locally (Figure 1).

Equipment used in this study, includes autoclave for sterilization and incubator was used for growing the fungi. Besides, the light microscope was needed for morphological studies on fungi. Macherey-Nagel pH test strips were used to measure the pH. Next, centrifuge (Kubota 28000, Japan) was used to separate immiscible liquids. In addition, D-Tube™ dialyzer tube together with floating rack was used for dialysis. Ultraviolet-visible (UV-Vis) spectrophotometer, Shimadzu Lambda 25 (Fisher Scientific Sdn. Bhd. Malaysia) was also used to measure the protein absorbance.

### 2.2 Isolation of Fungi-Keratin Baiting

*Feathe*r and hair baiting techniques were employed for the isolation of keratinophilic fungi. *Soil samples* with an amount of 200 g were collected from four different places, which include poultry farm, school, park and mosque. The soil samples were placed in polyethylene bags and transported to the laboratory for further processing. Chicken feathers and hairs were collected as the keratin bait. Feathers were defatted to remove the additional fat, which was done by immersing them f*or 24 hour*s *in a 500 mL beaker of diethyl ether. Feather*s *were later cleaned about four to five times with distilled water followed by air dried. Chicken feathe*rs were divided into segments of 1 cm in length while hairs were cut into the length of 4 cm. Both feathers and hairs were placed in centrifuge bottles and then were autoclaved to ensu*re *they are sterile*. Each soil sample was placed in four sterile Petri dishes. The feathers and hair segments were scattered on the Petri dishes containing soil. The plates were moistened with an antibiotic solution, which contains 0.5 mg/mL of cycloheximide, 0.05 mg/mL of chloramphenicol and also 1000 IU/mL of streptopenicillin by using a

micropipette. The plates were placed in the incubator for a period of 2 months at room temperature and were remoistened with sterile deionized water whenever required. *Figure 2 shows the Petri dishes of chicken feathers and hairs together with soils from four different places.*

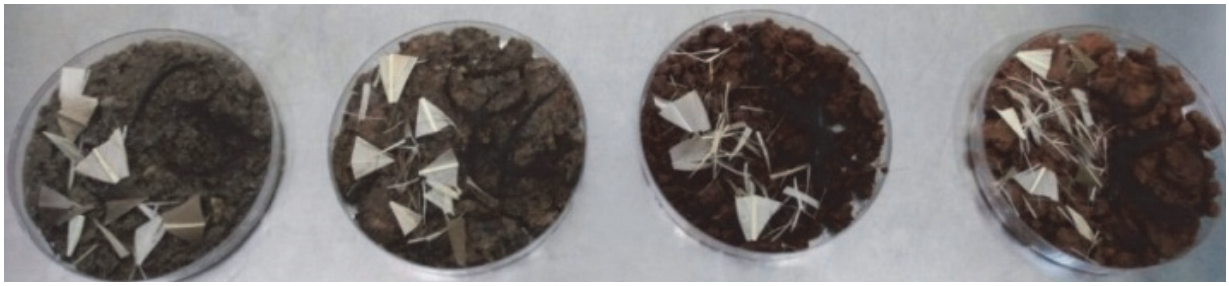

**Figure 2.** Keratin-baiting. Petri dishes filled with soil samples four different public places. Feather and hair were used as the bait.

### 2.3 Screening of Fungi

*To make sure the fungi* isolated, they w*ere* stained with Lactophenol cotton blue and obs*erved* under the light microscope. The pure fungal isolates w*ere* confirmed to have possible characters of keratinophilic fungi based on morphological studies. The baits were selected at random from each Petri dish once in a week and transferred to the plates containing Sabouraud dextrose agar (SDA) medium with 0.5 mg/mL of cycloheximide and 0.05 mg/mL of chloramphenicol. *The t*ransfer the baits to the plate, it was done by picking up the feathe*r* and hair using a clippe*r*, which was incubated later for a period of one week at room temperature. The developed colonies on the bait were examined.

The isolated strains were sub-cultured and then were stored on casein agar slants to obtain a pure culture. A 10 mL of SDA medium was poured into each test tube and all of them were slanted until become solidified. The sub-culture process was done by inoculating the isolates on the agar slants using the inoculating loop, which was sterilized by passing it through the flame of a Bunsen burner. The inoculating loop was then allowed to cool by imm*ersing* in 70% of the ethanol solution. The strains that produce clearing zones on the solid agar with keratin as the substrate will be selected for subsequent screening.

### 2.4 Extraction of Extracellular Keratinase

In the extraction of extracellular keratinase, submerged fermentation was performed by inoculating pure culture of isolate into 2 mL of LB media. Keratinase production was optimized at different pH of 5, 7 and 9 under a temperature 37 °C. Moreover, it was also optimized under different temperatures of 25 °C, 37 °C and 45 °C with the same optimal pH. The pH indication of the broth was determined by using pH test strips. The incubation was carried out for 3 days.

After incubation of 3 days, 1 mL was taken from each tube and the broth was centrifuged for 5 minutes at 10,000 x g. The supernatant was collected for the further process while the pellet was discarded. A volume of 1 mL of supernatant from each tube was transferred into a new tube. An amount of 0.7 g ammonium sulfate was added equally to all the tubes and shook until completely dissolved. The solution was cooled at 4 °C for 30 minutes and later centrifuged for 10 minutes at 10,000 x g. After that, the pellets were taken while the remaining solution was discarded. The pellet with the proteins was used as the crude enzyme extracts for further analysis.

Next, the pellet from each tube was diluted with distilled water. Dialysis process was then conducted to remove the salts from the proteins. An amount of 1 to 10 µL of the solutions was pipetted into the open end of the dialyzer tubes. The caps were closed and dialyzing tubes were

kept in an inverted position, so that the dialysis membrane is facing downward. The beaker with dialyzing tubes was kept in a cold room for 1 day.

### 2.5 Evaluation of Keratinase Product

The absorbance of Bovine serum albumin (BSA) and enzymes were determined at 280 nm using UV-Vis spectrophotometer using distilled water as the blank. By availing the extracted enzyme solutions, the feather was treated in 1 mL of protein solution for a few days in Eppendorf tubes and examined under a microscope with 10 x magnification.

## 3. RESULTS AND DISCUSSION

### 3.1 Isolation, Screening and Identification of Keratinolytic Fungi

In the present study, baiting technique was used to isolate the fungal strains from soil samples collected from a poultry farm, school, mosque and park. From the observation, the fungi showed their growth on the feathers and hair which were used as the baits which also serve as substrates. From the plates, molds can be seen on the surface of feathers and hair after 2 weeks of incubation at 25 ºC.

The feather and hair degrading organisms were isolated and maintained on the Sabouraud dextrose agar, the selective medium for the cultivation of fungi which contains nutrient. After 5 days of incubation at 25 ºC, colonies appeared on the agar medium with various colours. From the plates, different fungi produced variation in the appearance of colonies, they were circular, irregular, and filamentous in shape, some colonies may be colored and lastly, some colonies may be solid and spongy. The study shows the overall prevalence of keratinophilic fungi in four different places, which are poultry farm, school, mosque and park. The distribution data of these fungi are presented in Table 1.

**Table 1** Distribution of major keratinophilic fungi in four different places

| Isolated fungal strain | Site (number of colonies) | | | | Total | % occurrence |
|---|---|---|---|---|---|---|
| | Poultry farm | School | Mosque | Park | | |
| I | 10 | 7 | 3 | 5 | 25 | 34.25 |
| II | 5 | 5 | 3 | 4 | 17 | 23.29 |
| III | 3 | 4 | 2 | 4 | 13 | 17.81 |
| IV | 5 | 3 | - | 3 | 11 | 15.07 |
| V | 4 | 3 | - | - | 7 | 9.6 |
| Total | 27 | 22 | 8 | 16 | 73 | |

The presented data reveals that out of four soil samples collected, a total of 73 colonies of different keratinophilic fungi were isolated. The isolated keratinophilic fungi were in the following order of dominance; Strain I (34.25%), Strain II (23.29%), Strain III (17.81%), Strain IV (15.07%) and Strain V (9.6%). The strain I (34.25%) was the most predominant species with the maximum number of colonies and showed the positive growth in all places which was identified based on the

morphological study. The strain I appeared as a black colony on the feathers and hair. Out of the four places, poultry farm was recorded with a higher number of fungi species, compared to school, mosque and park. The excessive presence of feather (keratin) may attribute to the maximum number of keratinophilic fungi in the soil samples of a poultry farm. The difference in the organic matter of the soil may be the factor of the isolation of keratinophilic fungi from different places is not uniform. This means the presence of keratinophilic fungi in soil is affected by the organic matter content of the soil.

### 3.2 Optimization of Culture Conditions for Keratinase Production

The gelatinolytic activity of isolated fungi was monitored during growth in Lysogeny broth, the most common media for the growth of fungal cultures. The strain I was selected as the best fungi among others due to its higher number of colonies in all places and inoculated in the Lysogeny broth with different pH and temperatures.

### 3.3 Effect of pH on Keratinase Production

Keratinase production was optimized with different pHs, such as 5, 7 and 9. The effect of pH on keratinase production by Strain I is shown in Figure 3. From the figure, it was proved that pH 7 yielded the maximum enzyme production, which was 0.7899 U/mL. The lower production was observed at pH 5 and pH 9 with 0.7769 U/mL and 0.7169 U/mL of keratinase activity, respectively. The physiology of a microorganism is influenced by the pH of the medium itself (Kanchana & Mesta, 2013).

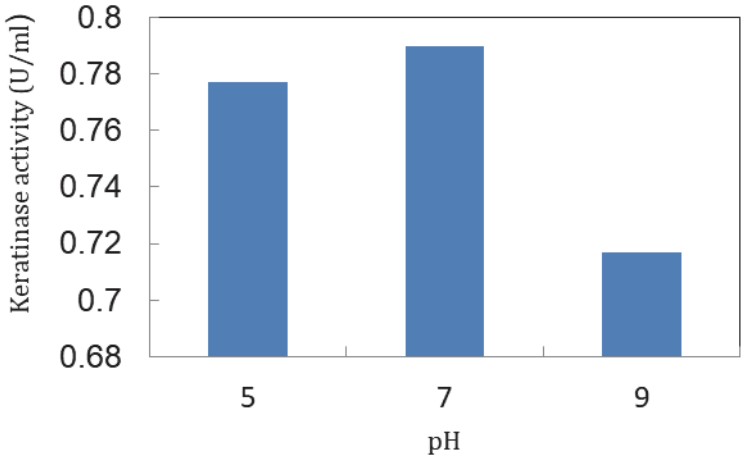


**Figure 3.** Effect of different pH on keratinase production.

### 3.4 Effect of Temperatures on Keratinase Production

The effect of temperatures on keratinase production by Strain I is shown in Figure 4. From the figure, the temperature of 37 ºC favoured the maximum enzyme production which was 0.8341 U/mL by Strain I.

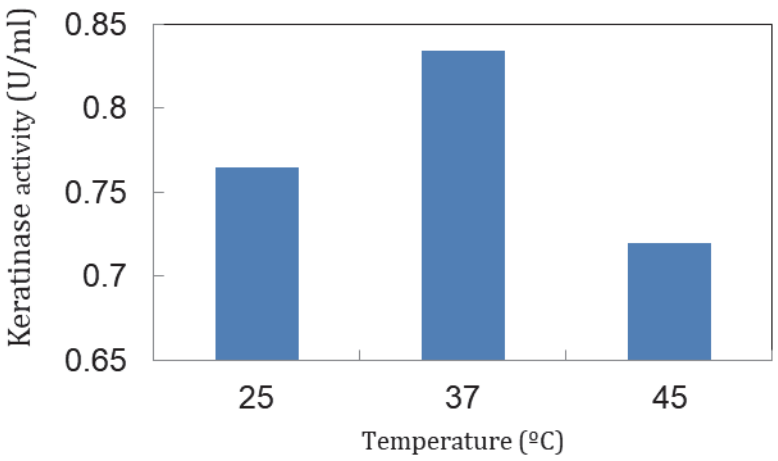


**Figure** Error! No text of specified style in document.**.** Effect of different temperatures on keratinase production.

### 3.5 Protein Estimation

The content of crude enzyme was estimated using Bovine serum albumin (BSA) as standard. From the standard curve (Figure 5), equation of y = 0.083x + 0.8239 was obtained. This equation was used to indicate the protein content of the samples of different pH and temperatures (Figure 4; Table 2).

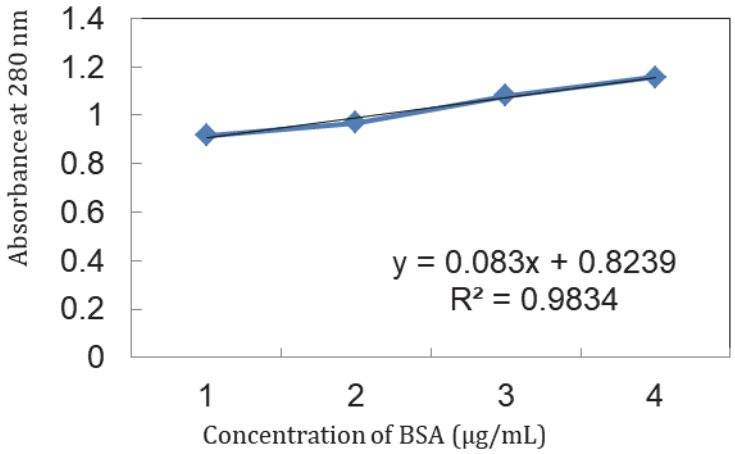


**Figure 5.** Protein standard curve of BSA.

**Table 2** Protein content of the samples at different pH

| Sample | Protein content (µg/mL) |
|---|---|
| Sample 1 (pH 5) | 2.85 |
| Sample 2 (pH 7) | 2.90 |
| Sample 3 (pH 9) | 3.49 |
| Sample 4 (pH 7) | 3.73 |
| Sample 5 (pH 7) | 3.08 |
| Sample 6 (pH 7) | 2.62 |

## 4. CONCLUSIONS

Baiting technique was used to isolate the fungal species from soil samples collected from a poultry farm, school, park and mosque. The strain I was to found to be highly keratinolytic and was selected for the keratinase enzyme assay. The strain I had a maximum keratinase activity of 0.7899 U/mL and 0.8341 U/mL for pH value of 7 and temperature of 37 ºC, respectively. This shows that Strain I has a great potential in degrading keratin thus will protect our environment. Hence, it is desirable to culture an organism using a low-cost and economical substrate, which results in low production cost. Simultaneously, industrial waste like chicken feather can be transformed into the required nutritional feed additives, thus conserve the environment by reducing wastage.

## REFERENCES

[1] Anbu, P., Hilda, A., Gopinath, S. C. B. (2004). Keratinophilic fungi of poultry farm soil and feather dumping soil in Tamil Nadu, India. Mycopathologia, 158, 303-309.

[2] Anbu, P., Gopinath, S. C. B., Hilda, A., Lakshmipriya, T., G. Annadurai. (2005). Purification of keratinase from poultry farm isolate-S*copulariopsis brevicaulis* and statistical optimization of enzyme activity. Enzyme and Microbial Technology, 36, 639-647.

[3] Anbu, P., Gopinath, S. C. B., Hilda, A, Lakshmipriya, T., Annadurai, G. (2007). Optimization of extracellular keratinase production by poultry farm isolate *Scopulariopsis brevicaulis.* Bioresource Technology, 98, 1298-1303.

[4] Godheja, J., & Shekhar, S. K. (2014). Biodegradation of keratin from chicken feathers by fungal species as a means of sustainable development. *Journal of Bioremediation & Biodegradation, 5*, 1-5.

[5] Gopinath, S. C. B., Hilda, A., Anbu, P. (2000) Screening methods for detecting lipolytic enzymes by Aspergillus species. Acta Botanica Indica, 28, 41-44.

[6] Gopinath, S. C. B., Hilda, A., Lakshmipriya, T., Annadurai, G. (2002) Purification of lipase from *Cunninghamella verticillata* and optimization of enzyme activity using response surface methodology. *World Journal of Microbiology and Biotechnology,* 18, 449-458.

[7] Gopinath, S. C. B., Hilda, A., Lakshmipriya, T., Annadurai, G., Anbu, P. (2003a) Purification of lipase from *Geotrichum candidum*: Conditions optimized for enzyme production using Box-Behnken design. *World Journal of Microbiology and Biotechnology,* 19, 681-689.

[8] Gopinath, S. C. B., Hilda, A., Lakshmipriya, T., Annadurai, G. & Anbu, P. (2003b). Statistical optimization of amylolytic activity by Aspergillus versicolor. *Asian Journal of Microbiology, Biotechnology and Environmental Sciences,* 5, 327-330.

[9] Gopinath, S. C. B., Anbu, P., Lakshmipriya, T., Tang, T.H., Chen, Y., Hashim, U., Ruslinda, A.R. Md Arshad, M. K**.** (2015). Biotechnological aspects and perspective of microbial keratinase production*.* BioMed Research International, 2015, 140726.

[10] Gugnani, H. C., Sharma, S., Gupta, B., & Gaddam, S. (2012). Prevalence of keratinophilic fungi in soils of St. Kitts and Nevis. *Journal of Infection in Developing Countries,* 6, 347-351.

[11] Kanchana, R., & Mesta, D. (2013). Native feather degradation by a keratinophilic fungus. *International Journal of ChemTech Research,* 5, 2947-2954.

[12] Kumar, R., Mishra, R., Maurya, S., & Sahu, H. B. (2013). Isolation and identification of keratinophilic fungi from garbage waste soils of Jharkhand region of India. *European Journal of Experimental Biology,* 3, 600-604.

[13] Kumarevel, T., Gopinath, S. C. B., Hilda, A., Gautham, N., Ponnusamy, M. N. (2005). Purification of lipase from Cunninghamella verticillata by step-wise precipitation and optimized conditions for crystallization. *World Journal of Microbiology and Biotechnology*, 21, 23-26.

[14] Sharma, R., & Rajak, R. C. (2003). Keratinophilic fungi: nature's keratin degrading machines. Resonance, 8, 28-40.

[15] Teresa, K. K., & Justyna, B. (2011). Biodegradation of keratin waste : theory and practical aspects. Waste Management, 31, 1689-1701.