Keratinase-producing fungi from local environmental samples of Far South Thailand and their efficiency in hydrolyzing keratinous wastes

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Abstract. Samples from around BangoRana, Bang oAsae, and Tanod, Narathiwat Province, Thailand were screened for keratinase-producing fungi. By using Horikoshi media and keratin (1%) agar plate, 3 isolates of fungi were shown to produce wider clear zone, and they were Aspergillus sp., Penicillin sp. and Cladosporium sp., and designated as Aspergillus KF1, Penicillin KF2 and Cladosporium KF3. Each isolate was growth in Feather Meal Medium with 37°C incubation in shaking condition. Results showed that these fungi had typical growth profile. Extracellular keratinase profile for Penicillium KF2, the keratinase release began at the late lag phase (1 U/ml), whereas those of Aspergillus KF1, and Cladosporium KF3 started at the log phase (U/ml: 3.9 and 2.9). Maximal keratinase production was observed in the fungi culture on day 4 and day 5 upon shaking incubation at 37°C. Different activities were observed in U/ml likes 70.7 for Aspergillus KF1; 83.5, Penicillium KF2; and 72.4, Cladosporium KF3. Aspergillus KF1 and Penicillin KF2 reached their maximum on day 4 (CFU/ml: 8.9 x 10^6 and 8.4 x 10^6) Cladosporium KF3 on day 5 were 8.95 x 10^6 CFU/ml. Keratinases were expected to be of primary metabolites, and hence best harvested at the beginning of stationary phase.

1. Introduction
Feathers constituting 5-7% of the total weight of mature chickens are slow degradable by nature as they are made up primarily of keratin, a fibrous and insoluble structural protein. Being in mechanical stability and resistance to common proteolytic enzyme such as pepsin, trypsin and papain [1], these feathers are often accumulated as an increasingly sizable waste disposal problem. Several different approaches have been used for disposing of feather waste, including land filling, burning, natural gas production and treatment for animal feed. Most feather waste is land filled or burnt which involves expense and can cause contamination of air, soil and water [2]. Still feathers are currently used to manufacture feather meal through thermal processing but with low nutritional value. Feathers hydrolyzed by microbial keratinases have been used as additives for animal feed [3, 4], as well as have potential use as organic fertilizers, production of edible films and rare amino acids [4, 5].

Many microorganisms such as bacteria, Actinomyces and fungi are known produce keratinases [6, 7, 8]. However, their action on keratin is still not clear despite using purified keratinases. These days,
industrial and environmental products often demands isolation of more and more new microbial enzymes. Keratinases as potential friendly degrader will find places in industrial and biotechnological processes, would solve disposal problem of poultry waste and the similar [9]. Among the microbes that cycle this protein in nature, keratinophilic fungi are very common and the most diverse. If keratinophilic fungi were not there to cycle this highly stable protein (keratin), then one can imagine the quantity of keratin that would have accumulated on earth, since a vast quantity of keratin is shed by the vertebrates. This work aimed in isolating keratinase-producing fungi from local waste-dumping soil in hoping to find these fungi for further research with high impact on local biotechnological industries.

2. Experimental

2.1 Isolation of chicken feather-hydrolyzing fungi

In enrichment step, 1 g of chicken-feather dumping soil from different localities was subjected to 10-fold serial dilution to reduce an initial microbial number. Appropriated dilution was then inoculated in Basal feather broth (BFB) supplemented with pre-washed and sterilized feather powder. Culture in BFB was incubated at 37°C in shaking condition 120 rpm for 24-48 hours or until the visible growth was observed. It was later sub-culture and re-subculture for several times to achieve the enrichment [10]. For isolation, this BFB culture was spread on keratin (1%) agar plate constituting (g/l) of agar: 15, MgSO4.H2O: 0.5, KH2PO4: 0.1, FeSO4.7H2O: 0.01, ZnSO4.7H2O: 0.005, and 1% (w/v) sterilized chicken feather powder, pH 7.5 [12]. Incubation was followed at 37°C for 5 days, and colonies with clear surrounding zone was selected to perform hyphal tip inoculation on potato dextrose agar (PDA) (HiMedia, India). It took 3-4 days at 37°C to make each hyphal tip developed into visible and mature colony. This purified colony was stored at 4°C until further use.

2.2 Fungal identification

Fungal isolates obtained were characterized and identified on the basis of their morphological assessment that is, macroscopic and microscopic features. Simplified fungi identification key of Jean Williams-Woodward, and online Dichotomous keys from Mould Laboratory, University of Toronto, Canada at http://labs.csb.utoronto.ca/moncalvo/malloch/Moulds/Moulds.html or/and online Mould: Isolation, cultivation and identification of New Brunswick Museum, Canada at http://website.nbm-nmb.ca/mycologywebpages/Moulds/Moulds.html, were used as a guide.

2.3 Growth and keratinase production profile

Growth of keratinase-producer fungi. Growth profile was followed by the procedure suggested by Wawrzkiewcz and co-workers [12]. Spore suspension from starting culture was cultured into 250-ml flask that containing 50 ml Feather meal broth (g/l of MgSO4, H2O: 0.5, KH2PO4: 0.1, FeSO4, 7H2O: 0.01, and ZnSO4, 7H2O: 0.005), supplemented with 1% sterilized feather powder, and pH 7.5. Culture was incubated at 37°C for 7 days in 120 rpm shaking condition. Daily drawn culture was subjected to colony counting on PDA plate (King at al., 1979), and spectrophotometric read at 600 nm wavelength using UV-VIS spectrophotometer (LIBRA S32, England) as well as keratinase activity and protein content measurement.

Keratinase production profile. In determining keratinase activity and protein content, technique suggested by Yu at al. (1968) was followed with modification. Growing fungal culture was drawn for 10 ml from Feather meal broth, filtered by using 0.45 µm pore size Nitrocellulose membrane to collect cell-free filtrate for determining keratinase activity and protein content. Using 16x150 ml tube, 0.002 g of feather powder was added into 3.8 ml Phosphate buffer, pH 7.8 and 3.0 ml filtrate. This hydrolysis mixture was kept at 37°C for an hour, after when it was dipped into ice cubes for 10 minutes, filtered using Whatman No. 4, and read the optical density at 280 nm of UV-VIS spectrophotometer against no-hydrolysis mixture as a blank. One U/ml of keratinase activity was defined as the amount of enzyme that
causes the hydrolytic change of optical density for 0.01 in 1 hour, as measured at 280 nm wavelength. Protein determination followed established procedure (Bradford, 1976) using Bovine serum albumin (BSA; Bio-Rad, USA) as a standard.

3. Results And Discussion
3.1. Isolation of keratinase-producing fungi
Results on keratin (1%) agar plate of isolating keratinase-producing fungi from chicken feather-dumping soil in localities of Narathiwat Province, Far-south of Thailand yielded totally 138 colonies, and out of these, 53 colonies of 3 isolates constituting 38.41% were keratinase producers as distinguishable by the clear zone surrounding each fungal colony on Feather meal agar (Table 1). They were designated as KF1, KF2 and KF3 (Figure 1).

Table 1. Population density of KF1, KF2 and KF3 grown on keratin (1%) agar plate after 5-day incubation at 28°C.

| Localities (Village) | Total (%) | Total colonies with surrounding clear zones (%) |
|----------------------|-----------|-----------------------------------------------|
|                      |           | KF1          |  | KF2          |  | KF3          |  | Total of clear zone-surrounding colonies |
| Bango Rana           | 46 (33.34)| 9 (19.57)    | 7 (15.22)| 3 (6.52)    | 19 (41.30) |
| Bango Asae           | 47 (34.06)| 8 (17.02)    | 5 (10.64)| 4 (8.50)    | 17 (36.17) |
| Tanod                | 45 (32.61)| 8 (17.78)    | 6 (13.33)| 3 (6.67)    | 17 (37.78) |
| Sum (%)              | 138 (100)| 25 (18.12)   | 18 (13.04)| 10 (7.25)   | 53 (38.41) |

Figure 1. KF1, KF2 and KF3 colonies that were surrounded by clear zone as a result of keratin hydrolysis. No keratin hydrolysis (KF0) was also shown as a comparison.
Kumar & co-workers [13] reported isolating keratinolytic fungi of *Absidia* sp. from 26 out of 80 soil sampling (28.9%), and only 6 (6.7%) of *Entomophthora coronate*. In fact, microbial isolation depends solely on chance and isolating technique [13]. Mini and co-workers [14] reported isolating fungi from rural areas Kerala, India. Six samples of soil collected in 60 poultry farms were plated on Keratin agar medium, and found only in 51 samples with fungal growth. Of these, eight isolates (15.7%) were of clear zone-surrounding colonies, and they were *Aspergillus niger*, *A. flavus*, *A. fumigatus*, *A. nidulans*, *Penicillium* sp., *Chrysosporrium keratinophylum*, *Microsporum gypseum* and *Trichophyton mentagrophytes*.

3.2 Fungi identification

By using online Dichotomous keys of Mould Laboratory, University of Toronto, Canada and Mould: Isolation, cultivation and identification of New Brunswick Museum, Canada, KF1, KF2 and KF3 were able to primarily allocate as *Aspergillus* sp, *Penicillium* sp., and *Cladosporium* sp., respectively (Table 2).

Isolating *Aspergillus* sp., *Penicillium* sp., and *Cladosporium* sp. as keratinolytic enzyme producers from soil were not new, especially from feather waste-dumping soil. Geethanjali [15] isolated fungi from soil in Madikeri, India by using the feather baiting technique, and was able to isolate 46 colonies and 10 isolates accounted for 21.7% with keratinolytic ability. These fungal isolates were later morphology-based identified as *Microsporum*, *Penicillium*, *Aspergillus*, *Fusarium*, *Cladosporium*, *Chaetomium*, *Staphyllichium*, *Gleocadium*, *Pithomyces* and *Saccharomyces*. Meanwhile, similar samples from Nanded, Maharashtra, India were reported to harbor as many as 22 fungi and 7 isolates (31.8%) when culturing on Sabouraud Dextrose Agar (SDA) plates [16]. Upon morphology-based identification, they were *Aspergillus niger*, *Alternaria alternate*, *Curvularia lunata*, *Fusarium oxsporum*, *Penicillium* sp., *Myrothecium roridum* and *Trichoderma hamatumand*. Poultry farm Soil samples from Kerala, India were reported to harbor among others *Aspergillus niger*, *A. flavus*, *A. fumigatus*, *A. nidulans*, *Penicillium* sp., *Chrysosporrium keratinophylum*, *Microsporum gypseum*, and *Trichophyton mentagrophytes* [14]. Keratinolytic *Aspergillus*, *Fusarium*, *Penicillium* and *Trichoderma* sp. were reported to isolated from soil in chicken farms in Egypt [11].

3.3. *Aspergillus KF1*, *Penicillium KF2* and *Cladosporium KF3* growth and keratinase production profile

In keratin broth, *Aspergillus KF1*, *Penicillium KF2* and *Cladosporium KF3* showed normal growth and metabolite production profile like that of keratinase. *Penicillium KF2* released extracellular keratinase at the end of the Lag phase of growth (1 U/ml). In contrast, keratinase of *Aspergillus KF1* and *Cladosporium KF3* were released at the beginning of the growth phase (U/ml/ml: 3.9 and 2.9). Maximal keratinase production was observed at the end of the log phase for *Aspergillus KF1*, *Penicillium KF2*, and *Cladosporium KF3* with U/ml/ml enzyme activity of 70.7, 83.5, and 72.4, respectively (Figure 2).

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On Day 4, Aspergillus KF1, Penicillin KF2 and Cladosporium KF3 demonstrated cfu/ml/OD growth rates of $8.9 \times 10^6/1.119$, $8.4 \times 10^6/0.814$, and $8.95 \times 10^6/0.085$, respectively (Figure 2).

On the issues of the OD difference, it was likely to be caused by several factors, such as their different biomass and viability. That is, viability represents cfu count but OD is measurement of total biomass regardless of viability or non-viability including spores, fibers and other elements of mold [17]. As regard to the production of keratin, our results were consistent with report of *Streptomyces gulbargensis* DAS 131, whose keratinase production was highest during the last log phase of growth [18, 19].

Despite the fact that fungi are rather more interesting, study of keratin-producing fungi was somewhat less than that of bacteria. Saber and co-workers [11] reported keratinase activity in *Alternaria tenuissima* and Aspergillus *nidulans* shaking broth basal medium culture, at pH 7.8 of Tris-HCL buffer, that *A. tenuissima* produced the highest enzyme (53.4 U/ml) in 5 days, and *A. nidulans* in 6 days (55.8 U/ml). *A. niger* were reported to produce the highest keratinase enzyme (75 U/ml) in 12-day liquid mineral medium with shaking condition [16]. In other reports, *Penicillium* sp. produced keratinase enzyme up to 64 U/ml in 11 days [14].
Table 2. Macroscopic and microscopic morphology of KF1, KF2 and KF3 isolates of keratinolytic fungi.

| Isolates/Description | Macroscopic/microscopic features | Designation        |
|----------------------|----------------------------------|--------------------|
| Macroscopically, KF1 showed green color colony with evenly growth on PDA medium. | ![Macroscopic image](image1) | Aspergillus sp.   |
| Microscopically, globular vesicles, conidiophores shaped translucent yellowish green, semi conidiaspore round to round-shaped light green to brownish green. | ![Microscopic image](image2) |                      |
| Macroscopically, KF2 was yellow-colored colonies with greenish black color inside. | ![Macroscopic image](image3) | Penicillium sp.   |
| Microscopically, single-celled spores were in chains with strigma arising from metula of the conidiophore. The branching conidiophores were arised from a septate mycelium. | ![Microscopic image](image4) |                      |
| Macroscopically, KF3 growth rate seen on potato dextrose agar at 25°C was moderate with velvety to powdery colony texture, and olivaceous green to black color from the front toward the reverse. | ![Macroscopic image](image5) | Cladosporium sp.   |
| Microscopically, feathers included septate brown hyphae, erect and pigmented conidiophores. Conidia were elliptical to cylindrical in shape, and pale to dark brown in color. | ![Microscopic image](image6) |                      |
Figure 2. Growth and production of extracellular keratinase profile of Aspergillus sp. KF1, Penicillium sp. KF2 and Cladosporium sp. KF3 in keratin broth medium.

Prerna and Kushwaha [20] reported keratinase enzymes in Cladosporium chlorocephalum (GPCK 3069) and Cla. Cladosporioides (MTCC 9983) cultures. Whereas Friedrich and co-workers [21] reported keratinase enzymes of A. flavus 7-day old culture (781 mU/ml), and of Cla. Cladosporioides 10-day old culture. (92 mU/ml)

These reports indicated that activity of keratinases, impure or coarse, depends on cultures as well as their age, their conditions, which include the conditions of the enzyme and substrate [22, 23].

4. Conclusion

By inoculating feather waste dumping soils from localities in Narathiwat Province on keratin (1%) agar plate, keratinase-producing Aspergillus FK1, Penicillium FK2 and Cladosporium FK3 were isolated. These fungi showed normal growth and enzyme production profile, constituting lag, log, stationary, and decline phases with distinguishable features. Aspergillus FK1, Penicillium FK2 and Cladosporium FK3 went through lag phase for 1 day or less. However, log phase was varied. Aspergillus FK1 and Penicillium FK2 were in log phase from day 2 to day 5. Cladosporium FK3 log phase extended to day 6. Keratinase production took place at the end of lag phase and reached its maximum at the end of log phase. Highest U/ml keratinase activity for Aspergillus KF1, Penicillium KF2, and Cladosporium KF3 were 70.7, 83.5, and 72.4, respectively. Growth and keratinase production was in most cases parallel.

References

[1.] R. Gupta and P. Ramnani, 2006 Microbial keratinases and their prospective applications: an overview. Appl. Microbiol. Biotechnol. 70, 21-33
[2.] S. G. Joshi, M. M. Tejashwini, N. Revati, R. Sridevi and D. Roma, 2007 Isolation, Identification and characterization of a feather degrading bacterium, Inter J. Poultry Sci. 6, 689-693
[3.] N. H. Odetallah, J. J. Wang, J. D. Garlich and J. C. H., Shih, Versazyme 2005 Supplementation of broiler diets improves market growth performance, Poultry Sci. 84, 858-864
[4.] A. Riffel, A. Brandelli, C. M. Bellaro, G. H. M. Souza, M. N. Eberlin and F. C. A Tavarez, 2007 Purification and characterization of a keratinolytic metalloprotease from Chryseobacterium sp. kR6, J. Biotechnol. 128, 693-703
[5.] A. Brandelli and A. Riffel, 2005 Production of an extracellular keratinase from Chryseobacterium sp. growing on raw feathers, Electronic J. Biotechnol. 8(1), 35-42
[6.] H. Gradisar, J. Friedrich, I. Krizaj and R. Jerala, 2005 Similarities and specificities of fungal keratinolytic proteases: comparison of keratinases of Paecilomyces marquandii and Doratomyces microporus to some known proteases, Appl. Environ. Microbiol. 71, 3420–3426

[7.] B. Jaouadi, N. Aghajari, R. Haser and S. Bejar, 2010 Enhancement of the thermostability and the catalytic efficiency of Bacillus pumilus CBS protease by site directed mutagenesis, Biochimie, 92, 360-369

[8.] S. M. L. Cedrola, A. C. N. Melo, A. M. Mazotto, U. Lins, R. B. Zingali, A. S. Rosado, R.S. Peixoto and A. B. Vermelho, 2012 Keratinases and sulfide from Bacillus subtilis SLC to recycle feather waste, World J. Microbiol. Biotechnol. 28, 1259-1269

[9.] A. Brandelli, D. J. Daroit and A. Alessandro Riffel, 2010 Biochemical features of microbial keratinases and their production and applications, Appl Microbiol Biotechnol. 85, 1735–1750

[10.] J. C. H. Shih and W.C. Michael, 1992 Purified Bacillus licheniformis PWD-1 keratinase, United States Patent 5171682

[11.] W. I. A. Saber, M. M. El-Metwally and M. S. El-Hersh, 2009 Keratinase Production and Biodegradation of Some Keratinous Wastes by Alternaria tenuissima and Aspergillus nidulans, Res. J. Microbiol. ISSN, 1816-4935

[12.] K. Wawrzkiewicz, T. Wolski and J. Lobazewski, 1991 Screening the keratinolytic activity of dermatophytes in vitro, Mycopathologia, 114, 1–8

[13.] P. Kumar, Y. F. Kazi and I. H. Soomro, 2012 A comparative characterization of indigenous keratinase enzymes from district Khairpur, Sindh, Pakistan, Pak. J. Pharm. Sci. 25(1), 73-79

[14.] K. D. Mini, K. P. Mini and J. Methew, 2012 Screening of fungi isolated from poultry farm soil for keratinolytic activity, Adv. Appl. Sci. Res. 3(4), 2073-2077

[15.] P. A. Geethanjali, 2011 Screening of Keratinolytic fungi from the Poultry Soil for the Degradation of Chicken Feather, J. Theoretical Experiment. Biol. (ISSN: 0972-9720), 8 (1 and 2), 53-56

[16.] S. S. Ingle, V. D. Kalyankar, G. M. Karadkhele and M. M. V. Baig, 2012 Biodegradation of Poultry feather by non dermatophytic filamentous Keratinolytic Fungi, Asian J. Biol. Biotechnol. 1(1), 102

[17.] R. W. Bauman, 2014 Microbiology: with diseases by taxonomy. 4th Edition, Pearson Education, Inc. Boston

[18.] D. G. Syed, J C. Lee, W-J. Li, C-J. Kim and D. Agasar, 2009 Production, characterization and application of keratinase from Streptomyces gulbargensis, Bioresource Technol. 100, 1868–1871

[19.] J. M. Kim, W. J. Lim and H. J. Suh, 2001 Feather-degrading Bacillus species from poultry waste, Process Biochem, 37, 287–291

[20.] A. Prerna and R. K. S. Kushwaha, 2011 Keratinase Activity of Some Hyphomycetous Fungi from Dropped off Chicken Feathers, Int. J. Pharmaceut. Biol. Arch. 2(6), 1745-1750

[21.] J. Friedrich, H. Gradisar, D. Mandin and J. P. Chaumont. 1999 Screening fungi for synthesis of Keratinolytic enzymes, Lett Appl Microbiol. 28, 127-130

[22.] H. J. Son, H. C. Park, H. S. Kim and C. Y. Lee, 2008 Nutritional regulation of keratinolytic activity in Bacillus pumilus, Biotechnol Lett. 30, 461–465

[23.] G-T. Park and H-J. Son, 2009 Keratinolytic activity of Bacillus megaterium F7-1, a feather-degrading mesophilic bacterium, Microbiol Res. 164, 478—485

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